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THE EFFECT OF AQUEOUS EXTRACTS OF NIGELLA SATIVA ON BREAST CANCER CELL LINE MCF-7: AN IN VITRO STUDY

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ABSTRACT

Nigella sativa (NS) or also known as black seeds is one of the traditional herbs that were identified to have anticancer properties due to the presence of an active compound, thymoquinone. In this study, the cytotoxic effect of the aqueous extracts of NS were tested on the breast cancer cell line, MCF-7. Aqueous extraction of NS using cold water producing 3.8% of yield. The cytotoxic effect of aqueous extracts of NS were tested after incubation at 5 hours, 24 hours and 48 hours by using a modified MTT colorimetric assay. Interestingly, the IC50 of aqueous extracts of NS on MCF-7 was at 11.5 µg/ml. Additionally, aqueous extracts of NS at the concentration beyond 11.5 µg/ml causing the swelling of MCF-7 and leads to cell burst. In conclusion, aqueous extracts of NS have been proved to have cytotoxic effect on MCF-7.

KEYWORDS

Nigella sativa, aqueous extracts, MCF-7, cytotoxic

1. INTRODUCTION

According to a study, medicinal plants were widely used during the ancient time. This was recorded in many ancient books such as Unani, Ayurveda, Chinese and Arabic Medicines [1-4]. Currently, those plants gain serious attention in modern science and western medicines because of the presence of phytochemical potential and being new discovered drugs with high selectivity on drug design, which that link the traditional and modern medicine [5]. Based on a research, ethnopharmacology have been playing major roles to contribute in the development of alternative medicines and they worked hard to produce on medicinal plant research [6,7]. A study shows, medicinal herbs nowadays are rising confirmation to show that medicinal plants contain synergistic or neutralizing side-effect, which are considered to be safe instead of modern drugs [8,9]. Nigella sativa (NS) or commonly known as habbatus-sauda is one of the well-known medicinal plants that has a lot of benefits to maintain health and cure various diseases.

In the report narrated by Imam Muslim stated that NS is recommended for using on a regular basis in Tibb-e-Nabwi (Prophetic Medicine) which is known as the therapeutic value of healing medicine [8]. All medicinal agents to cure diseases are owned by habatus-sauda except for a death through their studies identified that numerous pharmacological actions are possessed by NS such as antioxidant, anti-inflammatory, antimicrobial, antidiabetic, antineoplastic, as well as anticancer activity [10]. Based on a research, anticancer properties of NS are due to the presence of thymoquinone as active ingredients in methanolic extract [11]. This study investigates the potential cancer inhibition of aqueous extracts of *Nigella sativa* on breast cancer MCF-7 cell lines.

2. MATERIAL AND METHODS

2.1 Preparation of aqueous extracts of Nigella sativa

Nigella sativa seeds were powdered using a mechanical grinder for ten minutes or grounded using pestle and mortar, respectively. The NS powder were weighted for 80 g and placed into a beaker containing 100 ml of distilled water. The water-soluble compounds were let to diffused out from the powder by continuous mixing using a magnetic stirrer for 18 hours, at room temperature. The soluble extracts were separated from the remaining insoluble NS powder in the mixture by filtration using Whatman filter paper number 41. The filtrate was then transferred into an open dish and left to dry for overnight before been powdered by freeze drier. A total of 3.06 g of the freeze-dried powder was recovered. The freeze-dried powder was diluted in growth medium in the all subsequent experiments.

2.2 Maintenance of MCF-7 cell lines

2.2.1 Thawing of cells

Frozen stabilate were placed in 37o C water bath for 1 minute. Then, the thawed cells were resuspended in 3 ml pre-warmed growth medium (RPMI 1640 supplemented with 10% Foetal Bovine Serum) and centrifuged at 1500 rpm for 3 minutes. Later, the supernatant was discarded by aspiration and the cells pellet were again resuspended in 3 ml of pre-warmed growth medium. The recovered cells suspension then transferred into a 25 cm2 culture flask and incubated for minimum of 2 hours in humidified 37o C incubator with 5% (v/v) of CO2 for cells attachment. Following the initial incubation, the culture medium was replaced with fresh culture medium (RPMI 1640 supplemented with 10% Foetal Bovine Serum).

2.2.2 Changing of medium

MCF-7 is an adherent cell. The medium was changed in every 48 hours. To change the medium, first the old medium was removed and discarded by aspiration. Then, 3 ml of fresh pre-warmed growth medium were carefully added into the flask before been incubated until confluent.

2.2.3 Passage of cells

The cells were maintained at confluency of 70% to 80% for the optimal culture condition. Once the cells have achieved the optimal confluency, the cells were passage into a new flask. First, the cells were washed with pre-warmed sterile PBS to remove any dead cells and the carryover medium. Then, 1 ml of room temperature TrypLE was added directly to the cells and incubated at room temperature for maximum of 3 minutes. The proteolytic action of TrypLE were inhibited by the addition 2 ml of fresh growth medium. The detached cells were transferred into a sterile 15 ml centrifuged tube and centrifuged at 1500 rpm, 4°C for 3 minutes. The cells pellet was recovered and resuspended in 9 ml of growth medium before been transferred and divided equally into 3 unit of the same size culture flasks.

2.3 Cytotoxic test

2.3.1 Seeding of cells

An equal number of cells were used in all experiments of this studies. To achieve this, first a flask of MCF-7 at the confluency of 80% were subjected to the TrypLE procedure as above. The recovered cells pellet was resuspended in 500 µl of growth medium. Then, 10 µl of the cells suspension were mixed with 90 µl of 1X trypan blue giving the ratio of 1:10. Some of the stained cell suspension then been applied onto a Neubauer counting chamber. Appropriate dilution was done using growth medium, and the cells density were adjusted to 20,000 cell/well of 96 well plate.

2.3.2 Cells treatment

The cells were treated with a serial dilutions of NS extract starting from 500 µg/ml to 0.97 µg/ml for three different incubation duration, 5 hours, 24 hours and 48 hours. 70% ethanol were used as positive control while medium only as the negative control. All treatment was done in triplicate and repeated for 3 times. The same scheme of treatments was done for microscopy studies.

2.3.3 MTT Assay

Following incubation, the medium with treatments were carefully removed from each well by aspiration and replaced with 100 µl of fresh pre-warmed RPMI. This step is essential due to the reactivity of MTT reagent with the extract. Then, 20 µl of 5 mg/ml of prepared MTT reagent were added into each well and incubated for another 3 hours at 37°C. Then, the RPMI containing MTT were discarded by aspiration leaving the formazan crystal deposited on the cell surface. The crystal then dissolved in 100 µl DMSO and the color intensity were measured using a spectrophotometer (TECAN m4000) at 570 nm of wavelength.

2.3.4 IC50 determination

The absorbance that reflecting the viability of MCF-7 in relative to the negative control were plotted and the IC50 was determined by measuring the concentration of treatment at the point of inhibition of 50% of cells.

2.4 MCF-7 morphology assessment

2.4.1 Preparation of coverslip

In a sterile condition, round shaped coverslip was placed into the 24 well plate by using forceps. Then, 1 ml of absolute ethanol was added onto the coverslips and incubated for 30 seconds for sterilization. Later, the ethanol was removed and washed three times with 500 µl of sterile PBS. The coverslips were then washed with growth medium and kept in 37° C incubator for 5 – 10 minutes.

2.4.2 Preparation of cell on culture slide

A flask containing optimal confluency of MCF-7 were subjected for trypsinization as mentioned in 3.3.1. Due to the larger surface area of the coverslip compare to the 96 well plate, each well of the 24 well plate containing prepared coverslip were seeded with 80,000 cells and maintained in 1ml of growth medium.

2.4.3 Staining

Following the incubation with NS extracts, the old medium was discarded and washed with fresh growth medium. To visualize the cell membrane, a 500 µl of prepared Cell Mask ™ were added into each well and incubated for minimum of 10 minutes. The cells were then fixed using 4% paraformaldehyde for another 10 minutes at 37°C. The coverslip containing cell were then transferred onto a glass slide with the surface that containing cells are facing up. For nucleus staining, a 50 µl of Vectastain ™ DAPI we dropped onto the cells and covered with a thin rectangle coverslip.

2.4.4 Fluorescence microscopy and image analysis

The slides were immediately observed under an inverted microscope at 60x objective magnification. NIS- element D software was used to capture and measure the perimeter of the cell cytoplasm and nucleus. Nucleus was observed using DAPI filter (ex/em: 340/450), while the perimeter of cell cytoplasm was observed using FITC filter (ex/em: 450/515 nm). Three areas with well distributed of cells was searched for each slide and the images were captured.

3. RESULTS AND DISCUSSION

3.1 Aqueous extracts of Nigella sativa

Table 1: Total yield of Nigella sativa extraction

Preparation of powder	Ground sample weight (g)	Freeze dried extract weight (g)	Yield (%)
Mechanical grinder	80	3.05	3.81
Pestle and mortar	80	1.07	1.33

Table 1 demonstrates that little amount of extracts can be recovered by using cold water extraction methods. Seeds powder preparation also showed a significant determinant for the yield of extracts, where mechanical grinder gives up to three folds more yields compared to pestle and mortar. This might due to the size of the NS powder is finer when mechanically grounded compared to the use of pestle and mortar. According to a research, cold water extraction methods however, yields significantly low extracts as compared to the extraction using soxhlet [12].

3.2 Cell viability

Cell viability were affected by the addition of treatments in a uniform trend. Figure 1-3 shows the percentage of viable cells in response to the serial dilution of treatment concentrations after 5 hours, 24 hours and 48 hours of incubation.

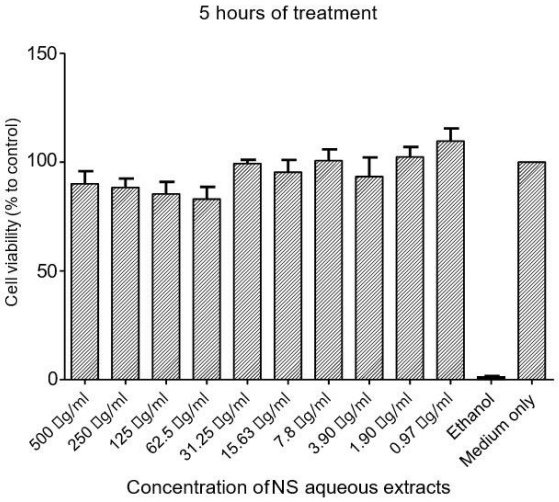

Figure 1: The percentage of viable cells in comparison to untreated cells following 5 hours of incubation. The data was the average of measurement from three different set of experiments. Single tailed error bar was the +3 S.E.M.

Based on a study, cells treatment for 5 hours did not show any sign of immediate cell death (Figure 1). This may indicate that the aqueous extracts of NS did not kills the MCF-7 immediately and some molecular events may be involved [13]. Although not significant, it is worth to emphasize that the concentration below 31.25 µg/ml did not cause any changes compared to untreated cells (medium only), but slight changes occurs in the cell viability if the concentration is over 62.5 µg/ml. In contrast to 5 hours, both 24 hours and 48 hours of incubation with NS extract shows significant changes in the cell viability in a pattern of proportional to the concentration (Figure 2 and Figure 3).

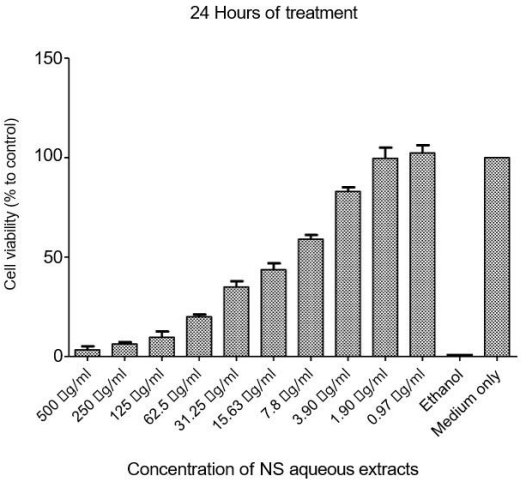

Figure 2: The percentage of viable cells in comparison to untreated cells following 24 hours of incubation. The data was the average of measurement from three different set of experiments. Single tailed error bar was the +3 S.E.M.

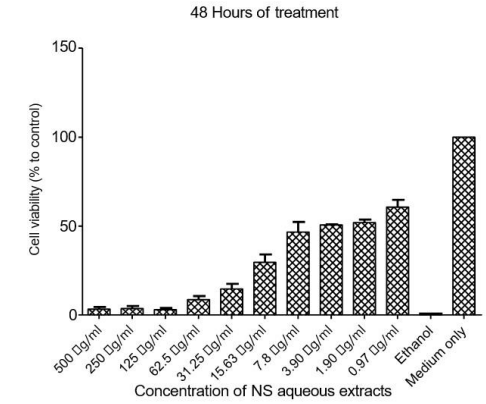

Figure 3: The percentage of viable cells in comparison to untreated cells following 48 hours of incubation. The data was the average of measurement from three different set of experiments. Based on a research, single tailed error bar was the +3 S.E.M. In comparison of 24 hours incubation, 48 hours incubation with aqueous extracts of NS shows more significant inhibition. For 48 hours incubation, the lowest concentration of extract shows inhibition near to 50% of MCF-7 population. It is in line with the doubling time for MCF-7 is every 24 hours [13,14].

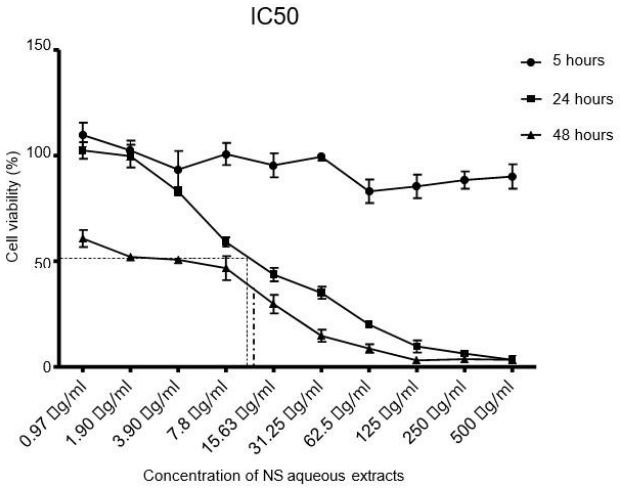

Figure 4: The percentage of viable cells in comparison to untreated cells following 5, 24 and 48 hours of incubation. The data was the average of measurement from three different set of experiments. Dotted line indicates the concentration equivalent of 50% of viable cells. Error bar was the +3 S.E.M. The optimal concentration was determined by using IC50 plot method. The percentage of viable cells were plotted in a line chart and the inhibition to 50% of the MCF-7 population was extrapolated. Figure 4 clearly shows that the IC50 for both 24 hours and 48 hours treatments are at the almost similar range of concentration. The IC50 for both falls between 11.7 to 12 µg/ml.

3.3 Morphological changes upon treatment with Nigella sativa

The morphological changes of MCF-7 breast cancer cell in response to Nigella sativa extract was evaluated quantitatively and qualitatively. The area of plasma membrane and nucleus of the cells was used to measure the ratio of the area. The mean and standard deviation of the ratio area were used to get the overall result of the treatments. All the calculated data of all treatment and controls are tabulated in Table 2.

Table 2: Mean and standard deviation of ratio area

Treatments	5 Hours	24 Hours	48 Hurs
Q5 medium	4.97 ±1.46	2.24 ±0.55	None
PBS	3.30 ±1.38	2.04 ±0.32	None
500 µg/ml *N.sativa*	None	None	None
15.63 µg/ml *N.sativa*	2.62 ±0.58	None	None
0.97 µg/ml *N.sativa*	2.22 ±1.07	None	None

The mean of ratio calculated showed that the size of the cells treated after hours is larger than 24 hours for the respective treatment. There is no calculated ratio obtained for all Nigella sativa treatments induced after 24 hours exposure. Nearly double the size of reduction from the positive control for the 15.63 µg/ml and 0.97 µg/ml of extracts in 5 hours treatment.

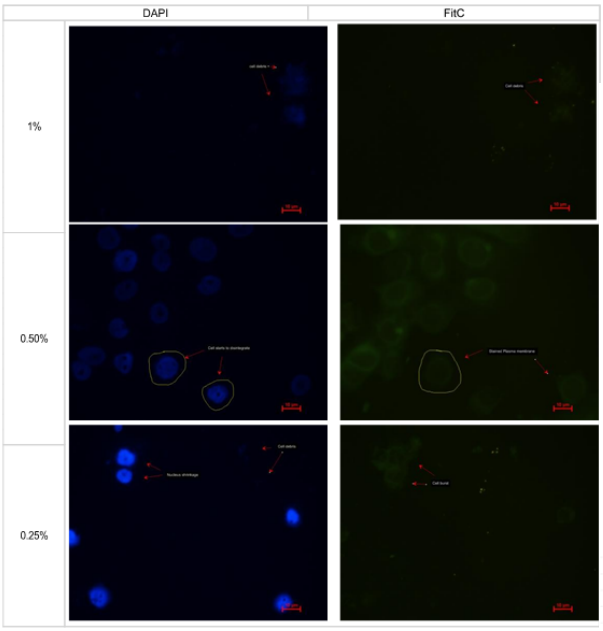

Figure 5: Morphology of the cells for 500, 15.63 and 0.97 µg/ml treatments after 5 hours exposure

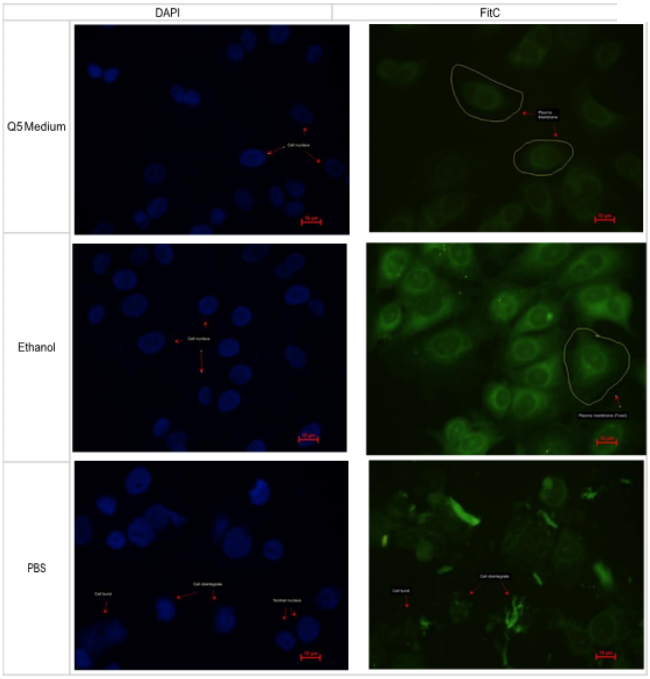

Figure 6: Morphology of the cells for all controls treatment after 5 hours exposure

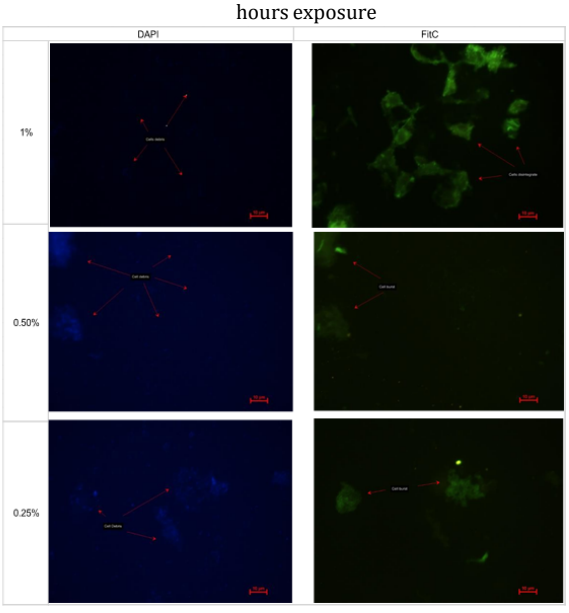

Figure 7: Morphology of the cells for 500, 15.63 and 0.97 µg/ml treatment after 24 hours exposure

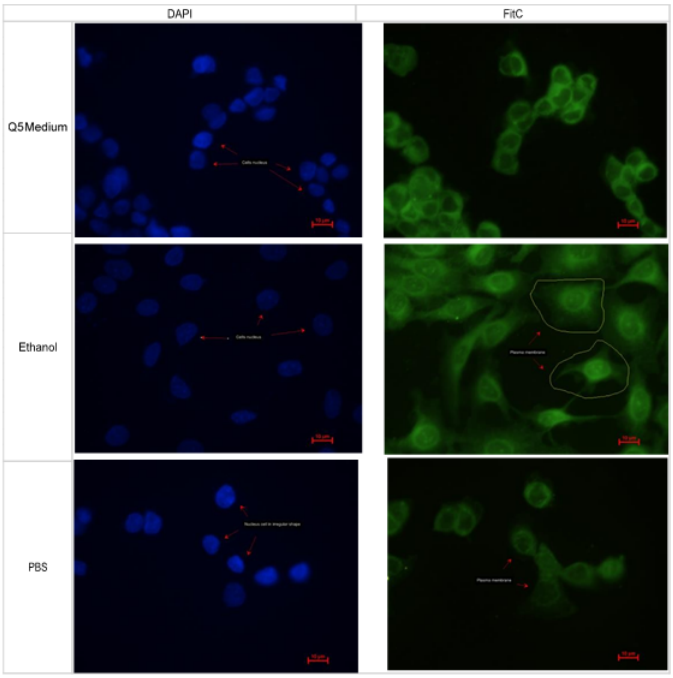

Figure 8: Morphology of the cells for all controls treatment after 24 hours exposure

Figure 5 to 8 shows the microscopy appearance of MCF-7 after the treatment with selected concentration of NS extract for 5 and 24 hours. No microscopy images were recorded for 48 hours of treatment due to the inability to locate live cells. Figure 5 and 7clearly indicates that the integrity of the cells were damaged following 24 hours and 48 hours of treatment with any concentration of NS extracts. In highlight, NS extracts was found to increase the average size of nucleus with slight fragmentation and decreasing the average size of MCF-7 cytoplasm which could be the indicator for cell death mechanism. Based on a study, NS extract was found to induce program cell death through caspase 3, 8, 9 and BAX pathway [15].

Interestingly, in all cell viability experiment the viability of MCF-7 treated with ethanol were reduced to near 1%, but microscopy images show an intact MCF-7. Research shows that this may due to the nature of ethanol that can fix the cells on the glass slide, thus keep the morphology intact although no metabolic activity as showed by MTT assay [4].

In general, aqueous extract of NS shows significant inhibition in the viability of MCF-7 in the pattern of concentration and incubation period dependent. Although the use of MTT has been used in many viability studies, it was recommended to include some microscopy analysis as it can supply the information of the cells integrity [16].

4. CONCLUSION

Aqueous extracts of Nigella sativa have water soluble active compound that can inhibit the growth and viability of MCF-7 in incubation period and concentration dependent relationship. The extract also can reduce the integrity of MCF-7 cell membrane and causing the swelling of its nucleus.

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