Immunomodulatory Effects of *Cyperus rotundus* Extract on 7,12-Dimethylbenz[a]anthracene (DMBA) Exposed BALB/c Mice

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Abstract

**Background:** The carcinogenic substance 7,12-Dimethylbenz[a]anthracene (DMBA) was commonly used to induce tumor formation in rodents. The development of tumor may trigger higher expression of pro-inflammatory cytokines, which in turn supports tumor progression. In this study, we examined the efficacy of *Cyperus rotundus* extract (CRE) that was reported to have anti-inflammatory properties. We focused on investigating the levels of activated T lymphocytes and the pro-inflammatory cytokines expressed by macrophages.

**Methods:** Female BALB/c were injected with DMBA subcutaneously. The DMBA exposed mice were given CRE orally in three different doses; 63.33, 158.4, and 316.8 mg/kg. After 14 days, the levels of activated T lymphocytes and pro-inflammatory cytokines were analyzed using flow cytometry. Graphical analysis was done with FlowJo v10 and followed by statistical analysis.

**Results:** The treatment of CRE reduced the population of CD4 and CD8 T cells. The number of activated CD4 and CD8 T cells were also significantly suppressed. The population of macrophages marked by CD11b cells was significantly reduced. Finally, the CRE treatment suppressed the levels of TNF-α, IFN-γ, IL-1β, and IL-6 expressed by macrophages.

**Conclusion:** Our findings suggest that CRE could be a potential agent useful in therapeutic approaches for curing the disease caused by aberrant cells.

Introduction

Cancer is one of the leading causes of death worldwide. To gain further insight, researchers have been trying to study cancer on animal models. The 7,12-Dimethylbenz[a]anthracene (DMBA) is a chemical agent that was used to study cancer in animal models. Previous studies revealed that DMBA may form mammary tumors with various routes of administration, doses, and exposure duration. Adenocarcinomas were observed from 3 to 7 weeks of multiple administration of DMBA on BALB/c mouse. The multiplicity, latency, and incidence of the tumors were affected by age, reproductive condition, and hormonal status of the host. However, the immunological perspective of the effects of carcinogen exposure, including DMBA, have not been studied well. Although the previous report showed that the administration of DMBA may cause pro-inflammatory cytokine to rise, its effects on lymphocytes and macrophages were not fully understood.

Also known as ‘wounds that do not heal’, cancer is relatively hard to cure due to its ability to escape the immune system. Cancer patients were known to have high levels of inflammatory cytokines. These cytokines were closely related to cancer development by promoting the infiltration of lymphocytes and were shown to have a bad prognosis for cancer patients. Under normal conditions, inflammation will be stopped when the agents causing the disease were eliminated. However, in such cases as cancer, inflammation becomes unregulated leading to chronic inflammation and constitutive expression of different kinds of cytokines. Some inflammatory cytokines were claimed to induce anti-tumor response during the early stages of cancer, but will eventually induce pro-tumor response during the later stages of cancer. Inflammatory cytokines promote tumor proliferation, increases tumor motility, and induces the formation of blood vessels through angiogenesis. Therefore, cancer cells have a higher chance to adapt and survive, because not only blood vessel carries nutrition for tumor cells, but also allows it to metastasize to different parts of the body. Studies have tried to overcome this problem by examining the effects of various herbal extracts to inhibit these cytokines. Although there has
been a limitation for cancer drug development, utilizing local herbs seems to be an alternative way to develop drug. Common Nut Sedge (*Cyperus rotundus* L.) is a well-known medicinal plant in Asian countries. Typically famous as weeds, this plant provides many anti-cancer bioactive compounds which have been clinically proven to treat several types of cancer cell lines, including MCF-7 (breast), HeLa (cervix), Hep-G2 (liver), PC-3 (prostate), and HT-29 (colorectal) cell lines by causing apoptosis. Besides having cytotoxic effects on cancer, CRE also has the potential to promote anti-inflammatory response. Several bio-compounds isolated from *Cyperus rotundus* were claimed to decrease pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α. Furthermore, the expression of NF-κB and STAT3 signaling pathways are suppressed, which allows other pro-inflammatory cytokines to be downregulated. Another research also showed that CRE significantly reduces the expression levels of IL-6 and IFN-γ in inflammatory bowel disease. Other nutrients in this plant are exploited for its bioactive compound with anti-cancer benefits. Ethanol extract of *C. rotundus* has shown promising effects in cancer, which urge this paper to rise. However, its efficacy in modulating the immune system of DMBA-induced mice have not been well understood. The anti-inflammatory effects of CRE might be a potential treatment to suppress the inflammatory cytokines, thus providing a better prognosis to cancer inhibition. This paper aims to observe the modulation of lymphocytes and the cytokine levels in macrophages on mice after the administration of CRE.

**Materials and Methods**

**Animals**

Sixty female BALB/c mice aged 8 weeks old were obtained from Gajah Mada University, Yogyakarta Province, Indonesia. Each mouse was fed with a nutritional pellet diet, given enough water, and morphological changes were recorded routinely. The experimental procedures were evaluated and proved by the Animal Care and Use Committee of Brawijaya University (Legal number: 1152-KEP-UB). The animals were caged separately in 5 different groups and kept at a free-pathogen facility provided by the Biology Department of Brawijaya University. After a week of acclimatization, the animals were randomly separated into 5 different groups (Figure 1). Group I (Vehicle): mice only received corn oil by subcutaneous injection representing control group (n=5). Group II (DMBA): mice were administered with DMBA by subcutaneous injection without any herbal treatment (n=5). Group III-V (CR1-CR3): mice were administered with DMBA by subcutaneous injection and were treated with *Cyperus rotundus* extract (CRE) orally (n=5). Not all mice survived until the end of the study and these mice were not counted in our data analysis. Previous studies reported that CRE had shown significant anti-inflammatory effects at a dose ranging from 50 to 300 mg/kg for mice models, and slightly higher for rat models. In this experiment, we formulated the lower, mild, and higher doses of CRE groups as 63.33, 158.4, and 316.8 mg/kg, respectively.

**DMBA injection**

Cancer induction was done using carcinogenic substance 7,12-Dimethylbenz[a]anthracene (DMBA) (Tokyo Chemical Industry Co. Ltd.). After a week of acclimatization, mice were injected with DMBA subcutaneously near the lower-left flank of the mammary glands. DMBA dose was given 15 mg/kg weight of mice. The administration of every DMBA dose was diluted with corn oil as a solvent. The injection was done weekly for 8 weeks. All mice in the vehicle group survived until the end of the study.

![Figure 1](image_url). Protocol of study. CR1 group (DMBA 15 mg/kg + CRE 63.33 mg/kg); CR2 group (DMBA 15 mg/kg + CRE 158.4 mg/kg); CR3 group (DMBA 15 mg/kg + CRE 316.8 mg/kg); DMBA: 7,12-Dimethylbenz[a]anthracene; CRE: *Cyperus rotundus* extract.
of study. Unfortunately, we found 23.33% of mortality rate in all mice that were exposed with DMBA.

**Herb extraction**

*Cyperus rotundus* rhizome (specimen no.: 074/84A/102.7/2019) was obtained and identified by botanists from UPT Materia Medika, Batu City, Indonesia. Rhizome was cut into small pieces and grounded until it became powder. Extraction was prepared by dissolving *Cyperus rotundus* rhizome powder with absolute ethanol with the proportion of 1:10 (drug: solvent, w/v) inside an aluminum-covered flask to keep it free from sunlight. Materials were shaken with magnetic stirrer using rotary shaker at 1000 rpm for 24 h at 70˚C. The mixture was then filtered and evaporated using an evaporator. The *Cyperus rotundus* extract (CRE) was freeze-dried at -20˚C for 1 week to remove the remaining ethanol. For storage, the extract was kept at 4˚C freezer. The extract was diluted with warm water and administered orally to mice daily for 14 days. It was worth noting that up to 316.8 mg/kg dose of PNE did not exhibit any toxicity.

**Flow cytometry**

Sacrificed mice were given 70% alcohol around the abdomen for sterile sectioning. The spleen was also isolated from sacrificed mice and grounded in Phosphate Buffer Saline (PBS). Homogenate was centrifuged 2500 rpm for 5 minutes at 10˚C and the obtained pellet was resuspended with 1 mL PBS. Antibody staining was done by adding cell suspension with specific intracellular (IL-1β, IL-6, TNF-α, IFN-γ) and extracellular (CD4, CD8, CD62L, CD11b) antibodies (BioLegend, San Diego, CA). Intracellular antibody staining was also added with 50 µL Cytofix & 500 µL Permeabilization wash buffer solution (BioLegend, San Diego, CA). Antibody staining was followed with 20 minutes incubation at 4˚C and was centrifuged 2500 rpm for 5 minutes at 10˚C. The centrifuged cell suspension was added with 400 µL PBS inside 1.5 mL microtube, homogenized using a vortex and transferred to a cuvet tube. The sample was followed with flow cytometry analysis (FACS Calibur™) and data was recorded using computer. Graphical analysis was done with FlowJo v10.

**Statistical analysis**

Statistics were performed using SPSS 25.0 software for ANOVA test and Post Hoc Tukey HSD Test. In this study, a value of p<0.05 was considered significant between two different groups. All data were shown in mean ± standard deviation (SD).

**Results**

*CRE treatment suppressed lymphocyte T cell population*

The population of CD4\(^+\) T cells in the DMBA group was significantly higher than the vehicle group (p<0.01) (Figure 2). In this study, we observed that the treatment of CRE reduced the levels of CD4\(^+\) T cells in a dose-dependent manner. The lower dose of CRE diminished the population of CD4\(^+\) T cells to 8.4% compared to the DMBA group (p<0.05). The mild and higher dose reduced CD4\(^+\) T cells up to 8.3% (p<0.05) and 7.7% (p<0.01), respectively. Similarly, the population of CD8\(^+\) T cells in the DMBA group was significantly higher than the vehicle group. Unlike CD4\(^+\) T cells, the reduction of CD8\(^+\) T cells were not obtained in a dose-dependent manner. However, each CRE doses have very similar amount of CD8 T cells ranging around 6.3~6.4% of the total lymphocytes (p<0.01). The

![Figure 2](image_url)

*Figure 2.* The relative number of CD4\(^+\) and CD8\(^+\) T cells. Vehicle group; DMBA group (DMBA 15 mg/kg BW); CR1 group (DMBA 15 mg/kg + CRE 63.33 mg/kg); CR2 group (DMBA 15 mg/kg + CRE 158.4 mg/kg); CR3 group (DMBA 15 mg/kg + CRE 316.8 mg/kg). **:** P<0.001, **:** P<0.01, *:** P<0.05. DMBA: 7,12-Dimethylbenz[a]anthracene; CRE: *Cyperus rotundus* extract.
results indicate that every dose of CRE have the potential to normalize the immense number of CD4 and CD8 T cells after DMBA exposure.

**CRE treatment inhibits T cell activation**
Activated T cells in this experiment were marked by the loss of CD62L marker on the surface of CD4 and CD8 T cells. The population of activated CD4 T cells was higher in the DMBA group compared to the vehicle group (Figure 3). The treatment of CRE reduced the number of activated CD4 T cells in a dose-dependent manner. The lower dose of CRE reduced activated CD4 T cells to 48.1% (p<0.01). The mild and higher doses of CRE further reduced the number of activated CD4 T cells up to 47.5% and 47.7%, respectively (p<0.01). Activated CD8 T cells in the DMBA group were also significantly higher compared to the vehicle group. The lower dose of CRE does not significantly reduce the CD8 T cells. However, the mild and higher doses of CRE reduced the number to 46.0% and 47.1%, respectively (p<0.01).

**CRE treatment suppressed macrophage population**
CD11b is a common marker used for detecting the presence of macrophages. In this experiment, we only observed 8.7% of CD11b+ cells in the vehicle group (Figure 4). The exposure of DMBA significantly increased the population to 22.7% (p<0.001), which is almost 3 times fold compared to the vehicle group. The lower CRE dose does not significantly reduce the CD11b+ cells. However, the mild and higher doses of CRE successfully reduced the population to 17.2% and 18.5%, respectively (p<0.05). This demonstrated that CRE treatment reduced the population of CD11b cells in the DMBA-exposed mice.

**CRE treatment inhibit TNF-α expression**
Based on the previous results, we suspect that CRE may have anti-inflammatory properties that resulted in the reduction of lymphocyte T cells. Therefore, we examined the effects of CRE treatment on the cytokine levels expressed by macrophages. TNF-α is regarded as one of the main pro-inflammatory cytokines in the immune system. The levels of TNF-α+ expressed by macrophages in the vehicle group is around 18.9% (Figure 5). The exposure of DMBA elevated the level of the cytokine up to 35.01% (p<0.001), which is almost twice the amount compared to the vehicle group. No significant difference was observed in the lower and mild doses of CRE compared with the DMBA group. However, at a higher dose, the CRE treatment has significantly reduced the expression of TNF-α+ to 23.7% (p<0.01).

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**Figure 3.** The relative number of activated CD4+ and CD8+ T cells. Vehicle group; DMBA group (DMBA 15 mg/kg BW); CR1 group (DMBA 15 mg/kg + CRE 63.33 mg/kg); CR2 group (DMBA 15 mg/kg + CRE 158.4 mg/kg); CR3 group (DMBA 15 mg/kg + CRE 316.8 mg/kg). ***: P<0.001, **: P<0.01, *: P<0.05, ns: non-significant. DMBA: 7,12-Dimethylbenz[a]anthracene; CRE: Cyperus rotundus extract.
CRE treatment inhibit IFN-γ expression

Macrophages were known to have important roles in inflammation. Another essential pro-inflammatory cytokine by macrophages is IFN-γ. In this experiment, IFN-γ expressed by macrophages was marked by CD11b⁺IFN-γ⁺ (Figure 6). The levels of IFN-γ⁺ in the vehicle group is 17.3%, and DMBA exposure increased the amount to 39.1% in the DMBA group (p<0.001). The administration of CRE suppressed IFN-γ⁺ levels in a dose-dependent manner. The lower dose of CRE reduced IFN-γ⁺ levels to 29.6% (p<0.01). At the mild and higher dose, CRE had its highest suppression potential causing IFN-γ⁺ being reduced to 25.8 (p<0.001) and 25.7% (p<0.001), respectively.

CRE treatment inhibit IL-1β expression

The levels of IL-1β secreted by macrophages were marked by CD11b⁺IL-1β⁺. The IL-1β level in the vehicle group was 17.9% and was elevated up to 31.0% due to the exposure of DMBA (p<0.001) (Figure 7). The treatment of CRE significantly reduced the IL-1β levels, although not in a dose-dependent manner. At the lower dose, CRE was able to reduce the cytokine level to 23.4% (p<0.05) compared with the DMBA group. The mild and higher dose of CRE further suppressed IL-1β levels to 22.4% (p<0.01) and 24.4% (p<0.05). The results demonstrated that the treatment of CRE could minimize the high levels of IL-1β after DMBA exposure.
**CRE treatment inhibit IL-6 expression**

Inflammation is often correlated with high expression of IL-6 cytokine. Although it may also be expressed by other immune cells, IL-6 was primarily secreted by macrophages. The levels of IL-6 in the vehicle group was 17.9% (Figure 8). After the exposure of DMBA, the levels of IL-6 significantly elevated around twice the level to 33.6% (p<0.001). Similar to the other cytokines, the treatment of CRE affected the expression of IL-6, although not observed in a dose-dependent manner. The lower dose of CRE reduced the levels of IL-6 to 23.7% (p<0.01). The mild and higher doses of CRE further reduced the levels of IL-6 to 27.8% (p<0.05) and 27.4% (p<0.05), respectively. This indicates that the treatment of CRE may be used to normalize the levels of IL-6 in the DMBA exposed mice.

**Discussion**

Polycyclic aromatic hydrocarbons (PAH) such as the 7,12-Dimethylbenz[a]anthracene (DMBA) were known to have carcinogenic effects and was previously reported to successfully trigger the formation of breast tumors in animal models. Several mechanisms were proposed to understand the carcinogenicity of DMBA from ras gene mutation to cellular oxidative damage that was important...
Figure 8. The relative number of IL-6+ expressed by CD11b+ cells. Vehicle group; DMBA group (DMBA 15 mg/kg BW); CR1 group (DMBA 15 mg/kg + CRE 63.33 mg/kg); CR2 group (DMBA 15 mg/kg + CRE 158.4 mg/kg); CR3 group (DMBA 15 mg/kg + CRE 316.8 mg/kg). ***: P<0.001 **: P<0.05, *: P<0.01. DMBA: 7,12-Dimethylbenz[a]anthracene; CRE: Cyperus rotundus extract.

in the progression of breast cancer. To enhance tumorigenesis, DMBA was injected to young rodents, when mammary glands were undifferentiated and highly proliferative. Interestingly, tumorigenesis in mice is strains dependent. In this study, we chose BALB/c mice because it was reported to be more sensitive to carcinogen substances compared to other strains. The administration of DMBA on BALB/c mice caused the pro-inflammatory cytokines expressed in CD4 T cells to rise. Similar to this finding, we found pro-inflammatory cytokines expressed in macrophages had significantly elevated. The population activated CD4 and CD8 T cells were higher compared to the normal state. This suggests that DMBA may have triggered the innate and adaptive immune response that caused the activation of immunocompetent cells.

In this study, we found that CRE treatment may normalize the population of CD4 and CD8 T cells in the DMBA exposed mice. The treatment of CRE reduced the levels of CD4 and CD8 T cells in a dose-dependent manner. We also found that the ratio of CD4+ to CD8+ T cells range from 1.33 to 1.77 in all groups. Ratios ranging from 1.5 to 2.5 is often considered normal, although it may depend on several factors such as age and sex. According to Yang et al, CD4/CD8 ratio >1.7 was associated with worse breast cancer prognosis. Studies have shown that a high ratio of CD4/CD8 T cells in tumors has been linked with lymph node metastasis and reduced patient survival in breast cancer. Increased CD4 T lymphocytes are usually correlated to lymph node metastasis, leading the patient to a worse outcome. CD8 T cell, on the other hand, was associated with a better outcome in breast cancer, as it is necessarily needed for inducing apoptosis in cancer cells. This allows a better prognosis for cancer suppression growth and development. Unlike CD4+ T cells which were able to differentiate into many different subsets, the CD8 T cells mainly differentiate into memory and effector cells. The CD4 T lymphocyte can differentiate into different subsets, including Th (T helper) 1, Th2, Th17, Th22, Treg (regulatory T cells), and Th (follicular helper T cells). The CD4 and C8 T cells can be characterized by different cytokine profiles, have distinct surface markers, and serves a different function in cancer development.

As part of the innate immune system, macrophages may function as antigen-presenting cells (APC). Polarization of macrophages involves uncommitted macrophage M0 and turns into either M1 or M2 subtypes. During cancer development, macrophages tend to infiltrate tumor microenvironment and support tumor metastasis. These macrophages are often called as tumor-associated macrophages. In this report, we showed that CRE may reduce the number of macrophages. To examine the effects of CRE on the pro-inflammatory cytokines, we measured several cytokines that were expressed by macrophages. We found that CRE treatment could reduce the expression
of TNF-α in the DMBA exposed mice. This may be advantageous for cancer treatment since higher TNF-α levels showed worse prognosis at the later stages of cancer.\textsuperscript{19} TNF-α is one of the main cytokines that is involved in inflammation and tumorigenesis. This cytokine induces cancer growth through NF-κB and AP-1-dependent pathways, which later promotes tumor survival.\textsuperscript{36-37} Interferon-γ (IFN-γ) also exerts the same function and plays an important role in tumor growth and development. A high level of IFN-γ is correlated with a more aggressive cancer phenotype. The presence of IFN-γ may upregulate PD-1 in the tumor microenvironment. This immune checkpoint caused cytotoxic CD8 T cells to be inactivated, which makes it ineffective to eliminate tumor cells. The reduction of IFN-γ shows a good prognosis in cancer and promotes cytotoxic T cells to eliminate tumor cells.\textsuperscript{38} Interleukin 1β (IL-1β) was secreted by most cell types and signals on cells of both hematopoietic and non-hematopoietic originated cells. The secretion of IL-1β generates the vast spectrum of biological responses ranging from the effects of the central nervous to hematologic and metabolic systems. Although IL-1β secretion is essential for immunity, overexpression may be highly detrimental and leads to autoimmune diseases. IL-1 should thereby be controlled and regulated to prevent the progression of diseases. In breast experimental mice, IL-1β contributes to the development and invasiveness of mammary tumors. High levels of IL-1β were shown to have a bad prognosis in cancer, especially in the later stages. Since IL-1β promotes tumor metastasis, studies revealed that inhibiting IL-1β shows a better prognosis for survival.\textsuperscript{39,40} Analogous to this case, similar effects were observed on interleukin-6 (IL-6). IL-6 was shown to have a good prognosis in the early stage of cancer, but will eventually promote tumor development during the later stages of cancer.\textsuperscript{7} Our study revealed that CRE significantly suppressed IL-6 in the DMBA exposed mice. The reduction of IL-6 will inhibit tumor proliferation and prevent the secretion of several pro-tumor cytokines such as the interleukin-17 (IL-17).\textsuperscript{21} Based on our results, Cyperus rotundus had the potential to promote an anti-inflammatory response in the DMBA exposed mice. The mechanisms underlying the therapeutic effects may be due to the inhibition of nitric oxide (NO) and superoxide (O\textsubscript{2}⁻) expression in a dose-dependent manner. We suspect this may be one of the therapeutic pathways of CRE since it was described that DMBA stimulates tumorigenesis and inflammation by manipulating the reactive oxygen species (ROS) pathways. Besides, CRE was also reported to suppress the activation of NF-κB and STAT3 signaling pathways which allows pro-inflammatory cytokines to be downregulated.\textsuperscript{13} For this reason, CRE had been noted to acquire anti-inflammatory properties and reduce the levels of IL-1β, IL-6, and TNF-α.\textsuperscript{10,12} Other anti-inflammatory effects of CRE were extensively studied in many reports.

**Conclusion**

The carcinogenic substance DMBA had elevated the levels of activated T cells and pro-inflammatory cytokines. In this study, we found that the CRE treatment was able to normalize the population of lymphocyte T cells and suppressed CD4 and CD8 T cell activation. We also found that CRE was able to suppress pro-inflammatory cytokines secreted by macrophages. It is worth noting that these reductions generally occur in a dose-dependent manner. We suspect that CRE may have certain compounds that were able to control and interfere with the pro-inflammatory cytokines pathways. Our findings suggest that CRE possessed anti-inflammatory properties and further experiments using purified bio-compounds should be put to investigate the mechanism.

**Ethical Issues**

Any experimental protocols for ethical clearance used in our study were approved by the Brawijaya University Animal Experimentation Ethics Committee (Legal number: 1152-KEP-UB).

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**Conflict of Interest**

The author declare there is no conflict of interest in this study.

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