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The Inhibitory Effects of *Nigella sativa* Nanocapsules on Increased NNK-induced Inflammation, Oxidative Stress, and Tumor-associated Macrophage Responses in Wistar Rats

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A R T I C L E I N F O	A B S T R A C T
	Introduction: Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a potent cancer-causing agent in cigarettes and is also associated with the induction of lung tumors by the stimulation of malondialdehyde (MDA) levels and expression of tumor-associated macrophages (TAMs). The present study aimed to examine the variations of MDA levels and TAM expression in the lung tissues of rats exposed to NNK following 12 weeks of *Nigella sativa* nanocapsule injection. **Methods:** In this study, 48 Wistar rats were randomly divided into five groups of supplement, supplement with NNK, NNK, control, and saline. For 12 weeks, NNK was injected subcutaneously per kilogram of the animals' body weight with the weekly dose of 12.5 milligrams. In addition, the nanocapsules were subcutaneously injected once a week per kilogram of the body weight with the weekly dose of 12.5 milligrams. The MDA levels and CD68-TAM expression in lungs were determined using the enzyme-linked immunosorbent assay and immunohistochemistry, respectively. **Results:** The injection of *Nigella sativa* nanocapsules for 12 weeks significantly decreased the MDA levels and CD68-TAM expression in the NNK group (P<0.001). Additionally, the injection of *Nigella sativa* nanocapsules along with the consumption of NNK significantly decreased the MDA levels and CD68-TAM expression in the lung tissues of the NNK group (P<0.001). **Conclusion:** According to the results, the orderly injection of *Nigella sativa* nanocapsules could significantly deter lung tissue inflammation induced by NNK through the reduction of MDA levels and CD68-TAM expression in rats.

Introduction

Lung cancer is the leading cause of cancer deaths worldwide, especially in the areas where cigarette smoking is prevalent (1). The secular trend in lung cancer histology indicates that the proportion or incidence of lung adenocarcinoma has been increasing markedly, which is partly due to the introduction of filter cigarettes and secondary smoking (2). Tobacco contains various carcinogens, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which has recently been reported to be significantly associated with lung cancer (3). In this regard, Barta et al. observed that persistent swelling and tumor formation increased in lung tissues following NNK consumption (4). Chronic inflammation and the subsequent cell proliferation may also cause neoplasia, angiogenesis, metastasis, apoptosis, and the release of immunosuppressive factors (5).

Tumor-associated macrophages (TAMs) are an inherent element of the tumor microenvironment (6), and CD68 is a marker used to identify TAMs (7). The recent findings of Sun et al. have demonstrated the overexpression of CsD68-TAMs in squamous cell carcinoma (8). Furthermore, malondialdehyde (MDA) is a lipid peroxidation product and the most commonly measured indicator of oxidative damage to membrane lipids (9). MDA may be involved in tumor promotion as it is able to interact with the functional groups of various cellular compounds (10). MDA elevation in lungs may reflect the inability of the lungs to eliminate the reactive oxygen species (ROS) produced by nicotine (11).

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Recent findings have indicated that the progression of lung cancer is possible due to changes in some key molecules or signaling pathways. In this regard, the use of molecularly targeted therapeutic and biological agents along with nutrition has been reported as a novel strategy for the prevention of lung cancer (12). The chemotherapy/radiotherapy regimens that are currently used for cancer treatment cause adverse side-effect and may alter gene functions, and natural products are considered to be generally safe, effective, and less costly alternatives to anticancer chemotherapeutics. According to the literature, *Nigella sativa* and its constituents are among widely accepted therapeutic options for the prevention of cancer (13). Although the mechanisms and signal changes of recent variables have been proposed, data regarding the correlation of *Nigella sativa* supplementation and NNK consumption are contradictory. In a study, Fadda et al. reported that treatment with a thymoquinone (TQ) combination was effective in the down-regulation of CD68 expression (14), while Wilson et al. stated that M2 macrophage chain expression significantly increased following TQ treatment (15). Furthermore, an *in-vivo* study demonstrated that the oral administration of *Nigella sativa* seeds for six months exerted protective effects against oxidative stress and colon carcinogenesis in rats through the reduced expression of MDA (16), and the findings of Assayed indicated the increased concentration of MDA due to the effect of *Nigella sativa* oil on rats (17).

Considering the effects of NNK on inflammation and the subsequent development of lung cancer (4) and the multiple roles of TAMs in inflammatory processes and tumor growth, the use of medicinal plants in the form of nanocapsules has been proposed to control the drug release system. Secondly, for a long time the concentration of drug in plasma be preserved without reaching the ineffective areas (18). Given the utmost importance of cancer prevention and the scarce data regarding the effects of black seed nanocoupled supplementation and NNK induction on biomarkers, the present study aimed to investigate the effects of *Nigella sativa* nanocapsules on CD68 and MDA in rats consuming NNK.

Materials and Methods
Experimental Animals
This study was conducted on 48 Wistar rats aged 6-8 weeks with the mean weight of 105.84±27.93 grams at Mazandaran University, Iran. After the adaptation process of the rats with the research environment for two weeks, they were randomly divided into five groups, including control (n=9), saline (n=10), supplement (n=9), NNK (n=10), and supplement with NNK (n=10). The rats were kept in polycarbonate cages, with the mean temperature set at 22±1.4°C and 55% humidity within a 12-hour light/dark cycle. The animals had free access to sufficient water and pellets. For 12 weeks, the treatment groups received distilled water and NNK, which were injected subcutaneously per kilogram of the rats' body weight with the weekly dose of 12.5 milligrams (19).

The study protocol was approved by the HRI Ethics Committee of Babol University of Medical Sciences (ethics code: MUBABOL.HRI.REC.1395.109).

Chemicals
NNK was purchased from Toronto Research Chemicals Inc. (North York, Canada).

Preparation of the Herbal Extract
Nigella sativa seeds were purchased from a local market, and stored as a voucher specimen. The seeds were crushed manually with a mortar and pestle. A volume of 20 mL PBS was added to 10 g powder. It was vortexed continuously until there was no further change in colour of the solution and stored overnight at 4°C in sterile tubes. Afterwards, it was centrifuged at 3,000 rpm for 15 minutes, and the liquid lying above the solid residue was purified through a Whatman filter and maintained at the temperature of 4°C in disinfected microtubes until use. In addition, the quantification of the total proteins was completed using Bradford reagent, and spectrophotometry was performed on a DU70 spectrophotometer (20).

Preparation of Nigella sativa Nanocapsules
In order to prepare the nanocapsules, 50 milligrams of human albumin serum were dissolved in one milliliter of water with the pH of 7.4, and 0.5% of Tween 80 was added to the samples. Following that, the samples were stirred at 300 rpm for 30 minutes using a magnet, and four milliliters of ethanol was

gradually added to the solution. In addition, 117 microliters of glutaraldehyde were added to the samples, and stirring at 500 rpm continued for 24 hours. With the addition of the *Nigella sativa* extract, the structure remained spherical and slightly stuck together, confirming the presence

of the extract within the synthetic nanocapsules (21). For 12 weeks, the nanocapsules were subcutaneously injected to the animals per kilogram of body weight at the weekly dose of 12.5 milligrams (22) (Figure 1).
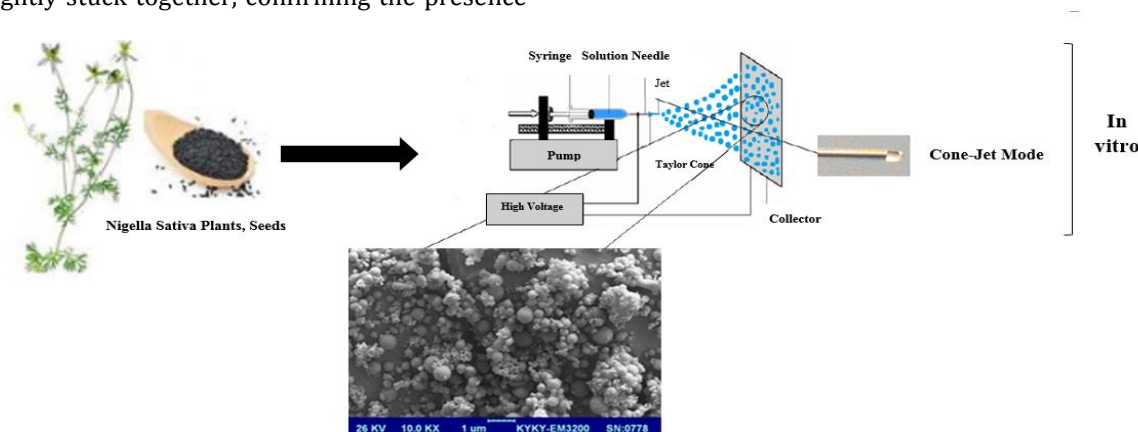

Figure 1. Preparation of nigella sativa nanocapsules

Sample Preparation

After surgery, the lung tissues were removed from the ribcage of the animals, placed inside polypropylene graduated microtubes, and kept frozen in an LN2 freezer at -70°C. To measure the dependent variables, one milliliter of the PBS buffer (100 millimolar) was mixed with 100 milligrams of the lung tissues and centrifuged for 15 minutes at 6,000 rpm (23). Afterwards, the buoyant was transferred to the research laboratory to measure the MDA levels.

Estimation of Lipid Peroxidation

At this stage, MDA stock (4 micromole) was prepared for use by hydrolyzing 9.6 microliters of CH_2 $(CH[OCH_3]_2)_2$; T9889, Sigma) in 10 milliliters of hydrochloric acid (100°C for five minutes). In addition, 100 microliters of the solution were liquefied to 10 milliliters in KCl-Tris buffer saline (24).

Immunohistochemical Staining

To recover the antigen, the sections were heated in trisodium citrate buffer at 100°C for 20 minutes. The sections were incubated with the CD68 antibody at normal laboratory temperature for two hours. Monoclonal Mouse Anti-Human CD68, Clone (1:100) (DakoAutostainer-Autostainer Plus) were purchased from Daco, Denmark. At the next stage, the sections were incubated for 30 minutes with second antibody at laboratory temperature, and PBS was used as the control

agent rather than the primary antibody. The positive expression report via light microscopy was obtained for the cytoplasm of the yellow-brown ingredient. Peroxidase obstructive buffer, trisodium citrate antigen, and PBS were obtained from Biotechnology Company (Sanfrancisco, USA) (25).

Evaluation of Immunoreactivity

Fewer per nuclear expression was observed in the stroma, and the affirmative expression of CD68-TAM was recognized by the brownish yellow colored patches in the tumor cells. Excess from 10% coloring was specified as an affirmative response, and less than 10% was perceived as non-affirmative. If 10-25% of the cells were affirmative, it would indicate a low expression level (+), while an average expression level (++) was confirmed with 26-50% of affirmative cells, and a considerable expression level (+++) was defined with more than 50% of affirmative cells. In the cytoplasm of macrophages, the affirmative expression of CD68-TAM appears as yellowish brown grains. In our study, a pathologist assessed the positive expression of CD68-TAM based on the average rate of a high-power field (26).

Statistical Analysis

Data analysis was performed in SPSS version 24.0 (SPSS Inc., Chicago, Illinois) using the Shapiro-Wilk test to examine the normal distribution of data, and Levene's test was

applied to control the homogeneity of the variances. Moreover, one-way analysis of variance (ANOVA) and Tukey's test were employed at the significance level of P<0.05.

Results

The obtained results indicated no significant difference in the weight of the animals between the groups.

MDA Levels

The results of one-way ANOVA indicated a significant difference in the MDA levels between the study groups (P<0.001). In addition, Tukey's

test showed that the MDA levels of the NNK group significantly improved compared to the saline group (81.76%) (P<0.001). Additionally, the consumption of the *Nigella sativa* nanocapsules significantly increased the MDA levels compared to saline injection (14.48%) (P<0.001), while the MDA levels significantly decreased in the NNK group (37.01%) (P<0.001). Furthermore, the consumption of *Nigella sativa* with NNK injection significantly reduced the MDA levels compared to the NNK injection alone (28.70%) (P<0.001) (Figure 2).
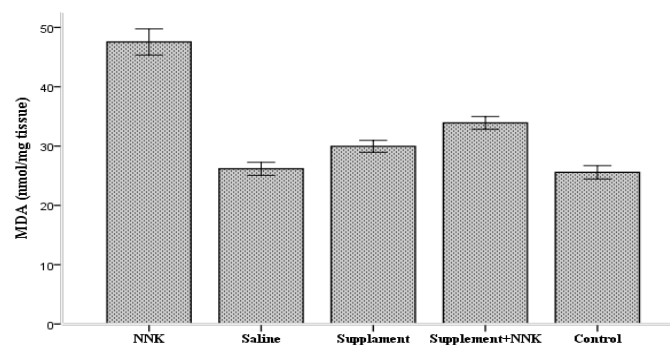

Figure 2. Changes in MDA values (nmol/mg tissue) in lung tissue of research groups.
* Signs of a significant changes compared to saline, # signs of a significant changes compared to NNK, ¥ signs of a significant changes compared to Supplement+NNK.

CD68 Expression

The results of one-way ANOVA indicated a significant difference in the CD68 expression between the study groups (P<0.001), while Tukey's post-hoc test showed no significant difference between the control and saline groups in this regard (P=0.707). Compared to the NNK and saline groups, CD68 expression increased significantly in the NNK group (766%) (P<0.001). Moreover, the consumption of the

Nigella sativa nanocapsules decreased the CD68 expression significantly compared to NNK injection (88%) (P<0.001). However, these changes were not considered significant compared to the solvent and supplement with NNK groups (P=1.00 and P=0.775, respectively). *Nigella sativa* consumption along with NNK injection could significantly reduce CD68 expression compared to NNK injection (92%) (P<0.001) (Figure 3).
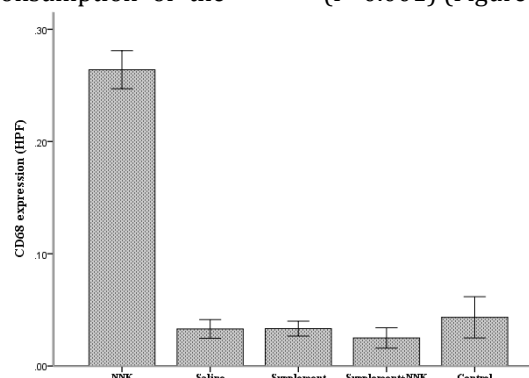

Figure 3. CD68 expression (high power field) in lung tissue of research groups.
* Signs of a significant changes compared to saline, # signs of a significant changes compared to NNK, ¥ signs of a significant changes compared to Supplement+NNK.

Histopathological studies in the present study demonstrated that lung tissue parenchyma was totally normal in the saline and control groups. In the *Nigella sativa* nanocapsule group, the parenchymal structure was largely preserved, while acute inflammation was also detected in this group. In the NNK group, we observed the aggregation of inflammatory and atypical cells, as well as the partial formation of tumor nodules. In the NNK group receiving the herbal supplement, a slight acute inflammation was also denoted despite the fact that the lung structure was totally conserved (Figure 4).
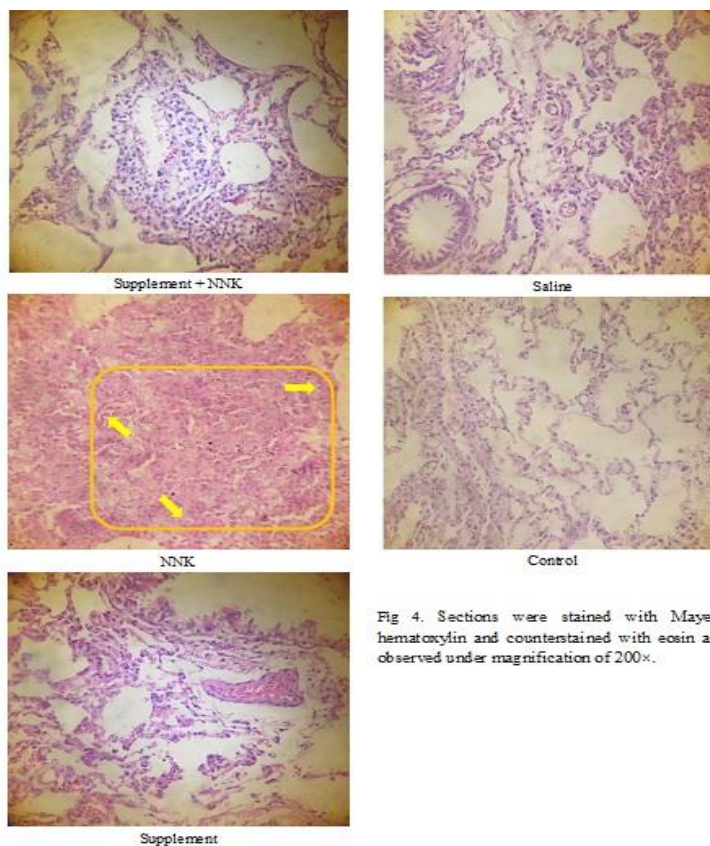

Fig 4. Sections were stained with Mayer's hematoxylin and counterstained with eosin and observed under magnification of 200×.

Furthermore, the negative response of CD68 was observed in the training groups (0.02 high-power field), as well as the training with NNK consumption (0.02 high-power field), control (0.04 high-power field), and saline groups (0.03 high-power field). However, the positive response of CD68 was denoted in the NNK group (0.26 high-power field) (Figure 5).

Discussion and Conclusion

The present study aimed to investigate the effects of *Nigella sativa* nanocapsules and NNK injection on MDA levels and CD68 expression in the lung tissues of rats. Compared to the NNK and saline groups, the MDA levels significantly increased in the NNK group. This is consistent with the previous studies in this regard, which have suggested that the accumulation of ROS may significantly increase lipid peroxidation at cellular and molecular levels, while also affecting tumor promotion (27, 29). Furthermore, ongoing lipid peroxidation within the affected tissues and the subsequent release of lipid peroxidation products into the circulation may possibly be induced by the enhanced generation of oxygen radicals or the deficiency of antioxidant defense mechanisms (29). However, one study in this regard reported no elevation in the MDA levels of smokers (30). The discrepancy could be due to narrowing of age range, differences in the rate of cigarette consumption, duration of smoking habits, dietary intake of fruits and vegetables or a combination of these factors in the mentioned study.
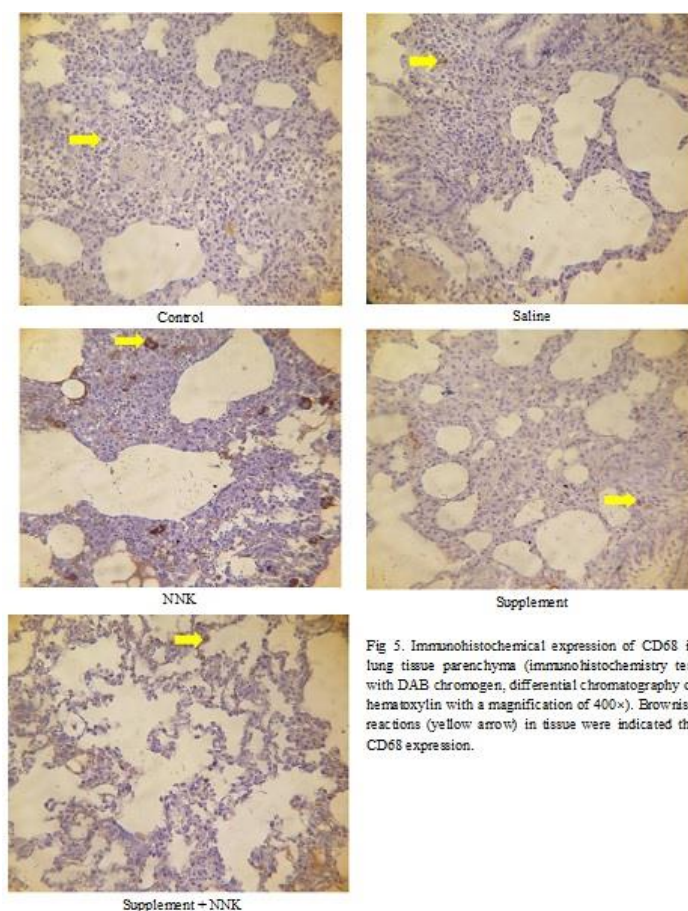
Fig 5. Immunohistochemical expression of CD68 in lung tissue parenchyma (immunohistochemistry test with DAB chromogen, differential chromatography of hematoxylin with a magnification of 400×). Brownish reactions (yellow arrow) in tissue were indicated the CD68 expression.

According to the results of the present study, CD68-TAM expression increased significantly in the NNK group compared to the saline group. Consistently, Proulx et al. stated that NNK consumption stimulated CD68-TAM expression. NNK activation inhibits the production of alveolar macrophages (TNFα, IL-12, and NO), while increasing IL-10 and PGE2 release. Therefore, it could be inferred that NNK favors the differentiation of alveolar macrophages into polarized type II cells (AM-2); interestingly, this macrophage has been associated with tumor growth and progression. On the other hand, the reduction of type I alveolar macrophages may limit the host defense capacity of smokers and secondhand-smoke-exposed subjects (31). M2 macrophages are able to tune inflammatory responses and adaptive Th2 immunity, promote angiogenesis, and tissue remodeling and repair (32).

In the current research, a period of *Nigella sativa* nanocapsule consumption significantly decreased the levels of MDA compared to the NNK group. In this regard, Avti et al. reported that using smokeless tobacco in rats induced ROS production and increased the MDA levels (33, 34). However, *Nigella sativa* consumption in rats administered with smokeless tobacco has been reported to significantly decrease MDA levels, which could be due to the fact that *Nigella sativa* supplementation seizes ingredients such as flavonoids and polyphenols, which hinder the induction of oxidative stress (35).

According to the results of the present study, the consumption of the *Nigella sativa* nanocapsules decreased the CD68-TAM expression more significantly than NNK. Consistently, Wilson et al. reported that after TQ injection for 10 days (3 days a week, 20 mg/kg) in mice with cervical cancer, anti-tumor effects were observed in the form of NF-kB inhibition in malignant tumors and reduced M2 macrophages (CD68-TAM). In contrast, long-term TQ treatment has been reported to increase tumorigenic changes in the tumor microenvironment (36). Previous findings have also confirmed that prolonged TQ

treatment (20 mg/kg, thrice weekly, IP, 30 days) stimulated pro-tumorigenic changes in the tumor environment. Enlarged ascites has also been associated with higher NF-KB operation, increased expression of the M2 macrophage, and the degree of pro-tumorigenic solvable agents, such as IL-10 and the vascular endothelial growth factor in the ascites liquid (13). The conflicting results of our investigation with the previous studies could be due to the composition of the nanocapsules and duration of *Nigella sativa* consumption. Another reason could be associated with the specs of the samples the study by Wilson et al. investigated a murine syngeneic model of ovarian cancer (29).

Our findings demonstrated that *Nigella sativa* consumption with NNK injection significantly reduced MDA levels compared to NNK injection alone. A similar study confirmed the effectiveness of treatment with various substances in inhibiting oxidation and enhancing the survival of the animals consuming lipopolysaccharide toxicants (37). In the present study, *Nigella sativa* consumption could reduce the MDA levels of the tissues and inhibit oxidation. Lipopolysaccharide has proven effective in the treatment of rats with significantly increased lung fluorodeoxyglucose absorption, which is associated with increased lung inflammation (38). Considering that nicotine-derived nitrosamine ketone increases the ROS formation and MDA, *Nigella sativa* supplementation could enhance the tissue capability for the removal of ROS (37).

According to the current research, *Nigella sativa* consumption along with NNK injection significantly reduced CD68 expression compared to NNK injection alone. In another study, Wilson et al. reported that TQ could actualize macrophage quantity and genotype in the ascites liquid of the tumor microenvironment. In the mentioned study, structural evaluation also showed a significant increase in the mononuclear cellules with TQ consumption although the comparative stimulatory efficacy was larger within 30 days. In contrast, decomposition of the macrophages separated from the ascites liquid after 10 days of TQ consumption has been shown to significantly decrease the expression of pro-tumorigenic M2 macrophages. On the other hand, 30 days of TQ consumption has been reported to significantly increase the expression of these M2 macrophages, while also leading to

the higher expression of antitumor genic M1 macrophages (12). Therefore, the discrepancies between our research and the previous studies in this regard could be due to the differences in the dose and the duration of the consumed supplement. Notably, *Nigella sativa* was injected in the form of nanocapsules in the present study. Some of the limitations of our study were the lack of control over the effects of anesthetic injection and measurement of NNK absorption in the subjects. Our findings indicated the capacity to evaluate the role of CD68-TAM expression in mediating cancer progression and the immune system. The ability to measure CD68-TAM expression and MDA levels allows multiple independent assays to confirm the changes in M2 macrophage activity in rats exposed to NNK. In conclusion, our findings indicated that the consumption of the *Nigella sativa* nanocapsules could decrease CD68-TAM expression and MDA levels, thereby suppressing the adverse effects of NNK.

Acknowledgments

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