The Effects of Thymus Plant Extracts on Single Breast Cancer Cell Morphology in the Microfluidic Channel

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***Abstract*— Microfluidics based systems could be useful for drug discovery as they allow for miniaturization and could potentially be run as multiple parallel cell based assays. Such miniaturization allows assays at single cell level and reduces the amount of test material needed, which, in the case of natural product extracts, simplifies the preparation. Thyme species extracts have been reported to show some promising anti-cancer effects. In the present work, we used a microfluidics based system to study the effects of Thymus kotschyanusm Boiss plant extract on two human breast cancer cells lines which are MDA-MB-231 and MCF-7. For better understanding a single cancer cell death mechanism and a flow control, a Polydimethylsiloxane (PDMS) microfluidic device has been fabricated. The morphology of single cancer cells in the microfluidic system showed that the higher concentration of plant Thymus extract (560 µg/ml) had a significant effect on the cell membrane compared to the lower concentration (15 µg/ml). In addition, the results showed that MDA-MB-231 cells were more sensitive to the cytotoxic effects of the extracts compared to the MCF-7 cells. These results concur with the MTT assay analysis that showed the IC_{50} values of the extract in MDA-MB-231 and MCF-7 cells to be 15 µg/ml and 60 µg/ml, respectively. This proof-of-principle study suggests the possibility of the use of microfluidics systems for natural product research. These systems could allow the development of miniaturized multiple parallel cell based assays which are measured with sensors and used for natural product drug discovery consistent with the needs of Industry 4.0.**

Keywords— ***Thymus; breast cancer cell; MTT assay; microfluidic.***

I. INTRODUCTION

Breast cancer is cancer that develops from breast tissue and the second leading cause of deaths in women [1]. Nowadays, several studies have been reported for breast cancer treatment such as chemotherapy, however this method, suffer a high percentage of healthy cells will be lost with cancer cells due to non-selectivity of medicines [2]. Recently, it seems that there is an urgent need for improvements in detection, diagnosis, and treatment of breast cancer. Currently, in cancer prevention and treatment, several groups are found the natural products are play major role for drug discovery [3-5]. The natural compound used in medicine was produce by plants as the main source. Thymus genus belongs to Lamiaceae family which some species extracts of it have remarkably been demonstrated to positively affect depression and cancer cells [6-8]. One of the species which is widely grown in Iran, is T. kotschyanusm Boiss [9].

Previous phytochemical analysis of Thymus species has confirmed the occurrence of phenolic compounds such as including carvacrol, thymol, p-cymene, and ɣ- terpinene [10, 11]. It has been shown to exhibit a range of biological activities such as antibacterial, antifungal, insecticidal, analgesic and antioxidant properties [12-14]. Other groups applying a different method to demonstrate antioxidant properties of oil and volatile extracts from Thyme [15]. However, minor alteration in cellular conditions may significantly influence the cell properties. For that reason, a faithfully explore the cellular response to the tested drug conditions in a quantitative format, a steadier and more quantifiable environment is essentially required [16].

Recently, microfluidics has shown a potential to become for real-time single cell analysis and repeatable media exchange [17]. The advance of microfluidic systems offers the possibility of measuring the real-time monitoring of individual cells [18]. Indeed, microfluidic based cell culture platform can mimic in vivo conditions as well as generate different environmental profiles. In addition, the microfluidic has capability to controlled precisely drug flow through inside culture chamber. Thus, it allows to monitor the cell's response to high concentrations of drug inside the culture chamber [19,20]. The progress of microfluidic technology has demonstrated the capability to capture a single cell by channel geometry and controlling the fluid flow.

In this research, the effect of Thymus plant extracts on a single breast cancer cell morphology has been investigated through an inverted microscope and Real-Time Monitoring in a microfluidic device. The fabricated device provides a trapping site that allows for manipulation of cancer cells using the fluid control by syringe pump. Two type of breast epithelial cell lines included MDA-MB-231 and MCF-7 were investigated. Alternatively, a fluorescence microscopy nuclear morphology assay and MTT assay were used to investigate the viability of two breast cancer cell lines.

II. Materials and Methods

A. Cell culture

MDA-MB-231 and MCF-7 cell line was cultured in DMEM medium (Dulbecco's Modified Eagle Medium) (Gibco® Life Technologies, NY, USA) containing 10% heat-deactivated Fetal Bovine Serum (Gibco® Life Technologies, USA) and 1x of Antibiotic/Antimycotic (Gibco® Life Technologies, USA).

MCF-10A cell line was cultured in DMEM/F12 medium (Invitrogen, CA, USA) containing 5% of Horse Serum (Invitrogen, CA, USA) and 1x of Antibiotic/Antimycotic. This media was supplemented by 10ng/ml of Epidermal Growth Factor (Gibco® Life Technologies, NY, USA), 1x of Insulin Transferrin Selenium-A (ITS-A) (Gibco® Thermofisher Scientific, USA) and 0.5ug/ml of Hydrocortisone (STEMCELL™ Technologies, Vancouver, Canada).

All cell lines were cultured in 10cm TPP plate (Merck KGaA, Darmstadt, Germany) and maintained at 37 °C and 5% CO_2 incubator. The growth of the cell was monitored every 2-3 days and media were changed every 3-4 days. Once the cells are 80-90% confluent, cells were trypsinized using TrypLE Express (Gibco® Thermofisher Scientific, USA) for 10 minutes.

B. MTT assay

MTT assay method was used to evaluate the cytotoxicity of the extract on MDA-MB-231 and MCF-7 cells as well as MCF-10A. The method is according to the ability of viable cells to produce purple formazan crystals from yellow tetrazolium salt MTT by the mitochondrial dehydrogenase. The cells were plated into 96-well plate (Nunc, Denmark) at a density of 10^4 cells/well/200 µl [21]. After 24h, a fresh complete medium containing different extract concentrations (16, 60, 120, 560 µg/ml) and 0.2% DMSO (Sigma, USA) as a negative control were replaced.

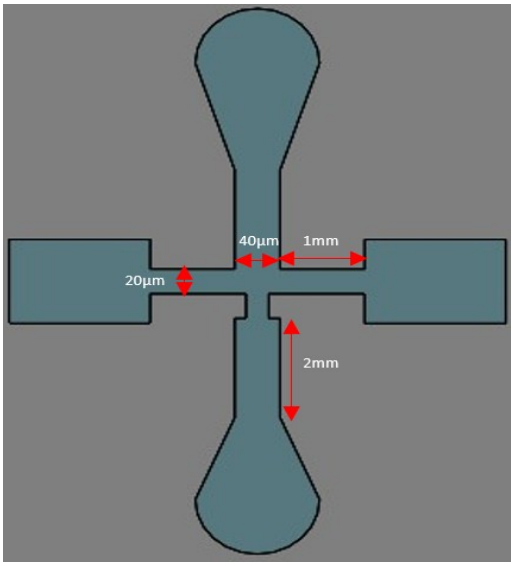

Fig. 1. The schematic of microfluidic device

Next, 10 ml of MTT labeling reagent was dispensed into each well, then the cells were administered with the extract for 24 h. The plates were incubated at 37 ºC and 5% CO_2 for 4 h. Afterward, supernatants were discarded and 100 ml of the solubilization solution was added to each well and plates were incubated overnight in a humidity atmosphere [21]. Finally, cytotoxicity was detected by measuring the absorbance at a wavelength of 570 nm using an ELISA plate reader (Lab System).

C. T. Kotschyanusm boiss extract preparation

T. kotschyanusm Boiss were obtained from Iran. They harvested from Thymus plant and rinsed with deionized water multiple times for removing dust, then left to dry for 15 days in room temperature and in the shadow. The desiccated Thymus was grinded and boiled for 10 minutes and was filtrated. Thereafter, the extract was centrifuged at 1200 RPMs for 5 minutes for separating heavy biomaterial within the extract. The resulting solution was filtrated with filter paper. Finally, the solution was condensed using rotary equipment down to 5 ml. The extract was then dispersed in thin films on glass plates which later dried in a temperature below 45 °C. Through weighing the plates once empty and once after the desiccation of the extract, the amount of the yielded extract was found. The extraction efficiency was obtained by dividing the yielded weight by the sample weight. After that, the extract was kept in the refrigerator until the time of the experiment.

D. Microfluidic fabrication

The standard soft lithography technique was utilized to fabricate the PDMS microchip [22]. The fabrication process consisted of mould fabrication and PDMS casting. The design was patterned using chrome mask on a silicon wafer substrate coated by SU-8 photoresist (MicroChem 3025, USA). These designs defined the dimensions of the micro-channels and inlet/outlet. The schematic and dimensions of the features are given in Fig. 1. PDMS (Sylgard 184A, USA) was poured onto SU-8 master mold after mixing with a curing agent (SYLGARD 184B, USA) in a ratio of 10:1 by weight. After curing at 80 °C for 2 h, the PDMS was peeled off from the master mold [23]. Then, the PDMS was permanent bonding onto a glass slide to form a closed channel after washing with Isopropanol and treated by Oxygen plasma (Plasma Etch PE-25) for 25 seconds [24,25]. A microfluidic funnel shaped channel was designed and fabricated in order to investigate the reaction of a single cancer cell in the microchannel [26].

E. Cell culture in microfuidic device

Before experiments, the microfluidic devices were sterilized using 70% weight ethanol aqueous solution for 30 minutes and sequentially replaced the ethanol solution by 30% weight ethanol and PBS aqueous solution to prevent bubble trapping in the channels. The PDMS device was sterilized under UV light for 15 minutes. The cells were passaged with standard 0.25% weight trypsin solution

(Gibco, Invitrogen), which was neutralized by a medium. The cell suspension solution was centrifuged at 1,500 rpm for 5 min and the cells were resuspended in cell culture medium. The cells were diluted to a density of 2×10^6 cells/mL after counting the cell number by using hemocytometer. Then the cell was infused into the microchannel by utilized a syringe pump for further culture.

III. RESULTS AND DISCUSSIONS

A. Elemental compositions

Fig. 2 shows the field-emission scanning electron microscopy (FESEM) image of the herb extract sample. Fig. 3 illustrates the Energy-dispersive X-ray spectroscopy (EDX), which determines the elemental compositions and spatial distributions of existent elements in the herb extract sample. The observed characteristics peaks in Fig. 3 confirmed the existence of chlorine with an atomic weight of 17.98%, 59.05% of oxygen, 1.04% of Magnesium, 0.61% of Phosphorus, 1% of Calcium and 20.28% of Potassium in the extracted sample. The EDX spectrum of element analysis was tabulated in Table 1. In addition, the EDX mapping analysis illustrates the uniform dispersion of all elements across the sample. Carl Zeiss Ultra 55 FE-SEM was utilized to perform the FESEM-EDX analysis.

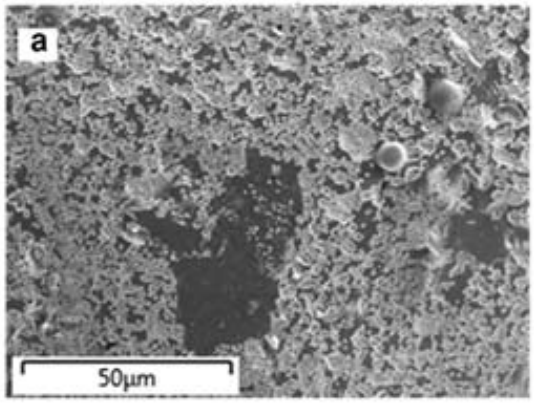

Fig. 2. FESEM image of the herb extract

TABLE 1: THE EDX SPECTRUM OF ELEMENT ANALYSIS

Element	Weight (%)	Atomic (%)
O	38.43	59.09
Mg	1.03	1.04
P	0.77	0.61
Cl	25.91	17.98
K	32.23	20.28
Ca	1.64	1.00
Total	100.00	100.00

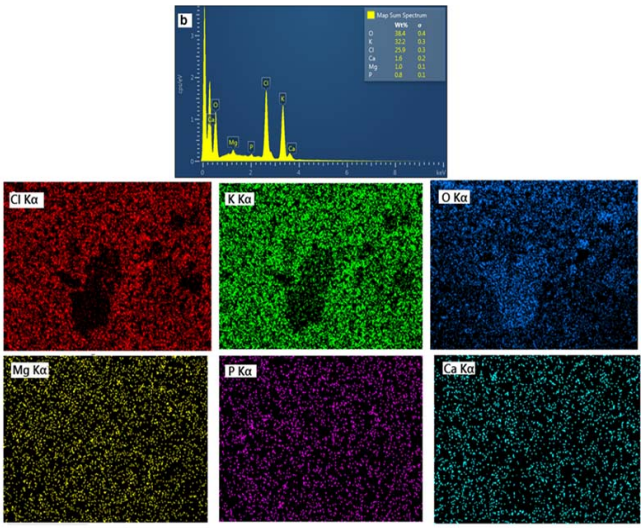

Fig. 3. EDX pattern and mapping analysis of the herb extract

B. Cell viability determination by MTT assay

The viability of cells was determined by MTT assay as described in section II (B). The cytotoxicity of the extract was evaluated on both cancer cells MCF-7 and MDA-MB-231 as well as MCF-10A cells by using the MTT method. The cells were treated with different concentrations (15-560 µg/ml) of extract for 24 h and then MTT assay was used to measure mitochondrial activity inviable cells. Fig. 4 shows that the Thymus extract could decrease cell viability in a dose dependent manner in both cancer cells. However, cytotoxicity of extract in MCF-7 was lower than MDA-MB-231.

The IC_{50} values of the extract in MDA-MB-231 and MCF-7 cells were found to be 15 µg/ml and 60 µg/ml, respectively. Because the IC_{50} value of Thymus extract in MDA-MB-231 cells are lower than MCF-7 cells, indicating that these cells are more sensitive to cytotoxic effects of the extract. In addition, cell viability assay shows that thymus extracts are nearly nontoxic for normal MCF-10A cells. Even at higher concentrations of extract (560 µg ml^{-1}), cells are viable, and the results are statistically significant ($p<0.05$).

C. Morphological analysis of an elongated single cancer cell in microfluidic device

The attached MDA-MB-231 cells and MCF-7 cells were injected into the microchannel with a syringe pump for analyzing a single cancer cell. The few cells were trapped inside an aggregation microchannel by controlling the fluid flow. Firstly, the flow rate was 0.05 µL/min and then it was stopped. In this condition, the cell flow can be controlled and reduced to attach the cell in the desired area in the microfluidic devices. In fact, by controlling the flow rate and microchannel shape, the few numbers of cells are trapped in a microfluidic system (Fig. 5a). The first attached cells are treated as an obstacle, which reduces the flow rate and attaching more cells. After 2 days culturing, cells are appeared thin and elongated after 24h culture in the

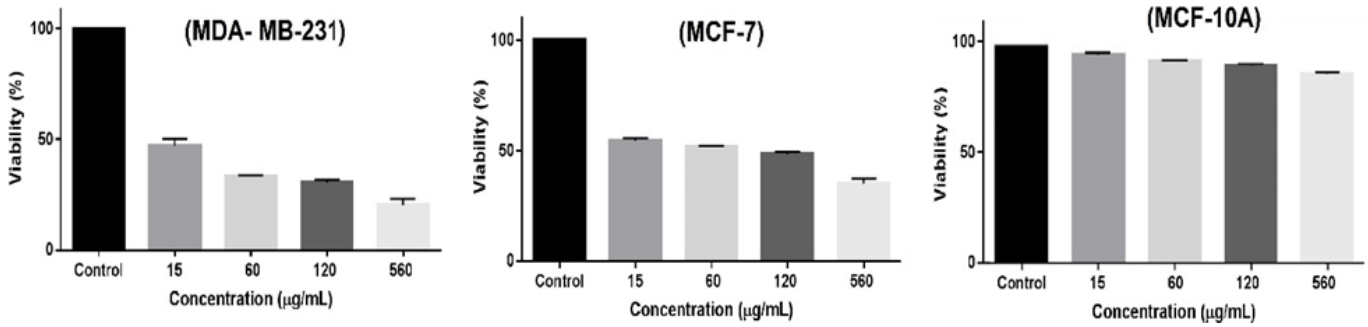

Fig. 4. Cell viability in treated cells versus untreated cells

microfluidic device inside an incubator.

MCF-7 has a cobblestone-like phenotype with strong cell-cell adhesion, whereas MDA-231 cells have an elongated fibroblast-like morphology and pronounced cellular scattering (Fig. 5b and c). The extract fluid was then infused into microfluidic channels then the reaction effect on the cancer cells was observed after 3h. Fig. 6 shows the optical microscopic images of cancer cells while treated during 3h with high (560 µg/ml) and low concentration (15 µg/ml) of extract. Both cell death mechanisms, though very different, can be observed and differentiated with extract concentration. Cell death mechanism is different for two cases of high and low concentration of the extract, as shown in Fig. 6. It is usually expected that very high toxic concentration of cytotoxic can induce a massive "physical" damage to most cell components including cell membranes leading to membrane leakage and death by necrosis (Fig. 6a and c).

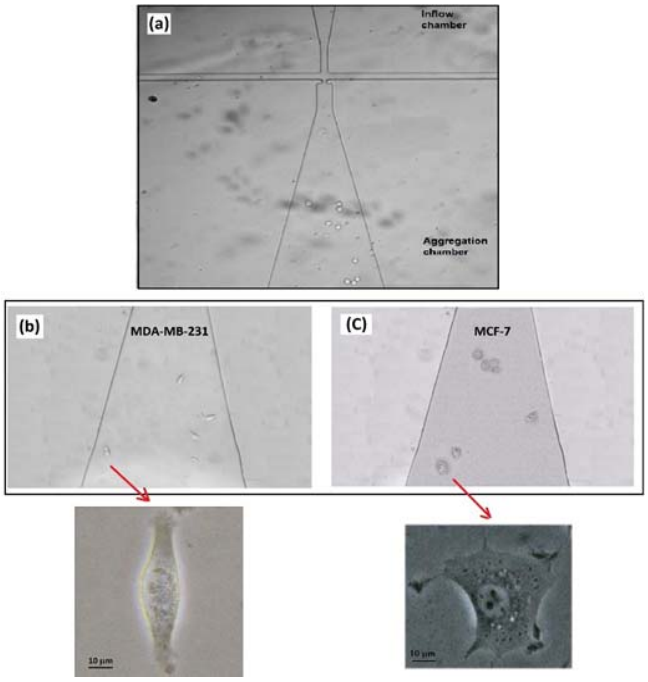

Fig. 5. The attached MDA-MB-231 cells in a microfluidic device

However, at low concentration of extract can cause the apoptotic on cells (Fig. 6b and d) [27]. Also, the morphology changes of MDA-MB-231 cancer cells are during 3h and for MCF-7 cells are during 6 h. In addition, MDA-MB-231 cells demonstrated to be more sensitive than MCF-7 cells, because of greater inhibitory effect in MDA-MB- 231 cells than MCF-7 cells in the same extract concentrations.

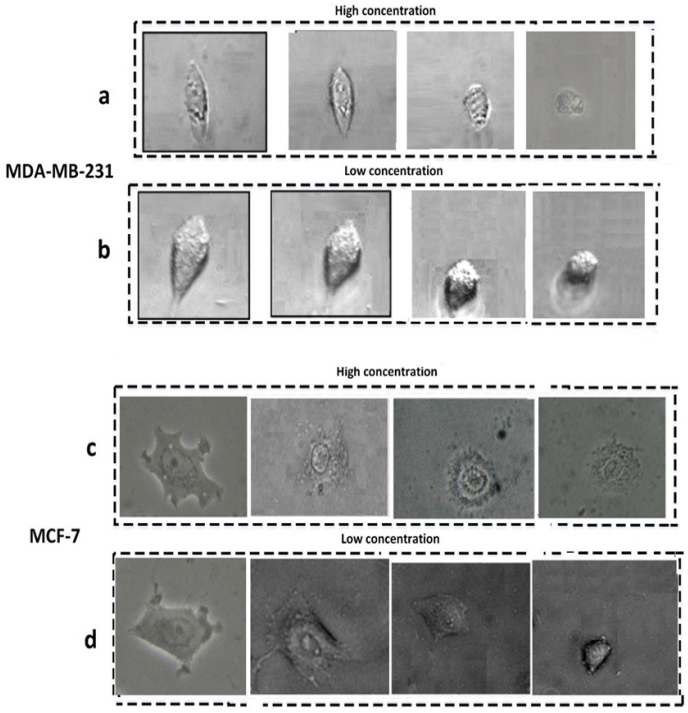

Fig. 6: Morphology changes in elongated MDA-MB-231 cancer cells after treatment 3 h with extract and 6h for MCF-7 cells

IV. CONCLUSION

This study investigated the anti-cancer activity of Thymus Kotschyanusm Boiss plant extract on breast cancer line (MDA-MB-231 and MCF-7). The results showed that the high concentration of thymus (560 µg/ml) has more effect on the cell membrane and has less effect for low concentration case (15 µg/ml). The results were completely in agreement with MTT assay analysis. In addition, the results demonstrated that the MDA-MB-231 cells are more sensitive to extract compared with MCF-7 cancer cell lines. In conclusion, the achieved results in this research are significant for breast cancer treatment since in chemotherapy method, a high percentage of healthy cells will be lost.

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