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Investigation of Cytotoxic Compounds in Ethyl Acetate and Chloroform Extracts of *Nigella sativa* by Sulforhodamine-B Assay-Guided Fractionation

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Abstract**—**A Sulforhodamine-B assay-guided fractionation on *Nigella sativa* seeds was conducted to determine the presence of cytotoxic compounds against human hepatoma (HepG2) cells. Initially, a freeze-dried sample of *Nigella sativa* seeds was sequentially extracted into solvents of increasing polarities. Crude extracts from the sequential extraction of *Nigella sativa* seeds in chloroform and ethyl acetate showed the highest cytotoxicity. The combined mixture of these two extracts was subjected to bioassay guided fractionation using a modified Kupchan method of partitioning, followed by Sephadex® LH-20 chromatography. This chromatographic separation process resulted in a column fraction with a convincing IC_{50} (half-maximal inhibitory concentration) value of 13.07 µg/ml, which is considerable for developing therapeutic drug leads against human hepatoma. Reversed phase High-Performance Liquid Chromatography (HPLC) was finally conducted for the same column fraction and the result indicates the presence of one or several main cytotoxic compounds against human HepG2 cells.

Keywords**—**Cytotoxic compounds, half-maximal inhibitory concentration, high-performance liquid chromatography, human HepG2 cells, *Nigella sativa* seeds, Sulforhodamine-B assay-guided fractionation.

I. Introduction

CHEMOPREVENTION is a widely used strategy that makes use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression to invasive cancer. Cancer chemopreventive agents, many of which are natural products of plant origin, can prevent or inhibit the process of carcinogenesis. The discovery of novel cytotoxic compounds from medicinal herbs and their combination formulas in traditional medicinal practices is an important area in anti-cancer drug development. So far, one of the most efficient methods of discovering such novel chemical prototypes is bioactivity-guided fractionation [1], [2].

A traditional herbal formulation, which is of our interest to isolate a cytotoxic compound, has been in use for treatment of human hepatocellular carcinoma (human hepatoma). This formulation is prepared as a hot-water extract from *Nigella sativa* seeds, *Hemidesmus indicus* roots, and *Smilax glabra* rhizome by boiling 20 g each of the plant material in 1.6 liters of distilled water until the final volume is reduced to 200 ml [3]. The anti-cancer potential of this formulation has been established against human hepatoma. The greatest inhibitory effects have been observed on DNA synthesis with both the herbal formulation and *Nigella sativa* plant extract even at low concentrations such as 5 mg/ml. It has also been noticed that the three individual plant extracts have the cytotoxic activity in the following order, *Nigella sativa* seeds > *Hemidesmus indicus* roots > *Smilax glabra* rhizome [4]. In a subsequent study, marginally higher cytotoxic potential has been identified in the standardized aqueous extract from the herbal formulation of *Nigella sativa* seeds, *Hemidesmus indicus* roots, and *Smilax glabra* rhizome than in its standardized ethanolic extract. HPLC analysis of these two extracts (aqueous and ethanolic) of the herbal formulation has shown the presence of both polar and non-polar compounds in their analysis. Furthermore, a higher aggregation of saponins has also been observed in the aqueous extract [5]. However, a concise bio-assay guided fractionation has not been carried out for the isolation of individual plant components of this standardized herbal formulation.

In the present study, an attempt is made to develop a Sulforhodamine-B (SRB) assay-guided fractionation that sequentially isolates cytotoxic compounds of *Nigella sativa* seeds into solvents of hexane, chloroform, ethyl acetate, and methanol, in the order of increasing polarity. Initially, primary screening is carried out for cytotoxicity of each of the individual plant components, *Nigella sativa* seeds, *Hemidesmus indicus* roots, and *Smilax glabra* rhizome. Subsequently, based on the cytotoxicity results from SRB assays, the study is extended to isolate and purify the chloroform and ethyl acetate extracts of *Nigella sativa* seeds using a modified Kupchan method of partitioning, followed by Sephadex® LH-20 chromatography. The resulting column fraction is then analyzed by reversed phase HPLC to visualize the presence of numerous cytotoxic compounds.

II. Materials and Methods

A. Plant Material

- *Nigella sativa* seeds
- *Hemidesmus indicus* roots
- *Smilax glabra* rhizome

B. Instruments and Other Material

- Filter papers: Whatman™ (18.5 cm, No: 1001-110).
- Thin-layer Chromatography (TLC) papers: pre-coated aluminum sheets ALUGRAM® SIL G/UV254, 20 cm × 20 cm.

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- Ultrasonic bath: TOCHO® UC-205-B.
- Rotary evaporator: BUCHI® Rota vapor R-200 (BUCHI Water bath B-481 & BUCHI Heating Bath: B-490 Made in Switzerland).
- Vacuum Pump: VACCUBRAND® -MZ-2C-NT Made in Germany.
- Analytical Balance: Precisa® XB 120A.
- Electric oven: Memmert® Beschckung Loading model 100-800.
- UV viewing system: CARMAG® UV Viewing System.
- Micro plate reader: Synergy HT Universal Micro Plate Reader (BIO-TEK INSTRUMENTS, USA).
- HPLC System: Schimadzu HPLC system (Japan) connected to a UV-Visible detector (Model SPD-10AVP).

C. Preparation of Total Extracts of Plant Materials for Primary Screening

A stock sample of 250 g of *Nigella sativa* seeds was carefully washed with distilled water (500 ml × 3) to remove debris of all types. The washed seeds were first air dried and then oven dried at a temperature below 40 °C. The oven dried seed sample was ground to a fine powder and then freeze-dried. A 10 g sample of freeze-dried ground *Nigella sativa* seeds was used to extract into a solvent mixture of 1:1 chloroform:methanol using ultrasound sonication. This extraction was carried out for a period of 45 minutes and the resultant mixture was filtered through Whatman® filter paper. The residue was further extracted using the same solvent system, for two more times, and the filtrates of each step were pooled together. The resultant filtrate is concentrated under reduced pressure in a rotary evaporator and completely dried under nitrogen atmosphere. This total extract was then weighed and stored at –20 °C for the later use in bioassays and TLC analysis. The same procedure was carried out with *Hemidesmus indicus* roots and *Smilax glabra* rhizome samples.

D. Sequential Solvent Extraction

A 100 g sample of the freeze-dried ground *Nigella sativa* seeds was used to sequentially extract into hexane, chloroform, ethyl acetate, and methanol by ultrasound sonication. Initially, 100 g freeze-dried ground *Nigella sativa* seeds was extracted into hexane (300 ml) by ultrasound sonication for a period of 45 minutes and the resultant mixture was filtered through Whatman® filter paper. Further, the filtered residue undergoes two consecutive extractions into two hexane samples (300 ml each) and the filtrates of these three extractions were pooled together. The resultant filtrate was concentrated under reduced pressure in a rotary evaporator and completely dried under nitrogen atmosphere. This dried hexane extract was then weighed and stored at –20 °C for the later use in bioassays and TLC analysis. The resultant residue was air dried until all the remnant solvent is evaporated. The final filtered out residue from the extraction process in hexane then undergoes sequential extraction into chloroform, ethyl acetate, and methanol, according to the order of increasing solvent polarity. The procedure for extraction in each of these solvents is same as the procedure for the extraction into hexane. All the resultant sequential solvent extracts of *Nigella sativa* were labeled, weighed, and stored for the later use in analyses.

E. Cell Culture

HepG2 cells were harvested by trypsinization, and plated (5×10^3 cells/well) in a 96-well cell culture plate and maintained in Dulbecco's Modified Eagle Medium (DMEM) for 24 hours at 37 °C in 95% air/5% CO_2 atmosphere with 95% humidity. Cell cultures were exposed only to medium Dimethyl sulfoxide (1% DMSO, controls) or medium containing different concentrations of solvent extracts dissolved in 1% DMSO (25 µg/ml–400 µg/ml) and incubated for 24 hours. At the end of this incubation period, cells were briefly washed with Phosphate-buffered saline (PBS) [6]. Fresh medium of 200 µl was then placed in each well and SRB assay was performed.

F. SRB Cytotoxicity Assay

The SRB assay for cytotoxicity was performed according to the procedure mentioned in [7]. Cell survival was determined after the exposure to different concentrations of the different extracts. Cells were fixed with 50 µl of ice-cold 50% trichloroacetic acid solution by gently adding on top of the medium overlaying the cells. The cell culture plates were incubated for 60 minutes at 4 °C. The wells were rinsed five times with running tap water and then the cells were stained with 0.4% SRB solution (100 µl stain/well) for 15 minutes at 25 °C. After staining, the SRB solution was poured off and unbound dye was removed by washing five times with 1% acetic acid solution and left to air dry. The bound SRB dye was then solubilized by adding unbuffered Tris-base solution (200 µl/well), and plates were placed on a plate shaker for 1 hour at 25 °C. Using a micro plate reader (Synergy HT Universal Micro Plate Reader, BIO-TEK INSTRUMENTS, USA), the OD_{450} (Optical Density measured with a 450 nm filter) values were taken.

G. Determination of IC_{50} Values

IC_{50} is the concentration of a compound required to reduce the rate of an enzymatic reaction by 50%. The IC_{50} value of an extract is determined by generating the dose response curve using nonlinear regression curve fitting (GraphPad Prism®) for OD_{540} values against logarithm of extract concentrations. Nonlinear regression curve fitting was conducted for the function $y = 100/[1+10^{\{(\text{LogIC50}-x)\times\text{HillSlope}\}}]$, where y, x, LogIC50, and Hillslope represent the OD_{540} value for an extract concentration, logarithm of an extract concentration, logarithm of half-maximal inhibitory concentration, and the steepness of the response curve, respectively. The outputs of this nonlinear regression curve fitting are the best-fit value of IC_{50} and Hillslope for an extract.

H. Thin-Layer Chromatography

Each sample to be analyzed was spotted onto TLC plates coated with silica gel (pre-coated, GF254) and separated using solvent systems of hexane and ethyl acetate with different volume ratios, and were visualized under UV light (366 nm, 254 nm), iodine chamber and anisaldehyde reagent spray. Reversed phase TLC was carried out using RP-18 TLC plates

coated with silica gel (pre-coated, GF254) to analyze the fractions from the Sephadex® LH-20 column chromatography. For each of the analyzed extracts, retardation factors (R_f) were calculated as the ratio of *Distance travelled by the spot* to *Distance traveled by the solvent* [8].

I. Kupchan Method of Partitioning

The modified Kupchan method of partitioning that was carried out for the combined extracts of chloroform and ethyl acetate of *Nigella sativa* seeds is illustrated in Fig. 1. All the solvents used were analytical grade, all the solvent fractions were completely dried, and the resultant extracts were stored at −20 °C until further use.

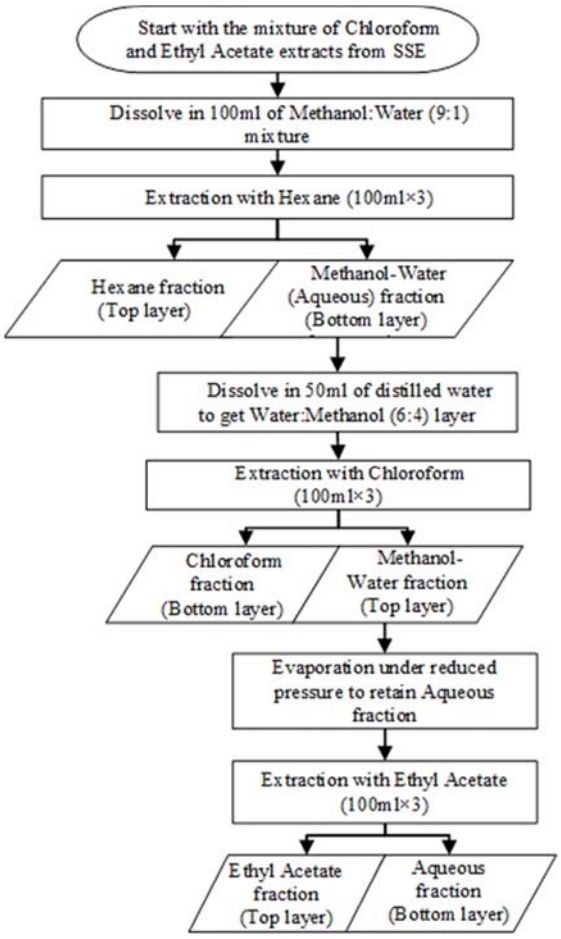

Fig. 1 Flow chart for Kupchan method of partitioning

J. Extraction Process of Nigella Sativa

A summarized flow chart for the extraction procedure of *Nigella sativa* seeds is presented in Fig. 2. It should be noted that the resultant extracts from sequential solvent extraction, Kupchan method of partitioning, and Sephadex® LH-20 column chromatography were tested by SRB assay and TLC analysis.

K. High-Performance Liquid Chromatography

The column fraction CC2 from Sephadex® LH-20 column chromatography was analyzed by reversed phase HPLC. The sample was dissolved in distilled water and 80% methanol respectively, so that the sample concentration is 20 mg/ml. After filtration through Millipore filters (pore size of 0.45 µm), 20 µl of the sample was injected into an Inertsil® ODS-3 C18, (250 mm × 4.0 mm, 5 µm) reversed phase column of a HPLC System (Schimadzu, Kyoto, Japan), connected to a UV-Visible detector (Model SPD-10AVP). The HPLC analysis was performed using a linear gradient of 80% water in methanol to 100% methanol for 30 minutes, followed by100% methanol at a flow rate of 0.5 ml/minute, and UV detection at 254 nm. All solvents were HPLC grade, which were purchased from Fisher Scientific International Company, United Kingdom.

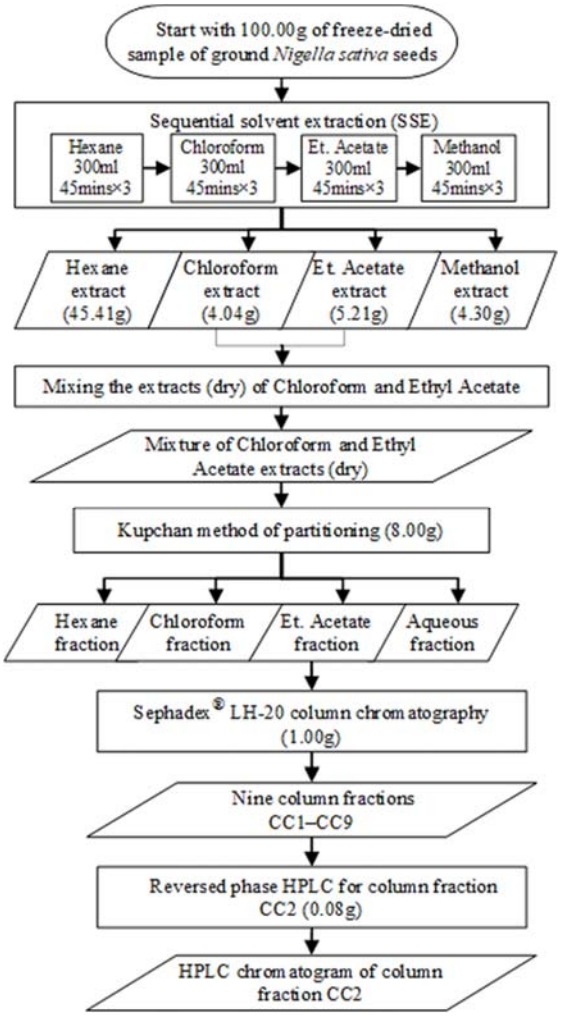

Fig. 2 Flow chart for the extraction process of *Nigella sativa*

III. Results and Discussion

In this study, the primary screening of individual plant materials was carried out by solvent extraction of freeze-dried samples (10 g each) of ground *Nigella sativa* seeds, *Hemidesmus indicus* roots, and *Smilax glabra* rhizome, in 1:1 chloroform:methanol solvent system. A SRB assay for cytotoxicity was performed for each of these total extracts on human HepG2 cells. The results from the SRB assay were analyzed using the Prism software version 6.0 and the half-maximal inhibitory concentration (IC_{50}) values were determined as 257.5 µg/ml, 335.8 µg/ml, and 938.1 µg/ml for

the total extracts of *Nigella sativa* seeds, *Hemidesmus indicus* roots, and *Smilax glabra* rhizome respectively. Since the lowest IC_{50} value implies the highest cytotoxicity, *Nigella sativa* shows the highest cytotoxicity than the other two plant components.

Based on these IC_{50} values, further extraction and purification was considered exclusively for *Nigella sativa* seeds. To begin with, a sample of 100 g of freeze-dried ground *Nigella sativa* seeds was sequentially extracted into hexane, chloroform, ethyl acetate, and methanol. These extracts were concentrated under reduced pressure in a rotary evaporator and dried completely under nitrogen atmosphere. The resultant sequential solvent extracts from hexane, chloroform, ethyl acetate, and methanol were tested for their cytotoxicity on HepG2 cells by SRB assay and the calculated IC_{50} values are 424.5 µg/ml, 169.7 µg/ml, 133.7 µg/ml, and 453.4 µg/ml respectively. In this bioassay process, the highest cytotoxicity was seen in chloroform and ethyl acetate extracts in comparison to that of the extracts of hexane and methanol.

Subsequently, several TLC analysis steps were carried out for these chloroform and ethyl acetate extracts and results show that the two extracts have much similar retardation factor (R_f) values. Based on R_f values from TLC analysis and IC_{50} values from SRB assay results of the chloroform and ethyl acetate extracts, an assumption was made that these extracts would share chemically similar compounds. Hence, the chloroform and ethyl acetate extracts were combined and subjected for further separation and purification using a modified version of Kupchan method of partitioning. In this method of separation, the combined mixture of the resultant extracts from chloroform and ethyl acetate was partitioned into solvents of increasing polarity, in the order of hexane, chloroform, ethyl acetate, and water. A detailed procedure for Kupchan method of partitioning is given under the Methodology section of this paper.

The extracts from Kupchan method of partitioning were tested on HepG2 cells using SRB assay and the calculated IC_{50} values are 70.62 µg/ml, 116.00 µg/ml, 53.09 µg/ml, and 92.56 µg/ml for hexane, chloroform, ethyl acetate, and aqueous extracts respectively. The SRB assay based relative cell survival response of human HepG2 cells when treated with the extracts (under different concentrations) from the Kupchan method of partitioning are shown in Fig. 3. Nonlinear curve fitting for each of these survival response data against extract concentration was conducted (using OriginLab®) for the decay function $y = A_1 \cdot exp(-x/t_1) + y_0$, where y, x, A_1, t_1, and y_0 represent survival response, extract concentration, amplitude, decay factor, and offset, respectively. The value of the decay factor t_1 is obtained from the nonlinear curve fitting with pre-determined values of A_1 and y_0 (see Fig. 3). Since the graph for ethyl acetate extract shows the lowest t_1 value, it further assures that the ethyl acetate extract shows the highest cytotoxicity against HepG2 cells.

TLC analysis for this ethyl acetate extract in several different solvent systems of hexane: ethyl acetate has shown indistinct spots on the TLC plate. Therefore, size exclusion chromatography was adopted as the next step of separation. In this process, Sephadex® LH-20 column chromatography was carried out for a sample of 1.00 g (dry mass) of ethyl acetate extract.

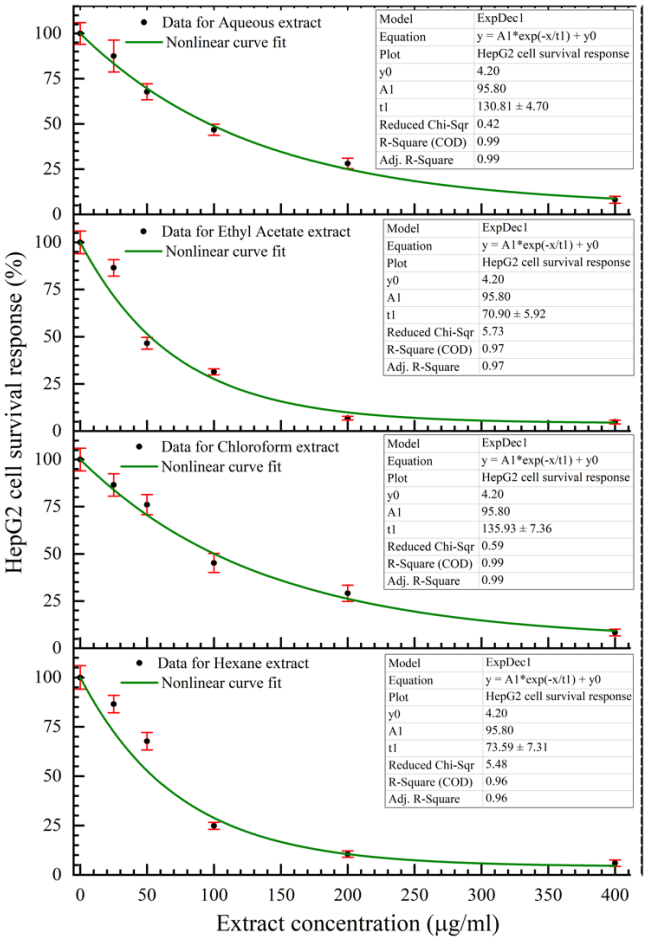

Fig. 3 Cell survival response of human hepatoma (HepG2) cells for fractions from Kupchan method of partitioning. Results are expressed as mean and ± standard deviation

Sephadex® LH-20 column chromatography resulted a total of 60 column fractions, which were then pooled into nine fractions based on their TLC studies. These nine column fractions were dried out and labeled as CC1–CC9 column fraction extracts. The SRB assay was carried out for each of these column fraction extracts and IC_{50} values were determined as 257.10 µg/ml, 13.07 µg/ml, 189.70 µg/ml, 4228.00 µg/ml, 1240.00 µg/ml, 65.75 µg/ml, 180.82 µg/ml, 810.10 µg/ml and 963.70 µg/ml, respectively, for CC1–CC9. Since, the column fraction extract CC2 noticeably shows the lowest IC_{50} value, the fraction extract CC2 is preferable for the use of cytotoxicity against human HepG2 cells.

To visualize the individual components of this cytotoxic column fraction extract CC2, a reversed phase TLC analysis was carried out and a spot of R_f value of 0.25 in methanol:water of 3:2 mobile phase was isolated. The isolated spot was UV active at both 264 nm and 366 nm wavelengths and appeared in a green spot when sprayed with anisaldehyde reagent. To detect any cytotoxic compounds, the column fraction extract CC2 was subjected to reversed phase HPLC analysis and the

chromatogram with percentage response is shown in Fig. 4.

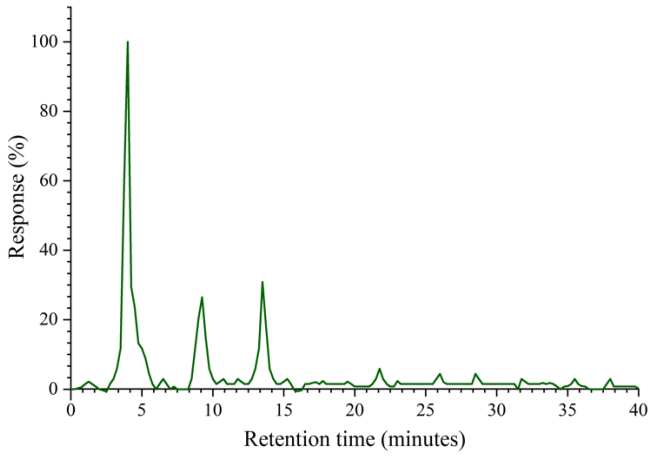

Fig. 4 HPLC chromatogram for the column fraction extract CC2 from Sephadex® LH-20 column chromatography

This HPLC profile for the column fraction extract CC2 shows several peaks, indicating the presence of one or several main cytotoxic compounds in it. The main peaks, which are well above 10% response, appeared at retention times less than 20 minutes and the corresponding compounds, individually or as a combination, could cause the cytotoxicity for HepG2 cells.

In order to completely identify the cytotoxic compounds of the column fraction extract CC2, further purification and complete structure elucidation should be carried out. However, the results obtained in the present study act as strong evidence for the presence of novel cytotoxic compounds in solvent extracts of *Nigella sativa* seeds. Also, the results would be useful in formulation of a standardized pharmaceutical product that can be used in the future for clinical therapy of hepatocellular carcinoma. The current study can also be extended to test the binding affinity of those cytotoxic compounds with fluorescently-labeled cellular receptors in microfluidic devices [9]–[11], which would further facilitate clinical research in testing cytotoxicity of *Nigella sativa* seeds.

IV. Conclusion

In this paper, we show that *Nigella sativa* seeds are highly cytotoxic to human hepatoma (HepG2) cells. We conclude that this high cytotoxicity can be present due to one main active compound solely, or as two or three main active compounds in combination. We further identify that the amount of cytotoxicity exhibited by the main active compound(s) in *Nigella sativa* seeds is substantial enough for formulation of a therapeutic drug against human hepatocellular carcinoma.

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