Estrogenic and Anticarcinogenic Properties of Kurarinone, a Lavandulyl Flavanone from the Roots of *Sophora flavescens*

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Kurarinone, a lavandulyl flavanone, was isolated from a polyphenolic extract of the roots of *Sophora flavescens* using fractionation guided by estrogenic activity, which was determined by recombinant yeast and Ishikawa Var-I bioassays. Kurarinone showed weak estrogenic activity both in the yeast screen and in the Ishikawa Var-I assay with EC_{50} values of 4.6 and 1.66 μM, respectively. Furthermore, kurarinone was found to have potent cytotoxic activity (IC_{50} value = 22.2 μM) against human MCF-7/6 breast cancer cells in the sulforhodamine-B assay.

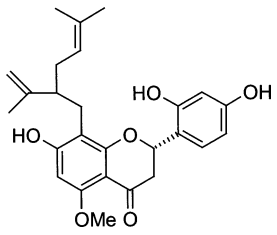
Despite decades of accumulated observational evidence, the balance of risks and benefits for hormone use in healthy postmenopausal women remains uncertain. Recent evidence convincingly demonstrated increased risks for breast cancer[1] and cardiovascular diseases[2] associated with hormone replacement therapy (HRT). There is a renewed interest in naturally occurring phytoestrogens as potential alternatives to HRT, and also in the use of selective estrogen receptor modulators. Various phytotherapeuticals with a claimed hormonal activity are recommended for prevention of discomforts related to a disturbed hormonal balance.[3]

Unlike xenobiotic estrogens (environmental pollutants with estrogenic activity) that have a negative impact on the development of human reproductive organs and male fertility,[4−6] phytoestrogens are believed to have mainly health-beneficial effects.[7,8] Although phytotherapy for prevention of menopausal ailments gained much interest and appreciation during the past few years, its benefits have been questioned.[9,10] It is clear that we need to know much more about the molecular mechanisms, safety, and efficacy of bioactive natural compounds before they can be generally applied to the benefit of postmenopausal women.[11]

Previous investigations on the health-protective effects of a soy-rich diet (soybeans and soy-derived products) have shown that isoflavone-type constituents are beneficial with respect to menopausal symptoms (hot flushes, osteoporosis), hormone-dependent cancers, and cardiovascular diseases.[7,12−14] Together with lignans, coumestans, flavones, and flavanones, isoflavones belong to the larger group of nonsteroidal phytoestrogens. Although phytoestrogens can function as typical estrogens through ERα, it was found that most representatives have a stronger binding affinity for ERβ.[15,16] Besides their estrogenic properties, phytoestrogens exert a wide variety of pharmacological effects in animal cells, including inhibition of tyrosine kinases and DNA topoisomerases, antioxidative effects, interference in a plethora of signaling pathways, cell cycle, and apoptosis events, synergism with growth factors by inducing synthesis or activating receptors, and modulation of important enzymatic activities.[17,18]

Sophora flavescens Ait. (Leguminosae) is a perennial shrub occurring wild as well as cultivated in Northeast Asia. The plant is known as "ku shen", which literally means "the bitter root". This oriental crude drug is used in traditional Chinese medicine as an antipyretic, analgesic, anthelmintic, and stomachic drug. Phytochemical studies of *S. flavescens* have described the isolation of quinolizidine alkaloids, triterpenoids, and a number of flavonoids.[19−22] The occurrence of flavonoids carrying one or multiple prenyl units incited us to investigate their potential estrogenic properties, since we discovered very potent estrogenicity in prenylated flavonoids present in hops, *Humulus lupulus* L.[23−25] Previous research has demonstrated favorable effects of these plant components in relation to hormone-dependent cancers,[26−29] cardiovascular diseases,[14] and (post)menopausal symptoms such as osteoporosis and hot flushes.[11,30,31]

Structure of kurarinone



Results and Discussion

Screening for Estrogenic Activity. Kurarinone, a lavandulyl flavanone present in *S. flavescens*, has been shown to exhibit antiandrogenic activity,[19] but its potential estrogenicity was not investigated. Thus, we embarked on a study aimed at identifying the estrogenic activity by bioactivity-guided fractionation of a polyphenolic fraction of *S. flavescens*, using an estrogen-inducible yeast screen and the Ishikawa Var-I bioassay. It was readily shown that estrogenic activity was present at concentrations above 0.02% with respect to the original polyphenolic extract

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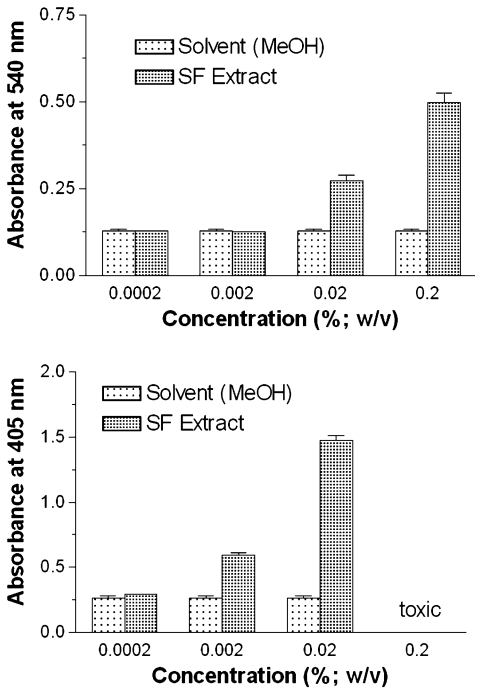

Figure 1. Dose−response data relating to the estrogenicity of a polyphenolic extract of *S. flavescens* (SF) according to an estrogen-inducible yeast screen (upper panel) and the Ishikawa Var-I assay (lower panel). For experimental details, see the Experimental Section.

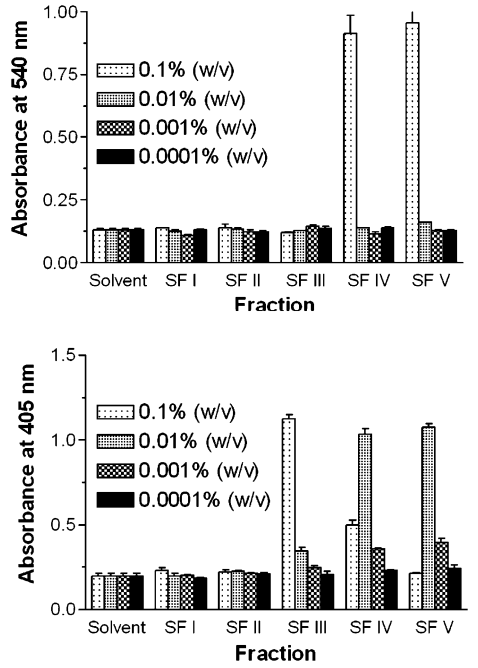

Figure 2. Dose−response data relating to the estrogenicity of five subfractions of a polyphenolic extract of *S. flavescens* (SF) [concentrations expressed as % (w/v)] according to an estrogen-inducible yeast screen (upper panel) and the Ishikawa Var-I assay (lower panel). For experimental details, see the Experimental Section.

(Figure 1). Due to the higher sensitivity of the Ishikawa Var-I-assay, slight differences in the results are apparent, particularly at lower concentrations. The reduced values observed in the Ishikawa Var-I assay at the highest concentration (0.2%) can be attributed to cellular toxicity.

Bioassay-guided fractionation of this polyphenolic extract led to five subfractions according to polarity, SF-I being most polar. As shown in Figure 2, estrogenic activity was present only in the less polar fractions SF-IV and SF-V.

The major compound, which proved to be estrogenically

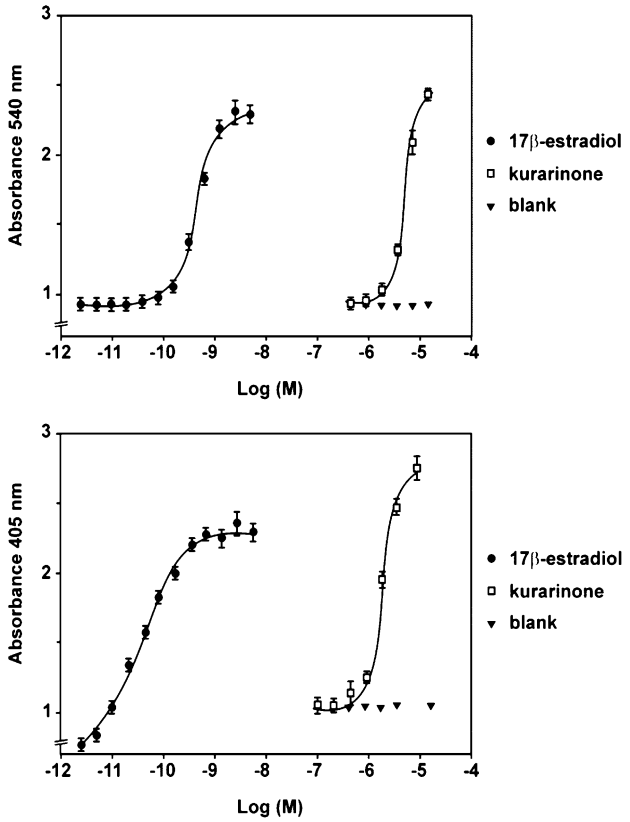

Figure 3. Dose−response curves relating to the estrogenicity of 17β-estradiol and of kurarinone according to an estrogen-inducible yeast screen (upper panel) and the Ishikawa Var-I assay (lower panel). For experimental details, see the Experimental Section.

active, was identified as kurarinone, a lavandulyl flavanone, following comparison of its spectroscopic data with those reported.[19,20] Dose−response curves of the estrogenic activity of kurarinone were assayed by the yeast screen (EC_{50} [kurarinone] = 4.6 μM; EC_{50} [17β-estradiol] = 0.37 nM) and by the Ishikawa Var-I assay (EC_{50} [kurarinone] = 1.66 μM; EC_{50} [17β-estradiol] = 0.047 nM) (Figure 3). The obtained results indicate that kurarinone is a very weak estrogen, in agreement with features of established phytoestrogens.

Screening for Anticarcinogenic Activity. Observations in populations that are daily exposed to phytoestrogens suggest a lower incidence of breast, endometrium, and prostate cancers.[13,32] The in vitro antitumor activity of kurarinone has been demonstrated in various cell lines including A549 (non-small cell lung), SK-OV-3 (ovary), SK-MEL-2 (skin), XF498 (central nerve system), HCT-15 (colon), and HL-60 (human myeloid leukemia).[20,33] We complemented these data with a study on the influence of a polyphenolic extract of *S. flavescens*, the estrogenically active subfractions SF-IV and SF-V, as well as kurarinone (data not shown) on the growth of MCF-7/6 breast cancer cells using the sulforhodamine-B assay. All samples have been examined in octaplicate.

A dose-dependent growth inhibition was established after incubation of the MCF-7/6 cells at particular concentrations of kurarinone. At a concentration of 80 μM, inhibition of growth exceeded 80%. At a concentration of 40 μM, growth was inhibited by approximately 60%. Further dilutions had no significant effect on the growth of MCF-7/6 cells. This screening clearly indicates that kurarinone has a growth-inhibitory effect on MCF-7/6 breast cancer cells with an IC_{50} value (concentration resulting in 50% inhibition of control growth) of 22.2 μM.

It needs to be further established to what extent these effects are estrogen-dependent or -independent.[34−38]

Conclusions

Estrogenicity-guided fractionation of a polyphenolic extract of the roots of *S. flavescens* led to the isolation of kurarinone as the main estrogenically active constituent. Compared to the endogenous (female) hormone 17β-estradiol, kurarinone exhibited a 10 000-fold weaker estrogenic activity. It was demonstrated that kurarinone inhibits the growth of MCF-7/6 breast cancer cells in a dose-dependent manner, with an IC_{50} value of 22.2 μM. Identification of compounds in medicinal plant extracts with estrogenic and anticarcinogenic activity might lead to the development of new drugs for clinical and endemic use. These nonsteroidal modulators may serve as lead compounds for developing new pharmaceuticals to be used in the treatment of hormonal disturbances associated with menopause and certain types of cancer. Further investigations will be necessary to prove whether kurarinone holds promise as a prophylactic compound for the treatment of various postmenopausal disturbances and neoplastic disorders.

Experimental Section

General Experimental Procedures. ^1H and ^{13}C NMR spectra were recorded on a Varian-300 [300 MHz] spectrometer in deuterated methanol. Mass spectra and exact mass measurements were performed on a quadrupole/orthogonal-acceleration time-of-flight [Q/oaTOF] tandem mass spectrometer [qT of 2, Micromass, Manchester, UK] equipped with a standard electrospray ionization [ESI] interface (samples were infused in a 2-propanol/water [1:1] mixture at 3 μL/min). HPLC analyses were performed on a Kontron HPLC pump, equipped with a Kontron photodiode array detector (DAD 440), using an Alltima RP-18 (5 μm, 250 × 4 mm) column (Alltech, Lokeren, Belgium) and a Hibar Lichrospher RP-18 (5 μm, 4 × 4 mm) precolumn (Merck, Overijse, Belgium) for analytical purposes. Preparative HPLC was carried out on a Gilson HPLC pump (Gilson Series SF3, Gilson Medical, Villiers-le-Bel, France), provided with a UV/vis detector using an Econosil RP-18 (10 μm, 250 × 22 mm) column (Alltech, Lokeren, Belgium).

Plant Material. Dried roots of *S. flavescens* were provided by SINECURA bvba, Ghent, Belgium (Source Batch Nr.: IPF/SMJ03312/04.17.03). A voucher specimen was deposited in the herbarium of Ghent University (ADN-SF).

Extraction and Isolation. Dried and chopped roots of *S. flavescens* (100 g) were extracted with isooctane (3 × 1 L) by refluxing during 60 min in a nitrogen atmosphere. After evaporation of the solvent, the residue was extracted with refluxing MeOH/H_2O (3:1, v/v) (3 × 1 L) during 60 min (nitrogen atmosphere). The combined MeOH/H_2O extracts were concentrated and freeze-dried, leaving a residue representing a total polyphenolic fraction of *S. flavescens* (20 mg).[24] Stock solutions were prepared by dissolving the powderous polyphenolic fraction (20 mg) in MeOH (1 mL) followed by serial dilutions.

The extract (1 mL diluted 10-fold in water) was subdivided into five fractions (SFI−V) using solid-phase extraction (Bond Elut 200 mg C_{18} cartridge, Varian, CA) by successive elutions (2 mL) with H_2O (SFI), MeOH/H_2O (1:3, v/v) (SFII), MeOH/H_2O (1:1, v/v) (SFIII), MeOH/H_2O (3:1, v/v) (SFIV), and MeOH (SFV). As fractions SFIV and SFV exhibited significant estrogenicity, several subfractions were isolated using semipreparative HPLC. Concentrated amounts (up to 50 mg) of the estrogenically active fractions were injected onto an octadecylsilica column, and the constituents of interest were collected using gradient elution with MeOH/CH_3CN (50:50, v/v) (solvent B) and 0.05% HCO_2H in H_2O (solvent A) (gradient profile: 0−3 min: 66% B in A [isocratic]; 3−17 min: 66% B in A to 81% B in A; 17−20 min: 81% B in A to 95% B in A; 20−23 min: 95% B in A [isocratic]; 23−25 min: 95% B in A to 66% B in A; 25−27 min: 66% B in A [isocratic]).

Estrogenicity-guided evaluation led to the recognition of a main estrogenically active compound, which was identified as kurarinone by comparison of its relevant spectroscopic data with those reported.[19,20]

Recombinant Yeast Bioassay. In the yeast assay, estrogenic activity was determined using an estrogen-inducible yeast screen (*Saccharomyces cerevisiae*) expressing the human estrogen receptor and containing expression plasmids carrying estrogen-responsive sequences controlling the reporter gene *lac*-Z (encoding the enzyme β-galactosidase). Estrogenic activity was determined from the metabolism of chlorophenol red β-D-galactopyranoside (CPRG) by monitoring the absorbance at 540 nm.[39]

The yeast cells were a gift from Dr. P. Sumpter, Brunel University, Middlesex, UK. The growth medium and yeast culture were prepared according to Routledge et al.[39] A stock solution of 17β-estradiol (E_2, 3.68 mM) was prepared by adding E_2 (10 mg) in EtOH (10 mL) in a sterile tube, from which E_2 concentrations ranging from 0.06 to 1000 nM were prepared. The polyphenolic extract of *S. flavescens* was serially diluted with EtOH, and aliquots (20 μL) of each concentration were transferred to a 96-well flat-bottom microtiter plate and then allowed to evaporate to dryness on the assay plate. Aliquots (200 μL) of the seeded assay medium (medium containing recombinant yeast) and the chromogenic substrate CPRG were then dispensed to each sample well. The 96-well plate also contained one row of blanks (assay medium only) as well as a set of standards of E_2. The plate was then sealed with autoclave tape, shaken for 2 min on a plate shaker, and incubated at 32 °C in a naturally ventilated heating cabinet for 3 days. During incubation, the plate was removed once daily and shaken again for 2 min to mix and disperse the cells. After 3 days of incubation, the absorbances were measured at 540 nm using a plate reader (Dynatech MR5000).

Ishikawa Var-I Bioassay. The Ishikawa Var-I bioassay is based on stimulation of the activity of alkaline phosphatase by estrogens in a human endometrial adenocarcinoma cell line, which was established by Nishida et al. (1985).[40] A variant of this cell line is unresponsive to estrogens by cell proliferation, but is sensitive to the stimulatory effect of the alkaline phosphatase activity. The estrogenic activity was determined from the metabolism of *p*-nitrophenyl phosphate to *p*-nitrophenol by monitoring the absorbance at 405 nm.[41−45]

Ishikawa Var-I cells, kindly provided by Dr. E. Gurpide (Mount Sinai School of Medicine, New York), were routinely maintained in MEM (Eagles minimum essential medium), containing 5% (v/v) FBS (fetal bovine serum) and supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 2.5 mg/mL amphotericin B, 2 mM glutamine, and 1 mM sodium pyruvate. Cells were plated at 1.5×10^6 cells/75 cm^2 flask and were passaged twice weekly. Twenty-four hours before the start of the experiment, nearly confluent cells were incubated in an estrogen-free basal medium (EFBM) of a 1:1 mixture of phenol red-free Ham's F12 and Dulbecco's modified Eagles medium, the supplements listed above, and 5% charcoal-stripped FBS. Just prior to the assay, cells were harvested with 0.25% trypsin-EDTA. After centrifugation, the cell pellet was resuspended in EFBM, then cells were counted and diluted appropriately in EFBM. Cells were plated out in 96-well flat-bottomed microtiter plates (ICN Biochemicals, Oxon, UK) in EFBM at a density of 2.5×10^4 cells/well in 100 μL aliquots.

Samples and E_2 were dissolved in EtOH and diluted appropriately in EFBM (final concentration of EtOH < 0.1%); 50 μL aliquots of the samples were added to individual wells in duplicate. The plates were incubated for 72 h in a humidified atmosphere of 5% CO_2 in air. At the end of the incubation period, the microtiter plates were inverted and the growth medium was removed. The plates were rinsed five times by gentle immersion in phosphate buffer saline (PBS: 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4). After the final rinsing, PBS was shaken out and the plates were frozen at −80 °C for 20−30 min to fracture the cell membranes. The

plates were placed on ice, and 50 μL of an ice-cold buffer solution containing 5 mM *p*-nitrophenyl phosphate (Sigma Fast pNPP tablet) was added to each well. The plates were incubated at 37 °C for 60 min, and the formation of *p*-nitrophenol was monitored using a plate reader (Dynatech MR5000) at 405 nm.

Sulforhodamine-B Bioassay. Human MCF-7/6 breast cancer cells, obtained from Dr. H. Rochefort (Unité d'Endocrinologie Cellulaire et Moléculaire, Montpellier, France), were investigated using the sulforhodamine-B (SRB) bioassay, a colorimetric in vitro anticancer drug screening test.[46−50] The cells, originally derived from a pleural effusion of a metastasized mammary adenocarcinoma, possess fully characterized estrogen receptors, and their proliferation in vitro is modulated by E_2 and by other ligands of the steroid/thyroid receptor superfamily.[51]

The bioassay estimates the inhibition rate of cell proliferation after continuous exposure of the MCF-7/6 cells, seeded at 5×10^3 cells/well in a 96-well microtiter plate, to varying concentrations of kurarinone. The cells were maintained in a 50:50 mixture of Dulbecco's modification of Eagle's medium (DMEM) and Ham F12 (Gibco, Merelbeke, Belgium), supplemented with 100 IU/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), 2.5 μg/mL amfotericin B (Bristol-Myers Squibb, Brussels, Belgium), and 10% fetal bovine serum (FBS). A stock solution [40 mM] of kurarinone was obtained by dissolving the lyophylized compound in methanol [control on solvent effects]. Dilutions (1/500−1/10^5) of this stock solution were prepared in the medium. After 72 h, the MCF-7/6 cells were fixed to the bottom of the wells by adding 50% trichloroacetic acid. After incubation for 1 h at 2 °C, the wells were washed (5×) with water and allowed to dry. SRB was added to the wells, and after 30 min, the wells were washed (4×) with 1% HOAc to remove unbound dye. Bound SRB was redissolved in Tris buffer (pH 10.8), and the absorbances were measured at 490 nm using a V_{max} plate reader (Molecular Devices, Palo Alto, CA).

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