Cytotoxicity of quercetin and curcumin combination against HeLa cells line

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ABSTRACT
Curcumin and quercetin combination are potential candidates for anticancer. Previous studies reported this combination active against several cancer cells including breast cancer, myeloid leukemia, and melanoma. However, the activity of this combination against cervical cancer has not been reported, yet. This study aimed to evaluate the cytotoxicity of the combination of curcumin and quercetin against HeLa and Vero cells. MTT assay was applied to evaluate cell growth inhibition. The inhibitory 50% concentration (IC₅₀) of curcumin and quercetin on HeLa and Vero cells was determined and the selectivity index (SI) was then calculated. Combination index (CI) was calculated after evaluation of the cytotoxicity of the curcumin and quercetin combination at various concentration of 1 IC₅₀, ½ IC₅₀, and ¼ IC₅₀. The IC₅₀ of the curcumin against the HeLa and Vero cells were 26.57 ± 2.00 μM and 73.89 ± 12.93 μM with a SI of 2.78. Whereas the IC₅₀ of the quercetin against the HeLa and Vero cells were 149.52 ± 21.09 μM and 1094.47 ± 15.68 μM with a SI of 7.32. The CI of combinations of curcumin and quercetin at concentrations of ¼ IC₅₀ was 0.78 indicating a mild-moderate synergistic effect, whereas the combination at a concentration of 1 IC₅₀ or ½ IC₅₀, the CI value was >0.9 indicating an antagonistic effect.

ABSTRAK
Kombinasi kurkumin dan kuersetin merupakan kandidat potensial antikanker. Penelitian sebelumnya melaporkan kombinasi ini aktif melawan beberapa sel kanker termasuk kanker payudara, leukemia myeloid, dan melanoma. Namun, aktivitas kombinasi ini melawan kanker serviks belum dilaporkan. Penelitian ini bertujuan untuk mengevaluasi sitotoksisisitas kombinasi kurkumin dan kuersetin terhadap sel HeLa dan Vero. Uji MTT dilakukan untuk mengevaluasi penghambatan pertumbuhan sel. Konsentrasi penghambatan 50% (IC₅₀) kurkumin dan kuersetin pada sel HeLa dan Vero ditetapkan untuk menghitung indeks selektivitas (SI). Indeks kombinasi (CI) dihitung setelah evaluasi sitotoksisisitas kombinasi kurkumin dan kuersetin pada berbagai konsentrasi yaitu 1 IC₅₀, ½ IC₅₀, dan ¼ IC₅₀. Nilai IC₅₀ kurkumin terhadap sel HeLa dan Vero adalah 26,57 ± 2,00 μM dan 73,89 ± 12,93 μM dengan nilai SI sebesar 2,78. Sedangkan IC₅₀ kuersetin terhadap sel HeLa dan Vero adalah 149,52 ± 21,09 μM dan 1094,47 ± 15,68 μM dengan nilai SI sebesar 7,32. Nilai CI kombinasi kurkumin dan kuersetin pada konsentrasi ¼ IC₅₀ sebesar 0,78 menunjukkan efek sinergis ringan-sedang, sedangkan kombinasi pada konsentrasi 1 IC₅₀ atau ½ IC₅₀ nilai CI >0,9 menunjukkan adanya efek antagonis.
INTRODUCTION

Cervical cancer has the highest mortality rate among cancers, accounting for 60% of all reported cases. In 2018, the incidence of cervical cancer ranked second only to breast cancer, with 348,809 reported cases and 207,210 casualties. Indonesia sees an estimated 180,000 new cancer cases annually, with a staggering 75% mortality rate. Various treatments have been implemented for cancer patients, including surgery, radiation, chemotherapy, and the use of herbal medicine. The advancement of herbal remedies involves rigorous testing through in vitro, in vivo, and in silico methods to scrutinize active compounds and their respective mechanisms of action.

Secondary metabolites of medicinal plants have been widely reported to have cytotoxic activity, namely quercetin, curcumin, and rutin. Even some of them have been successfully developed as anticancer and used in clinics such as vincristine, and vinblastine. Curcumin and quercetin were combined against breast cancer cells due to their synergistic effect. Moreover, through its antioxidant effects, this combination can modulate several inflammation-mediated signaling pathways (reactive oxygen species/ROS, nitric oxide, and pro-inflammatory cytokines) thereby helping to protect cells from molecular changes that promote HDFB cell migration. However, the combination of curcumin and quercetin has not been studied for its cytotoxicity against HeLa cells.

The anticancer activity of curcumin and quercetin compounds individually is based on their activity as antioxidants, immune system enhancers, as well as antiproliferation and apoptosis promotors. Curcumin is able to inhibit metastasis and invasion of cervical cancer cells so that it can prevent spread to other organs. However, curcumin is also reported to be toxic to normal cells. Therefore, its combination with quercetin could reduce its concentration and the toxic effect on normal cells.

Based on their IC\(_{50}\) value against cancer cells, the cytotoxic activity of a compound can be categorized into 4 groups. A compound can be categorized to have high cytotoxic activity if it has IC\(_{50}\) value of \(\leq 20\ \mu\text{g/mL}\), moderate cytotoxic activity if it has an IC\(_{50}\) value between 21-200 \(\mu\text{g/mL}\), weak cytotoxic activity if it has an IC\(_{50}\) value between 201-500 \(\mu\text{g/mL}\), and no cytotoxic activity if it has an IC\(_{50}\) value of >501 \(\mu\text{g/mL}\).

The effectiveness of a combination of compounds as an anticancer can be evaluated based on its combination index (CI) value. A combination of two compounds can produce synergistic or antagonistic effects depending on the CI value obtained in an experiment. The combination is considered as synergism potential if the CI value of <1, additive effect if the CI value of 1, and antagonism if the CI value of >1.

The combination of quercetin and curcumin has been reported in previous studies. This combination proved can reduce the proliferation of breast cancer cells, myeloid leukemia cells, and melanoma cells. However, the effect of the combination of curcumin and quercetin on cervical cancer has not been studied. This study aimed to evaluate the effect of the combination of quercetin and curcumin on HeLa cells. Furthermore, the selectivity index (SI) of this combination was also evaluated.

MATERIALS AND METHODS

Materials

This study was conducted after approval by the Ahmad Dahlan University Research Ethics Committee (Ethical Clearance number 012212203). The ingredients in this study were curcumin (MERCK-Schuchardt), quercetin (Sigma-Aldrich), FBS (Fetal Bovine Serum, Gibco), stopper solution in the form of SDS (UltraPure®) 10% in 0.1 N HCl, Trypsin-EDTA 0, 25% (Gibco),
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determined by its antilog to obtain a concentration that inhibits cell death by 50% (IC$_{50}$).

**Determination of selectivity index (SI)**

The SI value reflects how optimal the sample frequency is for the test cell. If the SI value exceeds 2, it indicates that the substance has a high level of selectivity against cancer cells. The SI is calculated by the following equation:

$$SI = \frac{IC_{50\text{ normal cell}}}{IC_{50\text{ cancer cell}}}$$  \hspace{1cm} (1)

**Determination of combination index (CI)**

Determination of the CI was carried out by transferring 100 µl of HeLa cells into 5x10$^3$ wells. Next, the cells were incubated at 37 °C for 24 hr. When the cells reached 80% confluence, the medium in each well was discarded and the cells were washed with PBS. Sample treatment in the CI test was carried out by adding 50 µL of curcumin and quercetin each with a concentration of 1 IC$_{50}$, ½ IC$_{50}$, ¼ IC$_{50}$ per well (TABLE 1). The next steps were the same as the cytotoxic test.

The CI is determined from a formula that contains the combination concentration, which produces a cell viability value produced by a concentration that is equivalent to a single concentration. The data started from the absorbance of the test results for a combination of curcumin and quercetin in TABLE 1 of 50 µL each. The formula used to find the CI is:

$$CI = \frac{(C)_1}{(Cx)_1} + \frac{(C)_2}{(Cx)_2}$$  \hspace{1cm} (2)

(Cx)$_1$ and (Cx)$_2$ are the combined concentrations that provide the same effect as single concentrations, namely curcumin and quercetin, and C$_1$, C$_2$ are the concentrations of the combination of the two compounds to provide the same effect.

**TABLE 1. Concentrations of curcumin and quercetin added to the combination test with a volume 50 µL each**

<table>
<thead>
<tr>
<th>Value of IC$_{50}$</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Curcumin</td>
</tr>
<tr>
<td>1 IC$_{50}$</td>
<td>26</td>
</tr>
<tr>
<td>½ IC$_{50}$</td>
<td>13</td>
</tr>
<tr>
<td>¼ IC$_{50}$</td>
<td>6.5</td>
</tr>
</tbody>
</table>

**RESULTS**

**Cytotoxicity single curcumin and quercetin on HeLa and Vero cells**

The cytotoxicity test of curcumin and quercetin against HeLa and Vero cells was performed using a MTT assay. The morphological changes of the HeLa and Vero cells after 24 h incubation with curcumin and quercetin under microscopic examination are shown in FIGURE 1. The relationship between the log concentration of curcumin or quercetin vs. % viability of the HeLa and Vero cells is shown in FIGURE 2.
FIGURE 1. Microscopic photographs of the morphological changes of cells induced by curcumin and quercetin after 24 hr treatment and comparison with untreated controls. A. HeLa cells and B. Vero cells: 1. Control; 2. Curcumin 50 µM; 3. Quercetin 50 µM.

FIGURE 2. Viability of HeLa and Vero cells after 24 hr incubation with curcumin and quercetin in various concentration.
Based on the relationship between the log concentration vs. % of the cell viability, the IC\(_{50}\) of curcumin and quercetin was calculated. The IC\(_{50}\) values of the curcumin against the HeLa and Vero cells were 26.57 ± 2.00 \(\mu\)M and 73.89 ± 12.93 \(\mu\)M, respectively, whereas the IC\(_{50}\) values of the quercetin against the HeLa and Vero cells were 149.52 ± 21.09 \(\mu\)M and 1094.47 ± 15.68 \(\mu\)M, respectively (TABLE 2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cells</th>
<th>IC(_{50}) (mean ±SD (\mu)M)</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>HeLa</td>
<td>26.57 ± 2.00</td>
<td>2.78</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Vero</td>
<td>73.89 ± 12.93</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>HeLa</td>
<td>149.52 ± 21.09</td>
<td>7.32</td>
</tr>
<tr>
<td>Quercetin</td>
<td>Vero</td>
<td>1094.47 ± 15.68</td>
<td></td>
</tr>
</tbody>
</table>

**Selectivity index assay**

The IC\(_{50}\) value of the tested compounds was then used to determine their SI. The selectivity index results for curcumin and quercetin in HeLa cells and Vero cells were 2.78 and 7.32 (TABLE 2).

**Combination test**

The CI determination was carried out at concentrations of 1 IC\(_{50}\), ½ IC\(_{50}\), and ¼ IC\(_{50}\) with a volume of 50uL each of curcumin and quercetin (TABLE 1). The results showed changes in the morphology of HeLa cells due to changes in concentration, namely that they appeared rounded and separated from the tissue culture dish at a ratio of ¼ IC\(_{50}\). Percent of cell viability was calculated using the MTT method based on the uptake of living cells by the influence of combinations of curcumin and quercetin at various concentrations at IC\(_{50}\) and below IC\(_{50}\) (TABLE 3). The CI values obtained from 9 combinations of curcumin and quercetin in HeLa cells at concentrations of ¼ IC\(_{50}\) curcumin (6.5 \(\mu\)M) and quercetin (32.5 \(\mu\)M) was 0.78 indicating a mild-moderate synergistic effect. Whereas the combination of curcumin and quercetin at a concentration of IC\(_{50}\) or ½ IC\(_{50}\), the CI value was >0.9 indicating an antagonistic effect.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Curcumin concentration ((\mu)M)</th>
<th>Quercetin concentration ((\mu)M)</th>
<th>150</th>
<th>75</th>
<th>32.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>26</td>
<td>1.52</td>
<td>1.51</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>1.3</td>
<td>1.2</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>1.33</td>
<td>1.08</td>
<td>0.78</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

**Cytotoxicity single curcumin and quercetin on HeLa and Vero cells**

The IC\(_{50}\) value of curcumin and quercetin a HeLa cells and in normal cells (Vero cells) was carried out to calculate the SI. The results showed that curcumin has moderate cytotoxicity on both HeLa cells (IC\(_{50}\) of 26.57 ± 2.00 \(\mu\)M) and Vero cells (IC\(_{50}\) of 73.89 ± 12.93 \(\mu\)M), although curcumin is more toxic on HeLa cells compared to Vero cells (TABLE 2). The cytotoxicity of curcumin is due to active \(\alpha\)-\(\beta\) unsaturated carbonyl bonds in MCF-7, HeLa, and CEMss cells. Curcumin can reduce cell proliferation by regulating the cell cycle in the mitosis phase.\(^6\) In line with curcumin, quercetin also has unsaturated \(\alpha\)-\(\beta\) bonds carbonyl, which has activity in MCF-7 cells and HeLa cells. Quercetin has anticancer activity by reducing metastasis and inhibiting proliferation. This is because quercetin has C\(_{3'4'}\) dihydroxy, C\(_{2-3}\) unsaturated carbonyl, as well as C\(_3\) and C\(_5\) hydroxy, which have antioxidant activity. The result of this activity is inhibition of telomerase, thereby reducing cell proliferation.\(^7\)

The cytotoxic activity of curcumin and quercetin through induction of apoptosis by damaging cancer cell DNA encourages research on the synergy of this combination.\(^6\) This is in line with the
potential of the flavonoid in this research, namely quercetin, to be combined with cytotoxic compounds. Another flavonoid, namely naringenin, has potential as an adjuvant because its structure is rich in chromophores. Likewise, curcumin has the potential to act as an anti-cancer agent when combined with other cytotoxic compounds, which are synergistic.

**Selectivity index assay**

These results indicate that curcumin and quercetin have a high level of selectivity against HeLa cells (TABLE 2). Curcumin has high safety in Vero cells, as indicated by a SI value most 3 (2.78). The IC₅₀ results of curcumin and quercetin on Vero cells show that although single curcumin has an effect on HeLa cells, it also has an IC₅₀ that is toxic to Vero (normal) cells. This supports the need to combine it with co-chemotherapy, in this case, quercetin. The CI is used to evaluate whether the combination is synergistic, additive, or antagonistic at a certain concentration.

**Combination test**

The CI value provides information concerning the interaction of two compounds as synergic, additive, or antagonistic. This study showed that the interaction of curcumin and quercetin at small concentration (¼ IC₅₀) provide a synergistic effect. In contrast, at higher concentration (1 IC₅₀, ½ IC₅₀) provide an antagonistic effect (TABLE 3).

Curcumin and quercetin single have various pathways in induce of apoptosis in cancer cells. The molecular mechanism of anticancer combination of curcumin and quercetin is through the Wnt/β-catenin pathway, which can produce various types of genes, some of which are cyclooxygenase 2 (COX₂) and cyclin D1. In various types of tumors, it is found that cyclin D1 is overexpressed so that it can facilitate cells from the Gap1 phase to the synthesis (S) phase. Curcumin and quercetin are known to influence the regulation of cyclin D1 and Axin2, which can then reduce β-catenin, thereby suppressing cell proliferation genes.

Combination of quercetin and curcumin increased BRCA1 histone H3K9 promoter acetylation. Quercetin regulates the expression levels of proinflammatory agents such as COX₂ and controls histone acetylation. Quercetin can also inhibit β-catenin expression. Cell death via apoptosis can be triggered via receptor-mediated signaling pathways or via mitochondria. Drugs with chemotherapy or other stimulant effects can change the inner properties of the mitochondrial membrane and cause the mitochondrial membrane to lose its potential and release cytochrome c from the mitochondria into the cytosol.

The combination of curcumin and quercetin induces apoptosis through the mitochondrial pathway and inhibits AKT and ERK phosphorylation. Similar research shows that quercetin and curcumin can trigger apoptosis through the mitochondrial pathway, making them potentially anticancer. Further research can be carried out to determine the mechanism of cell death by the combination of curcumin and quercetin at a ratio of 6.5 µM curcumin and 32.5 µM quercetin, as well as track the IC₅₀ of the combination on the synergistic effect.

A limitation of this study is that the IC₅₀ of the combination of curcumin and quercetin was not determined after determining the CI. It is necessary to determine the IC₅₀ of the combination to determine the level of toxicity obtained after the combination. To find out more about the mechanisms and pathways of cell death, it is also necessary to test using immunocytochemical or flow cytometer methods.

**CONCLUSION**

The combination of curcumin and quercetin higher than ¼ IC₅₀ has antagonistic activity with a CI value >0.9, whereas at concentrations lower than ¼
IC\textsubscript{50}, has a mild to moderate synergistic level with a CI value of 0.78. The curcumin and quercetin combination at this concentration have cytotoxic activity against HeLa cells with high SI.

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