

# Inhibition of Growth and Migration of Esophageal Squamous Cell Carcinoma Cells by Orange Peel Extract and Naringin

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**ABSTRACT**

**Background and Objectives:** Citrus fruits and their constituents especially naringin (NR), a natural predominant flavanone, have a wide range of pharmacological activities without toxicity against cancer cells. The aim of this study was to investigate the anticancer effects of orange peel extract (OPE) and naringin (NR) on esophageal squamous cell carcinoma (ESCC) cells.

**Methods:** Amount of phenol, flavonoid and antioxidants in OPE was determined using Folin-Ciocalteu procedure, aluminum chloride colorimetric and DPPH assays, respectively. Effects of NR and OPE on viability, wound healing assay and DNA fragmentation using DAPI were investigated. Data were analyzed by ImageJ software and GraphPad Prism 6.0 at significance of 0.05.

**Results:** Total amount of phenols, flavonoids and 1,1-diphenyl-2-picrylhydrazyl was 2.83, 2.143 and 60.76 g/100g of OPE. Amount of NR in the dried OPE was estimated to be 5.260 (µg/gr) using high-performance liquid chromatography. Treatment of ESCC cells with OPE or NR decreased viability of cancer cells in a dose-dependent manner. In addition, both OPE and NR were able to decrease cell migration and increase DNA fragmentation.

**Conclusion:** The findings of our study suggest that OPE and NR have anticancer effects on ESCC cells but the anticancer effects of OPE was better than that of NR alone.

**Keywords:** Orange peel extract, Naringin, Migration, Esophageal squamous cell carcinoma.

## INTRODUCTION

Cancer is a major public health problem worldwide. In 2012, there were 14.1 million new cancer cases and the number of new cancer cases per year is expected to rise to 23.6 million by 2030 (1). Esophageal cancer is an aggressive type of cancer that have a 5-year survival rate of 12–20% and is the sixth leading cause of cancer death worldwide (2). Natural products, especially plants, have been long used for treatment of various diseases, and more than 60% of approved drugs are of natural origin (3). As a rich source of compounds for drug discovery, natural products have been used for the treatment of various cancers without any toxicity (4, 5). Medicinal plants contain various anti-cancer compounds that can prevent formation of cancer cells by blocking metabolic activation, enhancing detoxification or prevention of target cells conversion to electrophilic metabolites (6). Citrus (*Citrus sinensis*) from the family *Rutaceae* and the *Aurantium* subfamily is among the most widely used natural products in the world due to the presence of various metabolites and bioactive compounds, such as phenols and carotenoids. In addition, citrus fruits contain nutrients including a variety of sugars, vitamin C, essential oils, folic acid, potassium and pectin (7).

The flavonoids in citrus consist of flavanone, flavone glycosides and highly methoxylated flavones, named polymethoxylated flavones. The flavanones in citrus are known as O-glycosides, depending on citrus species, such as neo-hesperidosides or rutinoides (8). Citrus flavonoids inhibited colon adenocarcinoma SW480 cell proliferation by inducing apoptosis through the intrinsic pathway (9). In addition, naringin (NR) inhibited the growth of hepatocellular carcinoma HepG2 cells through the induction of apoptosis (10). The use of orange peel extract (OPE) decreased cell viability and increased total apoptosis (early and late apoptosis) cell proportion in esophageal cancer stem cells (11). It has been widely considered that excessive cell proliferation and insufficient apoptosis are associated with tumorigenesis (12). Chemo-preventive effects of NR were accompanied by its anti-proliferative and pro-apoptotic properties.

The aim of the present study was to investigate the antioxidant properties of OPE, measure the

amount of NR in OPE and evaluate its effects on viability, migration and DNA fragmentation of esophageal squamous cell carcinoma (ESCC) cells.

## MATERIALS AND METHODS

Naringin and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Naringin was dissolved in 5% dimethylsulfoxide (DMSO) and stored as a stock solution. Fetal bovine serum was purchased from Gibco (Invitrogen Life Technologies, UK). Other reagents used in the experiments were of analytical grade and purchased from Sigma-Aldrich.

Mature oranges (*Citrus sinensis*) were purchased from a local farmer in north of Iran (36.9268° N, 50.6431° E). The fruits were peeled by hand, rinsed with water and then dried under sunlight for 72 h. The dried peels were ground to powder using an electric blending machine and sieved. The powder was kept in the dark before extraction. For extraction, 20 g of the citrus powder were mixed with 200 mL of methanol (80%) for 48 h on an orbital shaker. The mixture was filtered and evaporated to dryness under reduced pressure at 40 °C by a rotary evaporator. The concentrated extract was dried by freezing (13).

The total phenolic content was determined using the Folin-Ciocalteu reaction by reading absorbance at 765 nm using a UV-Visible (UV-VIS) spectrophotometer (14). The total phenolic content was calculated as mean  $\pm$  standard deviation and expressed as grams of chlorogenic acid equivalents (CAE) in 100 g of the extract and dried powder.

Total flavonoids were analyzed using aluminum chloride colorimetric method (14). The total flavonoid content was calculated as mean  $\pm$  standard deviation and expressed as grams of isoquercetin equivalents (IQE) in 100 g of the extract and dried powder.

The free radical scavenging activity of the extract and standard solutions (chlorogenic acid and isoquercetin) were investigated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method (14). The corresponding blank readings were also taken and percent inhibition was then calculated using the following formula:

$$\text{Percent inhibition} = \left( \frac{\text{Ablank} - \text{Asample}}{\text{Ablank}} \right) \times 100$$

Where Ablank is the absorbance of the control reaction (containing all reagents except the test compound) and Asample is the absorbance of the test compound.

High Performance Liquid Chromatography (HPLC) analysis of the samples was done on a Merck Hitachi HPLC system model L-7400 equipped with UV detector L-7400 and injector with a 20  $\mu\text{l}$  loop and diode array detector (10 ATVP).

For analysis, 20  $\mu\text{l}$  of each sample were injected into a HPLC column. Elution was performed using a mixture of 0.5% acetic acid in triple distilled water and acetonitrile (79.5:20). Both solutions were filtered and degassed before use.

Chromatography was performed at  $25 \pm 3$  °C and at a flow rate of 1 mL/min. The flavanone (NR) was quantified with isocratic conditions by reading the absorbance at 285nm.

The human esophageal cancer cell line (YM-1) was previously established in our lab (15). The cells were cultured in Dulbecco's modified Eagle's medium: F12 (DMEM: F12) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL) and streptomycin (100  $\mu\text{g}/\text{mL}$ ) at 5%  $\text{CO}_2$  and 37 °C under standard conditions.

Cell viability was assessed using MTT assay with slight modifications (16).

To determine the cytotoxicity of the extract, ESCC cells ( $1 \times 10^4$  cells/well) were treated with various concentrations of OPE (0-1600 mg/mL) and NR (0-1600  $\mu\text{M}$ ) in a 96-well plate for 24 h. Then, the cells were incubated with 100  $\mu\text{L}$  of MTT reagent [0.5 mg/mL in phosphate buffer saline (PBS)] in the dark at 37 °C for 4 h. The medium was removed, and 150  $\mu\text{L}$  of DMSO were added to each well to dissolve the MTT metabolic product. Reduction of MTT was assessed by reading absorbance at 570 nm and 630 nm using an ELISA reader. The mean percentage of viable cells was calculated based on the following formula: Percent viability = (A570 of treated

cells/A570 of control cells)  $\times$  100 (1). Moreover, 4,6-diamidine-2-phenylindole dihydrochloride (DAPI, Roche Boehringer Mannheim) staining was performed according to the manufacturer's instructions. In brief, cells were fixed with methanol: acetic acid (3:1) for 10 min. After washing with PBS, the cells were stained with DAPI working solution (1  $\mu\text{g}/\text{ml}$  in PBS) for 10 min. Slides were mounted with a mounting solution (PBS: glycerol/ 1:1). The chromosomal DNA morphology was observed by fluorescence microscopy with a 340 nm excitation filter.

The YM-1 cells were seeded onto a 6-well plate and allowed to grow to 90% confluency in complete medium. Cell monolayers were wounded by a plastic tip (1 mm) that touched the plate as described (17).

Wounded monolayers were then washed four times with medium to remove cell debris and incubated in serum-free medium in the absence or presence of OPE (400 mg/mL) and NR (300  $\mu\text{M}$ ) for 24 h. The cells were incubated at 37 °C and 5%  $\text{CO}_2$ . Migration of YM-1 cells to the wounded area was photographed at 0, 6, 12 and 24 h using an inverted microscope (Nikon, Japan). Finally, the wound area was photographed by the ImageJ software.

All experiments were done in triplicate. Data were acquired using a BD Accuri C6 flow cytometer (USA) and were analyzed using the Prism 6.0 software.

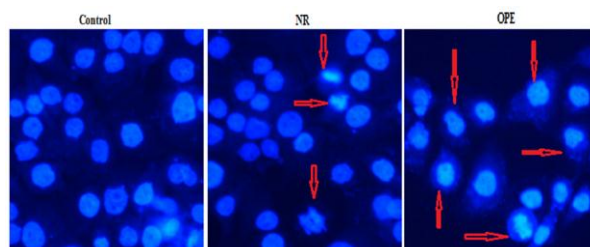
## RESULTS

The total phenolic content of the citrus extract was 2.83 mg CAE in 100 g and the amount of flavonoid was 2.143 mg IQE in 100 g. The highest phenolic content (4.20 g/100g) was recorded by MeOH: water extract of OPE (18). The antioxidant activity of citrus peel extract was 60.76% in the DPPH assay.

The retention times (RT) of NR (Figure 1B), according to NR standard peak (Figure 1A) was 13.60 min.

The amount of NR in the dried OPE was 5.260 ( $\mu\text{g}/\text{gr}$  dried extract).

**Figure 4- Chromatin condensation of YM-1 cells stained with DAPI after OPE and NR treatments. The cells were treated with 400 mg/Kg OPE and 300  $\mu\text{M}$  NR. Photomicrographs were taken by a fluorescence phase contrast microscope that showed fragmented and condensed nuclei.**



## DISCUSSION

Citrus fruits are commonly used for juice and the average of juice production of processed citrus fruits is about 50 percent of the total, on a weight basis. Citrus fruits are widely used natural products in the world. Hesperidin, NR and alkaloids, mainly synephrine are the most important active compounds in citrus plants (19). These compounds have beneficial physiological and pharmacological properties (20). Plants and plant extracts could improve the immune system and decrease the side effects of chemical treatments (3).

Similar to the results of our study, a previous study showed that 0.71-2.85 mmol of NR could prevent proliferation of HT-29 colon cancer cells (21).

Cell migration is an important aspect of cancer research and can also be applied in developmental, immunological and wound healing studies. The wound healing assay as well as the transwell cell migration and invasion assays reveal detailed information about cell migratory behaviors and can be used to investigate the molecular mechanisms of cell migration (22). In 2016, Tsai et al. reported that 1% citrus polyphenols can inhibit the migratory abilities of oral and cervical cancer cells (23). In our study, maximum chromatin condensation was observed in the cells treated with 400 mg/Kg of OPE. Treatment with 20–50  $\mu$ M of NR decreased DNA methyltransferase activity and 5-methyl-20-deoxycytidine level in the DU145 prostate cancer cell line, indicating that NR may be a DNA hypomethylating agent that has the

potential to modulate gene expression (24). In a previous study, treatment of SiHa cells with different concentrations of NR caused apoptotic cell death, internucleosomal DNA fragmentation, morphological changes and a decrease in the mitochondrial transmembrane potential through both death receptor and mitochondrial pathways in a dose-dependent manner (25).

## CONCLUSION

Our findings suggest that OPE and NR can inhibit the migration of esophageal cancer cell. In addition, the anticancer effects of OPE were better than that of NR alone. Based on these findings, the anticancer effects of these agents against ESCC cells can be further investigated in future studies.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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