

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

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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 ER β but not via ER α . 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 ER β . 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; ER α ; ER β ; 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, ER α and ER β (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)

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Table 1. Details of *C. genistoides* Plant Material Harvested, Dried Methanol Extracts (DMEs) Prepared from the Harvestings, and Extract Yield and Total Polyphenol (TPP) Content of DMEs

species	harvesting ^a	area harvested	date of harvesting	extract	extract yield (%) ^b	TPP content (%) ^c
<i>C. genistoides</i> (West Coast type)	P104	Koksrivier, Pearly Beach	15 March 2001	O ^d P104	13.35	22.31
				N ^e P104	16.93	23.53
	P105	Koksrivier, Pearly Beach	28 March 2001	O P105	13.41	21.99
				N P105	16.28	23.89
	P122	Koksrivier, Pearly Beach	31 March 2003	O P122	18.94	25.02
				N P122	16.43	24.87

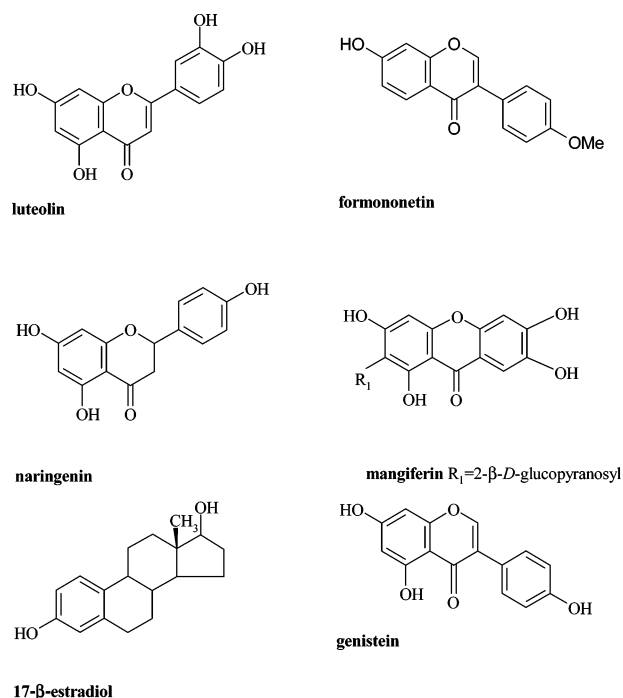
^a The abbreviations used for the harvestings are also used for the dry methanol extracts (DMEs) prepared from these harvestings. Although all harvestings were done on the same plantation, they were done at different times. Two extracts were prepared of each harvesting. ^b Yield = g of freeze-dried extract per 100 g of dried pulverized plant material. ^c TPP content = g of gallic acid equiv per 100 g of of freeze-dried extract. ^d First methanol extract of the same harvesting. ^e Second methanol extract of the same harvesting (prepared at a later stage).

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 ER β than for ER α (25, 26). In addition, phytoestrogens have been shown to induce transactivation via both ER subtypes (27), with an increased transcriptional response through ER β . 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 ER β subtype is believed to be a negative modulator of ER α -mediated activity as it has been demonstrated to inhibit transactivation and cell proliferation when coexpressed with ER α (32–34). ER β is thus believed to be the natural cellular protective mechanism against excessive cell proliferation mediated by ER α , 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 via ER β (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

**Figure 1.** Chemical structures of the plant polyphenols investigated together with that of E₂.

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 μ L/mL) mixture (penicillin–streptomycin). The ER α and ER β 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 2×10^6 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-hER α (a kind gift from D. Harnish, Womens' Health Research Institute, Wyeth-Ayerst Research, U.S.) or pSG5-hER β (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 hER α transfections, and the DEAE–Dextran transfection method was used for hER β transfections. The total DNA transfected for both transfection protocols was 6 μ g/10 cm dish that consisted of 0.72 μ g of receptor and 5.28 μ g of empty vector. The Fugene6 transfection protocol, used for hER α , was per the manufacturer's instructions with 12 μ L of Fugene6 reagent allowed to react with 6 μ g of DNA. The DEAE–Dextran transfection medium, used for hER β , consisted of 5 mL of DMEM, pre-heated to 37 °C, 0.1 mM chloroquine (stock solution 100 mM), 6 μ g 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 transfected COS-1 cells were pooled and seeded into 24-well tissue culture plates at a density of 5×10^4 cells/well and incubated for 24 h. The next day the cells were washed three times with 500 μ L of PBS/well (pre-heated at 37 °C). This was followed by a 2-h incubation of the transfected cells with 10^{-9} M radiolabeled estradiol (2,4,6,7-³H-17- β -estradiol from Amersham, Cape Town, South Africa, with specific activity 87.0 Ci/mmol and counting efficiency of 46%) and various concentrations, ranging from 2.7×10^{-13} to 7.94×10^{-3} mg/mL, of unlabeled competitors, that is, extracts and polyphenols (dissolved in DMSO) in DMEM without phenol red and FCS. All assays included a total binding point, which was in the presence of 0.1% DMSO, and E₂ and genistein as positive controls. After the 2 h incubation period, the cells were immediately placed on ice, and further work was done at 4 °C. Cells were washed three times with 1 mL of 0.2% bovine serum albumin–PBS with an interval of 15 min between washes to remove free ligand. Cells were then lysed with 50 μ L of lysis buffer (0.2% (v/v) Triton, 10% (v/v) glycerol, 2.8% (v/v) Tris-phosphate-EDTA, and 1.44 mM EDTA) per well. For effective lysis, plates were placed on a shaker for approximately 15 min and thereafter allowed to freeze at –20 °C. On thawing of samples, 5 μ L of lysate was used for protein determination using the Bradford method (39). Another 50 μ L of lysis buffer was added to the remaining lysate in the wells, and this was transferred to scintillation vials to which 3 mL of scintillation fluid (Quickszint FLOW 2; Zinsser Analytic, Cape Town, South Africa) was added. Radioactivity of the assay samples was determined using a Beckman LS 3801 Beta-scintillation counter. The protein concentrations were used to normalize radioactivity readings, and results are expressed as percentage of normalized control with total binding (in presence of 0.1% DMSO) taken as 100%. All binding experiments also included a control for ligand depletion. The ligand depletion for all whole cell ER binding experiments was less than 10%.

Transient Transfections and ERE-Containing Promoter Reporter Assays in COS-1 Cells. The cells were transfected using the Fugene6 reagent as per the manufacturer's instructions. For hER α transfection, COS-1 cells (5×10^4 cells/well) were directly transfected in 24-well dishes 24 h after plating. Briefly, 300 ng of total DNA/well, consisting of 5 ng of hER α (pSG5-hER α , a kind gift from F. Gannon, European Molecular Biology Laboratory, Heidelberg, Germany) expression plasmid, 200 ng of ERE-containing promoter reporter construct (ERE.vit2.luc, a kind gift from K. Korach, National Institute of Environmental Health Science, U.S.), 5 ng of pCMV- β -galactosidase (Stratagene, La Jolla, CA) for normalization of transfection efficiency, and 90 ng of empty vector (pGL2-Basic) were used with 0.6 μ L of Fugene6 for hER α transfections. Cells were induced 24 h after transfection. For hER β transfections, COS-1 cells were plated at a density of 2×10^6 cells/10 cm dish and transfected 24 h after plating. A total of 9.6 μ g of DNA consisting of 0.8 μ g of hER β (pSG5-hER β , also a gift from F. Gannon) expression plasmid, 8 μ g of ERE.vit2.luc, and 0.8 μ g of pCMV- β -galactosidase were transiently transfected using 19.2 μ L of Fugene6 reagent/dish. The following day cells were pooled and seeded at a density of 5×10^4 cells per well into 24-well tissue culture plates and incubated for 24 h before induction. Transfected cells were induced for 24 h with various concentrations of polyphenol compounds or DMEs (dissolved in DMSO) ranging from 2.7×10^{-13} to 7.94×10^{-3} mg/mL. All assays included a negative control, which consisted of 0.1% (v/v) DMSO only, and E₂ and genistein as positive controls. After induction the medium was aspirated, 50 μ L of lysis buffer (Tropix Inc. (Applied Biosystems, Bedford, MA)) was added, and cells were frozen at –20 °C overnight. Luciferase assay reagent (Promega Corp., Madison, WI) was used to quantify luciferase activity in accordance with the manufacturer's instructions. Briefly, 10 μ L of cell lysate was allowed to react with 50 μ L of luciferase assay reagent. The relative light units (RLU's) were measured using the Veritas luminometer. A further 5 μ L of cell lysate for each sample was used 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 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 μ L 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 a 1% penicillin–streptomycin mixture. On day five the medium was aspirated and cells were induced with increasing concentrations, ranging from 2.7×10^{-13} to 7.94×10^{-3} mg/mL, of test compounds or DMEs (in DMSO) prepared in DMEM without phenol red, but supplemented with 5% charcoal stripped FCS and a 1% penicillin–streptomycin mixture. Cells were then incubated for 48 h whereafter the colorimetric MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay was performed. The MTT assay entails that 5 h before the end of the incubation period the assay medium is changed to unsupplemented DMEM without phenol red whereafter 20 μ L of MTT solution (5 mg/mL) is added to each well. Cells were incubated for 5 h at 37 °C, the medium was then removed, and 200 μ L of solubilization solution (DMSO) was added to each well. The DMSO was pipetted up and down in the well to dissolve crystals until a uniform purple color had formed. The plate was then placed in a 37 °C incubator for 5 min, and the absorbance was read at 540 nm in a microtiter plate reader (Titertek Multiskan Plus, Titertek Instruments Inc., Huntsville, AL). All assays included a negative control, which consisted of 0.1% (v/v) DMSO only, and E₂ and genistein as positive controls. Results are expressed as fold induction with negative controls (0.1% DMSO) taken as 1.

Co-treatment by both E₂ (10^{-9} M) and the polyphenols (10^{-5} M except for genistein, which was tested at 10^{-7} M) or DME (9.8 μ g/mL) was investigated. In addition, induction with test compounds and DME was investigated in the presence of 10^{-9} M ER antagonist, ICI 182,780.

Table 2. Whole Cell Competitive Binding by E₂, Polyphenols, and DME to the hER Subtypes

test compounds	IC ₅₀ (mg/mL) ^a		RBA ^b (%)		β/α of RBA ^c	K _i ^d (M)		β/α of K _i ^c
	hERα	hERβ	hERα	hERβ		hERα	hERβ	
E ₂	3.7 × 10 ⁻⁷ (3.63) ^{##e}	7.3 × 10 ⁻⁷ (4.74)	100	100	1	0.37 × 10 ⁻⁹ M (5.44)	1.17 × 10 ⁻⁹ M (5.01)	0.3
genistein	4.2 × 10 ⁻⁵ (37.32) ^{##f}	9.0 × 10 ⁻⁷ (1.23)	0.73	81.11	111.10	43.1 × 10 ⁻⁹ M (8.99) [*]	1.01 × 10 ⁻⁹ M (4.98)	42.7
luteolin	1.5 × 10 ⁻² (4.88) ^{####}	1.4 × 10 ⁻⁴ (3.07) ^{####}	0.003	0.52	173.35	12.20 × 10 ⁻⁶ M (25.61) ^{**}	0.39 × 10 ⁻⁶ M (8.79) ^{**}	31.3
formononetin	4.1 × 10 ⁻⁵ (4.59) ^{**}	1.5 × 10 ⁻⁴ (0.45) ^{####}	0.93	0.48	0.52	34.51 × 10 ⁻⁹ M (9.51) [*]	0.14 × 10 ⁻⁶ M (7.52) ^{**}	0.25
naringenin	3.9 × 10 ⁻⁴ (8.33) ^{**}	1.5 × 10 ⁻⁴ (2.88) ^{####}	0.097	0.48	4.95	0.27 × 10 ⁻⁶ M (0.79) ^{**}	0.11 × 10 ⁻⁶ M (10.46) ^{**}	2.5
mangiferin	NB ^g	NB	NB	NB	NB	NB	NB	NB
N P104	2.1 × 10 ⁻⁴ (4.88) ^{####}	1.3 × 10 ⁻¹ (26.28) ^{####}	0.18	0.0006	0.003			
O P104	5.9 × 10 ⁻⁴ (18.07) ^{####}	2.3 × 10 ⁻¹ (19.24) ^{####}	0.05	0.0003	0.006			
N P105	NB	NB	NB	NB	NB			
O P105	NB	NB	NB	NB	NB			
N P122	NB	NB	NB	NB	NB			
O P122	NB	NB	NB	NB	NB			

^a The IC₅₀ and CV (coefficient of variation) values are calculated from the log IC₅₀ values from at least three independent experiments. ^b RBA or relative binding affinity is expressed relative to that of E₂ (100%) and was calculated as follows: 100 × IC₅₀ (E₂)/IC₅₀ (test compound). ^c β/α ratio of RBA or K_i is such that the ratio is >1 for compounds having a higher affinity for hERβ than hERα, <1 if compounds have a higher binding affinity for hERα than hERβ, and equal to 1 for compounds having a similar affinity for both ER subtypes. The β/α ratio of RBA is calculated by RBA hERβ/RBA hERα, and the β/α ratio of K_i is calculated by K_i hERα/K_i hERβ. ^d K_i values were determined from the K_d of E₂. The K_d values of E₂ for hERα and hERβ were 0.37 × 10⁻⁹ ± 0.38 M and 1.17 × 10⁻⁹ ± 0.18 M, respectively. ^e Statistically different from genistein with ^{##} representing *P* < 0.05, ^{###} representing *P* < 0.01, and ^{####} representing *P* < 0.001. ^f Statistically different from E₂ with ^{*} representing *P* < 0.05, ^{**} representing *P* < 0.01, and ^{***} representing *P* < 0.001. ^g 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 determined by the competitive SHBG binding assay as adapted from the method used by Hammond and Lähteenmäki (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 CA1 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 mixed 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 (10⁻⁵ M), DME (9.8 μg/mL), 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 unbound 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 the 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 K_i values were determined from the IC₅₀ values and K_d 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 (K_i value = 1.01 × 10⁻⁹ M) for hERβ and showed a strong preference for this subtype (K_i β/α ratio = 42.7). Formononetin, similarly to E₂, had a slight binding preference (K_i 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

Table 3. Potency (EC₅₀) and Efficacy (Maximal Fold Induction) Values As Determined from Transactivation of an ERE-Containing Promoter Reporter Gene Construct via hER α or hER β for E₂, Various Polyphenols, and the DME

test compounds or DME	potency (EC ₅₀) ^a mg/mL		efficacy (maximal fold induction)	
	hER α	hER β	hER α	hER β
E ₂	3.70 × 10 ⁻⁷ (0.49) ^{b##c}	1.39 × 10 ⁻⁷ (4.99)	1.3 (33.97)	2.34 (6.48)
genistein	9.03 × 10 ⁻⁵ (8.15) ^{**}	1.06 × 10 ⁻⁶ (4.93)	1.77 (14.90)	2.76 (17.18)
luteolin	1.97 × 10 ⁻³ (4.9) ^{**##}	3.53 × 10 ⁻³ (38.69) ^{####d}	2.41 (26.57)	3.69 (48.22)
formononetin	1.01 × 10 ⁻³ (4.36) ^{**}	4.29 × 10 ⁻⁵ (5.53) ^{**}	2.18 (17.45)	2.20 (18.42)
naringenin	N/A	1.04 × 10 ⁻⁴ (4.68) ^{***}	N/A	2.99 (33.94)
mangiferin	N/A	N/A ^e	N/A	N/A
N P104	N/A	1.51 × 10 ⁻⁵ (22.60) [*]	N/A	2.44 (36.78)
O P104	N/A	1.18 × 10 ⁻⁵ (21.36)	N/A	2.39 (21.11)
N P105	N/A	9.20 × 10 ⁻⁵ (12.31)	N/A	1.63 (3.25)
O P105	N/A	2.93 × 10 ⁻⁵ (22.66) [*]	N/A	2.53 (62.77)
N P122	N/A	6.90 × 10 ⁻⁵ (0.21)	N/A	1.90 (20.52)
O P122	N/A	2.48 × 10 ⁻⁶ (0.59)	N/A	1.94 (27.49)

^a EC₅₀ values calculated from the log EC₅₀ values of three independent experiments given as the mean (CV). ^b CV (coefficient of variation) calculated from the log EC₅₀ of at least three independent experiments performed in triplicate. ^c Statistically different from genistein with ^{##} representing $P < 0.05$, ^{###} representing $P < 0.01$, and ^{####} representing $P < 0.001$. ^d Statistically different from E₂ where ^{**} represents $P < 0.05$ and ^{***} represents $P < 0.01$. ^e N/A: test compound or DME did not induce the ERE-containing promoter reporter gene construct via the indicated hER subtype.

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 β/α ratio = 0.003 and 0.006 for N P104 and O P104, respectively) for the ER α subtype.

Transactivation of an ERE-Containing Promoter Reporter Construct via the hER Subtypes. E₂ transactivated hER α and hER β with similar potencies, while the polyphenols generally, with the exception of luteolin, transactivated more potently via hER β (Table 3). The order of potency via hER α was E₂ \gg genistein \gg formononetin = luteolin, while via hER β it was E₂ $>$ genistein $>$ formononetin $>$ naringenin $>$ luteolin. The potency of E₂ via hER α 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 E₂ via hER β 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 transactivational efficacy of the various polyphenols via hER α was luteolin $>$ formononetin $>$ genistein $>$ E₂, with luteolin and formononetin not significantly different ($P > 0.05$) from E₂ and genistein, with the latter not statistically different ($P > 0.05$) from each other. The transactivational efficacy of the various polyphenols via hER β did not differ significantly ($P > 0.05$) from each other or from that of E₂ and genistein (Table 3). Although both E₂ and genistein, in contrast to the polyphenols tested, displayed a relatively high potency for both binding and ERE-containing promoter reporter assays via hER β , the transactivational efficacy was approximately similar for all polyphenols and E₂ ($P > 0.05$).

The DMEs were only able to induce the ERE-containing promoter reporter construct via the hER β , but not via hER α (Table 3) despite the fact that some extracts (from the P104 harvesting) were able to displace ³H-E₂ from both hER β and hER α , with higher RBAs for hER α than for hER β (Table 2). The order of potency (EC₅₀) of E₂, genistein, and extracts was as follows for hER β : E₂ $>$ genistein $>$ O P122 $>$ O P104 $>$ N P104 $>$ O P105 $>$ N P122 $>$ N P105 (Table 3). Potencies of extracts, via hER β , were not significantly ($P > 0.05$) different from that of E₂, except for N P104 and O P105, while none of

the extracts were significantly different from genistein. The efficacy of the extracts, via hER β , was not significantly different ($P > 0.05$) from that of either genistein or E₂.

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 E₂ \gg naringenin $>$ genistein $>$ luteolin $>$ formononetin $>$ mangiferin (Table 4). All of the potencies of the polyphenols were significantly different ($P < 0.05$) from that of E₂ 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 $>$ E₂ $>$ naringenin $>$ mangiferin $>$ formononetin $>$ luteolin (Table 4). None of the efficacy values determined for the polyphenols were significantly ($P > 0.05$) different from that of E₂ or genistein except for luteolin and formononetin ($P < 0.01$). Neither the polyphenols nor E₂ 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: E₂ \gg 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 E₂ 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 $>$ E₂ $>$ 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 E₂ 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, E₂, 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 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

test compounds or DME	MCF-7-BUS cells		MDA-MB-231	
	potency (EC ₅₀) ^a mg/mL	efficacy (maximal fold induction)	potency (EC ₅₀) mg/mL	efficacy (maximal fold induction)
E ₂	2.79 × 10 ⁻¹⁰ (2.92) ^{b##c}	2.14 (8.46)	N/A ^d	N/A
genistein	1.02 × 10 ⁻⁶ (7.56) ^{##e}	2.35 (10.57)	N/A	N/A
luteolin	2.54 × 10 ⁻⁶ (15.77) ^{##}	1.26 (2.52) ^{###}	N/A	N/A
formononetin	1.48 × 10 ⁻⁵ (14.90) ^{##}	1.38 (4.15) ^{###}	N/A	N/A
naringenin	3.27 × 10 ⁻⁸ (1.60) [*]	2.08 (4.15)	N/A	N/A
mangiferin	3.13 × 10 ⁻⁴ (31.07) ^{##}	1.72 (3.44)	N/A	N/A
N P104	1.98 × 10 ⁻⁶ (7.34) ^{##}	2.07 (17.05)	2.47 × 10 ⁻⁹ (2.66)	1.62 (19.31)
O P104	1.34 × 10 ⁻⁴ (17.64) ^{###}	2.17 (18.21)	1.39 × 10 ⁻¹⁰ (18.87)	1.59 (25.56)
N P105	6.52 × 10 ⁻⁶ (25.71) ^{##}	1.82 (31.08)	2.62 × 10 ⁻¹⁰ (16.81)	1.81 (32.24)
O P105	1.47 × 10 ⁻⁴ (2.45) ^{##}	1.50 (13.37) [#]	2.79 × 10 ⁻¹⁰ (17.23)	1.38 (35.85)
N P122	N/A ^e	N/A	N/A	N/A
O P122	N/A	N/A	N/A	N/A

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

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 × 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 × 10⁻⁹ M E₂.

Binding to SHBG and Displacement of E₂. The percentage of ³H-E₂ displaced from SHBG by the polyphenols (Figure 5A) and *C. genistoides* DME (Figure 5B) was significant ($P < 0.05$), except in the case of mangiferin. Displacement by naringenin, which was higher than that of genistein, was not significantly different (statistical data not shown) from that of E₂, similar to what was found by others (44). In addition, N P104 and O P122 were also as effective as E₂ in competing with ³H-E₂ for binding to the SHBG (statistical data not shown).

HPLC and LC-MS Analysis. The polyphenols quantified in the *C. genistoides* DME included formononetin, luteolin, naringenin, and mangiferin, as their estrogenicity was tested in this study. In addition, these polyphenols had also been shown to be present in *C. intermedia* and *C. subternata* (2, 3). Levels of isomangiferin, eriocitrin, narirutin, hesperidin, hesperetin, and isosakuranetin were also evaluated as they had been shown to

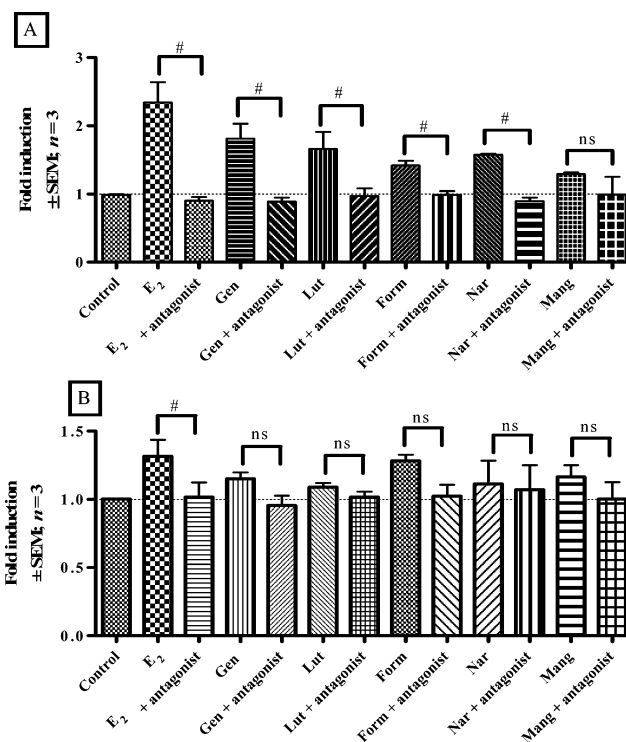


Figure 2. Cell proliferation of polyphenols and E₂ in (A) MCF-7-BUS and (B) MDA-MB-231 breast cancer cells. Co-treatment with ER antagonist, ICI 182,780, identifies if induced response is ER-dependent. All compounds were tested at 10 × 10⁻⁶ M except for E₂ and genistein, which were investigated at 1 × 10⁻⁹ and 0.1 × 10⁻⁶ M, respectively. The control represents vehicle (DMSO) only. Statistical analysis compared induction by a specific compound in the absence and presence of the ER antagonist using two-tailed *t* tests ([#] = $P < 0.05$; ns = $P > 0.05$ or not significantly different). Abbreviations: genistein (Gen), luteolin (Lut), formononetin (Form), naringenin (Nar), and mangiferin (Mang).

be present in some *Cyclopia* species, although a previous study (22) showed that only eriocitrin, narirutin, and eriodictyol bound to the ER β . Peaks corresponding to luteolin, eriocitrin, and narirutin were identified on the HPLC chromatogram (Figure 6). However, the peaks eluting at retention times similar to those of eriocitrin and narirutin 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

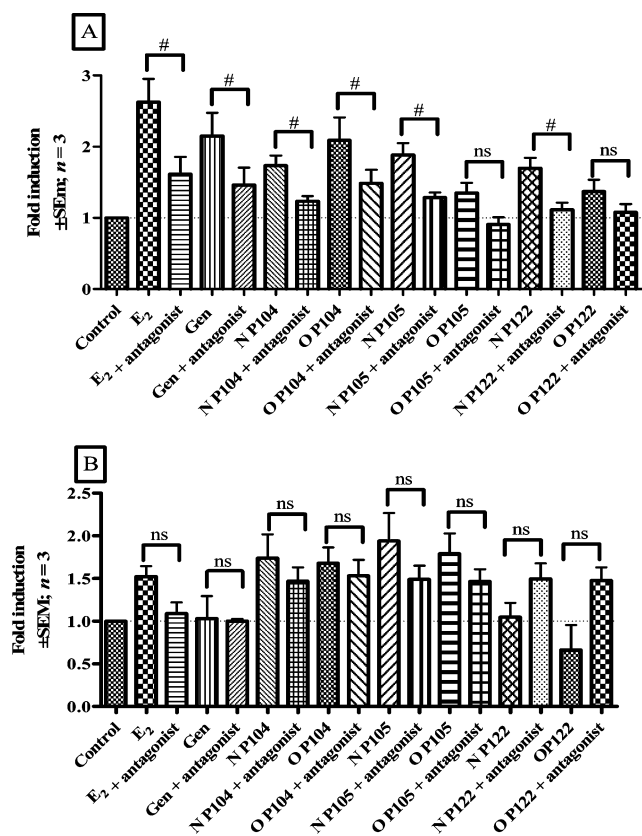


Figure 3. Cell proliferation of DME, genistein, and E₂ in (A) MCF-7-BUS and (B) MDA-MB-231 breast cancer cells. Co-treatment with ER antagonist, ICI 182,780, identifies if induced response is ER-dependent. All extracts were investigated at 9.8 $\mu\text{g/mL}$, and E₂ and genistein at 1×10^{-9} and 10×10^{-6} M, respectively. The control represents vehicle (DMSO) only. Statistical analysis compared induction by a DME in the absence and presence of the ER antagonist using two-tailed *t* tests (# = $P < 0.05$; ns = $P > 0.05$ or not significantly different). Abbreviations: genistein (Gen).

two compounds are flavanone glycosides with λ_{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).

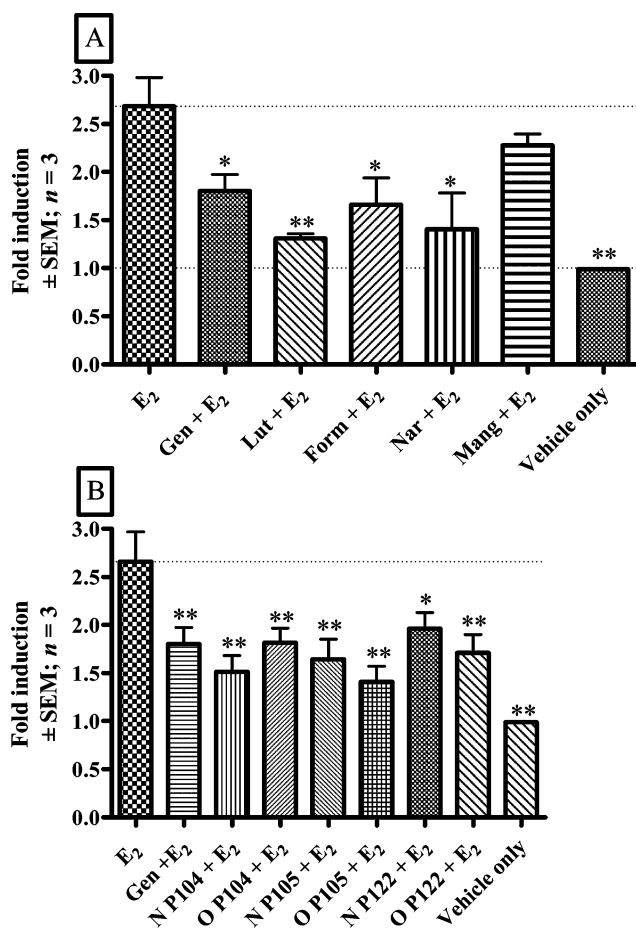


Figure 4. Effect of (A) various polyphenols and (B) *C. genistoides* DME on E₂ (1×10^{-9} M)-induced proliferation of MCF-7-BUS. All polyphenols were investigated at 10×10^{-6} M, and the DME was investigated at 9.8 $\mu\text{g/mL}$. Statistical analysis compared cell proliferation induced by 1×10^{-9} M E₂ only with that induced by 1×10^{-9} M E₂ plus polyphenols or DME using one-way ANOVA with Dunnett's multiple comparisons' post test (* $P < 0.05$; ** $P < 0.01$). Abbreviations: genistein (Gen), luteolin (Lut), formononetin (Form), naringenin (Nar), and mangiferin (Mang).

The *C. genistoides* extracts all induced transactivation via hER β , but not hER α , 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 ³H-E₂ 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

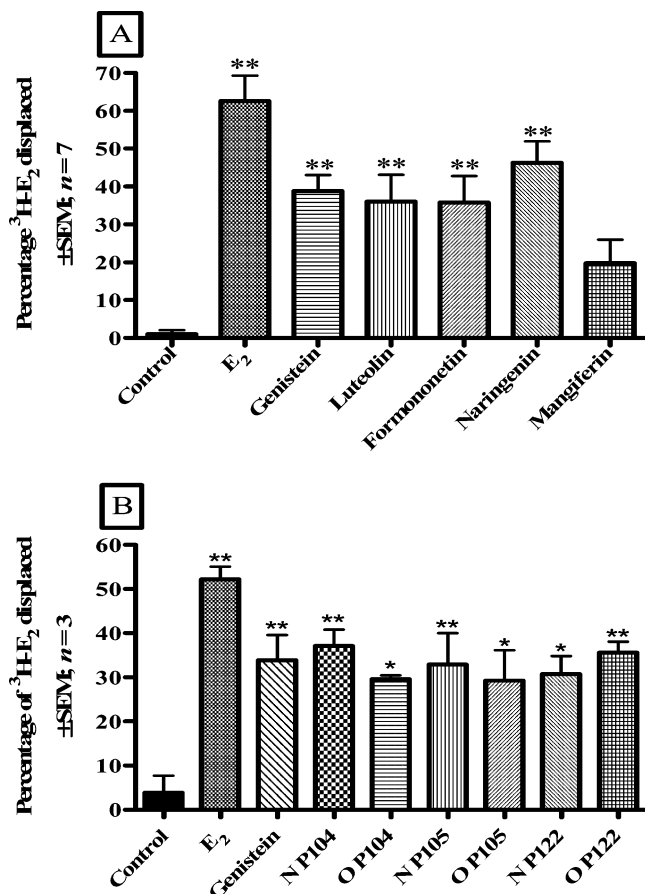


Figure 5. Competitive binding of (A) polyphenols and (B) *C. genistoides* DME to SHBG in DCC stripped human pregnancy serum incubated with 20×10^{-9} M $^3\text{H-E}_2$. Polyphenols and E_2 were used at a concentration of 10^{-5} M and the extracts at a concentration of $9.8 \mu\text{g/mL}$. The control in both represents vehicle (DMSO) only. For statistical analysis, one-way ANOVA was used with Dunnett's multiple comparisons' post test comparing percentage $^3\text{H-E}_2$ displaced to control. *P*-values are represented as follows: $P < 0.05$ by *, $P < 0.01$ by **.

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 $\text{ER}\beta$, 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 E_2 , 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 (10^2 – 10^5 times) potent than E_2 (Tables 2–4). Other studies have shown similar decreases in potencies for genistein as compared to E_2 in ER binding, transactivation, and proliferation studies (27, 55, 56).

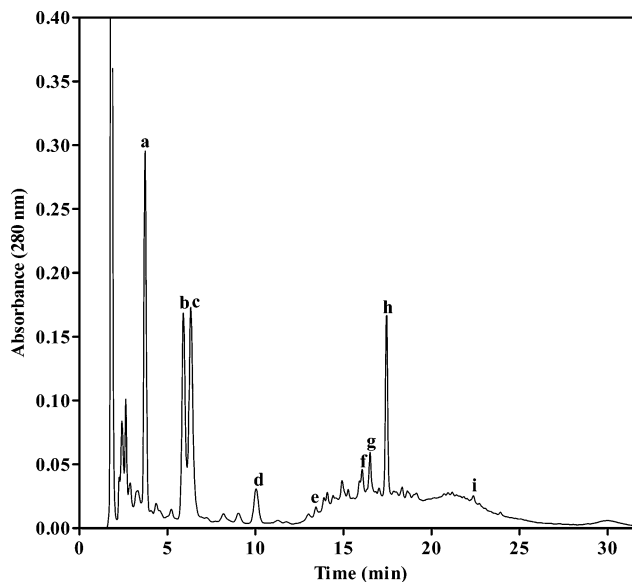


Figure 6. Typical HPLC chromatogram of a DME showing the polyphenols co-eluting at retention times similar to those of known standards: (a) unknown at 3.7 min, (b) mangiferin, (c) isomangiferin, (d) unknown flavanone glycoside at 10.0 min, (e) unknown flavanone glycoside at 13.4 min, (f) unknown flavanone glycoside at 16.0 min, (g) unknown flavanone glycoside at 16.5 min, (h) hesperidin, and (i) luteolin.

The $\text{hER}\beta$ is believed to be a modulator of $\text{hER}\alpha$ activity as it inhibits proliferation of breast cancer cells and immature rat uterus (34, 57, 58). It has been shown, in $\text{ER}\alpha$ containing T47D breast cancer cells, that $\text{ER}\beta$ inhibits E_2 -induced cell proliferation if the cells are transfected with $\text{ER}\beta$ to such an extent that the mRNA levels of the two ER subtypes were equal (34). These findings would suggest that either $\text{ER}\beta$ has an anti-proliferative effect on breast cancer cells or it quenches $\text{ER}\alpha$ activity (34, 59). Competitive binding with both ER subtypes was investigated as numerous studies have shown that phytoestrogens bind preferentially to the $\text{ER}\beta$ (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 $\text{ER}\beta$ subtype (Table 2), confirming results by others (25, 26, 60–63). Formononetin, however, had a slight binding preference for $\text{hER}\alpha$, 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 $\text{ER}\beta$, they also preferentially transactivated via $\text{ER}\beta$ (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 $\text{hER}\alpha$, like E_2 (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 $\text{ER}\beta$ (62, 64–66). It was therefore not expected that the *C. genistoides* methanol extracts would preferentially bind to the $\text{hER}\alpha$. However, despite binding preferentially to the $\text{hER}\alpha$ and binding to the $\text{hER}\beta$ with a potency significantly ($P < 0.001$) lower than that of either E_2 or genistein, P104 was able to transactivate an ERE-containing reporter promoter via $\text{hER}\beta$, but not via $\text{ER}\alpha$, with a potency similar to that of E_2 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 E_2 (Table 4). In addition, although P105 and P122 were unable to compete with $^3\text{H-E}_2$ for binding to $\text{hER}\beta$, both

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

DME	percentage of soluble solids ^a											
	mangiferin	isomangiferin	eriocitrin	narirutin	hesperidin	luteolin	eriodictyol	naringenin	hesperetin	formononetin	isosakuranetin	
O P104	3.606	5.094	nd ^b	nd	1.277	0.096	nd	nd	nd	nd	nd	
N P104	4.264	4.901	nd	nd	1.728	0.097	nd	nd	nd	nd	nd	
O P105	3.292	3.955	nd	nd	1.190	0.090	nd	nd	nd	nd	nd	
N P105	6.498	4.250	nd	nd	2.153	0.097	nd	nd	nd	nd	nd	
O P122	2.977	4.934	nd	nd	1.243	0.106	nd	nd	nd	nd	nd	
N P122	4.228	4.835	nd	nd	1.522	0.104	nd	nd	nd	nd	nd	

^a Quantities were expressed as a percentage of the extract. ^b nd = not detected.

extracts were able to induce transactivation via the hER β , but not the ER α , and P105 was also able to induce proliferation. These results seem to suggest that the *C. genistoides* extracts are disproportionately effective in activating the hER β . Further evidence for the activity of the extracts through hER β 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 hER β , 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 ER α and ER β , with preferential activation of ER β observed (66). In addition, extracts from *Moghania 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 antagonize 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 (0.096–0.106 g/100 g) is, however, too low to explain the fact that in MCF-7-BUS cell proliferation, for example, two DMEs (N P104 and N P105) show potencies similar to that of luteolin. Five unknown peaks (a, d–g) are observed in the HPLC chromatogram. Of these, four (d–g) are most probably unknown flavanone glycosides based on their UV-vis spectra (λ_{\max}) and relative retention time to the other flavanone glycoside. The fact 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 hER β , 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 ER β . 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.

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