Estrogen and Progestin Bioactivity of Foods, Herbs, and Spices (44247)

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Abstract. **In this study we report on the content and bioactivity of plant (phyto) estrogens and progestins in various foods, herbs, and spices, before and after human consumption. Over 150 herbs traditionally used by herbalists for treating a variety of health problems were extracted and tested for their relative capacity to compete with estradiol and progesterone binding to intracellular receptors for progesterone (PR) and estradiol (ER) in intact human breast cancer cells. The six highest ER-binding herbs that are commonly consumed were soy, licorice, red clover, thyme, tumeric, hops, and verbena. The six highest PR-binding herbs and spices commonly consumed were oregano, verbena, tumeric, thyme, red clover and damiana. Some of the herbs and spices found to contain high phytoestrogens and phytoprogestins were further tested for bioactivity based on their ability to regulate cell growth rate in ER (+) and ER (–) breast cancer cell lines and to induce or inhibit the synthesis of alkaline phosphatase, an end product of progesterone action, in PR (+) cells. In general, we found that ER-binding herbal extracts were agonists, much like estradiol, whereas PR-binding extracts, were neutral or antagonists. The bioavailability of phytoestrogens and phytoprogestins *in vivo* were studied by quantitating the ER-binding and PR-binding capacity of saliva following consumption of soy milk, exogenous progesterone, medroxyprogesterone acetate, or wild mexican yam products containing diosgenin. Soy milk caused a dramatic increase in saliva ER-binding components without a concommitant rise in estradiol. Consumption of PR-binding herbs increased the progestin activity of saliva, but there were marked differences in bioactivity. In summary, we have demonstrated that many of the commonly consumed foods, herbs, and spices contain phytoestrogens and phytoprogestins that act as agonists and antagonists *in vivo*.**

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Early recorded history documents the use of foods, herbs, and spices for treating illnesses and maintaining health (1, 2, 3). Today approximately 80% of the worlds population still relies on natural phytotherapy as a major source of medicine. Through thousands of years of human experimentation specific herbs and foods have become recognized as useful for treating symptoms associated with sex-hormone imbalance or the decline of estrogens, androgens, and progesterone during menopause (3, 4, 5).

A vast array of chemicals with estrogenic, progestogenic, and androgenic properties are found in natural components of foods, herbs, and spices (3), in derivatives from the petrochemical industry (6, 7, 8), and in pharmaceutical synthetic analogs of the parent steroids used for contraception and hormone replacement therapy (9, 10). These sex steroid hormone mimics are not detected by conventional RIAs for estradiol, progesterone, or testosterone, yet they can bind to the respective receptors for these sex hormones in target cells of the breast, brain, and reproductive tract and induce (agonist) or inhibit (antagonist) biological responses similar to those of the sex hormones.

Over the last several decades, *in vitro* cell culture bioassays have been developed and refined to determine the estrogenic content and bioactivity of foods, herbs, pharmaceuticals, and industrial products (11–13). While these bioassays have been quite useful for identifying the relative content of ''potentially'' estrogenic compounds consumed by humans, they suffer from several limitations. First, such bioassays offer no information on individual variability of absorption, metabolic disposition in the gut and liver, and bioavailability and target tissue access of an exogenous es-

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trogen once it is ingested, absorbed, or inhaled. Second, estrogenic potency predicted by *in vitro* bioassays may not accurately reflect *in vivo* biopotency since some estrogens may be present as precursors, or be highly unstable and undergo rapid transformation to a more potent or weaker estrogen. Third, estrogenic bioassays neglect the progestin, androgen, glucocorticoid, or mineralocorticoid content and bioactivity of exogenous chemicals consumed in foods, pharmaceuticals, and petrochemicals, which also may be responsible for some of the biological effects observed.

As a means to circumvent at least some of these limitations, we developed a series of *in vitro* bioassays to detect not only the estrogenic, but also the progestogenic activity of foods, herbs, and spices, before and after human consumption. Saliva was used as a simple, noninvasive means to assess the *in vivo* bioavailability and bioactivity of exogenous phytoestrogens and phytoprogestins consumed in the diet or taken as herbal medication.

In this study we report on the utility of using these bioassays to determine the estrogenic and progestogenic content and bioactivity of foods, herbs, and pharmaceuticals before and after human consumption. As estrogen paradigms we have investigated the estrogenic content of saliva following consumption of soy milk, a phytoestrogen-rich food source. As progestin paradigms we studied the progestogenic content and activity of saliva following use of progesterone, synthetic progestins, and wild yam (Dioscorea) herbal products containing diosgenin, which is reported to be a progesterone precursor.

Methods

Chemicals, Hormones, and Reagents. Tris base, sulforhodamine B, calf thymus DNA, sodium azide, triton X-100, fatty-acid-free bovine serum albumin, estradiol (E2), progesterone, trypsin/EDTA, and phenol-red-free RPMI-1640 medium were purchased from Sigma Chemical Co. (St Louis, MO). Diethylether (30,996-6, 99.9% HPLC grade inhibitor free) was purchased from Sigma-Aldrich (Milwaukee, WI). Charcoal-treated fetal bovine serum (A-1120-6) was obtained from Hyclone Labs, Inc. (Logan, UT). [^{125}I]-estradiol (NEX-144L, ca 2200 Ci/mmole) and [^{3}H]-progesterone (NET-555, 70–90 Ci/m*M*) were obtained from New England Nuclear (Dupont, Wilmington, DE). Fisher Scintiverse-B was obtained from Fisher Scientific (Pittsburgh, PA). The alkaline phosphatase Attophos kit was purchased from JBL Scientific, Inc. (San Luis Obispo, CA).

Preparation of herbal extracts (tinctures). Herbs were extracted in 50% ethanol/dH_2O (2 g herbs/10 ml) for 2 days, and the insoluble contents were sedimented by centrifugation at 1500× g for 15 min at room temperature. The soluble extract was removed and transferred to a 15-ml polypropylene tube and stored at 4°C. Unless otherwise indicated, 2 μl of herbal tinctures were combined with 1 ml of 5% charcoal-treated fetal bovine serum in RPMI growth medium containing 0.2 ng/ml bovine insulin (5%CT-FRI) for growth studies (11) and with 1% ct-FR (4 μl extract/ml) for RRA studies.

Collection and processing of saliva. Saliva was collected in 15-ml polypropylene tubes. Volunteers were asked to rinse throughly with water then chew sugarfree gum for about 5 min to stimulate saliva flow before collecting the saliva sample. Sodium azide was added to a final concentration of 0.1%. Saliva was then frozen, thawed, and clarified of mucosal debris by centrifugation at 1500× g for 10 min before aliquots were taken for assays. Saliva was stored at 4°C.

Extraction and reconstitution of saliva. For the estrogen and progesterone radioreceptor assays (RRAs), 1 ml of saliva was extracted twice with 1 ml of diethylether (DEE), and the ether was evaporated to dryness under nitrogen. The dried ether extract was then reconstituted in 1.0 ml of 0.1% BSA/dH_2O for the RRA, or in growth media (see below) for the cell culture bioassays. For the estrogen and progestin RRAs (see below), a single standard, containing both 10 ng/ml estradiol and 100 ng/ml progesterone, prepared in 0.1% CT-F/H_2O was extracted with DEE in parallel with saliva samples to normalize for extraction efficiency (80%–90%). After reconstitution in assay buffer, the 10/100 ng/ml standard was further diluted serially seven times to a final concentration of 0.003/0.03 ng/ml in assay buffer.

Cell lines. ER/PR positive (MCF7 and T47D) and ER/PR negative (MDA-468) breast carcinoma cell lines were purchased from American Type Culture Collection (Rockville, Maryland). The breast cancer cell lines were grown and passaged routinely as previously described (11, 14)

Radioreceptor assay. MCF-7 (ER) and T47D (PR) cells were fed with 5% CT-FRI at least 3 days prior to the RRA. Cells were removed with trypsin/EDTA and diluted in 1% CT-FR to 1.75 (± 0.5) × 10^6 cells/ml. Diluted cells (100 μl) were added to tubes, containing 50 μl of test compounds, standards, or controls and 50 μl of [^{125}I]-E2 (ER) or [^{3}H]-Pg (PR), which had been equilibrated in the tissue culture incubator beforehand. Tubes were incubated at 37°C with 5% CO_2 for 45 min and then plunged into an ice-water bath for 5 min. For the progestin assay, cells were centrifuged at 3000 RPM at 4°C, the supernatant discarded, and then washed once with ice cold PSG (PBS containing 0.1 *M* sucrose and 10% glycerol) and sedimented by centrifugation. The cell pellet was lysed in 3 ml of scintillation fluor and the contents poured into a scintillation vial and counted. For the estrogen assay, cells were sedimented as above and washed with 1 ml of ice-cold TPSG (0.2% Triton X-100 + PSG) as described (11). Radioactive [^{125}I]-E2 remaining in the nuclei was quantified in a gamma counter.

Estrogens and progestins extracted from saliva were quantified by interpolation from the standard curves for estradiol and progestin, respectively, and were expressed as estradiol and progesterone binding equivalents.

Cell growth studies. Cells were removed from their growth chamber with trypsin/EDTA and counted, and 250

μl containing 2000–5000 cells were seeded into the inner wells of 96-well tissue culture plates. One to two days later, media was removed and replaced with 250 μl/well of 5% CT-FRI with or without test compounds in a final ethanol concentration of 0.1%. Media was changed on Days 3, 5, 7, and 9, and growth was quantified *in situ* at times specified in the text by measuring total DNA content by propidium iodide staining and/or total protein by sulforhodamine B staining (11).

Alkaline phosphatase bioassay. About 20,000 T47D cells/well in 250 μl of 5% CT-FRI were plated into a 96-well microtiter plate. Two to three days later, the growth media was removed and replaced with appropriate standards and herbal or reconstituted saliva extracts. Eight standards of progesterone (0 and 30–10,000 pg/ml) were prepared in 5% CT-FRI. Cells were incubated for 2–3 days with 250 μl of the standards and test compounds and then assayed *in situ* for alkaline phosphatase activity as previously described (14). Briefly, growth media was removed and cells were fixed *in situ* with 250 μl of 10% formalin/PBS for 15 min. Formalin was removed and replaced for 10 min with 250 μl of 0.1% BSA in PBS, pH 7.4, then washed once with 250 μl of dH_2O. To each well was added 200 μl of the alkaline phosphatase substrate, Attophos, prepared as described by the manufacturers. The incubation was allowed to proceed for 30 min at room temperature (protected from fluorescent light) before the plate was monitored with a Cytofluor 2300 fluorometer at 485 nm excitation/590 nm emission wavelengths. The reaction was allowed to proceed for another 30 min and the plate read again. Progestins in the saliva extracts were quantified by interpolation from the progesterone standard curve and were expressed as pg/ml progesterone equivalents. Samples that fell above the standard curve were diluted and retested.

Statistics. Data are presented as the mean ± SD. Statistical significance was determined using Student's *t*-test with $P < 0.05$ evaluated as statistically significant.

Results

ER and PR Binding Properties of Herbal Extracts. One hundred and fifty different herbs, foods, and spices were extracted, diluted in cell growth media, and tested for their ability to bind to ER and PR in intact human breast cancer cell lines. The products tested are listed in Table I, and those containing the highest relative ER and PR binding activity are listed in Table II, along with their estradiol and progesterone binding equivalents, respectively.

Herbal Extracts on Cell Proliferation. Several of the herbs shown to contain high ER-binding activity (Table I) were tested for their ability to stimulate cell proliferation in ER(+) T47D breast cancer cells. Growth-inhibitory effects unrelated to the presence of ER were evaluated by testing the effects of the same herbs on the ER(−) breast cancer cell line MDA468. Other herbs (black cohosh and dong quai) reported to have estrogenic activity and to be useful for treatment of menopausal symptoms, but not found to have potent ER-binding activity in our initial screening tests, were also tested. Cells were grown for 9 days and growth was evaluated directly in the microwells as an increase/decrease in total cellular protein and/or DNA relative to untreated and estradiol-treated cells. Physiological concentrations of estradiol (10^{-9} *M*), estradiol plus a 100-fold molar excess of the antiestrogen hydroxytamoxifen, and 10^{-8} *M* coumestrol, a phytoestrogen, were used as controls.

The relative effects of herbs on protein and DNA were similar in both T47D and MDA468 cells and, therefore, for simplicity only the protein results are depicted in Figures 1 (T47D) and 2 (MDA468). In the ER(+) T47D cells, licorice, red clover, yucca, hops and motherwort demonstrated significantly higher growth than control, indicating possible estrogenic effects. Some of the herbal extracts (red clover, yucca) were found to be equipotent to estradiol at the concentrations tested. It is likely that the concentration of the estrogenic chemicals is substantially higher than that of estradiol because of the much lower affinity of phytochemicals for binding to ER. In the ER (−) MDA468 breast cancer cells, the same group of herbs either showed little difference from control, or estrogen treatment, or markedly inhibited cell growth (mandrake, bloodroot, juniper, mistletoe).

Progestin-Induced Alkaline Phosphatase as an Index of Progestin-Agonist Activity of Herbal Extracts. We have previously reported that progesterone, and other progestin agonists (R5020, MPA) induce the synthesis of alkaline phosphatase (14). Induction of alkaline phosphatase is dose dependent, occurs over a physiological range of progesterone, and is highly progestin specific as no other steroids (estrogens, androgens, glucocorticoids), induce this enzyme at a physiologic dose.

To determine if some of the high PR-binding herbs listed in Table II possess progestin agonist properties, we incubated diluted extracts from these herbs with T47D cells and monitored alkaline phosphatase activity. Progesterone, over a physiological concentration range, was used as control without and with a 100-fold molar excess of RU486, a potent antiprogestin. The antiprogestin activity of the PR-binding herbs was tested by including progesterone in a parallel series of wells with the herbal extracts. Alkaline phosphatase induction defines the PR-binding herb as an agonist, whereas no induction functionally defines the herb either as neutral or as an antagonist. If the herb blocks progesterone induction of alkaline phosphatase, it is defined as an antagonist, similar to the actions of RU486.

As seen in Figure 3 alkaline phosphatase activity increases in a dose-dependent manner by progesterone and is completely blocked by an excess of the competitive inhibitor RU486. None of the PR-binding herbs tested significantly increased alkaline phosphatase activity, thus categorizing them as either neutral or antagonists. To further subcategorize these PR-binding herbal extracts, their effects on progesterone induction of alkaline phosphatase was investigated. Four of the herbs (damiana, dong quai, yucca, and

Table I. Alphabetical Listing of Herbs Studied

Acacia (*Acacia senegal*), Agrimony (*Agrimonia eupatoria*), Unicorn root (*Aletris farinosa*), Alfalfa (*Medicago sativa*), Aloe (*Aloe vera*), Anise (*Pimpinella anisum*), Asparagus (*Asparagus officinalis*), Astragalus (*Astragalus membranaceus*), Barberry (*Berberis vulgaris*), Bay leaf (*Laurus nobilis*), Bayberry (*Myrica cerifera*), Black Cohosh (*Cimicifuga racemosa*), Black Current (*Ribes nigrum*), Blessed Thistle (*Cnicus benedictus*), Bloodroot (*Sanguinaria canadensis*), Blue Cohosh (*Caulophyllum thalictroides*), Blue Flag (*Iris versicolor*), Borage (*Borago officinalis*), Bryony root (*Bryonia alba*), Burdock root (*Arctium lappa*), Calamus root (*Acorus calamus*), Calendula (*Calendula officinalis*), Caraway seed (*Carum carvi*), Cascara Sagrada (*Rhamnus purshiana*), Catnip (*Nepeta cataria*), Cayenne (*Capsicum annum*), Celandine (*Chelidonium majus*), Chamomile (*Chamaemelum nobile*), Chaste-tree (*Vitex agnus-castus*), Cherry, wild (*Prunus avium*), Chickweed (*Stellaria media*), Cilantro (*Coriandrum sativum*), Cloves (*Syzygium aromaticum*), Crampbark (*Viburnum opulus*), Cumin (*Cuminum cyminum*), Damiana (*Turnera diffusa*), Devil's Club (*Olopanax horridum*), Dock (*Rumex obtusifolius*), Dong quai (*Angelica sinensis*), Echinacea (*Echinacea purpurea*), Endive (*Chicorium endivia*), Ephedra-Ma Huang (*Ephedra distachya*), Equisetum (*Equisetum arvense*), Evening Primrose (*Oenothera biennis*), Eyebright (*Euphrasia officinalis*), False Unicorn (*Chamaelirium luteum*), Fennugreek (*Trigonella foenum-graecum*), Fennel (*Foeniculum vulgare*), Flaxseed (*Linum usitatissimum*), Garlic (*Allium sativum*), Gelsium (*Gelsemium sempervirens*), Gentian root (*Gentiana macrophylla*), Ginger root (*Zingiber officinale*), Ginko (*Ginko bilboa*), Ginseng (*Panax ginseng*), Ginseng, Wild Siberian (*Eleutherococcus senticosus*), Goldenseal (*Hydrastis canadensis*), Gotu Kola (*Centella asiatica*), Gravel root (*Eupatorium purpureum*), Henna-Red (*Lawsonia inermis*), Hops (*Humulus lupulus*), Horehound (*Marrubium vulgare*), Hyssop (*Hyssopus officinalis*), Ironwood bark (*Ostrya virginiana*), Juniper berry (*Juniperus communis*), Kavakava root (*Piper methysticum*), Lavendar (*Lavandula angustifolia*), Lemon Grass (*Cymbopogon citratus*), Licorice (*Glycyrrhiza glabra*), Lobelia (*Lobelia chinensis*), Lovage root (*Levisticum officinale*), Mandrake (*Mandragora autumnalis*), Marapuama (*Dulacia inopiflora*), Marshmallow root (*Althaea officinalis*), Milkweed (*Asclepias tuberosa*), Mistletoe (*Viscum album*), Motherwort (*Leonurus cardiaca*), Mugwort (*Artemisia vulgaris*), Mullein (*Verbascum thapsus*), Nettle (Urtica dioica), Nutmeg (*Myristica fragrans*), Oatstraw (*Avena sativa*), Ocatillo (*Fouquieria splendens*), Onion (*Allium cepa*), Oregano (*Origanum vulgare*), Oregon Grape seed (*Berberis aquifolium Pursh.*), Passion fruit (*Passiflora incarnata*), Pau d'Arco (*Tabebuia spp.*), Pennyroyal (*Mentha pulegium*), Peony root (*Paeonia officinalis*), Periwinkle (*Vinca*), Pipsisewa (*Chimaphila umbellata*), Pokeweed root (*Phytolacca americana*), Pomegranate (*Punica granatum*), Poppy seed (*Papaver somniferum*), Gravel root (*Eupatorium purpureum*), Red Clover (*Trifolium pratense*), Red Raspberry (*Rubus idaeus*), Red Root (*Ceanothus americanus*), Rosemary (*Rosmarinus officinalis*), Rue (*Ruta graveolens*), Saffron (*Crocus sativus*), Sage (*Salvia officinalis*), Sarsaparilla (Smilax), Sassafras (*Sassafras albidum*), Savory (*Satureja hortensis*), Saw Palmetto (*Serenoa repens*), Senna (Senna), Sesame seed (*Sesamum indicum*), Shavegrass (*Equisetum arvense*),

Table I. Continued

sheep Sorrel (*Rumex acetosella*), Shephard's Purse (*Capsella bursa-pastoris*), Skullcap (*Scutellaria lateriflora*), Slippery Elm bark (*Ulmus rubra*), Smallage (*Apium graveolens*), Solomon's Seal (*Polygonatum officinale*), St. John's Wort (*Hypericum perforatum*), Strawberry leaf (*Fragaria vesca*), Sumac (*Rhus glabra*), Tansy (*Tanacetum vulgare*), Thyme (*Thymus vulgaris*), Turmeric (*Curcuma longa*), Uva Ursi (*Arctostaphylos uva-ursi*), Valerian (*Valeriana officinalis*), Vervain (*Verbena officinalis*), Vitex-chaste tree (*Agnus canthus*), Wild Pansy (*Viola tricolor*), Wild Yam (*Dioscorea villosa*), Wormwood (*Artemisia absinthium*), Yarrow (*Achillea millefolium*), Yellow dock (*Rumex crispus*), Yerba Mansa (*Anemopsis californica*), Yucca (*Yucca Spp.*)

Table II. Herbs and Spices Containing ER Binding Components

Micrograms of Estradiol Equivalents/200 cc or 2 g Dry Herb

Soy milk	8/200cc	Yucca	0.5/2g
Licorice	4/2 g	Tumeric	0.5
Clover (red)	3	Hops	0.5
Mandrake	3	Verbenna	0.5
Bloodroot	2	Yellow dock	0.5
Thyme	2	Sheep sorrel	0.5

Soy milk, herbs, and spices were extracted and tested for their ER-binding capacity as described in the Methods. Soy milk and 11 of the herbs and spices with the highest activity are listed and expressed in total estradiol binding equivalents per 200 cc of soy milk or 2 g of dried herb. Values were rounded to the nearest 0.5 micrograms of estradiol binding equivalents. Results are representative of at least two other assays.

Table III. Herbs and Spices Containing PR Binding Components

Micrograms of Progesterone Equivalents/2 g Dry Herb

Bloodroot	100	Thyme	4
Ocotillo	8	Calamus rt.	3
Mandrake	8	Red clover	3
Oregano	8	Goldenseal	3
Damiana	6	Licorice	3
Pennyroyal	5	Mistletoe	3
Verbena	5	Cumin	2
Nutmeg	4	Fennel	2
Tumeric	4	Camomille	2
Yucca	4	Cloves	2

Herbs and spices were extracted and tested for their PR-binding capacity as described in the Methods. The 20 herbs and spices with the highest activity are listed and expressed in progesterone-binding equivalents. Values were rounded to the nearest microgram of progesterone-binding equivalents. Results are representative of at least two other assays.

mistletoe), did not inhibit progesterone induction of alkaline phosphatase and, therefore, would be categorized as neutral. In contrast, red clover, licorice, goldenseal, pennyroyal, and nutmeg all either completely or partially blocked enzyme induction by progesterone, tentatively defining them as antiprogestins.

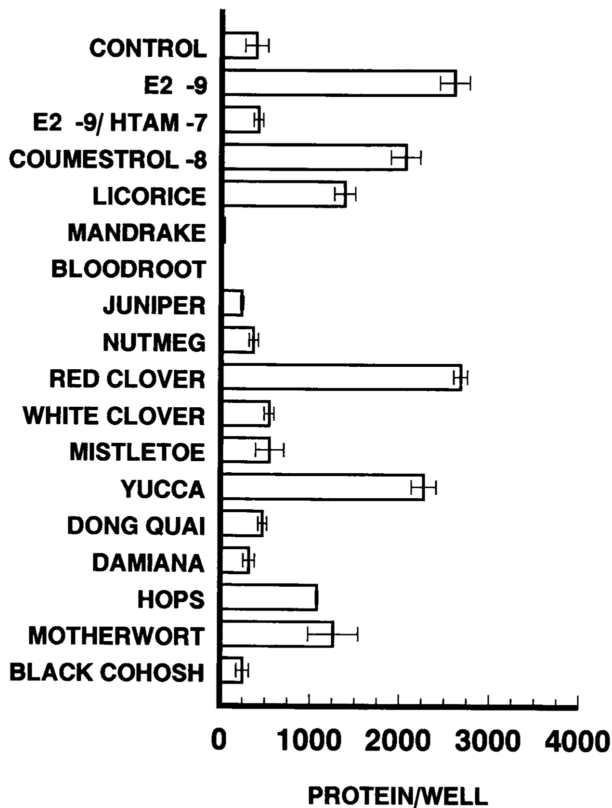

Figure 1. Effects of ER-binding herb extracts on growth of ER (+) breast cancer cells *in vitro.* Cells were grown in 96-well plates for 9 days in the absence (control) or presence of estradiol, estradiol plus hydroxytamoxifen, coumestrol, or herbal extracts (1/500 dilution). Growth was assessed by measuring protein directly in the wells as indicated in the Methods.

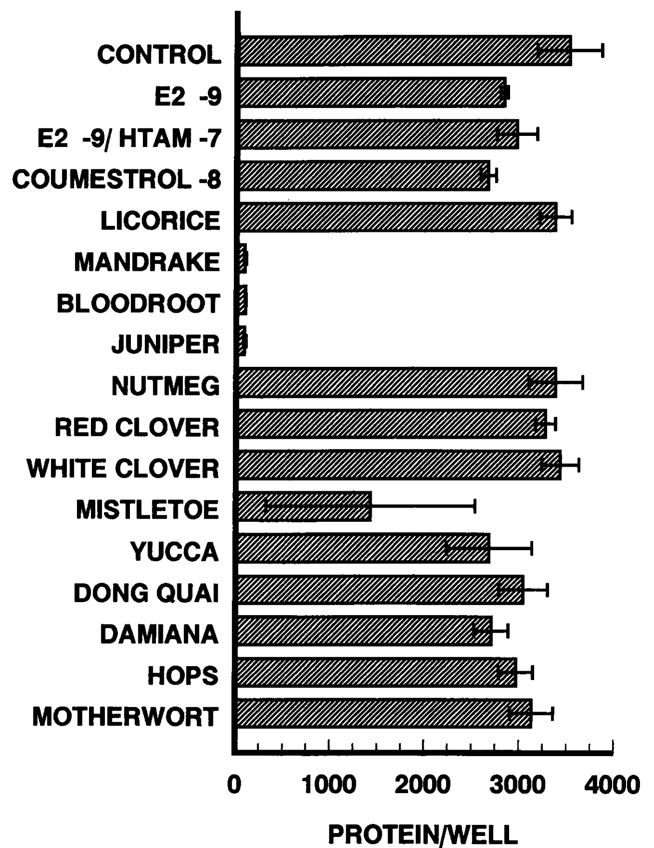

Figure 2. Effects of ER-binding herb extracts on growth of ER (–) breast cancer cells *in vitro.* Cells were grown in 96-well plates for 7 days in the absence (control) or presence of estradiol, estradiol plus hydroxytamoxifen, coumestrol, or herbal extracts (1/500 dilution). Growth was assessed by measuring protein directly in the wells as indicated in the Methods.

Progestin-Mediated Down Regulation of ER as an Index of Progestin-Agonist/Antagonist Activity of Herbal Extracts. One of the mechanisms by which progestins modulate estrogen action is by down regulating ER (15). Estrogens also down regulate ER, but the mechanism is different (16). ER in T47D cells is exquisitely sensitive to down regulation by progestins, and this action is blocked by antiprogestins like RU486. RU486 inhibits ER-down regulation by progestins, but not by estrogens (14), thereby allowing for the distinction between the effects of estrogens and progestins on ER down regulation.

To determine if the putative antiprogestin actions of the PR-binding herbs (Fig. 3) were operating *via* ER mechanisms, we incubated T47D cells with these herbs, with and without RU486. Progesterone at several concentrations, with and without RU486, was used as a control (Fig. 4). Whereas nearly all of the herbal extracts down-regulated ER, this effect was not reversed with RU486, indicating that ER down regulation by these herbs was unlikely to be PR-mediated.

Detection and Bioactivity of PR-binding Hormones in Human Saliva. The alkaline phosphatase assay described above has been modified to quantify the relative bioactivity of progestins present in human saliva. A representative dose-response curve for progesterone-spiked human saliva is depicted in Figure 5. The progesterone concentration in saliva ranges from about 10–400 pg/ml (Ref. 17, and unpublished results from Aeron LifeCycles), with postmenopausal and follicular levels ranging from 10–50 pg/ml, and mid-luteal ranging from about 100–400 pg/ml. As seen in Figure 5, reconstituted saliva extracts, when exposed to T47D cells for 48 hr, induced alkaline phosphatase from nearly undetectable levels at 30 pg/ml to maximum levels at about 300 pg/ml. When a 100-fold molar excess of RU486 was included with 3 ng/ml progesterone, alkaline phosphatase induction was completely suppressed to control levels. These results demonstrate the exquisite sensitivity of this assay, which spans the expected physiological range for saliva progesterone levels in premenopausal women (17) and in women taking exogenous progesterone or progestin therapy.

In an earlier report (14) we demonstrated the unique specificity of the alkaline phosphatase assay for detecting and differentiating PR-binding compounds with progestin agonist and antagonist activity. Not unexpectedly, pharmaceutical synthetic progestins like medroxyprogesterone acetate (MPA), are not detected by conventional RIAs for progesterone, but their PR-binding capacity and bioactivity

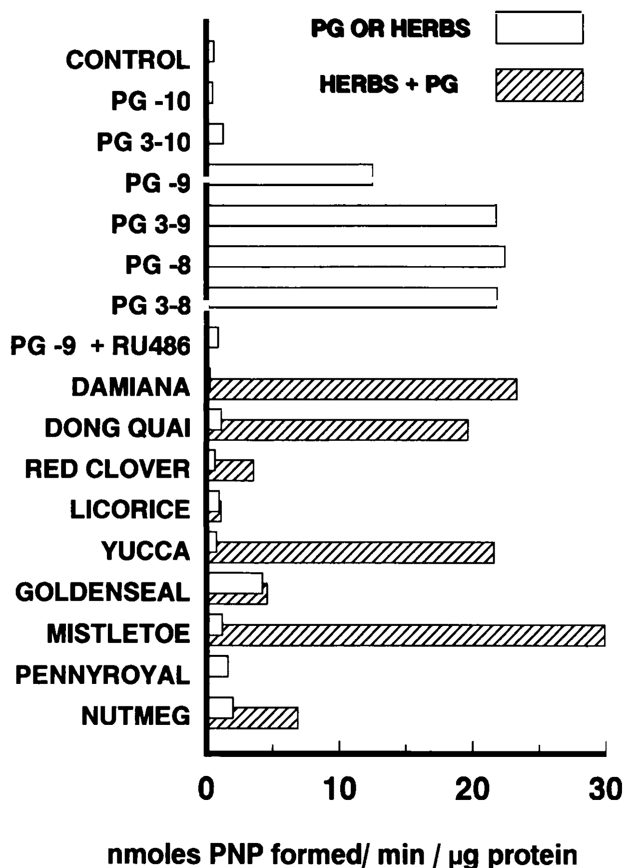

Figure 3. Induction of alkaline phosphatase by progesterone, progesterone plus RU486, and PR-binding herbal extracts, with and without progesterone. Near confluent cells in 96-well plates were exposed to increasing doses of progesterone, 10^{-9} *M* progesterone plus 10^{-7} *M* RU486, or 1/500 dilution of herbal extracts (as indicated) without and with 10^{-9} *M* progesterone. After 48 hr, cells were fixed and alkaline phosphatase measured directly in the plate as indicated in Methods.

can be measured by the RRA and the alkaline phosphatase bioassays, respectively (14).

We tested by Pg-RIA, Pg-RRA, and AP-bioassay the salivas of 35 women who were not taking any hormones (normal cycling or menopausal) or who were taking progesterone, synthetic progestins, or Mexican wild yam containing diosgenin as the active ingredient. The mean progesterone levels (determined by RIA) in the saliva of MPA and diosgenin users was very low; respectively, 17 pg/ml (n = 7, range 10–27) and 19 pg/ml (n = 10, range 5–34). These pilot results showing that MPA and diosgenin appear to suppress progesterone synthesis are very consistent with a much larger database from our laboratory.

When progesterone equivalents, determined by RRA and AP-bioassay, were compared (Fig. 6), women not using any exogenous hormonal products (cycling or menopausal) and those reporting use of progesterone (transdermal skin cream) showed comparable RRA/AP-bioassay values. In contrast, women reporting use of oral MPA and diosgenin-containing herbal products more often had low AP-bioassay values (one exception) despite a wide range of PR-binding RRA values.

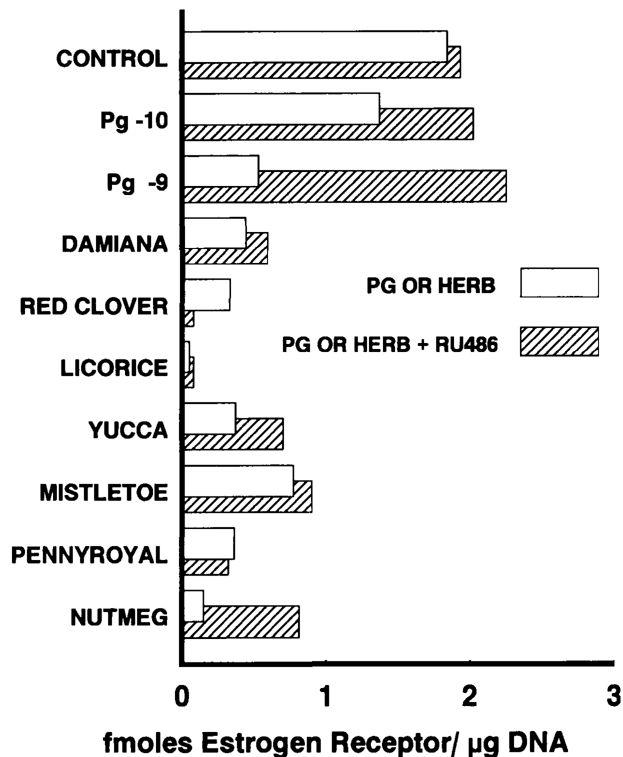

Figure 4. Down regulation of ER by progesterone and herbal extracts in the absence and presence of RU486. Near confluent cells in 24-well plates were exposed to progesterone and herbal extracts, without and with 10^{-7} *M* RU486. After 48 hr, ER was measured directly in the plate by incubating cells with 2 n*M* [^{125}I]-estradiol. Following incubation, the cells were removed from the plate with trypsin and the radioactive binding of [^{125}I]-estradiol was measured in nuclei as described previously (11).

Detection of ER-Binding Hormones in Saliva. Over the past several years, a great deal of emphasis has been centered on the role of soy foods in health issues such as prevention of cancer, heart disease, osteoporosis, and menopausal symptoms (18, 19). Asians have a very low incidence of these ''Western diseases,'' and it is becoming increasingly clear that many of these benefits may be derived from the very high consumption of isoflavones, such as genistein and daidzein, found in soybeans, but very few other legumes (20). The saliva estrogen RRA provides a unique noninvasive means to study net changes in circulating concentrations of bioavailable phytoestrogens following consumption of soy foods. We performed a pilot study with laboratory volunteers using soy milk to validate the utility of the RRA for measuring changes in ER-binding components (i.e., phytoestrogens) with consumption of a phytoestrogen-rich food source. Six volunteers consumed 200 cc (one cup) of soy milk and then collected saliva samples at hourly intervals over 24 hr. Saliva was processed as described above, and the total estrogens present determined by the total estrogen RRA.

Results depicted in Figure 7 demonstrate that soy milk consumption in most of the participants led to a dramatic rise from 1–3 hr in ER-binding components in saliva followed by a steep fall, and then a second rise, in some par-

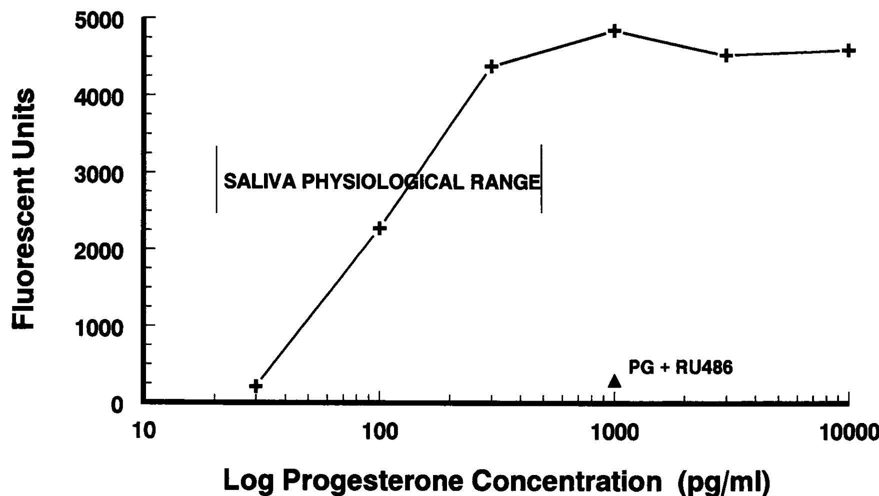

Figure 5. Induction of alkaline phosphatase in T47D breast cancer cells with extracts of saliva spiked with increasing doses of progesterone. Pooled saliva was charcoal-treated then spiked with increasing concentrations of progesterone as indicated. Saliva was then extracted in DEE, reconstituted in growth media and incubated with cells for 48 hr. Alkaline phosphatase was then determined as indicated in the Methods.

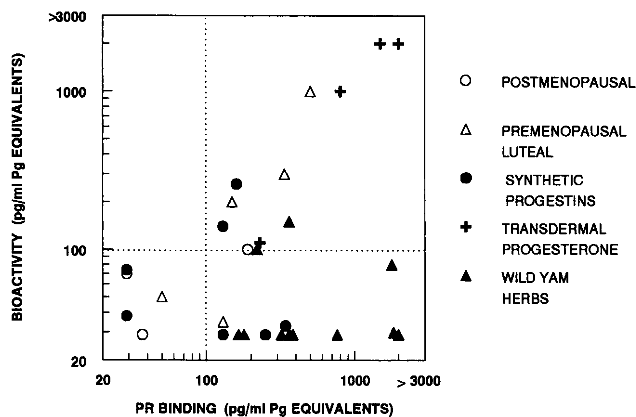

Figure 6. Induction of alkaline phosphatase in T47D breast cancer cells with extracts of saliva from premenopausal women, and women taking progesterone, MPA, and wild-yam (diosgenin-containing) herbs. Saliva was processed as described in Figure 5 and incubated with cells for 48 hr. Alkaline phosphatase was measured as described above.

ticipants from 5–12 hr. Although the ER-binding components showed dramatic fluctuations associated with soy milk consumption, saliva estradiol levels, determined by RIA did not change significantly in any of the six volunteers over this 24-hr time course (data not shown). Moreover, when cow's milk or other food products not found to contain phytoestrogens were consumed under the same conditions, there was no significant change in the baseline levels of ER-binding components in saliva.

Discussion

In this study we tested the estrogen and progestin content and bioactivity of various foods, herbs, spices, and pharmaceutical products, before and after human consumption, using bioassays developed or refined in our laboratory. Of the foods, herbs, and spices tested, the most potent ER-binding products were soy milk, licorice, and red clover. Licorice and red clover, but not soy milk, also had high levels of a PR-binding component(s) which had potent progestin antagonist properties based on their ability to block progesterone induction of alkaline phosphatase, an end product of progestin action. Interestingly, all three herbs belong to the leguminosa family, yet the active phytoestrogen is derived from different parts of the plant—bean, root, and flower, respectively. Of further interest is that all three have been suggested to have cancer-preventive properties (1–3). We recently have reported on the biphasic actions of genistein on cell proliferation in ER(+) human breast cancer cells *in vitro* (11). Genistein is one of the principal phytoestrogens in soy and thought to be present in other legumes like licorice and red clover. Soy and licorice are actively being studied by the US Designer Food Program for their anticancer effects (1–3). It has been suggested that the phytoestrogens present in these plants might be partly responsible for their antineoplastic actions in hormone-dependent tumors such as breast and endometrial cancers (11, 18–20).

Licorice, red clover, dong quai, damiana, black cohosh, verbena, and motherwort are common ingredients of traditional herbal remedies used as alternatives for treating menopausal symptoms (1–3). Our studies indicate that licorice, red clover, and motherwort could be direct-acting estrogen agonists based on the evidence that extracts from them bind ER and stimulate cell proliferation. Although direct extracts of dong quai, damiana, and black cohosh had little ER binding and estrogenic bioactivity, all three are constitutents of herbal therapies commonly used for correcting hormonal imbalance in women (2).

Saliva results from women taking dong quai (unpublished results) indicate that estradiol levels are consistently very low, suggesting that this herb may suppress estradiol synthesis. Soy foods, which contain high levels of the phytoestrogen genistein, also suppress estradiol synthesis by direct genistein inhibition of aromatase (21) and 17B steroid oxidoreductase (22), enzymes necessary for the conversion of androgens to estrone and estrone to estradiol, respectively. An extract of black cohosh, commercialized in the product Remifemin, has an extensive clinical history of use in Western Europe for relief of menopausal symptoms commonly associated with estrogen deficiency (23). Clearly,

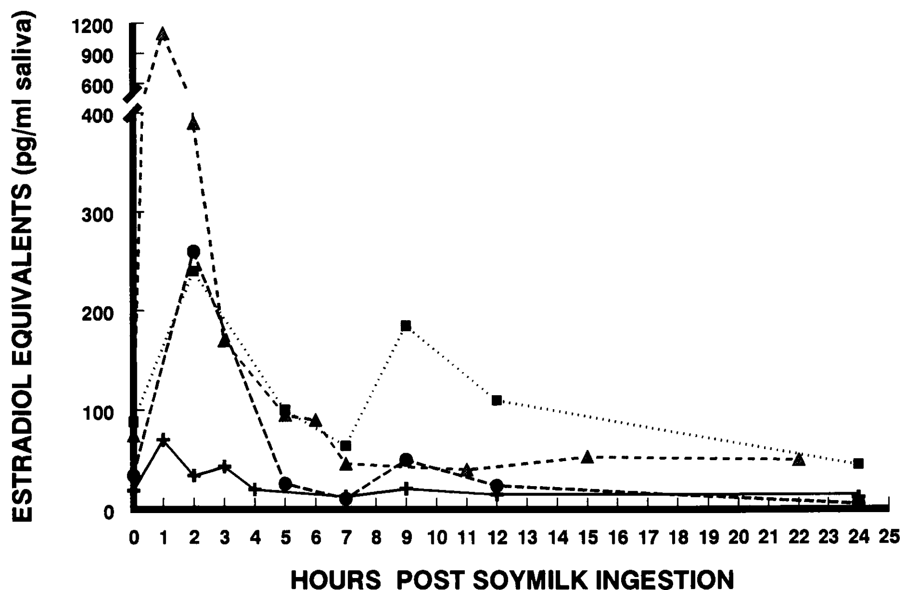

Figure 7. Phytoestrogen content of saliva following soy milk consumption. Volunteers drank 200 cc of soy milk and collected saliva samples at hourly intervals over 24 hr. Saliva was processed and assayed for estrogen receptor binding components and for estradiol by conventional radioimmunoassay as described in the Methods. Results are expressed in picograms (pg) of estradiol binding equivalents per ml of saliva.

further work with these herbs is needed to determine if they contain phytoestrogens or precursors that are converted into active estrogens *in vivo* or mediate their actions *via* other receptor systems or indirect mechanisms such as alteration in synthesis and metabolism of endogenous estrogens and progesterone.

Other spices (thyme, oregano, and tumeric) and herbs (hops) found to contain relatively high ER-binding activity are common ingredients in foods and beverages and may have weak estrogenic activity *in vivo.* Although we did not test all of them further for estrogenic bioactivity, these spices might be expected to have mild estrogenic effects in foods prepared with them. Tumeric is a spice commonly used in Middle Eastern and Mediterranean foods, whereas hops is an ingredient of beer. Anecdotal reports claim that women harvesting hops often begin menstruating within 2 days after commencing harvest, suggesting a hormonal effect (24). Studies on hop extracts and beer have identified phytoestrogens (24, 25). In contrast, other investigators have failed to identify significant levels of phytoestrogens in hops, based on the mouse uterine assay (26). Our study confirms that an ethanol extract of hops contains a significant amount of phytoestrogens with moderate estrogenic bioactivity.

Mandrake, bloodroot, juniper, and mistletoe were all potent inhibitors of cell proliferation in both ER(+) and ER(−) breast cancer cell lines, implying that their growth-inhibitory mechanism of action is not likely to be estrogen-regulated. Mistletoe had little effect on ER(+) cells, but was moderately growth-inhibitory to ER(−) cells. Bloodroot and mistletoe have an extensive history of use as herbal therapies for treatment of cancer (2). Bloodroot was used successfully for treatment of breast cancer in England over 100 years ago (2) and by Native Americans for treatment of surface tumors, including breast cancer (2). Today herbalists use bloodroot topically for nasal polps, cervical dysplasias, and breast cancers. Like bloodroot, extracts of mistletoe (ISCADOR) have been used for almost 100 years in Western Europe to treat a variety of different cancers, including breast cancer and leukemias (27). It is tempting to speculate that the estrogen receptor and progesterone receptor binding properties of mistletoe and bloodroot may underlie their reported clinical benefits in such nontraditional treatment of breast cancer.

Curiously, none of the PR-binding herbs studied were progestin agonists. All were either neutral (damiana, yucca, mistletoe), or were progestin antagonists (red clover, licorice, goldenseal, pennyroyal, nutmeg), based on their ability to inhibit progesterone induction of alkaline phosphatase. This is in sharp contrast to ER-binding phytoestrogens which we found instead to be primarily estrogen agonists (Table I and Figure 1). Of the herbs and spices found to contain PR-binding activity, bloodroot was by far the most potent. Bloodroot, mandrake, pennyroyal, yucca, and mistletoe, all of which had high PR-binding activity, are used as abortifacents, emmenagogues (to bring on the menses) and to facilitate child birth, but are dangerous when used during pregnancy (1–3).

Our studies on direct extracts of foods, herbs, and spices have provided useful information on their receptor binding activity and bioactivity *in vitro.* However, these results may not always be relevant to their effects as estrogen and progestin agonists and antagonists *in vivo* in humans because of individual differences in absorption, activation/inactivation, metabolic disposition, and bioavailability to target tissues. Saliva testing, coupled with the bioassays for phytoestrogens and phytoprogestins, was developed as a simple noninvasive means to circumvent these deficiencies, and to measure the *in vivo* bioactivity of the bioavailable fraction of phytohormones following their consumption in foods, herbs, and spices. Saliva is a natural ultrafiltrate of serum and, therefore, provides an excellent diagnostic medium in which to monitor the bioavailable or free fraction of phytochemicals in the bloodstream. Larger

glucuronide and sulfate conjugates of steroids and other small nonpolar molecules, including phytochemicals, do not freely diffuse into saliva; therefore, it is not possible to monitor their content or bioactivity in this biological fluid.

Results shown in Figure 6 clearly demonstrate the utility of the saliva bioassays for determining not only the bioavailability, but also the bioactivity of virtually any type of progestin in the bloodstream as a result of endogenous formation (i.e., progesterone) or from consumption of foods, pharmaceuticals, or herbal phytotherapy. Interestingly, salivas from only three of seven women taking the synthetic progestin MPA, and who had high PR-binding determined by RRA, demonstrated equivalent bioactivity. In all seven cases the saliva progesterone values, determined by RIA, were much lower (mean 17, range 10–27) than the mean levels we find in postmenopausal women who are not taking exogenous hormones. This is a very consistent characteristic we and others (28) have observed in women taking synthetic progestins such as MPA. The low bioactivity/progestin-RRA ratios of saliva from women using synthetic progestins may be associated with formation of weaker PR-binding metabolites such as medroxyprogesterone (MP), which has much less progestin bioactivity than MPA (29, 30).

Many of the diosgenin-containing herbal products (Dioscorea-wild yam) are being sold under the guise that they are progesterone precursors and will be converted *in vivo* into progesterone and other steroids (DHEA). None of the saliva from women reporting consumption of diosgenin-containing herbs was found to possess any progesterone bioactivity despite high levels of PR-binding components in some of them (20%–30%). Moreover, all of the saliva from the 11 women who were using diosgenin-containing products also contained very low levels of progesterone, determined by RIA (mean 19, range 5–34 pg/ml progesterone). Hence, our preliminary data support recent arguments (31, 32) and uncontrolled pilot clinical studies (3) that diosgenin is not converted to progesterone in the human body.

Our pilot study with phytoestrogen-rich soy milk demonstrates the feasibility of using the saliva estradiol-RRA for monitoring the bioavailable levels of phytoestrogens following consumption of foods or herbs containing ER-binding components. We are currently developing bioassays, similar to the progestin AP-bioassay to elucidate the estrogenic bioactivity of the ER-binding components of saliva. While it is unlikely that saliva phytoestrogens beyond 1 hr following soy milk consumption represent a contamination of residual phytoestrogens retained in the oral cavity, these experiments are being repeated with encapsulated forms of soy extracts. It is also worth noting that what we measure is binding to ER, relative to estradiol, and values are expressed as estradiol binding equivalents. Assuming that the saliva assay for phytoestrogens closely reflects its bioavailable fraction *in vivo,* as observed for most other steroid hormones (estradiol, progesterone, testosterone, and cortisol), these results would suggest that a very high concentration of these phytoestrogens are bioavailable, relative to estradiol. Our experimental results with soy milk, demonstrating a high level of saliva phytoestrogens following consumption, are entirely consistent with elevated ER-binding activity in the saliva of women reporting heavy use of soy foods (tofu, soy chips, soy protein isolate) and medications containing some of the herbs we find have high ER-binding activity (licorice, red clover, bloodroot).

Because herb extracts are composed of complex mixtures of phytochemicals, it cannot be excluded that some of their well-recognized physiological actions are mediated *via* other phytochemicals that mimic the actions of steroids other than estrogens or progestins, including phytoandrogens, phytomineralocorticoids, and/or phytoglucocorticoids. For example, licorice is one of the most common herbs used in traditional Chinese medicine and contains a mineralocorticoid-like activity that increases water retention (32, 33). The glycyrrhitinic acid found in licorice contributes to the aldosterone-like actions of licorice. We have not found that either purified glycyrrhizin or glycyrrhitinic acid bind to either ER or PR (data not shown). However, deglycerinized licorice (DGL) retains high ER-binding activity. These results clearly indicate that the estrogen and progestin activity present in licorice is mediated by phytochemicals other than glycyrrhizin or glycyrrhitinic acid. The specific phytochemical(s) responsible for the estrogenic and progestogenic actions of licorice, as well as other herbs, remains to be elucidated.

In summary, we have shown that many of the foods, and spices commonly consumed by humans as nutrients or phytomedicines contain phytoestrogens and phytoprogestins with both agonistic and antagonistic properties. Generally, most phytoestrogens were found to be estrogen agonists, whereas all phytoprogestins were progestin antagonists, consistent with their tradional use by phytotherapists. Phytoestrogens consumed in the form of soy milk were shown to be bioavailable *in vivo* based on their presence in saliva. The use of saliva, coupled with the bioassays described in this report, should prove useful for exploring the *in vivo* content, bioactivity, and bioavailability of phytochemicals in foods, herbs, and spices as well as synthetic pharmaceuticals.

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1. Duke JA. Handbook of Medicinal Herbs. Boca Raton, FL: CRC Press, 1985.
2. Erichsen-Brown C. Medicinal and Other Uses of North American Plants: A Historical Survey with Special Reference to the Eastern Indian Tribes. New York: Dover Publications, Inc., 1979.
3. Beckham N. Phyto-oestrogens and compounds that affect oestrogen metabolism. Aust J Med Herbalism **7**(1), 1995.
4. Farnsworth NR, Bingel AS, Cordell GA, Crane FA, Fong HHS. Po-

tential value of plants as sources of new antifertility agents II. J Pharmaceutical Sci **64**:717–754, 1975.
5. Avis NE, Kaufert PA, Lock MA, McKinlay SM, Vass K. The evolution of menopausal symptoms. Clin Endo Metab **7**:17–32, 1993.
6. Davis DL, Bradlow HL. Can environmental estrogens cause breast cancer? Scientific American **Oct**:169–172, 1995.
7. Safe SH. Do environmental estrogens play a role in development of breast cancer in women and male reproductive problems? Human and Ecol Risk Assessment **1**:17–23, 1995.
8. Safe SH. Environmental and dietary estrogens and human health: Is there a problem? Environ Health Perspect **103**:346–351, 1995.
9. Ojasoo T, Raynaud JP, Dore JC. Affiliations among steroid receptors as revealed by multivariate analysis of steroid binding data. J Steroid Biochem Mol Biol **48**:31–46, 1994.
10. Gupta C, Bullock LP, Bardin CW. Further studies on the androgenic, anti-androgenic, and synandrogenic actions of progestins. Endocrinology **120**:736–744, 1978.
11. Zava DT, Duwe G. Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells *in vitro*. Nutr Cancer **27**:31–40, 1997.
12. McLachlan JA. Functional toxicology: A new approach to detect biologically active xenobiotics. Environ Health Perspect **101**:386–387, 1993.
13. Soto AM, Lin T, Justicia H, Silvia RM, Sonnenschein C. An "in culture" bioassay to assess the estrogenicity of xenobiotics (E-Screen). In: Colburn T, Clement C, Eds. Chemically-Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection. Princeton, NJ: Princeton Scientific Publishing Co., pp295–309, 1992.
14. DiLorenzo DD, Albertini A, Zava DT. Progestin regulation of alkaline phosphatase in the human breast cancer cell line T47D. Cancer Res **51**:4470–4475, 1991.
15. Mauvais-Jarvis P, Kutten F, Gompet A. Estradiol/progesterone interaction in normal and pathologic breast cells. Ann N Y Acad Sci **464**:152–167, 1986.
16. Eckert RL, Katzenellenbogen BS. Effects of estrogens and antiestrogens on estrogen receptor dynamics and the induction of progesterone receptor in MCF7 human breast cancer cells. Cancer Res **42**:139–144, 1982.
17. Ellison PT. Measurements of salivary progesterone. Ann N Y Acad Sci **694**:161–176, 1993.
18. Adlercreutz H, Goldin BR, Gorbach SL, Hockerstedt KA, Watanabe S, Hamalainen EK, Markkanen MH, Makela TH, Wahala KT. Soybean phytoestrogen intake and cancer risk. J Nutr **125**:757S–770S, 1995.
19. Messina MJ, Persky V, Setchell KDR, Barnes S. Soy intake and cancer risk: A review of the *in vitro* and *in vivo* data. Nutr Cancer **21**:113–131, 1994.
20. Franke AA, Custer LJ, Cerna CM, Narala KK. Quantitation of phytoestrogens in legumes by HPLC. J Agric Food Chem **42**:1905–1913, 1994.
21. Wang C, Mäkelä T, Hase T, Adlercreutz H, Kurzer MS. Lignans and isoflavonoids inhibit aromatase enzyme in human preadipocytes. J Steroid Biochem Mol Biol, 1994.
22. Mäkelä S, Poutanen M, Lehtimaki J, Kostian ML, Santti R, Vihko R. Estrogen-specific 17β-hydroxysteroid oxidoreductase Type I as a possible target for the action of phytoestrogens. Proc Soc Exp Biol Med **208**:51–59, 1995.
23. Stoll W. Phytopharmacon influences atrophic vaginal epithelium: Double-blind study of Cimicifuga versus estrogenic substances. Therapeuticum **1**:23–31, 1987.
24. Chury J. Uber den phytoostrogengehalt einiger pflanzen. Experentia **16**:194–200, 1960.
25. Rosenblum ER, Campbell IM, Van Thiel DH, Gavaler JS. Isolation and identification of phytoestrogens from beer. Alcoholism. Clin Exp Res **16**:843–845, 1992.
26. Fenselau C, Talalay P. Is oestrogenic activity present in hops? Fd Cosmet Toxicol **11**:597–603, 1973.
27. Khwaja TA, Dias CB, Pentecost S. Recent studies on the anticancer activities of mistletoe (*Viscum albumin*) and its alkaloids. Oncology **43S**:2–7, 1986.
28. Prior JC, Vigna YM, Barr SI, Rexworthy C, Lentle BC. Cyclic medroxyprogesterone treatment increases bone density: A controlled trial in active women with menstrual cycle disturbances. Am J Med **96**:521–530, 1994.
29. Pollow K, Lubbert H, Boquoi E, Pollow B. Progesterone metabolism in normal human endometrium during the menstrual cycle and in endometrial carcinoma. J Clin Endocrinol Metab **41**:729–737, 1975.
30. Gupta C, Bullock LP, Bardin CW. Further studies on the androgenic, anti-androgenic, and synandrogenic actions of progestins. Endocrinology **102**:736–744, 1978.
31. Dentali S. Hormones and yams: What's the connection? The American Herb Assoc **10**:4–5, 1994.
32. Gaby A. Multilevel yam scam. Townsend Letter for Doctors and Patients **Jan**:96–97, 1996.
33. Kumagai A, Nishino K, Shimomora A, Kin T, Yamamura Y. Effect of glycyrrhizin on estrogen action. Endocrinol Japan **14**:34–38, 1967.
34. Kraus S. The anti-estrogenic action of beta-glycyrrhetinic acid. Exp Med Surg **27**:411–420, 1969.