Cytotoxicity of Crude Lectins from Red Macroalgae from the Southern Coast of Java Island, Gunung Kidul Regency, Yogyakarta, Indonesia

C Anam1, 3 E Chasanah2 B P Perdhana1 ND Fajarmingsih2 Yusro N F2 A M Sari1 A Nursiw1 D Praseptiangga4 A Yunus3

1 Department of Food Science and Technology, Sebelas Maret University (UNS), Jl. Ir. Sutami 36 A, Kentingan 57126, Surakarta, Indonesia
2 Research and Development Center for Marine and Fisheries Product Competitiveness and Biotechnology, Ministry of Marine Affairs and Fisheries, Republic of Indonesia, Jl. KS Tubun Petamburan VI, Slipi, Jakarta, Indonesia
3 Graduate School Program of Sebelas Maret University (UNS), Jl. Ir. Sutami 36 A, Kentingan, 57126, Surakarta, Indonesia

E-mail: ekowatichasanah@gmail.com

Abstract. Lectins or carbohydrate-binding proteins, are widely distributed in nature, including in marine algae. It may have been considered that binding specificity of lectins to some carbohydrates provokes to produce many unique biological activities, including cell agglutination, mitogenic activity, and antitumor activity. The aim of this study was to determine the cytotoxicity of crude lectins from red macroalgae collected from the southern coast of Java Island, Gunung Kidul Regency, Yogyakarta against MCF-7 and HeLa cancer cells. In vitro MTT assay was used in this study. The results showed that less than 50% of MCF-7 and HeLa cancer cells growth were inhibited by the crude lectins from five species of red macro algae used in this study. The highest inhibition ability shown in the red alga A. nana was able to kill 47.68% of HeLa cervical cancer cells.

1. Introduction
Lectins are carbohydrate-binding proteins of non-immune origin that agglutinates cells and/or precipitates polysaccharides or glycoconjugates [1]. Lectins were one of many bioactive compounds found in macroalgae [2]. Lectins algae have several advantages compared to most terrestrial plant lectins, i.e., they had a small molecular weight, had no affinity for monosaccharides or oligosaccharides but can be inhibited by glycoproteins [3]. Specific binding of lectins lead to a variety of unique biological activities such as cell agglutination, precipitation of polysaccharides, mitogenic activity, antitumor activity and toxicity [2]. Algal lectin research was first started by Boyd et al. [4]. Algal lectins have been widely reported from various areas, such as Japan [2], Spain [5,6], Brazil [7,8], China [9], Antarctica [10,11], and Vietnam [12]. More than 250 species of algae were reported contain lectin [13]. In this study, algal lectin from Gunung Kidul regency, Yogyakarta, Indonesia will be reported. The aim of this research was to investigate the cytotoxicity of crude lectins from red macro algae from the southern coast of Java Island, Gunung Kidul regency, Yogyakarta, Indonesia.
2. Experimental

The main materials used in this research were red macro algae from the southern coast of Java Island, Gunung Kidul, Yogyakarta, Indonesia namely Nitophyllum punctatum, Acanthophora spicifera, Acrocystis nana, Helminthora divaricata and Gloiocladia repens. All red macroalgae were cleaned from debris and sand, and were rinsed with cleaned water. Red algae were then transported in the laboratorium per species by putting in the cool box with ice outside the plastic bag of the macroalgal sample. All chemicals used in all analysis were in analytical grade.

Crude lectins fractions were prepared by procedures as follow: A hundred grams of frozen samples that have been identified were thawed and grounded to a powder using a mortar with addition of liquid nitrogen. The extraction process was conducted by adding a 20 mM phosphate buffer solution containing 0.85% NaCl (PBS, pH 7.0) to the sample with a ratio of 1:2 (w/v) [9]. Then the mixtures stirred overnight (at least 8 hours) at 4°C and centrifuged at 10,000 rpm for 30 minutes at 4°C. Supernatant was taken and to be precipitated by the addition of ammonium sulfate slowly to obtain a final concentration of 75% saturation. Samples were stored overnight at 4°C and centrifuged again (10,000 rpm) at 4°C. The precipitates dissolved by the addition of a minimum buffer and dialysed against PBS for 8 hours with replacement of PBS 3 times every 2 hours. Fraction (inner fraction) obtained was referred to the crude lectins fraction (salting-out fraction) which was then stored in a freezer at -200°C until used for further analysis [3,14].

Hemagglutination activity was determined on a 96-well microtiter V-plate using a 2% suspension (v/v) of trypsin-treated rabbit erythrocytes (TRBC) [15]. It was a routine assay to determine the hemagglutination activity on crude lectins and was expressed as a titer, the reciprocal of the highest two-fold dilution exhibiting positive hemagglutination.

Cell culture was prepared by seeding the cell in flask culture with complete medium. After cell culture 70-80% confluent (about one week), medium was removed and cell culture was washed by 5 ml PBS. Flask culture was added by trypsin then incubated in 37°C/5% CO₂ for 3 minutes to make the cells rounding up. The cells were then ready to be counted and used.

Cytotoxicity assay was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [16]. The assay was started with the preparation of 1x10⁴ cells/ml cancer cells and add 100 µl in 96-well micro liter plate then incubated for 12 hours at 37°C at 5% CO₂ condition. After the media was removed, add 100 µl crude lectin 100 µg/ml and incubated at 5% CO₂ incubator for 24 hours at 37°C. Then the culture medium was removed and 100 µl of MTT 5 mg/ml was added and incubated for 4 hours at 37°C at 5% CO₂. After 4 hours, 100 mL of SDS was add and immediately incubated in the dark at the ambient temperature for 12 hours. The calculation was conducted by measuring absorbance at λ=570 and 690 nm. Using data obtained from measurements of absorbance, determination of the percentage of cells inhibition was conducted by using the following formula:

\[ \% \text{Mortality} = \frac{\text{Control cells absorbance} - \text{treatment cells absorbance}}{\text{Control cell absorbance}} \times 100\% \quad (1) \]

3. Results and Discussion

Based on preliminary research, five (5) species of red algae (table 1) were considered as red algae with the highest hemagglutination activity. [17] Samples then were extracted to obtain crude lectins and their hemagglutination activity were tested (table 1.) [14].

Lectins activity can be ascertained from its ability to cause agglutination of erythrocytes. The ability of lectins in causing agglutination of erythrocytes is caused by cross-linking by lectins in the surface of erythrocytes, and therefore it causes precipitation [18]. Hemagglutination activity assay used erythrocytes of rabbit blood since rabbit erythrocytes have higher sensitivity compared to other animal erythrocytes [19]. Red macroalgae lectins crude fraction which was examined showed various
hemagglutination activity. The result of hemagglutination activity testing of *N. punctatum* showed that the hemagglutination activity was $2^6$. Meanwhile, other macroalgae showed hemagglutination titer of $2^{12}$, $2^6$, $2^{24}$, $2^{13}$ for *A. nana*, *H. divaricata*, *G. repens*, *A. spicifera* respectively.

| No. | Species          | Volume (ml) | Hemagglutination Activity (HA)* | Total Hemagglutination Activity (THA)* |
|-----|------------------|-------------|---------------------------------|---------------------------------------|
| 1   | *N. punctatum*   | 20          | $2^6$                           | 1280                                  |
| 2   | *A. nana*        | 11.5        | $2^{14}$                        | 188416                                |
| 3   | *H. divaricata*  | 6           | $2^6$                           | 384                                   |
| 4   | *G. repens*      | 10          | $2^{24}$                        | 167772160                             |
| 5   | *A. spicifera*   | 16          | $2^{13}$                        | 131072                                |

*HA*, hemagglutination titer, the reciprocal of the highest two-fold dilution exhibiting positive hemagglutination; THA, total hemagglutination titer (volume x HA).

Cytotoxicity assay of the crude lectins was analysed using MTT method. The principle of the MTT method was the reduction of yellow tetrazolium salt MTT (3-(4,5-dimetiltiazol-2-yl) 2,5-difeniltetrazolium bromide) by reductase system. Succinate tetrazolium of which included in the respiratory chain in the mitochondria of living cells would form insoluble purple formazan crystals. Dead cells were unable to metabolize the various kinds of tetrazolium salts [20]. *In vitro* testing using MTT was used because it was more specific, faster and required minimal test materials when compared with *in vivo* testing and could also limit the use of experimental animals [21].

![Figure 1. Result of Cytotoxicity Assay of Crude Lectins from Five Species of Red Macroalgae](image)

The cytotoxicity assay was conducted using HeLa cervical cancer cells and breast cancer cells MCF-7. HeLa cells were first taken from the cervix Henrietta Lacks, who died in 1951 because of a malignant cancer of the cervix [22]. Breast cancer cells MCF-7 is a good *in vitro* model to study the mechanism of tumor response or complex linkages between bond and biological reaction of hormones [23].
Figure 2. a) MCF-7 and b) HeLa Cancer Cells Morphology

Tests were performed at a concentration of 100 µg/ml for all samples against MCF-7 and HeLa cancer cells. The results showed that none of the crude lectins reached 50% inhibitory activities. National Cancer Institute (NCI) explained that the crude extract of natural materials is claimed to be active as anticancer agents if performing IC<sub>50</sub> < 30 µg/ml, moderate active when concentrations 30 µg/ml ≤ IC<sub>50</sub> < 100 µg/ml, whereas for the nature fractional with a concentration ≥ 100 µg/ml is considered as inactive [24,25]. Referring to the NCI, it can be concluded that all samples that tested were not performed potent cytotoxic effects on MCF-7 and HeLa cells. It may indicate that the crude fraction still contain impurities compounds. Thus, the crude lectins need to be further purified by a combination of purification procedures include conventional chromatography, such as gel-filtration chromatography, ion exchange chromatography, hydrophobic interaction chromatography, and/or affinity chromatography [26].

Of 5 samples tested, the highest activity was from sample A. nana which had an average inhibition of 47.68% against HeLa cells but giving lower inhibition against MCF-7 cells. It means that crude lectin from A. nana cell was more selective to HeLa, on the contrary, crude lectin extracted from G. repens was more selective to MCF-7. From this research, it may conclude that A. nana was potentially used as an anticancer compound, however purification steps is needed to obtain higher activity. Previous research reported that lectin from red algae Eucheuma serra was able to cause cell death when tested on some cancer cells. The mechanism of cell death was caused by lectins by apoptosis mechanisms [27]. Most lectins follow a general pathway involving carbohydrate recognition of receptors. Lectin-induced apoptosis occurs through binding of lectin molecules to a specific receptor, internalization into the cell via endocytosis, and further pathway cascades leading to apoptosis [26].

4. Conclusion
Based on this study it can be concluded that crude lectin from red algae studied showed selectitivity. The 5 crude lectins extracted were considered not potential as anticancer shown by less than 50% inhibition of breast cancer cells MCF-7 and HeLa cervical cancer cells. The highest inhibitory activity was detected by the crude lectins of red algae A. nana, showing its ability to inhibit HeLa cancer cell growth up to 47.68%. From this result, A. nana is needed to be purified to obtain higher activity of the lectin.

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References

[1] Praseptiangga D 2015 Squalen Bull. of Mar. & Fish. Postharvest & Biotech. 10(2) 89-98
[2] Hori K, Miyazawa K, Ito K, 1981 Bulletin of the Japanese Society Of Scientific Fisheries, 47(6) 793-798
[3] Hori, Kanji, Keisuke Miyazawa and Keiji Ito 1990 Hydrobiologia 204/205 561-566
[4] Boyd WC, Luis R, Almodovar, Lyle G, Boyd, 1966 Journal of Transfudion 6 82-83
[5] Fabregas J, Munoz A, Llvo J 1986 J. Exp. Mar. Biol. Ecol. 97 213-219
[6] Fabregas J, Llvo J, Munoz A 1985 J. of Bot. Mar. XXVIII 517-520
[7] Ainouz I, Lima, Sampaio AH 1991 J. of Bot. Mar. 34 211-214
[8] Ainouz I, Lima, Sampaio AH, Freitas LP 1995 J. of R. Bras. Fisiol. Veg 7(1) 16-19
[9] Yi Z, Hai-sheng L 2002 Chinese J. Oceanol. Limnol. 20 (3) 256-260
[10] Souza B, Inacio DA, Teixeira FBA, Andrade FK, Melo MRS, Munoz AM, Freitas ALP, 2007 11(1) 122-130
[11] Souza B, Inacio DA, Teixeira FBA, Mansila A, Freitas ALP 2010 J. of Polar Biol. 33 1311-1318
[12] Hung LD, Sato T, Shibata H, Hori K 2009 Fish Sci. 75 723–730
[13] Hung LD, Ly BM, Trang VTD, Ngeo NTD, Hoa LT, Trinh PTH. 2012 J Appl. Phycol. 24 227-235
[14] Praseptiangga D 2013 Jurnal Teknologi Hasil Pertanian VI (1) 1-6
[15] Praseptiangga D, Hirayama M, Hori K 2012 Biosci. Biotechnol, Biochem, 76 (4) 805-811
[16] Wahyuni F S, Sutma S, Aldi Y 2011 Jurnal Sains and Teknologi Farmasi, 16 (2) 209-215
[17] Nurhayati T 2015 Penapisan and Karakterisasi Awal Senyawa Bioaktif Lektin Alga Merah dari Pesisir Pantai Gunung Kidul, Yogyakarta (Skripsi) (Surakarta:Program Sarjana, Universitas Sebelas Maret)
[18] Lis H, Sharon N. 1998. Chem. Rev. 98 637-674
[19] Hori, Kanji, Miyazawa K and Keiji I 1981 Bull Jpn. Soc. Sci. Fish 47(6) 793-798
[20] Rode, H J 2008 Apoptosis, Cytotoxicity and Cell Proliferation 4th Edition (Germany: Roche Applied Science)
[21] Wikanta T, Gusmita D, Rahayu L, Marraskuranto E 2012 JPB Perikanan 7 (1) 1–10
[22] Lucey, Brendan P, Walter A, Nelson-Ress, Grover M. Hutchins 2009 Arch Pathol Lab Med 133
[23] Sethilraja P, Khatiresan K 2015 Journal of Applied Pharmaceutical Science 5 (03) 080-084
[24] Inthe MG, Tarman K, Safihr M 2014 J. Teknol. and Industri pangan 25 (1)
[25] Scheuer P J 1987 Bioorganic Marine Chemistry Volume 1. (USA: Springer)
[26] Yau T, Xiuli, Cheuk C, Ng W, Ng TB 2015 Molecules 20 3791-3810
[27] Suhagara T, Ohama Y, Fukuda A, Hayashi M, Kawabuko A, Kato K 2001 Cytotechnology 36 93–99