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Black cumin seed oil increases phagocytic activity and secretion of IL-12 by macrophages.

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Abstract

Interleukin-12 (IL-12) is one of cytokines that regulate the innate and adaptive immune responses produced by macrophages. This study was to investigate the effect of Black Cumin Seed Oil (BCSO) to the structure of peripheral blood, phagocytic activity, and secretion of IL-12 by macrophage peritoneal Sprague-Dawley (SD) rat. The 36 female Sprague Dawley (SD) rats divided into 6 groups. Normal group was only given standards food and drink. Treatments group were given BCSO 0.25, 2.5 and 5 ml/kg BW/day for 14 d. Positive control group was given thymoquinone 50 mg/kg BW/day for 14 d. Solvent control group was given DMSO. Blood test, phagocytic activity and secretion of IL-12 by macrophage in vitro performed on the 15th day. Latex and NBT assay method were conducted to measure the phagocytic activity of macrophages. The expression level of IL-12 and TLR4 were analysed by ELISA. Data were statistically tested by one-way ANOVA with significance 95%. BCSO was increase phagocytic activity, secretion of IL-12 by macrophage, and the expression of TLR4. The highest phagocytosis percent (56.83 ± 6.37%) and phagocytosis index (5.18 ± 0.39) was performed by 0.25 BCSO group and did not significantly different with thymoquinone group. The highest level of IL-12 was in 2.5 BCSO group (66.33 ± 2.11 µM).

Keywords: Black cumin seed oil, IL-12, Macrophage, Phagocytosis, Thymoquinone, TLR-4.

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Introduction

Interleukin-12 (IL-12) is one of the main cytokines in immune response regulation. It is produced by activated lymphocytes and accessory cells such as macrophage, dendritic cell, neutrophil, and monocyte. *In vivo* study proved that IL-12 increase the activity of CD4 and CD8 [1,2], lead the formation of CD4 T cell, induce the secretion of IFNγ by T and NK cell, anti-angiogenesis, and DNA repair [3,4]. The production of IL-12 by macrophage is increased by immunogen such as phytoimmunogen [5].

In Indonesia, black cumin seed is widely used as herbal medicine with the efficacy as a tonic, anti-cancer, anti-pain, and anti-asthma and reinforcing the body's defenses and antioxidant, but many people use the BCSO to maintain of fitness and health status. BCSO as guardians of fitness and health status generally is consumed every day in the time varying between 1-2 weeks with 2-3 × 1.5-2 ml/day. Black cumin seeds contain oil evaporate, fatty acids, sterols mainly β-sitosterol, thymoquinone, dithymoquinone and saponin [6]. Black Cumin Seed Oil (BCSO) inhibits inflammation of bronchi and decrease asthma by inhibiting mRNA expression of IL-4, IL-5, IL-6 and TGF-β in the allergens-induced experimental animals [7]. Thymoquinone, as the main active compound of BCSO, decreases inflammatory reactions in mice bronchi, IgE and IgG specific-OVA, IL-5, IL-4, IL-13 and increases IFN-γ in ovalbumin-induced mice [8,9]. Thymoquinone as a benzoquinone compound has a chemical structure identical to the active compounds as anti-inflammatory, so it is estimated that thymoquinone also has anti-inflammatory activity. However, the results of Finley et al. study [10,11] showed that through the Toll-Like Receptor 4 (TLR-4) thymoquinone is able to increase the activity of macrophages. The immunomodulatory effects of daily consumption of BCSO in 1-2 weeks in healthy people have not been clear, particularly against components cells of blood and macrophage activity.

In this study, we want to clarify the activity of BCSO as an immunomodulator by analyzing the phagocytic activity and the secretion of IL-12 by the macrophage.

Method

BCSO treatment

Black cumin seed oil has been prepared by the department of Phytochemistry and Pharmacognosy, Faculty of Pharmacy, University of Ahmad Dahlan. Thymoquinone levels in oil has been established which is 2.7% volume/volume (=21.6 mg thymoquinone per ml BCSO). Thirty six one-month-old female SD rats were divided into 6 groups randomly. Group I as a normal group was only given standards food and drink. Groups II-IV as the treatment group were given BCSO 0.25, 2.5 and 5 ml/kg BW/day (similar to 5.4 mg, 54 mg and 108 mg/kgbw thymoquinone) for 14 d. Group V as the positive control group was given thymoquinone 50 mg/kg BW/day. Group VI as a solvent control group was given DMSO7. Research procedures and the research protocol had been reviewed and approved by the ethics committee of the Gadjah Mada University (no: 222/KEC-LPPT/III/2015).

Blood test

A peripheral blood test conducted in the laboratory of Gadjah Mada University (GMU). It was done by hematology analyser Sysmeix kx. The blood of rats was taken through orbital sinus as much as ± 1.5 ml and collected in labeled eppendorf tubes that were already containing anticoagulants [12]. As suggested by the ethics committee, the blood sampling and isolation of macrophage cells is conducted under the animals test were decapitated.

Macrophage cell preparation

On day 15, all of the rats were euthanized using chloroform. They were sprayed with 70% ethanol and mounted on the styrofoam block on its back. The abdomen skin was opened and 10 ml cold Roswell Park Memorial Institute medium (RPMI) 1640 (Sigma-Aldrich) was injected into the peritoneal cavity using a 27 g needle. The peritoneum was gently massaged to dislodge any attached cells into the RPMI solution. The fluid was collected by inserting a 25 g needle into the peritoneal cavity and saved on the tube. It was centrifuged at 1200 rpm, 4°C for 10 min. Supernatant was discarded while pellet cell was resuspended by RPMI contained Fetal Bovine Serum (FBS) 10% (Sigma-Aldrich). The number of the cell was counted by hemocytometer. 5×10^5 cell/ml macrophage cells were cultured in 24 well microculture with a coverslip and incubated in CO_2 incubator 37°C for 30 min. Each well was added by 1 ml complete medium and phytoimmunogen, Phytohaemagglutinin (PHA) and incubated 2 h. Cells then were washed twice with 1 mL RPMI and incubated in complete medium for 24 h [13].

Macrophage phagocytosis activity test

Macrophage cells were washed twice with RPMI then added by 200 µL of 2.5×10^6/ml latex suspension/well. It was incubated in CO_2 incubator 5%, 37°C for 60 min. Cells were washed three times with PBS, air dried, fixed with absolute methanol for 30 s and stained with 20% Giemsa. The percentage of phagocytic activity and the phagocytic index was measured by light microscope with 400X magnification.

ROI and NO secretion activity test

The ability of macrophages cells in secreting ROI was measured by NBT reduction assay [13,14]. Determination of nitrite (NO_2) in the supernatant of macrophage culture was performed by Grees assay [15].

IL-12p40 secretion activity test and expression of TLR4

The level of IL-12p40 from the supernatant of splenocytes cell culture was measured by Enzyme-Linked Immunosorbent Assay (ELISA) sandwich. The kit used was rat IL-12 (IL-12p40, Biosource, USA, catalog KRC 4022). The expression of TLR-4 was also determined by ELISA according to Finley [14].

Statistical analysis

The number of blood cells, phagocytic activity, phagocytic index, ROI secretion activity, the levels of NO and IL12 and TLR-4 expression were analysed by normality test, homogeneity and mean difference with 95% confidence interval. Normality test was used Kolmogorov-Smirnov test and homogeneity test was used Levene test then followed by LSD.

Result and Discussion

The overview of peripheral blood cell

The blood tests showed that administration of BCSO for 14 d did not affect the number of erythrocytes, platelets, and hemoglobin and hematocrit levels, as shown in Table 1. The level of hemoglobin, hematocrit, MCV, MCH, MCHC, and the number of erythrocytes and platelet with the oral administration of BCSO were still in normal values. However, the lowest of platelet number was in 5 ml/kg BW BCSO and significantly different from the normal and the solvent group ($p<0.05$). The condition of the thirty-six SD rats were used for this study until the sampling are all in good health.

Table 1. The number (± Sd) of erythrocytes, hemoglobin, hematocrit, MCV, MCH, MCHC and platelet of SD rats after 14 d BCSO treatment.

Group	RBC (10^6/ml) (mean ± Sd)	Hb (g/dl) (mean ± Sd)	Hematocrit (%) (mean ± Sd)	MCV(um^3) (mean ± Sd)	MCH(pg) (mean ± Sd)	MCHC(g/dl) (mean ± Sd)	Platelet (10^3/ml) (mean ± Sd)

Normal	6.6 ± 0.04	13.0 ± 0.51	37.8 ± 1.78	56.9 ± 2.52	19.6 ± 0.76	34.5 ± 0.27	1011 ± 106
BCSO 0.25 ml/kgBW	6.5 ± 0.05	12.7 ± 0.15	36.7 ± 0.99	55.8 ± 1.14	19.4 ± 0.15	34.7 ± 0.67	801 ± 139
BCSO 2.5 ml/kgBW	6.6 ± 0.53	11.5 ± 2.38	37.0 ± 3.36	57.3 ± 1.44	17.8 ± 3.41	30.9 ± 4.73	850 ± 121
BCSO 5 ml/kgBW	6.9 ± 0.43	13.2 ± 1.11	37.7 ± 2.35	55.8 ± 1.85	20.0 ± 0.70	35.8 ± 0.25	640 ± 33
Thymoquinone	6.5 ± 0.40	13.1 ± 1.29	36.7 ± 3.35	56.5 ± 2.65	20.2 ± 0.98	35.8 ± 0.38	878 ± 40
Solvent	6.5 ± 0.26	11.9 ± 0.39	35.6 ± 1.23	54.8 ± 0.83	18.4 ± 0.47	33.5 ± 0.33	837 ± 91

Note: BCSO: Black Cumin Seed Oil.

The results of the number of leukocytes measurement (thousand/mm^3) after oral administration of BCSO for 14 d were presented in Table 2. BCSO did not affect the number of leukocytes but affects leukocyte counts especially for a dose of 5 ml/kg BW. 5 ml/kg BW decreased the number of neutrophils and increased the number of lymphocytes compared to the normal group ($p<0.05$). It did not affect the number of monocytes, eosinophil, and basophil.

Table 2. *The number (± Sd) of total leukocyte and composition of leukocyte counts of SD rats after 14 d treatment.*

Group	Leukocyte (10^3/ml)	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)	Eosinophil (%)	Basophil (%)
Normal	6.3 ± 0.70	28.0 ± 2.00	65.0 ± 1.00	5.7 ± 1.15	1.3 ± 0.58	0.0
BCSO 0.25 ml/kgBW	5.7 ± 0.96	27.3 ± 2.08	64.3 ± 0.58	5.3 ± 0.58	2.7 ± 2.08	0.0
BCSO 2.5 ml/kgBW	7.8 ± 0.83	35.7 ± 3.06	58.0 ± 2.00	6.7 ± 1.15	1.7 ± 1.15	0.0
BCSO 5 ml/kgBW	5.8 ± 1.05	23.7 ± 1.53a	71.0 ± 5.29a	6.0 ± 1.00	2.7 ± 2.08	0.0
Thymoquinone	7.4 ± 0.95	31.7 ± 3.76	61.0 ± 3.61	6.3 ± 0.58	1.3 ± 0.58	0.0
Solvent	7.6 ± 1.06	30.3 ± 3.95	62.3 ± 3.50	6.3 ± 0.50	1.5 ± 0.58	0.0

Note: [a]significantly different ($p<0.05$) from normal group; BCSO: Black Cumin Seed Oil.

The number of macrophage cell, phagocytic activity, secretion of ROI and NO

As shown in Table 3, BCSO and thymoquinone influence the number of macrophage cell, phagocytic activity and phagocytic index of macrophage culture cells. The number of macrophage cell, phagocytic activity and phagocytic index in BCSO and thymoquinone group were significantly increase compared to normal and solvent group. BCSO and thymoquinone were also increase the ROI secretion activity (Table 4). ROI secretion activity of macrophages culture cells from SD rat peritoneal which have been exposed by 0.25, 2.5 and 5 ml/kg BW BCSO and 50 mg/kg thymoquinone higher than control ($p<0.05$). The increasing of NO secretion activity only occurred in 5 ml/kg BW BCSO.

Table 3. *The number of peritoneal macrophage, phagocytic activity and phagocytic index of peritoneal macrophage of SD rats after 14 d BCSO treatment.*

Group	**Macrophage (1 × 10^6 cell/ml) (mean ± Sd)**	**Phagocytic activity (%) (mean ± Sd)**	**Phagocytic index (mean ± Sd)**
Normal	1.65 ± 0.20	49.67 ± 1.97	2.99 ± 0.54
BCSO 0.25 ml/kgBW	2.56 ± 0.68[a]	56.83 ± 6.37[a]	5.18 ± 0.39[a]
BCSO 2.5 ml/kgBW	2.57 ± 0.50[a]	52.50 ± 5.24[a]	4.92 ± ± 0.61[a]
BCSO 5 ml/kgBW	2.02 ± 0.35[a]	56.17 ± 6.52[a]	4.35 ± 0.43[a]
Thymoquinone	2.18 ± 0.47[a]	55.83 ± 4.88[a]	5.55 ± 0.58[a]
Solvent	1.53 ± 0.13	41.33 ± 1.51	2.97 ± 0.14

Note: [a]significantly different ($p<0.05$) from normal group; BCSO: Black Cumin Seed Oil

Table 4. The activity of ROI secretion (%) and NO levels in supernatant of peritoneal macrophage cells culture of SD rats after 14 d BCSO treatment.

Group	ROI secretion activity (%) (mean± Sd)	NO level (µM) (mean± Sd)
Normal	31.33 ± 5.32	2.86 ± 0.55
BCSO 0.25 ml/kgBW	52.33 ± 1.63[ab]	3.54 ± 0.39
BCSO 2.5 ml/kgBW	52.83 ± 5.88[ab]	3.58 ± 0.34
BCSO 5 ml/kgBW	65.17 ± 14.19[ab]	4.17 ± 0.36
Thymoquinone	44.67 ± 7.55	3.85 ± 0.12
Solvent	28.00 ± 1.55	2.86 ± 0.82

Note: [a]significantly different (p<0.05) from normal group; [b]significantly different (p<0.05) from thymoquinone group; BCSO: Black Cumin Seed Oil

Secretion of IL-12p40 and expression of TLR-4

BCSO or thymoquinone exposure for 14 d increased the activity of macrophage cells in IL-12 secretion and TLR-4 expression. Level of IL-12 the increasing of IL-12 secreted by macrophages and the expression of TLR-4 in BCSO groups did not differ with thymoquinone group (Table 5).

Table 5. The levels of IL-12 and the ratio of TLR-4 expression in peritoneal macrophage cells of SD rats after 14 d BCSO treatment.

Group	IL-12 levels (µL/ml) (mean ± Sd)	The ratio of TLR-4 expression (×10^2) (mean ± Sd)
Normal	18.83 ± 1.37	1.00 ± 0.00
BCSO 0.25 ml/kgBW	58.33 ± 7.95[a]	1.22 ± 0.17[a]
BCSO 25 ml/kgBW	66.33 ± 2.11[a]	1.22 ± 0.17[a]
BCSO 5 ml/kgBW	51.17 ± 2.25[a]	1.28 ± 0.25[a]
Thymoquinone (50 mg/kgBW)	52.83 ± 10.49[a]	1.33 ± 0.21[a]
Solvent	18.75 ± 1.25	1.00 ± 0.00

Note: [a]significantly different (p<0.05) from normal group; BCSO: Black Cumin Seed Oil

Discussion

Macrophages are professional phagocytes that act as APC and the main effectors in cellular innate and adaptive immune response [16]. This study proved that administration of BCSO and thymoquinone increased the activity of SD rat peritoneal macrophages. The number of peritoneal macrophages, phagocytic activity, macrophage phagocytosis index, secretory activity of ROI, NO and IL-12 in BCSO and thymoquinone groups higher than normal and solvent groups. One of the cytokines produced by activated macrophages is IL-12 [16]. The secretion of IL-12 by macrophages can be stimulated by the presence of parasitic intracellular microorganisms, endotoxins, and glycosides of the medicinal plant such as skrosafisida A of *Pikroriza skrofulariflora*. Secretion of IL-12 is inhibited by the presence of anti-inflammatory agents, immunosuppressants including anti-inflammatory cytokines, such as IL-10 or TGF-β [17].

Macrophages have TLR4 receptor which serves to identify the presence of natural antigen from herbal or pathogen. Black cumin (*Nigella sativa* L.) contain evaporated oil, fatty acids, rich in sterols especially β-sitosterol, thymoquinone, dithymoquinone and saponins [18] that can activate macrophages through TLRs, especially TLR-4. Thymoquinone proved increases the activity of TLR-4 [19,20].

IL-12 cytokine is produced by APC, has various functions, and plays an important role in enhancing immune response mediated by CD4Th1 cells [21]. IL-12 is a regulatory cytokine that mediates a natural immune response by the adaptive immune response. The main roles of IL-12 in regulating the immune response are: (i). activate macrophages and dendritic cells [22], (ii). Activate CD4Th0 cell and increase the proliferation and differentiation of CD4Th0 into CD4Th1, (iii). Increase the secretion of IFN-γ by T cells, macrophages and NK cells, (iv). Increase the proliferation and activity of macrophage and NK cells, (v). Increase the activity of CTL, and (vi). Activate B lymphocytes to produce antibodies. The biological effects of IL-12 are regulating the proliferation and differentiation of lymphoid, regulate the function of

macrophages and dendritic cells, regulate the tolerance, memory and lymphocyte homeostasis [21,23].

Conclusion

BCSO treatment (0.25, 2.5 and 5 ml/kg BW) for 14 d in SD rats did not affect the number of erythrocytes, hemoglobin concentration, hematocrit, MCV, MCH, MCHC and the number of leukocytes. Administration BCSO of 5 ml/kg BW decreased the number of platelet and neutrophils but increased the number of lymphocytes. All of BCSO dose increased phagocytic activity, secretion of ROI, NO and IL-12p40 and expression of TLR-4.

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