Phytoestrogenic Potential of *Cyclopia* Extracts and Polyphenols

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- Fabaceae
- *Cyclopia*
- phytoestrogen
- ER binding
- ERE promoter reporter assay
- E-screen
- uterotrophic assay

Abstract

Cyclopia Vent. species, commonly known as honeybush, are endemic to Southern Africa. The plant is traditionally used as an herbal tea but several health benefits have recently been recorded. This minireview presents an overview of polyphenols found in *Cyclopia* and focusses on the phytoestrogenic potential of selected polyphenols and of extracts prepared from the plant.

Introduction

Cyclopia species (family Fabaceae; tribe Podalyrieae) are part of the fynbos biome and endemic to the coastal and mountainous regions of the Western and Eastern Cape Provinces of South Africa. The plant may grow up to heights of 3 m in the wild and is distinguished by trifoliate leaves and sweet smelling deep yellow flowers with an indented calyx [1] (**Fig. 1**). Although more than twenty species of *Cyclopia* have been described [2], the commercially important species include *C. genistoides, C. sessiliflora, C. intermedia*, and *C. subternata*. Fermented (oxidised) *Cyclopia* is traditionally used as an herbal tea, called honeybush tea, which is acclaimed for its distinct sweet aroma and fragrant flavour. Recently, unfermented honeybush has also been added to the market. *Cyclopia* is one of the few South African plants to have made the transition from regional use to commercial product [3], and in 2011 a total of 174 tons of *Cyclopia* was exported, mostly to Germany (37%), the Netherlands (29%), USA (14%), and UK (12%) (data supplied by Soekie Snyman, SA Rooibos Council, 2012).

Cyclopia has traditionally also been used for medicinal purposes, including as a restorative, as an expectorant, and to promote appetite [4]. Research into the phenolic composition of *Cyclopia* spp. [5–7] has been crucial in identifying value-adding opportunities in the arena of health promoting attributes. Foremost amongst these have been the demonstration of antioxidant properties [8,9], inhibition of tumour development [10,11], and antidiabetic potential [12,13]. Furthermore, scrutiny of phenolic composition coupled to anecdotal claims of *Cyclopia* as of use in stimulating milk production [14] and alleviating menopausal symptoms has led to recent research on the phytoestrogenic potential of *Cyclopia*. This minireview will focus on the polyphenol content of *Cyclopia* and the phytoestrogenic potential of selected polyphenols identified in this genus and extracts from the shoots and leaves of the plant.

Phenolic Composition of *Cyclopia*

The phenolic composition of a number of commercially important *Cyclopia* species has been investigated due to the relevance of these constituents for bioactivity of their herbal teas and extracts. In-depth studies, making use of NMR to unequivocally elucidate chemical structures, deal only with *C. intermedia* and *C. subternata* [5–7, 15]. Generally, *Cyclopia* species are characterised by the presence of the xanthone, mangiferin, with the co-occurrence of its 4-*C*-glucoside regioisomer, isomangiferin, and the flavanone, hesperidin, an *O*-rutinoside of hesperetin, in relatively large quantities [16]. Other classes of compounds identified in *C. intermedia* are flavonols, flavones, isoflavones, and coumestans, as well as some C6-C1 and C6-C2 secondary metabolites [5,6]. Apart from luteolin, none of the latter compounds has been found in detectable quantities in *C. intermedia* extracts by HPLC analysis. The isoflavone orobol was isolated from *C. subternata* [7]. In an *in vitro* culture, *C. subternata* produces glucosides of the isoflavone aglycones, calycosin, pseudobapti-

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Fig. 1 Shoots of *C. subternata* (left) and *C. genistoides* (right) with distinctive yellow flowers having an indented calyx, characteristic of *Cyclopia* species. (Color figure available online only.)

genin, and formononetin, present in *C. intermedia* [5,15]. Recent investigations demonstrated the presence of benzophenones and dihydrochalcones in *C. subternata* [15,17]. An iriflophenone-di-*O,C*-hexoside, an eriodictyol-di-*C*-hexoside, 3-hydroxyphloretin-3,5-di-*C*-hexoside, and vicenin-2 (apigenin-6,8-di-*C*-glucoside) were tentatively identified in *C. subternata*, based on UV-Vis, LC-MS, and LC-MS/MS characteristics of the compounds [17]. **Fig. 2** depicts phenolic compounds present in *C. subternata*.

The abundance of *C*-glycosides, both in terms of content and number of compounds (**Fig. 1**, **Table 1**), has implications concerning stability during processing and *in vivo*. The *C-C* bond is very stable and resistant to acid and intestinal enzymes able to hydrolyse *O*-glycosides, but evidence of *C-C* bond-cleaving reactions by human intestinal bacteria is growing [18–20].

Relatively high levels of certain phenolic compounds are present in the leaves of *C. subternata* (**Table 1**). These values could vary substantially as recently demonstrated by De Beer et al. [17] for seedling plants. Several of the compounds, including mangiferin, isomangiferin, iriflophenone-3-*C*-glucoside, scolymoside, the 7-*O*-rutinoside of luteolin, and eriocitrin, the 7-*O*-rutinoside of eriodictyol, occur in higher levels in aqueous extracts prepared from the leaves, while hesperidin, the 7-*O*-rutinoside of hesperetin, and the dihydrochalcone *C*-glycosides are predominant in the stems. Although natural variation is a contributing factor, trace or undetectable quantities of luteolin by HPLC-DAD in aqueous extracts, whilst present in the methanol extract (**Table 1**), are attributed to poor solubility of this aglycone in water.

Phytoestrogenic Potential of *Cyclopia* Polyphenols and Extracts

Phytoestrogenic potential may be defined in terms of the mechanism of action of the endogenous hormone 17*β*-estradiol (E_2) [21]. According to this definition, compounds with phytoestrogenic potential would act through at least one of the main isoforms of the estrogen receptor (ER), namely ER*α* or ER*β* [22], and act as agonists, antagonists, or selective ER modulators (SERMS) via ER signalling pathways [21] (**Fig. 3**). Phytoestrogens are, however, also considered to be endocrine disruptors and as such the definition used by regulatory bodies in both the USA and Europe could be useful [23,24]. The European Commission State of the Art Assessment of Endocrine Disruptors, for example, defines estrogenicity in terms of "binding to the estrogen receptor(s) (ER), ER activation, cell proliferation in ER-competent cells and physiological responses (proliferation of uterine tissue in rodents, induction of vitellogenin in fish)" [24].

Although several assays have been suggested to evaluate estrogenic activity [25], for the purposes of this review we will evaluate the phytoestrogenic potential of both the polyphenols shown to be present in *Cyclopia* and extracts prepared from *Cyclopia* in terms of their *in vitro* ability to either bind to ER*α* or ER*β*, to induce or prevent activation of ER-responsive promoters, or to cause cell proliferation in ER-responsive cells (e.g., E-screen in MCF-7 cells, a breast cancer cell line) or in terms of their *in vivo* responses in known estrogenic tissues such as the uterus (**Fig. 3**, **Tables 2**, **3**, and **4**). In addition, where it was not apparent that the ER was involved, we used evidence of loss of activity via ICI 182,782, an ER antagonist, as confirmation of ER involvement.

Although *in vivo* studies have been considered the "gold standard" for the evaluation of estrogenicity, many authors have not conducted such studies, and thus we have to rely on *in vitro* results. In terms of *in vitro* results, it is important to establish that

Table 1 Phenolic composition of leaves and extracts (g · 100 g^{-1} dry basis) of unfermented *Cyclopia subternata*.

Compound	Leaves [92] (n = 6)	Aqueous extract [16] (n = 6)	Aqueous extract [17] (n = 64)	Methanol extract [44] (n = 1)
Mangiferin	1.22 ± 0.35	2.73 ± 1.65	0.93 ± 0.42	1.91
Isomangiferin	0.38 ± 0.05	0.86 ± 0.28	0.47 ± 0.12	0.77
Hesperidin		0.62 ± 0.17	0.64 ± 0.36	2.21
Eriocitrin	0.23 ± 0.06	0.32 ± 0.07	0.55 ± 0.15	1.25
Eriodictyol glucoside[a]		0.35 ± 0.07[b]		
Iriflophenone-3-*C*-*β*-glucoside	0.25 ± 0.06	0.82 ± 0.44[c]	0.47 ± 0.29	
3-Hydroxyphloretin-3,5-di-*C*-hexoside[a]			0.54 ± 0.13	
Phloretin-3,5-di-*C*-glucoside	0.41 ± 0.01	0.86 ± 0.20[d]	1.05 ± 0.34	1.22[f]
Scolymoside	0.48 ± 0.32	0.68 ± 0.62[e]	0.49 ± 0.24	2.04[g]
Luteolin				0.09

[a] Position and/or identity of glycosyl moiety not certain; previous designation, [b] compound **9**, [c] compound **8**, [d] compound **12**, [e] compound **11**, [f] unknown **2**, [g] unknown **1**

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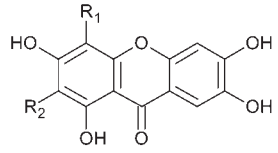
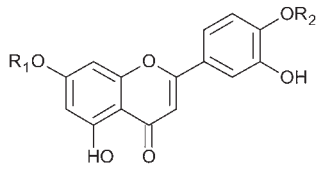


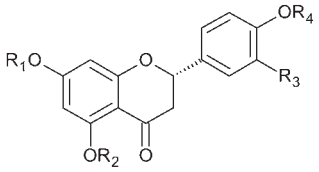
Xanthone

Mangiferin: R_2 = *C*-glucosyl; R_1= H
Isomangiferin: R_2 = H; R_1= *C*-glucosyl



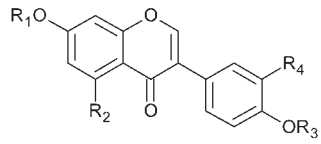
Flavone

Luteolin: R_1, R_2, = H
Diosmetin: R_1 = H; R_2 = CH_3
Scolymoside: R_1 = rutinosyl; R_2 = H



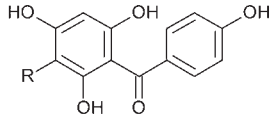
Flavanone

Naringenin: R_1, R_2, R_3, R_4 = H
Eriodictyol: R_1, R_2, R_4 = H; R_3 = OH
Hesperetin: R_1, R_2 = H; R_3 = OH; R_4 = CH_3
Narirutin: R_1 = rutinosyl; R_2, R_3, R_4 = H
Prunin: R_1 = glucosyl; R_2, R_3, R_4 = H
*Eriodictyol-*O*-glucoside: R_1 or R_2 = glucosyl or H; R_3 = OH, R_4 = H
Hesperidin: R_1= rutinosyl; R_2 = H; R_3 = OH; R_4 = CH_3
Eriocitrin: R_1= rutinosyl; R_2, R_4 = H; R_3 = OH



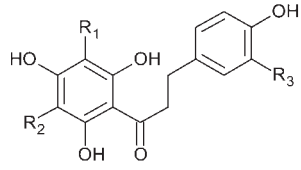
Isoflavone

Orobol: R_1, R_3 = H; R_2, R_4 = OH
Formononetin: R_1, R_2, R_4 = H; R_3 = CH_3
Calycosin: R_1, R_2 = H; R_3 = CH_3; R_4 = OH
Ononin: R_1, = glucosyl; R_2, R_4 = H; R_3 = CH_3
Calycosin-7-*O*-glucoside: R_1 = glucosyl; R_2 = H; R_3 = CH_3; R_4 = OH



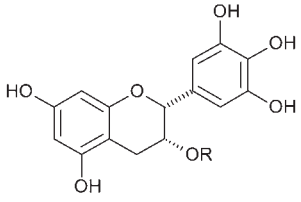
Benzophenone

Iriflophenone-3-*C*-β-glucoside: R = glucosyl



Dihydrochalcone

Phloretin-3',5'-di-*C*-β-glucoside: R_1, R_2 =*C*-glucosyl; R_3 = H
*3-Hydroxyphloretin-3',5'-di-*C*-hexoside: R_1, R_2 = *C*-hexoside; R_3 = OH



Flavanol

Epigallocatechin gallate: R = gallate

Fig. 2 Structures of major phenolic compounds of *C. subternata* and minor compounds with estrogenic activity present in the leaves and stems of some *Cyclopia* spp. (* indicates that the position or identity of the glycosyl moiety is not certain; bold text indicates the class of compound).

a hierarchy in terms of sensitivity has been established, with the E-screen generally considered the most sensitive assay [26–28]. Furthermore, although binding to the ER may be considered a prerequisite for estrogenic activity and is certainly the most characteristic mode of action of phytoestrogens [29], receptor binding assays cannot distinguish agonists from antagonists or SERMs [26]. Assays relying on the activation of ER-responsive promoters (both of artificial ERE-containing promoter reporters and endogenous ERE-containing estrogen responsive genes) and the E-screen are more appropriate assays to distinguish agonists from antagonists and SERMs [26]. Furthermore, to distinguish activation of ERα from activation via ERβ, cell lines expressing these receptors separately have to be utilised. MCF-7 cells, used in the E-screen, contain both ERα and ERβ and thus lack the ability to discriminate between the roles of the ER isoforms [25]. In addition, the uterotrophic assay is primarily an assay to verify ERα-mediat-

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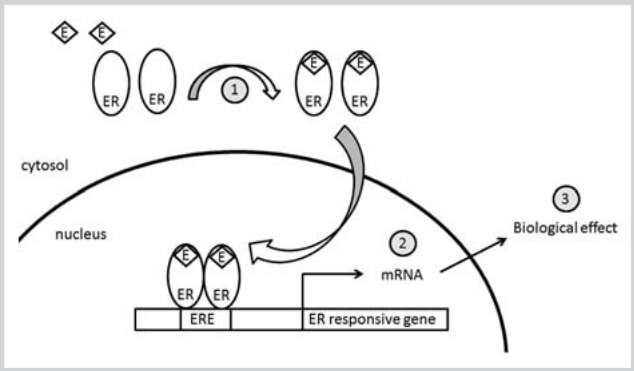

Fig. 3 Steps in ER signalling used to evaluate estrogenicity. E = estrogenic compound, ER = estrogen receptor, ERE = estrogen response element. (**1**) Binding of an estrogenic ligand to the ER may be evaluated by ligand-binding assays, (**2**) binding of ligand-activated ER to an ERE in the promoter of an estrogen responsive gene may be evaluated by promoter-reporter studies using an ERE-containing promoter reporter or by measuring mRNA levels of select ER-responsive genes, and (**3**) downstream biological effects such as cell proliferation or hypertrophy of the uterus may be measured using the E-screen or uterotrophic assay, respectively.

Table 2 Known [5–7, 15] *Cyclopia* polyphenols that have not been tested for estrogenic potential.

Class of compound	Specific compound(s)
Xanthone	isomangiferin
Flavanone	eriodictyol-5-*O*-glucoside, eriodictyol-7-*O*-glucoside, naringenin-5-*O*-glucoside, isosakuranetin
Flavone	5-deoxyluteolin, scolymoside, isorhoifolin, vicenin-2
Flavonol	kaempferol-5-*O*-glucoside, kaempferol-6-*C*-glucoside, kaempferol-8-*C*-glucoside
Methylinedioxyflavanol derivative	3′4′-methylinedioxyflavanol apiosyl-glucoside
Isoflavone	formononetin apiosyl-glucoside, afrormosin, rothindin, wistin
Methylinedioxyisoflavone derivative	pseudobaptigenin, fujikinetin
Coumestan	flemichapparin, sophoracoumestan B
Benzophenone	iriflophenone-3-*C*-*β*-glucoside
Dihydrochalcone	phloretin-3′,5′-di-*C*-*β*-glucoside
Benzaldehyde derivative	benzaldehyde apiosyl-glucoside
Phenylethanoid derivative	tyrosol,3-methoxy-tyrosol, 4-glucosyltyrosol, phenylethanol apiosyl-glucoside

ed *in vivo* effects, and no appropriate *in vivo* assay for ER*β* has been established [25].

Initially, we wanted to standardise our comparison of the estrogenic potential of polyphenols in *Cyclopia* using the relative binding affinity (RBA) and relative induction index (RII) where binding and activation are expressed relative to the values for E_2 (calculated as follows: $100 \times IC_{50}$ or EC_{50} (E_2)/IC_{50} or EC_{50} (test compound), however, we found that few papers provide quantitative data. Thus most of our comparisons of estrogenic activity of the polyphenols present in *Cyclopia* (**Table 3**) rest on qualitative and not quantitative data.

Most of the polyphenols present in *Cyclopia* have, to our knowledge, not been tested for estrogenicity (**Table 2**). For example, the dihydrochalcone phloretin-3′,5′-di-*C*-*β*-glucoside, the flavone scolymoside, and the benzophenone iriflophenone-3-*C*-*β*-glucoside, all present in relatively high concentrations in *C. subternata* (**Table 1**), have not been tested (**Table 2**).

Table 3 summarises data for compounds that have been tested for estrogenicity in different assay systems. Mangiferin, the major xanthone in *Cyclopia* species (**Table 1**), has been shown to have no estrogenic activity both via ER binding assays and ERE-promoter reporter assays (**Table 3**). Although isomangiferin has not been tested (**Table 2**), it is unlikely to have estrogenic activity as it is a regioisomer of mangiferin (**Fig. 2**). The phenolic acid p-coumaric acid and the coumestan medicagol have both been tested but found not to be estrogenic (**Table 3**).

Of the flavanones present in *Cyclopia*, most have been tested for estrogenicity. Prunin (naringenin-7-*O*-glucoside), one of the rarer flavanones, is estrogenic, while of the glycosylated flavanones present in relatively high concentrations in *Cyclopia* (**Table 1**), like eriocitrin and hesperidin, only eriocitrin is estrogenic (**Table 3**). Eriodictyol and naringenin, as well as their rutinosyl derivatives, eriocitrin and narirutin bind to ER, although therutinosyl derivatives bind with a lower affinity than their corresponding aglycones. Specifically, in a competitive binding assay, eriodictyol and naringenin displaced 44% and 70% of 1 nM tritiated E_2 from ER*β*, respectively, while their corresponding rutinosyl derivatives displaced 28% and 28%, respectively [30]. Naringenin is interesting as it has been shown to be estrogenic *in vitro* using the usual array of screening assays, namely ER-binding, activation of ERE-responsive promoters both in promoter reporter studies and with endogenous genes, yet *in vivo*, using the immature uterotrophic assay, it does not display estrogenicity (**Table 3**). This may suggest that naringenin is not absorbed or is inactivated, either during hepatic metabolism or by gut bacteria, and highlights the importance of validating these parameters [31]. On the other hand, it may also suggest that naringenin does not transactivate via ER*α*, the ER responsible for uterotrophic action, but rather via ER*β*, as borne out by some [32], but not by other [33–35] promoter reporter studies. Hesperetin and its rutinosyl derivative, hesperidin, do not bind ER, although hesperetin, but not hesperidin, does transactivate an ERE-containing promoter reporter, which can probably be ascribed to the lower activity of glycosalyted derivatives relative to their aglycones. Furthermore, hesperetin activates estrogen responsive genes and causes cell proliferation in the E-screen via an ER-mediated mechanism as ICI 182,782 antagonises the response. This suggests that the ER-binding assay may not be sensitive enough to evaluate weak estrogenicity, which is further borne out by the fact that in three studies where naringenin and hesperetin were directly compared, hesperetin was a weaker agonist [33, 34, 36]. Specifically, Breinholt and Larsen [36] report EC_{50} values of 89.6 µM and 0.3 µM, while Promberger et al. [34] report 2% and 80% efficacy for hesperetin and naringenin, respectively, in ERE-containing promoter reporter studies. Liu et al. [33] also clearly show that hesperetin is weaker than naringenin at causing both cell proliferation in the E-screen and activation in promoter reporter studies. The lower activity of hesperetin relative to naringenin may be ascribed to the methyl functional group found on the B-ring of hesperetin (**Fig. 2**). The flavanol (−)-epigallocatechin gallate, however, was found to be estrogenic by binding to ER and via the GAL4 promoter assay (a very artificial system in which the ER is fused to a GAL4 element), but not via the ERE-containing promoter reporter assay (**Table 3**). This suggests that, contrary to what we have suggested for hesperetin, namely that ER binding may not be sensitive enough to test for weak estrogenic activ-

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Table 3 Phytoestrogenic potential of polyphenols found [5–7, 15] in *Cyclopia*.

Polyphenol	Estrogenic effect	Test for estrogenic effect		Reference
		Test system	Test model	
Xanthones				
Mangiferin	No	ER binding assay	COS-1 cells + hERα or hERβ	[30, 32]
			Fluorescence ERα competitor assay kit	[45]
		ERE promoter reporter assay	COS-1 cells + hERα or hERβ	[32]
Flavanones				
Hesperetin	No	ER binding assay	COS-1 cells + hERα or hERβ	[30]
			MCF-7 cells	[93, 94]
	Yes	ERE promoter reporter assay	Yeast cells + hERα	[34]
			Yeast cells + hER	[36]
			U2OS cells + hERα or hERβ	[33]
		Estrogen responsive genes	PC12 cells ± ICI[a]	[95]
		Cell proliferation assay	MCF-7 cells ± ICI	[33]
Hesperidin	No	ER binding assay	COS-1 cells + hERα or hERβ	[30]
		ERE promoter reporter assay	MCF-7 cells	[43]
Eriodictyol	Yes	ER binding assay	COS-1 cells + hERα or hERβ	[30]
		ERE promoter reporter assay	Yeast cells + hER	[96]
Eriocitrin	Yes	ER binding assay	COS-1 cells + hERα or hERβ	[30]
Naringenin	Yes	ER binding assay	COS-1 cells + hERα or hERβ	[30, 32]
			Nonisotopic ERβ-based assay	[37]
		ERE promoter reporter assay	COS-1 cells + hERα or hERβ	[32]
			MCF-7 cells	[43, 97]
			U2OS cells + hERα or hERβ	[33]
			Yeast cells + hERα; hER; ERα or ERβ	[34, 35, 95]
		Estrogen responsive genes	BT-474 cells	[98]
		Cell proliferation assay	MCF-7 cells ± ICI	[32, 33]
	No	Uterotrophic assay	Immature rats; mice	[34, 84]
Narirutin	Yes	ER binding assay	COS-1 cells + hERα or hERβ	[30]
Prunin	Yes	ERE promoter reporter assay	MCF-7 cells	[43]
Flavones				
Luteolin	Yes	ER binding assay	COS-1 cells + hERα or hERβ	[30, 32]
			Nonisotopic ERβ-based assay	[37]
			MCF-7 cells	[46]
		ERE promoter reporter assay	MCF-7 cells	[43, 46]
			COS-1 cells + hERα or hERβ	[32]
		Estrogen responsive genes	BT-474 cells	[98]
		Cell proliferation assay	MCF-7 cells ± ICI	[32]
Diosmetin	Yes	ERE promoter reporter assay	Yeast cells + hERα	[34]
Isoflavones				
Formononetin	Yes	ER binding assay	hERα or hERβ	[38]
			ERα or ERβ	[99]
			COS-1 cells + hERα or hERβ	[30, 32]
			Nonisotopic ERβ-based assay	[37]
	No		Rabbit uterine estrogen receptor	[100]
	Yes	ERE promoter reporter assay	COS-1 cells + hERα or hERβ	[32]
			MCF-7 cells ± ICI	[43, 101]
			Yeast cells + hERα; hERα or hERβ	[34, 40, 102]
		Cell proliferation assay	MCF-7 cells ± ICI	[32, 101]
		Uterotrophic assay	Ovariectomised mice	[41]
Calycosin	Yes	ER binding assay	Erα and Erβ competitor assay kit	[38]
		ERE promoterreporterassay	MCF-7 cells	[42]
		Uterotrophic assay	Ovariectomised mice	[41]
Calycosin-7-*O*-glucoside	Yes	ERE promoter reporter assay	MCF-7 cells	[43]
Orobol	Yes	ER binding assay	ERα and ERβ competitor assay kit	[103]
			ERα or ERβ	[104]
		ERE promoter reporter assay	Yeast cells + hERα	[105]
			U2OS cells + hERα	[105]
Ononin (formononetin-7-*O*-glucoside)	Yes	ERE promoter reporter assay	MCF-7 cells	[43]

continued

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Table 3 *Continued*

Polyphenol	Estrogenic effect	Test for estrogenic effect		Reference
		Test system	Test model	
Flavanols				
(−)-Epigallocatechin gallate	Yes	ER binding assay	hERα or hERβ	[94]
			Mouse uterine estrogen receptor	[94]
		Gal4 promoter reporter assay	MCF-7 cells + hERα or mERβ + 17m5-G-Luc	[94]
	No	ERE promoter reporter assay	HeLa cells + hERα or hERβ	[95]
Coumestans				
Medicagol	No	ER binding assay	Rabbit uterine estrogen receptor	[100]
Phenolic carboxylic acid				
p-Coumaric acid	No	Uterotrophic assay	Ovariectomised rats	[106]

[a] ICI 182,782: an estrogen receptor antagonist

ity, some compounds may bind ER but not display estrogenicity in other assays.

Of the flavones present in *Cyclopia* only two, luteolin and diosmetin, have been tested for estrogenicity, and both are estrogenic (**Table 3**). Luteolin is present in a methanol extract from *C. subternata* (**Table 1**) and has been shown to be estrogenic via ER-binding, ERE-containing promoter assays, and estrogen responsive genes, as well as by stimulating cell proliferation in the E-screen. It has, however, not been tested *in vivo*. Work from our laboratory suggests that luteolin binds preferentially to ERβ, with an RBA of 0.52 % for ERβ, while for ERα the RBA is 0.0025 % [30, 32] and that it has a similar affinity for ERβ as naringenin [30, 32, 37]. In promoter reporter assays, luteolin has a lower potency but higher efficacy via ERβ than naringenin, specifically it has a potency of 3.53×10^{-3} mg/mL (12.3 µM) versus the potency of 1.04×10^{-4} mg/mL (0.0382 µM) of naringenin and a efficacy of 3.69-fold versus a 2.99-fold induction by naringenin. However, unlike naringenin it does transactivate via ERα, with a potency of 1.97×10^{-3} mg/mL (6.88 µM), which is just slightly higher than via ERβ. Yet, in the E-screen, it has a lower potency (2.54×10^{-6} mg/ml or 0.00887 µM) than naringenin (3.27×10^{-8} mg/ml or 0.00012 µM) suggesting that in terms of a biological response in physiologically relevant tissues, it may favour ERβ.

Although the isoflavones shown to be present in *Cyclopia* are not observed in quantifiable amounts (**Fig. 2**, **Table 1**), many of them are estrogenic (**Table 3**). Of these, formononetin and calycosin have been thoroughly tested, both *in vitro* and *in vivo*, and generally show a slight preference for ERβ in ER binding assays [30, 32, 38, 39]. These compounds differ only on the B-ring in that calycosin has a 3′-OH moiety. In promoter reporter studies, the ER isoform preference for formononetin is not so clear [32, 40], while both compounds are uterotrophic, with calycosin being more potent than formononetin [41, 42], suggesting that both must act via ERα. Here again we observe the phenomenon of the glycoside being less estrogenic than its corresponding aglycone, with calycosin showing greater estrogenic activity via a promoter reporter construct in MCF-7 cells than calycosin-7-*O*-glucoside [43]. Orobol, with OH groups at the 3′ and 4′ positions, and ononin, the 7-*O*-glucoside of formonentin, are also both estrogenic but here their activity appears to be similar to that of calycosin-7-*O*-glucoside and not to be preferentially via ERβ (**Table 3**).

The presence of polyphenols with phytoestrogenic capabilities in the plant material of *Cyclopia* species (**Table 3**) raised the question of whether extracts from the plant material will have phytoestrogenic capabilities. One cannot simply assume that the estrogenicity of the pure compounds will be transferred to extracts of the plant material as varying levels of polyphenols, as well as the presence of various polyphenols with varying levels of estrogenicity, might modulate the effects observed with pure polyphenols. To address this issue, examination of the phytoestrogenicity of crude extracts prepared from the plant material of various commercially cultivated *Cyclopia* species [30, 32, 44] as well as the HPLC analyses of these extracts to identify the polyphenols present is warranted. We chose two extracts for discussion (**Table 4**), P104 (methanol extract) from *C. genistoides* as it was found to have the highest binding affinity for both the ER subtypes [32], and SM6Met (methanol extract of plant material following extraction with ethyl acetate and ethanol) from *C. subternata* as it had the highest potency when compared to other extracts [44]. P104 bound to both ERα and ERβ, albeit with a lower potency than that of E_2, and had a higher affinity for ERα. This correlates with previous studies that showed a slightly higher displacement of E_2 from ERα than from ERβ by P104 [30]. Despite binding to ERα with a higher affinity, P104 was not able to activate an ERE containing promoter reporter construct through ERα, but was able to do so through ERβ with an efficacy similar to that of E_2, although its potency was much lower. In addition, P104 induced cell proliferation of MCF-7 cells, but it was less potent than E_2. SM6Met has also been shown to bind to the ER by performing whole cell binding assays in MCF-7 cells. Unfortunately, these results cannot distinguish between binding to specific ER isoforms as MCF-7 cells contain both ERα and ERβ. Similar to P104, SM6Met also activated an ERE containing promoter reporter construct and induced cell proliferation in MCF-7 cells and like P104, SM6Met had a lower potency than E_2 in both assays. The extracts were analysed with HPLC, and **Table 4** shows the polyphenols detected. Apart from these, the extracts were also screened for narirutin, eriodictyol, naringenin, hesperetin, and formononetin. Although these polyphenols were not present in quantifiable amounts, one cannot exclude the possibility of their presence and thus the effect they may have on the estrogenicity of the whole extract. The unidentified compounds in the extract of Mfenyana et al. [44] have since been tentatively identified (**Table 4**) as the flavone, scolymoside, and the dihydrochalcone, phloretin 3′,5′-di-*C*-β-glucoside. The presence of unidentified compounds was also previously indicated for P104 [32], but they were not quantified. Comparison of **Tables 3** and **4** may allow the deduction of which of the polyphenols might be causing the phytoestrogenicity of the extracts. Both extracts contain the xanthones mangiferin and isomangiferin, but as they are not phytoestrogenic [30, 32, 45] (**Tables 2** and **3**), it is unlikely that they are contributing. Hesperidin also does not bind to hERα or hERβ and is un-

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Table 4 Phytoestrogenic potential of polyphenols and extracts of unfermented *C. genistoides* and *C. subternata*.

Species		Extract	
		P104 [32]	SM6Met [44]
		C. genistoides	*C. subternata*
ER binding[a] (RBA[b] ± SEM[c])		ERα: 0.1195 ± 0.0567 % ERβ: 0.0004 ± 0.0001 %	0.0802 ± 0.0139 %
ERE promoter reporter assay[d] (RII[e])	Potency ± SEM	ERβ: 1.0490 ± 0.1287 %	0.0102 ± 0.0032 %
	Efficacy ± SEM	ERβ: 103.2 ± 1.1 %	57.6 ± 2.4 %
Cell proliferation assay[f] (RII)	Potency ± SEM	0.0072 ± 0.0069 %	0.0579 ± 0.0325 %
	Efficacy ± SEM	99.1 ± 2.3 %	78.5 ± 6.6 %
Polyphenols (g · 100^{-1} g dry extracts ± SEM)			
▶ Mangiferin		3.935 ± 0.329	1.85
▶ Isomangiferin		4.998 ± 0.097	0.75
▶ Eriocitrin		ND[g]	1.25
▶ Hesperidin		1.503 ± 0.226	1.87
▶ Luteolin		0.097 ± 0.001	0.04
▶ Scolymoside[h]		ND	1.82
▶ Phloretin-3,5-di-*C*-glucoside[i]		ND	1.27

[a] Whole cell bindings were performed in COS-1 cells transfected with hERα or hERβ [32] and in MCF-7 cells that contain both hERα or hERβ [44]. [b] RBA or relative binding affinity is expressed relative to that of E_2 (100 %) and was calculated as follows: 100 × IC_{50} (E_2)/IC_{50} (test compound). [c] Values represent an average of values from different extractions of the same plant material. [d] ERE promoter reporter assays were performed in COS-1 cells transfected with hERα or hERβ [32] or in T47D-KBluc cells that contain both hERα or hERβ [44]. [e] RII or relative induction index is expressed relative to that of E_2 (100 %) and was calculated as follows: 100 × EC_{50} (E_2)/EC_{50} (test compound) for potencies and 100 × fold (test compound)/fold (E_2) for efficacies. [f] Cell proliferation assays were performed in MCF-7 cells. Verhoog et al. performed assays in the presence and absence of ICI 182,782 [32]. [g] Not detected. [h] Previously 'Unknown **1**'. [i] Previously 'Unknown **2**'

able to induce an ERE containing promoter reporter construct [30,43], however, its aglycone hesperetin, despite showing no binding to ER, does transactivate ERE-containing promoters and causes cell proliferation in the E-screen (**Table 3**). As glycosides are likely to be metabolised to their aglycones *in vivo*, hesperidin should not be discounted for *in vivo* studies, however, for *in vitro* testing, it is unlikely to contribute to the estrogenicity of the extracts. Luteolin has been shown to bind to both ER isoforms [30, 32,37,46], to activate an ERE promoter reporter construct through both isoforms [32,43,46], and to induce proliferation of a breast cancer cell line (**Table 3**). The amount of luteolin present was, however, shown to be too low to explain the degree of phytoestrogenicity observed for the P104 [32] or SM6Met [44] extract. On the other hand, scolymoside, the 7-*O*-rutinoside of luteolin, may be important *in vivo*. The flavanone eriocitrin was quantified in SM6Met, but not in P104 (**Table 4**). Eriocitrin has been shown to bind to ERβ [30], but no further tests for estrogenicity have been performed (**Table 3**). To our knowledge, scolymoside and phloretin 3′,5′-di-*C*-β-glucoside tentatively identified in SM6Met have not been tested for phytoestrogenicity (**Table 2**). Taken together, no concrete conclusions regarding the polyphenols responsible for the phytoestrogenic effect of extracts of *Cyclopia* can be drawn. Some of the identified polyphenols still need to be tested for phytoestrogenicity, and the desired answer might be found in the results from these studies. We cannot, however, exclude the possibility that the effect seen with the *Cyclopia* extracts is the result of a fine balance between different polyphenols present in varying amounts with varying phytoestrogenic potential (agonistic, antagonistic, or SERM activity via either ERα or ERβ) and that synergism or antagonism could play a role with multiple polyphenols targeting multiple ER isoforms [47].

Blanket Claims for Phytoestrogenic Potential of *Cyclopia*

Caution should be exercised in making blanket claims for the phytoestrogenic potential of all harvestings of *Cyclopia*. Research indicates that variations in the polyphenol composition or content as well as the phytoestrogenic potential of individual harvestings of a specific *Cyclopia* species may differ (**Table 5**). For example, *C. genistoides* dried methanol extracts differed remarkably in their ability to induce cell proliferation in the E-screen assay with three out of the six harvestings displaying such low levels of activity that EC_{50} values could not be determined (**Table 5**). Even amongst the harvestings with higher activity, there was considerable variation with M7 and NP105 extracts displaying 1.4- and 3.3-fold less activity than NP104. In addition, the concentration of luteolin, a polyphenol with proven phytoestrogenic potential (**Table 3**), also varied between harvestings with a 2.6-fold difference between the harvesting with the highest concentration (M9) and that with the lowest concentration (NP104 or NP105) of luteolin (**Table 5**). This variability in polyphenol content is even more pronounced both quantitatively and qualitatively between species of *Cyclopia* with, for example, eriocitrin varying between undetectable in the *C. genistoides* aqueous extract to 0.47 % of the aqueous extract of unfermented *C. subternata* [8].

The lack of standardisation, both in terms of levels of active substances and activity levels, of botanical and dietary supplements plagues the industry. Combined with little to no regulation by national bodies regulating drug use in most countries, this has led to contrary and inconsistent findings relating to health benefits, which has damaged the credibility of the industry [48]. Thus for claims of phytoestrogenic activity in *Cyclopia,* individual harvestings would have to be tested for activity until such time as a marker compound(s) shown to be related to activity can be identified.

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Table 5 Variation in phytoestrogenic potential and polyphenol content of *C. genistoides* harvestings.

Farm	Harvesting date	Dried methanol extract	E-screen in MCF-7 cells RII[c]	Luteolin ($g \cdot 100^{-1}$ g dry extracts)
Koksrivier/Overberg[a]	22 January 2002	M7	9.8×10^{-5}	0.13
Reins/Albertina[a]	01 April 2003	M8	ND[d]	0.12
Reins/Albertina[a]	22 April 2004	M9	ND	0.25
Koksrivier/Overberg[b]	15 March 2001	NP104	1.4×10^{-4}	0.097
Koksrivier/Overberg[b]	28 March 2001	NP105	4.3×10^{-5}	0.097
Koksrivier/Overberg[b]	31 March 2003	NP122	ND	0.104

[a] Data from [44]; [b] data from [32]; [c] RII (relative induction index) = EC_{50} E_2/EC_{50} extract; [d] ND = RII could not be determined as activity was too low

Potential Usage of Phytoestrogens

Estrogen plays an important role in the development of the female reproductive tract, secondary sex characteristics, and in reproductive behaviour [49]. However, estrogen also influences the growth of hormone-dependent cancers such as breast cancer [50].

Hormone replacement therapy (HRT), which includes estrogen combined with or without progesterone, is given to alleviate the symptoms of menopause, and advocates of HRT believe that it also confers long-term benefits regarding cardiovascular disease, bone preservation, and general well-being [51,52]. Although the efficacy, superiority, and cost effectiveness of estrogen in the treatment of menopausal symptoms is accepted [53], recent large randomised clinical trials [54,55] and observational studies [56] on HRT have modified the risk/benefit perception. Specifically, increased risk of breast cancer and cardiovascular disease has raised concerns amongst the public [57], and the Endocrine Society statement of 2010 now recommends use of HRT with the lowest effective dose and for the shortest duration possible [58].

The double-edged sword of estrogen has prompted the search for alternatives in the management of menopause, and phytoestrogens have been suggested as a viable alternative, due to their potential to modulate estrogen action [59,60]. In addition, epidemiological studies suggest that Asian populations who consume 20–50 mg soy/day have fewer occurrences of hormone-dependent diseases, including menopausal symptoms, osteoporosis, and breast cancer and that this lower incidence is not due to under reporting or genotypic factors [53,61–63].

Pharmacological validation of claimed health benefits for phytoestrogens has, however, only recently been undertaken and most work has focused on *in vitro* assays to establish biological activity while large, well-designed *in vivo* studies have lagged behind [64]. Molecular aspects of phytoestrogens that have been heralded as positive regarding health benefits include the fact that phytoestrogens generally have orders of magnitude lower potency than estrogen [53,65], display estrogen agonist activities in the presence of low levels of estradiol (post-menopausal) and antagonistic activity in the presence of high levels of estradiol (premenopausal) [48], exhibit partial selectivity for ERβ, the ER isoform believed to attenuate the proliferative effect of ERα [66, 67], and many act like SERMs, making them safer for breast and endometrial tissue [29,48,68]. Furthermore, phytoestrogens have additional diverse beneficial biological effects, such as anti-inflammatory, antioxidant, and anticancer effects [65,69].

Several studies and reviews have evaluated the health potential of phytoestrogens for treating post-menopausal symptoms by maintaining bone density, decreasing cardiovascular disease and hot flashes, and in preventing or treating estrogen-dependent cancers such as breast, prostate, endometrial, and colon cancer [29,48,53,70–73]. Although there is contradictory scientific proof of the effectiveness of phytoestrogens, specifically soy and red clover isoflavones, for the treatment of vasomotor menopausal symptoms, such as hot flushes [29,73,74], for other symptoms, such as osteoporosis and cardiovascular disease, the data to date strongly suggests efficacy. Specifically, phytoestrogens, such as coumestrol, genistein, daidzein and its metabolite equol as well as extracts from soy, black cohosh, and red clover, appear to slow bone loss and improve bone density [29,48], which is positive for osteoporosis, while for cardiovascular disease, phytoestrogens, primarily from soy, are beneficial in decreasing LDL and triglycerides, while increasing HDL [48,53]. In addition, several studies have suggested that phytoestrogen use, mainly flavones and isoflavones from soy, is associated with a reduced risk of breast cancer [67,75–77].

Despite beneficial effects of phytoestrogens being reported, results have, however, not always been favourable or reproducible [73]. For example, although some studies suggest that soy food intake does correlate with reduced risk or recurrence of breast cancer [78,79], other studies have found no such association between isoflavone intake and breast cancer risk [80,81]. The diversity in results may be attributed to, amongst others, the fact that a wide variety and doses of botanicals have been used and the fact that standardisation of formulations are not currently required making comparison between studies difficult [29,48,70]. In addition, an evaluation of effects of phytoestrogenic preparations on health is complicated by the fact that exact formulations and concentrations of active constituents are not always known and studies are often retrospective (relying on recall of diet). Furthermore, the fact that there has never been a study comparable in size to the Million Women's or WHI studies investigating side effects of phytoestrogen use should encourage caution. This is especially relevant as many consumers base their beliefs of both efficacy and safety on source rather than evidence [29]. Despite this caveat, there is no current data suggesting that dietary phytoestrogens promote hormone-dependent cancers in humans, and thus phytoestrogens can probably be used safely on a long-term basis [53,73]. Finally, the fact that phytoestrogens are often not selected for specific attributes, such as acting only via ERβ, may have confounded studies on health effects. Some promising results regarding amelioration of hot flushes with liquiritigenin, an ERβ-selective agonist from a Chinese herbal extract, have, however, resulted in Phase 2 clinical trials to evaluate safety and efficacy for the treatment of menopausal symptoms [82,83].

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Conclusions

The increased public and industry interest in phytoestrogens suggests that validated health claims would contribute significantly to adding value to products such as honeybush tea. Certain extracts of *Cyclopia* undoubtedly display estrogenic activity (**Table 4**), and many of the major and minor polyphenols found in *Cyclopia* certainly have been shown to have phytoestrogenic potential (**Table 3**), but whether this translates into firm health recommendations for a "cup-of-tea" of honeybush is debatable. Firstly, harvestings of *Cyclopia* differ significantly in terms of estrogenic activity and polyphenol content (**Table 5**), and secondly, *Cyclopia* extracts have not been tested for estrogenicity *in vivo*. The importance of evaluating the bioavailability as well as the metabolic transformation of active compounds, both by gut microflora and hepatic enzymes, has been stressed [31,84]. *Cyclopia* extracts have been tested *in vivo* for absorption and metabolism [85,86]; however, the focus was on mangiferin and hesperidin, both compounds without estrogenic activity (**Table 3**). The aglycone of hesperidin, hesperetin, which does display weak estrogenic activity, was, however, one of the metabolites detected in urine [85]. This suggests that glycosylated polyphenols, of which several constitute the major polyphenols in *Cyclopia* extracts (**Table 1**), would probably be transformed to the corresponding aglycone with higher phytoestrogenic activity. Finally, the concept of either synergistic or even antagonistic formulations consisting of intelligent mixtures of natural products to treat disease is gaining ground [47,87–91] and thus, although we have focussed on the phytoestrogenicity of individual compounds found in *Cyclopia*, we should consider the possibility that it is the mixture of compounds found in *Cyclopia* extracts, rather than an individual compound, that confers the desired estrogenic activity.

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Conflict of Interest

The authors declare no conflict of interest.

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