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Research Article

CHEMOPREVENTION EFFECT OF *CURCUMA AERUGINOSA* IN DMBA-INDUCED CYTOKINES PRODUCTION

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ABSTRACT

The member of Zingiberaceae, *Curcuma aeruginosa Roxb*. (C. *aeruginosa*) has been recognized as medicine for cancer. However, not enough effort has been invested on this herb as chemoprevention agent on cancer development. In this current study, we investigated the influence of *C. aeruginosa* extracts as a chemo-preventive agent in Wistar rat induced by 7,12-Dimethylbenz[a]anthracene (DMBA). As methods, female Wistar rats were treated with three *C. aeruginosa* extract doses (CA1: 40mg/ 200g BW; CA2: 80mg/ 200g BW; CA3: 120mg/ 200g BW) in the entire course of the experiment, and were induced with DMBA after one week of administering the extract. As comparison, commercial drugs containing *Phyllanthus niruri* L. were being used as positive control group, while the group given only DMBA represents the negative control group. Based on experiment, we found that the administration of *C. aeruginosa* during experiment promotes an increasing in TNF-α, IL-2, and IL-12 levels. The dose of 80mg/ 200g BW (CA2) appeared to be best potential dose as chemo-preventive agent represented the smallest tumor incidence and tumor multiplicity at the end of the experiment. Moreover, this plant also efficient to eliminate the cancer cells at the beginning of the tumor until the metastatic phase.

Keywords: Chemoprevention, cancer, *C. aeruginosa*, DMBA, wistar, cytokine.

INTRODUCTION

Cancer remains the leading cause of death in the world. The International Agency for Research on Cancer (IARC) recently estimated that 7.6 million deaths worldwide were attributable to cancer, with 12.7 million new cases being reported on a yearly basis worldwide. This disease is increasing by a significant proportion in developing countries. A recent report indicates that 68% of cancer deaths are reported in developing countries. Indonesia is one of the countries reporting the highest number of cancer cases in South East Asia. Based on a national survey in 2013, there will be at least 170-190 new cancer cases per every 100,000 people annually. This therefore indicates that cancer has risen to the sixth rank in causes of deaths, below infectious disease, cardiovascular disease, traffic accident, nutritional deficiency, and congenital diseases. Presently, the various modalities of therapy for cancer being used are radiotherapy, surgery, and chemotherapy. However, most cancer patients (60-70%) do not seek medical treatment until it is too late[1–3].

One of the enormous potential means to solving cancer problems is through chemoprevention, which is defined as the use of natural, synthetic or biological agents to reserve, suppress or prevent either the initial phases of carcinogenesis or the progression of premalignant cells to invasive disease [4]. Natural dietary agents such as fruits, vegetables, and spices have been utilized as traditional medicines for thousands of years. Some phytochemicals derived from spices, herbs and other plants possess substantial cancer preventive properties [5-6]. A member of Zingiberaceae, *Curcuma aeruginosa* Roxb. (*C. aeruginosa*) has been recognized to be widely used as food, medicine, and traditional knowledge [7–9]. *C. Aeruginosa,* known as pink and blue ginger in English, is among the indigenous and underutilized ethno-medical plants in Southeast Asia (Indonesia, Thailand, and Malaysia), Papua New Guinea, and Northern Australia. A plant commonly referred to as "temu ireng" or "temu hitam" in Indonesia. The rhizome of *C. aeruginosa* is traditionally used for the treatment of gastrointestinal problems, as well as an anti-microbial and anti-inflammatory agent. Other recent studies have also reported this rhizome to possess pharmacological activities for treating various diseases, such as tumor, asthma, and bronchitis [10-11].

Unfortunately, insufficient efforts have been invested in this herb to know the effect of *C. Aeruginosa* as a chemoprevention agent. Whereas, pharmaceutical companies have recently been exploring extensively into biodiversity rich regions all over the world to develop their products in the line of plant materials containing valuable medicinal properties. This diversification to plant based products may be primarily due to safety issues as regards humans, more economical, and easily accessible compared to the synthetic counterparts [8]. This present study was aimed at investigating the influence of *C. aeruginosa* extracts as a chemoprevention agent in wistar rat induced by 7,12-Dimethylbenz[a]anthracene (DMBA) on cytokines production. The information obtained was an essential preliminary step to commence with the use of this herb as a source of health-related products such as, functional foods or pharmaceuticals to prevent cancer.

MATERIAL AND METHODS

Animals

4 weeks old female Wistar rats were purchased from Biofarma Inc. (Bandung, Indonesia) and housed in an environment control room maintained at a temperature of 23±2°C, 60-70% relative humidity, 12:12h light/dark cycle, and standard food. All animal experiments were approved by Ethic Committee for Health

Research, Medical School, University of Indonesia – Cipto Mangunkusumo Hospital, Jakarta, Indonesia.

Plant Extraction

C. aeruginosa rhizome were collected from a herb farm of Martha Thilaar. Dry rhizome were ground to powder form. 5Kg of simplicia were extracted with the use of ethanol food grade, filtered and evaporated with rotary evaporator (Axiovert). The extract was dissolved into CMC-Na 0.5% and divided into three doses (40mg/200g BW, 80mg/20g BW and 160mg/200g BW).

Experimental Design

Animals were allocated to six groups (n = 8 female rats each) as follows. An untreated group was used as control. Three other groups (CA1, CA2, CA3) were sequentially treated with 3 *C. aeruginosa* extract doses (CA1: 40mg/200g BW; CA2:80mg/200g BW; CA3: 120mg/200g BW). As comparison, negative Control: DMBA only treated group; and Positive control: treated by commercial drugs containing *Phyllanthus niruri* L were also used; Body weight were registered for the entire week through the course of the experiment. *C. aeruginosa* doses were given respectively to all groups, except normal groups since acclimatisation over to the end of experimental (20 weeks). After 2 weeks of extract administrations, rats were induced by DMBA for 1 week. Some animals of the treatment groups were eliminated to investigate the tumor incidence and multiplicity. The development of tumor was evaluated until the 18th week. Blood collecting was done 4 times to collect the serum, and evaluate the cytokines production. The protocols employed were in accordance to the Ethical Principles for Animal Research, and were approved by the local Ethical Committee for Animal Research Faculty of Medicine, University of Indonesia, Indonesia (Protocol No. 152/H2.F1/ETIK/2014).

Cytokine levels

The presence of cytokines (TNF-α, IFN-γ, IL2, IL-12) in blood serum was measured by enzyme-linked immunosorbent assay (ELISA) (Thermo Scentific,USA). The procedure was conducted acording to the manufacturer's instructions.

Statistical analysis

Tumor incidence was calculated by the proportion of mice that had one or more tumors. Chi-square or Fisher's exact tests were employed to test the differences in tumor incidence among groups. The distribution of tumor multiplicity was determined graphically and descriptively for skewness and normality. Analysis of variance was used to test for significant differences in tumor multiplicity. Post hoc pairwise comparisons were made using Tukey's honest significant differences. The production of cytokines were analysed by the Student's test or Mann–Whitney test at week 4 (initiation step) and by the analysis of variance (ANOVA) or Kruskal–Wallis test at week 30 (promotion step). A two-sided P-value <0.05 was considered statistically significant.

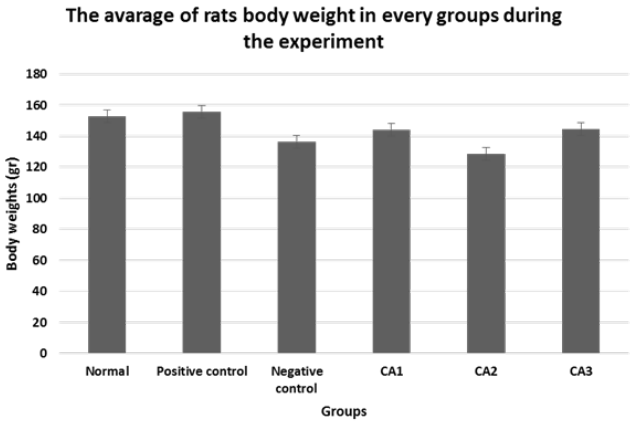

Figure 1: The average rat body weight gained during the experiment. N: Normal group; Negative control: DMBA only treated group; Positive control: treated by commercial drugs containing *Phyllanthus niruri* L; CA1: treated by 40mg/BW doses of *C. aeruginosa* extract; CA2: treated by 80mg/BW doses of *C. aeruginosa* extract; CA3: treated by 120mg/BW doses of *C. aeruginosa* extract.

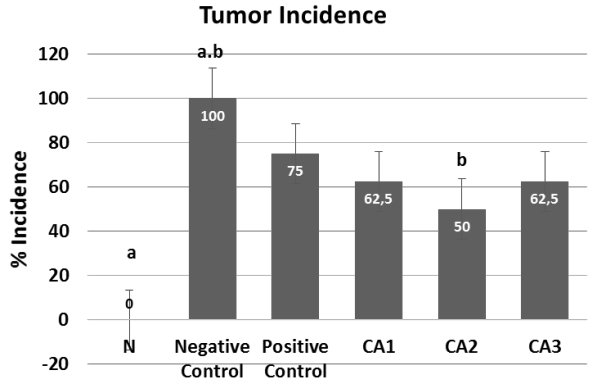

Figure 2: Percentage of tumor incidence. Tumor incidence is expressed as the proportion of rats who had one or more tumors from the total sample population of rats ± SE. N: Normal group; Negative Control : DMBA only treated group; Positive control: treated by commercial drugs containing *Phyllanthus niruri* L; CA1: treated by 40mg/BW doses of *C. aeruginosa* extract; CA2: treated by 80mg/BW doses of *C. aeruginosa* extract; CA3: treated by 120mg/BW doses of *C. aeruginosa* extract.

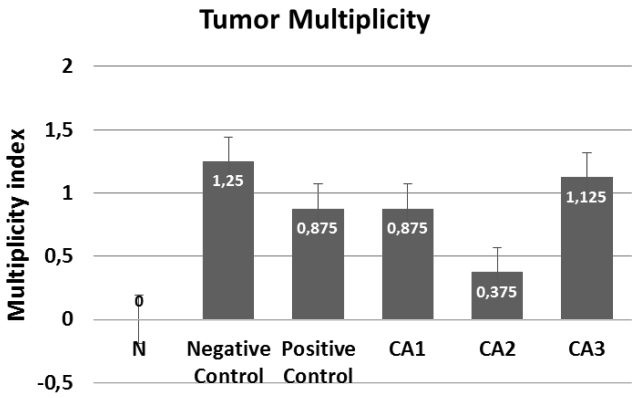

Figure 3: Average index of tumor multiplicity. The average tumor multiplicity for each treatment group is expressed as a number of tumors per-rats/total number of rats with tumors ± SE. N: Normal group; Negative Control : DMBA only treated group; Positive control: treated by commercial drugs containing *Phyllanthus niruri* L; CA1: treated by 40mg/BW doses of *C. aeruginosa* extract; CA2: treated by 80mg/BW doses of *C. aeruginosa* extract; CA3: treated by 120mg/BW doses of *C. aeruginosa* extract.

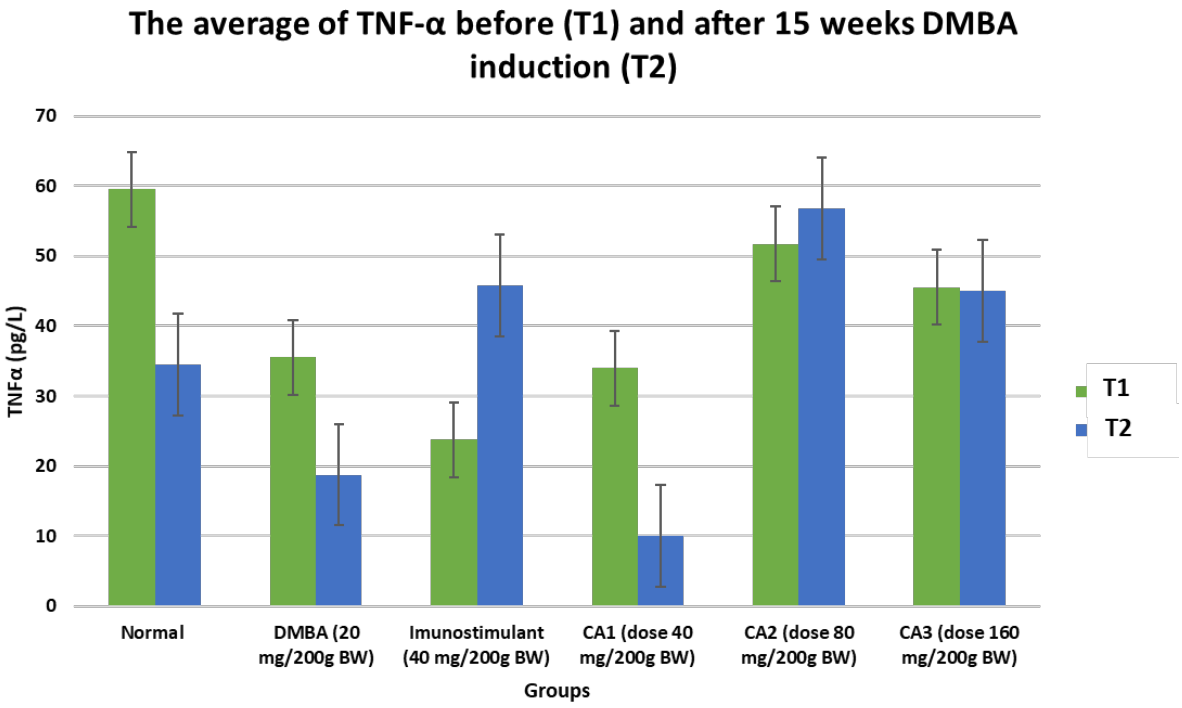

Figure 4: TNF-α levels during experiment (pg/ml). N: Normal group; Negative Control : DMBA only treated group; Positive control: treated by commercial drugs containing *Phyllanthus niruri* L; CA1: treated by 40mg/BW doses of *C. aeruginosa* extract; CA2: treated by 80mg/BW doses of *C. aeruginosa* extract; CA3: treated by 120mg/BW doses of *C. aeruginosa* extract.

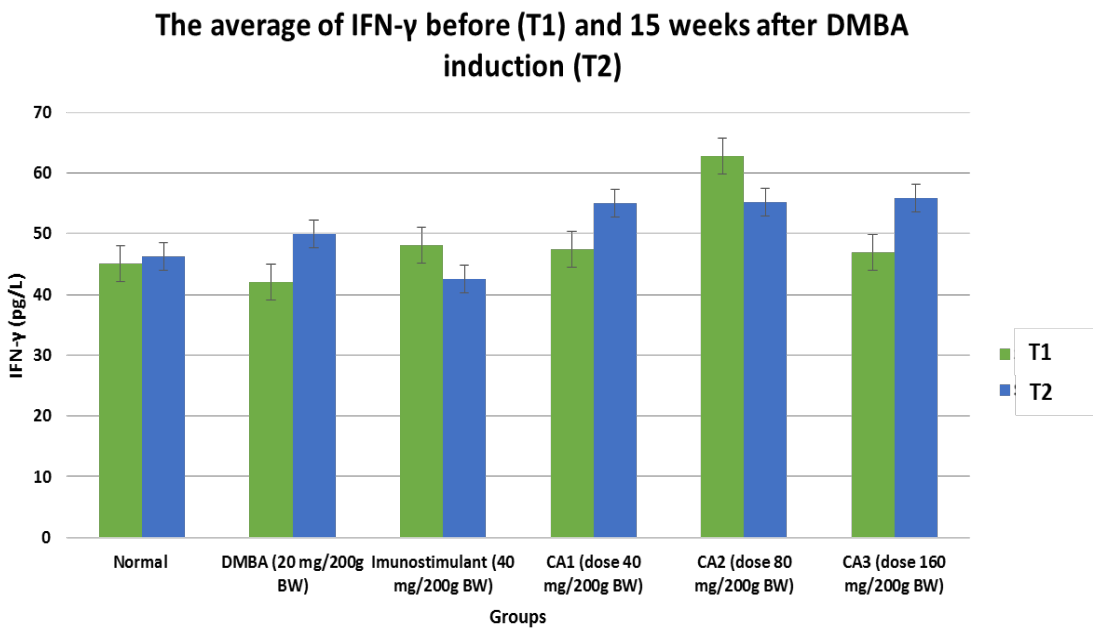

Figure 5: IFN-γ levels during experiment (pg/ml). N: Normal group; Negative Control: DMBA only treated group; Positive control: treated by commercial drugs containing *Phyllanthus niruri* L; CA1: treated by 40mg/BW doses of *C. aeruginosa* extract; CA2: treated by 80mg/BW doses of *C. aeruginosa* extract; CA3: treated by 120mg/BW doses of *C. aeruginosa* extract.

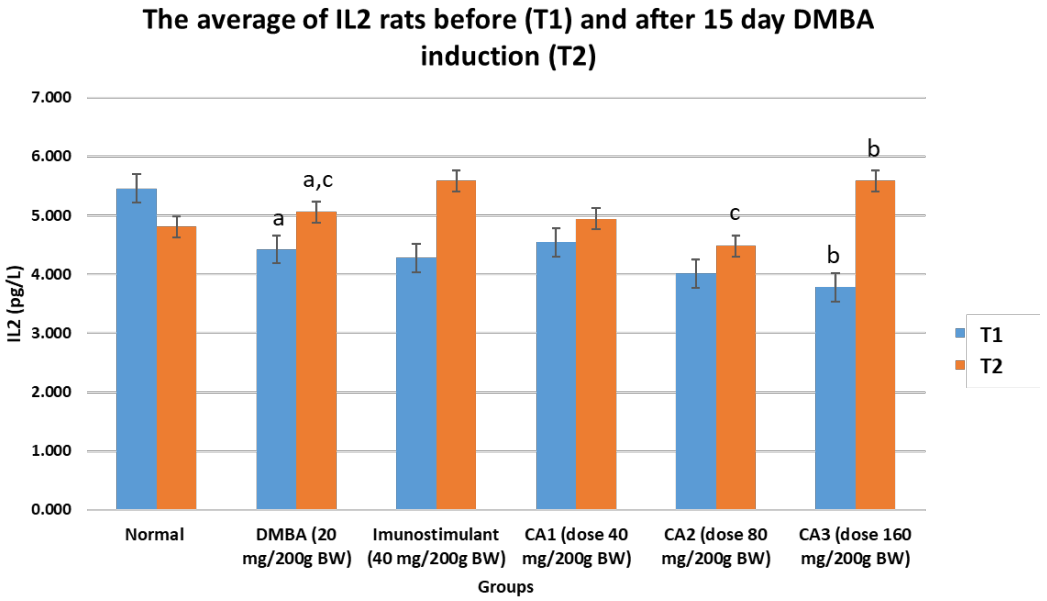

Figure 6: IL-2 levels during experiment (pg/ml). N: Normal group; Negative Control: DMBA only treated group; Positive control: treated by commercial drugs containing *Phyllanthus niruri L*; CA1: treated by 40mg/BW doses of *C. aeruginosa* extract; CA2: treated by 80mg/BW doses of *C. aeruginosa* extract; CA3: treated by 120mg/BW doses of C. aeruginosa extract.

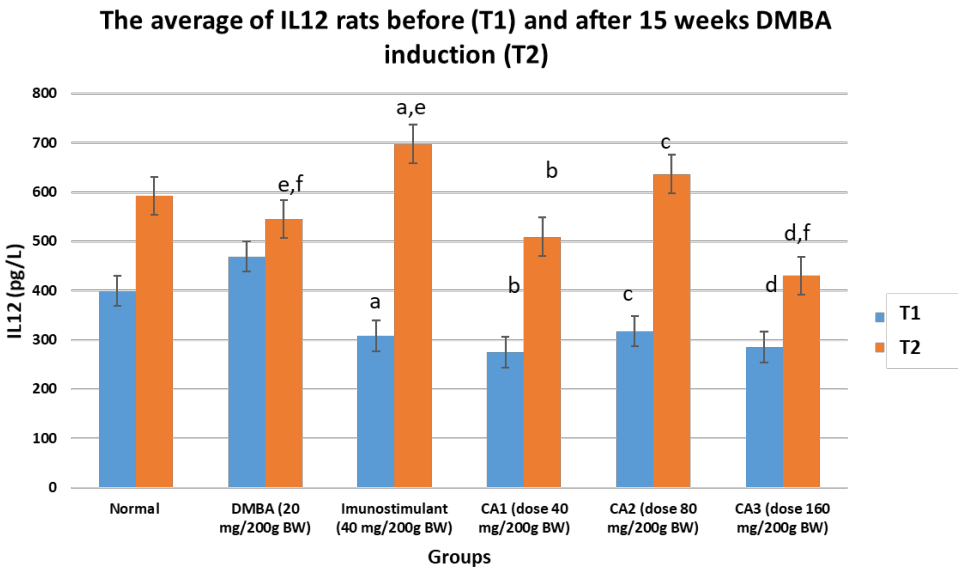

Figure 7: IL-12 levels during experiment (pg/ml). N: Normal group; Negative Control: DMBA only treated group; Positive control: treated by commercial drugs containing *Phyllanthus niruri* L; CA1: treated by 40mg/BW doses of *C. aeruginosa* extract; CA2: treated by 80mg/BW doses of *C. aeruginosa* extract; CA3: treated by 120mg/BW doses of *C. aeruginosa* extract.

RESULT

The present results indicate that the dietary administration of C. *aeruginosa* before, during and after the carcinogen treatment to the termination of the experiment, regulates the stability of rat's body weight. Moreover, no significant weight difference was perceived in each groups (P>0,05), and this signified that DMBA induction had no influence on the rats' weight (Figure 1).

The investigation regarding the tumor incidence and multiplicity discovered no tumor among rats in control group. The results illustrated in Figure 2 and 3 revealed negative group to be the highest group with tumor incidence (100%) and multiplicity (1,25). On the other hand, the CA2 was the least group with 50% of tumor incidence and multiplicity (0,375). In our experiment, CA2 with 80mg/200 BB. C. *aeruginosa* extract has been demonstrated to the best dose for overcoming the cancer incidence. Further analysis and experiment on rat cytokines disclosed the ability of this plant to suppress the carcinogenesis. After 1 week DMBA induction (T1), TNF-α had increased in all groups, but the normal group (Figure 4).

However, statistical analysis only revealed the difference in the CA3 group at T3 compared to T1 (p= 0,042). In addition, the increase of this cytokine did not occur until the end of the experiment. Moreover, comparison analysis before DMBA induction and at the end of experiment shown only CA1 (p≤0,005) and CA3 (p=0,042) were significantly different at those two times, though only the CA2 group which still controlled the TNF- α leveled at around 50pg/ml. Contrary to TNF-α, there was no escalation in IFN-γ serum (Figure 5). Based on statistics, there were no differences found between IFN-γ around the groups.

Apart from these, Interleukin-2 (IL-2) as an anti-inflammatory cytokine is also involved in tumor regression. Based on experiment, progressions found in IL-2 for all groups treated by C. *aeruginosa* after the induction by DMBA (Figure 6). The level pattern of IL-12 was also identical to that of IL-2 productions. The escalation occurred after the induction of DMBA all through to the end of the experiment (Figure 7). CA2 was the highest group with IL-12 level after control positive group.

DISCUSSION

The reduction of cancer in our research, especially in CA2 dose (80 mg/weight BW) can be attributed to the presence of numerous phytochemicals in C. *aeruginosa* which have been discovered to be anti-cancer agents. Studies have observed main active compounds from terpenoid, such as curzerene, cineole, germacrone, and β-element to exhibit anti-cancer activities towards gastric cancer cell lines. Zhong et al also reported in his study that sesquiterpenoid germacrone exhibited the best anti-cancer properties by inhibiting cell proliferation, increasing lactate dehydrogenase, and mediating G1 and G2 cell cycle arrest in human breast cancer[10]. Other sesquisterpenes which consist of β, α, and γ- element has also been reported to exhibit anti-tumor activities and approved by the Food and Drug Administration of China as anti-cancer adjuvant drug on cancer patients [8].

Further analysis and experiment on rat cytokines disclosed the ability of this plant to suppress the carcinogenesis. After 1 week DMBA induction (T1), TNF-α had increased in all groups, but no in the normal group. As a pro-inflammatory cytokine, TNF is secreted by inflammatory cells, which may be involved in inflammation-associated carcinogenesis. Therefore, the administration of DMBA in this experiment has been evidently capable of inducing a large quantity of macrophage in lymphoid organs such as a white pulp in spleen to produce high levels of TNF-α (data not shown).

Unfortunately, an increase in this cytokine did not occur until the end of the experiment. On the 18th week, the level of TNF-α dropped. Only the CA2 group kept the TNF- α levels at around 50pg/ml. This probably occurred because 80mg/BW *C. aeruginosa* extract had the potential to prevent the escalation of some tumor cells. This was proven by the minimal percentage of tumor incidence and tumor multiplicity observed in this group. A number of in-vivo studies have shown that curcumin in *C. aeruginosa* exhibits potent chemo preventive activities, especially preventing the initiation of tumors [12]. In contrast, there were no escalations in IFN-γ levels in the course of the experiment. It is hypothesized because IFN-γ levels were induced by antigens such as virus, not malignant cells [13].

Apart from these, Interleukin-2 (IL-2) as an anti-inflammatory cytokine is also involved in tumor regression [14]. Based on experiment, there were progressions in IL-2 for all groups treated by C. *aeruginosa* after the induction by DMBA. IL-2 exerts its effect on numerous cells, with the most prominent being the T lymphocyte. The production of IL-2 subsequent to the activation of DMBA on T cell receptors induces the expansion of T cells (CD4 and CD8), B cells, and NK cell. IL-2 helps CD4 T cells to differentiate between TH1 and Th2, promotes CD8 T cells to secrete cytokines, enhances antibody production through B cells, and promotes NK cells to proliferate and secrete cytokines or enhance cytolysis activity[15].

The level pattern of IL-12 was identical to that of IL-2 productions. The escalation occurred after the induction of DMBA all through to the end of the experiment. IL-12 is secreted as an early pro-inflammatory cytokine in response to infection[16]. Induction by DMBA resulted in the increase of this cytokine levels during the experiment. CA2 was the highest group with IL-12 level after control positive group. It showed that there were active compounds in C. aeruginosa and *Phyllanthus niruri* which potentially promoted the IL-12 secretions at the metastatic phase, even though these doses are still incapable of suppressing the occurrence of tumor.

Unfortunately, the role of IL-2 as a link between innate and adaptive was not proven in this study. It is indicated by the minor secretion of IFN-γ during the course of the experiment. Theoretically, IL-12 plays a central role in the immune system by driving the immune response towards the Th1 type, which is characterized by a high IFN-γ and low IL-4 production. The imbalance between high production of IL-12 and the least of IFN-γ probably occurred due to the mechanism of IL-12 in inducing anti-tumor responses involved in distinct effector cell types and cytokines depending on the tumor type and/or location [16].

CONCLUSION

We hereby conclude that the administration of C. *aeruginosa* as a chemo-preventive agent promotes an increase in TNF-α, IL-2, and IL-12 levels as a consequence of immune response after DMBA induction. The dosage of 80mg/200g BW in CA2 group was observed to be the optimal potential dose as a chemo preventive agent, as it had the least tumor incidence and multiplicity at the end of the experiment. C. *aeruginosa* was capable of serving as a chemo preventive agent in cancer development, which was evidently seen in the elimination of cancer cells at the beginning of tumor until the metastatic phase in these animal models.

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