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Original Article

Cyperus rotundus L. rhizome extract modulates immune system and induces apoptotic in 4T1-tumor bearing mice

[Extracto de rizoma de *Cyperus rotundus* L. modula el sistema inmunitario e induce la apoptosis en ratones portadores de tumores 4T1]

Wirdatun Nafisah[1,2], Nadia Wahyuningsih[3], Muhammad Faiz Maulana[3], Shella Zahra Kumala Azmi[3], Muhaimin Rifa'i[4], Nashi Widodo[4], Muhammad Sasmito Djati[4]*

[1]Doctoral Program of Biology Department, Brawijaya University, Malang, Indonesia.
[2]Bioinformatics Research Center, Indonesian Institute of Bioinformatics (INBIO), Malang, Indonesia.
[3]Undergraduate Program of Biology Department, Brawijaya University, Malang, Indonesia.
[4]Department of Biology, Brawijaya University, Malang, Indonesia.
*E-mail: msdjati@ub.ac.id

Abstract

Context: Triple-negative breast cancer (TNBC) is a type of breast cancer with the highest aggressiveness and malignancy characteristics.

Aims: To evaluate the possible anticancer potential of *Cyperus rotundus* rhizome extract (CRE) against 4T1-tumor-bearing mice.

Methods: *C. rotundus* rhizome was extracted using maceration methods with ethanol. *C. rotundus* extract (CRE) was then determined the total phenolic (TPC) and flavonoid content (TFC) using colorimetric analysis. The cytotoxic activity of CRE against 4T1 cells was carried out operating WST-1 assay, and the IC_{50} value was then used for *in vivo* study. The 4T1-tumor-bearing mice were treated with CRE at 72.5, 145, and 290 mg/kg body weight (BW) for two weeks or treated with cisplatin once a week for two weeks (4 mg/kg BW). The analysis of IL-2 cytokines production and the activation of CD4 and CD8 T cells was assessed using flow cytometry analysis. Histopathological analysis with hematoxylin and eosin (HE) staining was applied to determine the outcome of CRE on breast cancer tissue.

Results: CRE indicated a higher TPC (76.97 mg GAE/g) value than TFC (29.37 mg QE/g). This study demonstrated a significant reduction of IL-2 cytokines and CD4 T cell activation in treated groups than in the cancer group ($p<0.05$) and showed a good prognosis, further confirmed by histopathological data. The breast tissue of 4T1-tumor-bearing mice in treated groups showed apoptotic cells compared to the cancer group, which has more viable cells.

Conclusions: The high phenolic content in CRE can modulate mice's immune systems and induce cancer cell apoptosis.

Keywords: *Cyperus rotundus*; immune system; triple-negative breast cancer; 4T1 cells.

Resumen

Contexto: El cáncer de mama triple negativo (CMTN) es un tipo de cáncer de mama con las mayores características de agresividad y malignidad.

Objetivos: Evaluar el posible potencial anticancerígeno del extracto de rizoma de *Cyperus rotundus* (CRE) frente a ratones portadores del tumor 4T1.

Métodos: El rizoma de *C. rotundus* se extrajo mediante métodos de maceración con etanol. A continuación, se determinó el contenido total de fenoles (TPC) y flavonoides (TFC) del extracto de *C. rotundus* (CRE) mediante análisis colorimétrico. La actividad citotóxica del CRE contra las células 4T1 se llevó a cabo mediante el ensayo WST-1, y el valor IC_{50} se utilizó para el estudio *in vivo*. Los ratones portadores del tumor 4T1 fueron tratados con CRE a 72,5, 145 y 290 mg/kg de peso corporal (PC) durante dos semanas o con cisplatino una vez a la semana durante dos semanas (4 mg/kg PC). El análisis de la producción de citocinas IL-2 y la activación de células T CD4 y CD8 se evaluó mediante citometría de flujo. Se aplicó un análisis histopatológico con tinción de hematoxilina y eosina (HE) para determinar el resultado de la CRE en el tejido del cáncer de mama.

Resultados: La CRE mostró un valor de TPC (76,97 mg GAE/g) superior al de la TFC (29,37 mg QE/g). Este estudio demostró una reducción significativa de las citocinas IL-2 y de la activación de células T CD4 en los grupos tratados que en el grupo con cáncer ($p<0,05$) y mostró un buen pronóstico, confirmado además por los datos histopatológicos. El tejido mamario de los ratones portadores del tumor 4T1 en los grupos tratados mostró células apoptóticas en comparación con el grupo de cáncer, que tiene más células viables.

Conclusiones: El alto contenido fenólico de la CRE puede modular el sistema inmunitario de los ratones e inducir la apoptosis de las células cancerosas.

Palabras Clave: células 4T1; cáncer de mama triple negative; *Cyperus rotundus*; sistema inmune.

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AUTHOR INFO
ORCID: 0000-0001-9731-218X (WN)
0000-0002-7718-0789 (NW)
0000-0003-2030-2092 (MFM)
0000-0001-5526-3252 (SZKA)

INTRODUCTION

Breast cancer has an extremely high fatality rate and is one of the most common cancers worldwide (Abu Bakar et al., 2021). Breast cancer is divided into luminal A, -B, HER2-positive, and triple-negative subtypes based on its molecular characteristics. The difference is luminal A determined with the expression of ER and/ PR molecule and HER2/neu negative; luminal B is determined by the expression of ER and/or PR and HER/neu positive; HER2-positive due to the positive expression of HER2/neu only; and the ER, PR, and HER/neu molecules all express negatively in triple-negative breast cancer (TNBC) (Al-Thoubaity, 2020). TNBC is the most malignant and aggressive kind of breast cancer among these subtypes (Yin et al., 2020)

To have a representative TNBC model, the 4T1 cell line, a murine mammary carcinoma cell that shares a substantial future of human TNBC, such as highly metastatic activity, is often used for screening anti-breast cancer drugs (Kaur et al., 2012; Silva et al., 2016). Some factors make 4T1 cells suitable to become an experimental model, primarily animal models for human mammary cancer: (a) technically, it is effortless to be transplanted to the animal mammary gland; (b) it can develop spontaneous metastatic cancer from the primary site or tumor; and (c) it has a high similarity of progressive spread to the draining lymph nodes and other organ compared to human mammary cancer (Pulaski and Ostrand-Rosenberg, 2000).

Herbal medicine has been of concern to be used as an alternative strategy to develop an anticancer drug. The exploration of potential natural products for cancer treatment is widely done. In tropical countries, a wild plant named *Cyperus rotundus* L. (family *Cyperaceae*) or nut grass often is used to treat bowel and stomach disease by the ancestor (Peerzada et al., 2015). Recently, scientists revealed this plant's biological function and pharmacological attributes, including its anti-inflammatory, anticancer, and antioxidant benefits (Peerzada et al., 2015; Lin et al., 2019). This plant is often used in anticancer studies due to its anticancer properties *in silico*, *in vitro*, or *in vivo*. Based on our previous study, the bioactive components of *C. rotundus* extract (CRE) can inhibit PD-L1 protein through *in silico* study (Nafisah et al., 2022) and shows the immunomodulatory effect on DMBA (7,12-dimethylbenz[a]anthracene)-exposed mice (Ramadhani et al., 2020). To confirm our *in silico* study for the anticancer mechanism of this extract, further research is urgently needed. Here, this study analyzes the anticancer potential of CRE on 4T1-tumor-bearing mice.

MATERIAL AND METHODS

Plants extraction

C. rotundus rhizome was extracted using a previous method by Nafisah et al. (2022). The rhizome was obtained from UPT. Balai Materia Medica, Indonesia (7°52'01.2"S and 112°31'13.2"E). In addition, the taxonomic identification of this rhizome was deposited by UPT. Balai Materia Medica with determination number 074/ 536/ 102.7-A/ 2021.

Briefly, absolute ethanol (1:10, m:v) was added to the powdered *C. rotundus* rhizome and mixed constantly for 24 h at room temperature while stirred continuously. The mixture was next filtered and evaporated utilizing a rotary evaporator at 50°C (IKA® RV 10, IKA Works (Asia) Sdn Bhd, Malaysia). The extract yield from 500 g/5000 mL of rhizome powder and ethanol was 12.7 g. The *C. rotundus* extract (CRE) was then stored at 4°C prior to use.

Total flavonoid and phenolic content

The amount of phenolic and total flavonoid was assessed according to Budiono et al. (2022). A colorimetric assay determined the total flavonoid content (TFC) with an aluminum chloride reagent. Briefly, for 5 min at room temperature, a sample or standard (250 µL) was combined with distilled water (350 µL) and 5% sodium nitrite solution (75 µL). A 10% aluminum chloride solution (75 µL) was added, and the mixture was incubated for 6 min and added with 1 M sodium hydroxide solution 500 µL. Then, a UV-Vis spectrophotometer (Biochrom Libra S12, UK) was used to quantify the absorption with a wavelength of 510 nm. The analysis was done in triplicates. The standard linear curve was obtained using quercetin as the standard, results were expressed in mg QE/g of dried extract.

In addition, total phenolic content was measured in triplicates using the Folin-Ciocalteu technique (Budiono et al., 2022). Briefly, 400 µL of a diluted Folin-Ciocalteu reagent with water (1:1 v/v) was mixed with 80 µL sample extract and incubated for 5 min at room temperature. Then, the mixture was incubated for 2 h at room temperature with no light after adding sodium carbonate solution (7 g/L) for 300 µL. A UV-Vis spectrophotometer 740 nm wavelength was used to measure absorption. The standard used in this study was gallic acid, and the data were given as mg GAE/g or gallic acid equivalents.

CRE cytotoxicity analysis on 4T1 and TIG-1 cell lines

The cytotoxicity analysis was done according to Christina et al. (2022). A murine mammary carcinoma, 4T1 cell line, and normal human fibroblast cell line (TIG-1), obtained from Cancer Chemoprevention Research Center (CCRC) of Universitas Gajah Mada, Indonesia, used to be carried out for WST-1 assay. Briefly, DMEM or MEM complete medium (1% penicillin-streptomycin and 10% fetal bovine serum/FBS) was used to seed the cells in a 96-well plate for 24 h in an incubator at 5% CO_2 37°C. Cells were treated with CRE at concentrations (6.25, 12.5, 25, 100, 200, and 400 µg/mL) for 24 h. After that, the medium was removed and replaced with the completed medium containing 10 µl WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate) reagent (1:10) and incubated for 30 min. Using a Bio-Tek ELISA Microplate Reader (BioTek Instruments, Inc., Winooski, USA), the sample absorbance was determined at a 490 nm wavelength. Then, the growth inhibition (%) was obtained using the following formula [1] (Alam et al., 2017).

$$\text{Growth inhibition (\%)} = \frac{\text{(control-sample)}}{\text{control}} \times 100 \quad [1]$$

The 50% growth inhibition or IC_{50} was calculated from a linear equation of the percentage plot of growth inhibition against the extract log concentration. The IC_{50} value of the extract was then used for the treatment *in vivo* research. Then, the selectivity index (SI) of CRE against 4T1 and TIG-1 cells was calculated according to the formula [2] (Althaher et al., 2022).

$$SI = \frac{IC_{50} \text{ of CRE on normal cells (TIG-1)}}{IC_{50} \text{ of CRE on cancerous cell (4T1)}} \quad [2]$$

Animals

The BALB/c mice *(Mus musculus)* used in this research were obtained from Pusvetma Veterinary Center, Surabaya, Indonesia. The animal experiment protocol in this research was approved by the Committee of Animal Use and Care, Bioscience Institute, Brawijaya University, under the number of ethical clearance 150-KEP-UB-2022 and following EU Directive 2010/63/EU guidelines for animal experimentation. The mice must meet specific requirements, including being physically healthy and active without experiencing hair loss. Mice were subjected to acclimation for a week. Mice were provided access to food and water ad libitum. The husks in the animal cages were replaced every three days.

In vivo experimental study

This study used 30 BALB/c mice which divided into 6 groups: Normal (healthy mice and untreated group, n = 4), Cancer (4T1-tumor bearing mice and untreated group, n = 4), Cisplatin (4T1-tumor bearing mice and treated with cisplatin 4 mg/kg BW (Aminullah et al., 2022), CRE1 (4T1-tumor bearing mice and treated with 72.5 mg/kg BW), CRE2 (4T1-tumor bearing mice and treated with 145 mg/kg BW), and CRE3 (4T1-tumor bearing mice and treated with 290 mg/kg BW). The three CRE doses were used according to the IC_{50} (CRE2), ½ IC_{50} (CRE1), and 2 × IC_{50} (CRE3) value of the WST-1 assay on 4T1 cells. The treatment was done after the mice were confirmed to have breast cancer. Cisplatin treatment was done once a week for two weeks intraperitoneally, while CRE treatment was done every day for two weeks orally.

4T1-tumor bearing BALB/c mice

The 4T1 cells were grown in DMEM complete medium supplemented with 10% fetal bovine serum/FBS and 1% penicillin-streptomycin (Gibco™, Thermo Fisher Scientific, USA) and incubated in incubator CO_2 5% at 37°C. The mice's right flank mammary fat pad was injected subcutaneously once a week for two weeks with 7.5 × 10^5 4T1 cells. The tumor formation was monitored every 3 days by palpation, and three mice were chosen randomly to be sacrificed and used for confirmation in the last two weeks. The confirmation was done through histopathological analysis. After the three mice were confirmed to have breast cancer, the treatment was carried out on the mice in the treatment group.

Antibody staining and flow cytometry analysis

The spleens of the experiment's mice were isolated after they were sacrificed. The spleen was then crushed and homogenized with phosphate buffer saline/PBS (Biowest, USA). The mixture was centrifuged (HERMLE Z 326 K) for 5 min at 10°C and 2500 rpm. The pellet was collected and stained with antibodies, including fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 and CD8 (BioLegend) and PE/Cy7-conjugated anti-mouse IL-2 (BioLegend). After antibody staining, the sample was added by PBS and analyzed using a flow cytometer (BD FACSCalibur™, San Jose, CA) with BD Cell Quest Pro software.

Histopathological analysis of 4T1-tumor bearing

Mice's mammary tissue was isolated and fixed with 4% of formalin. The tissue was prepared using paraffin wax, cut 5-7 mm thick, and stained with hematoxylin and eosin (HE) staining. The histopatho-

logical analysis was done using an Olympus BX51 microscope and pictured using OptiLab 3.0 software.

Statistical analysis

One-way ANOVA and Post Hoc Tukey HSD test were performed for the statistical data analysis using the SPSS 26.0 program. Significant data was defined as $p<0.05$. All the data was displayed in mean ± standard deviation (SD).

RESULTS

CRE total flavonoid and phenolic content

The total flavonoid content (TFC) and phenolic content (TPC) were expressed as equivalent to each standard, such as in mg/g in QE (quercetin) and GAE (gallic acid), respectively. Based on the study using colorimetric analysis, CRE showed a total phenolic content of 76.97 mg GAE/g, with R^2 = 0.908, and a total flavonoid content of 29.37 mg QE/g, with R^2 = 0.997 (Fig. 1).

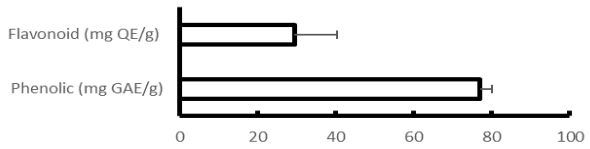

Figure 1. Total flavonoid (TFC) and phenolic (TPC) contents of CRE.

Table 1. Cytotoxic effect of CRE on 4T1 and TIG-1 cell lines.

CRE (µg/mL)	Growth inhibition (%)	
	4T1	TIG-1
6.25	13.06 ± 5.46*	12.9 ± 2.4*
12.5	22.77 ± 0.61*	18.27 ± 0.97*
25	19.48 ± 0.52*	19.35 ± 1.54*
100	53.94 ± 6.16**	20.43 ± 1.2*
200	75.28 ± 14.58**	21.50 ± 3.12*
400	96.94 ± 2.36***	31.18 ± 2.87**

Data were expressed as mean ± standard deviation (SD), and different notations showed significant differences based on the Tukey HSD test ($p<0.05$).

CRE cytotoxicity against cancerous (4T1) and non-cancerous (TIG-1) cells

The study demonstrated the percentage of growth inhibition as the effect of various CRE concentrations against 4T1 cells, ranging from 13 to 96% in a dose-dependent manner (Table 1). Concentrations 6.25 to 25 µg/mL showed insignificant differences compared to 100, 200, and 400 µg/mL ($p<0.05$). Besides, CRE demonstrated low cytotoxicity against TIG-1 cells with growth inhibition ranges from 12.9% to 31.18% (Table 1). The percentage of cell growth inhibition was then used to create a linear equation, and the IC_{50} for 4T1 and TIG-1 were obtained, such as 145.02 µg/mL and 1,347.04 µg/mL. Based on the IC_{50} of both cells, the selectivity index of CRE was 9.28.

IL-2 cytokine produced by T cells in 4T1-tumor-bearing mice

The study found insignificant differences in $CD4^+IL\text{-}2^+$ between normal and cancer groups, although $CD4^+IL\text{-}2^+$ of the cancer group tended to increase to 10.80% compared to the normal group (9.33%). However, $CD4^+IL\text{-}2^+$ tends to decrease significantly after treatment, both cisplatin and CRE ($p<0.05$) (Fig. 2). In addition, the IL-2 production from CD8 T cells differed significantly between the normal and cancer group. The study found a significant increase of $CD8^+IL\text{-}2^+$ in the 4T1-tumor-bearing mice (17.55%) compared to the normal group (13.55%). However, the treatment group showed a fluctuating level of $CD8^+IL\text{-}2^+$ where CRE1 has the lowest level (11.97%) and the highest was CRE2 and CRE3 (18.32% and 17.51%, respectively) (Fig. 2).

CD4 and CD8 T cell activation in 4T1-tumor-bearing mice

The analysis of T cell activation is crucial since T cell, especially CD8 T cell, is the leading player in the anticancer immune system. The level of CD62L molecule loss on the cell surface was used in this study to analyze the activation of CD4 and CD8 T cells. Based on the flow cytometry analysis, the cancer group experienced a significant increase in CD4 T cell activation ($CD4^+CD62L^-$). In addition, the study indicated a significant decrease after treatment with cisplatin and all doses of CRE ($p<0.05$) (Fig. 3). The treatment group's $CD4^+CD62L^-$ level was similar to the normal group indicated with the same notation of Tukey HSD Post Hoc test. Besides, the level of CD8 T cell activation ($CD8^+CD62L^-$) demonstrated a fluctuating level, but insignificantly different between normal, cancer, cisplatin, CRE1, and CRE3 (Fig. 3).

Histopathology of 4T1 tumor-bearing mice mammary tissue

Mice's mammary tissue was harvested and stained with HE staining to observe how CRE treatment affected breast cancer progression in mice. Based on microscopic observation, the mammary tissue of normal groups showed the appearance of adipocytes (A), duct (D), and blood vessels (BV) (Fig. 4). Compared with mammary tissue from the cancer group, adipocytes were replaced by 4T1 cancer cells with

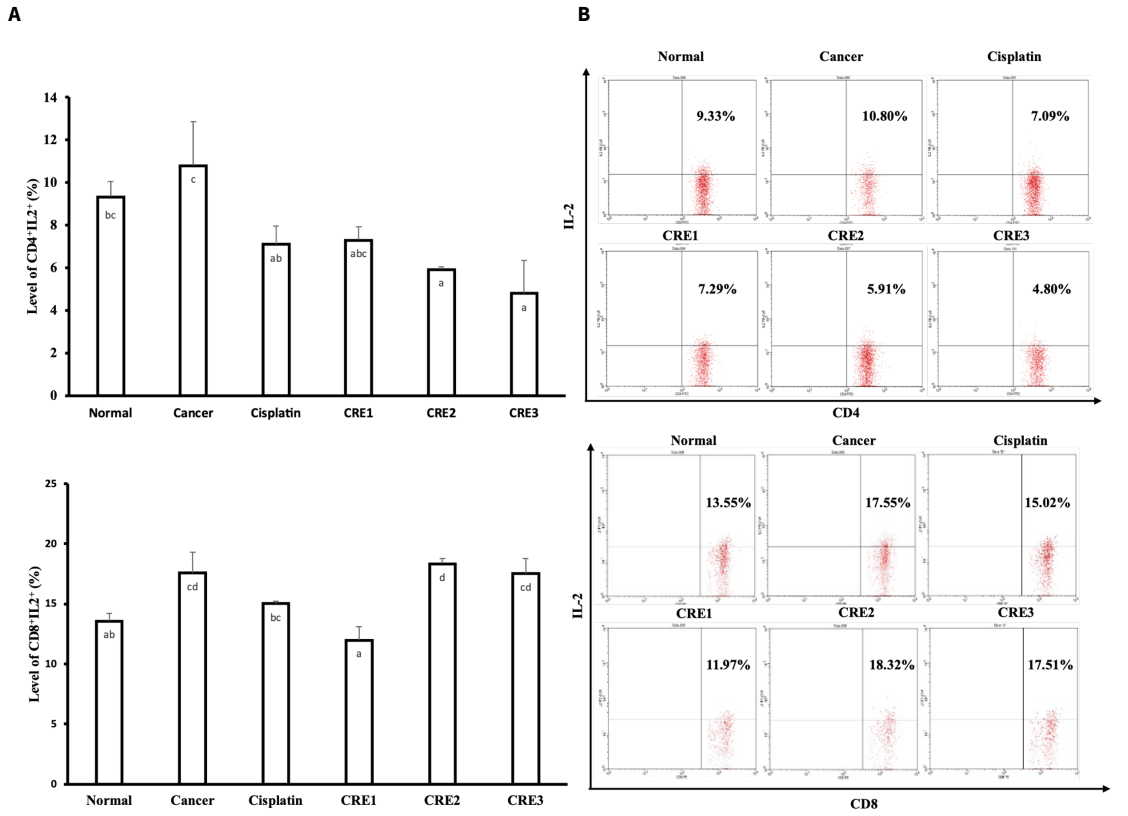

Figure 2. The level of IL-2 production from CD4 ($CD4^+IL\text{-}2^+$) and CD8 ($CD8^+IL\text{-}2^+$) T cells of 4T1 cancer cells bearing mice.

(A) The data were expressed as Mean ± standard deviation (SD), and different notations showed significant differences based on the Tukey HSD test ($p<0.05$). **(B)** Quadrant plots FACS analysis showed a relative number of IL-2 in various groups.

Normal: normal/healthy mice without 4T1 cells injection and treatment; Cancer: mice injected with 4T1 cancer cell lines; Cisplatin: mice injected with 4T1 cancer cell lines and treated with cisplatin drug; CRE1,-2, and -3: mice injected with 4T1 cancer cell lines and treated with CRE 72.5, 145, and 290 mg/kg BW, respectively.

pleomorphic characteristics and single or multiple nuclei (black arrows), which expanded tremendously. This phenomenon also occurred in treated-4T1-tumor-bearing mice. However, necrosis/late apoptotic cells characterized by cell swelling and cellular detail disappearance (green arrow) were found in the treatment group, such as cisplatin. The apoptotic appearance was also found in the CRE treatment group (red arrow), indicated with apoptotic bodies (Fig. 4).

DISCUSSION

Herbal medicine has been used since ancient times, but the concern about finding the bioactive compounds as the link to health, including disease prevention and treatment, has just emerged lately. The capability of bioactive compounds to modulate biological activities and physiological function become the main factor contributing to health (Gallo, 2022). Among the well-known bioactive compounds in CRE are flavonoid, alkaloid, and phenolic compounds. Flavonoid compounds have various anticancer effects, such as reactive oxygen species (ROS) modulation, which results in cell cycle arrest, induction of apoptosis, autophagy, and inhibition of cell growth and invasion (Kopustinskiene et al., 2020). A possible mechanism of alkaloid mechanism such as vincamine, where CRE contained vincamine derivative such as apovincamine, is the suppression of cancer growth through the reduction of cancer cells ion levels, induction of caspase-3, and interfering mitochondrial membrane (Dhyani et al., 2022). This study indicated that the total phenolic content was higher than the total flavonoid content. Phenolic compounds have several anticancer activities, including improving the immune system's ability to recognize and kill cancer cells (Wahle et al., 2010) and inducing cancer cell death through the suppression of oncogenes (Anantharaju et al., 2016). Studies show a significant link between high phenolic content and antioxidant activity (Qader et al., 2011) and associated with higher antiproliferative activity (Okafor et al., 2021).

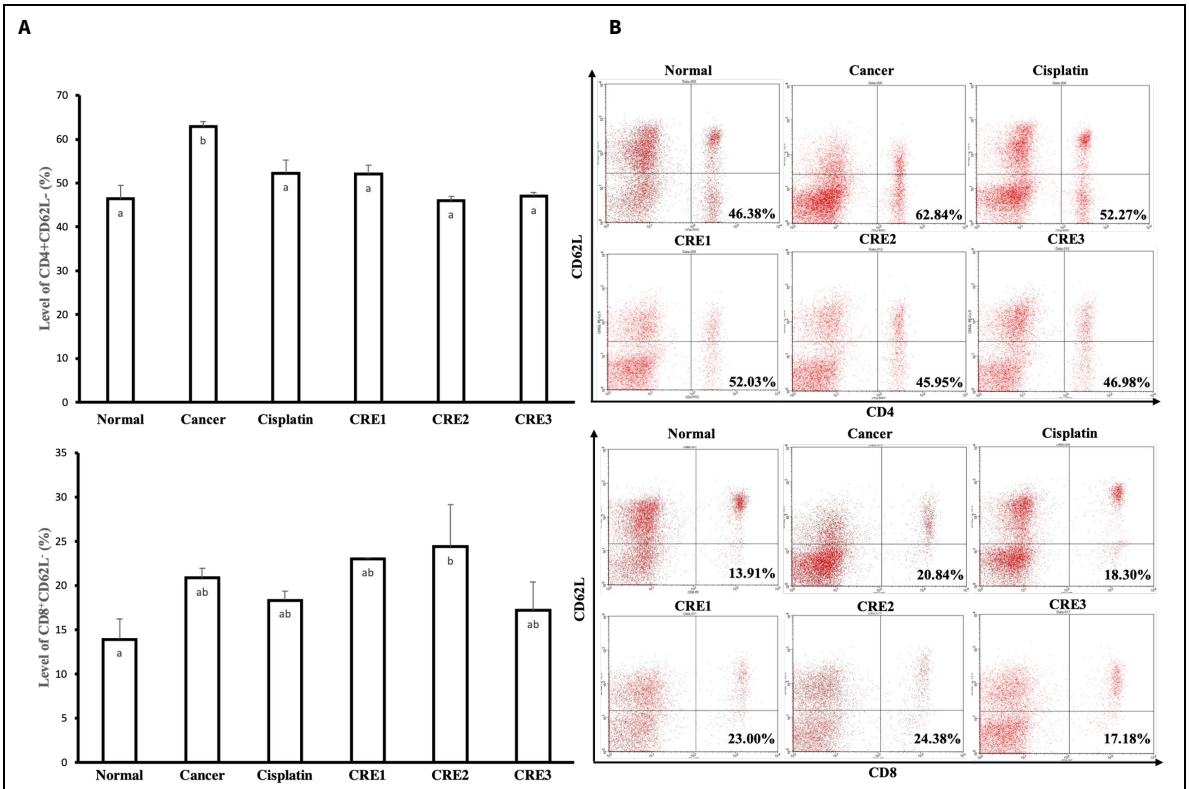

Figure 3. The level of CD4 and CD8 T cell activation ($CD4^+CD62L^-$ and $CD8^+CD62L^-$) of 4T1 cancer cells bearing mice.

(A) The data were expressed as Mean ± standard deviation (SD), and different notations showed a significant difference based on the Tukey HSD test ($p<0.05$). **(B)** Quadrant plots FACS analysis showed a relative number of activated T cells ($CD4^+CD62L^-$ and $CD8^+CD62L^-$) in various groups.

Normal: normal/healthy mice without 4T1 cells injection and treatment; Cancer: mice injected with 4T1 cancer cell lines; Cisplatin: mice injected with 4T1 cancer cell lines and treated with cisplatin drug; CRE1,-2, and -3: mice injected with 4T1 cancer cell lines and treated with CRE 72.5, 145, and 290 mg/kg BW, respectively.

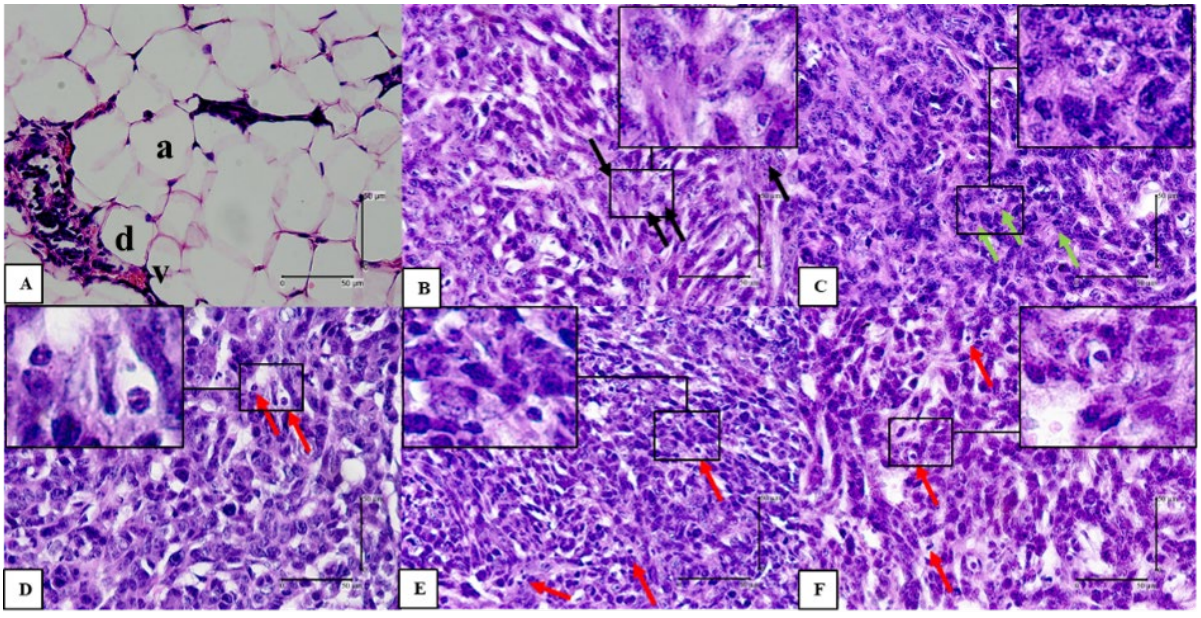

Figure 4. Mice mammary tissues stained with hematoxylin and eosin (HE).

a: adipocyte; **d**: duct; **v**: blood vessel; black arrow indicated pleomorphic cancer cells, green arrow; necrosis/late apoptosis cell; red arrow: apoptotic body.

(A) normal/healthy mice without 4T1 cells injection and treatment; **(B)** mice injected with 4T1 cancer cell lines; **(C)** mice injected with 4T1 cancer cell lines and treated with cisplatin drug; **(D-F)** mice injected with 4T1 cancer cell lines and treated with CRE1-3 with concentration 72.5, 145, and 290 mg/kg BW, respectively. Magnification 400×.

On the contrary, the cytotoxic analysis showed that CRE was categorized as a low cytotoxic agent according to the IC_{50} value (145.02 µg/mL) (Indrayanto et al., 2021). This result is in line with Masfria et al. (2018) that an ethanolic extract of the rhizome of *C. rotundus* showed only weak cytotoxic effects against 4T1 cells (139.92 µg/mL). *C. rotundus* displayed low cytotoxic activity when extracted with various solvents, such as the n-hexane fraction with IC_{50} value of 120.819 µg/mL but still has high anticancer activity, such as cell cycle arrest induction (Simorangkir et al., 2019). In addition, the IC_{50} of CRE obtained in this study was used for further *in vivo* analysis with two more doses variation (0.5 × IC_{50}, IC_{50}, and 2 × IC_{50}) to get the optimum dose as an anticancer *in vivo*. The cytotoxic analysis was also carried out on normal human fibroblast cells (TIG-1) to obtain the selectivity index of CRE. Based on this study, CRE was safe to normal cells (TIG-1) and classified as highly selective against 4T1 cells with SI 9.28. Based on Althaher et al. (2022), a drug with an SI value >3 is highly selective against cancerous cells. Based on Simorangkir et al. (2019), the SI of the n-hexane fraction of *C. rotundus* between MCF-7 and Vero cells was 1.831.

In tumor pathobiology, the immune system is almost there for every step. It was classified into two main groups according to the action mechanism, such as the pro- and anti-tumor immune systems. According to Gonzalez et al. (2018), the pro-tumor immune system involved in angiogenesis, ECM (extracellular matrix) modeling, and immune evasion are tumor-associated macrophages (TAMs), neutrophils (TANs), and immature dendritic cells (DCs). Besides, the immune system's anti-tumor cells, such as cytotoxic cells (T cells, macrophages, NK, and mature DCs), play a crucial role in eliminating cancer cells. In addition, a small molecule commonly produced by immune cells is also divided into two groups according to their mechanisms of action, such as pro-inflammatory (IL-1β, IL-2, IL-6, IL-12, TNF-α, chemokines—IL-8, MCP-1, and others) and anti-inflammatory cytokines (IL-4, IL-10, and TGF-β, among others) (Bel'skaya et al., 2022). One of the unique cytokines with a crucial function in becoming the factor of T cell growth, supporting T cells' differentiation and promoting their cytolytic function (Fasoulakis et al., 2018). Since then, the immune system has needed IL-2 to help eliminate cancer cells. Frequently, the prognosis of breast cancer patients is tracked using this molecule as a marker. Patients with breast cancer had higher levels of IL-2 in their blood and saliva than healthy control (Bel'skaya et al., 2022). This study indicated a significant reduction of IL-2 levels from CD4 T cells ($CD4^+IL\text{-}2^+$) in treatment groups in contrast with the untreated group ($p<0.05$), although there was an insignificant difference in $CD8^+IL\text{-}2^+$. The low level of CD4+IL-2^+ was in line with the low level of CD4 T cell activation ($CD4^+CD62L^-$). The treatment group had lower levels of $CD4^+CD62L^-$ than the untreated group ($p<0.05$). This study emphasized the positive influence of IL-2 in homeostasis and T cell development, especially CD4 T cells (Ross and Cantrell, 2018). A significant increase in CD4 T cells is consistent with the progression of the tumor in 4T1 cells (Huang et al., 2015). Thus, a decrease in $CD4^+CD62L^-$ levels in the treatment group in this study is expected to be a promising treatment effect which can be determined by confirming the result of the mammary histopathological analysis. Contrarily, CD8 T cell activation ($CD8^+$ $CD62L^-$) has the opposite function from CD4 T cells activation. The higher activation of CD8 T cells, a main player of cytotoxic molecules, become the marker of better survival in TNBC (Oshi et al., 2020). Insignificantly, more CD8 T cells were activated in the treatment group than in the cancer control group, according to this study.

The histopathological observation confirmed the positive prognosis of treatment groups compared to cancer control. The cancer group showed more viable cells than the cisplatin and CRE treatment groups. Commonly, cancer cells undergo apoptotic dysregulation to support the survival of the cancer cells (Call et al., 2008). The appearance of apoptotic cell characteristics in treatment groups indicated a promising effect of the treatment, especially CRE, since apoptosis is one target in cancer therapy. The apoptotic cells' characteristic was similar to Nigjeh et al. (2019), which demonstrated the citral apoptotic induction in 4T1-tumor-bearing mice breast tissue using HE staining.

CONCLUSION

This study found that CRE contains phenolic and flavonoids, which could be responsible for the extract activity. CRE is highly selective to induce cancer cell death (4T1) compared to non-cancerous cells (TIG-1). Phenolic compounds may be the main contributor in inhibiting or eliminating cancer cells through immune modulation, such as IL-2 cytokine production and the activation of CD4 and CD8 T cells, and directly induce apoptosis in cancer cells (shown by the apoptotic appearance in the breast tissue of treated mice). Thus, this study found a possible apoptotic pathway as the targeted therapy in which CRE can inhibit the growth of 4T1 cancer cells. However, this finding still needs to be confirmed using the specific apoptotic molecular marker.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Wirdatun N	Nadia W	Muhammad FM	Shella ZKA	Muhaimin R	Nashi W	Muhammad SD
Concepts or ideas	x				x	x	x
Design	x						x
Definition of intellectual content					x	x	x
Literature search	x	x	x	x			
Experimental studies	x	x	x	x			
Data acquisition	x	x	x	x			
Data analysis	x	x					
Statistical analysis			x	x			
Manuscript preparation	x	x	x	x			
Manuscript editing							x
Manuscript review	x	x	x	x	x	x	x

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