沙漠研究 **19**-1, 367- 370 (2009)
Journal of Arid Land Studies

Anti-Proliferative and Apoptotic Effects of Oleuropein and Hydroxytyrosol on Human Breast Cancer MCF-7 Cells

Junkyu HAN[1], Terence P.N. TALORETE[1], Parida YAMADA[1], Hiroko ISODA[1]

Abstract: Oleuropein or hydroxytyrosol decreased cell viability, inhibited cell proliferation, and induced cell apoptosis in MCF-7 cells. MTT assay guided 200 μg/ml of oleuropein or 50 μg/ml of hydroxytyrosol remarkably reduced cell viability of MCF-7 cells. Oleuropein or hydroxytyrosol decrease of the number of MCF-7 cells by inhibiting the rate of cell proliferation and inducing cell apoptosis. Also hydroxytyrosol and oleuropein exhibited statistically significant block of G_1 to S phase transition manifested by the increase of cell number in G_0/G_1 phase.

Key words: Apoptosis, Hydroxytyrosol, MCF-7 cells, Oleuropein

1. Introduction

The phenolic compounds of olive oil and leaf are a complex mixture of compounds that include 3, 4-dihydroxyphenylethanol (hydroxytyrosol), 4-hydroxyphenylethanol (tyrosol), 4-hydroxyphenylacetic acid, protocatechuic acid, caffeic acid and *p*-coumaric acid, among others (Caponio *et al*., 1999). The concentration of the phenolic fraction is several times more in olive leaf than in oil and varies depending on the cultivar, climate (Abaza *et al*., 2005). Even though anticancer properties of oleuropein and hydroxytyrosol were confirmed *in vitro* with different cell lines, studies of their protective effect from breast cancer have not been demonstrated. Since many epidemiological studies suggest the possible correlation between olive products consumption and incidence of breast cancer here we tried to elucidate the possible effect of the main phenolic compounds hydroxytyrosol and oleuropein of olive leaf on breast cancer using human breast cancer cell line MCF-7.

2. Materials and methods

2.1. Cell culture

MCF-7 cells were obtained from American Type Culture Collection (ATCC), and maintained in DMEM, supplemented with 10% FBS (Sigma), 1% 5000 units/ml penicillin, 5000 μg/ml streptomycin (Sigma). The cells were incubated in an atmosphere of 5% CO_2 at 37 ˚C. All cells used in this study were between passages 5 and 15.

2.2. Growth inhibition assay

MCF-7 cells were seeded in 96-well plates at 2×10^3 cells/well in 100 μl of DMEM with 10% FBS. After 24 h the cells were treated with olive leaf extract (0.001, 0.01, 0.02, 0.1% (w/v)) for 12 h. Also MCF-7 cells were seeded in 96-well plates at 2×10^3, 2×10^4 , 2×10^5 cells/well in 100 μl of DMEM with 10% FBS. After 24 h the cells were treated with oleuropein (25, 50, 100, 200 μg/ml) or hydroxytyrosol (6.25, 12.5, 25, 50 μg/ml) for 12 h. The treated cells were washed with PBS and 10 μl of MTT solution (5 mg/ml MTT in PBS) was added to each well. Cells were incubated for 12 h at 37 ˚C at the end of which 100 μl 10% SDS was added and incubated further for 6 h at 37 ˚C. Optical densities at 570 nm were measured using a plate reader (Powerscan HT; Dainippon Pharmaceutical, Osaka, Japan). The relative cell viability in percentage was calculated as (*A*570 of treated samples/*A*570 of untreated samples) ×100.

2.3. Cell proliferation assay

Cells were regularly seeded into 6-well plates at 2×10^4 cells/well and incubated at 37 ˚C for 24 h. Cells were treated in fresh medium with Cells were incubated with oleuropein (200 μg/ml) or hydroxytyrosol (50 μg/ml) for 3, 6, and 12 h. At the end of each time period, the cells were trypsinized to

[1] Alliance for Research on North Africa (ARENA), Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305-8572, Japan;

produce a singlecell suspension, and the viable cell number in each well was counted using the ViaCount Assay (Guava Technologies, Hayward, CA).

2.4. Multicaspase assay by flow cytometry

Apoptosis was assessed using a flow cytometry based multicaspase assay kits (Guava Technologies, Burlingame, CA). The multicaspase assay is based on measurement of caspase enzymes activated during apoptosis. MCF-7 cells (2×10^5 cells/plate) were plated on tissue culture dishes (100 mm) in 10% FBS medium. Cells were incubated with oleuropein (200 µg/ml) or hydroxytyrosol (50 µg/ml) for 3, 6 and 12 h. Cells were trypsinized, washed in PBS and stained with sulforhodamine-valyl-alanyl-aspartyl-fluoromethyl-ketone (SR-VAD-FMK), and 7-amino-actinomycin D (7-AAD) according to the manufactures instructions. Cell populations were quantified using Guava personal cytometer (Guava Technologies, Hayward, CA).

2.5. Cell cycle analysis

MCF-7 cells (2×10^5 cells/plate) were exposed to concentrations of oleuropein (100 µg/ml) or hydroxytyrosol (25 µg/ml) for 24 h and the treated cells were collected by trypsinisation. The cells were fixed with 70% ethanol and stored at -20 ˚C. On the day of analysis, the cells were washed with PBS and suspended in 250 µl of PBS. To the cell suspension 1.0 ml of phosphate-citrate buffer was added and the suspension was incubated at room temperature for 5 min to facilitate the extraction of low molecular weight DNA. Following centrifugation the cells were resuspended in 500 µl DNA staining solution (20 µg/ml propidium iodide, 200 µg/ml DNase free RNase and 0.1% Triton X-100) and incubated in the dark at room temperature for 30 min. Cell cycle distribution was determined by fluorescence-activated cell sorting analysis of propidium iodide-stained ethanol-fixed cells using a Guava EasyCyte (Guava Technologies, Hayward, CA).

3. Results and discussion

For the screening of anticancer effect of olive leaf we used olive leaf extract to treat breast cancer MCF-7 cells. MTT assay results have shown that after 48 h treatment with the extract at the dosage of 1% cancer cell proliferation was inhibited compared to the vehicle treated control cells (**Fig. 1**). The rest of treatment concentrations did not show cytotoxic effect. Since the major phenolic compounds of olive leaf are oleuropein and hydroxytyrosol, in subsequent anticancer assays we assumed that this cytotoxic effect could be mainly due to presence of these compounds and tried to elucidate their tumor suppressive effects (**Fig. 2**).

This data may suggest that phenolic compounds of olive leaf have health protective effect rather than healing effect. Constant consumption of olive products such as leaf tea and oil may enhance this protective effect thus, suggesting their daily consumption is more effective than the infrequent intake.

Since caspase activation is the classic feature of apoptosis we performed multicaspase assay using fluorochrome conjugated caspase inhibitor sulforhodamine-valyl-alanyl-aspartyl-fluoromethylketone (SR-VAD-FMK). This inhibitor is cell permeable and non-cytotoxic and binds to multiple caspases activated during apoptosis. Also the cell impermanent dye 7-aminoactinomycin D (7-ADD) was included in the

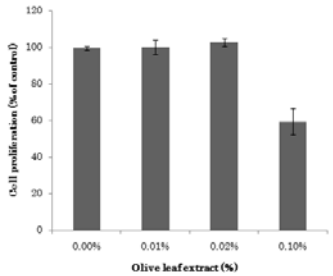


Fig. 1. Cytotoxic effect of olive leaf extract.

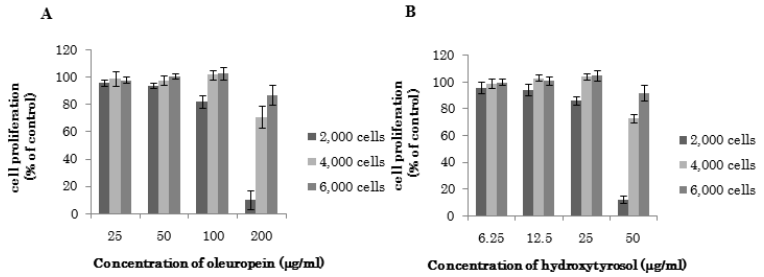
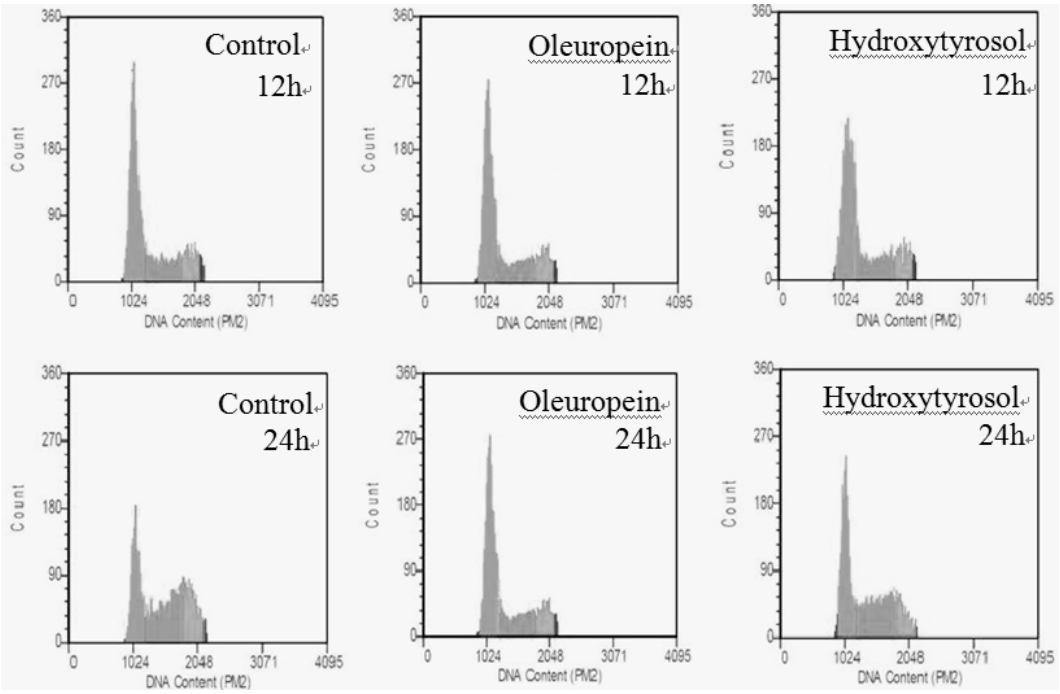


Fig. 2. Influence of initial cell number on the concentration-dependent decrease in viability of human breast cancer MCF-7 cells incubated with oleuropein (A) and hydroxytyrosol (B).



	12 h			24 h		
	Control	Oleuropein	Hydroxytyrosol	Control	Oleuropein	Hydroxytyrosol
GO/G1	57.9	60.6	58.6	32.5	41.1	45.7
S	22.7	21.4	23.4	39.9	37.6	36.7
G2/M	17.6	16.3	15.7	27.1	20.7	16.4

Fig. 3. Effect of hydroxytyrosol and oleuropein on MCF-7 cell cycle.

assay to detect dead cells from apoptotic ones. From the multicaspase assay we could observe that caspases were activated soon after treatment with both compounds, besides, induction of the enzymes' activation was greater in oleuropein treated cells compare to the hydroxytyrosol treated cells in the beginning of the treatment.

Because it has been reported that the cells undergoing apoptosis are arrested in G_1/G0 phase of cell division phase we could verify the induction of apoptosis of breast cancer cells by hydroxytyrosol and oleuropein using cell cycle analysis. In this assay we treated MCF-7 cells with 100 μg/ml of oleuropein and 25 μg/ml of hydroxytyrosol. When cells were treated for 24 h with changed concentration ranges, the cell cycle arrest at G1 phase was evident, accompanying a decrease in G2/M phase when compared with the untreated control cells. From this result we suggest that apoptotic cell death of MCF-7 cells after hydroxytyrosol and oleuropein treatment was accompanied with the block of cell cycle at G1 phase (**Fig. 3**).

Our data confirmed that the cells treated with hydroxytyrosol and oleuropein exhibited statistically significant block of G_1 to S phase transition manifested by the increase of cell number in G_0/G_1 phase. Although the oleuropein notably induced less cell cycle arrest than hydroxytyrosol it was still capable to significantly promote breast cancer apoptotic cell death.

4. Conclusion

Our results have shown that main phenolic compounds of olive leaf hydroxytyrosol and oleuropein induced apoptotic cell death of human breast cancer MCF-7 cells. Our findings may support the breast

cancer protective results of epidemiological studies of Mediterranean diet with correlation of olive products' consumption. Even though the cancer protective effect of the main olive oil component oleic acid in gene regulation was demonstrated further studies are needed to explore molecular mechanisms of tumor suppressive effect of other olive tree products such as olive leaf because they also could be the potent inducers of such effect if consumed frequently.

Aknowledgement

This study was supported in part by funds from the Grant-in-Aid for Scientific Research (Grant No. 17255011)

References

Abaza L., Taamalli W., Temine S.B., Daoud D., Gutierrez F., Zarrouk M. (2005): Natural antioxidant composition as correlated to stability of some Tunisian virgin olive oils. *Rivista Italiano Delle Sostanze Gresse*, **132**: 12-18.

Abaza L., Talorete T.P.N., Yamada P., Kurita Y., Zarrouk M., Isoda H. (2007): Tunisian Gerboui Olive Leaf Extract Induces Growth Inhibition and Differentiation of Human Leukemia HL-60 Cells. *Biosci. Biotech. Bioche.*, **71**: 1306-1312.

Caponio F., Alloggio V., Gomes T. (1999): Phenolic compounds of virgin olive oil: influence of paste preparation techniques. *Food Chem.*, **64**: 203-209.

Maourizio S., Maura B., Robert S., Alceo M., Gian Francesco M. (1999): Phenolic compounds of olive fruit: One- and two-dimensional nuclear magnetic resonance characterization of nuzhenide and distribution in the constitutive parts of fruit. *J Agric Food Chem.*, **47**: 12-18.

Owen R.W., Mier W., Giacosa A., Hall W.E., Spiegelhalder B., Bartsch H. (2000): Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene. *Food Chem. Toxicol.*, **38**: 647-659.

Visioli F., Bellomo G., Galli C. (1998): Free radical-scavenging properties of olive oil polyphenols. *Biochem. Biophys. Res. Commun.*, **247**: 60-64.

Yamada P., Zarrouk M., Kawasaki K., Isoda H. (2008): Inhibitory effect of various Tunisian olive oils on chemical mediator release and cytokine production by basophilic cells. *J. Ethnopharm*, **116**: 279-287.