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Black Cumin Seeds Extract Increase Lymphocyte Activity in IFN-γ Secretion in Sprague Dawley Rat (SD) Induced by Dimethylbenzantracene

Akrom[1*], Titiek Hidayati[2], Sagiran[3], Indrayanti[4]

[1]Pharmacology and Clinical Pharmacy Department, Universitas Ahmad Dahlan, Yogyakarta Indonesia
[2]Community and Family Medicine Department, Medicine and Health Science Faculty, Universitas Muhammadiyah Yogyakarta, Yogyakarta, Indonesia
[3]Surgeon Department, Medicine and Health Science Faculty, Universitas Muhammadiyah Yogyakarta, Yogyakarta, Indonesia
[4]Pathology Anatomy Department, Medicine and Health Science Faculty, Universitas Muhammadiyah Yogyakarta, Yogyakarta, Indonesia

Abstract

Interferon-gamma (IFN-γ) is one of the central cytokines in the anti-carcinogenesis immune response. Black cumin seeds (BCS) have an active content of thymoquinone and unsaturated fatty acids with biological activity as immunomodulators. This study aimed to determine the effect of administration of BCS extract on IFN-γ secretion activity by DMBA-induced SD rat lymphocytes. *In vivo* experimental study on DMBA-induced SD rats, BCS extract was given with three doses for two weeks before being induced and five weeks during DMBA induction. IFN-γ levels in lymphocyte culture supernatants were determined by the ELISA method. The difference in IFN-γ levels between groups was analyzed by ANOVA test, the significance of 95%. The results showed that administration of BCS extract for 14 days did not affect cellular composition toward the edge of the test animal. BCS extract can increase IFN-γ secretion activity by DMBA-induced SD rat lymphocytes.

Keywords: *black cumin seed, IFN-γ; DMBA: immunomodulator, carcinogenesis.*

INTRODUCTION

The immune system with immunosurveillance is a vital component that is responsible for the development of cancer of a neoplastic cell (Dembic, 2015; Disis, 2010; American Cancer Society, 2016; Li, *et al*., 2019). The development of proto-oncogenes into cancerous oncogenes that are evolutionarily is thought to be one of them caused by a decrease in the ability of the host immunosurveillance (Baj-Krzyworzeka, *et al*., 2004) (Ballestero Fêo, *et al.,* 2018). The host's response to cancer is a complex mechanism, involving components of the regulatory system, phagocyte effector activity and immune system mediators (Hayakawa, *et al*., 2002) (Ren, *et al*., 2019; Selinger, *et al*., 2018; Upadaya, *et al*., 2018). Interferon-

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*Corresponding author: akrom@pharm.uad.ac.id

gamma (IFN-γ) is one of the anti-carcinogenesis pro-inflammatory cytokines, is one of the regulatory system immune response, due to exposure to dimethylbenzanthracene (Castro, *et al.*, 2018). Unfortunately, until now, it has not been widely studied, primarily when associated with immunogens from plants that have the potential as immunomodulators and also as chemopreventives (Dunn, *et al*., 2004; Zekri, *et al*., 2018; Li, *et al*., 2002).

One of the plants that empirically and laboratory has been used as chemopreventive as well as immunomodulators is black cumin seed (BCS) or *Nigella Sativa* (Shaterzadeh-Yazdi, *et al.,* 2018; Hidayati, *et al*., 2019; Badr, *et al*., 2011; Randhawa, *et al.,* 2011; Soliman, *et al.*, 2017). *Nigella sativa* seeds (*N.sativa*) contain various active compounds which are thought to be immunomodulatory as well as cancer chemopreventives (El-Mahmoudy, *et al*., 2002). Mousa (2004) proved that BCS could provide chemopreventive effects in vivo in DMBA-induced mice carcinogens (Mousa, *et al.*, 2004). BCS administration for 14 days in mice induced with 7,12-di-methylbenz(a)anthracene (DMBA) reduces tumor markers and increases TNF α levels as a factor driving DNA apoptosis and fragmentation (Odhaib, *et al.*, 2018). In Swiss mice- infected plasmodium berghei (*P.berghei)*, the ethanolic extract of BCS was shown to increase the phagocytic activity of macrophages (Hidayati, 2006). Administration of BCS oil in mice induced with streptozotocin has been shown to increase phagocytosis of peritoneal macrophages (Fajar, *et al*., 2017). Immunomodulatory activity of the BCS can optimize the immune response to foreign substances or antigens, including neoplasmic antigens (el Aziz, 2005; Gholamnezhad, *et al*., 2019; (Hidayati, *et al.*, 2017).

DMBA compounds are genotoxic and immunotoxic. Genotoxic indirectly from DMBA has a mechanism through cytochrome P450-mediated enzymatic or biotransformation activation and glutathione-S-transferase activity (Parmar, *et al*., 2011). These conversions produced electrophilic, which can react with several nucleophilic compounds in proteins, DNA, and RNA. Cancer cells start on the occurrence of mutations of protooncogene into oncogenes (Barletta, *et al.*, 2004). The variation in the p53 gene will lead the mutant cells to avoid the mechanism of apoptosis so that it will stay alive and develop into cancer cells (Gao, *et al.*, 2008). Neoplastic cells will be recognized as non-self by the immune system, which will then generate an immune response. Peptides from H-ras gene mutations induce T cell proliferation *in vitro*. Single-point mutations in oncogenic H-Ras and p53 genes trigger tumors with the potential to form T-cell specific epitopes. Activation of cellular immune responses through the CD4 T cells increase the phagocytic activity of effector cells (CTL, macrophages, and NK cells) and increase the production of cellular immune response regulators, among others, IFN-γ, TNF α and IL-2. The immune system as a body surveillance system will prevent neoplastic cells towards tumor tissue formation and subsequent carcinogenesis (Soliman, *et al.*, 2017) (Ren, *et al.,* 2018).

Black cumin seeds are empirically, laboratories and clinically proven to be safe, and tolerable (Akrom, *et al.*, 2017) (Rachman, *et al.*, 2017). The administration of BCS is thought to increase the immune response in neoplasms (Akrom, *et al.*, 2017; Al Ghamdi, 2002. From previous studies it has been proven that administration of BCS oil in Balb c mice infected with cytomegalovirus has been shown to prevent infection and increase IFN-γ levels and CD4 number and activity (Shaterzadeh-Yazdi, *et al*., 2018). Administration of BCS ethanol extract has been shown to be effective in inhibiting carcinogenesis and improving immune responses (Hidayati, *et al.,* 2006; Randhawa, *et al*., 2011). The administration of BCS ethanolic extracts in doses of 250 mg/kg BW was proven to be able to inhibit carcinogenesis (Fathy, 2013). IFN-γ is a pleiotropic anti-carcinogenesis cytokine and plays an important role in regulating the anti-carcinogenesis immune response. IFN-γ is associated with antiproliferative, pro-apoptotic, and antitumor mechanisms (Ren, *et al.*, 2019). Until now how the effect

of BCS ethanolic extract on IFN-γ levels in SD rats induced by DMBA has not been studied. Black cumin seed (BCS) is expected to increase IFN-γ secretion activity by lymphocyte of DMBA-induced SD rats. The purpose of this study was to determine the effect of BCS administration to IFN-γ secretion activity of DMBA-induced SD mice lymphocytes.

METHODS

This study was an experimental laboratory with the control group. Test animals were divided into seven groups randomly, group one as a healthy control group (solvent group), group two as a sick control group (DMBA group), group three to five as the treatment groups, group six as Imboost group (positive control group 1), and group seven as a tamoxifen group (positive control group 2). The study protocol has been reviewed and declared ethically viable by the research ethics committee of Universitas Ahmad Dahlan, Yogyakarta, Indonesia (No. 043/KEP-UAD/II/2019).

Animal, Material and Equipment

The study was conducted at the animal breeding and experimental unit of Gadjah Mada University. We used 7,12 dimethylbenzanthracene (DMBA, Sigma-Aldrich, St Louis, USA) for inducing carcinogenesis. We obtained a DMBA from one of the official distributors in Yogyakarta. We used corn oil as solvent DMBA, as in previous studies (Hidayati, *et al*., 2019). Sprague Dawley (SD) rat test animals were obtained from the Animal Breeding and Experimental Unit, Gadjah Mada University. We used 105 female SD rats strain, aged 14-30 days with an average weight of 60-80 g. A twenty one female SD rats are for the preliminary test, and 84 are for the examination of BCS preparation. The BCS preparation is ethanolic extract of BCS. Ethanol extract of BCS has been provided by the Department of Pharmaceutical Biology, Pharmacognition and Phytochemistry, Faculty of Pharmacy, Ahmad Dahlan University. "Imboost" preparations, one of the immunomodulatory preparations and have obtained a marketing authorization from the Republic of Indonesia drug and food control agency, were obtained by prescription from a pharmacy, as positive control 1. Tamoxifen citrate (Nolvadex), a preparation for chemotherapy in breast cancer patients in Indonesia, is used as a positive control 2. Tamoxifen is obtained from a pharmacy using a prescription from a doctor. We used an IFN-γ (Quantikine, R & D system, Inc. Minneapolis, USA) elisa kit to determine IFN-γ levels in the supernatant culture of limpocytes.
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Experimental Procedure

Test animals with age as needed, after ensuring their health by trained personnel, then weighed in order to obtain test animals in accordance with the criteria. Test animals are then placed in plastic cages. During the experiment the test animals were kept at the appropriate temperature and humidity of the room, had adequate lighting and got standard food and drink. Test animal care is carried out by certified trained personnel in the Unit of care and breeding of test animals, Gajah Mada University. Test animals are acclimatized for one week before being used for experiments.

Test animals were divided into seven groups randomly, twelve animals each group.. Group one as solvent group, during the trial period the test animals get additional treatment viz 1x0.5 ml/day per oral of corn oil, but without inducing DMBA. Group two as a DMBA (sick) group, In the first two weeks of the experimental period the test animals have not received additional treatment, starting form third week of experimental perode the test animals were given preparations containing DMBA 20 mg/kg BW in 0.5 ml of solvent corn oil given 2x/week per peritoneal for five weeks. All test groups, except the solvent group, received DMBA preparations starting from the third week of treatment. Group three to five as the treatment groups, the test animal received BCS preparation in three doses. BCS preparations was carried out start-

ing from 14 days before the first DMBA administration and continued for five weeks during DMBA administration, *i.e.* from 4th weeks of age to 11th weeks of age, 1x/day orally at doses of 5.25 and 125 mg/kgBW/day. BCS preparations were given with three dose ratings based on the results of previous studies (el Aziz, *et al*., 2005; Karimi, *et al*., 2011). Group six as Imboost group, the test animals were given immunomodulatory (Imboost) preparations according to the dose in adults who were converted into rat doses. The preparation is given once a day two weeks before DMBA induction and two weeks during the DMBA induction period so that the total administration is four weeks. The preparation company recommends that Imboost be used no more than 4 weeks. Group seven as a tamoxifene group, the preparations was given as the way to administer other preparations, two weeks before DMBA induction and five weeks during DMBA induction. Six-week-old rats were given a DMBA solution in corn oil at a dose of 20 mg/kg BW orally. DMBA induction was repeated ten times with the frequency of administration twice a week. (Zekri, *et al.,* 2018).

Hemogram and Lymphocyte Activity in secreting IFN-γ Test Procedure

Hemogram measurements of the animal were carried out 14 days after administration of BCS extract. Blood was isolated from sinus orbitalis by professional trained. We examined hemogram with hemoanalyzer. In the 15th and 50th day, we sacrificed the animal study. The spleen organ was isolated after two and seven weeks administration of BCS preparation and after DMBA induction was completed. Lymphocytes were isolated and cultured following the procedures of previous researchers (Soliman, *et al*., 2017). IFN-γ levels of splenocyte culture supernatants were measured by Enzyme-Linked Immunosorbent Assay (ELISA) sandwiches. The kit used was quantikine M from R & D System, Inc. Minneapolis, USA. Examination procedures as per the procedures provided by the factory. The test results were read by ELISA reader at a wavelength of 450 nm, IFN-γ levels were expressed as pg/mL.

Data Analysis

The difference in IFN-γ secretion activity lymphocytes and the average number of blood cells between groups was analyzed by ANOVA, with a confidence level of 95%.

RESULTS

Hemogram of Experimental Animals

The results of blood tests after administration of BCS preparations for 14 days showed that there were no changes in the cellular composition of the blood of the test animals, as shown in Table 1.

From table 1, it is known that the administration of BCS for 14 days does not affect the composition of the hemogram. IFN-γ secretion lymphocytes activity before and after DMBA induction by the administration of BCS ethanolic extract Lymphocytes are isolated from the spleen organs. Table 2 presents the IFN-γ levels that secreted by lymphocyte culture. In this experiment as a baseline is the DMBA group before DMBA induced. Before the DMBA was induced, the DMBA group got standard food and drink. Before the DMBA was induced, the treatment group and the control group received additional treatment according to their group for two weeks besides getting standard food and drink. Research data shows that before being induced by DMBA, the DMBA group and the solvent group had equivalent IFN-γ levels, *i.e* 80±9 and 80 ± 9 pg/mL (p>0.05). Provision of corn oil for 2 weeks does not affect the activity of lymphocytes in secreting IFN-γ. In contrast to the solvent group, administration of BCS, thymoquinone and tamoxifen preparations for two weeks increase lymphocyte activity in secreting IFN-γ. The highest IFN-γ level was in the with 125 mg/kg BW/day BCS group (336+49pg/mL), then followed by a group of 25 mg/kg BW/day BCS (304+15pg/mL) and Imboost group (291.7+55p/mL). Before

induced DMBA, the treatment group and the positive control group had IFN-γ levels higher than the IFN-γ levels of the solvent and the DMBA group (p<0.05).

The administration of BCS dosages 5, 25, and 125 mg/kg BW/day for 14 days increase IFN-γ secretion activity by lymphocytes. The BCS dosage group with a dose of 125 mg/kg after giving 14 had the highest IFN-γ secretion activity, higher than the Imboost and Tamoxifen group but not statistically significant. In eexperimental group, the BCS group with a dose of 5 mg/kg BW/day had the lowest IFN-γ secretion activity but was higher than the DMBA group and solvent group.

Five weeks administration of corn oil during the DMBA induction period in the solvent group did not affect the lymphocyte activity in secreting IFN-γ. In the solvent group, IFN-γ levels before and after the DMBA induction period were not different (p>0.05). During the DMBA induction period the solvent group received additional treatment that is given corn oil once per day orally.

DMBA induction decreases lymphocyte activity in secreting IFN-γ. In the DMBA group, the IFN-γ levels after induction were lower than before DMBA induced and were statistically significant (72±2.7 pg/mL v.s. 87±11.2 pg/mL, p<0.05, p<0.05). Measurement of IFN-γ levels after five weeks of induced DMBA, the DMBA group had the lowest level. The IFN-γ level of DMBA group was lower than the solvent group (72±2.7 pg/mL v.s. 83±10.2, p<0.05). MBA induction reduced IFNγ levels in all experimental groups except the 125 mg/kg BW BCS group. In the 125 mg/kg BW BCS group, IFN-γ levels after DMBA induction were higher than before DMBA induced (690±54.1 pg/mL v.s. 336+49, p<0.05).

Administration of BCS, imboost and tamoxifen preparations increase lymphocyte activity. The research data showed that IFN-γ levels in 25 and 125 mg/kg BW BCS groups were higher than IFN-γ levels in the Imboost or Tamoxifene group (p <0.05). After the duration of DMBA induction, among the treatment groups, the 125 mg/kgBW BCS group had the highest IFN-γ levels, followed by the 25 mg/kgBW BCS group, the Imboost group, the 5 mg/kg BW BCS group and the lowest is tamoxifene group.

Table I. Composition of blood cells of test animals after obtaining 5, 25, and 125 mg/kgBW/day of BCS preparation for two weeks

Groups	Leukocytes ($x10^3$)	Eritrocytes ($x10^6$)	Hb	Platelet ($x10^3$)
Solvent group	5.30±2.1	6.18±2.1	13.01±2.1	786.66±24
DMBA group	5.28±2.1	6.12±2.1	12.20±1.1	562.33±25
BCS 5 preparation group	5.63±2.1	6.64±2.1	13.10±3.2	1011.00±31
BCS25 preparation group	5.66±2.1	6.57±2.1	12.70±3.1	699.33±23
BCS125 preparation group	5.76±2.1	6.46±2.1	11.50±1.7	845.00±34
Imboost groups	7.40±2.1	6.48±2.1	13.12±4.1	787.33±21
Tamoxifen group	6.50±2.1	6.49±2.1	12.21±2.4	837.25±22

Table 2. IFN γ secretion activity by Sprague Dawley rat lymphocytes after obtaining black cumin seed ethanolic extract for 14 days before and five weeks during DMBA-induction

Groups	IFN-γ level (mean±SD)	
	Before DMBA-induction (two weeks) (pg/mL)	After DMBA-induction (five weeks) (pg/mL)
Solvent group	80±9.2	83±10.2
DMBA group	87±11.2	72±2.7*
BCS 5 preparation group	125.4+14.3*	116±10.2b
BCS25 preparation group	304+15*	225±18.5b
BCS125 preparation group	336+49*	690±54.1a
Imboost groups	291.7+55*	140±16.9b
Tamoxifen group	150+132*	109±89b

note: *=p<0.05 to normal/solvent group; a=p<0.05 after DMBA-induction>before DMBA-induction; b=p<0.05 after DMBA-induction<before DMBA-induction.

DISCUSSION

DMBA induction in test animals decreases lymphocyte activity in secreting IFN-γ. The IFN-γ secreted by the negative control group (DMBA) was the lowest. IFN-γ level of a negative control (DMBA) was lower than the solvent group (p<0.05). Administration of BCS preparations can inhibit decreased lymphocyte activity in securing IFN-γ due to DMBA induction. In the group that had received BCS dosages of 5 and 25 mg/kg/day, imboost and tamoxifen decreased IFNγ secretion activity due to DMBA induction, but the activity of IFN-γ secretion by lymphocytes remained higher than the negative control group (p<0.05). The group of BCS preparations with a dose of 125 mg/kg BW/day did not decrease due to DMBA induction. The level of IFN-γ of lymphocyte culture supernatant in the BCS group dosage of 125 mg/kg BW/day after DMBA induction increased if compared to DMBA induced (p<0.05). Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants that are carcinogenic and immunosuppressive. Previous studies show that IP administration of DMBA to mice results in a substantial hypocellularity of the bone marrow at 48 h after exposure (Gao, *et al.*, 2008). This response was dependent on local metabolism of the DMBA by Cyp1b1 that is expressed in the bone marrow, spleen, thymus, and peripheral blood leukocytes, but not the liver parenchyma (Xiao, *et al.*, 2009) (Buters, *et al.*, 2003). The reduction in bone marrow cellularity was evident in both the lymphoid (B cell) and myeloid (largely granulocyte) populations (Ichihara, *et al.*, 2003; N'jai, *et al.*, 2010). Although these previous studies identified the adverse effects of DMBA on bone marrow hematopoiesis, they did not examine whether exposure to DMBA changes the ability of lineage-specific progenitor cells to proliferate and differentiate into mature bone marrow cell populations (Gao, *et al.*, 2008). Lymphocytes produce IFN-γ cytokines after getting stimulation from antigens, inflammatory mediators, or other cytokines produced by macrophages or neutrophils due to antigen exposure (Upadhyay, *et al.*, 2018). Gamma interferon plays a role in regulating natural and adaptive immune responses. IFN-γ has been shown to inhibit carcinogenesis, neoplasm formation, and activate antitumor immunosurveillance (Selinger, *et al.*, 2018). Thymoquinone and the active ingredient BCS are proven to be able to increase the activation of natural and adaptive immune responses and anti-carcinogenesis. Based on the results of this study, it is known

that activation of the immune response by thymoquinone and BCS active substances is one of them through increased lymphocyte activity (Shaterzadeh-Yazdi, *et al*., 2018; Mollazadeh, *et al*., 2017).

CONCLUSION

From this study, it can be concluded that the administration of ethanolic extract of BCS for 14 days did not affect the peripheral blood cellular composition and the administration of BCS preparation proved to increase IFN-γ secretion activity by lymphocytes and inhibit DMBA activity in suppressing lymphocyte activity. BCS dosages of 125 mg/kgBW/day have lymphocytes with the highest IFN-γ secretion activity.

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