

Antiproliferation Activity of *Nigella Sativa* on 7,12dimethylbenz[A] Anthracene Induced Carcinogenesis in Female Mice Breast

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Abstract

Breast cancer is the leading cause of death in the world. *Nigella sativa* is one of the plants that has anticancer activity. Previous study reported *Nigella sativa* has cytotoxic activity in T47D cell. This study aimed at observing the effect of chloroform extract of *Nigella sativa* seed (NSS) on mice breast cell after initiation of 7,12-dimethylbenz [a] anthracene. Sprague Dawley strain female rats were divided into five groups. Each group consisted of 12 mice. The experiment consisted of five mice groups, corn oil solvent control group, the DMBA dose 20 mg/kgBW p.o. twice a week during five weeks, DMBA+NSS dose 250 mg/kgBW, DMBA+NSS dose 500 mg/kgBW and DMBA+NSS dose 750 mg/kgBW. Extract dissolved in corn oil was administered daily by the oral route 1 week before and during the DMBA induction. At the end of the study, mice was sacrificed and breast organs were collected and stained with Haematoxylin and Eosin (H&E) and AgNOR methods. H&E staining showed that there were no histopathology changes in treated group compared with DMBA group. NSS was able to suppress breast cancer cell proliferation activity. mAgNOR value on DMBA control group, dose 250, 500, 750 mg/Kg BW NSS+DMBA and corn oil were 2.14 ± 0.33 ; 1.47 ± 0.55 ; 0.83 ± 0.24 ; 0.33 ± 0.23 ; 0.22 ± 0.19 , respectively. The results suggested that *N. sativa* can be developed into chemopreventive agent for breast cancer.

Keywords : Breast cancer, *Nigella sativa*, DMBA, Antiproliferation, AgNOR

INTRODUCTION

Cancer is a major health problem in the world. Cancer became the second highest cause of death in the United States after heart disease. The American Cancer Society estimates, more than 1.4 million cases of cancer occurred in 2005, which led to the deaths of more than 570,000 people (Rugo, 2006). In 2005, breast cancer ranked first with a high incidence of cancers in Indonesia, namely 16.9% of inpatients and the other 22.33% of outpatients (Anonymous, 2007).

Several methods for mammary cancer therapy such as surgery, chemo-

therapy, hormonal therapy, and radio-therapy had been established. Exploration of natural products is going on to find chemopreventive agent to be an attempt to reduce the number of cancer patients. Chemoprevention is defined as the use of substances of natural origin, phyto-chemical agents, synthetic, or chemical compounds to prevent or suppress cancer progression, reverse to normal physiological functions and to perform early detection of pathological cancer conditions. One plant that is empirically believed by the public as a chemopreventive agent is black cumin (*Nigella. sativa*).

N. sativa content in the form of oil is 95.5% fatty acids. The main components of these fats are linoleic acid, oleic acid, and

palmitic acid that have anticancer activity (Nickavar, 2003). Chloroform extract of black cumin have cytotoxic activity against T47D cells with IC₅₀ of 124.206 µg/ml (Ekowati *et al.*, 2011) *N. sativa* suppress the proliferation of human cervical carcinoma cells (Hela), hepatocellular carcinoma (HepG2), squamous cell carcinoma (SCC), fibrosarcoma (FsaR), colorectal carcinoma, breast adenocarcinoma, osteosarcoma, ovarian carcinoma, myeloblastic leukemia, dan pancreatic carcinoma (Yazan *et al.*, 2008; Hasan *et al.*, 2008; Ivancovic *et al.*, 2006; Aggarwal *et al.*, 2008). Our research was conducted to observed the activity of NSS extract on 7,12dimethylbenz [a] anthracene-induced carcinogenesis in mice. In this experiment we would like to explore the effect of NSS based on the histopathology staining.

MATERIAL AND METHODS

Materials

The chloroform extract of *Nigella sativa seed*, DMBA (7,12dimethylbenz[a]anthracene) (Sigma Chem., Steinherm), corn oil as a solvent of DMBA and solvent of extracts, buffer formalin 10 %, paraffin, Histological preparations dye (Hematoxylin and Eosin) and silver nitrate (AgNO₃) for AgNOR staining.

Animals

Sprague Dawley female mice (30 days old) weighed from 100 to 150 g were obtained from the Laboratory of Pharmacology and Toxicology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. The mice were kept for at least one week before use, and were given standard pellet diet and water *ad libitum*, and kept on a 12:12 h light/dark cycle.

Sixty mice were divided into five groups (12 mice per group). DMBA

control (group A) was administered with DMBA (DMBA was dissolved in corn oil), groups of treatment (groups B, C and D) intended for carcinogen were treated with single oral dose of DMBA (Sigma) (20 mg/kgBW), twice a week for 5 weeks. DMBA was dissolved in corn oil and NSS with 250 mg/kgBW, 500 mg/kgBW, 750 mg/kgBW dose. Group 2, 3, and 4 were administered orally one a day by NSS, respectively for 7 weeks, started 2 weeks before DMBA initiation. Solvent control (group 5) was administered with corn oil. Body weight was recorded weekly throughout the study.

H&E staining

At the end of the experiment (16 week) all mice were sacrificed. At autopsy, breast organ were removed and fixed in 10% buffered formalin. After 12-24 h fixation, 3-5 µm tissue slices were embedded in paraffin, and stained with hematoxylin and eosin for microscopy. H&E staining was used to observe histopathology changes in mice breast cells (Nisa, 2012).

AgNOR staining

The staining solution was prepared by mixing one part of 2% gelatin in 1% formic acid with two parts of 50% aqueous silver nitrate. All sections were cut to 3 µm in thickness from routinely processed paraffin blocks. Sections were immersed in sodium citrate buffered and incubated 20 min in autoclave. Sections then were covered with the AgNOR staining solution at room temperature in the dark for 15-20 min. The specimens were then washed with 5% sodium thiosulfate and distilled deionized water, dehydrated through graded ethanol to xylene, and mounted in synthetic medium.

AgNOR which appeared as black-dots were counted. Mean AgNOR was used as a parameter to evaluate antiproliferative activity. mAgNOR is a mean blackdots in a cell, counted from total number of blackdots divided with number of minimal 100 cells.

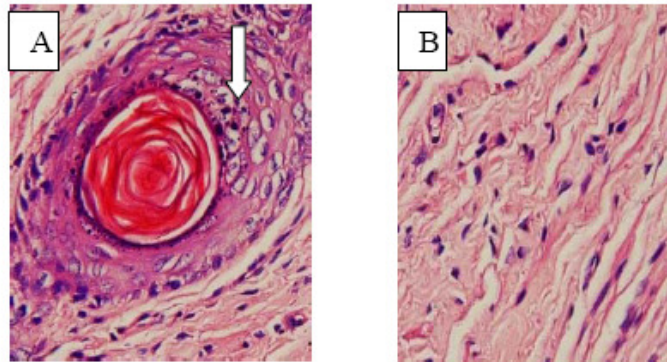


Figure 1. H&E staining. A: DMBA control group (20 mg/kgBW in corn oil) the white arrow showed pearl carcinomas, B : normal breast cells (in all groups except DMBA group). At autopsy, breast organ were removed and fixed in 10% buffered formalin. 3-5 μ m tissue slices were embedded in paraffin, and stained with hematoxylin and eosin Magnification x 600.

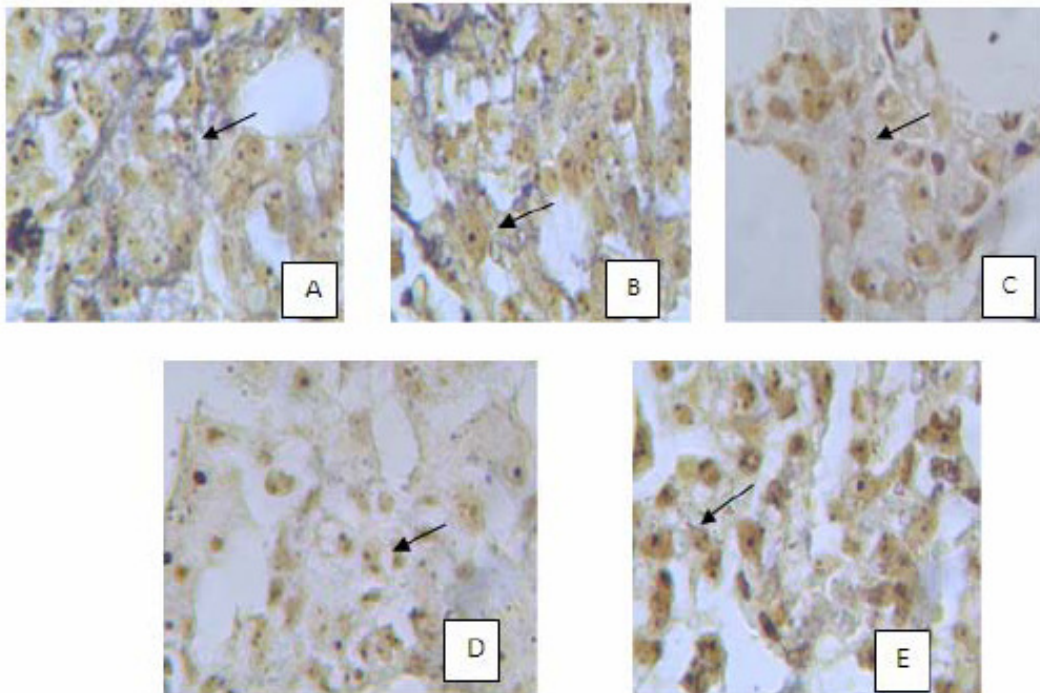


Figure 2. AgNOR staining. Mice were divided into 5 groups, (A) Corn oil, (B) DMBA control group (20 mg/kgBW in Corn oil), (C) DMBA+ 250 mg/kgBW NSS; (D) DMBA+500 mg/kgBW NSS, (E) DMBA+750 mg/kgBW NSS. At autopsy, breast organ were removed and fixed in 10% buffered formalin. 3-5 μ m tissue slices were embedded in paraffin, and stained with AgNO₃. Argyrophilic nucleolar organiser regions (AgNORs) are visible as dark dots within the plasma cell nuclei (pointed by black arrow). Magnification x100.

All specimens were observed on a binocular microscope (Olympus® DP12 microscope digital camera system, NY) with an immersion oil lens at magnification of x1000 (Nisa, 2012).

Statistical analysis

A statistically significant difference in average of black dot (mAgNOR) was evaluated by ANOVA continued with HSD. P < 0.05 between group was inferred as statistically significant using SPSS.

Table 1. mAgNOR = mean AgNOR score (average±SD) on breast cell mice

| No | Group | mAgNOR(Mean ± SD) |
|----|------------------------|-------------------|
| 1 | DMBA | 2,147±0.33 |
| 2 | DMBA + NSS 250 mg/KgBW | 1,103±0.11* |
| 3 | DMBA + NSS 500 mg/KgBW | 0,837±0,24* |
| 4 | DMBA + NSS 750 mg/KgBW | 0,337±0,23* |
| 5 | Corn oil | 0,227±0,19* |

*mAgNOR are statistically significant different from DMBA groups ($p < 0.05$) by *one way Anova* continued with *Tukey HSD*. (1) DMBA control group; (2) DMBA+250 mg/kgBW NSS; (3) DMBA+500 mg/kgBW NSS; (4) DMBA+750 mg/kgBW NSS; (5) corn oil.. The highest proliferation seen in the DMBA 20 mg/kg mice group and then was significantly decrease in group extract 750 mg/kg and corn oil mice group.

RESULTS AND DISCUSSION

Observation was terminated at 16 week after the last DMBA initiation. Then a necropsy was performed on mice. The histopatological appearance of mammary tumors in DMBA and DMBA+NSS-treated mice was depicted in figure 1. H&E staining showed carcinoma cells in DMBA group. Treatment groups and corn oil group did not showed carcinoma cells and changes in histopathology cell.

AgNOR staining on breast organ (figure 2) showed that DMBA group has higher blackdots than the other groups, with mAgNOR score (table 1) of corn oil and DMBA are 1.26 ± 0.125 and 1.63 ± 0.058 respectively. Treat-ment of extract NNS 750 mg/kgbw represented significant antiproliferative activity of DMBA with mAgNOR score 1.39 ± 0.049 mg/kgbw. While treatment extract *N. sativa* seed 250 mg/kgbw and 500 mg/kgbw showed no significance to DMBA group, with mAgNOR score 1.62 ± 0.086 mg/kgbw and 1.60 ± 0.101 mg/kgbw. The data showed mAgNOR score was contrary with extract concentration of NSS extract. The higher dose of NSS extract, the lower proliferative activity of the extract.

The results showed that the chloroform extract of *N. sativa* was able to reduce the activity of cell proliferation on DMBA-induced breast mice. mAgNOR value can be seen in table 1.

This study explored the effect of *N. sativa* seeds chloroform extract on DMBA induced female rats breast. In this study, NSS was given two weeks before and

during induction of DMBA. According Kubatka *et al.* (2002) induction of mammary carcino-genesis in female rats is influenced by age, strain, dose and timing of carcinogens, the pattern of the immune system, endocrine system and feed status. Therefore in this study we used Sprague Dawley strain female rats, which are more sensitive than Wistar to mammary tumor formation, with the age of 1.5 months, which is right for the induction of mammary carcinogenesis because it is at the beginning of puberty (Kubatka *et al.*, 2002; Singletary *et al.*, 1998). NSS treatment was intended to prevent metabolic activation of DMBA and also prevent initiation and progression of breast cancer. The results of mAgNOR showed that the chloroform extract *N. sativa* was able to reduce the activity of cell proliferation in DMBA-induced mice breast by Table 1 and Figure 2. It showed that the rate of cell proliferation in mice breast in DMBA control group was the highest, while the lowest was that of solvent corn oil group. Cellular proliferation requires an orderly progression through the cell cycle, primarily driven by protein complexes composed of cyclins and cyclin-dependent kinases (Cdks). Progression through the G1-S transition requires the activity of at least two different types of kinases, cyclin D-Cdk4/6 and cyclin E/A-Cdk2. *N. sativa* decrease the expression of cyclin D1 (Aggarwal *et al.*, 2008) also inhibit cell proliferation and

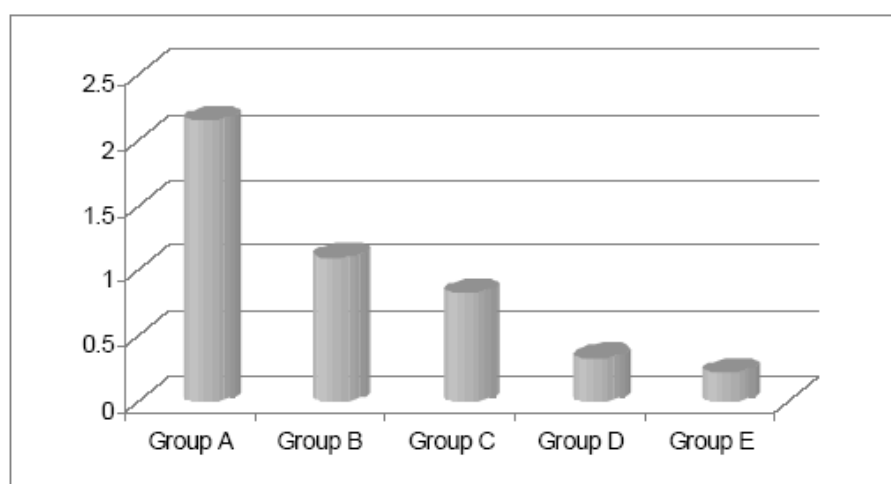


Figure 3. Effects of NSS extract against breast cell proliferation. A. DMBA 20 mg/kg, B. Extract 250 mg/kg + DMBA 20 mg/kg, C. Extract 500 mg/kg + DMBA 20 mg/kg, D. Extract 750 mg/kg + DMBA 20 mg/kg, E. Corn oil. The extract have the most antiproliferation activity at concentration 750 mg/kg BW with mAgNOR score 0.33 ± 0.23 .

causes cell cycle arrest (Ilaiyaraja and Khanum, 2010).

Tumor cells evolve a variety of strategies to limit or circumvent apoptosis. Most common is the loss of TP53 tumor suppressor function. Tumors may achieve similar ends by increasing expression of antiapoptotic regulators (Bcl-2, Bcl-xL) or of survival signals (Igf1/2), by down-regulating proapoptotic factors (Bax, Bim, Puma), or by short-circuiting the extrinsic ligand-induced death pathway (Hanahan and Weinberg, 2011). *N. sativa* stimulates apoptosis through increased expression of p53 (Yazan *et al.*, 2009; Ilaiyaraja and Khanum, 2010) and inhibits anti-apoptotic protein (Ivankovic *et al.*, 2006).

7,12-dimethylbenz [a] anthracene (DMBA) is one of Polycyclic Aromatic Hydrocarbons carcinogenic compounds (PAHs) which is metabolized by cytochrome P450 (Rajapaksa *et al.*, 2006). DMBA is a substrate of the enzyme cytochrome P450 (CYP), CYP1A1 and CYP1B1 [14]. In the phase I metabolism, DMBA is changed to 8.9-; 5.6-; 3,4-epoxide DMBA by CYP1A1 and 3,4-epoxide DMBA by CYP1B1 [15;16]. DMBA can enhance the production of

reactive oxygen species (ROS). DMBA can also decrease the body's antioxidant levels. This led to a radical attack can not be neutralized by the body. This will cause cell damage and cell necrosis (Patri *et al.*, 2009). *N. sativa* is a plant that has antioxidant activity so as to prevent the body from oxidative stress (Gilani *et al.*, 2004).

N. sativa can stimulates the expression of glutathione S-transferase (Ilaiyaraja and Khanum, 2010) which will conjugates DMBA compounds to prevent DMBA from binding with DNA, RNA or protein (Murray *et al.*, 2006). Glutathione S-transferase (GST) is a phase II metabolic enzyme, detoxifies carcinogens and facilitates their excretion by promoting the conjugation of electrophilic compounds with glutathione. GST deactivates and protects the surrounding tissues from mutagenesis and carcinogenesis. Most of GST inducers affect the activity of GST gene trans-cription via the Antioxidant Responsive Element (ARE), Xenobiotik Responsive Element (XRE), GSTP Enhancer 1 (GPE) or Glucocorticoid Responsive Element (GRE). The result

showed that *N. sativa* has the potential to prevent and treat breast cancer.

CONCLUSION

Nigella sativa seed extract was able to reduce the breast cell damage and proliferation. This study indicated that *Nigella sativa* can be develop into chemopreventive agent for breast cancer.

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