

## Protective effects of *Nigella sativa* against 7,12-dimethylbenz [á] anthracene (DMBA) induced carcinogenesis in rats

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### ABSTRACT

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#### BACKGROUND

Liver cancer is the third most common cause of death from cancer worldwide. Recently, natural products have been widely used as an alternative therapy for liver cancer. Previous studies have reported that *Nigella sativa* has chemopreventive activity *in vitro* and *in vivo*. The objective of this study was to evaluate the effect of a chloroform extract of *Nigella sativa* seeds (NSS) on female rat hepatocytes after administration of 7,12-dimethylbenz [á] anthracene (DMBA).

#### METHODS

The experimental design comprised five groups of rats. Group I (DMBA control group) received oral DMBA at a dosage of 20 mg/kgBW twice weekly for five weeks, while group V (solvent control group) was given corn oil only. The other three groups received DMBA + NSS at dosages of 250 mg/kgBW, 500 mg/kgBW, and 750 mg/kgBW, respectively. Each group consisted 12 rats. The NSS extract dissolved in corn oil was administered daily by the oral route for 2 weeks before and subsequently during DMBA tumor induction. At the end of the study, rat livers were collected and stained with hematoxylin and eosin (H&E) and silver staining by the the AgNOR method.

#### RESULTS

There was a difference in liver tissue histopathological profile between the NSS, DMBA control, and the solvent control group. AgNOR counts in the DMBA control group, the DMBA+NSS 250 mg/kgBW group, DMBA+NSS 500 mg/kgBW group, and DMBA+NSS 750 mg/kgBW group were 1.79, 1.51, 1.41, and 1.35, respectively.

#### CONCLUSION

*Nigella sativa* seed extract was able to reduce the liver damage and proliferation in rats induced by DMBA administration.

**Keywords:** *Nigella sativa*, liver, DMBA, cancer, female rats

## ***Efek protektif *Nigella sativa* terhadap karsinogenesis yang diinduksi 7,12-dimetilbenz [á] antrasena (DMBA) pada tikus***

### **ABSTRAK**

#### **LATAR BELAKANG**

Kanker hati merupakan penyebab ketiga kematian di dunia yang disebabkan kanker. Produk bahan alam digunakan secara luas akhir-akhir ini sebagai terapi alternatif untuk kanker hati. Penelitian sebelumnya melaporkan bahwa *Nigella sativa* mempunyai aktivitas kemopreventif *in vitro* dan *in vivo*. Penelitian ini bertujuan untuk menilai efek dari ekstrak kloroform biji *N. sativa* (NSS) terhadap sel hepar tikus betina setelah diinisiasi dengan 7,12-dimetilbenz(á)antrasene (DMBA).

#### **METODE**

Sebuah rancangan eksperimental mengikutsertakan lima kelompok tikus. Grup I adalah kelompok kontrol DMBA yang diberi DMBA oral dengan dosis 20 mg/kgBB dua kali seminggu selama lima minggu,, sedangkan grup V adalah kelompok kontrol pelarut yang diberi minyak jagung semata. Ketiga grup lainnya diberi DMBA + NSS, dengan dosis masing-masing 250 mg/kgBB, 500 mg/kgBB dan 750 mg/kgBB. Setiap grup terdiri dari 12 ekor tikus. Ekstrak NSS yang dilarutkan dalam minyak jagung diberikan tiap hari dengan rute peroral dua minggu sebelum dan selanjutnya selama inisiasi DMBA. Pada akhir percobaan, tikus dinekropsi dan dilakukan pengecatan hematoksin eosin (H&E) dan perak dengan metode AgNOR.

#### **HASIL**

Terdapat perbedaan pada profil histopatologi jaringan hati antara grup perlakuan ekstrak, grup DMBA dan grup minyak jagung. Hasil pewarnaan AgNOR menunjukkan bahwa pemberian NSS dapat menghambat proliferasi sel hepar pada kanker hati pada tikus betina yang diinduksi DMBA. Nilai rerata AgNOR pada grup kontrol DMBA; DMBA+NSS 250 mg/kgBB, 500 mg/kgBB dan 750 mg/kgBB berturut-turut adalah 1,79; 1,51; 1,41 dan 1,35.

#### **KESIMPULAN**

Penelitian ini mengindikasikan bahwa *N. sativa* dapat mengurangi kerusakan dan proliferasi hati pada kanker hati yang diinduksi DMBA.

**Kata kunci:** *Nigella sativa*, hati, DMBA, kanker, tikus

## **INTRODUCTION**

Cancer is the second most common cause of death in the United States,<sup>(1)</sup> and liver cancer is the third most common cause of death from cancer worldwide. Liver cancer is extremely difficult to treat and the overall 5-year survival rate (for all stages) is only about 7% in the United States and even lower in developing countries.<sup>(2)</sup> Several methods for liver cancer therapy such as surgery, chemotherapy, hormonal therapy, and radiotherapy have been established. However,

there are no recognized methods for prevention of liver cancer, and only recently has there been exploration of natural products to find chemopreventive agents, in an attempt to reduce the number of cancer patients. Chemoprevention is defined as the use of substances of natural origin, phytochemical agents, synthetic, or chemical compounds to prevent cancer or suppress cancer progression, effect a reversal to normal physiological functions, and perform early detection of pathological cancer conditions.<sup>(3)</sup> One of the medicinal plants that

has been extensively investigated is black cumin (*Nigella sativa* seeds), which contain fixed and essential (volatile) oils, that presumably have anticancer activity. The main components of the fixed oil are linoleic acid, oleic acid, and palmitic acid.<sup>(4)</sup> The effects of *N. sativa* have been evaluated in animal studies as well as *in vitro*. Chloroform extracts of black cumin have cytotoxic activity against T47D cells with IC<sub>50</sub> of 124.21 µg/mL.<sup>(5)</sup>

There are many reports in the literature on the biological activities of *N. sativa*, including immunopotentiating, anti-inflammatory, analgesic, antihypertensive, antiulcerogenic, respiratory stimulation, antibacterial, antifungal, anticestode, antinematode, antiglycemic, antitumor, and anticancer effects.<sup>(6-10)</sup>

An experimental study showed that orally administered *N. sativa* had hepatoprotective effects against dimethylaminoazobenzene (DAB)-induced cholangiocarcinoma in male Swiss albino mice.<sup>(11)</sup> *N. sativa* essential oils injected into tumors grafted in DBA2 mice presumably showed either antimetastatic activity, or an inhibitory or delaying effect on metastasis through rapid reduction of primary tumor volume at the site of induction.<sup>(12)</sup> Another study indicated that an ethanol extract of *N. sativa* effected a significant decrease in cell proliferation, DNA synthesis, mitosis and the extension of the percentage of live rats.<sup>(13)</sup> The administration of NS reduced the carcinogenic effects of DMBA in skin carcinoma, suggesting a protective effect.<sup>(14)</sup>

Cancers have a high proliferation rate that is positively related to the amount of argyrophilic proteins (AgNOR proteins) in the nucleolar organizer regions (NORs), which are DNA loops located on acrocentric chromosomes responsible for their synthesis. AgNOR proteins are visualized as black-brown dots by their binding to silver nitrate according the AgNOR method. A higher AgNOR count indicates a correspondingly higher proliferation rate (as in

cancer cells), and conversely, a lower proliferation rate (as in normal cells) is shown by a low AgNOR count.<sup>(15)</sup> The objective of our study was to evaluate the activity of a chloroform extract of NS seeds (NSS) on 7,12-dimethylbenz [á] anthracene (DMBA)-induced liver carcinogenesis in rats by examination of histopathologic sections stained with hematoxylin-eosin and with silver-staining by the AgNOR method.

## METHODS

### Animals

Thirty-day old female Sprague Dawley rats weighing from 100 to 150 g were obtained from the Laboratory of Pharmacology and Toxicology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia. Prior to the experiments, the rats were fed a standard pellet diet and water *ad libitum*, and acclimatized to a 12 h light-dark cycle. The study was conducted from March – October 2011.

### Study design

A total of 60 female rats was randomized into 5 groups (control and experimental groups) of twelve rats each. The minimum group size as derived from the formula  $(t-1)(r-1) \geq 15$ , where  $t$  = number of treatments and  $r$  = number of rats, was 4 rats per group. However, in this study we used 12 rats in each group. Group I (DMBA control group) received DMBA dissolved in corn oil (SIGMA) at a dosage of 20 mg/kg BW for 5 weeks. In group II, III and IV, two weeks before DMBA initiation, the animals were given a single oral dose of NSS daily at a dosage of 250 mg/kg BW, 500 mg/kg BW, and 750 mg/kg BW, respectively, followed by DMBA administration at a dosage of 20 mg/kg BW for 5 weeks. Group V (solvent control group) received corn oil for 5 weeks. After 16 weeks, all animals were sacrificed by ether as scheduled. Body weights of the animals were recorded weekly throughout the study.

### Histopathologic examination

At autopsy, the rat livers were removed and fixed in 10% buffered formalin. After 12–24 h of fixation, paraffin-embedded 3–5 µm sections were stained with hematoxylin-eosin (H&E) and AgNOR for histopathologic examination. Mean AgNOR scores were calculated by counting at least 100 cells per paraffin section at a magnification of 1000 x.

### Ethical clearance

Animals were handled according to the rules and regulations of the Animal Care Committee of the Faculty of Medicine, Gadjah Mada University.

### Statistical analysis

A statistically significant difference in the mean number of black dots (mean AgNOR score) was evaluated by one way ANOVA, continued with Tukey HSD. A p value of <0.05 between groups was considered statistically significant using SPSS.

## RESULTS

This study showed that the NS chloroform extract was capable of reducing cellular proliferation in DMBA-induced liver cancers in rats. As may be seen from Table 1, the mean AgNOR values in the five groups were statistically significantly different ( $p < 0.05$ ) by one way Anova continued with Tukey HSD. The cellular proliferation rate was found to be highest in the DMBA control group and to be

significantly decreased in the rats of group IV receiving 750 mg/kgBW NSS extract and corn oil. The NSS extract had an optimal antiproliferation activity at a concentration of 750 mg/kg with a mean AgNOR value of  $1.35 \pm 0.038$ .

Anova was followed by Tukey HSD to differentiate between groups, and the results showed significant differences in mean AgNOR between NSS extract groups (at dosages of 250 mg/kg, 500 mg/kg, and 750 mg/kg) and DMBA 20 mg/kg. By contrast, mean AgNOR values of the NSS extract groups and the corn oil control group showed no significant difference. Observation was terminated at 8 weeks after the last DMBA initiation. Macroscopic observation showed morphological differences of the livers in the five groups. The livers from the DMBA control group (group I) were nodular in appearance, while the livers in groups II-V did not show any nodules.

The histopathological appearance of liver tumors in DMBA and DMBA+NS treated rats are depicted in Figure 1. H&E staining showed necrosis (Figure 2). Treatment with corn oil did not induce cancer in the livers of the rats. The AgNORs appeared as intranuclear black dots (Figure 3).

## DISCUSSION

This study explored the effect of NS seed chloroform extract on DMBA-induced liver cancers in female rats. NSS extract was given two weeks before and also during tumor

Table 1. mAgNOR value of *N. sativa* on proliferation of DMBA-induced liver female rats

Group	AgNOR
DMBA 20mg/kg	1.79 ± 0.175
Extract NSS 250 mg/kg + DMBA 20 mg/kg	1.51 ± 0.031*
Extract NSS 500 mg/kg + DMBA 20 mg/kg	1.41 ± 0.055*
Extract NSS 750 mg/kg + DMBA 20 mg/kg	1.35 ± 0.038*
Corn oil	1.24 ± 0.047

mAgNOR=mean AgNOR; DMBA=7,12-dimethylbenz[*a*]anthracene; values are means ± SD; \* $p < 0.05$  compared with DMBA

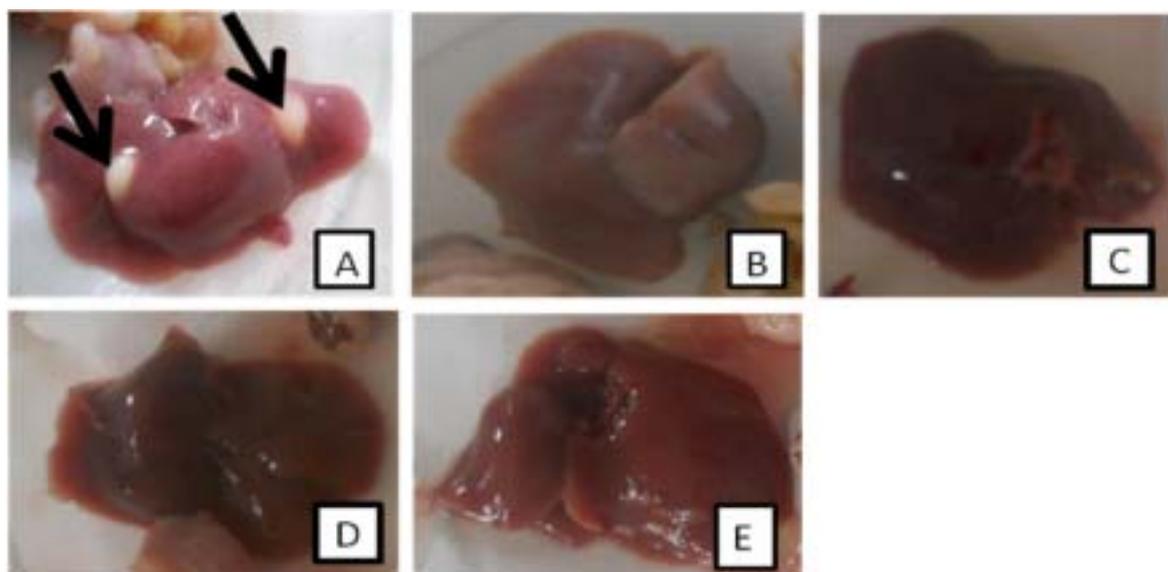


Figure 1. Morphological difference of liver treated with NSS in DMBA-induced female rats liver cancer. (A) DMBA control group; (B) DMBA+250 mg/kgBW NSS; (C) DMBA+500 mg/kgBW NSS; (D) DMBA+750 mg/kgBW NSS; (E) corn oil; (→) Nodule

induction by DMBA. NSS treatment was designed to prevent metabolic activation of DMBA and suppress liver cancer progression. The liver is exceedingly vulnerable to damage by chemical carcinogens, which is thought to result from its central role in the metabolism of

foreign substances (xenobiotics), including potential carcinogens.<sup>(16)</sup> DMBA is a polycyclic aromatic hydrocarbon (PAH) carcinogen that is oxidized to 7,12-DMBA-3,4-oxide by cytochrome P450 enzymes (CYPs), then hydrolyzed to its corresponding diol and finally

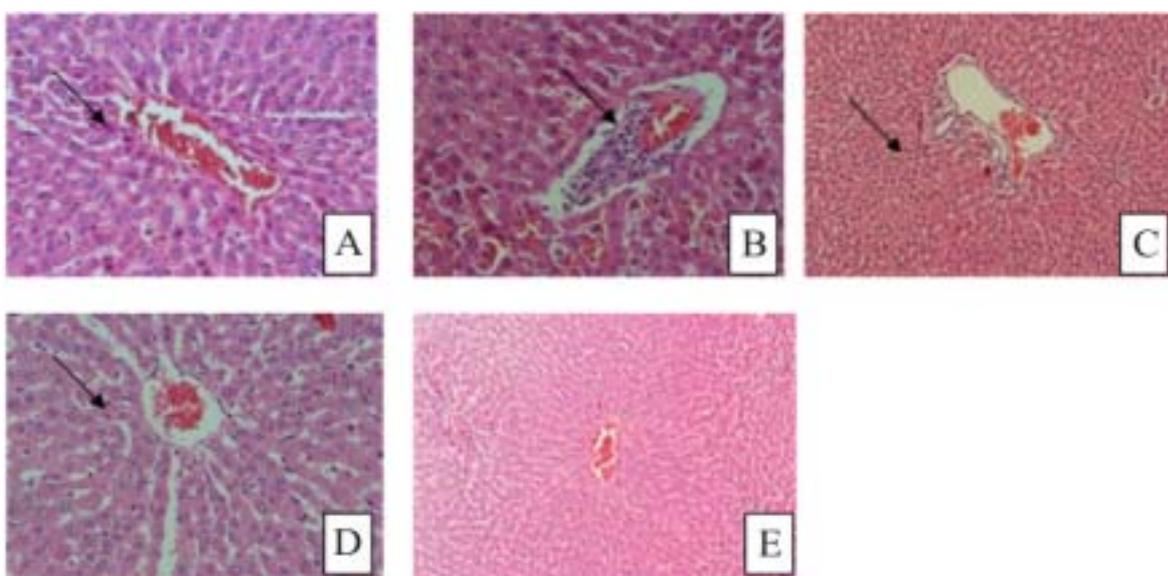


Figure 2. Histological evaluation in liver tissues of control and experimental group. Rats were divided into 5 groups, (A) Corn oil; (B) DMBA control group (20 mg/kgBW in Corn oil); (C) DMBA+ 250 mg/kgBW NSS; (D) DMBA+500 mg/kgbw NSS, (E) DMBA+750 mg/kgbw NSS; (→) necrosis magnification x600

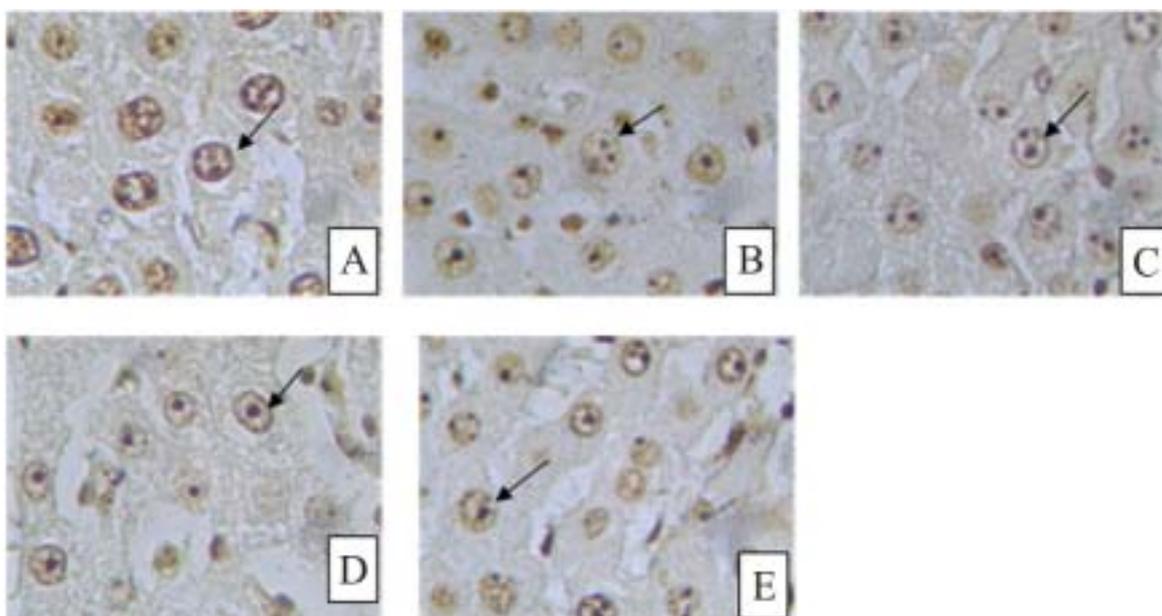


Figure 3. AgNO<sub>3</sub> stained liver tissues of control and experimental group. Rats were divided into 5 groups, (A) Corn oil; (B) DMBA control group (20 mg/kgBW in corn oil); (C) DMBA+ 250 mg/kgBW NSS; (D) DMBA+500 mg/kgbw NSS; (E) DMBA+750 mg/kgbw NSS. Argyrophilic nucleolar organiser regions (AgNORs) are visible as dark dots within the plasma cell nuclei (pointed by black arrow); Magnification x1000

oxidized by CYPs to 7,12-DMBA-3,4-oxide-diol-1,2-epoxide, which is the ultimate carcinogen.<sup>(17)</sup> Individual PAHs may affect their own metabolism, which is catalyzed by CYP 1A1, 1A2, and 1B1.<sup>(18)</sup>

DMBA metabolites are toxic and cause oxidative stress, leading to structural damage to cells and possibly cell necrosis.<sup>(19)</sup> DMBA administration to experimental animals causes an increase in lactate dehydrogenase (LDH), followed by necrosis.<sup>(20)</sup> Figure 1 shows that the livers in the DMBA control group were nodular in appearance. H&E staining showed necrosis in the DMBA control and DMBA + NSS groups, with the group treated with NSS at a dosage of 750 mg/kgBW showing negligible necrosis on H&E sections, if at all, which was similar to the solvent control (corn oil-only) group. Necrosis is defined as a type of cell death without apoptosis and autophagy, and is usually considered out of control.<sup>(21)</sup> Damage due to oxidative stress in sixty Swiss Albino rats weighing about  $40 \pm 5$  g, which were orally

administered of 0.5% DMBA solution demonstrated the presence of necrosis.<sup>(16)</sup> Necrotic hepatocytes release factors (damage signals or alarmins) that activate Kupffer cells (KCs), which in turn produce cytokines, such as interleukin 6 (IL-6), that promote compensatory hepatocyte proliferation.<sup>(22)</sup> Fractionation of the NSS chloroform extract yields linoleic and palmitic acids, and an indole compound which is an alkaloid that is tryptamine.<sup>(5)</sup> Linoleic acid (omega-3) and  $\alpha$ -linoleic acid (omega-6) can influence gene expression in experimental animals, have antiinflammatory activity, and can suppress interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and interleukin-6 (IL-6).<sup>(23)</sup>

The linoleic acid content of NS seeds can inhibit cell proliferation. Linoleic acid reduces cancer cell proliferation in lung, breast, prostate and colon cancers.<sup>(24)</sup> The linoleic and palmitic acid content of NSS has antiproliferative activity by induction of apoptosis in liver cells.<sup>(25)</sup>

Cancer is a complex disease that involves many regulatory proteins that differ with the

type of cancer. This study provides the information that NSS inhibits liver cancer growth. In the present study, NSS inhibited tumor incidence based on histopathological profile and mean AgNOR value. However, the proposed molecular mechanism involving CYP and glutathione S-transferases (GST) by the NSS extract needs to be further explored. GSTs may be potential targets on which to base the development of new antitumor compounds. These results suggest that NSS may be a promising new anticancer therapeutic agent and a potential candidate to be further evaluated.

## CONCLUSIONS

NS seed extract was able to reduce the hepatic cellular damage and proliferation in DNBA-induced liver cancers. This study indicated that NS can be developed into a chemopreventive agent for liver cancer.

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