Synergistic Cytotoxicity Effect by Combination Treatment of Polyketide Derivatives from *Annona muricata* Linn Leaves and Doxorubicin as Potential Anticancer Material on Raji Cell Line

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**Abstract.** Nasopharynx cancer is one of the most deadly cancer. The main priority of nasopharynx cancer treatment is the use of chemotherapeutic agents, especially doxorubicin. However, doxorubicin might also lead to diverse side effect. An approach recently develop to overcome side effect of doxorubicin is to used of combined chemotherapeutic agent. One of the compounds found efficacy as an anticancer agent on nasopharynx 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 raji cell line. The determination of cytotoxic combination activity from polyketide derivative and doxorubicin was evaluated using MTT assay to obtain the value of CI (*combination index*). Data analysis showed that combination of polyketide derivative from *Annona muricata* L. (14.4 µg/ml) and doxorubicin with all of concentration performed synergistic effect on raji cell line with CI value from 0.13 – 0.65.

1. **Introduction**

Monotetrahydrofuran annonaceous acetogenins is one of the active compound were isolated from *Annona muricata* L leaves. It was one of Indonesia's native plants widely used in traditional medicine, especially cancer therapy and parasitic infections [1]. Chloroform extract and fraction of *Annona muricata* L leaves acetogenins resulted in higher apoptosis rate compared to ethyl acetate solvent which was 91.86% at 2000 ng/mL concentration, while in ethyl acetate extract 23.79% [2]. Annonaceous acetogenin often referred to inhibit the growth of cancer cell. Acetogenin is a polyketide compound with structure C-35 or C-38 compounds, possessing one or two tetrahydrofuran rings and γ-lactone (either saturated or unsaturated) usually involving three carbon chain attached to long aliphatic chain and having unbranched aliphatic region [3]. Incidence of cancer caused by virus is increasing every year, with estimation of new cases of cancer caused by Epstein Barr Virus ranges from 3.1 cases per 100,000. Most (83%) nasopharyngeal cancer are diagnosed at regional or distant stages. The overall 5 year relative survival rate is 60% [4]. There are several ways to treat cancer caused by virus such as surgery, radiation therapy, chemotherapy, and combination of these. To date, the use of chemotherapeutic agent is the mostly used treatment beside neoadjuvant chemotherapy and concurrent chemoradiotherapy [5].
but it cause many side effects, such as severe nausea and vomiting, acute and chronic nephrotoxicity, anemia acute cochlear toxicity (ototoxicity) [6] and also developed resistance. Many studies was conducted on natural compound as an anticancer agent that has shown potentially of anti-cancer that have toxicity selectively without damaging in normal cells. One of the natural compound which has potential effect as anticancer agent is *Annona muricata* L. Liaw et. al., 2002 reported that four compound of *Annona muricata* L. have a potential anticancer namely are monotetrahydofuran acetogenins, muricin H, muricin I and cis-annomontacin [7]. The main focus of this study was to observe the synergistic cytotoxic activity of polyketide compound from *Annona muricata* L after treatment with doxorubicin on raji cell line. Chemotherapeutic combination could reduce doxorubicin side effects and cellular resistance by decreasing the concentration of doxorubicin for treatment [8]. Herbal medicines have been used as anti-cancer agents to overcome side effects and drug resistance [9].

2. Experimental

2.1 Sampel preparation

*Annona muricata* L. leaves isolate was obtained from previous research belongs to Professor. Okid Parama Astririn, Sebelas Maret University. It was obtained from Karanganyar, Central Java, Indonesia. Dried sample of *Annona muricata* L leaves was extracted with methanol and evaporated at 80°C. Thus methanol extract was partitioned with chloroform, n-hexane and ethyl acetate using vacuum liquid chromatography (VLC). Polyketide derivatives from *Annona muricata* L.leaves was isolated by chloroform and each sample from TLC process which have one spot is joined and evaporated again to FTIR and GC-MS analysis.

2.2 Cell culture

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

2.3 MTT assay

Cytotoxic activity of polyketide isolation from *Annona muricata* L. leaves was evaluated using MTT assay to obtain the value of IC$_{50}$. 12x10$^3$ Raji cells/well were grown in 96-well plate before being exposed to sample treatment. For cell viability assay, cells were treated for 24 hours with increasing concentration of isolate alone and in combination with doxorubicin. As negative control, only growth medium was added. At 100 μg/ml of MTT solution (0.5 mg/mlm PBS) was added to each well 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.

2.4 Statistical analysis

Single citotoxic assay was determined by linier regression between concentration and % cell viability giving the equation $y = Bx+A$ were used to calculate IC$_{50}$ value. 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. Synergistic effect was evaluated based on the Combination Index (CI).The interpretation of CI value was done based on classification listed in Table 1.
3. Result and Discussions

3.1 Effect of polyketide derivative from Annona muricata L. leaves on Raji Cells growth

Polyketide isolation from Annona muricata L. leaves shows potent cytotoxicity on Raji cells. Furthermore, IC₅₀ value acquired as the parameter of concentration that needed to yield 50% cells’ growth inhibition of isolate on Raji cells. The effect of Polyketide isolation from Annona muricata L. leaves 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 isolation from Annona muricata L. caused inhibition of cell growth in a concentration-dependent manner. It has performed cytotoxic effect on Raji cells with IC₅₀ of 71.96 µg/ml. Cell viability after treatment with polyketide isolation from Annona muricata L. was significantly decreased by IC₅₀ value is 71.96 µg/ml on Raji cells (Fig.1). According to Prayong et al. (2008) this active compound exhibited potent cytotoxicity (IC₅₀ ≤ 100µg/ml) [10]. These results above suggested that polyketide isolation from Annona muricata L. leaves indicate as the most potential concentration which has potential cytotoxic effect.

![Figure 1](image_url)

Figure 1. The effect of polyketide derivative from Annona muricata L. on the growth Raji cells. The cells were incubated in the 96 well plates and given the treatment the sample in the range 10 µg/ml - 100 µg/ml.

3.2 Synergistic effect of polyketide derivatives from Annona muricata L. combined with doxorubicin on Raji cell growth

Synergistic effect was done by combining ½ IC₅₀, ¼ IC₅₀, 1/₅ IC₅₀ of polyketide derivative from Annona muricata L. with ½ IC₅₀, ¼ IC₅₀, 1/₅ IC₅₀ of doxorubicin. The strongly synergistic effect obtained were combaining 1/₅ IC₅₀ and ½ IC₅₀ of polyketide derivative from Annona muricata L. with ½ IC₅₀ and 1/₅ IC₅₀ of doxorubicin. With CI value in range 0.1-0.3 (Fig. 2). The synergistic effect obtained were combaining ½ IC₅₀ of polyketide derivative from Annona muricata L. with ½ IC₅₀ and 1/₄ IC₅₀ of doxorubicin repectively with CI value in range 0.3-0.7. The synergistic effect of doxorubicin combine polyketide derivative from Annona muricata L. probably through the apoptotic pathway. This result indicates that polyketide derivative from Annona muricata L. can be used in combination with doxorubicin on Raji cells. Ability of it to increase apoptotic and necrosis cell death induced by doxorubicin.

Table 1. Combination Index (CI) value of polyketide derivative from Annona muricata L. combined with doxorubicin on Raji cells

| Polyketide derivative from Annona muricata L (µg/mL) | 1.4 | 1.75 | 3.5 |
|----------------------------------------------------|-----|------|-----|
| 14.4                                               | 0.21| 1.08 | 0.17|
| 18                                                 | 1.37| 0.22 | 0.03|
| 36                                                 | 0.28| 0.35 | 0.40|
**Figure 2.** The synergistic effect of polyketide derivative from *Annona muricata* L. after treatment with doxorubicin on Raji cells. The cells were incubated overnight in the 96 well plates and given the treatment of these two compound by combining $\frac{1}{2}$ IC$_{50}$, $\frac{1}{4}$ IC$_{50}$ and $\frac{1}{5}$ IC$_{50}$. The graph shows CI value on Raji cell after treatment (p<0.05)

*Annona muricata* L. reported to be utilises as remedies against cancer, however is not especially for cancer caused virus. Our result demonstrated that polyketide isolation from *Annona muricata* L. have growth inhibitory and cytotoxic effect on nasopharing cancer cell line. Decreasing cell viability may be because of either cell death or cell cycle arrest, so that the observation of these should be done. Therefore, polyketide derivative may be potential to develop as viral inhibitor agent and competitor of vaccine to prevent nasopharinx cancer. However, this speculation still needs further investigation by in vitro and in vivo study.

4. **Conclusions**

Polyketide derivative from *Annona muricata* L. has synergistic effect with doxorubicin. It means that polyketide derivative from *Annona muricata* L. potential to be developed as a co-chemotherapeutic agent on Raji cell lines. Further molecular target detection to investigate its cellular pathway needs to be conducted.

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