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Evaluation of black cumin seeds hexane extract as reactive oxygen intermediates (ROI) and phagocytic activity modulator in DMBA induced-rats.

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Abstract

Dimethylbenzanthracene (DMBA) compounds were proven to suppress bone marrow activity, immunosuppressive, and genotoxic and carcinogenic. Black cumin seed extract was used as an immunomodulator, which can be developed as a great therapeutic agent for adjuvant chemotherapy in cancer patients as an immunomodulatory agent. The aim of this study is to determine the effect of black cumin seed hexane extract (BCSHE) as an immunomodulator in SD rat's peritoneal macrophages induced by DMBA or without DMBA. *In vitro* experimental study was performed on rat's peritoneal macrophages cultures induced by DMBA and without DMBA. BCSHE were administered to cultures in five different doses; 1, 5, 25, 125 and 625 μg/ml with control media, tween, and positive control thymoquinone. The test substance was added after the macrophages were activated and then measured by using latex phagocytosis method while ROI secretion assay was measured by using NBT assay method. Results showed that macrophage activation by BCSHE solution increased ROI secretory activity in both SD rat's macrophage induced by DMBA and without DMBA. BCSHE could increase phagocytic activity *in-vitro*. It can be concluded that BCSHE can increase ROI secretion and phagocytic activity on SD rat's peritoneal macrophages induced by DMBA and without DMBA.

Keywords: Black cumin seeds hexane extract, Dimethylbenzanthracene, Immunomodulator, Macrophages.

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Introduction

Thymoquinone is the primary active substance of black cumin (Nigella sativa) seeds which is used as traditional medicine to repair the immune system, enhance stamina, and as an anti-inflammatory. Black cumin seed extract inhibited the cyclooxygenase pathway and a 5-lipooxygenase in arachidonic metabolism on leukocytes of rat's peritoneal cavity [1]. Furthermore, Akrom et al. proved that black cumin seeds extract administration to Sprague-Dawley (SD) rats before and during dimethylbenzanthracene (DMBA) induction prevented mammary carcinogenesis, increased the number of leukocytes, and the viability of experimental animals [2]. ROI is one of the pleiotropic compounds. Macrophages are activated by pathogens secreted by ROI to kill bacteria. However, the excessive level of ROI become a reactive radical and disrupt the normal cells. DMBA compounds are known as carcinogens, immunosuppressive, and a source of reactive radicals. Other study reported that DMBA induction inhibited inducible nitric oxide synthase (iNOS) expression, NO, and interleukin 12 (IL-12) secretion by macrophages [3]. NFκB and CCAAT/enhancer binding protein (C/EBP) depletion alongside with a decrease in function and activity of rat's peritoneal macrophages induced by DMBA [3]. DMBA exposure on *in vivo* and *in-vitro* proven to reduce the activity of macrophages [4].

Administration of thymoquinone to the *in vitro* macrophage cell cultures activated Toll-Like Receptor 4 (TLR-4) through the activation of the sialidase NEU-4 enzyme. Administration of thymoquinone in rats increase levels of IL-1, TNFα, and

IL-6, even without induced LPS, it proved that thymoquinone increased phagocytic activity of macrophages and pro-inflammatory in macrophage cells [5]. Thymoquinone and unsaturated fatty acid compound in black cumin seed extract are assumed to have anti-oxidative and immunostimulatory activity which expected to activate antioxidant enzymes of rat's macrophages exposed by DMBA and impede the immunosuppressive properties [6]. Although black cumin seed is a potential immunomodulatory agent, including for people with cancer, currently there is no evidence how it influences ROI secretion activity of SD rat's *in vitro* DMBA induced macrophages. This study is to determine the effect of black cumin seed hexane extract (BCSHE) on ROI secretion activity of SD rats *in vitro* DMBA induced macrophages.

Materials and Methods

Identification, extraction and standardization of black cumin seed with thymoquinone

Black cumin seeds obtained from a certified traditional medicine sellers in Semarang were identified at the Pharmacy and Biology Laboratory, Gajah Mada University. Extraction was done through maceration method. Different level of solvent was applied as maceration strategy. 500 g of black cumin seeds powder was processed maceration with 1 L hexane solvent for 24 hours. Thus, it was stirred for 30 minutes and incubated overnight. The extract then was filtered using Buchner funnel to get the filtrate. This maceration process was done with three replication. Three filtrates then were mixed and evaporated in a rotary evaporator at 50°C up to get gel extract. Gel extract then was evaporated in disk plate using water bath. Thus, the crude extract was collected in the bottle [2]. Standardization of black cumin seed hexane extract (BCSHE) with thymoquinone was done by KLT-densitometry method to get the specific grade of thymoquinone in black cumin seed extract [7]. The assay of unsaturated and saturated fatty acid of BCSHE was done by Gas Chromatography/Mass Spectroscopy (GCMS). Measured parameters on a standardized black cumin seed hexane extract were the percentage of thymoquinone and unsaturated fatty acids. Standardization procedure of N. sativa as the extract is as described by earlier researchers [7].

Effectiveness test of black cumin seed hexane extracts (BCSHE) as immunomodulator

Female SD rats aged 3-4 weeks old, weighed around 100-140 g were obtained from the Biological Sciences Laboratory, Gajah Mada University. Experimental animals were kept in 50 × 30 × 20 cm metal cages individually, fed with pellet 529, and water moderately [2]. Parameters measured on immunomodulatory and anti-oxidative activity test was the ROI secretory activity of macrophages on healthy SD rats and DMBA-induced SD rats. ROI secretory activity of macrophages is determined by NBT assay [2]. All activities and treatments on experimental animals were performed in accordance with procedures approved by the ethics committee on Animal Testing Laboratory Gajah Mada University. Peritoneal macrophage was taken from 11/12 months SD rats.

DMBA induction and macrophage isolation: Oral administration of DMBA (1 × 15 mg) was given to SD rats [7]. After three days, 5 rats from each group was dissected by using narcosis chloroform. Rats were placed in supine position, the abdomen was opened and peritoneum sheath was cleaned by using 70% ethanol, then 10 ml of cold RPMI medium was injected into the peritoneal cavity, waited for 3 minutes and shook it slowly. Peritoneal fluid was removed from the peritoneal cavity by pressing the cavity with two fingers, the fluid was aspirated with a syringe injection, the lean and non-fatty part was selected. The aspirate was centrifuged at 1200 rpm, 4°C, for 10 min. The supernatant was discarded and the bottom sediment was taken, and then the pellet was re-suspended in RPMI-1640 (Gibco) (contained 10% FBS, 1 mM sodium Bicarbonate, 2 mM L-Glutamine, 100 µl Penicillin and 0.5 mg streptomycin, 95% ethanol, heparin, sodium oxalate, and distilled water). The number of cells was calculated using a hemocytometer and the cell viability was determined by trypan blue solution, and then added to a complete medium to obtain a cell suspension with a 2.5×10^6 cell/ml. Microculture suspension cells were grown in 24 wells and given round coverslips. Each of the wells was filled by 200 microliters (5×10^5 cells) and incubated in a 5% CO_2 incubator, 37°C, for 30 minutes. After that, it was added by 1 ml complete medium and incubated in a 5% CO2 incubator, 37°C for 2 hours. Cells were washed twice with 1 ml RPMI for each well and incubated in complete medium for 24 hours.

Black cumin seed hexane extract (BCSHE) to macrophage cultures: Black cumin seed hexane extract (BCSHE) was dissolved in DMSO, vortex and sonicated for homogenisation before given to macrophage culture. Black cumin seed extract with five different concentrations (1, 5, 25, 125 and 625 µg/ml) were added in macrophage cultures, 5 µg/ml thymoquinone was added in positive control group macrophage cultures while the media control group got an additional growth media. It was incubated for 2 hours in a 5% CO_2 incubator, at 37°C. After that, the cells were washed twice with 1 ml RPMI each well and then phagocytic activity and the secretion of ROI examinations was done [2]. ROI secretion activity test using NBT assay: ROI secretion by peritoneal macrophage was measured by NBT reduction assay [8]. To induce superoxide anion secretion, macrophage cell cultures were stimulated with Phorbol Mystrate Acetate (PMA) 125 ng/ml final concentration.

Macrophages that was cultured for 24 hours were washed twice with RPMI and 500 µl 1 mg/ml NBT and 125 ng/ml PMA in PBS, incubated in 5% CO_2 incubator, 37°C, for 60 minutes. Subsequently, cells were washed with PBS three times, dried at room temperature, and fixated with absolute methanol for 30 seconds, and then dyed with 2% neutral red solution. The percentage of macrophage cells that showed NBT reduction was calculated using light microscopy with magnification 400X [2]. Phagocytic activity test using latex assay: Phagocytic analysis was done using by direct fixation

procedure in microplates. Macrophages that was incubated in 5% CO_2 incubator, 37°C, for 60 minutes. The cells were washed with PBS three times, dried at room temperature, and fixated with absolute methanol for 30 seconds. Then dyed with 20% Giemsa. The observation was done under the microscope with 400X magnification. Macrophage cells indicated with blue color. Percentage of phagocytic activity was measured minimum 100 observed cells. Phagocytic activity indicated by macrophage that phagocyte latex particles [7].

Data analysis

Data of ROI secretion was presented descriptively by stating the number, percentage, and average of each descriptive parameter but not followed by statistical analysis because the measurement was only performed twice.

Results

Determination, extraction, and standardization of black cumin seed

The result of maceration with hexane followed by distillation was clear yellow extract with fairly thin viscosity and distinctive volatile odor while the distillation method resulted in the yellowish brown extract with fairly thin viscosity and a distinctive volatile odor. Results of standardization of black cumin seed ethanol extract are presented in Table 1. Most of black cumin seed extract contents is vaporized extract (essential extract) and un-vaporized extract (fatty extracts).

Table 1. *The content of black cumin seed extract set with TLC for thymoquinone and GCMS for unsaturated fatty extract.*

Content of black cumin seed	N	Crude extract market	Black Cumin Seed Hexane Extract
Thymoquinone	3	2.72 ± 0.02	2.72 ± 0.03
Fatty acid	3	69.33 ± 1.16	78.00 ± 3.00
Caproic acid	3	0.21 ± 0.01	0.01 ± 0.00
Capric acid	3	0.06 ± 0.08	0.05 ± 0.00
Lauric acid	3	0.04 ± 0.05	0.02 ± 0.00
Myristic acid	3	0.18 ± 0.01	0.20 ± 0.00
Palmitate	3	12.28 ± 0.01	12.86 ± 0.08
Palmitoleic	3	0.29 ± 0.01	0.23 ± 0.01
Stearate	3	79.98 ± 0.02	11.30 ± 0.44
Oleic	3	0.07 ± 0.00	69.67 ± 0.68
Linoleic	3	2.86 ± 0.02	3.44 ± 0.09
Linolenic	3	0.11 ± 0.02	2.75 ± 0.10

More than 60% was fatty extract and less than 5% essential extract. Fatty extract of black cumin seed extract consisted of unsaturated fat and saturated fat. Most of the fatty acid was fatty extract macerated with hexane. Meanwhile, there were 74% distilled unsaturated fatty acid and 24% saturated fatty acid. The unsaturated fatty acids in the essential extract were: palmitoleic, oleic, linoleic and linolenic. Test results showed that maceration method with hexane, followed by steam distillation produced more favorable extract than the other methods.

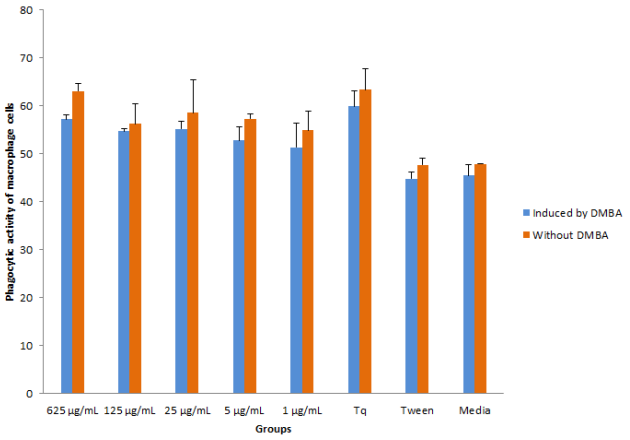

Figure 1. *Percentage of ROI secretory activity on SD rat peritoneal macrophages cultures induced by DMBA and with the administration of black cumin seed hexane extract.*

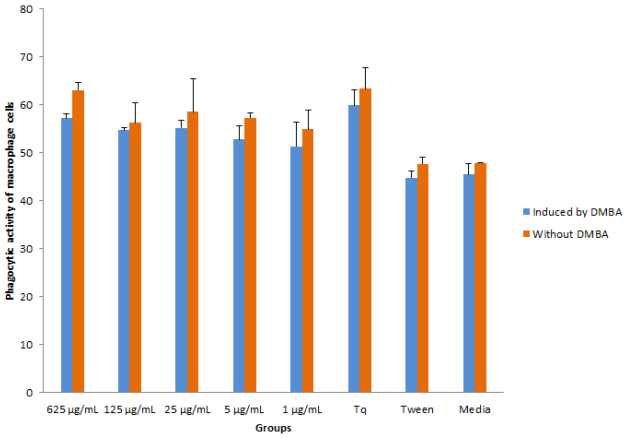

Figure 2. *Percentage of macrophage phagocytic activity on SD rat peritoneal macrophages cultures induced by DMBA and with the administration of black cumin seed hexane extract.*

Activity test of black cumin seed hexane extract against ROI secretory activity in macrophages

ROI secretion activity of SD rat macrophages induced by DMBA is presented in Figure 1, including the ROI secretory activity on SD rat peritoneal macrophages culture induced by DMBA or without DMBA with the administration of black cumin seed hexane extract. ROI secretory activity of SD rat peritoneal macrophages without DMBA was higher than those of induced by DMBA. Results showed that black cumin seed hexane extract (BCSHE) increased ROI secretion activity in peritoneal macrophages of SD rat either induced by DMBA or normal. It was higher than the phagocytic and ROI secretion activity of SD rat peritoneal macrophages induced by DMBA.

It means that BCSHE extract enhanced the ROI secretion activity peritoneal macrophages induced by DMBA or not.

Activity test of black cumin seed hexane extract against macrophage phagocytic activity

Phagocytic activity of SD rat macrophages induced by DMBA is showed in Figure 2, including the phagocytic activity on SD rat peritoneal macrophages culture induced by DMBA or without DMBA with the administration of black cumin seed hexane extract. The result showed that BCSHE could increase phagocytic activity *in-vitro*. Phagocytic activity of SD rat peritoneal macrophages without DMBA was higher than those of induced by DMBA. In general, BCSHE extract improve the phagocytic activity of macrophages induced by DMBA or not.

Discussion

The extraction methods and solvent substances determined the bioactive compound that can be extracted from black cumin seed hexane extract (BCSHE). Maceration method with hexane solvent followed by steam distillation produced an essential extract containing thymoquinone with the highest percentage of unsaturated fatty acid as 2.72% and the lowest percentage of unsaturated fatty acid was 24%. It also had 69% oleic acid. The previous study reported that the fatty extract of BCSHE mostly contains unsaturated fatty acids [9]. In addition, it also consists of triacylglycerol structure with typical triasil glycerol consists of 3 linolenic acid (LLL), 2 linolenic acid and 1 linoleic acid (LLO), 2 linolenic acid and one of palmitic acid (LLP), 1 linolenic acid and 2 linoleic acid (LOO) and 1 linolenic acid, 1 linoleic acid and 1 palmitic acid (LOP), which has a beneficial pharmacological effect to health. It also rich in sitosterol with beta-sitosterol as the main content. In 100 g of BCSHE contains 1 g of sterol, consist of 59.1% b-sitosterol, 16.5% stigmasterol, 14.4% isofucosterol and 10% kaempsterol.

The previous researcher has reported that the largest sitosterol content of BCSHE in Iran and Tunisia is beta-sitosterol (44-54%) and stigmasterol (11-20%) [6].

Sitosterol is a collection of plant sterol compounds that has various beneficial biological effects for health including antioxidant, antidiabetic, anti-hypercholesterolemia, and chemo-preventive [10,11]. B-sitosterol showed the chemopreventive effect on colon cancer by inhibiting lipid peroxidation in mice induced dimetilhidrazin [10,12]. Paniagua-Pérez (2008) revealed that it also protected cell damage from genotoxic and stimulate lymphocyte activity [13]. Six percents of black cumin seed essential extract is a ketone group monoterpenoid, consisting of karfon (4%), fenkon (1.1%), thymoquinone (0.6%) and dihidrokarfon (0.3%). The content of black cumin seed essential extract is nonterpenoid hydrocarbon group (4%), hydrocarbon monoterpenoid (26%), alcohol monoterpenoid (2.7%), hydrocarbon sesquiterpenoids (1%) and phenylpropanoid compound (64.1%) [14]. Although the content of thymoquinone in the black cumin seed volatile extract is low, it determines the pharmacodynamic effect of black cumin seed extract.

Nigellone is polymerized form of thymoquinone that can inhibit enzyme cyclooxygenase and lipoxygenase activity on the arachidonic metabolism, thus, might be used as an analgesic, anti-inflammatory and anticancer [15-17].

Macrophages are phagocytes that act as antigen presenting cell (APC) and effectors on natural and adaptive cellular immune response [18]. Research data showed that DMBA induction reduced the number and activity of peritoneal macrophages, phagocytic activity, and ROI secretion. In line with the results of this study, Torroella-Kouri et al. reported that DMBA-induced mice have a lower amount, less active and less reactive peritoneal macrophages [3]. It was associated with low expression of NFκB transcription factor and low level of E2 prostaglandin [3]. Exposure to DMBA has shown to be immunosuppressive and caused toxicity to the bone marrow [19], decreased the number of lymphocytes and inhibited spleen proliferation [20], iNOS and NO production by macrophages, and immunotoxic to macrophages [21].

Aside from thymoquinone, some unsaturated fatty acids such as sitosterol and terpenoids have also shown to increase the activity of macrophage phagocytosis and ROI secretion, which was exposed by xenobiotic. This is done through a cytoprotective mechanism by increasing the secretion of glutathione antioxidant by glutathione S-transferase (GST) due to the activation of the antioxidant responsive element (ARE). Results showed that the hexane extracts of black cumin seeds suppressed the activity of *CYP* genes and stimulated the activity of *GST* genes and enzymes.

Previous studies proved that black cumin seed extract served as a stimulant of phase II enzymes, both *in vitro* and *in vivo* assays [22,23]. Black cumin seed extract and thymoquinone serve as chemo-preventive through cytoprotective antioxidant effects by suppressing the activity of *CYP* genes (phase I) and increasing the activity of *GST* genes (Phase II) through Nrf2 activation, resulting in an increased production of GST enzyme for the detoxification process. *Nrf2* gene is a gene that is responsible for oxidative stress, along with ARE [24,25].

Conclusion

Black cumin seed hexane extract (BCSHE) contained thymoquinone and unsaturated fatty acids. Hexane improves ROI secretion activity of SD rat's peritoneal macrophages either DMBA-induced or without DMBA. Further research to assess the effectiveness of hexane as an *in vivo* antihepatotoxic immunomodulator along with acute and sub-chronic toxicity test is necessary to be done as a foothold for the development of drugs administration.

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