Screening of four *Cyclopia* (honeybush) species for putative phyto-oestrogenic activity by oestrogen receptor binding assays

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Phyto-oestrogens mediate an oestrogenic effect through binding to the oestrogen receptor (ER) subtypes, ERα and ERβ. Four commercially available *Cyclopia* (honeybush) species—*C. intermedia*, *C. subternata*, *C. genistoides* and *C. sessiliflora*—together with nine commercially obtainable polyphenols present in some or all of the species, were screened for phyto-oestrogenic activity, using a competitive whole-cell ER binding assay. Only naringenin, formononetin and luteolin were able significantly to displace ^{3}H-E$_2$ from hERα, whereas luteolin, naringenin, formononetin, eriodictyol, narirutin and eriocitrin displaced ^{3}H-E$_2$ from hERβ. Mangiferin, hesperidin and hesperetin did not bind to either receptor subtype. To our knowledge, this is the first time that binding of eriodictyol, eriocitrin and narirutin to the hERβ has been shown. Furthermore, both aqueous and methanol extracts from three independent harvestings of each *Cyclopia* species were screened. The results suggest that *C. genistoides* and *C. subternata* display significant phyto-oestrogenic activity and that methanol extracts from 'unfermented' (unoxidized) plant material generally display greater activity. Great variation exists within a species, however, with one *C. genistoides* harvesting displacing ^{3}H-E$_2$ from both ER subtypes, while another harvesting displaced ^{3}H-E$_2$ from only hERβ, and a third did not displace ^{3}H-E$_2$ from either receptor subtype.

Introduction

The genus *Cyclopia* (family: Fabaceae), comprising *c.* 24 species, is part of the rich fynbos plant kingdom of the Western Cape province of South Africa.[1] *Cyclopia intermedia, C. subternata, C. genistoides* and, to a lesser extent, *C. sessiliflora,* are species that are processed commercially. *Cyclopia,* commonly known as honeybush tea owing to its sweet distinct aroma and flavour, is growing in popularity. Traditionally, only 'fermented' honeybush tea was available to the public, but the 'unfermented' (green) tea has recently been introduced to local and international markets. The fermentation (oxidation) step during processing, which is necessary to release the characteristic sensory properties, reduces the total phenolic content of the plant material.[2] Several studies that investigated the chemical composition of *Cyclopia* reported that mangiferin and hesperidin are the main compounds present.[3–6] Ferreira *et al.*[4] studied the phenolic composition of fermented *C. intermedia* and found, amongst others, formononetin, mangiferin, naringenin, eriodictyol, hesperetin, hesperidin, medicagol, flemichapparin, sopharacoumestan B and luteolin. A subsequent study involving unfermented *C. subternata* revealed a phenolic composition that also included luteolin, mangiferin and hesperidin, but with the additional presence of narirutin and eriocitrin, amongst others, which were not present in *C. intermedia.*[5]

Recently, honeybush tea has been identified as displaying antioxidant and antimutagenic activity, which is thought to be due to its phenolic composition.[7–9] The phenols present in *Cyclopia,* however, may have additional biological activities. For example, formononetin, naringenin, eriodictyol and luteolin are known to have weak oestrogenic effects.[10–12] Phenolic plant compounds able to mediate an oestrogenic effect are commonly referred to as phyto-oestrogens, which are believed to alleviate menopausal symptoms and to protect against cardiovascular disease and osteoporosis.[13–15] Plant extracts of *Cimicifuga racemosa* (black cohosh)[16] and *Trifolium pratense* (red clover)[17] are available commercially in western countries for the treatment of menopausal symptoms.[18]

The public is more inclined towards the use of 'natural' products, which they believe have fewer side-effects, than synthetic drugs.[19] Their perceptions as related specifically to phyto-oestrogens are supported by both epidemiological studies and recent reports of health risks associated with conventional medicine. Epidemiological studies have shown that the incidence of breast cancer and prostate cancer is much lower in Asian than Western populations[20] and that this phenomenon is associated with the traditional Asian diet, which contains high levels of soya.[21] The isoflavone, genistein, present in soybean, was isolated and identified as the principal contributor to these findings.[22] Two clinical trials, involving hormone replacement therapy (HRT), by the Women's Health Initiative, had to be prematurely stopped due to health risks, in particular breast cancer, cardiovascular disease and stroke.[23,24] Alternatives to traditional HRT being investigated thus include phyto-oestrogens, believed to minimize the risks associated with the therapy, while retaining the health benefits and even protecting against hormone-related cancers. Although these beliefs are supported by the literature,[25–27] some studies suggest that phyto-oestrogens may not be effective in alleviating menopausal symptoms.[28] Despite this caveat, phyto-oestrogens are in demand by the public and new plant sources of these compounds are investigated regularly for exploitation by the nutraceutical industry.

Oestrogens are responsible for numerous physiological effects, especially in reproductive tissues.[29] Beneficial physiological outcomes include maintaining or increasing bone density[30] and protecting against cardiovascular disease.[31] However, because of their stimulating effect on growth and proliferation of certain cells, they are a risk factor for breast and endometrial cancer.[32,33] Oestrogen signalling is mediated mainly via the oestrogen receptors (ERs) to which oestrogens bind. Such binding induces a conformational change and dissociation of heat shock proteins. This is followed by dimerization and binding to the classic oestrogen response element, whereby it is able to mediate a biological response.[34]

Two ER subtypes, namely ER-alpha (ERα)[35] and ER-beta (ERβ),[36] have been identified. Both are capable of stimulating transcription of ER target genes. The degree of oestradiol (E$_2$)

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activation (fold induction) through ERβ in many cell types is, however, lower than that of ERα. Physiologically, ERβ decreases the overall sensitivity to E_2 in ERα-mediated gene transcription, and is believed to be the natural cellular protective mechanism against over-proliferation of cells that could lead to cancer formation.[37–39] Compounds that bind with a higher affinity to ERβ than ERα are thus of great interest pharmacologically. Phytooestrogens, which could be a natural alternative or supplement for the treatment of menopausal women, have been shown preferentially to bind to the ERβ.[40–42]

In the study reported here, selected commercially available polyphenols present in *Cyclopia* as well as extracts of the four most commonly available *Cyclopia* species, namely, *C. genistoides, C. subternata, C. sessiliflora* and *C. intermedia,* were investigated by evaluating their interaction with both ERα and ERβ. The polyphenols were screened for use as possible marker compounds of oestrogenicity in *Cyclopia*, while the extracts of the *Cyclopia* plants were screened to identify the species with the highest oestrogenicity. Both aqueous extracts (as a 'cup of tea'), from fermented and unfermented *Cyclopia* plant material, and methanol extracts from unfermented *Cyclopia* material, were tested. We also investigated three different harvestings of each species to eliminate false negatives and to include variations due to growth conditions and/or genetic effects. Phenolic compounds are especially known to occur at high concentrations in plants that are under stress.[43,44] Binding to the ER subtypes was chosen as a screening assay, because it constitutes the first step in the signal transduction pathway mediating an oestrogenic effect.

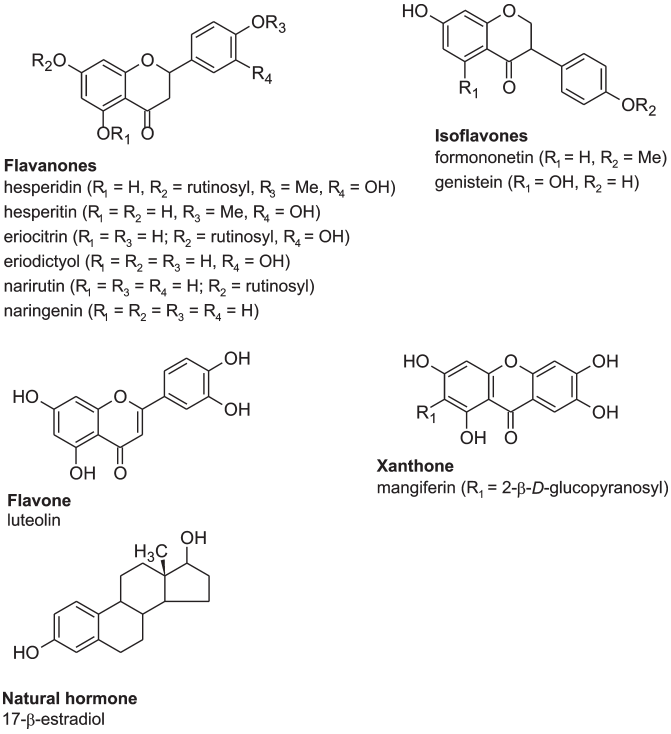

Fig. 1. Chemical structures of the polyphenols and E_2 investigated in this study.

Materials and methods

Test compounds

Test compounds included 17-β-oestradiol, genistein, mangiferin, hesperetin, hesperidin, and naringenin (Sigma-Aldrich) and luteolin, formononetin, eriodictyol, narirutin, and eriocitrin (Extrasynthese, France) (Fig. 1). Radiolabelled ligand, 2,4,6,7-[^{3}H]17-β-oestradiol (specific activity 87.0 Ci mmol^{-1}, counting efficiency of 46%) was from Amersham.

Plant material

Three randomly chosen independent harvestings of *C. intermedia, C. subternata, C. genistoides* and *C. sessiliflora* were selected. Plant material for the aqueous extracts (Table 1) was processed according to a standard method for fermented and unfermented tea.[2] Briefly, the leaves and stems were cut into small pieces. For the fermented extracts the shredded plant material was moistened with deionized water to *c.* 60% moisture content, after which fermentation at 70°C for 60 h, followed by drying at 40°C for 12 h to *c.* 10% moisture content, took place. Unfermented material was dried directly after shredding at 40°C for 12 h. The dried plant material was then sieved with an Endecott test sieve (2 mm) and the fraction smaller than 2 mm pulverized with a Retsch rotary mill (1-mm sieve), which was stored in plastic containers at room temperature. The plant material for the preparation of the methanol extracts (Table 2) was dried whole at 40°C to *c.* 10% moisture content, after which it was pulverized and stored in plastic containers at room temperature.

Dried aqueous extract preparation

Dried aqueous extracts (DAE) were prepared by E.S. Richards.[7] Briefly, 100 g of pulverized, processed (fermented and unfermented) plant material (Table 1) was steeped in 1 litre of freshly boiled deionized water for 5 min while stirring. Extracts were then filtered through 125-μm Polymon mesh cloth followed by filtration through Whatman No. 4 filter paper. The filtrate was stored at –20°C before freeze-drying. The freeze-dried DAE were stored at room temperature in sealed glass vials, covered with aluminium foil, in desiccators kept in the dark.

Dried methanol extract preparation

The plant material used for dried methanol extracts (DME) of unfermented *Cyclopia* material (Table 2) was not the same as that used to prepare the DAE. Dried, pulverized unfermented plant material (25 g) was extracted three times with 50 ml dichloromethane (UNIV AR, Merck) at room temperature for 20 h each 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 DME were ground in a darkened room until a fine homogeneous powder was obtained, which was stored in screw-cap glass vials, covered with aluminium foil, and placed in vacuum-sealed desiccators in the dark at room temperature.

Extract yield

The extract yield was determined by calculating the mass of dry extract (DAE or DME) per 100 g of initial processed plant material.

Determination of total polyphenol content of DAE and DME

The total polyphenol (TPP) content of the DAE and DME was quantified colorimetrically in triplicate. The method of Singleton and Rossi[45] was adapted for use in flat-bottomed, 96-well plates (B & M Scientific). Briefly, 20 μl of sample [gallic acid (Sigma-Aldrich) standards (0.01–0.1 mg ml^{-1}), or DAE and DME (0.25 mg ml^{-1})] were allowed to react with 100 μl 10% (v/v) Folin-Ciocalteau reagent (Merck) in the presence of 80 μl 7.5% (w/v) Na_2CO_3 at 37°C for 2 h. Absorbance was measured at 620 nm using a microtitre plate reader, recorded, and gallic acid equivalents obtained from the standard curve. The TPP content was calculated as gallic acid equivalents per 100 g of freeze-dried extract.

Binding to ER subtypes

The ability of the polyphenols and extracts to bind to the receptors was investigated by the competitive whole-cell receptor binding assay by transiently transfecting COS-1 cells, which do not endogenously express ERα or ERβ,[46] with either hERα or hERβ.

Cell culture conditions

COS-1 cells (ATCC) were maintained in DMEM (Sigma-Aldrich) supplemented with 10% (v/v) FCS (Highveld Biological, Johannesburg), and penicillin (100 IU ml^{-1}) and streptomycin (100 μl ml^{-1}) (penicillin–streptomycin) from Gibco-BRL Life Technologies. COS-1 cells were plated at a density of 2×10^6 cells per 10 cm tissue culture dish (Nunc).

Transient transfections

Twenty-four hours after plating, the COS-1 cells were transiently transfected with expression vectors for one of the ER subtypes, pcDNA3-

hERα (D. Harnish, Wyeth-Ayerst Research, USA) or pSG5-hERβ (F. Gannon, European Molecular Biology Laboratory, Germany), an expression vector for β-galactosidase, pCMV-βgal (Stratagene), and a filler vector, empty pGL2-basic (Promega Corp.), using the DEAE-Dextran transfection method[47] adapted as described.

Transfections were carried out in 10 cm tissue culture dishes and cells re-plated 24 h later into 12-well plates. Briefly, 4950 μl DMEM pre-heated to 37°C and containing 0.1 mM chloroquine (Sigma-Aldrich) (stock solution 100 mM) and 6 μg DNA (720 ng receptor, 480 ng β-galactosidase expression vector and 4.8 μg filler vector) was prepared per 10 cm tissue culture dish. To this mixture, 50 μl DEAE-Dextran (Sigma-Aldrich) solution (stock solution 10 mg ml^{-1}) was added to give a final concentration of 0.1 mg ml^{-1}. The transfection medium (5 ml per dish) was added to each 10 cm dish and cells were incubated for 1 h at 37°C, followed by washing of cells with pre-heated 10% DMSO/PBS. Finally, transiently transfected cells were incubated at 37°C overnight in maintenance medium. Twenty-four hours after transfection cells were re-plated at a density of 2×10^5 cells per well into 12-well plates and incubated for 24 h at 37°C.

Table 1. *Cyclopia* plant material harvested and used for the preparation of dried aqueous extracts.

Species	Processing	Harvesting*	Area harvested	Date of harvesting
C. genistoides west coast type	Unfermented	Gen 1G	Koksrivier, Pearly Beach	15 Mar 2001
		Gen 2G	Koksrivier, Pearly Beach	15 Mar 2001
		Gen 3G	Koksrivier, Pearly Beach	15 Mar 2001
	Fermented	Gen 1F	Koksrivier, Pearly Beach	15 Mar 2001
		Gen 2F	Koksrivier, Pearly Beach	15 Mar 2001
		Gen 3F	Koksrivier, Pearly Beach	15 Mar 2001
C. subternata	Unfermented	Sub 1G	Waboomskraal, Outeniqua	10 Feb 1999
		Sub 3G	Waboomskraal, Outeniqua	10 Feb 1999
		Sub 15G	Du Toitskloof	01 Oct 1998
	Fermented	Sub 8F	Waboomskraal, Outeniqua	10 Feb 1999
		Sub 10F	Waboomskraal, Outeniqua	17 Oct 1999
		Sub 13F	Waboomskraal, Outeniqua	17 Oct 1999
C. sessiliflora	Unfermented	Sess 2G	Helderfontein, Stellenbosch	15 Feb 2001
		Sess 3G	Helderfontein, Stellenbosch	15 Feb 2001
		Sess 4G	Helderfontein, Stellenbosch	15 Feb 2001
	Fermented	Sess 4F	Helderfontein, Stellenbosch	15 Feb 2001
		Sess 6F	Helderfontein, Stellenbosch	15 Feb 2001
		Sess 7F	Helderfontein, Stellenbosch	15 Feb 2001
C. intermedia	Unfermented	Int 1G	Haarlem, Langkloof	10 Mar 2000
		Int 2G	Haarlem, Langkloof	10 Mar 2000
		Int 5G	Haarlem, Langkloof	10 Mar 2000
	Fermented	Int 2F	Haarlem, Langkloof	10 Mar 2000
		Int 3F	Haarlem, Langkloof	10 Mar 2000
		Int 4F	Haarlem, Langkloof	10 Mar 2000

*The abbreviations used for the harvestings are also used for the dried aqueous extracts prepared from these collections. Although some harvestings were conducted on the same day and on the same plantation, different numbers indicate that they involved different plants or group of plants.

Whole-cell binding assays

Transfected COS-1 cells were washed three times with 500 μl PBS (pre-heated at 37°C) to remove any endogenous oestrogen-like compounds present in the culture medium.

This was followed by a 2-hour incubation at 37°C of the transfected cells with 1 nM radiolabelled oestradiol (^{3}H-E$_2$) and various unlabelled competitors, i.e. extracts or polyphenols in DMEM without phenol red, FCS or antibiotics. All unlabelled competitors, except for the DAE, were dissolved in absolute ethanol (Merck) and subsequently added to the culture medium, giving final concentrations of the phenolic compounds and DME of 10^{-5} M and 1.5 μg ml^{-1}, respectively, with the final concentration of ethanol not exceeding 0.1% (w/w). The DAE were dissolved directly into the culture medium at a concentration of 1.5 μg ml^{-1}. All assays included a negative control, which was in the presence of 0.1% ethanol, as well as positive controls, which consisted of incubations with E$_2$ (10^{-5} M) or genistein (10^{-5} M).

After the 2-hour incubation, cells were immediately placed on ice and further work was done at 4°C. Cells were washed three times with 1 ml ice-cold 0.2% bovine serum albumin/PBS (Roche Applied Science), with an interval of 15 min between washes to remove free ligand. Cells were lysed with 50 μl lysis buffer (Tropix Inc.) per well, placed on a shaker for approximately 15 min and thereafter allowed to freeze at –20°C.

Protein concentrations were used to normalize radioactivity readings and 5 μl lysate was used for the Bradford protein assay.[48]

Another 50 μl of lysis buffer was then added to the remaining lysate and this was quantitatively transferred to scintillation vials to which 3 ml scintillation fluid (Quickszint FLOW 2; Zinsser Analytic) was added.

Radioactivity of the all assay samples was determined by recording the counts per min for each measurement using a Beckman beta scintillation counter (model LS 3801).

High-performance liquid chromatography analysis of *Cyclopia* extracts

The quantification of specific phenolic compounds in DAE and DME was performed by reverse-phase HPLC as described by Joubert *et al.*[6] The HPLC system (Merck Hitachi, LaChrom system, Merck, Germany) consisted of a solvent pump (LC-7100), autosampler (L-7200), UV detector (L-7450), diode array detector (DAD) (L-7450), interface (D-7000) and D-7000 HPLC system manager version 4.1 for system control and data acquisition and analysis. Solvents were degassed in-line with a Phenomenex Degasser Model DG-4400 (Separations, South Africa). The Phenomenex RP/C$_{18}$ 5μm Jour Guard column, Phenomenex Synergy MAX-RP 80A column (C$_{12}$ reverse-phase with TMS end-capping, 4 μ m; 150 × 4.6 mm i.d.) and the Phenomenex Luna Phenyl-hexyl column (150 mm × 4.6 mm; 3 μm) were from Separations, South Africa. Only Int P125 was separated on the Luna Phenyl-hexyl column.

Table 2. *Cyclopia* plant material harvested and used for the preparation of dried methanol extracts.

Species	Harvesting*	Area harvested	Date of harvesting
C. genistoides west coast type	Gen P104	Koksrivier, Pearly Beach	15 Mar 2001
	Gen P105	Koksrivier, Pearly Beach	28 Mar 2001
	Gen P122	Koksrivier, Pearly Beach	31 Mar 2003
C. subternata	Sub PY1	Bien Donne, Simondium	1 Mar 2000
	Sub P111	Helderfontein, Stellenbosch	5 Mar 2001
	Sub P118	Helderfontein, Stellenbosch	28 Feb 2003
C. sessiliflora	Sess P69	Bien Donne, Simondium	1 Mar 2000
	Sess P108	Helderfontein, Stellenbosch	14 Nov 2001
	Sess P118	Helderfontein, Stellenbosch	28 Feb 2003
C. intermedia	Int P111	Helderfontein, Stellenbosch	19 Feb 2001
	Int PX1	Bien Donne, Simondium	Jan 2000
	Int P125	Helderfontein, Stellenbosch	14 Jan 2002

*The abbreviations used for the harvestings are also used for the dried methanol extracts prepared from these collections. Although some harvestings were conducted on the same plantation, this was done at different times.

Stock solutions (*c.* 4 mg ml^{-1}) dissolved in water for DAE and DMSO for DME were prepared. They were filtered through a 25 mm 0.45-μm Millipore Millex-HV Hydrophilic PVDF syringe filter (Microsep, South Africa) directly into an HPLC sample vial for injection (10 μl) in duplicate, without further dilution. Separations were carried out at 30°C with the temperature maintained by a column oven. Tentative peak identity was determined by means of retention time and comparison to UV spectra of external HPLC standards. Quantification of compounds at 280 nm was based on peak area, obtained with valley-to-valley integration, using external standards. Quantities were expressed as a percentage of the dried extracts. The concentration ranges of the external standards were based on the expected levels of compounds in the dried extracts.

Data manipulation and statistical analysis

The GraphPad Prism® version 4.00 for Windows® was used for graphi-

Table 3. Extract yield[a] and total polyphenol content[b] of *Cyclopia* dried aqueous (DAE) and dried methanol (DME) extracts.

Species	DAE from unfermented plant material[c]			DAE from fermented plant material[c]			DME from unfermented plant material[d]		
	Harvesting	Extract yield	TPP content	Harvesting	Extract yield	TPP content	Harvesting	Extract yield	TPP content
C. genistoides	Gen 1G	39.5	29.1	Gen 1F	35.5	21.8	Gen P104	13.4	22.3
	Gen 2G	38.8	30.5	Gen 2F	34.3	21.8	Gen P105	13.4	22.0
	Gen 3G	39.9	30.3	Gen 3F	36.8	22.3	Gen P122	18.9	25.0
	Average	**39.4**	**30.0**	**Average**	**35.5****	**22.0*****	**Average**	**15.2*****	**23.1****
C. subternata	Sub 1G	39.8	33.2	Sub 8F	21.1	19.2	Sub P118	13.0	22.2
	Sub 3G	39.7	32.4	Sub 10F	23.7	18.4	Sub P111	8.1	23.8
	Sub 15G	30.6	31.9	Sub 13F	20.8	17.5	Sub PY1	14.0	23.2
	Average	**36.7**	**32.5**	**Average**	**21.9****	**18.4*****	**Average**	**11.7****	**22.0*****
C. sessiliflora	Sess 2G	31.9	29.1	Sess 4F	22.2	16.3	Sess P108	12.8	29.1
	Sess 3G	33.3	29.8	Sess 6F	23.5	17.7	Sess P118	14.8	32.8
	Sess 4G	32.0	30.0	Sess 7F	23.2	19.8	Sess P69	16.0	28.9
	Average	**32.4**	**29.6**	**Average**	**23.05*****	**17.9*****	**Average**	**14.5*****	**30.2**$^{n.s.}$
C. intermedia	Int 1G	26.8	30.5	Int 2F	16.9	17.2	Int P111	12.8	22.9
	Int 2G	25.4	29.4	Int 3F	16.3	16.1	Int PX1	15.9	25.6
	Int 5G	29.1	30.6	Int 4F	ND[e]	ND	Int P125	16.9	30.7
	Average	**27.1**	**30.2**	**Average**	**16.6****	**16.6*****	**Average**	**15.2****	**26.4**$^{n.s.}$
Across species	**Average**	**33.9**	**30.6**	**Average**	**24.2**$^{\#}$	**18.7**$^{\#\#}$	**Average**	**14.2**$^{\#\#}$	**25.7**$^{n.s.}$

[a]Yield (g) of freeze-dried extract per 100 g dried pulverized plant material.
[b]Total polyphenol (TPP) content as grams gallic acid equivalents per 100 g freeze-dried extract.
[c]All aqueous extracts were prepared by E.S. Richards[7] from fermented and unfermented plant material as described in Materials and methods.
[d]All methanol extracts were prepared from unfermented plant material as described in Materials and methods.
[e]N.D., no data available.

The statistical analyses were conducted using two-tailed *t*-tests and comparing extract yields (and TPP content) within each species (unpaired) and across all species (paired). Within each species, the extract or TPP content was compared with the extract or TPP content of unfermented DAE ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; n.s., not significant). For comparison across species, the across-species average of fermented DAE and unfermented DME was compared with the corresponding average of unfermented DAE ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$; n.s., not significant).

cal representations and statistical analysis. One-way ANOVA and Dunnett's multiple comparisons test as post-test or paired two-tailed *t*-tests were used for statistical analysis. Pearson correlations (two-tailed) were performed and are expressed as Pearson correlation coefficient (*r*).

Results

Determination of extract yield and TPP content of DAE and DME

The results of DAE and DME yields and their TPP content are presented in Table 3. The DAE yield across all species was significantly ($P < 0.05$) higher for unfermented than for fermented plant material. For all species and unfermented plant material, aqueous extraction gave significantly ($P < 0.01$) higher yields of soluble solids than methanol extraction. A similar pattern, DAE from unfermented plant material > DAE from fermented plant material > DME from unfermented plant material, was observed when each species was considered separately.

Within species and across all species, DAE from unfermented plant material had a significantly ($P < 0.01$) higher TPP content than the corresponding extracts from fermented plant material. Within species, the TPP content of the DME from unfermented material was significantly lower ($P < 0.01$) than that of DAE of unfermented plant material from *C. genistoides* and *C. subternata,* but not from *C. intermedia* and *C. sessiliflora.* However, there was no significant difference between the DAE and DME from unfermented plant material across all species.

Extract yield and TPP content of DAE (Pearson $r = 0.34$) and DME ($r = 0.28$) from unfermented plant material did not correlate, whereas they did for fermented material ($r = 0.91$).

Binding to ER subtypes

All polyphenols investigated at a concentration of 10^{-5} M were able significantly ($P < 0.01$) to displace ^{3}H-E_2 from hERβ except for mangiferin, hesperidin, and hesperetin (Table 4). Naringenin, luteolin and formononetin displaced more than 50%. Luteolin, in displacing 92% of the ^{3}H-E_2 from hERβ, compared closely with the phyto-oestrogen control, genistein, that displaced 95% (Table 4). Eriodictyol correspondingly displaced 44% whereas both eriocitrin and narirutin displaced 28%.

The affinity of the polyphenols for hERα was significantly less, with eriodictyol, narirutin, eriocitrin, hesperidin, hesperetin and mangiferin unable significantly to displace ^{3}H-E_2 from hERα (Table 4). Although naringenin, formononetin and even luteolin were able to compete with radiolabelled E_2 for binding to both ER receptors, the extent of their displacement of ^{3}H-E_2 from hERα was much less (Table 4), with only luteolin displacing more than 50% of the ^{3}H-E_2. In addition, genistein also achieved a slightly lower displacement from hERα. Despite this, the order of ability to displace ^{3}H-E_2 was similar for both receptor subtypes: E_2 > genistein > luteolin > naringenin > formononetin (only for ERβ > eriodictyol > eriocitrin = narirutin).

The DAE of both fermented and unfermented *Cyclopia* spp. displayed no significant binding affinity for hERα (Table 5),

Table 4. Summary of percentage ^{3}H-E_2 displaced from ER subtypes by the polyphenols present in *Cyclopia.*

Test compounds (10^{-5} M)	Percentage of ^{3}H-E_2 displaced[a]	
	hERα	hERβ
E_2[b]	100^{**c}	100**
Genistein[b]	89**	95**
Luteolin	61**	92**
Naringenin	24**	70**
Formononetin	22*	58**
Eriodictyol	n.s.[d]	44**
Eriocitrin	n.s.	28**
Narirutin	n.s.	28**
Hesperidin	n.s.	n.s.
Hesperetin	n.s.	n.s.
Mangiferin	n.s.	n.s.

[a]Percentage ^{3}H-E_2 displaced calculated from 10^{-5} M E_2 set at 100% displacement and control set as 0% displacement.
[b]E_2 and genistein were used as positive controls.
[c]For statistical analysis, one-way ANOVA was used with Dunnett's multiple comparisons post-test comparing binding to control (0% displacement of ^{3}H-E_2). * $P < 0.05$; ** $P < 0.01$.
[d]n.s., Did not significantly displace ^{3}H-E_2 from ER receptor ($P > 0.05$).

although the natural ligand, E_2, and genistein displaced ^{3}H-E_2 to a significant degree. The same trend was not observed when binding to hERβ was investigated (Table 5). The DAE of unfermented and fermented *C. sessiliflora* and *C. intermedia* were unable significantly to displace 1 nM ^{3}H-E_2 from hERβ , whereas the DAE of unfermented and fermented *C. genistoides* and *C. subternata* were notably more consistent in binding to these receptors. Two harvestings each from unfermented and fermented *C. genistoides* DAE significantly displaced ($P < 0.05$) ^{3}H-E_2 from hERβ. In contrast, only two unfermented *C. subternata* DAE were able to bind significantly ($P < 0.05$) to hERβ.

Unfermented DME of *C. intermedia* and *C. sessiliflora* showed no significant binding affinity for either ERs (Table 5). As for the DAE, some methanol extracts of *C. genistoides* and *C. subternata* competed with ^{3}H-E_2 for binding to the hERβ. One *C. genistoides* extract (Gen P104) displaced the highest percentage of ^{3}H-E_2 (Table 5) from both hERα and hERβ. One *C. subternata* extract (Sub P118) bound significantly ($P < 0.01$) to both ER types. *Cyclopia genistoides* DME demonstrated the highest displacement of ^{3}H-E_2 and the highest consistency of binding (two out of three harvestings) to ERβ.

HPLC analysis of DAE and DME

The *Cyclopia* extracts were analysed by HPLC to ascertain if the polyphenols (luteolin, formononetin, naringenin, eriodictyol, eriocitrin, narirutin, hesperidin, hesperetin and mangiferin) tested, were present. Mangiferin and hesperidin were present in all species, whether fermented or unfermented, and in all extracts, whether DAE or DME (Tables 6, 7). Other compounds detected were hesperetin, eriocitrin and narirutin. Hesperetin and narirutin were present in trace amounts. Luteolin, formononetin, naringenin and eriodictyol were not detected by HPLC under the conditions used. The HPLC profile did, however, show several unknown peaks, whose identification deserves further investigation (data not shown).

Correlations

The correlation between TPP content and binding to hERα or hERβ was investigated as phyto-oestrogens are polyphenols (Fig. 2), but none was found for any of the extracts. Eriocitrin and narirutin were the only known compounds present in the extracts that were able to compete with ^{3}H-E_2 for binding to the hERβ. Since both eriocitrin and narirutin gave the same displacement (Table 4), their combined content (Fig. 3) in each extract was correlated with the percentage ^{3}H-E_2 displaced from ERβ. No significant correlation was found.

Table 5. Summary of percentage ^{3}H-E_2 displaced from ERα and ERα receptors by *Cyclopia* dried aqueous (DAE) and dried methanol (DME) extracts.

Extracts screened[a]	Percentage displacement of 1 nM ^{3}H-E_2[b]	
	hERα	hERβ
DAE: *C. genistoides*		
Gen 1G	NB[c]	NB
Gen 2G	NB	34**[d]
Gen 3G	NB	48**
Gen 1F	NB	35*
Gen 2F	NB	NB
Gen 3F	1	52**
DME: *C. genistoides*		
Gen P104	74**	70**
Gen P105	9	34**
Gen P122	12	NB
DAE: *C. subternata*		
Sub 1G	NB	NB
Sub 3G	NB	27*
Sub 15G	36	33*
Sub 8F	NB	19
Sub 10F	NB	11
Sub 13F	NB	NB
DME: *C. subternata*		
Sub P118	43**	69**
Sub P111	7	5
Sub PY1	NB	6
DAE: *C. sessiliflora*		
Sess 2G	NB	2
Sess 3G	5	17
Sess 4G	NB	0.2
Sess 4F	7	NB
Sess 6F	19	40
Sess 7F	16	7
DME: *C. sessiliflora*		
Sess P108	NB	NB
Sess P118	NB	2
Sess P69	NB	NB
DAE: *C. intermedia*		
Int 1G	NB	2
Int 2G	NB	NB
Int 5G	NB	28
Int 2F	2	NB
Int 3F	13	5
Int 4F	12	NB
DME: *C. intermedia*		
Int P111	NB	NB
Int PX1	NB	10
Int P125	3	3
Genistein	76**	91**
17-β-oestradiol	100**	100**

[a]DAE and DME were tested at a concentration of 1.5 µg ml^{-1}. The positive controls 17-β-oestradiol and genistein were both assayed at 10^{-5} M (E_2: 2.72×10^{-3} mg ml^{-1}; genistein: 2.70×10^{-3} mg ml^{-1}).
[b]Percentage ^{3}H-E_2 displaced calculated from 10^{-5} M E_2 set at 100% displacement and control set as 0% displacement.
[c]NB, non-binder (extracts were unable to displace ^{3}H-E_2 from respective ER).
[d]For statistical analysis, one-way ANOVA was used with Dunnett's multiple comparisons post-test comparing binding to control (0% displacement of ^{3}H-E_2). *$P < 0.05$; **$P < 0.01$.

Discussion

Epidemiological studies indicate that the low occurrence of certain cancers as well as less severe or no menopausal symptoms in women from Asian countries may be due to the intake of flavonoids, especially from soya, present in their diet.[20,21] The phenolic compounds are thought to act as weak oestrogens. In contrast, menopausal women using traditional HRT are believed to be more predisposed to breast cancer.[23]

Alternative therapies for HRT are thus being researched, as conventional therapy does not protect menopausal women from certain conditions such as cardiovascular disease, as was previously thought.[25] In addition, consumers are inclined to prefer 'natural' alternatives, which they consider to have fewer side-effects than synthetic drugs.[19] Plant flavonoids, such as those present in an Asian diet, are already marketed and available to women for use as an alternative or supplement to conventional HRT. These phyto-oestrogens are believed to alleviate menopausal symptoms and to protect against oestrogen-dependent cancers.[13–15,49]

Cyclopia is a possible source of phyto-oestrogens. The identification of some examples, naringenin, luteolin, eriodictyol and formononetin,[10,11] in *C. intermedia*[4] and anecdotal information from a woman in the Langkloof, who drinks a herbal infusion prepared from *C. intermedia* to alleviate menopausal symptoms (pers. comm. to E. Joubert by J. Nortje, Kouga, 1996) led to this investigation. Honeybush tea has previously been shown to be antimutagenic and to have antioxidant properties.[7,8]

Several of the polyphenols present in the *Cyclopia* spp. were screened for phyto-oestrogenic activity to serve as marker compounds for future experimental studies on the phyto-

Table 6. Phenolic content, as determined by HPLC, of the dried aqueous extracts of unfermented and fermented *Cyclopia* plant material.

Species	Harvesting	Percentage of soluble solids[a]								
		Mangiferin	Hesperidin	Hesperetin	Eriocitrin	Narirutin	Luteolin	Formononetin	Naringenin	Eriodictyol
C. genistoides	Gen 1G	10.040 ± 0.11[b]	1.010 ± 0.00	n.d.[c]	0.175 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.
	Gen 2G	8.880 ± 0.07	1.005 ± 0.01	n.d.	0.165 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.
	Gen 3G	10.04 ± 0.11	1.004 ± 0.01	n.d.	0.169 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.
	Gen 1F	3.910 ± 0.03	0.460 ± 0.00	0.01 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Gen 2F	2.975 ± 0.01	0.445 ± 0.01	0.01 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Gen 3F	5.845 ± 0.02	0.475 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C. subternata	Sub 1G	1.855 ± 0.01	0.730 ± 0.01	n.d.	0.455 ± 0.01	0.040 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Sub 3G	1.025 ± 0.02	0.460 ± 0.00	n.d.	0.395 ± 0.01	0.040 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Sub 15G	1.270 ± 0.01	0.390 ± 0.00	n.d.	0.405 ± 0.01	0.030 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Sub 8F	0.105 ± 0.01	0.365 ± 0.01	n.d.	0.290 ± 0.00	0.040 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Sub 10F	0.070 ± 0.00	0.180 ± 0.00	n.d.	0.380 ± 0.00	0.050 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Sub 13F	0.090 ± 0.00	0.380 ± 0.00	n.d.	0.190 ± 0.01	0.040 ± 0.00	n.d.	n.d.	n.d.	n.d.
C. sessiliflora	Sess 1G	3.875 ± 0.09	0.515 ± 0.01	n.d.	0.285 ± 0.01	0.030 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Sess 2G	4.205 ± 0.09	0.560 ± 0.00	n.d.	0.300 ± 0.00	0.030 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Sess 4G	4.240 ± 0.08	0.525 ± 0.04	n.d.	0.265 ± 0.01	0.025 ± 0.01	n.d.	n.d.	n.d.	n.d.
	Sess 4F	0.180 ± 0.00	0.485 ± 0.01	n.d.	0.100 ± 0.00	0.020 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Sess 7F	0.215 ± 0.01	0.400 ± 0.00	n.d.	0.155 ± 0.01	0.010 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Sess 6F	0.250 ± 0.00	0.430 ± 0.00	n.d.	0.200 ± 0.00	0.025 ± 0.01	n.d.	n.d.	n.d.	n.d.
C. intermedia	Int 1G	1.815 ± 0.05	1.130 ± 0.03	0.020 ± 0.00	n.d.	0.030 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Int 2G	2.085 ± 0.01	1.140 ± 0.00	0.030 ± 0.00	n.d.	0.025 ± 0.01	n.d.	n.d.	n.d.	n.d.
	Int 5G	3.300 ± 0.01	1.085 ± 0.01	0.010 ± 0.00	n.d.	0.065 ± 0.01	n.d.	n.d.	n.d.	n.d.
	Int 2F	0.200 ± 0.00	0.525 ± 0.01	0.060 ± 0.00	n.d.	0.010 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Int 3F	0.240 ± 0.00	0.455 ± 0.01	0.100 ± 0.00	n.d.	0.020 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Int 4F	0.245 ± 0.01	0.23 ± 0.00	0.090 ± 0.00	n.d.	0.010 ± 0.00	n.d.	n.d.	n.d.	n.d.

[a]Quantities are expressed as a percentage of the extract.
[b]Values represent the means (% of DAE) ± s.d. of two determinations.
[c]Not detected.

oestrogenic activity of honeybush. In addition, the four commercially available honeybush tea varieties, *C. genistoides, C. subternata, C. sessiliflora* and *C. intermedia*, were screened, through whole-cell competitive receptor binding assays, to ascertain which *Cyclopia* species contained the highest putative phyto-oestrogenic activity and would merit further investigation.

As the plant extraction procedure is tedious and time consuming, a high yield is considered an advantage and aimed for. Additionally, the solvent used is also important for selective and efficient extraction of flavonoids. Hesperidin, for example, is poorly soluble in water. Similarly, extraction of aglycones is enhanced using methanol instead of water. Fermentation substantially reduced the DAE yield ($P < 0.05$), as well as its TPP content ($P < 0.01$). This confirms previous studies.[2,50] Methanol was less effective than water in extracting soluble solids ($P < 0.01$), but higher levels of the flavanone rutinosides, hesperidin, narirutin and eriocitrin, were obtained. The absence of the aglycones, luteolin, formononetin, naringenin and eriodictyol in DAE or DME indicates either poor extraction or low levels in the plant material. Caution should, however, be exercised in evaluating these differences in yield and phenolic content, especially between water and methanol extraction. Although extracts were from the same plant species, they were not prepared from the same harvesting nor always the same area; these factors may contribute to variation. The plants were not monoclonal as they were propagated from seedlings and large genetic variation is to be expected.

Binding to the ER is the first interaction in the molecular pathway of the oestrogen-mediated biological response. Naringenin, formononetin, eriodictyol and luteolin, which are known phyto-oestrogens, and narirutin, eriocitrin, hesperidin and hesperetin, identified to be present in *C. intermedia*[4,51] and/or

Table 7. Phenolic content, as determined by HPLC, of the dried methanol extracts of unfermented *Cyclopia* plant material.

Species	Harvestings	Percentage of soluble solids[a]								
		Mangiferin	Hesperidin	Hesperetin	Eriocitrin	Narirutin	Luteolin	Formononetin	Naringenin	Eriodictyol
C. genistoides	Gen P104	2.77 ± 0.01[b]	1.22 ± 0.00	n.d.[c]	0.19 ± 0.00	0.35 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Gen P105	2.71 ± 0.01	1.21 ± 0.00	n.d.	0.19 ± 0.01	0.35 ± 0.01	n.d.	n.d.	n.d.	n.d.
	Gen P122	2.28 ± 0.04	1.21 ± 0.01	n.d.	0.22 ± 0.00	0.31 ± 0.00	n.d.	n.d.	n.d.	n.d.
C. subternata	Sub P118	4.25 ± 0.00	1.14 ± 0.00	n.d.	0.92 ± 0.01	0.04 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Sub P111	3.26 ± 0.00	0.69 ± 0.00	n.d.	0.65 ± 0.00	0.03 ± 0.01	n.d.	n.d.	n.d.	n.d.
	Sub PY1	1.705 ± 0.02	1.63 ± 0.02	n.d.	1.90 ± 0.01	0.09 ± 0.01	n.d.	n.d.	n.d.	n.d.
C. sessiliflora	Sess P108	3.69 ± 0.00	0.88 ± 0.01	n.d.	0.45 ± 0.00	0.03 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Sess P118	4.28 ± 0.02	1.16 ± 0.01	n.d.	0.36 ± 0.02	0.05 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Sess P69	4.61 ± 0.04	1.22 ± 0.02	n.d.	0.50 ± 0.03	0.06 ± 0.00	n.d.	n.d.	n.d.	n.d.
C. intermedia	Int P111	3.90 ± 0.02	1.28 ± 0.01	n.d.	0.21 ± 0.001	0.23 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Int P X1	5.21 ± 0.01	1.53 ± 0.00	n.d.	0.21 ± 0.00	0.22 ± 0.00	n.d.	n.d.	n.d.	n.d.
	Int P125[d]	7.04 ± 0.06	2.34 ± 0.05	n.d.	0.63 ± 0.01	0.10 ± 0.00	n.d.	n.d.	n.d.	n.d.

[a]Quantities are expressed as a percentage of the extract.
[b]Values represent the means (% of DME) ± s.d. of two determinations.
[c]Not detected.
[d]Harvesting was analysed on a Luna phenyl-hexyl column, which did not separate eriocitrin effectively, thus results are over-expressed.

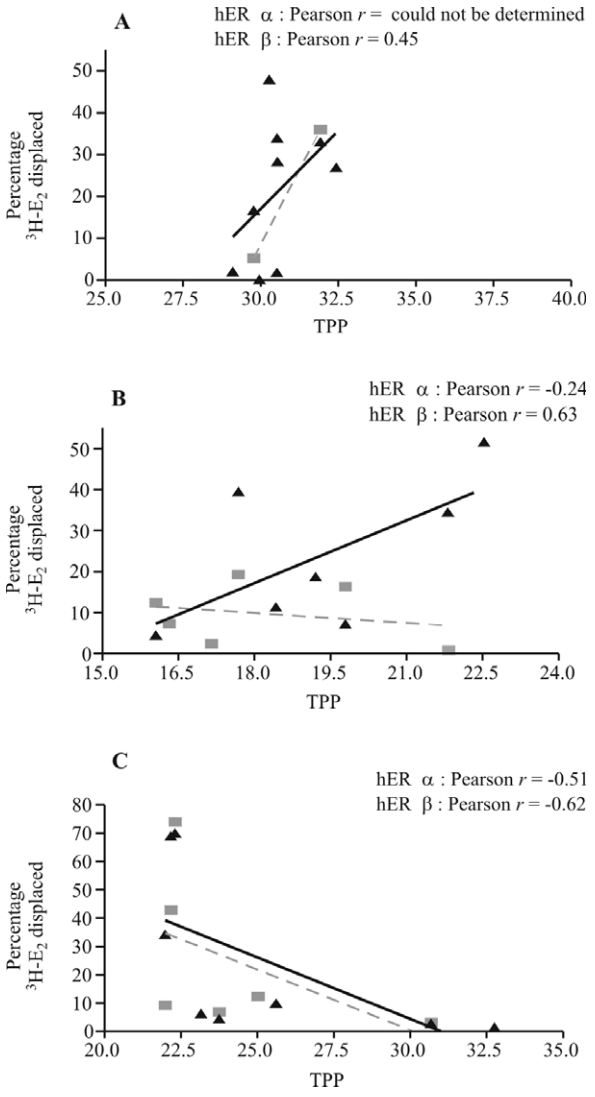

Fig. 2. Correlation between percentage ^{3}H-E_2 displaced from hERα (■) and hERβ (▲) and percentage total polyphenol content (TPP) of (**A**) DAE from unfermented plant material, (**B**) DAE from fermented plant material, and (**C**) DME from unfermented plant material. Pearson correlations (two-tailed) were performed using GraphPad Prism™ linear regression. All correlations are not significant.

C. subternata,[5] were screened at a concentration of 10^{-5}M for binding to the two receptor subtypes. Mangiferin, a xanthone glucoside, was also included in the investigation as it is present in high concentration in all *Cyclopia* species.[3,4,6,51]

Naringenin, formononetin and luteolin bound to both ERα and ERβ, with an order of displacement of luteolin > naringenin > formononetin. Similar to results of the present study, others have shown that luteolin binds to ERβ,[10] as does naringenin,[10,40] which also binds weakly to ERα.[40,52] Formononetin binds to ERβ and weakly to ERα.[40] To our knowledge, binding of luteolin to hERα has not previously been investigated. Others have shown that naringenin has a higher binding affinity for ERβ than luteolin,[10] which was not supported by our data. However, full dose–response curves were not investigated and therefore the results are not fully comparable to the IC_{50} values determined by Han *et al.*[10] However, we showed that naringenin displaced more ^{3}H-E_2 from hERβ than formononetin, as did Han *et al.*[10] Of the other polyphenols tested, narirutin, eriocitrin, and eriodictyol bound only to the ERβ, whereas hesperidin and its aglycone, hesperetin, like mangiferin, did not bind to either receptor subtype. Hesperetin has previously been shown to have no binding affinity for the endogenously expressed ER in MCF-7 cells.[53] To our knowledge, this is the first time that eriodictyol, narirutin, and eriocitrin have been shown to displace ^{3}H-E_2 from hERβ. Eriodictyol has, however, been found to demonstrate weak oestrogenic activity in stimulating both MCF-7 cell proliferation and transcriptional induction of an ERE-containing promoter.[11,12]

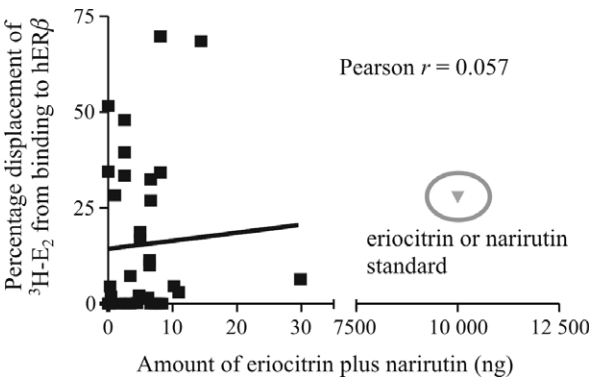

Fig. 3. Correlation between the percentage ^{3}H-E_2 displaced from hERα by all the extracts investigated and the amount of eriocitrin and narirutin present as quantified by reversed phase HPLC. Pearson correlation (two-tailed) was performed using GraphPad Prism™. Correlation is not significant.

Phyto-oestrogens have been shown to have a higher affinity for ERβ than for ERα,[40] which was also demonstrated in our study. The plant polyphenols tested, including genistein as control, all preferentially displaced ^{3}H-E_2 from hERβ.The ERβ may be a negative regulator of ERα activity and compounds with selective ERβ affinity are sought, as it is believed that they could be used for the treatment for oestrogen-dependent cancers resulting from ERα action.[54,55] The literature suggests that ERβ activity could protect against excessive proliferation of cancerous cells mediated by ERα.[5,6,57] This phenomenon may be the reason why phyto-oestrogens are considered an alternative to conventional HRT as they could still protect against osteoporosis and reduce cardiovascular disease, without inducing oestrogen-dependent cancers.

As with the commercially available polyphenols, we found that binding of *Cyclopia* extracts to ERβ was more significant than to ERα. None of the DAE bound significantly to ERα and only one out of three DME from *C. genistoides* and *C. subternata* did so. The binding of *C. genistoides* extracts to ERβ exceeded that of all other extracts, with two out of three harvestings giving significant displacement for all extracts. Of the other *Cyclopia* spp., only extracts from unfermented *C. subternata* (two out of three for DAE and one out of three for DME) displayed significant binding to hERβ. Only two extracts, both DME, from *C. genistoides* and *C. subternata*, showed significant displacement from both receptor subtypes. Extracts from *C. intermedia* did not bind significantly to either ER subtype, which was unexpected because of the presence of isoflavones and coumestans in this genus.[4] Poor extraction of aglycones due to low solubility in water, losses during clean-up with dichloromethane for subsequent methanol extraction, or initial low quantities of flavonoids present in the plant material, could explain the inability of *C. intermedia* to displace ^{3}H-E_2 from the two receptor subtypes.

Although the average TPP content of unfermented DAE was significantly higher than that of both fermented DAE and unfermented DME within species, ER binding was not correlated with TPP content. This was to be expected as the Folin-Ciocalteau assay measures only the presence and number of hydroxyl groups, which suggests that the structural requirements of the polyphenols are more important in determining oestrogenic effects than the number of these groups. Indeed,

Miksicek,[58] in comparing a wide variety of polyphenols, found that the diaryl ring structure common to all flavonoids and at least one hydroxyl group present on each of the aromatic rings are essential for oestrogenic activity.

Furthermore, despite the decrease in TPP content of the plant material and subsequently of their extracts after fermentation, both unfermented and fermented DAE of *C. genistoides* were able to bind to the hERβ, suggesting that the active compound(s) in *C. genistoides* is not highly susceptible to oxidation and thus is retained to a large extent during fermentation, or that oxidative changes did not result in changing the structure required for binding. Fermentation did, however, have an effect on binding of *C. subternata* extracts, as only unfermented DAE were able to bind significantly to hERβ. In this case, loss of the active compound(s) with oxidation is indicated, also suggesting that the active compound(s) differs from that of *C. genistoides.* These findings indicate that fermentation appears not to have such a clear-cut effect on ER binding and that identification of the active compound(s) is necessary to understand the influence of fermentation on the binding of ER subtypes by different species.

The quantification of phenolic compounds present in DAE and DME was carried out in an attempt to provide a chemical basis for the observed intra- and inter-species differences. However, the results did not explain the significant binding to the ER subtypes by some extracts. The two major compounds, mangiferin and hesperidin, did not bind to either receptor type. The phyto-oestrogens, formononetin, luteolin, eriodictyol, and naringenin, were not detected in any of the extracts (aqueous or methanol), whereas eriocitrin and narirutin, which bound to hERβ and could contribute to binding of some extracts, were present only in trace amounts. It is to be expected that the compounds that have binding affinity for the receptors would be present in higher amounts in the active species such as *C. genistoides* and *C. subternata,* or in extracts such as Gen 104 and Sub P118, which competed significantly for binding to both ER receptor subtypes. This, however, was not the case with Gen 104, which bound significantly to both receptors, and contained similar amounts of eriocitrin and narirutin as Int P11 bit which did not bind to either receptor subtype. The lack of correlation between the amount of eriocitrin and narirutin present in the extracts examined and the displacement of E_2 further confirmed that the low levels of eriocitrin and narirutin cannot be responsible for the binding activity observed in the extracts. This matter needs further investigation as there may be potentially novel compounds in *Cyclopia* mediating the oestrogenic activity observed.

This study indicates that not only is honeybush a source of phyto-oestrogens, but that both aqueous and methanol extracts preferentially bind to the hERβ. In another study conducted with methanol extracts of the roots of *Moghania philippinensis,* from the same plant family as *Cyclopia,* it was shown that these extracts induced proliferation of the human breast cancer cell line MCF-7.[59] Further studies with *Cyclopia* are therefore desirable to ascertain if the phyto-oestrogens present display potency in mediating oestrogenic or possibly anti-oestrogenic effects; these should include proliferative assays of human breast cancer cells. These assays are essential as receptor-binding studies do not differentiate between agonists and antagonists.

A high degree of variability in ER binding, even within the same species, was noticeable in our results. For example, DME from *C. genistoides* bound to both ER subtypes (Gen P104), only to ERβ (Gen P105), or to neither receptor (Gen P122). All three harvestings were from the same plantation, but were collected on different dates and not necessarily from the same plants. One explanation for the variability in binding ability may be that Gen P104 was harvested during a relatively dry season in contrast to the other two collections. Plants under stress tend to produce more secondary metabolites, such as polyphenols, as a protective mechanism,[43] which may contribute to the higher oestrogenicity (70% E_2 displaced from hERβ) of this particular harvesting, However, neither the TPP content nor HPLC data support this. Another explanation may be that as the *Cyclopia* industry is relatively new, there are large genotype and phenotype differences within one species and even within one plantation. Additionally, the phyto-oestrogens previously identified to be present in *C. intermedia* and *C. subternata* were not detected in either the DAE or DME of these species in our study. This variability was shown previously in terms of antimutagenicity by van der Merwe,[9] who examined the same DAE used in our study. The variability, both in ER binding ability and content of specific polyphenols, do however, highlight the importance of testing for oestrogenicity of individual harvestings as no blanket claims can at this stage be made concerning oestrogenic activity of individual *Cyclopia* species. Nevertheless, our results suggest that future studies should concentrate on investigating the oestrogenicity of *C. genistoides,* which displayed the most consistent phyto-oestrogenic activity. It is also important to identify the specific compounds responsible for this activity in *Cyclopia* as clearly the polyphenols thought to contribute to oestrogenicity are not present in sufficient quantities to explain the binding found with the extracts. In addition, at this early stage in the industry's history, it may be appropriate to concentrate on producing a nutraceutical product with enhanced oestrogenic activity from *Cyclopia* harvestings displaying initial high levels of oestrogenicity than to endorse blanket statements concerning oestrogenic activity in honeybush tea. Also required is a rapid screening method to ensure that the plant material used to prepare extracts displays the required activity.

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