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Original Article

Persicoimidate Isolated from *Allium ampeloprasum Subsp. Persicum* with Apoptotic Effects against Breast Cancer Cell Lines

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Abstract

Breast cancer is the most common malignancy among American women and the second leading cause of cancer death after the lung cancer. For this reason, trying to find new drugs for the treatment of this disease is essential. The aim of the present study was to evaluate the cytotoxic effects of three cinnamic acid derivatives isolated from *Allium ampeloprasum var. Persicum* including Caffeoyl tyramine, Feruloyl tyramine, and Persicoimidate on two breast cancer cell lines, MCF-7 and BT-474. Evaluation the cytotoxic effects of mentioned purified compounds against MCF-7 and BT-474 cells was performed using MTT assay and their IC50 was determined. Finally, flow cytometery analysis on MCF-7 cells was performed and used to determine the cell death mechanism of investigated compounds. The results exhibited that the cytotoxic effects of all three compounds against breast cancer cell lines was concentration-dependent. The IC50 of Caffeoyl tyramine, Feruloyl tyramine, and Persicoimidate was determined to be as 73, 56, and 40 µg/ml for MCF-7 and 100, 54, and 37 µg/ml for BT-474 cells, respectively. So, Perscicoimidate showed the most potent cytotoxic effects against two breast cancer cells. Finally, flow cytometery analysis showed that Persicoimidate caused approximately 48% of apoptosis in concentration of 40 µg/ml. According to the tyrosine kinase inhibitory activity of cinnamic acid derivatives, these compounds has the potential of being cancer drug candidates for complementary studies on breast tumors with highly expression of EGF receptor. However, evaluation of anti-cancer effects of these compounds against other breast cancer cell lines is suggested.

Keywords: Apoptosis, Breast cancer, Cytotoxicity, Persicoimidate, Persian Leek, MCF-7.

1. Introduction

Breast cancer is the most prevalent malignancy among American women and the second leading cause of cancer death after lung cancer. About 252,710 new cases of invasive breast cancer and 40,610 death due to

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this type of cancer occurred among US women in 2017 [1].

Treatment of breast cancer has been a major challenge in recent years due to the development of drug resistance. For this reason, finding of new drugs for the treatment of this disease is essential [2]. Among the various types of complementary and alternative medicine, phytotherapy is the most commonly used treatment for more than two thosands years to treat different diseases including cancer [3]. So far, various natural compounds have been evaluated for the treatment of breast cancer, and some of them have been very effective; Etoposide (derived from *Podophyllum peltatum*), vinblastine (from *Vinca rosea*) and Camptothecin (prepared from *Camptotheca acuminate*) are widely used chemotherapeutic agents in the treatment of breast cancer and other malignancies [2].

Persian Leek (*Allium ampeloprasum Subsp. Persicum*), as the most well-known and widely used *Allium* species, is an endemic plant called ‘‘Tarreh’’ in Iran and used as an edible and medicinal plant since the 10^{th} century. Phytochemically, the plant contains various phenolic, terpenoid, and organosulphuric compounds [4].

Cinnamic acid derivatives are among pharmacologically active structures found in various species of *Allium* [4]. Caffeoyl tyramine and Feruloyl tyramine are the most isolated compounds in this category [4], have been extracted from different plant species and shown to have pharmacological effects including anti-fungal, antioxidant and cytotoxic effects [4]. Persicimidate, on the other hand, has a much lesser distribution and was isolated from Persian leek for the first time and established to possess antifungal activity [4].

The anticancer effects of synthetic Caffeoyl tyramine and Feruloyl tyramine has been evaluated and it was shown that these compounds exert their anti-proliferative effects by inhibiting the activity of tyrosine kinase, associated with EGF (Epidermal Growth Factor) cell surface receptors [5].

Tyrosine phosphorylation is an important signaling pathway especially in the process of cell proliferation by increasing the expression of the epithelial growth factor receptor (EGFR) in various tumor cells [6]. Due to the role of tyrosine phosphorylation in the tumorigenic process, tyrosine kinase inhibitors have been considered to inhibit this signal and thereby inhibit the growth of tumor cells [7]. For instance, the results of Park *et al*. study have shown that Caffeoyl tyramine decreased the growth of tumor cells and increased the activity of caspase 3 and DNA fragmentation in a dose and time dependent manner [5].

According to the literature, Caffeoyl tyramine and Feruloyl tyramine inhibit phosphorylation of EGF receptors and are able to exert cytotoxic effects on the cancer cell lines with highly expression of these receptors. Persicoimidate, on the other hand, with the similar chemical backbone, could be also expected to possess similar activity [4].

The aim of this study was to evaluate the cytotoxic effects of the above mentioned structures against two breast cancer cell lines with moderate expression of EGF receptors on their surface [8]. Comparing the cytotoxic effects of these compounds, determining the relationship between structure and activity based on the type and position of functional groups. Determining the death mechanism of the structure with the most cytotoxicity, is also the other aim of the present study.

2. Materials and Methods

2.1. Plant Materials, Cells, and Reagents

The purified compounds (Caffeoyl tyramine, Ferrolyl tyramine, and Persicimidate) of the seeds of *A. ampeloprasum Subsp. Persicum*, Voucher specimens (N. 2751) are deposited at the Department of Pharmacognosy, Isfahan University of Medical Sciences, were provided from previous studies as explained bellow [4]. BT-474 and MCF-7 cell lines were purchased from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Annexin-V-FLUOS Staining Kit was obtained from Invitrogen® Company. All other chemicals were obtained from known commercial sources and in biological grades.

2.2. Sample Preparation for In Vitro Assays

The phenolic-rich fractions of *Allium ampeloprasum var. Persicum* chloroform-methanol (9:1) extract subjected to final purification by HPLC using a semi-preparative C18 column (Novapak® 7.8*300 mm) with H_2O: MeOH as mobile phase [4]. The pure compounds were dissolved in 1 ml DMSO and diluted by PBS to produce various concentrations including 1000, 500, 250, 125, 62.5, 31.25, and 15.6 µg/ml and used for biological tests after filtration.

2.3. Cytotoxicity Assay

In order to determine the cytotoxic effects of the mentioned purified compounds, MTT assay was used. Cell suspension with 2×10^4 cells/ml concentration of BT-474 or MCF-7 cells in RPMI 1640 in final volume 180 µl, was seeded to each well of a 96 well-plate and incubated at 37 ºC in a CO_2 incubator. In the next day, 20 µl of various concentrations of compound (final concentrations: 100, 50, 25, 12.5, 6.25, 3.125, and 1.56 µg/ml) was added to each well. After 48 hrs of incubation, 20 µl of MTT solution (5 mg/ml) was added to each well, and the plate was further incubated for 3 hrs. Finally, the formazan crystals were dissolved in 150 µl DMSO, and the plate was subjected to absorbance read at 570 nm using a microplate reader.

2.4. Flow Cytometery Analysis

About 5 × 10^5 cells/well of MCF-7 cells was cultured in a 6-well plate. After 12 hrs, the cells were incubated with the IC50 concentration of the compound with the highest cytotoxic effects for 24 hrs and subsequently subjected to flow cytometry analysis. Briefly, all cells were collected, washed with PBS and binding buffer (1X), then incubated with annexin-V-FITC according to the manufacturer's instructions (Invitrogen, US) for 10-15 min. Finally, the cells were centrifuged at 300×*g* and washed using binding buffer (1X), suspended in 200 µl binding buffer (1X) and treated with propidium iodide. At the end, all samples were analyzed by flow cytometry on a BD FACSCalibur (BD, USA) [9].

2.5. Statistical Analyses

Cytotoxicity assay was performed in three independent experiments and four replicate wells for each concentration of each compounds. PBS-treated cells containing 1 % of DMSO (0.1 % in each wells), were considered as negative control and results were expressed as cell viability % ± SD. SPSS 23 software was used for statistical analysis. Analysis of variance (ANOVA) followed by post hoc test was used to distinguish the differences between groups. The significance was assumed as $p < 0.05$. Finally, the IC50 of each purified compound was determined by drawing the graph of cell survival percent against concentration using GraphPad Prism 7.0 software.

3. Results and Discussion

3.1. Persicoimidate Showing the Most Cytotoxic Effects against Breast Cancer Cells

The aim of our study was to determine the cytotoxic effects of some cinnamic acid derivatives extracted from *A. ampeloprasum Subsp. Persicum* against two breast cancer cell lines MCF-7, and BT-474.

Cytotoxic effects of Caffeoyl tyramine, Feruloyl tyramine, and Persicoimidate on MCF-7 and BT-474 cells were measured *in vitro* by MTT assay. Cinnamic acid derivatives have been identified as interesting compounds with antioxidant, anti-inflammatory and cytotoxic properties [10]. Furthermore, natural analogues of cinnamic acid are known for their applications in the treatment of cancer for over centuries [11].

Our analyzed data showed that the cytotoxic effects of each compounds against both cell lines were concentration-dependent, i.e. increasing concentration of compounds leads to increased cytotoxic effects.

For BT-474 cells, significant differences were shown between the cytotoxic effects of three investigated compounds (P value < 0.05). Persicoimidate showed the most cytotoxic effects against these cells in comparison to the Caffeoyl tyramine and Feruloyl tyramine in the same concentrations (P value < 0.05). Persicoimidate showed significant cell survival reduction in concentration of 3.12 µg/ml and higher in comparison to the negative control (PBS treated cells). These results were repeated for MCF-7 cell.

However, there was no significant difference between the cytotoxicity of the

same compound against MCF-7 and BT-474 according to the calculated IC50. For example, the IC50 of Persicoimidate for BT-474 and MCF-7 were not statistically significant (P value > 0.05) (Figure 1).

Finally, based on the graph of concentration/percent of cell survival drawn by GraphPad Prism 7, the IC50 of Caffeoyl tyramine, Feruloyl tyramine and Persicoimidate against BT-474 was determined as 100, 54, and 37 µg/ml, respectively. The IC50 for these compounds against MCF-7, also determined as 73, 56, and 40 µg/ml, respectively.

Ekmekcioglu *et al*. investigated the effect of cinnamic acid derivatives on cell proliferation in human colon adenocarcinoma cells (Caco-2) [12] and their results revealed that cinnamic acid is an anti-proliferative agent in concentrations of 2.5–8.0 mM, and inhibits the DNA synthesis in growing cells. The anti-proliferative effect occurred rapidly after 2 hrs of treatment with 8.0 mM of cinnamic acid and reached nearly maximal values after 8 hrs of treatment [12].

However, In Pontiki *et al*. study, the antitumor properties of some synthetic cinnamic acid derivatives have been assessed against HT-29, A-549, OAW-42, MDA-MB-231, HeLa, and MRC-5 normal cell lines [13]. However, their results showed that these compounds had no cytotoxic effects against neither cancer nor normal cells [13].

In another study, the anti-proliferative effects of synthetic Caffeoyl tyramine and Feruloyl tyramine was investigated on HL-60, U937, and Jurkat cells [5]. The results of this study showed that Caffeoyl tyramine was the most potent with GI50 of 10 µM [5]. Treatment of the cells with Caffeoyl tyramine enhanced caspase-3 activity, and inhibited the growth of cells via decreasing in protein tyrosine kinase activity including epidermal growth factor receptor [5]. However, in the

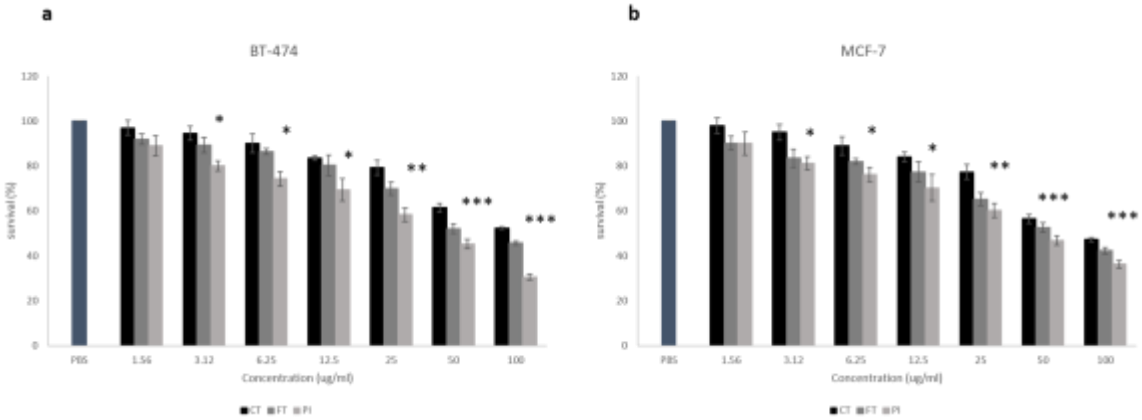

Figure 1. Cytotoxic effects of cinnamic acid derivatives against different cell lines. **a**: Treatment of $EGFR^+$ BT-474 cells, showed significant toxicity for all compounds except in 1.56, 3.12, and 6.25 µg/ml concentration of caffeoyl tyramine toward the negative control (PBS). **b**: Treatment of $EFGR^+$ MCF-7 cells, showed significant toxicity for all compounds except in 1.56 and 3.12 µg/ml concentration of caffeoyl tyramine toward the negative control. Data represent the mean percent of three independent experiments of triplicates. Error bars represent SD. CT: Caffeoyl tyramine, FT: Feruloyl tyramine, and PI: Persicoimidate.

present study, we used two breast cancer cell lines with moderate EGF receptor expression. As shown in the result section, the cytotoxic effects of the investigated compounds were similar in two cell lines. So it is concluded that the cytotoxic effects of compounds is exactly attributed to the amount of EGF receptor on the cell surface.

In Park *et al*. study, it is concluded that the presence of at least one OH group on the phenyl ring is essential for the cytotoxic effects of cinnamic acid derivatives [5]. In our study, however, there was an OH group on the phenyl ring for Feruloyl tyramine and Persicoimidate. For Caffeoyl tyramine, on the other hand, there was also two OCH3 groups on *Meta* and *para* positions, instead of OH. So, the less cytotoxic activity of Caffeoyl tyramine may be attributed to the absence of OH according to the results of the Park study. Furthermore, there were significant differences between the effects of Feruloyl tyramine and Persicoimidate. The only difference between the structures of these two compounds is on the chain between the phenyl groups (an OCH3 group on the C1 for Persicoimidate in comparison to Feruloyl tyramine with an =O in this position) and the significant differences in cytotoxicity between Feruloyl tyramine and Persicoimidate may be attributed to this structural difference. Finally, the results of Lee *et al*. study, surveyed the antifungal effects of some similar compounds including dihydro-N-caffeoyltyramine, trans-N-feruloyloctopamine, trans-N-caffeoyltyramine, and cis-N-caffeoyltyramine showed that trans Caffeoyl tyramine had anti-fungal effects without hemolytic activity against human erythrocyte cells in 5-10 µg/ml against *Candida albicans*, *Saccharomyces cerevisiae*, and *trichophyton Beigelii*. While the MIC of cis form of this compound against the mentioned organism was calculated as 40 µg/ml [14]. In the present study trans Caffeoyl tyramine showed IC50 as about 70 µg/ml which was higher than the calculated MIC against various fungal organism and this can attributed to the moderate presence of EGF receptors on these cell surface led to moderate sensitivity of these cells against Caffeoyl tyramine.

3.2. Persicoimidate Induced Cell Death via Apoptosis

The results of flow cytometry analysis of MCF-7 cells exposed with the concentrations of 30 and 40 µg/ml of Persicoimidate for 24 hrs showed that the percent of apoptotic cells increased with increasing the concentrations of Persicoimidate. In fact, the un-treated cells showed 69% of live cells, 30% of apoptotic cells, and approximately 0.01% of necrotic cells. In the case of the cells treated with 30 µg/mL of Persicoimidate for 24 hrs, results showed 42% of apoptotic cells, and 0.41% necrotic cells. The percent of live cells was calculated as 57%. Furthermore, the apoptotic and necrotic percent of the cells treated with the IC50 of Persicoimidate (40 µg/mL) after 24 hrs of incubation, was showed to be 48 and 0.13%, respectively (figure 2). Finally, the percent of live cells in sample treated with the IC50 concentration of Persicoimidate was calculated as 52.

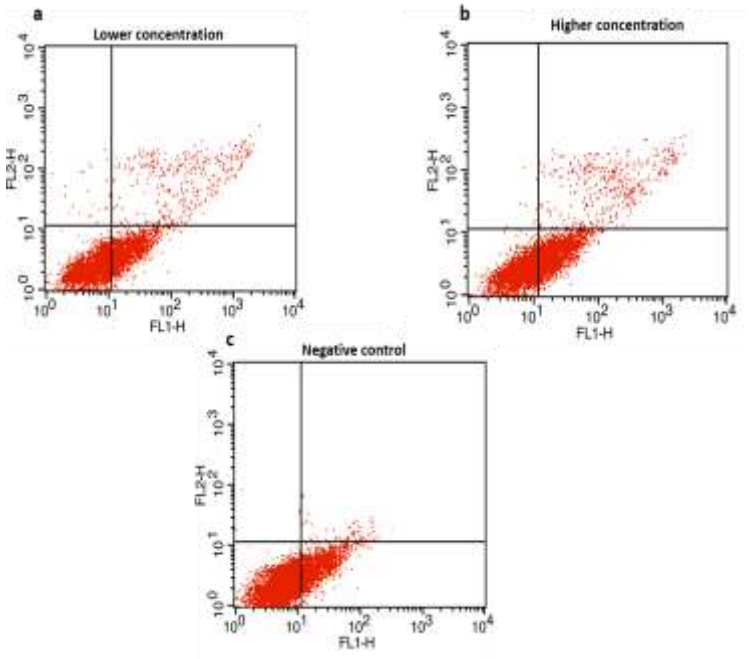

Figure 2. Determination of cell death mechanism of persicoimidate by flow cytometry after 24 hrs of incubation. **a**: cells treated with 30 µg/ml of persicoimidate. **b**: cells treated with 40 µg/ml of Persicoimidate for 24 hrs. **c**: un-treated control cells. Lower left chamber: live cells (annexin V^-/ PI^-); Lower right chamber: early apoptotic cells (annexin V^+/ PI^-); Upper left chamber: dead cells (annexin V^-/ PI^+); Upper right chamber: late apoptotic cells (annexin V^+/ PI^+).

It has been shown that cinnamic acid can decrease the percent of HT-144 cells in S phase in concentration of 3.2 mM, in comparison to the negative control [15]. On the other hand, in the mentioned study, it was confirmed that cinnamic acid can induce the apoptosis cascade after 24 hrs and increase the activity of caspase 9 in 0.4 mM concentration. However, the percent of apoptotic cells in treated sample was not significantly higher that the negative control [15].

In Park *et al*. study, the apoptotic effects of Caffeoyl tyramine (as the synthetic compound with the most cytotoxicity against Jurkat cell line) was established after 9 hrs of treatment with 30 µM concentration of the compound by measurement of caspase 3 activity. Furthermore, this concentration showed DNA fragmentation of mentioned cells as 70 % [5].

In our study, Persicoimidate showed the most cytotoxicity against MCF-7 and used for determining the cell death mechanism. The concentration induced apoptosis was four times higher than the value calculated in the Park study for Caffeoyl tyramine and this difference can be attributed to the more susceptibility of blood cancer cells such as Jurkat.

Persicoimidate, (1Z,2E)-methyl 3-(-p-hydroxy-m-methoxyphenyl)-N-(-p-hydroxyphenethyl) acrylimidate, was extracted for the first time from *Allium ampeloprasum Subsp. Persicum* and exhibited antifungal effects against *Penicillium italicum*,

Aspergillus niger, *Botrytis cinerea*, and *Trichoderma harzianum* fungi [4], while according to the results, Caffeoyl tyramine and Feruloyl tyramine had more potent antifungal effects than persicoimidate. However, our study showed that in cytotoxicity, Persicoimidate has more potent anti-proliferative effects against EGF receptor expressing mammalian cells.

4. Conclusion

In the present study, the cytotoxic effects of known natural compounds Caffeoyl tyramine and Feruloyl tyramine and also Persicoimidate as a new compound, were investigated against two $EGFR^+$ cells, BT-474 and MCF-7, as the first time. Persicoimidate had the potential to act as a drug candidate for the breast cancer treatment with tyrosine kinase inhibitory effects. However, it is suggested that the cytotoxic effects of this compound must be evaluated against other cell lines of breast cancer and even other cancer cells.

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