

## CYTOTOXICITY EVALUATIONS OF THE AQUEOUS EXTRACTS OF HERBAL MELANIN DRIVED FROM *NIGELLA SATIVA* SEEDS USING *IN VIVO* AND *IN VITRO* MODELS TESTS

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### ABSTRACT

**Background:** Many studies over millennia have been carried out to determine a widely used medicinal plant throughout the world. Of these, most of the therapeutic properties of *Nigella sativa* (*N. sativa*) seeds are due to the presence of thymoquinone which is major bioactive component of the essential oil outcome, whereas melanin was unknown to exist in *N. sativa* before despite the fact that the seeds have a widely used medicinal plant throughout the world still need more studies. **Objective:** To investigate the cytotoxic effect of aqueous extracts of melanin extracted recently from *N. sativa* (NSM) seed coats on *in vivo* and *in vitro* safety outcome and thus used as a new bioactive contents and/or a promising natural therapeutic extract from a plant.

**Materials and Methods:** Wistar rats and experimental mice *in vivo*

tests with different NSM aqueous extracts doses according to their weight through intraperitoneal injection (i.p.) have been undertaken. Also, histopathological *in vitro* section tests using rat's liver, intestines, kidney and testes segments were also observed and LD<sub>50</sub> was calculated. Either, different doses of NSM for *in vitro* viability of cells performed on Vero cells to analyses the cytotoxic activity of the extracts obtained. It was determined by cell death assay using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) colorimetric assay tests. Also, morphological changes and CC<sub>50</sub> were obtained and/or

calculated. The cytotoxic effects of NSM extracts in experimental animals found safely, inducing action of NSM extracts in dose-response curves for each cell. **Results:** study showed that NSM extracts were significantly high safety during cell growth in a dose-dependent manner without inducing damage of *in vivo* and *in-vitro* cells used. The obtained data indicated the possible therapeutic and safety actions of the NSM aqueous extracts and that might pave the ways of its using as new bioactive compound and/or a promising health extract derived from a plant. **Conclusion:** The present work is an effort to provide *in vivo* and *in vitro* detailed and safety margins in using melanin extracts of the literature on scientific researches of the pharmacy-toxicological guide and thus lightening presence of melanin from *N. sativa* seeds.

**KEYWORDS:** Cytotoxicity, Melanin, *Nigella sativa*, Vero cells, MTT assay.

## INTRODUCTION

*Nigella sativa* L. (*N. sativa*), of the botanical family Ranunculaceae, have been reported in the scientific literature to contain essential oil, fixed oil, saponins, alkaloids, flavonoids and polyphenolic compounds.<sup>[1]</sup> Hajhashemi and co-authors have been reported that the phenolic extracts of *N. sativa* seeds show considerable analgesic and anti-inflammatory pharmacological activities.<sup>[2]</sup> Many other authors have demonstrated therapeutic effects for *N. sativa* seeds extracts or its constituents such as thymoquinone and fixed seed oil.<sup>[3]</sup> Furthermore, seeds of *N. sativa* and their oil have a long history of folklore usage in various systems of medicines for a variety of conditions and treatments related to respiratory health, stomach and intestinal health, purgative, pityriasis, eye-scores, snakebite and scorpion stings<sup>[4,5]</sup> and it is used to treat many diseases such as hypercholesterolemia, diabetics, tumor and gynecological disorders for several years.<sup>[6,7,8,9]</sup>

Islamic medicine regards the *N. sativa* plant seeds as one of the greatest forms of healing medicine available. However, the Islamic prophet Mohammad once stated that the black seed can heal every disease except death. Avicenna, most famous for his volumes called The Canon of Medicine, refers to *Nigella* as the seed that stimulates the body's energy and helps recovery from fatigue and dispiritedness. It is also included in the list of natural drugs of 'Tibb-e-Nabawi'.<sup>[10]</sup> However, most of the studies on *N. sativa* did not take note of the rich presence of melanin in this plant. Literature reviews of the last few decades report the discovery of melanin biopolymers in many plant seeds.<sup>[11,12]</sup> However, several recent studies

have revealed a substantial occurrence of melanin in the seed coats of the *N. sativa* (NSM) plant.<sup>[13,14]</sup>

In these studies, melanin represents around 15% of the seed coat alone; amounting to around 2.5% of the total mass of the seed. Melanin was unknown to exist in *N. sativa* before despite the fact that the seeds have traditionally been used for thousands of years in the Middle East, Far East and Asia as a food additive and as a herbal health aid.<sup>[15]</sup> This study considers for the first time the cytotoxic effect of NSM aqueous extracts on *in vivo* and *in-vitro* and thus supports findings of scientific literature reported for melanins from other natural life sources, and suggests the use of *N. sativa* seeds and/or its melanin extracts as a rich source of Herbal Melanin for future applications of health protection and/or as a promising treatment of many diseases.

To the best of our knowledge, there are no reports of cytotoxic effects of NSM aqueous extracts using monkey Vero cell lines until now, however, in-depth survey of relevant scientific literature we found that NSM has been used in studies on human monocytic THP-1 cells and human embryonic kidney 293 (HEK293) cells in the induction of IL-8 by PBMC.<sup>[16]</sup>

## MATERIALS AND METHODS

### Morphology of the plant

*N. sativa* is an annual flowering plant which grows to 20-90 cm tall, with finely divided leaves, the leaf segments narrowly linear to thread-like. The flowers are delicate and usually colored white, yellow, pink, pale blue or pale purple, with 5-10 petals. The fruit is a large and inflated capsule composed of 3-7 united follicles, each containing numerous seeds.<sup>[17,18]</sup>

### A collection of plant material

The herbal melanin extracted from *N. sativa* seeds L. which used in this study has been supplied as a gift by its discoverer and manufacturer Professor Adil Magzoub Haseeb, College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia.

### *In vivo* methods

#### Rats' treatments

Twenty male albino Wistar rats, 4 – 5 months old weighing approximately 220 – 250 g, were used in the present study. Rats were maintained under controlled temperature, humidity and 12 hour light/dark cycles. The animals were fed standard rodent chow and allowed free

access to water ad libitum and were kept for one week prior to any procedure to allow proper acclimatization. Animal care and use were approved by the Ethics Committee of Faculty of Dentistry- King Saud University-Saudi Arabia.

The experiments were carried in accordance with the Guide for the Care and Use of Laboratory Animals. After acclimatization, rats were divided randomly into the following groups (5rat/group):

- Control group; animals in the control group were injected with normal saline single intraperitoneal (i.p) doses. Rats in the groups 2, 3, 4 and 5 were treated by a single intraperitoneal (i.p) injection of the aqueous extracts of the NS melanin at doses of 10, 20, 50 and 100 mg/kg, respectively, for 3 consecutive days. Control rats and NS melanin treated groups were all sacrificed either just before (0), or after 1, 2 and 3h following normal saline and/or aqueous extracts of NS melanin treatment.
- Initially, pilot experiments were performed in rats to find out the effectiveness of melanin in reducing blood glucose levels. Melanin was tested in (i.p.) at a dose up to 400 mg/kg (data not shown).

### **Preparation of the NSM solution**

The extraction and characterization of NSM extracts have been carried as described before.<sup>[19]</sup> Briefly, the *N. sativa* seeds were solubilized in an alkaline solution of NaOH (pH 12.5) for 3h. This solution was then centrifuged and filtered and melanin was precipitated from it at pH 2 using cons. HCl to regulate the pH to 7. However, this treatment procedure was repeated for almost 3 times to ensure a higher purity of melanin solutions wished for this experiment and thus it was thoroughly washed with distilled water and filtered out till it completely was dried and keep at 80°C and served as aqueous extracts of melanin until the time of using later.

### **Toxicological melanin Information**

#### **Determination of Lethal Dose<sub>50</sub> (LD<sub>50</sub>) in rodents**

Five grams of melanin powder was dissolved in 80 ml of 2M NaOH in water by the aid of thorough mixing. The solution was then neutralized, using 2M HCl, too (pH =7). The volume was then completed to 200 ml using distilled water (approximately 40 ml water) several batches of the solution were then prepared.

### **Mice treatment**

Male albino white mice (weight 25 g) and Wistar rats (weight 200 g) were divided into groups (n = 10 animals per group). For the determination of intraperitoneal (i.p.) LD<sub>50</sub> doses, the animals in the different groups were injected i.p. with doses of 20, 50, 100, 200, 400 and 800 mg/kg. Following treatment, all the animals were observed for six continuous h and thereafter at intervals 2–12 h. All behavioral changes and death during the observation period were recorded.

### **Skin allergenicity tests**

In Wistar rats (weight 200 g) (n = 6 animals per group), a skin area covering 20 cm<sup>2</sup> was cleared from hair in each rat using thioglycollate hair remover. Three h later, melanin solution (25 mg/ml) was applied to cover the whole area. The treated area was then observed for 60 minutes to note any redness or edema. The control group was treated with melanin vehicle: Skin irritation tests in rats: application of 1 ml of 2.5% aqueous solution (pH =7) did not produce any vasodilatation redness or edemas. Eye irritation tests white New Zealand rabbits: application of 0.5 ml of 0.25% aqueous solution (pH=7) produced only very slight redness but no lachrymation (all data are not shown).

### **Histological method**

The tissue (Kidney, liver, intestine, and testes) samples were collected and fixed by immersed them in 10% neutral buffered formalin (10% NBF) for 72 h. 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. To detect the histopathological changes might occur in sacrificed rats' cell sections; all cells were the stained laboratory with hematoxylin and eosin for that purposes, mounted with DPX before viewing under the light microscope with a mounted camera for interpretation and taking required images.

### ***In vitro* evaluation of the NSM extracts cytotoxicity**

#### **Experimental design**

Vero cells were exposed to various concentrations of NSM aqueous solutions (8-2500mg/ml) for a period of 72 h. Following the exposures of NSM, Vero cells cytotoxic responses were

assessed using MTT assays and cellular morphology using a phase contrast inverted microscope.

### **Preparation of NSM solutions**

For *in vitro* cytotoxicity studies, the NSM aqueous extracts were not completely soluble in natural Ph aqueous medium solution; therefore the stock solutions of all the extracts were prepared by dissolving in dimethylsulphoxide (DMSO) and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock concentration (of 1 mg/ml). The solutions were subsequently sterilized by filtration. Serial two-fold dilutions were prepared from this for carrying out cytotoxic studies.

### **Cell culture**

African green monkey kidney (Vero) cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% 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 treated with NS plant extracts were kept in maintenance medium containing 1% FBS, L-glutamine, and antibiotics. Cells were incubated at 37°C with 5% CO<sub>2</sub>.

### **Microscopic examination for morphological alterations**

Monolayer cultures of Vero 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 64µg/ml to 2500 µg/ml and added to cells in triplicates. Wells that received maintenance media only were served as cell (negative) controls. Wells that received methanol 70% were served as positive controls. All cultures were kept at 37°C in a CO<sub>2</sub> incubator for 72 h 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. Cellular alterations were recognized in the form of cell rounding, granulation, vacuolation, degeneration and lysis, as well as the detachment of the monolayer. The minimal toxic concentration (MTC) was identified as the last concentration that induces toxic effect(s) on culture cells as detected microscopically after 72 hr incubation.

### Cytotoxicity screening

Percent cell viability was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay developed by Mosmann.<sup>[20]</sup> The MTT assay with modification was used to screen the cytotoxic activity of the plant extract and thus measuring the extracts toxicity. Briefly, cells ( $1 \times 10^4$ ) were allowed to adhere for 24 h CO<sub>2</sub> incubator at 37°C in 96 well culture plates. After 72 h of incubation with crude extracts, the cells were rinsed with 1X PBS and incubated with 100 µL of 0.5 mg/mL MTT at 37°C. After 30 min of incubation, the dark blue crystals of formazan (MTT metabolites) were dissolved in 100 µL of DMSO. The level of reduced MTT was determined by measuring the difference in absorbance at 570 nm using a micro plate reader (Spectra Max M5, Molecular Devices). Untreated sets were also run under identical conditions and served as control.

### MTT Cell viability assay

The cytotoxicity of NSM aqueous extract was determined by using MTT assay (MTT-Cell Proliferation Assay Kit, Tervigen) tests. Vero cells (culture monolayer) are negative culture without treatments as control and/or other three including treated cells with NSM extracts. In this assay, the number of viable cells was determined calorimetrically in 96-well plates. In the independent set of experiment, following the incubation of confluent monolayer cultures of cells with two-fold dilution series of plant extracts for 72 hr. After 4 h of incubation at 37°C, the optical density (OD) was recorded at 570 nm in a microplate reader (BioTek, ELx800). Nonlinear regression analysis was performed in Excel software to determine the cell viability (%) using the following equation formula:

$$\text{Cell viability (\%)} = \frac{\text{OD(a)} - \text{OD(p)}}{\text{OD(c)} - \text{OD(p)}} \times 100$$

[Where OD(a) = absorbance of the assay well sample, OD(p) = the absorbance of positive control and OD(c) = is the absorbance of cell control, respectively].

### Cytotoxicity conditions

Using *in vitro* test, the 50% cytotoxicity concentration (CC<sub>50</sub>) was calculated as the concentration of the plant extract that induced the reduction in cell viability to 50%. Even though, the average of three assays with at least five concentrations within the inhibitory range of the compounds. The OD 570 nm of assay well, untreated and of treated cells, respectively. The CC<sub>50</sub> of each compound were obtained from dose-effect curves and the

absorbance of the extracts or fractions when compared with cell and cell controls (all data are not shown).

## RESULTS

### Determination of the *in vivo* LD<sub>50</sub>

The percentage of death in each group was then calculated. The Lethal Dose<sub>50</sub> (LD<sub>50</sub>) dose i.e. the dosage that kills 50% in a group of animals, was then calculated: Acute toxicity studies have been conducted on rats and mice. Acute i.p. LD<sub>50</sub> in Mice was found to be 400 mg/kg and in Wistar rats was found to be 412.5mg/kg. For determination of the oral LD<sub>50</sub> doses, the procedure used above was repeated except that the melanin solution was administered by gavage. Doses of volumes more than 2 ml were administered at intervals of 2h during the same day. The animals were then observed as indicated above and the LD<sub>50</sub> values were calculated: Acute oral lethal dose, given in 6 doses every 2 h, (LD<sub>50</sub>) for mice were found to be 1600 mg/kg and 1500 mg/kg for rats.

### Histopathological changes

As shown in Figure 1, the histopathological analysis indicated that an experimental rats which had been treated with NSM aqueous extracts and served as treated group (used only melanin extracts at high dose of 100 mg/kg (i.p.); all the sagittal sections such as kidney, liver, intestine and testes had no any percentage of changes when compared with the same sagittal section of those cells in control rats which had no any previous kinds of treatment, however, control rats were only received normal saline (i.p.) during study.

### Cell MTT assays

We have examined the cellular cytotoxicity effect of different concentrations of the aqueous extracts of NSM doses from 4 to 1000 µg/ml concentrations for 72 hr of each extract after exposing Vero cells line population growth *in vitro* using the MTT assay. Nevertheless, the percent cell viability of Vero cells against NSM extracts at a dose <1000 µg/ml and lower did not show any decrease in the cell viability. Thus, the cytotoxic effect of the NSM extracts regarding the percentages of the cell viability was suggested to be as higher than 1000 µg/ml doses as shown in Figure 2A.

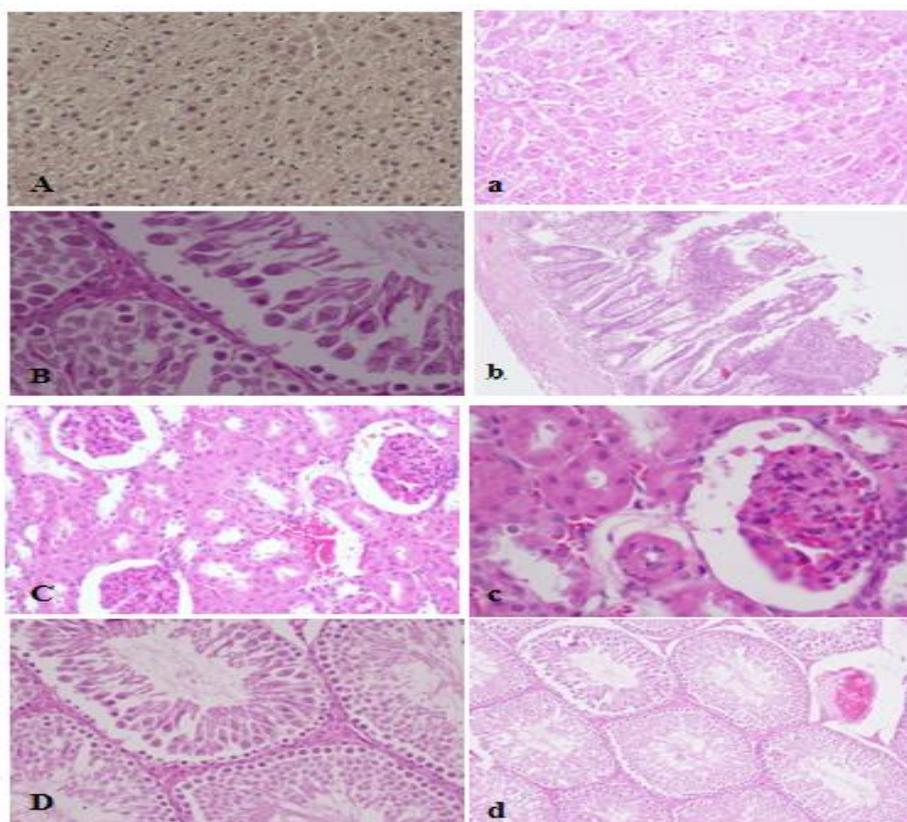
### *In vitro* cells microscopic cytotoxicity assay

In cytotoxic tested cell population growth of NSM concentration values 4, 8, 16, 32, 64, 125, 250, 500, 1000 and 2500 µg/ml regarding the percentages of the cell viability was found to be

lower than 2500  $\mu\text{g/ml}$  and higher than 1000  $\mu\text{g/ml}$  as shown in Figure 2A and Table 1, respectively. However, The minimal toxic concentration (MTC) was identified as the least concentration of the extract preparation that induce toxic effect(s) on culture cells as detected microscopically after 72 hr of incubation compared to 50% of cytotoxic concentration  $\text{CC}_{50}$  and the percent cell viability of Vero cells against NSM extracts at  $>2500 \mu\text{g/ml}$  was found to be toxic. Figure 2A and Table 2, respectively.

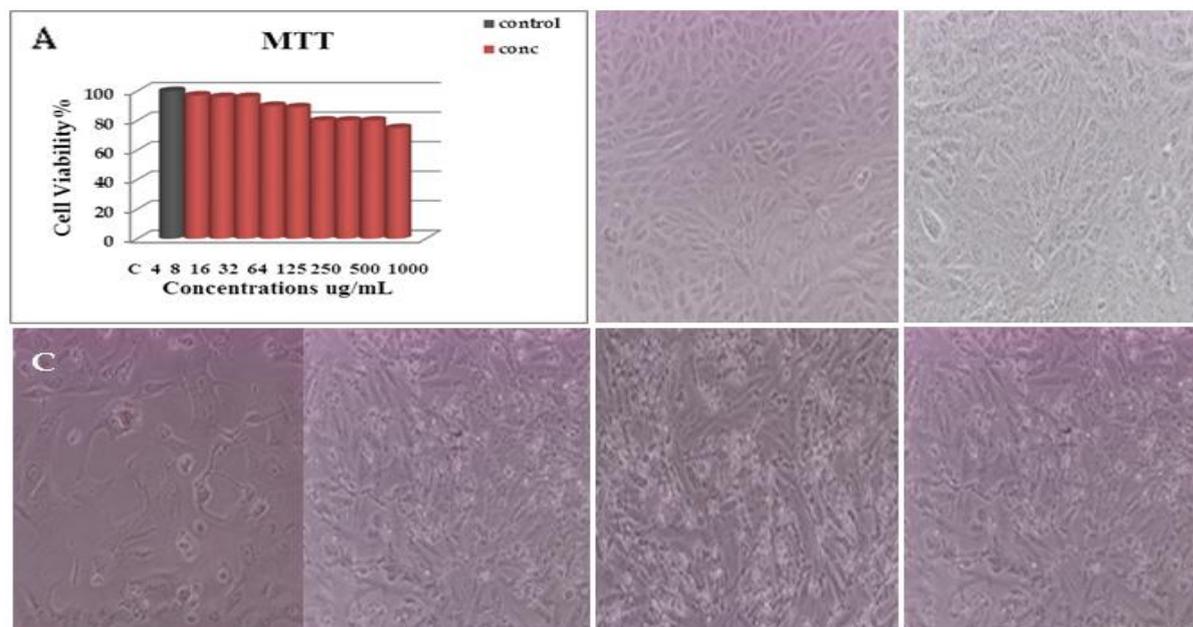
### Morphological changes

Alterations in the morphology of Vero cells exposed to aqueous extracts of the NSM were found to occur in a concentration dependent manner. The morphological changes observed in Vero cells are shown in Figures 2B-G. Cells exposed to  $>2500 \mu\text{g/ml}$  for 3 days started to reduce the normal morphology (shape) and cell adhesion capacity of Vero cells as compared to control. As shown most of the cells exposed to high concentration of NSM seeds lost their typical morphology and appeared smaller in size.



**Figure 1:**The sagittal sections of the histopathology issue from different structures of rat's *in vivo* tests. (A–D) Sections of: liver, intestine, kidney and testes showing cells for rats without any treatments. (a–d) Sections of: liver, intestine, kidney and testes of experimental rats after treated with NSM aqueous extracts injected i.p. at the high dose

of 100 mg/kg to each animal model. All control rats were received normal saline (i.p.) during the study. The sections were stained with hematoxylin and eosin stains (H&E). Scale bar= 200  $\mu$ m.



**Figure 2:** (A) MTT assay tests showing the amount of viable cells when the aqueous extracts of melanin derived from *Nigella sativa* (NSM) seeds treated with MTT at different concentration in ranging from (4–1000  $\mu$ g/ml). (B–G) showing a tracking record of morphological changes before/after NSM aqueous extracts used *in vitro* test by using Vero cell lines: (B) Vero cells without treatment as negative control group. (C) Vero cells with final concentration of NSM at (64 $\mu$ g/ml). (D) Vero cells with final concentration of NSM at (125  $\mu$ g/ml). (E) Vero cells with final concentration of NSM at (250  $\mu$ g/ml). (F) Vero cells with final concentration of NSM at (500  $\mu$ g/ml). (G)Vero cells with final concentration of NSM at (1000  $\mu$ g/ml). After 72 h, all the alteration of Vero cell lines and morphological changes were monitored and images were captured using camera bounded inverted microscope (100X).

**Table 1:** *In vitro* Vero cells microscopic cytotoxicity assay.

The plant	Concentrations ( $\mu$ g/ml)										
	Vero	4	8	16	32	64	125	250	500	1000	2500
Cell viability%	100	98	92	90	89	86	86	84	80	71	50
Extractions	-	-	-	-	-	-	-	-	-	-	+

Note: Vero cells = Normal cells of the African green monkey kidney *in vitro* cells which served in this work as control cell lines. (+) = positive (morphological alterations of Vero

cells when measured via **cytotoxicity assay cell lines growth**, (-) = negative (No morphological alterations in Vero cell lines growth).

**Table 2: The limit *in vitro* activity of the cytotoxicity assay of the extracts on Vero cells as indicated by MTC and CC<sub>50</sub>.**

The plant	Vero cells line	
	MTC*	CC <sub>50</sub> *
Extractions	2500	>2500

\*Concentration is expressed as µg/ml. MTC: Minimum toxic concentration. CC<sub>50</sub>: 50% cytotoxic concentration.

## DISCUSSION

In pharmaceutical development, several studies have revealed that screening on toxicity and deselection in an early phase of development of drugs may improve the success rate of new chemical entities.<sup>[21]</sup> Therefore medium or high throughput screening methods are necessary. The cytotoxicity could be identified for 70% of the compounds in these assays as compared with known toxicity in either *in vitro* assay. Notably, it had been reported that approximately 40% of the new drug candidates fail in the developmental phase due to toxicological side effects.<sup>[22]</sup> Nevertheless, the processes underlying basal cytotoxicity may account for the similarity in effects of toxins between different cell types. This may also reduce the need for more organ specific screening of chemicals that are structural, mechanistically and toxicologically unrelated.

Therefore, *in vitro* cell toxicity assay appears a realistic and attractive alternative for evaluating cytotoxic outcomes due to minor differences between the toxicity responses across the cell lines. Moreover, human cell lines can be used, which will increase the predictability with respect to rat, mice, hamster, dog or monkey cells. Moreover, natural products may serve as a source of many phytochemicals that possess various bioactivities including to protect and/or to prevent different types of diseases and illnesses.<sup>[23]</sup>

Melanin is a natural pigment, it could be found in human (occurs in different cells and could be found external and/or internal (e.g., hair, eye, skin, inner ear and neurons), animal, plants (in seed coats), fungus and microorganisms in cell wall and pathways.<sup>[24]</sup> The absence of this natural pigment in organs and/or tissues in living cells is correlated with the different disease. Melanin is important for the correct functioning of the cells, however, most of these functions

are related to protection against external insults and to confer environmental advantages to cells: protects sight, hearing and skin mainly in response to exposure to UV light, as an appropriate technique for the classification of bacteria spp., and physicochemical properties and thus melanin was identified as a natural pigment in life with a highly important roles in many biological systems.<sup>[24,25]</sup>

Melanin has been extracted from a few plants<sup>[26,27]</sup> and recently it has been extracted from the *N. sativa* plant seeds.<sup>[13]</sup> Indeed, a large body of studies has claimed an immunological role for the widely used of the NSM aqueous extracts.<sup>[9,16]</sup> The seeds of the plant are well-known for their pharmacological effect.<sup>[1,7,9]</sup> However, none of these studies has ascribed the biological toxic effects to the melanin, which is a constituent of the plant. The importance of determining the cytotoxic effects of this extracts in cell lines *in vitro* and/or *in vivo* experimental animals may pave the way for adding new methods in treatment using NSM extracts to help in preventing and/or protection from such disease that is associated with serious slow complications. Due to the fact that, plant extracts have beneficial activity in a different type of diseases producing in human beings. As per WHO, about 80% of the world's inhabitant's primary health care problems should be treated by medicinal herbal drugs.<sup>[28]</sup> Recently, literature data focusing on using natural products from plants or herbs have given of exceptional value in the control of cancer, cardiovascular disease, infection, and inflammation.<sup>[29,30]</sup> Many chemoprevention and anti-cancer agents currently used have their origins in plants.<sup>[31]</sup> Also, it's well known that using the natural products from plants or herbs has advantages, such as low side effect and low cost and also being easily accessible in comparison to common treatment methods.<sup>[32]</sup>

According to the United States National Cancer Institute (NCI), plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity of plant extracts are concentration which able to inhibit 50% viral replication at value  $\leq 20$   $\mu\text{g/mL}$ .<sup>[33]</sup> NSM aqueous extracts due to the fact that the elucidation of the mechanisms by which the anti-cancer properties derived from the natural products are of immense importance. Thus, NSM aqueous extracts could be considered as promising cytotoxic activity extract of novel bioactive compounds with anti-cancer properties. Pugh *et al.* reported that melanins also could be extracted from different botanicals other than *N. sativa* seeds which activate NF- $\kappa$ B and induce IL-1 $\beta$  production and they tentatively proposed TLR2 as a receptor of "botanical" melanins.<sup>[11]</sup> In correlations, NSM had been reported to be able to modulate

tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin 6 (IL-6), TLR4 transfected cells and vascular endothelial growth (VEGF) production in cell line study.<sup>[34]</sup>

Basically, in this study, aqueous extracts of melanin (NSM) *in vitro* cell viability data had been used in a different concentration in ranging from (0 - 1000  $\mu\text{g/ml}$ ) on Vero cells lines as a normal control kidney cells derived from a monkey. Nevertheless, the fewer amounts of viable cells and/or maximum significant death was detected at the concentration of 1000 $\mu\text{g/ml}$  as compared to low concentration. However, due to the morphological data, it was clearly found that any decreasing of NSM aqueous extracts concentrations was followed by significant increases at the viability of cell lines. Thus, we recorded here only the higher doses of NSM *in vitro* cells which range from 64 to 2500  $\mu\text{g/ml}$ . In correlations, in sagittal sections of the histopathology issue from different structures of rats *in vivo* tests comparing the rats sections tissue with the high dose of 100 mg/kg weight only as the maximum dose had been used, to found out they finally tissue changes might be occur due to using the higher doses of NSM aqueous extracts in rats tissues, because there were several reports suggested that in histopathological issues; almost the higher extracts doses might have some negative effects on the normal tissue functions particularly with the *N. sativa* seeds on different tissue functions of experimental rats and/or it may cause internal histopathological cell damages in some organs more than others.<sup>[35,36]</sup> Therefore, we select only the higher dose, however, the small doses of NSM extracts e.g. at 10, 20 and even 50 mg/kg. (i.p.), respectively, were not changing any of tissues similar to the high dose of the extracts.

The data presented in this study are in line with other recent studies which investigated the cytotoxic effects of the NSM aqueous extracts against different types of cell lines *in vitro*.<sup>[37,38]</sup> In this study, we investigated the effects of NSM on cytotoxic cell line by Vero cells. Our findings are:

**I-** NSM aqueous extracts was found to be safe and the toxic effects are lower than 2500  $\mu\text{g/ml}$  in Vero cell lines; the cytotoxic effects of the NSM aqueous extracts in Vero cell were found to increase in response to higher doses of extract concentrations. The data presented in this study are in line with other recent data indicate that the cytotoxic responses of the *N. sativa* seed extract stated that the cellular morphology by phase contrast inverted microscope significantly reduce the cell viability and alter the cellular morphology of A-549 cells in a concentration dependent manner.<sup>[39]</sup>

II- Acute (i.p.) LD<sub>50</sub> of NSM in mice has been found to be 350 mg/kg and in rats was 200 mg/kg, respectively.

III- Acute oral lethal dose, given in 6 doses every 2h, (LD<sub>50</sub>) in mice was=1600 mg/kg; Rats=1500 mg/kg.

IV- The results of the present study showed that cytotoxic effects and the CC<sub>50</sub> value on Vero cell lines tests with NSM aqueous extract was lower than that specified by NCI, for categorization of a pure compound as an anticancer agent. Therefore, we suggest NSM aqueous extracts as an alternative adjuvant for cancer immunotherapy that is safe for oral and/or other methods of application.

V- Experiments were carried in accordance with the Guide for the Care and Use of Laboratory Animals.<sup>[40]</sup>

## CONCLUSION

This study reveals that the *in vitro* cytotoxic activity of the aqueous extract of NSM when tested via *in vitro* using Vero cells and *in vivo* live cells of experimental animals are low. Thus it's possibly suggesting a new potential biopolymer-therapeutic agent for the treatment of certain diseases and illness with high safety. The results of this study provide the basis for further investigation of NSM aqueous extracts for potential identification of novel bioactive compounds with therapeutic properties. A more in-depth study will be necessary to determine whether the cytotoxic effect of NSM could be expressed fully to reveal more or less cytotoxic variations in the way different cell types respond to NSM. It will also be necessary to elucidate the mechanisms of enhancement and/or inhibition involved in the action of NSM aqueous extracts.

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## REFERENCES

1. Salem M. Immunomodulatory and therapeutic properties of the *Nigella sativa* L seed. Int J Immunopharmacol, 2005; 5: 1749–1770.
2. Hajhashemi V, Ghannadi A, Jafarabadi H. Black cumin seed essential oil, as a potent analgesic and antiinflammatory drug. Phytother Res, 2004; 18: 195–199.

3. Khan MD, Chen H, Tania M, Zhang D. Anticancer activities of *Nigella sativa* (Black cumin). *Afr J Trad Compl Alter Med*, 2011; 8(5 Suppl): 226–232.
4. Kirtikar K, Basu B. *Indian Medicinal Plants*, Alahabad. 1984; 1: 11.
5. Chopra R, Chopra I, Handa KD, Kapur LD. *Indigenous Drugs of India*, New Delhi. Kolkata. India. 2<sup>nd</sup> Ed. M/S Dhar VN and Sons: 1982.
6. Ali BH, Blunden G. Pharmacological and toxicological properties of *Nigella sativa*. *Phytother Res*, 2003; 17: 299–305.
7. El-Tahir KE, Bakheet D. The black seed *Nigella sativa* Linnaeus-a mine for multi-cures: A plea for urgent clinical evaluation of its volatile oil. *J Taibah Univ Med Sci*, 2006; 1: 1–19.
8. Ramadan M. Nutritional value, functional properties and nutraceutical applications of black cumin (*Nigella sativa* L.): an overview. *Int J Food Sci Tech*, 2007; 42: 1208–1218.
9. Al-Tayib OA, El Tahir K, Idriss MH, Haseeb A, Eram K. *Nigella sativa* L. seeds melanin: A new hypoglycemic agent. Comparison with insulin in alloxan-diabetic rats. *Sch Acad J Pharm*, 2014; 3: 332–335.
10. Ghaznavi K. *Tibb-e-Nabvi and Modern science*. Al-Faisal Nasheeran Wa., Tajeeran-e-Kutab. Urdu Bazar Lahore, Pakistan, 1991; 1: 334.
11. Pugh P, Balachandran P, Lata H, Dayan F, Joshi V, Bedir E, Makino T, Moraes R, Pasco DS. Melanin: dietary mucosal immune modulator from *Echinacea* and other botanical supplements. *Int J Immunopharmacol*, 2005; 5: 637–647.
12. Kerestes J, Andrejevna L. Biologically active fraction of vegetable melanin, process for its production and its use. US patent No. 6.576: 268, USA; 2006.
13. Hassib AM. *Nigella melanin*. Sudan Patent No. 451, 1998.
14. Hassib AM, Elhag H. Process for producing melanin using cultures of the genus *Nigella*. WO 2012125091 AI, (<http://www.Google.com/Patent/WO 2012125091A>): 2013.
15. Mohammad M, Mohamad M, Dradka H. Effects of black seeds (*Nigella Sativa*) on spermatogenesis and fertility of male albino rats. *Res J Med Sci*, 2009; 4: 386–390.
16. El-Obeid A, Hassib A, Ponten F, Westermarck B. Effect of herbal melanin on IL-6: a possible role of toll-like receptor 4 (TLR4). *Biochem Biophys Res Comm* 2006; 344: 1200–1206.
17. Goreja WG. *Black seed: nature's miracle remedy*. New York, NY: Amazing Herbs Press: 2003.
18. Warriar PK, Nambiar VP, Ramankutty. Chennai: Orient Longman Pvt Ltd; 2004. *Indian medicinal plants-a compendium of 500 species*; 2004; pp. 139–142.

19. Al-Tayib OA, Haseeb A, El-Tahir K, Idriss M. The aqueous extracts of the *Nigella sativa* melanin: Experimental *in vivo* test and *in vitro* HEP-2 cell lines cytotoxicity effects. *Agric Vet Sci J* 2016; 9: 84–90.
20. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immun Meth*, 1983; 65: 55–63.
21. Caldwell G, Ritchie D, Masucci J, Hageman W, Yan Z. The new pre-preclinical paradigm: compound optimization in early and late phase drug discovery. *Curr Top Med Chem* 2001; 1: 353–366.
22. Kola I, Landis J. Can the pharmaceutical industry reduce attrition rates. *Nat Rev Drug Discovery*, 2004; 3: 711–15.
23. Al Tayib OA, El Badwi SM. Assessment of ameliorative effects of aqueous extracts of *Moringa oleifera* on acetaminophen induced nephrotoxicity in rats. *IOSR J Human Soc Sci*, 2016; 21(9): 837–45.
24. Solano F. Melanins: Skin Pigments and Much More-Types, Structural Models, Biological Functions, and Formation Routes. *New J Sci*, 2014; Article ID: 498276.
25. Pathak M. Functions of melanin and protection by melanin, in: Zeise L, Chedekel MR, Fitzpatrick TB, (eds.), *Melanin: Its Role in Human Photoprotection*, American Society for Photobiology, Valdenmar Publishing Co, Overland Park, KS. 1985; pp: 125–134.
26. Buszman E, Kopera M, Wilczok T. Electron spin resonance studies of chloroquine–melanin complexes. *Biochem Pharmacol*, 1984; 33: 7–11.
27. Sava V, Yang S, Hong M, Yang P, Huang G. Isolation and characterization of melanic pigments derived from tea and tea polyphenols. *Food Chem*, 2001; 73: 177–184.
28. WHO. *Diet, Nutrition and the Prevention of Chronic Diseases*. World Health Organization, WHO, Geneva, Technical Report Series, 916: 2003.
29. Rabeta M, Chan S, Neda D, Lam K, Ong M. Anticancer effect of underutilized fruits. *Int Food Res J*, 2013; 20: 551–556.
30. Palasap A, Limpai boon T, Boonsiri P, Thapphasaraphong S, Daduang S, Suwannalert P, Daduang J. Cytotoxic effects of phytophenolics from *Caesalpinia mimosoides lamk* on cervical carcinoma cell lines through an apoptotic pathway. *Asian Pac J Cancer Prev*, 2014; 15: 449–454.
31. Hussain A, Sharma C, Khan S. *Aloe vera* inhibits proliferation of human breast and cervical cancer cells and acts synergistically with cisplatin. *Asian Pac J Cancer Prev*, 2015; 16: 2939–2946.

32. Listyawati S, Sismindari I, Mubarika S, Yosi M, Muthi I. Anti-proliferative activity and apoptosis induction of an ethanolic extract of *Boesenbergia pandurata* (Roxb.) schlecht against HeLa and Vero cell lines. *Asian Pac J Cancer Prev*, 2016; 17: 183–187.
33. Sriwiryajan S, Ninpesh T, Sukpondma Y, Nasomyon T, Graidist P. Cytotoxicity screening of plants of genus Piper in breast cancer cell lines. *Trop J Pharm Res*, 2014; 13: 921–928.
34. El-Obeid A, Al-Harbi S, Al-Jomah N, Haseeb A. Herbal melanin modulates tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin 6 (IL-6) and vascular endothelial growth factor (VEGF) production. *Phytomed*, 2005; 3: 324–333.
35. Modaresi M, Ghobadi M, Tabeidian A, Jalalizand A. Study of histopathologic changes of the effect of *Zingiber* extract on mice kidneys. *International Conference on Food Engineering and Biotechnology*. IPCBEE vol.9, IACSIT Press, Singapore: 2011.
36. Dollah M, Parhizkar S, Abdul Latiff L, Bin Hassan MH. Toxicity effect of *Nigella sativa* on the liver function of rats. *Adv. Pharm Bull*, 2013; 3: 97–102.
37. Jafri S, Glass J, Shi R, Zhang S, Prince M, Kleiner-Hancock H. Thymoquinone and cisplatin as a therapeutic combination in lung cancer: *In vitro* and *in vivo*. *J Exp Clin Cancer Res*, 2010; 29: 87.
38. Bourgou S, Pichette A, Marzouk B, Legault J. Antioxidant, anti-inflammatory, anticancer and antibacterial activities of extracts from *Nigella sativa* (black cumin) plant parts. *J Food Biochem*, 2012; 36: 539–546.
39. Al-Sheddi E, Farshori N, Al-Oqail M, Musarrat J, Al-Khedhairi A, Siddiqui MA. Cytotoxicity of *Nigella Sativa* seed oil and extract against human lung cancer cell line. *Asian Pac J Cancer Prev*, 2014; 15: 983–987.
40. The Guide for the Care and Use of Laboratory Animals published by the US National Institute of Laboratory Animal Resources, NIH Publication, revised in 1996, no. 85–23.