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Aqueous black rice (*Oryza sativa* L. *indica*) extract enhanced the activation of CD4⁺ and CD8⁺ T cells in mouse breast cancer model

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Abstract

Black rice is a functional food of Indonesia that has several pharmaceutical activities including anti-cancer. This study aimed to evaluate the immunomodulatory potential of aqueous black rice (ABR) extract on mouse breast cancer model. A carcinogen, 7,12-dimethylbenz[a]anthracene (DMBA), was applied for breast cancer induction in mice. The study was designed to be seven groups (six mice in each group), including Normal or control group (N), a Cancer (C; DMBA-induced mice) group, Cisplatin (Cis; DMBA-induced mice + Cisplatin) treatment group, and DMBA-induced mice + ABR extract (ABR1; dose 1 or 0.2 g/kg, ABR2; dose 2 or 0.3 g/kg, ABR3; dose 3 or 0.4 g/kg, and ABR4; dose 4 or 0.5 g/kg) treatment group. The cancer-bearing mice were given ABR extract for two weeks, and flow cytometry was used to assess the numbers of CD4⁺ and CD8⁺ T cells, as well as the production of inflammatory cytokines IL-17, TNFα, and IFNγ. The histopathology of breast tissue in mice was analyzed using hematoxylin and eosin staining and microscope observation. The induction of DMBA in mice lower the activation of CD4⁺ and CD8⁺ T cells, and induced IL-17, TNFα, and IFNγ production. The histology of the mammary tissue of the untreated mice demonstrated an infiltration of cancer cells toward stroma, whereas that of the treated groups, especially those under high doses of ABR extract, showed a better prognosis, exhibiting a reduction in cancer cells surrounded by a relatively larger number of adipose tissues. The activation of CD4⁺ and CD8⁺ T cells, as well as the reduction of cytokines (IL-17, TNFα, and IFNγ), were thought to be influenced by ABR extract. The activation of T cells optimized the elimination of cancer cells activity; moreover, the production of pro-tumor cytokines was inhibited.

Keywords

black rice, breast cancer, DMBA, cytokine, T cell

Introduction

Based on GLOBOCAN 2018 data, the new cancer cases and deaths in 2018 was about 18.1 and 9.6 million. The leading cause of death among all cancer types was lung cancer (18.4% of total deaths) followed by breast cancer (11.6%), which was mostly found in women. In Indonesia, the most diagnosed cancer type was breast cancer (16.7% in both sexes and 30.9% in females) (Bray et al. 2018). Conventionally, cancer diseases can be treated by chemotherapy that has various side effect such as vomiting (Nurgali et al. 2018), decreased quality of life, and gastrointestinal side effects as well as the radiation therapy and immunotherapy side effects (O'Reilly et al. 2020). However, at present, anti-cancer herbal medicine is being increasingly adopted for treatment. Therefore, a detailed examination of the anti-cancer activity of herbs is both significant and urgently needed (Zheng et al. 2018).

Black rice is a pigmented rice commonly found in Asian countries. Black rice is described as a functional food of several countries including Indonesia, due to its benefits in health (Pratiwi and Purwestri 2017; Thanuja and Parimalavalli 2018). Recently, much attention was received in the use of black rice extract due to the high bioactive compounds content, including tocopherols, oryzanols, polyphenols, B vitamins, and fibers, as well as a wealth of anthocyanins, a group of flavonoids that exhibit antioxidant properties (Dias et al. 2017). Other nutrients and bioactive components found in black rice include essential amino acids, functional lipids, dietary fiber, vitamins, minerals, anthocyanins, phenolic compounds, -oryzanols, tocopherols, tocotrienols, phytosterols, and phytic acid (Ito and Lacerda 2019). Anthocyanin, exhibiting anti-cancer activity, is one of most abundant compounds in black rice and has been well reported. Anthocyanins have anti-cancer properties such as cytotoxicity against breast cancer cell lines (MCF-7, MDA-MB-231, and MDA-MB-453) and the ability to induce apoptosis in MDA-MB-453 by activating its apoptotic intrinsic pathway (Chen et al. 2015). In addition, black rice anthocyanins also involved in the inhibition of MDA-MB-453 cells migration and invasion via the suppression of mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), and production of MMP-2 and MMP-9 (Chen et al. 2015). In addition, isolated anthocyanin also exhibit hepatoprotective activity in rats (Saati 2016). Although black rice has been shown to have anti-cancer properties, research on its relationship with cancer immunology is still restricted. Immune system plays an essential role in eliminating cancer especially anti-tumor immune effector such as CD8⁺ T cells (Nafisah et al. 2023). However, immune cells involved in all stages of cancer, to eliminate or promote cancer progression. Pro-inflammatory cytokines tend to increase in cancer and it was associated with tumor development through the induction of tumor proliferation, invasion, and angiogenesis (Ramadhani et al. 2020). Because the immune system plays an important role in cancer treatment, the goal of this study is to evaluate the effect of black rice extract on the immunological profile and histology of a mouse breast cancer model.

Materials and methods

Animals and treatment group

Our study used female Balb/c mice (6 weeks age), which were then acclimatized for one week. All experimental techniques were authorized by the Brawijaya University Experimental Ethics Committee (No: 1152-KEP-UB). Each mouse was kept in a cage and fed a conventional meal with plenty of water. Seven groups of mice were created (six mice of each group), including Normal or control group (N), Cancer group (C; 7,12-dimethylbenz[a]anthracene [DMBA]-induced), Cisplatin treatment group (Cis; DMBA-induced mice + Cisplatin), and DMBA-induced mice + aqueous black rice (ABR) extract treatment group (ABR1; dose 1 or 0.2 g/kg, ABR2; dose 2 or 0.3 g/kg, ABR3; dose 3 or 0.4 g/kg, and ABR4; dose 4 or 0.5 g/kg). The ABR extraction performed in this work was based on a method from a previous study by with some modification (Hartati and Andryanto 2022). Briefly, black rice extract powder was added to distilled water (1:10, m:v) and held for 12 h at room temperature. The extract was then filtered with filter paper before adding 4% maltodextrin. The addition of maltodextrin ensured the complete drying of the extract, so it could be stored longer.

After acclimatization, the mice were induced with DMBA, a carcinogenic substance, through subcutaneous injection for 4 weeks (once a week) and held for 6 weeks to allow tumor growth and development (10 weeks total for developing the mice breast cancer model), followed by ABR and Cisplatin treatment. The administration of ABR extract was done once in the morning, orally, for 2 weeks. This study used Cisplatin as the control drug through subcutaneous injection. Cisplatin is a well-known anti-cancer drug, suppressing breast cancer cell viability via extensive apoptosis induction (Zhu et al. 2020). The Cisplatin dose used in this study was 5 mg/kg of mouse body weight (BW) and DMBA dose was 15 mg/kg based on (Ramadhani et al. 2020).

Antibody staining and flow cytometry analysis

The evaluation of CD4⁺ and CD8⁺ was performed using flow cytometer, as well as the production of interleukin (IL)-17, tumor necrosis factor alpha (TNF α), and interferon gamma (IFN γ). Phosphate buffer saline was used to rinsed and homogenized the spleen of sacrifice mice. To obtain a pellet, the homogenate was centrifuged at 2500 rpm for 5 minutes at 10 °C. Several antibodies were used to label the cells, including anti-CD4, anti-CD8, anti-CD62L, anti-IL-17, anti-TNF α , and anti-IFN γ (Biolegend). The anti-CD62L antibody that was used in this study is also known as L-selectin, a homing molecule. Because L-selectin expression is downregulated when T-cell receptors are engaged, it is often utilized as a marker for T-cell activation (Watson et al. 2019). After antibody staining, the expression of the antibody or parameter was analyzed using an FACS Calibur™.

Tissue preparation for hematoxylin and eosin staining

The breast tissue of sacrificed mice was isolated to evaluate cancer progression using histological hematoxylin and eosin staining, beginning with deparaffinization procedures. The tissue section was then stained using hematoxylin and eosin. Finally, the samples were cleaned and dehydrated so that they could be mounted with malinol and covered by a cover glass.

Statistical analysis

The statistical analysis was carried out using SPSS 16.0 software, with the One-Way ANOVA test yielding a significant result of $p < 0.05$. Tukey's post-hoc HSD test was used to see if there was any significant difference between the groups.

Results

ABR extract enhanced the CD4⁺ and CD8⁺ T cells

Our study analyzed the effect of ABR on the activation of CD4⁺ and CD8⁺ T cells using flow cytometry. We found

that the activation of CD4⁺ (CD4⁺CD62L⁻) and CD8⁺ (CD8⁺CD62L⁻) were reduced in DMBA induction in mice, whereas, the activation of both cells was increased significantly upon administering ABR ($p < 0.05$), except CD4⁺CD62L⁻ of ABR1 group (Fig. 1).

ABR extract reduced the production of IL-17, TNF α , and IFN γ cytokine

A flow cytometry examination was performed to see how the ABR extract affected the production of cytokines. DMBA increased the level of CD4⁺IL17⁺, while it was significantly reduced after Cisplatin and ABR extract treatments ($P < 0.05$) (Fig. 2). The level of CD4⁺IL17⁺ in the C group was 8.45%, while that in the treatment groups was not higher than 1.0%. Further, the production of TNF α by CD4⁺ T cells was also analyzed. The results showed that DMBA increased the level of CD4⁺TNF α ⁺ (9.67%), yet it was reduced significantly after treating with Cisplatin and ABR extract ($P < 0.05$) (Fig. 2). The level of CD4⁺TNF α ⁺ in the Cisplatin, ABR1, and ABR2 groups was similar to that of the N group. The production of CD4⁺IFN γ ⁺ also increased in the cancer and Cisplatin groups, whereas their cytokine production was significantly ($P < 0.05$) reduced upon ABR extract treatment (Fig. 2).

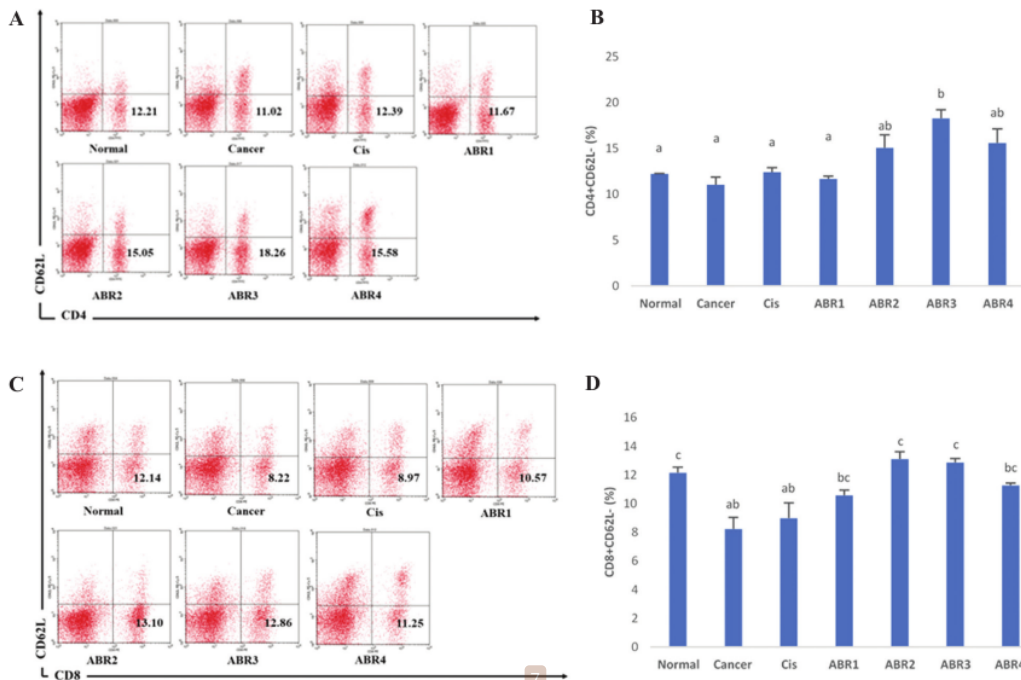


Figure 1. Aqueous black rice (ABR) extract increased the relative number of CD4⁺CD62L⁻ cells and CD8⁺CD62L⁻ cells. A, C. Show flow cytometry diagrams, and B, D. Show graphs of the flow cytometry results. The bars in the graphs show the calculated results as the mean \pm SD of the relative number of CD4⁺ and CD8⁺ cell activations. * $P < 0.05$ indicates a significant difference. The groups in this study included the following groups: Normal; Cancer, DMBA 15 mg/kg BW; Cis, DMBA 15 mg/kg BW + Cisplatin 5 mg/kg BW; ABR1, DMBA 15 mg/kg BW + ABR extract 0.2 g/kg BW; ABR2, DMBA 15 mg/kg BW + ABR extract 0.3 g/kg BW; ABR3, DMBA 15 mg/kg BW + ABR extract 0.4 g/kg BW; and ABR4, DMBA 15 mg/kg BW + ABR extract 0.5 g/kg BW.

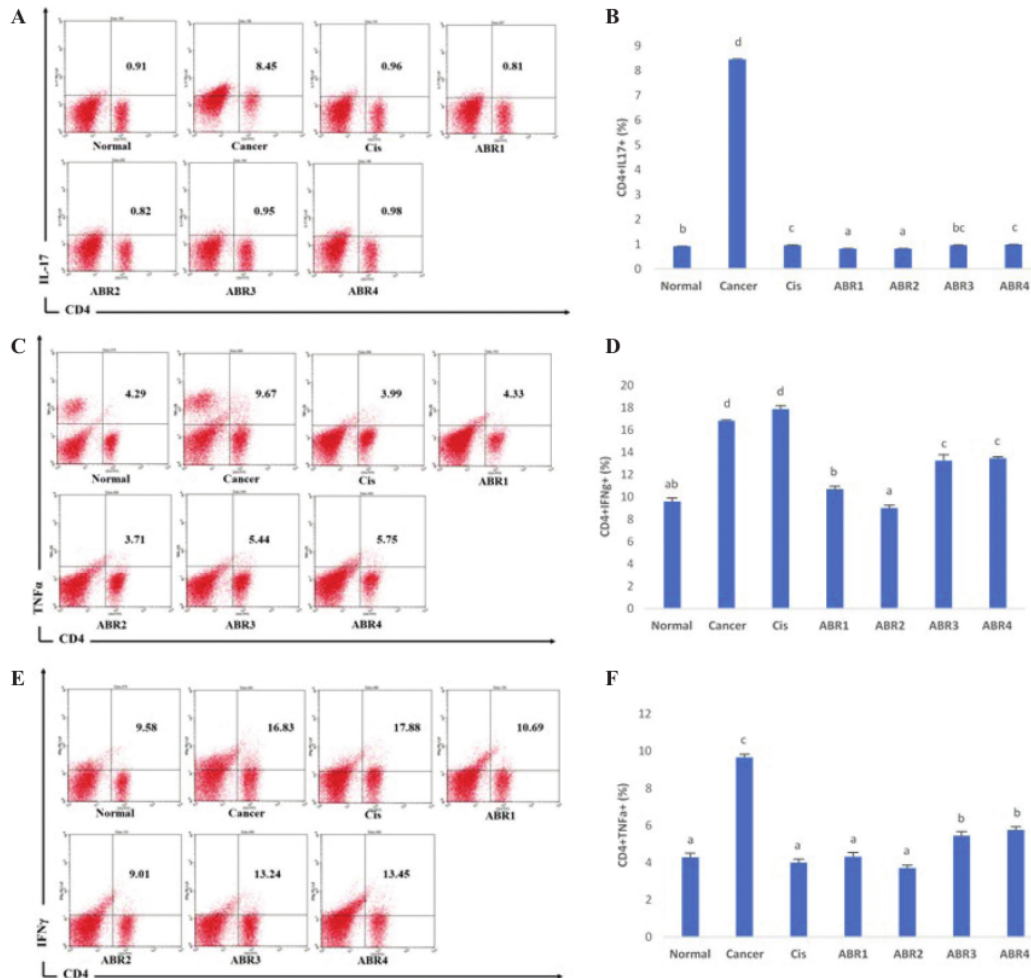


Figure 2. Aqueous black rice (ABR) extract reduced the relative number of CD4⁺IL17⁺, CD4⁺TNFα⁺, and CD4⁺IFNγ⁺ cytokine production. A, C, E, were flow cytometry diagram; B, D, F, were the graph of flow cytometry results. The bar in the graph shows the calculation results as the mean ± SD of the relative number of cytokine production. *P<0.05, indicate significant different. The group in this study were normal group; Cancer, DMBA 15 mg/kg BW; Cis, DMBA 15 mg/kg BW + Cisplatin 5 mg/kg BW; ABR1, DMBA 15 mg/kg BW + aqueous black rice extract 0.2 g/kg BW; ABR2, DMBA 15 mg/kg BW + aqueous black rice extract 0.3 g/kg BW; ABR3, DMBA 15 mg/kg BW + aqueous black rice extract 0.4 g/kg BW; ABR4, DMBA 15 mg/kg BW + aqueous black rice extract 0.5 g/kg BW.

Histopathological analysis

The development of cancer in the mice was characterized via a histopathological analysis. The normal group showed clear duct (D) that was surrounded by adipose tissue (AT) (Fig. 3). Significant differences were found in the mammary tissue of the Cancer group (C, Fig. 3), where cancer cells (arrow) infiltrated to stroma, indicating an invasive carcinoma. The mice treated with Cisplatin (5 mg/kg BW) showed microinvasive tumor cells toward stroma (Cis, Fig. 3). The histology of the ABR1 group exhibited an infiltration of cancer cells toward stroma, leading to the formation of tubules (arrow at ABR1, Fig. 3). While the

distribution of cancer cells in the ABR2 group (Fig. 3) was irregular, similar to the cancer group (C, Fig. 3), there was a relatively higher amount of AT (cells were not counted). The ABR extract treatment at doses 3 and 4 showed a relative abundance of AT with clearer ductal (ABR3, and ABR4, Fig. 3).

Discussion

The immune system role in killing cancer significantly influences the success of cancer therapy. The involvement of CD4⁺ and CD8⁺ T cells is critical in the response of

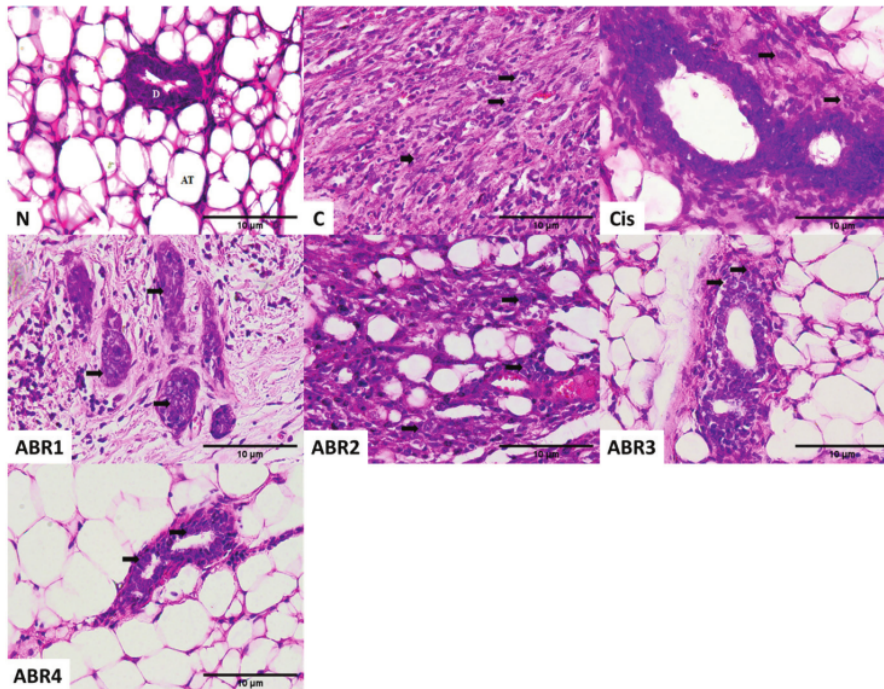


Figure 3. Aqueous black rice (ABR) extract effect on mammary mice histology based on Hematoxylin & Eosin staining (M: 400x). D, ductal; AT, adipose tissue; arrow, cancer cell. The group in this study were normal group; Cancer, DMBA 15 mg/kg BW; Cis, DMBA 15 mg/kg BW + Cisplatin 5 mg/kg BW; ABR1, DMBA 15 mg/kg BW + aqueous black rice extract 0.2 g/kg BW; ABR2, DMBA 15 mg/kg BW + aqueous black rice extract 0.3 g/kg BW; ABR3, DMBA 15 mg/kg BW + aqueous black rice extract 0.4 g/kg BW; ABR4, DMBA 15 mg/kg BW + aqueous black rice extract 0.5 g/kg BW.

effective immune anti-tumor activity (Bui and Schreiber 2007). Cancer causes the production of program death-ligand 1 (PD-L1), which can block T cells activation by interacting with programmed cell death protein 1 (PD-1) molecules on surface-activated T cells (Vareki et al. 2017). The activation of T cells, especially CD8⁺ T cells, is crucial and become most powerful effector for eliminating cancer cells. The killing mechanism of CD8⁺ T cells occur through several way such as the production of cytotoxic granules including perforin and granzyme, and death domain FASL activation which lead to DNA fragmentation on cancer cell (Raskov et al. 2021). Thus, the enhancement of CD8⁺ indicated the effect of immunotherapies through the blockade of PD-L1/PD-1 interaction (Nafisah et al. 2022). Phenolic compound was supposed to be the main ingredient of black rice extract and played as immunomodulator (Hartati et al. 2017). Dietary polyphenols can enhance B and T cell proliferation as well as inhibit pro-inflammatory factor through inducing Treg activity (Shakoor et al. 2021). The reduction of CD4⁺ and CD8⁺ T cell activation in the C and Cis groups was linked to increased IFN γ expression, according to our findings. The IFN γ receptor which binds to interferon regulatory factor 1 with the PD-L1 promoter, promoted PD-L1 expression in different cancer cells when IFN γ levels were high (Qian

et al. 2018). The IFN γ /PD-L1 axis enhanced the expression of PD-L1, which can eventually bind with PD-1 in T cells, causing T cell dysfunction and apoptosis. So that, the reduction of IFN γ production in the ABR treatment groups (Fig. 2) may be correlated with the induction of CD4⁺ and CD8⁺ T-cell activation (Fig. 1). The activation of CD8⁺ T cells via T-cell receptors that bind to class-1 major histocompatibility complexes (MHCs) can efficiently destroy cancer cells by inducing apoptosis using TNF-related apoptosis-inducing ligands (TRAILs) or the production of perforin and granzyme (Töpfer et al. 2011). This lack of CD8⁺ T cell activation affected the growth and development of cancer, as determined by the histopathological evaluation of the mouse breast tissue (Fig. 3). The histopathological analysis showed a relative reduction in cancer cells that sprouted up only at ductal areas, which indicated a better prognosis in both groups, ABR3 and ABR4.

In this study, the upregulation of IL-17 found in the C group was significantly reduced after treated with Cisplatin and ABR (Fig. 2). It was argued by (Welte and Zhang (2015) that the up regulation of IL-17 found in solid tumors is crucial in the development of cancer. IL-17 can bind to its receptor on cancer cell surfaces, which supports the growth and development of cancer through several mechanisms, including apoptosis suppression in

mammary cell carcinoma. Thus, the reduction in IL-17 in the treatment groups seems to be a promising sign for a good prognosis in cancer therapy. It was also reported by Zhao et al. (2020) that the inhibition of IL-17 suppressed cancer metastasis and development. Another study found that IL-17 and TNF α in human prostate and colon cancer can work synergistically to induce PD-L1 expression (Wang et al. 2017). TNF α is a central inflammation mediator that has become an interesting novel therapeutic target in developing cancer therapies. The role of this cytokines was reported to be take a part in the initiation, progression, and metastasis of breast cancer (Waters et al. 2013). TNF α is involved in the epithelial-to-mesenchymal (EMT) transition, which is crucial for cell migration and cancer metastasis (Mercogliano et al. 2020). In addition, pro-inflammatory cytokine IFN γ can promote cancer cell

metastasis and contribute to the failure of immunotherapy in cancer (Zaidi 2019; Beziaud et al. 2023). The reduction of these pro-inflammatory cytokines by black rice extract may contributed by anthocyanin which reduce NF- κ B signaling activity (Piazza et al. 2022).

Conclusion

This study concluded that ABR extract has immunomodulator effect by increasing T cell activation (CD4 and CD8) and reducing pro-inflammatory cytokines (IL-17, TNF α , and IFN γ) production. The enhancement of anti-tumor activity of T cell as well as the reduction of pro-inflammatory cytokines of ABR extract may a promising effect in cancer therapy.

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