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The Aqueous Extracts of the Nigella sativa Melanin: Experimental in Vivo Test and in Vitro HEp-2 Cell Lines Cytotoxicity Effects

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Abstract: *Medicinal plants have emerged as a promising source of novel therapeutic agent due to their higher structural diversity and potency as compared to standard synthetic chemistry. Recently, melanin pigments had been discovered and thus extracted from the Nigella sativa L. (N. sativa) seed coats, however, the natural protective role of the melanin in the different cell life had been well known. Melanins from Nigella used for several therapeutic activities and thus cytotoxic evaluation for this new extract derived from plant for therapeutic issues immensely need to be well investigated. In this report, to achieve that purpose we hereby investigate the cytotoxic effects and thus the safety margin using of melanin aqueous extracts from N. sativa both in vitro in human HEp-2 larynx carcinoma cells and in vivo tests in both Wistar rats and experimental mice was examined. The cytotoxic inducing (dose-response curves) action of the aqueous extracts of N. sativa melanin was evaluated in vitro according to their effect on cell morphology and the metabolic reduction of CellTietr-Blue (CTB) reagent HEp-2 cells. However, in vivo test of experimental rats live cells was determined by cell histopahological changes through microscopically outcomes. The results showed that melanin extracts was significantly considered highly safe in HEp-2 cells growth according the concentration had been used; indicated that generation of melanin extracted from N. sativa seeds in cell lines might pave the ways of using this herbal melanin as a new bioactive compound and/or a promising biological extracts which derived from plant for the treatment.*

Keywords: *Melanin; N. sativa seeds; HEp-2 Cells; Extract; Cytotoxicity*

I. Introduction

Medicinal plants have been used to treat diseases and illness for many centuries in different indigenous systems of medicine for people and animals as well as folk medicines. Furthermore, herbal medicine is still the main stay of 75-80% of the whole population and the major part of traditional therapy involves the use of plant extract and their active constituents [1, 2]. Following the advent of modern medicine, herbal medicine suffered a setback. In recent years, there has been increasing importance in another therapy and the therapeutic natural products, in particular those derived from plants.

This attention in drugs of plant source is due to some reasons, namely, the frequent inefficiency of conventional medicine, possible enhancement of region particular effects of synthetic drugs. In addition, the long history of use of folk medicine suggests that "natural" products are generally harmless and thus renewed the interest in herbal medicines [3, 4]. *Nigella sativa L.* botanical family Ranunculaceae, have been reported in the scientific literature to contain several active constituents playing protective and/or preventive role in the therapeutic effects of this plant [5]. Furthermore, Aftab and co-workers suggested that the phenolic extracts of *N. sativa* show considerable analgesic and anti-inflammatory pharmacological activities [6]. In correlation to this, another study stated that the therapeutic effects of the *Nigella sativa* (*N. sativa*) plant seed extracts are due to the presence of the thymquinone and fixed seed oil in the chemical constituents of the plant seeds [6, 7]. However, all these previous studies did not take note of the rich presence of melanin in the *N. sativa* seed coats. However, lately there are some reports in the scientific literature had been published and carried out that fact: in the last decades biopolymer melanin had been extracted and/or discovered from many seeds of different plants [8, 9]. However, several other studies discovered, isolated and thus using melanins have revealed a substantial

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occurrence of melanin in the seed coats of the *N. sativa* plant seeds; which considered as one of well known and widely used medicinal and culinary plant in the Arab area and/or at the Muslims area [10, 11].

Hassib and co-worker suggested that the melanin represents around 15% of the *N. sativa* seed coat alone; amounting to around 2.5% of the total mass of the plant seed [11]. On the other hand, it is well known that *N. sativa* seeds had been traditionally used for therapeutic and treatment purposes of several types of diseases and/or illnesses for more than a thousand of years in the Middle East, Far East and Asia [12]. Furthermore, the pharmacological investigations of the plant seeds protect and/or prevent role from several previous works had been revealed a potent anti-inflammatory actions, anti gastric ulcerogenic and anti-oxidant and anti-diabetic activities [13, 14]. Furthermore, recently the herbal melanin had been studied in the immunocellular investigations and found that melanin of the *N. sativa* could playing an amazing role to activate Toll-like receptor 4 (TLR 4) and to release interleukin 6 (IL-6) as well [15].

Many studies have shown a relatively good correlation between *in vitro* (basal cytotoxicity data) and *in vivo* (values). In the scientific methods, to evaluate the toxicological risk of pharmacological compounds without employing *in vivo* experiments, much emphasis has been put on the replacement of acute toxicity (LD_{50}) determinations; however, basically acute toxicity is related to a compound's cytotoxic effects [16], and thus, in previous reports the feasibility of the use of *in vitro* cytotoxicity data for the prediction of *in vivo* lethal doses was tested [17]. Nonetheless, in the literature reviews it was well established that, an early *in vitro* toxicity screening tests might improve the success rate of the new chemical entities in pharmaceutical development used. However, Kola and Landis, [18] found that approximately (around 40%) of the new drug candidates fail in the developmental phase due to toxicological side effects. Notably, Schoonen and co-authors found that the cytotoxicity assays could be identified for 70% of the compounds in compared with known toxicity in either *in vitro* cell lines assay tests [19]. Moreover, Caldwell and co-authors suggested that screening on toxicity and de selection in an early phase of development of drugs may improve the success rate of new chemical entities [20]. However, this strategy implies a large number of compounds for which only a small amount of material is available. Therefore medium or high throughput screening methods are necessary. In this studies, it was thought of interest to examine and/or to investigate for the first time the cytotoxic *in vivo* and *in vitro* effects of the aqueous extracts of NSM and thus supports scientific literature reported for melanins from its other natural life sources, and suggests the use of *N. sativa* seeds and/or its melanin extracts as a rich herbal-melanin source in future approaches for protects and/or as a promising treatment of such humans and animal illnesses and diseases, however, the cytotoxic effects of the NSM, prior to this study have not been yet totally investigated.

II. Materials And Methods

2.1. In vivo experiment

2.1.1. Preparation of the NSM solution

The extraction and characterization of melanin from *N. sativa* L. seeds have been carried to prepare an aqueous extract solution of melanin performed to injected I.P. The seed coats of the *N. sativa* plant were solubilized in an alkaline solution of NaOH (pH =12.5) for 3 h, which yielded a dark brown solution. The solution was dark but clear and did not indicate any suspension. This stock solution was then centrifuged and filtered and melanin was precipitated from it at (pH 2) using conc. HCl. This alkali–acid treatment was repeated 2–3 times to ensure a higher purity of melanin. The precipitate was thoroughly washed with distilled water, filtered out and dried at 80 ˚C. The dry powder was stored and used later to prepare solutions at pH 7 for biological studies by redissolving the desired amount of melanin powder (in w/w ratio) in NaOH solutions (at pH= 12.5) and using conc. HCl to adjust the pH to 7 to obtain a desired concentration to be injected into the animal models.

2.1.2. Rat's treatments

Male Wistar rats (235 ± 10 g body weight) were obtained from College of Pharmacy, King Saud University, Saudi Arabia. They were randomly divided into 4 groups (n= 5 animals per group) and then maintained in normal rat chow and water *ad libitum*. They were housed in cages in a room at temperature of 22 ± 2 ˚C and a relative humidity of 55 ± 5%. The light/dark cycle was 12/12 h. The dry powder of melanin used to prepare solutions at (pH 7) by redissolving the desired amount of melanin powder (in w/w ratio) in NaOH solutions (at pH 12.5) and using conc. HCl. The first group was injected intraperitoneally (i.p.) with normal saline (50 mg/kg i.p.) and served as control group. While, group 2, 3 and 4 were injected daily for three days with NSM extracts at 20, 50 and 100 mg/kg. i.p, respectively. After 3 days rats were anesthetized and liver tissues were collected.

2.1.3. Histological method

The tissue (liver and kidneys) samples were collected and fixed by immersed them in 10% neutral buffered formalin (NBF) for 72 hours. the samples were processed overnight by passing them in ascending

series of alcohols (dehydration), clearing with three changes of xylene and infiltrated in melted paraffin wax using automatic tissue processor (VIP tissue, Tek 5 Jr, Sakura, Japan). Tissue samples were embedded in paraffin blocks. Rotary microtome (RM2245, Leica, Germany) were used to cut four micron thickness sections. The sections were stained with hematoxylin and eosin stains (H&E), mounted with DPX before viewing under the light microscope with mounted camera for interpretation and taking required images.

2.2. In vitro experiment

2.2.1. Preparation of NSM for citotoxicity assay

For *in vitro* cytotoxicity studies, each weighed test drugs were separately dissolved in distilled dimethylsulphoxide (DMSO) and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock concentration (1 mg/ml) and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

2.2.2. Cell culture

Human larynx cancer (HEp-2) cells were obtained from the Virology Department, College of Science, King Saud University, Saudi Arabia. The HEp-2 cells were cultured in Dulbecco's modified agle's medium (DMEM) supplemented with 10% (v/v) of fetal bovine serum (FBS; Gibco, Grand Island, NY), 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml) (Sigma, St. Louis, MO). Cells were seeded at an appropriate cell density in a well plate. However, the HEp-2 cell lines were then treated with medicinal plant extracts in different concentration prepared already and kept in maintenance medium containing 1% FBS, L-glutamine and antibiotics. Cells were incubated at 37°C with 5% CO_2.

2.2.3. Determination of in vitro cyto/toxicity

Monolayer cultures of HEp-2 cells with (80-90% confluence) were prepared in 96 well plates. After removal of culture medium, cells were washed twice with phosphate buffered saline. Two-fold serial dilutions of the extract were prepared in maintenance medium starting from the concentration 8 to 1000µg/ml and added to cells in triplicates. Wells that received maintenance media only were served as cell (negative) controls. All cultures were kept at 37°C in CO_2 incubator for 72 hours with daily observation for morphological changes under phase contrast inverted microscope connected with a digital camera (Olympus IX51, Tokyo, Japan) at 20 and 40X magnification.

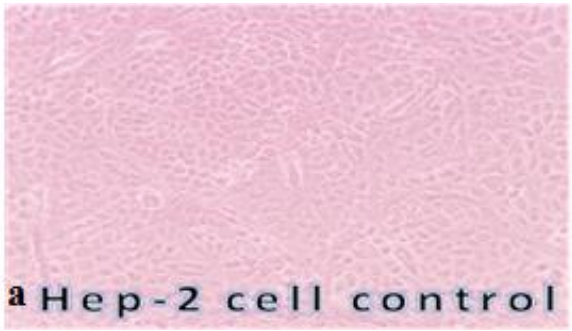
III. Results

3.1. Morphological changes

Alterations in the morphology of HEp-2 cells exposed to aqueous extracts of the NSM were found to be in a concentration dependent manner. The morphological changes observed in HEp-2 cells are shown in Figures 1. Cells exposed to >1000 mg/ml for 3 days reduced the normal morphology and cell adhesion capacity of HEp-2 cells as compared to control (Figure 1, 2 and Table 1). As shown in Figure, the most of the cells exposed to high concentration of NSM seeds lost their typical morphology and appeared smaller in size particularly with higher doses 1000 µg/ml. However, histopathological sections of liver for laboratory rats which treated with NSM extracts showed no histopathological changes and tissue feature shown in Figure 3.

3.2. Cell Titer-Blue (CTB) assays

The cytotoxicity of aqueous extracts of melanin which derived from *N. sativa* plant seed coats was assessed using CTB assays, after exposing the HEp-2 cells at 8 to 1000 µg/ml concentrations for 72 hr. The percent cell viability of HEp-2 cells against melanin extracts as observed by CTB assays. Result shows that NS melanin decrease in cell viability of HEp-2 cells in a concentration dependent manner. The HEp-2 cells exposed to Nigella melanin at >1000 µg/ml was found to be toxic. Nevertheless, the aqueous extracts of the Nigella melanin at <1000 µg/ml and lower did not show any decrease in the cell viability



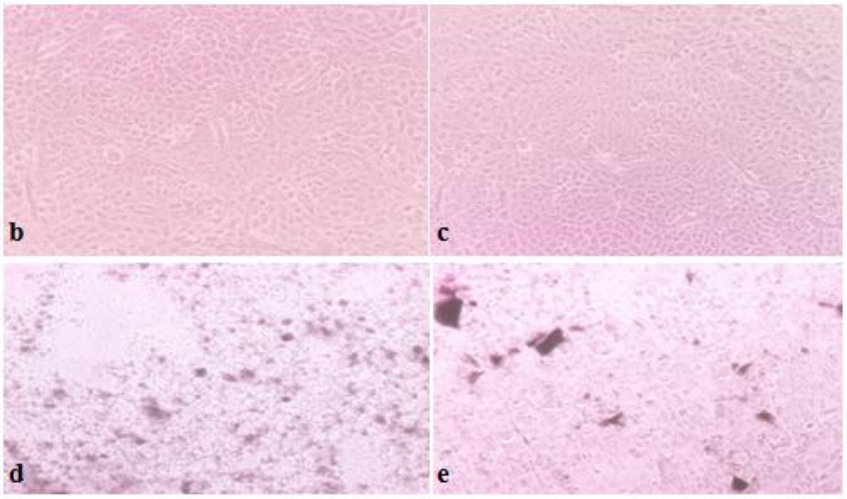


Figure 1. (**A**) The morphological changes before/after using aqueous extracts of melanin derived from *Nigella sativa* (**NSM**) seeds on HEp-2 el lines at a different concentration. (**a**) HEp-2 cells without treatment serve as negative **control** group. (**b**) HEp-2 cells with final concentration of NSM at (125µg/ml). (**c**) HEp-2 cells with final concentration of NSM at (250µg/ml). (**d**) HEp-2 cells with final concentration of NSM at (500µg/ml). (**e**) HEp-2 cells with final concentration of NSM at (1000µg/ml). After 72 h alteration of HEp-2 cells and morphological changes were monitored and images were captured using camera bounded inverted microscope (100X) (Eclipse TS100, Nikon, Japan).

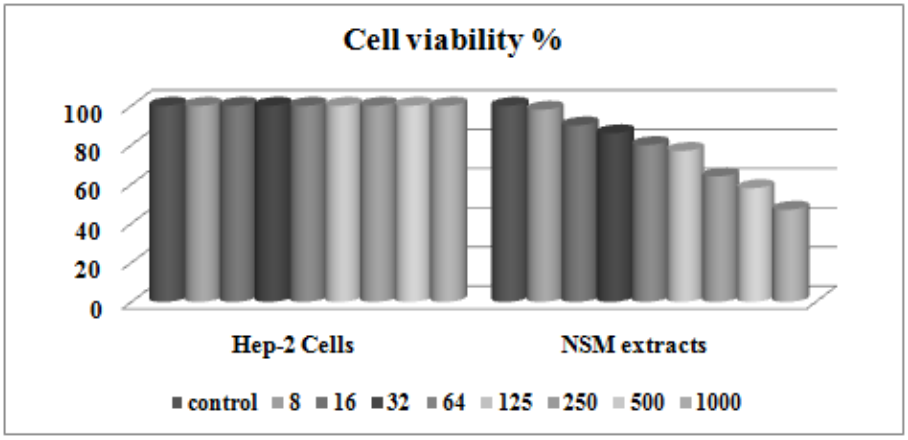


Figure 2. The aqueous extracts of NSM in different concentrations compared to HEp-2 control cell lines. NSM extracts caused a significant death with maximum amount of loss viable cells at 1000µg/ml. Very less amount of viable cells were detected at high concentration which shows the cell death and minimum lethal dose as compared to the low concentration which ranging from 8 –500 µg/ml.

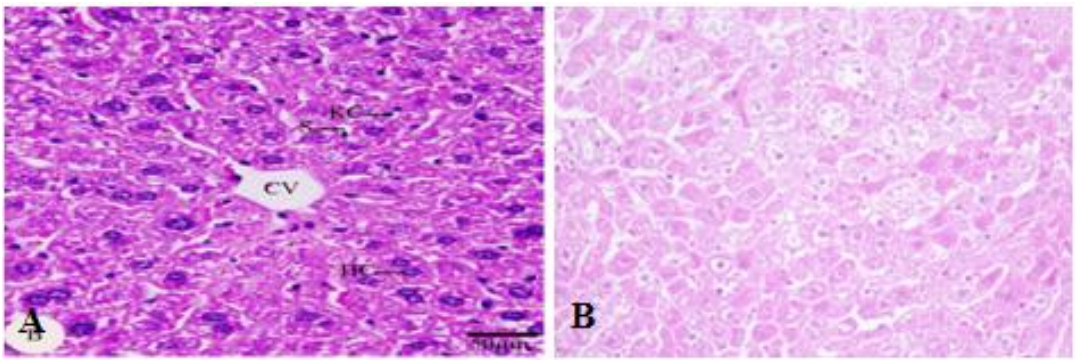


Figure 3. The sagittal sections of the histological structures of liver and kidney. (**A-B**) Sections of **liver** showing: central vein (CV). (**a**) **Control** group. All control rats were received normal saline (i.p.) during the study. (**b**) **NSM** aqueous extracts at high dose of 100 mg/kg group. Scale bar= 50 and/or 200 µm.

Table 1. *In vitro* HEp-2 cells microscopic cell-based cytotoxicity assay

The plant	**Concentrations (µg/ml)**								
	HEp-2	8	16	32	64	125	250	500	1000
Cell viability%	100	98	90	86	80	77	64	58	47
Extractions	-	-	-	-	-	-	-	-	+

Note: **HEp-2**= human larynx carcinoma in vitro cells as control cell lines.
(+)= positive (morphological alterations, cytopathic effect (CPE) forming);
(-) = negative (No morphological alterations no CPE).

IV. Discussion

Using cytotoxicity assay of the *in vitro* methods for around 110 compounds, it was shown that *in vitro* assay data resulted in good estimates for about 70% between basal cytotoxicity data of the *in vitro* of all these compounds and human lethal blood concentrations [19]. This implies that for about 30% of the cases the estimates on the basis of cytotoxicity data deviated from *in vivo* findings. However, this correlation means that a certain number of misclassifications have to be faced when using the existing tests [21]. These deviations from a simple linear relationship between effective concentrations *in vitro* and toxic doses *in vivo* can result from the fact that the effective concentrations *in vitro* are irrelevant for the concentrations that may cause toxicity at the target site in target organs *in vivo* [22].

Herbal medicines are culturally accepted among Greeks, Arabs, Indians, the Chinese in the old world and widely used in many countries for treatment of disorders and hence are of great importance as a mechanism to increase access to health care services. However, the use of wild herbs in folk medicine is old as man himself. Traditionally, such habits have been inherited by successive generations, and, thus, some of the plants became well known for their uses especially by herbalists. Lately, several studies regarding therapeutically effects of melanin which derived from the *N. sativa* seed coats (NSM) had been published [13, 23].

However, in-depth survey of relevant scientific literature regarding the *in vitro* cell line test for NSM there was only one previous report in human monocytic THP-1 cells and human embryonic kidney 293 (HEK293) cells in the induction of interleukin-8 (IL-8) by PBMC [15] and there was no any other report used *in vitro* cell line tests to evaluate the cytotoxicity assay of NSM extracts. Thus, and due to the importance of the cytotoxic assay test in pharmacological field, we report in this study the cytotoxic effects of the aqueous extracts of NSM on both *in vivo* refers to experimentation using a whole, living Wistar rats liver and *in vitro* refers to the technique of performing a given procedure in a controlled environment outside of a living organism using cell line tests of HEp-2 cells.

Basically, most of melanins is produced in life from different natural sources and playing a vital role in keeping cell healthy and highly safe and protected from all outside and/or each environmental badly side effects including oxidation, free radicals (e.g. peroxides, superoxide, hydroxyl radical, and singlet oxygen) acts and ultra violet and damage rays in human, animal, plant, fungus, bacteria and microorganisms [24, 25]. Therefore, melanin pigment was found in human cells, animals, plants and microorganisms which are found in skin, hair, eyes, inner ear, substantia nigra, fertilized ova, seed coats, and cell walls [26].

Recently, some bodies concerned about the evaluation of cytotoxicity of new chemical compounds via focusing in the positive results of *in vitro* testing which lead to follow-up *in vivo* tests. However, other bodies such as the cosmetics in EU no longer allow follow-up *in vivo* testing and thus a substance may be banned based on positive *in vitro* data only [27]. Our data are, however, the first to specify that NSM aqueous extracts from Nigella seeds has a direct cytotoxic effect on the cell lines *in vitro* and *in vivo* test. Nonetheless, regarding our cell line *in vitro* tests by human larynx carcinoma HEp-2 cells these findings were in line with Abdel-Hameed and co-workers [28] when stated that the American National Cancer Institute (NCI) guidelines set the limit of activity for crude extracts at 50% inhibition (IC_{50}) of proliferation of less than 30 µg/mL after the exposure time of 72 hours. However, the results of the IC_{50} value showed the potent cytotoxic effects of NSM extracts on HEp-2 cells and were found to be lower than that specified by NCI, thus, NSM due to these results might be applied, within novel approaches, for boosting of anticancer immunotherapy. Furthermore, our cell line *in vitro* result showed that there were concentration-dependant cytotoxic effects of extracts on the tested cell lines, however, the cytotoxic effects of the aqueous extracts of NSM was increased due to increasing of the doses of extracts. This was completely agree with *in vitro* study done by Tai and his colleague when demonstrated that a concentration-dependant antiproliferative effect of Sutherlandia on several tumor cell lines, and they also showed that Essiac and Flor-Essence herbal teas had demonstrated antiproliferative and differentiation inducing properties *in vitro* only at high concentrations [29]. The present results also were consistent with that reported in the study of Al- Jeboori and co-authors when studded with co-workers the cytotoxic effects of aqueous and ethanolic of *Artemisia herba alba* on human laryngeal carcinoma (HEp-2) cell line and other mammary adenocarcinoma cells and found that the HEp-2 cell line was showed high significant difference and affected after treatment with high concentrations of the aqueous extract of *Artemisia herba alba* for 48 hrs in

concentrations (1250,2500 and 5000 µg/ml) [30]. In addition, the present results were consistent with that reported in the study of Campbell and co-workers in which dose-response curves were obtained for several of the most potent crude extracts [31].

In correlation, a several work suggested that NSM could play an important role to modified cytokine production and was proposed as toll like reseptor-4 (TLR-4) ligand [23]. Also it degraded IκBα, induced caspase 8 cleavage and IL-8 and IL-6 production through NF-κB signaling in TLR4-transfected and TLR4-expressive cell lines; therefore, it may have a potential role in infectious diseases and cancer [32]. Notably, a previous *in vitro* study in (PBMC) cell lines had been suggested the ability of the NSM aqueous extracts to be considered as a powerful stimulant of TLR-4 expressing cells when it activate TLR-4 to stimulate the release of the IL-6 and thus could be considered as one of a very important promising anticancer agent in the very near future [15].

Finally, in our work, the aqueous extracts of melanin derived from *Nigella sativa* (NSM) seeds treated in a different concentration in ranging from (8 - 1000 µg/ml) on HEp-2 cells lines as normal control kidney cells derived from human larynx carcinoma patient's. Thus, the less amount of viable cells and/or maximum significant death was detected at concentration of 1000µg/ml as compared to low concentration. While decreasing the concentration of extracts increasing viability of cells showing negligible amount of cell death; we only select the high extracts concentrations treated in HEp-2 cells of 125-1000 µg/ml. In correlation to this, in histological *in vivo* test comparing the liver tissue with the high dose of 100 mg/kg body weight had been selected only, to found out the liver tissue changes due to using the higher doses of NSM aqueous extracts in live rats, because there was several reports suggested that in some studies regarding histological issues regularly the higher plant extracts doses might have some negative effects on the normal tissue functions particularly with the *N. sativa* seeds on the liver functions of experimental rats [33] and/or it may cause internal histopathological cell damages [34], so we select hereby the higher dose, however, the small doses of NSM extracts at 20 and 50 mg/kg. (i.p.), respectively, was not changing any of liver tissue similar to the high dose of the extracts (data not all shown here).

V. Conclusion

The results in this study was showed that cell growth is significantly lower in NSM extracts treated HEp-2 cell lines when histological and/or morphological changes were compared to untreated control. The effect of cells was higher in the treatment of both *in vivo* and/or *in vitro* cells when the concentrations of the NSM aqueous extracts were increased. Based on the results it is determined that NSM aqueous extracts driveled from *N. sativa* seed coats is a significant source of biologically active substances that have cytotoxic and anti proliferative activity *in vitro*. Thus, application of NSM is proposed as a promising clinical alternative in therapeutic and/or treatment issues. Further investigation on the detailed mechanism of NSM extracts versus protective, preventive and toxicity and/or cytotoxic activities and effects in experimental live cells and cell lines is underway.

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