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Report

Influence of *Cimicifuga racemosa* on the proliferation of estrogen receptor-positive human breast cancer cells

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Key words: black cohosh, *Cimicifuga racemosa*, hormone replacement therapy, *in vitro*, menopause, MCF-7, phytoestrogens, proliferation assay, SERM

Summary

Hormone replacement therapy, which is a common menopausal treatment, is contraindicated in women with breast cancers due to concerns regarding the potential for breast cell proliferation. As such, there is a need for alternative methods for treating menopausal symptoms. To determine the influence of one such alternative, black cohosh (*Cimicifuga racemosa* [CR]), on estrogen-dependent mammary cancers, we conducted an *in vitro* investigation of the effect of an isopropanolic CR-extract on the proliferation of estrogen receptor-positive breast cancer cells. The experiments were performed using the human breast adenocarcinoma (MCF-7) cell test system, an established *in vitro* model for estrogen-dependent tumors. The influence of CR-extract on the proliferation of the MCF-7 cells was determined by measuring the incorporation of radioactively labeled thymidine. Under estrogen-deprived conditions, the CR-extract (10^{-3}–10^{-5} dilutions) significantly inhibited MCF-7 cell proliferation. Additionally, application of the CR-extract inhibited estrogen-induced proliferation of MCF-7 cells. Moreover, the proliferation-inhibiting effect of tamoxifen was enhanced by the CR-extract. Such data that suggest a non-estrogenic, or estrogen-antagonistic effect of CR on human breast cancer cells lead to the conclusion that CR treatment may be a safe, natural remedy for menopausal symptoms in breast cancer.

Introduction

Hormone replacement therapy (HRT) is commonly prescribed to relieve menopausal symptoms, however this type of treatment is contraindicated in patients with estrogen-dependent cancers. Estrogen therapy avoidance is particularly recommended in individuals with a history of breast cancer due to the potential to stimulate breast cell proliferation [1]. Because of their inability to utilize HRT, estrogen-sensitive cancer patients often seek alternative methods to relieve hot flashes and menopausal symptoms.

In recent years, black cohosh (*Cimicifuga racemosa* [CR]), has gained acceptance as a natural alternative for the treatment of menopausal symptoms [2, 3]. The clinical efficacy of CR-extracts, particularly in comparison to HRT, has been demonstrated in both open and placebo-controlled studies, thus supporting CR as an effective treatment in relieving menopausal symptoms [2].

Although the precise mode of action of CR is not yet known, its primary pharmacological effects appear to involve interactions with the regulatory mechanisms of the endocrine system [3]. Early reports [4], which have been subsequently proven incorrect [5, 6], suggested that the activity of CR may be related to various phytoestrogenic isoflavonoids in CR, such as formononetin. Nevertheless, CR binding to estrogen receptors [4, 7] is a possible basis for its effectiveness in menopausal symptom management.

We evaluated the effect of CR-extracts on the proliferation of estrogen-dependent breast cancer cells (MCF-7) *in vitro* in an effort to further characterize its mechanism of action and to assess the safety of

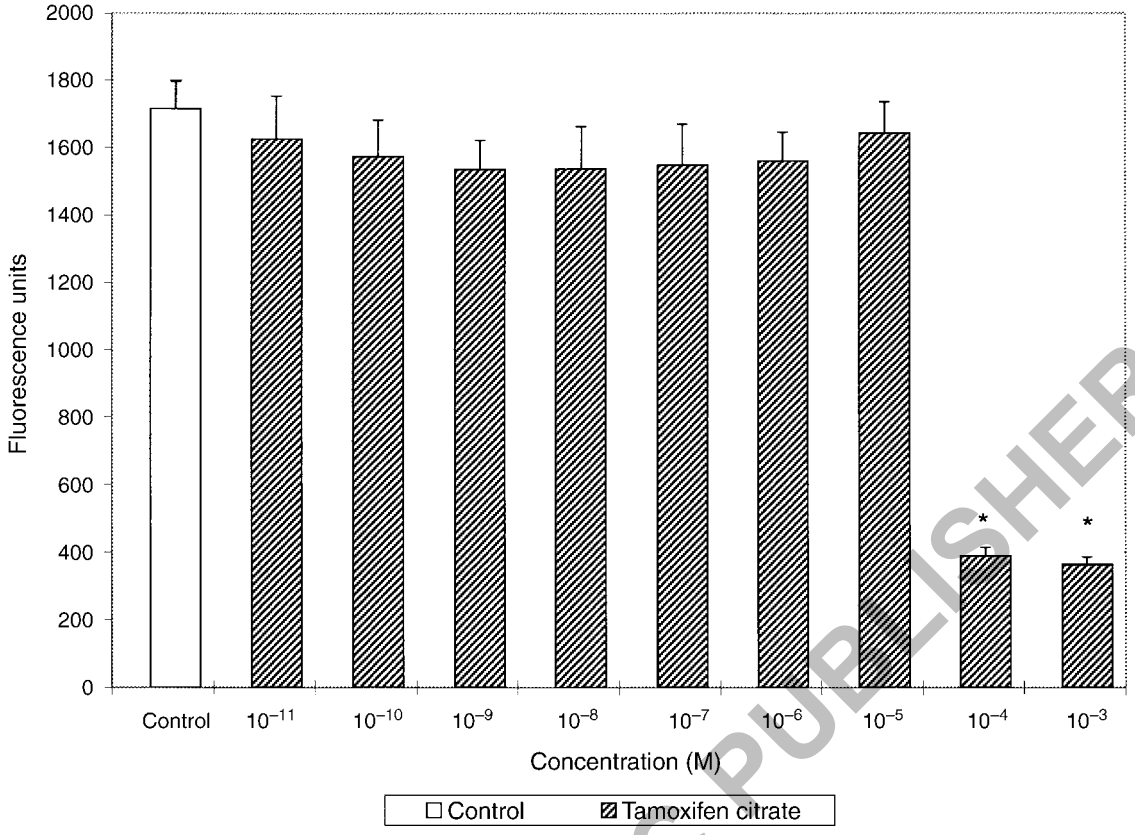

Figure 1. Toxicity assay: cytotoxic effects of tamoxifen citrate on MCF-7 cells. Control: cell culture medium; all data are presented as mean ± SD for $n = 6$. Key: $^{*}p < 0.05$ versus control (Student's *t*-test).

CR-extracts for the treatment of menopausal symptoms in patients with a history of estrogen receptor-positive mammary cancer.

Methods

Cell preparation

Estrogen receptor-positive MCF-7 cells (American Type Culture Collection HTB 22) were cultured in Eagle's MEM with non-essential amino acids (Biochrom KG, Berlin), 1 mM sodium pyruvate (Biochrom KG, Berlin), 10 µg/ml insulin (Sigma-Aldrich Chemie GmbH), 10% FCS (Biother GmbH, Kelkheim) and antibiotics.

Material preparation and identification

The aqueous isopropanolic extract of the rhizome of CR was prepared according to a validated manufacturing process submitted to the German BfArM. The extract contained a dry residue of approximately 10% (mass/mass). The CR-extract was diluted with cell culture medium to test dilutions of 10^{-3}–10^{-8}.

17β-Estradiol (β-estradiol-17-acetate, Sigma-Aldrich Chemie GmbH) and tamoxifen citrate (Sigma-Aldrich Chemie GmbH) were prepared by dissolving in DMSO and diluted in cell culture medium in a 1:10 dilution series to 10^{-7}–10^{-9} M and 10^{-3}–10^{-11} M, respectively.

Charcoal-stripped-FCS (CSF), used in the toxicity and proliferation assays, was either purchased from Sigma-Aldrich Chemie GmbH (Sigma C1696) or prepared by dissolving one dextran coated charcoal tablet (Steranti Separe®, Steranti Research Limited, St. Albans) in 100 ml FCS, inactivated in a water bath at 56°C for 2 × 45 min and washed by centrifugation at 3000 rpm for 10 min. The supernatant was removed and filtrated through a 0.2 µm sterile filter (Sarstedt Filtropur S, 0.2 µm).

Toxicity assay

To determine cytotoxicity, a fluorescence assay was performed using 4-methylumbelliferyl-heptanoate

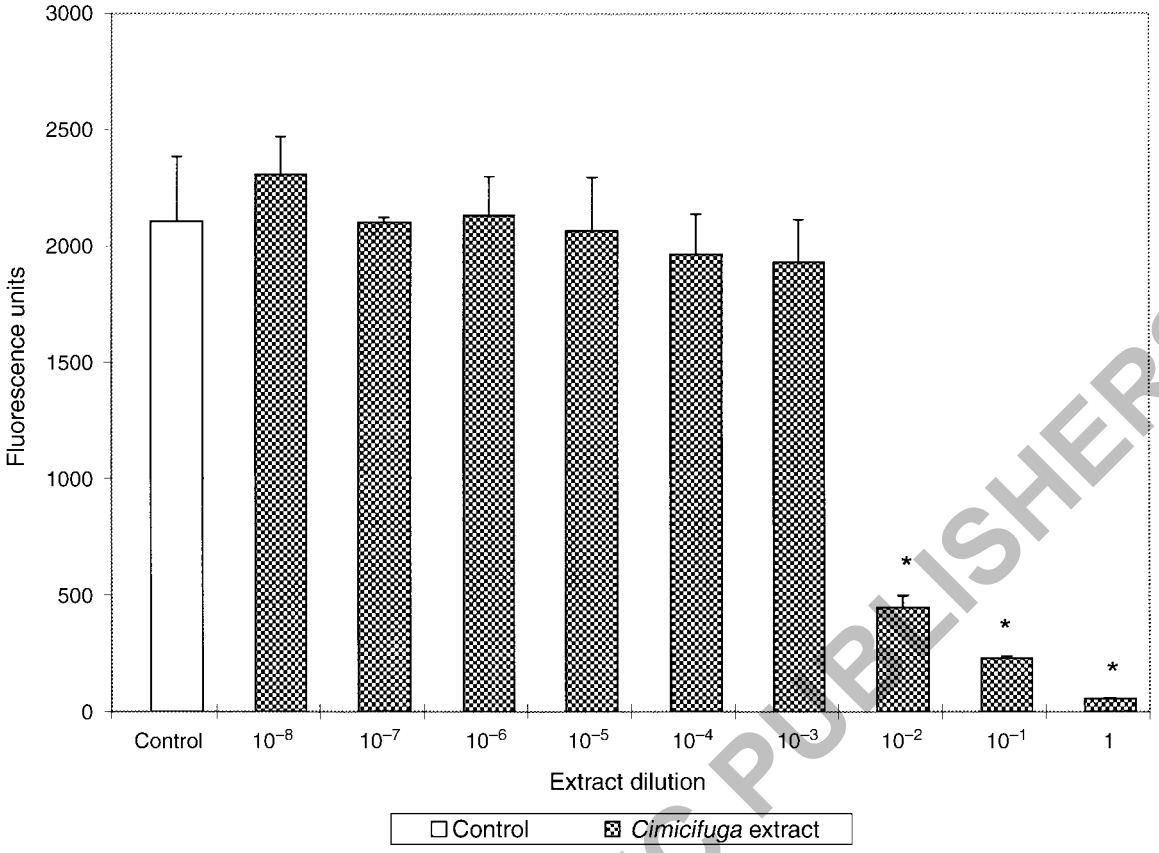

Figure 2. Toxicity assay: cytotoxic effects of CR-extract on MCF-7 cells. Control: cell culture medium; all data are presented as mean ± SD for $n = 6$. Key: $^{*}p < 0.05$ versus control (Student's *t*-test).

(4-MeUH, Sigma-Aldrich Chemie GmbH), a substrate that is non-fluorescent until taken up and cleaved by esterases in living cells. MCF-7 cells were plated at an initial cell density of 3×10^4 cells/well in Eagle's MEM without phenol red, supplemented with non-essential amino acids, 1 mM sodium pyruvate, 10 µg/ml insulin and 5% CSF. After incubation at 37°C and 5% CO_2 for 24 h, test substances (17β-estradiol, tamoxifen, and CR-extract) in cell culture medium were added. The microtiter plates were incubated for 48 h, then centrifuged at 800 rpm for 10 min. The supernatants were removed and 200 µl/well 4-MeUH (0.1 mg/ml in PBS) was added. The fluorescence units per well were measured in a microtiter plate fluorimeter (Fluoroskan II) after 120 min.

Proliferation assay

The MCF-7 test system, a well-established model for the *in vitro*-investigation of estrogenic activities [8, 9], was utilized to evaluate the effect of the test substances on cell proliferation. MCF-7 cells were cultured for at least one passage in Eagle's MEM without phenol red, supplemented with non-essential amino acids, 10 µg/ml insulin, 1 mM sodium pyruvate and 5% CSF. Cells were plated into 96 microtiter plates (200 µl MCF-7 cell suspension (5×10^4 cells/ml) per well), and incubated at 37°C and 5% CO_2 for 24 h. The supernatants were removed and 150 µl fresh culture medium was added. The test substances were dissolved, diluted in cell culture medium, and pipetted in four quadruplicate wells at 100 µl/well. Each microtiter plate contained control wells of the cell culture medium and test solvent diluents. After 48 h of incubation at 37°C and 5% CO_2, cells were pulsed with 25 µl/well [6-^{3}H] thymidine (Amersham Pharmacia, 2 Ci/mM, 1 mCi/ml) for 8 h. Cells were harvested onto glass fiber filters according to standard methods (Cell Harvester, Inotech) and counted in a liquid scintillation counter (Wallac). The method of cell quantification used in our study (incorporation of ^{3}H-thymidine)

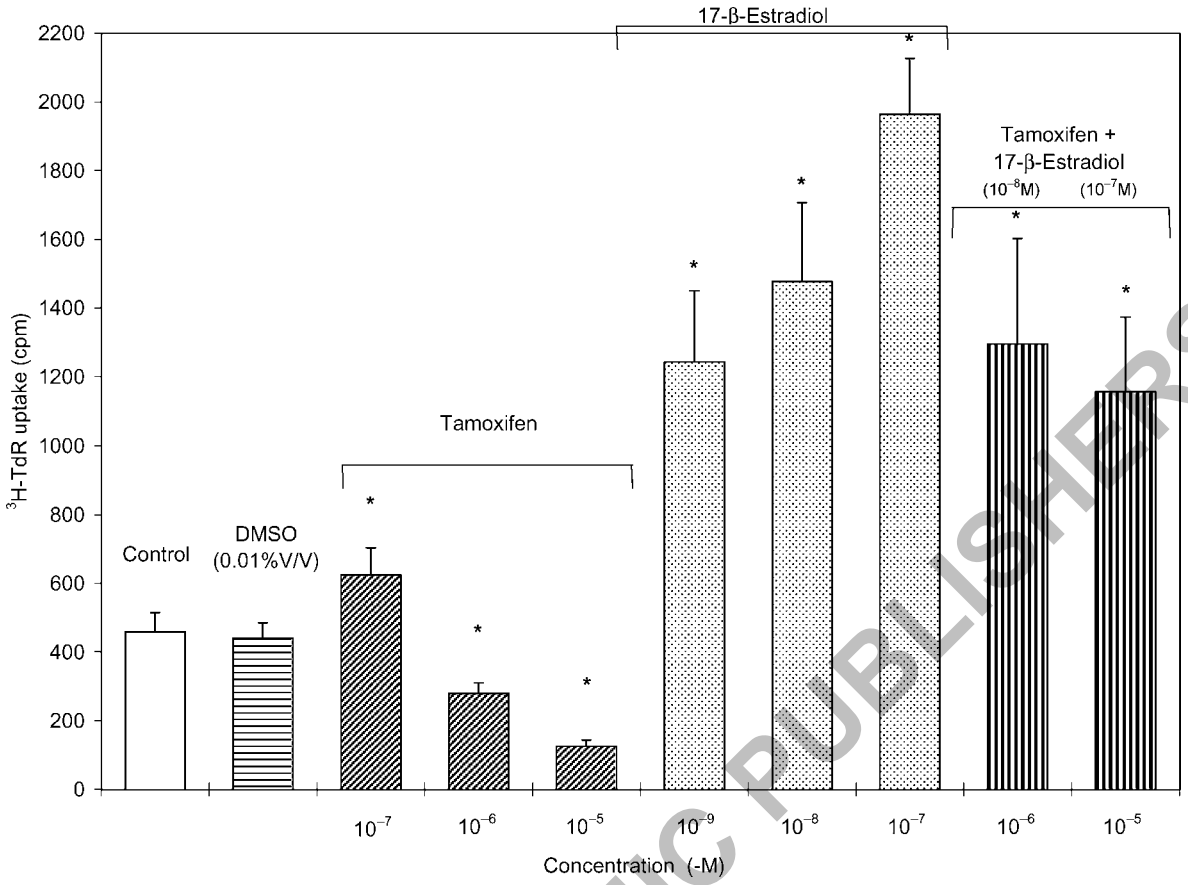

Figure 3. Influence of 17β-estradiol and tamoxifen on the proliferation of MCF-7 cells. Control: cell culture medium; DMSO: solvent control; all data are presented as mean ± SD for $n = 4$. Key: $^*p < 0.05$ versus control (Student's *t*-test).

permits the direct quantification of de novo-DNA-synthesis and is generally accepted as the most sensitive method for the measurement of cell proliferation [10].

Statistical analysis

The results are expressed as mean ± standard deviation (SD). The pharmacological data were analyzed by the Student's *t*-test and significance was assumed at $p < 0.05$.

Results

Toxicity assays

At the start of the study, the cytotoxic activity of the test substances was determined using MCF-7 and HeLa cells. 17β-Estradiol (10^{-7}, 10^{-8} or 10^{-9} M) was not found to be cytotoxic. The toxicity assay for the estrogen-antagonist tamoxifen revealed that tamoxifen concentrations $> 10^{-5}$ M were cytotoxic to MCF-7 cells; concentrations $\leq 10^{-5}$ M showed no cytotoxic activity (Figure 1). The cytotoxic activity of the CR-extract was also evaluated. CR-extract dilutions $\geq 10^{-2}$ induced toxic effects on the MCF-7 cells; dilutions $\leq 10^{-3}$ showed no toxic activities (Figure 2).

Proliferation assays

At the start of the study, the sensitivity of the MCF-7 test system was determined. Negative controls (medium and solvent controls) were carried out in each proliferation assay. In the maximum concentrations used, neither DMSO nor isopropyl alcohol influenced proliferation rates. As a positive control, we evaluated the estrogenic effect on cell proliferation of 17β-estradiol (10^{-7}, 10^{-8} or 10^{-9} M). Results show that all test concentrations of 17β-estradiol

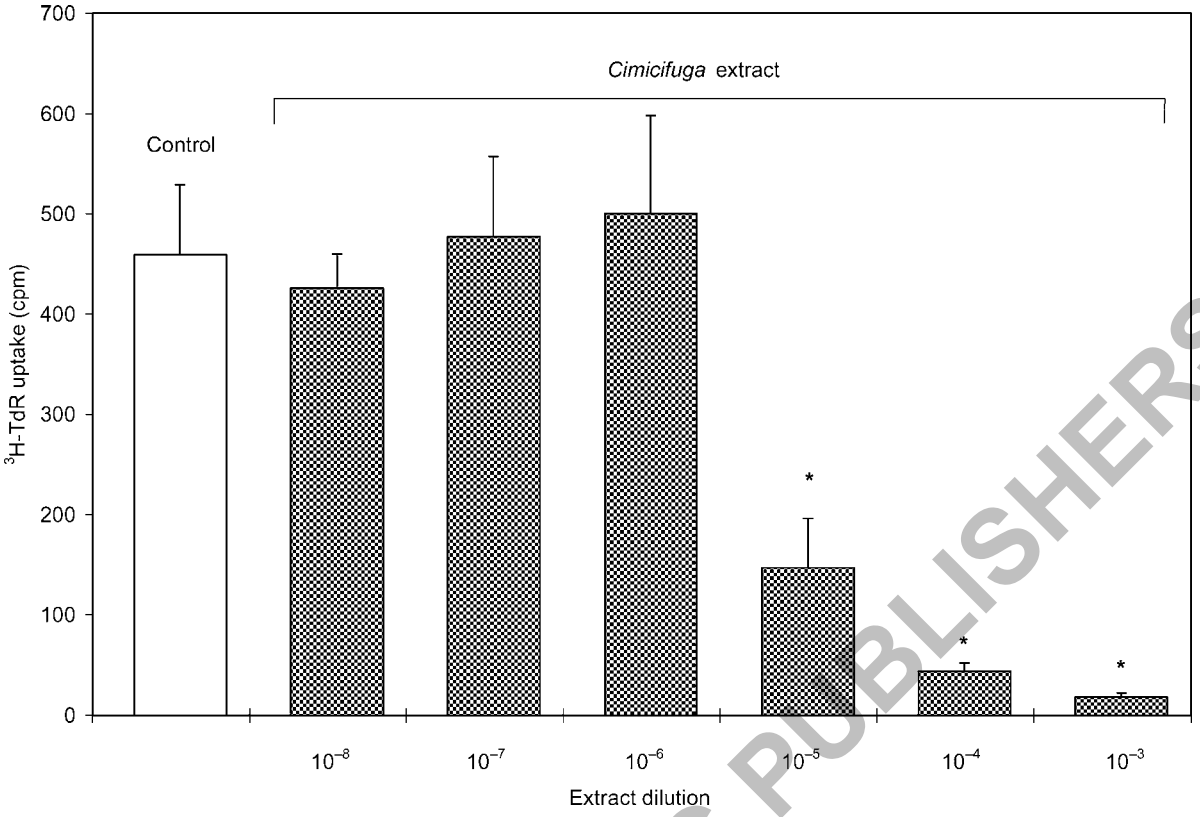

Figure 4. Influence of CR-extract on the proliferation of MCF-7 cells. Control: cell culture medium; all data are presented as mean ± SD for $n = 4$. Key: $^*p < 0.05$ versus control (Student's *t*-test).

induced proliferation of MCF-7 cells by 300–400% compared to control solvents (Figure 3). An assay evaluating the influence of non-steroidal, estrogen-antagonist tamoxifen (10^{-5} and 10^{-6} M; final concentration 4 and 0.4 μM, respectively) resulted in an inhibition of cell proliferation. At a concentration of 10^{-7} M, however, tamoxifen increased proliferation by 136%, as compared to the untreated controls, suggesting mixed agonist–antagonist activity (Figure 3). Co-incubating the cells with estradiol antagonized the anti-proliferative effect of tamoxifen, thus indicating an estrogen receptor mediated estrogen-antagonistic effect, rather than a cytotoxic effect (Figure 3). The results of these preliminary tests make it clear that the established system is suitable for the detection of both estrogen-agonistic and -antagonistic effects.

The CR-extract was tested in a dilution series of 10^{-3}–10^{-8} in the MCF-7 proliferation assay, corresponding to 100 μg/ml–1 ng/ml (final concentrations 40 μg/ml–0.4 ng/ml) native extract. The results are shown in Figure 4. CR-extract dilutions between 10^{-3} and 10^{-5} significantly inhibited the proliferation of MCF-7 cells. The incubation of MCF-7 cells with lower extract dosages neither increased nor decreased the proliferation rate, as compared with medium controls. The proliferation assay with the CR-extract at various concentrations was repeated using 14 different extract batches, and demonstrated similar findings, thus supporting that the CR-extract inhibits the rate of cell proliferation (Figure 5). The IC_{50} was calculated to be 0.3 μg/ml (final concentration).

We further investigated the effect of the CR-extract on an estrogen-induced increase in MCF-7 cell proliferation. CR-extract was applied in a dilution series from 10^{-3} to 10^{-8} with a constant 17β-estradiol (10^{-8} M) exposure. While an increase in the proliferation was achieved with 10^{-8} M estradiol without extract, the CR-extract inhibited the estradiol-induced effect at dilutions between 10^{-3} and 10^{-5}. Lower extract concentrations neither enhanced nor inhibited the estradiol-induced proliferation (Figure 6). An estradiol concentration of 10^{-7} M antagonized the

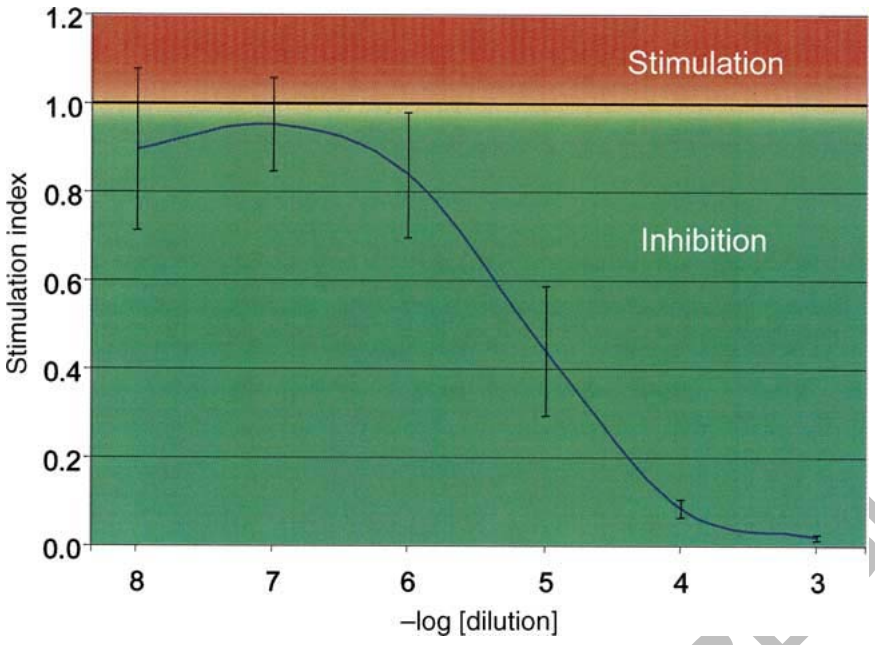

Figure 5. Influence of 14 different batches of CR-extract on the proliferation of MCF-7 cells. Stimulation index (SI) = cpm test/cpm medium control; all data are presented as mean ± SD for $n = 14$.

inhibitory effect of the CR-extract, while lowering the estradiol concentration in the system to 10^{-9} M resulted in a decrease in the rate of proliferation (Figure 7).

Similarly, we investigated the influence of the CR-extract on the tamoxifen-induced inhibition of the MCF-7 proliferation. As shown in Figure 8, the addition of CR-extract to a constant tamoxifen dose (10^{-6} M) enhanced the tamoxifen effect. While tamoxifen alone induced a decrease in the proliferation rate by 40%, the addition of the CR-extract in dilutions of 10^{-3} and 10^{-4} inhibited proliferation by 98 and 93%, respectively. Comparable results were also obtained after pre-incubation of the CR-extract with S9 Mix, a rat liver homogenate that mimics *in vivo* hepatic metabolism to assess whether CR-extract is converted by the liver to a metabolite with estrogenic activity (data not shown).

Discussion

While the efficacy of CR-extracts in the treatment of menopausal disorders has been noted in many clinical studies [3, 11], the mode of action and primary pharmacological effects of this herbal remedy are not yet fully understood. Based on its ability to modulate menopausal symptoms, similar to the effect of estrogen, questions have been raised as to whether CR-extracts similarly stimulate the proliferation of estrogen-dependent breast cancer cells, and should therefore be avoided in the treatment of patients with a history of estrogen receptor-positive mammary cancer.

Estrogen receptors (α and β) initiate both estrogenic and anti-estrogenic effects depending on specific target tissue [12]. Therefore the mere binding of a compound to an estrogen receptor, while essential for inducing an effect, does not allow for a determination of whether the compound's effects are agonistic or antagonistic. To clarify this question in light of the safety of CR-extract treatment in estrogen-sensitive menopausal women, our objective was to evaluate the influence of the CR-extract on the proliferation of estrogen receptor-positive breast cancer cells *in vitro*.

Some researchers have suggested that the positive effects of CR seen in clinical trials can be explained by an 'estrogen-like' activity; a claim that is supported by studies in ovariectomized rats in which LH levels decreased in response to parenteral administration of extracts [4, 13, 14]. Although it has been shown repeatedly that constituents of CR-extract bind to the estrogen receptor, recent investigations suggest that CR effects differ from a generalized 'estrogen-like'

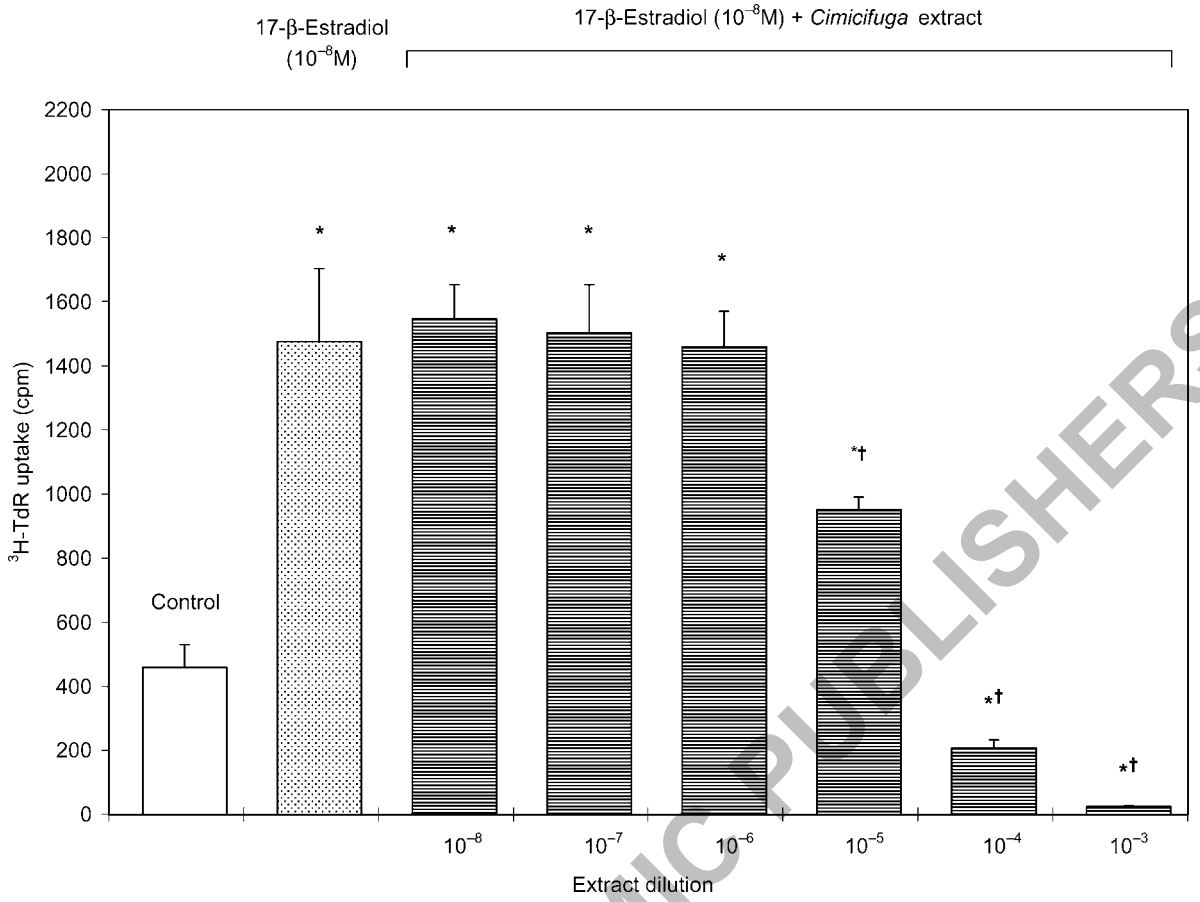

Figure 6. Influence of CR-extract on the estradiol-induced proliferation of MCF-7 cells. Control: cell culture medium; positive controls: estradiol 10^{-8} M; all data are presented as mean ± SD for $n = 4$. Key: $^{*}p < 0.05$ versus control, $^{\dagger}p < 0.05$ versus estradiol control (Student's *t*-test).

pathway [11, 15] or through a mechanism characteristic of classic isoflavonoid phytoestrogens [5, 6].

Prior to evaluating the effect of CR-extract on MCF-7 cell proliferation, we validated the sensitivity of our model to detect estrogenic and anti-estrogenic cellular effects. Our results show that estradiol (10^{-7}–10^{-9} M), as expected [8], induced cell proliferation while the estrogen receptor antagonist, tamoxifen (up to 10^{-6} M), caused a significant reduction of the proliferation rate. Dilutions of tamoxifen, $\leq 10^{-7}$ M, increased cell proliferation. This biphasic effect of tamoxifen has been observed in other studies [8].

CR-extract dilutions $\leq 10^{-3}$ did not exert cytotoxic activity in our assays. Under estrogen-deprived conditions, the CR-extract, even in very low concentrations, did not increase cell proliferation; but rather, significantly inhibited proliferation. Thus, it is evident from these data that the CR-extract does not have an estrogen-agonistic effect on breast cancer cells.

Other groups have similarly reported an absence of an estrogen-agonistic effect of CR on the proliferation of breast cancer cell *in vitro* using other cell lines [16–18]. Neßelhut et al. [16] evaluated an isopropanolic CR-extract on a mammary carcinoma cell line (MDA MB-435S) and found that the extract does not exhibit estrogenic activity. The validity of this test model was proven by a dose-dependent enhancement of proliferation by 17β-estradiol [16]. Zava et al. [17] investigated the influence of various plant extracts on the proliferation of estrogen- and progesterone receptor-positive T47-D cells. Under estrogen-free conditions, they found that a 50% ethanolic extract of CR (concentration of 0.2%, v/v) reduced T 47-D cell growth by 50%, whereas estradiol

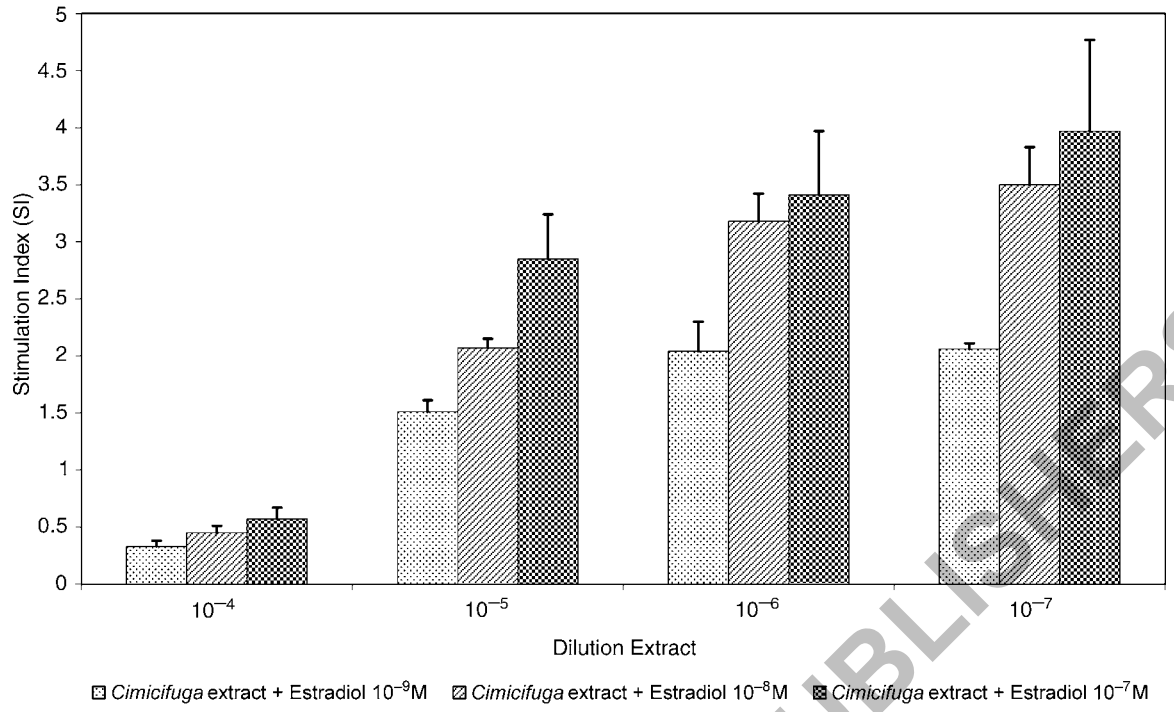

Figure 7. Influence of CR-extract on the estradiol-induced proliferation of MCF-7 cells at varying estradiol concentrations. Stimulation index (SI) = cpm test/cpm medium control; all data are presented as mean ± SD for $n = 4$.

(10^{-9} M) enhanced the proliferation rate by 500%. Similarly, Dixon-Shanies and Shaikh [18] demonstrated dose-dependent estrogen-antagonistic activity of several flavonoid phytoestrogens and plant extracts, including an ethanolic CR-extract (1–0.1%, v/v), on MCF-7 and T 47-D cells.

Contradictory to results suggesting an estrogen-antagonist effect, Kruse et al. [7] found that fukinolic acid, an isolated component from a 50% ethanolic CR-extract, induced a slight, but significant, increase in MCF-7 cell proliferation (126.3% ± 5.1). However, the cells used in this study had a very low response to estradiol, thus indicating a likely problem with the cells or culture conditions. It is known that culture medium containing estrogen may cause MCF-7 cells to lose their estrogen receptors, whereas estrogen-free culture medium may lead to an increased expression of estrogen receptors, therefore increasing their estrogen-responsiveness. In our investigation, we were able to demonstrate, through immunohistochemistry, that our MCF-7 cells expressed estrogen receptors (data not shown), thus indicating that the culture conditions of this study did not adversely affect the utility of the test system.

Differences in culture conditions have been proposed as a source of the variability of CR experimental results [8, 9, 18]. Discrepancies in data concerning the effect of CR-extract on cell proliferation may also be explained by varying methods for quantifying cell proliferation and various extraction techniques. This study is different from other similar studies in that our data, resulting from study of a large number of extract batches, are consistent, thus validating the range of manufacturing processes, from plant selection to preparation of the active ingredient, used to prepare our test CR-extract.

The vast majority of recent results from various laboratories suggest that CR-extract may act as a 'selective estrogen receptor modulator' (SERM) [11, 19, 20], an agent, like tamoxifen or raloxifene, that produces either an estrogen-agonistic or -antagonistic effect depending on the target tissue. For example, estrogen-agonistic effects of CR have been noted in studies of bone metabolism [21, 22], and it is suspected that CR-extracts exert estrogen-agonistic activity on vasomotoric centers of the brain, thus reducing the occurrence of hot flashes in menopausal women [23]. Alternatively, absence of estrogen-

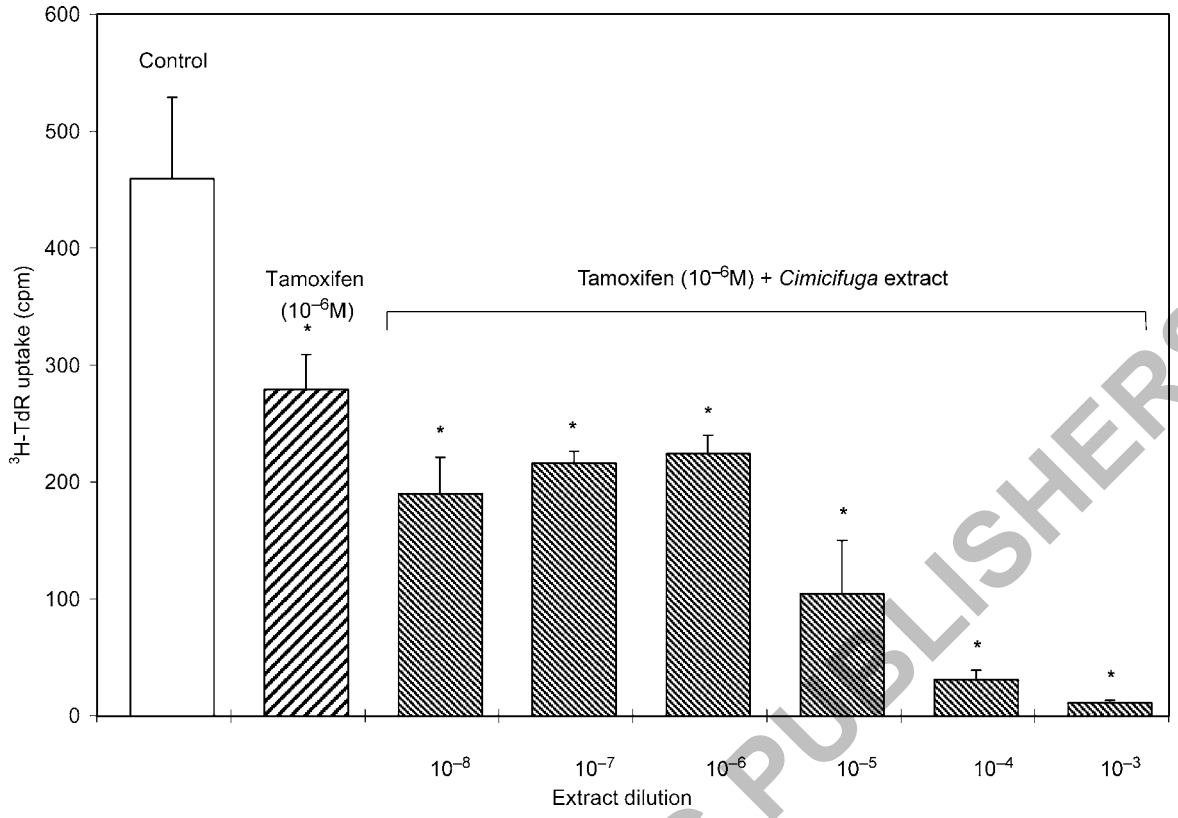

Figure 8. Influence of CR-extract on the tamoxifen-induced suppression of MCF-7 proliferation. Control: cell culture medium; positive control: tamoxifen 10^{-6} M; all data are presented as mean ± SD for $n = 4$. Key: $^*p < 0.05$ versus controls (Student's *t*-test).

agonistic effects has been noted in studies of the uterus [19, 20], and in studies of Sprague-Dawley-rats with induced dimethylbenz[a]anthracene-induced estrogen receptor-positive mammary tumors [24]. Similarly, our *in vitro* study results clearly indicate the absence of an estrogen-agonistic effect, and may point toward an estrogen-antagonistic effect, of CR-extract on estrogen receptor-positive breast cancer cells. In our study, estrogen-induced MCF-7 proliferation was reduced in the presence of the CR-extract. This anti-proliferative effect was reversible by increasing the concentration of estradiol. While this finding may be ascribed to an estrogen-receptor mediated effect, the data alone do not explain the strong anti-proliferative activity of CR-extract at high concentrations, as addition of estradiol (10^{-7}–10^{-9} M) did not restore cell proliferation to 100% of the control level. As such, it is likely that other estrogen receptor-independent signal transduction mechanisms, perhaps those that induce cell cycle arrest, play a role in the noted anti-proliferative effects.

The phyto-SERM theory, which suggests that a substance could have an estrogen-agonistic as well as an estrogen-antagonistic effect, may explain the contradictory results concerning the pharmacological activity of CR. Although the mechanism of action of SERMs is not completely understood, it appears that these agents induce conformational changes within the estrogen receptor that differs from those induced by estrogen [25]. To determine the mechanism of action of CR in menopausal symptoms itself, further research is needed to evaluate whether other signal transduction processes, such as inhibition of tyrosine protein kinases, DNA-topoisomerase or ribosomal S6 kinase, or induction of p21(WAF1/CIP1) expression [26–28], contribute to the overall activity of CR-extracts. Nevertheless, results from our study support other data suggesting that CR does not exert an estrogenic effect on either breast or uterine tissue, and therefore may be a safe alternative to HRT in menopausal women with estrogen-sensitive disorders.

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