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Cytotoxic selectivity of $MJC_{0.3}$ and $MJC_{0.5}$, acidic ribosome-inactivating proteins isolated from *Mirabilis jalapa* L. leaves against various cancer cell-lines

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ABSTRACT

Mirabilis jalapa L. contains basic (MJ30) and acidic (MJC) Ribosome-inactivating proteins (RIPs). Further purification of MJC has been found two RIPs, $MJC_{0.3}$ and $MJC_{0.5}$. This study is aimed to prove the cytotoxic selectivity of $MJC_{0.3}$ and $MJC_{0.5}$ against many cancer cell-lines and normal cell line. The two RIPs, $MJC_{0.3}$ and $MJC_{0.5}$ were tested their cytotoxic effect on 8 human cancer cell lines and normal cell using MTT assay compared with MJC protein. The highest cytotoxic activities of $MJC_{0.3}$ and $MJC_{0.5}$ were against EVSA-T followed by T47D, HeLa, WiDR, SiHa, Raji, NS1, and MCF7, with the IC_{50} of 59.3, 102.4, 162.9, 190.5, 249.5, and 304.5 μg/mL, respectively for $MJC_{0.3}$ and 32.8, 75.5, 86.0, 108.3, 346.7, and 220.06 μg/mL, respectively for $MJC_{0.5}$. Based on these IC_{50} values, $MJC_{0.3}$ and $MJC_{0.5}$ were specific to EVSA-T and T47D, whereas they were not selective against Raji and SiHa ($SI < 10.0$). It can be concluded that he acidic RIPs isolated from *M. jalapa* L. leaves was potential to be developed as anticancer agents for breast cancer.

Key words: *acidic RIPs – M. jalapa* L.*- cytotoxic selectivity - cancer cell lines.*

INTRODUCTION

Mirabilis jalapa L. is one of the medicinal plat that has been scientifically proven to contain ribosome-inactivating proteins (RIPs), a potential toxin isolated from plants that inhibits mamalian protein synthesis.[1,2] Seed and root of *M. jalapa* L. contain MAP (mirabilis antiviral protein), which is actively strive against mechanical transmission from TMV (Tobacco Mozaic Virus) in tobacco, pepper, and tomato.[3] Beside these activities, RIPs which are isolated from some plants have an antioxidant activity in the same level as Fe-superoxide dismustase from *Escherichia coli*,[4,5] and has a potential to be developed as immunotoxin, when conjugated with antibody for acquiring selective anticancer agent.[6]

Ribosome-inactivating proteins from *Annona squamosa* L. and *M. jalapa* L. have been identified,[7] as demonstrated for their ability to cleave super-coiled DNA and the adenine glycoside bond on 4324-rRNA of *Saccharomyces cerevisae*.[8] Total protein fraction isolated from the leaves of *M. jalapa* L. has cytotoxic effect and has been proven can induce the apoptotic process of HeLa cell line.[9] Ribosome-inactivating proteins isolated from the *M. jalapa* L. root also have cytotoxic activity against HeLa cell-line and relatively less cytotoxic against Raji cell line.[10] Based from the purification process, it was found that leaves of *M. jalapa* L. was demonstrated to contain more than one RIPs, basic and acidic RIPs respectively.[5,11]

Basic-RIP (MJ30) which was obtained from purification using CM-Sepharose CL-6B has cytotoxic activity against breast cancer T47D, HPV-18 induced uterine cervix cancer cell line HeLa, and blood myeloma cancer cell line NS1 with IC_{50} as

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0.36, 7.06 and 1.25 mg/mL respectively. This protein was able to induce apoptotic process as demonstrated by the appearance of DNA fragmentation.[12] The unbound fractions which are negatively-charged, called MJC, has been known to cleave super-coiled DNA[11] and has more powerfull cytotoxic effects compare to the MJ30.[13] The MJC inhibits carcinogenesis process as shown by its ability in decreasing the expression of oncogen RAS and COX-2 in HeLa cell-line.[5] Further purification step of MJC has been developed and gaining two acidic RIPs, $MJC_{0.3}$ and $MJC_{0.5}$.[5] However, the cytotoxic activity of these two acidic RIPs against cancer cell-lines has not been studied.

MATERIALS AND METHODS

Chemicals

Medium of RPMI 1640 (Sigma), DMEM (Sigma), M199 (Sigma), sodium bicarbonat (Sigma), and HEPES (Sigma), Fetal Bovine Serum (FBS) 0.5% v/v and 10% v/v (Gibco), penicillin-streptomycin 1% (v/v) (Gibco), and fungison 0.5% (v/v) (Gibco), sodium phosphat 5mM pH 6.5, sterile aquadest, trypsin 0.5%, Phosphat Buffer Saline solution, ethanol 70%, and MTT reagent 5 mg/mL were used in the study. The remaining chemicals were high purified grade (pro analysis) from Merck.

Cell Lines

Three kinds of cell lines (MCF7, EVSA-T, and WiDR) were gained from Erasmus Medical Center Netherlands, which were recultured by the Integrated Research and Testing Laboratory (IRTL), Universitas Gadjah Mada Yogyakarta. Other cell lines were the collections of the IRTL Universitas Gadjah Mada Yogyakarta. Cell lines were cultured from the stock which was stored at -60°C and freshly used post-culture. Cells were routinely maintained in their appropriate media supplemented with 10% FBS in 37°C and 5% CO_2 incubation.

MJC Proteins Preparation

$MJC_{0.3}$ and $MJC_{0.5}$ were prepared from fresh *M. jalapa* L. leaves using 5mM phosphate buffer pH 7.2 containing 0.14 M NaCl and precipitated using aceton. Total protein was then purified using CM-Sepharose CL-6B resins. Followed by DEAE-sepharose resins coloum for the unbound fraction. Two different active protein fraction ($MJC_{0.3}$ and $MJC_{0.5}$) were obtained from the purification processes.[5]

Cytotoxicity Studies

The study were started by preparing the appropriate media for each cell line and the $2x10^4$ cell/100µL was grown in the 96 well plates. These cells were then treated by a serial concentration of MJC's. The citotoxicity tests were performed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)-assay method. The formed formazans formed can not penetrate the life cell's membrane and accumulated in the live cells. This intracellular formazan can be dissolved with detergent, in this experiments we used sodium dodecyl sulphate (SDS). The intensity of the colour which appeared then can be measured with 96-well plate scanning spectrophotometer, where the percent of the colour intensity of the chambers treated over the control represents the amount of the live cells left by treatment over the cells before treatment, otherwise the percent of cells inactivated because of the treatment of the sample then can be assayed.

Value of IC_{50} was determined by regression correlation method, using protein concentration as the independent variable and percentage of inhibition as the dependent variable. Then using the regression equation, the IC_{50} value can be found as the concentration by putting 50% value as the y value at the regression. Value of p as the degree of significance was analyzed using SPSS statistical program version 16.0 on 95% confidence-interval.

RESULT AND DISCUSSION

Tested MJC, $MJC_{0.3}$, and $MJC_{0.5}$ proteins on the ability of cleaving supercoiled double stranded DNA indicated that the protein isolates were able to cleave double stranded pUC18 into nick-circular form as indicated in FIGURE 1. This result supported the previous study which using the crude extract.[8] Since the cleavage of supercoiled DNA is one of the characteristic of RIPs besides their N-glycosidase activity,[13-15] hence it was strongly suggest that these protein had RIPs activity.

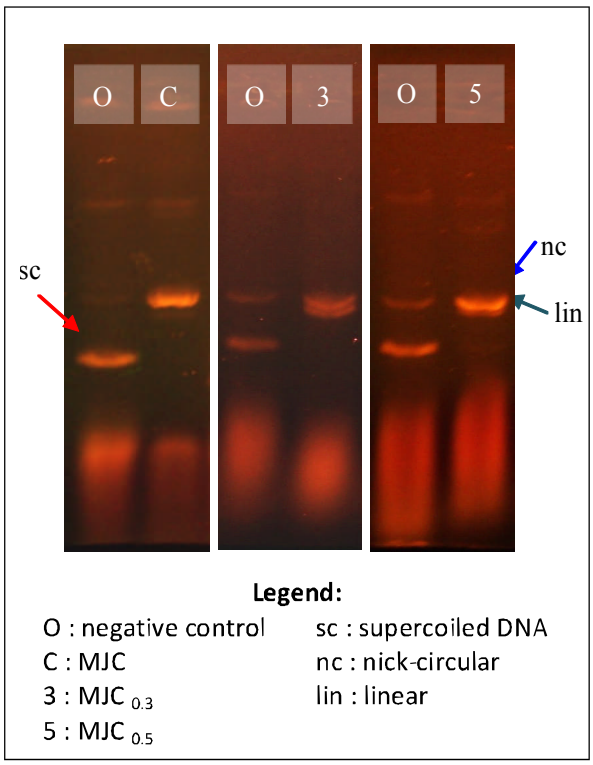

FIGURE 1. MJs cleavage activity study, shows that there were cleavage activities as the forming of linear and nick circular structure of the DNA

These MJC$_{0.3}$, and MJC$_{0.5}$ proteins demonstrated cytotoxic activity against EVSA-T, T47D and MCF7 (breast cancer cell-lines), WiDR (colon cancer cell-lines), SiHa and HeLa (uterine cervix cancer cell-lines induced by HPV-16 and HPV-18 respectively), Raji (naso-pharynx cancer cell-lines), NS1 (myeloma cancer cell-lines), but not on normal Vero cell-lines.

The induction of the cells death was characterized by the changing of the cells viability that was quantified by the intensity of the color formed by the formazans deposition. On the treated cancer cell-lines, the percentage of the cell death was higher compared to the untreated cells. The higher addition of protein samples, the higher the cancer cells death. For normal Vero cells, there were no differences on the cells viability between untreated and treated cells in the low concentration, however cytotoxicity was observed in very high concentrations.

Interestingly, these proteins showed different level of cytotoxicity in between cancer cell-lines. From the data TABLE 1, MJC$_{0.3}$ and MJC$_{0.5}$ gave best activities for EVSA-T as 32.8 µg/mL and 59.3 µg/mL respectively. Unfortunately, the exact IC$_{50}$ for MJC fraction was not determined. However, against other cell lines, T47D, we could get those all of the 3 MJs fractions gave best results, as 111.0 µg/mL for MJC, 102.4 µg/mL for MJC$_{0.3}$, and 75.6 µg/mL for MJC$_{0.5}$. These MJC$_{0.3}$ and MJ$_{0.5}$ showed various 2 or 3 times higher IC$_{50}$ values against another cell lines (TABLE 1).

TABLE 1. IC$_{50}$ of protein MJC, MJC$_{0.3}$, and MJC$_{0.5}$ against various cell lines

Cell Line	IC$_{50}$ (µg/mL)		
	MJC	MJC$_{0.3}$	MJC$_{0.5}$
HeLa	2517.3	162.9	86.0
Raji	691.7	304.5	220.1
SiHa	724.5	249.5	346.7
NS-1	749.3	*N/A*	*N/A*
MCF-7	880.8	*N/A*	*N/A*
T47D	111.0	102.4	75.5
EVSA-T	*N/A*	59.3	32.8
WiDR	1006.0	190.5	108.3
Vero	8775.8	2739.0	1166.1

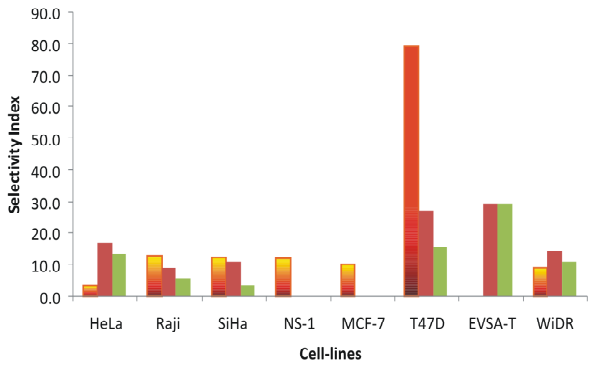

FIGURE 2. Selectivity index values show that MJC(orange), MJ$_{0.3}$(brown) and MJC$_{0.5}$ (green) were majority selective compared with Vero normal cell-lines. Selectivity indexes were measured by dividing IC$_{50}$ value against each cancer cell lines by the IC$_{50}$ value against the normal cell line Vero

Selectivity index is an important factor in the development of anticancer agent, because this index is the factor that ensure the safety of the xenobiotic. Selectivity index is the ratio between cytotoxic

activity of the xenobiotic against certain cancer cell line by the activity against normal cell line. In this study, Vero cell line was used as normal cell line comparator. After comparison of IC_{50} against cancer cell line with IC_{50} against normal cell line as a quantification for activity of protein MJC, $MJC_{0.3}$, and $MJC_{0.5}$, it was known that generally protein MJ was selective, except against some several cell lines (FIGURE 2). The value of a selectivity index can be accepted if it was higher than 10, which means that the xenobiotic is active to kill cancer cells 10 times stronger than to kill normal cells.

The selectivity index values of MJC were below the standard for HeLa and WiDR, but due to the activities of MJC against those two cell lines were very low, the IC_{50} values were very high. Same cases and discussion were applied for protein $MJC_{0.3}$, and $MJC_{0.5}$ against Raji and SiHa. For unmeasured IC_{50} values, selectivity index values also can not be measured.

Comparing this result with the result of the study performed by Ikawati *et al*.[10] in which the RIP total protein fraction which was isolated from the root of *M. jalapa* L. was cytotoxic against HeLa cell line but less cytotoxic against Raji cell line, it could be concluded that RIPs which were isolated from the leaves were more potent, because they were relatively more toxic against both HeLa and Raji cell-lines. This result can be explained by two probable reasons, the first is because the RIPs in the root and the leaves itself were different, and second because the concentration of RIPs in the root were smaller than the amount of RIPs in the leaves.

Furthermore, if we compare with the cytotoxicty of MJ-30,[12] against T47D, NS-1, and HeLa cell lines, with IC_{50} as 0.36, 7.06 and 1.25 mg/mL respectively, the results of this study for MJC gave smaller IC_{50} against T47D and NS-1. However, if this results are compared with the study by Sudjadi, *et al.*[13] where citotoxicity study of the fraction in the same level of MJC but purified by *ionenaustauscher type II*, gave IC_{50} values as 0.28 mg/mL (T47D), 0.007 mg/mL (NS1), and 0.014 mg/mL (HeLa), so that this study only gave smaller IC_{50} value for T47D as 111.0 µg/mL, but for NS-1 and HeLa were higher, as 0.749 and 2.517 mg/mL respectively (TABLE 1).

Those differences can be caused by various conditions, such as the purification method, and also the method of the study itself. Sudjadi, *et al.*[13] performed cytotoxicity analysis using tryphan-blue direct counting method, whereas this study was using MTT method. These can cause some perspective differences. With the result that, we can get actual values compilation which can be compared well just in this study where the method of isolation, purification, and also cytotoxicity analysis itself are in the same way, so that the results can show a valid comparison of the selectivity and the specificities of MJ proteins against each cancer cell line.

The next interesting discussion is that MJ proteins from the entire studies both for MJ-30 and MJC itself, showed a consistency of IC_{50} values that always relatively small against T47D cell line. For HeLa cell line, good result was shown by Sudjadi, *et al.*[13] and this study for $MJC_{0.3}$ and $MJC_{0.5}$ fractions. Contradictive result was shown for MJC fraction (TABLE 1). This fact strengthen the indication that MJ proteins were cytotoxic-specific against breast cancer, besides selective as shown by selectivity-index values (FIGURE 2).

CONCLUSION

From the study it can be concluded that MJC protein was relatively potent for T47D cells, $MJC_{0.3}$ for T47D and EVSA-T cells, and $MJC_{0.5}$ was potent for T47D and EVSA-T cells. Since both of T47D and EVSA-T cells are breast cancer cell-lines, the study showed that these protein fractions were potential against the breast cancer. Further study for other types of cancer cell-lines is needed. For the follow up of this study, further development of these isolates to be formulated as a potential anticancer agent against breast cancer will actuate research on anticancer therapy.

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