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Research Article

Synergistic Effect of *Imperata cylindrica* Root Extract and Erlotinib on A549 Lung Cancer Cell Viability

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ABSTRACT

Background: Erlotinib is typically used to treat lung adenocarcinoma and *Imperata cylindrica* extract has been shown to exert anti-cancer effects in various human cancer cell lines. Therefore, this study evaluated the combined anticancer effects of *Imperata cylindrica* ethanol root extract and erlotinib on the A549 lung cancer cell line. **Method:** The A-549 lung cancer cell line was treated with various combinations of 1/2 IC_{50}, 3/8 IC_{50}, 1/4 IC_{50}, and 1/8 IC_{50} of *I.cylindrica* root ethanol extract and erlotinib for 48 hours. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, analyzed and interpreted using CompuSyn software and a normalized isobologram curve. **Result:** Of the sixteen combinations, eleven combinations acted synergistically, four were additive, and one was in the antagonist zone. **Conclusion:** The combination of *I. cylindrica* root ethanol extract and erlotinib act synergistically to decrease A549 lung cancer cell viability, therefore they are potential lung cancer therapeutics.

Keywords: A549, Cell viability, Erlotinib, *Imperata cylindrica.*

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INTRODUCTION

Based on the 2020 Global Cancer Statistics (GLOBOCAN), lung cancer has the highest mortality rate (18% of total cancer deaths), followed by colorectal cancer (9.4%), liver (8.3%), stomach (7.7%), and breast cancer in women (6.9%).[1] The World Health Organization (WHO) classifies lung cancer based on the origin of the cancer cells and histological changes into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) which is the most common type of lung cancer.[2]

The current treatment for lung cancer is to use targeted therapy, with NSCLC patients showing good sensitivity to therapy according to the National Comprehensive Cancer Network (NCCN, 2021). Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) such as Erlotinib[3] inhibit EGFR activity by competing with ATP to occupy binding sites in the EGFR tyrosine kinase domain, thereby inhibiting cancer cell proliferation, invasion, metastasis, and angiogenesis and inducing apoptosis.[4]

Alang-alang (*Imperata cylindrica*) is a weed that thrives in Indonesia and has been used for generations as a medicinal plant for its anti-inflammatory, anti-oxidant, anti-bacterial, immunomodulator, and hepatoprotective properties.[5,6] Several studies have also shown that the leaf extract has anticancer effects on human oral squamous carcinoma cells (SCC-9,7) and exerts antioxidant and anticancer effects in human breast cells (MCF-7,8 and BT-549) and HT-299 colon cells.[7,8,9]

MATERIAL AND METHODS

Reagents

Erlotinib (Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Cell viability was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Cell culture

The A549 human lung adenocarcinoma cell line was purchased from the American Type Culture Collection (ATCC), USA and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% inactivated fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO_2 until 80 to 90% confluent. The cell monolayers were trypsinized (0.05% trypsin) and seeded into 96 well plates for 48 h at 37°C at a density of 1 x 10^4 cells/well for cytotoxicity studies.

Ethanol extract and erlotinib preparation

First, 100 g of powdered *Imperata simplicial* roots was mixed with ethanol for 24 h at 20–22°C and then filtered. The filtrate was evaporated and concentrated using a rotavapor at 50°C to produce an ethanol extract which was then serially diluted to prepare working stock (1000, 500, 250, 125, 62.5, 31.75, and 15.625 µg/mL). Erlotinib was serially diluted (100, 50, 25, 12.5, and 6.25 µM) to obtain the Inhibition concentration (IC) value of 50.[10]

MTT assay

A 5 mg/ml MTT (Sigma-Aldrich) stock solution was prepared by adding 50 mg MTT to 10 mL

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sterile phosphate-buffered saline (PBS) (Sigma-Aldrich) and pre-heated at 25–26°C and filter sterilized using a 0.2 µm pore sized syringe filter before use. The A549 cells were seeded (1 x 10^4 cells in 200 µL of complete culture medium) into TPP 96 well microplates (Thermo Fisher Scientific) and incubated for 12–24 hr at 37° C until they reached 80% confluency. The cells were treated in triplicate with the test solutions in culture media for 48 hrs, washed twice with pre-heated PBS before 90 µL of fresh media and 10 µL of MTT solution was added to each well to obtain a final MTT concentration of 0.5 mg/ml. After 4 hr, the MTT-containing medium was carefully aspirated and the resulting blue formazan crystals were solubilized with 100 µL of DMSO. The plates were shaken gently and the optical density was measured using an ELISA plate reader (Multiskan EX, Thermo Fischer Scientific) at a wavelength of 550 nm.

Combination test

The drug combinations were evaluated by the Combination Index (CI) and the Isobologram curve using CompuSyn version 1.0 software according to the following equation:

$$CI = (D)1/(Dx)1 + (D)2/(Dx)2$$

Where Dx is the concentration of one single compound needed to give the same effect as the combined effect, namely IC_{50} on A549 cell growth while (D)1 and (D)2 are the concentrations of the two compounds to give the same effect. The CI value is used to determine the additive effect of the two compounds (synergistic, additive, or antagonistic). The series of *Imperata cylindrica* ethanol extract and erlotinib concentrations tested were: 1/2 IC_{50}, 3/8 IC_{50}, 1/4 IC_{50}, and 1/8 $IC_{50,}$ and the combination test treatment map is shown below:[11]

	1	2	3	4	5	6	7	8	9	CONTROL
A	1/2 IC50 EAA	1/2 IC50 EAA	1/2 IC50 EAA	1/2 IC50 EAA 1/2 IC50 Erlo	1/2 IC50 EAA 1/2 IC50 Erlo	1/2 IC50 EAA 1/2 IC50 Erlo	1/2 IC50 EAA 1/4 IC50 Erlo	1/2 IC50 EAA 1/4 IC50 Erlo	1/2 IC50 EAA 1/4 IC50 Erlo	
B	3/8 IC50 EAA	3/8 IC50 EAA	3/8 IC50 EAA	3/8 IC50 EAA 1/2 IC50 Erlo	3/8 IC50 EAA 1/2 IC50 Erlo	3/8 IC50 EAA 1/2 IC50 Erlo	3/8 IC50 EAA 1/4 IC50 Erlo	3/8 IC50 EAA 1/4 IC50 Erlo	3/8 IC50 EAA 1/4 IC50 Erlo	
C	1/4 IC50 EAA	1/4 IC50 EAA	1/4 IC50 EAA	1/4 IC50 EAA 1/2 IC50 Erlo	1/4 IC50 EAA 1/2 IC50 Erlo	1/4 IC50 EAA 1/2 IC50 Erlo	1/4 IC50 EAA 1/4 IC50 Erlo	1/4 IC50 EAA 1/4 IC50 Erlo	1/4 IC50 EAA 1/4 IC50 Erlo	
D	1/8 IC50 EAA	1/8 IC50 EAA	1/8 IC50 EAA	1/8 IC50 EAA 1/2 IC50 Erlo	1/8 IC50 EAA 1/2 IC50 Erlo	1/8 IC50 EAA 1/2 IC50 Erlo	1/8 IC50 EAA 1/4 IC50 Erlo	1/8 IC50 EAA 1/4 IC50 Erlo	1/8 IC50 EAA 1/4 IC50 Erlo	
E	1/2 IC50 Erlo	1/2 IC50 Erlo	1/2 IC50 Erlo	1/2 IC50 EAA 3/8 IC50 Erlo	1/2 IC50 EAA 3/8 IC50 Erlo	1/2 IC50 EAA 3/8 IC50 Erlo	1/2 IC50 EAA 1/8 IC50 Erlo	1/2 IC50 EAA 1/8 IC50 Erlo	1/2 IC50 EAA 1/8 IC50 Erlo	Cell control
F	3/8 IC50 Erlo	3/8 IC50 Erlo	3/8 IC50 Erlo	3/8 IC50 EAA 3/8 IC50 Erlo	3/8 IC50 EAA 3/8 IC50 Erlo	3/8 IC50 EAA 3/8 IC50 Erlo	3/8 IC50 EAA 1/8 IC50 Erlo	3/8 IC50 EAA 1/8 IC50 Erlo	3/8 IC50 EAA 1/8 IC50 Erlo	
G	1/4 IC50 Erlo	1/4 IC50 Erlo	1/4 IC50 Erlo	1/4 IC50 EAA 3/8 IC50 Erlo	1/4 IC50 EAA 3/8 IC50 Erlo	1/4 IC50 EAA 3/8 IC50 Erlo	1/4 IC50 EAA 1/8 IC50 Erlo	1/4 IC50 EAA 1/8 IC50 Erlo	1/4 IC50 EAA 1/8 IC50 Erlo	Media Control
H	1/8 IC50 Erlo	1/8 IC50 Erlo	1/8 IC50 Erlo	1/8 IC50 EAA 3/8 IC50 Erlo	1/8 IC50 EAA 3/8 IC50 Erlo	1/8 IC50 EAA 3/8 IC50 Erlo	1/8 IC50 EAA 1/8 IC50 Erlo	1/8 IC50 EAA 1/8 IC50 Erlo	1/8 IC50 EAA 1/8 IC50 Erlo	

The absorbance was measured after 48 h at a wavelength of 550 nm and the percentage of live cells was calculated using the formula in CompuSyn ver 1.0 software:

$$\text{Percentage of live cells} = \frac{\text{Absorbance of treatment} - \text{Absorbance of media control}}{\text{Absorbance of cell control} - \text{Absorbance of media control}} \times 100$$

RESULTS

Effect of the *Imperata cylindrica* root ethanol extract and erlotinib on A549 cells

The anticancer potential of serial dilutions of the *Imperata cylindrica* root ethanol extract and erlotinib on A549 cells was assessed using the MTT assay. Figure 1A shows that the ethanol extract has a weak cytotoxic effect as evidenced by an IC_{50} of 541 µg/mL in comparison to erlotinib which has a moderate toxic effect (IC_{50} = 29 µM; Figure 1B).

Cytotoxicity of the combination of *Imperata* root ethanol extract and erlotinib on A549 cells

The cytotoxic effects of a series of concentrations (1/2 IC_{50}, 3/8 IC_{50}, 1/4 IC_{50}, and 1/8 IC_{50}) of the *Imperata cylindrica* root ethanol extract (Table 1) and erlotinib (Table 2) were evaluated.

Table 1. Effect of *Imperata cylindrica* root ethanol extract on A549 cells.

Dosage	Concentration (µg/mL)	Viability (%)
1/2 IC_{50}	270.5	68.53
3/8 IC_{50}	202.875	87.54
1/4 IC_{50}	135.25	80.99
1/8 IC_{50}	67.625	96.55

Table 2. Effect of erlotinib on A549 cells.

Dosage	Concentration (µM)	Viability (%)
1/2 IC_{50}	14.5	63.90
3/8 IC_{50}	10.875	65.32
1/4 IC_{50}	7.25	67.04
1/8 IC_{50}	3.625	88.53

Table 3. The Combination Index.[12]

CI value	Interpretation
<0.1	very strong synergism
0.1–0.3	strong synergism
0.3–0.7	synergism
0.7–0.9	moderate-slight synergism
0.9–1.1	nearly additive
1.1–1.45	slight to moderate antagonism
1.45–3.3	antagonism
>3.3	very strong antagonism

Table 4. CI of the combinations of *Imperata cylindrica* root ethanol extract and erlotinib on A549 cells.

Combination number	Ethanol extract concentration (µg/mL)	Erlotinib concentration (µM)	Viability (%)	CI
1	270.5	14.5	0.479	0.81693
2	202.875	14.5	0.515	0.88050
3	135.25	14.5	0.516	0.84250
4	67.625	14.5	0.684	1.38765
5	270.5	10.875	0.429	0.54355
6	202.875	10.875	0.533	0.73980
7	135.25	10.875	0.537	0.70090
8	67.625	10.875	0.676	1.03505
9	270.5	7.25	0.486	0.49200
10	202.875	7.25	0.679	0.96576
11	135.25	7.25	0.695	0.91193
12	67.625	7.25	0.731	0.91189
13	270.5	3.625	0.445	0.27089
14	202.875	3.625	0.529	0.33603
15	135.25	3.625	0.633	0.43811
16	67.625	3.625	0.737	0.55222

The effects of the test compounds on A549 cells were assessed using the Combination Index (CI) in CompuSyn software (Table 3) and the CI results of the combinations of root ethanol extract and erlotinib are shown in Table 4.

The isobologram in Figure 2 shows the synergistic, additive, and antagonistic effects of the two test compounds indicating that eleven combinations were in the synergistic zone, four were in the additive zone, and one was in the antagonist zone based on isobologram curve interpretation showed in Figure 3.

DISCUSSION

Erlotinib inhibits the activity of the tyrosine kinase of EGFR by competing with ATP to occupy the binding site in the EGFR

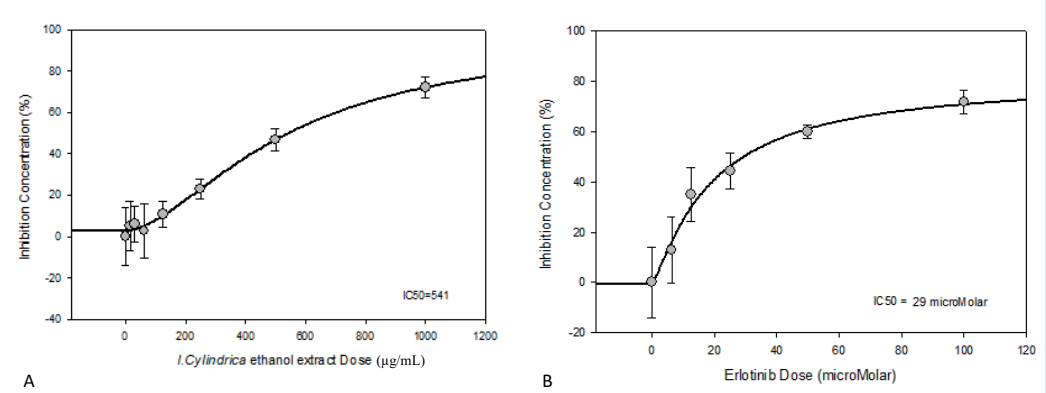
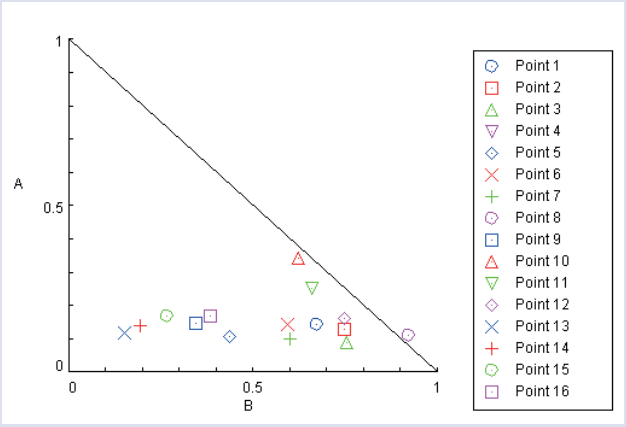


Figure 1. Cytotoxic effect of *I. cylindrica* root ethanol extract on A549 cells with IC_{50}=541 µg/mL (A), Cytotoxic effect of erlotinib on A549 cells IC_{50} = 29 µM (B).



270,5 IEE + 14,5 Erlo
202,5 IEE + 14,5 Erlo
135,25 IEE + 14,5 Erlo
67,25 IEE + 14,5 Erlo
270,5 IEE + 10,875 Erlo
202,5 IEE + 10,875 Erlo
135,25 IEE +10,875 Erlo
67,25 IEE +10,875 Erlo
270,5 IEE + 7,25 Erlo
202,5 IEE + 7,25 Erlo
135,25 IEE + 7,25 Erlo
67,25 IEE + 7,25 Erlo
270,5 IEE + 3,625 Erlo
202,5 IEE + 3,625 Erlo
135,25 IEE + 3,625 Erlo
67,25 IEE + 3,625 Erlo
A (D)2/(Dx)2 Erlo
B (D)1/(Dx)1 IEE

IEE: *I.cylindrica* ethanol extract

Figure 2. The combined effects of *Imperata cylindrica* root ethanol extract and erlotinib.

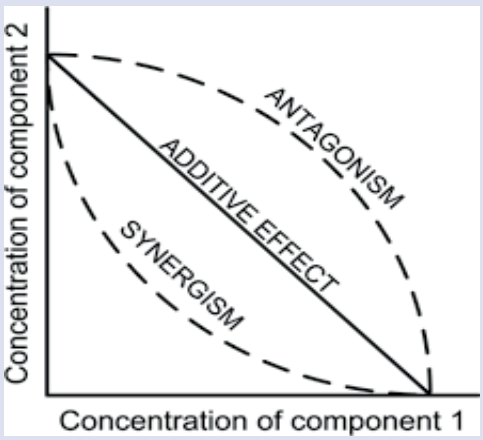


Figure 3. Isobologram Curve Interpretation.

tyrosine kinase domain, thereby inhibiting cancer cell proliferation, angiogenesis, invasion, and metastasis and inducing apoptosis.[4] Hence, erlotinib is widely approved for the treatment of NSCLC in the world, namely by the FDA in November 2004 and EMEA in November 2005.[13] *Imperata cylindrica* has many benefits due to its anti-inflammatory, antioxidant, antibacterial, immunomodulatory, hepatoprotective, and anticancer properties.[14]

The A549 cell line consists of alveolar basal epithelial cells and is typically used as a model of NSCLC.[15,16] In the development of new anticancer drugs, one of the most important aspects is their cytotoxicity. The cytotoxic test is usually a screening process to determine the effect or ability of a natural ingredient to inhibit cancer cells. A compound is considered active if it can inhibit the growth of 50% of the cancer cell population at a certain concentration. The requirements that must be met for a cytotoxicity test include being able to produce a usable and repeatable dose-response curve with low variability and showing a linear relationship between the dose given and the effects. The MTT method is widely used to determine the cytotoxicity of a compound (IC value). In this study, the ethanol extract exhibited a weak cytotoxic effect and the IC_{50} value for erlotinib was similar to previous studies.[17,18] The weak cytotoxic effect of the ethanol extract of *Imperata* root could be caused by a weaker ethanol extract containing less active compounds such as phenolic compounds, flavonoids, and alkaloids in a natural product compared to methanol extracts.[19] The extraction solvent will determine the amount and activity of the active compounds contained therein.[20]

Combination cytotoxic tests were then performed to determine if the *Imperata cylindrica* root ethanol extract and erlotinib could act synergistically as standard therapy in the lung cancer cell line A549. Drug synergistic effects can be produced when two compounds are given simultaneously and the resulting effect is greater than the sum of the effects of each drug individually.[21] According to the results, eleven combinations acted synergistically, four were additive zone, and one was in the antagonist zone. The highest synergism was exhibited by the combination of 1/2 IC_{50} of the *Imperata* extract and 1/8 dose of IC_{50} erlotinib which resulted in cancer cell viability of 44.5%. These results indicate that erlotinib at the smallest dose has a synergistic effect when given together with the ethanol extract of *I. cylindrica* roots. The synergistic effect of the active fraction contained in reed extract, namely the combination of sinensetin and imperatorin, has also been shown to have a synergistic effect in inhibiting the viability of A549 cancer cells in vitro when compared to administration separately.[22]

CONCLUSION

This study provided evidence supporting the potential anticancer effect of a combination of *Imperata cylindrica* root ethanol extract and erlotinib on A549 lung cancer cells.

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