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Apoptosis Cell Death Effect of Linoleic Acid from Nigella Sativa on Human Ovary Cancer Cells through Mitochondrial Intrinsic Pathway

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In this study, we evaluated the cytotoxic potential of fractions (F1-F5) isolated from hexane extract of the seeds of *N. sativa* on *human ovarian carcinoma cell line, A2780*. F2 showed an outstanding potent cytotoxic effect against A2780 cells. Next, this fraction was purified to obtain six sub-fractions (SF1-SF6) and their cytotoxic effects were then evaluated. The obtained results showed that SF2 had strong cytotoxic effect against A2780 cell line. The effective sub-fraction (SF2) was determined to be linoleic acid (LA) according to spectroscopic analyses. In the next set of experiments, the apoptotic potentials of LA were investigated. Induction of apoptosis by LA was accompanied by an increase in activation of caspase-3, -9 and reduction in mitochondrial membrane potential (MMP) in A2780 cells. It can be concluded that LA, inhibited the growth of human ovarian carcinoma cells, A2780 and induced mitochondrial-related apoptosis.

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Introduction

Ovarian cancer is the fifth leading cause of cancer-related death among women. Because of the absence of the symptoms at the early stages of the disease and poor prognosis, the overall 5-year survival rate of patients with ovarian cancer is only about 30% in most developed countries [1] .
Nigella sativa (Ranunculaceae) seeds are used as a condiment in food and claimed to have many therapeutic effects in traditional medicine such as anti-inflammatory and antitumor [2].
Ibn-Sina, the 11th- century Persian physician, used to use black cumin for the splenic tumors [3]. Injection of *N. sativa* oil into mice tumor reduced the size [4] and its oil could decrease 33% sarcoma growth in croton oil-induced tumor in mice [5]. On the other hand, seeds fatty acids showed anti-tumor activities against Ehrlich ascites carcinoma, Dalton's lymphoma ascites and Sarcoma-180 cells [6].Besides, its quinones and saponins exerted the anti-apoptotic effect on Hep-2 cell line [7]. The present study is designed to the bioassay‑guided isolation of cytotoxic constituents of hexane extract of the fatty acids extracted from seeds of *N. sativa* on human ovary carcinoma cell line, A2780. This cell line was established from an ovarian endometrial adenocarcinoma tumor from an untreated patient and extensively used for studies about ovarian cancer [8]. The underlying mechanism of the cytotoxic effect of the most potent fraction was also investigated. Moreover, the further phytochemical study on the effective fraction was carried out.

Materials and Methods

Plant material

Seeds of *Nigella sativa L*. were bought from Faculty of Agriculture, Razi University where the herbarium specimen was identified by Dr. Nastaran Jalilian, Botanist at Research Center of Agriculture and Natural Resources of Kermanshah Province, Iran and compared to the specimen No. 248008007. For herbarium sample preparation, seeds were grown in proper condition and plant material was dried and pressed.

Bioguided fractionation and purification of active constituents

Extraction and fractionation was performed as previously described [9]. In brief, ground seeds were extracted with hexane using a Soxhlet apparatus and fractionated by several Silica gel open column and HPLC analyses based on results of biological part.

Cell culture and cell viability assay with MTT test

The A2780 human ovary cancer cell line was purchased from the National Cell Bank of Iran (NCBI NO: C461). The cell maintained in DMEM-F12 medium (Sigma, St Louis, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco, USA). The cell viability was assayed by MTT assay as described by Mossman [4].

Measurement of caspase activity

Caspase-3 and 9 activity assays were carried out using the sigma colorimetric caspase kit. This assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate, Ac-DEVD-pNA (for caspase-3) and Ac-LEHD-pNA (for caspase-9) in equal amounts of cell protein.

Measurement of mitochondrial membrane potential (MMP)

In the current study, rhodamine 123 fluorescent dye, a good candidate to measure the actual mitochondrial membrane potential, was used for monitoring MMP. Briefly, at the end of treatment, cells were added with 5 mg/l rhodamine 123 for 30 min at 37 °C. The fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 520 nm using a fluorescence microplate reader (BioTek, H1M, USA).

Statistical analysis

Graph Pad Prism 3 software is used for analyzing the data and IC_{50} calculation. To examine the mean difference of groups, GraphPad InStat Version 3 statistical program and one-way ANOVA test and the relevant post-test are used.

Results

Cytotoxic effect of fractions and purified sub-fractions from active fraction

As shown in the Figure 1 exposure with fraction F2 potentially present an interesting cytotoxic activity towards human ovary cancer cell line (IC_{50} =10.89 µg/mL) . Fractions F1 (IC_{50} =23.8 µg/mL) and F4 (IC_{50} =74.2 µg/mL) showed a moderate cytotoxic effect against A2780 cell line. F3 and F5 (IC_{50} >100 µg/mL) did not show any significant cytotoxic effect against cells. Based on this information, F2 was further purified in three step to get six sub- fractions, SF1-SF6. As shown in the Figure 2 except for SF2 (IC_{50}= 26 µg/mL), all sub-fractions had no significant effect on the A2780 cell viability up to 200 µg/mL (IC_{50} >200 µg/mL). Morphological changes in treated cells with SF2 for 24 h showed that most of the cells were adhered to the culture flask in the control cells but after 24 exposure to SF2 cell population significantly decreased. Therefore, further phytochemical studies on this sub- fraction were carried out. Additionally, this sub-fraction was selected for identification of mechanisms of cytotoxic effect in A2780 cell line.

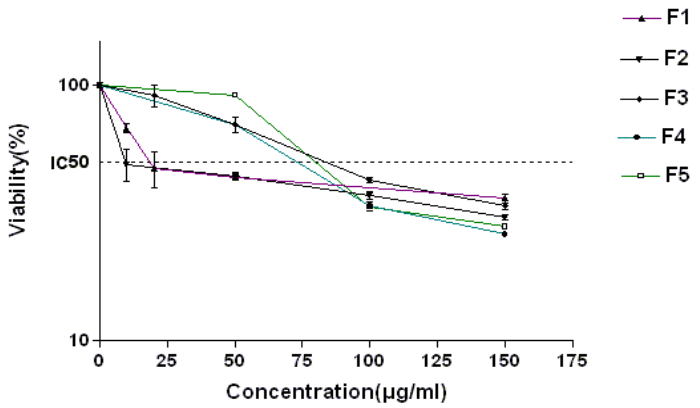

Fig. 1. The effects of fractions (0-150 *µ*g/ml) of hexane extract of the seeds of N. sativa on human ovary carcinoma cell line, cells. The cell viability was determined by MTT assay after 24 hr exposure as described in materials and methods. Data are expressed as the mean±SEM of three separate experiments.

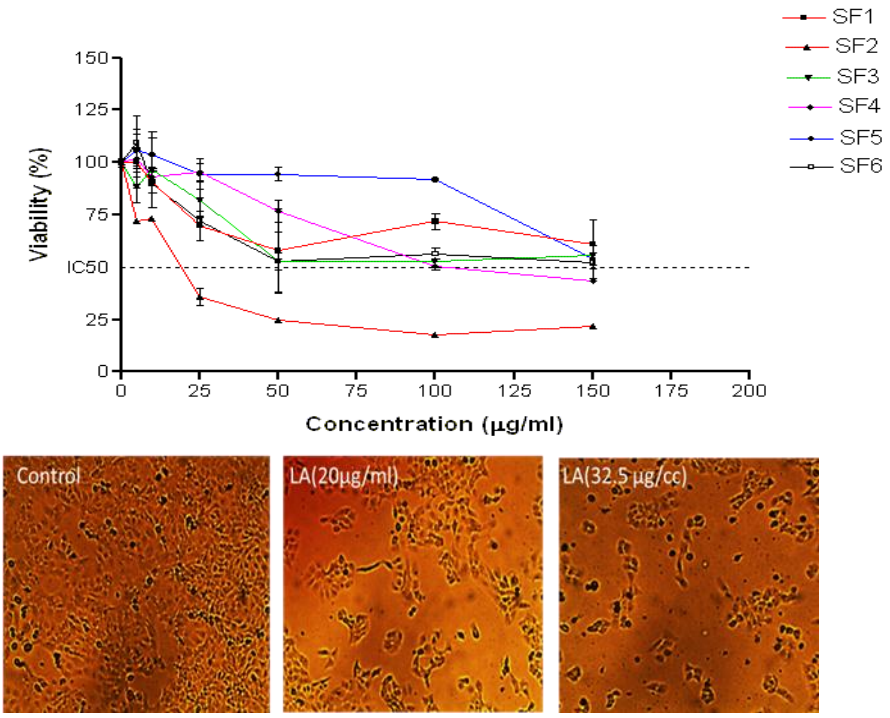

Fig. 2. A) The effects of purified fractions (0-150 μg/ml) from potent fraction(F2) of hexane extract of the seeds of N. sativa on A2780 cells viability. The cell viability was determined by MTT assay after 24 hr exposure as described in materials and methods. Data are expressed as the mean±SEM of three separate experiments. B) Representative photomicrograph shows morphological changes of A2780 after 24 hr treatment with LA(SF2).

Characterization of effective sub-fraction

Sub-fraction SF2 showed to be the most effective fraction. In chemical analyses including HNMR and GC experiment, it was determined to be linoleic acid (LA)(Figure 3) [10-12). Its retention time was compared to that of standard in GC apparatus. HNMR data showed unsaturated peaks at 5.4 ppm and aliphatic peaks at 1 to 3 ppm. In detail it is like the following :

Linoleic acid; 1HNMR (CDCl3, 400 MHz) δH 0.92 (3H, H18, t, J=1.2 Hz), 1.22 (8H, H4, H5, H6, H16, d), 1.35 (4H, H7, H15, d), 1.67 (4H, H3, s), 2.08 (4H, H8, H14, m), 2.38 (2H, H2, m), 2.81 (2H, H11, t), 5.40 (4H, H9, H10, H12, H13, m). EI MS: m/z 280 [M]+, 206 [M-74]+, 164 [M-116]+, 249 [M-31]+, m/z =74.

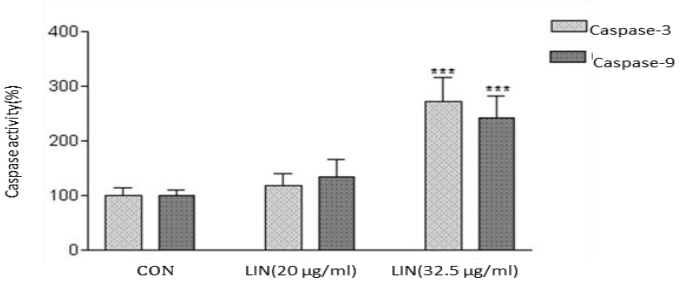

Fig. 3. Effect of LA on caspase-3 and 9 activity. Caspases activity was measured by colorimetric detection of p-nitroaniline and expressed as percent of control. Data are expressed as the mean± S.E.M of three separate experiments. *** P<0.001 vs. control treated cells

Effect of linoleic acid on caspase -3 and -9 activity

As seen in the Figure 3 After 24 h treatment of A2780 cells with LA a dose dependent increase in the activity of caspase 9 and 3 were detected in comparison to control (Figure 4). The caspase 3 and 9 activity increased 2.5 and 2.1- fold upon treatment with LA (32.5 µg/mL), respectively (p<0.001).

Effect of linoleic acid mixture on MMP

The collapse of MMP in A2780 cells was monitored with a cell permeable cationic fluorescent dye, rhodamine 123. Our results showed that fluorescence intensity was reduced after incubating cells with 20 µg/cc and 32.5 µg/cc of LA in a dose dependent manner. As shown in the figure 4, LA (32.5 µg/mL) was able to decrease significantly MMP to 63.2±5.1 compared to control (Figure 4).

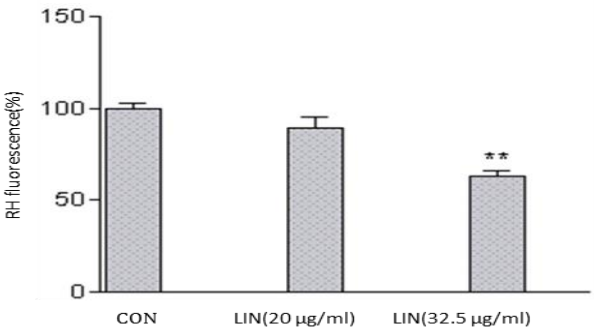

Fig. 4. Effect of LA on MMP collapse as detected by Rhodamine 123. Data are expressed as the mean± S.E.M of three separate experiments.Control, ** P<0.05 vs. control treated cells

Discussion

Poly unsaturated fatty acids are able to induce toxicity on cancer cells through apoptosis, either alone [13] or in combination with conventional

anticancer therapies [14]. Gamma linolenic acid (GLA), an essential fatty acid of omega-6 series, is produced in the body from linoleic acid by the enzyme delta-6-desaturase. It has been found that GLA potentiated cytotoxicity induced by paclitaxel in breast cancer in a dose-dependent manner [15]. Furthermore, it can regulate the levels of gene products including matrix proteins which are important in cancerous condition [16].Butyric acid derivatives in combination with currently used tumor therapeutic agents produced a synergistic effect on the growth of mouse neuroblastoma cells and rat glioma cells in culture [17]. Butyric acid is able to induce apoptosis in human colon carcinoma cells in vitro and in vivo [18]. In this study, the cytotoxic activities of a series of isolated from hexane extract of the seeds of *N. sativa*, against human ovary carcinoma cells, A2780, were evaluated. When the cytotoxic effect of five fractions was examined, we observed that F2 markedly decrease the viability of cells. Among the purified sub-fractions from effective fraction, SF2 showed the highest inhibitory potency against the growth of A2780 cells, whereas other sub-fractions failed to exhibit any cytotoxic activities. Spectroscopic data revealed that SF2 is linoleic acid (LA). Since, the induction of tumor cell apoptosis plays an essential role in cancer therapy in the next set of experiments; we investigated the apoptosis-inducing potential of the LA in A2780 cells. Our results showed that LA reduced proliferation was accompanied by in increased caspase-3 activation. Caspase-3 has been identified as being a key mediator of apoptosis in mammalian through extrinsic and intrinsic pathway of apoptosis [19]. To determine the role of intrinsic pathway in induction of apoptosis, we also evaluated caspase-9 activity and MMP, markers in mitochondrial pathway of apoptosis [19].LA was able to increase caspase-9 activity and also decrease MMP in A2780 cells, thus implying that mitochondrial pathway has a critical role in apoptosis induced by this compound. Several studies have demonstrated that poly unsaturated fatty acids have apoptogenic effect on tumor growth. Sharma et al evaluated the growth suppressive effect of omega-3 fatty acids (OM 6-FAs) on four epithelial ovarian cancer cell lines [20].OM 6-FAs inhibited cell growth of all cell lines and induced apoptosis in wild type p53 TOV-21G and IOSE-29 cells. It also has been sowed that exposure of breast, colon and leukemia cells with eicosapentaenoic acid (EPA) decreases the expression of the anti-apoptotic protein Bcl-2 [21]. Indeed in the human pancreatic cell lines, EPA treatment induced apoptosis and increased caspase-3 activation [22].

Conclusion

To summarize our in vitro study demonstrated that LA inhibited the growth of human ovarian carcinoma cells, A2780 and induced mitochondria-related apoptosis. The more detailed mechanism of cell apoptosis by LA remains to be explained.

Conflict of Interests

Authors certify that there is no actual or potential conflict of interest in relation to this article.

Acknowledgment

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