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Cytotoxicity assay for herbal melanin derived from Nigella sativa seeds using in vitro cell lines

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Abstract: Medicinal plants have formed the basis of health care over millennia and used to treat and prevent several diseases. The current study is to examine the cytotoxic effects and thus the safety margins in using melanin aqueous extracts which have recently been derived from Nigella sativa seed coats. In vitro tests performed on HEp-2 (Human epithelial cells derived from a larynx carcinoma) cells lines were undertaking and then the cell viability after adding the aqueous extracts of the herbal melanin exposed to various concentrations solutions to in vitro cells which range from 0 concentration to 1000 µg/ml for a period of 72h. Following the exposures of melanin extracts, HEp-2 cells cytotoxic responses were assessed and determined by using the MTT (3-(4, 5-dimetylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay using a phase contrast inverted microscope. The cytotoxic effects of the aqueous extracts of melanin on in vitro HEp-2 cell assay tests were found safely when lower concentrations had been used, however, the results also showed that melanin extracts was significantly high safety during cell growth in a dose- dependent manner without inducing damage up to high concentration of the different doses which had been used. our finding suggest that aqueous extracts of Nigella sativa melanin and regarding to its possible therapeutic effects is also with highly safety actions and that might pave the ways of its using as a new bioactive compound and/or a promising health extract derived from a natural source.

Keywords: Herbal melanin; N. sativa; seed extract; HEp-2 cells; MTT test

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I. INTRODUCTION

Globally, several infection might causes a wide spectrum of chronic liver disease. In recent times, alternative medicines and herbal extracts derived from different types of folkloric medicinal plants have got special attention in treating human diseases. A wide range of Nigella species, including *Nigella sativa* L. (*N. sativa*) has been demonstrated for its therapeutic values against several human metabolic and infectious diseases.

In connections to this, *N. sativa*, of the botanical family Ranunculaceae, have been reported severally in the scientific literature to contain essential oil, fixed oil, saponins, alkaloids, flavonoids and polyphenolic compounds [1]. However, the phenolic extracts of *N. sativa* seeds show considerable analgesic and anti-inflammatory activities [2]. Another study suggested seeds extract therapeutic effects due to its constituents such as thymoquinone and fixed seed oil [3]. Nevertheless, *N. sativa* seeds 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, snake-bite and scorpion stings [4, 5] and thus used for many other illness as hypercholesterolemia, diabetics, tumor and gynecological disorders for over 2000 years [6, 7, 8].

Islamic medicine regards *N. sativa* seeds considered 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. It is also included in the list of natural drugs of 'Tibb-e-Nabawi' [9]

Literature reviews of the last few decades report discovery of melanin biopolymers in many plant seeds [10, 11]. Moreover, several recent studies revealed a substantial occurrence of melanin in the seed coats of *N. sativa* plant [12, 13], which 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* despite the fact that its seeds traditionally used for thousands of years as herbal food for health aid additive in Middle East, Far East and Asia [14].

This study to our best of knowledge cytotoxic effect of the *N. sativa* melanin (NM) aqueous extracts on *in vitro* HEp-2 (human epithelial cells derived from a larynx carcinoma) cell lines tests using MTT assay yet published, and thus this data might supports findings of scientific literature reported for melanins from other natural life sources, and suggests the use of aqueous extracts of melanin as a future applications of therapeutic protector and/or as a promising treatment of many diseases. Nevertheless, using of *in vitro* HEp-2 cell lines which derived from a human larynx carcinoma, were found to be highly susceptible to infection. Even though, this cell lines were commonly used in clinical virology for the culture of different viruses from clinical specimens.

II. MATERIALS AND METHODS

2.1. Morphology of the plant

N. sativa (Figure 1) 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 [15]. Recently, melanin was known to exist in the outer coat of the *N. sativa seeds* which represents around 15% of the seed coat alone [12, 13].

2.2. Collection of plant material

Herbal NM aqueous extracts derived from the *N. sativa* seed coats. which used in this study has been supplied as a gift by its discoverer and manufacturer Professor Adil M. Haseeb, College of Science, King Saud University, Riyadh, Kingdom of Saudi Arabia.

2.3. Chemicals

Dublecco's Modified Eagle Medium (DMEM) was obtained from Gibco company (Invitrogen, USA). Fetal bovine serum (FBS) and trypsin-EDTA were from Gibco, USA, (The cell culture company). In addition, Dimethyl sulfoxide (DMSO) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from (TACSMTT Cell Proliferation Assay Kit, Trevigen) as per the manufacturer's instruction. (all chemical used in this study was a kind gift of Dr. Takayama Moawia, College of Science, Virology Department, King Saud University, Saudi Arabia.

2.4. Preparation of NM solutions

For *in vitro* cytotoxicity studies, the NM 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).

2.5. In vitro evaluation of the NM extracts cytotoxicity

2.5.1. Experimental design

HEp-2 (Human epithelial cells derived from a larynx carcinoma) cells were exposed to various concentrations of NM solutions range from 4 to 2500 µg/ml for a period of 72h. Following the exposures of NM, HEp-2 cells cytotoxic responses were assessed using MTT assays and cellular morphology using a phase contrast inverted microscope.

2.5.2. Preparation of NM solutions

For *in vitro* cytotoxicity studies, the NM 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).

2.5.3. Cell culture

HEp-2 *in vitro* 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 NM extracts were kept in maintenance medium containing 1% FBS, L-glutamine and antibiotics. Cells were incubated at 37°C with 5% CO_2.

2.6. Cytotoxicity screening
Percent cell viability and screen the cytotoxic activity of plant extract we assessed using the MTT (3-(4, 5-dimetylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) colorimetric assay [16]. Briefly, cells ($1x10^4$) were allowed to adhere for 24 h CO_2 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 with 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).

2.7. Cell viability MTT assay tests
Cytotoxicity of our extract was determined by using MTT assay tests. HEp-2 cells without treatments served as control and compare to other three including treated cells with herbal melanin extracts. In this assay, the number of viable cells was determined calorimetrically in 96-well plates. After 4 h of incubation at 37°C, the optical density was recorded at 570 nm in a micro plate reader (BioTek, ELx800). Dilution series of extracts for 72h. Nonlinear regression analysis was performed in Excel software to determine the cell line viabilities (%) using the following equation formula:

$$\text{Cell line viabilities (\%)} = \frac{\text{Optical density (a-b)}}{\text{Optical density (c-b)}} \times 100$$

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

2.8. Examine a morphological alterations
Monolayer cultures of HEp-2 cells with (80 - 90% confluence) were prepared in 96 well plates. Cells were washed twice with phosphate buffered saline. Two-fold serial dilutions of the extract were prepared in maintenance medium starting from the concentration 64 µg/ml to 1000 µg/ml and added to cells in triplicates. All cultures were kept at 37°C in CO_2 incubator for 72 h with daily observation for morphological changes under phase contrast inverted microscope connected with a digital camera (Olympus IX51, Tokyo, Japan).

2.9. Microscopy
A direct visual observation was made under an inverted microscope (Optica, 40x and 100x magnification) to observe any morphological changes in the cells cultured with different concentrations of NM aqueous extracts at 24, 48, and 72 h.

III. RESULTS

3.1. Cell MTT assays
The results of using different concentrations of NM aqueous extract concentration values ranging from 0 -1000 µg/ml, on HEp-2 cells population growth for 72 h. It was found to be lower than (>1000 µg/ml). However, NM extracts at concentration of 0 and up to 125 µg/ml did not show highly significant decreases in the viability of HEp-2 cells (available cells 100- 77%). While, the significance cell death were seen after 72 h after using the concentrations of NM extracts at 500 and 1000 µg/ml. The cellular cytotoxicity of herbal melanin aqueous extracts was assessed using MTT assays, after exposing on HEp-2 cells at different concentrations were reported in Figure 3.

3.2. Morphological changes
Alterations in the morphology of HEp-2 (control) cells exposed to NM extracts were found to occur in a concentration-dependent manner. Morphological changes in HEp-2 cell lines exposed to >1000 µg/ml for 3 days started to reduce their typical normal morphology accordingly with the treatment dose-dependent manners and thus treatment cells were appeared lower in viable cells as compared to control cell (Figure 3).

3.3. Toxic concentration
Minimal toxic concentration (MTC) was identified as the least concentration of NM extracts preparation that induce toxic effect(s) on culture cells as detected microscopically after 72 h of incubation compared to 50% of cytotoxic concentration CC_{50} (Table 1).

IV. DISCUSSION

Interestingly, several reports have revealed that screening on cytotoxicity and de selection in an early phase of development of drugs pave the way to improve the success rate of new chemical entities [17]. Furthermore, according to availability data from *in vitro* cell line tests the cytotoxic effectiveness might be identified for 70% of the compounds which compared with known toxicity *in vitro* assay [18]. Notably, about 40% of the new drug candidates fail in the developmental phase due to its highly toxic and/or un wanted side effects [19]. In correlation to all this facts, the processes underlying basal cytotoxicity may account for the similarity in effects of toxins between different cells, however, that might be a reasonable causes to reduce needs of more organ specific screening of chemicals that are structurally, mechanistically and toxicologically unrelated and thus evaluation of the cytotoxic effects of extracts by using cell line assay test still considered as the only realistic and attractive alternative for evaluating cytotoxic outcomes due to the very minor differences between the toxicity responses across experimental *in vivo* and *in vitro* cell lines [20].

The herbal NM aqueous extracts is considered as a biological natural pigment with defense and/or protective function and for that natural reasons it was found in almost all living cells of environmental biological systems [21]. The importance of determining the cytotoxic effects of NM aqueous extracts on *in vitro* HEp-2 cell lines might pave the way for addition new methods in treatment using this natural extracts from plant to help in preventing and protection of disease which associated with serious slow complications. As per WHO, about 80% of the world's inhabitant's primary health care problems could be successfully treated by using alternative medicine agents [22]. In line with this prospective, recently most of the literature data were focusing on using plant extracts which have given of exceptional value in the control of several disease such as hyperglycemia (untidiabetic agent), nephroprotective, cardiac disease, infectious and/or inflammation diseases and also successfully treated the different kind of cancer cells [8, 23, 24, 25]. In correlations to this, scientific researcher found that using of natural products which recently derived from different plants species has advantages, such as low side effects, low cost and also being easily accessible in comparison to common treatment methods [26]. Indeed, NM extracts and due to the elucidation of the mechanisms by which the anti-cancer properties derived from the natural products are of immense importance. Of these, NM extracted from different botanicals other than *N. sativa* was found to be able to activate NF-jB and induce IL-1b production and they tentatively proposed TLR2 as a receptor of ''botanical'' melanins [27]. In correlations NM extract was also found to be able to modulates tumor necrosis factor alpha (TNF-a), interleukin 6 (IL-6), TLR4 transected cells and vascular endothelial growth (VEGF) production in cell line study [28].

Basically, *In vitro* antioxidant activity of NM aqueous extracts and organic fractions revealed strong antioxidant activity on *in vivo* test (data not shown). However, the antioxidant activity of the plants such as which offered by NM aqueous extracts in this present work could be thus attributed to the presence of antioxidant and free radical scavenging factors, for example, phenolic compounds, flavonoids, and saponins, which were reported to have cell protective activity [29, 30]. Nevertheless, there is a linear relationship between the hepatoprotective and the antioxidant activity.

Our findings are: Accordingly to a previous biological *in vitro* cell line study revealed the numerous biological activities of NM aqueous extracts showed that a significant cytotoxic effects and the CC_{50} value on *in vitro* cell lines tests with the NM aqueous extract was lower than that specified by NCI, for categorization of a pure compound as anticancer agent [20]. Therefore, the authors suggest suggestion that NM extracts as an alternative adjuvant for cancer immunotherapy is considered safe for oral and/or other methods of application. Also, the data presented in this study were in line with recently data found that the cytotoxic responses of *N. sativa* seed extracts on cellular morphology by phase contrast inverted microscopy; was significantly reduce cell viability and alter the cellular morphology of A-549 cells in a concentration-dependent manner [31]. While, other report found that the acute (i.p.) LD_{50} of NM in mice and Wistar rats was in between 350 and 200 mg/kg, respectively, and acute oral LD_{50} of NM extracts in mice and rats 1600 and 1500 mg/kg, respectively. In the same line, these studies often involve the investigation of the effects of biologically active substances on cancer cells, however, they frequently originate from plants [32]. Thus, there is a great need to examine reliable and inexhaustible sources of natural substances. In addition, it is important to understand the mechanisms of anticancer agents for future application in cancer therapy [33].

This experiment investigated the cytotoxic activity of the aqueous extract of the NM (in a concentration range from 8 to 1000 μg/mL) in two HEp-2 cell lines using MTT assay.

A dose-dependent MTT reduction (or color change from yellow to purple) was observed in NM aqueous extract-treated cell lines in dependant manner according to the increases of the extract concentration. The shape of the curve shows significant inhibition of cell proliferation in HEp-2 cell line (Figure 2) in a dose-dependent manner after 24 and 72 h of treatment. The morphological growth of cell lines were significantly lower when compared to untreated control cells. After 72 h of NM aqueous extracts treatment, higher concentrations (1000 μg/mL) killed more than 50% of cells. After 72 h of treatment cytotoxic effect of those

concentrations was higher, but lower concentrations had weaker cytotoxic effects than after 24 h. Maximum inhibition of proliferation was achieved after 72 h at the highest concentration (1000 µg/mL) only.

The results indicated more dramatic effects of cytotoxic activity after the treatment with NM extract particularly at the highest concentration (500 and 1000 µg/mL) on cells. Only 47% of the HEp-2 cells remained viable 72 h after the treatment with extract from NM at a concentration of 1000 µg/mL. The same concentration of the plant extract (8-250 µg/mL) killed (2% and 36% of the cells (98 and 64% of viable cells) in the treatment of HEp-2 cell lines after 72 h. These results suggest greater safety of the extract from NM on HEp-2 cell line. Table 1 presents *in vitro* cytotoxic activity. The effects of extract were expressed by CC_{50} values (concentration which inhibit 50% of cell growth), as a parameter for cytotoxity.

Many plant extracts and natural products, with high antioxidant activity have shown cytotoxic effects in different cell lines [34]. On the other hands, several natural consumption of foods that contain phenolics and flavonoids has been shown to reduce the risk of such diseases particularly with cancer [35]. However, it is well known that using cytotoxicity assay of *in vitro* methods for around 110 compounds 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 [36, 37]. 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 [38]. 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* [39].

Based on these arguments, we can conclude that the high concentration of active contents of herbal extractions is most likely responsible for the significant cytotoxic/safety activities of the plants extract. Further research should be carried out to isolate and identify biologically active substances from the NM aqueous extracts which recently derived from the *N. sativa* outer seed coats, with an antiproliferative activity, as well as to conduct a detailed examination of the effects of these agents on various cancer cell lines and *in vivo* tests.

V. CONCLUSION

This study reveals that cytotoxic activity of NM aqueous extracts, tested on HEp-2 cells and animals were low and suggesting NM as new potential biopolymer-therapeutic agent with high safety. Hereby, we provide basis for further investigation of NM extracts as novel bioactive compounds with therapeutic properties. More in-depth study is necessary to elucidate the mechanisms of enhancement and/or inhibition involved in the action of NM extracts. Our results revealed very promising effective, safety and cell-proliferative effects of NM aqueous extracted from Nigella seeds on *in vitro* cell line conditions. Interestingly, further *in vivo* evaluations of NM aqueous extracts also exhibited anti-efficacy is such needed. Therefore, the therapeutic potential of NM aqueous extracts warrants further isolation of the active principle(s) and its phytochemical as well as biological studies.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Figure Legends

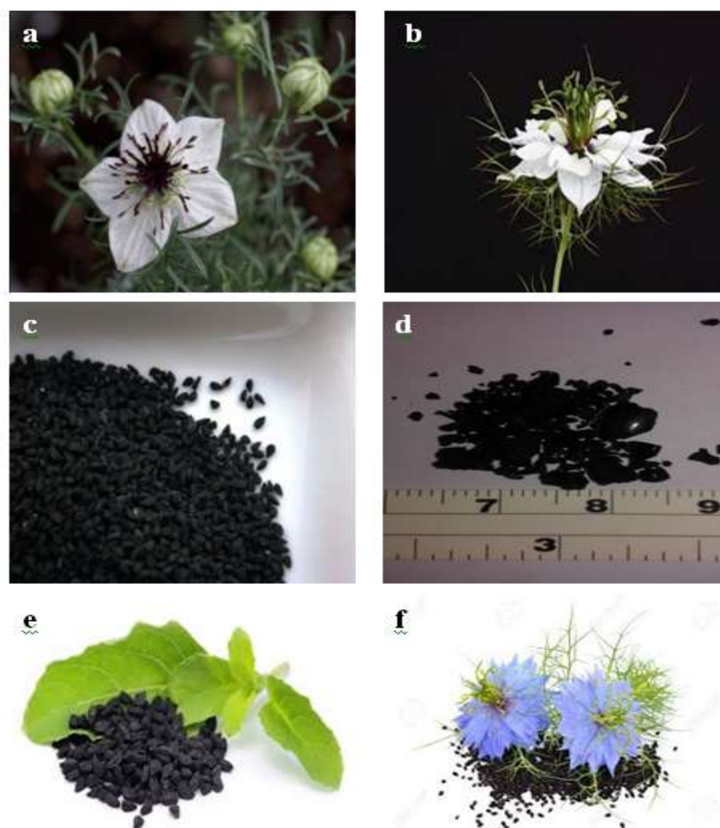

Figure 1. Hassib (1998) discovered that **melanin** exists in the seed coats of the well-known ***Nigella sativa*** (*N. sativa*) plant. Recently, Hassib and Elhag (2013) discovered and process the methods for producing melanin using cultures of the genous Nigella. A figure shows: the plant *N. sativa*: (**a-f**) Morphological features of *Nigella sativa* flowers, seeds, melanin, leaves and Fruit.

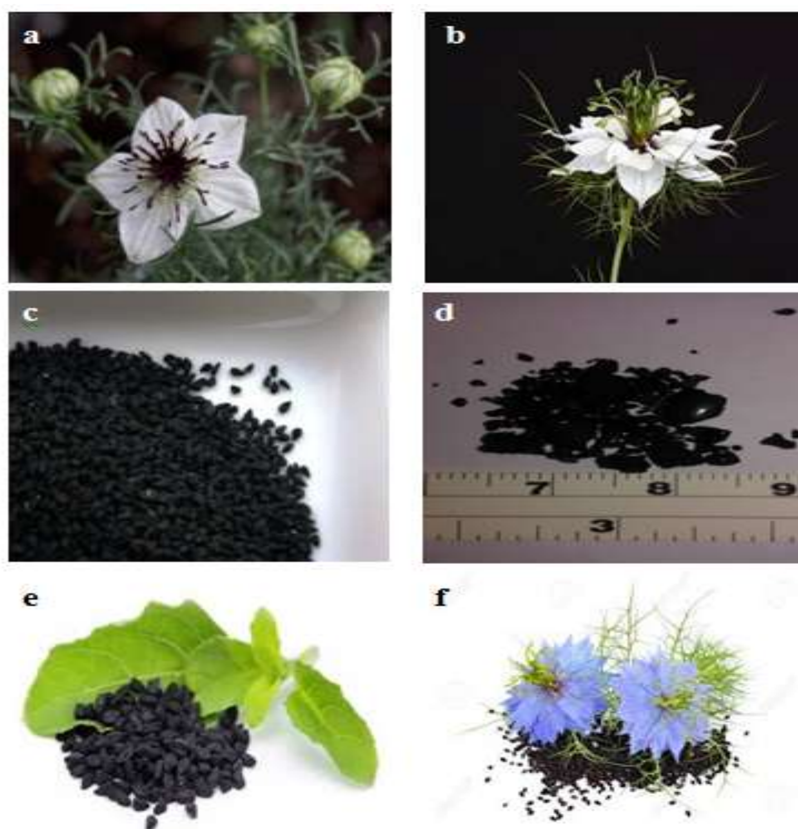

Figure 2.

(a) Morphological features of the *in vitro* HEp-2 (human epithelial cells derived from a larynx carcinoma) cells.

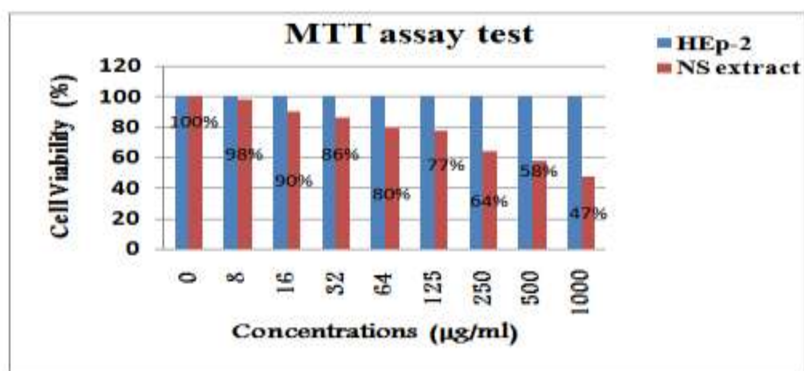

Figure 3. MTT assay tests and morphological changes tests when *N. sativa* melanin (NM) aqueous extracts compared with HEp-2 cell lines. (A) Aqueous extracts of NM derived from *N. sativa* seeds treated with MTT at different concentration in ranging from (0–1000 µg/ml) and HEp-2 cells as normal (control cells) lines. Less amount of viable cells and/or maximum significant death were detected at concentration of 1000 µg/ml as compared to low concentration. While decreasing the concentration of NM extracts increasing viability of cells showing negligible amount of cell death.

Table 1. The limit *in vitro* activity of the cytotoxicity assay of the aqueous extracts of melanin on HEp-2 cell line

The plant	HEp-2 cells line	
	MTC*	CC_{50}*
Cell viability after extracts	500	>1000

*Concentration is expressed as µg/ml. MTC: Minimum non toxic concentration. CC_{50}: Plants extract concentration which able to reduce 50% cell growth (50% cytotoxic concentration).

Omar A. Al-Tayib . "Cytotoxicity assay for herbal melanin derived from Nigella sativa seeds using in vitro cell lines." IOSR Journal Of Humanities And Social Science (IOSR-JHSS) , vol. 22, no. 10, 2017, pp. 43–51.