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Chemopreventive effect of black cumin seed oil (BCSO) by increasing p53 expression in dimethylbenzanthracene (DMBA)-induced Sprague Dawley rats

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Abstract
This study was conducted to investigate the chemopreventive effect of black cumin seeds oil (Nigella sativa) in 96 dimethylbenzanthracene (DMBA)-induced Sprague Dawley mice. The test animals were divided into eight groups. The normal group was given standard food and drink. The three black cumin seed oil (BCSO) treatment groups received BCSO dosages of 0.25, 2.5 and 5 ml/kgBW/day respectively and they were induced with DMBA. The thymoquinone and tamoxifen groups received 50 mg/kgBW/day thymoquinone and 0.6 mg/kgBW/day tamoxifen respectively and they were induced with DMBA.

The DMBA group was induced with 10x20mg/kgBW DMBA for five weeks. The solvent group received standard feeding and corn oil solutions. Starting from the third week of treatment, all groups except the normal and solvent groups were given 20 mg/kgBW DMBA twice a week for five weeks. Dissection and data retrieval were conducted at week 27. The chemopreventive effects are measured by nodules incidence, nodul multiplication, total weight and histopathology.

The p53 and H-Ras gene expression are assessed using densitometry after PCR. The mean intergroup difference was calculated using one-way ANOVA. The results showed that BCSO administration before and during DMBA induction could decrease nodule formation and count, decrease H-Ras gene expression and increase p53 gene expression. A dose of 0.25 ml/kgBW/day BCSO indicated a chemopreventive effect, increased p53 gene expression and decreased H-Ras gene expression, all of which were similar to a dose of 2.25 ml/kgBW/day but safer. It can be concluded that treatment of 0.25 ml/kgBW/day BCSO produces chemopreventive effects in DMBA-induced SD rats.

Keywords: Chemopreventive, thymoquinone, Black Cumin Seed Oil, p53 gene, H-Ras Gene.

Introduction
Breast cancer is a public health problem in Indonesia and the world. One of the factors associated with increased incidence of breast cancer in Indonesia is the high carcinogenic air pollutants including smoke from cigarettes, motor vehicles, industries and households[1-3]. These smoke sources contain carcinogenic polycyclic aromatic hydrocarbons, one of which is 7.12 dimethylbenzanthracene (DMBA)[4,5]. DMBA is genotoxic and immunotoxic[6]. Cancer cell formation due to exposure to DMBA is a complex phenomenon, as it involves the immune system and various enzymes in the metabolic system of the body[7-9]. DMBA can trigger the formation of breast, skin, blood, oral and pulmonary cancer through either the mechanism of DNA mutation by forming DNA adduct or chronic inflammatory device[10,11].

Cytochrome and epoxide microsomal hydrolyses enzymes metabolize DMBA into 7.12-dimethylbenzanthracene-3,4-diol-1,2, epoxide (DMBA-DE), the ultimate carcinogen that is both genotoxic and immunotoxic[12,13]. DMBA-DE binds to DNA to form DNA adducts with p53 and H-Ras genes as carcinogenesis initiators[14]. The formation of DNA adducts in the p53 gene by DMBA-DE causes inactive repair functions and the mechanism of proapoptosis against neoplasms. Conversely, the creation of DNA adducts in the H-Ras gene by DMBA-DE activates protooncogene; neoplasms undergo hyperproliferation[15,16]. p53 is a main tumor suppressor protein that has an essential role in anti polycyclic aromatic hydrocarbon-induced carcinogenesis.

In vivo study, DMBA-DE has been shown to suppress bone marrow activity[17], inhibit T cell activity[18], toxic agent to spleenocyte[7,8] and impede cytokine activity and stimulator IFN-γ gene (STING)[19]. These conditions undermine the immune system and allow neoplasms to develop into malignant tissues[20].

Phytochemistry chemopreventive compounds may be used to inhibit, delay, or reverse carcinogenesis[21]. One of the plants empirically used as an immunomodulatory agent in Indonesia is black cumin or Nigella sativa. In Indonesian traditional herbal medicine, empirically black cumin seed oil (BCSO) has been used as anti-inflammatory and immunostimulants[22]. As the primary active substance of BCSO, a dosage of 50 mg/day of thymoquinone is useful as

chemopreventive. Thymoquinone has also been shown to be anti-lung cancer[23], inhibiting lymphocyte proliferation in myeloma[24], inducing apoptosis in lymphoma[25], anticlastogenic[26], chemopreventive for fibrosarcoma[27] and increasing cytotoxic lymphocyte (CTL) activity[28, 29].

BCSO has been shown to increase p53 expression in the liver tissue of Sprague Dawley rats induced by alloxan. It has also been shown to increase lymphocyte proliferation[30] and macrophage phagocytosis activity[31] and proven as useful as chemopreventive in colonic cancer[32]. The BCSO is assumed as effective as anticarcinogenesis in DMBA-induced SD rats through increased p53 gene activity.

Material and Methods

This study was an experimental laboratory with a control group. The test animals used were Sprague Dawley (SD) (96) aged 3-4 weeks divided by eight groups. The treatment group received BCSO three dose ratings (0.25, 2.5 and 5 ml / kg BW / day), the control group I received thymoquinone (50 mg / kg BW/ day) (Sigma-Aldrich) and the control group II used tamoxifen (0.6 mg / kg BW / day) (Kimia Farma). The study was conducted at the animal breeding and experimental unit of Gadjah Mada University. We used 7,12 dimethylbenzanthracene (DMBA, Sigma-Aldrich) for inducing carcinogenesis. The study protocol has been reviewed and declared ethically viable by the animal research ethics committee of Gadjah Mada University, Yogyakarta, Indonesia (No 043/KEC-LPPT/II/2012).

Materials and equipment: The materials used in this research are BCSO made in Pharmaceutical Biology Laboratory, Faculty of Pharmacy, Ahmad Dahlan University. Thymoquinone, reagent and cell growth medium were obtained from Sigma. Hematoxylin-eosin and Giemsa are used for the painting of histopathologic preparations. Devices used for gene activity examination include PCR machine (Thermocycler Biometra, Göttingen, Germany), centrifuge (Heraeus Biofuge fresco Kendra, Hanau, Germany), Eppendorf tubes, micropipettes, electrophoresis (SE 260 Mini-Vertical electrophoresis-unit GE Healthcare, Munich, Germany), Mini-shaker (MS 2 IKA, Wilmington, USA), densitometry and spectrophotometer.

Materials for PCR include DNA markers (catalog No. 1721933, Roche Diagnostics GmBH), Trizol (catalog No. 10503-027, Invitrogen USA), RTG PCR and RTG-RT PCR kit (puReTaq Ready-To-Go RT-PCR beads , catalog No. 279557-01), room temperature beads with stabilizers, BSA, dATP, dCTP, dGTP, dTTP (catalog No. 27-9557-02), puReTaq DNA polymerase, (catalog No.27-9558- 01), dNTP (catalog No. 27-9559-01) GE Healthcare, UK), first strand cDNA synthesis kit for RT-PCR (Roche), loading buffer, agarose and other chemicals.

The p53 and H-Ras DNA primers are presented in table 1. The female Sprague Dawley (SD) rats aged 3-4 weeks weighing from 100 to 140 grams (uniform age and weight) are obtained from the Gadjah Mada University Biological Sciences Laboratory. The test animals were kept in a 50 x 30 x 20 cm individual-sized iron cage, fed 528 pellets and fed moderately.

Treatment, DMBA Induction and Anti Carcinogenesis Examination: Ninety-six of 2-week-old female SD line were treated in a 50 x 30 x 20 cm individual iron cage. Rats are kept by good animal testing standards in terms of cage conditions, foods and drinks. Before the study, test animals were put in a maintenance cage for one week for adaptation. During the study, the rats were weighed every week to measure weight development. Breast cancer mouse modelling was performed by administering DMBA with a procedure similar to previous researchers but with modification.

The 5-week-old mice were given DMBA solution in corn oil at a dose of 20 mg/kg BW perorally. The DMBA initiation is repeated twice a week, making it ten times in total[33]. After the adaptation period, the test animals were randomly divided into eight groups. Group I is the healthy group. The animals in this group only received regular feeding during the test. Group II, III and IV are the treatment group. They received a dosage of 0.25, 2.5 and 5 ml/kg BW/day BCSO for 14 days before induction and five weeks during the test. Group V is the thymoquinone group. It received 50 mg/kg BW/day pure thymoquinone for 14 days before induction and five weeks during the trial.

Thymoquinone is administered in the same preparation and manner as the treatment group. Group VI is the tamoxifen group. It received 0.6 mg/kgBW/day tamoxifen (34) for 14 days before induction and five weeks during the test. The tamoxifen was administered in the same preparation and manner as the treatment group. Group VII is the DMBA group. It received corn oil for 14 days before being induced with 10x20mg/kgBW DMBA for five weeks. Group VIII is the solvent control group. It received 100 mg/kgBW/day corn oil and was induced with DMBA in addition to regular feeding and drinking. The corn oil is administered in the same preparation and manner as the other groups. All test animals received 10x20 mg/kgBW DMBA orally within five weeks starting from the 3rd week of treatment except for the healthy group and the solvent control group.

Observation of BCSO chemopreventive effects: The chemopreventive effects are measured by nodules incidence, number of nodul, total weight and histopathology[7,34]. Examination of nodule appearance in the breast was done by palpation after the last DMBA induction i.e. ten weeks old or at 7th week of treatment and continued until 27 weeks old. Tumor nodules in the first gland examination are considered as nodule formation incidence. Some nodules are the number of nodules formed by each group. Incidence is a percentage of the number of mice that have tumors nodule in one group in rate (%). Histopathologic examination of nodules and mammary tissues was performed at week 27 of the trial. The

study aimed to investigate changes in the tissues and cell structures of tumor nodules and animal mammary. The removal of tumor nodules was done after the test animal was confirmed dead. The animal was decapitated before surgery. The tumor nodules that had been released from surrounding tissues and connective tissues were then weighed.

The histopathological preparation was done in the laboratory of Anatomical Pathology of Medical Faculty, Gadjah Mada University. Examination of the tissue preparation was done in the laboratory of Pathology of Veterinary Medicine Faculty, Gadjah Mada University. Tissue examination using light microscope was performed by anatomical pathology experts from Veterinary Medicine Faculty, Gadjah Mada University. Microscopic observations include the histological state of the mammary gland organs from the HE painting. HE- staining mammary tissue preparations were observed descriptively to determine the severity of cancer. Microscopic observation screening was based on the rate of epithelial cell proliferation categorizes tissues as normal, hyperplasia/dysplasia and adenocarcinoma according to histopathologic features by the veterinary pathologist.

Examination of P53 and H-Ras gene expression

RNA Isolation: Isolation of RNA from the tissue and the formation of cDNA; RNA isolation from mammary tissues was performed using an RNAzol kit with the procedure as recommended by the company. The 20 µL of isolated RNA solution was used for cDNA formation. The cDNA formation was done using the First Strand Synthesis Kit for RT-PCR version 09 (AMV, Roche). The 3 µg RNA concentration in 30 µL diethyl pyrocarbonate (DEPC) 0.1% was homogenized with a vortex in a microtube and then incubated at 65° C for 10 minutes.

The microtube was then inserted into the ice for 2 minutes and then the RNA was inserted into the first strand synthesis reaction tube containing two white cotton balls. Primary oligo (dT) 5'-GTA ATA CGA ATA ACT ATA GGG CAC GCG TGG TGT GCC CGG GCT GGT TTT TTT TTT TTT T-'3 with 1 µg / 3µL concentration was added into the reaction, then left for 1 minute. Microtube was incubated at 37° C for 1 hour and then SDW 50 µL was added to the cDNA formed.

Performing RT-PCR using RTG RT-PCR kit: The RT-PCR material preparation procedure was that 2 µL primer was added into RTG RT-PCR. We added cDNA template at a volume according to calculation, up to 25 µL H_2O DNase was added. The solution was then homogenized by beating the lower end of Eppendorf smoothly. We added 25 µL mineral oil to the homogeneous solution. The Eppendorf was then opened and the test material was ready for DNA amplification.

DNA amplification of the test material was carried out with the GeneAmp® PCR system thermal cycler. The prepared Eppendorf tube was fed into the PCR device and the temperature and number of cycles were adjusted as required. The denaturalization process was done for 5 minutes at 95ºC. The overall period, link and temperature settings are shown in table 1.

Electrophoresis product of RT-PCR test material with agarose gel is carried out by experienced technicians. The preparation procedure for 1.5% agarose is as follows: (a) 0.50 grams of agarose was put into Erlenmeyer; (b) 25 ml of 0.5X TBE was added; (c) after that, it was put into the oven for 150 seconds; (d) afterwards, 2 µL ethidium bromide was added; (e) then, after homogeneity, it was poured into the gel mold and (f) wait for 1 hour before the gel was used in order for the gel to harden.

The hardened gel is then fed into the electrophoresis chamber. In the next stage, the PCR DNA analyte was inserted into the electrophoresis well. Briefly, the DNA analysis procedure using the PCR method for ten µL of the PCR solution was piped and inserted in a sterile Eppendorf tube. One µL loading buffer was added and homogenized by beating the lower end of the tunnel. 2% agarose gel was introduced into the electrophoresis apparatus and 0.5X TBE was added until the agarose gel wholly immersed.

The PCR solution was piped and inserted in the agarose (no air in the pipette). The electrophoresis device was set to 100 volts voltage and 43 minutes of elution time if the buffer spot had reached the upper limit, the electrophoresis apparatus was turned off and the gene expression was seen under the UV light. The appearance of a visible gene with a UV lamp is then documented for analysis.

Table 1
Primary list for RT-PCR of p53 and H-ras gene

Gen	Sequencing	bp	T annealing	temperature	link
p53	F:5-GTCGGCTCCGACTATACCACTATC-3 B:5-CT GGA GTC TTC CAG CGT G-3[44]	138	55	72	33
H-Ras exon 1	F:5-TGG TTT GGC AAC CCC TGT AGA-3 B:5-AGTGGGATCATACTCGTCCAC-3[45]	305	62	72	49
b-Actin	F:5-AAGAGAGGCATCCTCACCCT-3 B: 5-TACATGGCTGGGGTGTTGAA-3[46]	815	55	72	33

Note F: forward; B: backward

Semi Quantification of gene expression by densitometry: Semiquantitative analysis of gene expression was performed with the aid of densitometry tools. Documentation photos of gene expression bands after electrophoresis were printed in black and white in postcard size. The difference in the size and thickness of the DMBA band (bend) image of PCR results from the gene is then read by densitometry at visible wavelength (420 nm). The magnitude of gene activity is indicated by the thin size or thickness of the bender image and by densitometry reading as the area under the curve (AUC). Comparison of DNA tape AUC and DNA bands AUC of the b-actin gene is referred to as gene activity.

Data analysis: Statistical analysis of the study results was accomplished using different methods for different data types. Data on the incidence tumor formation per group and the number and the total weight of nodule tumor per group were presented descriptively. Results of histopathological examination of nodules and mammary tissues were presented descriptively. Examination results of p53 and H-Ras gene expression were presented descriptively as the ratio of p53 or H-Ras gene expression to actin gene expression. Relative activities of p53 and H-Ras genes on actin genes were analyzed with mean difference test (ANOVA) with a 95% confidence level.

Results and Discussion

Data from observations on the number of nodule/group, the entire weight of nodule/group and frequency of nodule formation are presented in table 2. The results showed that DMBA induction caused tumor nodules formation. The number of nodules, the total weight of nodule and incidence of nodule formation in the DMBA group were highest, while nodules were not formed in the healthy group and the solvent group. All DMBA group animals had nodules (100%), followed by BCSO0.25 and BCSO2.5 (50%) and BCSO5 groups (45%). Black cumin seed oil, thymoquinone and tamoxifen have been shown to reduce tumor nodule formation. The thymoquinone group had the lowest of incidence, number and weight of tumor nodules, followed by the tamoxifen group. The researchers did not succeed in observing the multiplication of tumor nodules.

Chemopreventive Effects of BCSO: We found the chemopreventive effects of BCSO. The calculation results of the chemopreventive effect of BCSO administration in SD rats are shown in table 3. BCSO administration may decrease the percentage of nodule formation and number and total weight of nodule. The treatment groups BCSO0.25 and BCSO2.5 had the highest rate of nodule formation at 50%, while the lowest was BCSO5 (45%).

Based on these results, it can be proved that BCSO can inhibit and decrease the occurrence of nodule formation and the number and total weight of tumor nodules in DMBA-induced SD rats.

Histopathology and time formation of nodule: The observation results of the time of appearance and number of tumor nodules in DMBA-induced SD rats with BCSO administration are presented in figure 1. The results showed that DMBA induction leads to the appearance of tumor nodules. The presence of tumor nodules occurred in week 14. Among the groups receiving BCSO, the DMBA and BCSO 0.25 groups had the fastest time in nodule formation at week 14.

Based on the time of tumor nodule formation (Figure 1), the earliest formation of nodules occurred in the DMBA group at week 14 together with the BCSO 0.25 group followed by BCSO 5 (week 17), BCSO 2.5 (week 18) and tamoxifen (week 18). The latest group in the formation of tumor nodules was the thymoquinone group (week 20). The results indicate that BCSO, thymoquinone and tamoxifen administration can delay the formation of tumor nodules.

Table 2
A number of the nodule, the total weight of nodule/group and incidence of nodule tumor formation/group in DMBA-induced SD mice 2x20 mg/kgBW/week for five weeks after treatment of BCSO 0.25, 2.5 and 5 ml/kgBW/day for two weeks before and five weeks during DMBA induction.

Groups	n	Number of Nodule/group	Total weight nodul/group (gram)	Nodul incidence (percentage of nodul formation/group) (%)
Normal	12	0	0	0
BCSO0.25	12	8	2,51	50,00
BCSO2.5	12	8	3,25	50,00
BCSO5	12	6	4,17	45,00
Thymoquinone	12	3	1,20	30,00
Tamoxifen	12	5	1,40	40,00
DMBA	12	14	10,53	100,00
Solvent	12	0	0	0

Table 3
The results of observation on BCSO chemopreventive effect in DMBA-induced SD mice 2x20 mg/kgBW/week for five weeks after treatment of BCSO 0.25, 2.5 and 5 ml/kgBW/day for two weeks before and five weeks during DMBA induction.

Test Groups	n	Percentage of nodule formation (%)	Chemopreventive effect
Normal	12	0	0
BCSO0.25	12	50.00	50%
BCSO2.5	12	50.00	50%
BCSO5	12	45.00	55%
Thymoquinone	12	30.00	70%
Tamoxifen	12	40.00	60%
DMBA	12	100.00	0%
Solvent	12	0	0

Note: BCSO0.25=black cumin seed oil dose of 0.25 ml/kg BW/day; BCSO2.5=Black cumin seed oil dose of 2.5 ml/kgBW/day; BCSO5 = black cumin seed oil doses of 5 ml/kg BW/day.

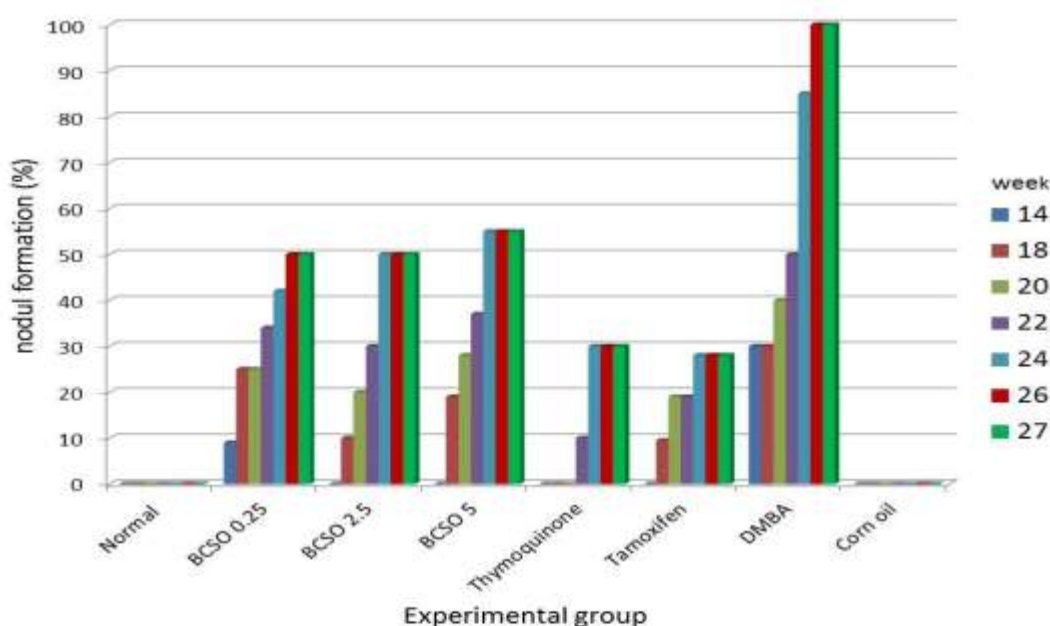

Figure 1: Time of nodule formation of DMBA- induced SD rats 2x20 mg/kgBW/week for five weeks. The test animals received 0.25, 2.5 and 5 ml/kgBW/day BCSO for two weeks before and five weeks during DMBA induction (twice a week)

In the solvent and healthy groups, none of the primary rats was diagnosed with carcinoma (adenocarcinoma) mammae. Microscopically, the mammary gland cells of the solution and normal groups show the histologic features of normal mammary tissues. There was no change in histologic pictures of mammary tissues characterized by hyperplasia, metaplasia and neoplasm.

Meanwhile, the DMBA group which was carcinogenesis and formed tumor tissues showed hyperplasia on connective tissues. The connective tissues were denser because of the enlarged tumor cells, neoplasm cells from ductal epithelial (adenocarcinoma) and asinus and presence of inflammatory cell gels and collection of necrotic cells. The results showed that the DMBA group had tumorigenesis (100%) and was 100% histopathologically diagnosed as adenocarcinoma. In the mammary gland tissues, there was adenocarcinoma of the *in situ* and invasive ducts.

BCSO treatment for two weeks before and five weeks during DMBA induction may inhibit the process of carcinoma formation. Histopathological examination of mammae tissues of the treatment groups with various doses of BCSO found tumor with adenocarcinoma, neoplasm without adenocarcinoma, hyperproliferation and normal. There were multiple forms of epithelial proliferation of asinus epithelium and ductal epithelium, but some of the hype related features cannot be said as mammae adenocarcinoma. Also, some primary rats gave a histopathologic picture of hyperplasia in the epithelial asinus.

The study identified 15 SD rats with histopathologic features similar to the adenocarcinoma mammae: seven from the DMBA group (100%), two from the BCSO0.25 (16.7%), two from the tamoxifen (20%), three from BCSO2.5 (30%) and one from BCSO5 (10%). The percentages of adenocarcinoma formation among SD rats receiving BCSO treatment were 10%, 20% and 30% for BCSO 5, BCSO 0.25 and BCSO 2.5 respectively. The DMBA group had the largest percentage with 100% while the tamoxifen group stood at 20%. The results proved that DMBA induction of 10x20 mg/kgBW twice a week for five weeks resulted in carcinogenesis, i.e. the process of adenocarcinoma mammae formation. The BCSO administration of 0.25, 2.5 and 5 ml/kg BW/day proved to inhibit carcinogenesis as BCSO can act as an anticarcinogenesis chemopreventive agent.

H-Ras and p53 gene expression: The results showed that DMBA induction increased H-Ras gene and decreased p53 gene expression. It can be seen that P53 gene expression was stronger than H-Ras. The normal group H-Ras gene expression was lower than the DMBA, but p53 gene expression was higher in the normal group than that in the DMBA group. Table 4 presents the relative activity of the p53 gene nodules of mammary tissues. The activity of the DMBA group p53 gene (1.06 ± 0.10) was higher than that of the normal group (0.84 ± 0.13) ($p < 0.05$) but lower than that of the thymoquinone group (1.26 ± 0.06) ($p < 0.05$) and tamoxifen group (1.12 ± 0.10) ($p < 0.05$). The results show that DMBA induction decreases the relative activity of the p53 gene.

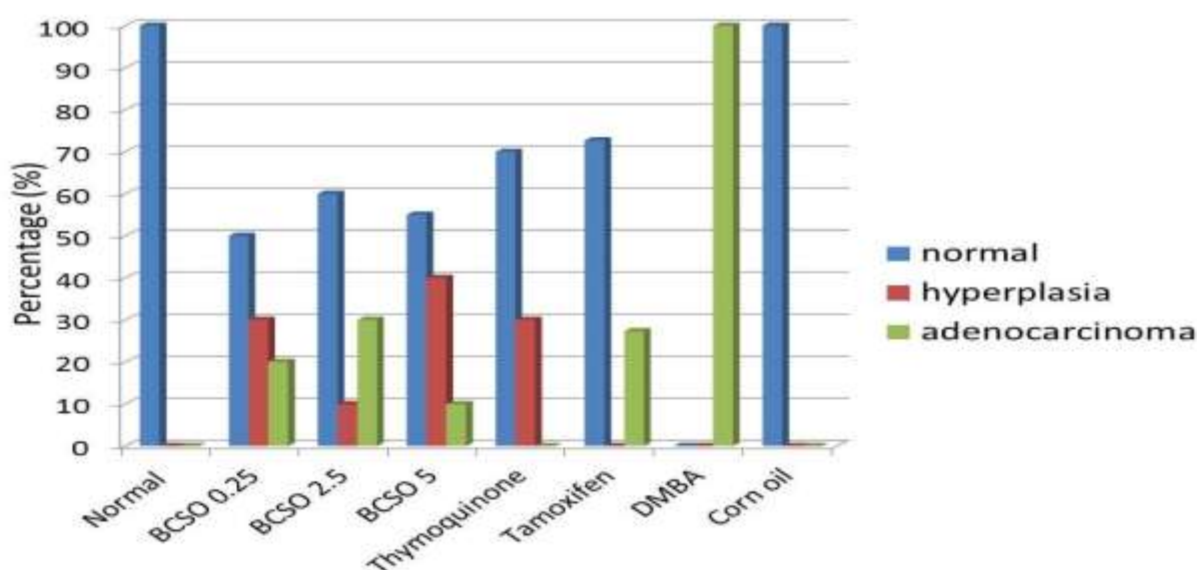

Figure 2: Histopathology of DMBA-induced rat tissues (10x20 mg/kg BW) for five weeks after treatment of BCSO 0.25, 2.5 and 5 ml/kg BW/day for two weeks before and five weeks during induction. Tissue retrieval was done at week 27.

Table 4
Relative activity of the H-Ras and p53 gene in DMBA-induced primary mice 10x20 mg/kg BW for five weeks after BCSO treatment of 0.25, 2.5 and 5 ml for two weeks before and five weeks during DMBA induction. Network retrieval was performed at week 27 of the experiment.

		Gene relative activity to B-Actin	
Groups	**N**	**H-Ras gene (mean±SD)**	**P53 gene (mean±SD)**
Normal	12	0.18±0.02	0.84±0.13
BCSO0.25	12	0.26±0.09*	1.30±0.21*
BCSO2.5	12	0.22±0.03*	1.24±0.15*
BCSO5	12	0.31±0.13*	1.30±0.09*
Thymoquinone	12	0.14±0.09*	1.26±0.06*
Tamoxifen	12	0.14±0.02*	1.12±0.10*
DMBA	12	3.28±0.62#	1.06±0.10#
Solvent	12	0.20±0.03*	1.10±0.03

Note: BCSO0.25=black cumin seed oil dose of 0.25 ml/kg BW/day; BCSO2.5=Black cumin seed oil dose of 2.5 ml/kg BW/day; BCSO5 = black cumin seed oil doses of 5 ml/kg BW/day; *=$p<0.05$ to the DMBA group;#=$p<0.05$ to normal group.

BCSO treatment increased p53 gene activity in DMBA-induced SD rats. The p53 gene activity of BCSO groups (0.25 (1.30 ± 0.21), 2.5 (1.24 ± 0.15) and 5 (1.30 ± 0.09)) was higher than the DMBA group (1.06 ± 0.10) ($p < 0.05$). The BCSO groups activity was proportional to p53 gene activity of positive control group of thymoquinone (1.26 ± 0.06) ($p > 0,05$) and tamoxifen (1.12 ± 0.10) ($p > 0,05$). BCSO administration two weeks before and five weeks during DMBA induction can inhibit the decrease of p53 gene activity in DMBA-induced mice.

The activity in the DMBA group (3.28 ± 0.62) was higher than that in the normal group (0.18 ± 0.02) ($p < 0.05$), thymoquinone group (0.14 ± 0, 09) ($p < 0.05$) and tamoxifen group (0.14 ± 0.02) ($p < 0.05$). The results prove that DMBA induction increased H-Ras gene activity. The results also proved that BCSO treatment two weeks before and five weeks during DMBA induction could inhibit the increased relative activity of H-Ras genes. The H-Ras oncogenesis activity of the BCSO treatment groups (0.25 (0.26 ± 0.09), 2.5 (0.22 ± 0.03) and 5 (0.31 ± 0.13)) was lower than that of the DMBA group (3.28 ± 0.62) 0.05). The H-Ras activity of the BCSO treatment groups was higher than that of the thymoquinone group (0.14 ± 0.09) ($p < 0.05$).

The results show that DMBA induction was confirmed to increase the risk of animal mortality and 10x20 mg/kgBW DMBA administration also proved to cause carcinogenesis. These results are consistent with previous research. The results of previous research have shown that DMBA metabolite (DMBA-DE) forms DNA adduct in p53 and H-Ras genes and initiates carcinogenesis. This study has demonstrated that DMBA induction can decrease p53 gene expression and increase H-Ras gene expression[14]. This study has also proved that BCSO treatment before and during DMBA induction can improve the survival rate of test animals and prevent or inhibit carcinogenesis.

The BCSO group with a dose of 5 ml/kgBW/day had the largest mean percentage of nodule formation with 55% while the doses of 0.25 and 2.5 had the same percentage at 50%. Based on the survival rate, it was found that the group receiving 0.25 ml/kgBW/day had the highest survival rate and the lowest percentage of deaths at 8%. Based on these results, it can be established that BCSO dose of 0.25 ml/kgBW/day is safer and able to reduce the cancer incidence in DMBA-induced test animals.

Breast cancer neoplasms express new molecules of malignant determinants as a result of gene dysregulation[4,10]. Gao et al[8] have proven that DMBA metabolites (DMBA-DE) successfully formed DNA adduct in the p53 gene resulting in immune toxicity. This study has shown that BCSO administration was useful to inhibit carcinogenesis caused by DMBA exposure. This finding is consistent with the results of previous studies. Mousa et al[35] and Mbarek et al[36] have proven that Black Cumin Seed extract is indigenous. BCSO mechanisms in inhibiting carcinogenesis caused by DMBA induction include the activation of NFKb pro-apoptosis pathway via the p53 gene.

Sethi et al[37] have demonstrated that thymoquinone activates the NF-kB pathway and thus suppressing the anti-apoptotic gene and enhancing apoptosis. BCSO has also been shown to restore wild-type p53 genes that are damaged by genetic stress due to DMBA-DE exposure so that the p53 regulating function is reactivated[35]. In line with data from this study, BCSO has been shown to increase p53 expression in streptozotocin-induced primary mice[22].

These study results have revealed that DMBA induction can decrease some gene expression, but conversely, BCSO administration can increase it. In addition to causing changes in the expression of various surface protein molecules, genetic dysregulation in neoplasms can also lead to transcriptional and translational disorders of intracellular protein molecules that are identified as non-self and immunogenic[36]. DMBA-DE, the metabolic of DMBA, has also been confirmed to be immune-toxic and suppressing bone marrow hematopoietic activity[8]. Cellular injury due to genetic stress and protein molecules of mutant gene products are due to p53 genetic dysregulation[38]. Environmental factors play a significant role in carcinogenesis, neoplasms and tumor tissue formation[39].

As the immune system weakens and the inflammatory reaction becomes excessive, cancer formation in the DMBA group is most successful[40]. BCSO administration can inhibit DNA damage caused by DNA adduct formation, prevent carcinogenesis and improve specific immune responses[41-43]. The dose of BCSO 0.25 or 2.5 ml/kg BW /day seems to have the same potential as chemopreventive, but the dose of 0.25 ml/kg BW/ day BCSO is safer. The BCSO contributes to lymphocyte proliferation and secretion of pro-inflammatory cytokines leading to the activation of cytotoxic lymphocytes (CD8). BCSO is believed to activate TLR in inhibiting carcinogenesis and increasing the number and activity of cytotoxic T lymphocytes[44-46].

Conclusion

Based the results of the study, it can be concluded that BCSO administration of 0.25, 2.5, or 5 ml/KgBW/day before and during DMBA induction in SD mice can inhibit carcinogenesis.

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