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Nigella sativa Methanol Extract Inhibits PC-3 Cell Line Colonization, Induced Apoptosis, and Modulated LC3-based Autophagy

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Abstract

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Keywords

Apoptosis; Autophagy; Nigella sativa; PC3; TIGAR protein

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RESEARCH PAPER

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Abstract

Nigella sativa **has various pharmacological properties and has been used throughout history for a variety of reasons. However, there is limited data about the effects of *N. sativa* (NS) on human cancer cells. This study aimed at observing the roles of methanolic extract of *N. sativa* on apoptosis and autophagy pathway in the Human PC3 (prostate cancer) cell line. The cell viability was checked by MTT assay. Clonogenic assay was performed to demonstrate clonogenicity and Western blot was used to check caspase-3, TIGAR, p53, and LC3 protein expression. The results demonstrated that PC3 cell proliferation was inhibited, caspase-3 and p53 protein expression was induced, and LC3 protein expression was modulated. The clonogenic assay showed that PC3 cell line colonization was restricted. To conclude: *N. sativa* methanol extract had potent anti-cancer effects that inhibited cell viability, induced apoptosis, and inhibited clonogenicity in the PC3 cell line.**

Keywords: Apoptosis, Autophagy, Nigella sativa, PC3, TIGAR protein

1. Introduction

Among men, prostate cancer is classified as a second common type of cancer and the sixth cause of death [1]. Approximately, 1.1 million new cases and 307,000 deaths occurred worldwide in 2012 [2]. Prostate cancer can metastasize to bones and other vital organs and lead to conversion from androgen-dependent growth to androgen-independent growth [3,4].

Programmed cell death is divided into two types: apoptosis and autophagy. Apoptosis is a genetically controlled programmed cell death that allows cells to die. These cells have either completed their function in the organism or have been destroyed without damaging the surrounding cells. Recently, many diseases have been known to be related to cell death. Therefore, the apoptotic process can bring about important treatment methods to treat diseases of uncontrolled cell division [5,6].

Autophagy is a mechanism in which the cell digests its building blocks through lysosomal enzymes when the cell is exposed to physiological factors such as hunger and stress. Many studies have shown that autophagy prevents the deterioration of homeostasis caused by cellular stress in the absence of nutrients. It is also known that autophagy has a role in many events such as morphogenesis, cell differentiation, and death in metabolic arrangements [7,8].

Extracted compounds from natural products have been used against many different types of cancers, including prostate cancer. Anti-cancer research has been carried out with compounds extracted from approximately 250,000 different species of over 1000 plants [9]. *Nigella sativa* belongs to the wedding

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flowers family (*Ranunculaceae*) [10,11]. *N. sativa* seeds have many benefits and are used for a variety of purposes. They are can be found in bread, buns, and some cheeses. Ancient Egyptian and Greek doctors used them to treat toothaches. Furthermore, it has been used in the treatment of nasal congestion and getting rid of intestinal worms [11]. The greatest *N. sativa* extract producers and consumers in the world are Southern Europe, Russia, North Africa, Sudan, Ethiopia, Syria, Turkey, Iran, Afghanistan, and India [12]. Twelve different kinds of *Nigella* species are grown in Turkey. The chemical and pharmacological properties of many of their extracts have not been studied yet [10]. Studies have reported that extracts obtained from *N. sativa* have anti-inflammatory, antioxidant, anti-diabetic, and anti-microbial activity, including anti-fungal and anti-bacterial effects [5,13]. In addition, some researchers found that *N. sativa* extracts have no significant effects on normal human cells. *N. sativa* extracts have no significant cytotoxic effects on noncancerous fibroblast cells [14]. In *Awad* et al.*, 2005*, *N. sativa* oil modulated the fibrinolysis formation and demonstrated the cytoprotective effect on human umblical endothelial cells, human uterine arterial cells (HUA), and endothelial cells (ECs) [15]. Published studies observed the cytoprotective effect of *N. sativa* extracts and decreased H_2O_2 induced cell death in Human Umbilical Vein Endothelial Cells (HUVEC) [16].

The TIGAR protein regulates apoptosis and induces TP53 based glycolysis. It is involved in blocking glycolysis and maintaining cellular metabolism through the pentose phosphate pathway [17] and is considered as part of the p53 tumor suppressor pathway. It has been reported that TIGAR reduces the level of cellular fructose-2,6-bisphosphate (Fru-2,6-P2), which inhibits glycolysis and increases the level of NADPH (Nicotinamide Adenine Dinucleotide Phosphate). It does so by controlling the ROS level and preventing oxidative phosphorylation-induced apoptosis [18,19].

Cancer treatment methods are improving these days. Prostate cancer treatment, on the other hand, is still under investigation. Studies have shown limited investigation about *N. sativa's* effects on human cancer cells and the methanolic extract of *N. sativa*. Traditional treatment methods such as *N. sativa*, however, are utilized in many countries as one of the treatment options for cancer patients. The role of NS extracts on many different types of diseases has been demonstrated, but the effect of methanolic extract of NS extract on human PC3 cells is still unclear. The question is, does a methanolic *N. sativa* extract have the same effect as a traditional pathway such as a *N. sativa* water extract? Or whether the *N. sativa* extract has an adverse effect on TIGAR protein, apoptosis, and autophagy? Does it influence the TIGAR protein to trigger autophagy? This study was conducted to investigate how the methanolic extract of *N. sativa* affected proliferation, apoptosis, and autophagy in the human PC-3 cell line. It also aimed to investigate the effect it had on TIGAR protein in relation to apoptosis and autophagy in the human PC-3 cell line.

2. Materials and methods

2.1. Methanol extraction of N. sativa

The seeds were collected from various local markets in Turkey's Konya province. Five hundred grams of seeds were weighed and placed in a volumetric flask. The seeds were extracted using a Soxhlet extractor and stored at −20 °C with methanol as the solvent. Then, the solvent was removed by a rotary evaporator at 55 °C under vacuum. The *N. sativa* extract was prepared in different concentrations of 10, 25, and 50 μg/ml using DMSO (Dimethyl Sulfoxide) as a solvent. DMEM (Dulbecco's Modified Eagle Medium) was used as a diluter and culture medium for the PC3 cell line.

2.2. Cell culture

The PC-3 cell line was acquired from American Type Culture Collection (ATCC, USA) in vials. The frozen cells were removed from liquid nitrogen and immediately placed in a sterile water bath (Nüve, Turkey) at 37 °C. To avoid ice crystal formation, the cells were thawed rapidly in less than 1 min. Before opening the vial, it was wiped with 70% ethanol and then dried. The cells were carefully transferred to a 15 ml falcon tube (ISO Lab Germany) containing 5 ml pre-warmed growth Dulbecco's Modified Eagle Medium (DMEM), then centrifuged for 3 min at 1500 rpm at 4 °C in a centrifuge (Hettich, Germany). The supernatant was discarded. The cells were gently resuspended in a fresh medium and seeded in a 75 cm^2 flask (Sigma−Aldrich, Germany). The cells were cultured at 37 °C in a humidified incubator (Esco, Singapore) with 5% CO_2. The cells were checked every 24 h and the medium was changed every 48 h.

2.3. Cell proliferation assay

MTT (3- (4,5-dimethylthiazole-2-yl) −2,5-diphenyltetrazolium bromide) cell proliferation kit (Roche, Germany) was used to analyze cell proliferation rate and viability [20]. A 96 well-plate (Sigma−Aldrich, Germany) was used to seed the cells (6×10^3 cells/well). The cells were incubated in humid conditions containing 5% CO_2 at 37 °C for 24 h. Then they were treated with various concentrations of *N. sativa* (10, 25, and 50 μg/ml) for 24 h. When the treatment time had finished, 10 μl of the first MTT labeling reagent (0.5 mg/ml concentration from Roche, Germany) was added to the cells, which were then incubated in the dark for 4 h in a humidified incubator (5% CO_2, 37 °C). The second MTT reagent kit (solubilization reagent from Roche, Germany) was then added (100 μl/well) and the plate was set aside overnight away from any light source. An ELISA reader (Molecular Devices LLC, USA) was used to measure the abosorbance of formazan crystals at a wavelength of 550−600 nm and a reference wavelength of more than 650 nm.

2.4. Colony formation assay

A 6-well plate was used to culture 500 cells/well and incubated for 24 h at 37 °C in humid conditions containing 5% CO_2. The cultured cells were treated with *N. sativa* extracts in a new DMEM medium for 24 h. The medium was changed repeatedly every 72 h and the cells were examined under the microscope. After 12 days of plating, the cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS) and fixed for 5 min in a methanol-acetic acid solution. Finally, the cells were incubated with 0.5% crystal violet for 15 min before being washed with water and dried at 25 °C. The colonies were counted, taking into account that each colony had at least 50 cells and was not in close proximity to one another.

2.5. Western Blotting assay

In a 6-well plate (Sigma−Aldrich, Germany), 500,000 cells/well were seeded and then treated by *N. sativa* extracts as previously mentioned. According to the standard protocol, ice-cold lysis buffer containing 0.01% protease and phosphatase inhibitors (Cell Signaling, USA) was used and poured into the microfuge tube containing the cells as a pellet, followed by 1 h incubation in an icebox and mixing 2−3 times. The debris, small particles, and non-degraded proteins were separated. The protein solution was boiled at 95 °C for 5 min with sample Laemmle buffer in a thermomixer (Eppendorf, Germany) in order to make the proteins linearized and negatively charged. The samples were loaded into a 12% polyacrylamide gel and placed into an electrophoresis chamber. The gel electrophoresis was initiated with the power supply (PowerPack, Singapore) at 20 mA for the first 30 min and then raised to 40 mA. The separated proteins were transferred to a PVDF membrane by a transfer system (Bio-Rad, USA). The membranes were blocked with 5% BSA dissolved in TBS-Tween 20 (0.05% TBS-T) for 60 min. The membrane was treated with the primary antibodies TIGAR, p53, caspase-3, LC3, and GAPDH overnight at 4 °C. The membrane was washed five times with TBS-T before adding the secondary antibody (Santa Cruz Biotechnology, Germany) for 60 min at room temperature. The membrane was immersed in ECL solution (Abcam, UK) for 3 min then stayed side by side with film (Konica, USA) in a dark room. A X-RAY cassette was used. The film was fixed and washed out with an X-Ray machine (Carestream, USA).

2.6. Statistical analyses

The results of this study were statistically analyzed and the IC50 result was extracted by the GraphPad Prism program. One Way ANOVA Tukey test (GraphPad Software, USA) was employed and the results were considered significantly different at P-value < 0.05. However, the result was obtained from three replicated trails ($n = 3$).

3. Results

3.1. Cell proliferation assay

This study investigated whether different concentrations of *N. sativa* extracts would show anti-proliferative activity against the Human Prostate Cancer cell line by MTT assay. PC-3 cells were treated with different doses of *N. sativa* extract (10, 25, and 50 μg/ml) for 24 h. As shown in Fig. 1., the PC3 Cell line was significantly affected and inhibited by the extracts in a dose dependent manner (****$p < 0.05$). In a way, *N. sativa* extracts dramatically inhibited cell proliferation after 24 h of exposure: 10 μg/ml inhibited approximately 29% of cell proliferation (***$P < 0.05$), 25 μg/ml was 36% (****$P < 0.05$), and 50 μg/ml dramatically inhibited approximately 63% of PC3 cell line proliferation (****$P < 0.05$). The IC50 of the treatments on the NS extracts was extracted by the GraphPad prism program. The IC50 of NS methanolic extract was 12.16 μg/ml after a 24-h treatment period.

3.2. Colony formation assay

Colony formation assay was performed to observe the effects of *N. sativa* extract on Human PC3 cell

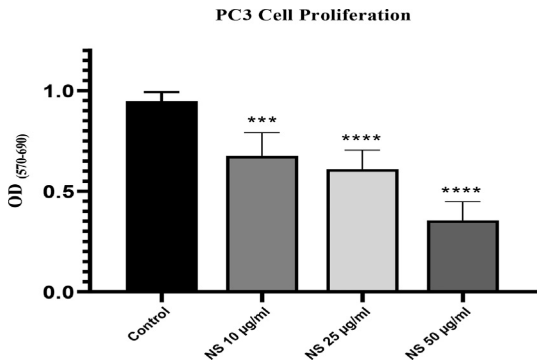

*Fig. 1. Nigella sativa methanol extract at different concentrations (10, 25, and 50 μg/m) affect the human PC-3 cell line and the result was obtained by cell proliferation assay (MTT assay). DMSO was used to dissolve NS extracts and the effect of DMSO on cell proliferation was extracted from the results of the treatments. The percents of inhibition were extracted (10 μg/ml ~32%, 25 μg/ml ~39%, and 50 μg/ml ~66%). The data are shown as mean values (***P < 0.05, ****P < 0.05, and ****P < 0.05). The IC50 was 12.16 μg/ml. The assay was replicated three times (n = 3).*

line colonization. According to the obtained results, *N. sativa* extracts inhibited PC3 cell colonization and the decrease in the number of colonies was dose-dependent. The number of colonies was 101 colonies in the control group, 70 colonies in the DMSO group, 62 colonies in the 10 μg/ml treatment group, 45 colonies in the 25 μg/ml treatment group, and 9 colonies in 50 μg/ml treatment group. According to the data, the colonies were inhibited when treated with 10 μl/ml of DMSO. The effect of DMSO on cell colonization was and the inhibition percentage of NS doses was extracted. *N. sativa* extracts (10, 25, and 50 μg/ml), respectively, inhibited PC-3 cell lines from colonizing after 24 h of exposure. NS extract of 10 μg/ml slightly inhibited the cell line colonization by approximately 8% and 25 μg/ml significantly inhibited approximately 25% of cell colonization. The highest effect of NS extracts was observed in the 50 μg/ml treatment group and cell colonization of the PC3 cell line was dramatically inhibited by ~61%. All results are shown in Fig. 2.

3.3. Expression levels of TIGAR and LC3 proteins

The expression levels of TIGAR and LC3 proteins were detected by the Western blot technique. The TIGAR protein was not significantly triggered when the cell line was treated with NS extracts in different concentrations. The expression level of the autophagic marker LC3 protein was modulated under the effect of NS extracts. A significantly increased level was observed only in one group that was treated with 50 μg/ml after 24 h of exposure when compared to the control group (**P < 0.05) and a noticeable conversion of LC3-I to LC3-II was detected in the same group. The results are shown in Fig. 3.

3.4. P53 and Caspase-3 proteins

To observe the effect of *N. sativa* extracts on autophagy in the PC3 cell line, the amount of p53 and caspase-3 proteins was determined by the

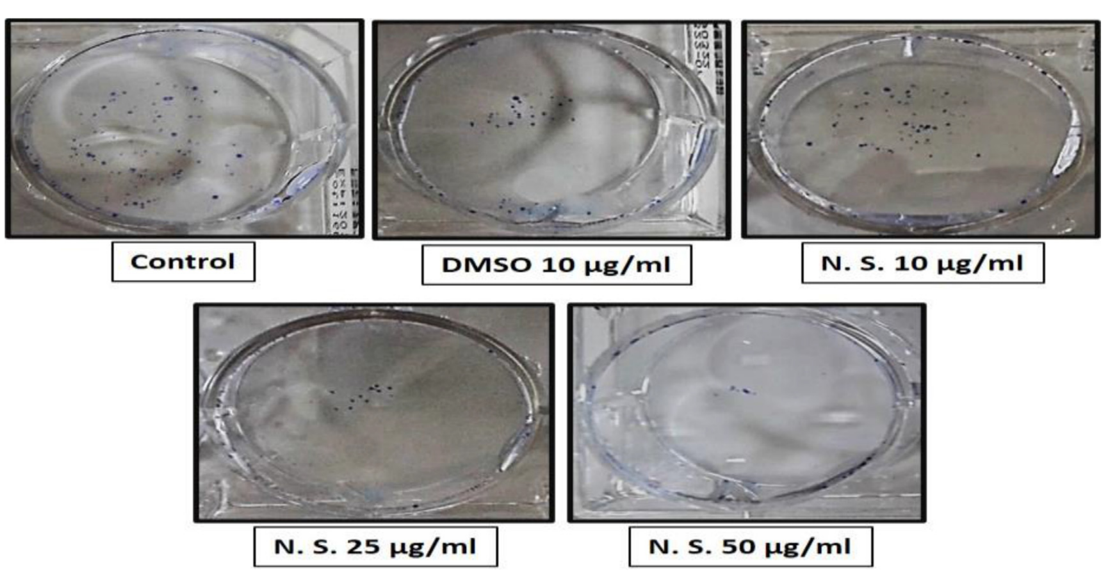

Fig. 2. The effect of various N. sativa concentrations (10, 25, and 50 μg/ml) on the PC-3 cell line was observed by colony formation assay. The percentage of inhibition (~8%, ~25%, and ~61%) of the different doses (10, 25, and 50 μg/ml) are respectively shown. The assay was replicated three times (n = 3).

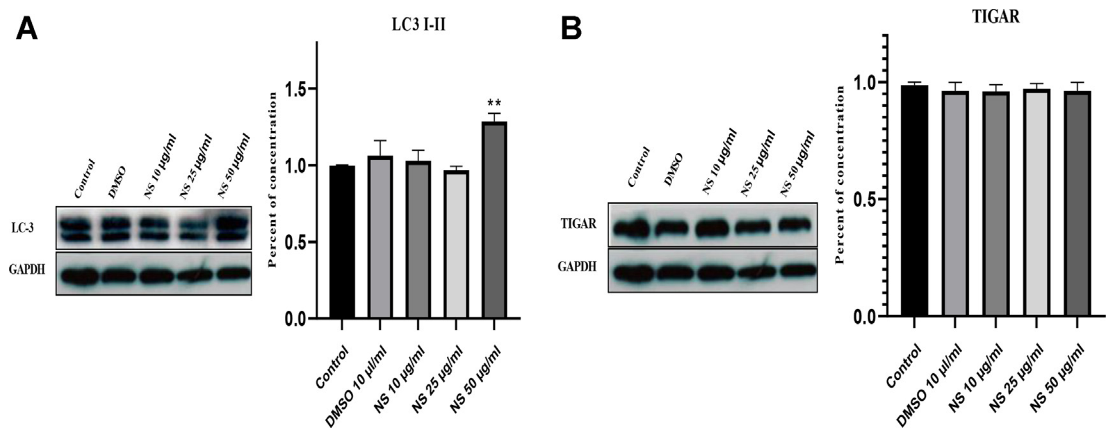

Fig. 3. (A) LC3 proteins expression level and (B) TIGAR protein expression level. The effects of Nigella sativa extract at different concentrations (10, 25, and 50 μg/ml) was observed by Western blot technique. The assay was replicated three times (n = 3).

Western blot technique. The result in this study exhibited that significant expression of p53 protein was observed at both 25 μg (*P < 0.005) and the highest dose 50 μg/ml (*p < 0.005) as shown in Fig. 4A. The expression of the caspase-3 protein was checked and the results demonstrated that low doses of *N. sativa* extracts (10 and 25 μg/ml) slightly, but not significantly, induced the expression of the caspase-3 protein. The significant change was only noticed at a higher dose of NS extract at 50 μg/ml (*P < 0.005), as shown in Fig. 4B.

4. Discussion

Prostate cancer is the uncontrolled growth of the prostate gland. The rate of prostate cancer is categorized according to age. The highest rate occurs in 60−69 years old people [21]. The study carried out by Schulz et al., emphasized the risk of getting prostate cancer increases more in elderly people and the risk of prostate cancer is 10-fold higher in developed countries than in less developed countries [22].

Medical plants have many benefits, including cancer treatment [11]. However, prostate cancer treatment is still in development. Thus, the purpose of this study was to observe some medical characterstics of *N. sativa* extract and focused on the effect of *N. sativa* methanol extract on apoptosis, autophagy, and TIGAR protein in prostate cancer PC3 cell line.

N. sativa is a perennial herbaceous plant, under the family name *Ranunculaceae* [11]. According to some studies, *N. sativa* and its components have beneficial effects against tumor cells, bacteria, and infection. They also exhibit anti-oxidant activity [23,24]. It has been reported that the most effective component of *N. sativa*, thymoquinone (TQ), can be used as an anti-convulsant in mild epilepsy [25]. *N.*

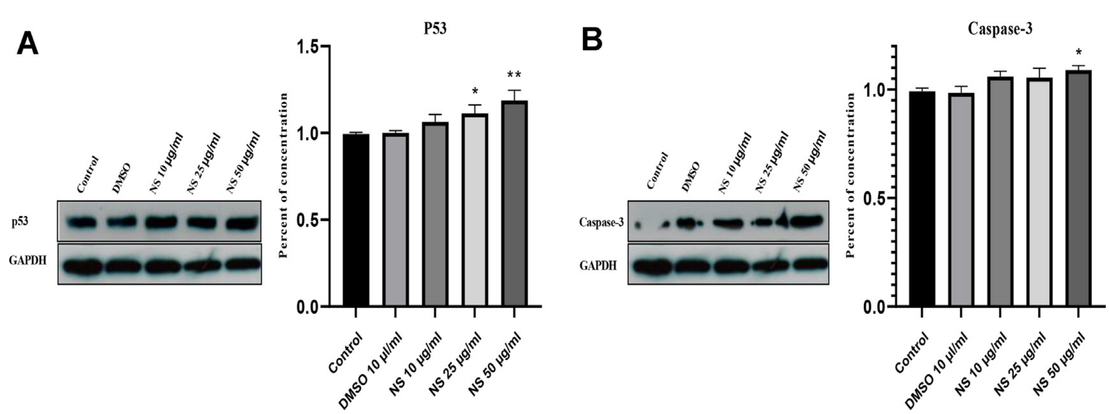

*Fig. 4. Nigella sativa extract enhanced p53 and caspase-3 proteins expression. (A) p53 protein and (B) caspase-3 protein expression results were examined by Western Blot assay. The significant expression of p53 protein was observed at both the 25 μg/ml (*P < 0.005) and the highest dose 50 μg/ml (**p < 0.005). In addition, Nigella sativa extracts induced caspase-3 protein expression at a higher dose of 50 μg/ml (*P < 0.005). The assay was replicated three times (n = 3).*

sativa extracts have been reported to strengthen the bone marrow immune system and stimulate myelopoiesis. Also, *N. sativa* has no toxic effects on normal cells [26]. *Swamy and Tan* (2000), aqueous extract of *N. sativa* seeds (50 μg/ml) have a significant cytotoxic effect against different cells such as: HepG2, MOLT4, and LL/2 [27]. According to studies, *N. sativa* prevents prostate enlargement and cancer [11,28].

In this study, PC-3 cell line proliferation levels were investigated after 24 h of exposure to *N. sativa* methanol extract. The different concentrations of *N. sativa* inhibited PC-3 cell proliferation in a dose depending manner (***$p < 0.05$, ****$p < 0.05$). This study showed that the highest dose (50 μg/ml) had more effect on decreasing cell viability than the other doses (****$p < 0.0001$). The IC50 result was extracted and it is 13.26 μg/ml. The result in our study agrees with recent publications and demonstrated that *N. sativa* extracts plays an anti-proliferation role against PC3 cancer cell lines. For instance, Baharetha et al. 2013, demonstrated the anti-proliferative activity of *N. sativa* against the MCF-7 cell line [29]. On the other hand, *N. sativa* seeds extract had a good anti-cell viability effect against the PC3 cell line [30]. *Farah* et al. *2003,* demonstrated the antiproliferative effect of *N. sativa* ethanolic and aqueous extract [31]. In RAW 264.7 (murine macrophage) and AGS (human gastric adenocarcinoma) cells, *N. sativa* extracts inhibited cell proliferation [32].

Autophagy is a mechanism in which a cell digests its building blocks through lysosome enzymes when exposed to physiological factors such as starvation and stress. LC3 proteins which are involved in autophagy, are thought to provide prolongation of the autophagic sac and its closure with oil [7,33,34]. LC3 Atg8 is an indicator for autophagy and required to form autophagosome. The two forms of LC3 (LC3-I and LC3-II) participate in the autophagic process [35]. Thymoquinone is *N. Sativa's* component, which induced LC3-I conversion to LC3-II in oral cancer cell lines [36]. The role of *N. sativa* methanol extract on LC3 conversion is not understood. So in this study, *N. sativa* extract on LC3 protein in PC3 cell line has demonstrated that the level of LC3-II protein and LC3-I expression and conversion in PC-3 cell line treated with *N. sativa* ethanol extract were noticeably increased at 50 μg/ml (**$P < 0.05$) when compared to the control group. In addition, LC3 protein expression was decreased, but not significantly, when treated with *N. sativa* extract 25 μg as shown in Fig. 3A. The result in this study showed that *N. sativa* ethanol extract triggered autophagy through LC3 protein.

TIGAR protein can inhibit cancer cell proliferation, delay the entry of the cell to the S-phase of the cell cycle, and promoting p53 based cell cycle arrest [37]. The result in this study showed *N. sativa* has no significant effect because the expression level of TIGAR protein has not significantly changed in the PC-3 cell line treated with *N. sativa* extract. Therefore, *N. sativa* extracts inhibited PC3 cell proliferation and triggered autophagy through LC3 protein conversion and expression. However, the level of TIGAR protein did not change significantly and maybe the methanol extract did not trigger TIGAR protein-based autophagy. The literature reported that TIGAR can inhibit autophagy through ROS levels down-regulation [38] and this result agress with our result because the expression of TIGAR protein was not induced when treated with *N. sativa* extracts. On the other hand, *N. sativa* extract may endeavor to induce autophagy through LC3 protein, but TIGAR protein had not participated in autophagy-induced cell death or *N. sativa* has no effect on TIGAR protein expression. On the other hand, maybe the methanol extract triggers autophagy through LC3 protein because TIGAR inhibits autophagy and has a protective effect on cancer cell survival [38]. In our study, TIGAR protein expression is not significantly changed when the cell was treated with *N. sativa* methanol extract. There is a published study showing that TIGAR, under normal conditions, had no significant effect on cell proliferation. In addition, TIGAR knockdown had inhibited cell growth on HepG2 cells treated with epirubicin [38].

Tumor cells are shed daily as part of their movement in blood circulation. Despite the daily hemodynamic stresses and body immunity, the cells continue to enter and colonize in the secondary sites. This depends on the cell migration and the cell invasion cascades [39]. The results of a recent study demonstrated that *N. sativa* methanol extract inhibited cell migration and colonization *in vitro* in the MCF7 cell line [29]. In this study, the clonogenic results demonstrated the inhibition of PC3 cell colony formation *in vitro* by *N. sativa* methanol extract and it indicates that the extract could probably inhibit PC3 cell metastasis. On the other hand, the result in this study coincides with other published articles such as, *Shafi* and colleagues in 2008, who demonstrated that *N. sativa* methanol extract has a role in inhibiting colony formation against PC3 cell line [30]. *N. sativa* CO_2 extraction inhibited cell migration and colony formation *in vitro* in breast cancer MCF-7 cell line [29].

Apoptosis is the mechanism by which the cell programmatically destroys itself. Especially in DNA damage, the cell prefers the apoptosis cell death pathway. P53 and caspase 3 are members of important proteins in the regulation of the apoptosis mechanism. P53 is a tumor suppressor protein. When cell damage occurs, p53 stops cell proliferation and thereby eliminating the damage. If the cell damage is too much to be removed, it directs the cell to apoptosis. Caspases are proteolytic enzymes that belong to the cysteine protease family in mammalian cells. When caspase-3 is activated, an apoptosome structure is formed by releasing cytochrome-C from the mitochondrial membrane to the cytoplasm. Then, the activation of caspase-3 causes protein laceration from aspartic acid regions and the apoptosis mechanism is terminated [40,41].

This study focused on p53 and caspase-3 protein expression to demonstrate whether *N. sativa* methanol extract can trigger apoptosis or not. Many studies show the effects of *N. sativa* extract and its components on apoptosis. Chu et al. in 2013, demonstrated that thymoquinone extracted from *N. sativa* induced apoptosis in the SASVO3 cell line [36]. *N. sativa* extracts (150 and 300 μg/ml) induced caspase-3 protein in RAW 264.7 (murine macrophage) and AGS (human gastric adenocarcinoma) [32]. In this study, p53 and caspase 3 proteins were examined in the PC-3 cell line treated with *N. sativa* methanol extract. A statistically significant increase in p53 and caspase-3 protein expression was detected at the highest dose of 50 μg/ml. Based on the results of this study, *N. sativa* extracts induced apoptosis in the PC3 cell line. Shafi with colleagues in 2008 demonstrated that methanolic extract of *N. sativa* induced apoptosis in the PC3 cell line [30].

Overall, apoptosis and autophagy were incited when the cells were treated with 50 μg/ml of *N.sativa* methanol extract and the cell viability was decreased in a dose dependent manner. *N. sativa* extract decreased the ability of PC3 cells to colonize. The results in this study demonstrated that *N. sativa* can trigger apoptosis, autophagy, decrease cell viability, and inhibit clonogenicity. However, TIGAR protein was not induced and not inhibited by *N. sativa* extract. In consideration of LC3 protein conversion, maybe TIGAR protein inhibited autophagy and then the cell underwent apoptosis. *N. sativa* treatment with TIGAR knockdown may be a viable option for treating cancer cells, resulting in induced autophagy and apoptosis or cytotoxicity-based cell death, thereby decreasing cancer clonogenicity. There are published results that corroborate the previous possibility which demonstrated that TIGAR expression leads to glycolysis inhibition and decreases intracellular (ROS) levels. These functions of TIGAR correspond to the ability to protect cells from apoptosis and autophagy-related ROS [19]. The knockdown of TIGAR protein mildly decreased the cell viability and increased apoptosis. However, knockdown of TIGAR enhanced anti-tumor effects and increased the rate of apoptosis, caspase-3, ROS level [38], and sensitized cells to p53-induced death [19].

5. Conclusion

In conclusion, our finding indicated that *N. sativa* methanol extract has a potent anti-cancer effect that inhibits cell viability, induces apoptosis via caspase-3 and p53 upregulation, induces LC-3-based autophagy, and has the ability to inhibit PC3 cell line colonization. In addition, the expression of TIGAR was not affected so it may be a good possibility to trigger autophagy. However, the role of TIGAR knockdown and *N. sativa* extract together is not well understood; thus, the subsequent study should shed light on TIGAR knockdown and *N. sativa* extract to trigger cancer initiation and progression.

Declaration of competing interest

None.

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