SELECTIVE EXTRACTION OF *CYCLOPIA* FOR ENHANCED *IN VITRO* PHYTOESTROGENICITY

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Declaration:

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree

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SUMMARY

Phytoestrogens are plant compounds whose ability to mimic the action of estrogens has resulted in their usage for the treatment of menopausal symptoms. Despite uncertainties about the safety and effectiveness of phytoestrogens in humans, the use of market phytoestrogenic nutraceuticals and botanicals is on the increase. Positive epidemiological study findings coupled to an entrenched belief in many societies about the superiority of what they view as "natural" remedies, as well as the reluctance of women to use the traditional hormone replacement therapy due to its association with detrimental health effects as reported by studies such as the World Health Initiative, the Million Women and the Kronos Early Estrogen Prevention studies, are thought to be instrumental in the growth of the phytoestrogen market.

As the subject of the current thesis, we investigated the candidacy of extracts of the honeybush plant (genus *Cyclopia*), which is used for the manufacture of popular tea beverages, for use in the formulation of a high quality phytoestrogenic nutraceutical with a competitive market edge. We evaluated four harvestings of *Cyclopia* (M6-9) available in bulk and selected 2 harvestings (M6 and M7) for further extraction using solvents of differing polarity and also mimicking the preparation of a cup of tea. Our findings clearly demonstrate that of the resultant 22 extracts the SM6Met and SM6EAc extracts had the highest *in vitro* potency and efficacy, respectively. Another exciting finding from our study is the unequivocal demonstration of phytoestrogenic activity by extracts prepared in the same manner as the traditional cup of honeybush tea. Additionally, our study has highlighted the importance and the influence of experimental variables such as the specific harvesting evaluated and the characteristics of the extraction solvent (e.g. polarity and temperature) on the yield and the estrogenic activity of the extracts. In addition, the advantage of certain *in vitro* assays over others for discriminating between estrogenic substances based on their efficacies and potencies was demonstrated with the alkaline phosphatase assay being most suitable for discriminating efficacy and the E-screen most suitable for discriminating potency.

Furthermore, our study has imparted a valuable lesson about the pharmacological behavior of estrogenic substances by presenting a conundrum in the form of the two desirable pharmacological parameters (potency and efficacy) occurring in different extracts, an outcome that complicates the central aim of our study, which is the preparation of an extract that embodies both parameters. Additionally, the low quantity of known putative phytoestrogens and the presence of unidentified polyphenols in M6, the source of our choice extracts (SM6Met and SM6EAc), makes the high estrogenic potency and efficacy of the choice extracts that much more intriguing. Nonetheless, benchmarking against four market phytoestrogen extracts indicate that the *Cyclopia* extracts have comparable estrogenicity suggesting potential as marketable phytoestrogenic preparations. The combination of the achievement of aims and the birth of new questions from that very achievement, which are the hallmark of scientific endeavors, have made this study a rewarding experience and we hope to share the feeling in its entirety with the reader.

OPSOMMING

Fitoestrogene is plant verbindings met die vermoë om die aksie van estrogene na te boots. Hierdie vermoë het gelei tot hul gebruik vir die behandeling van menopousale simptome. Ten spyte van onsekerhede oor die veiligheid en effektiwiteit van fitoestrogene in die mens is die gebruik van mark fitoestrogeen "nutraceuticals" en botaniesemiddels aan die verhoog. Positiewe epidemiologiese studie bevindings gekoppel aan 'n diep gewortelde geloof in baie samelewings oor die superioriteit van wat as "natuurlike" behandelings beskou word, te same met die huiwering van vroue om tradisionele hormoon vervangings terapie te gebruik as gevolg van 'n assosiasie met nadelige gesondheid effekte soos gerapporteer deur die World Health Initiative, die Million Women en die Kronos Early Estrogen Prevention studies, word beskou as instrumenteel in die groei van die fitoestrogeen mark.

As onderwerp van die huidige tesis het ons die kandidaatskap van ekstrakte van die heuningbosplant (genus *Cyclopia*), wat gebruik word vir die vervaardiging van populêre tee drankies, ondersoek vir die formulering van 'n hoe kwaliteit fitoestrogeniese "nutraceutical" met 'n kompeterende mark voordeel. Ons het vier oeste van *Cyclopia* (M6-M9) beskikbaar in grootmaat geevalueer en twee oeste (M6 en M7) geselekteer vir verdere ekstraksie met oplosmiddels van verskillende polariteite en ook deur die maak van 'n koppie tee na te boots. Ons bevindinge demonstreer duidelik dat vanuit die 22 resulterende ekstrakte die SM6Met en SM6EAc ekstrakte respektiewelik die hoogste *in vitro* potensie en effektiwiteit bevat. Nog 'n opwindende bevinding uit die studie is die onomwonde demonstrasie van fitoestrogeniese aktiwiteit deur ekstrakte voorberei op dieselfde manier as die tradisionele koppie heuningbostee. Daarby het ons studie die belang en effek van eksperimentele veranderlikes soos die spesifieke oes wat geevalueer word en die eienskappe van die ekstraksie oplosmiddel (bv. polariteit en temperatuur) op die opbrengs en estrogeniese aktiwiteit van die ekstrakte uitgelig. Verder is die voordele van sekere *in vitro* essais oor ander vir die diskriminasie tussen estrogeniese verbindings gebaseer op hul potensie en

effektiwiteit gedemonstreer met die alkaliese fosfatase essai die mees geskik om te onderskei op die basis van effektiwiteit en die E-sifting die mees geskik vir onderskeiding op die basis van potensie.

Ons studie het verder ŉ waardevolle les oor die farmakologiese gedrag van estrogeniese substanse verleen deur ŉ raaisel in die vorm van die bevinding dat twee verlangde farmakologiese parameters (potensie en effektiwiteit) in verskillende ekstrakte gevind is op te lewer, ŉ uitkoms wat die sentrale doelstelling van ons studie, die voorbereiding van ŉ ekstrak wat beide parameters insluit, kompliseer. Daarby maak die lae hoeveelheid van die bekende putatiewe fitoestrogene en die teenwoordigheid van ongeïdentifiseerde polifenole in M6, die bron van ons keur ekstrakte (SM6Met en SM6EAc), die hoe estrogeniese potensie en effektiwiteit van die keuse ekstrakte al te meer interessant. Nieteenstaande, toon vergelyking teenoor vier mark fitoestrogeen ekstrakte dat *Cyclopia* ekstrakte vergelykbare estrogeniese aktiwiteit bevat wat potensiaal as bemarkbare fitoestrogeniese preparate aandui. Die kombinasie van die bereik van doelstellings en die geboorte van nuwe vrae vanuit daardie prestasie, wat die kenmerk is van die wetenskaplike strewe is, het díe studie ŉ verykende ervaring gemaak en ons hoop om die gevoel in sy geheel met die leser te deel.

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Last but by no means least, glory, honor and eternal praise be to my Lord and Savior, Jesus Christ, for giving me strength and continuing to bless and shield me from all manner of physical and emotional harm during this and every other endeavor.

ALPHABETICAL LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADH	Antidiuretic hormone
AF	Activation factor
ARC	Arcuate nucleus
AVP	Arginine vasopressin
AVPV	Anteroventral periventricular nucleus
β-MSH	Melanocyte-stimulating hormone
BNST	Bed nucleus of the stria terminalis
CAT	chloramphenicol acetyltransferase
CBP	cAMP response element binding protein
cDNA	Complementary deoxyribonucleic acid
CITED	CBP/P300-Interacting Transactivator, with Glu/Asp-Rich Carboxy-Terminal Domain
DDT	Dichloro-diphenyl-trichloroethane
DME	Dried methanol extract
DBD	DNA-binding domain
E_1	Estrone
E_2	Estradiol
E_3	Estriol
ER	Estrogen receptor
ERE	Estrogen response element
ESPS	Early soybean production system
FSH	Follicle stimulating
GFP	green fluorescent protein
GH	Growth hormone
HAT	Histone acetyltransferase

HPLC	High-performance liquid chromatography
HRT	Hormone replacement therapy
HSP	Heat-shock protein
IFS	Isoflavone synthase
IL-6	Interleukin-6
KEEPS	Kronos Early Estrogen Prevention
LBD	Ligand-binding domain
LC-MS	Liquid chromatography-mass spectrometry
MAPK	Mitogen activated protein kinase
mPOA	Medial preoptic area
MTA1	Metastasis Associated-1
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NCoR	Nuclear Receptor Co-Repressor
NF-kB	nuclear factor-kB
NTS	Solitary tract nucleus
O-DMA	O-desmethylangolensin
PCBs	Polychlorinated biphenyls
PELP	Proline Glutamic Acid-Rich Nuclear Protein
POMC	Pro-opiomelanocortin
PPARγ	Peroxisome proliferator-activated receptor gamma
PSA	Prostate-specific antigen
RANKL	Receptor activator of nuclear factor kappa B-ligand
RBA	Relative binding affinity
RII	Relative inductive index

SERM	Selective estrogen receptor modulator
SFE	Super critical fluid extractions
SMRT	Silencing mediator of retinoid and thyroid receptors
SHBG	Sex hormone binding globulin
SRC	Steroid Receptor Co-activator
TBG	Thyroxine-binding globulin
TGF-β	Tumour growth factor-beta
TRAP	Tartrate-resistant acid phosphatase
TTR	Transthyretin
UV-VIS	Ultraviolet-visible spectrophotometry
WHI	World Health Initiative

TO

My mother Nonzame (Thooole!), father Phumzile (Bheeele!), brothers Phumza (Mboza!), Simbuyiselwe (Marompi!) and sisters, Lindelwa (Manqi!) and Bulelwa (Bullet!).

CONTENT PAGE

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Phytoestrogens are plant chemicals whose estrogen-like effects have resulted in their consideration as prospects for the treatment of hormone-dependent diseases such as breast and prostate cancers, immune dysfunction and climacteric symptoms (1-3). Despite conflicting and controversial findings concerning the effectiveness and safety of phytoestrogens as medicinal entities (4) there is a widespread and growing usage of commercial phytoestrogenic preparations for the treatment of menopausal symptoms (5) that is given impetus by society's perception of what it views as more "natural" and thus safer alternatives to conventional medicines (6-8). Recent findings such as by the Kronos Early Estrogen Prevention (KEEPS), the Million Women and the World Health Initiative (WHI) studies, which have suggested that the use of conventional hormone replacement therapy (HRT) by post-menopausal women promotes the onset of breast and ovarian cancers among other diseases (9-11), have also resulted in a degree of reluctance by women to use HRT and have increased the urgency of finding safer alternatives to traditional HRT (10; 12).

Some species of the honeybush plant (genus *Cyclopia*), a member of the fynbos biome that is endemic to the South African Western and Eastern Cape Provinces, and whose "fermented" (oxidized) products have been traditionally consumed as the fragrant caffeine-free honeybush tea beverage, have been reported to express phytoestrogenic activity *in vitro* (13; 14). Dried methanol extracts of the major species of *Cyclopia* used for making the herbal tea (*C. genistoides*, *C. subternata*, *C. intermedia* and *C. sessiliflora*) were tested for phytoestrogenic activity in a previous study (13) and from the results of the study it was determined that two species, *C. genistoides* and *C. subternata*, expressed higher degrees of estrogenic activity in comparison to the rest, with dried methanol extracts of *C. genistoides* expressing superior activity over *C. subternata* (13). Three harvestings of *C. genistoides* and one of *C. subternata* were hence chosen as subjects of the current

thesis with the aim of improving the phytoestrogenicity of the most active methanol extracts from the group through a system of activity guided extraction and to benchmark the activity of the improved extract against commercially available phytoestrogenic extracts. The rationale behind the study is to assess the viability of the select extract for the formulation of a phytoestrogenic nutraceutical.

In keeping with the aims of the current thesis, various methods used for the extraction of phytoestrogens, as well as *in vitro* assays used for screening test substances for phytoestrogenic activity, will be the focus of this literature review. Since the choice of *in vitro* assays used in our study is based on the molecular mechanism of action of estrogen, a presentation, and where necessary, a discussion of the current literature on the molecular mechanisms of estrogen and relevant phytoestrogens will also form part of this review. Because the principal motivating factor behind the current thesis is the desire to formulate a phytoestrogenic nutraceutical with a level of activity that would be effective for the treatment of climacteric symptoms, the clinical importance of relevant phytoestrogens, as well as the current trends in the phytoestrogenic nutraceuticals market will also form part of this review. The honeybush plant (*Cyclopia*), the very subject of our investigations, and its putative clinical benefits will also be discussed in this first chapter. Finally, in concluding the literature review we also present the aims of this thesis in the context of the literature discussed.

Chapter 2 of the text is an account of the methods, results and conclusions reached from the current study and because its contents have been submitted for publication, a necessary repetition of some information can be expected in that chapter. The following and last chapter draws conclusions from our findings and is the summary of the lessons learned from our journey, which we as the authors of this text found a rewarding experience and which we share with the hope that they will serve to enrich the scientist in the reader.

1.2 General overview of the action of endocrine hormones

The endocrine system is a conglomerate of organs and tissues that functions in partnership with the immune and the nervous systems to maintain homeostasis (15-16). In contrast to other systems, such as the nervous system which regulates relatively fast processes such as muscle movement (17), the endocrine system characteristically regulates slower-paced events such as mood, growth and development and sexual and reproductive processes (18). The endocrine system carries out its diverse functions by releasing chemical agents called endocrine hormones that act on target tissues (19). Endocrine hormones, derived either from amino acids (peptide hormones) (20) or sterols (steroid hormones) (21), are secreted by the eight major endocrine glands which include the hypothalamus, pituitary, pineal, thyroid, adrenal, pancreas, ovaries and the testes (Table 1 and Fig. 1) (2; 21-23). Other non-endocrine body organs which produce and secrete hormones and have important roles in the functioning of the endocrine system include the skin, thymus, heart, liver, kidneys, placenta, stomach and the intestines (24-30).

Upon receiving signals from the brain, endocrine hormones are secreted either into the same tissue of origin (autocrine), or into the blood plasma from where they are distributed to regulate functions of proximal (paracrine), or distant tissues (classical endocrine) (31-32). In blood plasma, hormones are bound to specific carrier proteins that protect the hormones from degradation by plasma proteases (peptide hormones) or that increase the solubility and plasma capacity of the hormones (steroid hormones) (33-34). Sex hormone binding globulins (SHBGs) for example transport the sex hormones progesterone, testosterone and estrogen (35), while thyroxine-binding globulin (TBG) and transthyretin (TTR) transport the thyroid homones triiodothyronine and thyroxine in the bloodstream (36).

Endocrine hormones are normally present in the plasma and interstitial tissue at low concentrations (approximately 0.15 to 2 nM for E_2 in healthy women) (37) hence the presence of sensitive protein receptors in target tissues to sense the presence of such weak signals (38).

Table 1. The major endocrine glands and the functions of their hormones

Endocrine gland	Function
Hypothalamus	Regulates the release of pituitary hormones by relaying brain-detected messages and signals to the pituitary gland (39).
Pituitary	Regulates the secretion of hormones by other endocrine glands (hence "the master gland") including the production of sex hormones by gonads (40-41). *Adenohypophysis*: regulates secretion of prolactin as well as luteinizing (LH), follicle stimulating (FSH), thyroid stimulating (TSH), and growth hormone (GH), as well as the production of pro-opiomelanocortin (POMC) which is cleaved into products such as β-MSH (melanocyte-stimulating hormone) and of adrenocorticotropic (ACTH) and other peptides (42-46). *Neurohypophysis*: releases (a) vasopressin (AVP) which regulates water retention and blood pressure (b) oxytocin, which, in addition to facilitatating physical changes in female reproductive organs during and after labor (47), has been implicated in the regulation of mammalian circadian rhythms (48-49).
Pineal	Secretes melatonin, a hormone that has been implicated in the regulation of the circadian rhythm in mammals (50-51).
Thyroid	Produces thyroxine and triiodothyronine (which control many body processes including nutrient fuel turnover, body temperature and growth) (52-53) as well as calcitonin, for the maintenance of calcium-related homeostasis (54).
Adrenals	*Adrenal cortex*: produces corticosteroids such as cortisol and aldosterone in humans, in addition to being a secondary site for androgen production, thereby influencing the body's response to stress, the immune system as well as sexual development and function (55-58). *Adrenal medulla*: produces catecholamines such as epinephrine (adrenaline) which increases blood pressure and heart rate under stress conditions (59)
Pancreas	Produces glucagons and insulin, which function to maintain a steady level of glucose for the maintainance of the body's energy stores and somatostatin which affects neurotransmission and cell proliferation (60-62).
Gonads	*Ovaries*: Female gonads which produce ova and secrete the female hormones estrogen and progesterone (63). *Testes*: Male gonads which produce androgens (androsteone, dehydroepiandosterone and testosterone) which regulate body changes associated with sexual development and the production of sperm cells by the testes (64).

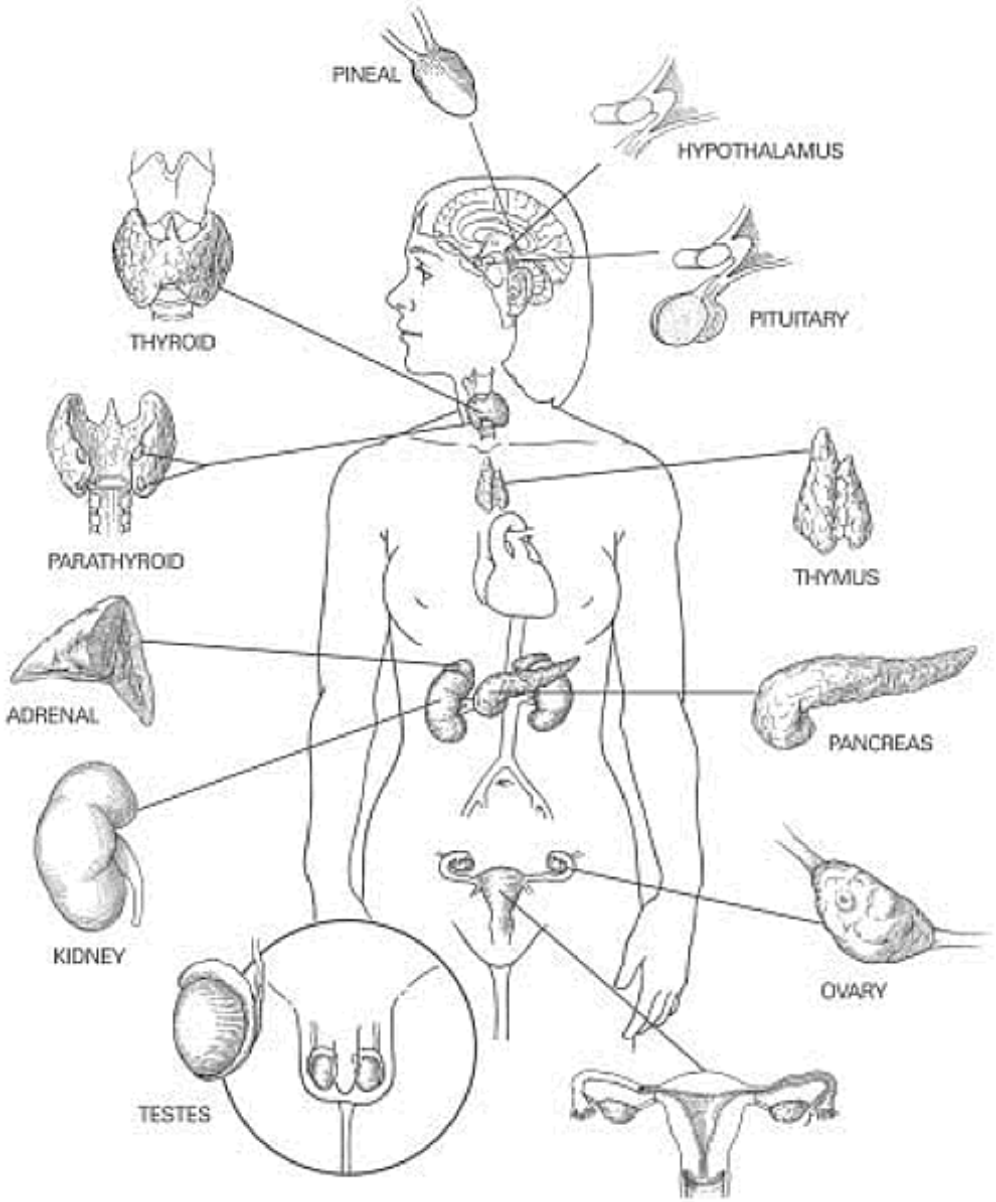


Figure 1. Location of endocrine glands and related organs. *Illustration created by Diane Abeloff (2002).*

The structural difference between peptide and steroid hormones requires different locations of their receptors in target tissues. Receptors for most peptide hormones are located on the plasma membrane (65-66) and induce the activation of intracellular secondary messenger cascades (67). Steroid and thyroid hormone receptors on the other hand are located intracellularly and act as transcripton factors (68).

1.3 The molecular mechanism of estrogen action

In order to understand the rationale behind the methodology applied in this study, a deeper understanding of the action of the steroid hormone estrogen is imperative. We shall therefore, in addition to describing the structure and expression of the estrogen receptor, also describe the molecular mechanisms of action of estrogen as well as its implication in estrogen-related diseases.

Although it has become customary to refer to the female sex hormone as estrogen, estrogen is in fact not one hormone, but rather a class of structurally similar compounds which may either be of natural or synthetic origins (69). In keeping with the objectives of this study, however, the focus will be on the mechanism of action of estradiol (E_2), the most physiologically active of the three ovarian estrogens, estrone (E_1), estradiol (E_2) and estriol (E_3), which are steroid hormones that regulate the function of a variety of mammalian physiological processes (70-72).

Primarily, E_2 is involved in the development of female secondary sexual features such as breast development and growth, and together with progesterone, is also involved in the regulation of other physiological processes which include the maintenance of pregnancy and regulation of the menstrual cycle (73-76). E_2, however, also affects the functioning of systems that are not related to sexual development and reproduction. Such systems include the cardiovascular, skeletal, muscle, immune, and nervous systems (77-85). It is possible for estrogen to affect these seemingly diverse activities, because estrogen receptors (ERs), whose content is decisive for the action of the estrogens (86-87), are located in various organs which include the brain, breast, heart, liver, vagina, lining of the uterus**,** cervix and in the bones (88).

Two main isoforms of ERs, designated ERα for the classical receptor and ERβ for the recently identified isoform, can be differentiated (Fig. 2) (70). ERα and ERβ are both composed of three independent but interacting functional domains, hence the modular structure of the ER. The domains of the ERs are, the activation function (AF-1)-containing NH_2-terminal or A/B domain, the C- or DNA-binding domain (DBD), and the C-terminal D/E/F or ligand-binding domain (LBD), that also contains the activation function (AF-2) (Fig. 2A) (89-91). Activation factor 1 (AF-1), is

thought to afford the ER its ability to function in the absence of a hormone, a phenomenon that the reader will learn more about when the mechanism of E_2 action is described in more detail (92-94).

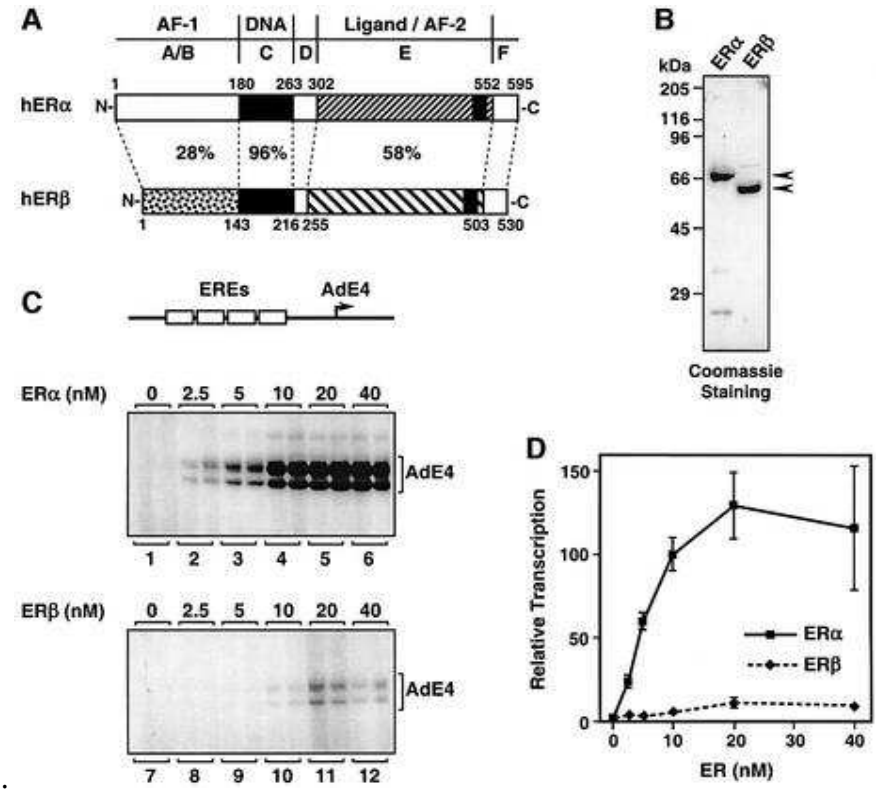


Figure 2. Structure and functional distinctions in estrogen receptors α and β. (A) Schematic diagrams of human ERα and β showing percentage homology between the different receptor functional domains. (B) SDS–PAGE analysis of purified, recombinant ERα and ERβ expressed in insect cells showing differences in molecular weights between the two ER isotypes. (C) Assessment of ERα and ERβ transcriptional activities in receptor dose–response experiments using an *in vitro* chromatin assembly and transcription system. A plasmid template containing four EREs upstream of the adenovirus E4 promoter (pERE; top) was assembled into chromatin using the S190 extract in the presence of increasing amounts of purified ERα or ERβ, The chromatin samples were subjected to *in vitro* transcription analysis in duplicate using a HeLa cell nuclear extract, and the resulting RNA products were analyzed by primer extension (bottom). (D) Quantification by PhosphorImager analysis of multiple experiments like those shown in (C). Each point represents the mean ± SEM for three or more separate determinations. ERβ is a weak transcriptional activator with chromatin templates (95).

The DBD, as its name implies, facilitates the binding of the ER to the DNA of target cells, a function which is mechanically facilitated by two zinc fingers in the DBD of the ER (96-97). The E region, which also contains a transactivation function (AF-2), is located in the LBD and is involved

in ligand binding and hormone-dependent dimerization (89; 98). Although the AF-2 is the main transactivation factor, maximum transcriptional activity requires the concerted actions of both the ligand-independent AF-1 domain and the ligand-dependent AF-2 domain (99-100).

Comparatively, the two ER isoforms share functional similarities in that they bind to E_2 with high affinity, and bind estrogen response elements in a similar manner (95). These two characteristics of the ER can be explained by the fact that the regions of highest homology between the two receptors are the DBD (96%) and the LBD (58%) (Fig. 2A) (95). ERα and ERβ do, however, also differ in many ways. Apart from structural differences, as observed in ERα (66 kDa) being composed of a total of 595 amino acids, while ERβ (52kDa) is slightly shorter with 530 amino acids (Fig. 2B) (95; 101-102), the two ER subtypes also differ with regards to tissue and organ distribution (Fig. 3), ligand specificity and transactivation potential (Fig. 2C and D). These differences lead to the ER subtypes mediating distinct phenotypes as observed in studies using mouse knockout models (103-104).

Regarding the expression of ERα and β, studies have led to the observation that, although the two ER subtypes are co-expressed in certain systems such as the vascular, breast, uterus and ovary, contrary to ERβ, ERα is much more highly expressed in the classical estrogen target tissues such as the adrenals, kidneys, testes, breast, uterus, and ovaries, which, with the exception of the kidneys, are related to reproductive functions (105-106). Estrogen receptor-β on the other hand, is mainly expressed in tissues that are not related to reproductive function such as the lung, thymus, prostate, bladder and bone (106-110) (Fig. 3).

An example of this general rule of ER expression has been demonstrated in a study of specific tissue ER isotype distribution in rat by Kuiper *et al*. (105). Kuiper *et al*. recorded a moderate to high ERα expression in the uterus, testis, pituitary, ovary, kidney, epididymis, and adrenals while ERβ was found to be expressed in relatively high amounts in the prostate, ovary, lung, bladder, brain, uterus, and the testis.

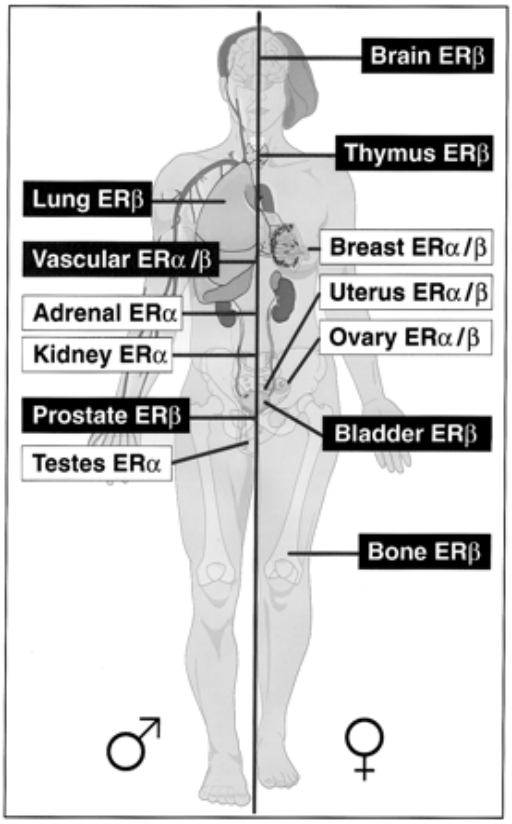


Figure 3. Anatomical distribution of ERs α and β (111).

Interestingly, certain physiological and psychological stimuli have been observed to influence or alter the expression of ERα and β in certain tissues, as reported in a study where the expression of ERα and β were altered in parts of female rat brains through systematic feeding and fasting (112). The changes in ERα and/or ERβ expression at various sites of the brain in this study are an indicator of the importance of expression of the ER subtype in mediating physiological and psychological parameters (such as reproductive physiology and behavior) and how profoundly the expression of the ER subtypes can be influenced or altered by environmental stimuli.

Differences in ligand specificity are another feature of the two ER isoforms. In the same study by Kuiper *et al*. (105), a substantial variance in affinities of various ligands for the ER isoforms was demonstrated *in vitro* (105; 113-114). E_2 has a significantly higher affinity for both ER subtypes when compared to synthetic estrogens (105). Specifically, E_2 has a K_i value of 0.13 nM for ERα and 0.12 nM for ERβ (105), while the well established plant derived estrogen

(phytoestrogen), genistein, has a K_i value of 2.6 nM for ERα and 0.3 nM for ERβ (105). Generally, phytoestrogens have a significantly higher affinity for ERβ in comparison to ERα with genistein, for example, displaying a 7.6 to 8.6-fold increased affinity for ERβ (105; 115-116). In addition, diphenolic ring-structured stilbene phytoestrogens, particularly coumestrol, binds with higher affinity to both ER subtypes when compared to other phytoestrogens (105; 117-118).

Differences in transactivational potential have also been reported between ERα and β. In a study by Cheung *et al*. (95), where an *in vitro* chromatin assembly and transcription system was used to compare the transcriptional activities of the two ER isoforms in the context of chromatin, ERα was reported to be a much more potent transcriptional activator than ERβ (Figs. 3 C and D). According to Cheung *et al*., this difference in transcriptional potential is attributable to the N-terminal region of ERα, which contains a transferable activation function that facilitates transcription specifically with chromatin templates. Interestingly, Cheung *et al*. observed that chromatin selectively restricts ligand-dependent transcriptional activation by ERβ under some conditions (e.g. with a closed chromatin architecture), while allowing it under other conditions (e.g. with an open chromatin architecture). These results reported by Cheung *et al*. define an important role for chromatin in determining distinct transactivational outcomes mediated by ERs α and β.

Differences in structure and distribution of the ER subtypes, ligand-specific affinities and transactivation potential is important because they allow E_2 to exhibit its characteristic pleiotropic effects and also allow other ligands the ability to behave as selective estrogen receptor modulators (SERMs) (105), meaning that they may behave as full or partial agonists or as antagonists depending on the expression of the ER isoforms in the tissue (119).

Regarding the mechanism of action of estradiol, two distinct models to describe the mechanisms by which estrogen regulates the transcription of target genes have been described. In the first one, generally referred to as the “classical pathway” of estrogen action, the ER directly binds to specific estrogen response elements (ERE) in the DNA while in other models, collectively

referred to as the “alternative pathways” of estrogen mechanism, the ER modulates the activity of other transcription factors leading to activation or repression of gene transcription (120-121).

1.3.1 The classical pathway of estrogen action

The ER is constitutively found in the nucleus when it is not bound to estradiol. The unbound nuclear ER is part of a multiprotein complex consisting of a dimer of Hsp90, a p23 monomer, and one of several immunophilins, including Cyp-40 and FKBP52. It has been proposed that this Hsp90-based chaperone complex inactivates the ER’s transcriptional regulatory capabilities and maintains the ER in a conformation that is competent for steroid binding (122-125).

Endogenous estrogens, synthetic estrogen analogs or phytoestrogens diffuse into the target cells and bind to the ER inducing a conformational change that leads to the dissociation of the ligand-receptor complex from the HSP-90 complex (126). In ERα and ERβ the positioning of helix 12 in the ligand binding pocket of the receptor plays a major role in ligand binding.

Upon binding of the ligand, the conformational changes that follow, rearrange the position of the ER helix-12 over the ligand, restricting the accessibility of the ER to other ligands and greatly diminishing the affinity of the ER for other ligands (127-129). The change in conformation strongly depends on the nature of the ligand and different ligands induce different conformations, which reflect either the agonist or antagonist characteristics of the ligands (128; 130-131).

According to the classical pathway of ER activity, upon binding of an agonist or antagonist to the ER and the subsequent dissociation of the heat-shock protein complex, the ER dimerizes with both ER subtypes capable of homo- and less frequently, heterodimerization (98; 132). The activated ER dimer complex then binds to a specific ERE sequence on the promoter DNA of estrogen responsive genes. Estrogen receptors bind to palindromic repeats on the ERE, the minimal consensus sequence of which is 5’-GGTCAnnnTGACC-3’, where n represents any nucleotide. At this point, other proteins (co-activators or co-repressors) are also recruited and interact with the activated ER complex (Fig. 4) (133).

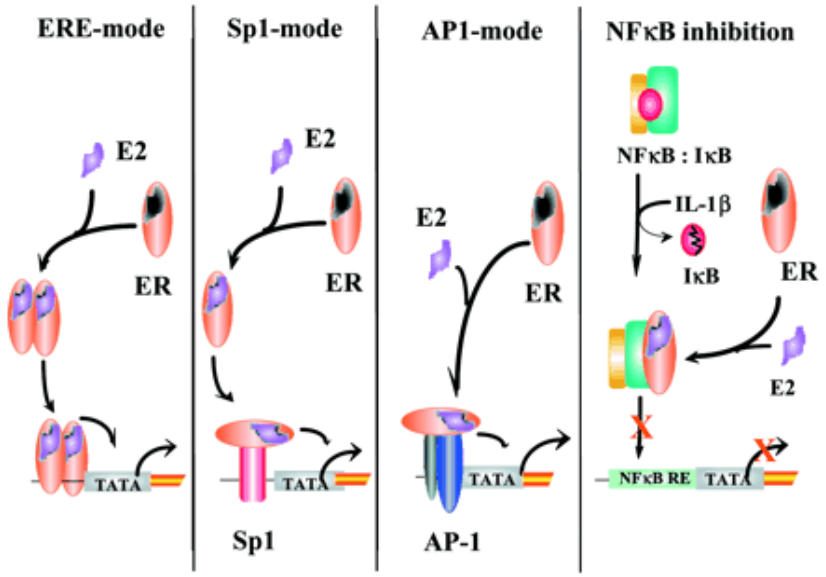


Figure 4**.** Model representing the various modes through which estrogen receptors can modulate transcription of genes. In the first panel is depicted the classical interaction of the activated receptor with estrogen response elements (EREs) on DNA. In the other three panels are representations of the indirect effects of estrogen receptors on transcription interactions. This occurs through protein-protein interactions with the Sp1, AP1, and NFκB proteins (70).

Members of the Steroid Receptor Co-activator (SRC) family, as part of their functions, facilitate the necessary unraveling of chromatin prior to transcription as seen in the actions of histone acetyltransferases (HATs) such as the p160 and CBP/p300 families (134). Apart from members of the p160 and CBP/p300 families, other co-activators in estrogen signaling include the TRAP/DRIP Coactivator Complex, Nuclear Receptor Coactivator-1 (SRC1/NCoA1), CREB-Binding Protein (CBP) as well as the CBP/p300-Interacting Transactivator, with Glu/Asp-Rich carboxy-terminal domain (CITED) protein family (86; 133; 135-136). Other structurally distinct co-activators whose function is not yet fully established in the estrogen signaling pathway include the peroxisome proliferator-activated receptor gamma (PPARγ), NSD1, TIF1, and PGC-1 (70).

Examples of co-repressors, which are thought to function through protein transcriptional silencing, include NCoR (Nuclear Receptor Co-Repressor), MTA1 (Metastasis Associated-1) and SMRT (silencing mediator of retinoid and thyroid receptors) (70; 137-138). Proteins such as PELP1 (Proline Glutamic Acid-Rich Nuclear Protein), which have been observed to behave both as co-activators and co-repressors of the ER also form part of the conglomerate of co-regulators that

facilitate estrogen signaling (139).

The ultimate result of the interaction between these various role players in the classical estrogen signaling pathway is either induction or repression of genes and an increase or decrease in protein synthesis, leading to cell growth and proliferation or apoptosis, respectively (140-141).

From observing the details of the classical pathway of estrogen action, the importance of balanced activities of receptors and various co-regulators in E_2 signaling is apparent, and the fact that the relative concentrations of these molecules is cell specific, could mean that steroid hormones can have vastly different functions in different tissues of the same organism, a phenomenon consistent with the pleiotropic effects exhibited by E_2 (142). Since the classical pathway of E_2 action-mechanism is not the only route to ER activation, we shall now consider the alternatives.

1.3.2 Alternative pathways of estrogen action

Alternative pathways of ERα and ERβ action have been reported by which the receptors influence gene transcriptional effects independently of the ER binding to its consensus ERE (143).

In one pathway, which is also referred to as “tethering”, ligand bound receptor complexes initiate transcription through interaction with other transcription factors (Fig. 4), such as the Sp1 or the AP-1 transcription factors (c-Fos and c-Jun), with the end result being the activation or repression of target genes (144). Alternatively, the ER may also interact with another important transcription factor, nuclear factor-kB (NF-kB) which may result in the suppression of genes such as the interleukin-6 (IL-6) gene, or an activation of genes, such as the serotonin receptor 1A gene (145) (Figure 4). IL-6 is a cytokine that is transcriptionally up-regulated by NF-kB which regulates bone metabolism and endothelial cell function (145-148). Evidently, the interaction of ER with the transcription factor NF-kB and the AP-1 is subtype and tissue specific and is considered to be involved in the inhibitory effects of estrogens on pro-inflammatory cytokine activity (149-150).

A rather interesting alternative pathway of ER signaling involves the activation of the AF-1

region of the ER by non-estrogens such as certain growth factors and neurotransmitters in the absence of a hormone ligand. An example of this phenomenon is the activation of the AF-1 region of ERα and ERβ by extracellular signals such as insulin via the mitogen activated protein kinases (MAPK) (151). This occurrence, which results in the transactivation of the ER without the involvement of the ligand involves, in most cases, the phosphorylation of the ER (70; 151).

Phosphorylation of the ER on serine, and less frequently, tyrosine residues, is regulated by enzymes called kinases and phosphatases that transfer or remove phosphate groups from ATP onto or off target proteins respectively (152). Although enhanced in the presence of E_2, phosphorylation is an important ligand-specific occurrence in alternative pathways of ER activation that occur in the absence of a hormone (70; 153), and as such, phosphorylation under these circumstances has been observed to be a major determinant of positive or negative transcriptional end results (153-155).

While the ERE-independent pathways of ER signaling may be efficient in driving key transcriptional processes, evidence of a necessary collaboration with the classical pathway has been elucidated by the use of the non-classical ER knock-in mutation (NERKI) mice (156-158). The use of NERKI mice, in which the selective abolishment of the classical ER signaling and the preservation of the non-classical pathway has been preserved by introducing a mutation into the proximal box (P-box) of the first zinc finger of the DBD of ERα (157), has revealed the importance of the maintenance of the balance between the classical and non-classical pathways with the observation of dire reproductive and skeletal conditions upon its perturbation. Infertility, decreased serum progesterone levels, hypoplastic mammary glands, anovulation, enlarged uteri with signs of cystic endometrial hyperplasia (157), and deficits in cortical bone (159) are some of the conditions that have been reported with NERKI female mice. Male NERKI mice have displayed acute axial and appendicular skeletal osteopenia of both the trabecular and cortical bone which were less prounced in their ERα-intact littermates (156). Paradoxical responses to estrogen, such as the administration of E_2 leading to the gaining of more bone mass by ovariectomized NERKI mice in comparison to their ERα-intact counterparts, the suppression of this increase by E_2 in the

ovariectomized NERKI mice while an augmentation is observed in the ERα-intact counterparts (159). E_2 suppressing and the ER antagonist, ICI 182,780, activating transcription via the AP1 reporter (158) is an interesting phenomenon observed with the attenuation of the classical pathway in the NERKI mice which has been speculated to be a result of the agonist recruiting co-repressors and the antagonist co-activators to the ligand-bound ER (158).

The phenomena that underlie the alternative pathways of estrogen mechanism may imply the possibility of a diverse range of transcriptional outcomes elicited by a range of other non-estrogens via the ER in response to diverse physiological signals (160). The estrogen-independent activation of estrogen receptors may be important as a contingency strategy to illicit necessary hormonal effects in cases where plasma estrogen concentrations are too low (144).

1.3.3 Estrogen receptor expression and its implications for estrogen-related cancers and menopausal symptoms

Because of the extensive spectrum of ER subtype distribution and the differences in their expression across the various tissues of the body, endogenous estrogens play an important role not only in the hypothalamic-pituitary-gonadal axis, but also in various non-gonadal systems, such as cardiovascular, bone and central nervous systems and lipid metabolism (161-164). As discussed previously, ERα and ERβ are variably distributed and co-expressed in many tissues and as such, there is an important role that the ER subtypes distribution plays in disease control and progression when estrogen imbalances occur (165-169).

One of the many lessons learned from the results of some of the studies is the possible suppressive role of ERβ on the action of ERα. Accumulating evidence supports the key role of ER subtypes in some cancers, and interestingly the expression of ERβ has been shown to be significantly reduced in breast, prostate and colon cancers (170-172). Malignant ovarian tumors originating from epithelial surface constitute about 90% of ovarian cancers and also express low

levels of ERβ compared to normal tissues (173). Another interesting observation is that restoration of ERβ in ovarian cancer cells leads to inhibition of E_2-induced proliferation and enhancement of apoptosis, thus suggesting that ERβ may play a possible tumor-suppressive role in ovarian carcinogenesis (173). A study by Strom *et al.* also reported on the role of ERβ in the inhibition of the proliferative effects mediated through ERα in the T47D breast cancer cell line (174). They observed that the induction of ERβ reduced the growth of exponentially proliferating cells with a concomitant decrease in components of the cell cycle associated with proliferation, namely cyclin E, Cdc25A (a key regulator of Cdk2), p45(Skp2) (a key regulator of p27(Kip1) proteolysis), and an increase in the Cdk inhibitor p27(Kip1). These observations may indicate a possible inhibitory action of ERβ on ERα-mediated gene expression which may point towards an inhibitory role by ERβ-selective ligands in chemo-protection. Although a recent review on the *in vivo* action of ERβ suggests that a role for ERβ-selective agonists are not supported by the current literature (175) and feeding studies, with soy-based products and purified isoflavones including genistein, have reported growth-induction with the more purified extracts (176) two recent studies suggest that ERβ-selective ligands do attenuate breast cancer cell proliferation both *in vitro* (177) and *in vivo* (174; 178-179).

The correlation between the natural depletion of endogenous E_2 and the symptoms of menopause is well established in the literature (180; 181). Substitution during menopause of endogenous estrogens with exogenous estrogens, widely known as hormone placement therapy (HRT), has been regarded as the most effective method of curbing the effects of climacteric symptoms (182). However, studies such as the Women's Health Initiative, Kronos Early Estrogen Prevention study and the Million Women study raised serious concerns about the safety of HRT (9-11), resulting in a reluctance by women to use HRT due to the fear of breast and uterus cancer development (183).

The use of SERMs is an avenue that is currently seen to bear promise towards solving some of the problems that arise with the use of traditional HRT methods (184). Selective estrogen

receptor modulators are compounds that display tissue specific agonist or antagonist activity. SERM activity may be influenced by the levels of ER-subtypes and specific co-factors present in different tissues and thus ligands that are able to discriminate between ERα and ERβ may elicit distinct estrogenic or anti-estrogenic transcriptional outcomes (185). Selective estrogen receptor modulators are considered a new alternative for postmenopausal therapy because they have been reported to improve symptoms of osteoporosis with minimal risk for breast and uterine cancer (186-187). An ideal SERM, however, would be one that targets a specific ER subtype in a specific tissue in the host, eliciting a specific transcriptional product by interacting with specific genes in the cell nucleus and in the end effectively alleviate a specific clinical symptom or group of symptoms without having a negative impact such as the promotion of mutagenesis on any system of the host organism (188).

Tamoxifen and Raloxifene are approved present-day SERM's that have been shown to improve some of the symptoms of menopause, such as improving bone mineral density and, perhaps more importantly, decreasing the incidence of breast cancer (189-190). Despite these features, Tamoxifen and Raloxifene have been shown to increase the incidence of hot flashes and thromboembolic events (191), meaning that these particular SERM's do not fit into the afore-defined mould of an ideal SERM, implying an unmet need for a non-mutagenic product for the holistic treatment of menopausal ailments (192-193).

1.4 Phytoestrogens

Herbs have a traditional history of medicinal use (194). Many ancient communities such as the Africans, Chinese and native Americans (195-197)have traditionally used plants like the Mexican wild yam (*Dioscorea barbasco*), kava (*Piper methysticum*), soybean (*Leguminosae glycine*), dong quai (*Angelica sinesis*) and black cohosh (*Cimicifuga racemosa*) for the treatment of various ailments including stimulation of red blood cell production, enhancement of cardiovascular function, muscle relaxation, topical agents for wound healing, blood pressure control, lung cancer

control and menopausal symptoms (198-202). Despite the long standing historical recognition of the ability of herbs to ease the severity of menopausal symptoms, it is only as recently as the 1980's that phytoestrogens, particularly their plant sources and their role in human and animal health, have become a major topic of scientific research (203).

Phytoestrogens are a family of plant-derived compounds that possess significant estrogen agonistic and antagonistic activity. They have been observed to elicit cell type and tissue-specific dose-dependent effects through ERα and ERβ and because of these characteristics, they are viewed as "natural SERMs" and could possibly have therapeutic effects on symptoms associated with estrogen perturbations such as postmenopausal osteoporosis and cardiovascular disease without an adverse effect on breast and uterus (204-208). Because the field of phytoestrogens is relatively new, uncertainties about their effectiveness and safety can be expected. However, because phytoestrogens exhibit medically significant characteristics such as a strong preference for the activation of ERβ and an inhibitory action on the effects of ERα-mediated proliferation of cancerous cells (179; 209), one is allowed to have realistic expectations about the effectiveness of phytoestrogens in the treatment of ailments related to estrogen perturbations, as in menopause and hormone related cancers. The amount of progress observed in the field of phytoestrogens, and the active engagement by many authors in the search for novel phytochemicals with estrogenic potential (210), reflect an expansion in the field of phytoestrogens and also bear testimony to the realization by many of the possibility of the use of phytoestrogens as medicinal entities.

The literature displays an impressive record of health benefits attributed to the ingestion of phytoestrogens by humans (211). The list includes the reduction of chronic coronary heart diseases and hormone-related cancers, the improvement of cognitive function and protection against atherosclerosis, osteoporosis, as well as the reduction of menopausal symptoms (5; 212-217). Although some health benefits, such as a delayed ageing of the skin may be attributed to non-estrogenic properties of phytoestrogens (e.g. antioxidant properties) (218), most of the clinical effects of phytoestrogens, particularly their anti-climacteric effects, results from their ability to

activate transcription factors that are important for steroid-hormone signaling, specifically ER-signaling, thus effectively influencing the transcription of medically-important genes (219). The ability of some phytoestrogens to inhibit the activities of key enzymes in the signaling pathways of estrogens such as 5α-reductase, 17β-hydroxysteroid dehydrogenase type-5, 17β-hydroxysteroid oxidoreductase type 1, tyrosine specific protein kinases, DNA topoisomerase-II and aromatase, is an example of the potential of phytoestrogens to influence important transcriptional outcomes such as the regulation of cell proliferation and cell transformation (220-223).

It may be intuitively inferred that the ability of phytoestrogens to intervene in the treatment of estrogen-dependent diseases requires that a significant amount of parallels be drawn between their molecular activities and those of the endogenous hormone, E_2. Establishing the existence of these parallels requires a study of the structures, the molecular mechanisms, the metabolism, as well as the pharmacological importance of phytoestrogens. In allegiance to the rationale and the intent of our study, the pharmacological aspect of phytoestrogens will mainly be discussed within the framework of their application as agents for the treatment of menopausal symptoms.

Phytoestrogens are dietary estrogens, and although the focus of this section is on phytoestrogens, a discussion of their relevance will not be complete without mention of other members of the broader class of dietary estrogens, which, because of similarities in biological activities, are often mentioned alongside the plant-borne chemicals that are of interest to our study. Broadly, dietary estrogens are ingestible chemical compounds that are capable of eliciting estrogenic or anti-estrogenic effects in mammals (224). These compounds can be divided into the naturally occuring and synthetic super-classes (Fig. 5). The synthetic agents (Fig. 6), differentiated into the classes of xenoestrogens and pharmaceutical estrogens, differ from the plant agents in that they are introduced into the environment as products of agricultural and chemical industries. Some xenoestrogens are associated with detrimental biological effects (such as negative sexual development and differentiation) (225) and are hence referred to as endocrine disruptors (226). Examples of industrially generated endocrine disruptors include (a) bisphenol A, a monomer of

polycarbonate plastic, (b) polychlorinated biphenyls (PCBs), which are insulating fluids for industrial transformers and capacitors, and (c) the two insecticides dichloro-diphenyl-trichloroethane, more commonly known as DDT, and methoxychlor (Fig. 5) (227-229).

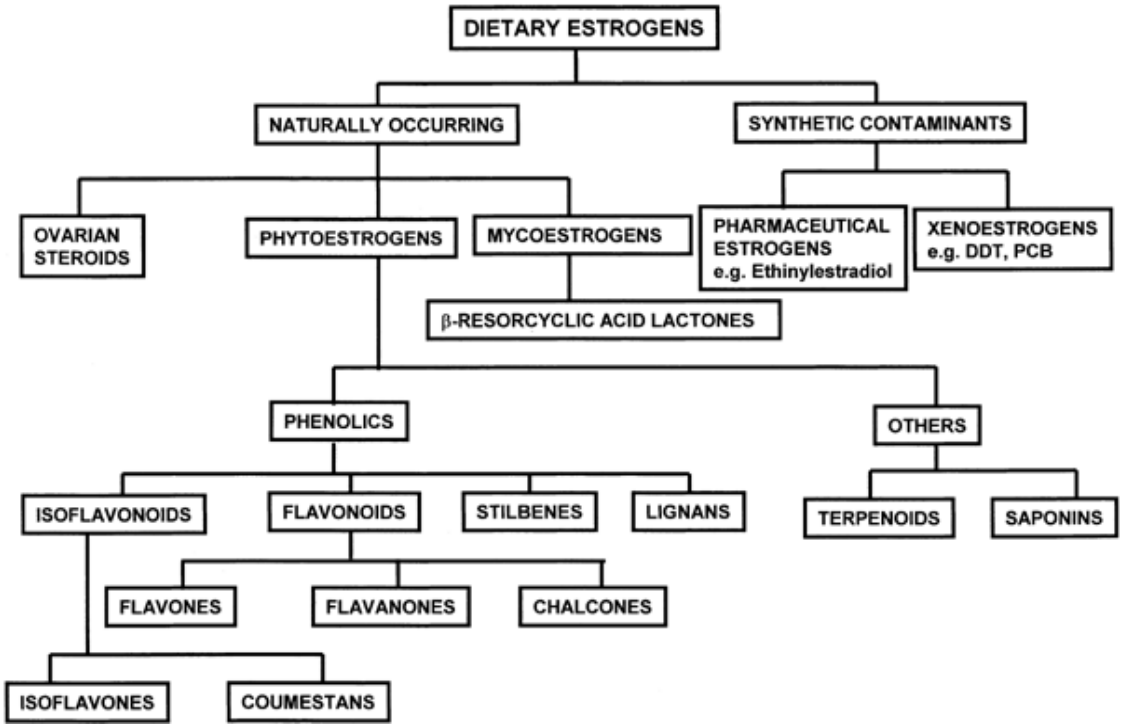


Figure 5. Classification of dietary estrogens (230).

The other class of synthetic estrogens, the pharmaceutical estrogens (Fig. 5), differs from xenoestrogens in that they have an intended medicinal use. Examples of pharmaceutical estrogens include estradiol ethinyl, an oral bio-active estrogenic component in most modern contraceptive pills and Quinestrol® (ethinyl estradiol 3-cyclopentyl ether), a synthetic estrogen used in hormone replacement therapy (231-233). Diethylstilbestrol is a synthetic estrogen that was approved for the treatment of disorders such as gonorrheal vaginitis, atrophic vaginitis, menopausal symptoms, and postpartum lactation, but is now classified as an endocrine disruptor after it was discovered to promote vaginal adenocarcinoma in human female offspring (234).

Phytoestrogens, which are the compounds of interest in our study, are members of the super-class referred to as naturally occuring estrogens. Naturally occuring estrogens comprise the three classes of fungal (mycoestrogens), plant (phytoestrogens) and ovarian (mammalian estrogens) estrogens (235-236). By definition, phytoestrogens are non-steroidal, non-nutrient plant molecules with estrogen-like bioactivity, a property which has resulted in their consideration as viable alternatives for the treatment of menopausal symptoms (6).

Phytoestrogens can be divided into the super-families of phenolic and non-phenolic compounds (Fig. 5). The non-phenolic compounds include the families of terpenoids and saponins while the phenolics comprise the families of flavonoids, isoflavonoids, stilbenes and lignans. Both phytoestrogen super-families are found ubiquitously in nature in over 300 plant species comprising various herbs, grains, and fruits (237-238).

Principally, phytoestrogens are produced by plants as secondary metabolites in response to environmental stress conditions where they may be useful as antimicrobials or fungicides, signal molecules for beneficial micro-organisms in the rhizosphere, herbivore deterrents and may afford the plant photo-protection against ultraviolet radiation (239-240). Although there is a variety of phytochemicals that structurally resemble mammalian estrogens in various plants species, only a limited number of these have been observed to exhibit phytoestrogenic activity in mammals (241).

The key attribute of phytoestrogens that is thought to allow them their estrogenic mimicry is their structural resemblance to the endogenous hormone, E_2 (Figs. 6 and 7) (242). This important structural resemblance of phytoestrogens to the endogenous hormone is, however, not the final determinant of the estrogenic potential of phytoestrogens. Another important structural feature of most phytoestrogens which makes them different from the endogenous E_2 and which affects their bioavailability and biological effects is that in nature, phytoestrogens mostly occur in glycosylated forms (243), an important consideration during the interpretation of animal model and cell culture study results where aglycosylated commercial products are often used as test substances.

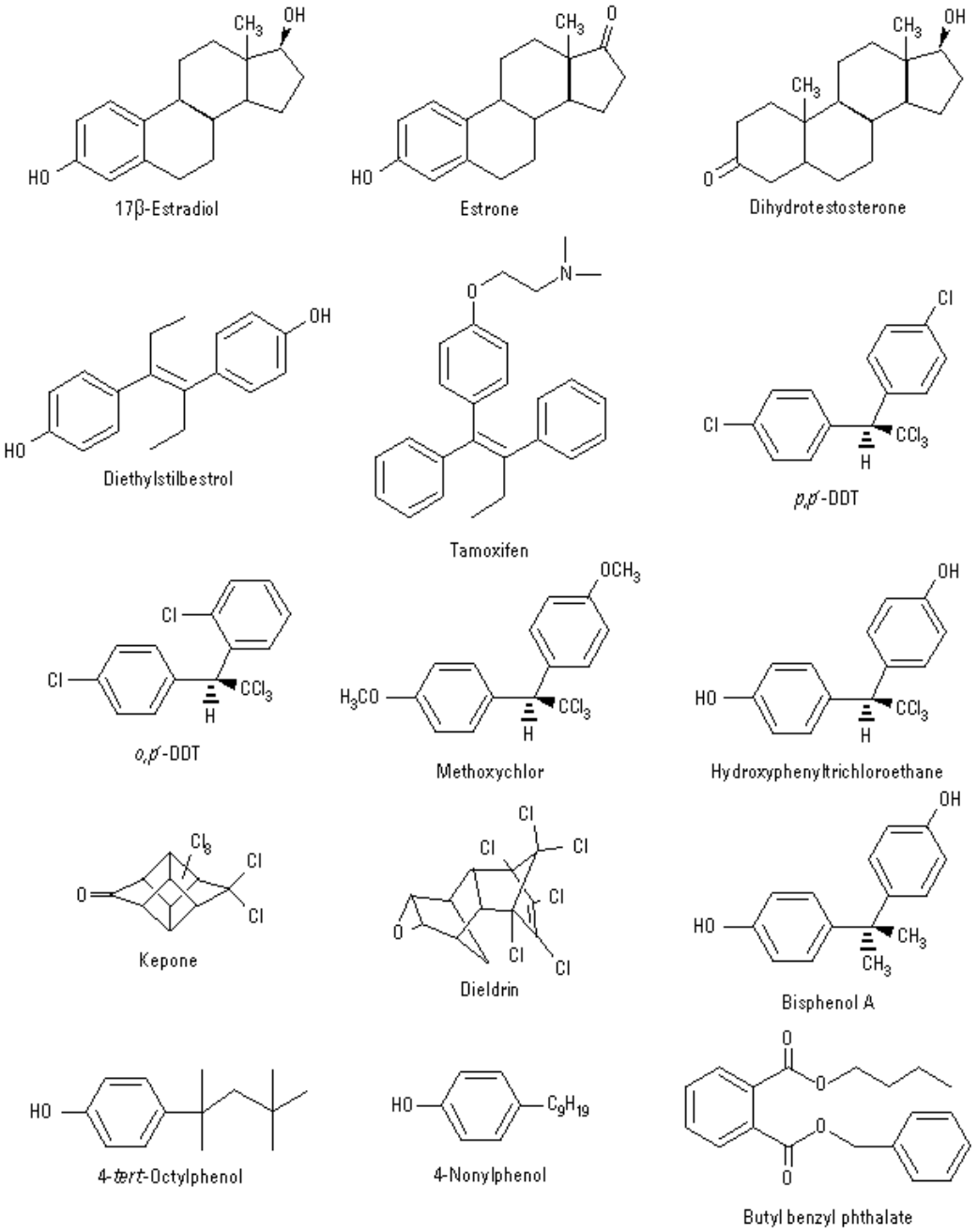


Figure 6. Structural comparison of natural and synthetic estrogens E_2 (244).

Another important consideration which relates to the bioavailability of phytoestrogens is the role of the gut microflora in transforming phytoestrogens from their natural state into a state that is conducive for the activation of transcription factors (243). It has been reported that the presence of an active gut microflora is essential for the bioavailability of soybean isoflavones (243) and interestingly, that the parent phytoestrogen may after metabolism by gut microflora yield a product

with distinctly different biological effects. An example of this phenomenon is the conversion of daidzein by the gut microflora to equol and O-desmethylangolensin (O-DMA) (245). Equol has been reported in the literature to be a more active estrogenic metabolite than it precursor daidzein (246). Equol, unlike the soy isoflavones daidzein or genistein, has a chiral center and can therefore occur as 2 distinct diastereoisomers, R and S (247). Human beings, through their gut microflora, are unique in their ability to synthesize S-equol from daidzein, which interestingly, is reputed to have a relatively high affinity for ERβ in comparison to the R enantiomer (248). When one considers that substantial inter-individual differences in daidzein metabolism exist, with approximately 30-50% of the human population producing equol, and approximately 80-90% producing O-DMA (249), one may be inclined to determine that there may be considerable inter-individual differences in clinical benefits from the use of phytoestrogens (242).

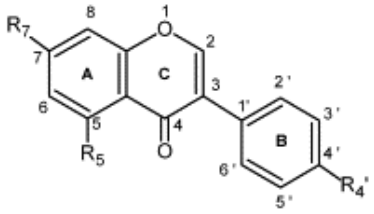
Phytoestrogens that are important for mammalian health belong to the super-family of phenolic phytoestrogens which include isoflavonoids, flavonoids, stilbenes and lignans (Fig.5). The best studied of these phenolic phytoestrogens are the isoflavonoids (isoflavones and coumestans) and lignans. The discussion will therefore focus on these classes of phytoestrogens.

Several plant foods have become important as rich sources of these important classes of phytoestrogens. Isoflavones (genistein, daidzein, glycitein, and equol), for example, are primarily found in soy, chickpeas, and other legumes while lignans (enterolactone and enterodiol) are found primarily in flaxseed, rye, cereal bran, legumes, and alcoholic beverages such as beer and bourbon. Coumestans (coumestrol, wedelolactone), on the other hand are found in alfalfa and clover sprouts. These three classes of phytoestrogens are of particular importance to our study because they have emerged as the most important for the treatment of menopausal symptoms (5; 250-251). Although an appreciable amount of research has been done on all three classes of clinically important phytoestrogens, the isoflavones, particularly the soy isoflavones, are by far the best studied compounds (203) due to their apparent usefulness in mediating against a diverse range of diseases (237; 252), a subject with which the text shall acquaint the reader with next.

1.4.1 Isoflavones

Isoflavones, an extensive family of structurally related phytochemicals (Fig.7), are synthesized in plants as part of the phenylpropanoid pathway from naringenin and liquiritigenin by the cytochrome P450 isoflavone synthase enzyme (IFS) (253).

Principally, isoflavones are chemo-attractants for nitrogen-fixing bacteria for the establishment of symbiotic relationships in root nodules as well as agents of disease resistance (255). In nature, isoflavones are mostly limited to the *Fabacae* family (subfamily *Papilionoideae*) and although amounts of isoflavones fluctuate across different plant species depending on genetics and environmental factors (256), the highest amounts are generally found in soybeans where the seeds accumulate high concentratons of daidzein and genistein plus smaller amounts of free and conjugated glycitein and coumestrol (257). Chickpeas, abundant in biochanin A, and alfalfa sprouts, with high concentrations of formononetin glycosides and coumestrol are other important sources of isoflavones (203; 258-259).



Isoflavonoid	R- 5	R- 7	R- 4'
Genistein	OH	OH	OH
Genistin	OH	O-glucose	OH
Daidzein	H	OH	OH
Daidzin	H	O-glucose	OH

Figure 7. Generic structure of isoflavones. The positions of the A, B and C rings and the functional groups are indicated for genistein. C-7 of ring A is attached with glucose moiety in genistin and daidzin. Daidzein does not have a hydroxyl group at position 5 of the A ring compared to genistein (254).

Isoflavones occur mostly as glycosidic conjugates in nature and studies suggest that the de-conjugation is not only important for the bioavalability of these compounds but also for their phytoestrogenicity as evidence suggests that it is the aglycones that show an affinity for estrogen receptors (260-261). Subsequent to the ingestion of soybean isoflavones, hydrolysis by the β-glycosidase enzyme takes place with the release of the aglycones, daidzein, genistein and glycitein, which are then absorbed or further processed to other metabolites. Examples of such further processing are the metabolism of daidzein to equol and *O*-desmethylangolensin and the conversion of genistein to *p*-ethyl phenol and 4-hydroxyphenyl-2-propionic acid (210; 261-262). The availability of a competent community of gut microflora in an optimum hydrocarbon milieu, which may enhance the fermentation process, is essential for the uptake of dietary isoflavones in the jejunum (210; 261). The resultant aglycones from deconjugated isoflavones are next absorbed from the intestinal tract by nonionic passive diffusion into the blood stream after which they are detoxified through conjugation mainly with glucuronic acid and to a lesser extent with sulphates in the liver (263). The deconjugated isoflavones in the blood stream, in a similar manner as E_2, are available for occupancy of the ER (264-265). In plasma, the free isoflavones, as is the case with E_2, are bound to serum albumin and SHBG, a major factor in their availability for occupancy of steroid receptor sites (261). Interestingly, phytoestrogens and xenoestrogens in general have less affinity for serum proteins in comparison to steroid hormones which allows for a greater proportion to be available to occupy the ER, a factor that should hypothetically favour an increased estrogenic effectiveness of isoflavones (261-262; 266).

In order for isoflavones, or phytoestrogens in general, to be effective in delivering pharmacological effects they need to occur in the plasma in sufficient concentrations and for sufficient amounts of time (267). Intuitively, exposure to higher or lower than optimum concentrations of isoflavones will lead to insufficient or high steady-state plasma concentrations which will inevitably affect the final biological effects elicited by the phytoestrogens (261). The importance of ingesting balanced and adequate amounts of isoflavones is the reason why there is a

concern about soy based infant formulas because although some studies indicate that exposure to soy-based infant formulas is a safe feeding option for most infants and does not appear to lead to different reproductive outcomes in later life in comparison to cow milk formula feeding (268), other authors have reported plasma genistein and daidzein concentrations that were over a 100 times higher in 4 month old infants that were fed soy-based formula in comparison to their counter parts that were either cow-milk based formula or breast fed (269). This infant exposure to high isoflavone concentrations could present health problems for the infants if it affects their sexual and reproductive development, immune function, visual acuity and cognitive development or thyroid functions, which are factors about which some authors feel more research is needed (270).

Complete elimination of phytoestrogens, mainly as glucuronide conjugates, has been recorded to occur between 2 to 3 days after ingestion mainly through the urine (261; 267; 271-272). The half-lives of plasma genistein and daidzein have been observed to be 8.36 and 5.79 h, respectively (267). This relatively short bodily retention period of isoflavones has been suggested to be the reason for their relatively weak estrogenicity and one of the reasons why phytoestrogens may be a safe alternative to HRT (273). The state of the food matrix is also a factor that affects elimination of isoflavones. More rapid elimination is observed for isoflavones in a liquid matrix than in a solid matrix food (261; 274).

The current propositions regarding the molecular mode of action of isoflavones are that their biological effects are as a result of either their mimicry of the normal estrogenic actions or competitive inhibition of the effects of the endogenous E_2, or both (275-276). The strong affinity of isoflavones for the ER is hypothesized to depend greatly on five structural attributes. These structural attributes include (a) the presence of a phenolic ring structure which is considered indispensable for binding to the ER, (b) the role of the A-ring of isoflavones mimicking the A-ring of estrogen at binding, (c) similar low molecular weights (272.39; 270.24 and 268.23 daltons for E_2, genistein and coumestrol, respectively), (d) the distance of approximately 11.5 Å between the hydroxyl groups at the 7- and 4´- carbon positions on isoflavones being very similar to the distance

between the hydroxyl groups positioned at carbons 3 and 17 in E_2, and (e) an optimal pattern of hydroxylations, i.e. a conserved pattern of hydroxylation of the 4′, 5 and 7 positions as is the case in genistein for example (Figs. 6-9) (203).

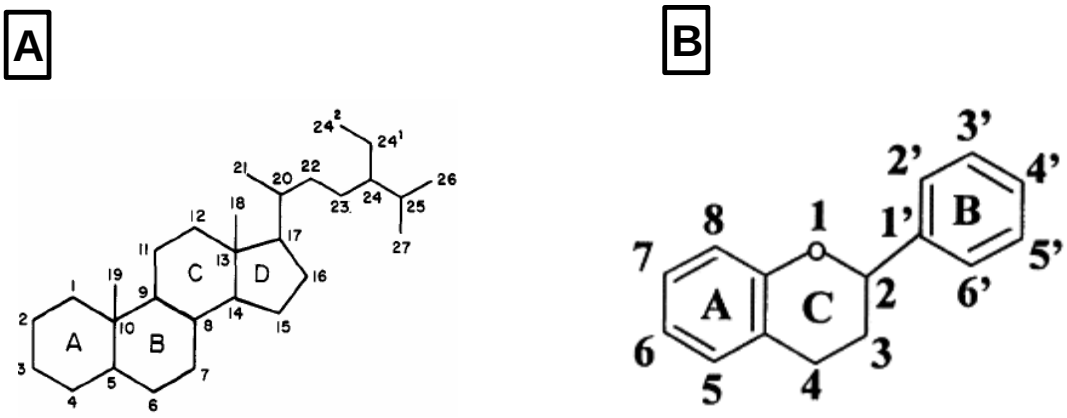


Figure 8. Steroid (A) and flavonoid (B) structures and nomenclature (277).

It is well established in the case of E_2 that binding of the endogenous ligand results in changes in ER conformation, particularly in the positioning of helix 12, a level of consensus that is yet to be established with the action of phytoestrogens (89; 91; 128-129; 278). Notably, isoflavones seem to preferentially bind to the ERβ isoform, whereas classic E_2 exerts its effects via both receptor isoforms, ERα and ERβ (209; 279). As such, isoflavones may act as natural SERMs that elicit distinct therapeutic effects by selectively recruiting specific coregulators to ERβ, which would then specifically affect important transcriptional end results (204; 279-280).

Although the preference by phytoestrogens for binding to ERβ and their resultant apparent inefficiency in eliciting uterine cell proliferation had awarded these compounds their status of non-estrogenicity (281), it is also important to note that none of the known phytoestrogens are absolutely discriminatory concerning binding to ERα and that in high concentrations, phytoestrogens will bind to ERα and will stimulate uterine and breast growth with a similar risk index as E_2 (281). Also an important fact to consider in deliberating on the mechanism of action of phytoestrogens is that although E_2 may be at least 1000 times more potent than phytoestrogens,

phytoestrogens are able to occur in plasma at higher concentrations that E_2, and they are also capable of exhibiting supra-agonist efficacies through ERα and β (282).

Viewed in entirety, although there is still a long way ahead in isoflavone and phytoestrogen research, these established hormonal characteristics of phytoestrogens may lead to significant and clinically important transcriptional end results (283).

The fact that the term phytoestrogens started appearing in the literature only in the late 1980s with less than 100 citations that year and that by the year 2000 almost 5 times the initial number of citations had been recorded (203) bears testimony to striking observations pertaining to suspected biological effects of phytoestrogens. Indeed, observations such as lower incidence of hormone-related cancers and decreased severity of menopausal symptoms in Eastern populations that consume phytoestrogen-rich diets in comparison to Western populations who have a low fiber, high fat diet were made (284-285).

Generally, the Asian consumption of legumes is assumed to supply 20–50 mg of isoflavones in the daily diet (284). This is not surprising considering that particular Japanese population groups may reach the highest intake of soy products, with levels up to 200 mg/day (286). This contrasts sharply with the typical Western diet that delivers a negligible amount of less than 1 mg isoflavones/day (287). Further, it was also observed that diet changes in migrant Asians to the Americas who had adopted more Western-like diets had correlated to migrant offspring being on the same risk level of contracting some hormone dependent cancers as the Western populations (203; 288). Since these observations pointed to diet-related factors rather than pure genetic factors contributing to the occurrence of these clinically important effects (289-290), research into the understanding of phytoestrogens and their molecular mechanisms had become the focal point of scientific investigations, and accordingly, the role of dietary soy phytoestrogens in health has gained importance (291-292).

Studies have presented evidence of the usefulness of soy isoflavones in improving bone mass in peri- and postmenopausal women (293) as well as in decreasing the incidence and severity

of vasomotor symptoms such as hot flashes (294). More studies, such as done by Anthony *et al.* (295), which had reported preventative effects of a soy based diet on atherosclerosis of coronary arteries in peri-pubertal male and *female* rhesus monkeys, and those by Crouse *et al.* (296), who had reported the effectiveness of a soy protein diet in significantly reducing cholesterol levels in mildly hypercholesterolemic volunteers, further support the beneficial effects of phytoestrogens on human health.

Isoflavones have also been reported to be effective in inhibiting tumour proliferation (297) as well as the proliferation of vascular endothelial cells thereby implying their potential in mediating against the chronic effects of extensive neovascularization (298). Moreover, soy seems to delay and protect against chemically induced mammary tumour formation in rodents (299) and from such findings it has been suggested that isoflavones as well as other classes of phytoestrogens may exert anti-carcinogenic properties through anti-aromatase, anti-proliferative and anti-angiogenic mechanisms (300). Despite findings which seem to point to possible beneficial human health effects by phytoestrogens, some reports propose that appreciable protection against diseases, such as breast cancer, is achievable only if soy intake is spread over a lifetime, with the pre-pubertal and adolescence stages being of particular importance (300-302). There are also concerns about the practicality of the attainment of chemo-protective effects of phytoestrogens since the observed effects have been achieved largely with supra-physiological concentrations of phytoestrogens. These may however, be balanced by evidence of dose responses of average diet and nutraceutical mixtures revealing synergistic effects at physiological doses (303-304).

Altogether, the literature seems to support the safety of isoflavones as consumed in diets based on soy products (303; 305-306). Although significant ground has been covered as far as the study of the beneficial effects of isoflavones, it is obvious that more still needs be done especially with regards to establishing the molecular mechanism, safety, minimal effective dosage, and efficacy before general application in cancer or hormone therapies can be undertaken.

1.4.2 Lignans

Lignans are a diverse class of phenylpropanoid oligomers that enjoy a wide distribution in seeds (such as flax seed), grains, vegetables and fruits, tea, coffee, and wine (307-308). Although widely distributed throughout the plant kingdom where they function primarily in plant defense (309), the major dietary sources of lignans are the outer layers of grains and cereal, with rye and flaxseed among the most important, meaning that the ingestion of whole grains is essential for the intake of lignans. This is demonstrated by the consumption of whole grains significantly elevating enterolactone serum levels in comparison to the intake of refined grains (310). Although their biosynthesis is poorly understood, it is established that the two phytoestrogenic mammalian lignans, enterodiol and enterolactone, are synthesized by the gut microflora from the precursors, secoisolariciresinol diglycoside and matairesinol (Fig. 9) (311-312).

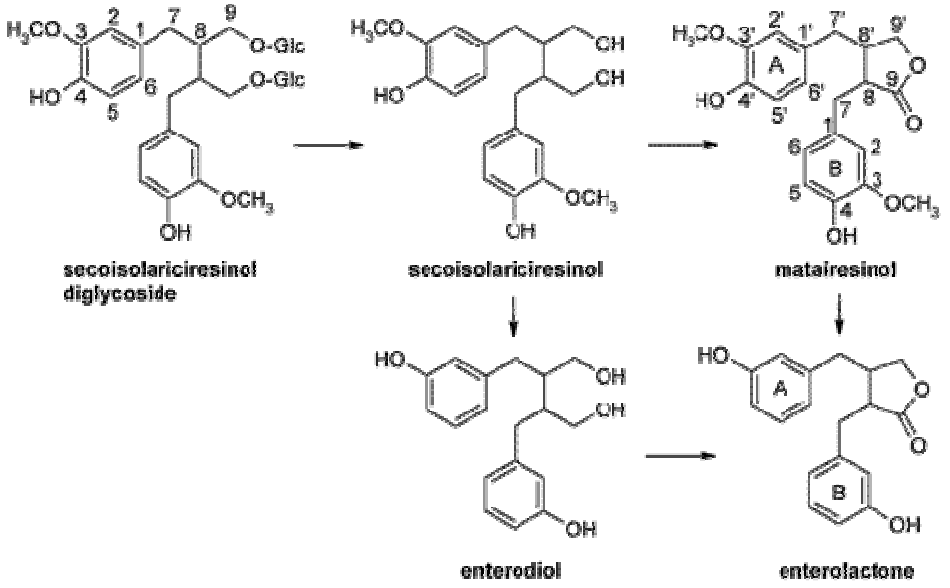


Figure 9. Bacterial transformation of the plant lignans secoisolariciresinol diglycoside and matairesinol to the mammalian lignans enterodiol and enterolactone (311).

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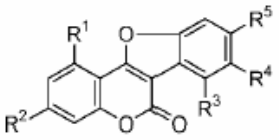
Chemoprotection (313) associated with antioxidant properties (314) and cardiovascular health (315) are among health benefits associated with the intake of whole grains with a significant content of lignans. The chemoprotective potential of lignans from traditional herbal medicines has

also been recorded in literature. Deoxyschizandrin and γ-schizandrin, bioactive lignans from *Schizandrae chinensis*, a traditional Chinese medicine herb, have been used to treat hepatitis B disease in Chinese hospital clinics (316).

Indirect cardiovascular, chemoprotective and antioxidant benefits of sesame seed lignans through vitamin E blood and tissue-level elevation has also been demonstrated in a study by Frank (317). Vitamin E has many reported health effects and is recognized as the most important lipid-soluble, chain-breaking antioxidant in the body (318). Vitamin E has also been reported to play a regulatory role in cell signalling and gene expression (319). Also, epidemiological studies show that high blood concentrations of vitamin E are associated with a decreased risk of cardiovascular diseases and certain cancers (320). Yet, high doses of supplemental vitamin E have been associated with an elevated risk of heart failure and mortality (321-322). Establishing alternative strategies to improve vitamin E status without potentially increasing mortality risk may, therefore, prove important for optimal nutrition. In the study by Frank (317), plasma and liver tissue concentrations of two important E vitamers, *α*- and *γ*-tocopherol, were observed to be significantly increased in male Sprague–Dawley rats after they were fed a standardized, semi-synthetic diet of sesame lignan (sesamin and cereal alkylresorcinols) for 4 weeks. Overall, their findings suggested that the tested dietary sesame lignans increased vitamin E concentrations through different mechanisms and thus have the potential to improve vitamin E status without the use of vitamin E supplements (317).

1.4.3 Coumestans

Although there are a large number of coumestans (Fig. 10), only a few, predominantly coumestrol and wedelolactone, has been shown to have biological activity (203). Coumestrol and wedelolactone are found in relatively high concentrations in legumes such as alfalfa and clover sprouts (203). *In vitro*, coumestans have been reported to exhibit a variety of biological activities that include, antibacterial, antifungal, antimycotoxic and phytoalexic in addition to phytoestrogenic effects (323-327).



$R^1 = R^2 = R^3 = H; R^4 = R^5 = OH$	1,12-Dihydroxy coumestan
$R^1 = R^4 = R^5 = OH; R^2 = OMe; R^3 = H$	Wedelolactone
$R^1 = R^3 = R^4 = H; R^2 = R^5 = OH$	Coumestrol
$R^1 = R^2 = R^5 = OH; R^3 = R^4 = H$	Aureol
$R^1 = R^3 = H; R^2 = OH; R^4 = R^5 = CH_2OCH_2$	Medicagol
$R^1 = R^4 = H; R^2 = R^3 = OH; R^5 = OMe$	Tifiliol
$R^1 = R^3 = R^4 = H; R^5 = OH; R^2 =$ isopentenyl	Psoralidin

Figure 10. Some naturally occurring coumestans (328).

Wedelolactone, first isolated from the extract of *Wedelia calendulaceae*, has been traditionally used both in China and India for the treatment of liver disorders including liver cirrhosis and infective heptatitis (329). Several studies have reported the effectiveness of wedelolactone in the intervention against menopausal symptoms and inflammatory disorders (330-332).

In a study by Annie *et al*. (330), an ethanol extract of *W. calendulacea,* at two different dose levels of 500 and 750 mg/kg/body wt. day was found to have a definite protective effect in the ovariectomized rat model of osteoporosis. In another study by Jayathirtha and Mishra (331), dose-dependent immunomodulatory activity of methanol extracts of the whole plant of *Eclipta alba* (1.6 % wedelolactone) was demonstrated. The *E. alba*, extracts were reported to significantly increase white blood cell count as well as the antibody titer in Swiss Albino mice.

Anti-carcinogenic properties and an effectiveness in the treatment of menopausal ailments have been reported for coumestrol (250; 333) . In a study by Kanno *et al.* (250), where they investigated the effects of coumestrol and other phytoestrogens on osteoclast differentiation using ER α-transfected RAW264.7 cells, they found that coumestrol has an inhibitory effect on the differentiation of osteoclasts, at least partially via ERK1/2 pathway.

In summary, it is clear from the literature that despite uncertainties about safety and

efficacy, there is a great potential for the use of phytoestrogens for the treatment of menopausal symptoms. The validity of this assertion is reinforced by the trends observed in the phytoestrogen nutraceutical market and the findings of studies that have assessed the phytoestrogenicity of plants extracts and phytochemicals reputed to confer such estrogenic effects in mammals. These aspects, as well as strategies and measures that have been utilized to establish the estrogenic effects of relevant plant extract preparations are topics that the following sections of this text shall engage with.

1.5 Nutraceuticals and the nutraceutical industry

In defining the concept of nutraceuticals, one needs to make a distinction between them and functional foods. Nutraceuticals are market medicinal foods that are packaged as pills, elixirs or powders and that play a role in maintaining well being, enhancing health, modulating immunity and thereby preventing as well as treating specific diseases (334). A functional food is a conventional food, consumed as part of a usual diet, but which is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions (335).

There is a growing demand for nutraceuticals that emanates from consumer's recognition of the diet-health link and a desire for what they perceive as "natural" treatment alternatives to drug treatments currently offered by the mainstream healthcare industry (336-338). These needs are driven by the idea that the non-conventional treatment methods have a higher safety value (336). In many countries, perhaps as a result of health professionals and governments not taking cognizance of the level of importance that the general public attaches to the use of nutraceuticals, nutraceuticals are not regulated and tested as tightly as pharmaceutical drugs are (338-339). This is a source of concern as it may expose the general public to potentially dangerous substances through self medication (8; 340). Although phytoestrogens are regulated under the Dietary Supplement Health and Education Act in the United States of America (341), the American Food and Drug

Administration organization does not evaluate the efficacy of herbal medicines due to the lack of substantial scientific evidence (342). Only four countries in the world (China, India, Germany and Japan) are known to have medical communities that actively prescribe herbal medicines and provide insurance coverage for such treatments (343).(316; 344-347)

The term nutraceutical, coined by Stephen DeFelice in 1989, is derived from the terms "nutrition" and "pharmaceuticals" (338; 348), and interestingly the nutraceutical industry embodies the characteristics of both the food and the pharmaceutical industries. The pharmaceutical industry, for example, is well known for the high cost of research and development associated with drug development and the use of patents to protect discoveries, which results in its high profit margins. The food industry on the other hand is known for lower profit margins and the commoditization of its products (349). Nutraceuticals and functional foods are somewhere in between (347). The interest in nutraceuticals and functional foods has also extended to institutions such as University departments (e.g. pharmacy, agriculture, medicine) where active compounds are being sought from plant and animal sources to be used as pharmaceuticals as well as food ingredients (347). Scientific research is currently leading to patentable discoveries of potential food ingredients as well as of extraction and purification processes that could allow entrepreneurial scientists a great competitive market advantage in time (350-351). Furthermore, entrepreneurs and manufacturers are always searching for higher value components from lower value raw material, an attainable aspiration in the case where the raw material is in a form of agricultural or wild plant populations (347).

As a word of caution however, harvesting from the wild may cause a loss of genetic diversity and habitat destruction (352). Controlled farming methods on the other hand is a viable alternative to overcome the problems that are inherent in herbal extracts such as misidentification (352-353), genetic and phenotypic variability (352), toxic components and contaminants (352-353) as well as extract variability (354). Obstacles to bringing medicinal plants into successful commercial cultivation include the difficulty of predicting which extracts will remain marketable and the likely market preference for what is seen as naturally sourced extracts (352).

Currently, an area of substantial concern regarding the nutraceutical and functional food industries is the unbalanced advertisement of health claims about the effectiveness of the products (355). It is important to emphasize that as a result of the lack of scientific consensus on the basic molecular mechanism by which nutraceuticals and functional foods operate, the claims which infer that nutraceuticals positively modulate nutrient metabolism and enhance the development and differentiation of body systems are at this stage not confirmed and no consensus on the claims has been reached (356). The establishment of health claims must be firmly based upon both scientific proof and legal regulation and efficient biomarkers related to specific biological responses must be found before health claims are presented to the general public. Furthermore, it is essential and indispensable to conduct valid studies on humans and in the case of epidemiological investigations the prime objective must be the diet as a whole (357).

1.5.1 Phytoestrogens as nutraceuticals

Since the discovery of phytoestrogens, through observations of negative effects of clover on cattle and sheep fertility (203), there has been a tremendous amount of growth in the field of phytoestrogens, with appreciable health benefits accredited to phytoestrogens. Of particular importance to our study however, is the use of phytoestrogens for the treatment of climacteric diseases, which is the reason why we shall dedicate the next section of the text to discussing phytoestrogens as nutraceuticals, particularly discussing plant sources for extracting phytoestrogens as well as some market products that are used for the treatment of menopausal symptoms.

As mentioned previously, the positive nature of the findings on the role of phytoestrogens in the control and treatment of climacteric symptoms and other diseases have given impetus to the creation of a considerable market demand and a resultant supply of a wide variety of standardized phytoestrogenic nutraceuticals and functional food preparations that cater for a wide spectrum of health needs of various age groups (357-358). Consumers of phytoestrogen supplements, however, tend to be peri- and postmenopausal women looking for an alternative to HRT for reduction of

vasomotor symptoms (359). As mentioned previously, the reluctance to use HRT is strengthened partly by the recently released reports from the WHI, KEEPS, and the Million Women studies which, apart from reporting no overall protection against heart attack or coronary death from the use of HRT, also reported a correlation between HRT and an increased risk of the onset of venous thromboembolic events and as well as breast, endometrial and ovarian cancers (9-11; 360-362). The movement to the use of alternatives to HRT by postmenopausal women is also reflected in the findings of a recent study of over 6000 Swedish women, where only 21% of them used HRT for the treatment of vasomotor symptoms while 45% took a non-hormonal variety (362-363).

Extracted phytoestrogens are the main active ingredients in nutraceuticals and estrogenic dietary supplements (364). Even though a variety of plant species that contain high concentrations of phytoestrogens serve as sources for the active ingredients of phytoestrogenic market products, the soybean, renowned for its high concentration of three of the most well researched and acclaimed isoflavones, i.e. genistein, daidzein and glycetein, is currently at the centre of the phytoestrogen and functional food markets with products which include soy-based infant formula, soy milk, baking mix, etc. (365-368).

Besides soy, other popular plant sources whose phytoestrogenicity has been exploited for the preparation of nutraceuticals include red clover, black cohosh, dong quai, wild yam, gingko, and American ginseng. Although research on specific phytoestrogenic nutraceuticals and botanicals is generally sparse (369), relatively substantial work has been done on investigating the effectiveness of some market products such as the black cohosh root and rhizome extract sold under the brand name Remifemin® and the red clover based extract branded Promensil®. Since we have already extensively discussed the action of soy isoflavones in the section dealing with isoflavones, we shall now focus on phytoestrogenic nutraceutical products based on other plant sources, namely black cohosh and red clover.

1.5.1.1 Black cohosh extracts

Black cohosh (*Cimifuga racemosa)* is native to North America and can be found in shaded woodlands located in the eastern United States and portions of southern Canada (370). The main bioactive components of black cohosh that are thought to confer phytoestrogenic properties are the triterpene glycosides, acetein and 27-deoxyactein (371). Although formononetin had been counted among the active phytoestrogens in black cohosh, several studies have not been successful in finding this isoflavone in the black cohosh plant (372).

Standardized black cohosh botanicals have been marketed as tablets for the relief of vasomotor symptoms under such names as Black Cohosh - Cimicifuga Extract Plus®, Nature's Resource Black Cohosh®, Schiff Menopause Nutritional System® and Remifemin®. The phytoestrogenic effects of black cohosh have been demonstrated in the literature and a positive safety rating by many of the studies on Remifemin and black cohosh botanicals has been given (373-374). We shall therefore, in the following section, discuss some of the literature on the safety and tolerability of black cohosh extracts in addition to providing some data on their efficacy for the treatment of menopausal ailments.

As far as the proliferative effects of black cohosh extracts on cancer cells is concerned, Bodinet and Freudenstein (375) demonstrated an inability by Remifemin to induce proliferation in MCF-7 breast cancer cells suggesting a positive safety profile for women with a personal or family history of breast cancer. Similar findings and conclusions were also reported in an epidemiological study by Rebbeck *et al*. (376) who reported that black cohosh botanicals and Remifemin exhibit breast cancer-protective effects. Also, Tian *et al*. (377) reported an anti-tumor action of the ethyl acetate fraction of black cohosh (*Cimicifuga foetida*) on hepatocytes through an induction of cell cycle arrest. These findings by Tian *et al.* are in line with the findings of Rebbeck *et al*. as well as those of Bodinet and Freudenstein as they reiterate the possibility of therapeutic effects of black cohosh extracts against hormone-related cancers such as of the breast and liver.

The effectiveness of Remifemin and black cohosh extracts in the intervention against

menopausal vasomotor symptoms has been documented (378-380). One such study by Vermes (378) reported a significant decrease in hot flashes, sweating, insomnia and anxiety in Hungarian women between the ages of 40 and 65 who had been treated with an isopropanol extract of black cohosh (*Cimifuga racemosa*). Also, in another study by Bai *et al*. (373), the efficacy of Remifemin for the treatment of mild to severe climacteric complaints was found to be on par with that of tibolone, a marketed synthetic hormone-type drug used mainly for hormone replacement therapy in post-menopausal women. Bai *et al*. (373) also demonstrated the safety and tolerability profiles of Remifemin to be clearly superior to those of tibolone.

Adverse effects associated with the use of black cohosh extracts, although deemed mild by some authors (374), have also been reported in the literature. Mahady (381), for instance, in a review of clinical data for the safety and efficacy of black cohosh on menopausal symptoms, while conceding the usefulness of black cohosh extracts in the treatment of vasomotor symptoms, has also cautioned against poor quality and follow up records of many such studies and has also reported instances (382) of adverse effects such as vomiting, dizziness, mastalgia and headaches associated with the use of black cohosh extracts. Other studies that have reported on the adverse effects associated with the use of Remifemin and black cohosh included induction of cutaneous pseudolymphoma and muscle damage respectively (381; 383).

1.5.1.2 Red clover extracts

The genus *Trifolium* (*Papilionoidae-Trifolieae*) includes over 250 species, the majority of which have not been phytochemically characterized. Only the species with agricultural significance such as *T. pratense*, *T. repens*, *T. resupinatum* and *T. incarnatum* have been studied for the occurrence of saponins, cyanogenic glycosides, and phenolics (384). Red clover (*Trifolium pratense*) dietary supplements and nutraceutials are currently used to treat menopausal symptoms because of their high content of the mildly estrogenic isoflavones, daidzein, genistein, formononetin, and biochanin A (385).

Estrogenic activities of red clover (*T. pratense*) standardized extracts such as Promensil have been investigated but the documented results show a lack of consensus about the estrogenic efficacy of these extracts. Van de Weijer and Barentsen (386) reported a correlation between the use of Promensil (80 mg/ day) and a significant (44%) decrease in incidence of hot flushes in post menopausal women suffering from more than 5 flushes per day, demonstrating the effectiveness of Promensil in the management of hot flushes. Rosenberg Zand *et al*. (387) also reported on the efficacy of Promensil in inducing steroid-hormone regulated proteins pS2 and the prostate-specific antigen (PSA), *in vitro*. They quantified pS2 and PSA in BT-474 human breast cancer cells using ELISA-type immunoassays in order to verify agonist and antagonist activity of Promensil among 19 other nutraceuticals and natural products. Only four of the products tested, two isoflavone preparations, Promensil and Estro-Logic, chamomile, and grapeseed extracts, exhibited appreciable estrogenic agonist activity, with the latter two also demonstrating weak progestational activity. Promensil exhibited the highest efficacy for the induction of pS2 of all the four products, with its ethanol stock solution (1:10) exhibiting an efficacy equivalent to that of the positive control (10^{-8} E_2). Promensil also exhibited a cytotoxic activity with its undiluted form that was similar to that observed with genistein.

In another study by Slater *et al.* (388), the estrogenicity of red clover extracts was demonstrated in the prostates of male Wistar rats by their propensity to induce the expression of ERβ as well as the adhesion protein E-cadherin, an estrogenically-induced marker of characteristics such as the maintenance of histological architecture, prostatic epithelial phenotype and a reduction of the potential for neoplastic transformation.

The results for the efficacy of red clover-derived isoflavone supplements is not always positive. Atkinson *et al*. (389), for example, observed no differences between decreases in mammographic breast densities in a group of women who had been treated with a red clover extract and the placebo group. Further, Atkinson *et al*. did not observe any effects by the red clover extract on lymphocyte tyrosine kinase activity or menopausal symptoms in their study. Tice *et al.* (390)

also reported similar findings from an investigation of the efficacy of Promensil and another red clover nutraceutical (Rimostil®). Tice *et al*. reported no decrease in the incidents of hot flushes in menopausal women experiencing an average of 35 hot flushes in a week, coming to a conclusion that although some evidence for the estrogenic effects of Promensil may exist *in vitro*, it does not translate to beneficial clinical effects against the symptoms of menopause in women (390).

These seemingly contradictory accounts of the efficacy of commercial phytoestrogen products bear testimony to the amount of research that still needs to go into the elucidation of the activities of phytoestrogens at molecular level. It should also serve as a word of caution against blanket suppositions about the efficacy of phytoestrogenic nutraceuticals.

1.6 Extraction of and testing for phytoestrogenicity

High quality demands on the phytoestrogenic nutraceuticals market as well as the increasing concern in both public and scientific communities about adverse effects that may arise from the use of phytoestrogenic nutraceuticals necessitates the optimization of high throughput *in vitro* assays as well as extraction methods that favour the preservation of the phytoestrogenically active components in plants, not only to identify active compounds, but also to analyze the short and long term effects of their possible endocrine disruptive properties (391). An understanding of the methods used for extraction of polyphenols and evaluation of estrogenicity will be important for discussing the findings of the current stud. The next section is dedicated to discussing methods used for extraction of polyphenols and evaluation of assays used to measure estrogenicity.

1.6.1 Factors affecting extraction efficacy of polyphenols

Investigation into the effects of agronomic practices and processing conditions on yields and the content of phytoestrogens in plant extracts have been the focus of many studies. Environmental factors such as harvesting season and irrigation, properties of the extraction solvent such as its polarity and acidification and the importance of water as part of or as the extraction solvent and

extraction variables such as equipment, ratio of extraction solvent to plant material, extraction temperature and time of extraction (392-397).

The fact that phytoestrogens are secondary metabolites produced by plants in response to environmental conditions (398) puts a significant degree of importance on monitoring the events that prevail during the growth of the plants and also on the timing of harvesting plants for the extraction of phytoestrogens. Tsai *et al.* (397) investigated the effects of harvesting season on the isoflavone yields of soy beans by assessing spring (February to June) and autumn (August to December) harvests of three soybean cultivars for their isoflavone content. Autumn harvests yielded a significantly ($P < 0.05$) higher isoflavone (daidzin, genistin, malonyldaidzin, and malonylgenistin) content in comparison to the spring harvest. These findings strongly suggest a link between prevalent weather conditions and the production of isoflavones by plants, particularly that autumn weather conditions may favour an increased production of phytoestrogens in soy beans.

An important aspect that ties in with the production of phytoestrogens by plants which depends on environmental conditions, is the content of phytoestrogens in the various parts of the same plant. As a control measure, it is imperative that the data for such an investigation be obtained from plants grown and harvested in the same season. Booth *et al.* (399) studied the expression of phytoestyrogens in various parts of the same plant and harvesting of red clover in north-eastern Illinois, particularly studying above-ground parts (leaves, stems, petioles, and flower heads). Booth *et al.* found that generally, autohydrolytic extracts of above-ground parts contained more isoflavones and exhibited more estrogenic activity in Ishikawa endometrial cells, compared with extracts of flower heads. Daidzein and genistein content peaked around June to July, while formononetin and biochanin A content peaked in early September. These observations by Booth *et al.* differ slightly with those of Tsai *et al.* (397) for soy bean in that the highest isoflavone content in red clover is expressed in the summer rather than the autumn. As the two studies did not use comparable seasonal divisions, nor investigate the same plant, direct comparison is difficult. However, the fact that Booth *et al.* reported biochanin A to peak around the autumn month of

September, as well as the fact that the two seasons follow each other in the year is significant, and it implies that environmental conditions that usually prevail over the two seasons may favour an increased production of phytoestrogenic polyphenols by plants.

Besides the effect of naturally occurring environmental events such as seasonal conditions, plants, particularly agricultural plants, are also subjected to man-controlled environmental events such as irrigation. The effects of such practices on the capacity of plants to produce phytoestrogens hence also warrant investigation. Bennet *et al.* (400) examined the effects of irrigation on the production of soybean isoflavones in a study which was part of a programme known as the "early soybean production system (ESPS)", developed in the mid-south of the United States. This programme entails early planting of short-season soybean varieties in an attempt to circumvent drought conditions that persist during the seed fill period. As a result of the hectareage of soybeans produced under the ESPS protocol being quite considerable, only a portion of the hectareage in is irrigated. HPLC analyses of methanol extracts of seeds harvested from different planting dates showed striking differences in isoflavone content. Bennet *et al.* found that the total isoflavone content was increased as much as 1.3-fold in early-planted soybeans. When comparing the irrigated and non-irrigated soybean crops. The results showed that irrigation enhanced the individual (daidzein and genistein) as well as the total isoflavone content of both early and late-planted soybeans as much as 2.5-fold (400). This information is quite relevant to our study as the honeybush plant, which is the source of phytoestrogens for our study, is harvested from non-irrigated plantations. While a certain measure of stressful weather conditions may be expected to form part of the summer and autumn seasons, which could lead to an increased production of phytoestrogens by plants, thereby giving credence to the findings of Tsai *et al*. (397) and Booth *et al*. (399), one would expect that irrigation of plants would ease the effects of environmental stress to the plants, such as would be expected in conditions of drought, which would be expected to lead to decreased production of phytoestrogens by plants, a reasoning that makes the findings of Bennet *et al.* (400) on the higher production of isoflavones by irrigated plants seem counterintuitive.

For any study, where the aim is to select the most phytoestrogenic extract of a plant, it is imperative that the choice of extraction method, of which variables such as extraction solvent and equipment form integral parts, be an informed one. While relatively little can be done in the short term, to control the production of phytoestrogens by plants in the field or plantation, use of optimal extraction techniques will maximize benefits from what nature yields.

A variable that affects yield and phytoestrogen content is the solvent used for extraction. Solvents commonly used include water and the organic solvents, acetone, actonitrile, ethyl acetate, diethyl formamide, ethanol, methanol, diethyl ether, *n*-hexane, methylene chloride, methyl formate, pentane (401) and their aqueous mixtures. From the results of several studies it appears that although the use of water as part of the solvent mixture may increase the yield of the plant extract due to the swelling effects of water on the plant matrix (402-403), the biological activity of the water extracts may not always be higher in comparison to the less polar solvent extracts. Tsai *et al.* (397), for example, reported that while methanol-water extracts of soybean isoflavones gave higher yields in comparison to acetic acid-acetonitrile extracts, a follow-up HPLC analysis could not detect the presence of malonyl conjugates of important phytoestrogens such as genistin, daidzin and glycetin in the methanol-water extracts. King *et al*. (395) also found higher yields from water extracts of the American ginseng root in comparison to methanol extracts. However, the water extracts could not induce cell proliferation of MCF-7 cells at low concentrations while the methanol extracts could, indicating lower estrogenicity in the water extracts. Turkmen *et al.* (404) on the other hand found that 50% aqueous solutions rather than absolute acetone or *N*,*N*-dimethyl formamide resulted in higher yields and antioxidant activities from black tea while less to no such activity was obtained with the extracts of the absolute solvents. It is worth mentioning though that Turkmen *et al.* did also test using pure water extracts which yielded no antioxidant activity.

Another consideration that closely relates to the use of water for extracting polyphenols is the effect of solvent polarity on the phytoestrogenic activity of the extract. Comparisons of the activities of extracts by solvents of different polarities have reported conflicting results in the

literature. For example, although Cheng *et al*. (392) found higher yields from lyophilized yam powders with the more polar methanol extracts, these extracts had little phytoestrogenic activity in comparison to the less polar ethyl acetate extracts, which had much lower yields. In support, Murphy *et al*. (396), while evaluating water mixtures of four organic solvents (53% organic solvent), namely, acetonitrile, acetone, ethanol and methanol, for their efficiency in extracting isoflavones from five different soy food matrices (soybean flour, texturized vegetable protein (TVP), tofu, tempeh and soy germ) also found that the less polar solvents produced extracts with higher activity. In addition, their findings clearly indicated that the four different solvents have different abilities to extract different isoflavone forms, and that the physical properties of the starting material affect the efficiency of the extraction. Specifically, they found that acetonitrile, the least polar solvent in their study, followed closely by acetone, was the superior solvent for the extraction of isoflavones from the various types of soy foods they used. Interestingly, the superiority of acetone over methanol and ethanol did not hold for all types of foods, indicating that the food matrix configuration (and perhaps the physical characteristics of plant parts) may have an impact on the extractability of isoflavones (396). Concerning their findings on the extraction capabilities of methanol on soy isoflavones, Murphy *et al.* came to another interesting conclusion. According to their ranking system, ethanol occupied the third position overall because, in soy flour, while ethanol was as efficient as acetonitrile and acetone in extracting the β-glucosides, it was less efficient in extracting the malonyl-β-glucosides compared to acetonitrile and acetone, meaning that it is possible for one solvent to discriminate between polyphenols of the same class that are conjugated to different moieties. This is significant as the glycosylation status of phytoestrogens play a role in determining their bioavailability, and an ability to select for a particular polyphenol-glycoside may be helpful in extracting particular fractions that will elicit particular biological effects (396).

The superiority of less polar solvents over more polar ones in extracting polyphenols does, however, not seem to apply for all cases. Case in point, is a study by Lin and Giusti (405), who,

upon testing for the effects of solvent polarity on extraction efficiency of soy isoflavones reported that the more polar 58% acetonitrile solvent had extracted higher amounts of isoflavones in comparison to the less polar 80% methanol and 83% acetonitrile solvents. Specifically, Lin and Giusti reported that for individual isoflavones, 58% acetonitrile (highest polarity) extracted either the highest amounts or no less than other solvents, while 83% acetonitrile (lowest polarity) extracted either the lowest amounts or no more than other solvents except, notably for the aglycone forms of isoflavones. The differences in findings between the studies of Cheng *et al*. (392), Murphy *et al*. (396) and Lin and Giusti's (405) are indeed interesting, because, although the individual groups came to different conclusions regarding which solvent polarity is optimal for extracting isoflavones, with the Cheng and Murphy groups opting for less polar and the Lin and Giusti group for more polar, a closer inspection of the results indicate that the two studies investigating soy, Murphy *et al*. and Lin and Giusti, actually agree that a water-acetonitrile mixture is best for extraction of total isoflavones from soy. The confusion arises from the fact that this mixture was designated to opposite sides of the polarity spectrum in the two studies. Therefore, although the studies do not concur in their conclusions, it is clear that a very polar solvent which gives a high polyphenol yield does not give a high phytoestrogen yield, and that lower polarity is a requirement for extraction of estrogenic compounds. This would appear to make sense as not all polyphenols are estrogenic, and the fact that estrogen, the natural ligand of the ER, is hydrophobic, suggests that the less polar extracts may be useful for estrogenicity.

A property of extraction solvents that was also investigated by Lin and Giusti (405) for its effects on the biological activities of extracts is that of acidification of the extraction medium. It significantly reduced the recovery of the malonylglucosides and total isoflavones from soybeans. This observation by Lin and Giusti about acidification of solvents being unfavourable for the extraction of polyphenols was shown not to be applicable to tannins. Chavan *et al*. (406), sought to extract tannins (polyphenols which are possibly estrogenic) from beach, green and grass peas using methanol or acetone at different concentrations with or without acidification. Their findings were

that on average, acidification of both acetone and methanol-water mixtures increased the concentration of tannins compared to un-acidified solvents. Overall, Chavan *et al*. found that, 70% acetone-water, containing 1% concentrated HCl, extracted a maximum amount of condensed tannins from all pea variants extracted.

With regards to the methods and equipment used for extraction of phytochemicals, several authors have presented data suggesting that in comparison to the simpler conventional method of stirring, the more modern use of microwaves and sonification, as well as extraction methods that are based on differences in solubilities of the samples such as the Soxhlet, pressurized fluid and the super critical fluid extraction (SFE) methods are more efficient for the extraction of isoflavonoids from plants (407-408). Judging by the sparse literature concerning the use of classical stirring methods, it would seem, perhaps as a result of the advantages just mentioned, that non-stirring methods are preferred at present. Although this may be the case, the use of the more modern non-stirring methods, although purported to be more efficient and time-saving, does carry the disadvantage of being more expensive and yielding complex extracts that are less specific with regards to targeting phytoestrogenic polyphenols. This complexity of extracts was one of the problems faced by He *et al*. (409) using micelle-mediated extraction of genistein from *Puerariae radix*, referred to as "Ge-Gen" in Chinese, a Chinese herb used to treat coronary heart disease, myocardial infarction, and hypertension (410-411). Also, while comparisons of the classical and super critical fluid extraction procedures have revealed that the composition of the phytoestrogens to be extracted (e.g. glycosylation) may be important for the efficiencies of these extraction methods (412), some authors have found no significant difference in the efficiency of extraction of total and individual isoflavones between the two kinds of methods (413).

Investigations of the effects of ratio of solvent volume to plant sample weight have resulted in conflicting observations. Yang and Zhang (414) found that by increasing the solvent volume relative to the weight of plant material more effective dissolution of constituents was obtained leading to an enhancement of the extraction yield. Zhang *et al.* (394), on the other hand, reported

that the ratio of 3:1 (mL ethanol: g soybean flour) resulted in the highest extraction of genistein and daidzein compared to conditions where the volume of ethanol was increased (5: 1 and 8:1). Rostagno *et al.* (413) also found that a decrease in the volume of solvent increases the activity of the extract. Rostagno *et al* compared a 5:1 to a 0.2:1 (mL ethanol or methanol: soy beverage) ratio, with the 0.2:1 ratio yielding an extract with a higher content of isoflavones. These findings are interesting mainly because they would seem counter-intuitive as one would expect more solvent to improve isoflavone yield.

Concerning the influence of time of extraction on the yield of phytoestrogens, although there is a wide variation of between 2h and 3 days used by various authors (407; 415-417), it appears that authors in general agree that in the case of extraction procedures that use the more conventional stirring methods, an increase in the duration of extraction favours an increase in the extraction of phytoestrogens. Zhang *et al*. (394), for instance, while investigating the extraction of isoflavones from soybeans using stirring methods, reported a correlation between an increase in time of extraction and increased extraction of genistein and daidzein, peaking at 7-8 h. In comparison, and as alluded to previously, the more modern, non-stirring methods are reported to be significantly more efficient and time-saving than the conventional stirring methods. In a study by He *et al*. (409), where ultrasonic-assisted extraction methods were employed for the extraction of daidzein from *P. radix*, optimum experimental conditions were established to be within 45 min. Yang and Zang (414) have also reported a decrease in the duration of extraction from 90 to 30min to favour increased extraction of rutin and quercetin from *Euonymus alatus*, a Chinese folk remedial plant with therapeutic actions mainly related to the heart and kidney.

It appears from literature that the effect of solvent temperature on improving the extraction of isoflavones depends largely on the method and equipment used. For the classical stirring method, literature suggests a correlation between an increase in solvent temperature and isoflavone yield. Zhang *et al*. (394) demonstrated this in a study using the stirring method and recorded a gradual increase in isoflavone yield from ethanol extraction of soybeans which peaked between 80 and

85ºC. With the use of rapid non-stirring methods, however, such as the ultrasonic bath, temperatures higher than 40°C appear to be unfavourable for the extraction of isoflavones. In this regard, Abad-García *et al.* (418) conducted an experiment where they evaluated the effects of several experimental variables including temperature (18, 25 and 40 °C) on the ultrasonic extraction of isoflavones from fruit using various aqueous mixtures of methanol. The temperature had no influence on the efficiency of the extraction process provided that the extraction time was less than 15 min (418). The exception, however, was the extraction of hesperetin-7-*O*-rutinoside and an unknown flavanone (XVI) of tangerine juice, for which higher recoveries were observed at 40°C. Furthermore, they observed that temperatures above 40°C produced a decrease in extraction yield due to possible degradation of polyphenolic compounds caused by hydrolysis, internal redox reactions or polymerisations (419). From these findings Abad-García *et al.* concluded that, 25 °C was the optimum temperature when extracting isoflavones using ultra-sonic methods.

The use of microwaves is another popular method for extraction. From another study by Rostagno *et al*. (413), it appears that an improvement of isoflavone yield can be reached at optimum temperatures slightly higher than 40°C. This study found that extraction temperature has a clear effect on isoflavone concentration with extractions performed at 50°C not affecting isoflavone concentration, whilst extractions performed at higher temperatures expose isoflavones to degradation. Specifically, extractions performed between 75 and 100 °C affects mainly malonyl isoflavones, temperatures between 100 and 125 °C also affect acetyl isoflavones, while higher temperatures sharply increase degradation of glucosides. From their overall results, Rostagno *et al.* inferred that 50 °C is a safe temperature to use for the development of a reliable isoflavone extract from soybeans (413) .

A subject that is related to the effect of solvent temperature on the extraction of polyphenols, which deserves a distinct mention because it was used in our study, is the effect of using boiled hot water as an extraction solvent. Much of the literature on this subject discusses either the more sophisticated high pressure water extraction methods such as pressurized-liquid

extraction and supercritical-fluid extraction (420-421) or the cooking of plant food material in water and its mostly negative effects on the phytoestrogen content of food (416; 422), which are different from extracting phytoestrogens from the plant material with freshly boiled water as we did in our study. Nevertheless, there are studies such as by Kammerer *et al.* (423), that reported on the significant improvement of polyphenol extraction from grape pomace through the use of hot (80-90ºC) water. Pandjaitan *et al.* (424) evaluated the efficiency of genistein extraction from soybean protein by comparing the effects of extraction with hot water (a method very similar to the one used in our study), acid (hydrochloric acid in de-ionized water, pH 4.5) and ethanol. The soy protein concentrates, extracted with the acidified water, gave the highest total genistin and genistein content compared to soy protein concentrates prepared with the hot-water. Hot-water extraction resulted in soy protein concentrates with a much higher genistin and genistein content (9.7 fold higher) than ethanol extracion. The results of these studies make a good case for the enhancement of phytoestrogen extraction by using hot water.

In summary, it appears that natural or man-induced environmental factors such as seasonal weather conditions and irrigation of crops, respectively, have an impact on the content and the activity of phytoestrogens produced by plants, with summer and autumn months as well as irrigation proving to enhance the production and the activity of phytoestrogens by plants. Also, while the literature suggests an increase in extract yield with water extraction, the resultant extracts are seldom phytoestrogenic. The polarity of the extraction solvent also seems crucial for the extraction of phytoestrogens, with solvents that are neither extremely polar nor extremely non-polar proving to be ideal for the extraction of phytoestrogens, perhaps as a consequence of the importance of a merger between hydrophilic (which the hydroxylated phytoestrogens tend to exhibits) and hydrophobic (which the endogenous E_2 exhibits) qualities that the extracts may need for estrogenic activity. Classical stirring methods, although reported to be more time consuming than the modern extraction methods such as the use of microwaves and ultra-sonication, are less expensive and have been shown by some authors to be just as efficient and more specific for the extraction of

phytoestrogens in comparison to the more modern methods. Although increased solvent temperatures and extraction time increase extract yield and activity, especially with regards to water as the extraction solvent, interestingly, and contrary to intuitive thought, a decrease in solvent: extract ratio has been shown to yield extracts with increased phytoestrogenic activity.

This overview suggests that although there is a lack of consensus on key issues concerning the optimization of extraction methods for the extraction of phytoestrogens, some guidelines are discernable. This is largely due to the fact that optimum extraction conditions are dependent on the compound(s) and on the matrix of the plant material to be isolated from (412). Because of the considerable diversity in these two areas in the plant kingdom, one can expect that the task of optimizing extraction methods in the laboratory would require a careful synchronization of the literature as well as a degree of trial and error, especially in a relatively new field of study such as is the case with the study of phytoestrogens.

1.6.2 Methods of testing for phytoestrogenicity

The conjecture made in the last paragraph leads to the discussion of the other half of the experimental techniques required to meet the aims of our study, which is the evaluation of the phytoestrogenicity of plant extracts. In order for a substance (or drug) to be considered as estrogenic it must behave like an estrogen to an appreciable degree. This appreciable degree means that the substance must be potent and efficacious enough to influence measurable estrogen-like effects on at least one of several possible measurable end points of the estrogen-signaling pathway. Besides the observation of clinical effects, the quantification of estrogen-like events elicited by substances can be studied at the molecular level using one or more sophisticated *in vitro* and *in vivo* laboratory assays. The diversity and the complexity of estrogen-signaling has led to the development of a great number of techniques designed to measure *in vitro* or *in vivo* estrogenic endpoints depending on the aims of the investigation. The quantification of the potency and

efficacy of estrogenic candidates must also be measured against the activities of a standard test compound(s), the choice of which, again, depends on the nature and aims of each investigation.

As the scope of the methods used in our study does not include *in vivo* models available to study estrogenic effects, the focus of the discussion will be on *in vitro* methods of interest to our study, particularly those methods useful in quantifying the estrogenic prowess of the test substances (E_2, genistein as standard test phytoestrogen, extracts of the honeybush plant and commercial phytoestrogenic extracts) in terms of potency and efficacy. The task of describing and justifying the methodology of our study will also requires an engagement with some aspects and terminology used in the field of pharmacology to describe the potency and efficacy of test substances.

1.6.2.1 In vitro methods for testing phytoestrogenicity

In vitro biochemical assays are useful for rapid screening of uncharacterized substances (as is the case with many phytoestrogens and xenoestrogens) for biochemical activities, but the convenience that they are renowned for must be balanced against accuracy and sensitivity. The availability of immortalized estrogen-responsive mammalian cells from various estrogen-responsive tissues (brain, breast, vaginal, ovarian, cervical and uterus), which are engineered for studying specific endpoints in the estrogen-signaling pathway (Fig. 10), makes it possible to investigate estrogenic effects induced by various substances *in vitro*. In our study we have opted to use breast and endometrial tissue because these tissues form part of the group of tissues regarded as “classical estrogen target tissues” and their engineering is such that they are useful in measuring some of the key endpoints in estrogen signaling.

Depending on the interests of the investigation, cell lines used to measure estrogenicity of substances or “estrogenic candidates” must, of course, express one or both of the two main ER subtypes, which may either be endogenously expressed in the cell line, or, in cases where the cells do not express the receptor, could be transiently transfected. Although the transfection of cells allows for the control of the ER subtype expressed in cells or tissue systems, the mechanical stress

that accompanies the forcing of foreign DNA into cells or tissues will most likely compromise the integrity of the very biochemical processes under investigation, a shortcoming that is eliminated with the use of cell that naturally express ER.

According to the classical pathway of estrogen action, several measurable end points in the estrogen signaling pathway, in their order of increasing complexity, include binding of ligand to receptor, the dimerization of the ER, the interaction of the ER-ligand complex with the ERE, the activation of estrogen responsive proteins through the transcription of estrogen responsive genes, and translation of mRNA to new proteins and cell proliferation as complex endpoints that incorporates both transactivation and transrepression of estrogen responsive genes (425) (Fig. 11).

In vitro methods for the measurement of the capacity of estrogenic candidates to bind to the ER, which may make use of either whole cells, cytosolic components or commercially available recombinant ER, allows for the measurement of the ER content (in whole cells or tissue systems) as well as the affinity of the estrogenic candidates for the ER. The assays used to measure the activation of ERE, other estrogen responsive genes and cell proliferation on the other hand provide information about the potency and the efficacy of estrogenic candidates, concepts which will be explained in greater detail when the various types of *in vitro* assays are discussed later in the text.

1.6.2.1.1 Receptor binding assays

The affinity of ligands for binding to the ER, which is the first step in the estrogen signalling pathway (Fig. 11 (1)), as well as the content of ER in a cell or tissue system, are usually quantified using competitive and saturation ER binding assays.

Competitive binding, which makes use of either radioactively-labelled or fluorescent ligands, measures the ability of various concentrations of the candidate estrogen or mixture to compete for binding to the ER, thereby providing the relative binding affinity of the candidate estrogen or mixture as compared to that of the labelled estrogen. Relative binding affinity may also be compared to an unlabelled known estrogen (the standard test compound).

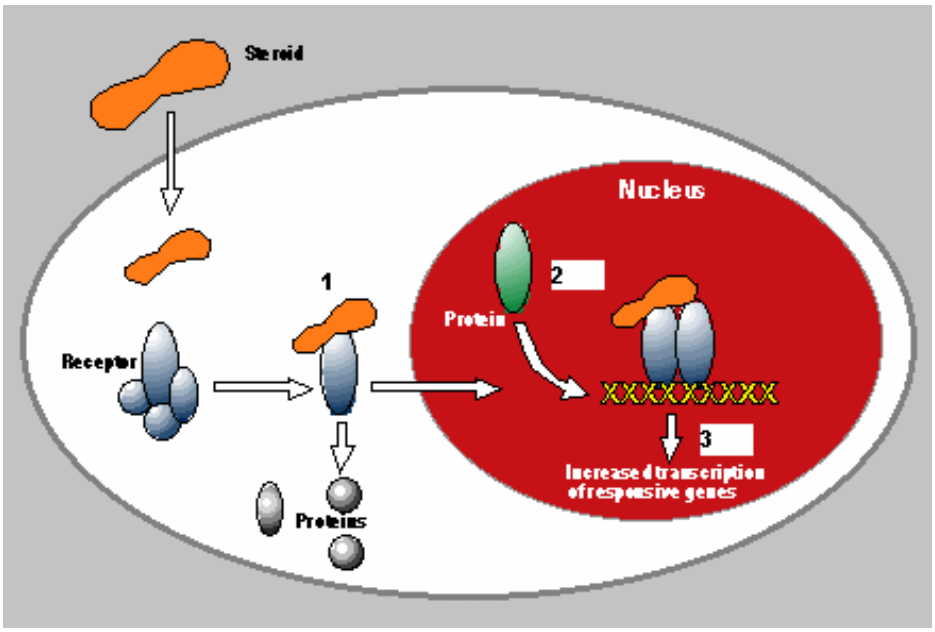


Figure 11. A model for steroid hormone signaling showing some of the endpoints that can be measured using *in vitro* assays. The endpoints indicated are: (1) binding of ligand to receptor leading to the formation or the ligand-receptor complex, (2) activation of estrogen responsive co-regulator proteins and (3) transcription of estrogen responsive genes and their translation into new proteins leading to cell proliferation (425).

Although both saturation and competitive binding assays may be used, competitive binding is usually preferred due to cost (426-427). In addition, use of crude extracts which contain a mixture of estrogenic compounds of either known or unknown origin requires the use of competitive rather than saturation binding techniques (426-427). The choice of whether to use optical spectroscopic assays such as fluorescent ligands (which may make use of the principles of fluorescence polarization, fluorescence resonance energy transfer and surface plasmon resonance) or to use radiometric methods needs to be an informed one as both methods have their pros and cons. Besides the advantage of eliminating the need for working in specialized laboratory environments with hazardous materials that produce radioactive waste, which occurs with the use of radiometric assays, fluorescent ligands are less expensive with a higher affinity for the ER as is the case with fluorescent E_2 in comparison to radioactively labeled E_2.

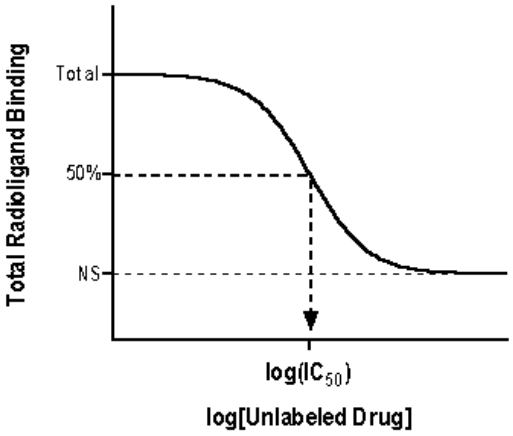


Figure 12. A competitive binding curve. The top of the curve is a plateau at a value equal to radioligand binding in the absence of the competing unlabeled drug. The bottom of the curve is a plateau equal to nonspecific binding. The concentration of unlabeled drug that produces radioligand binding half way between the upper and lower plateaus is called the IC_{50} (inhibitory concentration 50%) of the inhibitor (428).

Radiometric assays on the other hand are less cumbersome, highly reproducible and depending on the purpose of the investigation, can be quick (429).

Both the use of recombinant ER and transfection methods have the advantage of allowing for the testing of the affinity of ligands for particular ER isoforms,. However, the elimination of the intact cell environment in the former and the attempted coercion of the ER to function in a completely foreign environment in the latter are critical disadvantages. These disadvantages are eliminated with the use of whole cell binding assays, especially if the whole cells express both of the major ER subtypes, α and β, as is the case with MCF-7 cells (430).

Useful parameters that can be determined using *in vitro* saturation binding assays are the quantification of ER binding sites of any cell sample (B_{max}) as well as the concentration of ligand required to occupy 50% of the available binding sites (K_d), which, in the past, were expressed in a Scatchard plot (Fig. 13 (right)), but are nowadays generally plotted using nonlinear regression with sophisticated computer software such as the GraphPad Prism Software®.

Gutendorf and Westendorf (431), who conducted a study where they compared an array of *in vitro* assays for the assessment of the estrogenic potential of natural and synthetic estrogens,

evaluated the affinity of E_2 and genistein using a competitive binding assay with recombinant human ERα and β. They found the EC_{50} values of E_2 for ERα and β to be 3.5nM and 65nM, respectively, while genistein had EC_{50} values of 35 μM and 2 μM for ERα and β, respectively.

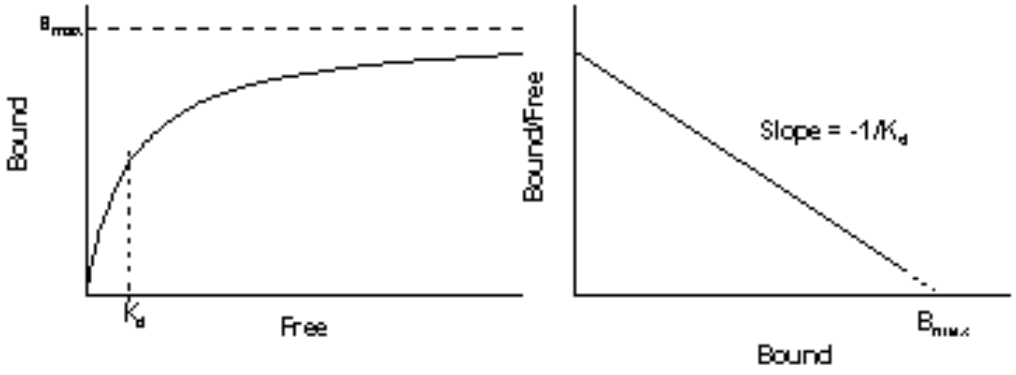


Figure 13. Schematic representation of the results of a saturation binding assay. The results of the saturation experiment can be plotted as the amount of radioactive ligand that is bound to the receptor (Bound) on the Y-axis and the free concentration of radioactive ligand (Free) on the X-axis (left) or as a Scatchard plot where the X-axis is specific binding and the Y-axis is specific binding divided by free radioligand concentration (right) (428).

Useful as the ER binding assays are for evaluating the affinity of candidate estrogens, they cannot be used to differentiate between agonists and antagonists, full or partial, and have been reported to be less sensitive than assays used for detection of the potency and efficacy of potential xenoestrogens such as reporter gene assays and cell proliferation (432).

1.6.2.1.2 Promoter- reporter gene assays

According to the classical pathway of ER signaling, the binding of the ligand to the receptor is followed by the binding of the ER-ligand dimer to an ERE in the promoter region of the estrogen responsive genes. To study these events, transfected (stably or transiently) yeast or mammalian cell lines which express the ERE linked to a reporter gene whose product is easy to visualize and measure, such as luciferase, β-galactosidase, chloramphenicol acetyltransferase (CAT) and the green fluorescent protein (GFP), are used to measure ligand-induced ER-mediated gene activation.

Addition of test substances induces dose-dependent transcription of the reporter gene that can be spectrophotometrically monitored and quantified, allowing for the determination of the transcriptional potency and efficacy of test substances. This dose-dependent expression is a principle which, as mentioned previously, also applies to assays that measure the mRNA or protein expression of endogenous ER-target genes as well as cell proliferation induction assays which will be discussed later.

The measurement of estrogenic or anti-estrogenic effects measured by dose response curves, whether using promoter-reporter assays, mRNA or protein expression of endogenous ER-target genes, or cell proliferation assays, seeks to quantify the pharmacological parameters of efficacy (maximal response or effect) and potency (EC_{50} or half maximal effective concentration, which is the concentration of a test substance which induces a response halfway between its baseline and maximum effect) (Fig. 14).

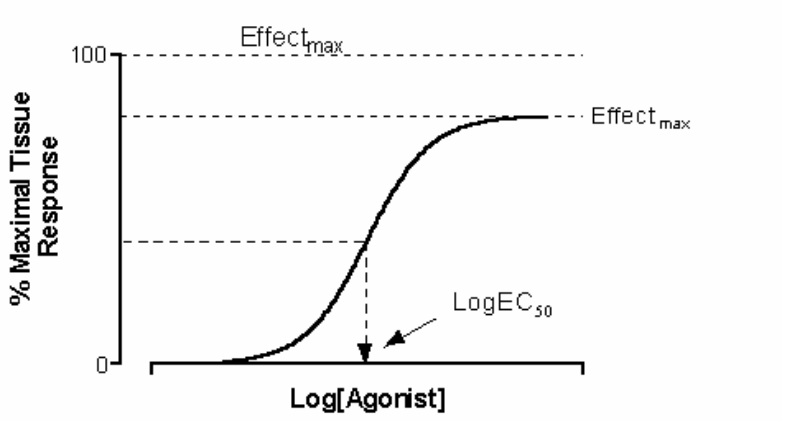


Figure 14. Schematic representation of an agonist curve (428).

Whether or not test substances are regarded as agonists or antagonists is determined by their behavior as compared to the behavior of a known or standard agonist or antagonist, which is usually a commercially available pure substance such as 17β-estradiol and ICI 182,780 which are ER agonists and antagonists respectively (433). Antagonists, as opposed to agonist, are characterized by their inability to induce a response in a cell or tissue system and they can be evaluated by their propensity to abrogate the activities of known estrogens. The comparison of the behavior of test

substances to the behavior of a standard test compound(s) also determines whether the test substances are partial or full agonists (Fig. 15).

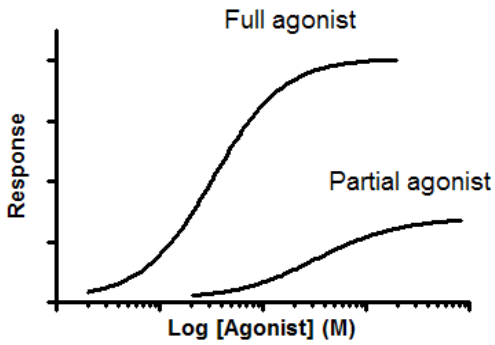


Figure 15. Schematic representation of a full and a partial agonist dose response curves (428).
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Comparisons between standard and test substances may be articulated as relative inductive indices (RII) or, relative binding affinities (RBA) in cases where the affinity of test substances for the receptor is evaluated against that of a standard estrogen in receptor binding assays (434).

In promoter-reporter assays, stably transfected cell lines that naturally express ER, such as breast cells, are a better option than cells that do not. The transfected ERE functions in its native environment in the former case, while there is a risk of the transfected ERE concentration not being optimal in the latter (432).

The usage of proper control methods is important as a guard against false positives and negatives in assays. For transiently transfected cells which do not naturally express the ER, co-transfection with vectors for proteins not normally expressed in the cells, such β-galactosidase and *Renilla* luciferase, are the most popular and widely used for normalization (435). Protein concentration determination methods such as the Bradford method (436) may be used for normalizing assays where stably transfected cells that naturally express ER are used.

On the subject of the use of the more reliable stably transfected cell lines, Wilson *et al.* (437) has described the development of the T47D-KBluc cell line from stably transfected T47D human breast cancer cells (which naturally express ERα and β). The advantage in using T47D-KBluc cells is that they are very sensitive, not only to potent estrogens like 17β-estradiol, ethynyl

estradiol, and diethylstibesterol, but also to weaker environmental estrogens like genistein, and pesticides such as 4-nonylphenol and HPTE (an estrogenic metabolite of methoxychlor). The EC_{50} value for estradiol was found by Wilson *et al.* (437) to be about 0.01 nM in this assay. The sensitivity of this assay to isoflavones was clearly demonstrated by genistein already beginning to show activity at the concentration of 1nM, and at 10 nM showing luciferase activity exceeding that of 0.1 nM E_2 (437). Gutendorf and Westendorf (431) also conducted luciferase-reporter-gene assay studies in ERα-expressing MVLN-cells (MCF-7-p-Vit-tk-Luc-Neo), derived from MCF-7 cells containing an estrogen regulated luciferase gene driven by an ERE in front of the vitellogenin-tyrosin-kinase-promoter. They found the EC_{50} values for E_2 and genistein to be 5 pM and 38 nM, respectively.

1.6.2.1.3 Assays measuring the mRNA or protein expression of endogenous ER-target genes

Because the ER signaling pathway is a complex process which includes several events including ER ligand binding, ER dimerization, nuclear translocation of the ER, binding of ligand-bound ER to response elements, recruitment of co-factors and transcription of genes, an assay that could be used to measure events that happen at or past these points would be useful. Events that take place at or after the point of transactivation are complex and hence, a number of points in the signal transduction pathway can be determined as end-points. Cell lines that endogenously express the ER, or transfected cells in cases where utilized cells do not naturally express the ER, can be used to measure the expression or up-regulation of end-point markers which may be mRNA expression or the up-regulation of estrogen-regulated proteins which include pS2, alkaline phosphatase, cathepsin D and the progesterone receptor, all of which are expressed under estrogen transcriptional control in a subclass of estrogen receptor-containing human breast cancer cells (438-441). The expression of these estrogen regulated proteins may be evaluated using Western blots, or, since enzymes may lose their activity over time while the total protein present may remain constant, enzyme activity assays, for which kits are commercially available. The expression of mRNA on the

other hand can be quantified using Q-PCR and DNA microarray methods (442-443). The quantification of relevant endpoint indicator(s) makes these assays valuable tools for the determination of specific estrogenic or anti-estrogenic tendencies of candidate estrogens. Markiewicz *et al*. (118) conducted a study using a variant (Variant 1) of the human Ishikawa cell line, which has been developed from endometrial carcinoma. The advantage of using this cell line is that, while it is unresponsive to E_2 with regards to proliferation, it is sensitive to the stimulatory effects of estrogens on the alkaline phosphatase activity. Markiewicz *et al*. found the EC_{50} values of E_2 and genistein to be 0.0673 ± 0.03 nM and 79.8 ± 11 nM, respectively. De Naeyer *et al.* (444) found the EC_{50} value of E_2 to be 0.6 ± 11 nM.when using the Ishikawa (Variant 1) cell line while investigating the estrogenic activity of a polyphenolic extract of leaves of *Epimedium brevicornum*, traditional Chinese medicine for the treatment of hormonal disturbances.

1.6.2.1.4 Cell proliferation assays

Estrogens are characterized by promoting the proliferation of cells, which itself is a reflection of complex transcriptional outcomes (445), while anti-estrogens in contrast are characterized by halting proliferation, depending on the cell line. This distinction between the two possible types of estrogen candidates is the basis of cell proliferation assays which assess the growth promoting effects of test substances. MCF-7 breast cancer cells, particularly the BUS strain, characterized by Villalobos *et al*. (446) as being the most sensitive to E_2 stimulation amongst four other MCF-7 strains (BUS, ATCC, BB, and BB104) tested, are widely used for the purpose of screening for the proliferative effects of natural and environmental estrogens, an assay referred to as the E-screen (446). Other cell lines used for measurement of cell proliferation include T47-D breast and BG-1 ovarian cancer cell lines (447-448). Also, as a control measure for cell proliferation assays, ER-negative carcinoma cell lines such as SKBR3 and MDA-MB breast cancer cells (231 or 435 variants are available with the MDA-MB line) (449-450) may be utilized. Quantification of cell proliferation assay results can be achieved with several methods which include the use of

radioactively labeled nucleic acid components (such as ^{3}H-thymidine). Although the method of choice for cell proliferation assays in the past, it has now been widely discounted with the availability of less hazardous, quicker, more accurate and reproducible methods such as the use of colometric assays such as the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay (451) which is based on the reduction of MTT to a blue formazan, a reliable reaction for assessing cell proliferation of only viable cells since it takes place only within live mitochondria. Other simpler but disadvantageous methods for their counting of both live and dead cells include the counting of cells using flow cytometry (hemocytometers or coulter counters) (452), determination of protein content using methods such as the Bradford (436), and counting of cells which have incorporated metabolizable dyes such as amido black (453).

Several authors have recorded the EC_{50} values of E_2 and genistein in various cell proliferation investigations. In a study by van Meeuwen *et al.* (438), which investigated the mixture effects of various estrogenic compounds on proliferation of MCF-7 BUS cells, the average EC_{50} values for E_2 and genistein were found to be approximately 5 pM and 74 nM, respectively. Gutendorf and Westendorf (431) also found very similar values (5 pM and 40 nM for E_2 and genistein, respectively), in the E-screen.

In summary, although these *in vitro* assays just discussed are useful in determining pharmacological effects of potential estrogens, they also have shortcomings which may stem from the lack of consideration for metabolism of compounds such as the necessary deconjugation of sugars from phytoestrogens by intestinal microflora which aides their absorption and their detoxification by glucoronidation in the liver. These shortcomings manifest in a lack of correlation between relative estrogenic potencies and efficacies of phytoestrogens as determined by *in vitro* assays (which are often reported to be much weaker in comparison to the endogenous E_2) and *in vivo* biochemical activities (which are often quite considerable and comparable to the effects of the natural ligand E_2) (432).

There is also often a lack of correlation between results obtained using different *in vitro*

assays. These difficulties were demonstrated clearly in the study by Booth *et al.* (399), where they found that extracts of flower-heads and total above-ground parts exhibited differential estrogenic activity in an Ishikawa cell-based alkaline phosphatase induction assay, whereas non-differential activity was observed for most extracts tested in an MCF-7 cell proliferation assay when tested at the same final concentrations. Booth *et al.* observed that Ishikawa assay results could be mapped onto the extracts' content of individual isoflavones, while MCF-7 results did not show such a pattern. These results suggest significant differences in signaling of isoflavones between MCF-7 and Ishikawa cells, and as such, caution is advised in the choice of bioassay used for the biological standardization of botanical dietary supplements (399).

Andersen *et al.* (454) and Feng *et al*. (455), who have compared the efficiencies of the various *in vitro* assays used for screening substances for estrogenic activity, recommended the use of the ligand binding assay (using a recombinant human ER) and the E-Screen, citing simplicity, high sensitivity and a usefulness in providing information on both receptor binding and a cellular response by these assays as their main reasons for their recommendations (456). Despite such observations and recommendations by various authors for the use of *in vitro* assays for assessing estrogenicity of substances, there are still considerable variability of results obtained using the various or same *in vitro* assays by various investigators (457). In the face of these difficulties, it is therefore advised that a combination of several *in vitro* test systems be used in order to validly predict the effects of test substances and to minimize the chances of generating false positives and negatives *in vitro.*

1.6.2.2 In vivo test systems for phytoestrogenic activity

Various *in vivo* assays (animal models) are useful in characterizing the estrogenic activities of phytoestrogens and their mechanism of action. In these studies, test phytoestrogens or mixtures (such as plant extracts) are orally or subcutaneously administered to immature, hypophysectomized or ovariectomized live animals such as rats, mice or rabbits, after which biological changes in

estrogen sensitive tissues such as the uterus (which is the most extensively used assay for *in vivo* testing of candidate estrogens), breast and brain are monitored and interpreted. Although indispensable for the evaluation of test phytoestrogens and a valuable tool for the confirmation of *in vitro* test results, *in vivo* assays too, have their shortcomings. The assessment of estrogenic activities of SERMs such as Raloxifen and Tamoxifen, for example, may not be possible with the use of uterotrophic assays since these SERMs have no effect on the uterus (458). Also, although end point *in vitro* assays fail to address the full range of putative phyto- and xenoestrogen effects in living systems, they carry the advantage of being less time consuming and much easier to study when compared to the much more technically involved, and expensive *in vivo* test methods. Further, because estrogenic candidates can interact directly or indirectly with the ER according to the classical and alternative pathways of E_2 mechanism of action, which are differences that are easier to determine and measure with *in vitro* methods, the use of *in vivo* methods makes it very difficult to detect, study or account for such differences. In addition, estrogenic candidates can elicit very distinct and variable biological effects depending on both the target tissue wherein they act and their interaction with other potentially estrogenic substances in the system, which are very important results-determining factors that can be very difficult if not impossible to control *in vivo* test methods. Also, subcellular genetic events as elicited by estrogen candidates may not be visible at organ level within the time limits of the experimental process, but may be noticeable at a much later stage which can make the interpretation of results based on observable organ alterations a confounding process. Further, as observed with rodent uteri, interspecies variation in organ sensitivity to estrogens occurs in mammals (459), which may present problems with results comparability, especially in cases where weak estrogens (as is the case with most phytoestrogens) are tested. These short comings of *in vivo* test systems, which are attributable to the sheer complexity of the endocrine system are however balanced against the fact that many of the mechanisms of the endocrine system are conserved among species (460), a reliable principle which has allowed for viable development and evaluation in the past. In conclusion, it appears that no one

assay type is adequate for the detection of estrogenic activity and hence the use of a suitable panel of different *in vitro* and *in vivo* test systems would be a good way of maximizing the chances of adequately predicting the estrogenic potential of test phytoestrogens.

1.7 The honeybush plant (genus *Cyclopia*)

The honeybush plant (genus *Cyclopia*), whose estrogenic activitiy is the subject of our investigation, is endemic to the Western and Eastern Cape Provinces of South Africa (461). The plant is a shrub of the *Fabaceae* family (*Leguminosae*) that grows in the fynbos (from the Dutch, meaning fine leaved plants) biome (Fig. 16), a narrow region along the coast, which has been studied for longer than any other part of sub-Saharan Africa and is renowned for being among the world's most diverse with an estimated 90 taxa of plants (462-464). *Cyclopia* is easily recognizable mainly by its trifoliate leaves, and bright yellow flowers (465).

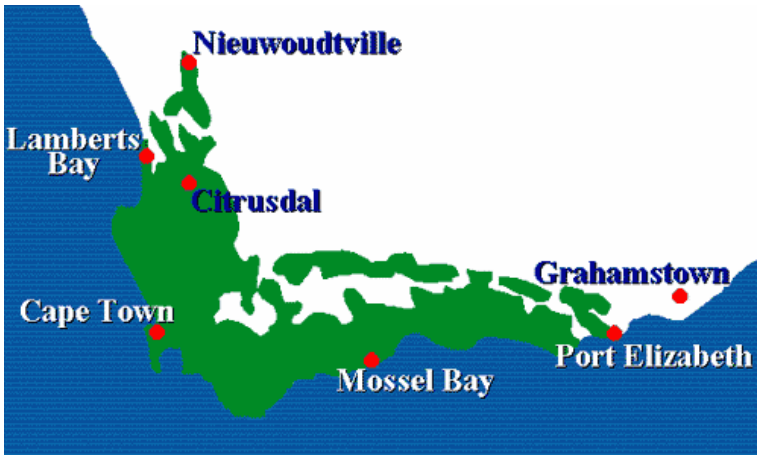

Figure 16. The Fynbos biome.

The honeybush plant is traditionally used for the manufacture of popular, sweetly-scented tea beverages with putative health benefits, and the tea itself is traditionally consumed with sugar and milk after steeping in boiled water (466-467).

Even though a minimum of 24 species of honeybush are suitable for manufacturing tea, only

four species of *Cyclopia* (*C. subternata, C. genistoides, C. sessiliflora* and *C. intemedia*) are used for manufacturing the commercial beverages (462; 467-468). Although most of the honeybush is still collected from wild populations, cultivation has become necessary with the rapid growth of the industry and the demand for more uniform products especially in the wake of evidence of the beneficial health effects of some of the plant species (466- 469)

The potential health benefits of polyphenol-rich beverages such as tea (from *Camellia sinensis*) have been known since it was discovered in China more than 5000 years ago and scientific evidence for such, which is attributed mainly to the polyphenol content of tea, is well documented in the literature (470-473). This is important considering that today tea comes second only to water as the most commonly drunk beverage on earth (470). Reported tea-associated health benefits include cardiovascular protection (474), inhibition of tumorigenesis (475), improved brain mineral content (476) and weight loss (477).

1.7.1 Potential health benefits of Cyclopia

The San population of South Africa was attuned to the health benefits of the honeybush plant and they passed their knowledge on to European settlers who then used it to treat common ailments, including sleeplessness and indigestion (465). Two attributes of the honeybush tea that may account for its putative beneficial health effects are its low tannin content and that it is naturally caffeine-free (478), meaning that the acute and chronic effects associated with caffeine dependency such as hyperreflexia, insomnia, respiratory alkalosis and increased risk of the development of peptic ulcers, erosive esophagitis, and gastroesophageal reflux disease may not be a factor with the usage of *Cyclopia*.

The presence of certain polyphenols in *Cyclopia* is associated with its beneficial clinical effects (479). Although much more still needs to be done with regards to the study and the validation of health claims associated with *Cyclopia*, significant progress has been made in

characterizing the polyphenol content of *Cyclopia* as (465; 480) well as the health benefits, mainly antioxidant and antimutagenic benefits, that can be expected from the usage of *Cyclopia* (469; 481).

Since our study investigates the phytoestrogenicity of *Cyclopia*, the discussion will focus on, some aspects of the putatively phytoestrogenic polyphenols found in *Cyclopia*, with a special reference to polyphenols identified by Verhoog *et al*. (13-14), who evaluated the estrogenic effects of some of the polyphenols found in the commercial variants of *Cyclopia* (14).

Sparked by the identification of known phytoestrogenic polyphenols (luteolin, naringenin, formononetin and eriodictyol) in *C. intermedia* and *C. subternata* (478; 480), as well as anecdotal evidence for the phytoestrogenic effects of honeybush (14), a study into the phytoestrogenic potential of the four main species of *Cyclopia* used for the manufacture of tea (*C. genistoides*, *C. subternata*, *C. sessiliflora* and C. *intermedia*), which was motivated by the possibility of improving the functional food status of honeybush tea, was undertaken by Verhoog *et al*. (13).

In their initial study (13), Verhoog *et al*. reported appreciable phytoestrogenic activity with two (*C. genistoides* and *C. subternata*) of the four *Cyclopia* species used to manufacture tea and further reported the presence of mangiferin, hesperidin, eriocitrin, narirutin and significant quantities of unknown polyphenols in dried methanol extract (DME) of these species. *Cyclopia genistoides* DME displayed the highest phytoestrogenic activity (74 and 70 % ability to displace 1nM ^{3}H-E_2 from hERα and β, respectively), while *C. subternata* DME displayed less phytoestrogenic activity in comparison (43 and 69 % abilities to displace 1nM ^{3}H-E_2 from hERα and β, respectively). Further, although not detected in *C. genistoides* and *C. subternata* in their study (13), hesperetin, luteolin, formononetin, naringenin, and eriodictyol, together with the detected mangiferin, hesperidin, eriocitrin and narirutin, were tested by Verhoog *et al*. for their potential to bind to ERα and β because other authors (478 ;480) had found the undetected compounds in *Cyclopia*. Formononetin, naringenin and luteolin were able to significantly bind to both ERα and β, while eriodictyol, eriocitrin and narirutin displaced ^{3}H-E_2 only from ERβ (13). Mangiferin, hesperidin and hesperetin did not bind to either of the ER subtypes (13).

In a follow-up study by Verhoog *et al.* (14) to evaluate the polyphenols and the phytoestrogenicity of *C. genistoides* DME, the most active extract in their first study (13), new information regarding the polyphenols came to light.. Firstly, in addition to eriocitrin and narirutin, which were the only known phytoestrogens detected in the first study, luteolin was the third phytoestrogen detected in the methanol extracts of *C. genistoides*, pointing to the evidence of varied intra-species phytoestrogen content of *Cyclopia*. More interestingly, it was learned in this follow-up study, through confirmatory liquid chromatography-mass spectrometry (LC-MS) methods that the peaks eluting at retention times similar to those of eriocitrin and narirutin were actually of unknown compounds as their mass was different from that of the pure standards. Their ultraviolet-visible spectrophotometry (UV-VIS) spectra and retention times suggested, rather, that these two compounds are flavanone glycosides with λ_{max} between 280 and 290 nm. Since these results bring under question the validity of the presence of eriocitroin and narirutin in the first study by Verhoog *et al.* (13), a summary of of the findings of both studies by Verhoog *et al*. would point to luteolin as the only deteactable source of phytoestrogenicity in *C. genistoides*. Such a hypothesis would, however, still be far from being credible, because the amount of luteolin present in *C. genistoides* (0.096-0.106 g/100g extract) was too low to explain the estrogenic proliferative effects elicited by *C. genistoides* in MCF-7 BUS cells. Comparison of the findings of the Verhoog *et al.* studies in relation to our own shall be discussed in Chapter 3.

Antioxidant and anti-inflammatory activities are also among health benefits attributed to various species of *Cyclopia*, and although our study focuses on the phytoestrogenic potential of *Cyclopia*, other health benefits of *Cyclopia* and its phenolic composition will also be discussed as further support of honeybush as potential nutraceutical product.

According to De Nysschen *et al.* (467) isosakuranetin, mangiferin and hesperetin are the major polyphenols found in the leaves of *Cyclopia*. In-depth studies of the phenolic composition of *C. intermedia* and *C. subternata* however did not show the presence of isosakuranetin (469, 483, 485). Further, hesperidin, and not hesperetin was found to be a major compound (482). Kamara *et*

al. (465), in addition to various flavonols, flavones, flavonones and isoflavones, reported the presence of two important antioxidants; 2-[4-[O-alpha-apiofuranosyl-(1"-->6')-β-d-glucopyranosyloxy]phenyl]ethanol and 4-[O-alpha-apiofuranosyl-(1"-->2')-β-d-glucopyranosyloxy]benzaldehyde, in the leaves and stems of *C. intermedia*.

McKay and Blumberg (474) reviewed the bioactivity of honeybush tea (*Cyclopia intermedia*) and its major polyphenols. Of particular interest is the antioxidative benefits for the liver, inferred in a study by Marnewick *et al.* (481). In this study, it was found that honeybush tea (*Cyclopia intermedia*) significantly ($P < 0.05$) enhanced the activity of the drug metabolizing enzymes; glutathione S-transferase alpha and microsomal UDP-glucuronosyl transferase in the liver of male Fischer rats. This observed modulation of phase II drug metabolizing enzymes and oxidative status in the liver may be important events in the protection against adverse effects related to oxidative damage.

An *in vitro* investigation into the antimutagenic properties of honeybush (*Cyclopia intermedia*) tea (481), antimutagenic activity against 2-acetylaminofluorene (2-AAF) and aflatoxin B_1-induced mutagenesis was demonstrated., Depending on the mutagen used, the unfermented rather than the fermented honeybush exhibited the highest mutagenic-protective effects against direct acting mutagens. A follow-up study by van der Merwe et al. (484), testing a number of harvestings for each species, showed antimutagenicity for *C. intermedia*, *C. subternata*, *C. sessiliflora* and *C. genistoides*.

Although no significant evidence to support their phytoestrogenic activity has been presented yet, mangiferin, hesperidin and hesperetin, found abundant in various species of *Cyclopia*, have other putative health benefits worth mentioning. Amazzal *et al.* (483) presented *in vitro* evidence of the potentially protective effects of mangiferin against Parkinson's disease using a mouse neuro-blastoma cell line. Evidence of immuno-protective effects of mangiferin in maintaining the survival of T-cell from the peripheral blood of healthy individuals has been found (492). In a study by Leiro *et al.* (484), mangiferin was found to enhance tumour growth factor-beta

(TGF-beta) mRNA expression, suggesting that this polyphenol might be of value in the prevention of cancer, autoimmune disorders, atherosclerosis and coronary heart disease. Mangiferin was also shown by Nair *et al.* (485) to significantly reduce cholesterol, triglycerol and free fatty acids levels in the serum and heart of cardiotoxic myocardial infracted rats, implying possible beneficial effects of mangiferin against cardiovascular diseases.

Hesperetin, the aglycone of hesperidin, has been identified by He *et al*. (486) to positively affect the secretory, motile, and mitogenic responses of inflammatory and immune cells. Interestingly, although generally deemed to have no phytoestrogenic activity, hesperetin was demonstrated *in vitro* and *in vivo* to inhibit the proliferation rate of the highly metastatic murine B16-F10 melanoma cells in a study by Lentini *et al*. (487). These findings by Lentini *et al*. (487) provide evidences for the potential anticancer properties of dietary hesperitin as chemopreventive agent against malignant melanoma.

The literature presents evidence that attests to the estrogenicity of putative phytoestrogens found in *Cyclopia*, viz. naringenin and its glycoside narirutin, formononetin, luteolin, eriodictyol and eriocitrin. The estrogenic and anti-estrogenic potential of naringenin, through its binding to both ERα and β (488-489) and suppression of estrogen induced MCF-7 cell proliferation (490), respectively, have been reported in literature. Naringenin and isosakuranetin showed a protective effect against tumorogenesis in male Wistar rats injected with a homogenate of the Yoshida's sarcoma tissue (310). A rather interesting observation concerning a relationship between disease state and the bioavailability of phytoestrogens which emerged in this study is that the total concentrations of naringenin metabolites reached 17.3 ± 2.7 μM in plasma 6 hours after the beginning of the meal in healthy rats and only 10.6 ±1.3 μM in tumour-bearing rats. The lower concentration of phytoestrogens in the tumour-bearing rats led Silberberg *et al*. to surmise that disease, and more particularly cancer, may affect the bioavailability of flavonoids (310). In another study by Schaefer *et al*. (491), a prenylated form of naringenin, 8-prenyl naringenin, first isolated from the heart wood of an indigenous tree in Thailand (*Anaxagorea luzonensis*), and later identified

as the estrogenic component in hops (*Humulus lupulus*) and beer (492), was found to show high affinity and strong selectivity for ERα. This is contrary to the norm as phytoestrogens are generally observed to have a stronger affinity for binding to ERβ (209). As a matter of significant interest, from their findings, using recombinant human ERα and ERβ *in vitro*, Schaefer *et al.* put forward that 8-prenyl naringenin is the strongest plant-derived ERα agonist identified so far, being about 10 times more potent than coumestrol and approximately 100 times more potent than genistein, an observation which also points to the possible importance of prenylation for the enhancement of phytoestrogenic activity of phytoestrogens. Surprisingly and in clear contrast to genistein, 8-prenyl naringenin was a much weaker agonist of ERβ than of ERα (491). Harai *et al.* (493), while investigating the relationship between obese adipose tissue and its enhanced infiltration by macrophages, observed that naringenin chalcone (the main flavonoid in tomatoes) inhibited the production of pro-inflammatory cytokines (TNF-α, MCP-1, and nitric oxide) by lipopolysaccharide-stimulated macrophages in a dose-dependent manner, and conclude that naringenin chalcone may be useful for ameliorating the inflammatory changes in obese adipose tissue. It has been proposed that the lipophilic nature of naringen may result in high concentrations of naringenin accumulating in the adipose tissue (494).

Extracts from red clover (*Trifolium pratense*), soybean (*Glycine max.*) and black cohosh (*Cimicifuga racemosa*) are frequently used as alternative compounds for HRT to treat menopausal disorders (305). Beck *et al.* (305), while investigating the estrogenic activities of fifteen commercially available products made either from red clover, soybean or black cohosh, observed formononetin-rich soy and red clover commercial products to exhibit a clear estrogenic activity through both ERα and ERβ in *in vitro* yeast-based transactivation assays, but black cohosh extracts showed no estrogenic activity. Also, in a review of the viability of formononetin-rich red clover extracts as alternatives for HRT (495), it is recorded that red clover extracts possess SERM-activity and interact with transcription factors such as NF-κB. In the same review, based on the daily intake of phytoestrogens in a traditional Japanese diet, it is recommended that formononetin, a metabolite

of daidzein, be part of a 40–50 mg daily intake of isoflavones as a measure to counteract the effects of diseases associated with estrogen perturbations. Other studies have also reported formononetin to bind, albeit relatively weakly, to the ER (489; 496). Formonetin is also found in high amounts in Xiao Yao Wan, a well-known ancient Chinese herbal formula used in ancient times for regulating menstruation. The tonic is still widely used today, not only for irregular menstruation, but also for distension pain in the chest and hypochondria (497).

The estrogenic effects of luteolin were confirmed in a study by Ise *et al.* (498), where expression profiling of estrogen responsive genes in response to phytoestrogens was studied. The expression of a total of 172 estrogen responsive genes was monitored with a customized DNA microarray and the comparison of their expression (up and down regulation of individual genes) in response to luteolin, among other phytoestrogens, was found to be comparable to that induced by E_2. Garai and Adlercreutz (499), who explored the possible relations between the steroid- and the flavonoid-signalling in animal and plant cells, reported signal transduction by luteolin on the estrogen-inducible type II estrogen binding sites of rat uteri. These sites are known to bind catecholic flavonoids with considerable affinity. Positive effects of luteolin on the function of osteoblastic MC3T3-E1 cells and the production of local factors in osteoblasts have been reported by Choi (500) implying that inflammatory mediators such as the cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in osteoblasts can be regulated by luteolin by stimulating osteoblastic function. Interestingly, luteolin has been implicated as being useful in treating oral and dental diseases in another study (501) by inhibiting the actions of *Porphyromonas gingivalis*, a Gram-negative bacterium obtained from the periodontal pocket of patients suffering from aggressive and chronic periodontitis, an oral disease marked by inflammation of the gingival and destruction of periodontal tissues which is accompanied by the loss of the alveolar bone and an eventual exfoliation of the teeth.

The estrogenic activity of eriodictyol, through stimulating MCF-7 cell proliferation and inducing transcription of an ERE containing promoter via the ER, has been demonstrated in a yeast-

based assays (502-503). Seo *et al.* (504) identified eridictyol as one of three major polyphenols in an extract that displayed cytotoxic activity against eleven human cancer cell lines among which were breast, colon and prostate cancer cell lines. The extract is of *Monotes engleri* leaves, a tree species which enjoys wide distribution in Africa and the Madagascan tropics. Van Zanden *et al.* (505) observed eriodictyol to exhibit a moderate glutathione *S*-transferase P1-1 inhibitory potential in MCF7 breast cancer cells. Glutathione *S*-transferases are a superfamily of xenobiotic metabolizing enzymes that catalyze the conjugation of various electrophilic compounds with glutathione and are associated with multi-drug resistance towards chemotherapeutic agents, a major obstacle in human cancer chemotherapy (505). Anti-inflammatory activity of eriodictyol has been demonstrated in a study by Clavin *et al.* (506), where eriodictyol was identified as an ingredient in ethanol extracts of *Eupatorium arnottianum*, a plant species distributed in Asia, Europe, and in America, which is widely exploited in phytotherapy for its choleretic and hepatoprotective effects. The finding of topical anti-inflammatory activity exerted by *Eupatorium arnottianum* extract and the identification of significant amounts of eriodictyol among the active ingredients in this plant could support a positive role by eriodictyol in the treatment of inflammatory affections.

While conceding the need for more research towards ascertaining the molecular mechanisms and the potential health benefits of *Cyclopia*, especially in the context of their phytoestrogenic activity, the findings detailed in this thesis as a collective suggest a significant potential for *Cyclopia* as an agent of medicinal use.

1.8 Conclusion and aims of thesis

Estradiol is an extremely important steroid hormone that affects and regulates, not only the functioning of tissues that are related to sexual development and function, but because its receptors are located in other important tissues such as brain, heart and bone, it also affects the functioning of important systems such as the nervous, cardiovascular and immune systems (80; 215; 507). Estrogen imbalances therefore result in an array of physiological and psychological discomforts that

warrant medical attention. Phytoestrogens are phytochemicals which are capable of eliciting estrogen-like effects when ingested by mammals, meaning that they can possibly be used for the alleviation of symptoms that arise from estrogen perturbations, such as is the case with the onset of menopause in women (508). The possibility of phytochemicals being useful in hormone-perturbation therapy has resulted in the creation of a lucrative market for phytoestrogenic nutraceuticals, which is primarily targeted at peri- and post-menopausal women who, in recent times, tend to shun the traditional HRT methods for what they perceive as safer, more "natural" therapeutic methods for the treatment of vasomotor symptoms (273). This perception of the safety of "natural" over conventional medicines by most communities is, however, not the only reason behind the growth of the phytoestrogen nutraceutical market. It is hypothesized that the recent findings by studies such as the KEEPS, WHI and the Million Women studies, which, apart from reporting no significant cardio-protective effects with the use of traditional HRT, have highlighted a link between HRT and an increased risk of the onset of hormone-related cancers and thromboembolic events, are also instrumental in the growth of the phytoestrogenic nutraceutical market. A concern, which has its roots in the less-than-adequate levels of understanding the molecular mechanisms by which phytoestrogens operate, does, however, exist around the increased usage of market phytoestrogens by the general public. This concern, however, seems to pale, at least from the public view-point, not only in the light of possible detrimental health effects that accompany the use of HRT, but also in the light of a considerable volume of *in vitro*, *in vivo* and epidemiological evidence of favourable health effects of phytoestrogens (215; 509-510). The aim of our study, however, was not concerned with settling the controversy around the safety and the efficacy of the use of phytoestrogens, but, our focus, based on the findings of Verhoog *et al.* (13-14), was rather on the utilization of *in vitro* methods to evaluate the enhancement of the estrogenicity of extracts of *Cyclopia* (*C. genistoides* and *C. subternata*), prepared using a range of extraction solvents, and the benchmarking of the activity of the most potent extract(s) against commercial phytoestrogens. The benchmarking was done because the rationale behind the study is

the evaluation of the validity of creating a high quality, uniquely South African nutraceutical with enhanced phytoestrogenic potency and efficacy.

Our methodology, for which the finer details are laid out in the following chapter, made use of a three-phase approach. Firstly (Phase 1), the evaluation of the estrogenicity of methanol extracts of four harvestings of *Cyclopia* (one of *C. subternata* designated M6, and three of *C.genistoides* designated M7-M9) available in bulk by making use of four *in vitro* assays, and the selection of the most estrogenically active harvesting for phytoestrogenicity-enhancement through further extraction methods in Phase 2. For the Phase 2 methodology, we followed a two-dimensional plan, using five solvents (ethyl acetate, ethanol, methanol, 50% methanol-distilled water and distilled water) of different polarities and two extraction methods (sequential and non-sequential) to prepare an extract with a higher estrogenic activity from harvestings identified in Phase 1 (M6 and M7). To make a determination of whether or not there are active phytoestrogens ingested with the traditional drinking of honeybush tea, we assessed the estrogenic potency and efficacy of extracts prepared from M6, in a manner akin to the preparation of a traditional hot cup of honeybush tea. Finally, Phase 3 of our study entailed benchmarking the activity of the extract chosen in Phase 2 (SM6Met) against the activities of four commercially available phytoestrogenic preparations (Phytopause Forte® capsules, a soy isoflavone extract, Promensil® tablets (Novogen), a red clover isoflavone extract, Remifemin® tablets, a black cohosh root and rhizome extract, and Femolene Ultra® tablets, a combination of herbs, vitamins and minerals including extracts from soy, black cohosh, Mexican wild, dong quai, chasteberry, and maidenhair tree (*Ginkgo biloba*).

The choice to evaluate the estrogenicity of *C. subternata* and *C. genistoides* methanol extracts in Phase 1 of our study was not only based on the fact that these harvestings were available in bulk for subsequent studies, but the choice of plant species was also inspired by the findings of a previous study by Verhoog *et al.* (13), who identified methanol extracts of *C. genistoides* and *C.subternata* as possessing significantly higher affinities for binding to the ER when compared to extracts of *C. sessiliflora* and *C. intermedia*.

The choice of *in vitro* assays for the quantification of estrogenic potencies and efficacies of extracts was based on the recognition of key points that can be measured in the estrogen signaling pathway. The assays were (i) radiometric whole-cell binding in breast MCF-7 BUS cells, (ii) luciferase promoter-reporter assays in T47D-KBluc human breast cancer cells, which naturally express ERα and β and are stably transfected with a triplet ERE–promoter–luciferase reporter gene construct, (iii) alkaline phosphatase induction assays in non-proliferative human endometrial Ishikawa (Variant 1) cells, and (iv) cell proliferation assays (E-screen) in MCF-7 BUS cells. Although the four assays were used in Phases 1 and 3, we tried to identify one or two assays from Phase 1 results which would be sufficient to discriminate between the potencies and efficacies of the various test substances in Phase 2 as we envisaged a large number of test extracts in Phase 2 of our study. Thus, it was determined from Phase 1 results that both the alkaline phosphatase induction assay and the E-screen were adequate for detecting differences in potencies and efficacies between test substances and were hence the only assays used in Phase 2.

The exploration of the potential use of *Cyclopia* as an agent for alleviating menopausal symptoms, for which positive results would mean added value to the already putatively health beneficial honeybush tea and a possible formulation of a uniquely South African phytoestrogen nutraceutical, a venture which would ogre well for the South African honeybush industry, in the end proved to be a worthwhile venture, and although exciting conclusions about whether or not *Cyclopia* has potential to mediate in the treatment of menopausal symptoms were reached, a few equally exciting questions were also borne from the study. We hope that, while proceeding to learn about the details of our experimental work and its results in the next chapter, the reader will share in the intrigue and the hope for clinically relevant breakthroughs that the findings of our study have sparked within us.

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CHAPTER 2

SELECTIVE EXTRACTION OF *CYCLOPIA* FOR ENHANCED *IN VITRO* PHYTOESTROGENICITY AND BENCHMARKING AGAINST COMMERCIAL PHYTOESTROGEN EXTRACTS

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2.1 Abstract

Previous work established the phytoestrogenicity of methanol extracts of "unfermented" *Cyclopia* (*C. subternata* and *C. genistoides*). The current study investigated the estrogenicity of four harvestings of *Cyclopia* (M6-9) available in bulk with a view to preparing extracts with enhanced phytoestrogenicity for benchmarking against commercial preparations. Dried methanol extracts from two harvestings (M6 – *C. subternata* and M7 – *C. genistoides*) were identified as having the highest estrogenic activity using estrogen receptor binding, an estrogen receptor response element containing promoter reporter assay, alkaline phosphatase activity, and E-screen. M6 and M7 were extracted using both sequential and non-sequential extraction methods and five different solvents of differing polarities (ethyl acetate, ethanol, methanol, 50% methanol-water and distilled water). In addition, two extracts were prepared to mimic the preparation of a cup of tea, the traditional way in which *Cyclopia* is consumed. The resultant 22 extracts were evaluated for estrogenicity. The sequentially extracted M6 methanol extract (SM6Met) had the highest potency and the sequentially extracted M6 ethyl acetate extract (SM6EtAc) had the highest efficacy of all the extracts. Benchmarking against four commercial phytoestrogenic preparations suggest that in terms of the assays used, *Cyclopia* extracts have comparable potency and efficacy to the commercial extracts and thus have potential as marketable phytoestrogenic nutraceuticals.

2.2 Introduction

The onset of menopause is triggered by a natural decrease in the endogenous levels of estradiol (E_2), which can result in vasomotor symptoms (hot flashes and night sweats) (1), sexual dysfunction (2), osteoporosis (3) and dementia (4-5). These symptoms bring a degree of physiological and psychological discomfort that women consider severe enough to seek medical intervention against. Hormone replacement therapy (HRT) has in the past been regarded as the best form of treatment against the symptoms of menopause until studies, such as done by the Women's Health Initiative, found that the treatment showed no improvement in cardiovascular conditions in post-menopausal

women and increased the risk of the onset of diseases such as venous thrombosis, coronary heart diseases**,** bone fracture and breast cancer (6). The serious nature of these findings created a great unmet need for safer alternatives to HRT (7).

Phytoestrogens are plant-borne compounds with estrogenic activity that are currently seen as a possible alternative to traditional HRT (8-9). The most important groups of phytoestrogens are isoflavones (e.g. genistein and daidzein) found in soybeans, chick peas and red clover and the lignans (e.g. enterolactone and enterodiol) found in flaxseed, cereal bran, legumes and fruit (10-11).

Although the molecular mechanism of action of phytoestrogens is not yet fully understood (12-;13), studies have suggested that they exert their actions through a preferential binding to the estrogen receptor (ER) β, resulting in their selective estrogen receptor modulator (SERM) properties, meaning an ability to display estrogenic or anti-estrogenic properties depending on the ER subtype distribution of the tissue (14-15). Epidemiological studies (16) have indicated lower incidence of hot flushes, coronary artery disease and estrogen-dependent cancers (e.g. breast and prostate) in oriental societies that consume phytoestrogen-rich diets. Although evidence for the effectiveness of phytoestrogens for the treatment of menopausal symptoms has been presented (17-18), there is still controversy concerning the clinical safety and efficacy of phytoestrogens (19-20). Despite this controversy, the use of phytoestrogenic nutraceuticals, driven by society's preference for what it considers to be a more "natural" and hence safer alternative to HRT, has gained popularity (21).

The honeybush plant (genus *Cyclopia*) is part of the rich fynbos biome that is endemic to the South African Western and Eastern Cape Provinces and its fermented (oxidized) form has been traditionally consumed as the fragrant, caffeine-free honeybush tea beverage (22). Dried methanol extracts (DMEs) of two honeybush species (*C. genistoides* and *C. subternata*) used for the manufacture of the commercial herbal tea beverage have been shown by a previous study to possess phytoestrogenic activity (23), suggesting that they may be suitable for the preparation of a phytoestrogenic nutraceutical. The goal of the current study was thus to improve the

phytoestrogenicity (potency and efficacy) of *Cyclopia* extracts and to compare activity with that of commercially available phytoestrogenic extracts. A three-phase approach was adopted. Phase 1 identified the most phytoestrogenic harvestings of *Cyclopia* out of four (M6 from *C. subternata* and M7-9 from *C. genistoides*) that were chosen because they were available in bulk for further extraction. Phase 2 was aimed at producing a more potent and efficacious extract from the harvestings selected from Phase 1 using a two dimensional approach that explored the use of five solvents of differing polarities (ethyl acetate, ethanol, methanol, 50% methanol-distilled water and distilled water) and two extraction methods (sequential and non-sequential). In addition, extracts that mimicked the preparation of a cup of tea, which is how *Cyclopia* is traditionally consumed, were also prepared. Phase 3 benchmarked the estrogenicity of the extract identified in Phase 2 against four commercial nutraceuticals, Phytopause Forte®, a soy isoflavone extract, Promensil®, a red clover isoflavone extract, Remifemin®, a black cohosh extract, and Femolene Ultra®, a combination of extracts from several plants including soy, black cohosh, Mexican wild yam, and maidenhair tree.

2.3 Materials and methods

2.3.1 Test compounds

Test compounds included the estrogenic standards, 17-β-estradiol (E_2) and genistein (Sigma-Aldrich). 2,4,6,7-^{3}H-17-β-estradiol (specific activity 93.0 Ci/mmol) was purchased from AEC-Amersham. Commercial phytoestrogen extracts purchased from a local pharmacy included: Phytopause Forte® capsules (Pharma Dynamics), a soy (*Glycine max*) isoflavone extract, Promensil® tablets (Novogen), a red clover (*Trifolium pratense*) isoflavone extract, Remifemin® tablets (Enzymatic Therapy, Inc.), a black cohosh (*Cimicifuga racemosa*) root and rhizome isopropanol extract, and Femolene Ultra® tablets (Kenza Health), a combination of herbs, vitamins and minerals including extracts from soy, black cohosh, Mexican wild yam (*Dioscorea villosa*),

dong quai (*Angelica sinensis*), chasteberry (*Vitex agnus-castus*), and maidenhair tree (*Ginkgo biloba*).

2.3.2 Plant material

Four independent harvestings, one from *C. subternata* (M6) and three from *C. genistoides* (M7, M8 and M9) were harvested at different locations and seasons (Table 1), dried whole at 40°C to ca. 10% moisture content in an experimental dehydrator tunnel with cross air flow, pulverized (Retch mill) to a particle size of less than 1 mm and stored in plastic containers at room temperature.

2.3.2.1 Dried methanol extracts (DMEs)

Using a magnetic stirrer, plant material (50 g of each harvesting, M6-9) was extracted four times with 200 ml dichloromethane (UNIVAR, Merck) in a 37ºC room for 30 min each and the filtrate was discarded. Thereafter methanol extraction (50 ml) of the air-dried plant material was performed three times in a 37ºC room for 30 min each. The methanol extracts were pooled with a small volume of water added and evaporated under vacuum before freeze-drying. The resultant DMEs were ground with pestle and mortar in a darkened room until a fine homogenous powder was obtained, which was stored in screw cap glass vials, covered with aluminium foil, and placed in a vacuum-sealed desiccator in the dark at room temperature.

2.3.2.2 Preparation of sequential, non-sequential and "cup-of-tea" extracts

Using a magnetic stirrer, M6 and M7 (500 g/harvesting) were each extracted four times with 300 ml dichloromethane at room temperature for 24 h/extraction and the filtrate was discarded. After overnight drying at room temperature the air-dried plant material from each harvesting was subjected to sequential and non-sequential extractions at room temperature.

Sequential extracts: Using a magnetic stirrer, air-dried dichloromethane-extracted plant material from the M6 and M7 harvestings (50 g) were each extracted with a sequence of five solvents (300

ml) in the order of increasing polarities of the solvents (ethyl acetate, ethanol, methanol, 50% ethanol-distilled water and distilled water). Each extraction in the sequence was performed three times at room temperature for 3 h/extraction and filtrates pooled. After extraction with the first solvent the air-dried plant material was extracted with the next solvent. Three additional M6 sequential methanol extracts (B1, B2 and B3) were prepared using the protocol detailed above.

Non-sequential extracts: The same five solvents and extraction protocol used for the preparation of the sequential extracts were used except that each solvent was used directly on the air-dried dichloromethane-extracted plant material.

"Cup-of-tea" extracts: M6 harvesting (2 g) was added to 150 ml of freshly boiled distilled or tap water and allowed to steep for 5min before filtration (Whatman no. 4 filter paper).

Except for water extracts, a small volume of water was added to the liquid extracts, which were evaporated under vacuum before freeze-drying. Freeze-dried extracts were ground into a fine homogenous powder, stored in aluminum foil-covered screw cap glass vials and placed in a vacuum-sealed desiccator in the dark at room temperature.

2.3.3 Preparation of test samples

Tablets or capsule contents of the commercial phytoestrogen extracts were ground into a fine homogenous powder. Stock solutions of all test samples were dissolved in DMSO at concentrations of either 9.8 mg/ml for plant extracts and commercial phytoestrogen extracts or 2.7 mg/ml for the standard estrogenic compounds, E_2 or genistein, and stored in amber screw-cap vials at -20ºC. Test samples were diluted 1000 times when added to assay media. Each assay consisted of a negative (0.1% DMSO) and two positive controls (E_2 and genistein).

2.3.4 Determination of extract yield

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

2.3.5 Total polyphenol (TPP) content of extracts

The TPP content of the extracts was quantified colorimetrically in triplicate. The method of Singleton and Rossi (24) was adapted for use in flat-bottomed 96-well plates (B & M Scientific). Briefly, 20 µl of sample (0.01-0.1 mg/ml gallic acid standards (Sigma-Aldrich) or 0.25 mg/ml dried plant extract) 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 microtiter plate reader (Titertek Multiskan Plus, Titertek Instruments Inc., USA), recorded, and g gallic acid equivalents obtained from the standard curve. The TPP content was expressed as g gallic acid equivalents per 100 g of freeze-dried extract.

2.3.6 Total flavonoid (TF) content of extracts

The method described by Zhishen *et al*. (25) was adapted for use in flat-bottomed 96-well plates. Briefly, 100 µl of sample (0.01-0.1 mg/ml rutin hydrate standards (Sigma-Aldrich) or 0.25 mg/ml dried plant extract) were allowed to react with 6 µl 5% (w/v) $NaNO_2$ for 5 min before 60 µl 10% (w/v) Al_2Cl_3 was added. The reaction was allowed to proceed for 6 min at room temperature before 34 µl NaOH (1M) was added and the reaction allowed to proceed for 20 min at room temperature. The absorbance was then measured at 450 nm using a microtiter plate reader, recorded, and g rutin equivalents obtained from the standard curve. The TF content was calculated as g rutin equivalents per 100 g of freeze-dried extract.

2.4 Tests for estrogenicity

2.4.1 Cell culture

T47D-KBluc human breast cancer cells (ATCC), which naturally express ERα and β and are stably transfected with a triplet ERE–promoter–luciferase reporter gene construct (26), were maintained in standard growth media (RPMI (Gibco) supplemented with 2.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, 1.5 g/l $NaHCO_2$, 10% charcoal treated fetal calf serum (FCS) (Hyclone) and 1%

mixture of penicillin-streptomycin). The cells were withdrawn from antibiotics for a week before the running of experiments.

The ER positive human endometrial cell line, Ishikawa Var-1 (a kind gift from S. Milligan, King's College, London, UK) (27), was maintained in phenol red-free DMEM/Hams F12 medium (Sigma-Aldrich) with 5% charcoal treated FCS, 1% penicillin-streptomycin and 1% plasmocin.

The ER positive MCF-7-BUS cells (28) (a kind gift from A. Soto, Tufts University, U.S.A.) were maintained in DMEM supplemented with 5% heat inactivated FCS, and 1% mixture of penicillin-streptomycin. The cells were withdrawn from antibiotics a week prior to the running of experiments.

All cells were maintained in a humidified cell incubator set at 97% relative humidity and 5% CO_2 at 37 °C.

2.4.2 Competitive whole cell ER-binding

MCF-7-BUS cells were seeded into 24-well tissue culture plates at a density of 1 x 10^5 cells/well and incubated for 24 h. The next day the cells were washed once with 500 μl of PBS/well and the medium replaced with phenol red-free un-supplemented DMEM with no antibiotics (pre-heated at 37°C) for 24 h. This was followed by a 4 h incubation of the cells with 20 x 10^{-9} M radio-labeled estradiol (2,4,6,7-^{3}H-17-β-estradiol) and a range of concentrations of unlabeled competitors (9.8 x 10^{-12} to 9.8 x 10^{-3} mg/ml for dried plant extracts and commercial phytoestrogen products and 2.7 x 10^{-12} to 2.7 x 10^{-3} mg/ml for standard test compounds) in DMEM without phenol red, antibiotics or FCS. All assays included a total binding point, which was in the presence of 0.1% DMSO. After the 4 h incubation period, the cells were immediately placed on ice and further work was done at 4°C. Cells were washed three times with 1 ml bovine serum albumin-PBS (0.2%) with an interval of 15 min between washes to remove free ligand. One hundred microliters of lysis buffer (0.2% (v/v) Triton, 10% (v/v) glycerol, 2.8% (v/v) Tris-phosphate-EDTA and 1.44 mM EDTA) was then added to each well after which the plates were shaken at room temperature for 15 min and frozen

overnight at -20°C. On thawing of samples, 5 µl of lysate from each well was used for protein determination using the Bradford method (29). The remaining lysate was transferred to scintillation vials to which 1 ml of scintillation fluid (Quickszint FLOW 2, Zinsser Analytic, South Africa) had been added. Radioactivity of the assay samples was determined using a Beckman LS 3801 Beta-scintillation counter. Results were normalized for protein content and expressed as percentage of normalized control with total binding (in presence of 0.1% DMSO) taken as 100%. Competitive binding curves were fitted (GraphPad Prism®) using non-linear regression and one site competition to determine IC_{50} values. Relative binding affinities (RBAs) were calculated using IC_{50} of E_2/IC_{50} of test samples. All binding experiments included a control for ligand depletion which was less than 10% for all experiments.

2.4.3 ERE-containing promoter reporter assay

T47D-KBluc cells were seeded into 96-well plates to a density of 1 x 10^4 cells/well and allowed to settle for a day. A range of concentrations of the test samples (as desribed under 2.4.2) were then added in phenol red-free DMEM with 1% penicillin-streptomycin mixture and 5% charcoal treated FCS and the induction was allowed to proceed for 24 hr. After induction the medium was aspirated, 50 µl lysis buffer (0.2% (v/v) Triton, 10% (v/v) glycerol, 2.8% (v/v) Tris-phosphate-EDTA, and 1.44 mM EDTA) was added to each well and cells were freeze-lyzed at -20°C overnight. The luciferase assay reagent (Promega Corp., Madison, WI) was used to quantify luciferase activity in accordance with the manufacturer's instructions. Briefly, 10 μl of cell lysate was allowed to react with 25 μl of luciferase assay reagent. The relative light units (RLU's) were measured using a Veritas luminometer (Turner Biosystems). A further 5 µl of lysate for each well was used for protein determination using the Bradford method (29). Results were normalized for protein content and expressed as fold induction with negative controls (0.1% DMSO) taken as 1. Dose response curves were fitted using GraphPad Prism® and non-linear regression and sigmoidal dose response

with variable slope to obtain potency (EC_{50}) and efficacy (fold-induction). Relative induction indices (RIIs) were calculated using EC_{50} of E_2/EC_{50} of test samples.

2.4.4 Alkaline phosphatase activity assay

Ishikawa Var-I cells were seeded at a density of 2.5 x 10^4 cells/well in 96-well plates and allowed to settle for a day. A range of concentrations of the test samples (as desribed under 2.4.2) were then added in DMEM without phenol red and antibiotics but supplemented with 5% charcoal stripped FCS. Induction was allowed to proceed for 96 h at 37˚C after which the medium was aspirated and the cells freeze-lyzed overnight at -20˚C. Lyzed cells were then placed at room temperature for 5 min, subsequently placed on ice and 50 µl ice-cold solution containing 5 mM *p*-nitrophenyl phosphate, 0.5 mM $MgCl_2$, and 1 M diethanolamine (pH 9.8) added. Plates were allowed to reach room temperature over 30 min, while allowing a yellow colour from the production of *p*-nitrophenol to develop. The colorimetric reaction was quantified by reading the absorbance at 405 nm in a mictrotiter spectrophotometer. Results are expressed as fold-induction with negative controls (0.1% DMSO) taken as 1. Data analyses were as for 2.4.3.

2.4.5 E-screen

MCF-7-BUS cells were plated at a density of 6 x 10^3 cells/well in 96-well plates and incubated for 24 h. The medium was then aspirated and cells induced with a range of concentrations of the test samples (as described under 2.4.2) prepared in DMEM without phenol red or antibiotics, but supplemented with 5% charcoal stripped FCS. Cells were then incubated for 96 h where after the colorimetric MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay was preformed. Four hours before the end of the incubation period the assay medium was changed to un-supplemented DMEM without phenol red where after 200 µl of MTT solution (5 mg/ml) was added to each well. Cells were incubated for 4 h at 37°C, the medium was then removed, and 200 µl of solubilizing solution (DMSO) added to each well. The DMSO was pipetted up and down in

the well to dissolve crystals until a uniform purple color had formed. The plate was then placed in a 37°C incubator for 10 min, and the absorbance was read at 450 nm in a micotiter plate reader. All assays included a negative control, which consisted of 0.1% (v/v) DMSO. Results are expressed as fold-induction with negative controls (0.1% DMSO) taken as 1. Data analyses were as for 2.4.3..

2.5 High-performance liquid chromatography (HPLC) analysis of *Cyclopia* DMEs

Reversed-phase HPLC analysis of the DME from M6-M9 was carried out on an Agilent 1200 series HPLC (Agilent Technologies, Waldbronn, Germany) consisting of quaternary pump, autosampler, column thermostat, diode-array detector and Chemstation software for LC 3D systems (Rev. B.02.01). The method of Joubert *et al*. (30) was slightly modified by changing to a Luna Phenyl-hexyl column (150 x 4.6 mm) with a Luna Phenylpropyl guard cartridge (4 x 3 mm) (Phenomenex, Torrance, CA, USA) for improved peak shape of the xanthones and the solvent gradient, starting with 0% organic modifier, was less steep. The flow rate and temperature were maintained at 0.8 ml/min and 30˚C, respectively. HPLC grade water was obtained by passing water sequentially through a Modulab Water Purification System (Continental Water Systems Corporation, San Antonio, TX, USA) containing in sequence carbon, reverse osmosis and deionizer cartridges and a Milli-Q académic water purifier (Millipore, Bedford, MA, USA).

Stock solutions (ca. 6 mg/ml) of the DME, dissolved in DMSO, were each filtered through a 33 mm 0.45 μm Millex-HV Hydrophilic PVDF syringe filter (Millipore) and without further dilution automatically injected (10 μl) in duplicate,. Calibration series of authentic standards i.e. mangiferin, hesperetin, hesperidin and naringenin from Sigma-Aldrich, and eriocitrin, narirutin, luteolin, eriodictyol, and formononetin from Extrasynthese, Genay, France, were used for quantification of peaks and determination of tentative peak identity according to retention time and UV-VIS spectra. Isomangiferin was quantified using mangiferin as standard. The xanthones and flavanones were quantificated at 320 and 280 nm, respectively, while the flavone and isoflavone were quantified at 255 nm. Quantities were expressed as a percentage of the dried extracts.

2.6 Data manipulation and statistical analysis

The GraphPad Prism® version 4.00 for Windows (GraphPad Software) was used for graphical representations and statistical analysis. One-way ANOVA and Dunnett's multiple comparisons' test as post-test were used for statistical analyses. For all experiments, unless otherwise indicated, the error bars represent the SEM of three independent experiments, where each point was determined in triplicate.

2.7 Results

2.7.1 Preliminary phytoestrogenicity screening to identify harvestings with high estrogenic potential (Phase 1)

Dried methanol extracts (DMEs) were prepared from 4 harvestings of *Cyclopia* (M6-M9), available in bulk, to identify harvestings with high estrogenic potential for further extraction. Before evaluating phytoestrogenic activity the DMEs were characterized as to yield, TPP and TF content and HPLC analysis conducted to identify polyphenols present in the DMEs.

2.7.1.1 Determination of extract yield, TPP and TF content and HPLC analysis of DMEs

The extract yield for the various DMEs varied from 17.5-26.3 g/100 g plant material (Table 1).

Table 1

Information on the original *Cyclopia* harvestings and the extract yield, total polyphenol (TPP) and total flavonoid (TF) contents of the DMEs prepared from these harvestings.

Species	Identity code[b]	Farm/Area	Harvesting date	Yield[c]	TPP[d]	TF[e]
C. subternata	M6	Kanetberg Flora/Barrydale	30/03/2004	18.6	33.0	1.7
C. genistoides[a]	M7	Koksriver/Overberg	22/01/2002	17.5	23.4	2.3
C. genistoides[a]	M8	Reins/Albertinia	01/04/2003	22.4	22.7	2.1
C. genistoides[a]	M9	Reins/Albertinia	22/04/2004	26.3	25.5	2.4

[a]West Coast type, [b]refers to harvesting and DME, [c]Yield expressed as g extract /100g plant material, [d]TPP expressed as g gallic acid equivalents/100g extract, [e]TF expressed as g rutin equivalents/100g extract.

The TPP and TF content (Table 1) indicated that M6 had the highest TPP content (33.0 g gallic acid equivalents/100 g extract), while M9 had the highest TF content (2.4 g rutin equivalents/100 g extract).

High performance liquid chromatography results (Fig. 1, Supplementary Figs. 1A-C, and Table 2) showed the presence of various amounts of mangiferin, isomangiferin, hesperidin, narirutin, luteolin, eriocitrin, eriodictyol, and naringenin in the DMEs. M6, in addition, to having a lower concentration of mangiferin and isomangiferin, is the only DME to contain eriocitrin and to lack eriodictyol and naringenin (Table 2 and Supplementary Fig. 1A). Although notable amounts of unidentified compounds were shown to elute between 5 and 20 min with all DMEs, M6 showed noticeably lower quantities of all the unidentified compounds, in comparison to the rest of the DMEs.

Table 2

Individual polyphenol content of DMEs of four *Cyclopia* harvestings, M6-M9, as determined by HPLC.

DME	**% of dry extract (g/100g dry extract)**							
	Mangiferin	Isomangiferin	Eriocitrin	Narirutin	Hesperidin	Luteolin	Eriodictyol	Naringenin
M6	2.04	0.79	2.38	0.13	2.77	0.09	-	-
M7	12.97	3.12	-	0.09	2.82	0.11	0.05	0.04
M8	8.99	3.84	-	0.12	3.07	0.12	0.04	0.04
M9	9.00	2.67	-	0.12	3.30	0.21	0.04	0.04

2.7.1.2 Evaluation of estrogenicity of DMEs

To ascertain the relative estrogenicity of the DMEs we performed whole cell ER-binding to evaluate binding affinity, and promoter reporter, alkaline phosphatase and E-screen assays to determine potency and efficacy relative to E_2 and genistein.

Results of competitive ER-binding in MCF-7 cells indicate that the relative binding affinity (RBA) and IC_{50} (Fig. 2A, Supplementary Figs. 2A and Table 3) of M6 and M7 DMEs are not

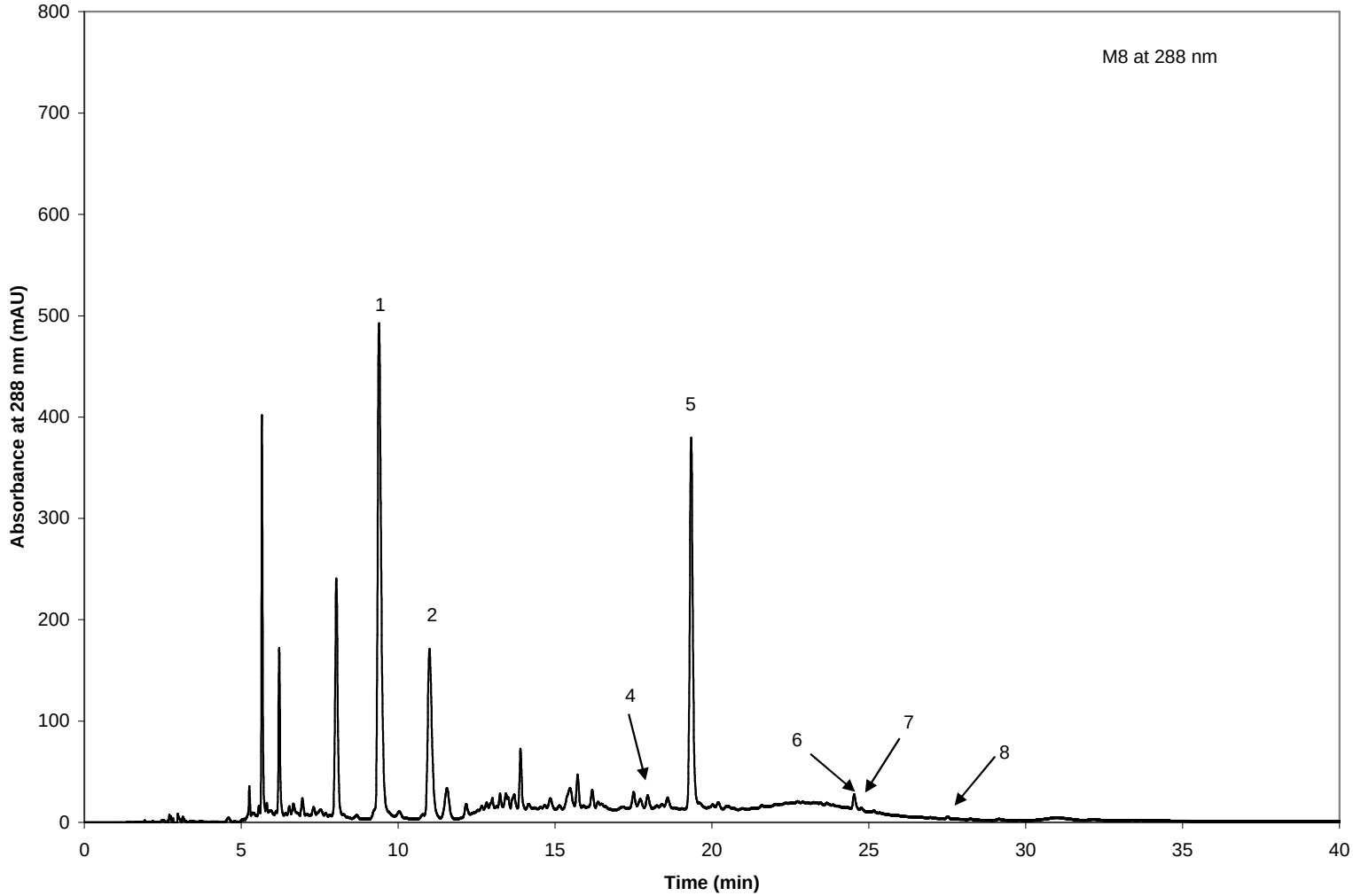

Fig. 1. HPLC of *Cyclopia* DME (M8) showing polyphenols (1-8) co-eluting at retention times similar to those of known standards, (1) mangiferin, (2) isomangiferin, (3) eriocitrin, (4) narirutin, (5) hesperidin, (6) luteolin, (7) eriodictyol, (8) naringenin, and unidentified polyphenols eluting between 5 and 20 minutes.

significantly ($P > 0.05$) different from that of genistein although, like genistein, lower than that of E_2. M8 DME could only displace ^3H-E_2 at 0.0098 mg/ml while M9 was unable to displace ^3H-E_2 indicating that these two DMEs do not have as high an affinity for the ER as M6 and M7.

Results of the promoter reporter assay (Fig 2B, Supplementary Figs. 2B and 3A), the alkaline phosphatase assay (Fig 2C, Supplementary Figs. 2C and 3B) and E-screen (Fig 2D and Supplementary Figs. 2D and 3D) indicate that M6 and M7 DMEs are more estrogenic than M8 and M9 DME.

Considering the potency (EC_{50}) of the extracts (Fig. 2 and Supplementary Fig. 2 and Table 3), no significant ($P > 0.05$) difference between the potency of the M6 and M7 DMEs, and genistein in the alkaline phosphatase assays was observed. However, in the promoter reporter assay and E-screen the potencies of M6 and M7 DMEs are significantly ($P < 0.01$) lower than that of genistein and E_2.

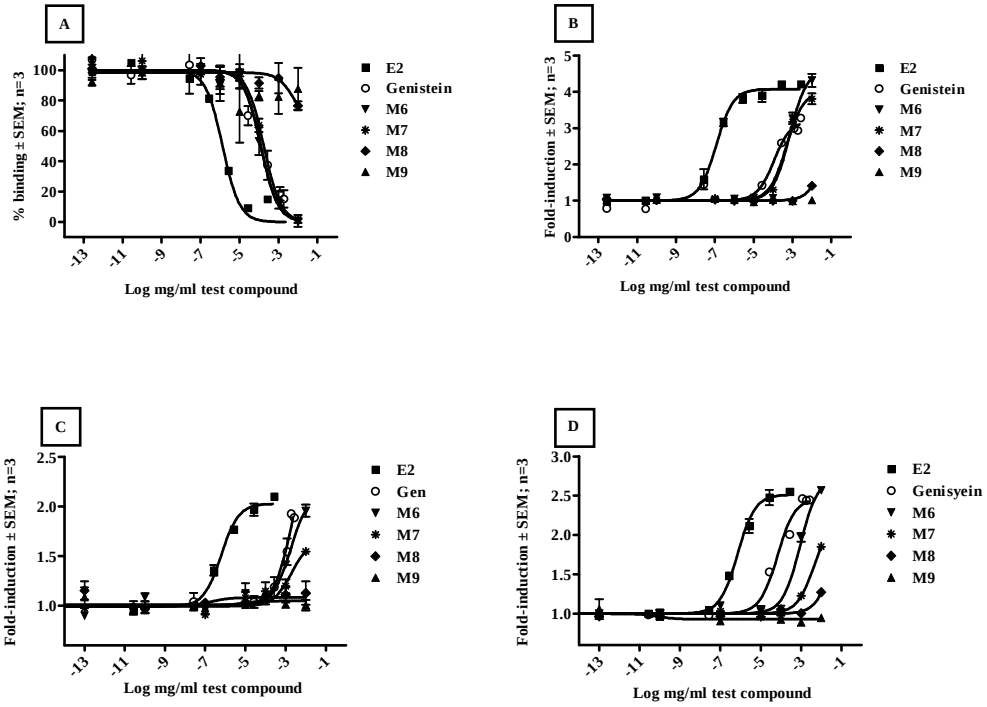

Fig. 2. Representative plots of (A) whole cell ER-binding, (B) promoter-reporter assay, (C) alkaline phosphatase activity and (D) E-screen for the four harvestings of *Cyclopia* (M6-M9) compared against E_2 and genistein.

Table 3

Comparison of the affinities (IC_{50} in mg/ml) and relative binding affinities (RBA) of DMEs, as determined by whole cell ER-binding assay, and the potencies (EC_{50} in mg/ml), relative induction indices (RII) and efficacies (fold induction) of DMEs, as determined by promoter reporter, alkaline phosphatase, and E-screen assays, respectively.

Assays		Test compounds					
		E_2	**Genistein**	**M6**	**M7**	**M8**	**M9**
WCB	IC_{50}	$1.51x10^{-6\#\#}$	$1.16x10^{-4**}$	$1.22x10^{-4**}$	$1.67x10^{-4**}$	$> 0.01^{**\#\#}$	$> 0.01^{**\#\#}$
	RBA[a]	1	0.0129	0.0124	0.00904	-	-
Promoter-reporter	EC_{50}	$1.00x10^{-7\#\#}$	$9.65x10^{-4**}$	$7.16x10^{-4\#\#**}$	$5.13x10^{-4\#\#**}$	$8.55x10^{-2\#\#**}$	$< 0.01^{\#\#**}$
	RII[b]	1	0.00010	0.00014	0.00019	-	-
	fold induction	$4.06^{\#}$	3.16^{*}	$4.6^{\#\#*}$	3.92	$1.38^{\#\#**}$	$1.07^{\#\#**}$
Alkaline phosphatase	EC_{50}	$5.69x10^{-7\#\#}$	$2.09x10^{-3**}$	$1.81x10^{-3**}$	$1.89x10^{-3**}$	$> 0.01^{**\#\#}$	$> 0.01^{**\#\#}$
	RII	1	0.00027	0.000314	0.000301	-	-
	fold induction	$2.14^{\#\#}$	2.98^{**}	$1.98^{\#\#}$	$1.54^{\#\#**}$	$1.05^{\#\#**}$	$1.03^{\#\#**}$
E-Screen	EC_{50}	$6.36x10^{-7\#\#}$	$6.66x10^{-5**}$	$2.11x10^{-3\#\#**}$	$6.48x10^{-3\#\#**}$	$> 0.01^{\#\#**}$	$> 0.01^{\#\#**}$
	RII	1	0.00955	0.000301	0.000098	-	-
	fold induction	2.31	2.21	2.52	2.21	$1.28^{\#\#**}$	$1.02^{\#\#**}$

[a]RBA = IC_{50} E_2/IC_{50} test compound, [b]RII = EC_{50} E_2/EC_{50} test compound, Statistical analysis was done using one-way ANOVA with Dunnett's Multiple Comparison Test as post test. *P<0.05, **P<0.01 if compared to E_2 and $^{\#}$P<0.05, $^{\#\#}$P<0.01 if compared to genistein.

Although the extracts have a fold-induction that is significantly ($P < 0.01$) lower than genistein according to the alkaline phosphatase assays, in the E-screen there is no significant ($P > 0.05$) difference in the fold-induction between the extracts (M6 and M7), genistein and E_2. In the promoter reporter assay the fold-inductions are actually higher than for genistein, although significantly higher ($P < 0.01$) only for M6 DME (Fig. 2, Supplementary Fig. 3 and Table 3).

2.7.2 Solvent extraction for the enhancement of the phytoestrogenicity of selected harvestings (Phase 2)

From Phase 1, DMEs from M6 and M7 were identified as possessing the highest phytoestrogenicity. In Phase 2, each of these two harvestings was subjected to extractions by sequential (S) and non-sequential (N) methods. In each method, 5 different solvents ethyl acetate (EAc), ethanol (Eth), methanol (Met), 50% methanol-distilled water (Hlf) and distilled water (Wat) were used. Freshly boiled water (H) extracts of M6 were also prepared (non-sequentially) with distilled (D) and tap (T) water in a process similar to the traditional manner of preparing a hot cup of tea resulting in the extracts NM6HDW and NM6HTW for the distilled and the tap water extracts, respectively. This undertaking resulted in a total of 22 extracts (Table 4) destined for phytoestrogenicity evaluation. Yields of extracts (Table 4) indicate that increased polarity of the solvent generally results in increased yield up to the water extracts where yield dropped significantly. In contrast, the “cup-of-tea” extracts using boiled water gave amongst the highest yields.

For this phase of the study we limited our assays to the alkaline phoshatase activity assay, which could discriminate between the efficacies of genistein and extracts, and the E-screen, which discriminated between the potencies of the extracts and genistein (Table 3 and Supplementary Figs. 2 and 3).

Both assays used in Phase 2 of this study (Figs. 3 and 4 and Supplementary Tables 1A and B, and 2) indicated that the water extracts, SM6Wat, NM6Wat, SM7Wat and NM7Wat, had no

phytoestrogenic activity while interestingly the “cup-of-tea” extracts, prepared by steeping the plant material in boiling water, did show appreciable phytoestrogenic activity.

Table 4.

Extracts investigated in Phase 2.

M6		M7	
Extract name	**Yield[a]**	**Extract name**	**Yield[a]**
SM6EAc	7.9	SM7EAc	6.2
SM6Eth	8.5	SM7Eth	11.6
SM6Met	15.4	SM7Met	13.8
SM6Hlf	35.0	SM7Hlf	29.0
SM6Wat	1.1	SM7Wat	0.8
NM6EAc	6.4	NM7EAc	7.3
NM6Eth	8.8	NM7Eth	7.6
NM6Met	17.4	NM7Met	17.4
NM6Hlf	26.4	NM7Hlf	26.2
NM6Wat	1.4	NM7Wat	1.0
NM6HDW	30.0		
NM6HTW	20.0		

[a]Yield expressed as g/100g plant material

The potencies were similar ($P > 0.05$) to that of genistein in the alkaline phoshatase assay (Fig. 3 and Supplementary Table 1A) although in the E-screen (Fig. 4 and Supplementary Table 1B) the potencies were significantly ($P < 0.01$) lower than that of genistein and the original M6 DME. The efficacies, however, were significantly ($P < 0.01$) lower than genistein in both assays.

It was noted that extraction of the M7 harvesting resulted in only one extract (SM7Hlf) that was significantly ($P < 0.01$) more potent, and only one extract (NM7EAc) that was significantly ($P < 0.01$) more efficacious than the original M7 DME from Phase 1 (Figs. 3 and 4C and D). In contrast, a total of two M6 extracts showed significantly ($P < 0.01$) improved potencies (SM6Met and SM6Eth) while three extracts showed significantly ($P < 0.05$) increased efficacies (SM6EAc, NM6EAc, and NM6Met) when compared to the original M6 DME (Figs 3A and B and 4A and B).

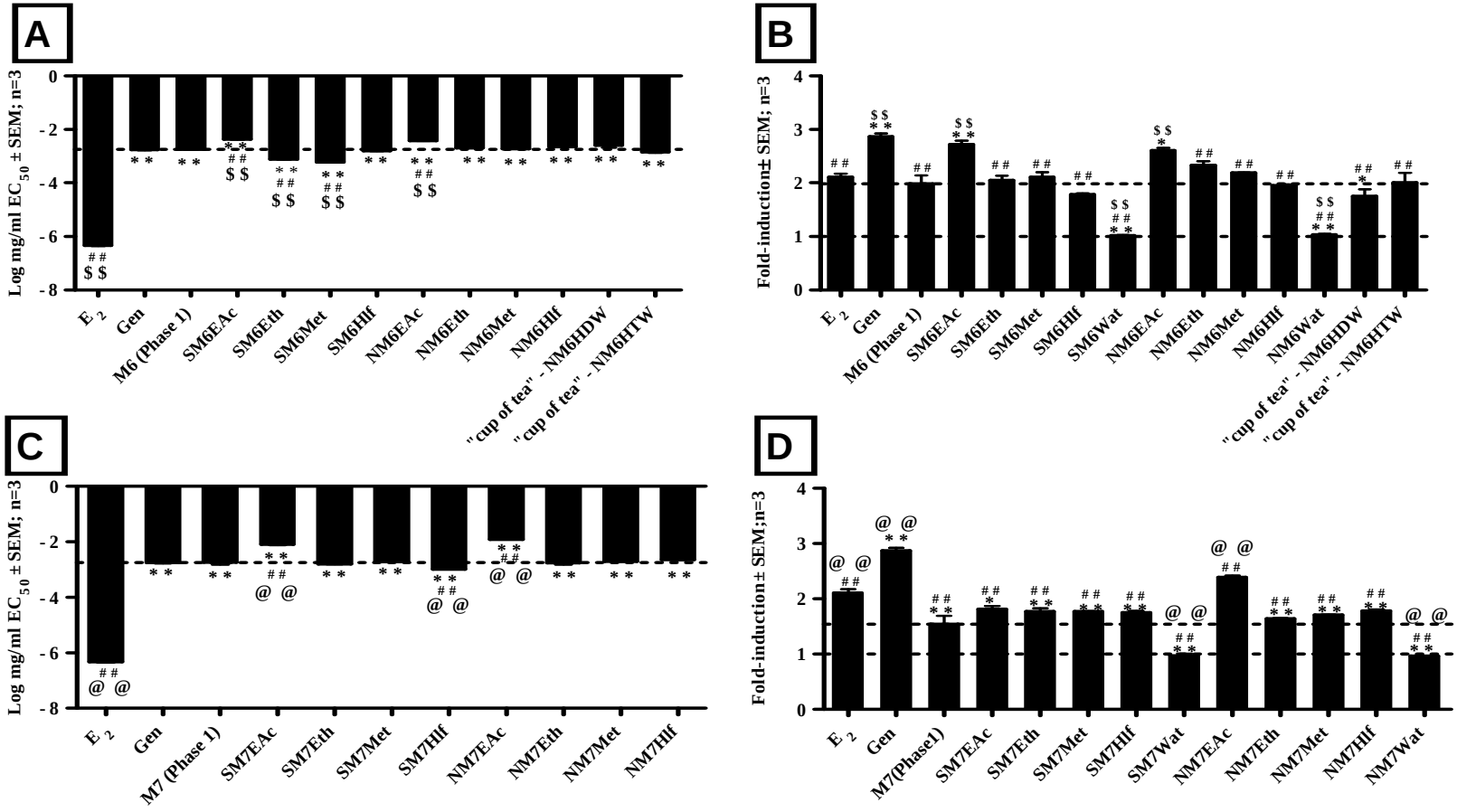

Fig. 3. Potency and efficacy data from the alkaline phosphatase assay for the sequential and non-sequential extracts of M6 (A and B, respectively) and M7 (C and D, respectively). Statistical analysis was done using one-way ANOVA with Dunnett's Multiple Comparison Test as post test. *P<0.05, **P<0.01 if compared to E_2, $^{\#}$P<0.05, $^{\#\#}$P<0.01 if compared to genistein, $^{\$}$P<0.05, $^{\$\$}$P<0.01 if compared to M6, and $^{@}$P<0.05, $^{@@}$P<0.01 if compared to M7. The dotted lines through the bars represent the log EC_{50} and efficacy values for M6, M7 and 1 for the control. .

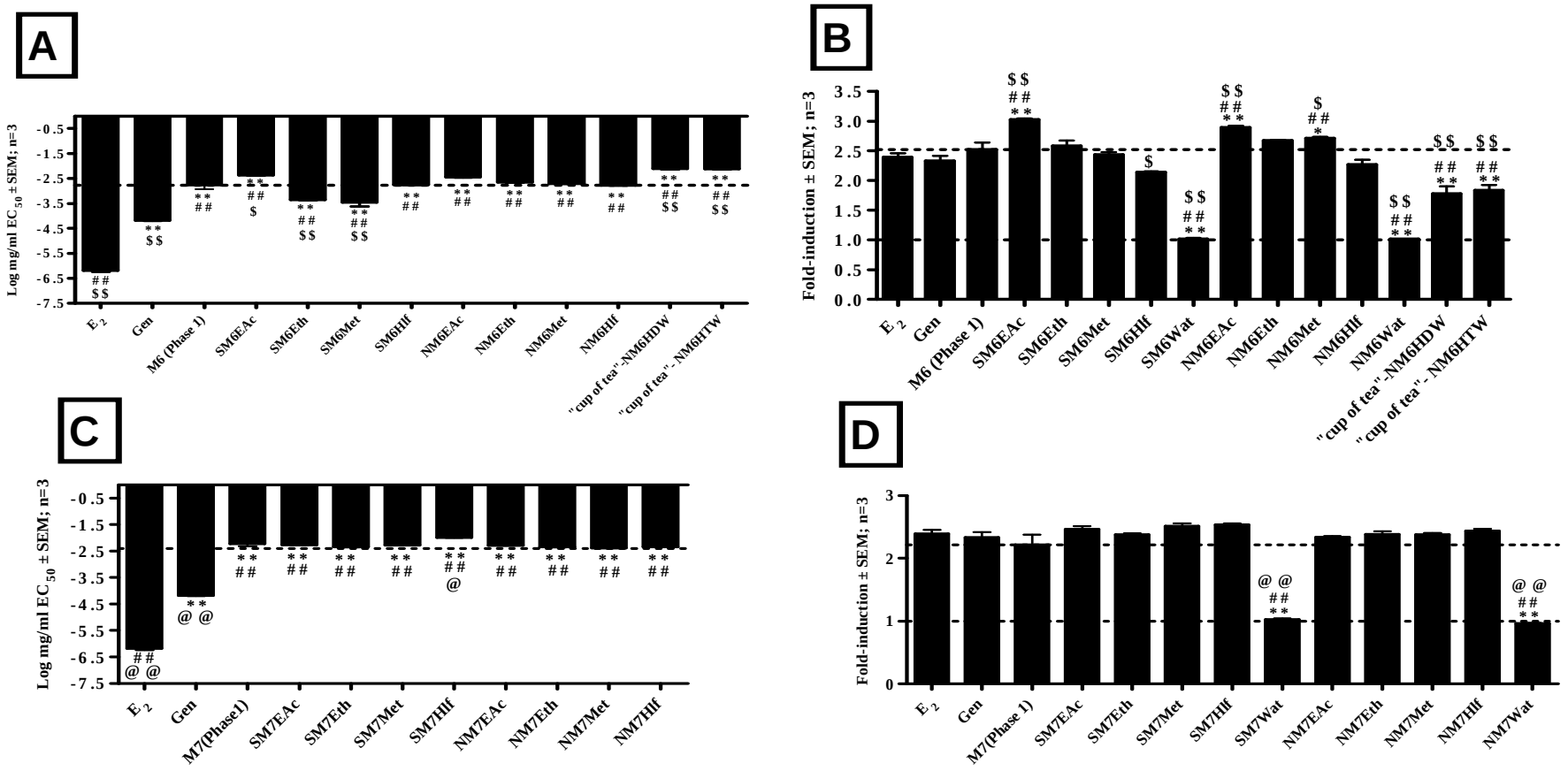

Fig 4. Potency and efficacy statistical from the E-screen for the sequential and non-sequential extracts of M6 (A and B respectively) and M7 (C and D respectively). Statistical analysis was done using one-way ANOVA with Dunnett's Multiple Comparison Test as post test. *P<0.05, **P<0.01 if compared to E_2, $^{\#}$P<0.05, $^{\#\#}$P<0.01 if compared to genistein, $^{\$}$P<0.05, $^{\$\$}$P<0.01 if compared to M6, and $^{@}$P<0.05, $^{@@}$P<0.01 if compared to M7.The dotted lines through the bars represent log EC_{50} and efficacy values for M6, M7 and 1 for the control.

Both assays for estrogenicity indicated that SM6Met and SM6Eth were significantly ($P < 0.01$) more potent than all of the other M6 and M7 extracts and the original M6 and M7 DMEs (Figs 3A and C and 4A and C). In contrast the two M6 ethyl acetate extracts, SM6EAc and NM6EAc, were the most efficacious of all the M6 and M7 extracts (Figs. 3B and D and 4B and D).

In an attempt to choose an extract to be used for benchmarking in Phase 3 we considered the fact that while the M6EAc extracts were 1.2-1.3 times more efficacious than SM6Met and SM6Eth extracts, the SM6Met extract was 7.2-10.5 times more potent than the M6EAc extracts. In addition, the fact that the SM6Met extract was more potent that the SM6Eth extract and it gave a higher extract yield made us decide in favour of SM6Met for benchmarking in Phase 3.

2.7.3 Benchmarking of SM6Met extract against commercial phytoestrogen extracts (Phase 3)

SM6Met was identified as the most phytoestrogenic extract in Phase 2 and all four assays (whole cell ER-binding, promoter reporter assay, alkaline phosphatase activity, and E-screen) used in Phase 1 were used in Phase 3 to benchmark SM6Met against commercial phytoestrogen products. The dilution series of the SM6Met extract used during Phase 2 (designated OSM6Met in Phase 3) was retested in Phase 3. In addition, three newly prepared SM6Met extracts (B1, B2 and B3) were also tested to evaluate the reproducibility of extraction.

Results from the whole cell ER-binding and alkaline phosphatase assays suggest that extraction is reproducible (Figs. 5 and 6 and Supplementary Tables 3 and 4) as results from new extractions (B1-B3) are not significantly ($P > 0.05$) different from results of the previous extraction in Phase 2 (OSM6Met) or from the previous testing in Phase 2 (SM6Met). However, the promoter reporter assay and E-screen

suggest that potency is significantly ($P < 0.01$) reduced in subsequent extracts as compared to the previous extraction and testing. Efficacy is only shown to be affected in the promoter reporter assay, not the E-screen, if we compare Phase 3 testing.

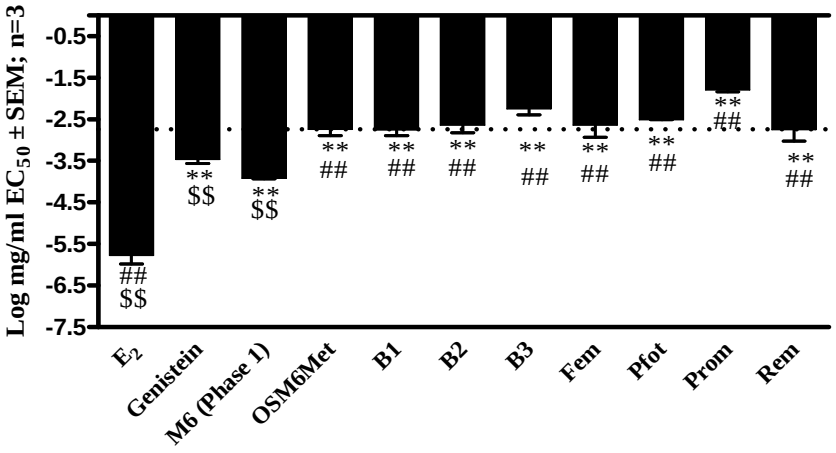

Fig. 5. Comparison of the affinity (log IC_{50}) of SM6Met extracts (OSM6Met, B1-B3) with that of commercial extracts, obtained for whole cell ER-binding. Statistical analysis was done using one-way ANOVA with Dunnett's Multiple Comparison Test as post test. *P<0.05, **P<0.01 if compared to E_2, $^{\#}$P<0.05, $^{\#\#}$P<0.01 if compared to genistein, and $^{\$}$P<0.05, $^{\$\$}$P<0.01 if compared to OSM6Met. The dotted line through the bars represents the log EC_{50} value for OSM6Met.

Comparison with results obtained during Phase 2, however, shows that extracts tested in Phase 3 had a significantly ($P < 0.01$) reduced efficacy in the E-screen.

Remifemin (Rem), a commercial phytoestrogenic extract, shows binding in the whole cell ER-binding assay (Fig. 5), but no estrogenic activity in the promoter reporter assay, the alkaline phosphatase assay and the E-screen (Fig. 6 and Supplementary Tables 3 and 4).

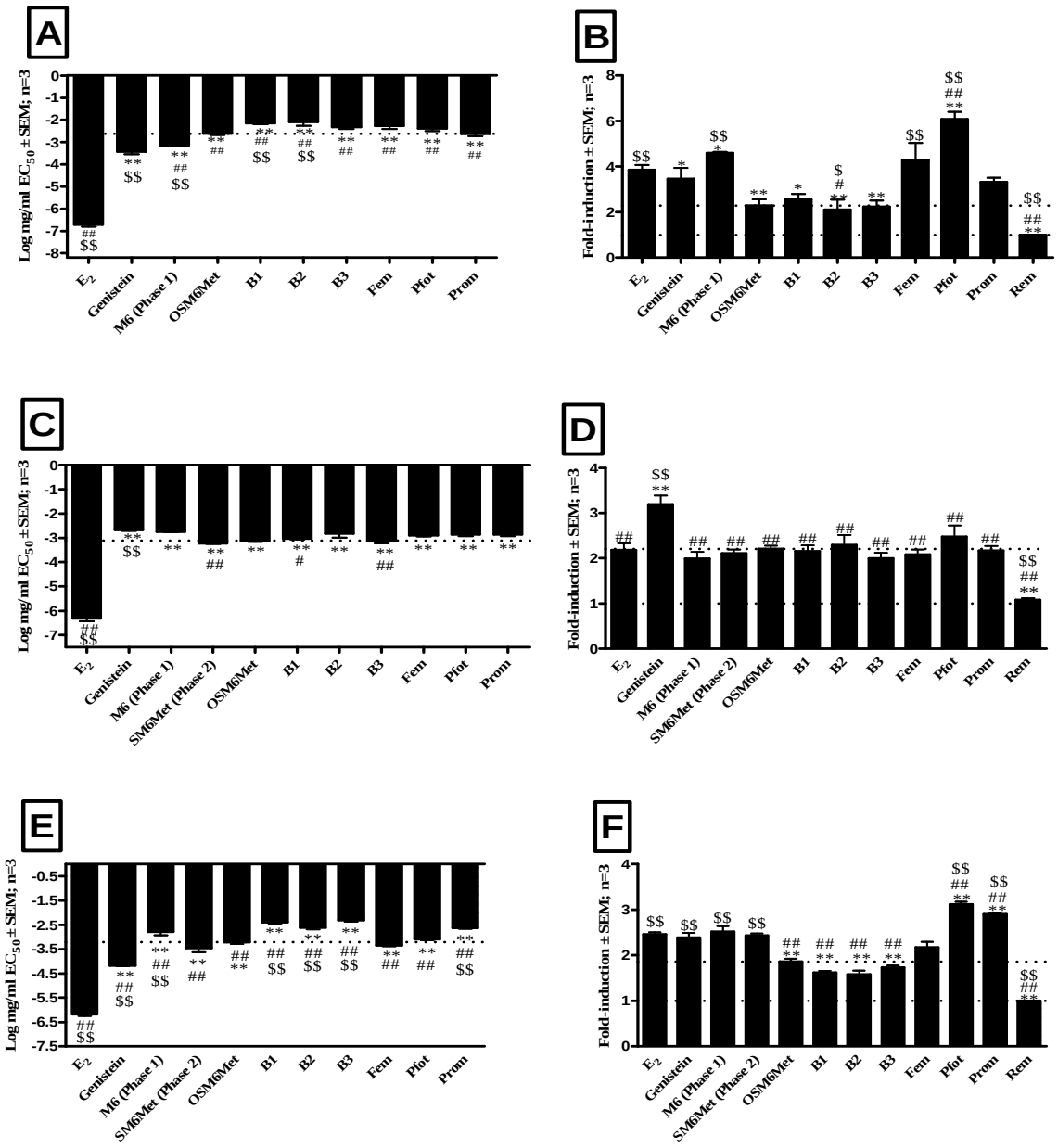

Fig. 6. Comparison of the potency (log EC_{50}) and efficacy (fold-induction) of SM6Met extracts (OSM6Met, B1-B3) with that of commercial extracts. Results were obtained during (A and B) the promoter reporter assay, (C and D) the alkaline phosphatase activity assay, and (E and F) the E-screen. Statistical analysis was done using one-way ANOVA with Dunnett's Multiple Comparison Test as post test. *P<0.05, **P<0.01 if compared to E_2, $^{\#}$P<0.05, $^{\#\#}$P<0.01 if compared to genistein, and $^{\$}$P<0.05, $^{\$\$}$P<0.01 if compared to OSM6Met. The dotted lines through the bars represent log EC_{50} and efficacy values for OSM6Met and 1 for the control.

Considering benchmarking of OSM6Met against the other three commercial extracts, results indicate that OSM6Met has a potency that is not significantly ($P > 0.05$) lower than that of Femolene (Fem), Phytopause Forte (Pfot), or Promensil (Prom) (Figs. 6A, C and E and Supplementary Table 3). Efficacy of OSM6Met is, however, significantly ($P < 0.01$) lower than that of Femolene and Phytopause Forte, but not Promensil, in the promoter reporter assay and significantly ($P < 0.01$) lower than Phytopause Forte and Promensil, but not Femolene, in the E-screen (Figs. 6B and F, and Supplementary Table 4).

Results from the alkaline phosphatase assay, however, indicate no significant ($P > 0.05$) difference between the efficacy of OSM6Met and the commercial extracts (Fig. 6 D and Supplementary Table 4). Similarly, whole cell ER-binding suggest no significant ($P > 0.05$) difference between the log IC_{50} values of OSM6Met and the commercial extracts (Fig. 5 and Supplementary Table 3).

2.8 Discussion

Menopausal women have shown an interest in the use of what they perceive as safer alternatives to standard HRT (31) and commercial phytoestrogenic extracts from plants such as soy, black cohosh and red clover have been seen as a viable option in meeting this need (32). *Cyclopia*, from which honeybush tea is prepared, is another plant with the potential to provide a phytoestrogenic extract as previous studies (23; 33) indicated that *Cyclopia* extracts have phytoestrogenic activity. In an attempt to improve the phytoestrogenicity of the original DME from the honeybush plant we investigated the use of several solvents and extraction methods in a three-phase study.

In Phase 1 we used four assays (whole cell ER-binding, promoter reporter assay, alkaline phosphatase activity and E-screen) to evaluate the estrogenicity of the

DMEs from harvestings M6-M9 and compared parameters to those obtained for the standards, E_2 and genistein. The values obtained for E_2 and genistein (Table 3) compared well with that discussed in the literature for the alkaline phosphatase assay (27; 34-35). Similarly for the whole cell ER-binding, the E-screen, and the promoter reporter assay values are within the ranges obtained by others (26; 36).

When the estrogenic potential of the DMEs are compared to those of the standard test compounds, it is clear from the results of the four assays used in our study that the only DMEs that showed appreciable phytoestrogenic activity were M6 and M7 and that on average M6 is quantitatively more efficacious than M7 (Figs. 2A-D, Supplementary Figs. 2 and 3, and Table 3). Although M8 showed a degree of phytoestrogenic activity in two of the assays (promoter reporter assay and E-screen), the activity was not high enough to obtain EC_{50} values. M9 showed no phytoestrogenic activity in any of the assays (Figs. 2A-D, Supplementary Figs. 2 and 3, and Table 3).

In summary, according to the four assays used in Phase 1, DMEs of only two of the four harvestings under investigation, i.e. M6 and M7, exhibited an appreciable degree of phytoestrogenic activity with regard to potency and efficacy and hence it followed that they were chosen for further analyses in Phase 2 of the study. It could also be determined from Phase 1 that of the four assays used, the E-screen was more useful in discriminating between potencies while the alkaline phosphatase assay was useful in discriminating between efficacies of the test compounds and extracts and it was thus decided that these two assays would be used in Phase 2 of our study.

In Phase 1 we also determined the extract yield and total polyphenol content (TPP) (Table 1) of our DMEs, which was slightly higher than previously reported (23; 33). Since the plant material was not from the same harvesting (harvest time, year and

age of bush differed), this difference could be expected, given the influence of environmental parameters on the phenolic composition of plants (37-38). While the DME of M6 had the highest TPP its total flavonoid content (TF) was the lowest of all the extracts. We would have expected TF, rather than TPP, to possibly predict estrogenicity but our results suggest no such correlation implying that the specific, rather than generic, flavonoid content is important for estrogenic activity.

HPLC analysis (Table 2) comparing the content of known phytoestrogenic polyphenols, eriocitrin (39) eriodictyol (40) narirutin (23) naringenin (41) and luteolin (42), in the DME from M6 with that of the other DMEs clearly indicate that eriocitrin is present in only M6 while eriodictyol and naringenin are absent. It, however, appears unlikely that eriocitrin could be the sole cause of the high estrogenic activity of M6 DME as previous work (33) suggested that eriocitrin can only partly displace ^{3}H-E_2 from ERβ, but not ERα, while in our study M6 DME is able to fully displace ^{3}H-E_2 in MCF-7 cells. In addition, we found that M7 DME also had a relatively high estrogenic activity as compared to that of M8 and M9 DMEs despite the fact that there is not much difference between the content of known phytoestrogenic polyphenols in these three extracts. A more plausible hypothesis to explain the estrogenic activity of the M6 and M7 DMEs is the presence of unidentified, polyphenolic compounds. The M6 and M7 HPLC chromatograms (Supplementary Figs. 1A and B) do indeed reflect the presence of several unidentified compounds that elute between 5 and 20 min, although comparison of all four chromatograms (Fig. 1 and Supplementary Figs. 1A-C) reflects this not to be unique to M6 and M7. Instead, the results show relatively lower quantities of all the unidentified compounds in M6 in comparison to the other DMEs. With the change in separation conditions from that previously used (22), the eriocitrin peak was separated into two in M6 although incomplete (Supplementary

Fig. 1A), showing the presence of a major unidentified compound. We thus support the suggestion of a previous study (23) that unknown polyphenols shown on the HPLC chromatogram (Fig. 1 and Supplementary Figs. 1A-C) probably contribute to the estrogenic activity of *Cyclopia* DME.

Following the selection of M6 and M7 for further analysis in Phase 2, four additional extraction solvents to methanol, i.e. ethyl acetate, ethanol, a 50% methanol-distilled water mixture and distilled water, and two different extraction protocols, sequential and non-sequential, were used to produce extracts of the M6 and M7 harvestings that would possess enhanced phytoestrogenic potencies and efficacies. This undertaking resulted in a total of 22 different plant extracts (Table 4).

For extracts prepared from the M7 harvesting only one, SM7Hlf, showed significantly ($P < 0.01$) improved potency while another, NM7EAc, showed significantly ($P < 0.01$) improved efficacy as compared to the M7 DME from Phase 1 (Figs. 3 and 4C and D, and Supplementary Tables 1A and B, and 2). In contrast, extraction of the M6 harvesting produced five extracts with improved activity (Figs. 3 and 4A and B, and Supplementary Tables 1A and B, and 2). SM6Met and SM6Eth showed significantly ($P < 0.001$) improved potency relative to the DME from M6 in Phase 1. Comparison of these extracts with the standards, E_2 and genistein, suggests that while they may be significantly ($P < 0.01$) less potent than E_2 they displayed a significantly ($P < 0.01$) higher potency than genistein in the alkaline phosphatase assay (Fig. 3A). In the E-screen, however, their potencies were significantly ($P < 0.01$) lower than that of genistein (Fig. 4A). SM6EAc, NM6EAc and NM6Met showed significantly ($P < 0.05$) improved efficacy relative to the DME from M6 in Phase 1 (Figs. 3B and 4B). SM6EAc and NM6EAc had efficacies significantly ($P < 0.05$) higher than E_2 in both assays while their efficacy was higher than that of

genistein only in the E-screen. NM6Met displayed higher efficacy relative to E_2 and genistein only in the E-screen.

It is worth mentioning that the potencies and efficacies of the ethyl acetate extracts, whether obtained sequentially or non-sequentially, were quantitatively similar, with the exception of efficacy values obtained with the alkaline phosphatase assay for SM7EAc and NM7EAc (Figs. 3 and 4). This is to be expected as the ethyl acetate extracts from the sequential and the non-sequential extraction processes are essentially the same, although presented separately, because they were prepared first during both the sequential and the non-sequential extraction processes.

Although the water extracts from the sequential and non-sequential extraction showed no estrogenic activity the "cup-of-tea" extracts from M6 displayed estrogenic activity (Figs. 3 and 4). This indicates that temperature, as by using freshly boiled water in the "cup-of-tea" extraction, could affect phytoestrogenic potential through improved extraction of some of the compounds with estrogenic activity. The beneficial effect of raising the temperature of extraction has been demonstrated for extracts from soybean (43) and should be considered in future studies with *Cyclopia*. In addition, these results suggest that the traditional consumption of *Cyclopia* as a beverage may result in intake of phytoestrogens.

In summary of the outcomes of the second phase of our investigation, SM6Met proved to be the most potent fraction while SM6EAc was the most efficacious. This result carries a disadvantage in terms of the rationale for our study, which is to explore the possibility of using a single extract of *Cyclopia* that is both highly efficacious and potent for the production of a competitive market phytoestrogenic nutraceutical, an outcome that minimizes the production costs of such a product. In an attempt to choose the most suitable extract for benchmarking we

considered the relative increases in potency and efficacy obtained through extraction. SM6EAc is only 1.2-1.3 times more efficacious than SM6Met while SM6Met is 7.2-10.5 times more potent than SM6EAc (Tables 1A and B, and 2). The higher yield obtained for SM6Met was also an important consideration as extract yield would greatly impact on the economic feasibility of producing a nutraceutical product from honeybush. It was thus decided to choose SM6Met as the most phytoestrogenically active extract for investigation in Phase 3.

Other authors have also investigated the use of different solvents for extracting phytoestrogens. For example, extraction of yam with ethyl acetate, but not methanol or *n*-hexane, produced extracts with estrogenic activity (44) while in other studies methanol or ethanol extracts of ginseng root or soybean, respectively, yielded estrogenic extracts (43; 45). Less polar solvents such as acetonitrile has been suggested by some to be more effective for soybean extraction (46) while other authors present data suggesting that more polar solvents, such as methanol-water, extract more estrogenic compounds from soybean (37). Our study suggests that less polar solvents extract more estrogenic activity. Interestingly, we found that ethyl acetate, the least polar solvent we investigated, gave the highest efficacy (SM6EtAc and NM6EtAc) while methanol and ethanol, mid range in terms of polarity of solvents tested, gave the highest potency (SM6Et and SM6Met). The polar solvents, 50% methanol-distilled water or water, extracted little to no estrogenic activity. Other parameters that could affect the effectiveness of extraction are ratio of solvent to plant material (43), extraction method (45; 47), and time and temperature of extraction (37; 43). A study of additional factors that could affect the extraction effectiveness of *Cyclopia* may be considered in future, especially an investigation of the effects of temperature in light of our results with the “cup-of-tea” extraction.

In Phase 3 SM6Met was benchmarked against four commercially available phytoestrogen extracts, Femolene Ultra, Phytopause Forte, Promensil and Remifemin, in order to assess whether a phytoestrogenic nutraceutical formulated from SM6Met would be able to compete, in terms of its potency and efficacy, with commercial phytoestrogen extracts. Remifemin, a black cohosh extract consisting of triterpene glycosides, had estrogenic activity only in whole cell ER-binding (Fig. 5 and Supplementary Tables 3 and 4). These seemingly conflicting results reflect the controversy in the literature regarding the estrogenic effects of black cohosh (48). Some authors report that black cohosh extracts do not bind to ER (49) while others suggest that they do (50). One study has found, as did we, that Remifemin, is not proliferative in the E-screen (51). Binding to ER without agonist effects may be due to anti-estrogenic effects of black cohosh and indeed some authors have shown this (52).

SM6Met was selected for benchmarking in Phase 3 on the basis of potency. Results indicate that the potency of OSM6Met is not significantly ($P > 0.05$) different from that of Femolene, Phytopause Forte, or Promensil in whole cell ER-binding, promoter reporter and alkaline phosphatase assays, and even significantly ($P < 0.01$) higher than Promensil, in the E-screen assay (Figs. 5 and 6, and Supplementary Table 3). Efficacy, however, is significantly ($P < 0.01$) lower than some of the commercial extracts in two assays. If we, however, compare the efficacies of SM6EAc and NM6EAc, which had the highest efficacies in Phase 2, with those of the commercial extracts, it is clear that the ethyl acetate extracts from Phase 2 compare favourably in terms of efficacy. Specifically, the ethyl acetate extracts had 2.6-2.7 and 2.9-3.0 fold-inductions versus the commercial extracts with 2.1-2.4 and 2.2-3.1 fold-inductions in the alkaline phosphatase assay and E-screen, respectively (Supplementary Tables 2 and 4). Thus, although we did not benchmark the ethyl acetate extracts, comparison of

our Phase 2 and 3 investigation results suggest that selection for efficacy would also have given us a favourable comparison as to fold-induction in benchmarking.

To conclude we found that sequential extraction of *C. subternata* (harvesting M6) with dichloromethane, followed by ethyl acetate, ethanol and methanol at a ratio of 6ml/g plant material for 3 hours at room temperature produced an extract (SM6Met) with potency comparable to that of the commercially available phytoestrogenic extracts tested. Efficacy similar to that of the commercial extracts tested could be obtained by following the same procedure, but omitting the ethanol and methanol extractions to produce SM6EtAc. Although other strategies are available to improve estrogenic extraction, extracts of *Cyclopia* prepared in this study certainly have potential for the development of a phytoestrogenic nutraceutical.

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2.10 Supplementary data:

Supplementary Table 1A
Potencies (IC_{50} and relative induction indices (RII)) of extracts of *Cyclopia* as reported by alkaline phosphatase assays in Phase 2.

Standards	**Potency EC_{50} (mg/ml)**		**RII ($IC_{50}E_2/IC_{50}$test compound)**	
E_2	$5.03x10^{-7}$		1	
Genistein	$1.77x10^{-3}$		$2.84x10^{-4}$	
Extracts	**M6**	**M7**	**M6**	**M7**
S-EAc	$4.35x10^{-3}$	$8.09x10^{-3}$	$1.16x10^{-4}$	$6.22x10^{-5}$
S-Eth	7.70×10^{-4}	$1.55x10^{-3}$	$6.53x10^{-4}$	$3.25x10^{-4}$
S-Met	$6.08x10^{-4}$	$1.90x10^{-3}$	$8.27x10^{-4}$	$2.65x10^{-4}$
S-Hlf	$1.60x10^{-3}$	$1.05x10^{-3}$	$3.14x10^{-4}$	$4.79x10^{-4}$
S-Wat	-	-	-	-
N-EAc	$3.24x10^{-3}$	$1.19x10^{-2}$	$1.55x10^{-4}$	$4.23x10^{-5}$
N-Eth	$2.08x10^{-3}$	$1.92x10^{-3}$	$2.42x10^{-4}$	$2.62x10^{-4}$
N-Met	$1.90x10^{-3}$	$2.02x10^{-3}$	$2.65x10^{-4}$	$2.49x10^{-4}$
N-Hlf	$2.27x10^{-3}$	$2.27x10^{-3}$	$2.22x10^{-4}$	$2.22x10^{-4}$
N-Wat	-	-	-	-
“cup of tea”-NM6HDW	$2.84x10^{-3}$		$1.77x10^{-4}$	
“cup of tea”-NM6TDW	$1.84x10^{-3}$		$2.73x10^{-4}$	

Supplementary Table 1B
Potencies (EC_{50} and relative induction indices (RII)) of extracts of *Cyclopia* as reported by E-screen in Phase 2.

Standards	EC_{50} (mg/ml)		RII($IC_{50}E_2/IC_{50}$test compound)	
E_2	$7.33x10^{-7}$		1	
Gen	$6.77x10^{-5}$		$1.01x10^{-2}$	
Extracts	**M6**	**M7**	**M6**	**M7**
S-EAc	$4.37x10^{-3}$	$5.46x10^{-3}$	$1.68x10^{-4}$	$1.35x10^{-4}$
S-Eth	$4.53x10^{-4}$	$4.48x10^{-3}$	$1.62x10^{-3}$	$1.64x10^{-4}$
S-Met	$4.15x10^{-4}$	$5.39x10^{-3}$	$1.77x10^{-3}$	$1.36x10^{-4}$
S-Hlf	$1.78x10^{-3}$	$1.03x10^{-2}$	$4.12x10^{-4}$	$7.16x10^{-5}$
S-Wat	-		-	-
N-EAc	$3.66x10^{-3}$	$5.17x10^{-3}$	$2.01x10^{-4}$	$1.42x10^{-4}$
N-Eth	$2.29x10^{-3}$	$4.53x10^{-3}$	$3.20x10^{-4}$	$1.63x10^{-4}$
N-Met	$1.99x10^{-3}$	$4.17x10^{-3}$	$3.69x10^{-4}$	$1.75x10^{-4}$
N-Hlf	$1.68x10^{-3}$	$4.58x10^{-3}$	$4.37x10^{-4}$	$1.61x10^{-4}$
N-Wat	-	-	-	-
“cup of tea”-NM6HDW	$5.68x10^{-4}$		$1.21x10^{-4}$	
“cup of tea”-NM6TDW	$7.63x10^{-3}$		$1.14x10^{-3}$	

Supplementary Table 2
Efficacies (fold-induction) of extracts of *Cyclopia* as reported by the alkaline phosphatase and E-screen assays in Phase 2.

Test compounds	Assays			
	Alkaline phosphatase		**E-screen**	
Standards				
E_2	2.50		2.39	
Gen	2.49		2.33	
Extracts	**M6**	**M7**	**M6**	**M7**
S-EAc	2.72	1.81	3.02	2.47
S-Eth	2.05	1.80	2.58	2.38
S-Met	2.11	1.78	2.43	2.52
S-Hlf	1.79	1.76	2.14	2.54
S-Wat	1.02	0.98	1.02	1.03
N-EAc	2.61	2.36	2.89	2.34
N-Eth	2.33	1.65	2.67	2.38
N-Met	2.18	1.71	2.70	2.37
N-Hlf	1.95	1.78	2.27	2.44
N-Wat	1.03	0.98	1.02	1.02
“cup of tea”- NM6HDW	1.75		1.78	
“cup of tea”- NM6TDW	1.91		1.66	

Supplementary Table 3

Potency data (EC_{50} (mg/ml) and relative binding affinities (RBA) or relative induction indices (RII)) of extracts of *Cyclopia* as reported by whole cell ER-binding assay (WCB), promoter reporter assay (promoter-reporter), alkaline phosphatase activity assay (AlkP), and E-screen in Phase 3.

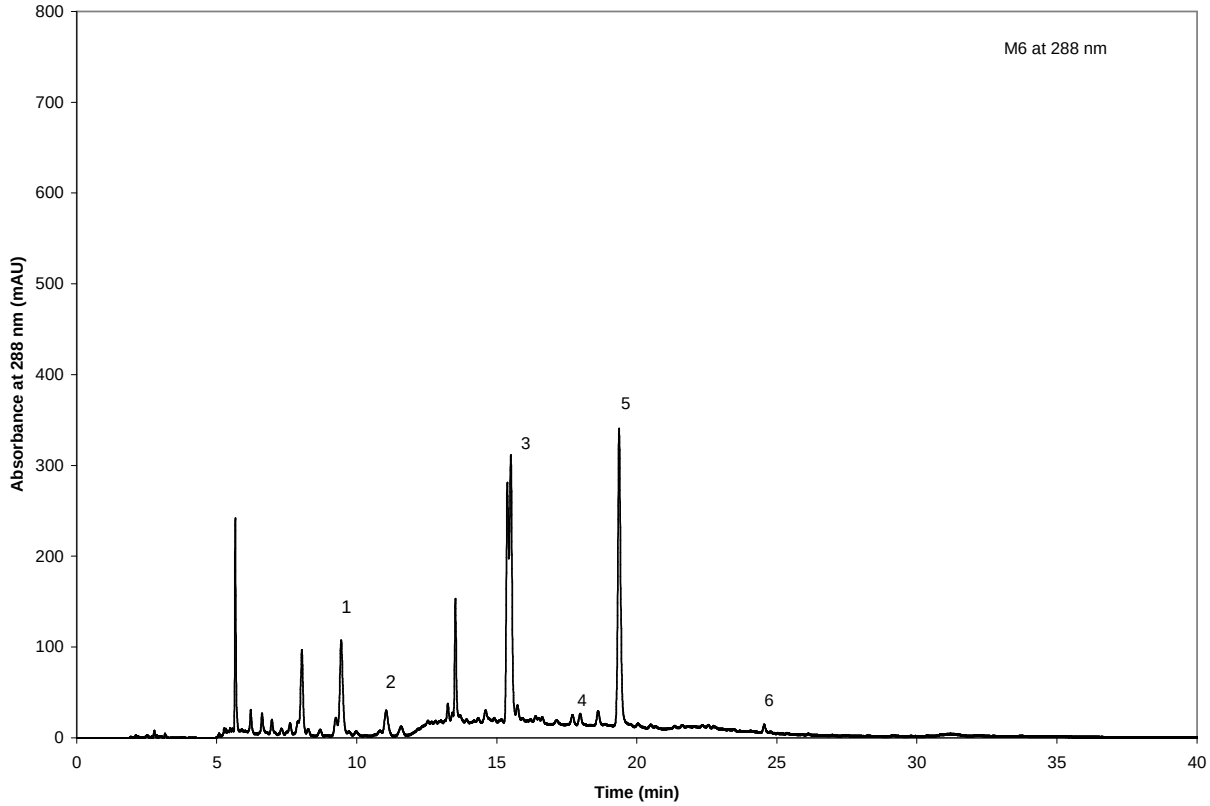
Test compounds	Assays							
	Potency (EC_{50}) mg/ml				**RBA/RII($EC_{50}E_2/EC_{50}$test compound)**			
	AlkP	**WCB**	**E-screen**	**Promoter-reporter**	**AlkP**	**WCB**	**E-screen**	**Promoter-reporter**
E_2	$5.75x10^{-7}$	$1.89x10^{-6}$	$7.00x10^{-7}$	$2.41x10^{-7}$	1	1	1	1
Gen	$2.85x10^{-3}$	$3.55x10^{-4}$	$7.43x10^{-5}$	$4.70x10^{-4}$	$2.02x10^{-4}$	$5.32x10^{-3}$	$9.42x10^{-3}$	$5.13x10^{-4}$
SM6Met	$6.08x10^{-4}$	-	$4.15x10^{-4}$	-	$9.46x10^{-4}$	-	$1.69x10^{-3}$	-
OSM6Met	$8.13x10^{-4}$	$1.89x10^{-3}$	$7.51x10^{-4}$	$1.58x10^{-3}$	$7.07x10^{-4}$	$1.00x10^{-3}$	$9.32x10^{-4}$	$1.53x10^{-4}$
B1	$9.61x10^{-4}$	$1.83x10^{-3}$	$1.24x10^{-1}$	$1.50x10^{-3}$	$5.98x10^{-4}$	$1.03x10^{-3}$	$5.65x10^{-6}$	$1.61x10^{-4}$
B2	$3.02x10^{-3}$	$2.55x10^{-3}$	$2.59x10^{-3}$	$4.90x10^{-3}$	$1.90x10^{-4}$	$7.41x10^{-4}$	$2.70x10^{-4}$	$4.92x10^{-5}$
B3	$8.02x10^{-4}$	$4.34x10^{-3}$	$5.24x10^{-3}$	$5.27x10^{-3}$	$7.17x10^{-4}$	$4.35x10^{-4}$	$1.34x10^{-4}$	$4.57x10^{-5}$
Fem	$1.33x10^{-3}$	$2.90x10^{-3}$	$4.66x10^{-4}$	$4.56x10^{-3}$	$4.32x10^{-4}$	$6.52x10^{-4}$	$1.50x10^{-3}$	$5.29x10^{-4}$
Pfot	$1.59x10^{-3}$	$3.13x10^{-3}$	$7.83x10^{-4}$	$5.02x10^{-3}$	$3.62x10^{-4}$	$6.04x10^{-4}$	$8.94x10^{-4}$	$4.80x10^{-5}$
Prom	$1.55x10^{-3}$	$8.15x10^{-3}$	$2.44x10^{-3}$	$2.45x10^{-3}$	$3.71x10^{-4}$	$7.19x10^{-4}$	$2.78x10^{-4}$	$9.84x10^{-4}$
Rem	-	$2.63x10^{-3}$	-	-	-	$5.75x10^{-4}$	-	-

Supplementary Table 4
Efficacies (fold-induction) of extracts of *Cyclopia* as reported by promoter reporter assay (promoter-reporter), alkaline phosphatase activity assay (AlkP), and E-screen in Phase 3.

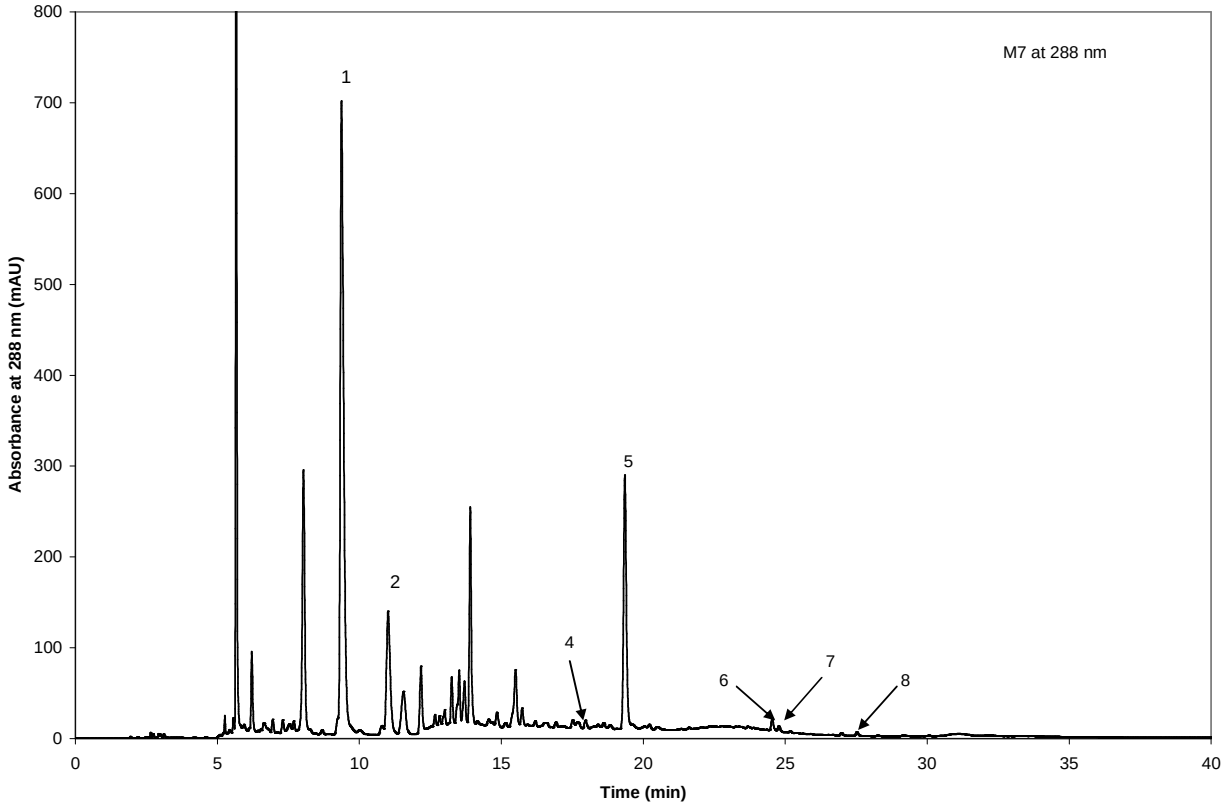
Test compounds	Assays		
	AlkP	**E-screen**	**Promoter-reporter**
E_2	2.18	2.35	3.98
Gen	2.67	2.21	3.42
SM6Met(Phase 2)	2.11	2.43	-
OSM6Met	2.21	1.86	2.28
B1	2.10	1.62	2.55
B2	2.29	1.58	2.10
B3	2.02	1.73	2.24
Fem	2.08	2.17	4.27
Pfot	2.42	3.12	6.08
Prom	2.07	2.90	3.32
Rem	1.08	-	-

Supplementary Figures

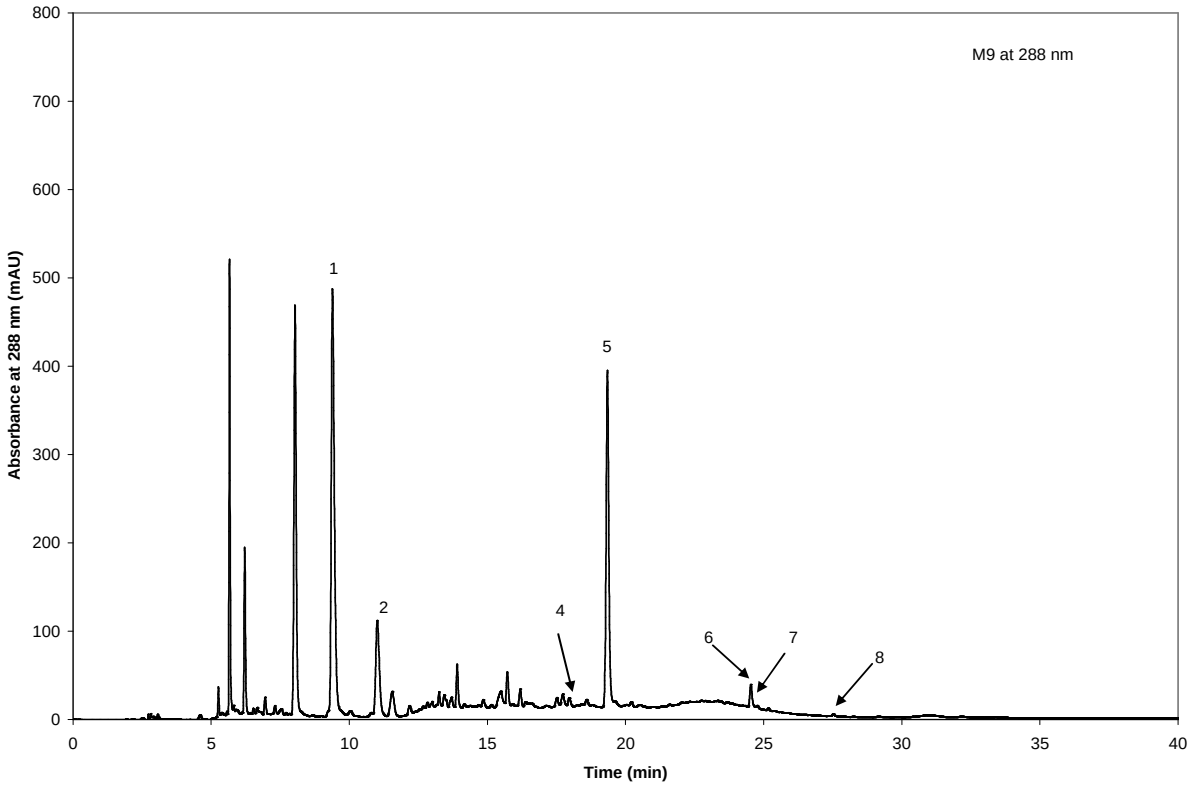
A



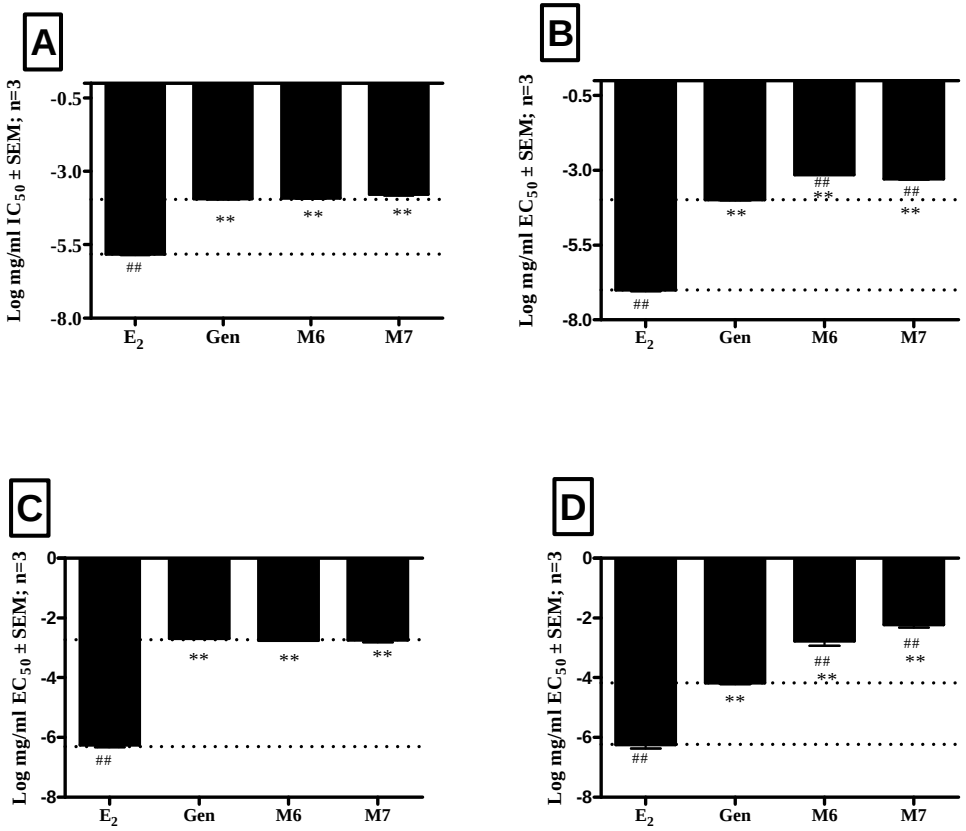
B


M7 at 288 nm
Absorbance at 288 nm (mAU)
Time (min)
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
40
1
2
4
5
6
7
8

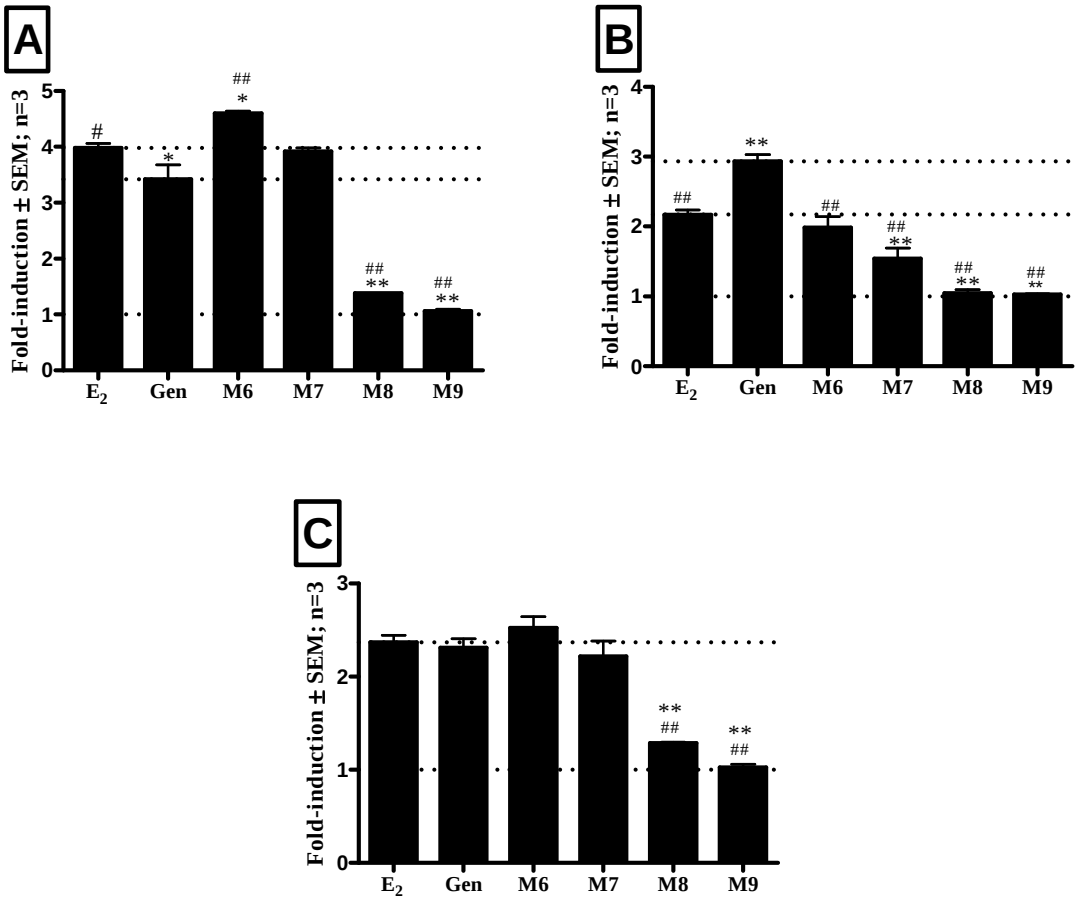
C



Supplementary Figs.1A-C. HPLC chromatograms of DMEs of three *Cyclopia* harvestings, (A) M6, (B) M7 and (C) M9, showing polyphenols (1-8) co-eluting at retention times similar to those of known standards: (1) mangiferin, (2) isomangiferin, (3) eriocitrin, (4) narirutin, (5) hesperidin, (6) luteolin, (7) eriodictyol, (8) naringenin and unidentified polyphenols between 5 and 20 minutes.



Supplementary Fig.2 . The (A) affinity and (B-D) potency data for the four DMEs from *Cyclopia* harvestings (M6-M9) as reported by (A) ER-whole cell binding assays, (B) promoter-reporter assays, (C) alkaline phosphatase activity assays and (D) the E-screen. Statistical analysis was done using one-way ANOVA with Dunnett's Multiple Comparison Test as post test. *P<0.05, **P<0.01 if compared to E_2 and $^{\#}$P<0.05, $^{\#\#}$P<0.01 if compared to genistein. The dotted lines through the bars represent log EC_{50} values for E_2 and genistein.



Supplementary Fig.3. The efficacy data for the DME of the four *Cyclopia* harvestings (M6-M9) as reported by (A) promoter-reporter assays (B) alkaline phosphatase activity assays and, (C) the E-screen. Statistical analysis was done using one-way ANOVA with Dunnett's Multiple Comparison Test as post test. *P<0.05, **P<0.01 if compared to E_2 and $^{\#}$P<0.05, $^{\#\#}$P<0.01 if compared to genistein. The dotted lines through the bars represent efficacy values for E_2, genistein and 1 for the control.

CHAPTER 3

GENERAL CONCLUSION AND DISCUSSION

17β-estradiol (E_2) is an endocrine hormone that regulates important male and female physiological functions related to sexual development and reproduction (1-3). Estrogens express their functions through estrogen receptor (ER) proteins α and β (4). Considering that these receptors are not only expressed in tissue related to sexual function and reproduction but also in other important tissues such as the brain (5), heart (6), liver (7) and bone (8), it is not surprising that imbalances in physiological estrogen levels result in a battery of physiological and psychological disorders (9-14).

Menopause, the natural state of estrogen depletion in women, results in symptoms that include vasomotor disturbances, bone mineral loss, severe fatigue and dementia, which warrant medical attention (15-18). The use of HRT, which has in the past been regarded as the most effective method in the intervention against climacteric symptoms, has recently been found by such studies as the Kronos Early Estrogen Prevention, the Million Women and the World Health Initiative studies to increase the risk of the onset of hormone related breast and ovarian cancers, in addition to promoting the onset of heart diseases, stroke, and pulmonary embolism (19-22). These findings have resulted in reluctance by women to use HRT and simultaneously increased the urgency for safer alternatives to HRT (19; 23). Selective estrogen receptor modulators (SERMs) such as Tamoxifen and Raloxifene, despite being found to be ineffective in the treatment of hot flashes (24-25) and desoite Tamoxifen having been identified as a risk factor for endometrial cancer {Garuti, Cellani, et al. 2006 3548 /id}, have been found to be successful in addressing other climacteric symptoms like osteoporosis without increasing the risk of heart diseases (26-27). Their actions are hypothesized to result from their selective estrogenic and anti-estrogenic actions on various tissues via the two ER isoforms (28).

Phytoestrogens are plant compounds that display estrogenic and anti-estrogenic activities *in vitro* and *in vivo* (29-31). These findings, coupled with epidemiological evidence that suggests their

usefulness in mediating against mutagenic and climacteric diseases, have resulted in their consideration as safer alternatives to HRT for the treatment of menopausal symptoms (31-32). As a result, the phytoestrogenic nutraceuticals market is currently experiencing a substantial growth as menopausal women, who are looking for safer alternatives to HRT, are turning to the use of phytoestrogens for the treatment of vasomotor symptoms (33).

The honeybush plant (genus *Cyclopia*) is an endemic South African fynbos plant that has traditionally been used to treat coughs and other respiratory ailments (34-35). The plant in its oxidized form is, however, popular as a fragrant caffeine-free tea beverage with putative health benefits such as anti-mutagenic and antioxidant effects (34-37). A possible addition to the list of putative health benefits of *Cyclopia* has emerged with the confirmation of the presence of a significant degree of phytoestrogenic activity in methanol extracts of the "green" (unoxidized) form of two species of *Cyclopia*, *C. genistoides* and *C. subtetrnata* (38-39).

The findings by Verhoog *et al*. carry a possibility of the usefulness of *Cyclopia* for the treatment of hormone-dependent diseases such as breast and prostate cancers as well as menopausal symptoms. The potential use of *C. genistodes* and *C. subternata* for the treatment of menopausal symptoms by the general public does of course hinge significantly on the phytoestrogenicity of extracts of these species of *Cyclopia* being comparable to those of existing market phytoestrogenic preparations. The quest to establish the viability of the use of the most active extract of *Cyclopia* from harvestings that were available in bulk so that follow-up studies such as identification of estrogenic compounds could also be carried out to form the basis for the formulation of a competitive market phytoestrogen extract thus served as the rationale and the principal motivating factor for the current thesis.

Our study was based on the use of five organic solvents with different polarities (ethyl acetate, ethanol, methanol, 50% methanol-distilled water and water) and two different extraction methods (sequential and non-sequential) to select an extract of *Cyclopia*, which exhibited the highest estrogenic potency and efficacy. The improvement of the activity of the selected extracts as

well as benchmarking that activity against four commercially available phytoestrogenic extracts were also part of the aims of our study. Also, to assess whether or not there is ingestion of a phytoestrogenic component with the traditional drinking of honeybush tea beverages, we tested the estrogenic efficacy and potency of extracts ("cup-of tea" extracts) prepared in a manner akin to the traditional way of making a cup of honeybush tea by steeping the plant material in freshly boiled water.

The results obtained in our study led to conclusions on key issues that may apply for the improvement of the biological activity of plant extracts in general. Properties of extracts of *Cyclopia*, such as the yield, total polyphenol (TPP) and flavonoid (TF) contents, as well as the presence and concentration of known phytoestrogenic polyphenols, which may influence the estrogenic activity of *Cyclopia* extracts, were investigated in our study. Conclusions drawn from the findings on the influence of these parameters, which are documented in this chapter, have taught us valuable lessons about the level at which the phytoestrogenicity of *Cyclopia* can be determined and about whether or not predictions on the phytoestrogenic activity of *Cyclopia* can, at present, be made for all harvestings of *Cyclopia*. Our study has also highlighted the importance of the properties of the extraction solvent, such as polarity, as well as the importance of water, particularly its temperature on the yield and the estrogenic activity of *Cyclopia* extracts. Also, the occurrence of enhanced efficacy and potency in different extracts of *Cyclopia*, as well as the usefulness of certain *in vitro* assays over others for the detection of differences in these two pharmacological determinants of estrogenic activity, were observed during the course of our study and as such, they were a valuable addition to the set of lessons learnt in our study about the phytoestrogenicity of *Cyclopia* extract. Comparison of the finding of our study with the findings of the previous studies by Verhoog *et al*. (38-39) have also led to several interesting conclusions about the phytoestrogenic activity of the *Cyclopia* extracts that were investigated in our study.

The average dried methanol extract (DME) yields (g/100g dry pulverized plant material) in Phase 1 of our study for both *C. genistoides* and *C. subternata* (Table 1) were higher than those of

the Verhoog *et al* study (39) (18.6 and an average of 11.7 g/100g for *C. subternata* in ours and the Verhoog study, respectively, and 17.5-26.3 and 13.4-18.9 g/100g for *C. genistoides* in ours and the Verhoog study, respectively). Comparing the differences in yields between the water and the methanol extracts between ours and the Verhoog studies, however, revealed similar results. For example, the Verhoog study (39) reported that freshly boiled water extraction resulted in yields that were on average twice their methanol extraction yields (13.5 and 28.7 g/100g for the methanol and the boiled water extracts, respectively), while the results comparing freshly boiled water and methanol extractions from Phase 2 of our study (Table 4) are similar, with freshly boiled water extract yields on average almost 1.7 times the methanol extraction yields (14.6 and 25.0 g/100g for the methanol and the water extracts, respectively). The literature suggests that water can be expected to increase the yield of the extraction process due to the swelling effects of water on the matrix of the plant tissue (40-41).

The comparison between the average total polyphenol content (TPP) of DMEs in Phase 1 of our study (Table 1) and the Verhoog *et al*. study (39) reported comparable TPP values for *C. genistoides* (23.9 and 23.1 in ours and Verhoog studies, respectively) while the *C. subternata* DMEs in our study were reported to have a TPP content that was approximately 1.5 times more than the values reported in the Verhoog *et al* study (39) (33.0 and 22.0 in ours and the Verhoog *et al*. study, respectively). Interestingly, although in Phase 1 of our study, M6 DME, the most phytoestrogenic extract, had the highest TPP value, M6 also had the lowest value of TF (Table 1). Even more intriguing is the observation that M9, the least phytoestrogenic DME in Phase 1 of our study had the highest value of TF content. These findings are indeed a valuable lesson on a lack of correlation between total polyphenol and flavonoid contents and phytoestrogenic activity, a principle that may not only apply to *Cyclopia*, but also to plant extracts in general. The concentration of individual compounds, and not classes of compounds are important, supporting future studies aiming at identifiying estrogenic compounds present in *Cyclopia*.

High performance liquid chromatography of DME in both our and the Verhoog study (39) revealed a common presence of (a) mangiferin, (b) narirutin, and (c) hesperidin in both *C. genistoides* and *C. subternata*. Also, while the Verhoog study (39) detected the presence of eriocitrin in both *C. genistoides* and *C. subternata* DMEs, our study detected eriocitrin only in *C. subternata* (M6). Further, while luteolin was detected in both *C.genistoides* and *C. subternata* in our study, none was detected in the Verhoog study. Verhoog *et al*., conducted a follow-up study (38), evaluating the phytoestrogenicity of DMEs of *C. genistoides* and relevant polyphenols and used LC-MS in addition to HPLC for the identification of putative phytoestrogenic polyphenols. Interestingly, contrary to the findings of their first study, the follow-up study by Verhoog *et al* revealed the presence of hesperidin, luteolin, mangiferin and isomangiferin in *C. genistoides* and no eriocitrin or narirutin as LC-MS revealed that the peaks eluting at retention times similar to those of eriocitrin and narirutin were actually of unknown flavanones with λ_{max} between 280 and 290 nm.

Comparing the HPLC results of *C. genistoides* DMEs between ours and the follow-up study by Verhoog *et al*. (38), it surfaced that although the average contents (percentage of soluble solids) of luteolin (0.13 and 0.098 for ours and the Verhoog studies, respectively) and isomangiferin (3.21 and 4.66 for ours and the Verhoog *et al* study, respectively) were comparable, our study interestingly reported an average content of mangiferin (10.32 and 4.14 for ours and the Verhoog *et al* studies respectively) and hesperidin (3.06 and 1.52 for ours and the Verhoog study, respectively) that were more than twice that reported by the Verhoog *et al* study (38). Also, while the HPLC analysis of *C. subternata* (M6) in our study reported the presence of eriocitrin, it also reported an absence of eriodictyol and naringenin, and a comparatively much lower content of isomangiferin. This is in contrast with the HPLC findings on the three *C. genistoides* (M7-M9) harvestings in our study, which reported a lack of eriocitrin, the presence of eriodictyol and naringenin, and a content of isomangiferin that is on average 5 times higher than the content in *C. subternata*.

As a matter of interest, the overall findings by the first Verhoog *et al*. study (39) were that *C. genistoides* DMEs exhibited significantly higher estrogenic activities that the *C. subternata*

DMEs while our study found the exact opposite, with the *C. subternata* DME (M6) in Phase 1 of our study exhibiting a significantly higher estrogenic potency and efficacy in comparison to a total of three *C. genistoides* DMEs which exhibited lower (M7 and M8) to no (M9) phytoestrogenic activities. More interestingly, the content of known phytoestrogenic polyphenols (narirutin, luteolin, and naringenin (42-45) in our study, and luteolin in the Verhoog study (38) was reported to be so low (0.04-3.30 and 0.09-1.06 g/100g extract in ours and the Verhoog studies, respectively) that it is thought unlikely that the observed phytoestrogenic activities could be attributed to their activities. This observation strongly suggests the presence of an estrogenic compound or compounds in *C. genistoides* and *C. subternata* that has not been detected or identified by the methods used in the two studies, and is the motivation for elaborate fractionation studies scheduled as part of future investigations in our group. Another important conclusion that has been drawn from observing the results of the two studies is that the phytoestrogenic activity of *Cyclopia* is determined at the level of the harvesting rather than at the species or higher levels. This is illustrated by the findings of higher estrogenic activities in *C. genistoides* by Verhoog *et al.* (39) while our study found the opposite result with *C. subternata* having the highest activity, despite significant similarities concerning the presence of known phytoestrogens in *C. genistoides* between the two studies. These results suggest therefore that the phytoestrogenic activity of *Cyclopia* cannot be predicted at this stage and hence a thorough investigation of the estrogenicity of every harvesting is still necessary before certain levels of biological activities can be expected. The unpredictability of *Cyclopia* may be the result of the fact that the plant was harvested from plantations established from seedlings (seeds collected from wild populations), which would results in phenotype and chemotype differences (46). Furthermore, the fact that phytoestrogens themselves are secondary metabolites produced by plants in response to variable environmental stress conditions (47) would aggravate variation in chemical composition.

Apart from learning about the importance of water and its usefulness in improving extract yield, other important conclusions that pertain to the importance of extraction solvent properties,

which were reached after observing the results of the use of 5 solvents with different polarities and two extraction methods (sequential and non-sequential) in our study, are that (i) the polarity of the extraction solvent seems to be important for the enhancement of the phytoestrogenicity of *Cyclopia* extracts, and (ii) the use of the sequential method of extraction yields more active extracts in comparison to the non-sequential method. Our study suggests that the less polar the extraction solvent the more phytoestrogenic the extract obtained. This is concluded from observing the higher phytoestrogenic activities of the extracts prepared using the less polar ethyl acetate, methanol and ethanol solvents in comparison to the 50% ethanol-water and the water extracts that exhibited significantly less to no phytoestrogenic activity. This observation, however, does not seem to hold for all extractions processes as some authors (48) concur with the conclusions from our studies, while others have observed more polar aqueous solvents yielding more biologically active extracts in comparison to less polar solvents (49).

Overall, consistently with the trend observed in Phase 1 of the study, the *C. genistoides* (M7) extracts in Phase 2 of our study were generally less phytoestrogenically active than the *C. subternata* (M6) extracts. As such, only two M7 extracts (SM7Eth and SM7EAc with significantly enhanced potency and efficacy, respectively) could have their phytoestrogenic activity significantly improved as compared to a total of four M6 extracts (SM6Eth and SM6Met with significantly improved potencies and SM6EAc and NM6EAc with significantly improved efficacies).

An exciting conclusion, which also highlights the importance of the influence of extraction solvent temperature, particularly the temperature of water as an extraction solvent, on the enhancement of the estrogenic activity of *Cyclopia*, has been reached from comparing the phytoestrogenic activities of the "cup-of-tea" extracts (NM6HTW and NM6HDW) with the activities of the cold water extracts (SM6Wat, NM6Wat, SM7Wat and NM7Wat) in Phase 2. The "cup-of-tea" extracts clearly demonstrated an enhancement of the phytoestrogenicity of *Cyclopia* extracts by using freshly boiled hot water as an extraction solvent in comparison with water extraction at room temperature. The estrogenic activities of the "cup-of-tea" extracts were

significantly higher than the activities of the cold water extracts, with potencies and efficacies comparable to that of the rest of the extracts tested in Phase 2 according to the alkaline phosphatase assay. The cold water extracts were in fact found to be completely non-estrogenic in contrast to the "cup-of-tea" extracts. These observations are an unequivocal suggestion that raising the temperature of the extraction solvent, particularly water, enhances the estrogenic activity of the extract obtained, and also that there is indeed an ingestion of phytoestrogens with the traditional manner of drinking tea made from the honeybush plant, if consumed as a "green" tea.. The literature also supports the enhancement of the phytoestrogenic activity of plant extracts by high solvent temperatures, as the extraction of isoflavones has been observed to peak at temperatures of 85˚C and above using stirring extraction methods (50). Interestingly, this observation seems to hold only for the classical stirring extraction methods such as the one we used in our study, and is abolished with the use of the more modern extraction methods, as temperatures higher than 40 and 50°C have been observed to be unfavourable for the extraction of isoflavones using methods such as the ultrasonic bath and microwaves, respectively (51-52).

Our study also led to several interesting conclusions about methods used for the detection and enhancement of the phytoestrogenic activity of *Cyclopia* as well as about the nature of the phytoestrogenic activity per se. The use of the four *in vitro* assays (whole-cell competitive binding, ERE-containing promoter reporter assay, alkaline phosphatase induction and E-screen) for the quantification of estrogenic activities of test extracts in Phase 1 of our study revealed the usefulness of the alkaline phosphatase and E-screen for discriminating between efficacies and potencies of test substances over the whole-cell binding and luciferase promoter reporter assays. Another valuable observation from the results of Phase 2 of our study is that the two pharmacological determinants of the biological activity of a test substance (within the context of the aims of our study), potency and efficacy, may not always both be observed equally within the same extract. As such, it has been a lesson in our study that the importance of each of the two parameters (potency and efficacy) may be independent of the other and that the decisions as to which parameter takes precedence over the

other may depend on the context of the desired outcomes from the use of the test substance. This conclusion has been reached after observing that each of the two most phytoestrogenic extracts in Phase 2 of our study possesses only one of the two determinants of phytoestrogenic activity, with SM6Met displaying the highest potency and SM6EAc displaying the highest efficacy. In our study, however, it was decided that the most potent of the two extracts, SM6Met, would be chosen for testing in the follow-up benchmarking in Phase 3 because although less efficacious than SM6EAc, the efficacy of SM6EAc was only 1.2-1.3 times more than the efficacy of SM6Met while the potency of SM6Met was 7.2-10.5 times more than that of SM6EAc.

The results of the benchmarking phase (Phase 3) of our study have led to exciting conclusions about the comparability of the estrogenic activity of our choice extract (SM6Met) to that of commercially available phytoestrogenic extracts. The choice of the market phytoestrogenic nutraceuticals in our study allowed for representation of the three plant species whose phytoestrogenic activity have been well documented in literature, i.e. soy (*Glycine max*) (53-54), red clover (*Trifolium pratense*) (55) and black cohosh (*Cimicifuga racemosa*) (56-57) and which were the main ingredients in Phytopause Forte®, Promensil® and Remifemin®, respectively. Additionally, a nutraceutical (Femolene Ultra®) that is prepared from a mixture of various phytoestrogenically important herbs that include soy, black cohosh, Mexican wild yam (*Dioscorea villosa*), dong quai (*Angelica sinensis*), and the maidenhair tree (*Ginkgo biloba*) was investigated.

The estrogen receptor whole cell binding assays in Phase 3 of our study revealed a curious displacement of radio-labeled estradiol by the black cohosh extract Remifemin, while interestingly, Remifemin showed no phytoestrogenic activity in the rest of the *in vitro* assays used in our study. The curiosity is in the fact that triterpene glycosides, which are supposed to be the active component of black cohosh are not known to bind to the estrogen receptor (58). These seemingly conflicting results may reflect the controversy in literature regarding the estrogenic effects of black cohosh. Authors such as Liu *et al*. (59) have, for example, reported that the constituents of black cohosh extracts do not bind to neither ERα or β while in contrast, Duker *et al* (60) have presented

evidence for the presence of compounds in a black cohosh that do compete *in vitro* with E_2 for binding to receptor sites in a mixed population of estrogen receptors. Bolle *et al.* (61) on the other hand, after observing the activation of ERα by the lipophilic sub-fraction of a black cohosh extract, while the complete extract showed no *in vivo* uterotrophic effects, proposed the presence of a yet unidentified ER isotype through which the SERM activity of the black cohosh extracts is mediated. The ability of Remifemin to bind to the estrogen receptor(s) in our study may therefore be the result of the action of the constituents of the lipophilic sub-fraction of the black cohosh binding to ERα in the mixed ER population of the MCF-7 BUS cells or may even suggest the presence of an unidentified ER isotype (62) present in the MCF-7 BUS breast cancer cells.

Concerning the comparison of the estrogenic activity of SM6Met against the commercial phytoestrogens, the whole cell binding results in MCF-7 BUS cells showed that the affinity of SM6Met for the estrogen receptor to be clearly comparable to the affinities of all the commercial nutraceuticals. Also, analysis of the potency results showed that according to the promoter reporter and the alkaline phosphatase assays, the potency of SM6Met is on par with the potencies of all four commercial phytoestrogens. Notably, the E-screen further revealed a significantly higher potency for our extract of choice in comparison to Promensil. These results suggest that the potency of SM6Met is indeed comparable or even higher than the potencies of the commercial phytoestogens.

The efficacy studies as reported by three assays, the promoter-reporter, the alkaline phosphatase and the E-screen, also revealed very interesting results about the efficacy of SM6Met. Although the alkaline phosphatase assays showed the efficacy of SM6Met to be comparable to that of the four commercial phytoestrogens, the results of the promoter reporter assays and the E-screen showed Phytopause Forte to be significantly more efficacious than SM6Met and all the other commercial phytoestrogens. Also, while the promoter-reporter assays reported Femolene to be significantly more efficacious than SM6Met, the assays also reported SM6Met to be as efficacious as Promensil. Interestingly, the E-screen gave a reversed account of the promoter-reporter assays results by revealing a significantly more efficacious Promensil than SM6Met while Femolene was

reported not to be significantly different to SM6Met with regards to efficacy. Also, although not benchmarked, a comparison of data from Phase 2 and 3 of our study showed that SM6EAc and NM6EAc demonstrated comparable efficacies to commercial phytoestrogenic nutraceuticals, with the alkaline phosphatase assays reporting fold induction values of 2.6-2.7 and 2.9-3 for the *Cyclopia* extracts and commercial nutraceuticals, respectively, and 2.1-2.4 and 2.2-3.1 for *Cyclopia* and the commercial extracts, respectively, according to the E-screen report (Supplementary Tables 2 and 4).

In summary, the overall results of our study demonstrated that the extract of *C. subternata* that was extracted sequentially with methanol (SM6Met) and the extract of *C. subternata* sequentially prepared with ethyl acetate (SM6EAc), have an estrogenic potency and efficacy, respectively, that is comparable to the activities of commercially available phytoestrogenic nutraceuticals and thus these extracts could be used to prepare a commercial phytoestrogenic nutraceutical that could be used to mediate in the treatment of diseases related to hormone perturbations. For future studies, fractionation of SM6Met and/or SM6EAc will be undertaken in order to identify the active ingredients in the extract. *In vivo* studies to test the phytoestrogenic activity of the active components of SM6Met and/or SM6EAc are also on schedule, as these will give an account of effects such as the bioavailability and absorption of the active phytoestrogens in SM6Met. Finally, the mechanism of action of the estrogenic component of *Cyclopia* will also be investigated as part of future studies.

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