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Nigella Sativa's Protection Against 7,12 Dimethylbenz [A] Anthracene -Induced Colon Carcinogenesis in Rats

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ABSTRACT. Colon cancer is the third most common cause of death from cancer worldwide. Recently, natural products have been widely used as an alternative therapy for colon cancer. Previous studies have reported that *Nigella sativa* has chemopreventive activity *in vitro* and *in vivo*.This study aimed to evaluate the effect of *Nigella sativa* seed (NSS) on rat-colon cell after initiation of 7,12-dimethylbenz [*a*] anthracene. Rats were divided into five groups, 12 rats in each group: Group I was given 7,12dimetilbenz [*a*] anthracene (DMBA) orally 20 mg/kgBW twice a week for five weeks, group V is the solvent control group was given corn oil. The other three groups were given DMBA + NSS, at the dosage of 250 mg/kgBW, 500 mg/kgBW and 750 mg/kgBW. NSS extract was dissolved in corn oil and administered daily per oral during the next two weeks before and during the initiation of DMBA. After 16 weeks, all rats were sacrificed. H&E staining showed that necrosis activity was lower in treated groups compared to DMBA group. AgNOR staining showed mAgNOR was significantly decrease following the increasing dose of NSS (250 mg/kgBW, 500 mg/kgBW and 750 mg/kgBW) were subsequently 1.62 ± 0.086, 1.60 ± 0.101 and 1.39 ± 0.049 ($p<0.05$). The results showed that NNS reduce the damage of colon cells and inhibit colon cell proliferation in DMBA induced rats.

Keywords: colon cancer, DMBA, *Nigella sativa*, rats

INTRODUCTION

Cancer is the second most common cause of death in the United States. In 2016, there were 9,678 new incidence of colon cancer, 6-7 people in 100,000 population died (Chu, 2018). Based on the data from the National Cancer Institute, the death rate from colon cancer is more than 33% in 2012 (Brody, 2015). Cancer therapy consists of surgery, radiation and chemoterapy. Chemoterapy still has a lot of constrains, because the huge of side effects such as anemia, thrombocytopenia, neutropenia and severe pain (Vogel, Eskicioglu, Weiser, Feingold, & Steele, 2017).

Currently, herbal is widely used as chemo-preventive to help solve the problems as complementary drug for cancer therapy. One of medicinal plant is black cumin (*Nigella sativa*) (Dajani, Shahwan, & Dajani, 2016). *N. sativa* contains 216 g protein, 406 g fat, 45 g ash, 84 g fiber, 249 g free nitrogen extract, 38 g moisture, 105 mg iron, 18 mg copper, 60 mg zinc, 527 mg phosphorus, 1. 860 mg calcium, 15.4 mg thiamin, 57 mg niacin and 160 µg folic acid per kg. Also, studies have shown the presence of different active pharmaceutical ingredients in the *N. sativa* seeds (NSS), including thymoquinone, thymol, limonene, carvacrol, p-cymene, alpha-pinene, 4-terpineol, longifolene, and t-anethole benzene (Kooti, Hasanzadeh-Noohi, Sharafi-Ahvazi, Asadi-Samani, & Ashtary-Larky, 2016). Thymoquinone is the main active constituent of the volatile oil of the *N. sativa*. The chemical composition of the black seed of Nigella sativa was found to contain a fixed oil (30%) and a volatile oil that is average 0.5%, maximum 1.5%. The volatile oil was found to contain 54% thymoquinone and many monoterpenes such as p-cymene and a-pinene, dithymoquinone and thymohydroquinone (Haseena, Aithal, Das, & Saheb, 2015).

In our previous study, cytotoxic activity of chloroform extract of NSS is higher than petroleum ether, alcohol and ethyl acetic extracts. The research suggests that chloroform is one of the semi-polar solvent commonly used to attract terpenoid compounds, phenols, and lipids (Harbone, 1998). Therefore, it is possible that thymoquinone largely attracted by the chloroform. The results obtained that the chloroform extracts of NSS have anticancer activity in vitro. Extract was said to have anticancer activity when the IC_{50} value of less than 1000 μg/mL after 24 h contact time. We continued to fractionated by column chromatography to separate the components of organic compounds in the sample. Hexane and chloroform were used with various comparisons and proceed according to different levels of polarity using

the other solvent that was ethyl acetate and methanol. Elution process performed to form ribbons of yellow and brownish red in stationary phase. Analyzing of active compounds in fractions using GC-MS method obtained linoleic acid (55.6%), oleic acid (23.4%), palmitic acid (12.5%) and tryptamine (0.01%) in the chloroform extract of *N. sativa* seed (Ekowati, Rahmani & Rastuti, 2011).

N. sativa can be used to inhibit carcinogenesis initiation and has protective against DNA damage caused by oxidative stress (Dajani et al., 2016). Nano emulsion of *N. sativa*'s essential oil can inhibit cell proliferation of MCF-7 breast cancer cell (Periasamy, Athinarayanan, & Alshatwi, 2016). The chloroform extract of *N. sativa* has cytotoxic activity on breast cancer T47D cells with IC_{50} of 124 µg/mL (Ekowati et al., 2011). Our previous study also reported that NSS chloroform extract was capable of reducing cellular proliferation in DMBA-induced liver cancers in rats (Alisah, Baroroh & Ekowati, 2012). An experimental study showed that orally administered of *N. sativa* had hepatoprotective effects against dimethylaminoazobenzene (DAB)-induced cholangiocarcinoma in male Swiss albino mice. *N. sativa*'s 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 (Mohamed, El-Sayed, & Moawad, 2010). 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. The administration of *N. sativa* reduced the carcinogenic effects of DMBA in skin carcinoma, suggesting a protective effect (Mbarek et al., 2007) Thymoquinone as the active compound of *N. sativa*, suppresses the proliferation of renal cell carcinoma cells via reactive oxygen species-induced apoptosis (Liou et al., 2019). p53, NF-κB, PPARγ, STAT3, MAPK, and PI3K/AKT signaling pathways are among the most significant pathways through which thymoquinone mediates its anti-cancer activity (Chen et al., 2017; Majdalawieh, Fayyad, & Nasrallah, 2017).

This study aimed to observe the activity of NSS extract on 7,12 dimethylbenz [a] anthracene-induced colon carcinogenesis in rat through histopathology and AgNOR staining of the colon cells.

EXPERIMENTAL SECTION

Materials

The chloroform extract of NSS, DMBA (7,12dimetilbenz [*a*] anthracene) was obtained from Sigma (Saint Louis, MO, cat.no D3254).

Preparation of NSS-Chloroform Extract

Nigella sativa seeds (NSS) are washed with water, dried and ground into powder. NSS powder were macerated using petroleum ether for 1 x 24 h. Extract was filtered and then evaporated by the evaporator. Then the filtrate was continued macerated with chloroform, ethyl acetate, and ethanol respectively, each for 3 x 24 h. The extract is filtered and then evaporated. Chloroform extract was used in our in vivo experiments based on previous study (Ekowati et al., 2011), reported that the chloroform extract is the most active extract in T47D cancer cell line.

Animals

Sprague Dawley female rats weighed 100 to 150 grams were obtained from the Laboratory of Pharmacology and Toxicology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. The rats were kept for at least one week before use, provided with standard pellet diet and water ad libitum, and was kept on a 12:12 h light/dark cycle (Faculty of Medicine UGM-MHREC/Ref: KE/FK/575/EC, 2013).

An experimental study design was set up with post only control group design using rats. Rats were divided into five groups, 12 rats in each group: Group I, the control group was given DMBA orally 20 mg/kgBW twice a week for five weeks, group V is the solvent control group was given corn oil. The other three groups were given DMBA + NSS, at the dosage of 250 mg/kgBW, 500 mg/kgBW and 750 mg/kgBW. NSS extract was dissolved in corn oil and administered daily per oral during the next two weeks before and during the initiation of DMBA. At the end of the experiment (16 week) all rats were sacrificed, observations of colonic cell proliferation was performed using hematoxylin and eosin staining (H&E) and AgNOR. Data were analyzed using one-way ANOVA, followed by Tukey HSD. Body weight was recorded weekly throughout the study.

H&E Staining

After 16 weeks all rats were sacrificed. At autopsy, the colon was removed and fixed in 10% buffered formalin. After 12-24 h of fixation, 3-5 µm tissue slices were embedded in paraffin, and stained with hematoxylin and eosin for microscopy examination.

AgNOR Staining

AgNOR staining was performed according to the modified method (D'Uva, Baci, albini, & Noonan, 2018; Kilari, Kotakadi, & Penchalaneni, 2016). 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 (pH 6.0) and incubated 20 min in autoclave (120°C,1.1-1.2 Bar). Sections were then 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.

AgNORs, which are appeared as dots both outside and within the nucleoli, were counted according to the

description of previous report (Gholamnezhad, Havakhah, & Boskabady, 2016). A minimum one hundred cells per specimen were observed randomly in three different views using High Power Field (HPF). Mean value of AgNOR (mAgNOR) was used as a parameter to evaluate antiproliferative activity. mAgNOR is a mean of black dots in a cell, computed from total amount of blackdots (min 100 cells) divided with amount of cells (min 100 cells) (Kilari et al, 2016). All specimens were observed by two observer on a binocular microscope (Olympus® DP12 *microscope digital camera system*, NY) with an immersion oil lens at magnification of 1000x.

Statistical analysis

A statistically significant body weight and mAgNOR score difference were evaluated by ANOVA, continued by HSD ($p<0.05$) using SPSS ver. 18.

RESULTS AND DISCUSSION

The NSS extract was prepared by maceration method. Maceration is a method to prepare extract by using appropriate solvent with several times shaking or stirring at room temperature. Maceration suitable for compounds that do not withstand heating at high temperatures. The aim is to attract the chemical components based on the principle of mass transfer of substance into the solvent component, where the movement began to occur at the interface layer and then diffuses into the solvent (Anonymous, 2011).

Petroleum ether is a non-polar solvent used to attract non-polar compounds such as wax, fat and lipids which can interfere during the separation process. In addition, petroleum ether was used to eliminate the resins which can interrupt the extraction. After the filtrate macerated with petroleum ether, macerate aerated and then macerated with chloroform for 3 x 24 h. Chloroform is one of the semi-polar solvent commonly used to attract terpenoid compounds, polyphenols, fatty acids, fats, oils, essential oils, phenols and lipids (Harbone, 1998). The study reported that potential as an anticancer compound from the extract is thymoquinone. Thymoquinone was a component of essential oil (Haseena, Aithal, Das, & Saheb, 2015).

Effect of DMBA on body weight female rats

There was no direct evidence of toxicity due to NSS treatment. Body weight changes of the animal treated with corn oil, DMBA and DMBA+NSS were compared (**Table 1).** In the beginning of the study, the body weight of corn oil group was different from the other groups ($p < 0.05$). Final body weight showed that DMBA goup was no significant different from DMBA+NSS groups ($p > 0.05$) (**Table 1**). This showed us that there is no toxicity due to NSS treatment related to body-reducing weight as we could find in the treatment of an anticancer drugs (Chu, 2018). Previous study, showed that the chloroform extract of NSS had cytotoxic activity on breast cancer cells T47D, with IC_{50} value 161.131 μg/mL (Ekowati et al., 2011). Our study aimed to asses the effect of NSS chloroform extract on DMBA induced rat colon cancer. In this study, extract chloroform of NSS was given two weeks before and during induction of DMBA (**Figure 1**).

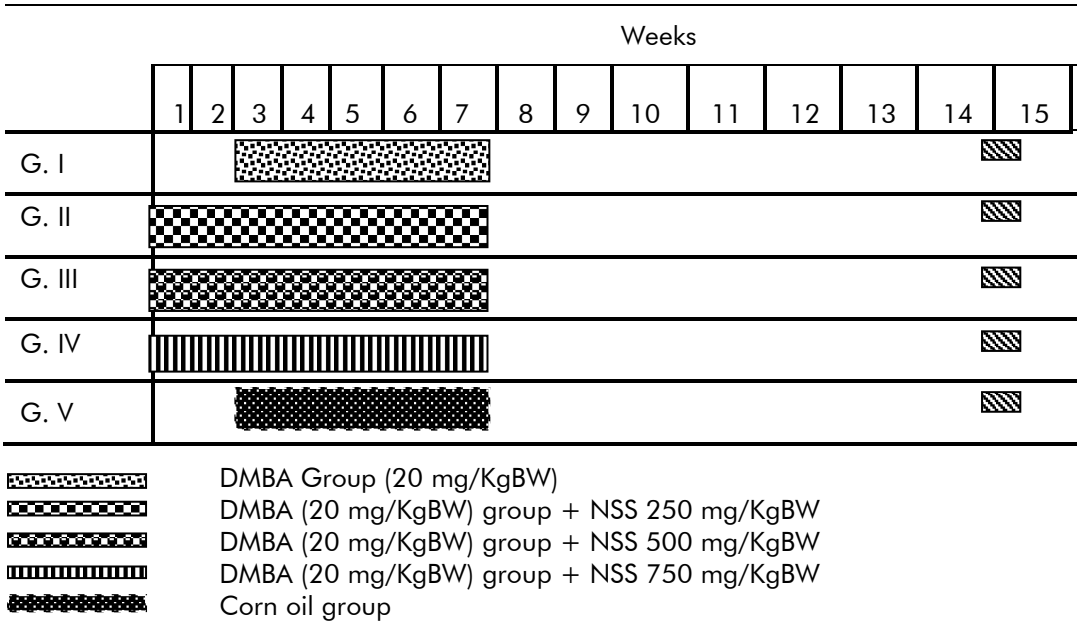

Figure 1. Experimental time line. NSS extract prevents directly the metabolic activation of DMBA, prevent tissue damage and also prevent cancer carcinogenesis at the initiation stage.

Table 1. Effect of DMBA on body weight

Groups	Body weight in the beginning of experiment (g)	Body weight in the end of experiment (g)
DMBA	90.00 ± 15.37	121.43 ± 68.95
DMBA + *N. sativa* 250 mg/Kg BB	90.00 ± 20.45	112.00 ± 58.98
DMBA + *N. sativa* 500 mg/Kg BB	78.33 ± 17.49	121.43 ± 63.88
DMBA + *N. sativa* 750 mg/Kg BB	64.17 ± 15.05*	82.00 ± 46.21*
Corn oil	54.17 ± 9.00*	90.00 ± 35.71*

Values are expressed as mean±SD; n= 12, * When compared with DMBA Group, Statistical significance different: $p < 0.05$

Histopathological Studies of Colon

Histopathology profile of colon cell of DMBA and DMBA+NSS treated rats is depicted in **Figure 2**. At autopsy, colon organ were removed and fixed in 10% buffered formalin. As thick as 3-5 µm tissue slices were embedded in paraffin, and stained with hematoxylin and eosin. In general, hematoxylin and eosin staining showed differences in morphology between DMBA+NSS and DMBA. DMBA group showed necrosis cells in the colon more severe than DMBA+NSS groups.

Our previous study showed that treatment of extract NSS 750 mg/kgbw significantly decreased proliferation of colon cells induced by DMBA, which in line with the result of the study by Andini, Baroroh, and Ekowati (2013) on renal cells. Treatment of NSS extract can induce the expression of p53 protein in WiDr cell with IC_{50} 3.31 µg/mL. Antiproliferative activity of extract chloroform of NSS is mediated by metabolic enzyme CYP and *glutathione S-transferase* (GST) (Darakhshan, Bidmeshki, Hosseinzadeh, and Sisakhtnezhad, 2015). DMBA needs to be metabolized to become a reactive metabolite. DMBA is a substrate of the enzyme cytochrome P450 (CYP), CYP1A1 and CYP1B1 (Kilari et al., 2016). After metabolized, DMBA will produce ultimate carcinogens, dihydrodiol epoxide and radical cation that are highly reactive (D'Uva et al., 2018). The epoxide metabolite also interact with GST to generate glutathione conjugates that are readily metabolized to the mercapturic acid and will be eliminated from the body (Gholamnezhad et al., 2016).

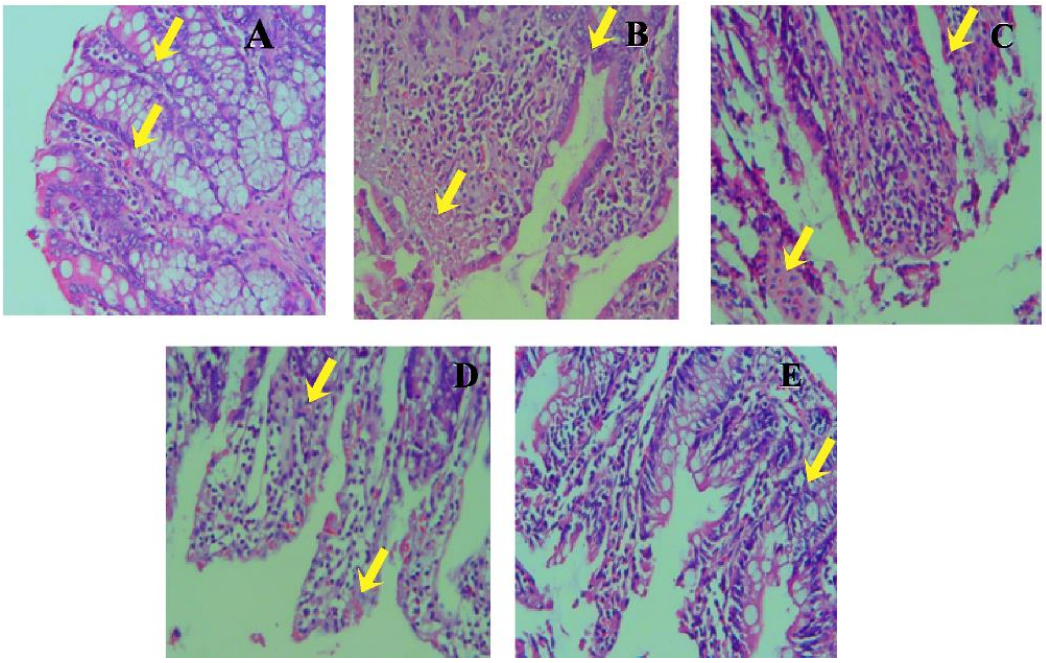

Figure 2. Histological evaluation in colon tissues of control and experimental group. Rats were divided into 5 groups, (**A**) Corn oil, (**B**) DMBA control group (20 mg/kgBW in Corn oil), (**C**) DMBA+ NSS 250 mg/kgBW; (**D**) DMBA+ NSS 500 mg/kgBW, (**E**) DMBA+750 NSS mg/kgBW. (↑=necrosis). Magnification x 600.

Antiproliferative Activity of NSS on Female Rats Colon

AgNOR staining on solven colon (**Figure 3**) showed that DMBA group has higher blackdots than the other groups. Cancers have a high proliferation rate that is closely related to the amount of argyrophilic proteins (AgNOR proteins) in the nucleolar organizer regions (NORs). The nucleolar organizer regions 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. Our study was to evaluate the activity of a chloroform extract of NSS extract on 7,12-dimethylbenz [á] anthracene (DMBA)-induced colon carcinogenesis in rats by examination with silver-staining by the AgNOR method.

At autopsy, colon organ were removed and fixed in 10% buffered formalin. As thick as 3-5 µm tissue slices were embedded in paraffin, and stained with $AgNO_3$. Argyrophilic Nucleolar Organiser Regions (AgNORs) are visible as dark dots within the plasma cell nuclei. The scores of mAgNOR (**Table 2**) of corn oil and DMBA are 1.26 ± 0.13 and 1.63 ± 0.06 respectively.

Treatments of NSS at the dosage of 250 mg/kgBW, 500 mg/kgBW and 750 mg/kgBW showed significant antiproliferative activity of DMBA (Table 2) with mAgNOR scores 1.62 ± 0.09, 1.60 ± 0.10, and 1.39 ± 0.05 ($p < 0.05$) respectively. This result is in line with our previous study, the NSS chloroform extract was capable of reducing cellular proliferation in DMBA-induced liver cancers in rats (Alisah et al., 2012). 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 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 oxidized by CYPs to 7,12-DMBA-3,4-oxide-diol-1,2-epoxide, which is the ultimate carcinogen. Individual PAHs may affect their own metabolism, which is catalyzed by CYP 1A1, 1A2, and 1B1(Shimada and Kuriyama, 2004).

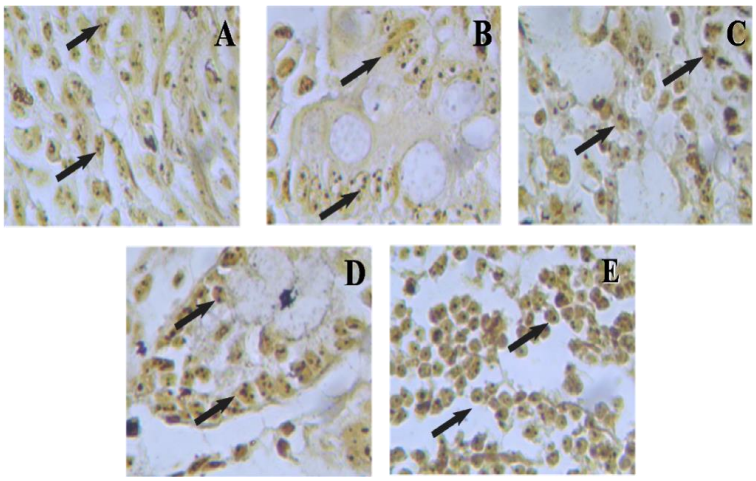

Figure 3. AgNO3 stained colon tissues of control and experimental group. Rats were divided into 5 groups, (A) Corn oil, (B) DMBA control group (20 mg/kgBW in Corn oil), (C) DMBA+ NSS 250 mg/kgBW; (D) DMBA+ NSS 500 mg/kgBW, (E) DMBA+ NSS 750 mg/kgBW. Argyrophilic Nucleolar Organiser Regions (AgNORs), pointed by black arrow. Magnification x1000

Table 2. Effect of *N. sativa* on proliferation of DMBA-induced colon female rats

Group Rats	mAgNOR
DMBA	1.63 ± 0.06
DMBA + NSS 250 mg/Kg BB	1.62 ± 0.09
DMBA + NSS 500 mg/Kg BB	1.60 ± 0.10
DMBA + NSS 750 mg/Kg BB	1.39 ± 0.05*
Corn oil	1.26 ± 0.13*

mAgNOR = mean AgNOR, * are statistically significant different from DMBA groups ($p < 0.05$) by *one way Anova* continued with *Tukey HSD*.

In general, treatment of NSS inhibit proliferation of colon cells in rats induced with DMBA. Fractionation of the NSS chloroform extract yields linoleic and palmitic acids, and an indole compound which is an alkaloid that is tryptamine (Ekowati et al., 2011). The linoleic acid content of NSS seeds can inhibit cell proliferation. Linoleic acid reduces cancer cell proliferation in lung, breast, prostate and colon cancers. The linoleic and palmitic acid content of NSS has antiproliferative activity by induction of apoptosis in liver cells (Zhang, Xue, Zhang, Yang, & Shi, 2012). Linoleic acid (omega-3) and á-linoleic acid (omega-6) can influence gene expression in experimental animals, have antiinflammatory activity, and can suppress interleukin-1â (IL-1â), tumor necrosis factor-á (TNFá) and interleukin-6 (IL-6). Linoleic acid can enhance the expression of pro-apoptotic genes such as *Bax* and can reduce expression of anti-apoptotic genes such as Bcl-2, which finally will activate caspase 3 and 9 pathway and enhance apoptosis. Linoleic acid has also been known to induce the activity of the p53 gene (Mericli et al., 2017; Zhang et al., 2015; Lu, Yu, Ma, Shen, & Das, 2010). p53 gene was a regulatory protein which trigger the expression of CKI (p21), resulting in stops of cell cycle at G1 phase. *N.sativa* stimulates apoptosis by increasing p53 expression (Kotowski et al., 2017).

While palmitic acid induces reactive oxygen species (ROS), which will oxidizing the mitochondrial membrane and would disrupted the mitochondrial membrane potential, give the pores (Liu et al., 2018). Pore would facilitate the entry of calcium, releasing cytochrome c. Cytochrome will bind Apaf-1 and activating procaspase-9 become caspase-9. Caspase-9 will excite caspase 3 to break down cell protein like cytoskeleton, enzyme and chromosomes (Fouad and Aanei, 2017).

Based on H&E and mAgNOR data, there is relationship between histopathology of cell and antiproliferative activity. The lower cell damage and necrosis, the higher anti-proliferative activity will be. This may be caused by linoleic acid activity of the NSS chloroform extract, that suppress the expression of bcl-2, then suppress COX-2 and the activity of proinflammatory cytokines (TNF-α, IL-6 and IL-1) (Da Boit, Hunter, & Gray, 2017).

CONCLUSIONS

It is conclude that chloroform extract of *N.sativa* seeds is able to reduce cell proliferation of DMBA-induced colon in rats, subsequently the chloroform extract of *N. sativa* is potential to be developed as chemopreventive agent.

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