Effect of Linoleic Acid of Nigella Sativa on MDA-MB-231 Human Breast Cancer Cells

Hasanzadeh GK[1], Latiffah AL[1], Hanachi P[2], Hj Lajis N[3]

Abstract

Background: Breast cancer is one of the main life-threatening diseases which a woman may face during her life. Several lifestyle factors such as weight gain, obesity, fat intake and decreased level of physical activity are associated with breast cancer risk. In vitro and vivo studies showed that, Linoleic acid (LA) is one of the main fatty acids composition of Nigella sativa.

The objective was to investigate inhibitory and anti-cancer effects of Linoleic acid on the MDA-MB-231 human breast cancer cells.

Methods: The apoptosis and cytotoxic activity assay was used in order to find toxic effects and the results were supported by flow cytometry (Cell cycle analysis).

The results showed the cytotoxic effect of Linoleic acid on the breast cancer cell can be considered as an anti-cancer effect of LA.

Results: According to our findings, when the concentration of lionleic acid was increased, compared with the concentrations currently being reported, it showed an anti-cancer effects. The IC50 was 84.72µl/ml. There was a significant ($p<0.05$) effect between the treatment groups which are more than IC 50 and the control group.

Conclusion: We came to this conclusion that Linoleic acid has an inhibitory effect on human breast cancer cell lines which can be due to its two double-bandings molecular structure.

Key words: Linoleic acid; Nigella sativa; MDA-MB-231

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Introduction

Overweight women are the most commonly observed to be at increased risk of postmenopausal breast cancer and at a reduced risk of pre-menopausal breast cancer. Obesity and a high intake of meat, dairy products, fat, and alcohol may increase risk of breast cancer but a high intake of fiber, fruits, vegetables, anti-oxidants and phytoestrogens may reduce the risk of breast cancer [1].

Considering hazards in treatment failure, drug resistance, heavy costs and other problems associated with current cancer therapy, medicinal plants have attracted interest of many researchers in this field. The use of the medicinal herbs for diseases has been practiced in all civilizations e.g. in ancient Egyptian, ancient Chinese, Indian Ayurvedic and Unani medicine [2].

Fatty acids have previously been shown to modulate eicosanoid metabolism both in vivo and in vitro [3-4]. Epidemiological and experimental studies have revealed an association between dietary fat and the incidence of breast cancer [5-6].

Linoleic acid (LA) has been shown to have an inhibitory effect on MCF-7 cells [7] whereas it has been indicated to have low efficacy on MDA-MB-231 proliferating activity [8]. According literature LA inhibits cancer cell proliferation in different manners according to the cell line and experimental model [9, 10]. It was found out omega 6 family LA did not have any effect on cell proliferation [11, 12, and 13]. LA has been shown to inhibit the initiation and promotion stages of chemical-induced mammary carcinogenesis in animal models [14, 15] and to

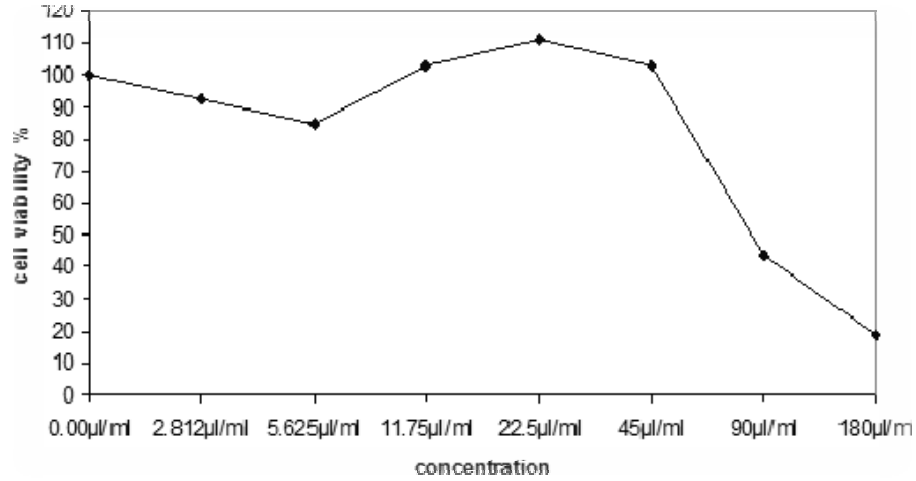

Figure 1. Percentage of cell viability in different concentrations of LA on MDA-MB-231

exert an inhibitory activity on human breast cancer cell growth in vitro [16, 17]. The objective was to investigate inhibitory and anti-cancer effects of Linoleic acid on MDA-MB-231 human breast cancer cell lines.

Materials and Methods

Materials

Human breast cancer cell lines, MDA-MB-231(HTB-26), were obtained from ATCC.DMEM Dulbecco's Modified Eagle's Medium – high glucose (tissue culture medium), (SIGMA.CHEMICAL. Co. Louis, MO, USA), Fetal bovine serum Mycoplex (FBS), (PAA Laboratories GmbH); Penicilin/Sterptomycin (100X) (PAA Laboratories GmbH); Phosphate Buffered Saline (PBS), (SIGMA.CHEMICAL. Co. Louis, MO,USA), Parafilm (4INX125FT.ROLL), (Menasha, WI, USA), Trypsin-EDTA (1X), (PAA Laboratories GmbH); Dimethyl sulfoxide minimum 99.5% GC (DMSO), (SIGMA, Louis, MO, USA); LINOLEIC ACID 99% (ALDRICH, Louis, MO, USA); CellTiter 96® AQueous One Solution Reagent, tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS], (Promega, Madison, WI, USA), RNase A (Novagen, WI, USA),Propidium Iodide(Novagen, WI, USA)were purchased from Sigma (St. Louis, MO).

Cell Culture

All experiments had 4 replications and were repeated 4 times, observing for both ER and ER- breast lines MDA-MB-231 human breast cancer cell lines. The estrogen receptor negative human breast cancer cells lines MDA-MB- 231, were grown in DMEM with 10% bovine calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 -C in a 5% CO2 atmosphere. Before start, cells were washed with phosphate-buffered saline (PBS) twice and placed in DMEM with 0.5% fetal bovine serum (FBS).

Treatment Procedure

For each experiment, seven doses were carried out. They can be made from the highest to the lowest concentrations by diluting them to half of its original up to 6 times. In each experiment, one control group was considered as well which was the only provided with growth media and received no treatment at all. The highest concentration of linoleic acid was 180µl/ml which was diluted to half 6 times i.e. 90, 45, 22.5, 11.75, 5.625, and 2.812µl/ml as the treatment groups. The control group received no treatment.

The treatment groups and the control group were in one 96-well plate at quite the same condition. The cells were seeded in 96-well assay plate at a density of $1x10^5$ cells/cm2 and left them to adhere overnight. Before experiments, cells were washed with phosphate-buffered saline (PBS) twice. For treatments, the cells placed in DMEM with 0.5% fetal bovine serum (FBS) containing LA and supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin. 18 mg of linoleic acid 99% were weighed by digital scale (Shimazu model, Japan), and solved in 1 ml DMSO. The first row (A row) of 96-well plate was the highest concentration (180µl/ml) and diluted to half to get the lowest concentration at 7^{th} row (G row); last row (H row)

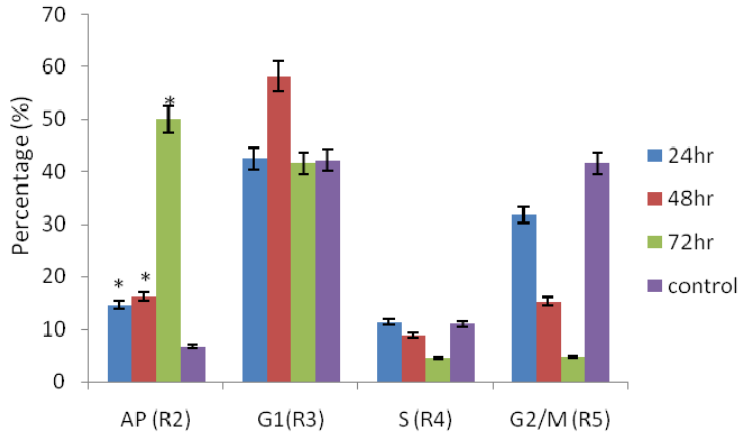

Figure2. Low dose (2.812µl/ml) effect of Linoleic acid on cell cycle progression of MDA-MB-231cells. *Significant level was set at P< 0.05. Data are expressed as Mean±SEM

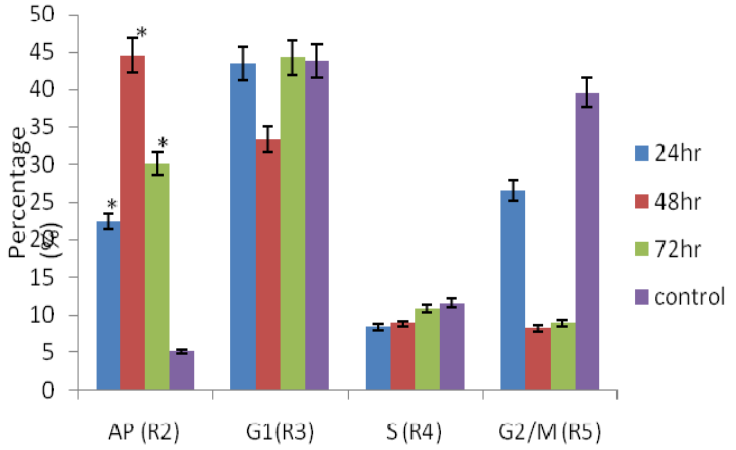

Figure3. Medium dose effect of Linoleic acid on cell cycle progression of MDA-MB-231cells. *Significant level was set at P< 0.05. Data are expressed as Mean±SEM

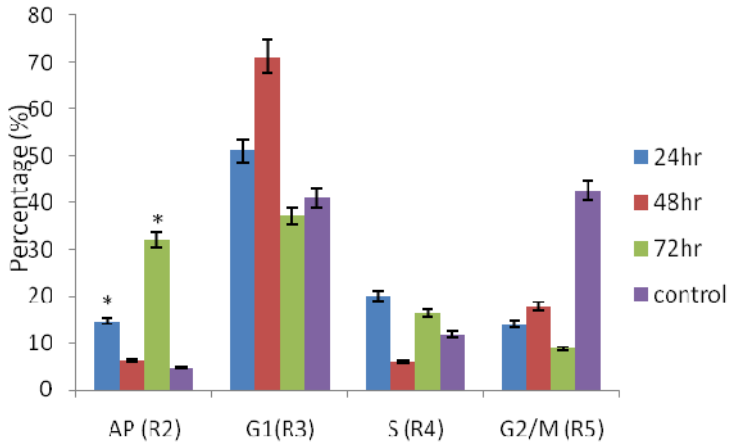

Figure4. High dose (90. 45 µl/ml) effect of Linoleic acid on cell cycle progression of MDA-MB-231cells. *Significant level was set at P< 0.05. Data are expressed as Mean±SEM

had growth media, without any treatment and was considered as a control group for 24, 48, and 72 hours.

Cell Viability Determination (MTS assay) Growth-Inhibition Assay

After 24, 48 and 72 hours of treatment, Cell viability was examined using CellTiter 96 Aqueous One Solution® (Promega, Charbonni`eres, France) according to the manufacturer instructions. 20µl of CellTiter 96® Aqueous One Solution Reagent was added to each of the 96-well assay plate containing the samples in 100µl of culture medium containing treatment solutions for the treatment groups and the control groups. Multichannel pipettes were employed for convenient delivery of uniform volumes of CellTiter 96® Aqueous One Solution Reagent to the 96-well plate. The plates were incubated at 37°C in incubator, 5% CO2 for 4 hours. The absorbance was recorded at 490nm using a 96-well plate reader (Bio-Rad, Richmond, CA, USA). The Reed-Muench Method was employed for counting IC_{50} [18].

Flow Cytometry

Flow Cytometer is for analyzing the distribution of cells in different cell cycle phases. Flow cytometry method is implemented to measure, identify cells and micro particles. Viable, apoptotic (cells present at subG0/G1 peak) or necrotic (cells with DNA content below subG0/G1) cells and percentages of cells in different phases of the cell cycle were evaluated by determining the DNA content after Propidium Iodide staining. The cells were suspended and counted at a density of $2x10^5$ cells/cm2 intoT-25 flask. They were left to adhere overnight. The cells took various concentrations of linoleic acid and incubated for 24, 48 and 72hs. After treatments, cells were harvested with 1ml of Trypsin-EDTA, washed in PBS and centrifuged at 1200 rpm for10 min. The pellets were fixed in 500 µl of cold PBS and were slowly added with 5 ml of 70% cold ethanol in 20^0C for 2 hours. The pellets were centrifuged at 1200 rpm for 10 min and the supernatant was carefully discarded to wash out the ethanol. The pellets were re-suspended in 10 µl RNase + 40 µl PI + 950 µl PBS. The final volume was 1 ml. The cells were finally incubated for 30 min on ice in the dark. The PI and RNase concentrations and volumes were referred Ormerod, 1992 [19] and then analyzed with a flow cytometer (CyanADP, Dako, Denmark) equipped with a 488 nm argon laser. Data were recorded on a Macintosh computer (G3), using software Summit V4.3 (Dako, Denmark).

Statistical Analysis

Statistical analyses were performed by one-way analysis of variance (ANOVA), Tukey's multiple comparison and Student's t-test using SPSS version 15.0. $P<0.05$ was considered as statistically significant difference.

Results

Growth-Inhibition and Assay Effect of Kinoleic Acid

MDA-MB-231: The result of linoleic acid effect, the main fatty acid of N. sativa seed oil, is given at Figure 1. The highest concentration of linoleic acid is 180µl/ml which is diluted to half its original 6 times i.e. 90, 45, 22.5, 11.75, 5.625, and 2.812µl/ml, as treatment groups. Control group received no treatment.

Effect of Linoleic Acid on MDA-MB-231 Cells

Based on the results, Linoleic acid has a growth promoting effect on the cells in concentrations between 11.75µl/ml and 22.5µl/ml but when the concentration is increased to more than 22.5µl/ml, linoleic acid showed cell growth inhibitory effect. The results of cell viability are given in figure 1. Based on the statistical results, there is a significant ($p<0.05$) difference between the treatment groups which are more than IC_{50} and the control group. The IC_{50} was 84.72µl/ml, however, linoleic acid showed an anti-cancer effect on the MDA-MB-231 human breast cancer cell lines.

Influence of Linoleic acid on Cell-Cycle Distribution and Apoptosis

Cell cycle analysis of Linoleic acid shows considerable increase in apoptosis phase of the cells lines. Figures 2-4 shows the changes in the cell cycle distribution of MDA-MB-231 treated with linoleic acid at different incubation hours and different concentrations and the control group which caused 50% cell at each time point. According to fluorescent intensity distribution obtained from Figs. 2-4, the first large peak represents cells in G0/G1 Phase (resting or protein synthesis phase) while the second phase represents the G2 phase (construction of mitotic apparatus phase). In normal cell profile, the G2 peak has twice the amount of DNA which gives twice fluorescent intensity. The area between G0/G1 and G2 peak belongs to S phase (DNA synthesis phase). When the cells undergo apoptosis, sub cellular of the cells will appear before G0/G1 peak and was referred as Sub-G0/G1 phase.

The reduction in cell proliferation rate was due to a higher percentage of cells which can enter to Sub G1 at different concentration of LA. Figures 2-4 shows the apoptotic rate after 24, 48 and 72 hours treatment on MDA-MB-231.

Discussion

Nigella sativa L., an annual plant from Ranunculaceae, has been traditionally used in Middle East, part of Asia, Africa and India [20, 21]. Apoptosis pathways and targeting cell cycle has emerged as an attractive approach for the treatment of cancer. Apoptosis can be modulated by targeting pro-apoptotic or pro-survival pathways. In this study the apoptosis effect of linoleic acid [22-23] as well as conjugated linoleic acid [24-27] was well documented. It is known that high concentration of certain fatty acids can cause cell death via apoptosis or necrosis [28]. The results obtained from the cell cycle analysis of Linoleic acid shows considerable increase in apoptosis phase of cells lines. Newly founded points in this study indicate that LA reveals much acceptable cytotoxic effects while in the previous studies these effects were only related to conjugate LA; and some studies mentioned that LA leads to promotion or LA has not any effect on the growth of cell line [29]. According to previous studies different kinds of conjunct LA have anti-cancer effects [30-37] and the LA itself had not shown a noticeable anticancer effect.

Conclusion

All in all, LA has cytotoxic effects and thus can be listed as an anti-cancer medicine. Since there is a remarkable amount of omega 6 of LA in cooking oil, it can be considered as a supplement. Furthermore, this research indicates that cytotoxic effect of the extract and active ingredients was more noticeable in lower concentration in MDA-MB-231 cells. However, when the LA concentration was increased compared to the former concentration, it showed a considerable anti-cancer effect. This was quite a new finding not being observed in the former studies. In this study with MDA-MB-231 reduced viability and the reaction were dose and time dependent manners as well.

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Conflicts of Interest

The authors have no conflict of interests in this article.

Authors' Contribution

HGK contributed data analysis and wrote the paper, LAL designed the study and writing-up process, HP contributed data entry and literature review while NHJ provide the equipments and materials. All authors read and improved the final manuscript.

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