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Original Article

Anti-leukemic activity of *Cyperus rotundus* L. on human acute myeloid leukemia HL-60 cells *in vitro*

[Actividad antileucémica de *Cyperus rotundus* L. sobre células humanas de leucemia mieloide aguda HL-60 *in vitro*]

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Abstract

Context: Acute myeloid leukemia (AML) is the most common form of acute leukemia. Currently, many people use medicinal herbs for the treatment of cancers. Nut grass (*Cyperus rotundus* L.) is a medicinal plant widely used in conventional medicine due to its role as an anti-cancer.

Aims: To evaluate the effect of *Cyperus rotundus* tuber (CRT) ethanolic extract on cell proliferation, differentiation, cell cycle, and apoptotic of HL-60 cells.

Methods: HL-60 cells line as a model for AML was cultured under the influence of CRT extract concentrations (35.4, 354, and 3540 µg/mL) for 48 h. Furthermore, the cell was subjected to carboxyfluorescein succinimidyl ester staining for proliferation, using a CD117 marker for differentiation. Annexin V-FITC and PI for cell cycle and apoptosis. The effect of this herb was studied by flow cytometry. The data were statistically analyzed with one-way ANOVA ($p \leq 0.05$) and the Tukey test using SPSS version 16 for Windows.

Results: The results showed that the CRT inhibited proliferation activity, differentiation, cell cycle arrest, and apoptosis.

Conclusions: The data clearly showed the potential anti-cancer activity of CRT on HL-60 cells and suggested that it could help develop promising therapeutic agents for AML treatments.

Keywords: acute myeloid leukemia; apoptosis; cell cycle; *Cyperus rotundus*; HL-60 cells line.

Resumen

Contexto: La leucemia mieloide aguda (LMA) es la forma más común de leucemia aguda. En la actualidad, muchas personas utilizan hierbas medicinales para el tratamiento de los cánceres. La hierba de la nuez (*Cyperus rotundus* L.) es una planta medicinal ampliamente utilizada en la medicina convencional debido a su papel como anticancerígeno.

Objetivos: Evaluar el efecto del extracto etanólico del tubérculo de *Cyperus rotundus* (CRT) sobre la proliferación celular, la diferenciación, el ciclo celular y la apoptosis de las células HL-60.

Métodos: Se cultivó la línea celular HL-60 como modelo de LMA bajo la influencia de concentraciones de extracto de CRT (35,4; 354 y 3540 µg/mL) durante 48 h. Además, las células se sometieron a tinción con éster succinimidílico de carboxifluoresceína para la proliferación, y con un marcador CD117 para la diferenciación. Annexin V-FITC y PI para el ciclo celular y la apoptosis. El efecto de este extracto se estudió mediante citometría de flujo. Los datos se analizaron estadísticamente con ANOVA unidireccional ($p \leq 0,05$) y la prueba de Tukey utilizando SPSS versión 16 para Windows.

Resultados: Los resultados mostraron que el CRT inhibió la actividad de proliferación, diferenciación, detención del ciclo celular y apoptosis.

Conclusiones: Los datos mostraron claramente la potencial actividad anticancerígena del CRT en células HL-60 y sugirieron que podría ayudar a desarrollar agentes terapéuticos prometedores para tratamientos de LMA.

Palabras Clave: apoptosis; células HL-60; ciclo celular; *Cyperus rotundus*; leucemia mieloide aguda.

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INTRODUCTION

Acute myeloid leukemia (AML) is a hematological malignancy with heterogeneous genetic disorders. AML hematopoietic cell activity is disrupted in various cell activity pathways, namely uncontrolled proliferation, cell cycle, and differentiation, resulting in maturation arrest and decreased apoptosis in the bone marrow (Yohe, 2015). Data from the National Center for Health Statistics (NCHS) in the USA shows the estimated incidence of new cases of leukemia in men is 4% or 30,900 cases, higher than that in women, which is 3% or 23,370. Estimated leukemia mortality in males is 5% or 14,210, and female deaths are 4% or 10,240 of the total incidence of cancer (Siegel et al., 2015). The molecular pathogenesis of AML is shown by the presence of genetic and epigenetic changes with abnormalities in hematopoietic progenitor cells (Karacaer, 2022).

Currently, AML treatment with individualized therapy approach has been developed based on genetic and epigenetic characteristics to explain the biomolecular pathophysiology. Therapeutic approaches with molecular targets are expected to reduce the incidence of AML (Rice and de Thé, 2014). Although chemotherapy achieves complete remission, many patients still experience relapse and death. Chemotherapy has high side effects and does not yet have a specific mechanism against cell targets (Yan et al., 2020). AML chemotherapy drugs can damage healthy cells and tissues and produce side effects such as anemia, infection, and bleeding (Bashmail et al., 2020). Therefore, a new therapeutic agent with fewer side effects needs to be found to treat AML.

Some natural compounds from plants have shown their potent anti-cancer property against various cancers. Phytochemicals have become more popular in both developed and developing nations due to many advantages such as having no severe adverse effects, lower cost, and being easy to get. Some natural compounds, including flavonoids, alkaloids, saponins, and many compounds, possess anti-cancer properties (Subramaniam et al., 2019). One type of plant that can be used as an anti-cancer is *Cyperus rotundus* L. (CR), family *Cyperaceae*, also known commonly as nut grass.

C. rotundus is a plant that is easy to grow in tropical and subtropical areas and is considered a nuisance plant. Herbal plant medicines have been widely developed as local wisdom that has the potential to treat several diseases (Badgujar and Bandivdekar, 2015). The nut grass tuber extract contains various types of compounds that have the potential as antioxidants and anti-cancers. The composition of chemical compounds contained in the tuber of *Cyperus rotundus* (CRT) has been identified and used in traditional medical medicine (Babiaka et al., 2021).

Therefore, the present study aimed to test the potential anti-cancer activity of CRT as a new targeted therapy for the management of human AML HL-60 cells. For this purpose, we evaluated the effect of CRT crude extract on cell proliferation, differentiation, cell cycle, and apoptotic of HL-60 cells.

MATERIAL AND METHODS

Plant material

CRT was collected from Tirtomarto Village, Ampelgading, Malang, Indonesia (8°15'00.1"S; 112°53'00.6"E). It was determined by the certification institution of East Java Provincial Government Health Office, UPT Materia Medica Batu No.074/118/102.7/2017.

Extraction

CRT selected only part of the tuber. The tubers were obtained and washed thoroughly. The tuber was cut into small pieces and dried. Next, ground the tuber until it became powder. Extraction was prepared by dissolving CRT powder with absolute ethanol. A total of 100 g CRT powder with 500 mL absolute ethanol inside an aluminum-covered flask to keep it free from sunlight was carried out. Materials were shaken using a rotary shaker at 120 rpm for 24 h. After incubation, once the mixture had been stirred repeatedly and allowed to settle, the transparent liquid above the solid layer was then isolated. The solution was filtered with Whatman No. 1, and the solvent was evaporated with a rotary evaporator until a concentrated extract was obtained. Samples were then stored at 4°C for further use.

Cells, cell culture, and treatment

Cell line HL-60 APL (subtype AML) is a progenitor promyelocytic cell line HL-60 obtained from stem-cell and Cancer Institute (SCI), KalGen (Kalbe Genomics) Laboratory PT. Kalbe Farma Tbk Pulomas, Jakarta Indonesia 13210. The cell line was maintained in RPMI 1640 (Rosewell Park Memorial Institute) and supplemented with $NaHCO_3$ 0.22 g, 10% FBS, and 1% penicillin/streptomycin. HL-60 in this study was only grown in a culture medium as an untreated cell (control). Cisplatin (3 µg/mL) was used as a drug control. CRT extract with various concentrations (35.4, 354, 3540 µg/mL) was prepared in a culture medium, incubated with HL-60, and the IC_{50} value was calculated (354 µg/mL). These cells were cultured in an incubator with 5% CO_2 at 37°C.

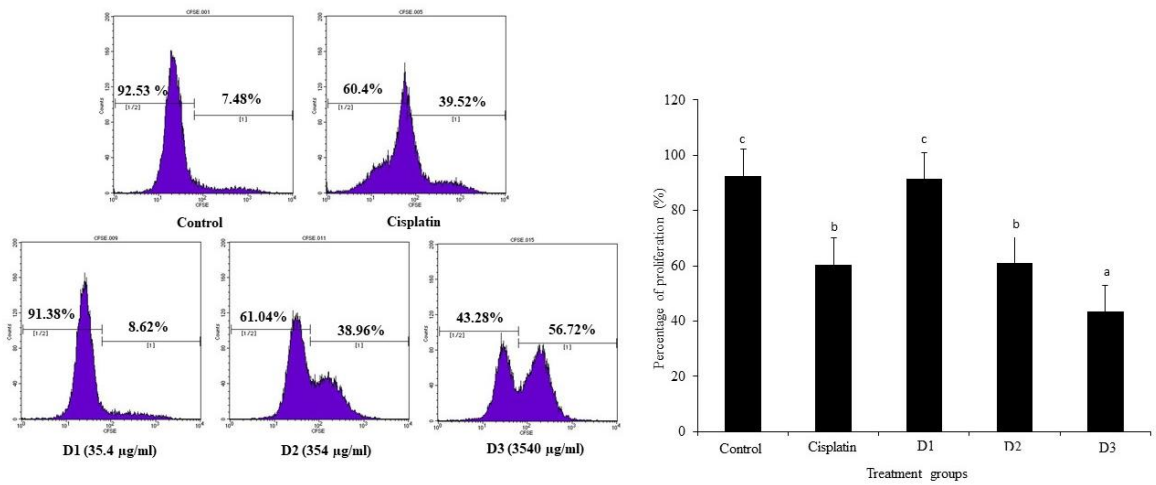

Figure 1. Inhibitory effect of CRT on the proliferation of HL-60 cell line with various treatment groups (control, cisplatin, D1, D2, D3).
The analysis showed that the proliferation rate significantly decreased after the addition of the CRT. The data are mean ± SD in each group with p≤0.05. Cisplatin concentration (3 µg/mL).

Flow cytometry analysis

After being treated with the CRT for 48 h, the cells were trypsinized and washed with phosphate buffer saline (PBS). Then, the cells were stained with carboxyfluorescein succinimidyl ester (CFSE) for proliferation analysis, annexin V-FITC, and PI for 15 min at room temperature under dark conditions, preparing it for cell cycle and apoptotic analysis and the further analysis of differentiation using CD117 marker.

Statistical analysis

Data from flow cytometry were statistically analyzed by using SPSS version 16. This analysis used a parametric one-way ANOVA and was followed by Tukey's *posthoc* test with a significance of 0.05%.

RESULTS

CRT extract reduces HL-60 cell proliferation

Proliferation analysis was performed using CFSE staining within 48 h and showed a significant difference between the control and CRT. HL-60 cells were grown in a fresh culture medium without being added with cisplatin, and CRT was used as a control. The control showed that HL-60 cells were grown better than other groups. CRT extract significantly reduced the cell number of HL-60 cells after 48 h on D2 (354 µg/mL) and D3 (3540 µg/mL) compared with the control. This study used cisplatin as a standard drug. Based on the results of the analysis, CRT administration showed that the higher concentration, the lower proliferation would be. The percentage number of proliferation in control, cisplatin (3 µg/mL), and CRT (34.5, 354, and 3540 µg/mL) treatments were 92.53, 60.48, 91.38, 61.04, and 43.28%, respectively (Fig. 1).

The effect of CRT on differentiation

Differentiation analysis was performed using CD117 staining and showed a significant difference between control and CRT. CRT extract significantly reduced the differentiation rate of HL-60 cells after 48 h on CRT D1, D2, and D3 (35.4, 354, and 3540 µg/mL), respectively compared with the control. The percentage number of differentiation in control (97.19%), cisplatin (80.82%), and CRT treatments were 94.18% (D1), 26.43% (D2), and 29.27% (D3) (Fig. 2).

HL-60 cells treated with CRT extract show cell cycle arrest

To elucidate the mechanisms underlying the inhibitory effect of CRT extract on cell growth, the cell cycle distribution of HL-60 cells was analyzed by flow cytometry. As shown in Fig. 3, flow cytometry analysis indicated that among the untreated cells (control), 17.54% were distributed in the G_0/G_1 phase, 44.97% were accumulated in the S phase, and 17.43% were in the G_2/M phase. Incubation of HL-60 cells with CRT extract in various concentrations for 48 h gave varying effects depending on the concentration. Only CRT D3 (3540 µg/mL) on HL-60 gave a significant effect (p≤0.05) on arresting cell cycle compared to other groups.

CRT extract induces apoptotic in HL-60 cells

It has been proven that in cancer, the apoptotic process is abnormal. To understand the molecular mechanisms based on the inhibitory effects of CRT extract clearly on HL-60 cell growth, we examined the

effects of CRT extract on apoptosis. The results showed that the CRT administration (354 and 3540 µg/mL) was significantly different compared to control, cisplatin, and CRT 35.4 µg/mL to induce apoptosis. Control groups had the lowest apoptosis expression compared to others, except for 35.4 µg/mL. The average percentage of apoptosis on control, cisplatin, and CRT (34.5, 354, and 3540 µg/mL) were 0.91, 5.55, 0.21, 49.24, and 49.58%, respectively (Fig. 4).

DISCUSSION

Cancer is currently one of the disease problems facing the world. AML is a type of cancer that causes abnormal differentiation and proliferation of hematopoietic stem cells that are systemic and malignantly transformed, leading to suppression and replacement of normal spinal cord components (Arnone et al., 2020). In most cases of AML, the body produces too many white blood cells called myeloblasts that are still immature. These immature cells are not as good as mature white blood cells for fighting infection. In AML, myelocytes, which normally develop into granulocytes, become malignant and soon replace normal bone marrow cells (Kabel et al., 2017). Therefore, this disease is the center of attention of researchers to develop effective anti-cancer drugs. So, the identification of new anti-cancer drugs is of paramount importance.

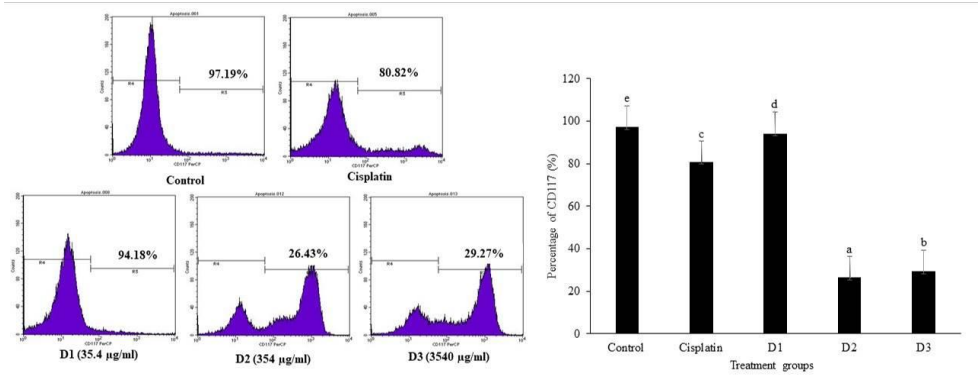

Figure 2. Inhibitory effect of CRT on differentiation (CD117) on HL-60 cell line.

The analysis showed that the differentiation rate significantly decreased after the addition of the CRT. The data are mean ± SD in each group with p≤0.05. Cisplatin concentration (3 µg/mL).

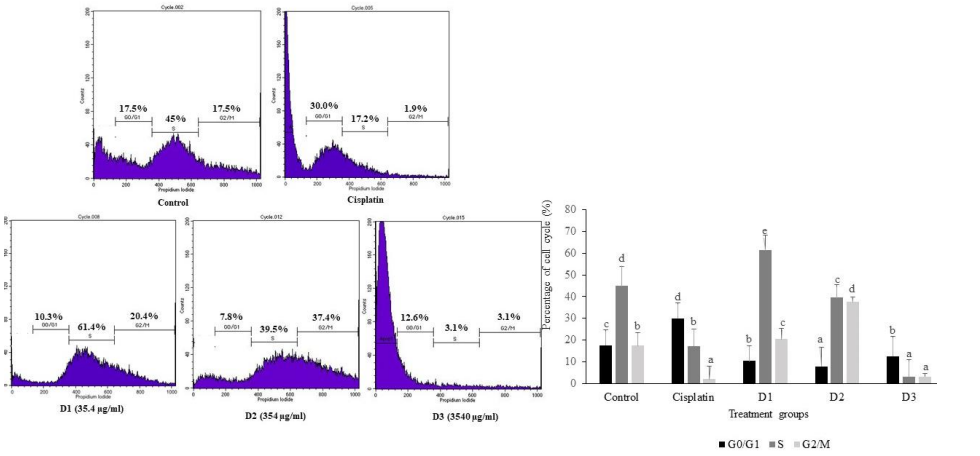

Figure 3. Effect of CRT on induction of HL-60 cell cycle arrest.

The analysis results showed that the CRT significantly induced cell cycle arrest. The data are mean ± SD in each group with p≤0.05. Cisplatin concentration (3 µg/mL).

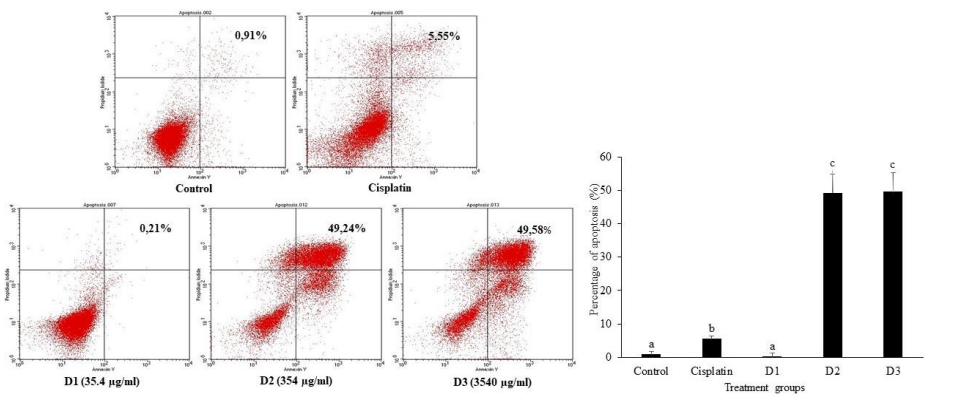

Figure 4. Results of apoptosis analysis on HL60 cell line.

The analysis results showed that the CRT significantly induced apoptosis. The data are mean ± SD in each group with p≤0.05. Cisplatin concentration (3 µg/mL).

Pharmaceutical plants are good sources of biologically active compounds that contain antioxidant and immunomodulatory features, making them potential candidates for anti-cancer agents (Majolo et al., 2019). In addition to being edible (Samarghandian et al., 2019), natural compounds are also gaining attention as cancer therapeutic agents due to their more effective but less toxic profile (Gupta et al., 2013).

This research was carried out through *in vitro* test using cell line HL-60 APL (subtype AML) and *C. rotundus* for treatment. This study showed that nut grass was able to perform anti-cancer activities through inhibition of proliferation, differentiation, cell cycle, and induction of apoptosis against cell line HL-60 APL (AML subtype). Thus, in this case, nut grass can be proven to use as an anti-cancer candidate. This finding is reinforced by previous studies that stated that *C. rotundus* has effectiveness as an anti-cancer (Park et al., 2014; Bajpay et al., 2018; Babiaka et al., 2021).

C. rotundus, also known as *Rumput teki* in Indonesia or nut grass in English, is a perennial weed plant. CR is widely used as herbal medicine because it has pharmacological effects such as anti-cancer, wound healing activity, anti-inflammatory, anti-malarial, anti-obesity (Bajpay et al., 2018), antioxidant, antidiabetic, antibacterial, and tranquilizing effects (Ullah and Hassan, 2022). The content of bioactive compounds in *Cyperus* is flavonoids, phenols, terpenes, and alkaloids (Abo-Altemen et al., 2019). Previous studies reported that CR has cytotoxic effects on some cancer cells, such as HeLa cells and SiHa cells in cervical cancer (Susianti, 2009), lymphoma cells of L5178 mice (Sayed et al., 2007), leukemia cells K562 and L1210 (Kilani et al., 2008).

The result of this study showed that the CRT extract has an effectivity as an anti-cancer to inhibit proliferation (Fig. 1), differentiation (Fig. 2), cell cycle (Fig. 3), and apoptotic induction (Fig. 3). The decreased proliferation of the HL-60 APL cell line (subtype AML) in this study is assumed due to the presence of flavonoids in *C. rotundus*. Zhang et al. (2018) showed that most flavonoids could inhibit cell proliferation. Flavonoids inhibit the expression of topoisomerase I and topoisomerase II enzymes that catalyze DNA playback and relaxation. Topoisomerase enzyme inhibitors will stabilize the topoisomerase complex and cause DNA to be cut and damaged. DNA damage can cause the expression of pro-apoptotic proteins such as Bax and Bak and decrease the expression of anti-apoptotic proteins, namely Bcl-2 and Bcl-XL (Rashidi et al., 2021; Zhang et al., 2018).

The compounds of *C. rotundus* tuber extract, which have the potential to inhibit the proliferative pathway, are beta-sitosterol, catechin, and quercetin (Srivastava et al., 2016). Quercetin is a natural compound that is considered an attractive candidate for cancer treatment and prevention. Quercetin is a flavonoid group that is a plant pigment. Currently, more than 6,000 different compounds are included in the flavonoid group. The mechanisms of inhibiting cell proliferation and inducing apoptosis in time by quercetin in chemotherapy depend on the concentration. Furthermore, quercetin decreases the expression of the anti-apoptotic protein Bcl-2 and regulates the expression of the pro-apoptotic protein Bax. Caspase-3 is also activated by quercetin, which initiates the mitochondrial caspase-3-dependency pathway to induce apoptosis (Niu et al., 2011). The role of luteolin compounds is known to prevent tumor development in large part by inactivating several signals and transcriptional pathways that are important for cancer cells (Tuorkey, 2016). Previous research reported that CR contained a terpenoid, a compound that had high anti-cancer activity, that was tested through the ability to block nuclear factor-*kappa* B, induces apoptosis, activate transcription and angiogenesis, which can be useful in the treatment of various types of cancer (Simorangkir et al., 2019).

Apigenin, quercetin, and luteolin in *Cyperus* have the potential to increase expression and induce the activation of the P53. Activation of p53 is crucial in cancer treatment (Gupta et al., 2019). The activation process of p53 causes the activity of cell cycle regulatory proteins such as p21. The p21 protein will inhibit cyclin E Cdk2 then the inhibition causes the cell cycle will stop at the G_1 phase. This series of processes occur inside the cell. In addition, p53 also stimulates the permeability of the mitochondrial outer membrane, causing a loss of membrane potential and the subsequent release of several pro-apoptotic proteins, such as Bax. The process of apoptosis also depends on the presence of protein caspase-3. This protein has a crucial role in the execution of apoptosis. Caspase-3 can bind to caspase-8 and caspase-9 in carrying out their functions. Caspase-3 activates cell apoptotic processes extrinsically and intrinsically (Rubio et al., 2019). Increased expression of caspase-3 and activation induced by the active compound is very beneficial for cancer treatment.

CONCLUSION

Cyperus rotundus tuber ethanolic extract has activity as an inhibitory proliferation, differentiation, induced cell cycle arrest, and apoptosis. These results provide new insights into the benefit of *Cyperus rotundus*, indicating its potential as a promising chemopreventive agent for cancer treatment.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Agustini SM	Widjajanto E	Rifa'i M	Haryana SM	Nurdiana	Lyrawati D	Sukorini U	Lestari ND
Concepts or ideas	x							
Design	x	x	x					
Definition of intellectual content		x	x					
Literature search	x					x		x
Experimental studies		x	x					
Data acquisition	x							x
Data analysis	x							
Statistical analysis		x	x					
Manuscript preparation				x	x		x	
Manuscript editing				x	x		x	
Manuscript review	x	x	x	x	x	x	x	x

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