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Cooperative Impact of Curcuma longa and Phyllanthus niruri Extracts on Cytotoxicity in HCT-116 Cells

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ABSTRACT

Background: The combination of herbal extracts has the potential to enhance cytotoxic effects against cancer cells. This study explores the combined cytotoxic effects of Curcuma longa and Phyllanthus niruri extracts on HCT-116 colorectal cancer cells. **Objective:** To assess the cytotoxic effects of *Curcuma longa* and *Phyllanthus niruri* extracts when used in combination and to determine the most effective ratio for inhibiting HCT-116 cell growth. **Methods:** HCT-116 cells were treated for 24 hours with varying concentrations of *Curcuma longa* and *Phyllanthus niruri* extracts based on the IC50 values of each extract administered individually. The concentrations for *Phyllanthus niruri* were 164 µg/mL (one part), 82 µg/mL (half part), and 41 µg/mL (quarter part), while for *Curcuma longa* the concentrations were 47 µg/mL (one part), 24 µg/mL (half part), and 12 µg/mL (quarter part). Cytotoxicity was assessed using the MTT assay. **Results:** The combination of *Phyllanthus* niruri and *Curcuma longa* extracts demonstrated varied cytotoxic effects. The most effective combination was identified as *Phyllanthus niruri* to *Curcuma longa* extract ratio of 1:0.25, resulting in a 13.5% cell viability rate. Interaction studies using the Chou-Talalay method indicated that the combination index (CI) revealed the most synergistic effect at a ratio of 0.25:0.50. **Conclusion:** The study identifies that the combination of *Phyllanthus niruri* and *Curcuma longa* extracts exhibits synergistic cytotoxic effects on HCT-116 cells, with the optimal combination showing significant inhibition of cell growth. These findings support further investigation into the synergistic potential of these extracts for colorectal cancer therapy.

Keyword : Curcuma longa, Phyllanthus nirusi, Combination Index, Colorectal Cancer, MTT-assay

INTRODUCTION

Colorectal cancer has been declared as the third most frequent type of cancer and the second leading cause of cancer-related death globally in 2020, there has been a surge in cases, with 1.9 million confirmed and 0.9 million reported deaths[1]. Colorectal cancer incidence is expected to increase by over 60% in 2030, resulting in more than 1.1 million mortality and 2.2 million new cases. There is a rising trend of CRC among young individuals, particularly in developed nations, attributed to life style and environmental factors. Chemotherapy regimens for CRC patients encompass anti-VEGF, topoisomerase, anti-EGFR, and fluoropyrimidines drug in particular Bevacizumab, Cetuximab, Irinotecan, and 5-fluorouracil, where the drugs have adverse effects on human health[2]. These circumstances provide a prospect for other therapies such as herbs medicine, to enhance treatment efficacy and improve quality life of patients.

The utilization of conventional remedies from natural components has been extensively practiced based on empirical evidence. Herbal remedies are known to possess various properties that can specifically target the eradication of cancer cell, without interfering the surrounding healthy cells, this is the reason to investigated plant extracts like *Curcuma longa* and *Phyllanthus niruri* on cancer cells[3,4,5].

Curcuma longa contains curcumin compounds that exhibit anti-cancer properties across various types of cancer cells[6,7]. Previous studies have revealed that curcumin operates through multiple mechanism in relation to cancer cell, including anti-proliferative effect through the inhibition of regulatory pathway AP-1 and STAT3, leading to decreased cancer cell growth. Additionally, curcumin hinders NF-κB activity, impacting the regulation of Bcl2, COX2, and MMP9, resulting in cell inhibition, suppression cell growth, and cell mortality. Moreover, curcumin can induce apoptosis by upregulating the expression of p53 gene, leading to mortality on G2 phase. Activation of p53 significantly enhance the expression of pro-apoptotic genes such as Bax [8,9,10].

Phyllanthus niruri is rich in various chemical compounds including phyllantin, hypophyllanthin, nirantin, isolintetralin, quercetin, astragilin, alkaloid, tanin, triterpenoids, and phenolic substances[11]. Studies have shown that *Phyllanthus niruri* has the potential to inhibit cell growth and disrupt signal cascade in cancer cells such as lung cancer, prostate cancer, breast cancer, and hepatocellular cancer[12]. Recent research indicates it has apoptosis mechanism in cancer cell by activating caspase-3, producing TNF-α, and suppressing IL-8 and COX-2 expression[13]. Furthermore, the upregulation of granzyme expression has been observed through the perforin-granzyme pathway in previous research studies involving the administration of *Phyllanthus niruri* extract[14].

MATERIALS AND METHOD

Plant material

The *Curcuma longa* rhizomes and *Phyllanthus niruri* herbs are obtained from supplier in B2P2TOOT (Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional) in Tawangmangu, Central Java, Indonesia. Subsequently, the plant material is dried using cabinet dryer at 40 - 50°C.

Extraction procedure

The dried *Curcuma longa* rhizomes and *Phyllanthus niruri* herbs undergo water content testing to ensure compliance with Farmakope Herbal Indonesia 2nd Edition standards before undergoing size reduction using a 60-mesh grinder. The *Curcuma longa* powder and *Phyllanthus niruri powder* separately extracted through maceration using an ethanol solvent in 1 : 10 ratio for 3 days, followed by re-maceration process. The filtrate is subsequently evaporated using vacuum evaporator at 40 - 50°C to yield a concentrated extract.

Cell material

Colorectal cancer HCT116 cells were acquired from the European Collection of Authenticated Cell Cultures (EACC, 91091005).

Cell culture and treatments

The HCT116 cells were cultured and maintained in F12 Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1.5% Penicillin-streptomycin antibiotic solutions (Gibco, USA) at 37°C and 5% CO2. After the cultured cells reached 80% confluency, they were treated with CLE and PNE in serial dose.

Cell Viability Test

The MTT assay was utilized to assess cell viability with a minor adjustment. In summary, 5 x 103 cell/well were plated in 96-well plate and kept at 37°C with 5% CO2 for 24 hours. Following this, cell was exposed to combination of CLE (5 – 200 µg/mL) and PNE (5 – 200 µg/mL) for 24 hours. Cells that were not treated served as negative control. Post – treatment, cells were exposed to 0.5 mg/mL of MTT and further incubate for 4 hours. After incubation, 100 µL of DMSO was added in the well and then incubated for 15 minutes. Subsequently, the absorbance was determined using ELISA reader (Biorad iMark TM Microplate Reader) at wavelength 595 nm. The absorbance readings were converted into percentages of cell viability by comparing the treated group with untreated group at specific time points. The IC50 value was determined by performing linear regression between the concentration (x) and % cell viability (y), resulting in the equation y = Bx + A. By using the linear equation, the x value at y = 50% was identified as the IC50 value, representing the concentration that inhibits 50% of cell proliferation. The experimental data for this research was obtained from three independent replication experiments.

Cytotoxic assay combination herbs

We assessed the efficacy of a specific concentration of *Curcuma longa* extract (CLE) and *Phyllanthus niruri* extract (PNE), and their combination on HCT116 cell in the initial combination research using the MTT assay. HCT116 cell were plated in a 96-well microplate and incubated at 37°C with 5% CO2 for 24 hours. Subsequently, the cells were exposed to one part, half part, and quarter part of IC50 CLE, PNE, and their combination for 24 hours. Following the treatment, the percentage of viable cells was determined utilizing the aforementioned in vitro cytotoxicity assay technique.

RESULT

Cytotoxic effect of Curucma longa and Phylanthus niruri extract on HCT166 cells

The cytotoxicity of CLE and PNE on HCT116 cells was evaluated at various concentrations and incubated for 24 hours, followed by assessment using the MTT assay. The toxic properties were found to be concentration – dependent, with higer concentrations showing greater effectiveness in reducing the growth of HCT116 cells. Treatment with PNE alone demonstrated the most potent activity, with an IC50 value of 163.33 µg/mL (Figure 1a). Additionally, morphological changes in HCT116 cells were observed, including a decrease in cell population, presence of apoptotic bodiesa, and cells with dark coloration indicative of apoptosis. This study reveals that PNE has a clear cytotoxic effect on HCT116 cells by inducing apoptosis and inhibiting cell growth in dose-dependent manner.

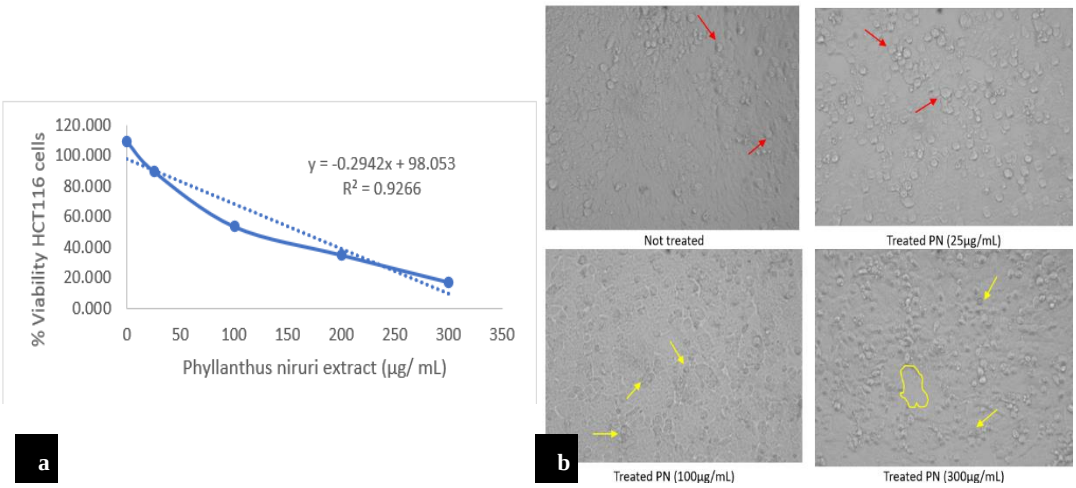


Figure 1. Correlation between the concentration of *Phyllanthus niruri* extract and the inhibition of HCT116 cell growth, showing: a) Linear suppression as the concentration of Phyllanthus niruri increases. b) The morphological changes in HCT116 cells are evident when comparing untreated cells to those treated with Phyllanthus niruri extract, indicating variations in cell population based on the concentration levels applied. The red arrow represents the typical state of HCT116 cells, while the yellow/ yellow-shaped arrow signifies harm to both the morphology and population of HCT116 cells.

The treatment of HCT116 cells with CLE, a similar outcome was achieved, resulting in a cell growth inhibition value of IC50 46.606 µg/mL (Figure 2a). Upon microscopic examination, it was observed that some cells underwent morphological changes, such as shrinkage, indicating a decrease in size, and pyknosis characterized by purple chromatin condensation or dark cytoplasmic eosinophils, suggesting apoptosis. Apoptosis was noticable at a concentration of 60 µg/mL (Figure 2b). Furthurmore, at a concentation of 100 µg/mL, CLE induced the formation of apoptotic bodies containing cytoplasm densely packed with organelles, along with nuclear fragments. These apoptotic bodies are formed through a process known as "budding", involving plasma membrane swelling followed by karyorrhexis[16].

Synergistic effect of cytotoxic activity Curcuma longa and Phyllanthus niruri extract on HCT116 cell

HCT116 celss underwent a 24-hour treatment with combination of CLE and PNE, with concentrations determined based on single extract IC50 values. Subsequently, an MTT assay was conducted to assess the cytotoxic effects of the combination. The concentration of PNE for combination test inculded one part (164 µg/mL), half part (82 µg/mL), and quarter part (41 µg/mL), while for CLE, it was one part (47 µg/mL), half part (24 µg/mL), and quarter part (12 µg/mL). The outcomes of various combination test are illustrated in Figure 3, higlighting that the most effective concentration in inhibiting cell growth of HCT116 cells is combination of CLE:PNE (0.25 : 1) with 13.5% viability rate. The assessment of interactions between CLE and PNE was carried out through variation test utilizing the Chou-Talalay method[15], particularly focusing on the combination index. These interactions can exhibit synergistic, additive, or antagonist properties. The evaluation result will identify the most synergistic combinatin of CLE and PNE to achieve the optimal effect on inhibiting cell growth or promoting cell apoptosisi in HCT 116. The evaluation of the combination index (CI) revealed that the most synergistic combination is PNE: CLE (0.25 : 0.50).

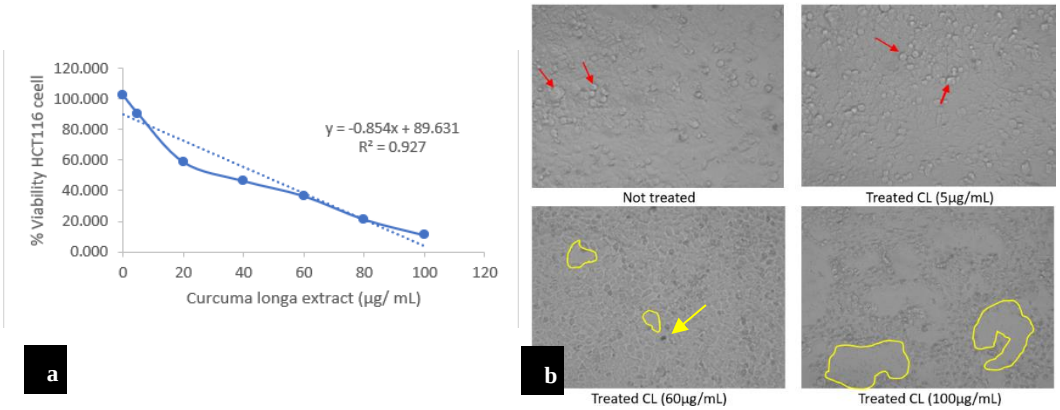
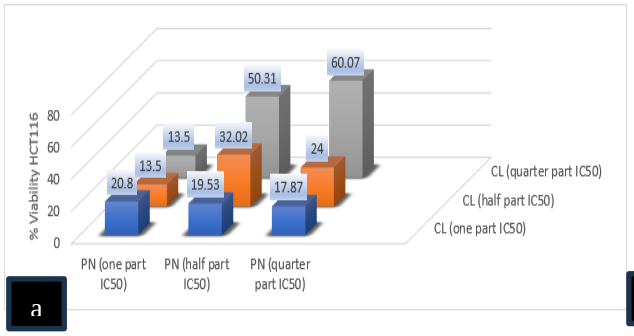
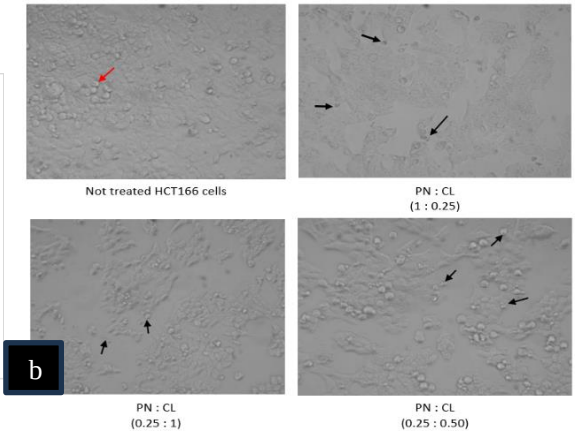


Figure 2. The concentration of CLE has direct impact on the inhibition of HCT116 cells growth, demonstrating: a) Consistent decrease in cells growth as the concentration of CLE increases. b) Observable changes in the morphology of HCT116 cells when comparing untreated cells to those treated with CLE, suggesting differences in cell population based on the levels of concentration used. The red arrow indicates the typical state of HCT116 cells, while the yellow/ yellow-shaped arrow indicates damage to both the morphology and population HCT116 cells.





Combination Index	Intepretation
< 0.1	Very strong synergism
0.1 – 0.3	Strong synergism
0.3 – 0.7	Synergism
0.7 – 0.9	Moderate to slight synergism
0.9 – 1.1	Nearly additive
1.10 – 1.45	Slight to moderate antagonism
1.45 – 3.30	Antagonism
> 3.3	Strong to very strong antagonism

c

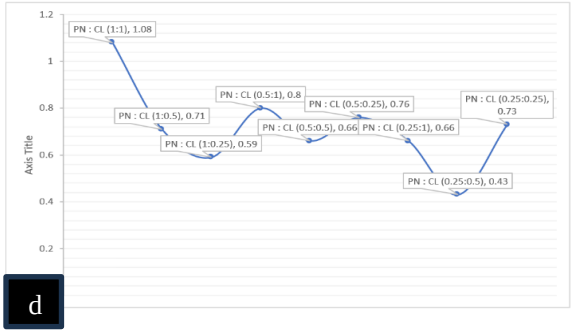


Figure 3. Effect of the *CLE* and *Phyllanthus niruri* extract combination on HCT116 cells at various concentrations. a) analysis of cell viability following a 24-hours treatment with a blend of CLE and PNE extract. The most significant growth inhibition of HCT116 cells is observed with the PNE : CLE (1 :0.25) combination. b) Examination of morphological alterations in HCT116 cells post exposure to the combined extracts, revealing apoptosis characteristics such as cell shrinkage, pyknosis, and the presence of apoptotic bodies (black arrow). c) Tabel of combination index (CI) interpretations. d) Evaluation of the combination index profile across different PNE:CLE combinations, highlighting the most synergistic combination by the lowest value.

DISSCUSION

Based on research findings, the combination of PNE:CLE has demonstrated cytotoxic effects on HCT cells, resulting in synergistic and robust outcomes at a concentration ratio of 0.25 : 0.5 (Figure 3a and 3d). This supports previous studies suggesting that CNE and PNE possess the capability to inhibit or eliminate cancer cells and tumors through various mechanism.

The process of apoptosis triggered by PNE through the enhancement of the immune system is characterized by an increased infiltration of lymphocytes, as well as an increase in perforin secretion by NK and CTL. The heightened presence of competent immune cells boosts the immunological status, leading to a more effective immune response against tumor cells and ensuring the elimination of cancerous cells[17]. Effector cells first express the TNF ligand group, while the second pathway involves perforin-funded granzyme pathway, releasing destructive cytotoxic granules from CTl/NK to target cells [18, 19, 20, 21] . Previous studies have shown that PNE significantly increase granzyme expression, thus promoting apoptosis through the perforin-granzym pathway. Granzyme B triggering caspase – 3 and other apoptosis carriers like caspase – 6, dan caspase – 7. On other hand, Granzyme A activates a parallel pathway that induces cell death independently of caspases, aiding in the destruction of single – stranded DNA[22, 23].

Curcumin compound found in CLE is flavonoid that offer a range of beenefits, including antioxidant, anti-inflammatory, chemoctherapeutic, chemopreventife, anti-mutagenic, anti – metastatic, and anti- angiogenetic effects[24]. Research findings indicate that curcumin plays a role in the apoptosis of colorectal cancer cells through various molecular targets, including enzyme (COX-2), transcription factror(NF-κB, Ap-1), ROS, Bcl-2 compounds group (Bak, Bcl-2, Bax), protease enzymes (caspase 2, caspase 8), and signaling pathways such p53, P13K/AKT, JNK, and ER stress. Additionally, curcumin has been found to suppress the level of Bcl-2 and upregulate Bax, leading to cytotoxic effects in HCT116 and COLO-205 colorectal cancer cells by affecting Ca2+ release through the ER (endoplasmic eticulum) membrane, ultimately inducing apoptosis [25].

Curcumin not only exhibits cytotoxic properties but also demonstrates the ability to enhance the sensitivity of chemotherapy-resistant cancer cells. For instance, research findings indicate that curcumin can render colorectal cancer cells sensitive to capecitabine by modulating the expression of cyclin D1, COX-2, MMP-9, VEGF, and chemokine receptor CXCR4 in orthotopic mouse models[26]. Additionally, another study revealed that curcumin effectively increased the sensitivity of HCT-8/VCR resistant cells to Vincristine (VCR), Cisplatin (DDP), Fluorouracil (5FU), and Hydroxycamptothecin (HCPT) [27].

After analyzing the outcomes and cytotoxic process of PNE and CLE, it is evident that the joint utilization of these extracts results in a synergistic impact. This is supported by alterations in cell structure leading to apoptosis (such as shrinkage, pyknosis, karyorrhexis, and apoptotic bodies) and the viability percentage of HCT116 post 24-hour treatment. Apart from serving as an alternative to chemotherapy, this combination has the potential to enhance the immune system and boost the susceptibility of resistant cancer cells, making it a valuable supportive therapy for cancer patients. However, further research is required to validate the molecular mechanism behind this combination.

CONCLUSION

According to the research, the combination of *Phyllanthus niruri* and *Curcuma longa* extract had a synergistic impact on inhibiting the growth of HCT116 cells, indicating potential for enhancing cancer treatment strategies.

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CONTRIBUTORS

SI conceptualized and designed the study. NH and FNPR conducted the experiments, while DG also performed the data analysis. The manuscript was written and edited by DG, with FNPR also contributing to the manuscript review.

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