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Article

Estrogenic Activities of Ten Medicinal Herbs from the Middle East

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Traditional medicinal plants have long been recognized as remedies and important sources of treatment for developing countries. In the present study, we report on a detailed study to quantify the presence of five known phytoestrogens in 10 widely used herbs used in the Middle East. Surprisingly some of these plants were almost devoid of tested phytoestrogens, whereas others were very rich in known phytoestrogens. For example, *Hibiscus sabdariffa* was found to be the richest in quercetin and daidzein, whereas *Cyperus conglomeratus* had the highest concentrations of kaempferol and genistein. On the other hand, *Salvadora persica* was almost devoid of the screened phytoestrogens. Ethanolic extracts were further tested for their proliferative activities in cell-culture using estrogen-responsive breast cancer cell lines (MCF-7) and were found to fall into three distinct groups based on their estrogenic activities. The most potent herbal extract (*O. vulgare*) was further fractionated and the fractions were analyzed again for phytoestrogenic content (using high-performance liquid chromatography) and proliferative activity. Our results indicate that the proliferative activities of some of the extracts and fractions are not completely attributable to the phytoestrogens screened, thus it is likely that some of these plants may have other (perhaps yet unknown) phytoestrogens.

Introduction

It has been known for some time that certain natural compounds derived from plant sources, called phytoestrogens, have estrogen-like activities and may in fact be physiologically active. To date, more than 300 plants have been found to contain estrogenic compounds (1, 2). Not surprisingly, recent epidemiological studies have suggested a correlation between high dietary intake of these phytoestrogens and lower rates of certain cancer, cardiovascular diseases and post-menopausal symptoms (3–5). It has long been known that phytoestrogens can bind to intracellular estrogen receptors and compete with estradiol for binding (1). Although inconclusive, a growing body of literature suggests that phytoestrogens may have both estrogenic and anti-estrogenic activities. For example, it has been shown that phytoestrogens may be beneficial to the skeletal and cardiovascular systems (6). Furthermore, other studies have suggested that phytoestrogens may also be effective in preventing and treating prostate cancer (6, 7). However, many important questions still remain about the benefits of various phytoestrogens for human health (8–10). Not surprisingly, a great deal of research is underway in this exciting field and an increasing number of papers are published each year on this topic.

A recent report has highlighted the fact that rates of breast cancer is rising and that it is the most common form of cancer in the population of the United Arab Emirates (UAE) (11); moreover, that women from the Arab countries, including the UAE, tend to develop breast cancer at least a decade sooner than in Western countries (12). Even on a global scale, breast cancer is one of the most common forms of cancer (new cases) in women and a leading cause of death (in women), and accounted for approximately 500,000 deaths worldwide in 2008 (13). Despite many drugs and approaches available for the treatment and prevention of breast and other forms of cancer, there is clearly a need for new and novel classes of drugs. An increasing body of literature suggests that natural products, including phytoestrogens, may have important anticancer properties (14, 15), and hence there is strong interest in performing detailed analysis of phytoestrogenic contents of medicinal plants and herbs. Of the numerous medicinal herbs that have been historically and currently used for diverse therapeutic uses, only a few have been thoroughly characterized for their estrogenic content. In this report, we examined the estrogenic activity of 10 commonly used plants by measuring the concentrations of six well-known phytoestrogens, as well as by quantifying the proliferative activity of their ethanolic extracts.

Materials and Methods

Plants and extraction

Dried forms of the 10 chosen medicinal plants were purchased from the local market and confirmed by the UAEU Biology department. The plants chosen for this study were as follows:

Cyperus conglomeratus is a member of the Chenopdiaceae family and is known to exist in the eastern Arabian Desert. It is widely found in the UAE, especially the Al-Ain region (16). It is commonly known as Ramath in the Arabian culture and is highly aromatic with some medicinal uses.

Fagonia bruguieri and *Fagonia indica* belong to the Zygophyllaceae family and are compact perennial shrubs that are much branched from the base. They are common on low rocky bluffs along the Arabian Gulf coast, especially further inland in the Ras al Khaimah area. They are commonly used in the Arab culture and have been reported to have medicinal uses (17).

Salvadora Persica (SP) (Savadoraceae) is often called the arak tree or miswak. Its branches and roots are widely used as tooth cleaning sticks in the Middle East and some Asian and African cultures. It has been reported to have antimicrobial (18) and anti-ulcer (19) activities, in addition to numerous other beneficial effects.

Roselle (*Hibiscus sabdariffa*) is a species of hibiscus native to the Old World tropics. It is an annual or perennial herb or woody-based shrub, growing to 2–2.5 m tall. The leaves are deeply lobed, 8–15 cm long, arranged alternately on the stems. Various therapeutic activities attributed to roselle include antitumor, antihypertensive, antioxidant and antiammonemic activities (20).

German chamomile (*Matricaria recutita*) is the major type of chamomile used for health conditions (21). Although chamomile is widely used, there is not enough reliable research in humans to support its use for any condition. Chamomile has been used medicinally for thousands of years and is widely used in Europe and the Middle East, including UAE. It is a popular treatment for numerous ailments, including sleep disorders, anxiety, digestion/intestinal conditions, skin infections/inflammation (including eczema), wound healing, infantile colic, teething pains and diaper rash.

Fava bean (*Vicia faba*) is a species that belongs to the Fabaceae family. It is native to North Africa and Southeast Asia and is cultivated in many countries in the world. These beans are rich in flavonoids and have been shown to have numerous beneficial properties (22). The dried fruits of fava beans are widely used to prepare many important dishes all over the world, especially in most of the Arab countries.

Oregano (*Origanum vulgare*) is an aromatic herb native to the Mediterranean that belongs to the Lamiaceae family. The dried leaves are used as a seasoning in many dishes in Mediterranean countries. In Eastern Mediterranean countries, the ground leaves are also used with sumac (*Rhus coriaria*) and olive oil to prepare a dip mixture, usually eaten on breakfast with bread. Oregano has been shown to be rich in antioxidants and various phenolic compounds (23).

Anise (*Pimpinella anisum*) is a plant that belongs to the Apiaceae family and is native to the eastern Mediterranean and Southwest Asia region. Anise seeds are sweet and very aromatic, distinguished by their licorice-like flavor. Therefore, it is used as flavoring agents for many sweets, candies and pharmaceutical products. Anise boiled water extract is used in some Arab countries to make a special hot drink called Yansoon, which is usually given to nursing mothers and has been shown to be rich in antioxidants (24).

Zallouh (*Ferula hermonis*) is a plant that grows at a height of approximately 2,000 meters above sea level on the side of Mount Hermoun between Lebanon and Syria. Its dried roots have been traditionally used aphrodisiac agents. *Ferula hermonis* extract has been found to contain compounds such as sesquiterpenes, which show both proliferative and antiproliferative activities (25).

Herbal extracts were prepared using the published protocol of Franke *et al.* (26). Briefly, 7–10 g of finely ground powder of each selected herb was soaked in 150 mL of aqueous ethanol (70%) for 72 h at 4°C and then filtered using gravity filtration. The solvent was removed by rotary evaporation at 35°C under vacuum. The extract was then dissolved in 10 mL DMSO and stored at −80°C until further analysis. A known quantity of each extract (400 µL) was adjusted to 1.6 mL with methanol, filtered using Minisart filters (pore size 0.45 µm) and subjected to high-performance liquid chromatography (HPLC) analysis and cell culture assay.

Silica gel column fractionation

The dried ethanolic extract obtained of *O. vulgare* (dissolved in DMSO) was subjected to fractionation on silica gel. Approximately 97 g of activated silica gel was loaded to a column and cleaned with approximately 200 mL of hexane (HPLC grade). Approximately 3 mL of the extract was loaded onto the column and eluted with hexane–ethyl acetate (1:1 v/v). Three fractions were separated during silica gel fractionation, which were assigned as OV-1 (250 mL elution volume), OV-2 (350 mL elution volume) and OV-3 (600 mL elution volume) on the basis of their polarity (non-polar to polar). The purification of these components were monitored by thin-layer chromatography (TLC) using ethyl acetate and hexane (1:1) and subsequently dried by rotary evaporation at 40°C under vacuum, then dissolved again in DMSO. A known quantity of each fractionation (400 µL) was adjusted to 1.6 mL with methanol, filtered using Minisart filters (pore size 0.45 µm) and subjected to HPLC analysis and cell culture assay.

HPLC analysis

All injections were conducted in HPLC coupled with two detectors, dual channel diode array and fluorescence (Agilent Technologies, Santa Clara, CA). The column used in all chromatographic analyses was a Waters Symmetry C18, 5 µm column (250 × 4.6 mm i.d.) with a directly connected guard column. The flow rate was fixed at 0.8 mL/min with the following solvent systems: Solvent A, 10% acetic acid–water and solvent B, 100% acetonitrile. The gradient scheme was as follows: 100% solvent A (0% solvent B) for 5 min, 23% solvent B (77% solvent A) to 100% solvent B (0% solvent A) over 20 min, followed by 100% solvent B for five more minutes, and then to 0% solvent B (100% solvent A) for the next 5 min. For all analyses, 20 µL of the MeOH–DMSO (4:1) diluted samples were injected directly into the HPLC system. Both selected herb extracts and

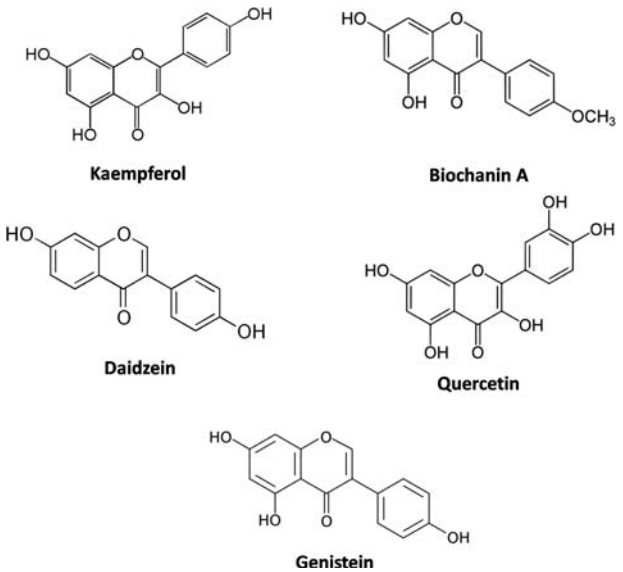

Figure 1. Names and chemical structures of the five phytoestrogens screened in the 10 medicinal plants.

fractions were concurrently screened with a dual channel diode array detector at 254, 266, 210 and 370 nm, and peaks were scanned between 200 and 800 nm.

Measurement of MCF-7 cell proliferation

The breast cancer cell line MCF-7 was propagated using standard cell-culture techniques using DMEM/HIGH glucose media (HyClone from Thermo) containing 10% fetal bovine serum (HyClone from Thermo), 100 μg/mL penicillin, 100 μg/mL streptomycin and 50 μg/mL gentamicin (HyClone from Thermo) at 37°C inside a humidified incubator with 5% CO_2 and 95% room air. Cells were subcultured every 3–4 days using Trypsin–EDTA as follows: the culture medium was removed and the residual serum was eliminated by rinsing monolayers with 10 mL of PBS. Then, 2 mL of 1*x* (0.05%) trypsin–EDTA (Invitrogen) solution was added to cover the cell monolayer and the cells were incubated at 37°C for 4 min. The cells were detached from the culture plate by gently pipetting with 8 mL of DMEM–10% FBS media and counted using a hemocytometer, after which the cells were plated again on new culture plates.

Cells were treated with several concentrations of the extracts for two days, after which their proliferation was quantified using the Cell Counting Kit (CCK-8 from Fluka/BioChemika). This kit is based on the reduction of the highly water-soluble tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4- nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], which produces a water-soluble formazan dye that is measured at 460 nm using a microplate reader.

Table I
Concentrations of Various Phytoestrogens in the Ethanolic Extracts of the 10 Medicinal Plants (Average of Triplicate Measurements)

Plants	Phytoestrogen concentration (μg/g of dried plant)				
	Daidzein Mean ± SD	Quercetin Mean ± SD	Genistein Mean ± SD	Kaempferol Mean ± SD	Biochanin A Mean ± SD
Pimpinella anisum	n.d.	5.20 ± 0.01	1.92 ± 0.01	107.7 ± 8.5	n.d.
Origanum vulgare	2.74 ± 0.02	25.1 ± 0.2	n.d.	177.7 ± 1.5	2.21 ± 0.03
Hibiscus sabdariffa	3.70 ± 0.02	49.3 ± 0.5	n.d.	13.5 ± 0.2	n.d.
Matricaria recutita	n.d.	15.3 ± 0.6	n.d.	122.3 ± 6.5	n.d.
Cyperus conglomeratus	n.d.	n.d.	5.2 ± 0.3	525.1 ± 11.7	n.d.
Vicia faba	0.23 ± 0.01	2.3 ± 0.4	n.d.	n.d.	0.14 ± 0.01
Salvadora persica	n.d.	0.49 ± 0.03	n.d.	n.d.	n.d.
Fagonia indica	n.d.	3.77 ± 0.04	n.d.	22.5 ± 0.2	n.d.
Fagonia bruguieri	1.67 ± 0.01	2.84 ± 0.2	1.01 ± 0.001	12.4 ± 0.4	n.d.
Ferula harmonis	0.29 ± 0.04	1.12 ± 0.01	n.d.	7.7 ± 0.3	0.08 ± 0.001

Results and Discussion

Phytoestrogenic content

There is a growing body of literature indicating that traditional and herbal plants commonly used in many communities may contain significant levels of phytoestrogens (1). Because these phytoestrogens may be physiologically relevant to humans, we wanted to quantify the actual amounts of five specific phytoestrogens in 10 traditional plants widely used in the UAE. The five specific phytoestrogens that we investigated were biochanin A, daidzein, genistein, kaempferol and quercetin (Figure 1 shows the chemical structures of these compounds). We employed the widely used HPLC–ultraviolet (UV) coupled

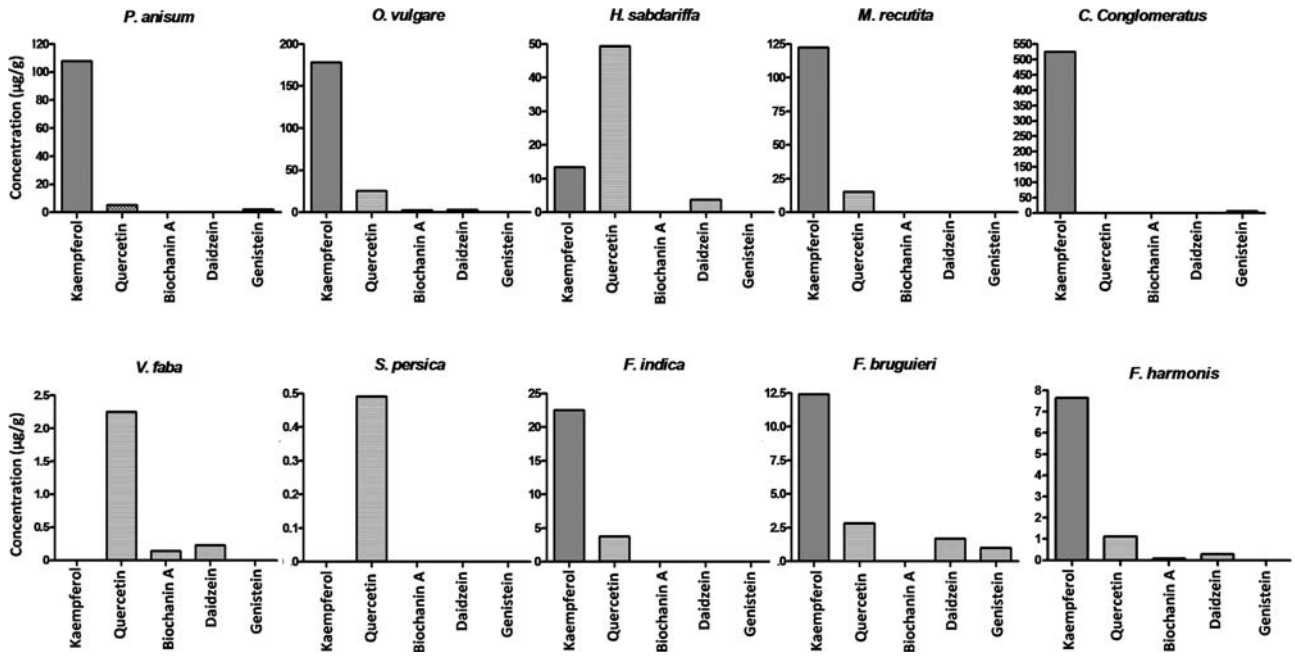

Figure 2. Concentrations of various phytoestrogens (μg/gram of dried plants) in the ethanolic extracts of the 10 medicinal plants. Extraction and quantitation details are explained under "Materials and Methods."

with diode array detector to establish a fast and reliable system to detect and quantify the various phytoestrogens. Using appropriate calibration curves (data not shown) and retention times, we were able to quantify the amounts of five isoflavone phytoestrogens in the 10 chosen plants. In our HPLC protocol, genistein, biochanin A and daidzein were found to give linear responses from 12.5 ng to 1.25 μg, while quercetin gave a linear response from 37.7 ng to 3.7 μg, and kaempferol was linear from 110 ng to 11 μg. Table I shows the summary of the phytoestrogen analysis; as shown, the plants varied greatly in their phytoestrogen content. For example, of the 10 plants analyzed, *Hibiscus sabdariffa* was found to be most rich in quercetin and daidzein, whereas *Cyperus conglomeratus* had the highest concentrations of kaempferol and genistein. On the other hand, *Salvadora persica* was devoid of any of the screened phytoestrogens, except for trace amounts of quercetin. Figure 2 shows the distribution of these phytoestrogens in each of the 10 plants and again, the differences in the phytoestrogen content of these medicinal plants is apparent. For example, of the five phytoestrogens examined, *C. conglomeratus* appeared to have only kaempferol; similarly, *S. persica* only appears to have quercetin. In contrast, plants like *O. vulgare*, *F. bruguieri* and *F. harmonis* contained four of the five tested phytoestrogens. Similarly, *V. faba*, *H. sabdariffa*, and *P. anisum* had at least three of the five screened phytoestrogens (Table I).

To confirm that our quantitation of these phytoestrogens was accurate and not due to artifacts, we implemented two additional layers of scrutiny in our analyses. First, we performed spiking studies of the plant extracts with the five known phytoestrogens. A representative result from this is shown in Figure 3, which shows that spiking *H. sabdariffa* extract spiked with the standard phytoestrogens resulted in the increase of only three of the existing peaks in the extract (as indicated by arrows), and hence, those three peaks were assumed to be the same as three of the phytoestrogens. The second approach to confirm the identity of the phytoestrogens in the extract was the full UV/Vis spectrum analyses from the HPLC diode array detector data. Figure 4 shows the full UV/Vis spectrum of the genistein peak in *P. anisum* HPLC trace and the full spectrum of the genistein standard peak (from a separate chromatogram). As clearly shown in this representative figure, both of the full UV/Vis spectra were identical, thus confirming that the peak at 15.4 min was indeed genistein.

To our knowledge, this is the first time such detailed and careful analysis of isoflavone class of phytoestrogens in the chosen 10 medicinal plants has been performed. Although the data are not very surprising, they clearly show that commonly used medicinal plants are quite rich in phytoestrogens (e.g., *C. conglomeratus* and *O. vulgare*) and should be used with caution. Furthermore, the analysis also underscores the importance of careful screening of the phytoestrogenic contents of popular medicinal plants.

MCF-7 proliferation activity

We were interested in also testing whether these medicinal plant extracts were physiologically active, and hence, the well-established estrogen-responsive breast cancer cell line MCF-7 was used to determined whether the various plant extracts had

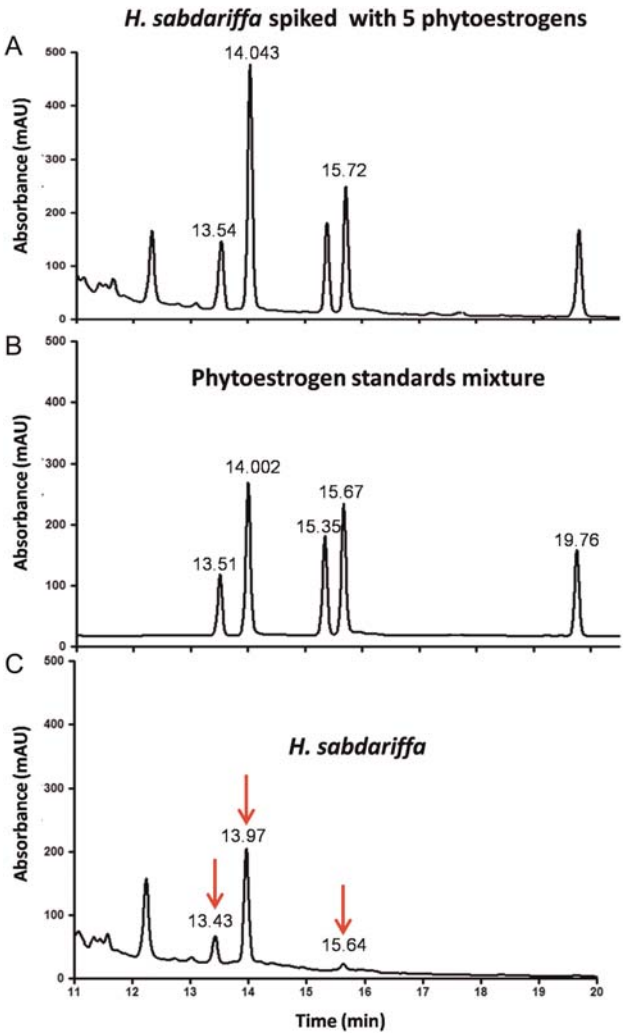

Figure 3. Confirmation of the presence of phytoestrogens in medicinal plants using standard spiking. A typical and representative figure shows: *H. sabdariffa* extracts (A); standard phytoestrogens (B); spiked extract (C).

proliferative effects. Figure 5 shows the effect of increasing concentration of *P. anisum* extract on the proliferation of MCF-7 cells. As shown in the figure and as expected, incubation of 17-beta Estradiol (E_2) caused a very potent increase in the proliferation of these cells, compared to control (DMEM media alone). Incubation of 5 μg/μL *P. anisum* extract caused a significant increase in the proliferation rate, which increased steadily as the concentration of the extract increased to 40 μg/μL, eventually reaching almost the same level as that observed with E_2 (Figure 5). These results confirm that *P. anisum* extract contains physiologically active phytoestrogenic compounds, some of which could be responsible for the proliferative effect observed on MCF-7 cells. Similar results were obtained when the other plant extracts were similarly tested for their proliferative activities (Figure 6). As shown in Figure 6, the 10 plants could be divided into three groups based on their proliferative potency. The first group (Figure 6A) represented the most active and proliferative extracts and included *C. conglomerates*, *F. bruguieri*, *O. vulgare* and *M. recutita*. The second group (Figure 6B)

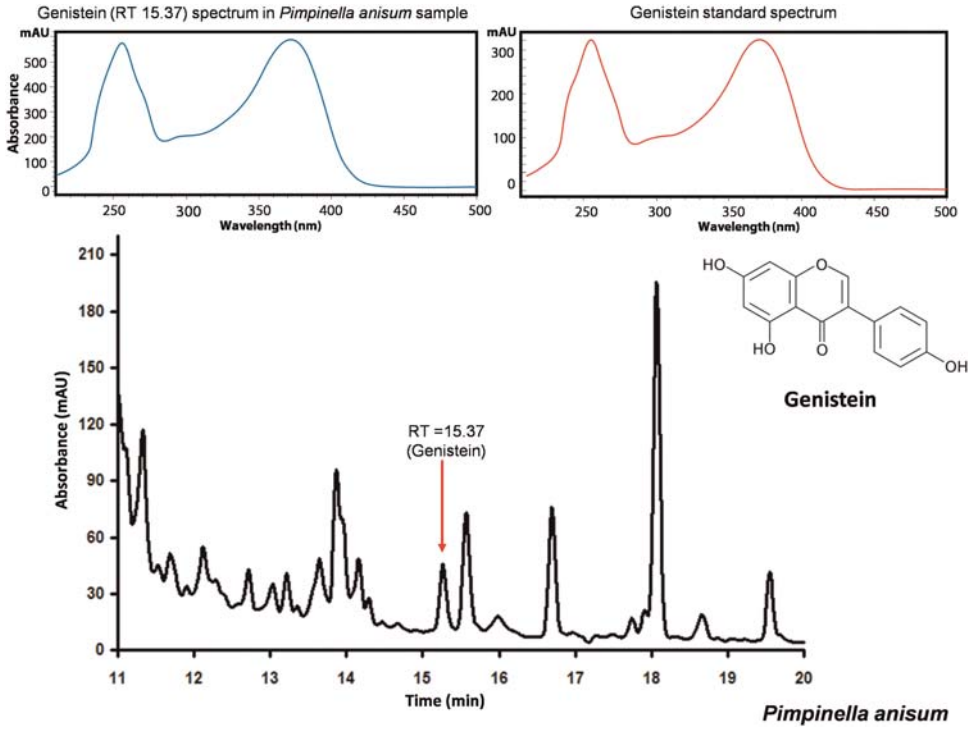

Figure 4. Confirmation of the presence of phytoestrogens in medicinal plants using diode array full-spectrum analysis. A typical and representative figure shows that the genistein peak in *P. anisum* extract (HPLC chromatogram) has the exact same full UV-Vis spectrum as the pure genistein standard peak (from a separate HPLC chromatogram).

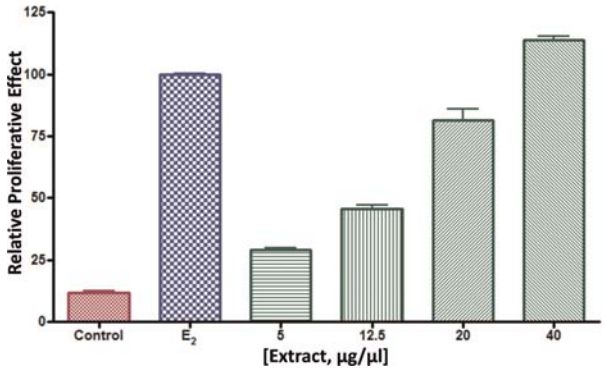

Figure 5. Cell proliferation activity of *P. anisum* extract on MCF-7 breast cancer cell line. Approximately 2,000 cells were exposed to *P. anisum* extract for three days, after which the cell proliferation was measured using a cell counting kit, as explained under "Materials and Methods." Control refers to DMEM (media) alone and E_2 refers to 375 nM 17-beta estradiol.

included three plants (*H. sabdariffa*, *F. harmonis* and *P. anisum*), all of which showed modest and significant proliferative effect. The last group (Figure 6C) had very low proliferative effects on MCF-7 cells and included *V. faba*, *F. indica* and *S. persica*. These results are very interesting in not only showing the different MCF-7 proliferative abilities of the plant extracts tested, but also lack of a direct relationship between the five phytoestrogenic contents of these plants (Table I) and their proliferative effects (Figure 6). For example, *F. bruguieri* appears to have strong proliferative effects on MCF-7 cells, yet has significantly lower concentrations of the five phytoestrogens than other plants. This result also clearly suggests that part of the proliferative effect of *F. bruguieri* (and maybe other plants) is most likely due to other phytoestrogens besides the ones that we quantified in Table I.

Fractionation and partial purification of Origanum vulgare *extract*

To further study the active components in the plant extracts that could be responsible for the proliferative activity in MCF-7, we carried out fractionation and partial purification of *Origanum vulgare* extract and tested them in the MCF-7 cell proliferation assay. As shown in Figure 7 and Table II, the three fractions of *O. vulgare* (named OV-1, OV-2 and OV-3) had very different chromatographic profile activities and phytoestrogenic content. Of the five phytoestrogens screened, the OV-1 fraction contained only biochanin A, OV-2 had both kaempferol and biochanin A and OV-3 had kaempferol and quercetin (these fractions also contained many other compounds, according to their HPLC chromatograms). Interestingly, testing of the estrogenic (proliferative) activities of these extracts on MCF-7 cells showed that all three had significant activities (Figure 8). However, OV-3, which was highly enriched in kaempferol, had the highest proliferative activity. It cannot be ascertained at this point whether the very high proliferative effect of OV-3 on MCF-7 was attributable to the high concentrations of kaempferol and quercetin alone. However, the fact that OV-1, which was devoid of kaempferol or quercetin (but was enriched in biochanin A), had the same

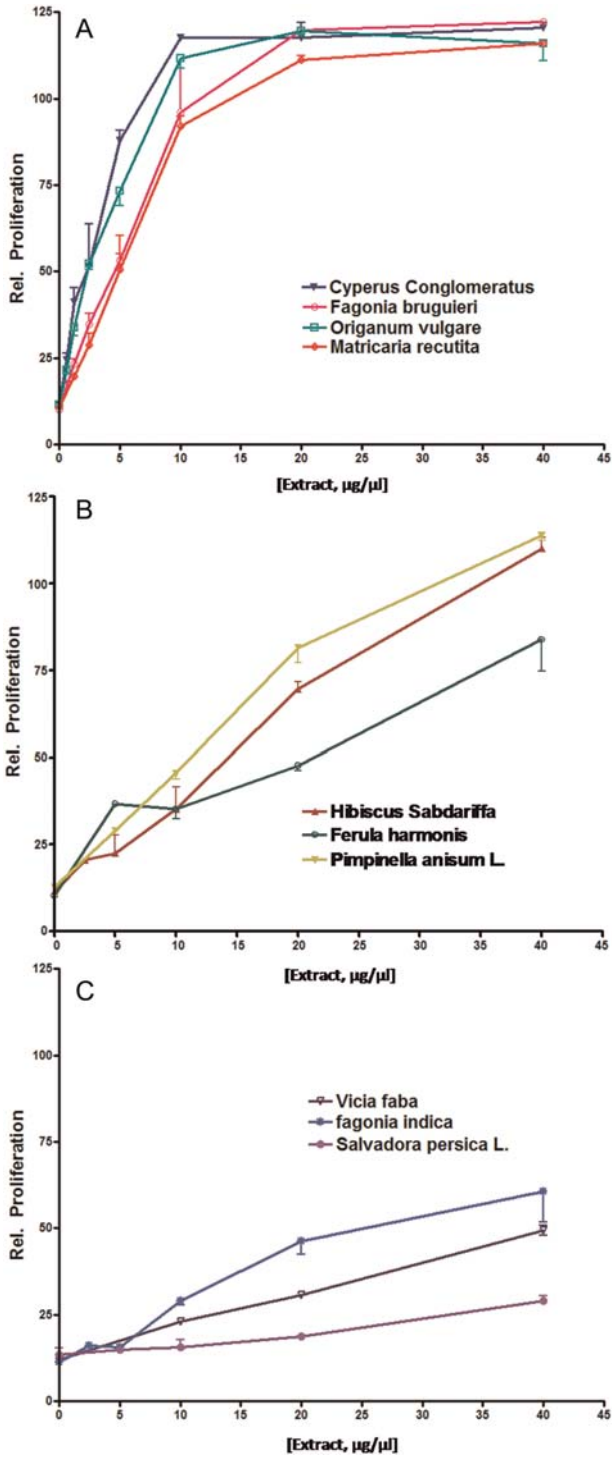

Figure 6. Cell proliferation activity of the ethanolic extracts of the 10 extracts on MCF-7 breast cancer cell line. Approximately 2,000 cells were exposed to the different concentrations of the extracts for three days, after which the cell proliferation was measured using a cell counting kit, as explained under "Materials and Methods." The data are plotted as relative proliferation based on the proliferation obtained from 375 nM E_2 as 100%.

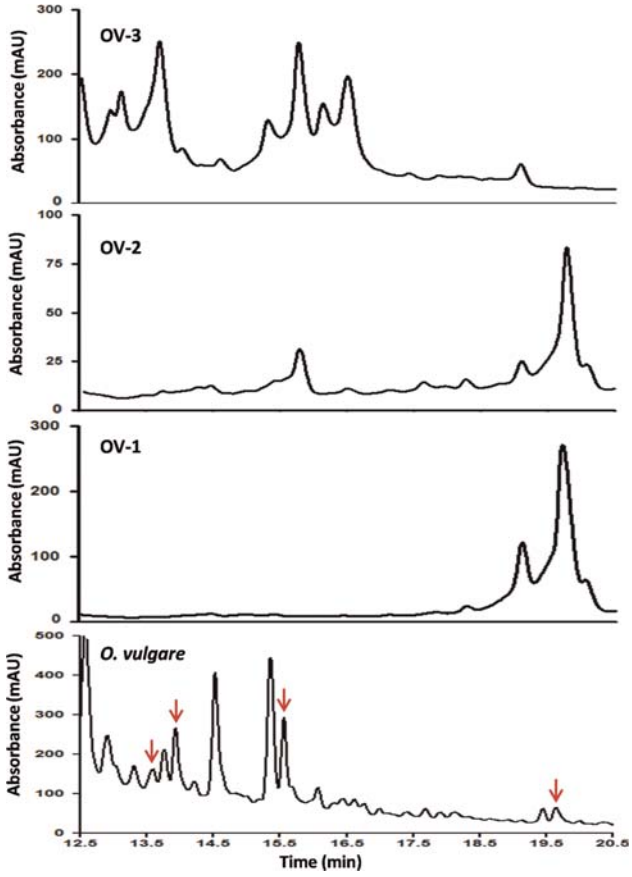

Figure 7. HPLC chromatograms of the partially purified and fractionated *O. vulgare* extract, as described under "Materials and Methods." OV-1, OV-2 and OV-3 are three silica column fractions eluted using increasingly polar solvents. The four arrows show the positions of four known phytoestrogens in *O. vulgare* extract.

Table II
Approximate Concentrations of Various Phytoestrogens in *O. vulgare* and its Three Fractions

Plants / Fractions	Phytoestrogen concentration (µg/g of dry weight of fractions)				
	Kaempferol	Quercetin	Biochanin A	Daidzein	Genistein
Origanum vulgare	178	25	2	3	n.d.
OV-1	n.d.	n.d.	1029	n.d.	n.d.
OV-2	106	n.d.	90	n.d.	n.d.
OV-3	41,740	2,275	n.d.	n.d.	n.d.

activity as *O. vulgare* extract suggests that proliferative effect of OV-3 was not only due to kaempferol and quercetin. Similarly, OV-2 which contained approximately 400 times less kaempferol than OV-3, had proliferation activity that was only approximately 15 times less than OV-3, which suggests that the proliferative activities of these extracts are not only attributable to the five estrogens. These preliminary results are extremely provocative and interesting, which warrants further detailed analysis of these extracts, especially *O. vulgare* and *C. conglomeratus*.

Conclusion

In summary, we report here, for the first time, the careful analysis and quantification of five well-known phytoestrogens in 10 widely used herbs. In addition, we show that these extracts

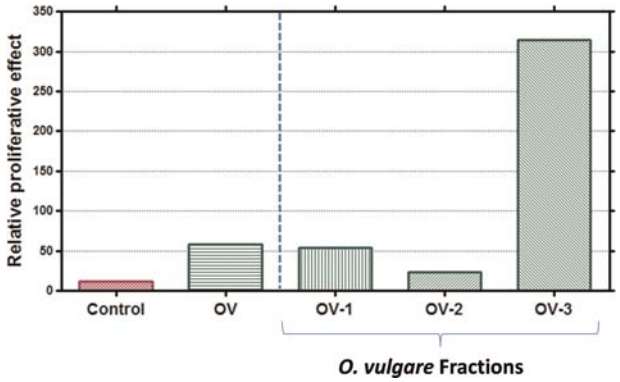

Figure 8. Cell proliferation activity of *O. vulgare* extract and partially purified fractions on MCF-7 breast cancer cell line. Approximately 2,000 cells were exposed to the extracts for three days, after which the cell proliferation was measured using a cell counting kit, as explained under "Materials and Methods." Control refers to DMEM (media alone) and the data are plotted as proliferation normalized by equal weight (1 mg) of dry *O. vulgare* extract or OV-1, OV-2 or OV-3 fractions.

possess significant estrogenic activities, as measured by MCF-7 proliferation assays. Finally, fractionation studies suggest that for some of these herbs, the proliferative/estrogenic activities are not only attributable to the five known phytoestrogens, but perhaps due to other physiologically active components.

Because the significant phytoestrogenic content of some of the herbs and their potent proliferative activities, especially *C. conglomeratus*, *F. brugguieri*, *O. vulgare* and *M. recutita*, these herbs must be used with caution and further studies should be performed to completely classify the active components in these plants.

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