Evaluation of the Phytoestrogenic Activity of Cyclopia genistoides (Honeybush) Methanol Extracts and Relevant Polyphenols

Nicolette J. D. Verhoog,† Elizabeth Joubert,‡,§ and Ann Louw*,†

Department of Biochemistry, and Department of Food Science, Stellenbosch University, Stellenbosch 7600, South Africa, and Post-Harvest & Wine Technology Division, ARC Infruitec-Nietvoorbij, Stellenbosch 7600, South Africa

Unfermented C. genistoides methanol extracts of different harvestings and selected polyphenols were evaluated for phytoestrogenic activity by comparing binding to both ER subtypes, transactivation of an ERE-containing promoter reporter, proliferation of MCF-7-BUS and MDA-MB-231 breast cancer cells, and binding to SHBG. The extracts from one harvesting of C. genistoides (P104) bound to both ER subtypes. All extracts transactivated ERE-containing promoter reporters via ERβ but not via ERα. All extracts, except P122, caused proliferation of the estrogen-sensitive MCF-7-BUS cells. Proliferation of MCF-7-BUS cells was ER-dependent as ICI 182,780 reversed proliferation. Physiologically more relevant, extracts antagonized E2-induced MCF-7-BUS cell proliferation. Furthermore, all extracts, except P122, induced proliferation of the estrogen-insensitive MDA-MB-231 cells, suggesting that the extracts are able to induce ER-dependent and ER-independent cell proliferation. Binding to SHBG by extracts was also demonstrated. These results clearly show that C. genistoides methanol extracts display phytoestrogenic activity and act predominantly via ERβ. HPLC and LC–MS analysis, however, suggests that the observed phytoestrogenic activity cannot be ascribed to polyphenols known to be present in other Cyclopia species.

KEYWORDS: Phytoestrogens; ERα; ERβ; MCF-7-BUS cell proliferation; MDA-MB-231 cell proliferation; SHBG; honeybush; Cyclopia genistoides

INTRODUCTION

Cyclopia genistoides, a fynbos shrub, together with C. subternata, C. intermedia, and to a lesser extent C. sessiliflora, are commercially available as the fragrant caffeine-free honeybush tea. Honeybush tea has already been identified as having both antioxidant and antimutagenic activity, which adds value to this herbal infusion (1). The presence of the known phytoestrogens, formononetin, eriodictyol, and naringenin, in C. intermedia (2) and luteolin in both C. intermedia and C. subternata (2, 3) plus anecdotal evidence that honeybush tea helps alleviate menopausal symptoms led to the investigation of putative phytoestrogenic activity in Cyclopia spp.

Phytoestrogens are plant polyphenols able to mediate weak estrogenic or anti-estrogenic activity (4). Most research investigating phytoestrogens has concentrated on soybean and the isoflavone, genistein, a well-documented phytoestrogen abundantly present in soy (5). Epidemiological studies suggest that an Asian diet rich in soy is protective against hormone-induced cancers such as breast and prostate cancer (6–9). In addition, phytoestrogens are thought to be useful for the treatment of menopausal symptoms and to protect postmenopausal women against cardiovascular disease and osteoporosis, without the risks associated with traditional hormone replacement therapy (HRT) (10–14). However, some studies have failed to show significant alleviation of menopausal symptoms, such as hot flushes, while other studies, although showing some efficacy, suggest that phytoestrogen treatment is not as effective as traditional HRT (15–17). Recently, the safety of long-term use of traditional HRT has been questioned by several studies (18–20). This and the general increase in popularity of natural medicine have lent impetus to the search for and investigation into alternative treatments (21).

A previous study by our group (22), which screened extracts from the four commercially available Cyclopia species for estrogenic activity through binding to the ER subtypes, identified methanol extracts from C. genistoides as consistently having the highest binding affinity for both ER subtypes.

The biological responses to estrogen are mediated mainly via the estrogen receptor (ER) subtypes, ERα and ERβ (23). The ERs are ligand-activated transcription factors (24) that dissociate from heat shock proteins on activation by ligand. Activation also involves a conformational change, which allows dimerization and binding to estrogen response elements (EREs)
situated in the promoter region of estrogen responsive genes thereby activating or inhibiting transcription (23). Phytoestrogens are able to compete with 17-β-estradiol (E2) for binding to the ER subtypes and are able to act as either agonist or antagonist when bound to the ERs (25). Phytoestrogens generally bind to the ER subtypes with a much lower affinity than E2 and display, unlike E2, a higher affinity for ERβ than for ERα (25, 26). In addition, phytoestrogens have been shown to induce transactivation via both ER subtypes (27), with an increased transcriptional response through ERβ. They are, however, less potent than E2 via both ER subtypes (27, 28).

Estrogens are responsible for the proliferation and differentiation of a number of tissues (29), and this property is often used to evaluate estrogenicity (30). Hyper-proliferation can cause or enhance the spread of cancer (31). The ERβ subtype is believed to be a negative modulator of ERα-mediated activity as it has been demonstrated to inhibit transactivation and cell proliferation when coexpressed with ERα (32–34). ERβ is thus believed to be the natural cellular protective mechanism against excessive cell proliferation mediated by ERα, and numerous studies concentrate on compounds, such as phytoestrogens, which are able to distinguish between the two ER subtypes with preferential binding to and/or transactivation through ERβ (33).

Estrogens circulating in the blood are transported primarily bound to serum albumin or sex hormone-binding globulin (SHBG) (35). Only unbound estrogens are able to diffuse across the cell membrane and mediate an estrogenic response (36). It has been suggested that phytoestrogens may alter the concentration of biologically active endogenous estrogens, by either binding to SHBG and displacing bound estrogens or by stimulating SHBG synthesis (37). It is thus clear that phytoestrogens not only have a direct effect on estrogen signaling through binding to the ER subtypes, but also an indirect effect through altering the concentrations of biologically active estrogens.

In the present study, methanol extracts from *C. genistoides* (Table 1) as well as known polyphenols present in Cyclopia spp., which either were shown to bind to both ER subtypes (luteolin, formononetin, and naringenin) or were present at very high concentrations such as mangiferin, were further investigated (Figure 1). Although useful as an initial screening technique, binding to the ER subtypes alone does not distinguish agonist from antagonist activity, and thus the present study extends the initial investigation (22) by including a number of other in vitro assays such as the transactivation of an ERE-containing promoter reporter construct, cell proliferation of two breast cancer cells, and binding to SHBG. In addition, HPLC and LC–MS analysis was done on the specific methanol extracts investigated to quantify and confirm the identity of the polyphenols known to be present in other Cyclopia species.

### MATERIALS AND METHODS

**Test Compounds Used.** 17-β-Estradiol, genistein, mangiferin, and naringenin were purchased from Sigma-Aldrich (Cape Town, South Africa), and luteolin and formononetin were from Extrasynthese (Genay, France).

**Dried Methanol Extract (DME) Preparation.** Two methanol extracts of unfermented *C. genistoides* were prepared from each of three independent harvestings. The extraction was repeated to compare different methanol extractions of the same plant material (Table 1). *Cyclopia genistoides* plants were chosen randomly in a plantation, and several bushes were harvested on each occasion. The harvested plant material (Table 1), comprising intact stems and leaves, was dried whole at 40 °C to less than 10% moisture content, whereafter it was milled (1 mm sieve) and stored at room temperature in a sealed container. Dried, pulverized, unfermented plant material (25 g) was extracted three times with 50 mL of dichloromethane at room temperature for 20 h each, filtered through Whatman No. 4 filter paper with a Buchner funnel, and the filtrate was discarded. Thereafter, methanol extraction (50 mL) of the air-dried plant material was performed twice at room temperature for 20 h each. The methanol extracts were pooled with a
small volume of water added and evaporated under vacuum before freeze-drying. Freeze-dried DMEs were ground in a darkened room to a fine homogeneous powder, which was stored in glass vials, covered with aluminum foil, and placed in vacuum-sealed desiccators in the dark at room temperature.

**Cell Culture.** COS-1 cells (ATCC) and estrogen-insensitive MDA-MB-231 cells (**38**) (a kind gift from G. Haegemann, University of Gent, Belgium) were maintained in DMEM supplemented with 10% (v/v) fetal calf serum (FCS) and a penicillin (100 IU/mL) and streptomycin (100 μg/mL) mixture (penicillin-streptomycin). The ERα and ERβ positive MCF-7-BUS cells (**38**) (a kind gift from A. Soto, Tufts University, U.S.) were maintained in DMEM supplemented with 5% (v/v) heat-inactivated FCS, but without antibiotics. All cells were maintained in a humidified cell incubator set at 97% relative humidity and 5% CO2 at 37 °C.

**Transient Transfections and Whole Cell Binding Assays in COS-1 Cells.** COS-1 cells were plated at a density of 2 × 10⁶ cells per 10 cm tissue culture dish. Twenty-four hours after plating, COS-1 cells were transiently transfected with expression vectors for the ER subtypes, pcDNA3-hERα (a kind gift from D. Harnish, Women’s Health Research Institute, Wyeth-Ayerst Research, U.S.) or pcDNA5-hERβ (a kind gift from F. Gannon, European Molecular Biology Laboratory, Heidelberg, Germany) and a filler vector, pGL2-basic (Promega Corp., Madison, WI). Two different transfections methods were used to transfect the ER subtypes. The Fugene6 transfection reagent was used for the hERα transfections, and the DEAE-Dextran transfection method was used for hERβ transfections. The total DNA transfected for both transfection protocols was 6 μg/10 cm dish that consisted of 0.72 μg of receptor and 5.28 μg of empty vector. The Fugene6 transfection protocol, used for hERα, was per the manufacturer’s instructions with 12 μL of Fugene6 reagent allowed to react with 6 μg of DNA. The DEAE-Dextran transfection medium, used for hERβ, consisted of 5 mL of DMEM, pre-heated to 37 °C, 0.1 mM chloroquine (stock solution 100 μM), 6 μg of DNA, and finally 0.1 mg/mL DEAE-Dextran solution (stock solution 10 mg/mL). Cells were incubated with the DEAE-Dextran transfection medium for 1 h at 37 °C after which they were shocked with 10 mL of pre-heated 10% DMSO—PBS for about 2 min. Finally, transiently transfected cells were incubated at 37 °C overnight in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin mixture. The following day the transient COS-1 cells were pooled and seeded into 24-well tissue culture plates at a density of 5 × 10⁴ cells/well and incubated for 24 h. The next day the cells were washed three times with 300 μL of PBS/well (pre-heated at 37 °C). This was followed by a 2-h incubation of the transfected cells with 10⁻⁷ M radiolabeled estradiol (2,4,6,7-³H-17β-estradiol from Amersham, Cape Town, South Africa, with specific activity 87.0 Ci/g of DNA; and counting efficiency of 46%) and various concentrations, ranging from 2.7 × 10⁻¹³ to 7.94 × 10⁻⁹ mg/mL, of unlabeled competitors, that is, extracts and polyphenols (dissolved in DMSO), to measure β-galactosidase activity with the β-galactosidase chemiluminescent Galacto-Star reporter gene assay system for mammalian cells (Tropix Inc. (Applied Biosystems, Bedford, MA)). Luciferase assay reagent (Promega Corp., Madison, WI) was used to quantify luciferase activity in accordance with the manufacturer’s instructions. Briefly, 10 μL of cell lysate was allowed to react with 50 μL of luciferase assay reagent. The relative light units (RLU’s) were measured using the Veritas luminometer. A further 5 μL of lysis buffer (Tropix Inc. (Applied Biosystems, Bedford, MA)). Luciferase RLU’s were normalized with β-galactosidase readings, and results were expressed as normalized fold induction with negative controls (0.1% DMSO) taken as 1.

**MTT Cell Proliferation Assay.** MCF-7 BUS and MDA-MB-231 cells were plated at a density of 2500 cells/well in 96-well plates and incubated for 24 h. The cells were then washed with 200 μL of PBS, pre-warmed to 37 °C, followed by steroid starving for 72 h through addition of DMEM pre-warmed to 37 °C without phenol red, but supplemented with 5% charcoal stripped FCS and 1% penicillin-streptomycin mixture. On day five the medium was aspirated, 50 μL of lysis buffer (Tropix Inc. (Applied Biosystems, Bedford, MA)) was added, and cells were frozen at −80 °C overnight. Luciferase assay reagent (Promega Corp., Madison, WI) was used to quantify luciferase activity in accordance with the manufacturer’s instructions. Briefly, 10 μL of cell lysate was allowed to react with 50 μL of luciferase assay reagent. The relative light units (RLU’s) were measured using the Veritas luminometer. A further 5 μL of lysis buffer (Tropix Inc. (Applied Biosystems, Bedford, MA)). Luciferase RLU’s were normalized with β-galactosidase readings, and results were expressed as normalized fold induction with negative controls (0.1% DMSO) taken as 1.
Table 2. Whole Cell Competitive Binding by E2, Polyphenols, and DME to the hER Subtypes

<table>
<thead>
<tr>
<th>test compounds</th>
<th>IC50 (mg/mL)²</th>
<th>RBA (%)</th>
<th>β/α of Kd</th>
<th>Kd (M)</th>
<th>β/α of Kd</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>3.7 × 10⁻⁹ (3.69)**</td>
<td>7.3 × 10⁻⁸ (7.47)</td>
<td>100</td>
<td>1</td>
<td>0.37 × 10⁻³ M (6.44)</td>
</tr>
<tr>
<td>genistein</td>
<td>4.2 × 10⁻⁵ (37.32)**</td>
<td>9.0 × 10⁻⁷ (1.23)</td>
<td>0.73</td>
<td>111.10</td>
<td>43.1 × 10⁻³ M (8.99)**</td>
</tr>
<tr>
<td>luteolin</td>
<td>1.5 × 10⁻⁴ (4.88)**</td>
<td>1.4 × 10⁻⁴ (3.07)**</td>
<td>0.00 3</td>
<td>0.52</td>
<td>173.35</td>
</tr>
<tr>
<td>formononetin</td>
<td>4.1 × 10⁻⁴ (4.59)**</td>
<td>1.5 × 10⁻⁴ (0.45)**</td>
<td>0.93</td>
<td>0.48</td>
<td>0.52</td>
</tr>
<tr>
<td>naringenin</td>
<td>3.9 × 10⁻⁴ (8.38)**</td>
<td>1.5 × 10⁻⁴ (2.88)**</td>
<td>0.09 7</td>
<td>0.48</td>
<td>4.95</td>
</tr>
<tr>
<td>mangiferin</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>N P104</td>
<td>2.1 × 10⁻⁴ (4.88)**</td>
<td>1.3 × 10⁻⁴ (26.28)**</td>
<td>0.18</td>
<td>0.0006</td>
<td>0.0006</td>
</tr>
<tr>
<td>O P104</td>
<td>5.9 × 10⁻⁴ (19.07)**</td>
<td>2.3 × 10⁻⁴ (19.24)**</td>
<td>0.05</td>
<td>0.0003</td>
<td>0.0006</td>
</tr>
<tr>
<td>N P105</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>O P105</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>N P122</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
<tr>
<td>O P122</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
</tbody>
</table>

² The IC50 and CV (coefficient of variation) values are calculated from the log IC50 values from at least three independent experiments. β RBA or relative binding affinity is expressed relative to that of E2 (100%) and was calculated as follows: 100 × IC50 (E2)/IC50 (test compound). β/α ratio of RBA or K is such that the ratio is >1 for compounds having a higher affinity for hERα than hERβ, < 1 if compounds have a higher binding affinity for hERβ than hERα, and equal to 1 for compounds having a similar affinity for both ER subtypes. The β/α ratio of RBA is calculated by RBA hERα/RBA hERβ, and the β/α ratio of K is calculated by K i hERα/K i hERβ. K values were determined from the Kd of E2. The Kd values of E2 for hERα and hERβ were 0.37 ± 0.03 ± 0.38 M and 1.17 ± 0.38 ± 0.18 M, respectively. Statistically different from genistein with * representing P < 0.05, ** representing P < 0.01, and *** representing P < 0.001. Statistically different from E2 with * representing P < 0.05, ** representing P < 0.01, and *** representing P < 0.001. NB = non-binder polyphenols or extracts were unable to displace ³H-E2 from ER subtype.

Competitive SHBG Binding Assay. Displacement of 20 × 10⁻⁹ M ³H-E2 by test compounds and DME from SHBG was examined by the competitive SHBG binding assay as adapted from the method used by Hammond and Lahteenmaki (40). Pooled human pregnancy serum with a SHBG concentration of 408.6 ± 10⁻⁹ M was diluted (1:100) with dextran-coated charcoal (DCC; 1.25 g of activated charcoal Norit C1A and 0.125 g of T70 dextran were added to 500 mL of 0.02% gelatin-PBS mixture). Briefly, 20 μL of pregnancy serum was added to 2 mL of DCC-slurry and mix at room temperature for 30 min. Following centrifugation at 5000g at room temperature, the supernatant was collected, and 100 μL of diluted serum was added to 100 μL each of unlabeled E2 (10⁻⁵ M), polyphenols (10⁻⁷ M), and DMSO vehicle only (negative control) as competitors. This was followed by the addition of 100 μL of PBS containing 60 × 10⁻⁹ M ³H-E2. The mixture was allowed to incubate for 1 h at room temperature followed by 15 min incubation in an ice-water bath kept at 4 °C. The bound ³H-E2 was then removed by incubating with 750 μL of ice-cold DCC-slurry for 10 min followed by centrifugation at 3000g for 3 min at 4 °C. The supernatant was quickly decanted, and a constant volume (750 μL) was added to scintillation vials containing 3 mL of scintillation fluid. Radioactivity was read on the Beckman LS 3801 scintillation counter. Results are expressed as the percentage 20 × 10⁻⁹ M ³H-E2 displaced from SHBG. The total bound, that is, in presence of vehicle (DMSO) only, represents 0% ³H-E2 displaced from the SHBG.

HPLC and LC–MS Analysis. DAD-HPLC analysis of the extracts was carried out according to Verhoog et al. (22) on a Phenomenex Synergy MAX-RP 80A (C12 reversed-phase with TMS end-capping) column using an aqueous acetic acid–acetonitrile gradient with quantification at 280 nm. For further confirmation of peak identity, the extracts were subjected to LC–MS analysis, using a Waters API Quattro Micro apparatus with a Waters 2690 quaternary HPLC pump and 996 photodiode array detector, and electrospray ionization operating in the negative mode. The operation conditions entailed: desolvation gas temperature 350 °C; nebulizing gas (nitrogen) flow rate, 500 L/h; source temperature, 120 °C; capillary voltage, 3500 V; and cone voltage, 25 V. Separation conditions was the same as for the HPLC analysis, except that the 2% acetic acid was replaced by 0.1% formic acid as the mobile phase. The same authentic standards of compounds tentatively identified by DAD-HPLC were analyzed for further confirmation of peak identity.

Data Manipulation and Statistical Analysis. The GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA) was used for graphical representations and statistical analysis. One-way ANOVA and Dunnett’s multiple comparisons’ test as post-test were used for statistical analysis. P-values are represented as follows:

- statistically different from E2 by * (P < 0.05), ** (P < 0.01), and *** (P < 0.001) and statistically different from genistein by * (P < 0.05), ** (P < 0.01), and *** (P < 0.001). Nonlinear regression and one-site competition curve fitting were used to graph the data from the whole cell binding assays and to determine IC50 values. The relative binding affinity (RBA) is expressed relative to that of E2 (100%) and was calculated as follows: 100 × IC50 (E2)/IC50 (test compound). The Kd values were determined from the IC50 values and Kd for E2 according to the equation by Cheng and Prusoff (41). Nonlinear regression and sigmoidal dose response curve fitting were used to graph the data from the ERE-containing promoter reporter and proliferation experiments and to determine fold induction and EC50. For all experiments, unless otherwise indicated, the error bars represent the SEM of three independent experiments done in triplicate.

RESULTS

Binding to ER Subtypes. All polyphenols were able to bind to both ER subtypes, except for the xanthone, mangiferin (Table 2). The order of potency for hERα (IC50 values) was E2 ≫ formononetin > genistein > naringenin ≫ luteolin. Generally, all polyphenols, including genistein, bound to ERα displayed significantly (P < 0.01) weaker binding than E2 with RBA values ranging from 0.93% for formononetin to 0.003% for luteolin. The order of potency for hERβ was E2 > genistein ≫ luteolin > formononetin = naringenin. All polyphenol IC50 values for binding to hERβ were significantly (P < 0.01) lower than those for E2 and genistein with RBA values ranging from 0.48% for naringenin to 0.52% for luteolin. All of the polyphenols that bound, except formononetin, had a higher binding affinity for the hERβ, in contrast to E2 that had a slightly higher affinity for ERα. Genistein, especially, had a very high binding affinity (Kd = 1.01 × 10⁻³ M) for hERβ and showed a strong preference for this subtype (Kd/β/α ratio = 42.7). Formononetin, similarly to E2, had a slight binding preference (Kd/β/α ratio = 0.25) for hERα.

The DME, even though from the same species, portrayed large variations in binding to the ER subtypes with only the two extracts from the P104 harvesting able to significantly (P < 0.01) compete with ³H-E2 for binding to the ER subtypes (Table 2). O P104 displayed a lower potency than N P104. The binding potencies measured for hERα and hERβ of N P104 and O P104 were significantly different (P < 0.01) from those of E2 and genistein. Although N P104 in comparison to O P104
had higher potencies for both ER subtypes, they were not significantly ($P > 0.05$) different from each other (statistical data not shown). In contrast to most of the polyphenols investigated, formononetin and mangiferin excluded, P104 had a higher RBA and a stronger preference ($RBA_{eta/\alpha}$ ratio = 0.003 and 0.006 for N P104 and O P104, respectively) for the ER$\alpha$ subtype.

**Transactivation of an ERE-Containing Promoter Reporter Construct via the hER Subtypes.** E$_2$ transactivated hER$\alpha$ and hER$\beta$ with similar potencies, while the polyphenols generally, with the exception of luteolin, transactivated more potently via hER$\beta$ (Table 3). The order of potency via hER$\alpha$ was E$_2$ $>$ genistein $>$ formononetin $>$ luteolin, while via hER$\beta$ it was E$_2$ $>$ genistein $>$ formononetin $>$ naringenin $>$ luteolin. The potency of E$_2$ via hER$\alpha$ was significantly different ($P < 0.01$) from that of genistein, luteolin, and formononetin, while only the potency of genistein was significantly different ($P < 0.05$) from that of luteolin. The potency of E$_2$ via hER$\beta$ was significantly ($P < 0.01$) higher than that of the polyphenols, except genistein, while the potency of genistein, however, was only significantly different ($P < 0.05$) from that of luteolin and naringenin, but not formononetin. The transactivation efficacy of the various polyphenols via hER$\alpha$ was luteolin $>$ formononetin $>$ genistein $>$ E$_2$, with luteolin and formononetin not significantly different ($P > 0.05$) from E$_2$ and genistein, with the latter not statistically different ($P > 0.05$) from each other. The transactivational efficacy of the various polyphenols via hER$\beta$ did not differ significantly ($P > 0.05$) from each other or from that of E$_2$ and genistein (Table 3). Although both E$_2$ and genistein, in contrast to the polyphenols tested, displayed a relatively high potency for both binding and ERE-containing promoter reporter assays via hER$\beta$, the transactivational efficacy was approximately similar for all polyphenols and E$_2$ ($P > 0.05$).

The DMEs were only able to induce the ERE-containing promoter reporter construct via hER$\beta$, but not via hER$\alpha$ (Table 3) despite the fact that some extracts (from the P104 harvesting) were able to displace $^{3}H$E$_2$ from both hER$\beta$ and hER$\alpha$, with higher RBAs for hER$\alpha$ than for hER$\beta$ (Table 2). The order of potency (EC$_{50}$) of E$_2$, genistein, and extracts was as follows for hER$\beta$: E$_2$ $>$ genistein $>$ O P122 $>$ O P104 $>$ N P104 $>$ O P105 $>$ N P105 (Table 3). Potencies of extracts, via hER$\beta$, were not significantly ($P > 0.05$) different from that of E$_2$, except for N P104 and O P105, while none of the extracts were significantly different from genistein. The efficacy of the extracts, via hER$\beta$, was not significantly different ($P > 0.05$) from that of either genistein or E$_2$.

**Proliferation of Breast Cancer Cells.** All polyphenols investigated were able to induce cell proliferation of the MCF-7-BUS cells in a dose-dependent manner with the order of potency being E$_2$ $>$ naringenin $>$ genistein $>$ luteolin $>$ formononetin $>$ mangiferin (Table 4). All of the potencies of the polyphenols were significantly different ($P < 0.05$) from that of E$_2$ but not significantly ($P > 0.05$) different from that of genistein. The order of efficacy for the cell proliferation of the MCF-7-BUS cells was genistein $>$ E$_2$ $>$ naringenin $>$ mangiferin $>$ formononetin $>$ luteolin (Table 4). None of the efficacy values determined for the polyphenols were significantly ($P > 0.05$) different from that of E$_2$ or genistein except for luteolin and formononetin ($P < 0.01$). Neither the polyphenols nor E$_2$ were able to induce significant proliferation of the MDA-MB-231 cells (Table 4).

DMEs from harvestings P104 and P105 were able to induce cell proliferation of both human breast cancer cells, whereas DMEs from harvesting P122 were unable to induce proliferation of either of the two cell lines tested (Table 4). The rank order of potency (Table 4) in MCF-7-BUS cells was as follows: E$_2$ $>$ genistein $>$ N P104 $>$ N P105 $>$ O P104 $>$ O P105. The potency of the DMEs in MCF-7-BUS cells (Table 4) was significantly ($P < 0.01$) lower than that of E$_2$ with only O P104 and O P105 having a significantly ($P < 0.05$) lower potency than genistein. The rank order of efficacy (Table 4) was as follows: genistein $>$ O P104 $>$ E$_2$ $>$ N P104 $>$ N P105 $>$ O P105. The efficacy of the DMEs in MCF-7-BUS cells was not significantly ($P > 0.05$) different from that of E$_2$ or genistein with the exception of O P105, which was significantly ($P < 0.05$) different from that of genistein.

Similar to results with MCF-7-BUS cells, P104 and P105 were able to induce, albeit to a lesser extent, cell proliferation of the estrogen-insensitive MDA-MB-231 cell line (Table 4). However, P122, E$_2$, and genistein were unable to induce proliferation. The rank order of potency (Table 4) was as follows: O P104 $>$ N P105 $>$ O P105 $>$ N P104. The potency values for the extracts were not significantly ($P > 0.05$) different from each other (statistical data not shown). The rank order of efficacy (Table 4) was as follows: N P105 $>$ N P104 $>$ O

---

Table 3. Potency (EC$_{50}$) and Efficacy (Maximal Fold Induction) Values As Determined from Transactivation of an ERE-Containing Promoter Reporter Gene Construct via hER$\alpha$ or hER$\beta$ for E$_2$, Various Polyphenols, and the DMEs

<table>
<thead>
<tr>
<th>test compounds or DME</th>
<th>potency (EC$_{50}$) mg/mL</th>
<th>efficacy (maximal fold induction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E$_2$</td>
<td>$3.70 \times 10^{-7}$ (0.49)**</td>
<td>$1.39 \times 10^{-7}$ (4.99)</td>
</tr>
<tr>
<td>genistein</td>
<td>$9.03 \times 10^{-5}$ (8.15)**</td>
<td>$1.06 \times 10^{-6}$ (4.93)</td>
</tr>
<tr>
<td>luteolin</td>
<td>$1.97 \times 10^{-3}$ (4.93)**</td>
<td>$3.53 \times 10^{-3}$ (38.69)**</td>
</tr>
<tr>
<td>formononetin</td>
<td>$1.01 \times 10^{-3}$ (4.36)**</td>
<td>$4.29 \times 10^{-3}$ (5.53)**</td>
</tr>
<tr>
<td>naringenin</td>
<td>N/A</td>
<td>$1.04 \times 10^{-4}$ (4.68)**</td>
</tr>
<tr>
<td>mangiferin</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>N P104</td>
<td>1.51 $\times 10^{-3}$ (22.60)**</td>
<td>N/A</td>
</tr>
<tr>
<td>O P104</td>
<td>1.18 $\times 10^{-3}$ (21.36)**</td>
<td>N/A</td>
</tr>
<tr>
<td>N P105</td>
<td>9.20 $\times 10^{-3}$ (12.31)</td>
<td>N/A</td>
</tr>
<tr>
<td>O P105</td>
<td>2.93 $\times 10^{-3}$ (22.66)**</td>
<td>N/A</td>
</tr>
<tr>
<td>N P122</td>
<td>6.90 $\times 10^{-3}$ (0.21)</td>
<td>N/A</td>
</tr>
<tr>
<td>O P122</td>
<td>2.48 $\times 10^{-4}$ (0.59)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*$^*$ EC$_{50}$ values calculated from the log EC$_{50}$ values of three independent experiments given as the mean (CV). $^*$ CV (coefficient of variation) calculated from the log EC$_{50}$ of at least three independent experiments performed in triplicate. $^*$ Statistically different from genistein with $^*$ representing $P < 0.05$, $^{**}$ representing $P < 0.01$, and $^{***}$ representing $P < 0.001$. $^*$ Statistically different from E$_2$ where $^*$ represents $P < 0.05$ and $^{**}$ represents $P < 0.01$. N/A: test compound or DME did not induce the ERE-containing promoter reporter gene construct via the indicated hER subtype.
P104 > O P105. None of the efficacies were significantly different from each other (statistical data not shown).

To establish whether induced cell proliferation was ER dependent, cells were co-treated with an ER antagonist, ICI 182,780. In MCF-7 BUS cells, co-treatment with $1 \times 10^{-9}$ M ICI 182,780 reduced the response induced by all polyphenols (Figure 2A), DMEs (Figure 3A), and E2, suggesting that the proliferation response in these cells is ER-dependent as has been previously suggested (42, 43). Similarly, in the MDA-MB-231 cells, ICI 182,780 reduced the minimal induction by all of the polyphenols (Figure 2B) and E2 to that of the level of the control. Induction by the DME in MDA-MB-231 cells, however, was only partially reversed by ICI 182,780 in the case of P104 and P105, while in the case of P122 the antagonist appeared to stimulate induction (Figure 3B).

In addition, the effect of the polyphenols or C. genistoides DMEs on E2-induced proliferation in MCF-7 BUS cells was investigated. Physiologically more relevant, this would establish how the polyphenols and extracts would react in the presence of the endogenous ligand. E2 proliferation in MCF-7-BUS cells was significantly ($P < 0.05$) prevented by co-treatment with all of the polyphenols, except mangiferin (Figure 4A) and all of the DMEs (Figure 4B), including P122, despite the fact that P122 did not induce cell proliferation on its own (Table 4). The polyphenols, genistein, luteolin, formononetin, and naringenin, and the DME, therefore antagonized E2-induced proliferation and appeared to act as anti-estrogens in the presence of $1 \times 10^{-9}$ M E2.

**Table 4.** Potency (EC50) and Efficacy (Maximal Fold Induction) Values Determined for E2, Various Polyphenols, and DME from Cell Proliferation Assays in MCF-7-BUS and MDA-MB-231 Cells

<table>
<thead>
<tr>
<th>Test Compounds</th>
<th>MCF-7-BUS Cells</th>
<th>MDA-MB-231 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Potency (EC50) mg/mL</td>
<td>Efficacy (Maximal Fold Induction)</td>
</tr>
<tr>
<td>E2</td>
<td>2.79 ± 10^{-10} (2.92)**</td>
<td>2.14 (8.46)</td>
</tr>
<tr>
<td>Genistein</td>
<td>1.02 ± 10^{-6} (7.56)**</td>
<td>2.35 (10.57)</td>
</tr>
<tr>
<td>Luteolin</td>
<td>2.54 ± 10^{-6} (15.77)**</td>
<td>1.26 (2.52)**</td>
</tr>
<tr>
<td>Formononetin</td>
<td>1.48 ± 10^{-5} (14.90)**</td>
<td>1.36 (4.15)**</td>
</tr>
<tr>
<td>Naringenin</td>
<td>3.27 ± 10^{-5} (1.60)*</td>
<td>2.08 (4.15)</td>
</tr>
<tr>
<td>Mangiferin</td>
<td>3.13 ± 10^{-4} (31.07)**</td>
<td>1.72 (3.44)</td>
</tr>
<tr>
<td>N P104</td>
<td>1.98 ± 10^{-4} (7.34)**</td>
<td>2.07 (17.05)</td>
</tr>
<tr>
<td>O P104</td>
<td>1.34 ± 10^{-4} (17.64)**</td>
<td>2.17 (18.21)</td>
</tr>
<tr>
<td>N P105</td>
<td>6.52 ± 10^{-4} (25.71)**</td>
<td>1.82 (31.08)</td>
</tr>
<tr>
<td>O P105</td>
<td>1.47 ± 10^{-4} (2.45)**</td>
<td>1.50 (13.37)</td>
</tr>
<tr>
<td>N P122</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>O P122</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

- EC50 values calculated from the log EC50 values of three independent experiments given as the mean (CV).
- CV or coefficient of variation calculated from the log EC50 of at least three independent experiments performed in triplicate.
- Statistically different from genistein with “*" representing $P < 0.05$ and "**" representing $P < 0.01$.
- N/A not applicable as it could not be determined.
- Statistically different from E2 with “#” representing $P < 0.05$ and "##" representing $P < 0.01$.

Note: The polyphenols quantified in the C. genistoides DME included formononetin, luteolin, naringenin, and mangiferin, as their estogenicity was tested in this study. In addition, these polyphenols had also been shown to be present in some Cyclopia species, although a previous study (22) showed that only eriocitrin, narinrutin, and eriodictyol bound to the ER/β. Peaks corresponding to luteolin, eriocitrin, and narinrutin were identified on the HPLC chromatogram (Figure 6). However, the peaks eluting at retention times similar to those of eriocitrin and narinrutin are of unknown compounds as their mass was different from that of the pure standards (Table 5). Their UV–vis spectra and retention times suggest that these
two compounds are flavanone glycosides with $\lambda_{\text{max}}$ between 280 and 290 nm. Three other unknown peaks were observed at retention times of 3.7, 10.0, and 16.5 min (Figure 6). The latter two peaks also had UV–vis spectra similar to those of flavanones.

**DISCUSSION**

The presence of the phytoestrogens, formononetin, naringenin, and luteolin, in Cyclopia, coupled to anecdotal evidence of its use for the treatment of menopausal symptoms, led to the investigation of phytoestrogenic activity in Cyclopia as a potential source of phytoestrogens indigenous to South Africa (2, 3). A previous study (22) identified C. genistoides, among the four species of Cyclopia tested, as the most consistent in demonstrating phytoestrogenic activity through binding to the ER subtypes. Thus, in the present study, DMEs from unfermented C. genistoides were chosen for further in-depth study using several estrogenic endpoints to establish and evaluate estrogenicity and to compare estrogenicity with that of the known phytoestrogen, genistein, and the natural ligand, E2.

Luteolin, formononetin, naringenin, and mangiferin were included in the study as plant polyphenols previously shown to be present in Cyclopia species (2, 3, 45), and all, except mangiferin, demonstrated ability to bind to both ER subtypes (22). Mangiferin was chosen as it is the most abundant polyphenol present in honeybush (45, 46).

The C. genistoides extracts all induced transactivation via hER$\beta$, but not hER$\alpha$, despite the fact that only one harvesting, P104, bound to the ER (Tables 2 and 3). Proliferation studies in MCF-7 cells (Table 4) showed that all but one harvesting, P122, induced proliferation with potency similar to that of genistein.

By using the ER antagonist, ICI 182,780, proliferation by polyphenols in MCF-7 cells was established to be via the ER (Figure 2). MCF-7 cell proliferation induced by DMEs was only partially, although significantly, reversed by ICI 182,780 (Figure 3A), while all of the extracts, except P122, induced cell proliferation in the MDA-MB-231 cells (Figure 3B), which could not be effectively blocked with the ER antagonist. This suggests that, in addition to an ER-dependent mechanism of action, the extracts may also display an ER-independent mechanism of action. Confirmation of this would, however, require further study.

In addition to measuring and validating phytoestrogenic activity, SHBG binding was also measured. All of the polyphenols, except mangiferin, and DME were able to significantly ($P < 0.01$) compete with $^3$H-E$_2$ for binding to SHBG implying that they can be transported in the bloodstream through binding to SHBG, which would consequently decrease metabolic clearance rate and subsequent excretion as was proposed for...
endogenous estrogens (37, 47). For future studies, it would be interesting to investigate whether extracts of C. genistoides and relevant polyphenols would increase the secretion of SHBG from liver cells as it has been shown that phytoestrogens can increase the synthesis of SHBG (48, 49), and an increase in the concentration of SHBG would affect the amount of biologically free steroid (36, 47).

Two attributes of phytoestrogens, weak estrogenicity and preference for ERβ, have been linked to their beneficial health effects (7, 50–52). Both attributes are to be discussed here as they pertain to the results obtained with C. genistoides extracts. To facilitate evaluation, we will also compare activities with that of E2, the endogenous estrogen linked to both adverse (53, 54) and beneficial health effects (53), and genistein, a well-studied phytoestrogen (5).

It has been suggested that the weak estrogenic potential of phytoestrogens may contribute to health-promoting effects such as protecting against the onset of osteoporosis, cardiovascular disease, and certain hormone dependent cancers (7, 50–52). The DMEs and polyphenols tested were consistently less (102–105 times) potent than E2 (Tables 2–4). Other studies have shown similar decreases in potencies for genistein as compared to E2 in ER binding, transactivation, and proliferation studies (27, 55, 56).

The hERβ is believed to be a modulator of hERα activity as it inhibits proliferation of breast cancer cells and immature rat uterus (34, 57, 58). It has been shown, in ERα containing T47D breast cancer cells, that ERβ inhibits E2-induced cell proliferation if the cells are transfected with ERβ to such an extent that the mRNA levels of the two ER subtypes were equal (34). These findings would suggest that either ERβ has an anti-proliferative effect on breast cancer cells or it quenches ERα activity (34, 59). Competitive binding with both ER subtypes was investigated as numerous studies have shown that phytoestrogens bind preferentially to the ERβ (26, 27, 60, 61). The present study indeed demonstrated that the phytoestrogens, genistein, luteolin, and naringenin, but not formononetin, bind with a higher affinity to the ERβ subtype (Table 2), confirming results by others (25, 26, 60–63). Formononetin, however, had a slight binding preference for hERα, which is contrary to what others have shown (61, 62) and differs from what is found for most phytoestrogens (26, 27, 60).

Not only did all polyphenols, except mangiferin, bind to ERβ, they also preferentially transactivated via ERβ (Table 3) and induced cell proliferation of MCF-7-BUS cells (Table 4). Of the three harvestings of C. genistoides tested, only one, P104, bound to the ER subtypes. Unlike the phytoestrogens, however, it bound preferentially to the hERα, like E2 (Table 2). Other plant extracts such as red wine, Ginkgo biloba, kudzu root, and red clover extracts have been shown to have binding affinity for both ER subtypes, but with a higher binding affinity for ERβ (62, 64–66). It was therefore not expected that the C. genistoides methanol extracts would preferentially bind to the hERα. However, despite binding preferentially to the hERα and binding to the hERβ with a potency significantly (P < 0.001) lower than that of either E2 or genistein, P104 was able to transactivate an ERE-containing reporter promoter via hERβ, but not via ERα, with a potency similar to that of E2 and genistein and to induce MCF-7 cell proliferation with a potency similar to that of genistein but significantly (P < 0.01) lower than that of E2 (Table 4). In addition, although P105 and P122 were unable to compete with 3H-E2 for binding to hERβ, both
extracts were able to induce transactivation via the hERβ, but not the ERα, and P105 was also able to induce proliferation. These results seem to suggest that the C. genistoides extracts are disproportionally effective in activating the hERβ. Further evidence for the activity of the extracts through hERβ comes from their ability to antagonize E2-induced cell proliferation of MCF-7-BUS cells (Figure 4B), also seen with the polyphenols, genistein, luteolin, formononetin, and naringenin (Figure 4A) and as shown by others (67, 69, 70). Polyphenols and extracts, which are able to act preferentially via hERβ, could be of physiological importance as this could play a role in the prevention of excessive cell proliferation, which is associated with cancer formation (31).

Investigations into the estrogenic activity of other plant extracts have yielded results similar to those found in the present study. Kudzu root, soybean, red clover, and alfalfa sprout displayed agonist activity through the ERE-containing promoter reporter assays by activating both ERα and ERβ, with preferential activation of ERβ observed (66). In addition, extracts from Moghanita philippinensis (71), kudzu root, red clover, alfalfa sprout, and soybean (66) could also induce MCF-7 cell proliferation. Additionally, Ginkgo biloba extracts were shown to induce proliferation of MCF-7 cells that is ER-dependent as the response could be blocked with an ER antagonist (65, 66). The Ginkgo biloba extracts could, however, not induce cell proliferation of MDA-MB-231 cells (65). On the other hand, methanol extracts from M. philippinensis have previously been shown to antigenize MCF-7 cell proliferation induced by E2 (71).

HPLC and LC−MS analysis (Table 5 and Figure 6) shows that of the polyphenols tested for estrogenicity only luteolin is present in detectable quantities. The amount of luteolin present that in MCF-7-BUS cell proliferation, for example, two DMEs (N P104 and N P105) show potencies similar to that of luteolin. That the DME behaved differently from the polyphenols tested in that they induced proliferation via the estrogen-insensitive MDA-MB-231 cell line, which was only partially reversed by the ER antagonist ICI 182,780, suggests that these unknown peaks may represent novel compounds present in the DMEs with biological activity that differs from that of the polyphenols tested. Confirmation of the estrogenic potential of these unknown peaks awaits further study.

To summarize, the present study showed that the polyphenols, luteolin, formononetin, and naringenin, present in Cyclopia spp. and some DMEs from C. genistoides are estrogenic in vitro through binding to both ER subtypes, inducing transactivation via hERβ, and by inducing cell proliferation of the estrogen sensitive MCF-7-BUS cells. Proliferation of the estrogen-insensitive MDA-MB-231 cell line was, however, only stimulated by DMEs. Although the present study showed that C. genistoides is a potential source of phytoestrogens, caution should, however, be exercised as variation within the species does exist. DME from only one harvesting (P104) was able to displace 3H-E2 from the ER subtypes, and DMEs of only two harvestings (P104 and P105) were able to induce proliferation of the MCF-7-BUS cells, while all three DMEs (P104, P105, and P122) portrayed estrogenicity through induction of the ERE-containing promoter reporter via ERβ. The variations in estrogenicity may be ascribed to polyclonal plant material and stress factors such as temperature and soil requirements (72, 73). Therefore, each individual batch of plant material available at this stage in the industry would probably have to be screened if it is to be used to prepare a nutraceutical.

ACKNOWLEDGMENT

We sincerely thank C. Langeveldt for her skillful technical assistance, especially for the maintenance and culturing of the COS-1 cells, and Dalene de Beer for the HPLC and LC−MS analysis. We thank Fritz Joubert of Koksrivier, Pearly Beach, Overberg, for providing honeybush plant material.

LITERATURE CITED


Table 5. Phenolic Content, As Determined by HPLC, of the DME

<table>
<thead>
<tr>
<th>DME</th>
<th>mangiferin</th>
<th>isomangiferin</th>
<th>eriocitrin</th>
<th>narirutin</th>
<th>hesperidin</th>
<th>luteolin</th>
<th>eriodictyol</th>
<th>naringenin</th>
<th>hesperetin</th>
<th>formononetin</th>
<th>isosakuranetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>O P104</td>
<td>3.696</td>
<td>5.094</td>
<td>nd</td>
<td>nd</td>
<td>1.277</td>
<td>0.096</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>N P104</td>
<td>4.264</td>
<td>4.901</td>
<td>nd</td>
<td>nd</td>
<td>1.728</td>
<td>0.097</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>O P105</td>
<td>3.292</td>
<td>3.985</td>
<td>nd</td>
<td>nd</td>
<td>1.190</td>
<td>0.090</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>N P105</td>
<td>6.498</td>
<td>4.250</td>
<td>nd</td>
<td>nd</td>
<td>2.153</td>
<td>0.097</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>O P122</td>
<td>2.977</td>
<td>4.934</td>
<td>nd</td>
<td>nd</td>
<td>1.243</td>
<td>0.106</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>N P122</td>
<td>4.228</td>
<td>4.835</td>
<td>nd</td>
<td>nd</td>
<td>1.522</td>
<td>0.104</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

* Percentages were expressed as a percentage of the extract. 
* nd = not detected.


(41) Cheng, Y.; Prusoff, W. H. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50% inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 1973, 22, 3099–3108.


Phytoestrogenic Activity of Cyclopia genistoides


Received for review December 11, 2006. Revised manuscript received March 13, 2007. Accepted March 14, 2007. This work was supported in part by grants to A.L. and E.J. from the Medical Research Council, National Research Foundation (NRF) (GUN 2053898), THRIP (project 2634), National Department of Agriculture (ARC project 270030), and the Western Cape Department of Agriculture (ARC project 270030). Any opinion, findings, and conclusions or recommendations expressed in this material are those of the author(s), and therefore the NRF does not accept any liability in regard thereto.