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Greek Plant Extracts Exhibit Selective Estrogen Receptor Modulator (SERM)-like Properties

EVA KASSI,[†] ZOI PAPOUTSI,[†] NIKOLAOS FOKIALAKIS,[‡] IOANNA MESSARI,[§] SOPHIA MITAKOU,[‡] AND PARASKEVI MOUTSATSOU*,[†]

Department of Biological Chemistry, Medical School, University of Athens, 115 27 Athens, Greece, Department of Pharmacognosy, Department of Pharmacy, University of Athens, 115 27 Athens, Greece, and Department of Experimental Pharmacology, Medical School, University of Athens, 115 27 Athens, Greece

To prevent bone loss that occurs with increasing age, nutritional and pharmacological factors are needed. Traditional therapeutic agents (selective estrogen receptor modulators or SERMs, biphosphonates, calcitonin) may have serious side effects or contraindications. In an attempt to find food components potentially acting as SERMs, we submitted four plant aqueous extracts derived from Greek flora (*Sideritis euboea, Sideritis clandestina, Marticaria chamomilla, and Pimpinella anisum*) in a series of in vitro biological assays reflective of SERM profile. We examined their ability (a) to stimulate the differentiation and mineralization of osteoblastic cell culture by histochemical staining for alkaline phosphatase and Alizarin Red-S staining, (b) to induce, like antiestrogens, the insulin growth factor binding protein 3 (IGFBP3) in MCF-7 breast cancer cells, and (c) to proliferate cervical adenocarcinoma (HeLa) cells by use of MTT assay. Our data reveal that all the plant extracts studied at a concentration range 10−100 μg/mL stimulate osteoblastic cell differentiation and exhibit antiestrogenic effect on breast cancer cells without proliferative effects on cervical adenocarcinoma cells. The presence of estradiol inhibited the antiestrogenic effect induced by the extracts on MCF-7 cells, suggesting an estrogen receptor-related mechanism. In conclusion, the aqueous extracts derived from *Sideritis euboea, Sideritis clandestina, Marticaria chamomilla*, and *Pimpinella anisum* may form the basis to design “functional foods” for the prevention of osteoporosis.

KEYWORDS: Sideritis; Anisum; Chamomilla; selective estrogen receptor modulators; plant extracts

INTRODUCTION

There is a continuously increasing interest in assessing the role of nutritional constituents, present in small quantities in food, to prevent the risk of chronic disease (*1*, *2*). In the food industry, there is a general policy to encourage food designers to produce health-promoting foods known as functional foods (*3*−*5*). Many bioactive compounds have been discovered in food, among them phenolic compounds, that is, flavonoids, phenolic acids, and phytoestrogens, which show antioxidant properties and may act as estrogen agonists/antagonists with beneficial health effects, for example, reducing the risk of cancer, osteoporosis, and cardiovascular disease (*6*). There are numerous reports regarding the effects of individual nutritional constituents present in plant-derived food; however, a few reports only exist on the effects of extracts of wild plants on human health. The Greek flora is very rich in aromatic plants, among which are the mountain tea (*Sideritis sp*), the *Pimpinella anisum*, and *Marticaria sp.* These plants contain essential oils and secondary metabolites, as polyphenols, flavonoids, terpenoids, and phytoestrogens with particular pharmacological and nutritional interest (*7*−*16*). Studies concerning the main constituents present in the above plants reveal that apigenin, an important constituent, is a flavonoid with a potent antioxidant activity and antiinvasive and apoptotic properties, playing a significant role in osteoporosis (*17*−*19*). Moreover, the bioactive compound verbascoside is a phenolic compound known for its antiproliferative, cytotoxic, antioxidant, and antimetastatic properties (*20*). Terpenoids, on the other hand, are substances known for their antiinflammatory and antiproliferative properties (*21*). Herbals have played an important role in Greek nutrition since the ancient times, whereas herbal extracts have been used as traditional medicines for a thousand years in Greece and the Mediterranean area. However, there are only a few data on the bioactivity of the Greek plant extracts. Existing studies concern mainly their antimicrobial and antioxidant activity, as well as their anti-*Helicobacter pylori* activity (*7*, *8*, *22*, *23*).

* Corresponding author. Mailing address: Department of Biological Chemistry, Medical School, National University of Athens, 75 M.Asias str., Goudi, 115 27 Athens, Greece. Tel +30-210-7462682, fax +30-210-7462703/+30-210-6563773, e-mail pmoutsatsou@med.uoa.gr/ladikos_d_v@ath.forthnet.gr.
[†] Department of Biological Chemistry, Medical School.
[‡] Department of Pharmacognosy, Department of Pharmacy.
[§] Department of Experimental Pharmacology, Medical School.

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In an attempt to find nutrients in plant extracts derived from Greek flora potentially acting as SERMs, that is, to prevent bone loss without serious side adverse effects on breast cancer and uterus, we submitted four plant extracts (*Sideritis sp., Chamomilla sp., Pimpinella anisum*) to a series of in vitro biological assays reflective of SERM profile and examined their ability (a) to stimulate the differentiation and mineralization of osteoblastic cell culture by histochemical staining for Alizarin Red-S (AR−S), (b) to induce, like antiestrogens, the insulin growth factor binding protein-3 (IGFBP3) in MCF-7 breast cancer cells, and (c) to inhibit cell proliferation of cervical adenocarcinoma (HeLa) cells by use of MTT assay.

MATERIALS AND METHODS

Plant Material. All plant materials were collected between May and July of 2002 from Greek mountains. The voucher specimens were deposited in the Department of Pharmacognosy and Natural Products Chemistry, School of Pharmacy, University of Athens (*S. clandestina subsp. clandestina* KL034, *S. euboea* KL009, *M. recutita* F007, *P. anisum* F023). The flowers of *Sideritis euboea,* were collected at mountain Dirfis in central Evia. The flowers of *Sideritis clandestina subsp. clandestina* were collected from mountain Parnonas in east Peloponnese. The flowers of *Matricaria recutita (Chamomilla recutita or Marticaria chamomilla)* and the seeds of *Pimpinella anisun* were collected at east Peloponnese.

Aqueous Extracts. For the preparation of the extracts the collected plant materials were air-dried at room temperature, pulverized, and extracted with distilled water for 5 min at 100 °C. Then they were filtrated, concentrated at 50 °C under reduced pressure using a rotavapor, and lyophilized. Those materials were kept at −15 °C until they were used in experiments. Yield of final extract in terms of starting crude materials was determined to be 10% for all aqueous extracts.

Bone Study. *Cell Culture.* The KS483 cell line is a nontransformed stable subclone of a parental cell line KS4 that has the ability to form mineralized nodules in vitro. The KS483 cells were grown in phenol-red-free α-minimum essential medium (α-MEM, Gibco-BRL) supplemented with 10% fetal bovine serum (Gibco-BRL) and penicillin/streptomycin (Gibco-BRL) in a CO_2 incubator (5% CO_2−95% air) at 37 °C and subcultured every 3−4 days at a dilution 1:5 to 1:6 using trypsin 0.125%/EDTA 0.01% solution.

For our experiments, the KS483 cells were seeded in 12-well plates (at a density of 45 000 cells per well) in α-MEM (Gibco BRL Life Technologies) supplemented with 10% FBS, DCC-treated. For the experiments, cells were seeded in 12-well plates at a density of 15 000/cm^2 and cultured in α-phenol-red-free MEM supplemented with 10% FBS, dextran-coated charcoal slurry-treated (DCC-treated). Three days after plating, cells reached confluence and were subsequently induced to differentiate by the addition to the culture medium 50 μg/mL ascorbic acid (Sigma) and in the absence or presence of plant extracts at four different concentrations (100, 50, 25, and 10 μg/mL). 17b-Estradiol was also used as positive control at four different concentrations (10^{-6}−10^{-9} M). B-Glycerophosphate (Sigma) was added after day 10. The medium with the reagents was refreshed every 3−4 days for 24 days in total.

Alkaline Phosphatase Activity. At the end of the culture period, the cell layer was washed with PBS and then frozen (−20 °C). For the determination of alkaline phosphatase activity, cells were sonicated for 15 s in 0.1 M Tris buffer, pH 7.2, containing 0.1% Triton X-100. Alkaline phosphatase activity was measured by using *p*-nitrophenol phosphate as substrate (kinetic alkaline phosphatase/German Society of Clinical Chemists (ALP/DGKC) method). Total protein was also determined by Bradford method.

Assay for Mineralization. After 24 days, the cultures in 12-well plates were rinsed with PBS, followed by fixation with 5% formalin for 10 min, and stained for calcium deposition with Alizarin Red-S (solution 2%, pH = 5.5) (Sigma) for 5 min. Mineralized nodules were counted by light microscopy at a 10-fold magnification.

Breast Cancer Study. *Cell Culture Stimulation−IGFBP3 Assay.* Cultures of MCF-7 cells (ATCC Cell Bank) were grown in Dulbecco's minimal essential medium (DMEM) (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL), 50 units/mL penicillin, and 50 μg/mL streptomycin (Gibco BRL) in T-75 cm^2 flasks at 37 °C, 85% humidity, and 5% CO_2 atmosphere. Stock cultures were subcultured every 4−5 days using a trypsin 0.25% and EDTA 0.02% solution (Gibco BRL). Cells were plated at an initial density of 250 000 cells/well in 24-place multiwell plastic culture dishes; cells were then grown in the same medium as above until confluence (3 days approximately). After 3 days of cell culture, the media were changed to DMEM without phenol red supplemented with 2% FBS DCC (dextran-coated charcoal slurry)-treated and 50 units/mL penicillin and 50 μg/mL streptomycin (total volume 1 mL).

After 24 h of cell culture, estradiol (Sigma), ICI 182780 (Tocris), or plant extracts were added, and incubation followed. A time-dependent and dose-dependent course was carried out. Each time, the culture fluids were collected from the dish and centrifuged at 12 000 rpm for 10 min. The supernatants were used for IGFBP3 measurement. The cells were removed carefully from the dish using cell-scrapers, collected in approximately 200 μL of PBS buffer, and counted using a hemocytometer plate.

Time-Dependent Course. IGFBP3 was measured at zero time (before the addition of estradiol or ICI182780), as well as after 6, 24, and 48 h of incubation with estradiol (10^{-10} M) or ICI182780 (10^{-8} M).

Dose-Dependent Course. Estradiol (10^{-7}−10^{-9} M), ICI 182780 (10^{-8}−10^{-9} M), or plant extracts (50, 25, and 10 μg/mL) were tested in MCF-7 cells. MCF-7 cells were also incubated with vehicle or plant extracts (10 μg/mL) in the presence of 10^{-8} M estradiol. ICI 182780 (10^{-8} M) was also incubated in the presence of 10^{-8} M estradiol. IGFBP3 was measured after 24 h of incubation.

IGFBP3 Measurement. IGFBP3 was measured in all samples using an enzymatically amplified "two-step" sandwich-type immunoassay (DSL Diagnostic System Laboratories, Inc). Briefly, standards and samples were incubated in microtitration wells coated with anti-IGFBP3 polyclonal antibody. After incubation and washing, the wells were treated with another anti-IGFBP3 polyclonal antibody labeled with the enzyme horseradish peroxidase (HRP). After the second incubation and washing step, the wells were incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution is then added, and the degree of enzymatic turnover of the substrate is determined by wavelength absorbance measurement at 620 nm.

Cervical Adenocarcinoma Study. *MTT Assay or [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide] Method.* Cultures of Hela cells (ATCC Cell Bank) were grown in Dulbecco's minimal essential medium (DMEM) (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), 50 units/mL penicillin, and 50 μg/mL streptomycin (Gibco BRL) in T-75 cm^2 flasks at 37 °C, 85% humidity, and 5% CO_2 atmosphere. Subcultures were carried out every 3−4 days using a trypsin 0.25% and EDTA 0.02% solution (Gibco BRL). Cell viability was estimated by a modification of the MTT assay (*24*). Briefly, cells were plated in their growth medium at density of 10 000 cells/well in 96 flat-bottomed well plate. Twenty-four hours after plating, test substance was added at concentrations ranging from 200 to 10 μg/mL in DMEM phenol-red-free. After 48 h incubation, the medium was replaced with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) dissolved at a final concentration of 1 mg/mL in serum-free, phenol-red-free medium for a further 4 h incubation. Then, the MTT−formazan was solubilized in 2-propanol, and the optical density was measured at a wavelength of 550 nm and a reference wavelength of 690 nm.

Statistical Analysis. All values are expressed as the mean ± standard deviation of four measurements. Statistical analysis was performed using analysis of variance, one-way and post-Hoc Scheffe. A $p < 0.05$ value was considered statistically significant.

RESULTS

Bone Study. Figure 1 demonstrates the effect of vehicle control (in the absence of compounds), estradiol, and plant extracts from *Sideritis euboea*, *Sideritis clandestina*, *Marticaria chamomilla*, and *Pimpinella anisum* on the alkaline phosphatase activity. Plant extracts from *S. euboea* increased significantly

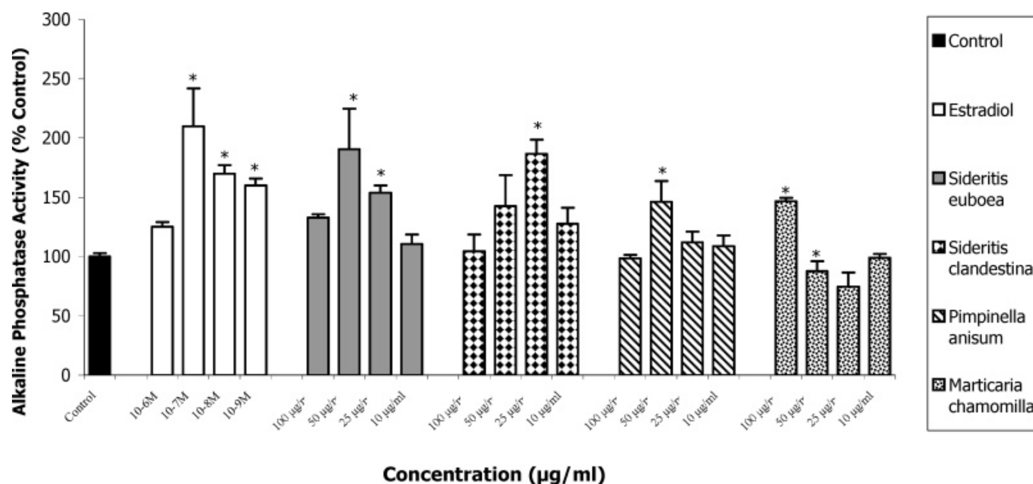

Figure 1. Effect of estradiol and plant extracts on alkaline phosphatase activity (ALP) in KS483 cells. Cells were treated with vehicle control, estradiol (10^{-6}–10^{-9} M), or plant extracts (100–10 μg/mL) for 24 days, and alkaline phosphatase activity was assessed. Results are expressed as percentage of control (vehicle). The data are expressed as mean ± standard deviation of four determinations. Asterisk denotes $p < 0.05$ compared to control.

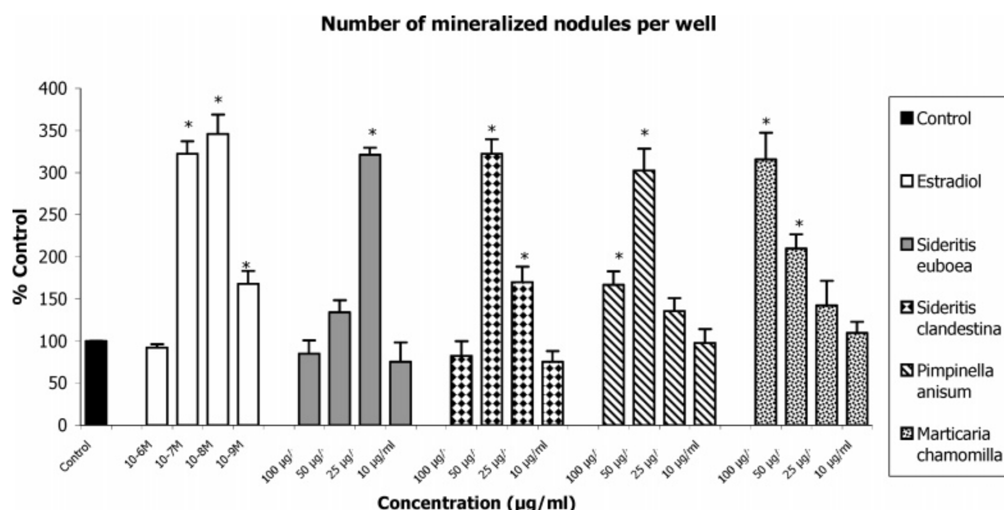

Figure 2. Effect of estradiol and plant extracts on the mineralization of extracellular matrix of KS483. Cells were treated with vehicle, estradiol (10^{-6}–10^{-9} M), or plant extracts (100–10 μg/mL) for 24 days. Mineralization was determined by AR-S staining. Results are expressed as percentage of control (vehicle). The data are expressed as mean ± standard deviation of four determinations. Asterisk denotes $p < 0.05$ compared to control.

($p < 0.05$) alkaline phosphatase activity at the concentration range 50−25 μg/mL with the higher effect at a concentration of 50 μg/mL, whereas plant extracts from *S. clandestina* and *P. anisum* caused a significant ($p < 0.05$) increase in alkaline phosphatase activity at concentrations 25 and 50 μg/mL respectively. Plant extracts from *M. chamomilla* increased signifficantly ($p < 0.05$) alkaline phosphatase activity at a concentration range 100−50 μg/mL with the higher effect at a concentration of 100 μg/mL.

Figure 2 shows the effect of vehicle control (in the absence of compounds), estradiol, and plants extracts from *S. euboea*, *S. clandestina, M. chamomilla*, and *P. anisum* on the mineralized nodules formation. Treatment with *S. euboea* at a concentration of 25 μg/mL stimulated the mineralization to 321% ($p < 0.05$) of control. Treatment with *S. clandestina* stimulated significantly ($p < 0.05$) the formation of mineralized nodules at a concentration range 50−25 μg/mL with the higher effect (322% of control) at a concentration of 50 μg/mL, whereas *M. chamomilla* and *P. anisum* increased significantly ($p < 0.05$) the formation of mineralized nodules at a concentration range of 100−50 μg/mL. *M. chamomilla* exerted its higher effect at a concentration of 100 μg/mL (315% of control) and *P. anisum* at a concentration of 50 μg/mL (302% of control).

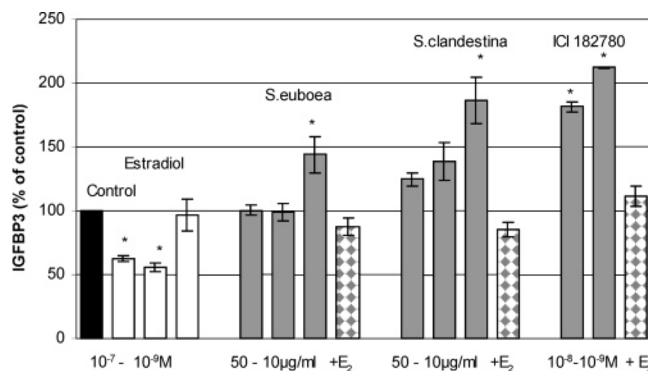

Figure 3. Effect of estradiol, *Sideritis euboea, Sideritis clandestina,* and ICI 182780 on $IGFBP_3$ levels in MCF-7 cells. MCF-7 were cultured with vehicle control, estradiol (10^{-7}–10^{-9} M), plant extracts (50, 25, 10 μg/mL), or ICI 182780 (10^{-8}–10^{-9} M) for 24 h. Cells were also treated with plant extracts (10 μg/mL) or ICI 182780 (10^{-8} M) in the presence of estradiol (E_2) (10^{-8} M). Results are expressed as percentage of control (vehicle). Columns and bars represent mean ± SD of the results of four cultures. Asterisk denotes $p < 0.05$.

Breast Cancer Study. Figure 3 demonstrates the effect of vehicle control (in the absence of compounds), estradiol,

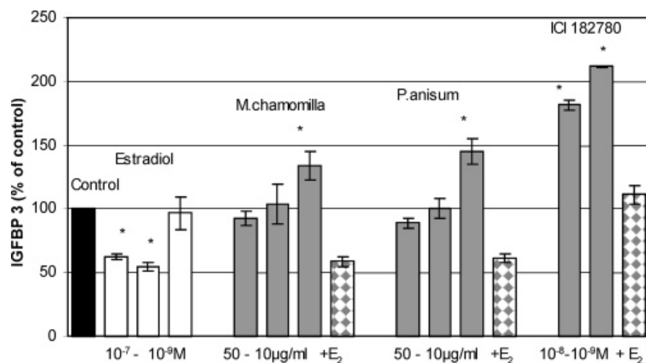

Figure 4. Effect of estradiol, *Marticaria chamomilla*, *Pimpinella anisum*, and ICI 182780 on $IGFBP_3$ levles in MCF-7 cells. MCF-7 were cultured with vehicle control, estradiol (10^{-7}–10^{-9} M), plant extracts (50, 25, 10 μg/mL), or ICI 182780 (10^{-8}–10^{-9} M) for 24 h. Cells were also treated with plant extracts (10 μg/mL) or ICI 182780 (10^{-8} M) in the presence of estradiol (E_2) (10^{-8} M). Results are expressed as percentage of control (vehicle). Columns and bars represent mean ± SD of the results of four cultures. Asterisk denotes $p < 0.05$.

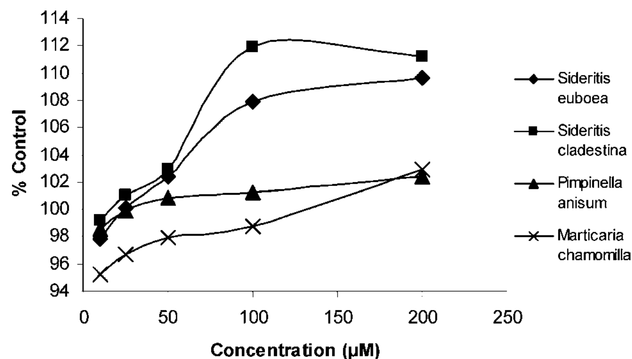

Figure 5. Effect of plant extracts on cell viability. HeLa cells were incubated with the indicated concentrations of plant extracts (200–10 μg/mL) for 48 h. Cell viability was determined by the MTT assay. Each point of the dose response curve is the average of four experiments. Standard deviation was less than 4% of the average values.

faslodex (ICI 182780), and plant extracts from *S. euboea* and *S. clandestina* on the IGFBP3 levels secreted in MCF-7 cells. The dose response curve showed that estradiol reduced IGFBP3 levels significantly ($p < 0.05$) at a concentration range $10^{-7}-10^{-8}$ M. On the other hand, the pure antiestrogen ICI 182780 increased significantly ($p < 0.05$) the IGFBP3 levels at a concentration range $10^{-8}-10^{-9}$ M, whereas this effect was abolished when co-incubation with estradiol 10^{-8} M occurred. Plant extracts from *S. euboea* and *S. clandestina* increased significantly ($p < 0.05$) the IGFBP3 levels in MCF-7 cells at a concentration of 10 μg/mL, and co-incubation with estradiol 10^{-8} M abolished the stimulatory effect of substances on IGFBP3 levels implicating an estrogen receptor (ER)-mediated effect. *S. euboea* and *S. clandestina* seem to have an antiestrogenic effect on MCF-7 cells.

Figure 4 shows the effect of vehicle control (in the absence of compounds), estradiol, faslodex (ICI 182780), and plant extracts from *M. chamomilla* and *P. anisum* on the IGFBP3 levels secreted in MCF-7 cells. *M. chamomilla* and *P. anisum* increased significantly ($p < 0.05$) the IGFBP3 levels in MCF-7 cells at a concentration 10 μg/mL, whereas coincubation with estradiol 10^{-8} M abolished their stimulatory effect on IGFBP3 levels, implicating an antiestrogenic effect in MCF-7 cells.

Cervical Adenocarcinoma Study. The dose response curve concerning the cytotoxicity of plant extracts on HeLa cells revealed that at low concentrations there was no stimulation above control and no inhibitory effect on cell proliferation. However, *S. cladestina* showed a proliferative effect, statistically significant ($p < 0.05$), at high concentrations (200 and 100 μg/mL) and *S. euboea* at concentration 200 μg/mL (**Figure 5**).

DISCUSSION

Greek flora is very rich in aromatic plants, such as *Sideritis euboea*, *Sideritis clandestina*, *Marticaria chamomilla,* and *Pimpinella anisum*. The water extracts of the above plants are very rich in constituents such as flavonoids, phenolic compounds, and phytoestrogens. Many studies in vivo and in vitro concerning their individual constituents support that they show beneficial effects on humans' health, because they are potent antioxidants, demonstrate antimetastastic and antitumor activity, and prevent bone loss (*17*−*20*). Despite the plethora of data regarding the properties of the individual plant constituents, very little is known about the effects of their aqueous extracts on humans' health. The main current concerns of the food industry, however, are the properties of the plant extracts as a whole since these may form the basis for the development of "functional foods".

The objective of our study was to determine the healthy effects of Greek plant aqueous extracts and in particular their SERM-like effects on bone, breast, and uterine cells. Phytochemicals may be a natural alternative to conventional hormone replacement therapy for the prevention of bone loss during menopause; therefore the discovery of such compounds is highly desirable. Thus, in the present study, we investigated the ability of the aqueous extracts derived from *Sideritis euboea*, *Sideritis clandestina*, *Marticaria chamomilla,* and *Pimpinella anisum* to induce osteoblast differentiation, as well as their anticancer effects on breast cancer and uterine cancer cells. Our study is the first to demonstrate that Greek plant extracts stimulate osteoblastic cells and suppress breast cancer cells without exhibiting proliferative effects on uterus and therefore are potential SERM-like candidates.

We observed the biological effects on osteoblasts and MCF-7 cells at concentrations of 10−100 μg/mL, which are well within the reported in vitro concentration range of other plant extracts (*25*, *26*). To assess the effects of extracts on bone cells, we used the KS483 cell line cloned from mouse calvaria, which is a nontransformed stable subclone of a parental cell line KS483 and undergoes in vitro osteoblast differentiation in association with formation of a bone-like mineralized extracellular matrix (ECM) (*27*). Alkaline phosphatase activity measurement and Alizarin Red-S staining are indicative of the differentiation and mineralization process, respectively (*28*, *29*). Our study therefore, based on reliable biological tests and biochemical markers reflective of osteoblastic activity, reveals that the aqueous extracts of *Sideritis euboea*, *Sideritis clandestina*, *Marticaria chamomilla*, and *Pimpinella anisum* stimulated differentiation and mineralization significantly at a concentration range 50−25 μg/mL.

The use of the IGFBP3 in MCF-7 cells is a well-established biological response to estradiol and antiestrogens such as ICI 182780 and is a useful marker for screening compounds that may function as estrogen agonists/antagonists and cell growth promoters (*30*, *31*). We treated cells with either the studied plant extracts or ICI 182780 in the absence and in the presence of estradiol to assess the extent to which the plant extracts inhibit the lowering effect (estrogenic) of estradiol on IGFBP3 levels. The stimulatory effect of the studied extracts on IGFBP3 levels support that they act as estrogen antagonists and implicate an

ER-mediated action. Moreover, the presence of estradiol (a known inhibitor of IGFBP3 levels) inhibited the stimulatory action of plant extract in a similar way to that observed when the antiestrogen ICI 182780 was coincubated with estradiol, speaking also for an ER-mediated activity. The antiestrogenic effect of plant extracts on IGFBP3 levels was observed at a concentration of 10 μg/mL, whereas at higher concentrations (25−50 μg/mL), there was no effect. This is in agreement with previous reports, which demonstrate that plant extracts may exhibit differential activities over a physiological concentration range (being agonist or antagonist) suggesting an estrogen-receptor-mediated effect at low concentration and an ER-independent effect at higher concentration (*32*).

In human cells, in addition to high-affinity, low-capacity ERs, there are also type II estrogen binding sites (low-affinity, high-capacity), which have been shown to regulate significantly normal and malignant uterine growth via their binding to endogenous estrogens, environmental estrogens, and particularly flavonoids (*33*−*36*). Due to the importance of type II binding sites (type II EBS) in regulating uterus function (an ER-independent growth effect), we were interested in our study whether the EBS type II is a possible mediator of the activity of the plant extract; therefore we used HeLa cells (ER-negative) in our experiments. The absence of proliferative effects of the extracts on HeLa cells implicates that their action is neutral on these cells, at least via an EBS type II dependent mechanism.

In our experiments, we cannot attribute the biological effects observed to particular constituents, because many other compounds are present in the plant extracts. Our study investigates the effect of the plant aqueous extracts as a whole on bone metabolism, as well as on breast and uterine tumor cells. Some extracts derived by other plants, such as licorice root extract, soy extract, Chinese herbal extracts, and white and red wine extracts, demonstrate estrogen-like effects on osteoblasts and estrogenic or anticancer effects on breast cancer cells, findings that are in agreement with our results (*25*, *26*, *37*−*41*). Moreover, our findings provide additional support to clinical trials that tea extracts inhibit osteoporosis (*42*). Current data support that some extracts may show greater effect than the phytoestrogen genistein alone, implicating that combinations of constituents present in plant extracts may be highly important in the final biological activity. In addition, different fractions of the herbs (aqueous or organic) contain compounds of different chemistry and therefore may exhibit different types of activities.

We certainly wish to characterize the active compounds in the Greek plant extracts studied. We are currently undertaking extensive experiments necessary for the identification of compounds and their active effects on bone and breast cancer cells, as well as experiments to delineate their mechanism of action, that is, via the estrogen receptor signaling system or via other ER-independent pathways. An important goal in our future studies will be to look for the possibility of synergistic combinations, which may actually exist naturally in plants, and extend this to clinical practice by use of combinations of cancer chemopreventive drugs, as well as combinations of drugs with SERM-like effects. Furthermore, in vivo trials are warranted for further elucidation of the multiple effects of the tested extracts on other systems such as cardiovascular, immune, and nervous system.

ABBREVIATIONS USED

SERM, selective estrogen receptor modulator; IGFBP3, insulin growth factor binding protein 3; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; α-MEM, α-minimum essential medium; FBS, fetal bovine serum; DCC, dextran-coated charcoal; ALP, alkaline phopshatase; PBS, phosphate-buffered saline; DMEM, Dulbecco's minimal essential medium; EDTA, ethylenediamine tetracetic acid; HRP, horseradish peroxidase; TMB, tetramethylbenzidine.

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