

## Korespondensi Jurnal

Judul Artikel : Anticancer Activity of Linamarin From Cassave Leaves (Manihot Esculenta Cranz )  
on Raji Cells

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# 2. Evaluation Report\_ Reviewer 1

Anticancer Activity of Linamarin on Raji Cells

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7	Abstract	• Overall Ok	
8	Keywords	• Overall Ok	
9	Introduction	• Overall Ok	
10	Materials and Methods	<ul style="list-style-type: none"><li>• Author is advised to cite the reference from where the protocols of Immuno-cytochemical test in Raji cells methodology were adopted i.e.</li><li>• Please provide the citation of reference from where you</li></ul>	MTT reagent (Sigma Aldrich, Darmstadt, Germa laminar air flow (Nuairie), incubator (Nuairie), phase cont microscope (Olympus, Japan), electric scales (Sartori micropipette (Socorex), centrifuge (B. Braun bio Internasional), vortex (Genie), waterbath (Labec), pH m (TOA ), Tissue Culture Flask (TCF) 25 cm2 (Nunclon microplate 96 (Nunclone), hemocytometer (Neubau

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		<p>have acquired this methodology in your study.</p> <ul style="list-style-type: none"> <li>Please do not provide whole protocol as you have presented in authors comment just add the reference of this protocol.</li> </ul>	<p>filter millipore 0,2 um (Labec), fluorescence microscope (Olympus, Japan), glass object (Sigma Aldrich), deck glass (Sigma Aldrich), light microscope (Olympus, Japan), <b>p53 Assay kit (Colorimetric) (Novocastra), liquid DAB substrate kit (Novocastra)</b></p>
11	<b>Results</b>	<ul style="list-style-type: none"> <li>Overall Ok</li> </ul>	
12	<b>Figures</b>	<ul style="list-style-type: none"> <li>Overall Ok</li> </ul>	
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14	<b>Discussion</b>	<ul style="list-style-type: none"> <li>Overall Ok</li> </ul>	
15	<b>Conclusion</b>	<ul style="list-style-type: none"> <li>Overall Ok</li> </ul>	
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17	<b>Significance Statement</b>	<ul style="list-style-type: none"> <li>Overall Ok</li> </ul>	
18	<b>References</b>	<ul style="list-style-type: none"> <li>Overall Ok</li> </ul>	

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Anticancer Activity of Linamarin on Raji Cells

19 **Anticancer Activity of Linamarin from Cassava Leaves**

20 **(*Manihot esculenta* Cranz) on Raji Cells**

21 **Running Title: Anticancer Activity of Linamarin on Raji Cells**

22  
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31 **Author contributions**

32 **Dwi Sutiningsih:** Performed literature review, developed research proposal, conducted

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34 Participated in research design and manuscript writing. **Henry Setyawan Susanto:** Reviewed

35 research proposal and contributed to cytotoxic examination and data analysis.

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

37 **Mustofa:** Conducted the experiments and wrote the manuscript.

38

39 **ABSTRACT**

40 **Background and Objective:** Linamarin is an active compound isolated from the leaves of

41 cassava (*Manihot esculenta* Cranz) that has a cytotoxic effects on HT-29, MCF-7, and HL-60

42 cells. This study was aimed to determine the cytotoxic and antiproliferation activity and

43 induction of p53 protein in Raji cells, after administration of various concentrations of

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47 while antiproliferation activity was tested using a *doubling time* test. P53 protein expression  
48 was observed by immunocytochemical tests. The cytotoxic activity of Raji cells was  
49 expressed by the value of Inhibitory Concentration 50 ( $\mu\text{g/ml}$ ). The *doubling time*  
50 was calculated by comparing the *slope* values of the log graphs of the number of cells at  
51 various times. Raji cells that were positive for p53 protein showed brown painted nuclei or  
52 cytoplasm. **Results:** Linamarin from cassava leaves can inhibit cytotoxic activity and  
53 proliferation on Raji cells. The higher the linamarin concentration, the longer the doubling  
54 time of Raji cells. The expression of p53 protein on Raji cells after linamarin administration  
55 was higher than the control. P53 protein expression was found in the nuclei (91.05%) and  
56 cytoplasm (8.95%). **Conclusions:** Based on those findings, linamarin from cassava leaves has  
57 the potential to be developed as an anticancer agent.

58

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

61

## 62 INTRODUCTION

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

68 In Indonesia, cassava has considerable economic value compared to other tubers. Not  
69 only is cassava (*Manihot esculenta* Cranz) one of the world's principal food staples after  
70 grains and corn,<sup>1</sup> their leaves, widely consumed in Indonesia and  
71 elsewhere, are rich in vitamins A, C, K, among others, and minerals, including iron, calcium,

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72 and phosphorus. The energy content of cassava leaves is greater than most other green  
73 vegetables<sup>2</sup>. Cassava also contains cyanogenic glucoside compounds, which consist of  
74 linamarin and lotaustrain at a ratio of 10:1<sup>3, 4</sup>. Linamarin has potential use as an anti-  
75 neoplastic compound<sup>5,6</sup>. The mechanism of linamarin in the treatment of cancer using  
76 linamarase gene therapy has been investigated.

77 Meanwhile the Idibie<sup>6</sup> study states linamarin in root tubers has been proven in  
78 vitro to have cytotoxic effects on HT-29, MCF-7, and HL-60 cells. From the results of this  
79 study, Inhibitor Concentration 50 (IC<sub>50</sub>) was obtained in the amounts of > 300 µg/ml, 235.96  
80 ± 9.87 µg/ml, and 246.51 ± 10.12 µg/ml after incubation for 48 hours. In this study, linamarin  
81 was obtained from cassava leaf extracted with methanol. The study of Yusuf *et al.*<sup>7</sup> using  
82 linamarin isolated from cassava leaves also showed cytotoxic effects on Caov-3 cells and  
83 Hela cells. The IC<sub>50</sub> value of the two cell lines is 38 µg/ml and 57 µg/ml respectively. Cancer  
84 cell death has been caused by the linamarin content found in cassava plants<sup>8-11</sup>. Carotene and  
85 vitamin C compounds found in cassava leaves are thought to have anticancer properties<sup>12-15</sup>.  
86 Research by Enger *et al.*<sup>16</sup>, stated that carotene is protective toward colon adenoma rather than  
87 other carotenoids in the early stages of tumor formation. Kontek *et al.*<sup>17</sup> stated that vitamin C  
88 had a positive effect on the damage level of oxidative DNA in colon cancer cells.

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

93

## 94 MATERIALS AND METHODS

95 Cassava leaves (*Manihot esculenta* Cranz) were obtained from the local market in  
96 Yogyakarta, Indonesia, then identified at the Laboratory of Pharmaceutical Biology, Faculty

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97 of Pharmacy, Gadjah Mada University. This research project was conducted from June 4,  
98 2018 to December 4, 2018. Raji cells were obtained from the collection of the Laboratory of  
99 Parasitology, Faculty of Medicine, Gadjah Mada University. This cell is a continuous cell line  
100 that grows floating, similar to lymphoblast cells (B lymphocytes) from Burkitt's  
101 lymphoma infected by Epstein-Barr Virus (EBV). Materials for growing Raji cells are RPMI  
102 solution (Sigma-Aldrich), Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich),  
103 HEPES, Fetal Bovine Serum (FBS)(Gibco), penicillin-streptomycin(Gibco), DMSO (Sigma-  
104 Aldrich), tripan blue (E-Merck), and 3-(4-, 5 dimethylthiazol-2-yl) -2.5-diphenyl tetrazolium  
105 bromide (MTT) (Sigma-Aldrich).

106 **Linamarin isolation from cassava leaves:** A 5 g batch of cassava leaves was cut into small  
107 pieces, then pounded in a mortar. The result was blended thoroughly with a total of 10 ml of  
108 0.1M HCl solution. The mixture solution was centrifuged at 3500 rpm to obtain the  
109 supernatant. The supernatant liquid obtained was transferred to the Falcon tube  
110 (Nunclone). The supernatant liquid mixture with 0, 1 M HCl was linamarin extract of cassava  
111 leaf, which was then isolated. Finally, the linamarin extract was frozen at - 20°C<sup>18</sup>.

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



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121

122 **Antiproliferation test (doubling time) in Raji cells:** Cells were fasted for 24 hours in culture  
123 media containing 0.5% of FBS. Afterwards, they were grown in a plate with a medium added,  
124 with linamarin at a non-lethal concentration of three series below the IC<sub>50</sub> value. Then it was  
125 incubated in a 5% CO<sub>2</sub> incubator (Nuairie) at 37 °C for 24, 48, and 72 hours. Each well was  
126 calculated by the number of cells living using hemocytometrics (Neubauer).

127

128 **Immuno-cytochemical test in Raji cells:** Immuno-cytochemical staining was performed  
129 using the avidin-biotin-peroxidase complex with monoclonal antibodies against p53  
130 according protocols of p53 Assay kit (Colorimetric) (Novocastra). In a microculture, 96 wells  
131 containing 100 µl of test cells, with a density of 2 x 10<sup>4</sup> cells/well, 100 µl of the test  
132 compound were added at concentrations of 10 µg/ ml. They were then incubated  
133 with 5% CO<sub>2</sub> flow at 37 °C for 24 hours. After being incubated overnight, 200 µl of cells  
134 from each well were taken and inserted in eppendorf tubes, then centrifuged to 1200rpm x 5  
135 minutes. The supernatant liquid was removed, leaving the pellet, and then re-suspended. The  
136 cell suspension was extracted and placed on a glass object that had been coated with poly-  
137 lysine. The cells were fixated with acetone for 10 minutes. Later, they were washed with PBS  
138 (Phosphate Buffered Saline)(E.Merck) x 5 minutes and etched with hydrogen  
139 peroxidase 0, 1% for 10 minutes. After washing them with running water, they were rinsed  
140 with PBS for five minutes, dripped with 100 µl normal horse serum for 10 minutes, and  
141 cleaned without water. Finally, they were dripped with anti-p53 protein primary antibodies  
142 (Novocastra) and left for 24 hours.

143 The next day the suspension was:

- 144
- washed twice with PBS x 5 minutes each;
  - dripped with biotinylated secondary antibodies (Novocastra) x 10 minutes;
- 145

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- 146 • washed x 2 with PBS x 5 minutes each;
- 147 • dripped then incubated with Avidin Biotin reagent enzyme (Novocastra) x 10 minutes;
- 148 • washed x 2 with PBS x 5 minutes each;
- 149 • incubated with a peroxidase substrate (DAB) (Novocastra) x 10 minutes or until the
- 150 coloring appeared;
- 151 • washed with running water;
- 152 • counterstained with hematoxylin for 10 to 20 seconds, then washed with running water; and
- 153 • Dehydrated using 95% ethanol and xylen x 10 minutes each.
- 154 • The mounting media was dripped, and then covered with a glass deck.

155 The results were observed under a light microscope (Olympus, Japan)

156 with 400x magnification. Cells positive for p53 protein showed nuclei or cytoplasm painted

157 brown.

158

159 **Data analysis:** Raji cell cytotoxicity was analyzed using probit analysis to determine 50%

160 Inhibition Concentration (IC<sub>50</sub>). Probit analysis was obtained from the conversion of the

161 percentage of inhibition to the probit value. Percentage of inhibition was calculated

162 as follows:

163 
$$\% \text{ Cell inhibition} = \left[ \frac{(\sum A - \sum B)}{\sum A} \right] \times 100\%$$

164  $\sum A$ : The number of living cells in untreated controls

165  $\sum B$ : The number of living cells due to the treatment of compounds at various

166 concentrations

167 The difference in percentages of cell inhibition between each treatment group was

168 tested statistically using a one-way ANOVA test with 95% Confidence Interval. Analysis of

169 doubling times was calculated by comparing the slope of the log graphs of the number of cells

170 at different observation times. To find differences between groups, the average number of

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171 cells living at the various times was analyzed statistically using the one-  
172 way ANOVA test with a 95% confidence level. Expression of p53 protein was analyzed by  
173 observing its percentages as expressed in Raji cells after immuno-histochemical  
174 treatment. Cells that were positively stained with p53 protein showed nuclei or cytoplasm  
175 painted brown. The proportion of cells that were positively p53 protein was determined by  
176 calculating the presence of stained nuclei or cytoplasm per 100 cells.

177

### 178 **RESULTS and DISCUSSION**

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

185 The parameters used to express the potency of linamarin toxicity from cassava leaves  
186 are  $IC_{50}$  values. The results of calculating cell inhibition percentage of Raji cells after  
187 linamarin administration from cassava leaves are presented in **Table 1**. The **Table 1** shows  
188 that at the highest linamarin concentration (1000  $\mu\text{g/ml}$ ), the percentage of Raji cell inhibition  
189 was 97.550%, while at the lowest concentration (31.25 $\mu\text{g/ml}$ ), the percentage was 27.194 %.

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

196

197 **Antiproliferation activity of linamarin in Raji cells:** The concentration of the test  
198 compound used in the doubling time test was three concentrations below the  
199  $IC_{50}$  value (15.63; 31.25; 62.50  $\mu\text{g/ml}$ ). Cell counts are carried out at 0, 24, 48, and  
200 72 hours. Raji cells had been previously fasted (starved) for 24 hours using RPMI 1640 media  
201 containing FBS 0.5 %. Data of doubling time analysis of Raji cells after linamarin treatment  
202 and control (without treatment) can be seen in Table 2.

203 Data from Table 2 shows how the multiplication times of Raji cells after linamarin  
204 treatment, at concentrations below the  $IC_{50}$  value, run greater than the control  
205 times. Linamarin concentration of 62.50 $\mu\text{g/ml}$  can delay the doubling times of Raji  
206 cells by  $\pm 2$  x those of the Raji control cells.

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

212

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

216 According to those results, there is a tendency for greater p53 protein expression in the  
217 treatment group compared to the control group. Linamarin concentration of 62.5  $\mu\text{g/ml}$  shows  
218 increased positive p53 protein expression in Raji cells by 77.5%,  $\pm 3.07\%$ , while linamarin  
219 concentration was 31.25  $\mu\text{g/ml}$  at  $40 \pm 1.87\%$ . The one-way ANOVA test results showed a  
220 significant difference in the number of p53 protein expression in Raji cells at various

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221 linamarin concentrations ( $p = 0.000$ ). Details pertaining to the expression of p53 protein in the  
222 nuclei and cytoplasm of Raji cells are presented in Table 4 and Fig. 1.

223 From Fig.1 it can be seen that in the Raji control cell there was a tendency to decrease  
224 the positive p53 protein expression, whereas in the Raji cells with linamarin, 32.5 and 62.5  
225  $\mu\text{g/ml}$  concentrations appeared to increase positive p53 protein expression, with most located  
226 in the nuclei (Table 4).

227

228 **Linamarin cytotoxic activity in Raji cells:** The cytotoxicity test determined the value of  
229  $\text{IC}_{50}$ , which is a concentration capable of inhibiting cell growth, such as Raji cells, by up to 50  
230 percent. The smaller the  $\text{IC}_{50}$  value, the more toxic the compound is. The potential for  
231 linamarin toxicity from cassava leaves (*Manihot esculenta* Cranz) to Raji cells is indicated by  
232  $\text{IC}_{50}$  values of  $71.865 \pm 0.229 \mu\text{g/ml}$ . At its highest concentration (1000  $\mu\text{g/ml}$ ), the  
233 percentage of Raji cell growth inhibition was  $97.550\% \pm 0.005\%$ , while the lowest  
234 concentration of linamarine (31.25  $\mu\text{g/ml}$ ) was  $27.194 \pm 0.096\%$  (Table 1). From Table 1,  
235 shows that at the higher the concentration of linamarin, the greater the percentage of Raji cell  
236 growth inhibition, with a significant statistical difference ( $p < 0.05$ ). This proves that linamarin  
237 obtained from cassava leaves (*M. esculenta* Cranz) can suppress the growth of Raji cancer  
238 cells. Linamarin is found in all parts of cassava plants (*M. esculenta* Cranz), but most  
239 abundantly at the roots, leaves, and root tuber skin<sup>5</sup>.

240 Yusuf *et al.*<sup>7</sup> found that linamarin from cassava leaves can inhibit the growth of Caov-  
241 3 cancer cells and Hela cells with  $\text{IC}_{50}$  values of 38  $\mu\text{g/ml}$  and 57  $\mu\text{g/ml}$ , respectively. Idibie *et*  
242 *al.*<sup>6</sup> in his research, stated that  $\text{IC}_{50}$  values decreased when pure linamarin compounds and  
243 crude extracts of cassava tubers were given along with linamarase enzymes on MCF-7 cancer  
244 cells (adenocarcinoma breast cancer), HT-29 (adenocarcinoma colon), and HL-60 (cell line  
245 leukemia). Meanwhile, the  $\text{IC}_{50}$  values of crude extracts are higher than linamarin if not given

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246 along with the linamarase enzyme. Likewise, the results of Alfourjani's<sup>5</sup> study showed that the  
247 IC<sub>50</sub> values of MCF cells after treatment with raw cassava leaf extract and boiled cassava  
248 leaves were 63.1 and 79.4 µg/ml, respectively.

249 Crude extracts are said to have strong potential as anticancer agents if the IC<sub>50</sub> value  
250 is less than 30 µg/ml<sup>19</sup>. The results of this study showed IC<sub>50</sub> value of Raji cells after  
251 linamarin administration to be greater than 30 µg/ml. In fact, they registered as high as 71.865  
252 ± 0.229 µg/ml, meaning that the potency of linamarin toxicity in active Raji cells was weaker,  
253 or only moderately active ( $30 \leq IC_{50} < 100$  µg/ml). This was presumably due to differences in  
254 the characteristics of cancer cells used in the study.

255 Raji cells are found in the Burkitt's lymphoma cell line in humans. Burkitt's  
256 lymphoma at the molecular level is characterized by synergistic Bcl-2 and c-  
257 myc expressions. C-myc is upregulation Bcl-2, so the increase in c-myc expression can also  
258 increase the expression of Bcl-2. As a result of this increase in expression, cells do not  
259 experience apoptosis<sup>20,21</sup>. Burkitt's lymphoma has chromosome translocation that activates c-  
260 myc. In some patients it also shows the occurrence of mutations in p53 which result in the  
261 inhibition of the apoptotic process in these cancer cells. Activating p16INK4a resulted in loss  
262 of CDK inhibitory function, diminishing loss of cell control of its growth. Changes  
263 (mutations) also occur in the expression of pRb and p53, which are gene suppressor  
264 tumors, and in other genes, such as Bax, p73, and Bcl-6, which provide sufficient growth  
265 signals and inhibit apoptosis in cancer cells<sup>22-24</sup>. Mutations also occur in downstream  
266 Caspase-3 which causes Raji cells to be resistant to apoptosis<sup>25,26</sup>.

267 The protein expression of the Epstein-Barr Nuclear Antigen 1 (EBNA1) in Burkitt's  
268 lymphoma, infected by Epstein-Barr Virus (EBV), can also inhibit the occurrence of  
269 apoptosis in cancer cells<sup>27</sup>. Through this mechanism, it is suspected that Raji cells can avoid  
270 the apoptotic mechanism triggered by linamarin compounds from cassava leaves. This is why

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271 the suspected cause of cassava leaf extract cytotoxicity against Raji cells is considered  
272 moderate.

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

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

287 Inhibition of Raji cell growth by linamarin can also be influenced by vitamin C.  
288 Cassava leaves contain vitamin C of 103 mg, higher than other green vegetables<sup>16</sup>. Vitamin C is  
289 known to act as an antioxidant in preventing infection, helps the absorption of iron and  
290 calcium, and is associated with the synthesis of collagen, carnitine, noradrenaline, and  
291 serotonin in the body<sup>29-32</sup>. Besides its function, vitamin C also plays an important role in  
292 activating genes involved in DNA repair, as well as modulating DNA damage in ROS-  
293 affected cells. The results of the Kontek *et al.*<sup>17</sup> study prove that vitamin C has  
294 a positive effect on the level of oxidative DNA damage. Vitamin C provides a protective effect

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295 for normal tissue to counteract the activity of toxic substances and their  
296 metabolites, thus affecting the extent of colon cancer cell inhibition<sup>33,34</sup>.

297

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

310 From Table 2 it can be seen that the doubling time value of Raji cells with linamarin  
311 treatment concentrations of 62.5 µg/ml is greater than the doubling time value of Raji cells  
312 with linamarin treatments of 32.5 µg/ml and 15.63 µg/ml. This is supported by the linamarin  
313 curve slope value of 62.5 µg/ml, which is smaller than the linamarin slope curve of the  
314 treatment with 32.5 µg/ml and 15.63 µg/ml. This means that linamarin 62.5 µg/ml has a better  
315 chance of postponing cell doubling time of Raji cells than linamarin 32.5 µg/ml and 15.63  
316 µg/ml. It is suspected that the linamarin in cassava leaf extract can inhibit genes or proteins  
317 that regulate cell division. It may inhibit signal transduction through inhibition of growth  
318 signals or through inhibition of cell cycle progression by inhibiting proto-oncogenes such as



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319 CycD, cdk 4/6 and c-myc. Similarly, it may activate suppressor tumors such as caspase 3/8/9,  
320 p53, pRb, and Bcl2 inactivation<sup>5,6</sup>.

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

333

334 **Expression of p53 protein in Raji cells with linamarine treatment:** Immunocytochemical  
335 analysis is intended to determine the expression of p53 protein in Raji cells. In this study  
336 antibodies can be used to detect both wild and mutant type p53 proteins in cancer  
337 cells. Positive expression of p53 protein is indicated by brown color in the cell nucleus or  
338 cytoplasm; wild or mutant types cannot be distinguished. The results showed that linamarin  
339 could increase the expression of p53 protein in Raji cell. Linamarin concentrations of 62.5  
340 µg/ml can increase positive p53 protein expression ( $77.5 \pm 3.07\%$ ) greater than linamarin  
341 31.25 µg/ml ( $60\% \pm 1.87\%$ ) (Table 1). In Raji control cells or with linamarin treatment from  
342 cassava leaf extract, most p53 protein expressions are located in the cell nucleus, although  
343 some are located in the cytoplasmic part (Table 3). The control cells also shown have positive

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344 p53 protein expression but the amount was less than the treatment with linamarin  
345 concentrations of 31.25 µg/ml and 62.5 µg/ml (Fig.1). This shows that Raji cell  
346 death occurred through the mechanism of inhibition of Raji cell proliferation, by  
347 activating suppressor gene tumors such as p53. The presence of stress or DNA damage  
348 can spur the expression of p53 protein in Raji cells<sup>39</sup>.

349 The increase in p53 protein expression in Raji cells after the linamarin treatment  
350 proved several possibilities: first, the increase was an increase in wild type p53  
351 expression. P53 protein is encoded by p53 tumor suppressor genes and has an important role  
352 in cell regulation and proliferation<sup>22</sup>. The wild type of p53 protein is expressed very little in  
353 normal conditions, but there will be an increase in response to normal cells if there is DNA  
354 damage<sup>40</sup>. Increased expression of wild-type p53 will be activated through the p21 protein to  
355 stop DNA replication and cell division when DNA damage occurs. This happens because an  
356 increase in p53 protein will stimulate p21 gene transcription. The p21 protein is an inhibitor  
357 of CDK and has the ability to inhibit phosphorylation of pRB, thus blocking the release of  
358 E2F transcription factors and DNA replication. However, if DNA damage is too severe and  
359 cannot be repaired, p53 will induce apoptosis by stimulating Bax transcription, which will  
360 then inhibit the activity of the Bcl2 gene<sup>41</sup>. The Bcl2 gene functions to inhibit the response of  
361 apoptosis to various cell types caused by various stimulations related to apoptosis. Thus, p53  
362 plays an important role in preventing the accumulation of cells with DNA abnormalities that  
363 can mutate into cancer cells<sup>42</sup>.

364 If the p53 expression is the wild type, then DNA damage will cause a rapid rise in p53  
365 protein expression, thus inducing a resting phase of the cell cycle during the G1 phase. Wild-  
366 type p53 will cause a cessation of growth in the G1 phase,<sup>43</sup> thus providing sufficient time for  
367 the DNA repair genes such as MLH, MSH<sub>2</sub>, PMS<sub>1</sub>, PMS<sub>2</sub>, Mdm2, BRCA<sub>1</sub>, and BRCA<sub>2</sub><sup>44</sup>. If

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368 the DNA damage can be repaired, the cell will continue to divide into the S phase; if this  
369 improvement is not possible, then p53 will induce apoptosis<sup>45</sup>.

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

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

380 The presence of positive p53 protein expression in the cytoplasm shows that inhibition  
381 of Raji cell growth occurs in the G1 phase of the cell cycle. Linamarin from cassava leaves  
382 can increase the expression of p53 protein in the cytoplasm compared to the control cells.  
383 Linamarin is thought to inhibit cell division in the G1 phase of the cell cycle by increasing the  
384 expression of p53 protein in the cytoplasm. According to Groeger,<sup>48</sup> most of the p53 genes act  
385 as 'the guardian of the genome': (1) p53 levels increase rapidly in response to DNA damage,  
386 (2) cause cell cycle inhibition during the G1 phase, (3) give cells time to repair DNA damage,  
387 (4) if damage cannot be repaired, p53 will induce programmed cell  
388 death (apoptosis). Both wild type and mutant proteins migrate in the cell nucleus known  
389 as Nuclear Localization Signals (NLS) that are attached to their primary  
390 sequences<sup>49</sup>. According to Burck *et al.*<sup>50</sup> and McManus *et al.*,<sup>51</sup> p53 wild-type causes growth  
391 inhibition in the G1 phase, so that it can be interpreted that in order to enter S phase of the  
392 cell, p53 must be inactive.

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

403

#### 404 **CONCLUSION**

405 Linamarin isolated from cassava leaves (*M. esculenta* Cranz) has the potential to be  
406 developed as an anticancer agent. Linamarin from cassava leaves (*M. esculenta* Cranz) has  
407 cytotoxic activity on Raji cells with IC<sub>50</sub> values of  $71.865 \pm 0.229$  µg/ml, antiproliferation  
408 activity on Raji cells with a doubling time value of 40.723 hours on linamarin concentration  
409 of 62.5 µg/ml and can increase the expression of p53 protein in the nuclei and cytoplasm of  
410 Raji cells.

411

#### 412 **SIGNIFICANCE STATEMENT**

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

418

419 **CONFLICT OF INTEREST STATEMENT**

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

422

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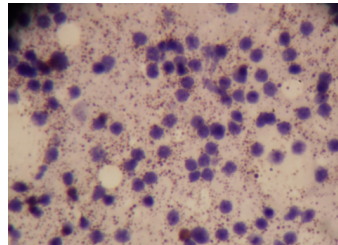
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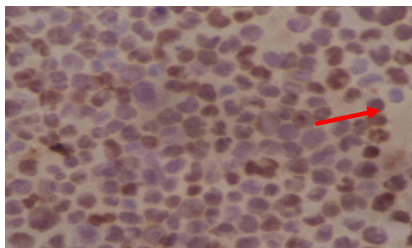


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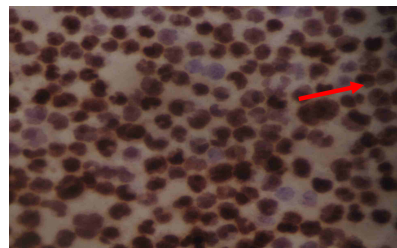
Anticancer Activity of Linamarin on Raji Cells



A. Raji control cell (without treatment)



B. Raji cells with linamarin 32.5µg/ml



C. Raji cells with linamarin 62.5µg/ml

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656 **Figure1.** Microscopic photo of Raji cells with treatment of linamarin 32.5 and  
657 62.5 µg/ml and control (without treatment) with immunocytochemical staining  
658 (magnification 400x). Information: (i) positive cells with expression of p53 protein have  
659 brown nuclei or cytoplasm; (ii) cells that are negative for p53 protein expression have purple  
660 nuclei or cytoplasm.

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Anticancer Activity of Linamarin on Raji Cells

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

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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

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

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674 **Table: 2. Doubling time of Raji cells after treatment with various concentrations of linamarin**  
 675 **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

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Anticancer Activity of Linamarin on Raji Cells

681 **Table: 3.** Percentage of p53 protein expression on Raji cell control and linamarin  
 682 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

683 SEM: Standard error of the mean

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

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689

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

690 SEM: Standard error of the mean

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# 3. Evaluation Report\_Reviewer 2

Anticancer Activity of Linamarin on Raji Cells

## 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	• Reread the article to avoid the Grammatical, typographical & spelling mistakes and personal pronouns (I, we, our etc), use past tense and third person throughout the manuscript.	Ok
3	Title	• Overall Ok	
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	• Author has just mentioned the manufacturer name with all materials used. Please indicate both the manufacturer's name and location (including city, state, and country) for all specialized equipments, kits,	RPMI solution (Sigma-Aldrich, Sain Louis, Missou USA), Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Sain Louis, Missouri, USA), HEPES, FBS, Bovine Serum (FBS)(Gibco, Grand Island, N.Y, US

Anticancer Activity of Linamarin on Raji Cells

		<p>software, incubators, instruments, pH meter, and reagents used in the experiment. The following example will serve to illustrate the style to indicate both the manufacturer's name and location. To detect AFB1, 20 g of the diet was mixed with 100 ml of methanol (Fisher, Pittsburgh, PA, USA):water (30/70 v/v) and shaken for 3 minutes. Then, the supernatant of the mixture was filtered through a Whatman filter (Whatman Clifton, NJ, USA). The filter was collected, and the AFB1 concentration was measured using an ELISA kit (Agra Quantum Aflatoxin B1 Assay, Romer, Singapore).</p> <ul style="list-style-type: none"> <li>• What is the source of the equation? Quote its reference</li> </ul>	<p>penicillin-streptomycin)(Gibco, Grand Island, N.Y, USA), DMSO (Sigma-Aldrich, Sain Louis, Missouri, USA), tripan blue (E-Merck, Darmstadt, Germany), and 3-(4-, 5 dimethylthiazol-2-yl) -2.5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, Sain Louis, Missouri, USA), Falcon tube (Thermo Fisher Scientific, Waltham, MA USA), CO<sub>2</sub> incubator (Nuaire, Plymouth, MN 5547, USA), Neubauer haemocytometry (EMS, 1560 Industry Road, Hatfield, PA), p53 Assay kit (Colorimetric) (NCL-L-p53-DO7, Novocastra, Benton Lane, Newcastle, United Kingdom)), PBS (E.Merck, Darmstadt, Germany)</p> <ul style="list-style-type: none"> <li>• Quote its reference no. 19</li> </ul>
11	<b>Results</b>	• Overall Ok	
12	<b>Figures</b>	• Overall Ok	
13	<b>Tables</b>	• Overall Ok	
14	<b>Discussion</b>	• Overall Ok	
15	<b>Conclusion</b>	• Overall Ok	
16	<b>Acknowledgement</b>	• Overall Ok	
17	<b>Significance Statement</b>	• Overall Ok	
18	<b>References</b>	• Overall 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.

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Anticancer Activity of Linamarin on Raji Cells

19 **Anticancer Activity of Linamarin from Cassava Leaves**

20 **(*Manihot esculenta* Cranz) on Raji Cells**

21 **Running Title: Anticancer Activity of Linamarin on Raji Cells**

22  
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31 **Author contributions**

32 **Dwi Sutningsih:** Performed literature review, developed research proposal, conducted

33 experiments and data analysis, and wrote manuscript. **Mohamad Arie Wuryanto:**

34 Participated in research design and manuscript writing. **Henry Setyawan Susanto:** Reviewed

35 research proposal and contributed to cytotoxic examination and data analysis.

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

37 **Mustofa:** Conducted the experiments and wrote the manuscript.

38

39 **ABSTRACT**

40 **Background and Objective:** Linamarin is an active compound isolated from the leaves of

41 cassava (*Manihot esculenta* Cranz) that has a cytotoxic effects on HT-29, MCF-7, and HL-60

42 cells. This study was aimed to determine the cytotoxic and antiproliferation activity and

43 induction of p53 protein in Raji cells after administration of various concentrations of

## Anticancer Activity of Linamarin on Raji Cells

47 while antiproliferation activity was tested using a *doubling time* test. P53 protein expression  
48 was observed by immunocytochemical tests. The cytotoxic activity of Raji cells was  
49 expressed by the value of Inhibitory Concentration 50 ( $\mu\text{g/ml}$ ). The *doubling time*  
50 was calculated by comparing the *slope* values of the log graphs of the number of cells at  
51 various times. Raji cells that were positive for p53 protein showed brown painted nuclei or  
52 cytoplasm. **Results:** Linamarin from cassava leaves can inhibit cytotoxic activity and  
53 proliferation on Raji cells. The higher the linamarin concentration, the longer the doubling  
54 time of Raji cells. The expression of p53 protein on Raji cells after linamarin administration  
55 was higher than the control. P53 protein expression was found in the nuclei (91.05%) and  
56 cytoplasm (8.95%). **Conclusions:** Based on those findings, linamarin from cassava leaves has  
57 the potential to be developed as an anticancer agent.

58

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

61

## 62 INTRODUCTION

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

68 In Indonesia, cassava has considerable economic value compared to other tubers. Not  
69 only is cassava (*Manihot esculenta* Cranz) one of the world's principal food staples after  
70 grains and corn<sup>1</sup>, their leaves, widely consumed in Indonesia and  
71 elsewhere, are rich in vitamins A, C, K, among others, and minerals, including iron, calcium,



## Anticancer Activity of Linamarin on Raji Cells

72 and phosphorus. The energy content of cassava leaves is greater than most other green  
73 vegetables<sup>2</sup>. Cassava also contains cyanogenic glucoside compounds, which consist of  
74 linamarin and lotaustrain at a ratio of 10:1<sup>3, 4</sup>. Linamarin has potential use as an anti-  
75 neoplastic compound<sup>5,6</sup>. The mechanism of linamarin in the treatment of cancer using  
76 linamarase gene therapy has been investigated.

77 Meanwhile the Idibie et al<sup>6</sup> study states that linamarin in root tubers has been  
78 proven in vitro to have cytotoxic effects on HT-29, MCF-7, and HL-60 cells. From the results  
79 of this study, Inhibitor Concentration 50 (IC<sub>50</sub>) was obtained in the amounts of > 300 µg/ml,  
80 235.96 ± 9.87 µg/ml, and 246.51 ± 10.12 µg/ml after incubation for 48 hours. In this study,  
81 linamarin was obtained from cassava leaf extracted with methanol. The study of Yusuf *et al.*<sup>7</sup>  
82 using linamarin isolated from cassava leaves also showed cytotoxic effects on Caov-3 cells  
83 and Hela cells. The IC<sub>50</sub> value of the two cell lines is 38 µg/ml and 57 µg/ml  
84 respectively. Cancer cell death has been caused by the linamarin content found in cassava  
85 plants<sup>8-11</sup>. Carotene and vitamin C compounds found in cassava leaves are thought to have  
86 anticancer properties<sup>12-15</sup>. Research by Enger *et al.*<sup>16</sup>, stated that carotene is protective toward  
87 colon adenoma rather than other carotenoids in the early stages of tumor formation. Kontek  
88 *et al.*<sup>17</sup> stated that vitamin C had a positive effect on the damage level of oxidative DNA in  
89 colon cancer cells.

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

94

## 95 MATERIALS AND METHODS

## Anticancer Activity of Linamarin on Raji Cells

96 Cassava leaves (*Manihot esculenta* Cranz) were obtained from the local market in  
97 Yogyakarta, Indonesia, then identified at the Laboratory of Pharmaceutical Biology, Faculty  
98 of Pharmacy, Gadjah Mada University. This research project was conducted from June 4,  
99 2018 to December 4, 2018. Raji cells were obtained from the collection of the Laboratory of  
100 Parasitology, Faculty of Medicine, Gadjah Mada University. This cell is a continuous cell line  
101 that grows floating, similar to lymphoblast cells (B lymphocytes) from Burkitt's  
102 lymphoma infected by Epstein-Barr Virus (EBV). Materials for growing Raji cells are RPMI  
103 solution (Sigma-Aldrich, Sain Louis, Missouri, USA), Dulbecco's Modified Eagle's Medium  
104 (DMEM) (Sigma-Aldrich, Sain Louis, Missouri, USA), HEPES, Fetal Bovine Serum  
105 (FBS)(Gibco, Grand Island, N.Y, USA), penicillin-streptomycin)(Gibco, Grand Island, N.Y,  
106 USA), DMSO (Sigma-Aldrich, Sain Louis, Missouri, USA), tripan blue (E-Merck,  
107 Darmstadt, Germany), and 3-(4-, 5 dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide  
108 (MTT) (Sigma-Aldrich, Sain Louis, Missouri, USA).

109 **Linamarin isolation from cassava leaves:** A 5 g batch of cassava leaves was cut into small  
110 pieces, then pounded in a mortar. The result was blended thoroughly with a total of 10 ml of  
111 0.1M HCl solution. The mixture solution was centrifuged at 3500 rpm to obtain the  
112 supernatant. The supernatant liquid obtained was transferred to the Falcon tube (Thermo  
113 Fisher Scientific, Waltham, MA USA). The supernatant liquid mixture with 0, 1 M HCl was  
114 linamarin extract of cassava leaf, which was then isolated. Finally, the linamarin extract was  
115 frozen at -20°C<sup>18</sup>.

116 **Cytotoxic test on Raji cells:** The cytotoxicity test was done colorimetrically using MTT  
117 reagents (Sigma-Aldrich, Darmstadt, Germany). Linamarin of 10 µL at various concentrations  
118 was added to Raji cell culture the day after transplantation. The concentration of linamarin  
119 used for treatment of Raji cells was 31.25, 62.5, 125, 250, 500, and 1000 µg/ml. Cells that

**Commented [11]:** 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. The following example will serve to illustrate the style to indicate both the manufacturer's name and location. To detect AFB1, 20 g of the diet was mixed with 100 ml of methanol (Fisher, Pittsburgh, PA, USA);water (30/70 v/v) and shaken for 3 minutes. Then, the supernatant of the mixture was filtered through a Whatman filter (Whatman Clifton, NJ, USA). The filter was collected, and the AFB1 concentration was measured using an ELISA kit (Agra Quantum Aflatoxin B1 Assay, Romer, Singapore).

#### Anticancer Activity of Linamarin on Raji Cells

120 were not treated were used as controls. On the third day, 20  $\mu$ L of MTT reagent was added to  
121 approximately 5 mg/ml per well. After four hours of incubation, 100  $\mu$ L of 0.1 N HCl-  
122 isopropanol was added to each well to dissolve the formazan crystals that had  
123 formed. Absorbance (A) was measured using a microplate reader at a wavelength of 595  
124 nm. All steps were carried out three times.

125

126 **Antiproliferation test (doubling time) in Raji cells:** Cells were fasted for 24 hours in culture  
127 media containing 0.5% of FBS. Afterwards, they were grown in a plate with a medium added,  
128 with linamarin at a non-lethal concentration of three series below the IC<sub>50</sub> value. Then it was  
129 incubated in a 5% CO<sub>2</sub> incubator (Nuairé, Plymouth, MN 5547, USA) at 37 °C for 24, 48, and  
130 72 hours. Each well was calculated by the number of cells living using hemocytometrics  
131 (Neubauer haemocytometry, EMS, 1560 Industry Road, Hatfield, PA).

132

133 **Immuno-cytochemical test in Raji cells:** Immuno-cytochemical staining was performed  
134 using the avidin-biotin-peroxidase complex with monoclonal antibodies against p53  
135 according protocols of p53 Assay kit (Colorimetric) (NCL-L-p53-DO7, Novocastra, Benton  
136 Lane, Newcastle, United Kingdom). In a microculture, 96 wells containing 100  $\mu$ l of test  
137 cells, with a density of  $2 \times 10^4$  cells/well, 100  $\mu$ l of the test compound were added at  
138 concentrations of 10  $\mu$ g/ ml. They were then incubated with 5% CO<sub>2</sub> flow at 37 °C for 24  
139 hours. After being incubated overnight, 200  $\mu$ l of cells from each well were taken and inserted  
140 in eppendorf tubes, then centrifuged to 1200rpm x 5 minutes. The supernatant liquid was  
141 removed, leaving the pellet, and then re-suspended. The cell suspension was extracted and  
142 placed on a glass object that had been coated with poly-lysine. The cells were fixated with  
143 acetone for 10 minutes. Later, they were washed with PBS (Phosphate Buffered  
144 Saline)(E.Merck, Darmstadt, Germany) x 5 minutes and etched with hydrogen

#### Anticancer Activity of Linamarin on Raji Cells

145 peroxidase 0, 1% for 10 minutes. After washing them with running water, they were rinsed  
146 with PBS for five minutes, dripped with 100 µl normal horse serum for 10 minutes, and  
147 cleaned without water. Finally, they were dripped with anti-p53 protein primary antibodies  
148 (Novocastra, Benton Lane, Newcastle, United Kingdom) and left for 24 hours.

149 The next day the suspension was:

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

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

164

165 **Data analysis:** Raji cell cytotoxicity was analyzed using probit analysis to determine 50%  
166 Inhibition Concentration (IC<sub>50</sub>). Probit analysis was obtained from the conversion of the  
167 percentage of inhibition to the probit value. Percentage of inhibition was calculated  
168 as follows<sup>19</sup>:

169 % Cell inhibition =  $[(\sum A - \sum B) : \sum A] \times 100\%$

**Commented [12]:** What is the source of this equation?  
Quote its reference

## Anticancer Activity of Linamarin on Raji Cells

170  $\sum$  A: The number of living cells in untreated controls

171  $\sum$  B: The number of living cells due to the treatment of compounds at various  
172 concentrations

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

183

## 184 **RESULTS and DISCUSSION**

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

191 The parameters used to express the potency of linamarin toxicity from cassava leaves  
192 were IC<sub>50</sub> values. The results of calculating cell inhibition percentage of Raji cells after  
193 linamarin administration from cassava leaves are presented in **Table 1**. The **Table 1** shows

#### Anticancer Activity of Linamarin on Raji Cells

194 that at the highest linamarin concentration (1000 µg/ml), the percentage of Raji cell inhibition  
195 was 97.550%, while at the lowest concentration (31.25µg/ml), the percentage was 27.194 %.

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

202

203 **Antiproliferation activity of linamarin in Raji cells:** The concentration of the test  
204 compound used in the doubling time test was three concentrations below the  
205  $IC_{50}$  value (15.63; 31.25; 62.50 µg/ml). Cell counts are carried out at 0, 24, 48, and  
206 72 hours. Raji cells had been previously fasted (starved) for 24 hours using RPMI 1640 media  
207 containing FBS 0.5 %. Data of doubling time analysis of Raji cells after linamarin treatment  
208 and control (without treatment) can be seen in Table 2.

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

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

218

#### Anticancer Activity of Linamarin on Raji Cells

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

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

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

233

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

#### Anticancer Activity of Linamarin on Raji Cells

244 cells. Linamarin is found in all parts of cassava plants (*M. esculenta* Cranz), but most  
245 abundantly at the roots, leaves, and root tuber skin<sup>5</sup>.

246 Yusuf *et al.*<sup>7</sup> found that linamarin from cassava leaves can inhibit the growth of Caov-  
247 3 cancer cells and Hela cells with IC<sub>50</sub> values of 38 µg/ml and 57 µg/ml, respectively. Idibie et  
248 al<sup>6</sup> in his research, stated that IC<sub>50</sub> values decreased when pure linamarin compounds and  
249 crude extracts of cassava tubers were given along with linamarase enzymes on MCF-7 cancer  
250 cells (adenocarcinoma breast cancer), HT-29 (adenocarcinoma colon), and HL-60 (cell line  
251 leukemia). Meanwhile, the IC<sub>50</sub> values of crude extracts are higher than linamarin if not given  
252 along with the linamarase enzyme. Likewise, the results of Alfourjani's<sup>5</sup> study showed that the  
253 IC<sub>50</sub> values of MCF cells after treatment with raw cassava leaf extract and boiled cassava  
254 leaves were 63.1 and 79.4 µg/ml, respectively.

255 Crude extracts are said to have strong potential as anticancer agents if the IC<sub>50</sub> value  
256 is less than 30 µg/ml<sup>20</sup>. The results of this study showed IC<sub>50</sub> value of Raji cells after  
257 linamarin administration to be greater than 30 µg/ml. In fact, they registered as high as 71.865  
258 ± 0.229 µg/ml, meaning that the potency of linamarin toxicity in active Raji cells was weaker,  
259 or only moderately active (30 ≤ IC<sub>50</sub> < 100 µg/ml). This was presumably due to differences in  
260 the characteristics of cancer cells used in the study.

261 Raji cells are found in the Burkitt's lymphoma cell line in humans. Burkitt's  
262 lymphoma at the molecular level is characterized by synergistic Bcl-2 and c-  
263 myc expressions. C-myc is upregulation Bcl-2, so the increase in c-myc expression can also  
264 increase the expression of Bcl-2. As a result of this increase in expression, cells do not  
265 experience apoptosis<sup>21,22</sup>. Burkitt's lymphoma has chromosome translocation that activates c-  
266 myc. In some patients it also shows the occurrence of mutations in p53 which result in the  
267 inhibition of the apoptotic process in these cancer cells. Activating p16INK4a resulted in loss  
268 of CDK inhibitory function, diminishing loss of cell control of its growth. Changes



#### Anticancer Activity of Linamarin on Raji Cells

269 (mutations) also occur in the expression of pRb and p53, which are gene suppressor  
270 tumors, and in other genes, such as Bax, p73, and Bcl-6, which provide sufficient growth  
271 signals and inhibit apoptosis in cancer cells<sup>23-25</sup>. Mutations also occur in downstream  
272 Caspase-3 which causes Raji cells to be resistant to apoptosis<sup>26,27</sup>.

273 The protein expression of the Epstein-Barr Nuclear Antigen 1 (EBNA1) in Burkitt's  
274 lymphoma, infected by Epstein-Barr Virus (EBV), can also inhibit the occurrence of  
275 apoptosis in cancer cells<sup>28</sup>. Through this mechanism, it is suspected that Raji cells can avoid  
276 the apoptotic mechanism triggered by linamarin compounds from cassava leaves. This is why  
277 the suspected cause of cassava leaf extract cytotoxicity against Raji cells is considered  
278 moderate.

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

284 Inhibition of Raji cell growth is also due to  $\beta$ -carotene content in cassava leaves.  $\beta$ -  
285 carotene has an anticancer mechanism by its carcinogen-modulating metabolism and  
286 antioxidant activity, thus modulating the immune system, increasing cell differentiation,  
287 stimulating communication gap cell junctions to cells and affecting retinoid-dependent  
288 signals<sup>29</sup>.  $\beta$ -Carotene is also directly related to inhibition of cell proliferation, increased  
289 apoptosis, induces cell cycle arrest<sup>14</sup>. In his research, Enger *et al.*<sup>16</sup> stated that  $\beta$ -carotene is  
290 protective toward colon adenoma in the early stages of tumor formation. The same thing was  
291 determined by Gloria *et al.*,<sup>14</sup> who proved that carotenoids were able to increase breast cancer  
292 cell apoptosis.

## Anticancer Activity of Linamarin on Raji Cells

293 Inhibition of Raji cell growth by linamarin can also be influenced by vitamin C.  
294 Cassava leaves contain vitamin C of 103 mg, higher than other green vegetables<sup>16</sup>. Vitamin C is  
295 known to act as an antioxidant in preventing infection, helps the absorption of iron and  
296 calcium, and is associated with the synthesis of collagen, carnitine, noradrenaline, and  
297 serotonin in the body<sup>30-33</sup>. Besides its function, vitamin C also plays an important role in  
298 activating genes involved in DNA repair, as well as modulating DNA damage in ROS-  
299 affected cells. The results of the Kontek *et al.*<sup>17</sup> study prove that vitamin C has  
300 a positive effect on the level of oxidative DNA damage. Vitamin C provides a protective effect  
301 for normal tissue to counteract the activity of toxic substances and their  
302 metabolites, thus affecting the extent of colon cancer cell inhibition<sup>34,35</sup>.

303

304 **Antiproliferative activity of Raji cells:** Analysis of cell proliferation inhibition can be done  
305 by the doubling time test. Compounds that delay the multiplication times of cells can inhibit  
306 genes or proteins that regulate the cell cycle. The doubling time test is done by counting the  
307 number of cells treated in a time unit (e.g., 24 hours). Each sample is calculated by a  
308 hemocytometer, and then a curve with cell number versus incubation time is  
309 made. Differences in cells' doubling times can be determined from the slope of the curve or  
310 calculated by extrapolation<sup>36</sup>. Raji cells were previously fasted (starved) for 24 hours using  
311 RPMI 1640 media containing FBS 0.5 percent. Reducing this growth signal is necessary  
312 because it reduces the speed of cell growth, which causes the cell to be in the same initial  
313 start, or G0 phase. Without fasting when treated, the cells remain in different phases  
314 which makes it difficult to observe the inhibition properties of linamarin on cell cycle  
315 progression<sup>37</sup>.

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

#### Anticancer Activity of Linamarin on Raji Cells

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

327 The data in Table 2 shows that the doubling time value of Raji cells with linamarin  
328 treatment concentrations of 62.5 µg/ml is twice the doubling time value of Raji cells without  
329 treatment (control). This means that linamarin concentration of 62.5 µg/ml can cut the  
330 doubling time of Raji cells to half that of Raji cells doubling times without treatment  
331 (control). The price of doubling time for linamarin treatment is greater than that for control.  
332 This indicates that linamarin has the ability to inhibit Raji cell proliferation and possess  
333 cytotoxic activity. The higher the linamarin concentration, the longer the doubling time of  
334 Raji cells. A linamarin construction of 31.25 µg/ml can inhibit cell proliferation better than  
335 linamarin 15.63 µg/ml. This inhibition may occur in signal transduction through inhibition of  
336 growth signals or through inhibition of cell cycle progression by inhibiting proto-oncogenes  
337 such as CycD, cdk 4/6, and c-myc. Or, it may be able to activate suppressor tumors such as  
338 caspase 3/8/9, p53, pRb, and Bcl2 inactivation<sup>38,39</sup>.

339

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

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343 cells. Positive expression of p53 protein is indicated by brown color in the cell nucleus or  
344 cytoplasm; wild or mutant types cannot be distinguished. The results showed that linamarin  
345 could increase the expression of p53 protein in Raji cell. Linamarin concentrations of 62.5  
346  $\mu\text{g/ml}$  can increase positive p53 protein expression ( $77.5 \pm 3.07\%$ ) greater than linamarin  
347  $31.25 \mu\text{g/ml}$  ( $60\% \pm 1.87\%$ ) (Table 1). In Raji control cells or with linamarin treatment from  
348 cassava leaf extract, most p53 protein expressions are located in the cell nucleus, although  
349 some are located in the cytoplasmic part (Table 3). The control cells also shown have positive  
350 p53 protein expression but the amount was less than the treatment with linamarin  
351 concentrations of  $31.25 \mu\text{g/ml}$  and  $62.5 \mu\text{g/ml}$  (Fig.1). This shows that Raji cell  
352 death occurred through the mechanism of inhibition of Raji cell proliferation, by  
353 activating suppressor gene tumors such as p53. The presence of stress or DNA damage  
354 can spur the expression of p53 protein in Raji cells<sup>40</sup>.

355 The increase in p53 protein expression in Raji cells after the linamarin treatment  
356 proved several possibilities: first, the increase was an increase in wild type p53  
357 expression. P53 protein is encoded by p53 tumor suppressor genes and has an important role  
358 in cell regulation and proliferation<sup>23</sup>. The wild type of p53 protein is expressed very little in  
359 normal conditions, but there will be an increase in response to normal cells if there is DNA  
360 damage<sup>41</sup>. Increased expression of wild-type p53 will be activated through the p21 protein to  
361 stop DNA replication and cell division when DNA damage occurs. This happens because an  
362 increase in p53 protein will stimulate p21 gene transcription. The p21 protein is an inhibitor  
363 of CDK and has the ability to inhibit phosphorylation of pRB, thus blocking the release of  
364 E2F transcription factors and DNA replication. However, if DNA damage is too severe and  
365 cannot be repaired, p53 will induce apoptosis by stimulating Bax transcription, which will  
366 then inhibit the activity of the Bcl2 gene<sup>42</sup>. The Bcl2 gene functions to inhibit the response of  
367 apoptosis to various cell types caused by various stimulations related to apoptosis. Thus, p53

#### Anticancer Activity of Linamarin on Raji Cells

368 plays an important role in preventing the accumulation of cells with DNA abnormalities that  
369 can mutate into cancer cells<sup>43</sup>.

370 If the p53 expression is the wild type, then DNA damage will cause a rapid rise in p53  
371 protein expression, thus inducing a resting phase of the cell cycle during the G1 phase. Wild-  
372 type p53 will cause a cessation of growth in the G1 phase,<sup>44</sup> thus providing sufficient time for  
373 the DNA repair genes such as MLH, MSH<sub>2</sub>, PMS<sub>1</sub>, PMS<sub>2</sub>, Mdm2, BRCA<sub>1</sub>, and BRCA<sub>2</sub><sup>44</sup>. If  
374 the DNA damage can be repaired, the cell will continue to divide into the S phase; if this  
375 improvement is not possible, then p53 will induce apoptosis<sup>46</sup>.

376 The second possibility is that the increase in p53 expression is an accumulation of  
377 mutant type p53. P53 mutations will cause the protein to be more stable and have a longer  
378 half-life than the wild type. This causes the mutant type of p53 protein to be more easily  
379 detected immunocytochemically, although positive expression of p53 is not always associated  
380 with its gene mutation<sup>47</sup>.

381 P53 mutation is the most common genetic lesion in neoplasms. P53 mutations are  
382 associated with increased cellular proliferation and transformation toward malignancy<sup>48</sup>. They  
383 will cause changes in the encoded protein products, so they cannot stimulate the transcription  
384 of p21 and Bax,<sup>42</sup> thus causing the accumulation of cells with DNA damage, which can turn  
385 into cancer cells<sup>23</sup>.

386 The presence of positive p53 protein expression in the cytoplasm shows that inhibition  
387 of Raji cell growth occurs in the G1 phase of the cell cycle. Linamarin from cassava leaves  
388 can increase the expression of p53 protein in the cytoplasm compared to the control cells.  
389 Linamarin is thought to inhibit cell division in the G1 phase of the cell cycle by increasing the  
390 expression of p53 protein in the cytoplasm. According to Groeger,<sup>49</sup> most of the p53 genes act  
391 as 'the guardian of the genome': (1) p53 levels increase rapidly in response to DNA damage,  
392 (2) cause cell cycle inhibition during the G1 phase, (3) give cells time to repair DNA damage,

#### Anticancer Activity of Linamarin on Raji Cells

393 (4) if damage cannot be repaired, p53 will induce programmed cell  
394 death (apoptosis). Both wild type and mutant proteins migrate in the cell nucleus known as  
395 Nuclear Localization Signals (NLS) that are attached to their primary  
396 sequences<sup>50</sup>. According to Burck *et al.*<sup>51</sup> and McManus and Alessi<sup>52</sup> p53 wild-type causes  
397 growth inhibition in the G1 phase, so that it can be interpreted that in order to enter S phase of  
398 the cell, p53 must be inactive.

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

409

#### 410 CONCLUSION

411 Linamarin isolated from cassava leaves (*M. esculenta* Cranz) has the potential to be  
412 developed as an anticancer agent. Linamarin from cassava leaves (*M. esculenta* Cranz) has  
413 cytotoxic activity on Raji cells with IC<sub>50</sub> values of  $71.865 \pm 0.229$  µg/ml, antiproliferation  
414 activity on Raji cells with a doubling time value of 40.723 hours on linamarin concentration  
415 of 62.5 µg/ml and can increase the expression of p53 protein in the nuclei and cytoplasm of  
416 Raji cells.

417

418 **SIGNIFICANCE STATEMENT**

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

424

425 **CONFLICT OF INTEREST STATEMENT**

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

428

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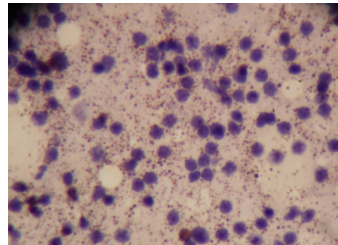
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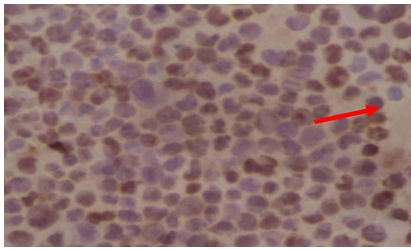
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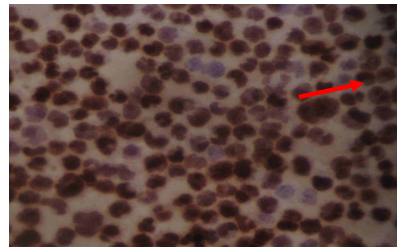
Anticancer Activity of Linamarin on Raji Cells



A. Raji control cell (without treatment)



B. Raji cells with linamarin 32.5µg/ml



C. Raji cells with linamarin 62.5µg/ml

691

692 **Figure1.** Microscopic photo of Raji cells with treatment of linamarin 32.5 and  
693 62.5 µg/ml and control (without treatment) with immunocytochemical staining  
694 (magnification 400x). Information: (i) positive cells with expression of p53 protein have  
695 brown nuclei or cytoplasm; (ii) cells that are negative for p53 protein expression have purple  
696 nuclei or cytoplasm.

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Anticancer Activity of Linamarin on Raji Cells

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

707

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

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

709

710 **Table: 2. Doubling time of Raji cells after treatment with various concentrations of linamarin**  
 711 **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

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Anticancer Activity of Linamarin on Raji Cells

717 **Table: 3.** Percentage of p53 protein expression on Raji cell control and linamarin  
 718 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

719 SEM: Standard error of the mean  
 720

721

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

724  
 725

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

726 SEM: Standard error of the mean  
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# 4. Answer Comment & Revised Article

Cytotoxic, Antiproliferative and Induction of p53 on Raji

## 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.	<a href="#">Activity anticancer of Linamarin from Cassava Leaf (Manihot esculenta) on Raji Cells</a>
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,	<a href="#">MTT reagent (Merck, Darmstadt, Germany), CO2 Incubator (Biospherix, Parish, NY) microplate reader (BioT Tokyo, Japan)</a>



Cytotoxic, Antiproliferative and Induction of p53 on Raji

		and reagents used in the experiment.	
11	<b>Results</b>	• Overall Ok	
12	<b>Figures</b>	• Author is advised to label the figure 1 correctly by which a reader can easily understand the results obtained in figure 1.	
13	<b>Tables</b>	• This value is not present in table 1. Author is advised to provide the correct value in table 1, in its description and discussion.	
14	<b>Discussion</b>	• Overall Ok	
15	<b>Conclusion</b>	• Overall Ok	
16	<b>Acknowledgement</b>	• Overall Ok	
17	<b>Significance Statement</b>	• Overall Ok	
18	<b>References</b>	• Author is advised to Provide DOI or URL of all listed references.	

**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.

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Cytotoxic, Antiproliferative and Induction of p53 on Raji

18 Plagiarism checked  
19 101171-IJCR-AJ

20 Research Article

21 **Cytotoxic and Antiproliferative Activity and Induction of p53 Protein on**  
22 **Raji Cells after Treatment with Linamarin from Cassava Leaves**

23 **(*Manihot esculenta* Cranz)**

24

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41 LiveDNA\*: 62.16151

42 **Author contributions**

43 Dwi Sutiningsih: Performed literature review, developed research proposal, conducted

44 experiments and data analysis, and wrote manuscript.

**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.

Cytotoxic, Antiproliferative and Induction of p53 on Raji

49 Mustofa: Conducted the experiments and wrote the manuscript.

50

51 **ABSTRACT**

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

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

65 **Results:** Linamarin from cassava leaves can inhibit cytotoxic activity and proliferation on  
66 Raji cells. The higher the linamarin concentration, the longer the doubling time of Raji  
67 cells. The expression of p53 protein on Raji cells after linamarin administration was higher  
68 than the control. P53 protein expression was found in the nuclei (91.05%) and cytoplasm  
69 (8.95%).

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

72

Cytotoxic, Antiproliferative and Induction of p53 on Raji

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

## 75 INTRODUCTION

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

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

90 Meanwhile the Idibie <sup>6</sup> study states linamarin in root tubers has been proven in  
91 vitro to have cytotoxic effects on HT-29, MCF-7, and HL-60 cells. From the results of this  
92 study, Inhibitor Concentration 50 (IC<sub>50</sub>) was obtained in the amounts of > 300 µg/ml, 235.96  
93 ± 9.87 µg/ml, and 246.51 ± 10.12 µg/ml after incubation for 48 hours. In this study, linamarin  
94 was obtained from cassava leaf extracted with methanol. The study of Yusuf *et al.* <sup>7</sup> using  
95 linamarin isolated from cassava leaves also showed cytotoxic effects on Caov-3 cells and  
96 Hela cells. The IC<sub>50</sub> value of the two cell lines is 38 µg/ml and 57 µg/ml respectively. Cancer  
97 cell death has been caused by the linamarin content found in cassava plants<sup>8-11</sup>. Carotene and

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Cytotoxic, Antiproliferative and Induction of p53 on Raji

104 vitamin C compounds found in cassava leaves are thought to have anticancer properties<sup>12-15</sup>  
105 Research by Enger *et al.*<sup>16</sup>, stated that carotene is protective toward colon adenoma rather than  
106 other carotenoids in the early stages of tumor formation. Kontek *et al.*<sup>17</sup> stated that vitamin C  
107 had a positive effect on the damage level of oxidative DNA in colon cancer cells.

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

## 112 113 MATERIALS AND METHODS

114 Cassava leaves (*Manihot esculenta* Cranz) were obtained from the local market in  
115 Yogyakarta, Indonesia, then identified at the Laboratory of Pharmaceutical Biology, Faculty  
116 of Pharmacy, Gadjah Mada University. [This research project was conducted from June 4,  
117 2018 to December 4, 2018.](#) Raji cells were obtained from the collection of the Laboratory of  
118 Parasitology, Faculty of Medicine, Gadjah Mada University. This cell is a continuous cell line  
119 that grows floating, similar to lymphoblast cells (B lymphocytes) from Burkitt's  
120 lymphoma infected by Epstein-Barr Virus (EBV). Materials for growing Raji cells are RPMI  
121 solution, Dulbecco's Modified Eagle's Medium (DMEM), HEPES, Fetal Bovine Serum  
122 (FBS), Steptomycin, penicillin, DMSO (Aldrich), tripan blue, and 3-(4-, 5 dimethylthiazol-2-  
123 yl) -2.5-diphenyl tetrazolium bromide (MTT). All media and solvents used in this study were  
124 obtained from commercial providers Sigma-Aldrich.

125 **Linamarin isolation from cassava leaves:** A 5 g batch of cassava leaves was cut into small  
126 pieces, then pounded in a mortar. The result was blended thoroughly with a total of 10 ml of  
127 0.1M HCl solution. The mixture solution was centrifuged at 3500 rpm to obtain the  
128 supernatant. The supernatant liquid obtained was transferred to the Falcon tube. The

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Cytotoxic, Antiproliferative and Induction of p53 on Raji

132 supernatant liquid mixture with 0, 1 M HCl was linamarin extract of cassava leaf, which was  
133 then isolated. Finally, the linamarin extract was frozen at - 20°C<sup>8</sup>.

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

143  
144 **Antiproliferation test (doubling time) in Raji cells:** Cells were fasted for 24 hours in culture  
145 media containing 0.5% of FBS. Afterwards, they were grown in a plate with a medium added,  
146 with linamarin at a non-lethal concentration of three series below the IC<sub>50</sub> value. Then it was  
147 incubated in a 5% CO<sub>2</sub> incubator at 37 °C for 24, 48, and 72 hours. Each well was calculated  
148 by the number of cells living using hemocytometrics.

149  
150 **Immuno-cytochemical test in Raji cells:** In a microculture, 96 wells containing 100 µl of  
151 test cells, with a density of 2 x 10<sup>4</sup> cells/well, 100 µl of the test compound were added at  
152 concentrations of 10 µg/ ml. They were then incubated with 5% CO<sub>2</sub> flow at 37 °C for 24  
153 hours. After being incubated overnight, 200 µl of cells from each well were taken and inserted  
154 in eppendorf tubes, then centrifuged to 1200rpm x 5 minutes. The supernatant liquid was  
155 removed, leaving the pellet, and then re-suspended. The cell suspension was extracted and  
156 placed on a glass object that had been coated with poly-lysine. The cells were fixated with

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Cytotoxic, Antiproliferative and Induction of p53 on Raji

163 acetone for 10 minutes. Later, they were washed with PBS (Phosphate Buffered Saline) x 5  
164 minutes and etched with hydrogen peroxidase 0, 1% for 10 minutes. After washing them with  
165 running water, they were rinsed with PBS for five minutes, dripped with 100 µl normal horse  
166 serum for 10 minutes, and cleaned without water. Finally, they were dripped with anti-p53  
167 protein primary antibodies and left for 24 hours.

168 The next day the suspension was:

- 169 • washed twice with PBS x 5 minutes each;
- 170 • dripped with biotinylated secondary antibodies x 10 minutes;
- 171 • washed x 2 with PBS x 5 minutes each;
- 172 • dripped then incubated with Avidin Biotin reagent enzyme x 10 minutes;
- 173 • washed x 2 with PBS x 5 minutes each;
- 174 • incubated with a peroxidase substrate (DAB) x 10 minutes or until the coloring appeared;
- 175 • washed with running water;
- 176 • counterstained with hematoxylin for 10 to 20 seconds, then washed with running water; and
- 177 • [Dehydrated](#) using 95% ethanol and xylen x 10 minutes each.
- 178 • The mounting media was dripped, [and](#) then covered with a glass deck.

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179 The results were observed under a light microscope with 400x magnification. Cells  
180 positive for p53 protein showed nuclei or cytoplasm painted brown.

181

182 **Data analysis:** Raji cell cytotoxicity was analyzed using probit analysis to determine 50%  
183 Inhibition Concentration (IC<sub>50</sub>). Probit analysis was obtained from the conversion of the  
184 percentage of inhibition to the probit value. Percentage of inhibition was calculated  
185 as follows:

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

187  $\sum A$ : The number of living cells in untreated controls

Cytotoxic, Antiproliferative and Induction of p53 on Raji

190  $\Sigma$  B: The number of living cells due to the treatment of compounds at various  
191 concentrations

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

202

## 203 **RESULTS and DISCUSSION**

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

210 The parameters used to express the potency of linamarin toxicity from cassava leaves  
211 are  $IC_{50}$  values. The results of calculating cell inhibition percentage of Raji cells after  
212 linamarin administration from cassava leaves are presented in Table 1. The table shows that at  
213 the highest linamarin concentration (1000  $\mu\text{g/ml}$ ), the percentage of Raji cell inhibition was

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Cytotoxic, Antiproliferative and Induction of p53 on Raji

215 97.550%, while at the lowest concentration (31.25µg/ml), the percentage was 27.194  
216 percent. IC<sub>50</sub> Raji cell values were 71.865 ± 0.229 µg/ml.

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

223

224 **Antiproliferation activity of linamarin in Raji cells:** The concentration of the test  
225 compound used in the doubling time test was three concentrations below the  
226 IC<sub>50</sub> value (15.63; 31.25; 62.50 µg/ml). Cell counts are carried out at 0, 24, 48, and  
227 72 hours. Raji cells had been previously fasted (starved) for 24 hours using RPMI 1640 media  
228 containing FBS 0.5 %. Data of doubling time analysis of Raji cells after linamarin treatment  
229 and control (without treatment) can be seen in Table 2.

230 Data from Table 2 shows how the multiplication times of Raji cells after linamarin  
231 treatment, at concentrations below the IC<sub>50</sub> value, run greater than the control  
232 times. Linamarin concentration of 62.50 µg/ml can delay the doubling times of Raji  
233 cells by ±2 x those of the Raji control cells.

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

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Cytotoxic, Antiproliferative and Induction of p53 on Raji

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

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

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

255  
256 **Linamarin cytotoxic activity in Raji cells:** The cytotoxicity test determined the value of  
257 IC<sub>50</sub>, which is a concentration capable of inhibiting cell growth, such as Raji cells, by up to 50  
258 percent. The smaller the IC<sub>50</sub> value, the more toxic the compound is. The potential for  
259 linamarin toxicity from cassava leaves (*Manihot esculenta* Cranz) to Raji cells is indicated by  
260 IC<sub>50</sub> values of 71.865 ± 0.229 µg/ml. At its highest concentration (1000 µg/ml), the  
261 percentage of Raji cell growth inhibition was 97.550% ± 0.005%, while the lowest  
262 concentration of linamarine (31.25 µg/ml) was 27.194 ± 0.096% (Table 1). The higher the  
263 concentration of linamarin, the greater the percentage of Raji cell growth inhibition, with a  
264 significant statistical difference (p <0.05). This proves that linamarin obtained from cassava  
265 leaves (*M. esculenta* Cranz) can suppress the growth of Raji cancer cells. Linamarin is found

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### Cytotoxic, Antiproliferative and Induction of p53 on Raji

270 in all parts of cassava plants (*M. esculenta* Cranz), but most abundantly at the roots, leaves,  
271 and root tuber skin.<sup>5</sup>

272 Yusuf *et al.*<sup>7</sup> found that linamarin from cassava leaves can inhibit the growth of Caov-  
273 3 cancer cells and Hela cells with IC<sub>50</sub> values of 38 µg/ml and 57 µg/ml, respectively. Idibie<sup>6</sup>  
274 in his research, stated that IC<sub>50</sub> values decreased when pure linamarin compounds and crude  
275 extracts of cassava tubers were given along with linamarase enzymes on MCF-7 cancer cells  
276 (adenocarcinoma breast cancer), HT-29 (adenocarcinoma colon), and HL-60 (cell line  
277 leukemia). Meanwhile, the IC<sub>50</sub> values of crude extracts are higher than linamarin if not given  
278 along with the linamarase enzyme. Likewise, the results of Alfourjani's study<sup>5</sup> showed that  
279 the IC<sub>50</sub> values of MCF cells after treatment with raw cassava leaf extract and boiled cassava  
280 leaves were 63.1 and 79.4 µg/ml, respectively.

281 Crude extracts are said to have strong potential as anticancer agents if the IC<sub>50</sub> value  
282 is less than 30 µg/ml<sup>19</sup>. The results of this study showed IC<sub>50</sub> value of Raji cells after  
283 linamarin administration to be greater than 30 µg/ml. In fact, they registered as high as 71.865  
284 ± 0.229 µg/ml, meaning that the potency of linamarin toxicity in active Raji cells was weaker,  
285 or only moderately active (30 ≤ IC<sub>50</sub> < 100 µg/ml). This was presumably due to differences in  
286 the characteristics of cancer cells used in the study.

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

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

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

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

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

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327 Inhibition of Raji cell growth by linamarin can also be influenced by vitamin C.

328 Cassava leaves contain vitamin C of 103 mg, higher than other green vegetables<sup>16</sup>. Vitamin C is

329 known to act as an antioxidant in preventing infection, helps the absorption of iron and

330 calcium, and is associated with the synthesis of collagen, carnitine, noradrenaline, and

331 serotonin in the body<sup>29-32</sup>. Besides its function, vitamin C also plays an important role in

332 activating genes involved in DNA repair, as well as modulating DNA damage in ROS-

333 affected cells. The results of the Kontek *et al.*<sup>17</sup> study prove that vitamin C has

334 a positive effect on the level of oxidative DNA damage. Vitamin C provides a protective effect

335 for normal tissue to counteract the activity of toxic substances and their

336 metabolites, thus affecting the extent of colon cancer cell inhibition<sup>33,34</sup>.

337

338 **Antiproliferative activity of Raji cells.** Analysis of cell proliferation inhibition can be done

339 by the doubling time test. Compounds that delay the multiplication times of cells can inhibit

340 genes or proteins that regulate the cell cycle. The doubling time test is done by counting the

341 number of cells treated in a time unit (e.g., 24 hours). Each sample is calculated by a

342 hemocytometer, and then a curve with cell number versus incubation time is

343 made. Differences in cells' doubling times can be determined from the slope of the curve or

344 calculated by extrapolation<sup>35</sup>. Raji cells were previously fasted (starved) for 24 hours using

345 RPMI 1640 media containing FBS 0.5 percent. Reducing this growth signal is necessary

346 because it reduces the speed of cell growth, which causes the cell to be in the same initial

347 start, or G0 phase. Without fasting when treated, the cells remain in different phases

348 which makes it difficult to observe the inhibition properties of linamarin on cell cycle

349 progression<sup>36</sup>.

350 From Table 2 it can be seen that the doubling time value of Raji cells with linamarin

351 treatment concentrations of 62.5 µg/ml is greater than the doubling time value of Raji cells

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### Cytotoxic, Antiproliferative and Induction of p53 on Raji

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

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

381  
382 **Expression of p53 protein in Raji cells with linamarine treatment:** Immunocytochemical  
383 analysis is intended to determine the expression of p53 protein in Raji cells. In this study  
384 antibodies can be used to detect both wild and mutant type p53 proteins in cancer

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388 cells. Positive expression of p53 protein is indicated by brown color in the cell nucleus or  
389 cytoplasm; wild or mutant types cannot be distinguished. The results showed that linamarin  
390 could increase the expression of p53 protein in Raji cell. Linamarin concentrations of 62.5  
391  $\mu\text{g/ml}$  can increase positive p53 protein expression ( $77.5 \pm 3.07\%$ ) greater than linamarin  
392  $31.25 \mu\text{g/ml}$  ( $60\% \pm 1.87\%$ ) (Table 1). In Raji control cells or with linamarin treatment from  
393 cassava leaf extract, most p53 protein expressions are located in the cell nucleus, although  
394 some are located in the cytoplasmic part (Table 3). The control cells also shown have positive  
395 p53 protein expression but the amount was less than the treatment with linamarin  
396 concentrations of  $31.25 \mu\text{g/ml}$  and  $62.5 \mu\text{g/ml}$  (Fig.1). This shows that Raji cell  
397 death occurred through the mechanism of inhibition of Raji cell proliferation, by  
398 activating suppressor gene tumors such as p53. The presence of stress or DNA damage  
399 can spur the expression of p53 protein in Raji cells<sup>39</sup>.

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

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417 plays an important role in preventing the accumulation of cells with DNA abnormalities that  
418 can mutate into cancer cells<sup>42</sup>.

419 If the p53 expression is the wild type, then DNA damage will cause a rapid rise in p53  
420 protein expression, thus inducing a resting phase of the cell cycle during the G1 phase. Wild-  
421 type p53 will cause a cessation of growth in the G1 phase,<sup>43</sup> thus providing sufficient time for  
422 the DNA repair genes such as MLH, MSH<sub>2</sub>, PMS<sub>1</sub>, PMS<sub>2</sub>, Mdm2, BRCA<sub>1</sub>, and BRCA<sub>2</sub>.<sup>44</sup> If  
423 the DNA damage can be repaired, the cell will continue to divide into the S phase; if this  
424 improvement is not possible, then p53 will induce apoptosis.<sup>45</sup>

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

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

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

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

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

465

#### 466 CONCLUSION

467 Linamarin isolated from cassava leaves (*M. esculenta* Cranz) has the potential to be  
468 developed as an anticancer agent. Linamarin from cassava leaves (*M. esculenta* Cranz) has  
469 cytotoxic activity on Raji cells with IC<sub>50</sub> values of  $71.865 \pm 0.229$  µg/ml, antiproliferation  
470 activity on Raji cells with a doubling time value of 40.723 hours on linamarin concentration  
471 of 62.5 µg/ml and can increase the expression of p53 protein in the nuclei and cytoplasm of  
472 Raji cells.

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475 **SIGNIFICANCE STATEMENT**

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

481

482 **CONFLICT OF INTEREST STATEMENT**

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

485

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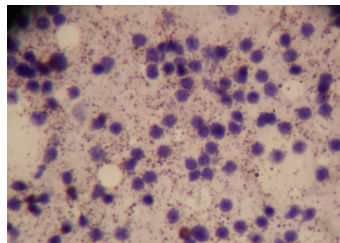
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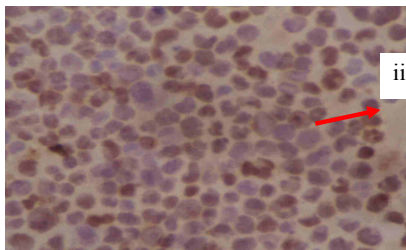
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Cytotoxic, Antiproliferative and Induction of p53 on Raji

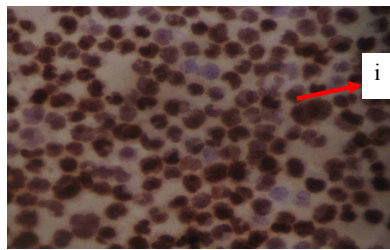
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A. Raji control cell (without treatment)



B. Raji cells with linamarin 32.5µg/ml



C. Raji cells with linamarin 62.5µg/ml

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

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**Commented [WU5]:** Author is advised to label the figure 1 correctly by which a reader can easily understand the results obtained in figure 1.

Cytotoxic, Antiproliferative and Induction of p53 on Raji

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

674

No	Linamarin concentration µg/ml	Absorbance				Average	% of Rajicell 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

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

676

677 **Table: 2.** Doubling time of Raji cells after treatment with various concentrations of linamarin  
678 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

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Cytotoxic, Antiproliferative and Induction of p53 on Raji

684 **Table: 3.** Percentage of p53 protein expression on Raji cell control and linamarin  
 685 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

686 SEM: Standard error of the mean

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

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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

693 SEM: Standard error of the mean

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Feb 04, 2020

Ms. Dwi Sutiningsih,  
Tropical Medicinical Medicine from Gadjah Mada University, Indonesia

**Subject:** Acceptance Letter for Article No. 101171-IJCR-AJ

It's a great pleasure for us to inform you that below mentioned manuscript has been accepted for publication in International Journal of Cancer Research as Research Article on the recommendation of the reviewers.

Title: Cytotoxic and Antiproliferative Activity and Induction of p53 Protein on Raji Cells after Treatment with Linamarin from Cassava Leaves (*Manihot esculenta* Cranz)

Author's Name: Dwi Sutiningsih, Mohamad Arie Wuryanto, Henry Setyawan Susanto, Sujud Hariyadi and Mustofa

Receiving Date: January 13, 2020

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Publication Manager

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

Dr. Sulungah, , Mahamad Aze Wuryanta, Henry Setyawan Susanto, Sujal Haryadi and Mubala

**Abstract: Background and Objectives:** Linamarin is an active compound isolated from the leaves of cassava (*Manihot esculenta* Cranz) that has cytotoxic effects on HT-29, MCF-7 and HL-60 cells. This study was aimed 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. The p53 protein expression was observed by immunocytochemical tests. The **cytotoxic activity** of Raji cells was expressed by the value of inhibitory concentration 50  $\mu\text{g mL}^{-1}$ . 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 stained 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. The p53 protein expression was found in the nuclei (91.03%) and cytoplasm (8.93%). **Conclusion:** Based on these findings, linamarin from cassava leaves has the potential to be developed as an anticancer agent.

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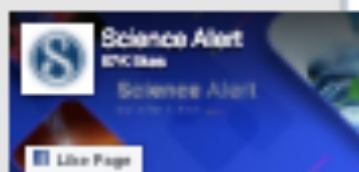
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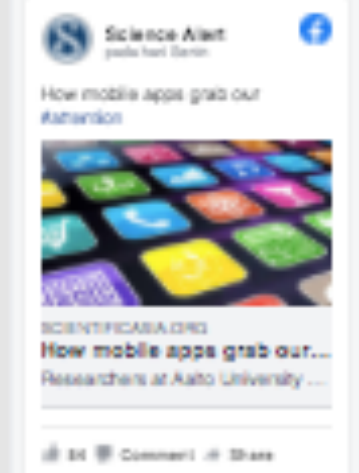
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