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# Differential effects of esculetin and daphnetin on in vitro cell proliferation and in vivo estrogenicity

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ABSTRACT

Esculetin (6,7-dihydroxycoumarin) and daphnetin (7,8-dihydroxycoumarin) are secondary metabolites of plants used in folk medicine. These compounds have showed great antiproliferative activity in several tumor cell lines and have been proposed as potential anticancer agents. However, the estrogenic potential of these two compounds has to date not been reported. The present study compared esculetin and daphnetin on the inhibition of cell proliferation and cell cycle progression of the MCF-7 estrogen-responsive human carcinoma cell line. In vivo and in vitro estrogenic activity for both compounds was also evaluated. Esculetin inhibited cell proliferation after 72 h exposure (IC50 = 193 ± 6.6 μM), while daphnetin evidenced inhibiting effects starting at 24-h exposure (72 h, IC50 = 73 ± 4.1 μM). Both effects showed changes in cyclin D1 gene expression. In non-estrogenic conditions (E-screening assay), esculetin produced biphasic response on proliferation of the MCF-7 cells; at $10^{-8}$–$10^{-6}$ M, concentrations induced proliferative effects as EC50 = $4.07\times10^{-9}$ M ($E_2 = 2.91\times10^{-12}$ M); at higher concentrations ($10^{-5}$–$10^{-4}$ M), cell proliferation was inhibited. Relative proliferative effect at $E_2$ was 52% ($E_2 = 100$), relative proliferative potency was 0.072 ($E_2 = 100$). Additionally, esculetin tested in vivo showed estrogenic effects at 50–100 mg/kg doses; relative uterotrophic effect at $E_2$ was 37%, with relative uterotrophic potency registered at 0.003. In contrast, daphnetin did not induce estrogenic effects in vitro or with in vivo models. The low estrogenic activity of esculetin could prove useful in postmenopausal therapy but not as a safe antitumor agent in estrogen-dependent tumors. Daphnetin-based antiproliferative selectivity with MCF-7 cells showed that daphnetin is a promising antitumoral agent also acting on estrogen dependent tumors.



## 1. Introduction

Clinical studies have reported antitumor activity of coumarin in several cancer types (Marshall et al., 1991; Mohler et al., 1992; Thornes et al., 1994). Coumarin acts as a prodrug creating active metabolites which could be responsible for observed effects (Egan et al., 1990). Experimental assays showed that coumarin producing antiproliferative effect in tumor cell lines with mM concentrations. However, 7-hydroxycoumarin (its main biotransformation product in humans) has greater antiproliferative activity (Jiménez-Orozco et al., 1999, 2001; Lopez-Gonzalez et al., 2004). The addition of two hydroxyl groups in the meta- or para-positions of the coumarin nucleus increases antiproliferative activity to ranges of μM concentration (Kolodziej et al., 1997).

Dihydroxy-coumarin derivatives, esculetin (6,7-dihydroxycoumarin) and daphnetin (7,8-dihydroxycoumarin), are secondary metabolites of plants used in folk medicine to counter inflammatory and allergic diseases (Fylaktakidou et al., 2004; Riveiro et al., 2010). Esculetin possesses diverse pleiotropic actions (Chu et al., 2001): it inhibits smooth vascular muscle cell proliferation through the inhibition of kinases and mitogen signal pathways mediated by Ras protein (Pan et al., 2003). The antiproliferative effect of esculetin has been reported to be greater in tumor cells than in non-malignant cells (Finn et al., 2002; Kawaii et al., 2001; Kawase et al., 2003). Esculetin has been considered a promising anticancer agent due to its multiple properties (Lacy and O'Kennedy 2004).

Daphnetin has been clinically used in the treatment of coagulation disorders, rheumatoid arthritis and has been shown to possess anti-

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malarian and anti-pyretic properties (Liang et al., 2010). The daphnetin compound also induces cell differentiation (Finn et al., 2004) and inhibits kinase activity (Yang et al., 1999). The antiproliferative effects of daphnetin have been studied to a lesser extent than those induced by esculetin. However, the daphnetin is also capable of producing notable antiproliferative activity among different malignant cell lines (Finn et al., 2001, 2002).

Therapeutic strategies for breast cancer-dependent hormone tumors typically involve the inhibition of the estrogen receptor by selective estrogen receptor modulators (SERMs), such as tamoxifen. A new 3, 4 substituted coumarin-based SERM was designed based on the structural similarity of coumarin molecules to the AB ring of natural estradiol hormones (McKie et al., 2004). A second complementary strategy in breast cancer therapy consists in the inhibition of enzymes found within the steroid biosynthetic cascade. In this respect, some 7-hydroxycoumarin analogs have been considered to be promissory anti-breast cancer agents for their sulfatase and aromatase inhibitory activity (Musa et al., 2008).

While the estrogenic effect of certain coumarin derivatives such as coumestrol has been firmly documented, the estrogenic properties of esculetin and daphnetin have yet to be reported. Evaluation of esculetin and daphnetin estrogenic activity should be obligatory, given that high estrogen levels or exposition to estrogenic substances are recognized factors in breast cancer (Fernández and Russo, 2010). The aim of the present study was to compare the effects of esculetin and daphnetin on MCF-7 cell proliferation under estrogenic and non-estrogenic conditions and their effects on the cell cycle. Estrogenicity for both compounds was also tested by means of uterotrophic assays.

## 2. Materials and methods

### 2.1. Reagents

17β-Estradiol ($E_2$; 1,3,5(10)-estratrien-3,17-β-diol), esculetin, (6,7-dihydroxycoumarin) and daphnetin (7,8-dihydroxycoumarin) from Sigma (St. Louis, MO, USA) were dissolved in an ethanol absolute (Merck, Darmstadt, Germany). Anti-cyclin D1 mouse monoclonal, anti-UBF, anti-cyclin E, and anti-p27 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All cell culture reagents, media, as well as the TRIzol reagent, were purchased from Gibco (Invitrogen Corporation, USA).

### 2.2. Cell culture conditions

The human carcinoma cell line MCF-7 was purchased from the American Type Culture Collection (Manassas, VA, USA) and was routinely cultivated at 37 °C in a humid, 5% $CO_2$ atm using Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, 1% (v/v) pyruvate, and a 1% (v/v) antibiotic–antimycotic mix (penicillin G sodium, streptomycin sulfate and amphotericin B). The cytostatic assays were carried out under estrogenic conditions using fetal bovine serum and red phenol DMEM. In the estrogen-screening assays (non-estrogenic conditions), cells were cultivated and supplemented with 5% (v/v) charcoal: dextran stripped fetal bovine serum (CDFBS) and phenol-red free DMEM, as has been described by Soto et al. (1991).

### 2.3. Cytostatic MTT assay

The cytostatic effect of compounds tested on the tumor cells was estimated using the microculture MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The assay is based on the reduction of soluble tetrazolium salt by mitochondria of viable cells. The reduced product, an insoluble purple-colored formazan, was dissolved in dimethyl sulfoxide and measured spectrophotometrically (570 nm). Under the experimental conditions of this study, the amount of formazan formed was proportional to the number of viable cells. Cells ($3\times10^3$) were seeded in each of the 96 microplate wells in a 200 μL medium containing the corresponding concentration of esculetin and daphnetin. The compounds were tested at five concentrations (12.5, 25, 50, 100, 200 μM). After 24-, 48-, and 72-h exposure, the percentage of proliferative inhibition of treated cells was estimated against the solvent-treated control cells (PI% = [(T/C) − 1] × 100). PI = proliferation inhibition; T = treated, C = control. IC50 was calculated from the least square concentration-response regressions.

### 2.4. Immunoblot analysis

Esculetin and daphnetin were dissolved in ethanol and diluted in culture media. MCF-7 cells ($5\times10^5$) were grown in 100-mm dishes containing 15 mL DMEM, supplemented with 10% FBS and treated with either ethanol as a control medium (0.1 v/v) or esculetin or daphnetin at concentrations of 50, 100, or 200 μM. Following 72-h exposure, cells were washed twice with ice-cold 0.1 M PBS, harvested by scraping and centrifuged at 3000 rpm for 5 min. Whole cell extracts were lysed in a cold lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS) also containing a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Samples containing 60 μg of total protein were resolved in 12% sodium dodecyl sulfate polycralymide gels (SDS-PAGE) and transferred to nitrocellulose membranes. The level of protein expression was determined using specific primary antibodies, followed by peroxidase-conjugated secondary antibodies and visualization using a chemiluminescent substrate (Luminol) and exposure to X-ray film. The densitometric analysis was carried out using Image J NIH software (National Institutes of Health, Washington, DC, USA).

### 2.5. Real time PCR

Cells were treated for 72-h as described above. The total RNA was isolated using the TRIzol reagent as recommended by the supplier. RT PCR was performed using LightCycler® 2.0 Fast Start DNA Master SYBR Green I Kit as recommended by the supplier (Roche). The primers of Cyclin D1 (cat. no 04688660001) and GAPDH (cat no. 05190541001) were purchased commercially from Roche.

### 2.6. Cytometric DNA flow analysis

Cells were treated for 72-h as described before. The cells were harvested using 0.25% trypsin, washed with PBS, fixed in ethanol, and incubated at −20 °C for 24-h. The cells were pelleted at 400×*g*, washed in PBS and re-suspended in a solution containing 0.5% RNase A and 1 μg/mL propidium iodide in PBS. After 30 mins incubation under dark conditions at −4 °C, the samples were analyzed using flow cytometry with a FACScalibur (Becton Dickinson, Mountain View, CA). Cell cycle analysis was performed on list mode data files using Modfit software (version 2.0).

### 2.7. E-screening assays

MFC-7 cells ($2\times10^3$) were seeded in each of 96-well microplates in 200 μL DMEM, supplemented with 10% FBS and allowed to attach. Following overnight incubation, the medium was replaced every 3 days with fresh phenol-red free DMEM supplemented with 5% CDFBS and containing the corresponding treatment. After 8 days of exposure, the proliferation of MCF-7 cells was estimated by the MTT microassay. The estrogenic calibration curve was assessed at $E_2$ in concentrations of $10^{-15}$ to $10^{-7}$ M, using ethanol as a solvent (0.001% v/v). Esculetin and daphnetin were tested at concentrations of $10^{-9}$ to $10^{-4}$ M. In each case, proliferation percentages for each treatment were calculated in relation to corresponding control cells. No differences were observed between the non-treated control cells