Original Article

# Effects of Flavonoid Fractions from *Calendula officinalis* Flowers in Parent and Tamoxifen Resistant T47D Human Breast Cancer Cells

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## Abstract

Three major flavonoid fractions were separated from a methanol extract of *Calendula officinalis* flowers by preparative TLC. These fractions were evaluated for the inhibition of parent and tamoxifen resistant T47D human breast cancer cells. We also examined the effect of quercetin and isorhamnetin on the growth of parent and resistant T47D cells in the presence and absence of tamoxifen. It was found that quercetin increased cell proliferation of the resistant T47D cells at the presence of tamoxifen but no effect was detected by using quercitin alone. The fractions isolated from *Calendula officinalis* did not show any inhibitory effects on the cells. Isorhamnetin did not have any proliferative or anti-proliferative activity on the both cell lines.

## 1. Introduction

The risk of human breast cancer is concerned to cumulative exposure of the breast cells to endogenous estrogens [1]. Experimental evidence indicates that estrogens play important roles in the breast cancer development [2]. Strategies aiming at reducing the production of estrogens may be useful for the prevention of estrogens-related breast cancer [1, 2]. The incidence rates of these disorders are higher in populations consuming high-fat and low fiber diets than in populations with diets rich in fruits and vegetables [3]. Several natural products with plant origin have the potential value as chemo-preventive or therapeutic agents in cancer. Flavonoids, the natural polyphenol compounds, are present with high concentrations in grains, legumes, fruits and vegetables. These compounds have shown antiviral, antiinflammatory, antimutagenic and anticarcinogenic activities [3-6]. *Calendula officinalis*, a member of compositae family, is well known for its pharmacological effects such as antiinflammatory, antiviral, anti-HIV, antitumor, antimutagenic and cytotoxic properties [7-10]. The flowers contain a variety of compounds including flavonoids, phenolic acids, terpenoids,

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carotenoids and sterols [11]. Most of the flavonoids present in the plant are the glycoside derivatives of quercetin and isorhamnetion [12]. Mechanism of the possible chemo-preventing action of flavonoids has not yet been completely understood. They may exert their chemo-preventive and anti-carcinogenic effects in estrogen-dependent cells by inhibiting aromatase,17β-hydroxysteroid oxidoreductase and other enzymes involved, or by possessing estrogenic and anti-estrogenic activities [13-17].

Aromatase (CYP19), a cytochrom P-450, is the enzyme that converts androgen compounds such as androstendione or testosterone to estrone and estradiol, respectively. It is expressed at a higher level in human breast cancer tissue than in normal breast tissue [2]. The enzyme inhibition lowers 17β-estradiol concentrations in the target cells, which then decreases the risk of breast cancer. Besides, the flavonoids which are structurally similar to estrogen are able to bind to the estrogen receptor and compete with endogenous substrates. Tamoxifen, an anti-estrogen pharmaceutical agent, is used for the treatment of severe breast cancer. Combination of tamoxifen with some flavonoids as aromatase inhibitors may reduce drug resistance in breast cancer.

In this study, we examined three flavonoid fractions from *Calendula officinalis* flower extract and evaluated their effect on proliferation of the parent estrogen-dependent cancer cells (T47D) and T47D cells which are resistant to tamoxifen.

## 2. Materials and methods
### 2.1. Chemicals
Quercetin (3,3',4',5,7-pentahydroxyflavone) was purchased from Sigma Chemical Co. Isorhamnetin (3,4',5,7-tetrahydroxy-3'-methoxyflavone) was kindly donated by Dr. Joerg Heilmann (ETHZ, Switzerland). RPMI 1640 was obtained form

Biotech GmbH (PAN) (Germany). Fetal bovine serum (FBS), trypsin-EDTA and penicillin-streptomycin stock solution were purchased from GIBCO BRL (Germany). Natural product (2-aminoethyldiphenylborate) reagent was obtained from Merck (Germany). All other reagents and solvents were of analytical grade and purchased from Merck (Germany).

### 2.2. Plant materials
The flowers of *Calendula officinalis* were prepared from the Botanical Garden of Giah Essence Phytopharm Co. (Gorgan, Iran).

### 2.3. Extraction and partial purification of Calendula officinalis
The dried and powdered flowers of *C. officinalis* (250 g) were extracted for 6 hours with petroleum ether by a Soxhlet apparatus to eliminate fatty materials. The extraction was continued by methanol/water (80:20) to obtain a total extract. The extract was then filtered and concentrated under reduced pressure. Methanol extract was fractionated by preparative TLC using silicagel plates with ethyl acetate/water/glacial acetic acid/formic acid (100:26:11:11) as the solvent system. The flavonoid fractions were detected using Natural Product reagent. Three major fractions with the $R_f$ value of 0.23, 0.45 and 0.85 were then selected for further purification. Re-chromatography was performed with preparative TLC by ethyl acetate/water/glacial acetic acid/formic acid (115:15:12:4). The flavonoid fractions were hydrolyzed with 10% HCl to obtain the aglycone moieties. The aglycones were then analyzed by TLC and were compared with pure quercetin and isorhamnetin followed by visualizing with Natural Product reagent.

### 2.4. Cell culture
Parent T47D cells and tamoxifen-resistant T47D (resistant to $10^{-6}$ M tamoxifen) were kindly provided by the Molecular Research

Laboratory, Department of Toxicology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. The cells were routinely maintained at 37 °C in a 5% $CO_2$- enriched humified air atmosphere and were cultured in RPMI 1640 medium containing 10% FBS and penicillin/ streptomycin (1%). Tamoxifen ($10^{-6}$ M) was added to the culture of the resistant cells in order to keep their entity. The cells were cultured toward confluency, then detached with 1X trypsin-EDTA solution and plated at the desired confluence or used for experiments.

### 2.5. Cell proliferation assay

The cells were cultured in 24-well plates in a density of $4 \times 10^4$ cells/well. The samples in various concentrations were added after 24 hours. Controls received no additional substance. Media were exchanged after 3 days, and viability and cell count were determined using trypan blue exclusion test after one week. The flavonoid fractions were added to the media in a range of 0.05-50 μg/ml and the concentrations of pure quercetin and isorhamnetin were in the range of 1-100 nM.

### 2.6. Statistical analysis

The analyses of differences were carried out by one way ANOVA, and Newman Keuls post test using Prism program. A *p*-value of less than 0.05 was considered significant.

### 3. Results

The effect of different concentrations (0.05-50 μg/ml) of the flavonoid fractions from *Calendula officinalis* on the proliferation of the parent T47D cells after one week of incubation is shown in Figure 1. No significant difference was observed between the samples and the controls. The effects of flavonoid fractions on the parent and the tamoxifen-resistant T47D cells at the presence of tamoxifen ($10^{-6}$ M), are presented in Figures

2 and 3. No marked difference has been observed in this experiment. We also evaluated the effects of quercetin and isorhamntine as the plants' major flavonoid aglycones in the range of 1-100 nM on the parent and tamoxifen-resistant T47D cells in the presence or absence of tamoxifen 1 μM (Figures 4 and 5). Results showed that quercetin significantly increased the proliferation of the tamoxifen-resistant T47D cells at the presence of tamoxifen. Isorhamnetin had no significant effect on the two cell lines in the presence or absence of tamoxifen.

### 4. Discussion

In the present study, we investigated the effects of flavonoid fractions extracted from *C. officinalis* and their aglycones -quercetin and isorhamnetin- on the proliferation of the parent and the tamoxifen-resistant T47D cells at the presence and absence of tamoxifen. The flavonoid extracts (0.05-50 μg/ml) did not cause significant effect on the proliferation of the two cell lines. It may be related to the attached sugar molecule at the position 3 of flavone that can reduce its ability to bind aromatase and other enzymes [18].

As other studies indicated, quercetin, daidzein, genistein, rutin, catechin, equol and β-naphtoflavone (113 μM) did not affect aromatization, but β-naphtoflavone, chrysin
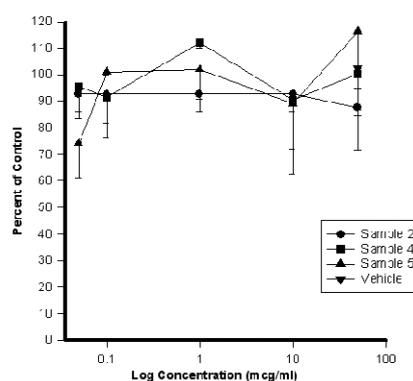


**Figure 1.** The effect of different fractions from *Calendula officinalis* on the T47D parent cells after a week of incubation. Sample 2, Rf 0.23; sample 4, Rf 0.45; sample 5, Rf 0.85 (n=3).
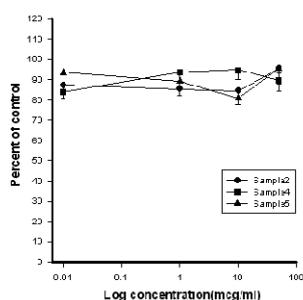
**Figure 2.** The effect of flavonoid fractions from *Calendula officinalis* on the T47D parent cells in the presence of tamoxifen after 7 days of exposure. Sample 2, Rf 0.23; sample 4, Rf 0.45; sample 5, Rf 0.85. Data are mean ± SD of two determinations.

and biochanin inhibited aromatase activity. They had no effect on the viability of preadipocyte cells after 24 hours [19].

No literature report was found on the effects of isorhamnetin with aromatase activity or proliferation of T47D cells. The chemical structure of isorhamnetin is similar to quercetin with a difference in methoxyl group instead of hydroxyl at the position 3' in the molecule.

Flavonoids can affect cell proliferation by many mechanisms. For example in estrogen-dependent cells (T47D), they can alter cell proliferation by inhibition of aromatase 17β-HSOD or 3β-HSOD that caused estradiol reduction or by estrogenic and anti-estrogenic activities [18, 20, 21]. In the JEG-3, human choriocarcinoma cells and HEK293 cells stably expressing aromatase, flavone and quercetin showed no aromatase inhibition ($IC_{50}$>100 μm) [22]. It is established that the number and location of hydroxyl groups in flavones are important for the aromatase inhibition. As 4'-hydroxylation decreases the inhibition of aromatase, the hydroxyl group at the position 3 of flavones (quercetin *vs* luteolin) and 3', 4'-dihydroxy, reduces their inhibition efficacy. The presence of hydroxyl group in the position 3 of flavone significantly reduces its ability to bind aromatase [18, 22].

Other studies revealed that in the cultured breast cancer cells (T47D), 17β-HSOR type 1 enzyme is primarily a reductase, converting the weak endogenous estrogen (estron) to the more potent estrogen, 17β-estradiol. The 4' and 5-hydroxyl groups in the flavone chemical structure are essential for 17β-HSOR inhibition but not for aromatase inhibition.

Structural demands for the inhibition of aromatase differ from 17β-HSOR, but in both cases the hydroxyl group at the position 3 of flavones (quercetin *vs* luteolin) and 3',4'-dihydroxy reduce their inhibitory activity [21, 23]. In addition, the structural requirements for aromatase inhibition do not correlate closely with the structures known for their participation in the estrogenicity of flavonoids [9].

The main feature required to confer estrogenicity is the presence of hydroxyl substituents in the positions 4' and 7 of the flavone nuclei [21]. An additional hydroxyl group at the position 5 may increase estrogenic activity in some cases. Two hydroxyl groups in position 4' and 5 are not essential structural features for the compounds as aromatase inhibitors. Furthermore, the flavanol structure may increase estrogenicity while decreasing the capacity of flavones for inhibiting aromatization. These differences in the structural requirements for aromatase inhibition and estrogenicity may be explained by a difference in the binding orientation between flavones and steroids in the binding sites of aromatase and ERs [18]. For example curcumin and genistein did not exhibit any
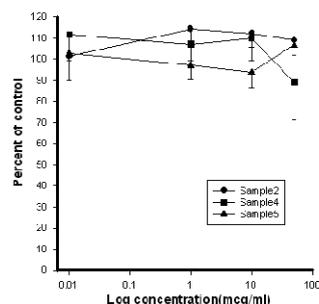


**Figure 3.** The effect of flavonoid fractions from *Calendula officinalis* on the tamoxifen-resistant T47D parent cells in the presence of tamoxifen after a week of exposure. Sample 2, Rf 0.23; sample 4, Rf 0.45; sample 5, Rf 0.85. Data are mean±SD of two determinations.
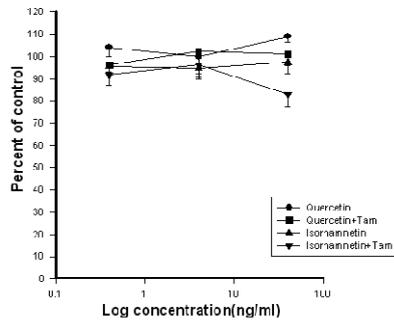
**Figure 4.** The effect of quercetin and isorhamnetin on the parent T47D parent cells in the presence and absence of $10^{-8}$ M tamoxifen after a week of exposure.

appreciable proliferative effect at concentrations 10 μM and 25 μM, respectively, but compeletly reduced the proliferative potency of estradiol (5 nM) and estrogenic pesticides. These compounds may either block the estrogen receptor binding sites or interfere with the processes that are induced after binding of estrogenic pesticides or estrogens with receptor [24]. In contrast, genistein, coumestrol and didzein recovered the cell growth of MCF-7 cell that was suppressed by the addition of toremifen at the concentrations of about 0.1-1 μM. This effect is related to estrogenic activity of these flavonoids, because of the similar chemical structures [25].

In another study, comparison between the effect of different flavonoids on the MCF-7 (ER$^+$) and MDA-MB231 (ER$^-$) human breast
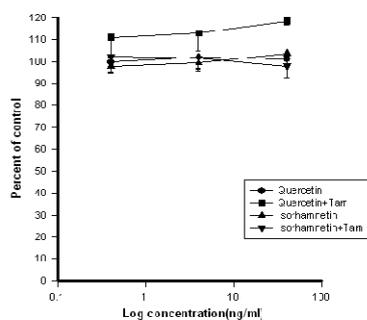


**Figure 5.** The effect of quercetin and isorhamnetin on the tamoxifen-resistant T47D cells in the presence and absence of $10^{-6}$ M tamoxifen after a week. Data are mean±SD of two determinations.

cancer cell lines, through estrogenic response and cell proliferation, was showed that 7-methoxyflavanone and 7,8-dihydroxyflavone, possess biphasic activity. At 50 μM in the presence and absence of 0.1 nM estradiol, estrogenic activity was reversed. In these concentrations they had anti-estrogenic activity [9].

Proliferation of MCF-7 in response to several flavonoids at concentrations ranging from 1 nM to 50 μM was biphasic. Generally low concentrations (1 nM-1 μM) stimulated the growth of MCF-7 cells and the potential flavonoids to increase cell proliferation has been shown to correlate with their affinity for the estrogen receptor. At high concentration (50 μM), all flavonoids appeared to inhibit cell proliferation in the presence or absence of estradiol (0.1 nM), with the exception of 7-hydroxyflavone, 7, 8-dihydroxyflavone and kaempferol, which did not show antiproliferative activity [9]. Flavonoids at 50 μM are able to inhibit the proliferation of MDA-MD231 cells which do not express ER [9]. It is related to other mechanisms of flavonoids on the malignant cells [26, 27]. Besides, estrogen deprivation such as exposure to tamoxifen causes tumors to develop hypersensitivity to estradiol. This estradiol hypersensitivity is associated by an up-regulation of MAP-kinase and PI-3 kinase pathways as well as increased usage of a membrane associated estrogen-receptor alpha [28]. These documents can help to understand why quercetin increases the cell proliferation of resistant T47D cells in the presence of tamoxifen. It may be related to hypersensitivity of resistant cells after exposure to tamoxifen; and quercetin revealed their weak estrogenic activity.

**Acknowledgment**

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