

Black cumin seed oil increase leucocyte and CD4Thelper number in sprague-dawley rats induced with dimethylbenzanthracene

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ABSTRACT

Cigarette smoke contains 7, 12 dimethylbenzanthracene (DMBA). Metabolic of DMBA is immunosuppressive. Black cumin seed oil (BCSO) is immunomodulation. This study aimed to determine the effect of BCSO on leukocyte, CD4Th and CD4CD25Treg in Sprague-Dawley (SD) mice induced with DMBA. The 96 SD rats were divided into eight groups of 12. Group I received aquadest and regular feeding. Groups II, III and IV received BCSO (an equivalent of 6.8, 68 and 136 mg/kg BW/day thymoquinone, respectively). Group V received thymoquinone (50 mg/kg BW/day) and group VI received tamoxifen (60 mg/kg BW). Group VII (DMBA) was induced with DMBA (10x20mg/kg BW for five weeks). Group VIII received regular feeding and corn oil treatment. In the third week, all groups began to be induced with DMBA (20 mg/kg BW twice per week for five weeks). Data collection of leukocytes, CD4Th and CD4CD25Treg, were performed at week 27th. The mean difference of CD4Th and CD4CD25Treg counts between groups was calculated with one way ANOVA. The administration of BCSO, thymoquinone, and tamoxifen had increased leukocytes and CD4 Th cell count. The CD4Th cell count of the treatment groups was higher than that of the DMBA group ($p < 0.05$). BCSO equivalent doses of 6.8 and 68 mg/kg BW/day thymoquinone showed immunoprotective effects. It can be concluded that the BCSO administration at doses of 6.8 and 68 mg/kg B/day shows immunoprotective effects due to DMBA induction.

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1. INTRODUCTION

A cigarette is one of the causes of community health problems in the world. Cigarette smoke, along with kitchen and motor vehicle smokes, contains polycyclic aromatic hydrocarbon (PAH) compounds that are harmful to health [1]. One of the PAH compounds, 7, 12 dimethylbenzanthracene (DMBA), is a carcinogenic immunosuppressive agent [2]. In the body, DMBA is metabolized by cytochrome enzymes and microsomal epoxide hydrolase into the ultimate carcinogen, i.e. 7, 12-dimethylbenzanthracene-3,4-diol-1,2, epoxide (DMBA-DE), that is immunotoxic [3-4]. DMBA metabolites through DNA methylation can trigger the formation of various cancers such as breast,

skin, blood, mouth, and lung [5]. As an immunotoxic carcinogen, DMBA has been shown to suppress bone marrow activity and thus inhibit erythropoiesis and lymphocyte development [1, 6].

CD4 T helper lymphocytes are a significant component in the administration of adaptive immune responses. Immune system's inability to recognise non-self-pathogens (neoplasms, cancer cells, pathogens, xenobiotics) is the beginning of the damage to the body's defence system against pathogens [7-8]. One component of the cellular immune system that plays a vital role to regulate the immune response due to exposure to immunosuppressor carcinogens is CD4 T helper (Th) and T regulatory cells (Treg) [8]. As a regulator of the adaptive immune response, T helper lymphocytes play an essential role in identifying and resisting the development of pathogens or neoplasms in the body. Weak positive immune reactions, particularly cellular immune responses, to pathogens increase the organism morbidity and mortality [9]. T helper cells play a role in designing cell-specific immune responses, whereas T regulator plays a role in regulating tolerance response to prevent excessive immune reactions [10]. Because effective cancer therapeutic methods have not been found, the search continues to find chemopreventive compounds that can be used to inhibit or delay carcinogenesis due to DMBA exposure. Antioxidants, proapoptosis agents or anti-proliferative agents, and immunomodulation are thought to impede carcinogenesis or prevent neoplasm formation [11-12]. Antioxidants play a role in inhibiting the formation of DMBA-DE [13-14]. Antioxidants play a role in inhibiting the formation of reactive metabolites DMBA-DE so as not to be carcinogenic, hepatotoxic or immunosuppressive. One of the plants empirically already used as an oxidative chemopreventive and immunomodulation is black cumin seed or *Nigella sativa* (Sativa). The content of black cumin seeds, thymoquinone, is proven laboratorically as immunomodulation because it can increase cellular and humoral immune response. Ethanol extract of black cumin seeds can increase the activity of phagocytosis and microbicidal macrophages [15-17]. Black cumin seed oil (BCSO) is thought to increase helper T cell function and affect T regulator, but until now there has been no research on the effect of BCSO on the number and activity of Th and Treg lymphocytes in DMBA-induced Sprague-Dawley (SD) rats.

2. RESEARCH METHOD

2.1. Design

The study used a randomized, experimental design with the control group in 96 female SD rats. Based on the results of previous studies it is known that the impact of DMBA exposure is influenced by gender [18] hence test animals are uninformed by females for reasons to reduce gender bias due to endocrine factors. This research protocol has obtained ethical clearance (No 043/KEC-LPPT/II/2012) from a research ethics committee from Gadjah Mada University (UGM), Yogyakarta, Indonesia.

2.2. Materials and reagents

The material used in this research is black cumin seed oil produced in the laboratory of Pharmaceutical Biology, Faculty of Pharmacy, Ahmad Dahlan University, Yogyakarta, Indonesia. Test results of bioactive content from BCSO showed fatty acids (78%) and small amounts of thymoquinone. As an immunosuppressive inducing agent used 7, 12-dimethylbenz [a] anthracene (DMBA) (>95%) with synonym 1,4-Dimethyl-2,3-benzophenanthridine, 9,10-Dimethyl-1,2-benzanthracene (C₂₀H₁₆) obtained from Sigma-Aldrich (cat. D3254). As a positive control, two ingredients were used, namely, thymoquinone and tamoxifen. Thymoquinone used as a positive control was 2-Isopropyl-5-methyl-1,4-benzoquinone (>98%) Obtained from Sigma-Aldrich (cat. 274666) agent, while tamoxifen (Tamoxifen, Kalbe Farma) was purchased from a pharmacy. Sardjito's Hospital with prescribing from the doctor. Corn oil as DMBA (lipophilic) solvent was obtained from Sigma-Aldrich (cat. C8267). All materials originating from Sigma-Aldrich were obtained through official agents in Yogyakarta.

Materials for the test of CD4T helper and CD4CD25Treg cell count were FITC CD4, and RPE CD25 monoclonal antibody eBioscience ® Flow Cytometry Staining (eBioscience cat. No. 00-4222). Samples were analyzed in a Becton Dickinson FACS-Calibur flow cytometer, with machine flow cytometry Beckman Coulter FC500. CO₂ incubator (incubator combine ESCO, Catalog-051817-LR), centrifuge (Thermo Scientific™ centrifuges) microplate reader (Synergy™ HTX Multi-Mode Microplate Reader), an inverted microscope (Olympus, BX53 microscope) are used for the preparation and culture of lymphocyte cells. Glassware, hemocytometers, glass objects, blenders, strainers, surgical instruments, and surgical tables are used for material weighing, treatment preparation, and animal cage.

This study used female Sprague Dawley rats aged 3-4 weeks, weighing 100-140 grams obtained from the experimental animal breeding unit, UGM. Based on previous reviews, it was found that DMBA induction in rats aged less than 16 weeks had a better success rate [19] so that in this study, SD rats aged 3-4 weeks were used. The test animals were kept in a 50x30x20 cm individual iron cage, fed 528 pellets, and

supplied moderately-handling of research test animals by international ethical standards for handling test animals as applied in UPHP UGM.

2.3. Treatment and DMBA induction

All rats were kept with the same condition and feed. The test animals were randomly divided into eight groups of 12. Group I (normal) received regular feeding during the test. Group II (BCSO6.8), III (BCSO68), and IV (BCSO136) were the treatment groups, each of which received BCSO doses of 6.8, 68, and 136 mg/kgBW/day respectively for 14 days before and during DMBA induction. Group V (positive control I) received thymoquinone at a dose of 50 mg /kg BW/day for 14 days before and during DMBA induction. Group VI (positive control II) received tamoxifen at a dose of 60 mg/kg BW for 14 days before and during DMBA induction. Group VII (negative control) received corn oil for 14 days before and during DMBA induction. Group VIII (solvent control) received standard feeding and corn oil at a dose of 100 mg/kg BW/day for 14 days before and during DMBA induction. The DMBA compound is dissolved in corn oil at a dose of 20 mg/kg BW and administered personally by trained personnel. The DMBA induction is repeated ten times with the frequency of administration of twice a week [18]. DMBA induction was performed in all groups except the healthy group (group I) and the solvent control group (group VIII). At week 27, peripheral blood was taken through an orbital vein to collect data.

We measured the number and type of leukocyte, the number of CD4 T helper and CD4CD25 Treg lymphocytes. Blood was taken from an orbital vein by a trained laboratory staff at the 27th week of the trial. Blood was then fed into a vacutainer tube containing anticoagulants ready for use to measure the number and count of leukocyte types and T helper and Treg lymphocyte counts with a flow cytometer. The number of CD4Th and CD4CD25 T cells (Treg) was measured by the following standard procedure as has been done by previous research. Fifty μ L specimens were piped into the falcon tube; (ii) 10 pL of CD4 FITC/CD25 tritest reagents per CP were added. The samples and reagents were mixed using a vortex mixer until homogeneous and then incubated for 15 minutes at a temperature of 20-25°C in the darkroom. While waiting for the incubation time, 50 pL of FACS solution was diluted by mixing with 450 pL of distilled water and then stirred until homogeneous. After the incubation time was complete, 450 μ L of the FACS reagent was added to the tube and mixed until homogeneous; After homogeneous, the sample was incubated for 15 min at a temperature of 20-25°C in the darkroom. After the incubation time was complete, BD Biosciences FACS and Cell Quest were used to determine CD4 and CD4CD25 counts.

2.4. Data analysis

The mean difference of the number of leukocyte cells, the number of CD4, and CD4CD25 lymphocytes between the groups were done by ANOVA test and followed by multiple comparison tests with a 95% confidence level.

3. RESULTS AND DISCUSSION

3.1. Number and type of leukocytes

The measurement results of the number of leukocyte types are presented in Table 1. Leukocytes are the first natural defences responsible for eliminating pathogens that enter the bloodstream. The normal leukocyte count in Sprague Dawley rats, according to Schalm's veterinary haematology [19] is 9.810 ± 1.789 (106 cells/mm³). The number of leukocytes in the healthy group at week 27 was 6.33 ± 1.37 (106 cells/mm³), which was lower than the number of leukocytes by Schalm.

Table 1 shows that DMBA induction decreases the number of leukocytes. The leukocytes in the normal group (6.33 ± 1.37 (106 cells/mm³)) were different from those in the DMBA group (2.80 ± 1.10 (106 cells/mm³)) ($p < 0.05$). The DMBA group had the lowest average leukocyte count. The BCSO group had higher average leukocyte count than that in the DMBA group ($p < 0.05$). The BCSO groups had relatively the same number of leukocytes as the normal, thymoquinone, and tamoxifen groups. The BCSO6.8 group had the highest leukocyte count, higher than the normal, thymoquinone and tamoxifen groups ($p < 0.05$).

DMBA induction reduces the number of lymphocytes and increases the number of neutrophils and monocytes. The number of lymphocytes and monocytes in the DMBA group was lower than that of in the healthy and solvent groups ($p < 0.05$). However, the number of neutrophils in the DMBA group was higher than that of the healthy group (50.80 ± 5.54 compared to 30.67 ± 4.23) ($p < 0.05$). BCSO, thymoquinone, and tamoxifen prevent a decrease in the number of lymphocytes and monocytes due to DMBA induction. The number of lymphocytes and monocytes in the BCSO, thymoquinone and tamoxifen groups was higher than that of in the DMBA group ($p < 0.05$).

Table 1. The measurement results of the number and count of leukocyte types in the DMBA-induced SD rats at a dose of 2x20 mg/kg BW/week for 5 weeks after receiving BCSO (at doses of 6.8, 68 and 136 mg/kg BW), thymoquinone (50 mg/kg BW/day) and tamoxifen (60mg/kg BW/day) for 14 days before and during induction. Blood collection was done at week 27. The solvent group received corn oil at a dose of 100 mg/kg BW/day without DMBA induction

Groups	n	number of leukocytes (x10 ⁶) (mean±SD)	percentage of neutrophils (mean±SD)	percentage of lymphocytes (mean±SD)	percentage of monocytes (mean±SD)	percentage of eosinophils (mean±SD)
Normal	12	6.33±1.37	30.67±4.23	61.67±3.61	6.33±0.52	1.33±0.52
BCSO6.8	12	9.86±0.90 ^b	33.57±0.98 ^b	53.71±1.80 ^b	10.86±1.07 ^b	1.86±1.07
BCSO68	12	6.29±0.49 ^b	27.14±3.63 ^b	65.14±3.58 ^b	6.14±0.89 ^b	1.57±0.53
BCSO136	12	7.00±0.93 ^b	27.75±1.67 ^b	65.13±0.83 ^b	5.75±1.04 ^b	1.38±0.52
Thymoquinone	12	6.86±0.90 ^b	28.14±1.68 ^b	64.71±1.11 ^b	5.71±0.95 ^b	1.43±0.53
Tamoxifen	12	7.00±2.68 ^b	35.53±8.64 ^b	57.50±7.12 ^b	5.67±1.86 ^b	1.33±0.52
DMBA	12	2.80±1.10 [*]	50.80±5.54 [*]	44.40±2.51 [*]	3.20±1.64 [*]	1.60±1.52
Solvent	12	6.67±1.03 ^b	30.67±4.23 ^b	61.67±3.72 ^b	6.33±0.52 ^b	1.33±0.52

note:*=p<0.05 to normal group; b=p<0.05 to DMBA group

3.2. Number of CD4 Th and CD4CD25 Treg

The CD4 Th is a lymphocyte that plays a role in regulating specific cellular immune responses while CD4CD25Treg acts as a regulator of tolerance response and prevents hypersensitivity reactions. CD4 Th and CD4CD25 Treg test results with flow cytometer are presented in Table 2.

Table 2. The measurement results of the absolute number of CD4 T Helper & CD4CD25 T regulator and percentage of CD4CD25Treg in the DMBA-induced SD rats at a dose of 2x20 mg/kg BW/week for 5 weeks after receiving BCSO (at doses of 6.8, 68 and 136 mg/kg BW), thymoquinone (50 mg/kg BW/day) and tamoxifen (60mg/kg BW/day) for 14 days before and during induction. Blood collection was done at week 27. The solvent group received corn oil at a dose of 100 mg/kg BW/day without DMBA induction

Group	n	Absolute Count Of CD4 (x103/mm ³)	Absolute Count Of CD4CD25 (x103/mm ³)	Percentage of CD4CD25 to CD4Th (mean±sd)
Normal	12	1575.67±131.70 ^b	70.50±11.76 ^b	4.41±0.01 ^b
BCSO 68	12	1619.57±519.86 ^b	80.86±17.78 ^b	4.19±0.01 ^b
BCSO 68	12	1868.57±382.55 ^b	113.32±20.58 ^b	6.14±0.01 ^b
BCSO 136	12	1668.75±398.01 ^b	92.50±20.53 ^b	5.66±0.01 ^b
Thymoquinone	12	1799.83±429.90 ^b	97.50±21.69 ^b	5.62±0.02 ^b
Tamoxifen	12	1940.00±203.76 ^b	84.50±13.46 ^b	4.34±0.00 ^b
DMBA	12	484.17±33.98 [*]	45.17±9.07 [*]	11.50±0.02 [*]
Solvent	12	1490.33±508.98 ^b	109.33±64.06 ^b	7.35±0.04 ^b

note:*=p<0.05 to normal group; b=p<0.05 to DMBA group

The DMBA-induced group experienced a decrease in absolute CD4 Th count. The absolute number of CD4 Th in the DMBA group was lower than that of in the healthy group (p<0.05). Flow cytometer analysis showed that CD4 Th cell count in normal SD rats was 1575 cells/ml, while for DMBA-induced rats DMBA the count was 484.17±233.98 cells/ml. DMBA induction at a dose of 10x20 mg/kg BW in female SD mice suppressed CD4Th cell count.

The DMBA induction decreases absolute CD4CD25 Treg count but increases the percentage of CD4CD25Treg. The absolute number of CD4CD25 in the DMBA group (45.17±9.07) was lower than that of in the healthy group (70.50±11.76) (p<0.05), it is not the same as the percentage of CD4CD25Treg. The DMBA group had a higher CD4CD25 Treg percentage than the healthy group (11.50±0.02 v.s.4.41±0.01) even the highest among the study group (p<0.05). Box plots of CD4 T helper and CD4CD25 Treg examination results are presented in Figure 1.

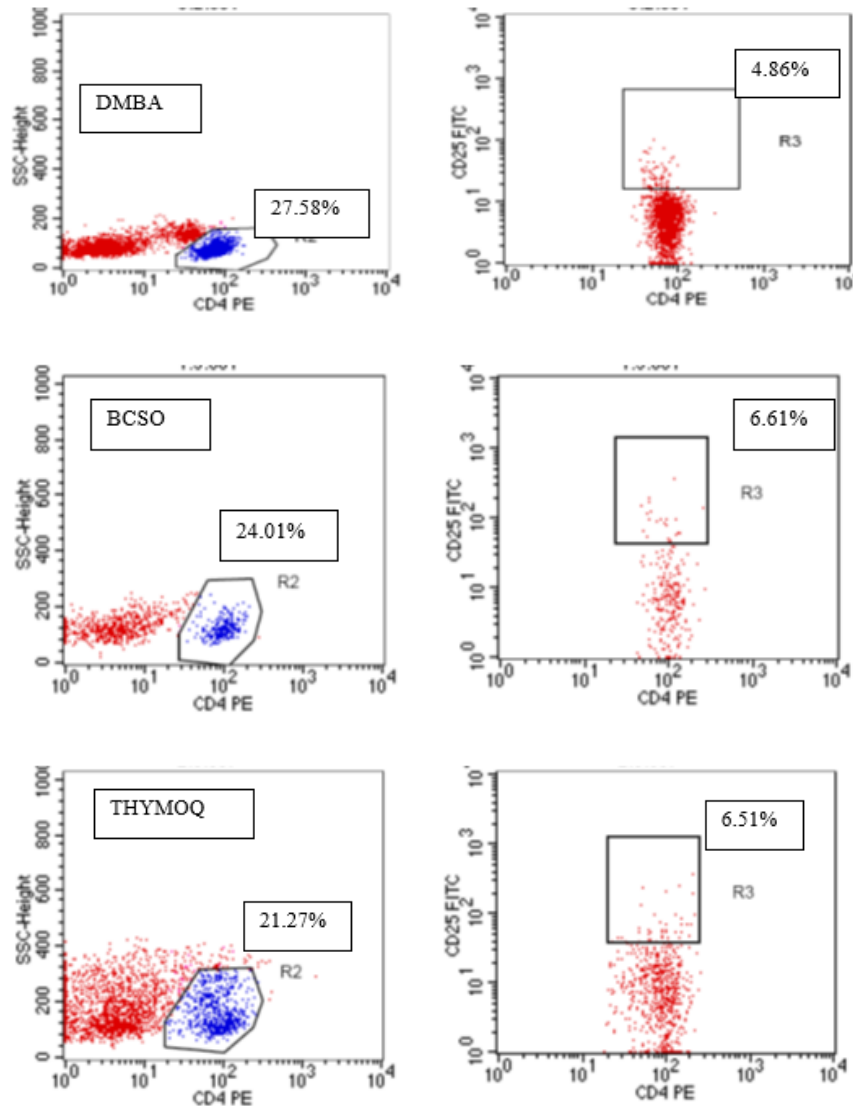


Figure 1. Box plots of CD4 lymphocyte measurements on peripheral blood of SD rats after receiving BCSO at doses of 6.8, 68 and 136 mg/kg BW, tamoxifen and thymoquinone for 14 days before and during DMBA induction (at a dose of 20 mg/kg BW in corn oil, twice a week perorally). The measurement was performed at week 27.

The administration of BCSO, thymoquinone, and tamoxifen had increased CD4 Th and CD4CD25Treg count. The CD4Th and CD4CD25 Treg count of the treatment groups receiving BCSO, thymoquinone, and tamoxifen was higher than that of the DMBA group ($p < 0.05$). The group receiving BCSO at a dose of 68 mg/kg BW/day had the highest absolute number of CD4 T helper followed by the BCSO group of 6.8 mg/kg BW/day and the BCSO group of 136 mg/kg BW/day. The group receiving tamoxifen had a higher absolute number of CD4 T helper than the thymoquinone and solvent control groups.

It can be seen that BCSO, thymoquinone and tamoxifen administration had increased absolute CD4CD25Treg counts in DMBA-induced SD rats but BCSO, thymoquinone and tamoxifen decrease CD4 CD25 Treg percentage. Total absolute CD4CD25 Treg count of the BCSO, thymoquinone and tamoxifen groups was higher than the DMBA group ($p < 0.05$). The BCSO6.8 group had the lowest CD4CD25Treg percentage. The percentage of CD4CD25 Treg in the BCSO, thymoquinone and tamoxifen groups was smaller than the CD4CD25Treg percentage in the DMBA group ($p < 0.05$). The BCSO6.8 group had the lowest CD4CD25Treg percentage.

The administration of multiple BCSO doses before and during DMBA induction increased the number of CD4 T helper and decreased CD4CD25 T reg counts in DMBA-induced SD rats. BCSO treatment

within two weeks before and five weeks during DMBA induction can reduce the immunotoxic effects of DMBA. The ability of BCSO with doses of 6.8, 68 and 136 mg/kg BW/day in inhibiting the immunotoxic effects of DMBA is equivalent to the strength of thymoquinone at a dose of 50 mg/kg BW ($p > 0.05$).

Based on the results of this study, it can be seen that the administration of BCSO before and during DMBA induction can inhibit the decrease in CD4 Th count. Based on the immune response test results, BCSO administration can increase the number of leukocytes, CD4 Th and CD4CD25 Treg count. The BCSO group of 6.8 mg/kg BW/day was able to improve the immune response to the same level as the BCSO group of 68 and 136 mg/kg BW/day and may be safer with smaller doses. The results of this study are consistent with previous studies showing that BCSO is both antioxidative and immunomodulatory [16-17, 20-22]. Data from this study support the results of previous studies, namely the effect of BCSO on the number of CD4CD25Treg. CD4CD25Treg lymphocytes have an essential role in peripheral tolerance response, so this information opens a gap for research on the effect of BCSO on autoimmune disease. The data from this study support the data from the previous research, which is about the impact of BCSO on the number of CD4CD25Treg. CD4CD25Treg lymphocytes have an essential role in peripheral tolerance responses, so this information opens a gap for research on the effect of BCSO on autoimmune disease.

The results of this study also support the claim that has developed so far that BCSO can act as an immunosurveillance amplifier [8, 15, 20]. First developed by Paul Ehrlich in the early 20th century, the concept of immune surveillance is based on the assumption that the immune system has a role in preventing and limiting tumour growth [7, 8, 23] including the process of carcinogenesis due to exposure to carcinogens. Research in the field of immunology, laboratory, and clinic then justify the assumption. One of the cellular components that play a crucial role in immunosurveillance against exposure to immunosuppressive carcinogens is CD4 Th lymphocytes. Immunosurveillance activity performed by CD4 Th lymphocytes is associated with CD4Th ability to activate CD8 cytolytic lymphocytes in destroying neoplasms and pathogens. As CD4 Th increases, the production of IL-2, IL-12 and IFN- γ cytokines also increases, thus increasing the number and activity of CD8 cytolytic lymphocytes [8]. It has been demonstrated that BCSO decreases eNOS activity, lymphocyte proliferative activity and differentiation increases the production of IL-12 cytokines by macrophages and inhibits inflammation through inhibition of prostaglandin production [15, 21-22, 24].

A reasonable amount of CD4CD25 Treg serves to regulate the tolerance response, but excessive CD4CD25Treg count is immunosuppressive [25-26]. Data from this study indicate that DMBA induction decreases the total amount of CD4CD25Treg but increases the percentage of CD4CD25 to CD4Th cells. CD4CD25Treg lymphocytes are activated to prevent excessive and destructive immune responses. CD4CD25Treg activation is performed in conditions where lymphocyte proliferation activity is excessive [9, 25-26], but if the CD4CD25 Treg count is too large, it will be immunosuppressive. Based on the results of previous studies, the average percentage of CD4CD25Treg to CD4Th is lower or equal to 10% [27]. In these ideal conditions, Treg can play a role in maintaining the homeostasis of the immune response, preventing the occurrence of an excessive immune response or immunodeficiency. Based on the results of previous studies, the average percentage of CD4CD25Treg to CD4Th is lower or equal to 10% [27]. In these ideal conditions, Treg can play a role in maintaining the homeostasis of the immune response, preventing the occurrence of an excessive immune response or immunodeficiency. Based on the results of this study, it can be proved that DMBA induction decreases CD4CD25 Treg count.

The low CD4Th count seems to be related to this CD4CD25Treg number due to DMBA metabolites, which are immunosuppressive and hematotoxic [15, 24]. BCSO administration increased the absolute amount of CD4CD25 Treg but decreased the percentage of CD4CD25Treg to CD4. The BCSO, thymoquinone and tamoxifen groups had higher CD4CD25 Treg absolute numbers than the DMBA group but had a lower CD4CD25Treg percentage than the DMBA group. BCSO administration increased the absolute amount of CD4CD25 Treg but decreased the rate of CD4CD25Treg to CD4. The BCSO, thymoquinone and tamoxifen groups had higher CD4CD25 Treg absolute numbers than the DMBA group but had a lower CD4CD25Treg percentage than the DMBA group.

4. CONCLUSION

BCSO administration before and during DMBA induction in SD rats can increase cellular immune response, especially the absolute number of CD4 T helper and CD4CD25 Treg count. BCSO at a dose of 6.8 mg/kg BW/day was able to increase CD4 T helper count to the same level as the doses of 68 and 136 mg/kg BW/day doses.

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