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The Potential of *Centella asiatica* Herb and *Cyperus rotundus* L. Rhizomes Extract as a Chemoprevention Agent for Lung Cancer

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Abstract

BACKGROUND: The number of smokers is increasing in Indonesia. Cigarette smoke can cause many diseases, such as lung cancer. *Centella asiatica* (CAS) and *Cyperus rotundus* L. rhizomes (CRR) exhibit anti-cancer effects.

AIM: The study examined the cytotoxic effects of CRR and CAS extracts in lung cancer cells.

METHODOLOGY: This research used the maceration method to extract the CAS and CRR powder and methanol solvent. Extraction was tested at 600μl, 400μl, 200 μl, 100 μl, and 50 μl to determine the effect of compound cytotoxicity causing 50% cell death or IC50. Cytotoxicity tests used the MTT method to obtain purple formation crystals and used an ELISA reader to obtain absorbance values.

RESULTS: CRR and CAS extract shows a low cytotoxicity effect. IC50 of CRR and CAS methanol extracts was 235 μg/ml and 279 μg/ml, respectively.

CONCLUSION: CRR and CAS extracts were proven to show chemopreventive activities against lung cancer cells.

Introduction

The number of smokers worldwide has reached 1.3 billion people [1], with Eastern European and Asian countries holding the highest number of smokers [2]. Indonesia is one of the ASEAN countries with the highest number of smokers [3]. Smoking is a risk factor for various degenerative diseases in the community [4]. Along with the high prevalence of smokers, the incidence of cancer in Indonesia has increased. Cigarettes contain many carcinogenic compounds. Exposure to polycyclic aromatic hydrocarbons from cigarette smoke causes gene mutations through DNA adducts formation [5]. Lung cancer ranks first as a cause of death after colorectal cancer (10%) and breast cancer (16%). Lung cancer in Indonesia ranks 3rd and it is most often found in hospitals [6]. Lung cancer is the uncontrolled growth of cancer cells in lung tissue caused by several carcinogens, especially cigarette smoke. The cell cycle is a division program that involves four phases, namely, G1 (and G0), S, G2, and M. phases. Controlling apoptosis in the cell cycle is very important so that cells remain in homeostasis. If the cell cycle is not controlled, cell proliferation will occur continuously in cancer cells [7]. Common lung cancer treatments include surgery, radiotherapy, and chemotherapy [8]. Chemotherapy or radiotherapy has the effect of inhibiting the proliferation and development of cancer cells and also killing cancer cells, so it is the main therapy for the treatment of lung cancer. However, in addition to its benefits in inhibiting the development or killing of cancer cells, both methods of therapy are often accompanied by side effects. Many active compounds from medicinal plants have the potential as proapoptosis and inhibitors of cancer cell proliferation so that they can be used as agents for prevention and complementary therapy in lung cancer [9].

Natural ingredients are proven to have antioxidant and immunomodulatory activities beneficial for cancer prevention [10]. As an agricultural country, Indonesia is rich in medicinal plants. There are various medicinal plants in the forest and yard, including *Cyperus rotundus*, *Centella asiatica*, *Curcuma xanthorriza*, ginger (*Zingiber officinale*), turmeric

(*Curcuma longa*), noni (*Morinda citrifolia*), moringa (*Moringa oleifera*), meniran (*Phyllanthus niruri*), and srikaya (*Annona squamosa*). Some medicinal plants can be used to prevent cardiovascular and degenerative diseases, cancer, diabetes mellitus, and aging/senility [11], [12]. These plants have shown antioxidative [13], immunomodulatory [14], anti-hypertensive [15], antibacterial, hypoglycemic [16], anti-dyslipidemic, cytotoxic, and antiproliferative activities [17]. Transforming the plants into medicinal preparations in herbs and standardized herbal medicines will increase their use-value and economic value. Several plants that can be transformed into preparations for cancer prevention are *Cyperus rotundus* L. rhizomes (CRR) and *Centella asiatica (CAS*) [18]. The CRR and CAS are easy to grow and cultivate for so long as they grow naturally. Many new agents (natural ingredients) studies are expected to be more effective with the lower side effects than chemotherapeutic drugs. Some of the natural ingredients used are the CRR and CAS. CRR and CAS contain phenolic acid compounds, alkaloids, hydrolyzable tannins, essential oils (α-longipinane, selinene, cyperene, and caryophyllene oxide), and flavonoids (anthocyanidins, catechins, flavans, flavones, and flavanols,) [19], [20]. This study examined the cytotoxicity activity of CRR and CAS methanol extracts on the lung cancer line cells.

Methods

Design

We conducted an experimental laboratory study. We used the MTT assay to determine the IC50 values of CAS and CRR extracts [18].

Material, instrument, and participant

We used micropipettes 20, 200, and 1000 l, small test tubes, small test racks, vortexes, conical tubes, 96-well plates, ELISA-readers, waste bins for used media, and PBS [18].

The materials used were sample stock (10 mg) in Eppendorf, DMSO solvent, culture media (MK), phosphate buffer saline (PBS) 1X, MTT 0.5 mg/ml, 10% SDS stopper in 0.1 N HCl, tissue paper (box), aluminum foil (Cancer Chemoprevention) [18], Simplicia CRR, CAS, and methanol solvent; we need for the manufacture of extracts.

Research procedures

Extraction method

Before being extracted, the test materials (CRR and CAS) have been determined by the Department of Pharmacy Biology, Faculty of Pharmacy, University of Muhammadiyah Yogyakarta. Extraction using CRR and CAS simplicia, which has been made into powder. A total of 500 g of CRR and CAS powder were soaked in 3.75 L of methanol and macerated for 3 × 24 h. The maceration results were then filtered to obtain a solution. The solution obtained is then evaporated until it becomes thick, which is called the extract.

Preparation of culture media for lung cancer cells

Preparation of lung cancer cell media (DMEM) was made using DMEM powder for 1 L dissolved into aquabidest of approximately 800 ml, plus 2.2 g of sodium bicarbonate. Aquabidest was then increased to 1 L and stirred using a stirrer until it dissolved. The solution was made to reach PH 7, and HCl 4N was added when the PH was too alkaline. The media was sterilized using a membrane filter and stored at 4°C for subsequent use [18].

Procurement of lung cancer cells

Lung cancer cells from the liquid nitrogen tank were thawed at room temperature. They were then inserted into sterile conical tubes filled with DMEM culture media and centrifuged at 1500 rpm for 5 min. The supernatant was discarded and pellets were taken to be grown on some tissue culture disc (TCD) in DMEM media that had been given 10% FBS. The disc was incubated in an incubator at 37°C [18].

Lung cell harvesting and calculation

The media was discarded using a micropipette after cells met TCD. 500 μl trypsin was added and incubated for 3 min. The solitary cell was inserted into a conical tube and centrifuged at 3000 rpm for 5 min. The supernatant was removed, and then the pellet was also removed and washed using the media. Cells were suspended and counted using a hemocytometer. The number of cells can be calculated using the formula written in Eq. (1) [18].

$$\text{A number of cells} = \frac{\text{Number of cells in 4 chambers}}{4} \times \text{Desired cell suspension}$$

Cytotoxicity test

Concentrations of methanol extracts of CRR and CAS were made in series: 600 g/ml, 400 g/ml, 200 g/ml, 100 g/ml, and 50 g/ml. Lung cancer cells were taken from a CO_2 incubator and observed under a microscope. Then, the media was removed from the well plate. Cells were washed with PBS 1 time, and cells were suspended for 500/ml. The treatment was given according to the series of concentrations, and the cells were put into an incubator for 24 h. MTT reagents were administered at a concentration of 0.5 mg/ml and then incubated for 3 h. After the formation crystal was formed, a 10% SDS stopper was immediately given, and the plate was wrapped in aluminum foil and incubated in a dark place (at room temperature) overnight. Cell absorbance was read using an ELISA reader [18].

Cytotoxicity test analysis

We made a log graph of concentration versus percentage of living cells. The linear regression equation from the graph was sought by displaying a trendline-linear regression. The parameter r was seen in the linear regression equation. If r is greater than the r table, the linear regression equation meets the standard for finding IC50. In the linear regression equation, we inputted y=50% and looked for the x value, and then the antilog of the concentration was calculated to obtain IC50 [18].

Results

Cytotoxicity test of CRR

Cytotoxicity test is a test carried out to determine the toxicity potential of CRR methanol extracts against lung cancer line cells. Cytotoxicity test will produce an IC50 value. The IC50 is the concentration value that causes 50% inhibition of cell proliferation. If the IC50 value is small, a compound's toxic potential is said to be good [18].

We used the MTT method in this study. An MTT (3-(4 – 5 – dimethyltiazol – 2-yl)-2,5-diphenyl tetrazolium bromide) is tetrazolium salt with water-soluble properties by giving a yellow color to the solution. MTT can only be reduced by living cells and cannot be reduced by dead cells because enzymes in these cells are no longer active. The principle that works is that the enzyme in the mitochondria of cells actively metabolizes tetrazolium salts. The enzyme dehydrogenase breaks the tetrazolium ring and causes changes to formazan, purple, and not water-soluble. The number of living cells affects the intensity of the color purple [18]. In Figure 1, we can see the difference before treatment and after treatment, both before and after being given MTT under an inverted microscope.

ELISA reader with a wavelength of 600 nm was used to measure the amount of absorbance and purple color intensity. The intensity is formed according to the number of living cells. The absorbance is more significant if the intensity of the purple color is increasingly more vital. The formazan formed is proportional to the number of cells that react and live with tetrazolium salts. The absorbance produced is then used to calculate the percentage of deaths [18].

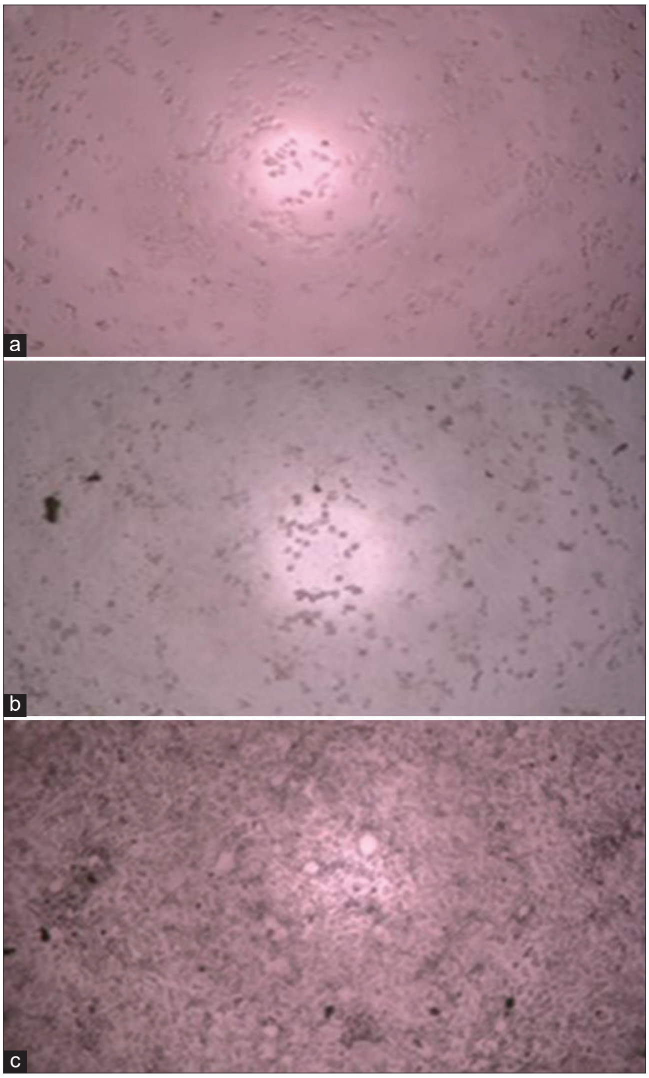

Figure 1: Morphology of lung cancer cells in the wells before the treatment (a), after incubation 24 h with methanol extract CRR (b), and after incubation 24 h with the treatment of methanol extract CRR and given MTT (c)

Table 1 shows the cytotoxicity test results of CRR methanol extract on lung cancer cells. The greater the levels of the methanol extract of the CRR, the greater the percentage of lung cancer cell death produced.

The IC50 value indicates a concentration value that results in a 50% inhibition of cell proliferation and indicates the potential toxicity to cells. This value is a benchmark for conducting cell kinetics observation tests. Table 1 shows that IC50 levels caused 50% inhibition of cell proliferation by converting log construction data and percentage cell viability data

Table 1: The cytotoxicity test results of methanol extract of CRR rhizomes against lung cancer

Concentration (μg/ml)	Log concentration	Mean absorbance	Mean cell viability (%)	IC_{50}
600	2,78	0,061	6,59	235 (μg/ml)
400	2,60	0,071	11,03	
200	2,30	0,242	84,38	
100	2,00	0,267	95,13	
50	1,70	0,276	99,00	

into linear regression equations. Based on the log concentration and mean cell viability data in Table 1, the regression equation obtained was y = −96.811x + 279.57 with the r = 0.8375. The calculation of the straight-line equation can be used to calculate the value of x and antilog of the value of x, which is the value of IC50. The IC50 of CRR was 235 μg/ml, which means that 235 μg/ml of the CRR extract causes 50% inhibition of cell proliferation and leaves a living cell as much as 50% of the number of lung cancer cells that are treated.

Figure 2 shows that the lowest lung cancer cell viability percentage of 6.59% occurred in treating lung cancer cells with methanol extract of CRR rhizomes at 600 μg/ml. The percentage of cell viability decreases if the level of methanol extract decreases. Based on Table 1, the methanol extract of CRR rhizomes had an IC50 value of 235 (μg/ml).

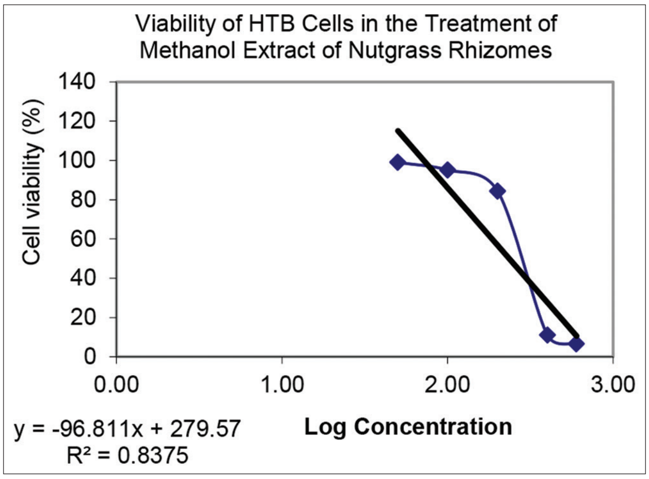

Figure 2: Relationship between the levels of CRR methanol extract versus % cell viability

The IC50 value can indicate the potential of a compound as cytotoxic, the higher the IC50 value, the less toxic the compound. The methanol extract is deemed to be non-toxic to lung cancer line cells if IC_{50} value is > 1000 μg/ml and toxic if IC_{50} value is < 1000 μg/ml. Based on the IC50 CRR value of 235 g/ml, the methanol extract CRR was categorized as weak[21].

The toxicity of the CRR methanol extract is caused by several compounds contained in the plant. CRR tubers contain flavonoids, tannins, glycosides, furochromones, monoterpenes, sesquiterpenes, sitosterols, alkaloids, saponins, terpenoids, essential oils, starch, carbohydrates, proteins, and amino acids. The active components of essential oils are monoterpenes ($C10H16$) and sesquiterpenes ($C15H24$) that are oxygenated. The essential oils found in teki grass bulbs include cyperone, cyperene, cyperotundone, cyperol, selinene, caryophyllene, valerenal, sugeonyl acetate, copaene, patchoulene, transpinocarveol, patchoulenenone, aristrol-9-en-3-one, selina-4, 11-diene, aristrol-9-en-8-one, kobusone, sugetriol, isokobusone, isocyperol, sugeonol, and sitosterol. Methanol solvent has been proven to be effective in extracting active substances from the sisquiterpene, polyphenol, and flavonoid groups where the active substances are antiproliferative. The flavonoid content has an antiproliferation effect [22], and the phenolic acid content repairs DNA damage, cell proliferation, apoptosis, and invasion [7], [23]. The content of alkaloids can inhibit the proliferation and induction of apoptosis [24]. The tannin content has several pharmacological effects, including antioxidant and anti-free radicals and antimicrobial, anti-cancer, anti-nutritional, and cardioprotective effects [4]. Other research results showed that *in vitro* water extract and methanol extract of CRR had cytotoxic and antioxidant activity. In a previous study, the CRR methanol extract on various types of cancer cells other than HTB had an IC50 of 4.52±0.57 to 9.85±0.68μg/ml. The cytotoxic activity of the CRR methanol extract was stronger than that of the aqueous extract. One of the active compounds of CRR which is suspected as a cytotoxic agent is orientin [25].

Cytotoxicity test of Centella asiatica herb extract

The potential cytotoxicity activity of CAS herb extract was tested on lung cancer cells. The morphology of lung cancer cells in the control and treatment of test compounds is shown in Figure 3. The control cells appeared to be polygonal and bright and stuck to the bottom of the well, while the treatment cells appeared to become rounded, and the dead cells looked dark.

The results of the cytotoxicity test of the CAS herb extract are presented in Table 2. Table 2 shows that IC50 levels caused 50% inhibition of cell proliferation by converting log construction data and percentage cell viability data into linear regression equations. Based on the log concentration and mean cell viability data in Table 2, we determine the equation of the linear regression line as shown in Figure 4. The regression equation obtained was y = −0.1915x + 103.57 with the r = 0.9835, where it shows that there is a linear relationship between log concentration and cell viability. The straight-line equation can be used to calculate the value of x and antilog of x, which is the value of IC50.

The CAS IC50 value was 279 μg/ml. This means that 279 μg/ml of the CAS extract can cause 50% inhibition of cell proliferation and leaves a living cell as much as 50% of the number of lung cancer cells treated.

Table 2: The results of the cytotoxicity test of methanol extract of CAS against lung cancer cells

Concentration (mcg)	Log concentration	Absorbance (mean)	Viability (mean)	IC_{50}
500	2.70	0.065	4.53	279 (μg/ml)
250	2.40	0.322	63.31	
125	2.10	0.392	79.40	
62.5	1.80	0.432	88.40	
31.25	1.49	0.468	96.69	

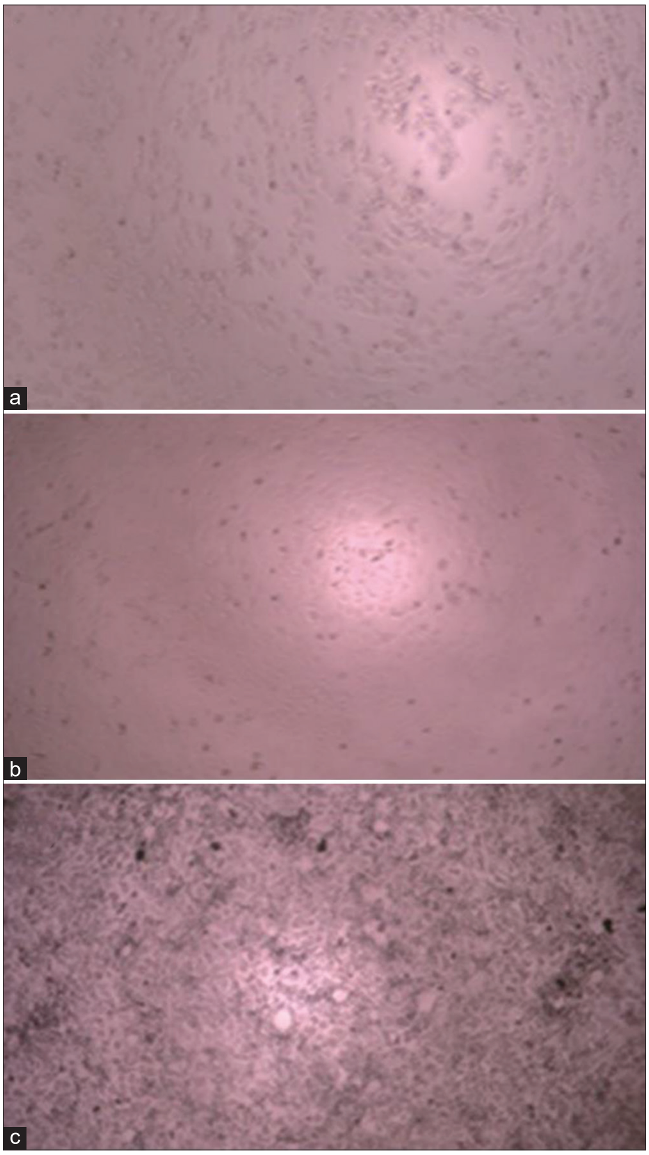

Figure 3: Morphology of lung cancer cells in the wells before the treatment (a), after incubation 24 h with CAS extract (b), and after incubation 24 h with the treatment of CAS extract and given MTT (c)

The cytotoxicity effect can be interpreted as an antiproliferative effect and the cytotoxicity effect was expressed as IC50 values. An agent with a lower IC50 value has a more remarkable ability to reduce the ability of cells to proliferate and increase cell death. Based on research data where the IC50 values of CRR and CAS are 235 and 279 μg/ml, respectively, the ability to inhibit HTB cell proliferation CRR is greater than CAS.

The treatment with CRR and CAS methanol extract can inhibit the proliferative activity of lung cancer. The greater the extract is given, the smaller the doubling time produced. Antiproliferation and death of lung cancer line cells are thought to be caused by the presence of flavonoids, phenolic acids, alkaloids, and tannins [26].

Some of the active compounds of CRR are flavonoids, alkaloids, sesquiterpenoids, tannins, saponins in the tubers, and leaves. Flavonoids especially are one of the main active ingredients. The results of the previous studies showed that per gram, CRR contained 108.37 mg of flavonoids [27]. Based on the previous research data, flavonoid compounds are one of the leading biomarkers to search for the primary active substance in CAS [28].

Similar to CRR, CAS also contains flavonoids, polyphenolic acids, alkaloids, and tannins. Flavonoids and polyphenols are the main ingredients of CAS. The results of the screening of active substances suspected of being proapoptotic and antiproliferative are sesquiterpenes and flavonoids [29]. Flavonoid compounds have often been used as guiding biomarkers to obtain the main active substances. The combination of CAS and CRR extracts is thought to synergize and increase chemopreventive, antioxidant, and anti-inflammatory activities. It has been proven that the antioxidant and neuroprotective activity of the combination of CRR extract and ginger extract is better than the single preparation of CRR extract or ginger extract [30]. The higher the incidence of lung cancer is, the greater the demand to immediately obtain safe and effective chemopreventive agents to inhibit disease progression [27], [30].

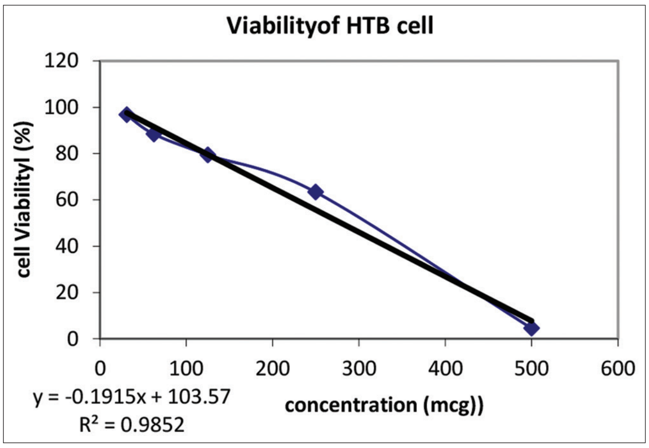

Figure 4: Relationship between the levels of methanol extract of CAS versus % cell viability

Conclusion

The methanol extract of *Cyperus rotundus L*. rhizomes and *Centella asiatica* herb exhibits a low cytotoxicity effect on the lung cancer cells. The IC50 value indicates antiproliferative activity. The results of this study have proven that the *Cyperus rotundus* L rhizomes and *Centella asiatica* can be used as alternative chemopreventive agents for lung cancer.

This research can be further developed into chemopreventive testing of the combination of CRR and CAS accompanied by an action mechanism, which can open the next research stage.

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