Polyketide Derivatives from *Annona muricata* Linn Leaves as Potencial Anticancer Material by Combination Treatment With Doxorubicin on Hela Cell Line

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**Abstract.** One of the compounds found effication as an anticancer agent on cervical cancer is acetogenin, a polyketide compound that is abundant in *Annona muricata* L. leaves. This study has been done to examine polyketide derivatives was isolated from *Annona muricata* L. which has potency to induce apoptosis by p53 expression on hela cell line. An approach recently develop to overcome side effect of chemoterapeutic agent is used of combined chemoterapeutic agent, i.e doxorubicin. The determination of cytotoxic combination activity from polyketide derivative and doxorubicin was evaluated using MTT assay to obtain the value of CI (combination index). The expression of p53 profile was evaluated by immunohistochemistry on hela cell line. Data analysis showed that combination of polyketide derivative from *Annona muricata* L. (38.5 µg/ml) and doxorubicin with all of concentration performed synergistic effect on hela cell line with CI value from 0.33 – 0.65. The analysis on immucytochemistry showed that polyketide derivative from *Annona muricata* L. leaves could enhance p53 pathway significantly on hela cell line.

1. **Introduction**

Monotetrahydrofuran annonaceous acetogenins is one of the active compound were isolated from *Annona muricata* L leaves. They exhibit a broad range of biological properties such as cytotoxic, immunosuppressive, antiparasitic and antimicrobial [1]. Especially, their potential to inhibit tumor cells and the activity as anticancer agent. Annonaceous acetogenin often referred to inhibit the growth of cancer cell. Acetogenin is a polyketide compound with structure C-34 or C-37 unbranched carbon chain attached to the 2-propanol group at C-2 to from a lactone [2]. The main focus of this study was to observe the citotoxic activity of polyketide derivative from *Annona muricata* L by combination treatment with doxorubicin on Hela cell line. Cervical cancer is the second most common found in women all over the world. There are 532,232 cases of cervical cancer in 100 million women [3]. The viral factor associated with oncogenic include the persisten infection, elevated viral load, virus genotype chemotherapy, and combination of these. To date, the use of chemotherapeutic agent is the mostly used treatment [4] but it cause many side effects, such as severe nausea and vomiting, acute and chronic nephrotoxicity, anemia [5] [6], acute cochlear toxicity (ototoxicity) [7] and also developed resistance [8]. Many studies was conducted on natural compound as an anticancer agent [9] that has shown potentially of anti-cancer that have toxicity selectively without damaging in normal cells [10]. One of the natural compound which has potential effect as anticancer agent is *Annona muricata* L.
There are four compound of *Annona muricata* L. have a potential anticancer namely are monotetrahydofuran acetogenins, muricin H, muricin I and cis-annomontacin [11]. Therefore, the aim of this research was to develop a new approach of combination treatment using polyketide derivatives (Me (CH$_2$)$_7$O C(O) (CH$_2$)$_4$C(O) O (CH$_2$)$_7$ Me) from *Annona muricata* L. [12] and doxorubicin as co-chemotherapeutic agent. Hela cells are known to express 2 kinds of oncogenes, E6 and E7. E6 bounds with p53 phosphorilated protein meanwhile the E7 bounds with pRb protein [13]. The bonding of p53 protein by the E6 causing the increase of protein’s degradation rate. Polikeptide derivatives from *Annona muricata* L reported to have ability in increasing the p53 expression thereby activates apoptosis process on Raji cell line [12].

2. Experimental

2.1 Sampel preparation

*Annona muricata* L. leaves was obtained from Karanganyar, Central Java, Indonesia. It was extracted with ethanol, evaporated and partitioned with chloroform, n-hexane and ethyl acetate by vaccum coloumb chromatography (VLC). The fractions was evaporated by using rotary evaporator and identified with KLT. Polyketide was isolated by chloroform fraction using vaccum coloumb chromatography (VLC). Each sample from TLC process which have one spot is joined and evaporated again to FTIR.

2.2 Cell culture

Hela cells were obtained from LPPT, Gadjah Mada University and were grown in RPMI (Gibco), supplemented with 10% Fetal Bovine Serum (FBS Qualified, Gibco, Invitrogen USA), 1.5% (w/w) penilsil-streptomisin (Gibco, Invitrogen USA), and 0.5 % fungizone (Gibco, Invitrogen USA). Cells were incubated at 37°C and 5% CO$_2$.

2.3 Cytotoxic assay

The cytotoxic activity were done by MTT assay to obtain the value of Combination Index (CI). Hela cells were distributed in 96-well plate before being exposured to sample treatment with 12000 in cells each well. For cell viability assay, cells were treated for 24 hours with polyketide derivate from *Annona muricata* L both for the single and combinational cytotoxic assay. As negative control, only growth medium was added. In the end of the incubation time, each well were added by 100 μg/ml of MTT solution (0.5 mg/ml PBS) continued with incubation for 3 hours at 37°C. There action was stopped by dilution with 10% (w/v) Sodium Dodecyl Sulphate in 0.01 N HCl, and cells were incubated overnight. The absorbance was determined by using ELISA reader at $\lambda$ 595 nm. [14].

2.4 Immunohistochemistry

Hela cells were grown with the density of 50000 cells/cover slip in 24-well plate for 24 hours prior to treatment of Cells were treated for 15 hours and then fixed with cold methanol p.a for 10 minutes and washed twice using PBS and sterile water. Cells were blocked with H$_2$O$_2$ and then prediluted blocking serum were added, continued with incubation for 10 minutes. Cells were washed using PBS and incubated with primary monoclonal antibody antip53 (mouse monoclonal anti-p53 antibody, BioGenex) overnight. Cells were washed using PBS, then universal detection kit was added (Star Trek Universal HRP Detection Kit, Ref STUHRP 700L10-KIT, Biocare Medical). Cells were then incubated with biotinylated universal secondary antibody for 20 minutes, followed by streptavidin-enzyme horse radish peroxidase for 10 minutes.Substrate solution DAB used as a chromogenic agent was exposed for 10 minutes and cells were counterstained with Mayer’s Haematoxylin (Dako) for 1 minute and fixed with ethanol and xylol. Between each immunostaining step, cells were washed briefly in PBS pH 7.4. Negative controls were prepared by replacing the primary antibody with PBS.
2.5 Data analysis

Single citotoxic assay was determined by linear regression between concentration and % cell viability to calculate IC_{50} value so that can be determined the cytotoxic potency. It was analyzed statistically by using Microsoft Excel and statistical significance was estimated by using ANOVA test. Statistical significance was placed at p<0.05. Combinational treatment was evaluated by calculating the Combinational Index (CI) value which has the formula as follows: CI = D1/Dx1 + D2/Dx2

D1 and D2 represent concentrations used in combinational treatment, while Dx1 and Dx2 are single treatment concentration giving same response as D1 and D2, respectively. The interpretation of CI value was done based on classification listed in Table 1.

**Table 1. Interpretation of CI value representing potency of combination application**

| CI value  | Interpretation       | CI value  | Interpretation       |
|-----------|----------------------|-----------|----------------------|
| < 0.1     | Very strong synergist| 0.9 – 1.1 | Closely additive     |
| 0.1 – 0.3 | Strongly synergist   | 1.1 – 1.45| Middle antagonist    |
| 0.3 - 0.7 | Synergist            | 1.45 – 3.3| Antagonist           |
| 0.7 – 0.9 | Middle synergist     | >3.3      | Strongly antagonist  |

3. Result and Discussions

3.1 Fourier Transform - InfraRed (FTIR)

There are 16 points uptake representing 16 functional groups contain in isolates of *Annona muricata* L. The absorption interpretation was done up to maximum wavelength 500 cm\(^{-1}\) and 15 points uptake that appropriate with standard table. The absorbance at 3.397,76 cm\(^{-1}\) indicates alcohol O-H groups. Other typical uptake absorbance 2.928,07 cm\(^{-1}\) indicates the chain C-H asymmetric and 2.851,88 cm\(^{-1}\) indicates CH symmetric, both groups showed adjacent C-H vibration. The absorbance at 1.465,96 cm\(^{-1}\) indicates vibration C-H as the form of vibration cut off [15]. The absorbance at 1.743,72 cm\(^{-1}\) indicates the absorpion of the lactone groups (Fig.1). It was an special absorbance that comes from the group C=O in γ-butyrolactone which estimated as lacton of acetogenis [16]. The existence of these different groups with ester lactones which have a longer absorption. This is reinforced by the absorption at 1.095,61cm\(^{-1}\) as the O-C-C that indicates the presence of a lactone group. According Blessing et al. (2015), alkena has absorbance in 1.680-1.600 cm\(^{-1}\)[17]. In Table 1, the group C = C bonds (alkenes) are shown in absorbance 1.614,49cm\(^{-1}\). This existence by the absorption at wave numbers 960.59 nm, 799.53nm dan 720.44nm which indicate the presence of aromatic C-H bending. Molecular masses of polyketide isolate from *Annona muricata* L are consisted of 20 or 22 carbon atoms apart for few exception such as fatty acid esters.

![Figure 1. FTIR interpretation of polyketide isolate from Annona muricata L.](image)
3.2 Effect of polyketide derivatives from Annona muricata L. on Hela Cells growth

Polyketide derivatives from *Annona muricata* L. leaves shows potent cytotoxicity on Hela cells. Furthermore, IC\textsubscript{50} value acquired as the parameter of concentration that needed to yield 50% cells’ growth inhibition of Polyketide derivatives from *Annona muricata* L. leaves on Hela cells. The effect of polyketide derivatives to cell viability of 24-hours treatment. Treated cells showed cytotoxic effect with change of cell morphology and decreased cell viability. Viable cells had epithelial shape, but after being treated with certain concentration of samples they gave spherical-shape and shrunk cell wall. Polyketide derivatives from *Annona muricata* L. caused inhibition of cell growth in a concentration-dependent manner (Fig. 1). Cell viability after treatment with polyketide derivatives from *Annona muricata* L. was significantly decreased by IC\textsubscript{50} value is 77.09 µg/ml on Hela cells (Fig. 2). According to Prayong *et al.* (2008) this active compound exhibited potent cytotoxicity (IC\textsubscript{50} ≤ 100μg/ml) [18]. These results above suggested that polyketide derivatives from *Annona muricata* L. leaves indicate as the most potential concentration which has potential cytotoxic effect.

**Figure 2.** The effect of polyketide derivative from Annona muricata L. on the growth Hela cells. The cells were incubated in the 96 well plates and given the treatment the sample in the range 15 µg/ml - 100 µg/ml.

Based on the study, the IC\textsubscript{50} of doxorubicin on Hela cells is 8.88 µM. Hela cells has potency resistance of doxorubicin by the antiapoptotic protein in Hela cells and efflux mechanism of doxorubicin from Hela cells [19]. Therefore, the observation of combinational cytotoxicity of polyketide derivatives from Annona muricata L and doxorubicin is needed to be done.

3.3 Effect of polyketide derivatives from *Annona muricata* L combined with doxorubicin on Hela cell growth

Combination assay was done by combining ½ IC\textsubscript{50}, ¼ IC\textsubscript{50}, 1/8 IC\textsubscript{50}, 1/10 IC\textsubscript{50} of polyketide derivatives from Annona muricata L with ½ IC\textsubscript{50}, ¼ IC\textsubscript{50}, 1/8 IC\textsubscript{50}, 1/10 IC\textsubscript{50} of doxorubicin. The cell viability were quantified by Combination Index (CI). The optimum of CI value were obtained were combining ½ IC\textsubscript{50} polyketide derivatives from *Annona muricata* L with ¼ IC\textsubscript{50}, 1/8 IC\textsubscript{50}, 1/10 IC\textsubscript{50} respectively and the combination of 1/8 IC\textsubscript{50} polyketide derivatives from Annona muricata L with ¼ IC\textsubscript{50} of doxorubicin with CI value in range 0.3 – 0.7 (Table 1). the synergistic effect of doxorubicin in the combination with polyketide derivatives from Annona muricata L. probably through the apoptosis induction mechanism so that the observation of these should be done.

**Table 2.** Combination Index (CI) value of polyketide derivatives from *Annona muricata* L. and doxorubicin

| Polyketide derivatives (µg/mL) | Doxorubicin (µM) |
|-------------------------------|------------------|
|                               | 0.9              | 1.125 | 2.25 | 4.5 |
| 7.7                           | 0.40             | 0.87  | 1.34 | 0.78|
| 9.625                         | 0.85             | 1.08  | 1.76 | 0.35|
| 19.25                         | 1.22             | 0.60  | 2.61 | 2.97|
| 38.5                          | 0.71             | 0.34  | 0.57 | 4.06|
Figure 3. The effects of combination treatment of polyketide derivatives from Annona muricata L. with doxorubicin on Hela cells viability. Cells were incubated overnight and given the treatment of these two compound by combining $\frac{1}{2} \text{IC}_{50}$, $\frac{1}{4} \text{IC}_{50}$, $\frac{1}{8} \text{IC}_{50}$, $\frac{1}{10} \text{IC}_{50}$. The graph shows Hela cells viability after the treatment ($p<0.05$).

3.4 Effect of polyketide derivatives from Annona muricata L. and Doxorubicin on p53 expression
To confirm the mechanism of polyketide derivatives from Annona muricata L. in Hela cells, therefore molecular target protein p53 was investigated. Hela cells have the characteristic of degrade p53 and loss of function as tumor suppressor protein causing uncontrolled proliferative cells [20]. As shown in Fig 4, immunocytochemistry evaluation indicated that p53 level increased significantly ($p<0.05$) with intensive brown colour in nucleus after being treated with 77.09 µg/ml Annona’s polyketide derivatives. The result suggest that Annona’s polyketide derivatives has cytotoxicity efficacy on Hela cells through p53 stabilization. Besides, Annona’s polyketide derivative addition to doxorubicin enhance cytotoxic efficacy on Hela cell line.

Figure 4. Doxorubicin in combination with Annona’s polyketide derivative increased p53 level in nucleus. Immunocytochemistry of (A) cell control overnight treatment with (B) 77 µg/ml Annona’s polyketide derivative (C) 8 µM doxorubicin (D) 8 µM doxorubicin in combination with 77 µg/ml Annona’s polyketide derivative.

The study for anticancer agent from natural source has been successfull worldwide. Active constituenes have been purified and nowadays used to treat in human tumours. The etnopharmacological knowledge is helpful to lead the study for plant which has potential cytotoxic activity. Annona muricata L. reported to be utilises as remedies against cancer, however is not especially for cancer caused virus. Our result demonstrated that polyketide derivatives from Annona muricata L. have growth inhibitory and cytotoxic effect on cervical cancer cell line. Polyketide derivatives from Annona muricata L. leaves performed potent cytotoxic effect on Hela cells with IC$_{50}$ value of 77.09 µg/ml.
Decreasing cell viability may be because of either cell death or cell cycle arrest. The mechanism of cell cycle distribution is also associated with some of cellular protein especially p53 protein. p53 is a tumor suppressor protein. In this study, we observed that polyketide derivative from *Annona muricata* L. treatment increased p53 level in nucleus. Therefore, polyketide isolation may be amenable as viral inhibitor agent and as competitor of vaccine to prevent the development of cervical cancer. However, this speculation still needs further investigation by in silico study. In conclusion, polyketide derivatives from *Annona muricata* L. leaves indicate has potential to be developed as a co-chemotherapeutic agent on HeLa cell lines, it can exhibit potential ability with p53 stabilization. Further molecular target detection to investigate its cellular pathway needs to be conducted.

4. Conclusions
The combination of polyketide derivative from *Annona muricata* L. (38.5 µg/ml) and doxorubicin with all of concentration performed synergistic effect on heLa cell line with CI value from 0.33 – 0.65. The analysis on immunocytochemistry showed that polyketide derivative from *Annona muricata* L. leaves could enhance p53 pathway significantly on heLa cell line.

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