

Cytotoxic Assay of *Nigella sativa* Leaf Callus Extract (Thymol) on Hep-2 Cell Line Using ELISA Assay

Zaynab S. Abdel Gany*¹ and Mayasaa F. Mahdi*

* Iraqi Center for Cancer and Medical Genetics Researches, Al Mustansiriya University Baghdad , Iraq

Abstract

Extract from cell culture of medicinal plant like *Nigella sativa* have been assessed for its cytotoxic properties. Thymol is likely responsible for the therapeutic effects of *Nigella sativa* leaf callus extract. In this short study the inhibitory effect of *Nigella sativa* leaf callus extract (Thymol) has been studied on Human Lorgnx Epidrmoid Carcinoma (Hep-2) cell line during different exposure period of time (24, 48 and 72 hrs.) using different concentration of the extract (1000, 500, 400, 300, 200 and 100 µg/ml). The optical density of the Hep-2 cells has been readed on 492 nm wave length. Thymol – induced cytotoxicity was (500 µg/ml) which inhibit cell growing compared to the control and this ratio increased at the 48 hrs of exposure and stopped at 72 hrs.

Key wards: *Nigella sativa*, callus extract, cell line, ELISA assay.

الخلاصة

تم عمل مستخلص من النسيج الزرعي للنبات الطبي الحبة السوداء لمعرفة خصائصه السمية للخلايا. الثايمول يعتبر هو المسؤول عن التأثيرات العلاجية لنبات الحبة السوداء من مستخلص أوراق الكالس. تم في هذه الدراسة القصيرة دراسة تأثير مبرتلخص أوراق كالس الحبة السوداء (الثايمول) على نمو الخلايا السرطانية Hep-2 ضمن أوقات تعريض مختلفة (24 و 48 و 72 ساعة) باستخدام تراكيز مختلفة من المستخلص (1000, 500, 400, 300, 200 و 100 µg/ml). تم قياس O.D للخلايا على الطول الموجي 492 nm. وجد ان الثايمول يكون ساما للخلايا السرطانية عند التركيز 500 µg/ml والذي تبط نمو الخلايا بالمقارنة مع معاملة السيطرة و زادت هذه النسبة في المدة 48 ساعة من التعريض وتوقف عند اليوم الثالث من التعريض.

Introduction

The main inspiration of black seed comes from the famous saying (Hadith) of our Prophet Mohammed; (God peace be upon him), that “Habbat Al-soda is remedy for all disease except death.”⁽¹⁾ It is an annual herbaceous plant believed to be endogenous to the Mediteranean region but has been cultivated in other parts of the world including India and Pakistan⁽²⁾. Black seed oil contains about 0.5-1.5 % volatile oil including nigellone and thymoquinone used as anti-histaminic, antioxidant, antiinfective and bronchodilating effects⁽³⁾. Thymol, is one of the active compounds in *N. sativa* extract, plays important role in the inhibition of cancer cells, and can attach with the mutagenic substance, because thymol is one of the antioxidant phenolic compounds⁽⁴⁾. Plant tissue culture techniques inters in several applications like plant micropropagation, genetic study, plant improvement, study of plant cell physiology, the production of secondary metabolites in addition to production of viruse free plant

diseases (bacterial and fungal)⁽⁵⁾. So, to increase the production of this compound (thymol) all the year round without depending on the mother plant, plant tissue culture techniques formed callus and then increased the production of thymol. The anticancer activity of *N. sativa* was first revealed by⁽⁶⁾ who observed enhancement of natural killer (NK) cell activity ranging from 200-300% in advanced cancer patients receiving multimodality immunotherapy programme in which *N. sativa* was one of these components. Thymoquinone and dithymoquinone, active principles of *N. sativa*, had cytotoxic effect against parental and multi-drug resistant human tumour cell lines which were over 10-fold more resistant to doxorubicin and etoposide⁽⁷⁾. Radiation protection activity of *N. sativa* in mice against induction of chromosomal aberrations by gamma ray was also reported⁽⁸⁾

1Corresponding author : E-mail : zaynabsaad@yahoo.com

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The using of plant tissue culture techniques make the easy of pharmaceutical compounds production instead of depending on the mother plant and become possible to produce these compounds at high amount and at high rate of pure may be over than these isolated from the complete mother plant, and its production may be quickly and independent on the season, also limit the surface area that is used in the medicinal plant culturing⁽⁹⁾. The objective of the present study was to assess the cytotoxic properties of this extract from cell culture of *N. sativa* leaf callus using against Hep-2 cell line using ELISA (enzyme linked immunosorbant assay) assay.

Material and Methods:

Collection of plant material:

Seeds of *Nigella sativa* gotten from Dr. Aws Al-Ani (Directorate of Agriculture Research and Food Technology/ Ministry of Science and Technology/ Baghdad/ Iraq) to be used.

Callus culture condition:

The establishment and maintenance of callus were carried out using the procedures described before⁽¹⁰⁾.

Extraction preparation:

For preliminary screening, the seeds were cultured and callus induced and material from callus culture were lyophilized and extracted by a method described elsewhere⁽¹¹⁾. In short, one g of callus was mixed with 30 ml of NaOH solution 5% and then diethyl ether was then added in a ratio of (2:1) (v/v) and mixed well as described elsewhere⁽¹²⁾. The extract was then filtered and concentrated in vacuum at 45°C and then kept in the dark at 4°C until tested.

Cytotoxicity test using ELISA assay:

For this test, the extract were weighed (0.05 g) and dissolved in phosphate buffer saline (PBS) and dimethylsulphoxide (DMSO) to prepare extract solution at 1000 µg/ml. The following dilutions of extract were then prepared: 500, 400, 300, 200 and 100 µg/ml. Hep-2 cell line, obtained from Iraqi Center For Cancer and Medical Genetics Researches at the passage level 326 were used in this study. The origin and description of this cell line was mentioned by⁽¹³⁾. It was a human laryngeal carcinoma excised from 57 years old man, then transplanted in immune suppressed rat by cortisone. After growth of the tumour in the rat, it was then excised and transplanted as an *in vitro* tissue culture. It was kept at -169°C (in liquid nitrogen). In preparation to any *in vitro*

assay, the frozen cell line was withdrawn and maintained in RPMI-1640 containing 10% bovine calf serum. When the *in vitro* cells culture forms a monolayer. These cells were treated with trypsin/ versine mixture in order to pursue subculture process. The percentage of inhibition was calculated according to the following equation:⁽¹⁴⁾

$$\text{Inhibition \%} = \frac{[(OC - OT) / OC] \times 100}{}$$

Where :

OC: optical density of control wells

OT : optical density of test wells

From the above calculation, a graph was plotted for the percentage of growth inhibition against each extract concentration. Activity against Hep-2 cell line was determined by the inhibition assay using an ELISA assay. In short, cell cultures in the microtitration plate were exposed to a range of plant extract concentrations during the log phase of growth and the effect determined after recovery time. The following protocol as described in⁽¹⁵⁾ was performed the extracts of *Nigella sativa* leaf callus :

- After trypsinization, cell suspension seed in a micro titration plates at 50000 cells/ml RPMI-1640 growth medium with serum 5% was used for seeding.
- Plates then incubated for 24 hours.
- By using maintenance medium, two-fold serial dilution were prepared starting from 1000 µg/ml ending with 100 µg/ml.
- After incubation for 24 hrs, cells exposed to different extract dilutions. Only 200 µl of each concentration added for each well (6-replicates for each tested concentration). 200 µl of maintenance medium added to each well of control group. The times of exposure were (24, 48 and 72 hrs). The plates sealed with self adhesive film then returned to the incubator at 37°C.
- After the end of the exposure period, the medium and the cells decanted off and replaced by 200 µl of 0.01% crystal violet dye. After 20 min. the stain was washed gently with tap water for three times. The plate was left until become dry.

The optical density of each well was read by using a micro-ELISA reader at 492 nm transmitting wave length^(15,16).

Statistical analyses:

A one-way analysis of variance was performed to test whether group variance was significant or not, the comparison between groups were used analyses of variance Least Significant Differences Test (L.S.D.)⁽¹⁶⁾.

Results

Cytotoxic effect of *Nigella sativa* leaf callus extract (thymol) on Hep-2 cell line:

The result of the cytotoxic effect of the extract readed using ELISA micro reader with wave length 492 nm indicate the presence of a relation ship between color density of the stain and the number of the viable cells. The result showed presence of significant at level ($p \leq 0.05$) differences between the concentrations compared with control started with the concentration (1000, 500, 400, 300, 200 and 100 $\mu\text{g/ml}$) with inhibition value ranged (67.2%, 75.4%, 74%, 75%, 72.2% and 74%) respectively at 48 hr of exposure periods, while there is no significant differences at level ($p \leq 0.05$) between the concentration itself as shown in figure (1).

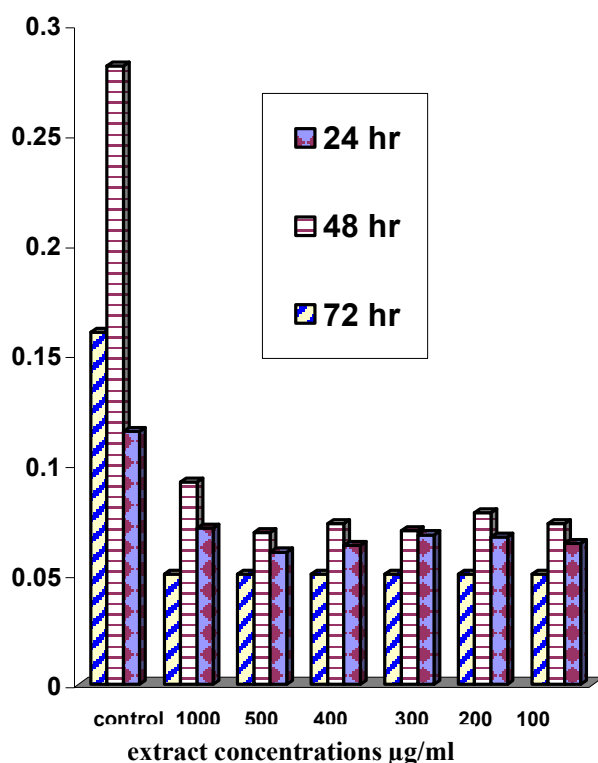


Figure (1): Effect of extract concentrations on the growth of Hep-2 cell line during different exposure preiod.

The results also showed the best exposure period was 48 hr than the other periods (24 and 72 hr), the inhibition to cell line begins at 48 hr, there is no significant differences at level ($p \geq 0.05$) in inhibition when compared with the period 72 hr. This means exposure the extract to cell line at 48 hr with lowest concentration showed significant differences. After that result we choose the inhibitory concentration which inhibit the growth of Hep-2 cell line depending on the changes that appears on the

optical density and the changes in the color that appears on the plate itself from the stain reaction with the cells⁽¹⁷⁾. So, the concentration 500 $\mu\text{g/ml}$ has inhibitory effect compared to other concentration that also showed a minimum inhibition on the growth of Hep-2 cell line at 48 hr exposure period as shown in table (1), and the inhibition ratio was shown in table (2).

Table 1: A comparison optical density of growth inhibition of Hep-2 cell line, by using different concentration of callus extracts of *Nigella sativa* during three periods of exposure.

Type of extract	Conc. ($\mu\text{g/ml}$)	Optical density (OD)		
		24hrs	48hrs	72hrs
Callus extract (thymol)	1000	0.071	0.092*	0.05
	500	0.060	0.069*	0.05
	400	0.063	0.073*	0.05
	300	0.068	0.070*	0.05
	200	0.067	0.078*	0.05
	100	0.064	0.073*	0.05
	control	0.115	0.281	0.16

*) means the presence of significant differences at level ($p \leq 0.05$) between the concentrations.

Table 2: A comparison of growth inhibition percentage of Hep-2 cell line, by using different concentration of callus extracts of *Nigella sativa* during three periods of exposure.

Type of extract	Conc. ($\mu\text{g/ml}$)	Inhibition%		
		24hrs	48hrs	72hrs
Callus extract (thymol)	1000	38.2	67.2	68.7
	500	47.8	75.4	68.7
	400	45.2	74.0	68.7
	300	40.8	75.0	68.7
	200	41.7	72.2	68.7
	100	44.3	74.0	68.7
	control			

Discussion

Although the quinone thymol has demonstrated significant *in vitro* and *in vivo* antineoplastic activities against different tumor cell lines^(7,18). In this study , thymol demonstrated different cytotoxicity *in vitro* toward Hep-2 cell line according to its concentration. This study appeared that the concentration 500 $\mu\text{g/ml}$ affect on the inhibition ratio when compared with the lowest concentrations which show a minimum inhibition compared with the control. This inhibition increased when reached 48 hr of

exposure and stopped at the 72 hr of exposure. The using of these concentrations (1000, 500, 400, 300, 200 and 100 µg/ml) in this study affect on the growth of Hep-2 cell line with slight differences between them , the OD of Hep-2 cell line ratio was the lowest on the concentration 500 µg/ml compared with other concentrations. So, the extract make an inhibition on the growth of Hep-2 cell line compared the control depending on the concentration that is used and the length of incubation period. The result of this study suggest that thymoquinone inhibited proliferation of tumor cell line by a mechanism that involves cytotoxicity, in fact, it is known that thymoquinone (a quinone from *Nigella sativa*) inhibited the proliferation of COS 31 (canine osteosarcoma) at concentration 100µM by inducing apoptosis and cell cycle arrest at G1. Non-cancerous cells are relatively resistance to thymoquinone ⁽¹⁹⁾. *Nigella sativa* and other plants were tested on human hepatoma Hep G2 cell line, the effect were determined on 24 hr of incubation. The greatest inhibitory effects were observed on *Nigella sativa* plant extract even at low concentration ⁽²⁰⁾.

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