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CHAPTER 1

Review of the Literature

1.1. Introduction

Nutraceuticals are a billion dollar industry because the public is more inclined to use natural (herbal) medicine than conventional drugs, as it is believed to have fewer side-effects than synthetic drugs.¹ Unfortunately in the nutraceutical industry, health claims are frequently not supported by the necessary standardisation, safety, quality and efficacy data, as this is not currently required by law. Fortunately this is changing as many countries, including South Africa, are considering altering their legislation pertaining to health claims made for functional foods and nutraceuticals.^{2;3;4} In addition to formulation standardisation, safety and quality assurance, a more thorough understanding of the biological mechanism of action of nutraceuticals and the active compound(s) involved, as well as their efficacy will become a prerequisite for the marketing and associated health claims of new nutraceuticals.

The popular usage of phytoestrogens as an alternative or complimentary herbal treatment in particular has grown in recent years as they are thought to protect against cardiovascular disease, osteoporosis, and a range of hormone dependent cancers and to alleviate menopausal symptoms.^{5;6;7;8;9;10;11;12;13} Epidemiological evidence furthermore supports the usage of phytoestrogens for hormone related conditions. The incidence of breast, endometrial, and prostate cancer, as well as cardiovascular disease, is notably lower in Asian countries as compared to Western populations.^{14;15} This phenomenon is mainly attributed to the Asian diet, especially the high intake of soy, although genetic differences could also have an effect. The fact that second-generation Asian emigrants to the West, who consume a more Western diet, have an increased occurrence of breast cancer as compared to their parents would, however, suggest that the Asian diet does play a more significant role in the prevention of certain cancers.¹⁶ The isoflavone, genistein, present in high abundance in soy, is thought to be one of the key compounds responsible for the low incidence of hormonally induced cancer in the Asian population.¹⁷ Because of the fear of cancer and personal preferences many patients do seek natural alternatives to conventional medicines. In addition, recent reports of increased occurrence of breast cancer and other health risks in menopausal women associated with using conventional hormone replacement therapy (HRT) have gained wide public interest. The two Women's Health Initiative

(WHI) studies, involving clinical trials of menopausal women using HRT, had to be terminated prematurely due to a number of risks associated with HRT.^{18;19} An increased incidence of breast cancer and cardiovascular disease was associated with the estrogen plus progestin treatment, while the occurrence of stroke was found to increase with an estrogen alone treatment.^{18;19} Concerns related to the side-effects of conventional HRT has thus given additional impetus to the use of alternative medicines, as well as sparking renewed scientific investigation into genistein and similar plant phenolic compounds. Caution concerning the health benefits of phytoestrogens should, however, be exercised as the results of smaller clinical trial studies investigating the effectiveness of phytoestrogens for mostly hormone dependent cancers and menopausal symptoms have not been unequivocally positive and as yet no large scale clinical studies have been conducted.^{5;20} Despite this caveat numerous phytoestrogens are already available as nutraceuticals.⁵ These include the ever-popular soy, red clover and black cohosh preparations, all of which contain flavonoids thought to be responsible for their biological effects.^{21;22;10;23;24}

The putative health promoting effects of phytoestrogens have been attributed to a variety of biological processes and mechanisms. The best known mechanism involves that of phytoestrogens mimicking endogenous estrogens through binding to the estrogen receptor (ER) and thereby mediating a weak estrogenic effect. Additionally, they can also bind to other hormone receptors, have antioxidant activity, modulate or interact with steroidogenic and detoxifying enzymes, inhibit angiogenesis, modulate apoptosis and inhibit protein kinases.^{25;26;27;28;29;30} To understand the mechanisms whereby phytoestrogens are able to mediate an estrogenic effect one must firstly understand the role of endogenous estrogens found in the human body. This chapter will thus discuss the literature pertaining to estrogen signalling pathways, followed by an overview of phytoestrogens and their pleiotropic functions in health and disease.

Cyclopia or honeybush, which is part of the rich fynbos kingdom found in the Western Cape, is traditionally consumed as a fragrant herbal infusion and is marketed as such. Honeybush tea has a low tannin content and contains no caffeine.^{31;32} The xanthone, mangiferin, and the flavanone, hesperidin, are the major phenolic compounds present in *Cyclopia*³³. Honeybush has been identified as having putative phytoestrogenic activity through anecdotal evidence as well as through its phenolic composition.^{34;35} Formononetin, naringenin and eriodictyol present in *C. intermedia* and luteolin present in both *C. intermedia* and *C. subternata*^{34;35} have for example

been shown to have phytoestrogenic activity.^{36;37;38} The honeybush tea industry is still a relatively young industry with commercial plantations only recently being established (Personal communication, Dr E Joubert, 2004) to replace or supplement harvesting from the wild. This is especially important when considering phytoestrogens present in harvested plant material, as conditions and treatments of plants affect their phenolic composition.^{39;40;41;42} Honeybush tea has already been shown to have numerous other biological properties such as antioxidant and antimutagenic activity^{43;44;45;46} and evidence of phytoestrogenic activity in *Cyclopia spp.* would substantially increase and expand the range of its functional food status and usage. In addition, it could be important to the future of the industry as concentrated *Cyclopia* extracts rich in phytoestrogens could be made available to the public as a uniquely South African nutraceutical. As the thesis aims to investigate the phytoestrogenic activity of *Cyclopia* the most commonly used assays and techniques used to evaluate estrogenicity will be discussed in this chapter, as will be *Cyclopia*, both in general and as a possible source of phytoestrogens. Finally, the literature will be summarised within the context of the aims of the thesis.

1.2. Estrogen and estrogen signalling

Estrogen, a steroid hormone, is mostly known for its role in regulating female reproductive activity and is most commonly known as the female sex hormone. However, estrogens are responsible for numerous key functions in growth and differentiation in both sexes. They exert their physiological role in various target tissues, such as the male and female reproductive tracts and the skeletal and cardiovascular systems.⁴⁷ Estrogens have a protective effect against osteoporosis, coronary heart disease, and Alzheimer's disease. They do, however, also negatively influence diseases such as breast, endometrial, ovarian and prostate cancer.⁴⁸ This steroid hormone has the ability to affect the rate of cell proliferation, which in turn is hypothesised to increase the amount of errors during deoxyribonucleic acid (DNA) replication, which has been implicated in hormone dependent cancers.⁴⁹ Estrogens therefore have widespread reproductive and non-reproductive actions both in health and disease.

Estrogens occur naturally in the female body as 17- β -estradiol (E_2), estrone (E_1), and estriol (E_3) (Figure 1). All are C_{18} steroids and are derived from cholesterol. E_2 is the most potent of these hormones as it has the highest affinity for the estrogen receptors (ERs). In women, E_2 is

1.2.1 Bioavailability of estrogens and SHBG

Like all steroids, estrogens are highly lipid soluble and are able, after synthesis, to diffuse through cell membranes into the bloodstream. In the bloodstream the majority of estrogens are bound to sex hormone binding globulin (SHBG) or albumin with only about 2% of estrogens present in the plasma unbound. SHBG, also known as testosterone-estrogen binding globulin (TeBG) or sex binding protein (SBP), is a 93.4 kDa glycoprotein synthesized by liver cells and contains 373 amino acid residues.^{51;52} This protein is able to bind endogenous sex hormones with high affinity with between 40-70% of estrogens and androgens bound to the SHBG in humans.⁵³ Dihydroxytestosterone has the highest affinity for SHBG, followed by testosterone and E₂. The binding equilibrium constant of E₂ is equal to 4 nM for human SHBG and 80 nM for rabbit SHBG.⁵⁴

SHBG is thought to influence the bioavailability of sex hormones as it regulates the plasma concentration of unbound or free steroids. Only free (unbound) estrogens are biologically active and are able to diffuse through the cell membrane into target cells.^{55;56} SHBG is thus traditionally thought of as regulating the concentration of free or unbound steroids by acting as a reservoir and subsequently influencing the equilibrium between free and bound steroids, important in estrogen signalling. Furthermore, by acting as a reservoir SHBG is also considered to protect circulating sex hormones from degradation and excretion.⁵⁷

The role of SHBG as carrier and regulator of free sex steroid concentrations, commonly referred to as the free hormone hypothesis, has been challenged as it has been suggested that if only unbound steroid is available to mediate a biological response, this would imply that all tissues would be exposed to similar concentrations of steroid.⁵⁸ In addition, as SHBG concentration in plasma is quite substantial, not enough steroid would be available for maximal induction.⁵⁸ Thus the function of the SHBG may be more complex and it has been proposed that SHBG-bound steroid is available to mediate a biological response.^{58;59} The discovery of plasma membrane SHBG receptors, present on hormone sensitive tissue, lends support to the idea that SHBG may have additional regulatory functions such as directing steroids to their target cells as only unliganded SHBG is able to bind to these receptors.^{60;61} In addition, the subsequent binding of ligands to the SHBG-membrane receptor complex induces intracellular effects such as an increase in intracellular cyclic adenosine monophosphate (cAMP)^{60;62;63} and inhibits E₂-induced proliferation of MCF-7 breast cancer cells.⁶⁴ A recent paper has shown that pre-incubation of

SHBG with MCF-7 cells causes inhibition of the anti-apoptotic effect of E₂ by preventing the E₂-induced phosphorylation of extracellular regulated kinase (ERK)-1/-2, a member of the mitogen-activated protein kinase (MAPK) family that mediates the anti-apoptotic effect of E₂ in breast cancer cells, without affecting nuclear ER-mediated transcription.⁶⁵ Numerous factors, which include SHBG concentration, the binding affinities and concentrations of various steroids for SHBG, and most importantly the number of steroids or compounds, both endogenous and exogenous that compete for binding to the SHBG influence the equilibrium of free *vs.* bound steroids. Thus it is important to determine whether exogenous steroid-like compounds are able to bind to SHBG and compete with endogenous steroids as this could influence endogenous steroid concentrations, by affecting metabolic clearance rates, and subsequent biological activity, by affecting free or unbound concentrations of endogenous steroids. Similarly, compounds, which are able to modulate the concentration of SHBG levels, can influence the equilibrium of free *vs.* bound steroids by affecting the amount of biologically active steroids.⁶⁶ Therefore SHBG plays an important role in the subsequent molecular function of estrogen signalling.

1.2.2. Molecular mechanism of estrogen signalling

Estrogen is able to elicit a biological response essentially through four signalling mechanisms as depicted schematically in Figure 2. They are (1) the classical pathway of estrogen signalling, which in short entails ligand binding to the ER, which in turn interacts with the estrogen response element (ERE) situated in the promoter region of estrogen responsive genes, (2) the ERE independent pathway, (3) the ligand independent pathway, and lastly (4) the non-genomic pathway. Although these pathways differ from each other mechanistically, they are all essential for estrogenic signalling and more than likely to act synergistically.^{67,68}

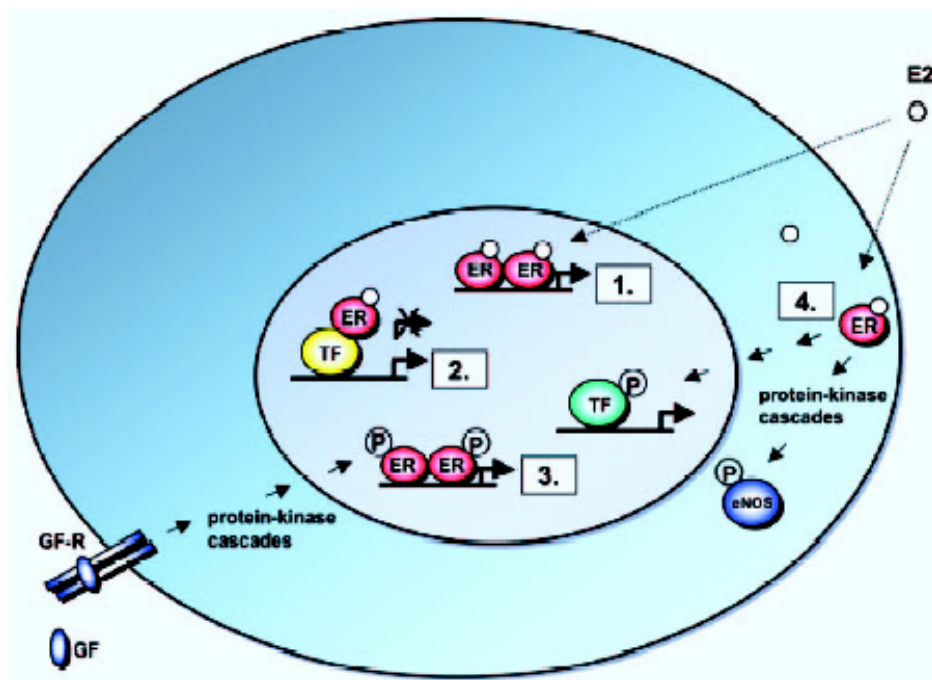


Figure 2: The numerous pathways through which estrogens and the ERs are able to mediate a biological response: (1) Classical ER pathway, (2) ERE-independent pathway, (3) ligand-independent pathway and (4) non-genomic pathway.⁶⁷ Abbreviations: GF-R: Growth factor receptor; GF: Growth factor; TF: Transcription factor; ER: Estrogen receptor; P: phosphorylated; E₂: 17-β-estradiol; eNOS: endothelial nitric oxide synthetase.

1.2.2.1. The classical estrogen signalling pathway

The classical estrogen signalling pathway (Figure 2) depends on the ligand bound ER interacting directly with the promoters of estrogen responsive genes, which results in an estrogenic response.

The biological effects of estrogens are mainly mediated through the two ERs, ER α and ER β . These two receptors are members of the steroid receptor family, together with the glucocorticoid receptor, androgen receptor, mineralocorticoid receptor, and the progesterone receptor (PR). The steroid receptor family is part of the nuclear receptor superfamily.^{69,70} The steroid receptors are ligand-dependent transcription factors and can translocate between the nucleus and cytosol where they are chaperoned by heat shock proteins until they are activated through binding of an appropriate ligand. On ligand binding the receptors undergo a conformational change, which results in the translocation of the receptor-ligand complex to the nucleus if the receptor is cytoplasmic. The ER, unlike the other steroid receptors is already mainly

present in the nucleus even in the absence of ligand.⁷¹ The activated ligand bound receptor can then dimerize and bind to specific DNA response elements, the ERE. The ERE, which is 13 base pairs long, consists of two identical sequences arranged as palindromic inverted repeats with a 3 base pair spacing of variable bases. The consensus sequence of the ERE is 5'-GGTCAnnnTGACC-3'⁷², however, most EREs present in the human genome are non-consensus sequences. The ERE sequence differ from other steroid receptor response elements, allowing only the ER to recognise it. These response elements are *cis*-acting enhancers located within the promoter region of target genes. Receptors bound to DNA are able to recruit general transcription machinery either directly or indirectly *via* co-factor proteins. The expression of the downstream target genes could then either be positively or negatively affected.⁷³ The two ER subtypes present are essential for an estrogen response *via* the classical signalling pathway and will be discussed in more detail.

1.2.2.1.1. Structure and functional domains of the ER subtypes

The ER α subtype was first cloned in 1986⁷⁴ whereas the ER β was only identified ten years later in rat prostate⁷⁵, human testes⁷⁶, and in mouse ovary.⁷⁷ Two distinct genes situated on different chromosomes encode each receptor subtype. The human ER α is located on chromosome 6⁷⁸, whereas ER β is situated on chromosome 14⁷⁹. The human ER α gene, ESR1, is 140 kb long and contains 8 exons and 7 introns and the hER α protein contains 595 amino acids (66.2 kDa).^{74;80;81} The gene for the human ER β , ESR2, is only 40 kb long, but also consists of 8 exons and 7 introns, while the protein consists of 530 amino acids (59.2 kDa), which is thus shorter than the ER α subtype protein.^{76;81} The two ERs, do share a high degree of homology in certain domains regardless of being encoded on different genes and therefore translating into different proteins (Figure 3).

The two ER subtypes are highly homologous in the DNA- and ligand-binding domains.⁸¹ The DNA-binding domain (DBD) is the most conserved region in all the steroid receptors. It is situated centrally and is responsible for interacting with DNA, i.e. the ERE in the case of the ERs. It contains two zinc finger motifs, involved in specific DNA-binding and receptor dimerization. The proximal box, or P-box as it is known, a motif situated within the DBD, is critical for receptor-DNA recognition and specificity. The P-box, similar in both ER subtypes, consists of six

amino acids, CEGCKA, at the C-terminal of the first Zn finger.⁷⁰ The DBD also contains the motif called the dimerization-box or D-box situated at the N-terminal half of the second Zn finger, which assists in stabilizing the receptor-DNA complex after dimerization. The DBD is greatly conserved between the two ER subtypes, having 97% amino acid similarity and thus, both receptors recognize and bind to the same DNA response elements (EREs).

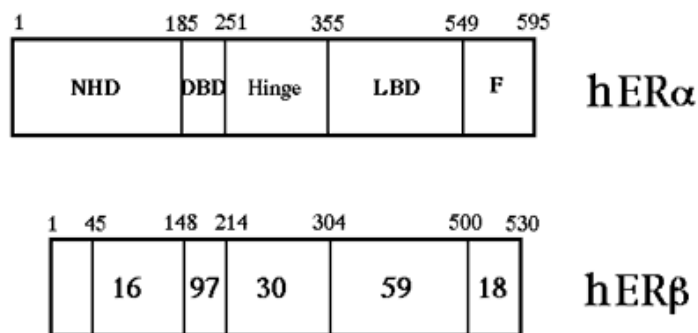


Figure 3: Comparison of the amino acid sequences of the two human ER subtypes. The separate functional domains are identified within the ER α diagram while the numbers within the ER β diagram show the sequence homology as a percentage. The numbers shown above the diagrams of the receptors represent the position of the amino acids. Abbreviations: NHD: N-terminal domain; DBD: DNA-binding domain; LBD: ligand-binding domain.⁸¹

The ligand-binding domain (LBD) is situated at the carboxy-terminal end of the receptor and is to some extent conserved between the two ER subtypes, with 59% amino acid homology. Both ER subtypes are able to bind E₂ with similar affinity, however, this does not hold for all ligands. Phytoestrogens, for example, bind to both ERs, but with a higher affinity to the ER β subtype.⁸² This selectivity is a result of the different amino acid compositions of the LBDs in ER α and ER β . The LBD is important not only for ligand binding as its name suggests, but also for receptor dimerization, nuclear translocation and interaction with transcriptional co-regulators. The LBD also contains the transcription activation-function-2 (AF-2) site. The AF-2 site contains a highly conserved C-terminal amphipathic α -helix, helix H12. This helix is important for ligand recognition and binding and subsequent recruitment of co-activators, and is present in both ER subtypes.^{83;84} Binding of both agonist and antagonist ligands induces a conformational change of the ER. The conformational change induced by agonist allows for the recruitment of co-regulators, while binding of antagonists results in a conformational change where H12 is positioned in such a manner that it is unable to interact with co-regulators.^{70; 83;84}

The N-terminal is less conserved between the two ER subtypes. This domain contains a transcription activation-function-1 (AF-1) site. The AF-1 is important for site-specific phosphorylation and is involved in ligand-independent activity of the receptor (discussed in section 1.2.2.3). It is, however, promoter and cell specific.⁸⁵ The AF-1 and AF-2 regions act synergistically to recruit various co-regulator proteins to the DNA-ER complex to induce full activation of transcription⁸⁶, furthermore, only partial activation of transcription *via* ER is observed when only the AF-1 region is involved in recruitment of co-activators.⁸⁷

1.2.2.1.2. Mechanism of action and functions of the ER subtypes

As transcription factors both ER subtypes are capable of stimulating transcription of ER target genes in a similar manner through binding to the classical ERE followed by the recruitment of co-regulators. This is not surprising as they are highly homologous in the DBD (section 1.2.2.1.1) and thus expected to recognise the same DNA sequences. ER α , however, usually exhibits higher ERE transactivation activity than ER β in many cell types.^{88;89;90} The level of transcriptional activation *via* the ER β is dependent on the cellular and promoter context and the type of ligand.⁹¹ Although some ligands have different binding specificity for the two ER subtypes, the natural ligand, E₂, appears to have a similar affinity for both ERs (Table 3) while phytoestrogens preferentially bind to the ER β .⁸²

The induction of gene transcription is dependent on ER dimerization. The ER subtypes are able to form functional heterodimers in cells that contain both subtypes such as the mouse mammary gland^{89;92;93} with heterodimers pre-dominating over the homodimers.⁹⁴ The heterodimers bind to the consensus ERE sequence with similar affinity to that of the ER α homodimers and with greater affinity than that of the ER β homodimers.⁹⁴ Additionally, it has been demonstrated that when co-expressed, ER β exercises an inhibitory effect on ER α mediated transcriptional activation.^{89;95} It has been suggested that the antagonistic effect of ER β on ER α and its lower transactivation activity is due to differences in the transactivation regions of the two subtypes.⁹⁶ The N-terminal AF-1 region of ER β is transcriptionally weaker than that of ER α , which could suggest its repressive action^{90;91}, and when removed, the transcriptional activity of ER β increases⁸⁹ further substantiating its repressive role. It has been proposed that when both ER subtypes are co-expressed in cells, the net estrogen responsiveness of these cells is dependent on

the ER α :ER β ratio.⁸⁹ Hence, to summarize, ER β appears to reduce gene transcription up-regulated by ER α probably through preferential formation of ER α :ER β heterodimers. These heterodimers compete effectively with ER α homodimers in binding to DNA and display a lower transactivation potential than the ER α homodimers.⁹⁴ These findings are substantiated by *in vivo* studies.

By comparing gene expression levels, using DNA microarray analysis, of wild type mice with α ERKO (ER α knockout) mice, it was shown that ER β reduces the overall gene expression in bone and liver tissue in the presence of ER α .⁹⁷ In the absence of ER α , i.e. α ERKO mice, ER β can fulfil the role of ER α , although gene transcription is not as high as in wild type mice, which express both ER subtypes.⁹⁷ However, not all pathways are compensated for by ER β in the absence of ER α .⁹⁸ Although physiologically the α ERKO mice of both sexes are infertile and estrogen insensitive, certain pathways in the α ERKO mice remain intact.⁹⁹ In contrast to the α ERKO mice, β ERKO mice remain fertile although the females do have some limitations in ovarian function.¹⁰⁰ Some phenotypes in these mice were less pronounced than expected e.g. the testes in the β ERKO and the bone tissue of both α ERKO and β ERKO mice. These results suggest that the two ER subtypes can compensate for each other to a certain extent.

In cells or tissue, which co-express both ER subtypes the expression levels of the ER subtypes have been found to be altered in certain tumour types. Healthy human mammary tissue predominantly expresses ER β mRNA while most ER-positive breast tumours express increased ratios of ER α :ER β .^{101;102} This phenomenon was also observed in ovarian cancer cells as compared to normal tissue.¹⁰³ This further substantiates the need for investigation into compounds such as phytoestrogens, which bind with a higher affinity to ER β than ER α , to modulate the proliferative effect of ER α in estrogen-dependent cancers.

1.2.2.1.3. Tissue distribution of the ER subtypes

Though some functional domains are highly conserved between the two ER subtypes and therefore exhibit similar roles, the ER subtypes also have distinctive functions in estrogen action as discussed above. Tissue localization studies showed different distribution patterns for the ER subtypes (Figure 4). A study conducted examining the distribution of the ER subtypes in rats revealed that the ER α is expressed mainly in the uterus, testis, pituitary, kidney, epididymis, and

adrenals, whereas ER β is expressed mainly in the prostate, lung, bladder, and brain.¹⁰⁴ They do, however, have some overlapping tissue distributions such as the ovaries, mammary gland, epididymis, thyroid, adrenal, and bone.^{75;76;104} Tissue distribution in humans is demonstrated in Figure 4. As discussed earlier (section 1.2.2.1.2) ER subtype levels tend to change with the development of tumour growth.¹⁰¹

Expression in the same tissue might, however, not indicate that they are co-expressed in the same cell type.¹⁰⁵ Nevertheless, it does not exclude co-expression as neurons⁹² and the epithelial cells of the mammary gland¹⁰⁵ have been shown to co-express both ER subtypes. The time of expression may also differ as in the case of the uterus and pituitary gland where ER β is expressed during development and ER α is only expressed in mature tissue.¹⁰⁶

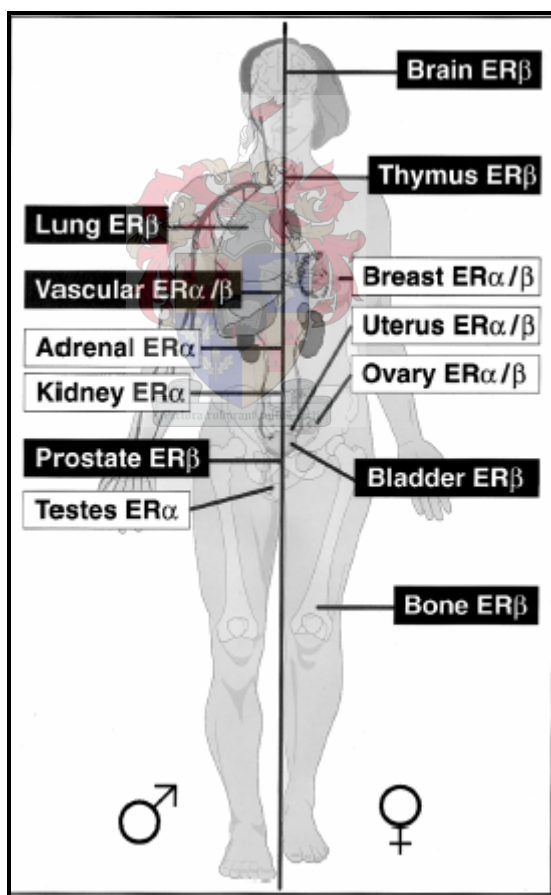


Figure 4: The tissue distribution of the ER subtypes in humans. A simplified diagram as per Setchell and Cassidy.¹⁰⁷

1.2.2.2. ERE-independent genomic actions of the ER

A wide variety of genes regulated by estrogens do not contain an ERE or ERE-like sequence in their promoter regions. The ER can still regulate gene expression independently from the ERE. Firstly, without direct DNA binding, depicted as pathway (2) (Figure 2) or secondly, by binding to other DNA response elements. In pathway (2) ligand-bound ER is able to interact with other transcription factors *via* protein-protein interactions thereby regulating gene transcription without DNA binding (also called a tethering mechanism). The second ERE-independent mechanism, however, does not involve protein-protein interaction. The ER binds to response elements other than the ERE. The mechanism involved is similar to that of ERE recognition, except a different response element is involved. The following section will discuss both the tethering mechanism and recognition of a DNA response element other than the ERE.

The ER through protein-protein interactions with other transcription factors can mediate gene transcription. These transcription factors include nuclear factor κ B (NF- κ B), Fos-Jun and steroidogenic factor 1 (SF-1) that directly recognise and interact with their specific DNA sequences. Transcription factor NF- κ B, amongst others, is responsible for interleukin-6 (IL-6) expression. Interaction of the ER with NF- κ B inhibits the expression of IL-6 with ER β demonstrated to be more potent in its repression of NF- κ B than ER α .¹⁰⁸ Similarly, the ER is able, through protein-protein interactions, to interact with Fos and Jun, two transcription factors that recognises activating protein-1 (AP-1) binding sites. However, at these sites ER α transactivates, while ER β inhibits transcription. It was suggested that because ER β contains a weaker AF-1 domain it is unable to activate gene transcription of genes that contain an AP-1 responsive element in their promoter region in the presence of an agonist. In fact, E₂ when bound to the ER β , acts as an antagonist when positioned at AP-1- binding sites. The ER β , however, in the presence of an antiestrogen, such as ICI 164,384, is able to activate transcription.¹⁰⁹ Such AP-1 activation by the traditional ER antagonist was suggested to be an AF-1- and AF-2 independent mechanism that only requires an intact DBD¹¹⁰ again highlighting the diverse physiological actions of the ER subtypes. In addition, the ERs have also recently been shown to interact with the SF-1 transcription factor.¹¹¹ Genes activated by an ER-SF1 complex in the presence of E₂ include the low-density lipoprotein (LDL)-receptor, *c-Fos*, and cyclin D genes and depends on the

ER subtype, ligand, and cell type.¹¹¹ The ER subtypes have therefore been shown to interact with other transcription factors and do therefore not act *via* DNA interactions alone.^{108;109;111}

The ER subtypes are also able to interact with DNA response elements other than the ERE although the same mechanism applies. The liganded-ER complex is able to bind directly to the antioxidant response element (ARE). The ARE is a *cis*-acting DNA element situated in the promoter region of some of the phase II detoxifying enzymes such as the glutathione-S-transferases (GSTs) and quinone reductase (QR). The phase II detoxifying enzymes protect the cells from oxidative damage by conjugation or reduction of reactive species, metabolites of the phase I enzymes, which are members of the P450 superfamily. In COS-1 cells E₂ and genistein repress gene transcription through both ER α and ER β , while the antiestrogen, ICI 182,780, induces transcription of ARE regulated genes through both ER subtypes with ER β being a stronger inducer of the ARE.¹¹² Selective ER modulators (SERMs) such as tamoxifen, however, show receptor subtype selectivity by activating *via* ER β , while transrepressing *via* ER β . Although ARE mediated activity *via* endogenous estrogens appears to be highly dependent on the cell type¹¹³ administration of E₂ to mice also decreased phase II detoxifying enzyme activity in the uterus, which confirms endogenous estrogen activity on AREs *in vivo*.¹¹³ The interaction of the ER subtypes with the ARE will subsequently affect the oxidative levels of cells or tissues and one would expect that ligands preferentially activating *via* the ER β would have an important role to play in protecting cells from oxidative damage and consequent cancer formation.

In summary, E₂ genomic signalling includes both DNA-protein interactions and protein-protein interactions (tethering mechanism). Not only are the ER subtypes able to recognize the ERE they are also able to interact with the ARE with ER β being a stronger activator of gene expression in the latter case but not in the former. The ER subtypes are also able to regulate gene transcription through tethering by binding to other transcription factors such as NF- κ B, Fos-Jun, and SF-1. ER β is more potent than ER α in repressing gene transcription *via* NF- κ B whereas *via* Fos-Jun it inhibits AP-1 induced gene transcription while ER α induces transcription. The responses of the ER subtypes in both mechanisms seem to differ and are an important avenue for future investigation.

1.2.2.3. Ligand-independent actions of the ER

The ERs can be activated in the absence of ligand by means of phosphorylation through cellular signals such as growth factors, neurotransmitters, cyclins and protein kinase activating agents¹¹⁴, which in turn are able to cross talk with other signalling pathways (pathway 3, Figure 2).

The growth factors, insulin-like growth factor-I (IGF-I)¹¹⁵ and epidermal growth factor (EGF)^{114;116;117}, have been shown to activate the ER in the absence of ligand. Both growth factors were shown to induce protein kinases, which subsequently activated the ER through phosphorylation.^{115;116} It was shown that EGF activates the ER in the absence of ligand through the MAPK pathway^{114;116}, which directly phosphorylates Ser¹¹⁸ positioned at the N terminal of the ER. Phosphorylation of the serine residue at position 118 (Ser¹¹⁸) is necessary for the activation of AF-1 and the N terminal transcriptional AF-1 is required for the activation of unliganded ER by the EGF.^{114;117} Specifically, EGF was shown to mimic the actions of estrogen in that the ER was able to translocate to the nucleus whereby it induced uterine DNA synthesis in ovariectomized mice.¹¹⁸ In addition, it has been shown that in the presence of E₂ the transactivational response is greater than that achieved in the absence of ligand suggesting that both ligand independent and ligand dependent activation can concur.¹¹⁷ Serine residues in ER β similarly can also be phosphorylated *via* the MAPK pathway.¹¹⁹

Similarly, the neurotransmitter dopamine, and Cyclin A induce ER dependent transcription in the absence of ligand through phosphorylation.^{120;121;122} Cyclin D1, expression of which is regulated by E₂, also induces ligand-independent ER activation; however, this occurs independently of phosphorylation.¹²³ Ligand-independent activation of the ER can also occur *via* cAMP. An increase in cAMP activates protein kinase A (PKA) and ER activation *via* the AF-2 is thought to be dependent on PKA.¹¹⁷ This is rather interesting as the AF-2 region is thought to be the classical ligand dependent activation region.¹²⁴

To summarise, activation of the ERs in the absence of ligand does occur, which results in increased expression of ER targeted genes. Phosphorylation appears to be the main mechanism of ligand-independent activation identified to date (Table 1).

Table 1: Summary of mechanisms of ligand independent activation of ERs

Activator	Mechanism
Peptide Growth Factors: IGF-I ¹¹⁵ EGF ¹¹⁷	Activates second messenger systems e.g. MAPK that cause phosphorylation of ERs
Neurotransmitters: Dopamine ¹²⁰	Might be phosphorylation, the exact mechanism is yet unsure
Cyclins: cyclin-D1 ¹²³ cyclin-A ¹²²	Does not require phosphorylation Activates the ER through phosphorylation of the AF-1 domain
Other: MAPK ¹¹⁶ cAMP ^{115;117}	Phosphorylation Phosphorylation of the AF-2 domain

1.2.2.4. Non-genomic effects of estrogens

The classical estrogen-dependent pathway (1.2.2.1), which involves ligand-binding to the ER, dimerization, binding to the ERE, and the recruitment of co-factors in addition to chromatin remodelling takes minutes or even hours to induce transcription of a target gene that will in effect deliver a biological response.¹²⁵ Similarly, the ERE-independent (1.2.2.2) and ligand-independent actions of the ER (1.2.2.3) require transcriptional activation and mRNA translation and the subsequent biological response occurs over a period of time. The time frame of these transcriptional pathways thus does not explain the rapid biological effects induced by estrogens.^{67;125;126} Hence, estrogens are also involved in non-transcriptional, non-genomic mediated biological responses that are independent of transcriptional activation by the nuclear ERs but may be mediated by membrane bound receptors (Figure 5). Estrogen can stimulate signalling pathways or cascades that recruit second messengers such as calcium and nitric oxide. Receptors involved include receptor tyrosine kinases [e.g. epidermal growth factor receptor (EGF-R) and insulin-like growth factor receptor (IGF-R)] and G-protein coupled receptors (GPCR) that activate several protein kinases [e.g. phosphatidylinositol-3' kinase (PI3K), MAPK family members, serine-threonine kinase (Akt) and protein kinases (PKA and PKC, respectively)]. This phenomenon is believed to be responsible for the rapid cellular activities of estrogen in cell

types such as breast cancer and bone cells.¹²⁵ Activation of these signalling pathways set of a cascade of events, which could intertwine with other signalling pathways including other ER-mediated pathways, which makes this rapid biological response of estrogen quite complex and vast.¹²⁵ The non-genomic effects of estrogen will thus only be discussed briefly as it is beyond the scope of this thesis. Receptors located in or close to the plasma membrane are expected to mediate the rapid biological effects of estrogen, whether the classical ERs or other receptors is still a matter of controversy, and will be elaborated on. The identification of a membrane ER is, however, thought to be an important contributor to understanding the non-genomic effects of E₂. The non-transcriptional ER signalling is still very much a mystery and under investigation, however, some signalling pathways have been identified as being related to the non-genomic signalling of the ERs and two, relating to cell proliferation and vasodilatation, will be very briefly discussed. Cell proliferation is crucial in the spread of cancer and vasodilatation is critical in cardiovascular disease.

The steroid hormone E₂ was shown to elicit a biological effect at the membrane when it was demonstrated that an impermeable conjugated E₂ (E₂-BSA) activates the MAPK kinase and induces phosphorylation and subsequent activation of both ERK-1 and ERK-2 in the neuronal cell line SK-N-SH.¹²⁷ A membrane bound ER has been identified by numerous groups, however, with conflicting results as to its origin. Some studies hypothesise that the nuclear ER subtypes locate to the membrane and behave like membrane receptors.^{128;129} In support of this hypothesis it has been shown that antibodies raised to the ER α react with the membrane situated receptor suggesting structural similarity¹²⁸ while a subsequent study showed that the membrane ER and nuclear ER are transcribed from the same mRNA and have the same molecular weight when the ER subtypes complementary DNA (cDNA) is transfected into Chinese hamster ovary (CHO) cells.¹²⁹ In addition, either ER subtype transfected into CHO cells can localize to the membrane where it can activate MAPK or increase cAMP levels.¹²⁹ The ER specific antagonist, ICI 182,780, has also been shown to inhibit the E₂ induced nitric oxide intracellular pathway thus substantiating a link between the nuclear ER and membrane ERs¹³⁰. However, numerous studies disagree with the hypothesis that the nuclear ERs are related to the membrane bound ER^{131;132;133} as the ability of E₂ to potentiate kainite-induced currents in hippocampal CA1 neurons was not affected in ER α knock-out mice nor did the co-treatment with the antagonist, ICI 182,780, which would eliminate residual ER β activity, reduce the response¹³². Recently, GPR30, a membrane bound receptor with

a high affinity for E₂, thought to be unrelated to the nuclear ERs, has been identified. The GPR30 is a GPCR capable of activating adenylyl cyclase activity upon E₂ binding.¹³⁴

The induction of cell proliferation by E₂ is a well-known biological response that is even exploited to identify new estrogens.¹³⁵ Cell proliferation entails activation of a wide variety of signalling pathways, which involve the ER-E₂ complex. For example, the ER α -E₂ complex activates the ERK/MAPK and PI3K/AKT, which are involved in cellular growth and cell death prevention. Interestingly and in contrast to ER α , the ER β -E₂ complex is responsible for the rapid induction of phosphorylation of the p38/MAPK, which sets of a cascade of reactions to induce cell apoptosis. The ER β -E₂ complex does not interact with the signalling pathways induced by the ER α -E₂ complex, which are involved in preventing cell apoptosis and promotion of cell growth.¹³⁶ Additionally, E₂ decreases MAP kinase phosphatase 1 activity, which in turn leads to an increase in ERK activity in breast cancer cells and as previously mentioned up regulation of ERK activity activates MAPK with downstream activation of cell proliferation.¹³⁷ In addition to indirectly activating MAPK through ERK activation the E₂-ER complex is also able to activate MAPK directly.¹³⁸

A rapid onset of vasodilatation occurs when endothelial cells are stimulated with E₂ and is not dependent on gene expression. The stimulation with E₂ leads to the activation of endothelial nitric oxide synthase (eNOS), which in turn leads to a cascade of cellular activity. The activation of eNOS activity results in nitric oxide release in a Ca²⁺ dependent manner¹³⁹ and is mediated by the PI3K/Akt signalling pathway. The E₂-ER α complex has been shown to interact with the p85 α regulatory subunit of PI3K. This in turn would lead to the downstream activation of eNOS through the phosphorylation of Akt. ER β is also able to mediate a rapid non-genomic increase in eNOS activation in response to E₂, however, ER β is unable to interact with the p85 α regulatory subunit of PI3K.¹⁴⁰ As ER β does not activate the PI3-kinase/Akt pathway it was suggested that it induces eNOS activation through a MAPK pathway.¹⁴⁰ Nitric oxide is responsible for the relaxation of vascular smooth muscle cells and although ER β may play a role, the activation of eNOS by E₂ is mainly mediated by ER α situated in the plasma membrane.¹⁴⁰

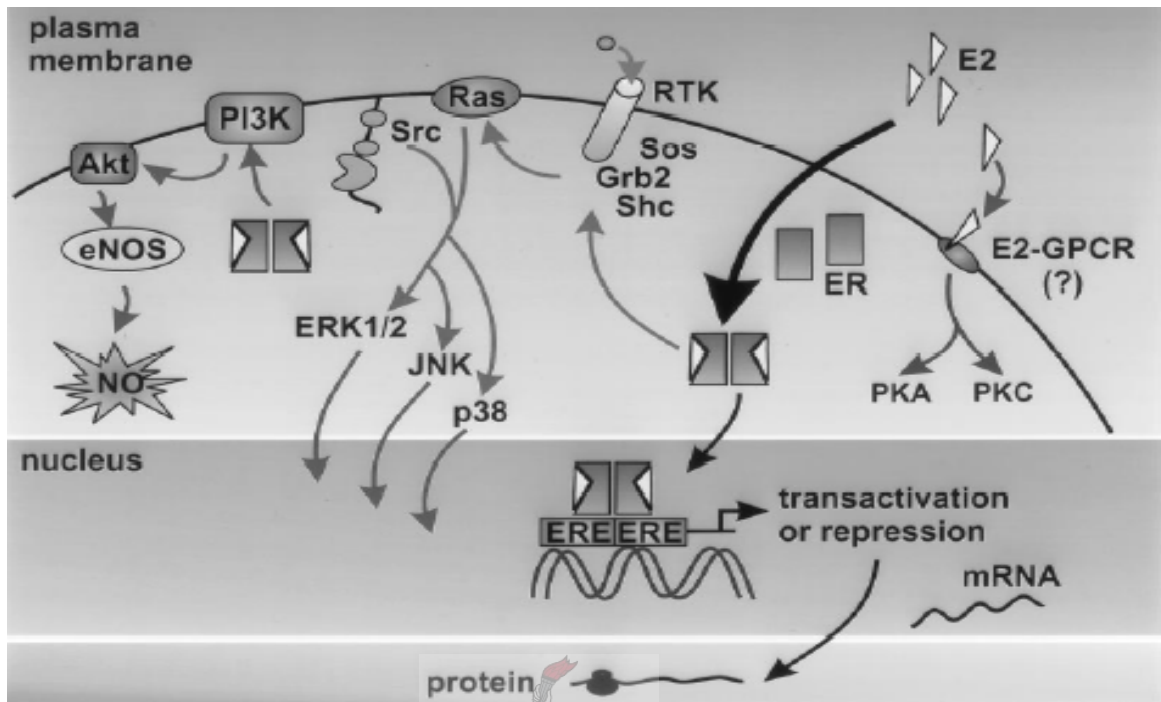


Figure 5: Selected nuclear and non-nuclear activities of ER α ¹²⁵. Abbreviations: endothelial nitric oxide synthase (eNOS), nitric oxide (NO), phosphatidylinositol-3' kinase (PI3K), 2-son of sevenless (Sos), growth factor receptor binding protein 2 (Grb2), G protein coupled receptor (GPCR), protein kinase A (PKA), protein kinase C (PKC), extracellular-regulated kinases 1 and 2 (ERK-1/-2), c-Jun N-terminal kinase (JNK), 38-kDa isoform of MAPK (p38), and estrogen response element (ERE), Src homology/collagen (Shc), receptor tyrosine kinase (RTK), Src is a tyrosine kinase; Ras is a GTP-binding protein.

Table 2: A brief summary table of some of the non-genomic actions of estrogen as per Karen J. Ho and James K. Liao.¹²⁵

Recruitment of second messengers calcium and nitric oxide

Receptor Tyrosine kinases

- EGF-R
- IGF-R
- GPCR

Protein kinases

- PI3K
 - Akt
 - MAPK
 - PKA and PKC
-

As clearly indicated in Figure 6, genomic and non-genomic pathways involving the ERs can converge through signal transduction pathways. The activation of protein-kinase cascades by membrane ER-E₂ complexes leads to phosphorylation of numerous transcription factors and nuclear ERs themselves. ER phosphorylated by non-genomic signalling is believed to enhance and complement the classical ER mediated pathway.⁶⁷

1.2.3. The role of estrogens in cancer formation

The numerous pathways estrogens can follow to mediate a biological response are complex, especially if one would consider cross talk with other pathways. Accordingly, there are numerous suggested pathways whereby estrogen is responsible for estrogen-dependent cancers. As discussed above (1.2.2.4) estrogen itself is able to act as a mitogenic agent in certain cell types by inducing cell proliferation and cell differentiation through activation of certain estrogen responsive genes such as cyclin D-1, tumour necrosis factor α (TNF α), and cathepsin D.¹⁴¹ Hyperproliferation is associated with genomic instability. It has been suggested that as E₂ increases the rate of cell proliferation it forces the cells to rapidly pass through the cell cycle bypassing set checkpoints within the cell cycle increasing the risk of DNA mutations.⁴⁹ In addition, proliferation is enhanced by a decrease in apoptosis. E₂ has been shown to inhibit apoptosis in a rapid non-genomic manner through activation of ERK.¹⁴²

Further mechanisms involve production of mutagenic compounds during the metabolism of estrogen or repression of cellular systems involved in the inactivation of mutagenic compounds. The metabolism of E₂ to 4-hydroxyestradiol has for example been reported to be responsible for estrogen-induced cancers.¹⁴³ More recently E₂ was found to have a repressive action on the ARE.¹¹³ It was demonstrated that gene expression of GST and QR, phase II detoxification enzymes, is repressed at physiological concentrations of E₂ (10 nM).¹¹³ Both GST and QR are driven by an ARE containing promoter. These enzymes are responsible for metabolising the metabolic products of phase I enzymes, which are able to induce DNA damage and mutations. Thus the body's internal defence mechanism against the carcinogenic metabolites of phase I enzymes are the phase II detoxification enzymes, which through conjugation or reduction reactions are able to protect against oxidative damage.¹⁴⁴

In summary, estrogen plays a role in cancer formation and progression through both ER-dependent (genomic and non-genomic) and ER-independent mechanisms. The exact process is

still elusive as numerous signalling pathways and genomic responses are involved. One concluding factor, however, does exist and that is that estrogens are associated with disease, both as a causative (e.g. breast cancer) and preventative (e.g. osteoporosis) agent. No-one is more affected by the good and bad of estrogens than menopausal women as both risk and prevention of disease come into play. Safer alternatives for hormone replacement therapy (HRT) are essential and need more attention so that menopausal women can have peace of mind. Alternative HRTs such as the plant derived estrogen mimics known as phytoestrogens need further investigation and will be discussed in the following section.

1.3. Phytoestrogens

Phytoestrogens are non-steroidal phenolic compounds derived from plants, which are able to interact directly with the ER subtypes. They are able to compete with E₂ for binding to both ERs and to act as both agonist or antagonist depending on the cell or tissue type, the ER subtypes present, or the presence or absence of endogenous estrogens.^{7;14;145} Research into these non-steroidal compounds is becoming increasingly popular especially when discussing alternatives to HRT and protection against hormone-induced cancers.^{14;145;146} Their biological roles in plants vary and include pigmentation¹⁴⁷, protection against UV-B radiation¹⁴⁸, antioxidant¹⁴⁹, antimicrobial¹⁵⁰ and antifungal¹⁵¹. Phytoestrogens can be divided into six main categories according to Miksicek¹⁵²: coumestans, resorcylic acid lactones, isoflavones, flavones, flavanones and chalcones (Figure 7). Most authors, however, use the main classification of phytoestrogens as isoflavones, coumestans and lignans as these are the most researched.^{146;153} Scientific research to date has mostly focused on the soy isoflavone, genistein, because of epidemiological studies, which have shown that Asian populations have fewer incidences of hormone-related cancers as compared to the West and that this phenomenon has been contributed to by the high soy intake of Asian populations.^{14;154} However, other plant phenolic compounds with phytoestrogenic activity deserve more attention and in addition the fact that a single plant could contain more than one type of phytoestrogen also warrants further investigation.

Phytoestrogens are able to activate the ER subtypes through binding to the receptors and as a result are able to mediate an estrogenic response.⁸² In addition phytoestrogens are also known to act independently of the ER through activation or inhibition of certain enzymes^{25;155}, modulation of endogenous estrogens levels¹⁵⁵, and by scavenging free radicals¹⁵⁶. Furthermore,

similarly to endogenous estrogen, phytoestrogens are also able to bind to SHBG and only when unbound is capable of entering the cell through passive diffusion.^{157;158;159} However, because most flavonoids occur in the form of glycosides, they have to be firstly metabolised to become more biologically active. Therefore in the following section, the bioavailability of phytoestrogens will firstly be discussed followed by the ER-dependent and ER-independent activity of phytoestrogens. Numerous plants have already been identified as having phytoestrogenic activity and some would be briefly mentioned within the context of clinical usage as will phytoestrogenic supplements available to the public.

1.3.1. Bioavailability of phytoestrogens

The bioavailability of phytoestrogens is important, as they must be present in a form that is easily absorbed by the body and available to the cell, as it is only then that they are able to be biologically active.¹⁶⁰ Chemically, phytoestrogens are low molecular weight hydrophobic compounds, which affects their water solubility. However, phytoestrogens are most commonly introduced into the body as glycoside conjugates (β -glucosides, malonylglucosides, acetylglucosides) (Figure 8).¹⁰⁷ The glycosides are more water-soluble, but are biologically less active, as they are less readily absorbed and unable to passively diffuse across the cell membrane.¹⁶⁰ For example, Setchell *et al.*¹⁶¹ showed that isoflavone glycosides are not absorbed intact across the enterocyte and that removal of the sugar is essential for the phenolic compound to become biologically active.

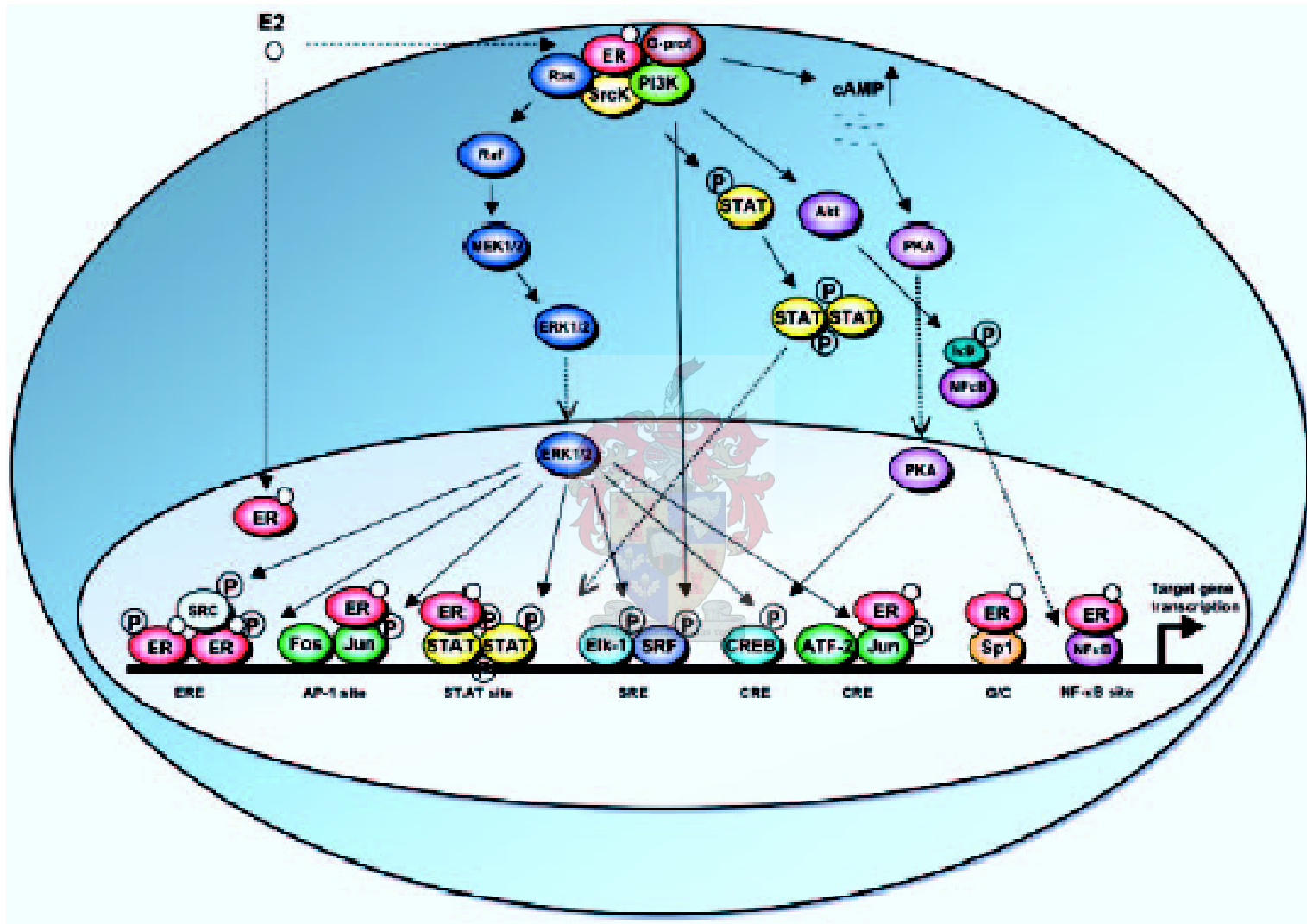


Figure 6: Schematic diagram of genomic (tethering and direct DNA interaction) and non-genomic actions of the ERs as illustrated by Björnstrom and Sjöberg.⁶⁷

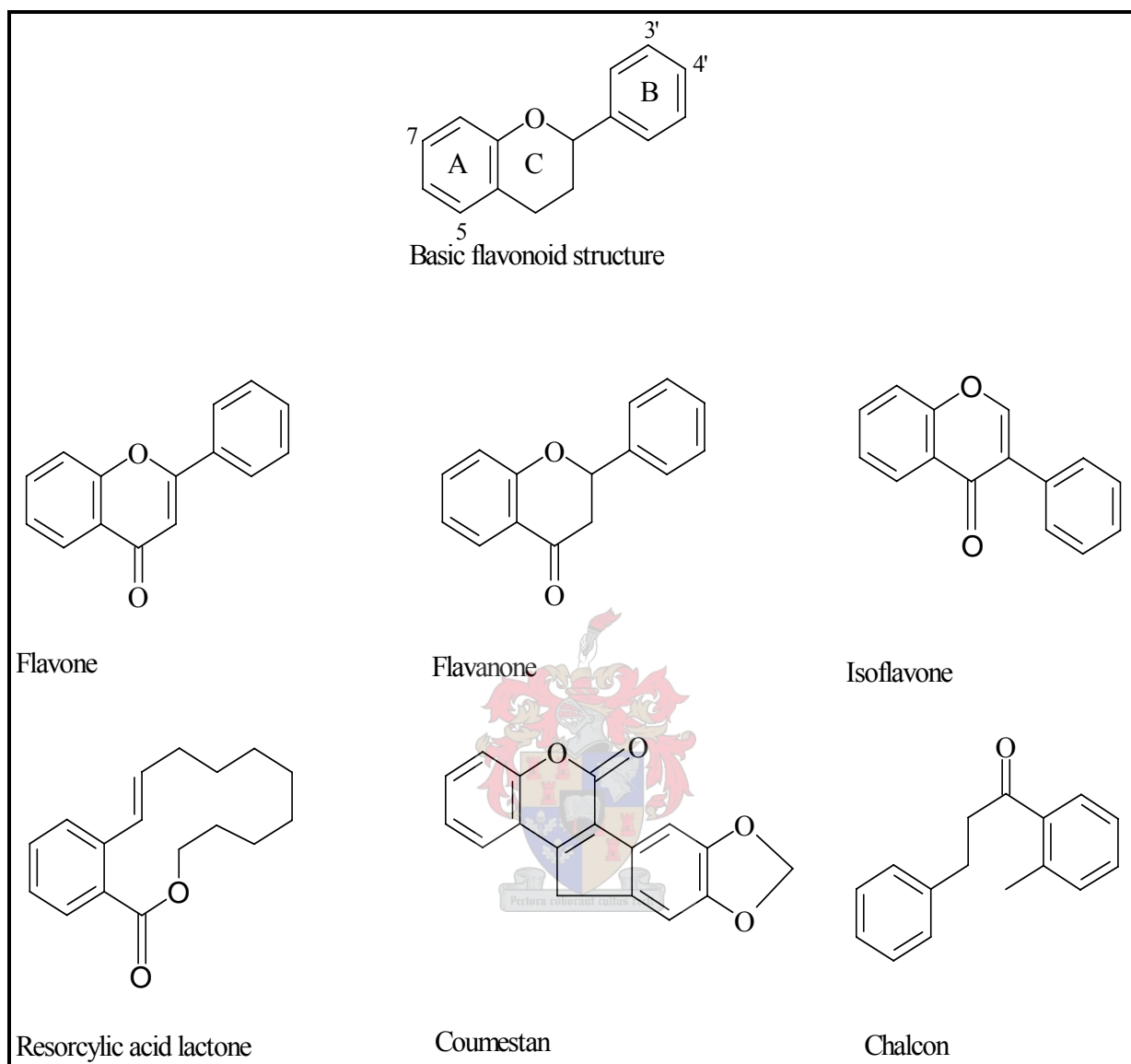


Figure 7: The basic chemical structures of the six main types of phytoestrogens together with the basic flavonoid structure.¹⁵²

The removal of the sugar residue occurs through enzymatic conversion by glucosidases present in the small intestine mucosal cells and the gut microflora.^{160;162} Hydrolysis of the glycoside results in the formation of the more biologically active aglycone that can be absorbed in the gut. In plants, for example, the inactive glycoside genistin is mainly present and only becomes activated when the sugar residue is removed, and genistein is formed. The aglycones can be absorbed and may be further metabolised or not depending on the intestinal environment. Further

metabolism could entail demethylation, sulfation, glucuronidation or a combination of these either in the intestine or liver.¹⁶⁰ Formononetin, for example, is demethylated to form daidzein, which is reduced to equol that has a higher estrogenic activity than the parent compound, formononetin.¹⁶³ The metabolism and absorption of phytoestrogens vary between individuals as the deconjugation of the glycoside is highly dependent on the composition of the intestinal flora and the mucosal cells, the time spent in the intestine, redox potential and dietary factors.¹⁶⁴ All these factors may thus contribute to the variability of phytoestrogen effects in humans. For example, it was found that not all humans can produce equol from daidzein.¹⁴

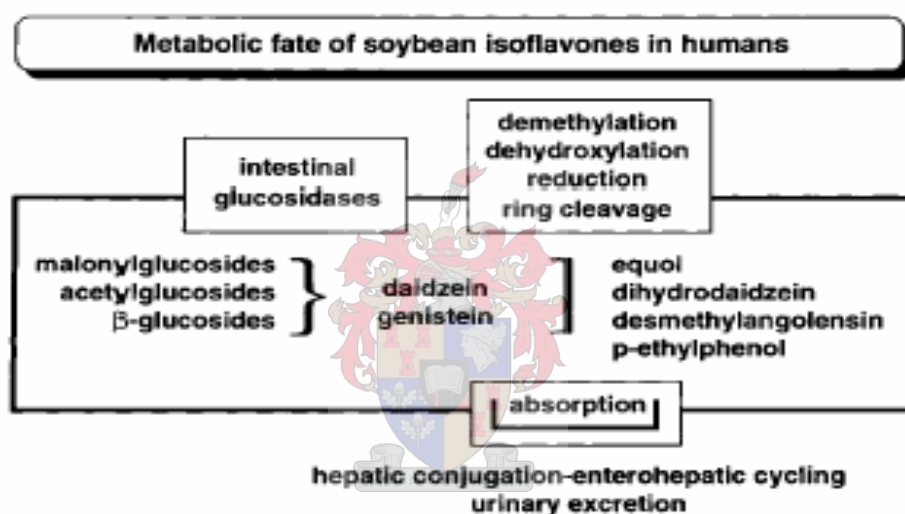


Figure 8: Simplified schematic representation of the metabolism of some common isoflavones in humans and animals.¹⁰⁷

Metabolism of glycosides is not the only process involved in the bio-availability of phytoestrogens. They can also be sequestered by SHBG. Phytoestrogens have been shown to bind to the SHBG^{157;158;159}, which can influence its bioavailability as similar to endogenous estrogens, only unbound or free phytoestrogens are available to diffuse through the cell membrane. Genistein and naringenin were both reported to have affinity constants for human SHBG equal to $6.3 \times 10^5 \text{ L}\cdot\text{mol}^{-1}$ and $8.1 \times 10^5 \text{ L}\cdot\text{mol}^{-1}$ respectively.¹⁵⁹ Although 3 orders of magnitude lower than that for E_2 they are able to displace endogenous E_2 from SHBG and to modulate SHBG levels as will be discussed under ER-independent mechanisms of phytoestrogens. It has been suggested that binding to SHBG could, like for the endogenous sex steroids, regulate free bioactive fraction

of phytoestrogens, protect them against further metabolism and subsequent excretion, and target them to responsive cells *via* the SHBG receptor.¹⁵⁹ Bioavailability of phytoestrogens is important and both the metabolism of the glycosides and the transport of the biologically active compounds by SHBG to the target tissues are essential for phytoestrogens to be able to exert their biological effect.

1.3.2. Biological effects of phytoestrogens

The beneficial health-effects of phytoestrogens have been noted in numerous epidemiological studies and clinical trials.¹⁶⁵ Their estrogenic activity is demonstrated by the mere fact that they are capable of interacting with the ERs and activate downstream effectors. In addition, scientific evidence would suggest that interacting with the ERs is not their only mode of action. Phytoestrogens are also able to act as antioxidants¹⁵⁶, able to inhibit a wide variety of enzymes²⁵, and reduce levels of LDL, cholesterol and triglycerides.¹⁶⁶ These pleiotropic effects combined are likely to contribute to the favourable health-effects of phytoestrogens and will be discussed under ER-dependent and –independent mechanisms.

1.3.2.1. ER dependent

Numerous studies have shown that phytoestrogens are able to compete with E₂ for binding to the ER subtypes, where after they are able to mediate a biological response similar to that described as the classical genomic estrogenic effect (1.2.2.1). The classical genomic estrogenic pathway entails the following: on binding of the ligand to the ER a cascade of events are initiated that include dissociation of heat shock proteins, dimerization of the ERs, recognition of an ERE situated in the promoter region of estrogen responsive genes, and finally the activation or repression of an estrogen targeted gene. The mechanism is similar for either exogenous or endogenous estrogens. However, unlike E₂ which binds with similar affinity to both ER subtypes, phytoestrogens preferentially bind to the ER β (Table 3).⁸² In addition, it has been demonstrated that although phytoestrogens were able to compete with E₂ for binding to both ER subtypes the relative binding affinities were all lower than that of E₂ (Table 3) which allows for the further classification of these plant phenolic compounds as weak estrogens.^{82;167;168} The relative binding affinity (RBA) determined by Mueller *et al.*¹⁶⁷ for E₂ with both ER subtypes were set to 100%

which gave RBAs for genistein of 1% and 31% for ER α and ER β , respectively. Coumestrol showed slightly higher RBAs of 12% and 77% for ER α and ER β , respectively.¹⁶⁷ Similarly, Muthyala *et al.*¹⁶⁸ also showed that genistein has much lower RBAs for the ER subtypes than E₂.¹⁶⁸ Both studies, however, showed that all phytoestrogens investigated had a higher RBA for the ER β subtype, therefore supporting the trend observed in Table 3.^{167;168}

Despite much lower binding affinities for the ER subtypes, phytoestrogens are able to regulate estrogen dependent genes through reacting with EREs (Table 4). The phytoestrogens, genistein, coumestrol and equol, for example, were shown to preferentially induce transcription *via* the ER β with higher efficacy (fold induction) and potency (EC₅₀, ligand concentration yielding half-maximal activation) values.¹⁶⁷ Their relative potencies (with EC₅₀ for E₂ set as 100%) were also higher for ER β than for ER α with genistein and coumestrol showing relative potencies for ER β of 73% and 63%, respectively. Resveratrol and enterolactone, in contrast, had very low relative potencies for both ER subtypes ranging from 0.09 to 0.02%.¹⁶⁷ In addition, although weaker agonists than E₂, due to their lower potency values, genistein, coumestrol, equol and resveratrol induced maximal fold induction similar to that of E₂ for both ER subtypes and were defined as full agonists.^{82;167} Similarly, An *et al.*¹⁶⁹ demonstrated that the phytoestrogens, genistein, daidzein and biochanin A, preferentially activated transcription *via* ER β . However, unlike the previous study mentioned, genistein was unable to induce a maximal response *via* ER α although, like in the previous study, both genistein and daidzein were full agonists *via* ER β .¹⁶⁹ The differences seen by these two independent studies could be due to the different cell types used or the different promoter reporter constructs containing different numbers of ERE repeats or different ERE sequences. Nevertheless, both showed that, similar to E₂, phytoestrogens were able to transactivate, albeit not to the same extent, *via* both ER subtypes. In addition, An *et al.* showed that genistein, daidzein and biochanin A, were able to repress the transcriptional response of a TNF α induced promoter *via* ER β but not *via* ER α , although E₂ was able to repress transcription *via* both ER subtypes.¹⁶⁹ Thus both studies mentioned as well as the data contained in Table 4, substantiate the fact that phytoestrogens not only preferentially bind to ER β but also preferentially induce or repress transcription *via* ER β . Interestingly, Pike *et al.*⁸⁴ when investigating the crystal structure of the LBD of ER β found that the conformational change induced by genistein was similar to that induced by antagonists, raloxifene, which differed from the agonist induced

conformation. This does not appear to explain the induction of transcription *via* ER β by phytoestrogens nor their lack of antagonism in most cases.^{82;167;169} Some phytoestrogens, however, have been shown to have antagonist activity *via* the ER subtypes. Zearalenone and resveratrol, for example, show antagonist activity at high doses *via* both the ER α and ER β subtypes.¹⁶⁷ Nonetheless, phytoestrogens in the main appear to have not only a higher binding affinity for ER β , but they also preferentially transactivate *via* ER β .

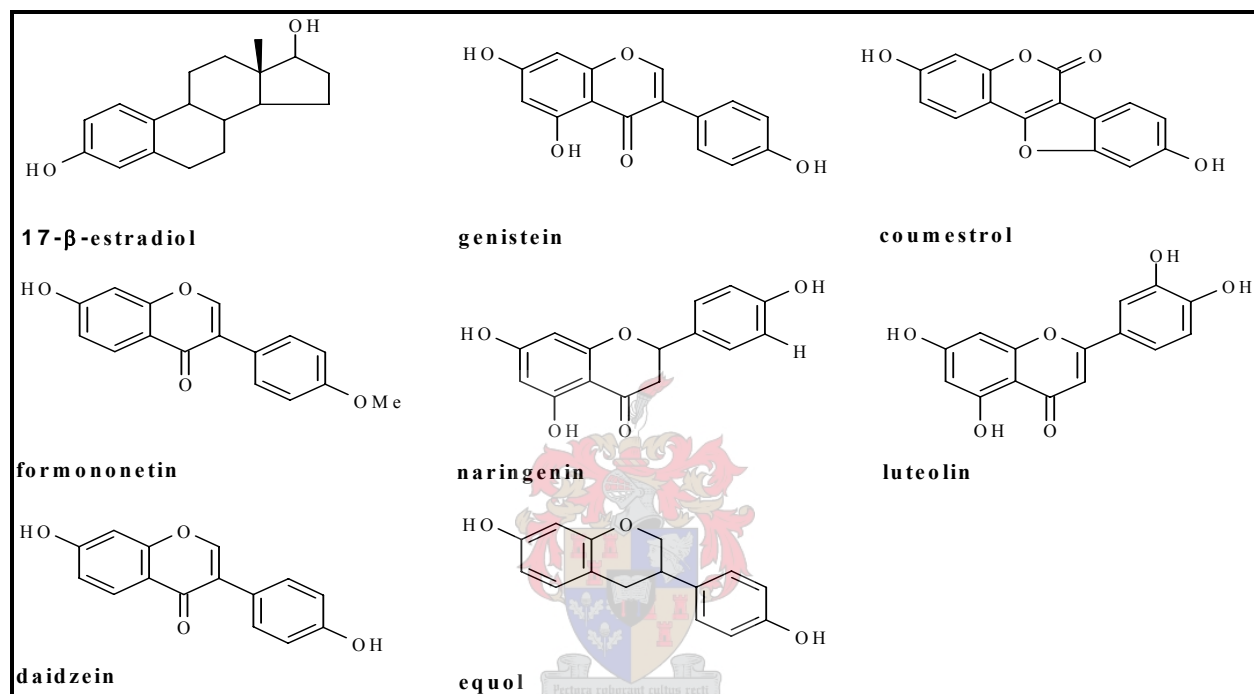


Figure 9: Structures of some common phytoestrogens and the endogenous ER ligand, E₂, listed in Tables 3 and 4.

The more effective transactivation of phytoestrogens through ER β has been ascribed to more effective co-activator recruitment. Phytoestrogens have been shown to selectively recruit co-regulators to ER β and not ER α , which would suggest that phytoestrogens would induce more effective ER β -mediated transcription, unlike E₂ which is able to recruit co-regulators to both ER subtypes.¹⁶⁹ Genistein in its interaction with ER β was able to induce a conformational change in the receptor so that the AF-2 domain was able to recruit co-regulators. This further substantiates the higher transcriptional activation observed with ER β and would suggest that the generally higher binding affinity of phytoestrogens for ER β allows a conformational change of the receptor, which favours the recruitment of co-regulators.

Table 3: Estrogen receptor subtypes binding constants of common phytoestrogens (Figure 6).

Assay system	Test compounds							
	E ₂	Genistein	Coumestrol	Formononetin	Luteolin	Naringenin	Daidzein	Equol
Purified hER α and hER β used for competition binding assays against fluorescein labelled E ₂ (1 nM) ¹⁶⁷	hER α : • RBA = 100% • ^b IC ₅₀ = 4.3 nM hER β : • RBA = 100% • IC ₅₀ = 5.7 nM	hER α : • RBA = 1% • IC ₅₀ = 0.3 μ M hER β : • RBA = 31% • IC ₅₀ = 15 nM	hER α : • RBA = 12% • IC ₅₀ = 15 nM hER β : • RBA = 77% • IC ₅₀ = 3.6 nM					hER α : • RBA = 0.3% • IC ₅₀ = 1.5 μ M hER β : • RBA = 3% • IC ₅₀ = 0.2 μ M
Saturation binding analysis with solubilised human receptor & 3 nM ³ H-E ₂ using gel filtration for separation of free and bound steroid ⁸²	hER α : • ^c Kd = 0.05-0.1 nM hER β : • Kd = 0.05 -0.1 nM	ER α : • RBA = 4 • IC ₅₀ = 145 nM ER β : • RBA = 87 • IC ₅₀ = 8.4 nM		ER α : • RBA = < 0.01 ER β : • RBA = < 0.01		ER α : • RBA = 0.01 ER β : • RBA = 0.11 • IC ₅₀ = 590 nM		
Purified full-length hER α and hER β used for competition binding studies with 10 nM ³ H-E ₂ ¹⁶⁸	hER α : • RBA = 100% • ^d Ki = 0.2 nM hER β : • RBA = 100% • Ki = 0.5 nM	hER α : • RBA = 0.017% • Ki = 1200 nM hER β : • RBA = 7.4% • Ki = 6.7 nM				hER α : • RBA = 0.01% • Ki = 2000 nM hER β : • RBA = 0.04% • Ki = 1300 nM	hER α : • RBA = 0.20% • Ki = 100 nM hER β : • RBA = 1.60% • Ki = 31 nM	
Pure human recombinant ER α or ER β in competition ER binding assay with 400 nM ³ H-E ₂ ¹⁷⁰	hER α : • IC ₅₀ = 0.0065 μ M hER β : • IC ₅₀ = 0.0024 μ M	hER α : • IC ₅₀ = 0.3 μ M hER β : • IC ₅₀ = 0.018 μ M		hER α : • IC ₅₀ = 104 μ M hER β : • IC ₅₀ = 60 μ M		hER α : • IC ₅₀ = 17 μ M hER β : • IC ₅₀ = 1.2 μ M		
Non-radioactive ELISA based assay using 1 nM E ₂ -BSA and 10 pm hER β ³⁶		IC ₅₀ = 50 nM	IC ₅₀ = 40 nM	IC ₅₀ = 10 μ M	IC ₅₀ = 0.5 μ M	IC ₅₀ = 0.3 μ M	IC ₅₀ = 0.1 μ M	
Competitive binding assay with pure human recombinant ER α and ER β (0.5 pmol) and 400 nM ³ H-E ₂ ¹⁷¹	hER α : • IC ₅₀ = 0.021 μ M hER β : • IC ₅₀ = 0.015 μ M	hER α : • IC ₅₀ = 0.30 μ M hER β : • IC ₅₀ = 0.020 μ M		hER α : • IC ₅₀ = 104 μ M hER β : • IC ₅₀ = 60 μ M		hER α : • IC ₅₀ = 17 μ M hER β : • IC ₅₀ = 1.20 μ M		
Cell extracts of COS-7 cells expressing hER and 10 mM ³ H-E ₂ was used. RBA was expressed as the concentration competitor relative to E ₂ required to give 50% inhibition of specific binding of ³ H-E ₂ ¹⁵²	RBA = 100%	RBA = 0.4%	RBA = 13.3%			RBA = 0.1%		

^aRBA values were calculated by $100 \times \text{IC}_{50}(\text{E}_2) / \text{IC}_{50}(\text{test compound})$, with E₂ set at 100.

^bIC₅₀ value is the ligand concentration yielding 50% inhibition of binding of E₂ to ER.

^cKd or dissociation constant was calculated as the free concentration of radioligand at half-maximal specific binding

^dKi or equilibrium binding competition constant values was calculated from the Kd of E₂: $(\text{Kd}(\text{E}_2) / \text{RBA} \times 100)$

Table 4: Transactivation potencies (EC₅₀s) and efficacies (maximal fold induction) of common phytoestrogens (Figure 6)

Assay system	Test compounds							
	E ₂	Genistein	Coumestrol	Formononetin	Luteolin	Naringenin	Daidzein	Equol
A yeast transactivation system transfected with hER and a β-galactosidase reporter plasmid ¹⁷²	hERα: • ^a EC ₅₀ = 0.41 nM • ^b Efficacy = 100% hERβ: • EC ₅₀ = 0.16 nM • Efficacy = 100%	hERα: • EC ₅₀ = 0.9 μM • Efficacy = 107% hERβ: • EC ₅₀ = 4.2 nM • Efficacy = 95%		hERα: • EC ₅₀ = 2.0 μM • Efficacy = 76% hERβ: • EC ₅₀ = 1.5 μM • Efficacy = 77%			hERα: • EC ₅₀ = 10.0 μM • Efficacy = 14% hERβ: • EC ₅₀ = 0.96 nM • Efficacy = 129%	
Transactivation assay in transfected HEC-1 cells with either hERα or hERβ ¹⁶⁸	hERα: • EC ₅₀ = 0.021 nM hERβ: • EC ₅₀ = 0.11 nM	hERα: • EC ₅₀ = 80 nM hERβ: • EC ₅₀ = 66 nM					hERα: • EC ₅₀ = 250 nM hERβ: • EC ₅₀ = 100 nM	hERα: • EC ₅₀ = 200 nM hERβ: • EC ₅₀ = 74 nM
Transactivation study using chimeric human ER containing the D, E, and F domains transfected into MCF-7 cells ¹⁷⁰	Gal4-hERαdef: • EC ₅₀ = 9.5 M	Gal4-hERαdef: • EC ₅₀ = 4.0 M	Gal4-hERαdef: • EC ₅₀ = 6.1 M			Gal4-hERαdef: • EC ₅₀ = 3.0 M		
Transactivation assay in transfected HepG2 cells. Cells were transfected with rERα and rERβ respectively ¹⁷³	rERα: • EC ₅₀ = 0.32 nM; rERβ: • EC ₅₀ = 1.5 nM	rERα: • EC ₅₀ = 34 nM rERβ: • EC ₅₀ = 4.9 nM					rERα: • EC ₅₀ = 390 nM rERβ: • EC ₅₀ = 87 nM	
Stably transfected BG1Luc4E ₂ were used for the transcriptional activation assay of the ER. Unfortunately the source of ER is not mentioned ¹⁷⁴	EC ₅₀ = 0.0023 ng/ml	EC ₅₀ = 0.19 μg/ml				EC ₅₀ = 1.22 μg/ml	EC ₅₀ = 0.52 μg/ml	
Co-transfected HeLa cells with wild type recombinant ER and ERE reporter construct ¹⁵²	EC ₅₀ = 0.01 nM	EC ₅₀ = 90 nM	EC ₅₀ = 15 nM	EC ₅₀ = 300 nM			EC ₅₀ = 90 nM	
Transactivation assay with Ishikawa and MCF-7 cells co-transfected. Only fold induction was established with test compounds tested at 100 nM ¹⁷¹	Ishikawa cells: • ^b Fold induction = 3.5 MCF-7 cells: • Fold induction = 4.3	Ishikawa cells: • Fold induction = 3.5 MCF-7 cells: • Fold induction = 4.0		Ishikawa cells: • Fold induction = 1.1 MCF-7 cells: • Fold induction = 1.2			Ishikawa cells: • Fold induction = 1.1 MCF-7 cells: • Fold induction = 1.6	
Transactivation assay with Ishikawa-hERα and Ishikawa-hERβ (stably transfected) ¹⁶⁷	hERα: • EC ₅₀ = 100% hERβ: • EC ₅₀ = 100%	hERα: • EC ₅₀ = 0.8% hERβ: • EC ₅₀ = 73%	hERα: • EC ₅₀ = 8% hERβ: • EC ₅₀ = 64%				hERα: • EC ₅₀ = 1% hERβ: • EC ₅₀ = 5%	
Transactivation assay in transfected U937 cells. Cells were transfected with hERα and hERβ respectively ¹⁶⁹	hERβ: • EC ₅₀ = 0.13 nM	hERβ: • EC ₅₀ = 55 nM					hERβ: • EC ₅₀ = 50 μM	

^aEC₅₀ (potency) value is the value required to produce half maximal induction

^bEfficacy or maximal fold induction induced by test compound

Apart from acting through EREs, phytoestrogens furthermore, have been shown to interact, through the ERs, with the ARE. Unlike E₂, which represses the expression of ARE driven genes, phytoestrogens induce the transcription of the phase II detoxification enzymes. As mentioned previously an ARE is situated in the promoter region of the GST and QR genes, both phase II detoxification enzymes. It has been demonstrated that genistein, biochanin A and resveratrol, interact with the ARE when bound to the ER and induces expression of QR²⁶. QR reduces quinones and similar compounds thereby protecting cells from reactive oxygen species. Transcriptional induction of the QR gene *via* the ER α was not as effective as that observed with ER β . The authors hypothesise that the protective effects of phytoestrogens against DNA damage caused by mutagens are partially mediated by binding to the ER β and inducing the expression of phase II detoxifying enzymes. Notably, however, was that the response was specific for a particular phytoestrogen and not all phytoestrogens induced a similar response. Only resveratrol and biochanin A were effective in protecting MCF-7 cells against estrogen-induced oxidative damage through inducing QR expression. Genistein was shown to be more protective in the absence of ER β than resveratrol, an indication that the protective effects against oxidative DNA damage *via* ER β is dependent on a particular phytoestrogen.

Within this context it is clear that not all phytoestrogens activate the ER subtypes with the same magnitude (Table 4). As phytoestrogens are able to induce different responses it would suggest that the structure of an individual phytoestrogen is important. A particular structure would induce a specific conformational change within a particular ER subtype and this would affect the recruitment of co-regulators. The structural requirements for phytoestrogenic activity would suggest that the diphenolic structure common to all flavonoids (Figure 7 ring A and B and Figure 9) is essential for estrogenic activity with at least one hydroxyl group present on each of the aromatic rings. Additionally, flavonoids with hydroxyl groups present at positions 4' and 7, such as genistein and naringenin (Figure 9) are almost certainly phytoestrogens. If the number of hydroxyl groups exceeds four estrogenic activity is eliminated.^{175;176} Furthermore, methoxy groups situated at the 4' or 7 positions, as in formononetin (Figure 9), seem to decrease or even diminish binding of the flavonoid to the ER.¹⁷⁷ Phytoestrogens are also referred to as natural SERMs due to the fact that they have different binding affinities for the ER subtypes and evoke different responses in different tissues and cell types. Similarly, they are also able to mediate a biological response independently of the ER subtypes that could further explain the different

responses obtained in different cell and tissue types, which will be discussed in the following section.

1.3.2.2. ER independent

Although most focus has been on the weak ER agonist and antagonist activity of phytoestrogens, it must be remembered phytoestrogens are pleiotropic in nature. The biological responses they induce can also be mediated independently from the ER, some of which can indirectly influence estrogenic activity. The following section will discuss some of the ER-independent mechanisms of phytoestrogens such as the inhibition of enzymes involved in estrogen metabolism, the inhibition of protein tyrosine kinase and topoisomerase II, as well as the antioxidant activity of phytoestrogens and their possible role in angiogenesis.

Endogenous estrogen synthesis and metabolism are dependent on numerous steriogenic enzymes some depicted in Figure 10. Phytoestrogens are able to have an effect on estrogen hormone metabolism by influencing the levels of some of these enzymes. Flavonoids, such as naringenin and formononetin, but not genistein, were shown to be aromatase inhibitors at low concentrations in one study.²⁵ Others were, however, unable to confirm the inhibitory effect of formononetin¹⁵⁵ while a recent study showed that genistein is, a weak inhibitor of aromatase, contradicting earlier evidence.¹⁷⁸ Aromatase is the enzyme responsible for the conversion of testosterone to E₂ and androstenedione to E₁ thus inhibition of aromatase would result in a lowering of estrogen levels. Furthermore, naringenin, coumestrol, genistein, daidzein, biochanin A and to a lesser extent formononetin are able to inhibit 17β-hydroxysteroid dehydrogenase (17β-HSD)¹⁵⁵, which catalyses the reversible conversion of E₁ to the more potent E₂.

In addition, coumestrol but not genistein can influence endogenous estrogen levels by repressing FSH and LH.¹⁷⁹ Coumestrol was found to repress the pulsatile secretion of LH in addition to the LH response to exogenous gonadotrophin-releasing hormone (GnRH). This response was blocked when rats were co-treated with the ER antagonist, ICI 182,780, thus establishing that the response is *via* the ER and should technically not be discussed under the ER-independent mechanism of phytoestrogens. However, as inhibition of LH indirectly influences estrogen levels it is more appropriate to discuss it here. Levels of endogenous estrogens are mainly controlled by the secretion of GnRH from the hypothalamus. GnRH further activates the pituitary gland to increase secretion of the gonadotrophins, FSH and LH. LH and FSH are

responsible for the maturation of the ova that allows for the conversion of testosterone to E_2 by aromatase. When a sufficient concentration of E_2 has been reached, a negative feedback effect is exerted on the hypothalamus, which inhibits further secretion of GnRH. Thus phytoestrogens can decrease effective estrogen levels by reducing the total levels of endogenous estrogens by inhibiting the stimulus for synthesis (LH) and synthesis itself (aromatase and 17β -HSD). For most postmenopausal women E_2 concentrations decrease significantly as production and secretion is not primarily *via* the ovaries anymore and E_2 is generated mainly from dehydroepiandrosterone (DHEA) and androstenedione produced in the adrenal gland resulting in much lower concentrations.¹⁸⁰ In addition, estrogens are also made bioavailable through the release of biologically active E_1 from estrone sulfates by steroid sulfatase. Genistein, daidzein, formononetin, luteolin, and to a lesser extent naringenin, are able to inhibit sulfotransferases responsible for the reconjugation or sulphation of free endogenous E_1 .¹⁸¹ The authors hypothesized that phytoestrogens may be able to increase endogenous estrogen levels, which could be associated with a risk in estrogen dependent cancers.¹⁸¹ They supported their theory with a study that linked low sulfotransferase concentration or high steroid sulfatase concentration to poor prognosis for disease progression¹⁸² and concluded that high doses of phytoestrogens could lead to an increase concentration of endogenous estrogens, which could be detrimental.¹⁸¹ The fact that no changes in estrogen levels were found in postmenopausal women taking high doses of phytoestrogens could suggest that the net effect of phytoestrogens on E_2 levels is negligible¹⁸³. However, another study found a negative correlation between phytoestrogen levels and plasma estrogen levels that was mainly ascribed to a group of women that have a polymorphism in their $ER\alpha$ gene (ESR1).¹⁸⁴ Thus effects on endogenous estrogen levels may be influenced by diet-gene interactions and effects would not be the same in all women.

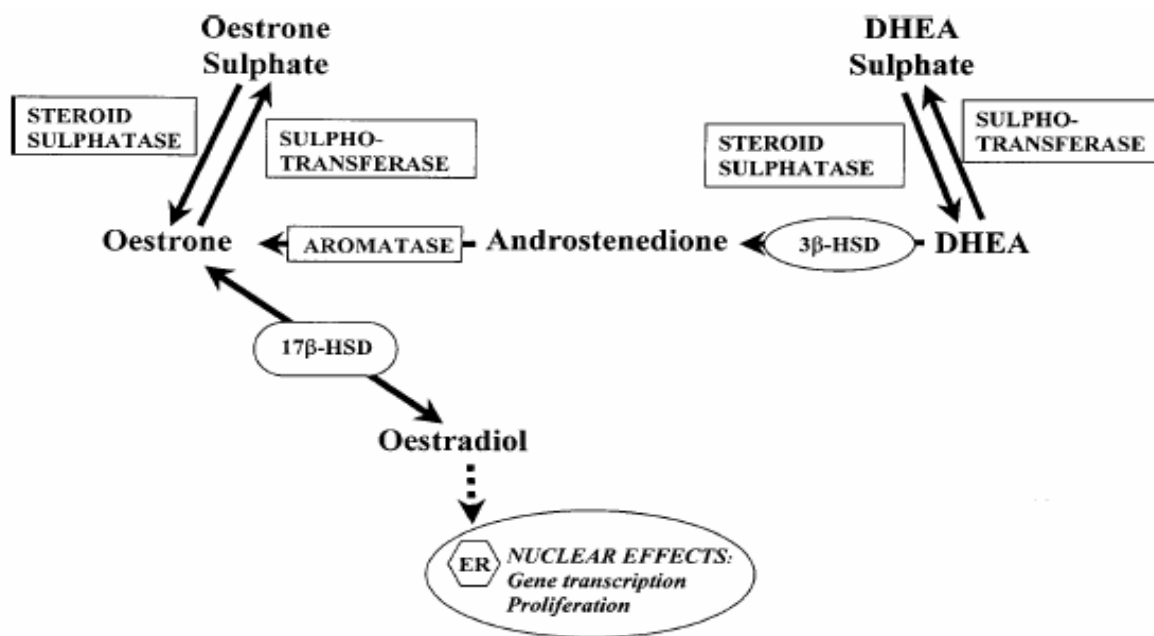


Figure 10: Diagram of the bio-synthesis of E₂ and E₁ from circulating steroids.¹⁸⁰ Abbreviation: 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; ER, estrogen receptor; DHEA, dehydroepiandrosterone

Phytoestrogens besides potentially influencing the total amount of endogenous estrogen present can also decrease the bioavailability of endogenous estrogens through the stimulation of SHBG from the liver. Genistein was shown to increase SHBG secretion from HepG2 (liver cells) cells *in vitro*^{185;186} with *in vivo* data obtained from 20 postmenopausal women taking 30 mg soy milk for 10 weeks supporting this result. A significant increase in SHBG levels was found especially in those women with initial low SHBG concentrations.¹⁸⁷ An increase in SHBG levels would increase the amount of bound estrogen and less endogenous estrogen would be available to mediate a biological response as only the free, unbound fraction of estrogen can diffuse through the membrane and enter a cell.⁵⁵ However, if phytoestrogens themselves bound to SHBG it could also serve as a reservoir by reducing their metabolic clearance rate as was proposed for steroids^{55;188} or could result in displacement of endogenous steroids from SHBG, increasing their free concentration.¹⁸⁸ Naringenin, coumestrol and to a lesser extent, genistein, are indeed able to bind to the SHBG as determined with competitive binding assays with radiolabelled E₂.^{157;159} Binding resulted in displacement of the endogenous steroid, E₂. At 100 μM naringenin and genistein were shown to increase non-SHBG bound E₂ by 46 and 11%, respectively¹⁵⁹ while in another study at 200 μM naringenin and genistein displaced E₂ by 49 and 58%, respectively.¹⁵⁷

Thus phytoestrogens through binding to and displacing endogenous estrogens, by stimulating the secretion of SHBG, and by inhibiting steroidogenic enzymes involved in estrogen biosynthesis are able to influence the bioavailability of endogenous estrogens. They, however, also mediate biological responses independently from estrogens.

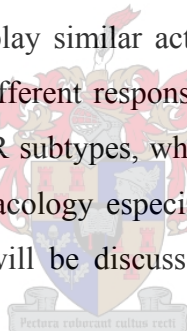
Genistein, for example, has been shown to have a biphasic effect on the cellular proliferation of MCF-7 breast cancer cells.²⁵ At low concentrations genistein induces cell proliferation that is dependent on the ER, while at high concentrations genistein is able to inhibit cell proliferation of MCF-7 cells, which is believed to be independent of the ER.¹⁴⁶ Furthermore, genistein has been shown to inhibit cell proliferation of both ER positive and ER negative cells.¹⁸⁹ These results would suggest that not all effects of phytoestrogens are mediated *via* the ER subtypes and that phytoestrogens are also able to exert biological effects independently from the ERs. These could include effects such as the inhibition or induction of other signalling pathways and antioxidant activity as will be briefly discussed.

As early as 1987 the inhibitory action of genistein on protein tyrosine kinase was established and it was shown that genistein was able to interact with the catalytic domain of tyrosine kinases.³⁰ In addition, genistein can suppress insulin induced tyrosine kinase activity resulting in the suppression of cell proliferation of MCF-7 cells.¹⁹⁰ As protein tyrosine kinase activation is associated with cell proliferation, inhibition could be important in preventing cancer.¹⁴⁶ The antiproliferative effect of genistein at high concentrations could also be aided by its ability to inhibit both topoisomerase II and angiogenesis.^{191;192} Genistein can also stimulate eNOS through a PKA-dependent pathway not inhibited by an ER antagonist¹⁹³ and increase intracellular cAMP, independently of the ER subtypes, in vascular endothelial cells which the authors suggest could have a protective function in vascular function.¹⁹³ Coumestrol was also shown to induce phosphorylation of ERK-1 and ERK-2 *via* membrane ER α in a pituitary tumour cell line GH3/B6/F10.^{194;195} ERK and cAMP both are thought to be involved in numerous cellular activities including cell proliferation.¹⁹⁶ Phytoestrogens are therefore also capable of mediating biological responses through interacting with other cell signalling pathways besides the ER pathways. However, the pleiotropic nature of phytoestrogen action does not end with genomic or non-genomic signalling. Phytoestrogens are also commonly referred to as antioxidants.¹⁹⁷

Due to their polyphenolic structure and the presence of hydroxyl groups, phytoestrogens are also able to act as antioxidants. They are able to scavenge free radicals¹⁹⁸ in addition to their

ability to induce the expression of phase II detoxifying enzymes.²⁶ Furthermore, phytoestrogens are also able to inhibit the oxidation of LDL, which is also attributed to their antioxidant activity.¹⁶⁶ One of the purported advantages of HRT is that estrogen can decrease the levels of LDL cholesterol and total cholesterol by increasing LDL-receptor activity thereby removing LDL from plasma¹⁹⁹. Phytoestrogens such as biochanin A, formononetin, daidzein, enterolactone, enterodiol, and coumestrol, are able to increase LDL receptor activity in HepG2 cells.¹⁶⁶ The response evoked by coumestrol, a coumestan found in alfalfa, was similar to that of E₂. Thus both the estrogenic (increase in LDL receptor levels) and antioxidant (inhibits oxidation of LDL) activity of phytoestrogens could, in concert, have an effect on the increase of LDL receptor activity resulting in the removal of LDL from plasma.

In summary, it is safe to assume that the biological responses evoked by phytoestrogens are complex in including not only ER-dependent, but also ER-independent mechanisms and that these mechanisms require further study for full elucidation. One important issue that has become clear is that not all phytoestrogens display similar activities, and that each individual phenolic plant compound potentially evokes a different response that is difficult to predict. Nevertheless they are all capable of binding to the ER subtypes, which is by definition their common feature, and thus are of great interest in pharmacology especially as pertaining to conditions involving estrogen hormones. These conditions will be discussed in the next section under the clinical effects of phytoestrogens.



1.3.3. Clinical effects of phytoestrogens

As described previously, phytoestrogens can mediate a biological response through both ER-dependent and ER-independent mechanisms each of which are complex systems. These biological properties coupled to epidemiological data on the health promoting effects of phytoestrogens have sparked renewed interest in clinical applications.^{14;200} Although no large-scale clinical trials have been conducted, results from smaller pilot studies are promising. Clinically phytoestrogens are believed to help alleviate menopausal symptoms, decrease the risk of certain types of cancer e.g. breast and prostate, and decrease the likelihood of osteoporosis and cardiovascular disease.^{14;146;165;176} The clinical effects of dietary phytoestrogens as relating to these conditions will be discussed as will be the effects of some plant extracts currently available.

1.3.3.1. Menopausal symptoms

The onset of menopause is ascribed to increasingly lower concentrations of E_2 , which may be due the decrease in ova present in the aging ovaries or to the surgical removal of both ovaries. The conversions of androgens to estrogens in fat cells supply postmenopausal women with some estrogen although concentrations are much lower than that of menstruating women.²⁰¹ General menopausal symptoms during the early onset of menopause include irregular menstrual cycle, hot flushes, headaches, dizziness, hypoglycaemic symptoms, atrophic vaginitis or thinning and drying of the vaginal wall, bloating of the upper abdomen, urinary tract infections, insomnia, depression and fatigue.²⁰¹ For many women HRT alleviates these symptoms thus improving quality of life. In addition, long-term usage of HRT has been credited with protecting against osteoporosis and cardiovascular disease.^{202;203} This will be discussed in sections 1.3.3.3 and 1.3.3.4, respectively. HRT formulations currently available include conjugated equine estrogens either alone (for women who have had a hysterectomy) or in conjunction with progestins, such as medroxyprogesterone, or human estrogens (E_1 , E_2 and E_3) in a ratio naturally found in the body along with progesterone.²⁰¹ Many women, however, do not tolerate conventional HRT or are concerned about side effects and thus seek alternative treatments such as phytoestrogens.

The treatment of menopausal symptoms, especially hot flushes, by phytoestrogens is contentious with some clinical studies finding no evidence that dietary phytoestrogens improve menopausal symptoms, whereas others have found it to be effective.²⁰⁴ Many studies did not include a placebo, which makes it difficult to interpret results. To complicate matters, epidemiological studies have shown fewer complaints of menopausal symptoms by Asian women who have a diet rich in isoflavones.^{8;9} Nevertheless, there are several studies, which showed phytoestrogen intake to be associated with either the reduction of hot flushes or a decrease in the severity. In a double-blind placebo-controlled trial with 104 postmenopausal women 76 mg isoflavones daily had no effect on vaginal dryness but did reduce the number of hot flushes by 45% as compared to 30% in the placebo group.²⁰⁵ Similarly, in comparing hot flushes of 145 postmenopausal women on either a diet high in phytoestrogens or on one containing less phytoestrogens, it was found that the diet high in phytoestrogens was effective in decreasing hot flushes by 50% over a 12 week period and it also improved vaginal dryness, contrary to the findings of the previous study.^{205;206} The results are, however, difficult to interpret as this study was not conducted as a placebo-controlled trial and the placebo response could have an effect on

hot flushes. Furthermore, a randomized double-blind placebo-controlled study investigating the efficiency of a red clover dietary supplement found a significant decrease in hot flushes compared to that of the placebo group.¹⁰ Although the abovementioned studies suggest that phytoestrogens may be useful in the reduction of menopausal symptoms several double-blind, placebo-controlled studies found isoflavonoids to be insufficient in alleviating menopausal symptoms^{207;208}. In a three month double-blind placebo trial consisting of 94 postmenopausal women, 44 women randomised to using 118 mg isoflavones per day reported no climacteric symptom reduction, however, vaginal dryness did improve.²⁰⁵ A similar result was found in another randomized double-blind study conducted for 24 weeks with 69 women 24 of which were on an isoflavone rich diet (80.4 mg per day), 24 on a isoflavone poor diet (4.4 mg per day) and 21 women on a whey protein control diet.²⁰⁹ Cornwell *et al.*¹⁶⁵ in reviewing a number of clinical and epidemiological studies recently stated that there is a lack of clinical evidence demonstrating the effectiveness of phytoestrogens in reducing menopausal symptoms as only 4 out of 17 published studies reported relief of menopausal symptoms.

Although there are contradictory results regarding the effectiveness of using phytoestrogens to alleviate menopausal symptoms, the use of alternative treatments by consumers are becoming increasingly popular.¹ This is mainly due to the public's concern of the risks involved in using conventional HRT, which include increased risk of stroke, breast cancer and endometrial cancer.^{18;19} However, none of the clinical studies investigating the effects of phytoestrogens on menopausal women comes close to the size of the studies done by WHI and the NIH Million Women study^{18;19;210} nor have they focussed specifically on side-effects evaluation.

1.3.3.2. Cancer

The cancer causing properties of estrogen is well known.²¹¹ The carcinogenic activity of estrogens could be through the stimulation of cell proliferation as hyper-proliferation could very likely increase genetic mutations.²¹¹ In addition, the metabolism of estrogen generates free oxygen radicals and quinines that could produce unstable DNA adducts, which could result in genetic mutations.^{211;212;213} The effects of estrogen metabolism could be exacerbated by the fact that E₂, as previously described, is not only unable to induce phase II detoxifying enzymes but in fact down regulates these enzymes.¹¹² Epidemiological studies have shown that Asian women

have a lower incidence of breast cancer than women in Western populations.^{200;214;215} This has been attributed to the high intake of soy in their diet, which contains high concentrations of phytoestrogens such as genistein and daidzein.¹⁴ The effect of phytoestrogens on cancer is complex and yet to be fully understood. Although a diet rich in phytoestrogens suggests a protective effect against the occurrence of hormone dependent cancers, the manner in which phytoestrogens elicit such a protective effect is still unclear. The favourable effects observed by phytoestrogens could be *via* a wide variety of mechanisms, which could include both ER-dependent and ER-independent mechanisms.

Phytoestrogens are able to protect against hormone-dependent cancers by decreasing the bioavailable endogenous estrogen levels through inhibition of aromatase²⁵, the finale enzyme in estrogen synthesis, and through the stimulation of SHBG levels, the plasma binding protein regulating the free concentrations of estrogen in the bloodstream.^{185;186} In addition, phytoestrogens can also act as antioxidants¹⁵³ and induce ARE regulated genes such as phase II detoxifying enzymes²⁶ thus inhibiting DNA damage from oxidative challenge.

Numerous *in vitro* studies have shown that genistein has a biphasic effect on cell proliferation, with stimulation at low concentrations and inhibition of cell proliferation at much higher concentrations.^{25;216;217} More than likely ER-dependent and ER-independent pathways are involved with possible crosstalk. Cell growth at low concentrations is thought to be mediated through interaction with ER α , while inhibition of cell proliferation is thought to be *via* signalling pathways that do not require ER and are involved with the specific arrest of the cell cycle more specifically the G2/M phase of the cell cycle.²¹⁸ Inhibition of protein tyrosine kinase^{30;190} and topoisomerase II¹⁹¹ appears to be important for the anti-proliferative effect of phytoestrogens mediated through ER-independent mechanisms. The collective preferential binding of phytoestrogens to the ER β , which is thought to regulate of ER α induced activity, could also be a protective mechanism of phytoestrogens against estrogen induced cancers.^{82;219;220;221} As previously discussed, they bind preferentially to ER β , although they have weak agonist activity when bound to the ER α .⁸²

Additionally, phytoestrogens have also been shown to inhibit angiogenesis.²⁷ Angiogenesis is simply the formation of new capillaries allowing for blood supply and thus nutrients to a particular site such as the endometrium or placenta. This vital mechanism, however, also affects growth and spread of tumours as it provides nutrients to tumours thus supporting

growth. E₂ has been shown to induce angiogenesis by increasing the expression of vascular endothelial growth factor (VEGF) an essential angiogenic stimulus and vital in wound healing.²²² However, phytoestrogens such as genistein and luteolin were found to inhibit angiogenesis.²⁷

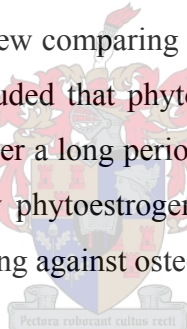
Numerous animal studies have shown that phytoestrogens do protect animals from prostate and mammary cancer.^{223;224;225} Exposure of Lobund–Wistar rats to genistein from conception to necropsy at 11 months protected the rats from induced prostate invasive adenocarcinomas, in a dose-dependent manner.²²⁴ Similarly, in Sprague-Dawley rats, which were either chemically induced to develop prostate cancer or genetically modified to develop prostate cancer, a diet rich in genistein inhibited the development of invasive prostate adenocarcinomas in a dose-dependent manner by regulating specific sex steroid receptors and growth factor signalling pathways.²²⁵ In the same study, Sprague-Dawley rats fed a genistein rich diet showed a decrease in the number of induced mammary tumours both in prepubertal and combined prepubertal and adult genistein treatments. However, genistein was not able to protect rats treated after prenatal- or adult-only treatments. It was concluded that the time of exposure to genistein was important for mammary cancer chemoprevention with the adolescent period probably being most important in humans.²²⁵ This was supported by the fact that EGF receptor, a tyrosine kinase, was shown to be up regulated during early postnatal life, associated with proliferation and differentiation of breast tissue, but down regulated at day 50, which was associated with reduced susceptibility to chemically induced carcinogenesis.²²⁵ To sum up, Adlercreutz reviewed the significance of a phytoestrogen rich diet and its role in the prevention of breast cancer and concluded that a soy-rich diet is slightly protective against the occurrence of breast cancer and that it would be most effective if exposure occurred from an early stage i.e. puberty.²²⁶

1.3.3.3. Osteoporosis

Osteoporosis is a chronic disease affecting numerous post-menopausal women. The cause of this disease is a lack of bone formation by osteoblasts coupled to an increase in bone resorption by osteoclasts that result in diminished bone density that could increase the risk of fractures.¹²⁴ E₂ has been shown to inhibit bone resorption by osteoclasts²²⁷ and thus estrogen therapy is believed to protect against the occurrence of osteoporosis in women. Additionally, excessive production of the cytokine, TNF- α , after menopause is believed to cause osteoporosis and as ER β represses the expression of the TNF- α gene this may contribute to the protective mechanism mediated by

estrogens.²²⁸ As mentioned previously (1.3.2.1.1), phytoestrogens have been demonstrated to be more effective in inducing transcriptional repression than activation¹⁶⁹ and its preference for binding to ER β could be a molecular mechanism through which phytoestrogens can protect against osteoporosis.

The protective mechanism of action of phytoestrogens on postmenopausal osteoporosis has been intensely studied *in vitro* and in animal models. Phytoestrogens have been shown to inhibit osteoclast differentiation *via* an ER-independent protective mechanism that includes the inhibition of tyrosine kinase and topoisomerase II.²²⁹ A double-blind placebo-controlled clinical trial involving 66 postmenopausal women showed that the women had an increase in bone density and in bone mineral content when treated daily with a dose of 90 mg of isoflavones for six months.¹² Similarly, a randomized placebo-controlled study investigating the effect of 144 mg isoflavonoids on bone resorption and bone formation in 55 postmenopausal women with a history of breast cancer concluded that isoflavonoid intake inhibited bone resorption thus protecting against osteoporosis¹³. An intensive review comparing results from numerous *in vitro* and *in vivo* (both animal and human) studies concluded that phytoestrogens do have a protective effect on bone formation and thus osteoporosis over a long period of usage.²²⁹ Similarly, comparison of 15 clinical studies on the effect of dietary phytoestrogens found that 11 gave positive results in sustaining bone density and thus protecting against osteoporosis.¹⁶⁵



1.3.3.4. Cardiovascular disease

Estrogens protect against cardiovascular disease and it is well known that with the onset of menopause, women are vulnerable to cardiovascular disease.²⁰² HRT is believed to protect women against cardiovascular disease²⁰³, however, it was found that HRT does not protect women against cardiovascular disease.¹⁸ These findings were questioned in a recent report suggesting that HRT initiated at the onset of menopause could decrease the risk of developing cardiovascular disease over time.

Both ER-independent and ER-dependent mechanisms are involved in this protective feature of estrogen, which include altering serum lipid concentrations, coagulation and fibrinolytic systems, antioxidant activity and the production of vasoactive molecules such as nitric oxide and prostaglandins.²³⁰ Estrogens are also able to inhibit atherosclerosis, which is a causative agent of cardiovascular disease.²⁰² Phytoestrogens are thought to be able to exert a similar

protective mechanism as estrogen. Similar to estrogens, phytoestrogens are able to decrease LDL concentrations.²³¹

The Framingham study, which involved 939 postmenopausal women, compared phytoestrogen intake with various parameters that could influence cardiovascular disease.¹¹ A food questionnaire together with a medical examination formed part of the study. The authors concluded that a correlation does exist between dietary intake of phytoestrogens and a lower risk of cardiovascular disease. However, a randomized control study with healthy men and postmenopausal women found that an isoflavonoid supplementation did not alter serum lipid concentrations in humans.²³² The discrepancy between these two studies could be due to fact that both men and women were investigated in the second study whereas in the first study only postmenopausal women were investigated. In addition, the sample size of the Framingham study (939 women) greatly outnumbers that of the second study (59 participants of which only 13 were women).

The wide discrepancies within clinical studies investigating the effects of phytoestrogens not only in cardiovascular disease but also in cancer and osteoporosis, and for menopausal symptoms need to be investigated further. One major concern is the number of people participating in a study, as an effective conclusion cannot be drawn when the sample size is very small. In addition, placebo controls must always accompany such clinical trials to control for the placebo effect. Despite the fact that conclusive evidence of the protective action of phytoestrogens against diseases such as osteoporosis, cancer and cardiovascular disease has not been provided, herbal remedies containing phytoestrogens are already available to the public.

1.3.4. Plant extracts shown to have phytoestrogenic activity

There are a number of plant extracts shown to have phytoestrogenic activity (Table 5). Most in-depth research has concentrated on *Glycine max* or soybean as epidemiological studies would suggest that a diet high in soy and soy products does protect against certain cancers.^{14;15} Genistein and daidzein are the most abundant phytoestrogens in soybean.¹⁷ In recent years, however, investigations of other plant sources of phytoestrogens have become increasingly popular and some of these will be briefly discussed.

Leguminosae plants, to which soybeans belong, are known to contain high levels of phytoestrogens.^{14;233} A study investigating other not so well known leguminosae plants found that

many plants belonging to the Leguminosae family could be a source of phytoestrogens.²³⁴ Although soybeans are the most well known, other leguminosae plants with phytoestrogenic activity include *Trifolium pretense* (red clover), and *Medicago sativa* (alfalfa sprout) both of which are also rich in genistein and daidzein. Red clover was first thought to contain hormone-disturbing compounds when in the 1940's it was noticed that sheep that grazed mainly on subterranean clover in Australia became infertile.²³⁵ Subsequently, biochanin A, formononetin, genistein and daidzein were shown to be the main phytoestrogenic components of red clover.¹⁷² In contrast alfalfa sprouts contains coumestrol, apigenin, luteolin and quercetin all of which are known phytoestrogens.²³⁶ The concentration of coumestrol and apigenin, however, differs depending on the stage of the plants development i.e. vegetative vs flowering stages.²³⁶ Coumestrol is known as a potent phytoestrogen exhibiting strong estrogen agonist activity.⁸² Despite containing coumestrol, alfalfa sprout extracts were shown to have the lowest binding affinity for ER β , when compared to six other leguminosae plants, and had very low binding affinity for ER α . It was nonetheless able to significantly induce MCF-7 cell proliferation at the highest concentration tested.²³⁷ Although soybean, red clover and alfalfa sprouts are thought of as the plants with the highest phytoestrogenic activity a comparative study of seven leguminosae plants identified kudzu root (*Pueraria lobata*) as having higher estrogenic activity than these plants as it had the highest binding affinity for ER β and was the only plant extract to display significant binding to the ER α . Similarly, it was more effective than all the others in inducing MCF-7 cell proliferation.²³⁷ Similarly to red clover it also contains genistein, daidzein, biochanin A and formononetin, in addition to coumestrol²³³ substantiating again the strong phytoestrogenic activity of coumestrol.⁸²

Common alcoholic beverages also contain phytoestrogens as they were shown by means of *in vitro* assays to have estrogenic activity. Resveratrol, commonly found in grapes, is able to bind, and subsequently activate the ER of MCF-7 cells.²³⁸ It was also shown to induce cell proliferation of T74D breast cancer cells.²³⁸ In addition, red wine extracts were shown to have agonist activity with both ER subtypes with a higher activity with ER β .²³⁹ Hops (*Humulus lupulus*), primarily used as a preservative and flavouring agent in beer, has phytoestrogen activity.^{240;241} It contains a potent phytoestrogen, 8-prenylnaringenin. Similar to other phytoestrogens, 8-prenylnaringenin was shown to have a stronger binding affinity for ER β than ER α .²⁴¹ A comparative study investigating plant extracts of red clover and hops determined the

binding potencies of the two plant extracts and found that the EC₅₀ values for binding to ER α and ER β was 15 μ g/ml and 27 μ g/ml, respectively, for the hops extracts, and 18 μ g/ml and 2 μ g/ml, respectively, for the red clover extracts.¹⁷¹ Additionally, both extracts were able to induce ERE reporter expression in both MCF-7 and Ishikawa cells, induce PR mRNA expression levels in both cell lines, and were also able to induce alkaline phosphatase activity in Ishikawa cells.¹⁷¹ These findings suggest that not only is soybean or red clover sources of phytoestrogens, but also to consider that other plants and plant based products like wine and beer deserve similar attention.

Cabbage, for example, was shown to induce MCF-7 cell proliferation, bind to the ER (lamb uterus) and induce transcriptional transactivation through both ER α and ER β .²⁴² *Ginkgo biloba* was also shown to bind to both ER subtypes with preferential binding to ER β and was able to induce MCF-7 cell proliferation and pS2 mRNA levels.²⁴³ Liu *et al.*¹⁷⁰ evaluated numerous plant extracts for the possible treatment of menopausal symptoms using a wide variety of *in vitro* screening assays. Some plant extracts that portrayed estrogenic activity included licorice (*Glycyrrhiza glabra*), ginseng (*Panax ginseng*), hops (*Humulus lupulus*) and chasteberry (*Vitex agnus-castus*) as well as red clover (*Trifolium pratense*).¹⁷⁰ This again substantiates that not only soybean needs to be investigated for phytoestrogenic activity but that other plants need to be recognised and investigated, such as *Moghania philippinensis*, a herb found in southwestern China, and black cohosh (*Cimicifuga racemosa*). Roots of the *Moghania philippinensis* herb both induced MCF-7 cell proliferation and β -galactosidase activity in a yeast two-hybrid assay.²⁴⁴ Black cohosh (*Cimicifuga racemosa*) extract on the other hand although shown to compete with radiolabelled E₂ for binding to cytosolic ER prepared from both porcine uteri and human endometrium²⁴. Similarly, the comparative study of Liu *et al.*¹⁷⁰ also showed that black cohosh did not display any binding affinity for the human recombinant ER subtypes. Black cohosh contains none of the common phytoestrogens²⁴⁵ such as isoflavones, flavonoids or coumestans, which may explain these results. Nonetheless, black cohosh extracts are commercially available for use in alleviating menopausal symptoms.

Plant extracts that have significant estrogenic activity are becoming increasingly popular as an alternative for the treatment of menopausal symptoms.

Table 5: Plant extracts evaluated for estrogenic activity and the assays used to determine estrogenicity

Plant extract	Assay used
<i>Humulus lupulus</i> (hops, Cannabaceae)	ER binding assays: both ER subtypes ^{171;241} ; Rat uterine cytosol ²⁴⁰ ERE reporter gene assays in either Ishikawa, MCF-7 ¹⁷¹ and yeast cells ²⁴¹ PR mRNA expression level determination ¹⁷¹ Alkaline phosphatase induction ^{171;240}
<i>Trifolium pratense</i> (red clover, Fabaceae)	ER binding assays: both ER subtypes ¹⁷¹ ERE reporter gene assays in both Ishikawa, MCF-7 ¹⁷¹ and yeast cells ¹⁷² PR mRNA expression level determination ¹⁷¹ Alkaline phosphatase induction ¹⁷¹
<i>Moghania philippinensis</i> (Fabaceae)	MCF-7 cell proliferation assay ²⁴⁴ Uterotrophic assay ²⁴⁴ Antiestrogen assay: cell proliferation of MCF-7 cells co-treated with E ₂ and various concentrations of extracts ²⁴⁴ Bone turnover assay ²⁴⁴ β -galactosidase activity in a Yeast Two-Hybrid Assay ²⁴⁴
<i>Ginkgo biloba</i>	ER binding assays: both ER subtypes ²⁴³ MCF-7 cell proliferation assay ²⁴³ pS2 mRNA expression level determination ²⁴³
Cabbage (Cruciferae)	ER binding assays: ER present in lamb uterus ²⁴² MCF-7 cell proliferation assay ²⁴² pS2 mRNA expression level determination ²⁴²
<i>Cimicifuga racemosa</i> (black cohosh)	ER binding assays: both ER subtypes ²⁴
<i>Glycine max</i> (soybean)	ER competitive binding assays ²³⁷ MCF-7 cell proliferation assay ²³⁷ Reporter gene assay in MCF-7 cells ²³⁷
<i>Medicago sativa</i> (alfalfa sprout)	ER competitive binding assays ²³⁷ MCF-7 cell proliferation assay ²³⁷ Reporter gene assay in MCF-7 cells ²³⁷
<i>Pueraria lobata</i> (kudzu root)	ER competitive binding assays ²³⁷ MCF-7 cell proliferation assay ²³⁷ Reporter gene assay in MCF-7 cells ²³⁷
Wine extracts	Yeast transactivation assay ²³⁹ Reporter gene assay in transiently transfected CHO cells ²³⁹ MCF-7 cell proliferation assay ²³⁹

1.3.5. Phytoestrogenic products available to the public

A soy isoflavone extract Phyto Soya™, which contains genistin (glycoside form of genistein) and daidzin (glycoside form of daidzein), was shown to be effective in reducing hot flushes in a double-blind, randomized, placebo-controlled study with 75 patients. Randomly chosen women with complaints of hot flushes were allowed to take 70 mg of soy isoflavone extracts or placebo for 4 months. Patients already reported a 61% reduction in hot flushes at week 16.²¹

Menoflavon™ and Promensil™ are registered standardised red clover (*Trifolium pratense*) products that contain concentrated amounts of isoflavones. The recommended daily dose is 40 and 40-80 mg isoflavone, respectively, and suggested usage is for the alleviation of menopausal symptoms such as hot flushes.^{21;22} Standardised red clover Menoflavon™ extracts were shown to display typical phytoestrogenic activity through binding preferentially to ERβ in a recombinant yeast based system.¹⁷²

Black cohosh (*Cimicifuga racemosa*) is also already available as an alternative to HRT under the registered name of Klimadynon®.²⁴ Although black cohosh extracts were unable to increase uterotrophic weight, a common indicator of estrogenic activity in rats, its estrogenic effects were comparable to that of conjugated estrogens in the hypothalamus, bone and in the vagina²⁴, highlighting the SERM activities of phytoestrogens.

The biological responses mediated by phytoestrogens are not yet fully understood with much contradictory evidence regarding health benefits. However, what is clear is that their effect is pleiotropic and that not all phytoestrogens are able to elicit the same responses (Tables 3 and 4). Epidemiological studies nevertheless do suggest that they have a protective effect. Lamartiniere postulates that early exposure to phytoestrogens is critical for the protective biological response mediated by phytoestrogens.²⁴⁶ This theory is supported by studies investigating and comparing breast cancer incidence amongst first- and second-generation Asian emigrants to the West.²⁴⁷ Additionally, there has been no major large scale study, comparable to that of the WHI studies on traditional HRT, to establish health benefits and examine any side-effects of phytoestrogens for the treatment of hormone dependent cancers or menopausal symptoms. This is important as previously mentioned clinical trials evaluating phytoestrogens for the treatment of hormone dependent cancers and menopausal symptoms have not been unequivocally positive. Nevertheless, phytoestrogenic products claiming to alleviate menopausal symptoms are already available on the market.

Most phytoestrogenic products are, like the majority of herbal medicines, available without prescription from a doctor and usage is solely dependent on the individual's preference. The general public is thought to be more inclined towards natural products as they are believed to have less side-effects than conventional synthetic drugs.¹ Specifically as pertaining to phytoestrogens, concerns over safety following the fact that conventional HRT has been found to increase the risk of certain cancers^{18;19} coupled to the evidence of epidemiological studies also

plays a major role in the public's perception of the effectiveness of a flavonoid rich diet. Unfortunately, there is a limited scientific basis for the effectiveness of many nutraceuticals as health claims are not by law required to be substantiated, nor is risk assessment or even quality control and standardisation relating to formulation. However, countries have and are changing legislation with respect to the nutraceutical and functional food industry, as less and less health claims without scientific research are allowed.^{2;4} In South Africa only medicinal claims for products registered with the Medicines Control Council are allowed. Currently, nutraceuticals are being considered for classification as a class of medicine (according to a draft regulation to the Medicines and Related Substances Control Act, which was published in the South African Government Gazette of 16 July 2004). Consequently, this would require substantiation of health claims through scientific research and clinical trials and product standardisation to ensure that each batch is identical and that the amount of active compounds, such as plant polyphenols, present is consistent from batch to batch, a difficult task considering that the concentration of plant polyphenols present in plants often depends on the growth conditions such as environmental stress and time of harvesting.

1.4. Techniques used to measure phytoestrogenic activity

Currently there are a number of experimental methods used to identify potential phytoestrogens most of which are molecular techniques as estrogen mediates its effect on a molecular level. The most commonly used *in vitro* methods include ER binding assays, reporter gene assays, cell proliferation assays, analysis of changes in gene expression, mRNA and protein expression, which are estrogen dependent. Also *in vivo* techniques are applied to evaluate estrogenicity of possible phytoestrogens. In 2000 the American Environmental Protection Agency proposed a two-tiered test system for the screening of endocrine disrupters, under which phytoestrogens are also grouped. The first test system or Tier 1 basically only identifies through both *in vitro* and *in vivo* screening methods substances or compounds able to bind to the estrogen, androgen or thyroid hormone receptors. This is followed by the second test system or Tier 2 that only consists of *in vivo* assays examining the compounds identified in the first test system, which causes unfavourable effects in animals. The effects are identified and followed by the identification of a quantitative relationship between the dose and the adverse effect.²⁴⁸

As previously described, estrogen enters the cell and binds to the ERs, that sets off a chain of events. All experimental methods used to measure the estrogenicity of a compound basically exploits one or more of these events, measuring endpoints involving either specific steps in the molecular mechanism of estrogens or subsequent cellular or organism responses (Figure 11). Upon ligand binding to the ER, the ER-ligand complex dimerizes and recognises an ERE situated in the promoter regions of target genes and recruits cell-specific co-activators. Binding to the ERE results in either activation or inhibition of gene transcription of estrogen-targeted genes. Gene transcription and translation, results in a cascade of events such as cell proliferation and the expression or repression of certain enzymes. Thus the *in vitro* or *in vivo* techniques employed to investigate estrogenicity targets one or more responses or actions in the “estrogen pathway”.

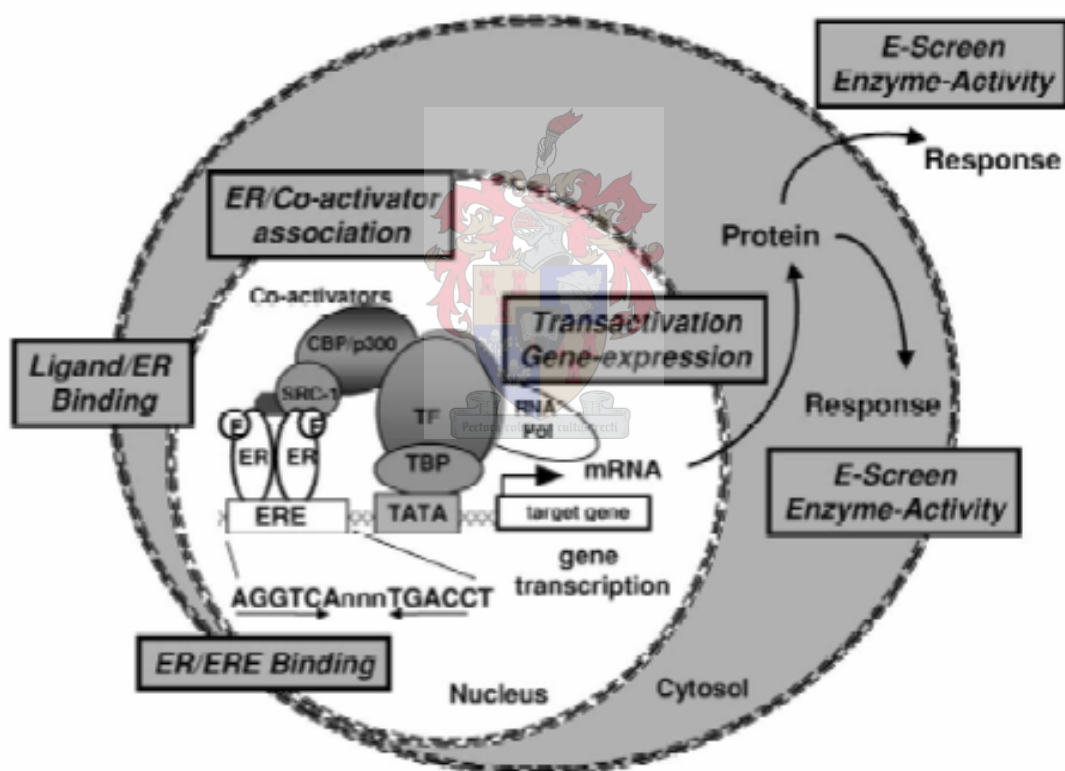


Figure 11: The different endpoints that can be measured to determine estrogenicity.²⁴⁹

1.4.1. *In vitro* techniques used to determine estrogenicity of putative phytoestrogens

There are numerous *in vitro* techniques used to determine whether plant phenolic compounds have phytoestrogenic activity. All techniques would generally include the endogenous

hormone E₂ as reference compound. The most frequently used *in vitro* techniques used are ER binding studies, transactivation assays and cell proliferation assays. Within each group of assays there also exist variations. For example for ER-binding studies ER α and/or ER β may be used, ERs may be from divergent sources such as recombinant, transfected or endogenous, and assays systems may differ. Seldom, if ever, only one technique is employed, as assays differ in sensitivity and limited information is obtained from only one assay. As a result binding studies, for example, which only identify ligand binding to the ER subtypes and cannot discriminate between agonistic or antagonistic activity, are commonly accompanied by transactivation studies or cell proliferation studies. Other techniques that will also be discussed include measuring the levels of estrogen dependent proteins or mRNA levels inducible by estrogens. All these methods not only identify new phytoestrogens but also give an idea of their potency relative to that of E₂.

1.4.1.1. Estrogen receptor binding assays

Estrogens, entering a cell from the bloodstream, mediate their biological effects by means of binding to the ERs. As binding to the ER initiates biological responses induced by estrogens, this assay is generally the first assay used for the identification of possible phytoestrogens.²⁴⁹ A common definition of phytoestrogens in the literature is plant phenolic compounds with a structural similarity to E₂ that are able to bind to the ERs.¹⁴⁶

The ER-binding assay simply measures the specific interaction of possible phytoestrogens with the ERs. It provides a quantitative measurement of estrogenic activity at receptor level and allows comparison of activity relative to that of E₂. It is a relatively easy *in vitro* assay to perform and more importantly it allows for selection of ER subtype (phytoestrogens bind preferentially to the ER β) and thus makes possible discrimination between binding affinities to the different ER subtypes. Binding assays used include saturation and competitive binding assays, with the latter more commonly used.

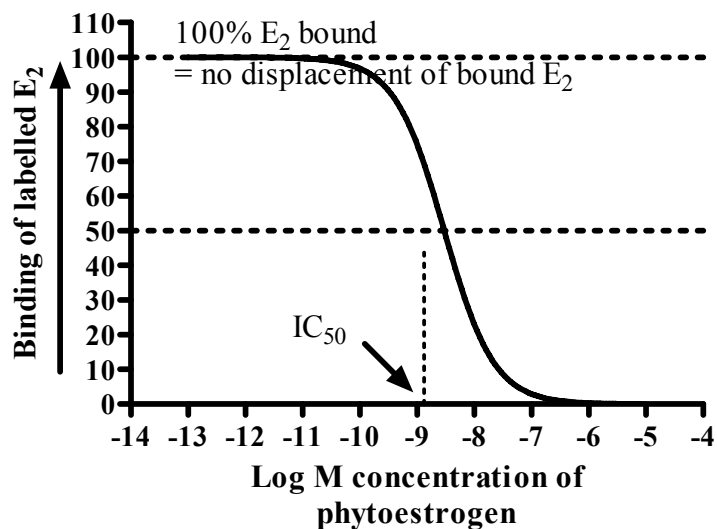


Figure 12: A schematic representation of a competitive binding curve. The binding affinity of a putative phytoestrogen is represented as an IC_{50} value. The IC_{50} value is the concentration of phytoestrogen required to inhibit 50% of radiolabelled E_2 binding.

Competitive binding assays conventionally make use of radiolabelled E_2 that competes with increasing concentrations of unlabelled ligand for binding to the ER. The radiolabelled E_2 concentration is kept constant. Binding affinity is normally expressed as the concentration of phytoestrogen required to inhibit E_2 binding by 50% (EC_{50} or IC_{50}) (Figure 12). For comparison between different types of assays binding affinity is often expressed relative to that of E_2 . The RBA for a phytoestrogen is calculated as the fraction or percentage of the IC_{50} for E_2 .

$$RBA = IC_{50} (E_2) / IC_{50} (\text{test compound}) \times 100$$

Not all competitive binding assays are, however, the same, as the source of ER commonly differs. Binding assays can be performed in either whole cells expressing the required ER subtypes, with purified or recombinant ERs, or in cytosolic extracts of cells or tissues.

Ovariectomized rat or mouse uteri are commonly used as a source for ERs for tissue specific binding assays. The uteri are homogenized and cytosolic preparations are used. Although one is able to investigate ER binding in a specific tissue the species difference is a disadvantage. For example, it has been shown that the phytoestrogens, genistein and coumestrol, have different affinities for recombinant human $ER\alpha$ than for the mouse ER uterine cytosol.²⁵⁰ In addition, the

tissue used may express both ER subtypes, often not at the same levels, which further complicates the interpretation of results especially when using cytosolic preparations

Recombinant ERs used in competitive binding assays are helpful and extremely useful as this allows for selection between the ER α and ER β , important as proliferation of estrogen dependent cancers is believed to be mediated *via* the ER α subtype.^{219;221;251} Recombinant ERs in addition allows for choice between species (rat *vs* human). However, variations in the ER subtype and variants i.e. full length ER *vs* only the LBD used in assays from different laboratories makes it difficult to compare RBA for a single phytoestrogen (Table 3).

The whole cell binding assay, although time consuming, with duration of up to five days (in our case), does offer the advantage of being in an intact cellular environment. Whole cell binding assays using MCF-7 breast cancer cells or other cell lines, which express endogenous ER, need not be transfected but limit options as to the ER subtype as both ER α and ER β are expressed in MCF-7 cells.²⁵² However, cell lines, which do not endogenously express ERs, present the option of discriminating between RBAs for the two ER subtypes through transfection of either cDNA.

Recently, an alternative to using radiolabelled E₂ as competitor in competitive binding assays has been developed and is commercially available.²⁵³ The fluorescence labelled E₂ has a high affinity for purified recombinant human ERs and commercial assay kits using recombinant ERs are available. Although a more artificial system it is a high-throughput system, which especially when screening for a great number of phytoestrogens is especially useful.

Binding experiments whether whole cell, cytosolic, or using purified receptor simply measure the affinity of phytoestrogens for the ERs. It, however, does not provide an indication of receptor activation nor does it differentiate agonists from antagonists. Thus once a phytoestrogen has been identified as binding to the ER subsequent *in vitro* or *in vivo* assays are required to establish an estrogenic response.

1.4.1.2. Reporter gene assay or transactivation assays

The reporter gene assay is an indirect measurement of estrogenic activity whereby the expression of a reporter gene construct under the promotional control of an ERE is measured and quantified.²⁴⁹ A recombinant assay with a simple design, it usually relies on a selected mammalian cell line or yeast strain being transfected with an expression plasmid containing the

ER cDNA if the cell line is lacking endogenous ER, together with a promoter-reporter construct plasmid consisting of an ERE-containing promoter cDNA linked to a reporter construct such as luciferase or chloramphenicol acetyltransferase (CAT) or in yeast systems, β -galactosidase (Figure 13).

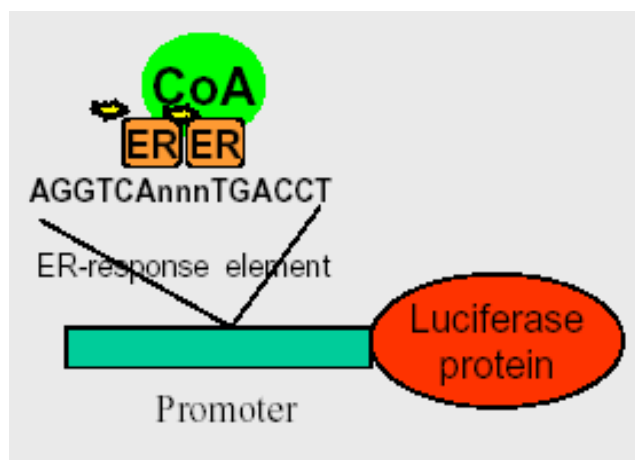


Figure 13: Simplified schematic diagram of the reporter gene assay.

Binding of a ligand to the ER will result in the ER-ligand complex binding to the ERE followed by the formation of a gene product, normally an enzyme, in a dose dependent manner that is easily measurable and quantified.²⁴⁸ The gene product is dependent on the ER-ligand complex interacting with the ERE and in the absence of ligand or receptor should not be activated. The reporter gene assay consequently not only measures ER-ERE interaction and thus transcription but it is also dependent on ER-ligand binding. This *in vitro* method is widely used to measure ligand induced ER-mediated gene transcriptional activation or inhibition.^{135;249} Unlike receptor binding assays the reporter gene assay can identify whether phytoestrogens will mediate a biological response through acting either as an ER (full) agonist, partial agonist, weak agonist or antagonist (Figure 14). The degree of activation comparable to that of a reference agonist, such as E_2 , determines whether a phytoestrogen would be defined as a weak agonist, partial agonist or agonist. A weak agonist generally has a much higher EC_{50} value than the reference agonist and a partial agonist even at the highest concentration examined does not reach maximal activation seen with the reference agonist. An antagonist is a ligand that, in a dose response manner, inhibits the transcription of the reporter gene in the presence of a known agonist like E_2 (Figure 14).

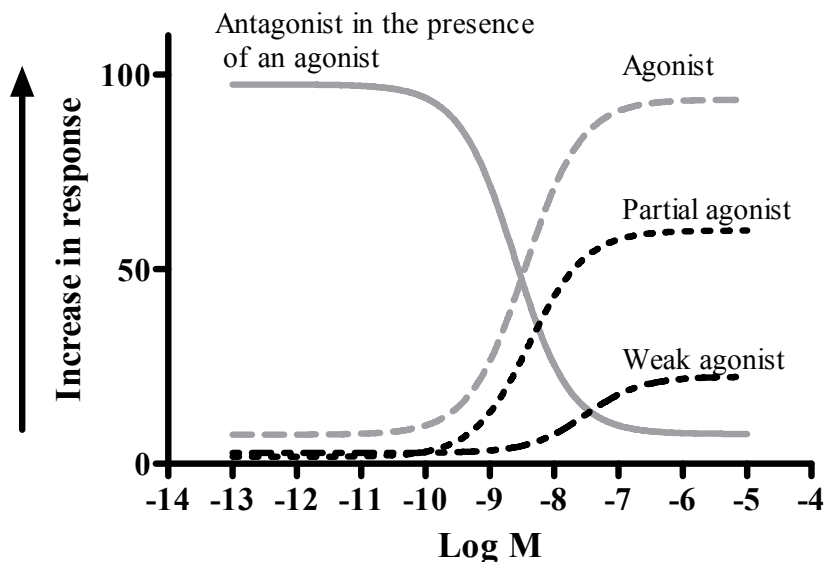


Figure 14: A schematic representation of dose response curves of different types of agonists and an antagonist.

The reporter gene assay is a very versatile screening method in that either mammalian or yeast cells can be used.²⁴⁹ Because some cell lines do not express endogenous ER either ER α or ER β or functional ER domains can be transfected into these cell lines. The promoter region of the reporter gene can also contain the entire promoter region of an ER specific target gene or any consensus or nonconsensus EREs.²⁴⁹

The potency of a phytoestrogen can be measured and quantified using this recombinant cell based assay. Specifically, the EC₅₀ values or potency values can be determined and compared relative to the EC₅₀ value of E₂.²⁴⁹ The potency or EC₅₀ value is basically the concentration of ligand that induces a response halfway between the maximum response and the baseline (Figure 15). Similarly, the efficacy values can be measured, quantified, and compared to that of E₂. The efficacy value is the maximal response induced by the ligand (Figure 15). Relative potency and efficacy may also be calculated to compare between different types of assays and is normally calculated as a percentage or fraction of the EC₅₀ or maximal induction, respectively, induced by a reference compound such as E₂.

$$\text{Relative potency} = \text{EC}_{50}(\text{E}_2) / \text{EC}_{50}(\text{test compound}) \times 100$$

$$\text{Relative efficacy} = \text{efficacy}(\text{test compound}) / \text{efficacy}(\text{E}_2) \times 100$$

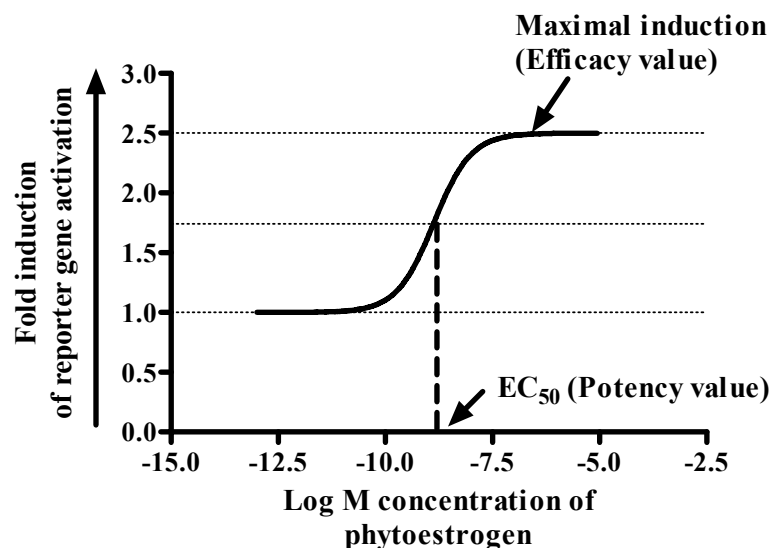


Figure 15: A schematic representation of a sigmoidal dose response curve of a common transactivation result. The potency (EC_{50} value) and the efficacy values are indicated.

Use of either mammalian or yeast systems does, similarly to binding assays, complicate comparison between studies. Factors that could explain the differences in yeast systems *vs* mammalian assays include differences in cell wall permeability, differences in receptor levels, and metabolic capabilities of the two different cell types.²⁴⁸ Even the use of different mammalian cell lines and reporter assays complicates comparison. According to the National Health Institute (NIH, United States of America) cell lines most commonly used in reporter assays, are HEK293, HEC-1, HeLa, HepG2, and MCF-7 cell lines while those used less frequently include CHO, BG-1, COS-1, ELT3, MDA-MB-231, T47D, and Ishikawa cell lines. In addition to using different cell lines, the source of the ERE and type of plasmid or reporter differs and can influence the measured estrogenic activity of a compound.²⁴⁸ Consequently, potency and efficacy values obtained from different transactivation systems used depend on numerous factors like experimental conditions, ER concentrations, and response element used. Recently various *in vitro* assays for use in screening of compounds for estrogenicity were compared.²⁵⁴ The most interesting result was the comparison of two stably transfected cell lines, MVLN (a breast cancer cell line stably expressing an estrogen regulated luciferase gene driven by an ERE in front of the vitellogenin-tyrosin-kinase-promoter) and HGELN (a cell line derived from the HeLa cells stably cotransfected with a plasmid conferring resistance to neomycin, p17m5- β Glob-Luc and the chimeric receptor expression plasmid GAL4-ER. Although there was a good correlation ($R^2 =$

0.9993) between the potencies values calculated from the reporter gene assays using the MVLN and HGELN cells, the EC₅₀ values obtained with the MVLN cells were much lower. Thus the MVLN luciferase reporter gene assay was more sensitive to stimulation by estrogens than the HGELN luciferase reporter gene assay. Cell type and reporter construct thus played a definite role in determining the potencies of the various compounds tested.

Despite the fact that different cell lines, reporter constructs, and receptor types are used that complicates comparison of results from different laboratories the reporter gene assay is still the most widely used assay because of its accuracy and sensitivity.

1.4.1.3. Analysis of estrogen induced mRNA and protein expression

Cell lines or tissues expressing endogenous estrogen sensitive genes can also be used to measure phytoestrogens for estrogenicity. This method allows for investigation of tissue or cell specific activation of estrogen responsive genes. Estrogenic activity is measured by quantifying mRNA or protein expression using either real-time polymerase chain reaction (PCR) or Northern-blot for quantification of mRNA or Western blot or activity assays for quantification of protein levels. A variety of estrogen regulated genes or proteins can be measured using these techniques. Estrogen regulated genes that have been used as bio-markers are pS2, a protein present in breast cancer and in MCF-7 cells²⁵⁵, the PR, present in breast tissue and in the uterus^{256;257} and cathepsin D expressed in the MCF-7 breast cancer cells.²⁵⁸ All these proteins are up regulated by estrogen and thus can be used as bio-markers to identify possible phytoestrogens. Monitoring the mRNA and protein expression in the absence or presence of E₂ can also distinguish agonist from antagonists.

As an extension of measuring protein levels the activity of estrogen sensitive enzymes can also be measured. Alkaline phosphatase activity can be stimulated by estrogen and is easily measured in the human endometrial Ishikawa cell line.²⁵⁹ This assay measures the physiological response of estrogens and can also be used to distinguish agonists from antagonists. The alkaline phosphatase assay is an easy and simple screening method for phytoestrogens and as a high throughput screening assay it is effective in identifying the presence of phytoestrogens in plant extracts.²⁶⁰

A new and innovative method to screen for estrogen-induced genes is DNA microarray analysis.²⁶¹ As estrogen is involved in numerous biological effects in the human body, this

technique allows one to simultaneously monitor the response of a wide variety of estrogen responsive genes in the presence of the compound of interest (phytoestrogen) in comparison to the natural ligand. DNA microarray analysis has been used to measure the response of a wide variety of phytoestrogens (13 in total) on 172 estrogen responsive genes in comparison to the response of the natural ligand E₂.²⁶²

1.4.1.4. Cell proliferation assays

“an estrogen is a substance that can elicit the mitotic stimulation of the tissues of the female genital tract; therefore, measuring cell proliferation is of key importance in assessing estrogenicity. To determine whether chemical “X” is an estrogen, one must test its ability to induce proliferation of estrogen-responsive target cells, even though not all estrogen responses or target tissues respond with proliferation.”

Roy Hertz (1985)

Cell proliferation is the most popular test system for the measurement of estrogenicity of putative phytoestrogens and other compounds thought to have estrogenic activity.²⁴⁹ A relatively easy assay to perform, it is a downstream effect of estrogen. This classical assay measures one of the physiological outcomes of estrogens and depends highly on estrogen-sensitive ER-positive cell lines, which proliferate when stimulated by estrogens or estrogen-like compounds. According to the NIH the MCF-7 breast cancer cell line is the most commonly used cell line, although the T47-D breast cancer cells and BG-1 cell line are also frequently used to measure proliferation.²⁴⁸ The estrogen insensitive cell line, MDA-MB-231, which does not proliferate in response to E₂, is generally used as a negative control for estrogenic activity. Numerous methods are used to measure the end point of the assay, cell proliferation. These include methods such as colometric or spectrophotometric assays using metabolizable dyes^{37;190;263;264;265;266} or determining protein content²⁶⁷, counting cells or nuclei with a Coulter counter or hemocytometer^{268;269} and measuring radiolabelled thymidine incorporation.²⁷⁰ The use of the DNA precursor, ³H-thymidine, to measure cell proliferation, however, is time consuming and the handling of a radiolabelled substance involves risks. The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium

bromide) assay is a colometric assay, which is relatively simple and easy to perform and is dependent on the cellular reduction of MTT to a blue formazan product by the mitochondrial succinate dehydrogenase of viable cells.²⁷¹ The formazan product is quantified spectrophotometrically at 570 nm.²⁷¹ Phytoestrogens and plant extracts have, however, been found to interfere with the MTT tetrazolium reduction and adequate washing of the cell is advisable before the addition of the MTT solution.²⁷² Another colometric assay commonly used to measure cell proliferation is the sulforhodamine B (SRB) assay, which uses SRB, to stain trichloroacetic acid fixed cells. The water-soluble anionic SRB dye binds electrostatically to the basic amino acids of cellular proteins and colour formation provides an estimate of the total cellular protein that is related to the cell number.²⁶⁷ The main advantage of using a colometric assay is the lack of using a radioisotope whereas counting of cells with a Coulter counter is fast and accurate but requires specialised equipment.

Cell proliferation is generally measured in response to increasing concentrations of test compound, which makes it possible to determine efficacy and potency from the dose response curve and to distinguish agonists from antagonists. However, it is advisable that cell proliferative assays be accompanied by ER binding studies or that proliferative induction by phytoestrogens is conducted in the presence of a known ER antagonist, such as ICI 182,780. This will establish whether the response, proliferative or antiproliferative, is *via* binding to the ERs as cell proliferation can occur independently from the ERs as has been reported for phytoestrogens.²⁷³

A simple assay in design, cell lines do differ in their response to estrogens and assays in each cell line should therefore be optimized as to specific conditions and requirements. Variants do exist of the most commonly used MCF-7 cell line, such as the MCF-7-BOS (sometimes called BUS), BB, and BB104 sublines.²⁷⁴ All these variants do differ in their response to estrogenic compounds, both in their potency and efficacy parameters and this should be taken into account when comparing potencies obtained from the literature.²⁶⁶ Unfortunately, some authors do not define the subtype of MCF-7 cells used, which makes comparison difficult.

The cell proliferation assays also do not distinguish between the ER subtypes as it is executed in cell lines, which normally contain both ER subtypes. Practical limitations include the relatively long incubation periods and the modest response although potencies measured are relatively high.²⁷⁵ Nevertheless, cell proliferative assays were found to be a consistent and valid test system for the physiological response of estrogens.²⁴⁹

Table 6: Summary of the most commonly used *in vitro* assays for the measurement of estrogenicity

***In vitro* assay**

Competitive ER binding assays

- Rat uteri cytosolic ER binding system²⁷⁶
- Mouse uteri cytosolic ER binding system²⁵⁰
- Purified recombinant ERs^{104;168;170;241;243;277}

Reporter gene assays

Mammalian based assays

- Endogenously expressed ERs²³⁷
- Stably transfected cell lines^{217;174;254;278}
- Transiently transfected cell lines^{168;277}

Yeast based assays

- Transfected cells³⁷

Protein or mRNA expression

- pS2^{170;243}
- PR^{170; 265;270}
- Alkaline phosphatase activity^{170;234}

Cell proliferation assays

- MCF-7 cell line^{217;242;37;243;270;277;279}

1.4.2. *In vivo* measurements for estrogenicity

Both mammalian and non-mammalian *in vivo* assays can be used to investigate possible phytoestrogens. As no *in vivo* assays were used in evaluating estrogenic potential in this thesis only the most commonly used assay, the uterotrophic assay, will be discussed.

The rodent uterotrophic assay is the most extensively used *in vivo* assay used to determine estrogenicity of possible estrogenic compounds.²⁸⁰ It resembles a true living system and takes into account absorption and metabolism of estrogenic compounds. This assay measures uterine growth in response to estrogenic compounds such as phytoestrogens. The uterotrophic assay uses either ovariectomized, immature intact, or hypossectomized female rats or mice to ensure the absence of endogenous estrogen production, which will guarantee that all results measured would be from the estrogenic potential of the administrated phytoestrogens.²⁸⁰ Administration of the phytoestrogens are either orally or subcutaneous. The uterine weight is expressed as either wet weight or dry weight. Potencies can be determined using this *in vivo* assay although to obtain a complete dose response is extremely time consuming and expensive and it would be difficult to screen a large amount of putative phytoestrogens. In contrast to *in vitro* assays that are much less time consuming and are inexpensive. It is important to note that the uterine weight assay only

measures the estrogenic effect in one tissue type and that the compound investigated could exert an estrogenic effect on selective tissue(s), which would be difficult if not impossible to identify with this type of assay. Investigation of vaginal opening after the immature rodents are sacrificed often accompanies the uterotrophic assay. A premature vaginal opening would suggest and strengthen positive increases in uterine weight, however, it has been found that premature vaginal opening is not as sensitive as an increase in uterine weight and that the route of administration of E₂ also has an effect on the vaginal opening²⁸¹. Numerous other endpoints, such as morphological, histological, molecular, and biochemical end points are becoming integrated into the classical uterotrophic assay.²⁸⁰ As the uterotrophic assay only measures uterine weight increase, the inclusion of other endpoints would increase the sensitivity of the uterotrophic assay and extend measurements of target organ or tissue selectivity. The target organ would thus not necessarily only include the uterus and vagina but the mammary gland, liver and bone tissue, the cardiovascular system, and the brain could all be investigated as additional endpoints.²⁸⁰ The measurement of the expression of estrogen inducible genes is common in determining the estrogenic potential of compounds *in vitro*. For example, PR expression levels in the mammary gland or uterus is sensitive to estrogen levels and PR mRNA or protein expression levels have also been measured *in vivo* through Northern-blot or real time PCR analysis, immunohistochemistry or Western-blot analysis.²⁸⁰

A study comparing the behaviour of various phytoestrogens in the *in vitro* transactivation assay with the *in vivo* uterotrophic assay incorporating additional endpoints such as the uterine epithelial cell height and gland formation, and expression levels of the estrogen sensitive lactoferrin protein present in the epithelial cells was conducted.¹⁷⁴ All phytoestrogens assayed, except for taxifolin, were able to induce transcription through the ER *in vitro*. The *in vivo* results were of much interest as some compounds, such as daidzein and naringenin, were unable to induce an increase in uterine wet weight although they tested positive for estrogenicity at other endpoints measured such as an increase in gland number and induction of a transcriptional response. This study underlines the complexity of screening for phytoestrogens especially when one considers that *in vivo* screening methods involve tissue sensitivity and selectivity and that signalling pathways activated may differ for each phytoestrogen tested.

1.4.3. Analytical screening methods

Analytical screening methods are usually employed to assist with the identification of known phytoestrogens in a complex mixture such as plant extracts. Analytical methods available for the determination of phytoestrogens can be separated into two groups: methods involving chromatographic separation such as high-performance liquid chromatography (HPLC) and those that do not depend on chromatographic separation such as UV spectroscopy and immunoassays.²⁸²

1.4.3.1. Chromatographic screening methods

Chromatographic methods in fact does not screen or identify potentially novel phytoestrogens but rather identifies and quantifies known active phytoestrogens ²⁸². Chromatographic screening methods are therefore commonly used to identify known phytoestrogens present in plant extracts through separation. The separation of phytoestrogens within a mixture by chromatography and the choice of a particular method depend on the sensitivity required and the complexity of the extracts or compounds of interest. Methods include gas chromatography-mass spectrometry, HPLC and liquid chromatography-mass spectrometry.

1.4.3.2. Non-chromatographic methods

Non-chromatographic methods available for the detection of phytoestrogens include immunoassays and spectroscopic methods to identify known phytoestrogens in mixtures according to Wang *et al.*²⁸². The immunoassays are specific, high throughput, and inexpensive but rely on the production of specific antibodies targeted at known phytoestrogens. The selectivity of the spectroscopic methods has been questioned and needs to be combined with chromatographic separation when working with plant extracts.²⁸²

1.4.4. Comparison of different methods

Estrogenicity test results obtained with different testing methods or more importantly with the same method but by different laboratories can and more than likely do differ significantly (Tables 3 and 4).²⁴⁸ This makes it extremely difficult to compare binding parameters and potency

and efficacy values for induction obtained from various sources as these are dependent on the test system itself and the conditions under which it was conducted. Regardless, these methods form an essential part of determining the estrogenic potential of plant polyphenols and comparisons of the effectiveness and reliability of methods and standardisation of procedures are required.²⁴⁸ A study comparing various *in vitro* assays in the testing of an assortment of compounds for estrogenic activity was conducted.²⁵⁴ Specifically, results from (i) two luciferase reporter gene assays using the stably transfected cell lines, MVLN (breast cancer cell line stably expressing an estrogen regulated luciferase gene driven by an ERE in front of the vitellogenin-tyrosin-kinase-promoter) and HGELN (derived from the HeLa cells stably cotransfected with a plasmid conferring resistance to neomycin, p17m5-βGlob-Luc and the chimeric receptor expression plasmid GAL4-ER), (ii) the proliferative assay (E-screen) with MCF-7 cells, and (iii) competitive binding assays of recombinant ER α and ER β , were compared. In comparing the potencies determined for E₂ the authors found that the sensitivity of the assays decreased in the order: MVLN-cells = E-Screen > HGELN-cells >> binding to recombinant ER- α or ER β . In addition, there was a good correlation between the EC₅₀ values obtained with transactivation assays using tissue culture (all in the pM range) and the competitive binding assays (EC₅₀ values in the nM-range) even though the source of ER is different, however, the correlation was lower than the correlation of potencies obtained with the transactivation assays and the cell proliferative assay. Thus the authors concluded that the various *in vitro* test systems investigated (reporter gene assays, receptor binding assays and cell proliferative assays) are all suitable for the determining estrogenic capability although they differ in sensitivity.²⁵⁴

In addition, a study comparing the behaviour of eight phytoestrogens in different *in vitro* assay systems using only the MCF-7 breast cancer cells has been conducted.²⁸³ By comparing ER-binding, estrogen-responsive reporter gene transcription, and cell proliferation in the same cell line it was possible to make direct comparisons of potencies obtained due to assay type excluding cell specific confounders. The reporter gene activation and cell proliferative assays showed no difference in rank order of potencies obtained for the phytoestrogens tested, however, the ER binding study did not correlate well with the other *in vitro* assays for all compounds. This agrees with the results of the previous study. Whole cell binding assays were not used but rather cytosolic preparations that may reflect differences in the fate of the ligand within the cell, which could be influenced by cellular uptake and/or metabolic processes.

When comparing *in vitro* transactivation assays in mammalian and yeast systems and *in vivo* assays for measuring the estrogenic activity of various compounds it was found that the stably transfected MCF-7 cells line, MVLN cells, were more sensitive (3-15 times depending on the compound tested) than the yeast reporter gene assay. In addition, there was a small difference between the potencies measured in the *in vitro* assays and the *in vivo* assay measuring vitellogenin induction in a zebrafish system.²⁸⁴

Generally then there seems to be a good correlation between assay systems compared especially in the rank order of potencies. However, the degree of sensitivity between the assays does differ with selective promoter reporter assays and the proliferative E-Screen appearing to be most sensitive. Identification of phytoestrogens that by definition are weak estrogens would require a sensitive screening assay and thus choice of assay is important. There are a wide variety of phytoestrogen screening methods available. The choice and combination of different techniques in an individual research laboratory depends mainly on cost and throughput needs. However, from the comparisons of various techniques discussed here it is clear that not all phytoestrogens act in a similar manner, which is an extremely important consideration when considering results from *in vivo* screening assays as tissue selectivity of phytoestrogens has been established.⁷ Also, even though phytoestrogens by definition are able to bind to the ER subtypes, ER binding studies alone do not suffice if the biological responses induced by a particular phytoestrogen are of interest, although it is a good indicator that the phytoestrogen might have estrogenic or anti-estrogenic activity. Thus, although determination of phytoestrogen binding is essential to be able to define a particular phytoestrogen as a ligand for the receptor it should be followed by an assay determining agonist or antagonist activity. An effective test strategy would be binding assays combined with either transactivation or cell proliferation assays. To further validate findings one could measure the mRNA levels of estrogen responsive genes such as pS2. If plant extracts are investigated, it would be constructive and interesting to identify the active compound(s) thus activity-guided fractionation through HPLC analysis could be useful. However, binding assays and some form of measurement of estrogenic activity either through transactivation experiments or cell proliferation assays should be sufficient for screening of putative phytoestrogens.

1.5. Honeybush (*Cyclopia*)

The genus *Cyclopia* belonging to the Fabaceae family is indigenous to South Africa and part of the bio-diverse fynbos kingdom. It is predominantly found in the coastal region of the Western and Eastern Cape Provinces with ca. 24 species growing wild, each within specific growth regions.²⁸⁵ The woody shrub is a leguminosae similar to soy bean (*Glycine max*), alfalfa sprout (*Medicago sativa*), and red clover (*Trifolium pratense*). The leaf shape and size differ between species with some species having a needle-like to elongated leaf while others have a broader leaf. With its small trifoliate leaves *Cyclopia* is easily recognizable during spring when its prominent yellow flowers bloom. Traditionally the plant was only harvested for the manufacture of a herbal tea during the flowering season to facilitate recognition in the wild. Because of its honey scented yellow flowers and the sweet, honey-like aroma that develops during the traditional manufacturing process, *Cyclopia* are more commonly known and referred too as honeybush. The leaves, stems, and flowers of honeybush are traditionally used to manufacture a herbal tea. Presently, *Cyclopia genistoides* (kustee), *C. subternata* (vleitee), *C. intermedia* (bergtee), and to a lesser extent *C. sessiliflora* (Heidelbergtee) are available commercially to the public. *Cyclopia subternata* is available either as fermented- or unfermented (green) tea (Personal communication, Dr E Joubert, 2004) and can be seen on the shelves of local supermarkets. This herbal infusion is caffeine free and has a very low tannin content which makes it ideal for consumers avoiding stimulants such as caffeine.³¹

1.5.1. Adding value to honeybush tea and nutraceutical products

Anecdotal evidence would suggest that honeybush is a diuretic, able to stimulate milk production in lactating woman, and prevent ulcers.²⁸⁶ Additionally, honeybush has been used to treat infants with colic and as a cough syrup for treatment of lung infections.²⁸⁷ Honeybush is not only an enjoyable beverage, but it also serves as a functional health tea as it was identified as having *in vitro* antioxidant^{43,44} and antimutagenic^{44,45} activity. Flavonoids found in honeybush were shown to act as antioxidants in scavenging the synthetic 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonate) radical cation, reducing a ferric-tripyridyltriazine complex and inhibiting Fe²⁺ induced lipid peroxidation in *in vitro* assay systems.⁴⁴ They were shown to donate an electron accompanied by a hydrogen nucleus from the OH group attached to their

phenolic rings to a free radical thus stabilizing and inactivating the damaging radical. These phenolic compounds are also thought to reduce the frequency of spontaneous or induced mutations against 2-acetylaminofluorene and aflatoxin B₁ induced mutagenesis in the *in vitro* *Salmonella* mutagenicity assay⁴⁵, which could lead to cancer chemoprevention. These health protective mechanisms add additional nutritional value to honeybush tea with both antioxidant and antimutagenic activity important in cancer chemoprevention and could be used to further popularise honeybush tea with the public. Additionally, further marketing in the ever-popular nutraceutical industry could concentrate on health products containing concentrated honeybush extracts.

1.5.2. Polyphenols present in honeybush

The xanthone, mangiferin, and the flavanone, hesperidin, were found to be the major phenolic compounds present in unfermented *C. genistoides*, *C. sessiliflora*, *C. subternata* and *C. intermedia*.⁴⁴ This agrees with a previous study reporting that the major phenolic compounds present in *Cyclopia* species are mangiferin, hesperetin, and isosakuranetin.³³ In addition, the coumestans, medicagol, flemichapparin and sophoracoumestan, the flavanones, hesperidin, naringenin, and eriodictyol, the xanthone, isomangiferin, a flavone, luteolin, and the isoflavones, formononetin, afrormosin, pseudobaptigen, fujikinetin, and calycosin, were identified as being present in fermented *C. intermedia*.³⁴ Phenolic compounds found to be present in unfermented *C. subternata* were an isoflavone, orobol, a flavanol, epigallocatechin 3-*O*-gallate, a flavan glycoside, a kaempferol glycoside, the flavones, luteolin and scolymoside, and the flavanones, hesperidin, hesperetin, narirutin, and eriocitrin.²⁸⁸ Comparison between the *C. intermedia* and *C. subternata* indicates that there exists up to an 80% difference in their phenolic composition with luteolin, mangiferin, and hesperidin the only identified phenolic compounds present in both.^{34;35}

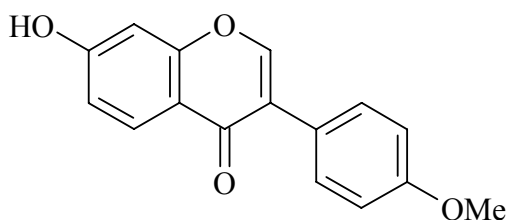
The flavonoids, formononetin, naringenin, and luteolin, present in *Cyclopia* have been described as weak estrogens acting *via* the ERs³⁶ and anecdotal evidence (J. Nortje, Kouga, 1996; personal communication) would suggest that honeybush tea alleviates menopausal symptoms. Together these facts were the spur for the present study investigating the phytoestrogenic potential of *Cyclopia*.

1.5.3. Putative phytoestrogens in *Cyclopia*

Plants often contain a variety of different phytoestrogens. Four phenolic compounds present in *Cyclopia*, formononetin, naringenin, eriodictyol and luteolin have been shown to have estrogenic activity.^{82;36} These flavonoids together with other commercially available plant polyphenols, i.e. eriocitrin, narirutin, hesperidin, hesperetin, and the xanthone, mangiferin, present in *Cyclopia*, were chosen in the present study as possible marker compounds for phytoestrogenic activity in the honeybush species. Eriocitrin and narirutin are structurally related to naringenin and eriodictyol, while mangiferin, hesperidin and hesperetin were included as they are the most abundant phenolic compounds present in honeybush and thus require investigation as to potential phytoestrogenic activity. The biological properties of all polyphenols selected for investigation will be briefly discussed.

1.5.3.1. Formononetin

Formononetin, an isoflavone and 4'-*O*-methyl derivative of daidzein, displays weak phytoestrogenic activity through binding to the ER.^{36;289} Daidzein, shown to have a stronger estrogenic activity than formononetin can be further metabolized to equol, a potent phytoestrogen.¹⁴ In addition, formononetin, like E₂, can up regulate the LDL receptor in HepG2 cells.¹⁶⁶



formononetin

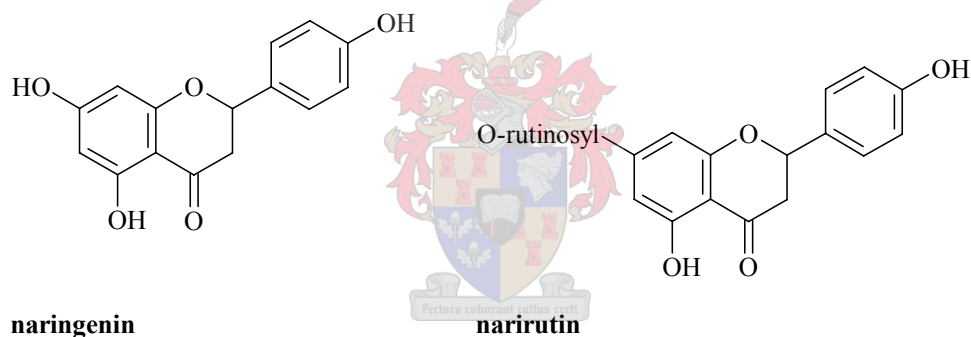
1.5.3.2. Naringenin and narirutin

Naringenin, a flavanone, is most commonly found in citrus fruit, as such or in its glycosidic form (naringin or narirutin). The aglycone is almost insoluble in water.²⁹⁰ Hydrolysis of the glycosides, naringin or narirutin, is required for absorption from the small intestine.

Conjugated forms can form either in the small intestine or liver.¹⁰⁷ Naringenin can thus be further metabolised in the liver by hepatic enzymes with the major metabolite being eriodictyol, which will be discussed later in this section.²⁹⁰

It has been suggested that because of the lipophilic nature of naringenin, accumulation in adipose tissue can occur and therefore very high concentrations can be reached within the organism.²⁹⁰ Naringenin is commonly referred to as a phytoestrogen as it competes with E₂ for binding to both ER subtypes^{82;36} and induces transcription *via* the ER (the ER subtype was not identified by the authors).¹⁷⁴

Not only has naringenin been shown to have estrogenic activity it can also act as an antiestrogen in the presence of E₂ by repressing estrogen induced cell proliferation of MCF-7 cells.²⁷⁰ The naringenin derivative 8-prenylnaringenin common in hops (*Humulus lupulus*) is a potent phytoestrogen.²⁴⁰ Naringenin also affects endogenous E₂ levels as, unlike genistein, it was shown to be a potent aromatase inhibitor.²⁵

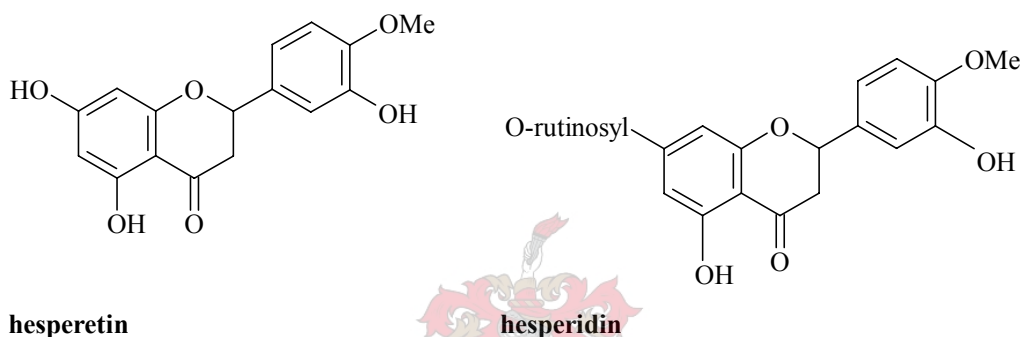


1.5.3.3. Hesperetin and hesperidin

Hesperidin, a flavanone glycoside, is most commonly found in citrus²⁹⁰, and is abundantly in honeybush.^{290;44} Its aglycone, hesperetin, is structurally related to naringenin and eriodictyol. Hesperetin and hesperidin, due to their lipophilic nature, is almost insoluble in water.^{35;290} Hesperetin does not bind to the ER, although it belongs to the same group (flavanone) as naringenin, which is able to interact with the ERs.²⁹¹ A single hydroxyl group at the 4' position of the B-ring (Figure 7) is hypothesized to be the one of the main features required for estrogenicity of a compound.¹⁵² The 4-methoxy group at this very important position in hesperetin is likely to influence its binding to the ER.¹⁷⁷ Even though hesperetin is unable to bind to the ERs, it is

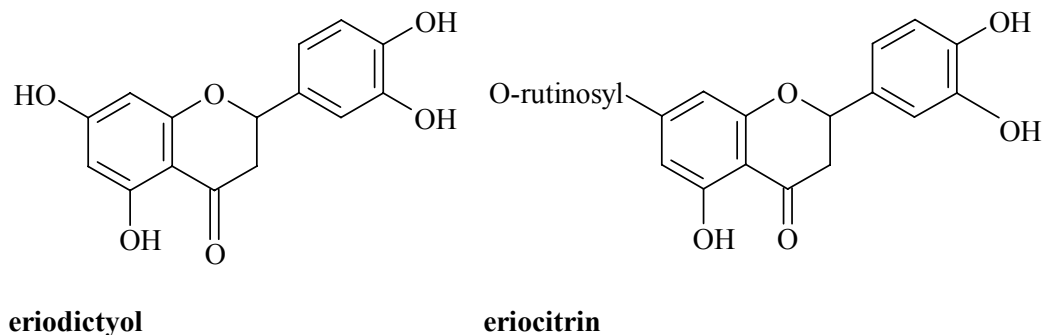
effective in inhibiting cell proliferation of ER negative human breast cancer cells and its activity in this regard is comparable to that of naringenin.²⁹² It would thus appear that hesperetin interacts in an ER-independent manner with signal transduction pathways involved in cell cycle progression.

Hesperidin is a potential anti-inflammatory agent and is associated with anti-atherosclerotic activity.²⁹⁰ It is also able to lower cholesterol levels and prevents bone loss in ovariectomized mice by decreasing the osteoclast number without affecting the uterus.¹⁷⁷ In addition, hesperidin has antioxidant activity and is believed to decrease the risk of cardiovascular disease, since it decreases the levels of plasma and hepatic cholesterol and triglycerides.²⁹³



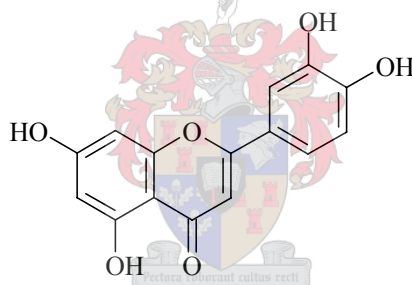
1.5.3.4. Eriodictyol and eriocitrin

Eriodictyol, a flavanone and antioxidant²⁹⁴ is commonly found in lemons. Eriocitrin, a 8-rutinoside, is metabolised *in vivo* to eriodictyol, methylated eriodictyol, 3,4-dihydroxyhydrocinnamic acid, and their conjugates.²⁹⁵ Eriodictyol has been shown to have weak estrogenic activity through stimulating MCF-7 breast cancer cell proliferation and inducing transcription of an ERE containing promoter *via* the ER in a yeast based assay (subtype not specified).^{37;38} Eriodictyol has also been shown to act as an antioxidant.



1.5.3.5. Luteolin

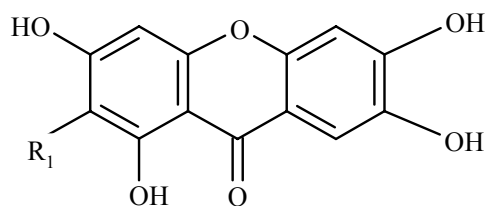
Luteolin, a flavone, is a primary yellow pigment present in flowers and has been historically used as a dye. Its medicinal properties include antimutagenicity²⁹⁶, antioxidant activity^{297;298}, anti-inflammatory properties²⁹⁹ and the ability to act as a hypoglycemic agent.³⁰⁰ It has anticarcinogenic potential due to its ability to inhibit the effects of EGF and protein tyrosine kinase.³⁰¹ In terms of estrogenic potential, luteolin is both estrogenic and antiestrogenic as it inhibits E₂-induced cell proliferation of MCF-7 breast cancer cells, acts as a weak estrogen through the induction of cell proliferation of MCF-7 cells, and increases uterine wet weight in immature 21-day old female rats.^{37;279;302} In addition to inhibition of breast cancer proliferation luteolin inhibits growth of the cancer cells P388, OVCAR-3, SF-295, A498, NCI-H460, KM20L2 and SK-MEL-5 cells.³⁰³ It is cytotoxic to MCF-7 and MDA-MB-231 cells²⁹⁸ and can inhibit angiogenesis which is vital for tumour growth.^{27;304}



luteolin

1.5.3.6. Mangiferin

Mangiferin, a xanthone, is well characterized for its antioxidant and anti-inflammatory properties.³⁰⁵ It is the most abundant phenolic compound found in *Cyclopia*⁴⁴ and has no known phytoestrogenic activity. Mangiferin is ineffective as an antimutagen⁴⁴, but it increases the expression of tumour growth factor β genes, which is reported to be of interest for treatment in cancer prevention and coronary heart disease.³⁰⁵ Mangiferin is able to decrease expression levels of inducible nitric oxide synthase and TNF- α genes, which would explain its anti-inflammatory action.^{305;306}



mangiferin $R_1=2\text{-}\beta\text{-D-glucopyranosyl}$

1.6. Conclusion and Aim of thesis

Phytoestrogens are plant polyphenols able to mediate a weak estrogenic or antiestrogenic response.^{14;162;153} Endogenous estrogens have a wide range of biological responses mediated mainly *via* the classical ER dependent pathway.¹²⁴ In addition to the classical estrogen signalling pathway, which is dependent on the ER and involves binding to an ERE in the promoter of inducible genes, other signalling pathways also exist. Briefly, in addition to the classical ER dependent pathway there is also an ERE independent pathway, a ligand independent pathway and a non-genomic or non-nuclear pathway. All these pathways can potentially cross-talk with one another. Similarly, to the endogenous ligand, phytoestrogens can also induce biological responses employing a wide variety of pathways. Therefore, phytoestrogens could potentially mediate a wide variety of responses not all necessarily estrogenic.

Phytoestrogens are also preferential ER β binders⁸² and, because ER β modulates the proliferative activity of ER α ^{97;219;220;221}, are of biological interest. In addition, the recent HRT scare has created great interest in developing new, alternative treatments for HRT with phytoestrogens considered as an attractive natural option.¹⁶²

However, conflicting results of clinical studies examining the protective effects of phytoestrogens, especially concerning breast cancer and menopausal symptoms, should be considered and deserves further attention.²⁰⁵ On the other hand, to our knowledge, no side effects have been reported for the use of phytoestrogens though this may be due to the fact that no large and extensive studies, similar in scope to the WHI study, have been conducted to evaluate the potential side-effects of phytoestrogens. Epidemiological studies do, however, suggest that the soy rich diet in Asian countries provides some sort of health protection as the

occurrence of hormone-dependent cancers, such as breast and prostate cancer, are much less as is the incidence of menopausal symptoms.^{14;200;307}

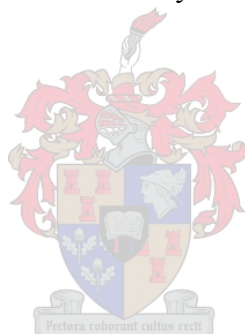
Although most phytoestrogen research has focussed almost solely on soy and the isoflavone, genistein, numerous other plants warrant investigation and research on these is slowly trickling through. Anecdotal evidence, together with the presence of the known phytoestrogens, luteolin, naringenin, eriodictyol and formononetin, in *Cyclopia spp.*^{34;35} suggests that an investigation into the potential phytoestrogenic activity of *Cyclopia* is warranted. Thus, in the present study, extracts from four commercially available *Cyclopia* species, *C. genistoides*, *C. subternata*, *C. sessiliflora* and *C. intermedia*, were initially screened for estrogenicity by means of binding studies to the ER subtypes, hER α and hER β (Chapter 2). This determined the species with the highest estrogenicity for further in-depth investigation. After the identification of the most active species, *C. genistoides*, full competitive dose response binding studies to both ER subtypes as well as transactivation studies with hER β only were chosen to establish whether *C. genistoides* extracts are able to compete with ³H-E₂ for binding to the ER subtypes and if through binding they could activate hER β and thus induce transcription (Chapter 3). Cell proliferative assays in both MCF-7 BUS and MDA-MB-231 cells were chosen to measure a more complex estrogenic endpoint, cell proliferation (Chapter 3). Cell proliferation can also, like transactivation assays, distinguish agonists from antagonists, which binding assays cannot do.

In addition to the extracts investigated, commercially available plant polyphenols known to be present in *C. intermedia* and *C. subternata* were also screened for phytoestrogenic activity (Chapters 2 and 3). Again, screening of these compounds was by means of ER binding assays with both ER subtypes investigated. The polyphenols with affinity for both ER subtypes were chosen for further in-depth studies similar to that of the extracts. Mangiferin was included in all in-depth studies as this polyphenol occurs in high concentrations in all *Cyclopia* species tested.⁴⁴

Finally, SHBG binding studies of the extracts and commercially available polyphenols were also performed to determine the degree of binding to the SHBG protein. Binding of extracts and polyphenols to the SHBG (Chapter 3) could decrease the excretion of these plant compounds and extracts thus increasing their availability to mediate a biological response.

Establishing that *Cyclopia spp.* have phytoestrogenic activity could be beneficial as not only would it be adding nutritional value to the currently available herbal infusion, known as honeybush tea, but it could also precipitate the development of a local South African nutraceutical. New research opportunities would be made possible as per identifying the active compounds, examining the extent of the phytoestrogenic activity (*in vitro vs in vivo*), and finally enriched fractions of *Cyclopia* extracts could be made available to the public as a nutraceutical. Thus the findings pertaining to the experimental work of this thesis and their implications will also be generally discussed in Chapter 4.

The experimental results have been written in the form of publications and thus inevitably repetition of information will occur. We trust that the reader will not find this tedious but rather useful in recapping the rather complex field of estrogen signalling and phytoestrogen activity. Data from this study has been presented in part at several national conferences and at the end of the thesis a list of conference abstracts may be found.



1.7. References

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CHAPTER 2

Screening of four *Cyclopia* (honeybush) species for putative phytoestrogenic activity through estrogen receptor binding assays

2.1. Abstract

Phytoestrogens are plant polyphenols, which are able to mediate an estrogenic effect through binding to the estrogen receptor (ER) subtypes, ER α and ER β . Four commercially available *Cyclopia* (honeybush) species, *C. intermedia*, *C. subternata*, *C. genistoides*, and *C. sessiliflora*, together with nine commercially available polyphenols present in some or all of the species, were screened for phytoestrogenic activity, using a competitive whole cell ER binding assay. Only naringenin, formononetin and luteolin were able to significantly displace tritiated E₂ from hER α , while luteolin, naringenin, formononetin, eriodictyol, narirutin and eriocitrin bound to hER β . Mangiferin, hesperidin and hesperetin did not bind to hER α and hER β . To our knowledge this is the first time that binding of eriodictyol, eriocitrin and narirutin to the hER β was shown. Furthermore, both aqueous and methanol extracts from three independent harvestings of each *Cyclopia* species were screened. The results suggest that *C. genistoides* and *C. subternata* display significant phytoestrogenic activity and that methanol extracts from “unfermented” plant material generally display greater activity. Great variation does, however, exist within a species, with one *C. genistoides* harvesting (Gen P104) significantly ($P < 0.01$) displacing tritiated E₂ from both ER subtypes, while the other *C. genistoides* harvestings either only significantly ($P < 0.01$) displaced tritiated E₂ from hER β (Gen P105) only, or were unable to significantly displace tritiated E₂ from either ER subtype (Gen P122).

2.2. Introduction

The genus *Cyclopia* comprising ca. 24 species, is part of the rich fynbos plant kingdom of the Western Cape.¹ *Cyclopia intermedia*, *C. subternata*, *C. genistoides* and to a lesser extent *C. sessiliflora*, are the four species commercially processed. *Cyclopia* is traditionally consumed as a caffeine-free herbal tea, which has a very sweet distinct flavour and aroma, and is more

commonly known as honeybush tea. Traditionally only “fermented” honeybush tea was available, but the “unfermented” (green) tea has recently been introduced to the public. The fermentation (oxidation) step during processing, which is necessary for development of the characteristic sensory properties, reduces the total phenolic content of the plant material.²

Several studies investigating the chemical composition of *Cyclopia* reported that mangiferin, a xanthone, and the flavanone, hesperidin are the major compounds present.^{3;4;5} Ferreira *et al.*⁴ investigated the phenolic composition of fermented *C. intermedia*, and found amongst others, the isoflavone, formononetin; the xanthone, mangiferin; flavanones, naringenin, eriodictyol, hesperetin, and hesperidin; the coumestans, medicagol, flemichapparin, and sopharacoumestan B; and a flavone, luteolin.⁴ A subsequent study involving unfermented *C. subternata* revealed a phenolic composition that also included luteolin, mangiferin and hesperidin, but with the additional presence of the flavanones, narirutin and eriocitrin amongst others, which were not present in *C. intermedia*.⁵

Recently, honeybush tea has been identified as having antioxidant and antimutagenic activity, which is thought to be due to its phenolic composition^{2;6} The phenolic compounds present in *Cyclopia*, however, may have additional biological activities. For example, formononetin, naringenin and eriodictyol present in *C. intermedia*⁴ and luteolin present in both *C. intermedia* and *C. subternata*^{4;5} are known to have weak estrogenic effects.^{7;8;9} Phenolic plant compounds able to mediate an estrogenic effect, are commonly referred to as phytoestrogens. Phytoestrogens are believed to alleviate menopausal symptoms and to protect against cardiovascular disease and osteoporosis.^{10;11;12} Plant extracts of *Cimicifuga racemosa* (black cohosh)¹³ and *Trifolium pratense* (red clover)¹⁴ are already commercially available in western countries for the treatment of menopausal symptoms, with red clover containing the phytoestrogens biochanin A, formononetin, genistein and daidzein.¹⁵

The public is more inclined towards natural product use that they believe have fewer side-effects than synthetic drugs.¹⁶ Their perceptions, as related specifically to phytoestrogens, are supported by both epidemiological studies and recent reports of health risks associated with conventional medicine. Epidemiological studies have shown that the incidence of breast cancer and endometrial cancer is much lower in Asian than Western populations¹⁷ and that this phenomenon is associated with the traditional Asian diet, which consist mainly of soy.¹⁸ A very well known and intensively studied phytoestrogen, the isoflavone, genistein, present in soybean,

was isolated and identified as the major contributor to these findings.¹⁹ Two clinical trial studies, involving hormone replacement therapy (HRT), by the Women's Health Initiative, had to be prematurely terminated due to a number of health risks.^{20;21} The estrogen plus progestin treatment increased risk of breast cancer and cardiovascular disease, while estrogen alone was found to increase the occurrence of stroke. Alternatives to traditional HRT being investigated thus include phytoestrogens. Phytoestrogens are believed to minimize the risks associated with HRT, while retaining the health benefits and even protecting against hormone related cancers. Although these beliefs are supported by the literature^{22;23;24} some studies suggest that phytoestrogens may not be effective in alleviating menopausal symptoms.²⁵ Despite this caveat phytoestrogens are in demand by the public and new plant sources of phytoestrogens are continually investigated for exploitation by the nutraceutical industry.

Estrogens are responsible for numerous physiological effects especially in reproductive tissues. Better known for its function in females, it is also essential for the normal development and reproductive physiology in males.²⁶ Positive physiological effects include maintaining or increasing bone density²⁷ and protecting against cardiovascular disease.²⁸ However, because of its stimulating effect on growth and proliferation of certain cells it is a risk factor for breast and endometrial cancer.^{29;30} Estrogen signalling is mainly mediated *via* the estrogen receptors (ERs) to which estrogens bind. The ERs are members of the nuclear receptor superfamily, more specifically the steroid hormone receptor family, and are mostly known as ligand-activated transcription factors. Binding of the ligand to the ER induces a conformational change and dissociation of heat shock proteins. This is followed by dimerization and binding to the classical estrogen response element (ERE) whereby it is able to mediate a biological response.³¹

Two ER subtypes, namely estrogen receptor-alpha ($ER\alpha$)³² and estrogen receptor-beta ($ER\beta$)³³, have been identified. The two ER subtypes are homologous, especially in the DNA binding domain (DBD) with 95% amino acid homology and to a lesser extent, 58% homology, at the C-terminal ligand binding domain.³⁴ The $ER\alpha$ has a broader expression pattern compared to $ER\beta$. They do, however, have some overlapping tissue distributions.^{33,34,35}

Both ER subtypes are capable of stimulating transcription of ER target genes. The degree of E_2 activation (fold induction) through $ER\beta$ in many cell types is, however, lower than that of $ER\alpha$. Physiologically, $ER\beta$ decreases the overall sensitivity to E_2 in $ER\alpha$ mediated gene transcription, and is believed to be the natural cellular protective mechanism against over-

proliferation of cells that could lead to cancer formation.^{36;37;38} Compounds that bind with a higher affinity to ER β than ER α , are thus of great interest pharmacologically, as ER α induced hyper-proliferation of estrogen-dependent cancer cells may be constrained by ER β . Phytoestrogens, which could be a natural alternative or supplement for the treatment of menopausal women, have been shown to preferentially bind to the ER β .³⁹

In this study, selected commercially available polyphenols present in *Cyclopia* as well as extracts of the four most commonly available *Cyclopia spp.*, i.e. *C. genistoides*, *C. subternata*, *C. sessiliflora* and *C. intermedia*, were investigated by evaluating their interaction with both ER subtypes (ER α and ER β). The polyphenols were screened for use as possible marker compounds of estrogenicity in *Cyclopia*, while the extracts of the *Cyclopia spp.* were screened to identify the species with the highest estrogenicity. Both aqueous extracts, from fermented and unfermented *Cyclopia* plant material, and methanol extracts, from unfermented *Cyclopia* plant material, were tested. It was also thought necessary to investigate three different harvestings of each species to eliminate false negatives and to include variations due to growth conditions and/or genetic variation as this could influence the amount of phenolic compounds present in the plant material. Phenolic compounds are especially known to occur at high concentrations in plants that are under stress.^{40;41} Binding to the ER subtypes was chosen as a screening assay, because binding to the receptor would constitute the first step in the signal transduction pathway mediating an estrogenic effect.

2.3. Materials and Methods

Test compounds used

Test compounds used in this study included 17- β -estradiol (1, 3, 5(10)-estratriene), genistein (4', 5, 7-trihydroxyisoflavone), mangiferin (2- β -D-glucopyranosyl-1,3,6,7-tetrahydroxy-9H-xanthene-9-one), hesperetin (5,7,3'-dihydroxy-4'-methoxyflavanone), hesperidin (5,3'-dihydroxy-4'-methoxy-7-O-rutinosylflavanone), and naringenin (5,7,4'-trihydroxy-flavanone) and were purchased from Sigma-Aldrich, South Africa. Luteolin (5,7,3',4'-tetrahydroxyflavone), formononetin (7-hydroxy-4'-methoxyisoflavone), eriodictyol (5,7,3',4'-tetrahydroxyflavanone), narirutin (naringenin-7-O-rutinoside), and eriocitrin (eriodictyol-7-O-rutinoside) were purchased

from Extrasynthese, France. The aqueous extracts of *Cyclopia spp.* were prepared by E.S. Richards and kindly provided by Dr. E. Joubert, ARC Infruitec-Nietvoorbij. Radiolabelled ligand, 2,4,6,7-³H-17-β-estradiol, (specific activity 87.0 Ci/mmol, counting efficiency of 46% in our system) was purchased from Amersham, South Africa.

Plant material

Various species of *Cyclopia* were harvested as described in Tables 1 and 2. Harvested plant material (Table 1) was processed according to the standard processing method for fermented and unfermented tea.⁴² Briefly, for the aqueous extracts, the leaves and stems were cut into small pieces using a modified fodder cutter. The shredded plant material was moistened with deionised water to ca. 60% moisture content, after which fermentation at 70°C for 60 hours, followed by drying at 40°C for 12 hours to ca. 10% moisture content took place. Unfermented material was dried directly after shredding at 40°C for 12 hours. The dried plant material was then sieved with an Endecott test sieve (2 mm). The plant material (≤2 mm) was pulverized with a Retsch rotary mill (1 mm sieve) and stored in plastic containers at room temperature. The plant material for the preparation of the methanol extracts (Table 2) was dried whole at 40°C until dry where after it was pulverized with a Retsch rotary mill (1 mm sieve) and stored in plastic containers at room temperature.

Aqueous extract preparation

Aqueous extracts were prepared by E.S. Richards.² Briefly, 100 g of pulverized processed (fermented and unfermented) plant material from three randomly chosen independent harvestings of *C. intermedia*, *C. subternata*, *C. genistoides* and *C. sessiliflora* (Table 1) were steeped in 1 L freshly boiled deionised water for 5 minutes while stirring. Extracts were then filtered through a Buchner filter using 125 µm Polymon mesh cloth to remove most of the plant material followed by further filtration through Whatman No. 4 filter paper for the removal of finer particles. The filtrate was stored at -20°C before freeze-drying. The dried aqueous extracts, after freeze-drying, were stored at room temperature in sealed glass vials, covered with aluminium foil, in desiccators

kept in the dark. Yield of extract was determined by calculating the mass (g) of dry aqueous extract (DAE) per 100 g of initial processed plant material.

Methanol extract preparation

Methanol extracts of unfermented *Cyclopia* species namely, *C. intermedia*, *C. subternata*, *C. sessiliflora*, and *C. genistoides* were prepared. Extracts were made of three randomly chosen independent harvestings of each species (Table 2). The plant material used was not the same as that used for the preparation of the aqueous extracts.

Dried, pulverized unfermented plant material (25 g) was placed in a 250 ml Erlenmeyer flask with 50 ml dichloromethane (UNIV AR, Merck, South Africa). The plant material was allowed to stir continually for 20 hours at room temperature after which it was removed by filtering through Whatman No. 4 filter paper and the filtrate was discarded. Extraction with dichloromethane was repeated three times. This preliminary extraction procedure was to remove the chlorophyll and some of the non-polar components. Dichloromethane extraction was followed by addition of 50 ml methanol (Pro-analyti GR, Merck, South Africa) to the air-dried plant material and again this was allowed to stir continually for 20 hours. Methanol extraction was performed twice, the methanol extracts were pooled with a small volume of water added and evaporated under vacuum on a rotary evaporator before freeze-drying. Freeze-dried methanol extracts were ground with a mortar and pestle in a darkened room until a fine homogenous powder was formed. The powders were stored in screw cap glass vials, covered with aluminium foil, and placed in vacuum-sealed desiccators in the dark at room temperature. All glassware used during the extraction process was covered with aluminium foil to protect the extracts from light at all times. Yield of extract was determined by calculating the mass (g) of dry methanol extract (DME) per 100 g of initial processed plant material.

Determination of total polyphenol content of aqueous and methanol extracts

The total polyphenol content of the DAE and DME was quantified colorimetrically in triplicate and expressed as g gallic acid equivalents per 100 g of extract. The method of Singleton and Rossi was adapted for use in flat-bottomed 96 well plates (B & M Scientific, South Africa).⁴³ Briefly, 20 µl of sample [gallic acid standards (0.01-0.1 mg/ml) from Sigma-Aldrich, or DAE and

DME (0.25 mg/ml)] were allowed to react with 100 μ l 10% (v/v) Folin-Ciocalteu reagent (Merck, South Africa) in the presence of 80 μ l 7.5% (w/v) Na_2CO_3 at 37°C for 2 hours. Absorbance was measured at 620 nm using a microtiter plate reader, recorded, and g gallic acid equivalents obtained from the standard curve. Total polyphenol content was calculated as g gallic acid equivalents per 100 g of freeze-dried extract.

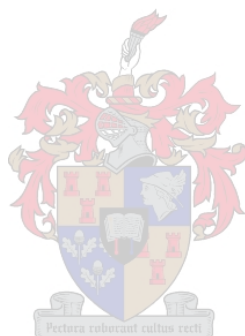


Table 1: Details of *Cyclopia spp.* plant material harvested and used for the preparation of aqueous extracts.

Species	Processing	Harvesting*	Area harvested	Date of harvesting
<i>C. genistoides</i> West Coast type	Unfermented	Gen 1G	Koksrivier, Pearly Beach	15 March 2001
		Gen 2G	Koksrivier, Pearly Beach	15 March 2001
		Gen 3G	Koksrivier, Pearly Beach	15 March 2001
	Fermented	Gen 1F	Koksrivier, Pearly Beach	15 March 2001
		Gen 2F	Koksrivier, Pearly Beach	15 March 2001
		Gen 3F	Koksrivier, Pearly Beach	15 March 2001
<i>C. subternata</i>	Unfermented	Sub 1G	Waboomskraal, Outeniqua (3-yr-old plantation)	10 February 1999
		Sub 3G	Waboomskraal, Outeniqua (3-yr-old plantation)	10 February 1999
		Sub 15G	Du Toitskloof	01 October 1998
	Fermented	Sub 8F	Waboomskraal, Outeniqua (3-yr-old plantation)	10 February 1999
		Sub 10F	Waboomskraal, Outeniqua (2-yr-old plantation)	17 October 1999
		Sub 13F	Waboomskraal, Outeniqua (2-yr-old plantation)	17 October 1999
<i>C. sessiliflora</i>	Unfermented	Sess 2G	Helderfontein, Stellenbosch (experimental plantation)	15 February 2001
		Sess 3G	Helderfontein, Stellenbosch (experimental plantation)	15 February 2001
		Sess 4G	Helderfontein, Stellenbosch (experimental plantation)	15 February 2001
	Fermented	Sess 4F	Helderfontein, Stellenbosch (experimental plantation)	15 February 2001
		Sess 6F	Helderfontein, Stellenbosch (experimental plantation)	15 February 2001
		Sess 7F	Helderfontein, Stellenbosch (experimental plantation)	15 February 2001
<i>C. intermedia</i>	Unfermented	Int 1G	Haarlem (wild harvested)	10 March 2000
		Int 2G	Haarlem (wild harvested)	10 March 2000
		Int 5G	Haarlem (wild harvested)	10 March 2000
	Fermented	Int 2F	Haarlem (wild harvested)	10 March 2000
		Int 3F	Haarlem (wild harvested)	10 March 2000
		Int 4F	Haarlem (wild harvested)	10 March 2000

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

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

Species	Harvesting*	Area harvested	Date of harvesting
<i>C. genistoides</i> West Coast type	Gen P104	Koksrivier, Pearly Beach	15 March 2001
	Gen P105	Koksrivier, Pearly Beach	28 March 2001
	Gen P122	Koksrivier, Pearly Beach	31 March 2003
<i>C. subternata</i>	Sub P118	Helderfontein, Stellenbosch	28 February 2003
	Sub P111	Helderfontein, Stellenbosch	5 March 2001
	Sub PY1	Bien Donne, Simondium	1 March 2000
<i>C. sessiliflora</i>	Sess P108	Helderfontein, Stellenbosch	14 November 2001
	Sess P118	Helderfontein, Stellenbosch	28 February 2003
	Sess P69	Bien Donne, Simondium	1 March 2000
<i>C. intermedia</i>	Int P111	Helderfontein, Stellenbosch	14 January 2002
	Int PX1	Bien Donne, Simondium	January 2000
	Int P125	Helderfontein, Stellenbosch	19 February 2001

*The abbreviations used for the harvestings are also used for the methanol extracts prepared from these harvestings. Although some harvestings were done on the same plantation they were done at different times.

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

The quantification of specific phenolic compounds in DAE prepared from unfermented and fermented *Cyclopia spp.* plant material and DME prepared from unfermented *Cyclopia spp.* plant material was performed by reverse-phase HPLC as described by Joubert *et al.*⁴⁴ and kindly done by C. Malherbe. The HPLC system (Merck Hitachi, LaChrom system, Merck, Germany) consisted of a solvent pump (LC-7100), autosampler (L-7200), UV detector (L-7450), diode array detector (DAD) (L-7450), interface (D-7000) and a D-7000 HPLC system manager version 4.1 for system control and data acquisition and analysis. Solvents were degassed in-line with a Phenomenex Degasser Model DG-4400 (Separations, South Africa). The Phenomenex RP/C₁₈ 5µm Jour Guard column, Phenomenex Synergy MAX-RP 80A column (C₁₂ reverse-phase with TMS end-capping, 4µm; 150 x 4.6 mm ID) and the Phenomenex Luna Phenyl-hexyl column (150 mm x 4.6 mm; 3 micron) were purchased from Separations, South Africa. Only Int P125 was separated on the Luna Phenyl-hexyl column.

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

Cell culture conditions

African green monkey kidney fibroblast (COS-1) cells (ATCC, United States of America) were cultured in 175 cm² culture flasks (Greiner Bio-One International, Austria) in Dulbecco's modified Eagle's medium (DMEM) from Sigma-Aldrich, South Africa, supplemented with 10% (v/v) fetal calf serum (FCS) from Highveld Biologicals, South Africa, and penicillin (100 IU/ml) and streptomycin (100 µl/ml) (penicillin- streptomycin) from Gibco-BRL Life Technologies,

United Kingdom. The cells were maintained in a cell incubator at a humidified atmosphere of 97% relative humidity and 5% CO₂ at 37°C. COS-1 cells were plated at a density of 2 x 10⁵ cells per well into 12 well plates (Nunc, Denmark) or 2 x 10⁶ cells per 10 cm tissue culture dish (Nunc, Denmark). Cells were allowed to adhere and were therefore incubated for 24 hours before transfecting.

Transient transfections

Twenty-four hours after plating COS-1 cells were transiently transfected with expression vectors for one of the ER subtypes, pcDNA3-hER α (D. Harnish, Womens's Health Research Institute, Wyeth-Ayerst Research, Radnor, United States of America) or pSG5-hER β (F. Gannon, European Molecular Biology, Laboratory Heidelberg, Germany), a constitutively expressed expression vector for β -galactosidase, pCMV- β gal (Stratagene, United States of America), and a filler vector, empty pGL2-basic (Promega Corp, United States of America), using the DEAE-Dextran transfection method⁴⁵ adapted as described. For association and homologous competitive binding assays, 12-well plates were used and the following transfection protocol was followed. Briefly, 495 μ l, DMEM pre-heated to 37°C and containing 0.1 mM chloroquine (Sigma-Aldrich, South Africa) (stock solution 100 mM) and 300 ng DNA (36 ng receptor, 18 ng β -galactosidase expression vector and 246 ng filler vector) was prepared per well. To this mixture, 5 μ l DEAE-Dextran (Sigma-Aldrich, South Africa) solution (stock solution 10 mg/ml) was added to give a final concentration of 0.1 mg/ml. The transfection medium (500 μ l per well) was added to each well and cells were incubated for 1 hour at 37°C in a humidified incubator, followed by removal of transfection medium and washing of cells with pre-heated 10% DMSO/PBS. Finally, transiently transfected cells were incubated at 37°C overnight in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin mixture.

For screening of polyphenols and dried extracts, COS-1 cells were transfected in 10 cm tissue culture dishes. Briefly, 4.95 ml DMEM pre-heated to 37°C and containing 0.1 mM chloroquine and 6 μ g DNA (720 ng receptor, 480 ng β -galactosidase expression vector and 4.8 μ g filler vector) was prepared to which 50 μ l DEAE-Dextran solution was added (final concentration of 0.1 mg/ml). The transfection medium, 5 ml, was added and COS-1 cells were incubated for 1 hour at 37°C in a humidified incubator, followed by removal of transfection

medium and washing of cells with pre-heated 10% DMSO/PBS. Finally, transiently transfected cells were incubated at 37°C overnight in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin mixture.

The following day transiently transfected cells were trypsinized, pooled and plated at a density of 2×10^5 cells per well into 12 well plates and incubated for 24 hours in at 37°C in a humidified incubator.

Whole cell binding assays

On the day of whole cell assay (day three for association and homologous competitive ER binding and day four for screening of polyphenols and extracts) transiently transfected COS-1 cells were washed three times with 500 µl PBS (pre-heated at 37°C) to remove any endogenous estrogen-like compounds present in the culture medium.

This was followed by a two-hour incubation of the transfected cells with 1 nM radiolabelled estradiol ($^3\text{H-E}_2$) and various unlabeled competitors, i.e. extracts or polyphenols in DMEM without phenol red, FCS or antibiotics. All unlabeled competitors, except for the aqueous extracts, were dissolved in absolute ethanol (Merck, South Africa) and subsequently added to the culture medium, giving final concentrations of the commercially available phenolic compounds and methanol extracts of 10^{-5} M and 1.5 µg/ml, respectively, with the final concentration of ethanol not exceeding 0.1% (w/w). The aqueous extracts were dissolved directly into the culture medium at a concentration of 1.5 µg/ml. All assays included a negative control, which was in the presence of 0.1% ethanol, as well as positive controls, which consisted of incubations with E_2 (10^{-5} M) and genistein (10^{-5} M).

After the 2-hour incubation period the cells were immediately placed on ice and further work was done at 4°C to ensure that the ligand remained bound to the receptor. Cells were washed three times with 1 ml ice-cold 0.2% bovine serum albumin/PBS (bovine serum albumin from Roche Applied Science, South Africa), with an interval of 15 minutes between washes to remove free ligand. Cells were lysed with 50 µl lysis buffer (Tropix Inc., United States of America) per well. For effective lysis, plates were placed on a shaker for approximately 15 minutes and thereafter allowed to freeze at -20°C. The same general protocol for whole cell binding was followed for the association (Figure 3) and homologous (Figure 4) competitive

binding experiments with a few modifications. For the association experiments transfected COS-1 cells were incubated at various time points with DMEM without phenol red containing 1nM $^3\text{H-E}_2$ and either 10^{-5} M E_2 or 0.1% DMSO. For homologous competitive binding assays, transiently transfected COS-1 cells were incubated for 2 hours with 1nM $^3\text{H-E}_2$ and increasing concentrations of E_2 .

On thawing of samples, 5 μl lysate was used for protein determination. Protein concentrations were determined using the Bradford protein assay method.⁴⁶ Protein concentrations were used to normalise radioactivity readings for plating efficiency (screening of polyphenols and dry extracts).

For association and homologous competitive ER binding β -Galactosidase Relative light units (RLU's) were used to normalise radioactivity readings for transfection efficiency. On thawing of samples 5 μl lysate was used to determine β -galactosidase activity using the chemiluminescent Galacto-StarTM reporter gene assay system for mammalian cells (Tropix Inc., United States of America). RLU's were measured using a luminometer (Labsystems Luminoskan luminometer, Global Medical Instrumentation Inc., United States of America).

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

Radioactivity of the all assay samples was determined by recording the counts per minute (CPM) for each measurement using a Beta-scintillation counter (Beckman scintillation counter LS 3801, Beckman, South Africa).

Data manipulation and statistical analysis

The GraphPad Prism[®] version 4.00 for Windows (GraphPad Software, USA) was used for graphical representations and statistical analysis. One-way ANOVA and Dunnett's multiple comparison's test as post-test or paired two-tailed t-tests were used for statistical analysis. P-values are represented as follows: P < 0.05 by *, P < 0.01 by **, and P < 0.001 by ***. For all experiments, unless otherwise indicated, the error bars represent the SEM of three independent experiments, where each point was determined in triplicate. Pearson correlations (two-tailed) were done and are expressed as Pearson correlation coefficient (r).

2.4. Results

2.4.1. Determination of extract yield and total polyphenol content of aqueous and methanol extracts

The results of DAE and DME yields and their total polyphenol content are presented in Table 3. The extract yield across all species was significantly ($P < 0.05$) higher for unfermented plant material compared to fermented plant material in aqueous extracts. When comparing the extraction solvents across all species for unfermented plant material, aqueous extraction gave significantly ($P < 0.01$) higher yields of soluble solids than methanol extraction. A similar pattern was observed when each species was considered separately with extract yield highest for DAE from unfermented plant material, followed by DAE from fermented plant material, and with DME from unfermented plant material giving the lowest yields.

On comparing the total polyphenol content of the DAE within species, DAE from unfermented plant material had a significantly ($P < 0.001$) higher total polyphenol content than DAE from fermented plant material for all species. The same pattern was observed when comparing across all species. Within species the total polyphenol content of the DME from unfermented plant material was significantly lower ($P < 0.01$) than that for DAE of unfermented plant material from *C. genistoides*, and *C. subternata*, but not from *C. intermedia* and *C. sessiliflora*. However, when comparing across all species there was no significant difference between the DAE and DME from unfermented plant material. In terms of solvent, DME yields were significantly lower for all species while the total polyphenol content was only lower for *C. genistoides* and *C. subternata*.

Extract yield and total polyphenol content did not correlate with the DAE (Pearson $r = 0.3416$) and the DME (Pearson $r = 0.2796$) from unfermented plant material. However, DAE yield from fermented plant material did correlate with total polyphenol content (Pearson $r = 0.9145$) (Figure 1).

Table 3: Extract yield^a and total polyphenol content^b of aqueous and methanol *Cyclopia spp.* extracts.

Species	DAE - from unfermented plant material ^c			DAE - from fermented plant material ^c			DME - from unfermented plant material ^d		
	Harvesting	Extract yield (%)	TPP ^e content (%)	Harvesting	Extract yield (%)	TPP content (%)	Harvesting	Extract yield (%)	TPP content (%)
<i>C. genistoides</i>	Gen 1G	39.52	29.14	Gen 1F	35.5	21.82	Gen P104	13.35	22.31
	Gen 2G	38.84	30.54	Gen 2F	34.29	21.83	Gen P105	13.41	21.99
	Gen 3G	39.89	30.28	Gen 3F	36.75	22.33	Gen P122	18.94	25.02
	Average	39.42	29.99		35.51**	21.99***		15.23***	23.11**
<i>C. subternata</i>	Sub 1G	39.79	33.24	Sub 8F	21.09	19.21	Sub P118	13.02	22.17
	Sub 3G	39.69	32.44	Sub 10F	23.7	18.42	Sub P111	8.14	23.75
	Sub 15G	30.62	31.93	Sub 13F	20.77	17.47	Sub PY1	14.02	23.16
	Average	36.7	32.53		21.85**	18.37***		11.73**	22.03***
<i>C. sessiliflora</i>	Sess 2G	31.85	29.10	Sess 4F	22.18	16.32	Sess P108	12.83	29.12
	Sess 3G	33.25	29.78	Sess 6F	23.5	17.68	Sess P118	14.77	32.76
	Sess 4G	32.01	29.96	Sess 7F	23.16	19.79	Sess P69	16.01	28.93
	Average	32.37	29.62		22.95***	17.93***		14.54***	30.17 ^{ns}
<i>C. intermedia</i>	Int 1G	26.75	30.53	Int 2F	16.89	17.15	Int P111	12.83	22.93
	Int 2G	25.43	29.43	Int 3F	16.31	16.06	Int PX1	15.92	25.61
	Int 5G	29.1	30.55	Int 4F	No data available	No data available	Int P125	16.93	30.69
	Average	27.09	30.17		16.60**	16.61***		15.23**	26.41 ^{ns}
Across species average		33.9	30.58		24.23 [#]	18.72 ^{##}		14.18 ^{###}	25.70 ^{ns}

^aYield = g freeze-dried extract per 100 g dried pulverized plant material.

^bTotal polyphenol content = g gallic acid equivalents per 100 g of freeze-dried extract.

^cAll aqueous extracts were prepared by E.S. Richards² from fermented and unfermented plant material as described in Materials and Methods.

^dAll methanol extracts were prepared from unfermented plant material as described in Materials and Methods.

^eTPP abbreviation for total polyphenol.

The statistical analyses were done using two-tailed t-tests and comparing extract yields (and total polyphenol content) within each species (unpaired) and across all species (paired). Within each species the extract or TPP content was compared to the extract or TPP content of unfermented DAE (*P < 0.05, ** P < 0.01, ***P < 0.001, ns = non significant). For comparison across species, the across species average of fermented DAE and unfermented DME was compared to the across species average of unfermented DAE ([#]P < 0.05, ^{##}P < 0.01, ^{###}P < 0.001, ns = non significant).

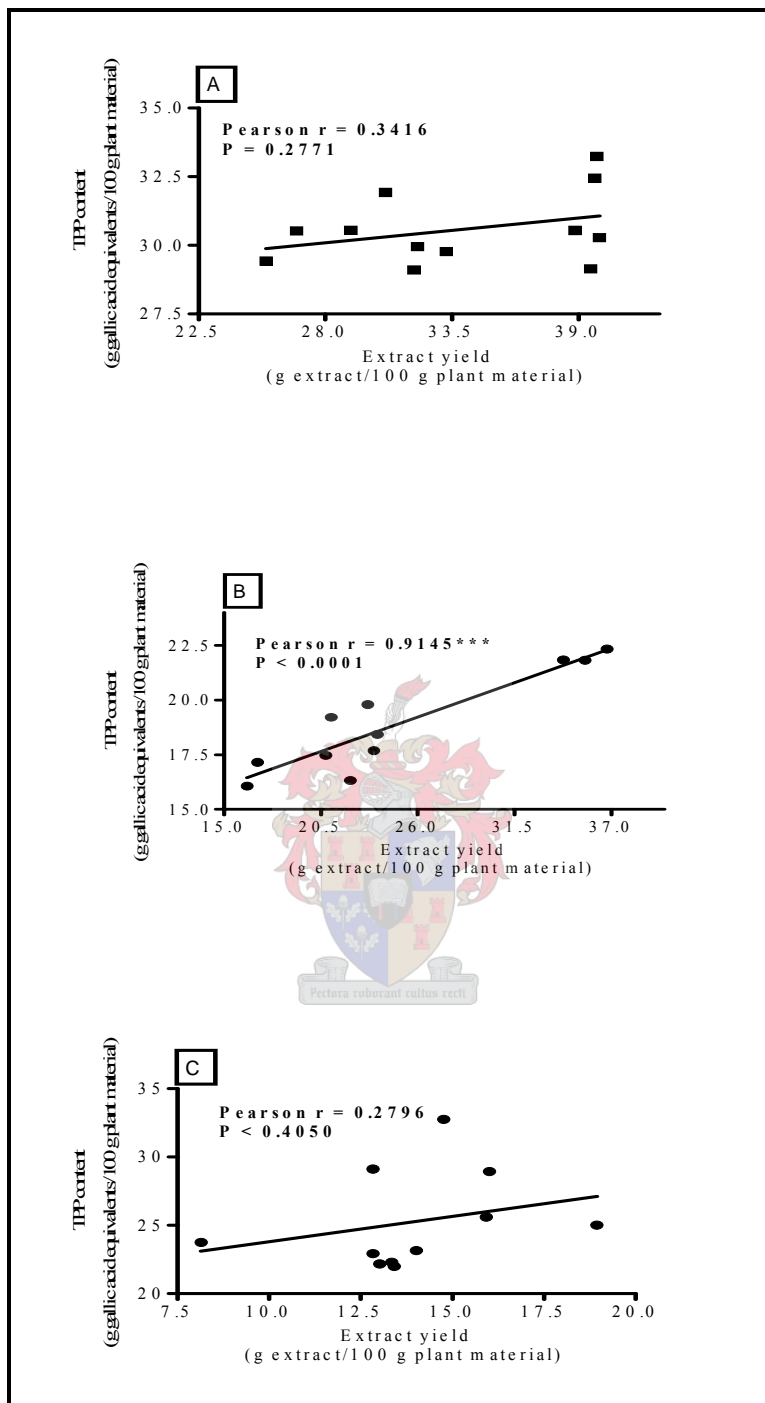


Figure 1: Correlation between the extract yield and total polyphenol content for (A) DAE from unfermented plant material, (B) DAE from fermented plant material and (C) DME from unfermented plant material. Pearson correlations (two-tailed) were done using GraphPad Prism™.

2.4.2. Binding to ER subtypes

Phytoestrogens have been shown to compete with E₂ for binding to both ERs.^{39;47;48} By transiently transfecting COS-1 cells with either hER α or hER β it would be possible to determine whether the commercially available polyphenols known to be present in *Cyclopia*^{4;5} and the DAE and DME bind to the ER subtypes. Optimization and validation of the whole cell binding assay were carried out before screening of extracts and polyphenols.

2.4.2.1. Optimisation of whole cell ER-binding assay

COS-1 cells do not endogenously express ER α or ER β .⁴⁹ This is clearly shown in Figure 2, whereby whole cell binding of cells transfected with empty vector confirmed the lack of receptor expressed. It is therefore essential to transiently transfect these mammalian cells with plasmid DNA expressing either ER subtype to evaluate binding to ER. It is also clear from Figure 2 that the hER β is expressed at a higher level than ER α with 1820 fmol of ³H-E₂ binding to ER β per well while only 889 fmol of ³H-E₂ binds to ER α per well. This difference may be due to the different expression vectors used for ER α and ER β . We also determined inter- and intra-assay variability. The coefficient of variation (CV) calculated within each individual experiment was under 10% with an average CV of 2.3% for hER α and 6.0% for hER β . The coefficient of variation for hER α binding experiments performed in triplicate and repeated thrice was only 10%, whereas the coefficient of variation for hER β binding was 23%.

There is a presumption that for competitive ligand binding experiments equilibrium has been achieved, thus determining the time to reach binding equilibrium to the receptor is important in order to interpret results accurately. Thus before screening for putative phytoestrogenic activity of polyphenols and *Cyclopia* extracts commenced, the incubation time for equilibrium to be reached with 1nM ³H-E₂ had to be determined. Equilibrium for hER α -E₂ binding was reached at approximately 60 minutes with a half-life (t_{1/2}) at 9.3 minutes (Figure 3A). Binding equilibrium for hER β , however, is already reached at approximately 40 minutes with a t_{1/2} equal to 6.5 minutes (Figure 3B). For the present study it was decided to use a two-hour incubation period to ensure that equilibrium had been reached for both ER subtypes.

After determining the equilibrium time homologous competitive binding experiments were carried out to determine K_d values of E₂ for both receptor subtypes. The K_d values

calculated for hER α and hER β were 0.2 nM and 1.6 nM, respectively. The CV for the Kd was 5.9% and 3.6% for hER α and hER β , respectively, for three independent experiments done in triplicate. From the specific binding (SB) determined in the absence of cold ligand the amount of receptor levels for hER α and for hER β was calculated ($SB = B_{max}[L]/K_d + [L]$) to be 1165 fmol and 1677 fmol, respectively.

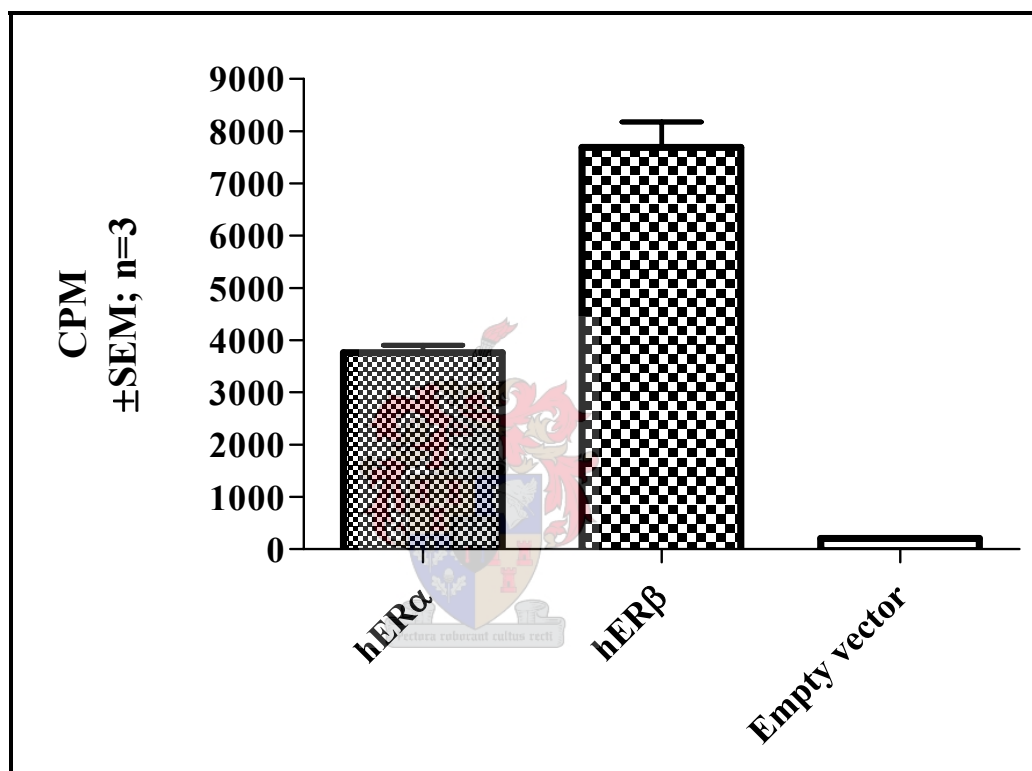


Figure 2: Total binding of $^3\text{H-E}_2$ (1 nM) in COS-1 cells transfected either with hER α (pcDNA3-hER α), hER β (pSG5hER β) or empty vector (pGL2-basic) as assayed by whole cell binding. Incubation time was two hours.

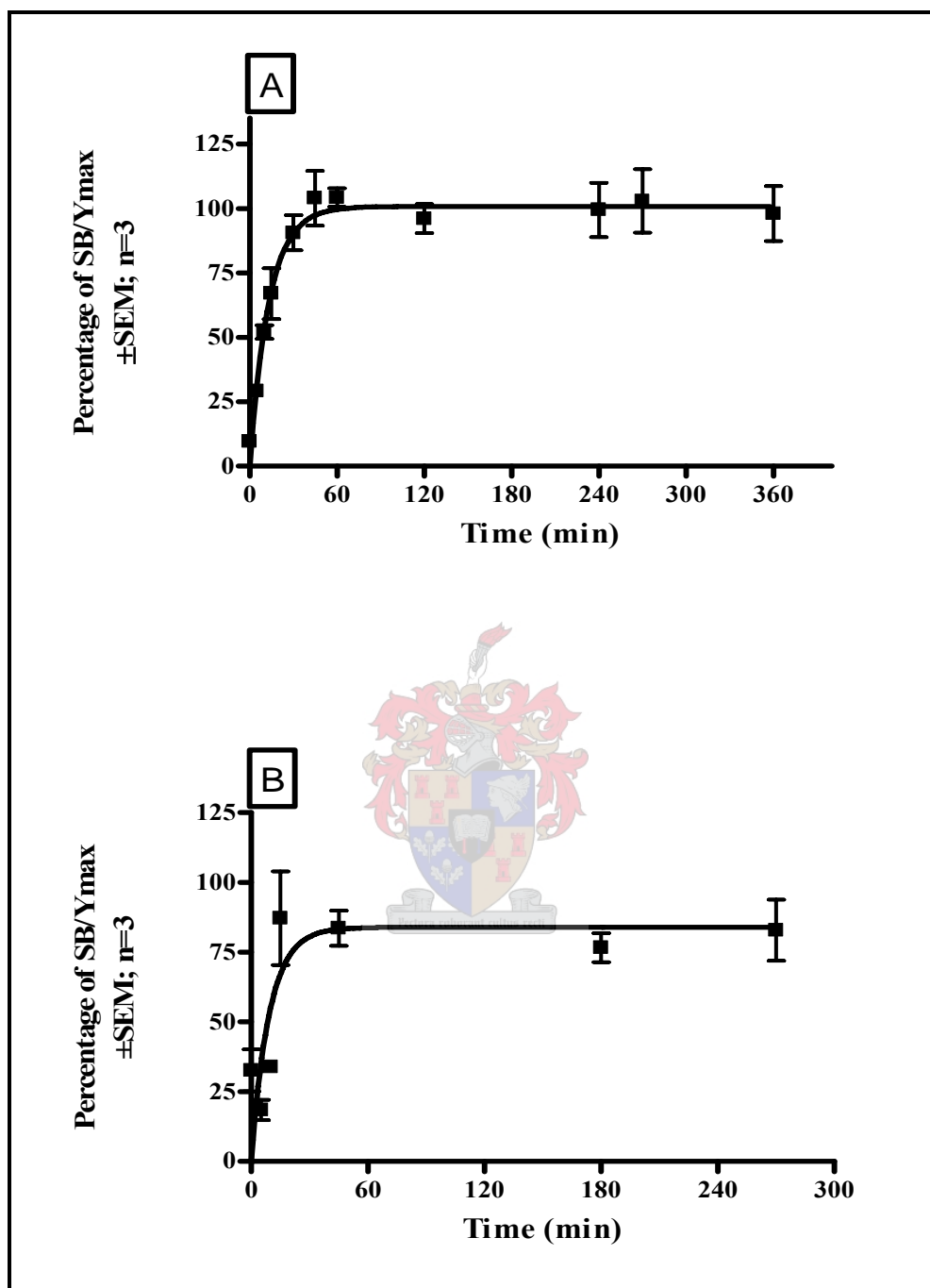


Figure 3: Association or equilibrium-binding experiments where COS-1 cells transiently transfected with either (A) hER α or (B) hER β were incubated with 1 nM $^3\text{H-E}_2$ for various times until receptor-ligand binding had reached equilibrium. Specific binding (SB) was calculated by subtracting the non-specific binding from the total binding. One phase exponential association curve-fit from GraphPad PrismTM was used to calculate $t_{1/2}$. Y-axis: SB (CPM)/Ymax (CPM) x 100.

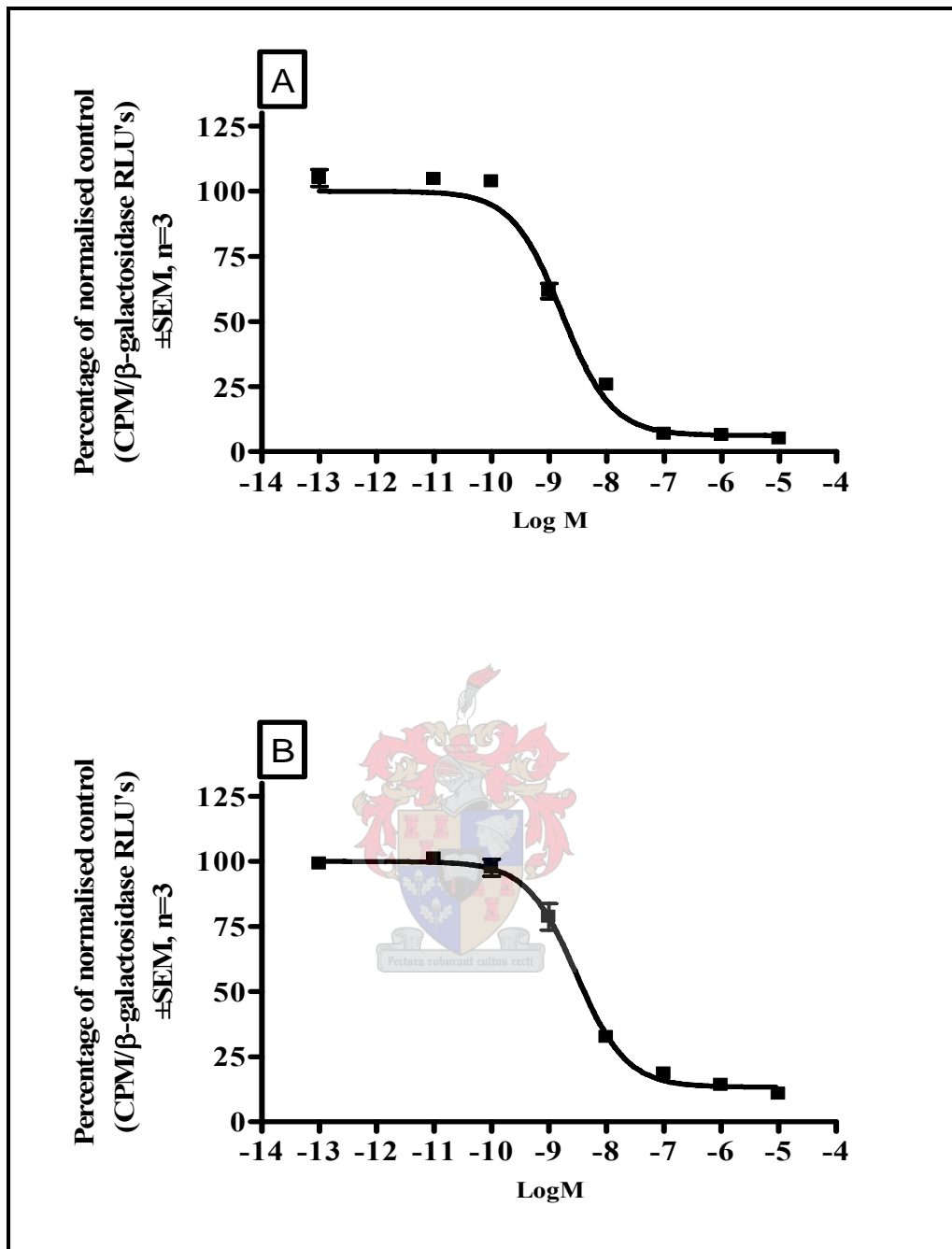


Figure 4: Homologous competitive binding curve for COS-1 cells transiently transfected with either (A) hER α or (B) hER β . A representative curve of each ER subtype is shown whereby transfected COS-1 cells were incubated in the presence of 1 nM $^3\text{H-E}_2$ and increasing concentrations of unlabelled E_2 for 2 hours. Curve fit was done by means of non-linear regression, homologous competitive binding curve with one class of binding sites using GraphPad Prism to determine K_d and B_{max} .

2.4.2.2. Binding of commercially available phenolic compounds present in *Cyclopia spp.*

The ability of the polyphenols, formononetin, luteolin, naringenin, narirutin, eriodictyol, eriocitrin, hesperetin, hesperidin and mangiferin, present in *Cyclopia*^{4;5} (Figure 5), to bind to the ER subtypes were investigated by the competitive whole cell receptor binding assay. The compounds displacing the highest percentage of tritiated E₂ could then serve as marker compounds for future studies investigating the phytoestrogenic activity of honeybush.

All polyphenols investigated at a concentration of 10⁻⁵ M were able to significantly (P < 0.01) displace tritiated E₂ from hERβ, except for mangiferin, hesperidin, and hesperetin (Figure 6B and Table 4). Naringenin, luteolin and formononetin were able to displace more than 50% of tritiated E₂ from hERβ. More known for its antimutagenic and antioxidant properties^{2;6}, luteolin was able to displace 92% of the radiolabelled E₂ from hERβ and compared well with genistein that displaced 95% (Table 4). Eriodictyol displaced 44% tritiated E₂ while both eriocitrin and narirutin were able to displace 28% tritiated E₂ from hERβ.

Affinity of the polyphenols for hERα was significantly less, with eriodictyol, narirutin and eriocitrin, hesperidin, hesperetin and mangiferin unable to significantly displace tritiated E₂ from hERα. Although naringenin, formononetin and even luteolin were able to compete with radiolabelled E₂ for binding to both ER subtypes, the extent of their displacement of radiolabelled E₂ from hERα was greatly less (Table 4) with only luteolin displacing more than 50% of the tritiated E₂ from hERα. In addition, the phytoestrogen control, genistein, also displayed a slightly higher displacement from hERβ. Despite this, interestingly enough the order of ability to displace labelled E₂ from the ER subtypes was similar for both receptor subtypes: E₂ > genistein > luteolin > naringenin > formononetin (only for ERβ: > eriodictyol > eriocitrin = narirutin).

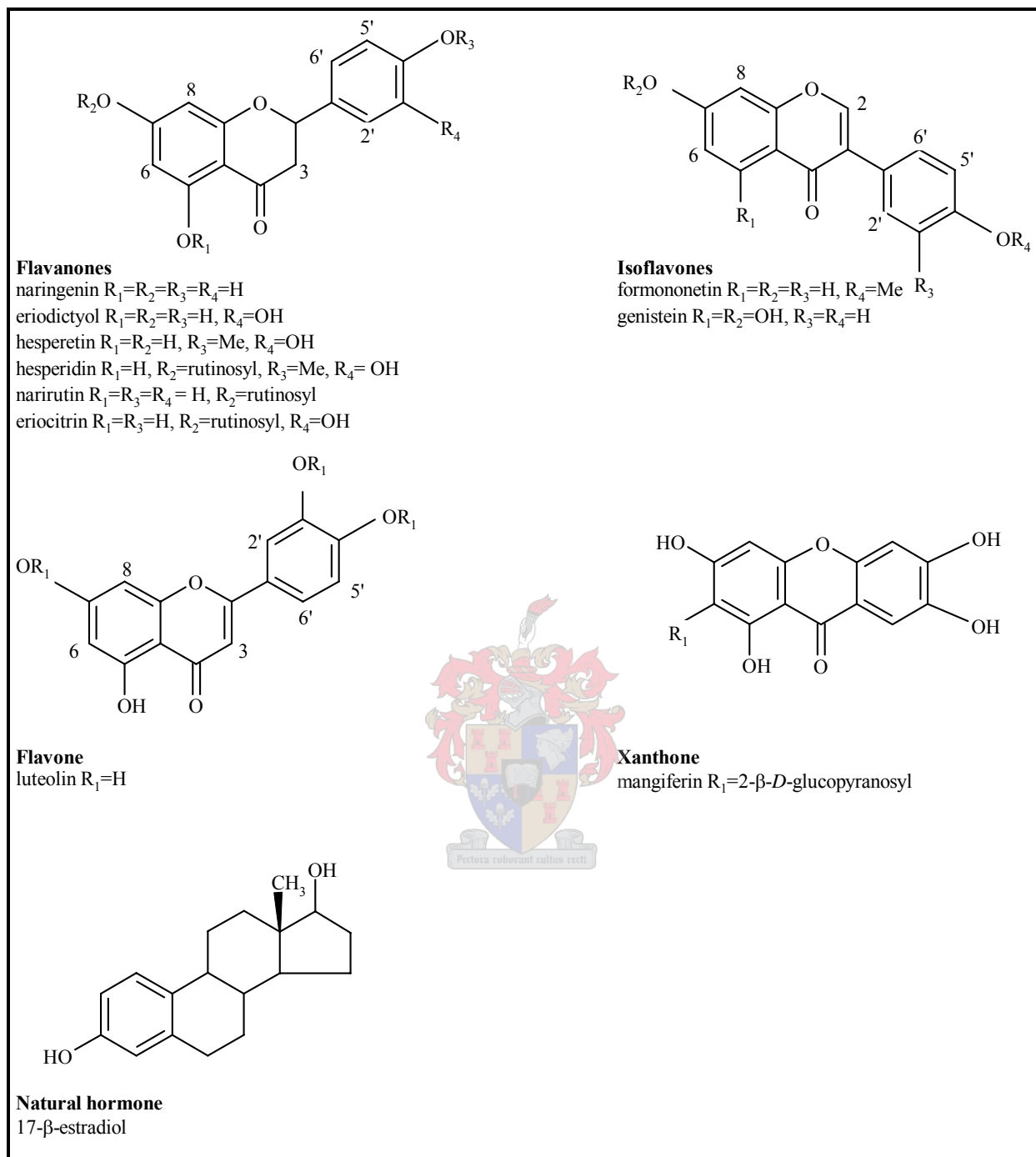


Figure 5: Chemical structures of the polyphenols and E₂ investigated in this study.

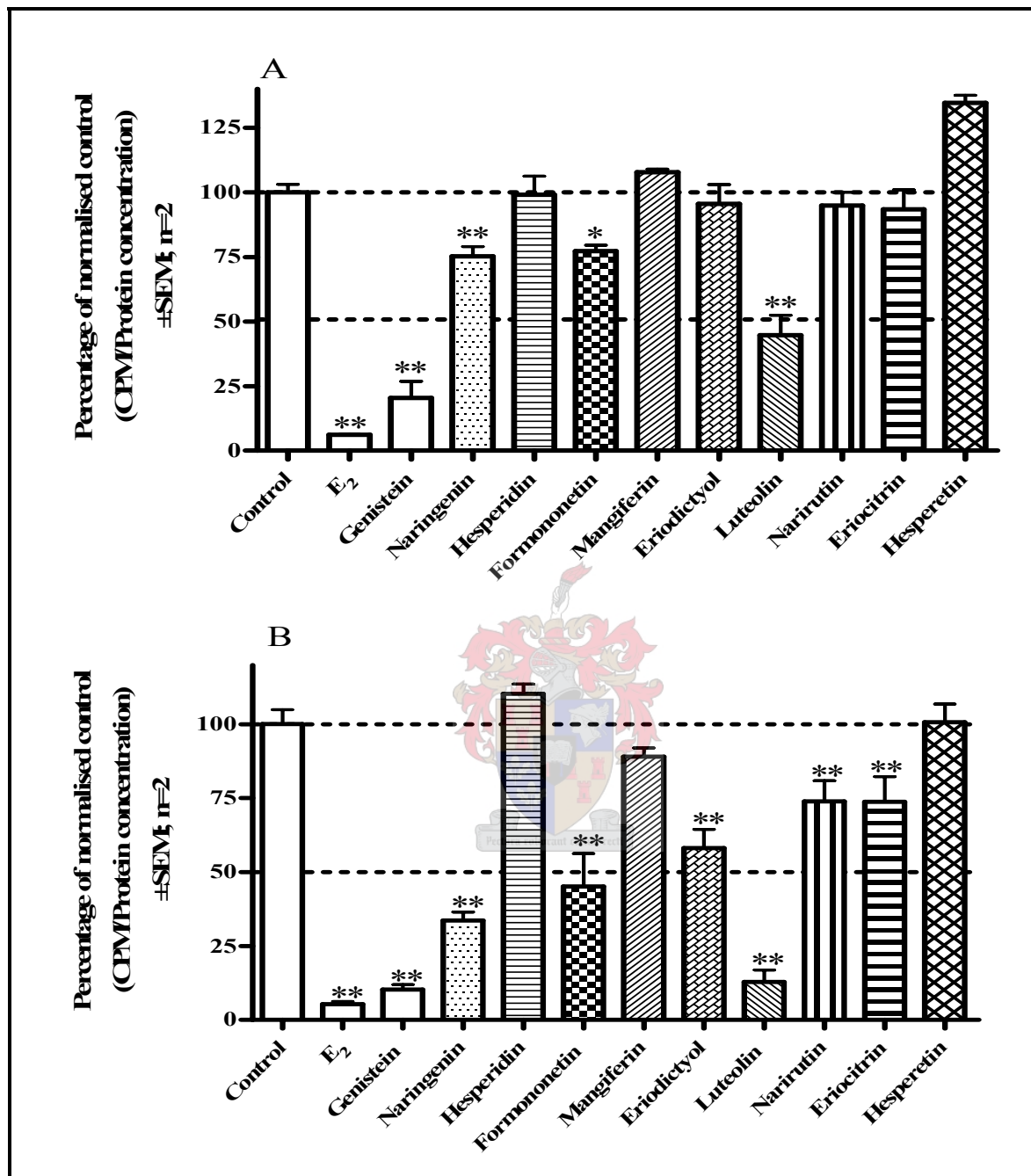


Figure 6: Commercially available phenolic compounds present in *Cyclopia* together with genistein and unlabelled E₂ were tested at 10⁻⁵ M for displacement of 1 nM ³H-E₂ from (A) hER α or (B) hER β expressed in COS-1 cells. Genistein and 17- β -estradiol (E₂) are used as positive controls. Control represents total binding of 1 nM ³H-E₂ to the ER subtypes and is set at 100%. For statistical analysis one-way ANOVA was used with Dunnett's multiple comparison's post test comparing binding to control. P-values are represented as follows: P < 0.05 by * and P < 0.01 by **.

Table 4: Summary of percentage $^3\text{H-E}_2$ displaced from ER subtypes by the polyphenols present in *Cyclopia*

Test compounds (10^{-5} M)	Percentage of $^3\text{H-E}_2$ displaced ^a (%)	
	hER α	hER β
E ₂ ^b	100	100
Genistein ^b	89	95
Luteolin	61	92
Naringenin	24	70
Formononetin	22	58
Eriodictyol	ns ^c	44
Eriocitrin	ns	28
Narirutin	ns	28
Hesperidin	ns	ns
Hesperetin	ns	ns
Mangiferin	ns	ns

^aPercentage $^3\text{H-E}_2$ displaced calculated from 10^{-5} M E₂ set at 100% displacement.

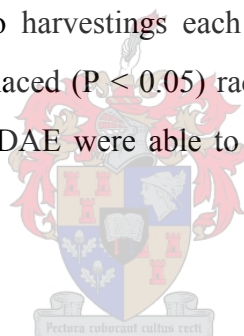
^bE₂ and genistein were used as positive controls.

^cns = Did not significantly displace $^3\text{H-E}_2$ from ER receptor subtype.

2.4.2.3. Binding of DAE from *Cyclopia*

DAE from *Cyclopia* assayed represents not only three harvestings of each species, but also fermented and unfermented plant material (Table 1). All extracts were assayed for binding to ER subtypes at a concentration of 1.5 µg/ml.

The DAE of fermented and unfermented *Cyclopia spp* portrayed no significant binding affinity for hER α (Figure 7 and Table 5), although both the natural ligand E₂ and the phytoestrogen, genistein, displaced tritiated E₂ to a significant degree. The same trend was not observed when binding to hER β was investigated (Figure 8 and Table 5). Although the DAE of unfermented and fermented *C. sessiliflora* and *C. intermedia* were unable to significantly displace 1 nM labelled E₂ from either ER subtype (Figure 7B and Figure 8B; Table 5), *C. genistoides* and *C. subternata* were notably more consistent in binding to the hER β (Figure 8A and Table 5), even though neither species were able to significantly displace tritiated labelled E₂ from hER α (Figure 7A and Table 5). Two harvestings each from the unfermented and fermented *C. genistoides* DAE significantly displaced ($P < 0.05$) radiolabelled E₂ from hER β . In contrast, only two unfermented *C. subternata* DAE were able to bind significantly ($P < 0.05$) to hER β (Figure 8A).



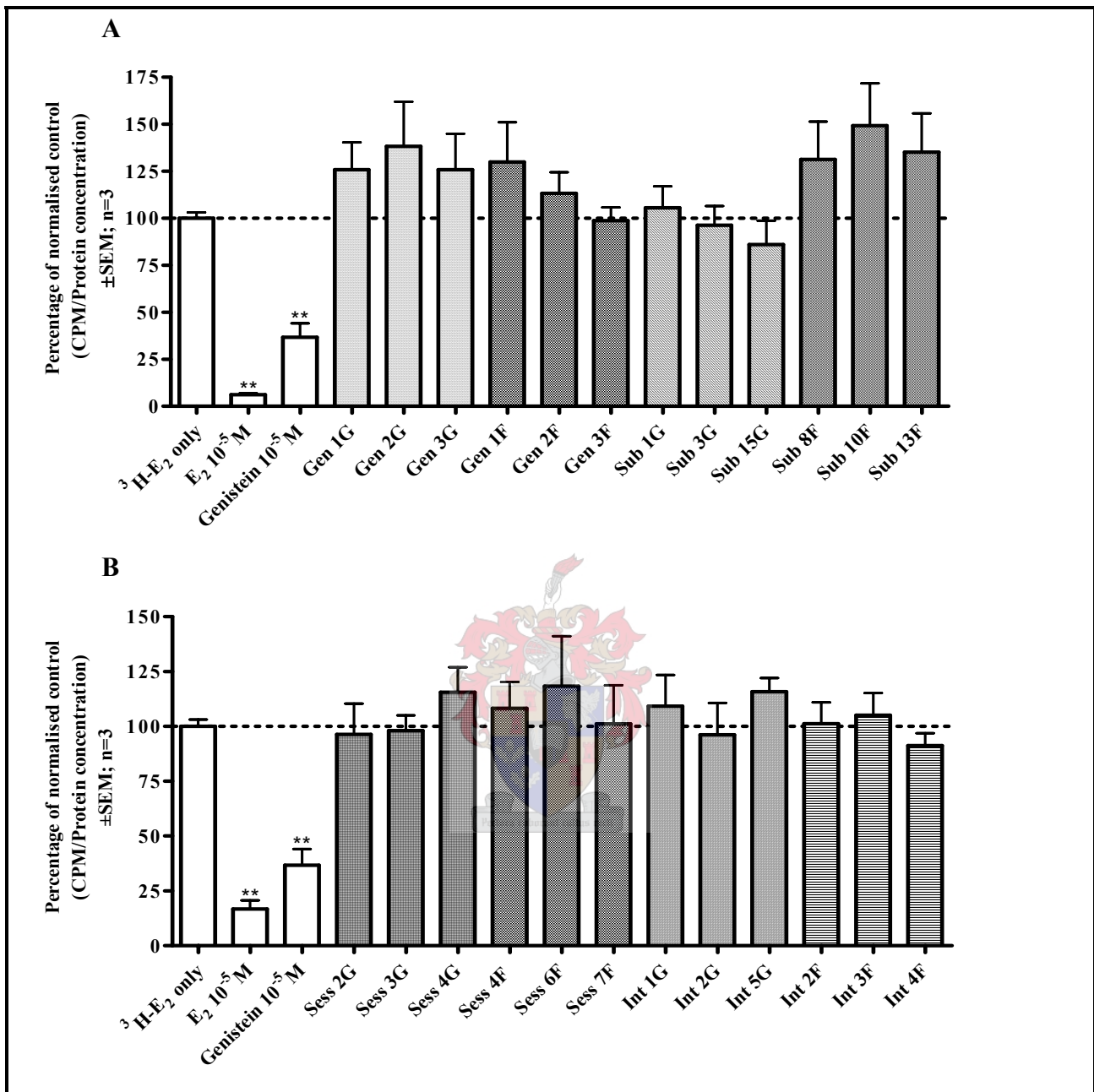


Figure 7: Percentage binding of 1 nM ³H-E₂ to hER α , expressed in COS-1 cells, by DAE of (A) *C. genistoides* and *C. subternata* (fermented and unfermented) and (B) *C. sessiliflora* and *C. intermedia* (fermented and unfermented) at a concentration of 1.5 μ g/ml. As positive controls 17- β -estradiol (E₂) and genistein were used at 10⁻⁵ M. Control represents total binding of 1 nM ³H-E₂ to hER α set at 100%. For statistical analysis one-way ANOVA was used with Dunnet's multiple comparison's post test comparing binding to control. P-values are represented as follows: P < 0.05 by * and P < 0.01 and by **.

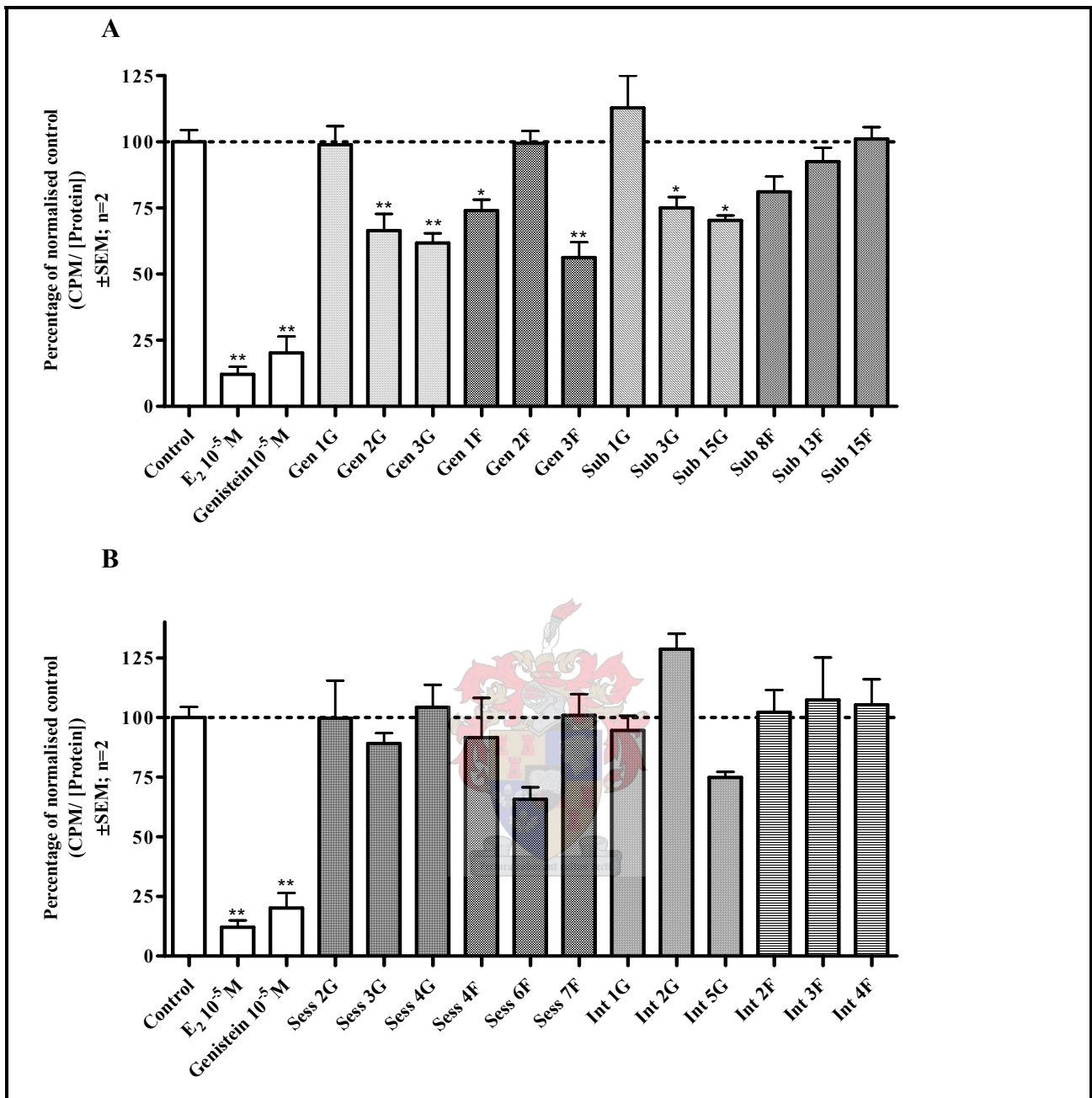


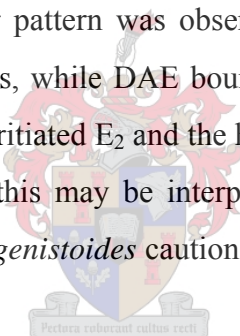
Figure 8: Percentage binding of ³H-E₂ to hERβ, expressed in COS-1 cells, by DAE of (A) *C. genistoides* and *C. subternata* (fermented and unfermented) and (B) *C. sessiliflora* and *C. intermedia* (fermented and unfermented) at a concentration of 1.5 μg/ml. As positive controls 17-β-estradiol (E₂) and genistein were used at 10⁻⁵ M. Control represents total binding of 1 nM ³H-E₂ to hERβ set at 100%. For statistical analysis one-way ANOVA was used with Dunnet's multiple comparison's post test comparing binding to control. P-values are represented as follows: P < 0.05 by * and P < 0.01 by **.

2.4.2.4. Binding of DME from *Cyclopia*

Screening of the various DME (Table 2) for binding to the estrogen receptor was assayed in a similar manner as for the DAE. However, based on the results of the aqueous extracts, only unfermented plant material was investigated for ER binding ability. All extracts were assayed at a concentration of 1.5 µg/ml.

In this study unfermented DME of *C. intermedia* and *C. sessiliflora* showed no significant binding affinity for either ERs (Figure 9). This agrees with the results obtained for the DAE of *C. intermedia* and *C. sessiliflora* (Table 5).

Similarly to the DAE investigated, some methanol extracts of *C. genistoides* and *C. subternata* were able to compete with tritiated E₂ for binding to the hERβ. One *C. genistoides* extract (Gen P104) was able to displace the highest percentage tritiated E₂ (Figure 9 and Table 5) from both hERα and hERβ while the DAE of fermented and unfermented *C. genistoides* were only able to bind to hERβ. A similar pattern was observed for *C. subternata* in that a DME (Sub P118) bound to both ER subtypes, while DAE bound only to hERβ. *Cyclopia genistoides* portrayed the highest displacement of tritiated E₂ and the highest incidence of binding (two out of three harvestings) to ERβ. Although this may be interpreted as less variation in estrogenicity between different harvestings from *C. genistoides* caution should be exercised, as the sample size was small (Figure 9 and Table 5).



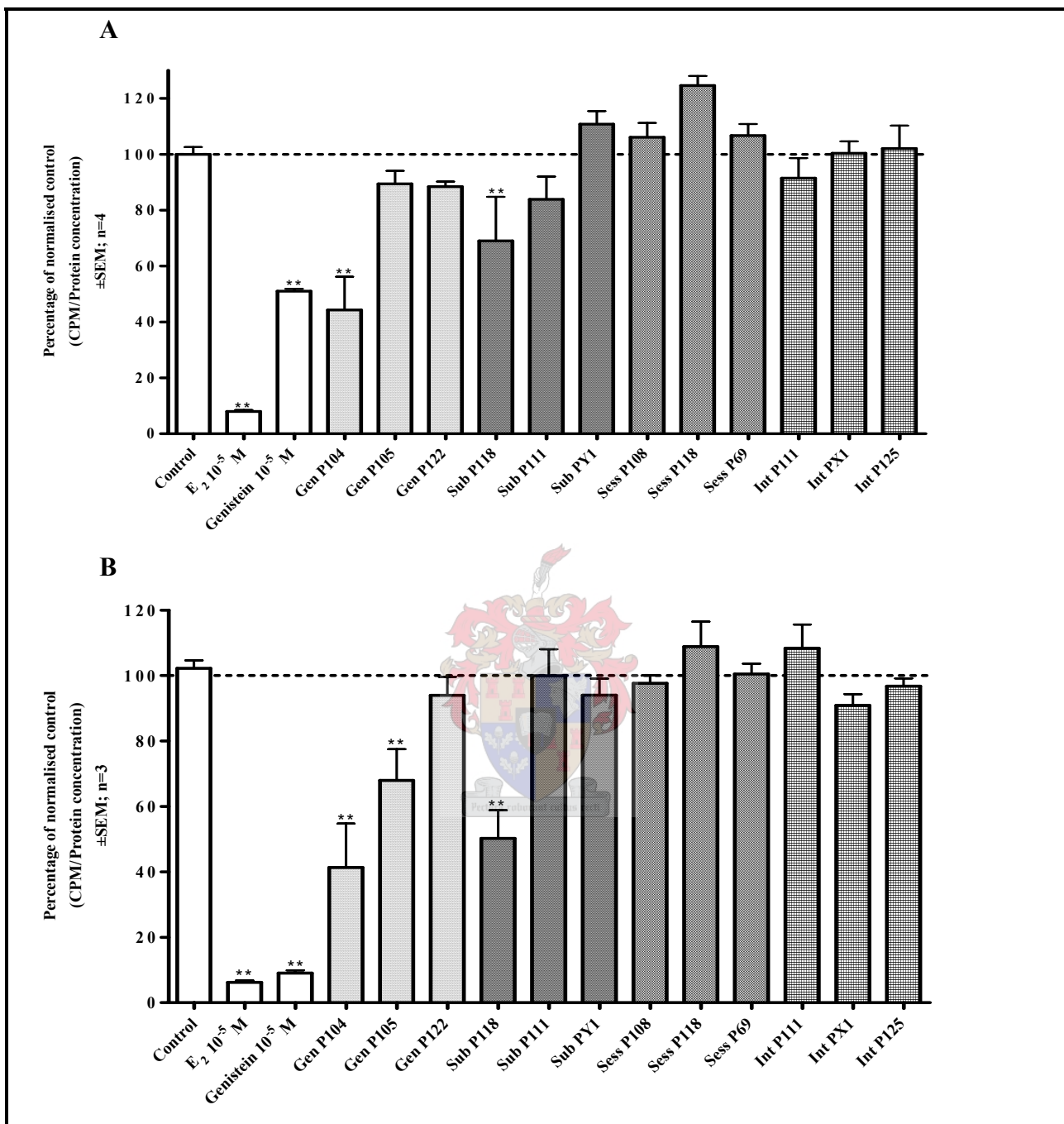


Figure 9: Percentage binding of ³H-E₂ to (A) hER α and (B) hER β expressed in COS-1 cells, by binding of DME of unfermented *Cyclopi*a species at a concentration of 1.5 μ g/ml. As positive controls 17- β -estradiol (E₂) and genistein were used at 10⁻⁵ M. Control represents total binding of 1 nM ³H-E₂ to hER β set at 100%. For statistical analysis one-way ANOVA was used with Dunnet's multiple comparison's post test comparing binding to control. P-values are represented as follows: P < 0.05 by * and P < 0.01 by **.

Table 5: Summary of percentage $^3\text{H-E}_2$ displaced from ER subtypes by both aqueous (DAE) and methanol (DME) *Cyclopia* extracts.

Extracts screened:	Percentage Displacement of $1\text{nM } ^3\text{H-E}_2$ (%) ^a	
	hER α	hER β
DAE: <i>C. genistoides</i> Gen 1G Gen 2G Gen 3G Gen 1F Gen 2F Gen 3F	NB ^b NB NB NB 1 NB	NB 34** 48** 35* NB 52**
DME: <i>C. genistoides</i> Gen P104 Gen P105 Gen P122	74** 9 12	70** 34** NB
DAE: <i>C. subternata</i> Sub 1G Sub 3G Sub 15G Sub 8F Sub 10F Sub 13F	NB NB 36 NB NB NB	NB 27* 33* 19 11 NB
DME: <i>C. subternata</i> Sub P118 Sub P111 Sub PY1	43** 7 NB	69** 5 6
DAE: <i>C. sessiliflora</i> Sess 2G Sess 3G Sess 4G Sess 4F Sess 6F Sess 7F	NB 5 NB 7 19 16	2 17 0.2 NB 40 7.26
DME: <i>C. sessiliflora</i> Sess P108 Sess P118 Sess P69	NB NB NB	NB 2 NB
DAE: <i>C. intermedia</i> Int 1G Int 2G Int 5G Int 2F Int 3F Int 4F	NB NB NB 2 13 12	2 NB 28 NB 5 NB
DME: <i>C. intermedia</i> Int P111 Int PX1 Int P125	NB NB 3	NB 10 3
Genistein 10^{-5} M (2.702×10^{-3} mg/ml)	76**	91**
17-β-estradiol (2.724×10^{-3} mg/ml)	100**	100**

^aDAE and DME were tested at a concentration of 1.5 $\mu\text{g/ml}$. The positive controls 17- β -estradiol and genistein were both assayed at 10^{-5} M (E_2 2.724×10^{-3} mg/ml; genistein: 2.702×10^{-3} mg/ml). The total percentage of $^3\text{H-E}_2$ displaced by E_2 was set at 100% displacement and all other displacements calculated relative to this.

^bNB = non-binder extracts were unable to displace $^3\text{H-E}_2$ from ER subtype.

* and **These extracts or compounds significantly (* $P < 0.05$ and ** $P < 0.01$) displaced $^3\text{H-E}_2$ from the indicated ER subtype.

2.4.3. HPLC analysis of DAE of fermented and unfermented *Cyclopia spp.* and DME of unfermented *Cyclopia spp.*

The extracts prepared from *Cyclopia spp.* (Table 3) were analysed by HPLC to quantify the polyphenols, luteolin, formononetin, naringenin, eriodictyol, eriocitrin, narirutin, hesperidin, hesperetin and mangiferin (Figure 5), previously shown to be present in *Cyclopia*^{4,5}. Mangiferin and hesperidin were ubiquitously present in all species, whether fermented or unfermented, and all extracts, whether DAE or DME (Tables 6 and 7). Mangiferin, a xanthone, was present at the highest concentration in most extracts analysed followed by the flavanone, hesperidin. The only known polyphenols with binding affinity for the ER β (Table 4) that were detected during the HPLC analysis were eriocitrin and narirutin, however, they were, in most cases, only present in trace amounts as compared to mangiferin and hesperidin. Luteolin, formononetin, naringenin and eriodictyol, all shown to bind to ER β (Table 4), were not detected by HPLC analysis under the conditions used. The HPLC profile (Figure 10) did, however, show unidentified peaks, which deserves further investigation as to their identity. These peaks may contain more than one compound that was eluted at the same time (Personal communication, Dr E Joubert, 2005).

2.4.4. Correlations

As phytoestrogens are polyphenols the correlation between total polyphenol content and binding to hER α or hER β was determined (Figure 11). There was, however, no significant correlation between percentage total polyphenol content and binding to hER α or hER β for any of the extracts. Eriocitrin and narirutin were the only known compounds detected in the extracts that were able to compete with ³H-E₂ for binding to the hER β . The amount of eriocitrin (Figure 12A) or narirutin (Figure 12B) present in each extract was correlated with the percentage ³H-E₂ displaced from ER β . There was no significant correlation between the binding observed for these extracts and the amount of eriocitrin or narirutin present. As the amounts of eriocitrin and narirutin present in the extracts did not correlate with binding to the ER β we hypothesised that unknown polyphenols in *Cyclopia* extracts might be responsible for binding to the receptor. Two sizeable unknown peaks, retention times on HPLC of 4.4 and 6.7 minutes, respectively, were observed (Figure 10) and correlations between these and the percentage ³H-E₂ displaced from ER β was done (Figure 13). No correlation between the amounts of these unidentified compounds present in the various dry extracts and binding to the hER β subtype was, however, found.

Table 6: Phenolic content, as determined by HPLC, of the dry aqueous extracts (DAE) of unfermented and fermented plant material from *Cyclopia spp.*

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

^aQuantities were expressed as a percentage of the extract i.e. g per 100g extract.

^bValues represent the means (% of DAE) ± SD of two determinations.

^cnd = Not detected.

Table 7: Phenolic content, as determined by HPLC, of the dry methanol extracts (DME) of unfermented plant material from *Cyclopia spp.*

Species	Harvesting s	Percentage of soluble solids ^a								
		Mangiferin	Hesperidin	Hesperetin	Eriocitrin	Narirutin	Luteolin	Formononetin	Naringenin	Eriodictyol
<i>C. genistoides</i>	Gen P104	2.77 ± 0.012 ^b	1.22 ± 0.002	nd ^c	0.19 ± 0.003	0.35 ± 0.004	nd	nd	nd	nd
	Gen P105	2.71 ± 0.005	1.21 ± 0.003	nd	0.19 ± 0.008	0.35 ± 0.010	nd	nd	nd	nd
	Gen P122	2.28 ± 0.036	1.21 ± 0.011	nd	0.22 ± 0.003	0.31 ± 0.000	nd	nd	nd	nd
<i>C. subternata</i>	Sub P118	4.25 ± 0.000	1.14 ± 0.000	nd	0.92 ± 0.007	0.04 ± 0.000	nd	nd	nd	nd
	Sub P111	3.26 ± 0.001	0.69 ± 0.002	nd	0.65 ± 0.001	0.03 ± 0.010	nd	nd	nd	nd
	Sub PY1	1.705 ± 0.021	1.63 ± 0.021	nd	1.90 ± 0.014	0.09 ± 0.007	nd	nd	nd	nd
<i>C. sessiliflora</i>	Sess P108	3.69 ± 0.002	0.88 ± 0.014	nd	0.45 ± 0.002	0.03 ± 0.003	nd	nd	nd	nd
	Sess P118	4.28 ± 0.015	1.16 ± 0.008	nd	0.36 ± 0.016	0.05 ± 0.000	nd	nd	nd	nd
	Sess P69	4.61 ± 0.042	1.22 ± 0.019	nd	0.50 ± 0.028	0.06 ± 0.002	nd	nd	nd	nd
<i>C. intermedia</i>	Int P111	3.90 ± 0.020	1.28 ± 0.007	nd	0.21 ± 0.001	0.23 ± 0.000	nd	nd	nd	nd
	Int P X1	5.21 ± 0.014	1.53 ± 0.002	nd	0.21 ± 0.001	0.22 ± 0.002	nd	nd	nd	nd
	Int P125 ^d	7.04 ± 0.057	2.34 ± 0.050	nd	0.63 ± 0.007	0.10 ± 0.000	nd	nd	nd	nd

^aQuantities were expressed as a percentage of the extract i.e. g per 100g extract.

^bValues represent the means (% of DME) ± SD of two determinations.

^cNot detected.

^dHarvesting was analysed on Luna phenyl-hexyl column, which did not separate eriocitrin effectively thus results are over expressed.

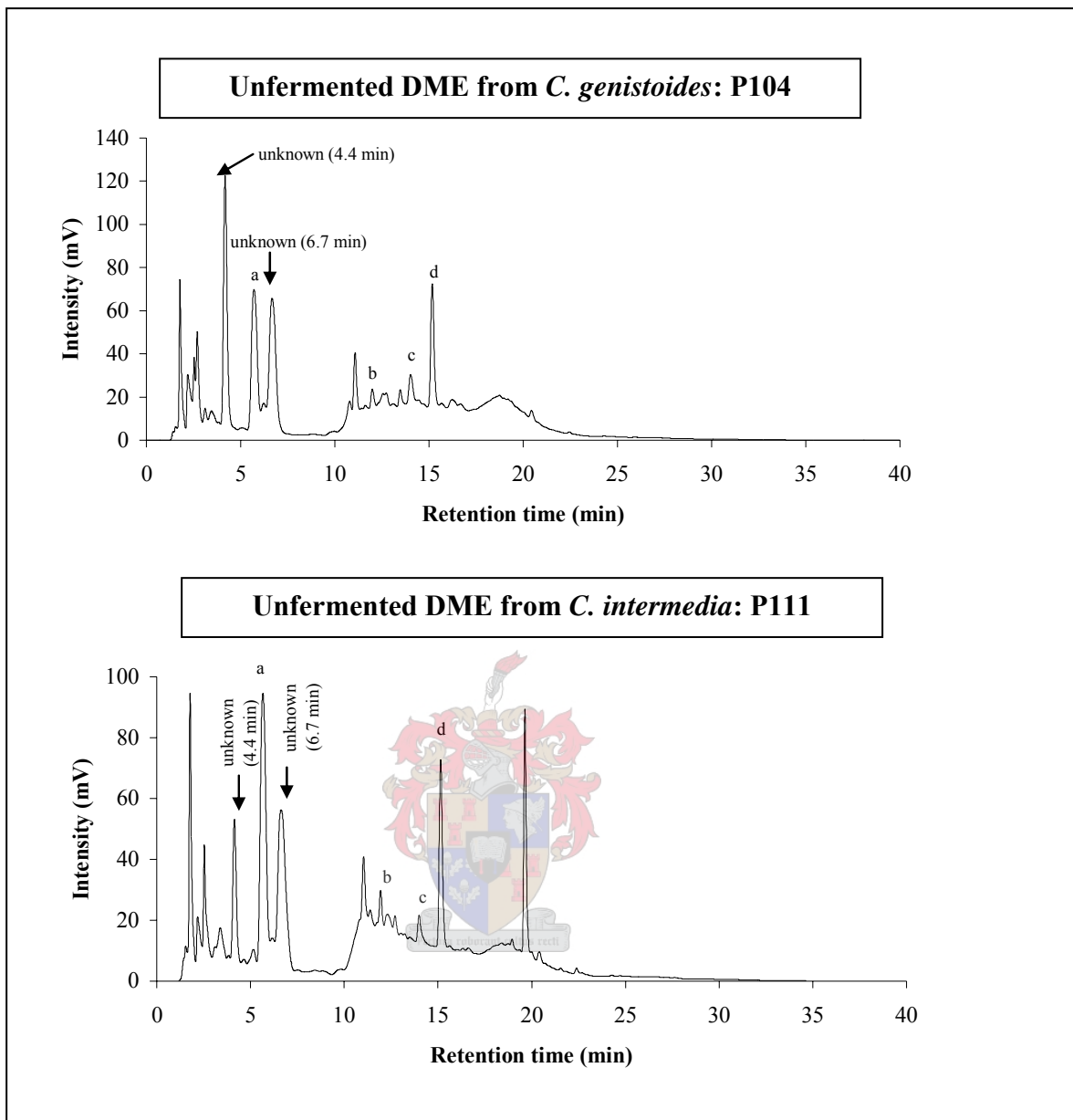


Figure 10: Examples of typical HPLC chromatogram profiles of *Cyclopia* spp. Polyphenols quantified and shown on profile are: (a) mangiferin, (b) eriocitrin, (c) narirutin and (d) hesperidin.

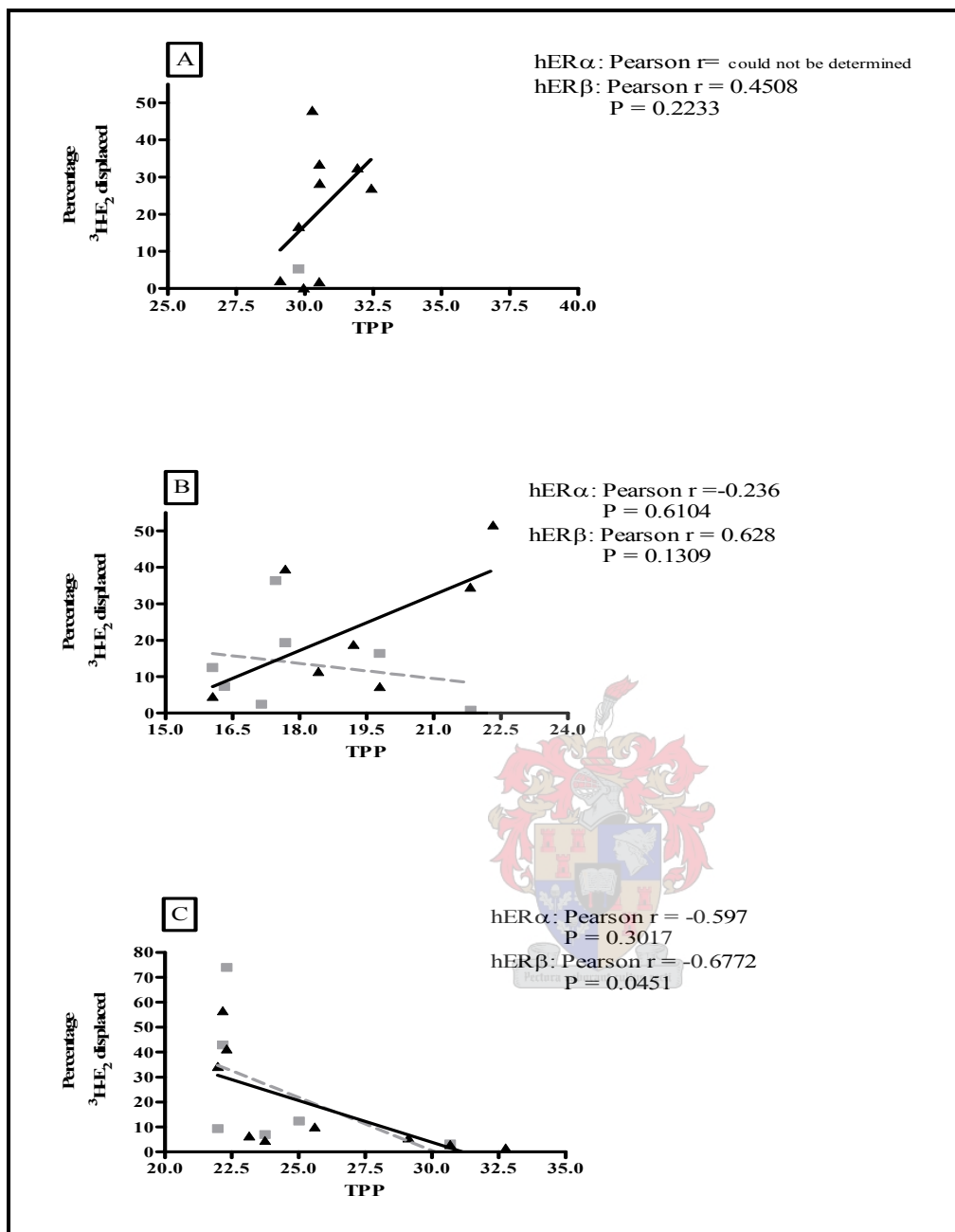


Figure 11: Correlation between percentage tritiated E₂ displaced from hER α (■) and hER β (▲) and percentage total polyphenol content (TPP) of (A) DAE from unfermented plant material, (B) DAE from fermented plant material and (C) DME from unfermented plant material. All correlations are not significant. Pearson correlations (two-tailed) were done using GraphPad Prism™.

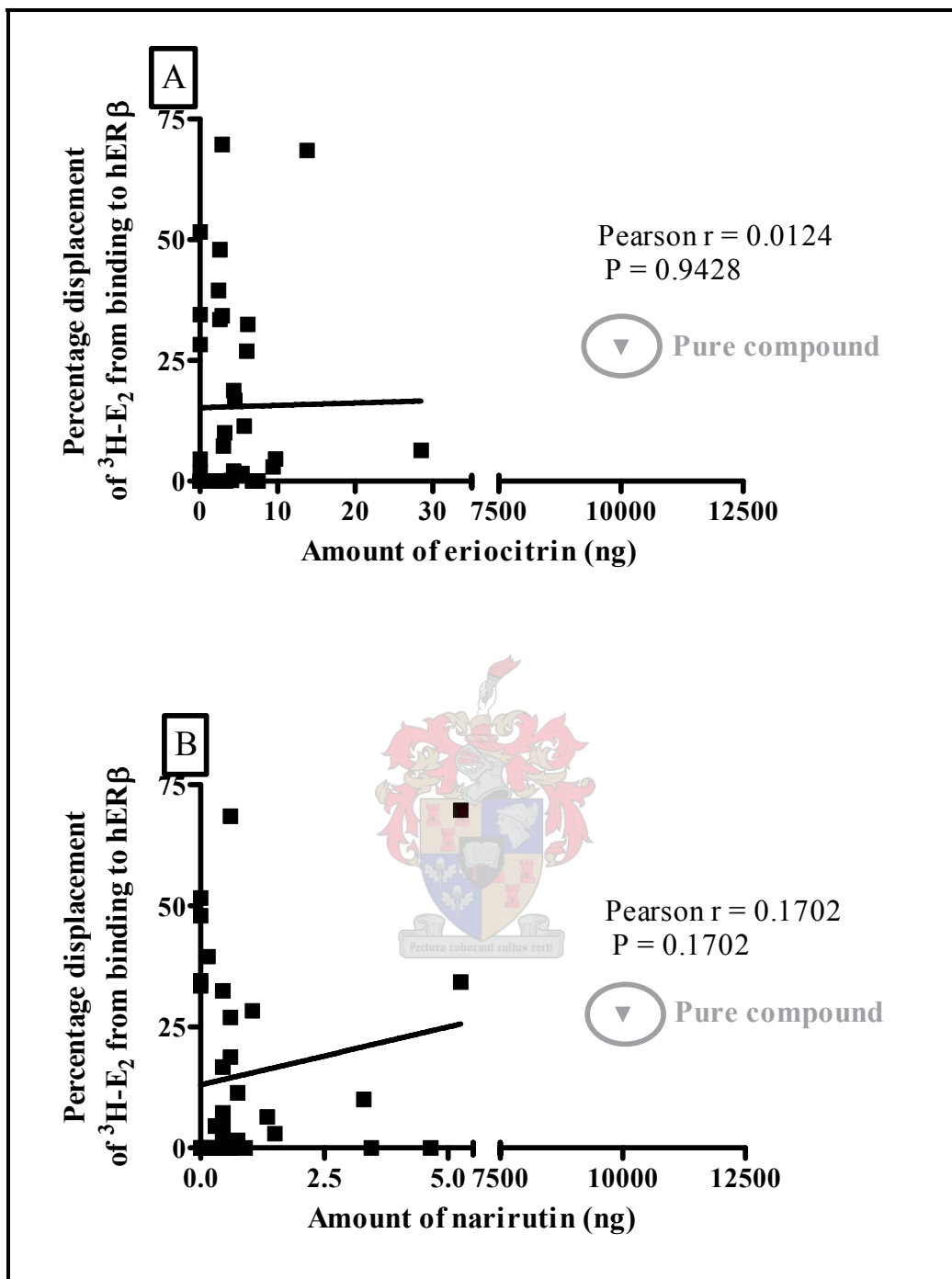


Figure 12: Correlation between the percentage tritiated E₂ displaced from hER β by all the extracts investigated and (A) amount of eriocitrin present; (B) amount of narirutin present as quantified by reversed phase HPLC. Pearson correlations (two-tailed) were done using GraphPad Prism™.

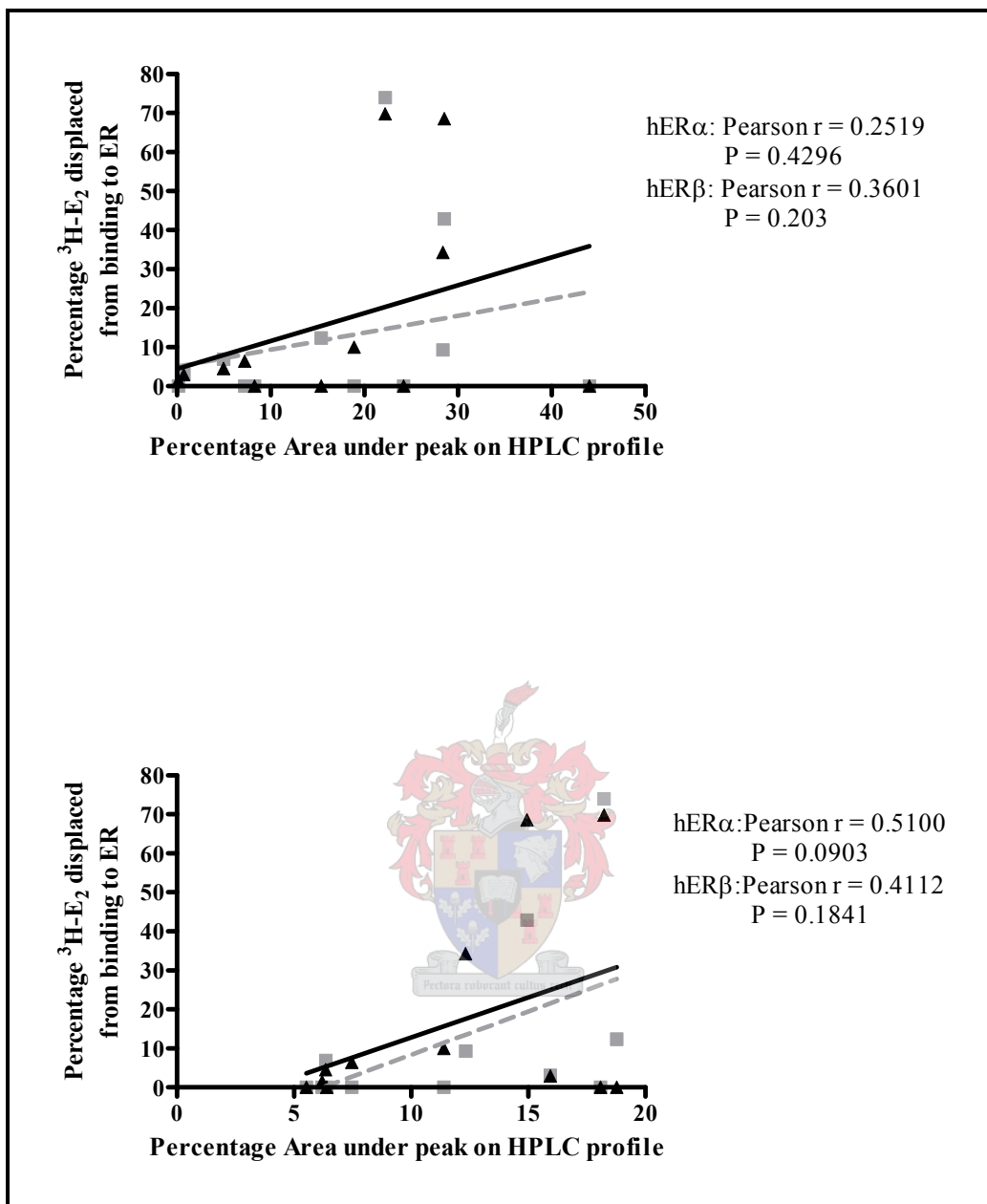


Figure 13: Correlation between the percentages tritiated E₂ displaced from the ER subtypes, hER α (■) and hER β (▲), by the unfermented *Cyclopia spp.* DME and the percentage area under the peak of unknown HPLC peaks at (A) a retention time of 4.4 minutes and (B) a retention time of 6.7 minutes. Pearson correlations (two-tailed) were done using GraphPad Prism™.

2.5. Discussion

Epidemiological studies propose that the low occurrence of certain cancers as well as less severe or no menopausal symptoms in women from Asian countries are due to the intake of flavonoids, especially soy flavonoids, present in the diet of these individuals.^{17;18} The phenolic compounds are thought to mediate their effect by acting as weak estrogens. In contrast, menopausal women using traditional HRT are believed to have a higher likelihood for the occurrence of breast cancer.²⁰

Alternative therapies for HRT are thus being researched, as conventional HRT is not protecting menopausal women from certain conditions such as cardiovascular disease, as were previously thought.²² In addition, consumers are inclined to prefer natural alternatives, which they consider to have fewer side-effects.¹⁶ Plant flavonoids, such as those present in an Asian diet, are already marketed and available to women for use as an alternative or supplement to conventional HRT. These phenolic compounds or phytoestrogens are believed to alleviate menopausal symptoms and protect against estrogen-dependent cancers.^{10;11;12}

Honeybush that belongs to the Fabaceae (legume) plant family is a possible source of phytoestrogens. The identification of known phytoestrogens, naringenin, luteolin, eriodictyol and formononetin^{7,8} in *C. intermedia*⁴ and anecdotal communication from a woman in the Langkloof who drinks a herbal infusion prepared from *Cyclopia* to alleviate menopausal symptoms (Personal communication to E. Joubert by J. Nortje, Kouga, 1996) led to this investigation. Scientific evidence supporting honeybush tea as a supplement to alleviate menopausal symptoms, could add value to this aromatic herbal infusion as it is already shown to be antimutagenic and having antioxidant properties.^{2,6}

Several of the polyphenols present in the *Cyclopia spp.* were screened for phytoestrogenic activity to serve as marker compounds for future experimental studies on the phytoestrogenic activity of honeybush. In addition, the four commercially available honeybush tea varieties, *C. genistoides*, *C. subternata*, *C. sessiliflora* and *C. intermedia*, were screened, through whole cell competitive receptor binding assays, to ascertain which *Cyclopia spp.* contained the highest putative phytoestrogenic activity.

As the plant extraction procedure is rather tedious and time consuming, a high extract yield is considered an advantage and aimed for. Additionally, the solvent used is also important for selective extraction and better extraction of flavonoids. In comparing the extract yield within a

species (paired two-tailed t-test) and across all species (unpaired two-tailed t-test) (Table 3) there was a significant difference between unfermented and fermented DAE yield ($P < 0.05$) and total polyphenol content ($P < 0.01$). Furthermore, in comparing the extract yield of unfermented DME within a species and across all species with the unfermented DAE there was a significant difference ($P < 0.01$). However, for the total polyphenol content the pattern differs with no significant difference between DME and DAE from unfermented *Cyclopia* across species. Caution should, however, be exercised in evaluating these differences in extraction yield and total polyphenol content. Although extracts were prepared from the same species they were not always prepared from the same harvesting or area and these may contribute to variation. The plants were not monoclonal as they were propagated from seedlings and large genetic variation is to be expected. It is still interesting, however, that extracts of unfermented samples do give higher yield and total polyphenol content compared to similar fermented samples.

Binding to the ER is the first interaction in the molecular pathway of the estrogen-mediated biological response. By using COS-1 cells, which do not endogenously express ER (Figure 2), selective transient transfection with either hER α or hER β , made distinguishing binding affinity of the compound or extract for a specific ER subtype possible.

It was essential to optimise and validate the whole cell binding assay before screening of polyphenols and *Cyclopia* extracts commenced. The time for binding equilibrium to be reached had to be determined. The two subtypes did not reach binding equilibrium at the same time (Figure 3), with hER β binding reaching equilibrium faster. This was expected, as the expression levels of hER β in COS-1 cells were higher than those for hER α (Figure 2). For convenience sake and to keep experimental conditions consistent, it was decided to standardise the incubation time for both hER α and hER β at 2 hours. The K_d values obtained from the literature in many cases show little difference between the affinity of the natural ligand E₂ for the ER subtypes. For example, Kuiper *et al.*³⁴ determined with competitive binding that the K_i for hER α and hER β is 0.13 nM and 0.12 nM, respectively, while with saturation binding³⁹ K_d values between 0.05-0.1 nM were obtained for both hER α and hER β . These literature values compare well with the K_d values of 0.2 nM (hER α) and 1.6 nM (hER β) determined in this study. It does, however, appear that E₂ had a higher affinity for the hER α in our system. This has also been shown by some investigators, for example, Sun *et al.*⁵⁰ showed that binding affinities for hER α and hER β were 0.3nM and 0.9nM, respectively, using competitive binding.

Having established the validity of the whole cell binding assay selected flavonoids, available commercially and present in *Cyclopia*^{4,5}, were investigated for binding to ER subtypes, since flavonoids have been shown to display ER binding. Specifically, naringenin formononetin, eriodictyol and luteolin, known phytoestrogens, and narirutin, eriocitrin, hesperidin and hesperetin, identified to be present in *C. intermedia*^{4,51} and *C. subternata*⁵, were screened at a concentration of 10⁻⁵M for binding to the ER subtypes. Mangiferin, a xanthone, was also included in the investigation as it is present in high concentrations in all *Cyclopia spp.*^{3,4;44;51}

Naringenin, formononetin and luteolin bound to both ER α and ER β , with an order of displacement of luteolin > naringenin > formononetin. Similar to results of the present study, others have shown that luteolin binds to ER β ⁷, that naringenin binds to ER β ^{7,39} and weakly to ER α ^{39,52}, and that formononetin binds to ER β and weakly to ER α .³⁹ To our knowledge binding of luteolin to hER α has not previously been investigated. Others have shown that naringenin has a higher binding affinity for ER β than luteolin⁷, which was not supported by data from the present study. However, full dose response curves were not investigated and therefore the results are not fully comparable to the IC₅₀ values determined by Han *et al.*⁷ Similarly to Han *et al.*, the present study showed that naringenin was able to displace more tritiated E₂ from hER β than formononetin. Again it is important to note that for the present study a full dose response was not investigated and compounds were only tested at 10⁻⁵ M. Of the other polyphenols tested the flavanones narirutin, eriocitrin, and eriodictyol only bound to the ER β , while the flavanones, hesperidin and hesperetin, like mangiferin, a xanthone, did not bind to either ER subtype. Hesperetin has previously been shown to have no binding affinity for the endogenously expressed ER in MCF-7 cells.⁵³ To our knowledge this is the first time it has been shown that eriodictyol, narirutin, and eriocitrin are able to displace radiolabelled E₂ from hER β (Figure 6B). Eriodictyol has, however, previously been shown to have weak estrogenic activity in stimulating both MCF-7 cell proliferation and transcriptional induction of an ERE containing promoter.^{8,9} A comparative study by Miksicek⁵⁴ with various plant flavonoids suggests that flavonoids with hydroxyl groups at positions 4' and 7 are more than likely estrogenic, while if the number of hydroxyl groups exceeds 4 estrogenic activity is reduced.⁵⁴ This appeared to hold true for naringenin, formononetin, luteolin, and eriodictyol investigated in this study. Although hesperetin contains a hydroxyl group at position 7 the methoxy group at the 4'-position is likely to prevent hesperetin from binding to the ER.⁵⁵ A similar argument would thus also be relevant for its glycoside,

hesperidin. The methoxy group at the 4'-position, however, does not prevent formononetin from binding to the ER subtypes.³⁹ However, formononetin is not only a isoflavone, with the B-ring in position 3, but the unsaturated C2-C3 bond imparts planarity.

Phytoestrogens have been shown to have a higher affinity for ER β than for ER α ³⁹ which was also demonstrated in this study. The plant polyphenols tested, including the control genistein, all preferentially displaced tritiated E₂ from hER β (Table 4). The ER β is suggested to be a negative regulator of ER α activity and compounds with selective ER β affinity are sought, as it is believed that they could be used for the treatment for estrogen dependent cancers resulting from ER α action.^{56,57} The literature would suggest that ER β activity could protect against excessive proliferation of cancerous cells mediated by ER α .^{58,59} This phenomenon could be the reason why phytoestrogens are considered an alternative to conventional HRT as it could still protect against osteoporosis and reduce cardiovascular disease, without inducing estrogen-dependent cancers.

Similar to the results with the commercially available polyphenols we found that binding of *Cyclopia* extracts to ER β was more significant than to ER α (Table 5). None of the DAE bound significantly to ER α and only one out of three DME from *C. genistoides* and *C. subternata* each bound significantly to ER α . Binding to ER β of *C. genistoides* extracts surpassed that of all other extracts with two out of three harvestings giving significant displacement for all extracts (DAE from fermented and unfermented plant material and DME from unfermented plant material). Of the other *Cyclopia* spp only extracts from unfermented *C. subternata* (two out of three for DAE and one out of three for DME) gave significant binding to hER β . Only two extracts, both DME, from *C. genistoides* and *C. subternata*, showed significant displacement from both ER α and ER β . Extracts from *C. intermedia* did not bind significantly to either ER subtype, which was unexpected due to the presence of isoflavones and coumestans in this genus.⁴ Poor extraction of aglycones due to low solubility in water, losses during cleanup with dichloromethane for subsequent methanol extraction or initial low quantities of flavonoids present in the plant material could explain the inability of *C. intermedia* to displace tritiated E₂ from both ER subtypes.

Although the average total polyphenol content of unfermented DAE was significantly higher than both fermented DAE and unfermented DME when compared within species, ER binding was not correlated with the total polyphenol content (Figure 11). This was to be expected as the Folin-Ciocalteu assay only measures the presence and number of hydroxyl groups, which would suggest that the structural requirements of the polyphenols is more important in

determining estrogenic effects than the number of hydroxyl groups. In fact Miksicek⁵⁴, in comparing a wide variety of polyphenols, found that the diaryl ring structure common to all flavonoids and at least one hydroxyl group present on each of the aromatic rings are essential for estrogenic activity. Richards showed a significant ($P < 0.0001$) correlation between the total polyphenol content and the antioxidant activity of *Cyclopia spp.*, but not for antimutagenicity.² This difference in correlation results was attributed to the differences in the requirements of the polyphenols where the number of hydroxyl groups is important for antioxidant activity, but the structure of a polyphenol determines antimutagenicity.²

Furthermore, despite the decrease in total polyphenol content of the plant material² and subsequently of their extracts with fermentation (Table 3), both unfermented and fermented DAE of *C. genistoides* were able to bind to the hER β (Figure 8), suggesting that the active compound(s) in *C. genistoides* is not highly susceptible to oxidation and thus retained during fermentation. Fermentation did, however, have an effect on binding of *C. subternata* extracts, as only unfermented DAE were able to bind significantly to hER β . In this case, loss of the active compound(s) with oxidation is indicated, also suggesting that the active compound(s) differs from that of *C. genistoides*. These findings indicated that fermentation appears not to have such a clear-cut effect on ER binding and identification of the active compound(s) is necessary to understand the effect of fermentation on the binding of ER subtypes by different species.

The quantification of phenolic compounds present in DAE and DME (Tables 6 and 7) was carried out in an attempt to provide a chemical basis for the observed intra- and inter-species differences. However, the results also did not explain the significant binding to the ER subtypes by some extracts. The two major compounds, mangiferin and hesperidin, did not bind to either ER subtype. The known phytoestrogens, formononetin, luteolin, eriodictyol, and naringenin were not detected in any of the extracts (aqueous or methanol), while eriocitrin and narirutin, which bound to hER β , and would contribute to binding of some extracts, were present only in trace amounts. It would be expected that the compounds that have binding affinity for the ER subtypes would be present in higher amounts in the active species such as *C. genistoides* and *C. subternata*, or in extracts such as Gen 104 and Sub P118, which were able to significantly compete for binding to both ER subtypes. This, however, was not the case with Gen 104, which bound significantly to both ER subtypes, containing similar amounts of eriocitrin and narirutin as Int P111 that did not bind to either ER subtype (Table 7). The lack of correlation between the

amount of eriocitrin and narirutin present in the extracts examined and the displacement of E₂ further confirmed that the low levels of eriocitrin and narirutin cannot be responsible for the binding activity observed in the extracts (Figure 12). This would suggest that unidentified polyphenols present in the extracts are mainly responsible for the binding activity. Correlations between two large unidentified HPLC peaks and ER binding of DME were done, but unfortunately did not suggest that these peaks contribute to the binding activity of the *Cyclopia* extracts (Figure 13). However, HPLC separation was not optimized for separation of the peaks, and several compounds, some with activity and others without activity, could have co-eluted. This matter needs further investigation.

This study would suggest that not only is honeybush a source of phytoestrogens, but that both aqueous and methanol extracts preferentially bind to the hER β . In another study conducted with methanol extracts of the roots of *Maghania philippinsis* also from the same plant family, Fabacea, it was shown that these extracts induced proliferation of the human breast cancer cell line MCF-7.⁶⁰ Further in-depth studies with *Cyclopia* are therefore important to ascertain if the phytoestrogens present in *Cyclopia* display relevant potency in mediating estrogenic or possibly antiestrogenic effects and these should include proliferative assays in human breast cancer cells. These assays are essential as receptor-binding studies do not differentiate between agonists and antagonists.

A high degree of variability in ER binding even within the same species is noticeable from our results. For example, DME from *C. genistoides*, showed binding to both ER subtypes (Gen P104), only to ER β (Gen P105), or to neither subtype (Gen P122) (Table 5). All three harvestings were from the same plantation (Table 2), but were collected on different dates and not necessarily from the same plants. One explanation may be that Gen P104 was harvested during a low-rainfall season in contrast to the other two harvestings (Personal communication, Dr. E. Joubert, 2004). This may contribute to the higher estrogenicity (78% E₂ displaced from hER β) of this particular harvesting, as plants under stress tend to produce more secondary metabolites i.e. polyphenols, as a protective mechanism.⁴⁰ However, neither the total polyphenol content nor HPLC data supports this. Another explanation may be that as the *Cyclopia* industry is relatively new, there are large geno- and phenotype differences within one species and even within one plantation (Personal communication, Dr. E. Joubert, 2005). Additionally, the phytoestrogens previously identified to be present in *C. intermedia* and *C. subternata*, were not

detected in either the DAE or DME of these species in the present study. This variability was also previously shown by van der Merwe⁶¹ who examined the same DAE used in the present study. The variability, both in ER binding ability and content of specific polyphenols does, however, also highlight the importance of testing for the estrogenicity of individual harvestings as no blanket claims can at this stage be made concerning estrogenic activity of individual *Cyclopia* species. Nevertheless, our results do suggest that future in-depth studies should concentrate on investigating the estrogenicity of *C. genistoides*, which was shown to have the most consistent phytoestrogenic activity. It is also important to identify the specific compounds responsible for the phytoestrogenic activity in *Cyclopia* as clearly the polyphenols thought to contribute to estrogenicity are not present in sufficient quantities to explain the binding found with the extracts. In addition, it may, at this stage within the industry, be more worthwhile to concentrate on producing a nutraceutical product with enhanced estrogenic activity from *Cyclopia* harvestings shown to have initial high levels of estrogenicity than to endorse blanket statements concerning estrogenic activity in honeybush tea. This also highlights the necessity of a rapid screening method to ensure that the plant material used for preparation of extracts displays the required activity.



2.6. Acknowledgements

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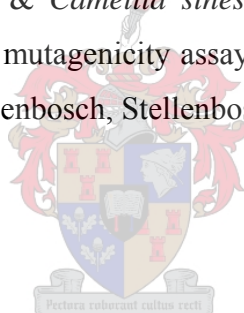
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CHAPTER 3

Evaluation of the phytoestrogenic activity of *Cyclopia genistoides* extracts and relevant polyphenols

3.1. Abstract

Cyclopia genistoides, used to prepare honeybush tea, had previously been identified to bind to the ER subtypes, however, no further information concerning its estrogenic activity is available. Unfermented *C. genistoides* methanol extracts and selected polyphenols were thus evaluated for phytoestrogenic activity by comparing (i) potency (IC_{50}) and binding affinity (K_i) in whole cell competitive binding assays to both hER α and hER β , (ii) potency (EC_{50}) and efficacy (fold induction) in ERE containing promoter reporter studies and proliferation assays in MCF-7-BUS and MDA-MB-231 cells, and (iii) displacement of 3H -E $_2$ from human SHBG. One harvesting of *C. genistoides* (P104) and all polyphenols tested, except mangiferin, bound to both ER subtypes. P104, as well as formononetin and naringenin had EC_{50} values for ER α , but not for ER β , similar to that of the soy isoflavone, genistein, although significantly lower than E $_2$. All the polyphenols that bound, except formononetin, had a higher K_i for ER β . In contrast, P104 and E $_2$ displayed a higher K_i for ER α . All the *C. genistoides* extracts could transactivate ERE containing promoter reporters with potencies and efficacies similar to that of genistein and E $_2$, while in the MCF-7-BUS proliferation assay all extracts, except P122, had potencies and efficacies similar to that of genistein, although the potencies were significantly lower than that of E $_2$. In contrast the potencies, but not efficacies, of the polyphenols luteolin, formononetin and naringenin were significantly lower than that of E $_2$ in the ERE containing promoter reporter studies and in the MCF-7-BUS proliferation assays. Luteolin and naringenin similarly had significantly lower potencies than genistein in the ERE containing promoter reporter studies, but not in the MCF-7-BUS proliferation assays. Proliferation of MCF-7-BUS cells induced by *C. genistoides* extracts and polyphenols was ER-dependent as the ER antagonist, ICI 182,780, reversed proliferation. Physiologically more relevant, *C. genistoides* extracts antagonised E $_2$ induced MCF-7-BUS cell proliferation. Furthermore, *C. genistoides* extracts, with the exception of P122, were able to induce cell proliferation of the estrogen insensitive MDA-MB-231 breast cancer cell line, suggesting that the extracts used in the present study are able to induce ER-dependent and ER-

independent cell proliferation. Binding to SHBG by *C. genistoides* extracts was also demonstrated. The various *in vitro* assays used in the present study to evaluate the estrogenic potential of unfermented *C. genistoides* methanol extracts and *Cyclopia* polyphenols, clearly showed that these extracts and compounds display phytoestrogenic activity.

3.2. Introduction

Phytoestrogens are non-steroidal plant polyphenolic compounds able to mediate weak estrogenic or anti-estrogenic activity.^{1;2} Most research investigating phytoestrogens has concentrated on soybean and the isoflavone, genistein, a well-documented phytoestrogen abundantly present in soy.³ Epidemiological studies suggest that an Asian diet rich in soy is protective against hormone-induced cancers such as breast and prostate cancer, while neonatal administration of genistein protects rats against chemically induced tumours.^{4;5;6;7;8} In addition, phytoestrogens, such as those found in the Asian diet, are thought to be useful for the treatment of menopausal symptoms and to protect postmenopausal women against cardiovascular disease and osteoporosis, without the risks associated with traditional hormone replacement therapy (HRT).^{9;10;11;12;13;14;15} However, some studies have failed to show significant alleviation of menopausal symptoms, such as hot flashes, while other studies, though showing some efficacy, suggest that phytoestrogen treatment is not as effective as traditional HRT.^{16;17;18} Recently, the safety of long term use of traditional HRT has been questioned by several studies.^{19;20;21} This and the general increase in popularity of natural medicine have lend impetus to the search for and investigation into alternative treatments involving menopausal symptoms.²²

The biological responses to estrogen are mediated mainly *via* the estrogen receptor (ER) subtypes, ER α and ER β .²³ The ERs are ligand-activated transcription factors and members of the nuclear receptor superfamily, more specifically the steroid hormone receptor family.^{24;25} On activation they dissociate from heat shock proteins and undergo a conformational change, which allows them to dimerize and bind to specific DNA sites termed estrogen response elements (EREs) situated in the promoter region of estrogen responsive genes thereby activating or inhibiting transcription.²³ Phytoestrogens are able to compete with 17- β -estradiol (E₂) for binding to the ER subtypes and are able to act as either agonist or antagonist when bound to the ERs.^{26;27} Phytoestrogens generally bind to the ER subtypes with a much lower affinity than E₂ and display, unlike E₂, a higher affinity for ER β than ER α .^{26;27} Phytoestrogens have been shown to induce

transactivation *via* both ER subtypes²⁸, with an increased transcriptional response through ER β . They are, however, less potent than E₂ *via* both ER subtypes.^{29;28}

Estrogens are responsible for a wide variety of physiological responses in both females and males, however, most notably are the effect on cell proliferation and cell differentiation of a number of tissues.³⁰ Hyper-proliferation can cause or enhance the spread of cancer.³¹ The ER β subtype is believed to be a negative modulator of ER α mediated activity as it has been demonstrated to inhibit transactivation and cell proliferation when co-expressed with ER α .^{32;33;34;35} ER β is thus believed to be the natural cellular protective mechanism against excessive cell proliferation mediated by ER α , and numerous studies concentrate on compounds, such as phytoestrogens, which are able to distinguish between the two ER subtypes with preferential binding to ER β .³⁴

Estrogens circulating in the blood are transported primarily bound to serum albumin or the glycoprotein, sex hormone-binding globulin (SHBG).³⁶ Only unbound estrogens are able to diffuse across the cell membrane and mediate an estrogenic response.^{37;38} It has been suggested that phytoestrogens may alter the concentration of unbound or biologically active endogenous estrogens, by either binding to SHBG and displacing bound estrogens thus increasing the unbound estrogen concentrations, but also increasing the metabolic clearance rate of estrogens or by stimulating SHBG synthesis by the liver, thereby reducing the unbound endogenous estrogen concentrations.³⁹ It is thus clear that phytoestrogens not only have a direct effect on estrogen signalling through binding to the ER subtypes, but also an indirect effect through altering the concentrations of biologically active estrogens.

Cyclopia genistoides, a woody fynbos shrub, together with *C. subternata*, *C. intermedia* and to a lesser extent *C. sessiliflora*, are commercially available as the fragrant caffeine-free honeybush tea.⁴⁰ Honeybush tea has already been identified as having both antioxidant and antimutagenic activity, which adds value to this herbal infusion.^{41;42} The presence of the known phytoestrogens, formononetin, eriodictyol and naringenin, in *C. intermedia*⁴³ and luteolin in both *C. intermedia* and *C. subternata*^{43,44} plus anecdotal evidence (Personal communication to Dr. E. Joubert by J. Nortje, 1996) that honeybush tea helps alleviate menopausal symptoms led to the investigation of putative phytoestrogenic activity in *Cyclopia spp.* A previous study, which screened different extracts from the four commercially available species for estrogenic activity through binding to the ER subtypes, identified methanol extracts from *C. genistoides* as

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

Table 1A: Details of *C. genistoides* plant material harvested and used for the preparation of unfermented *C. genistoides* methanol extracts.

Species	Harvesting ^a	Area harvested	Date of harvesting
<i>C. genistoides</i> (West Coast type)	Gen P104	Koksrivier, Pearly Beach	15 March 2001
	Gen P105	Koksrivier, Pearly Beach	28 March 2001
	Gen P122	Koksrivier, Pearly Beach	31 March 2003

^a The abbreviations used for the harvestings are also used for the dry methanol extracts (DME) prepared from these harvestings. Although all harvestings were done on the same plantation they were done at different times. Two extracts were prepared of each harvesting.

Table 1B: Extract yield^a and total polyphenol (TPP) content^b of DME from unfermented *C. genistoides*.

Harvesting	Extract yield (%)	TPP content (%)
Gen O ^c P104	13.35	22.31
Gen N ^d P104	16.93	23.53
Gen O P105	13.41	21.99
Gen N P105	16.28	23.89
Gen O P122	18.94	25.02
Gen N P122	16.43	24.87

^aYield = g freeze-dried extract per 100 g dried pulverized plant material

^bTPP content = g gallic acid equivalents per 100 g of freeze-dried extract

^cFirst methanol extract of the same harvesting

^dSecond methanol extract of the same harvesting (prepared at a later stage).

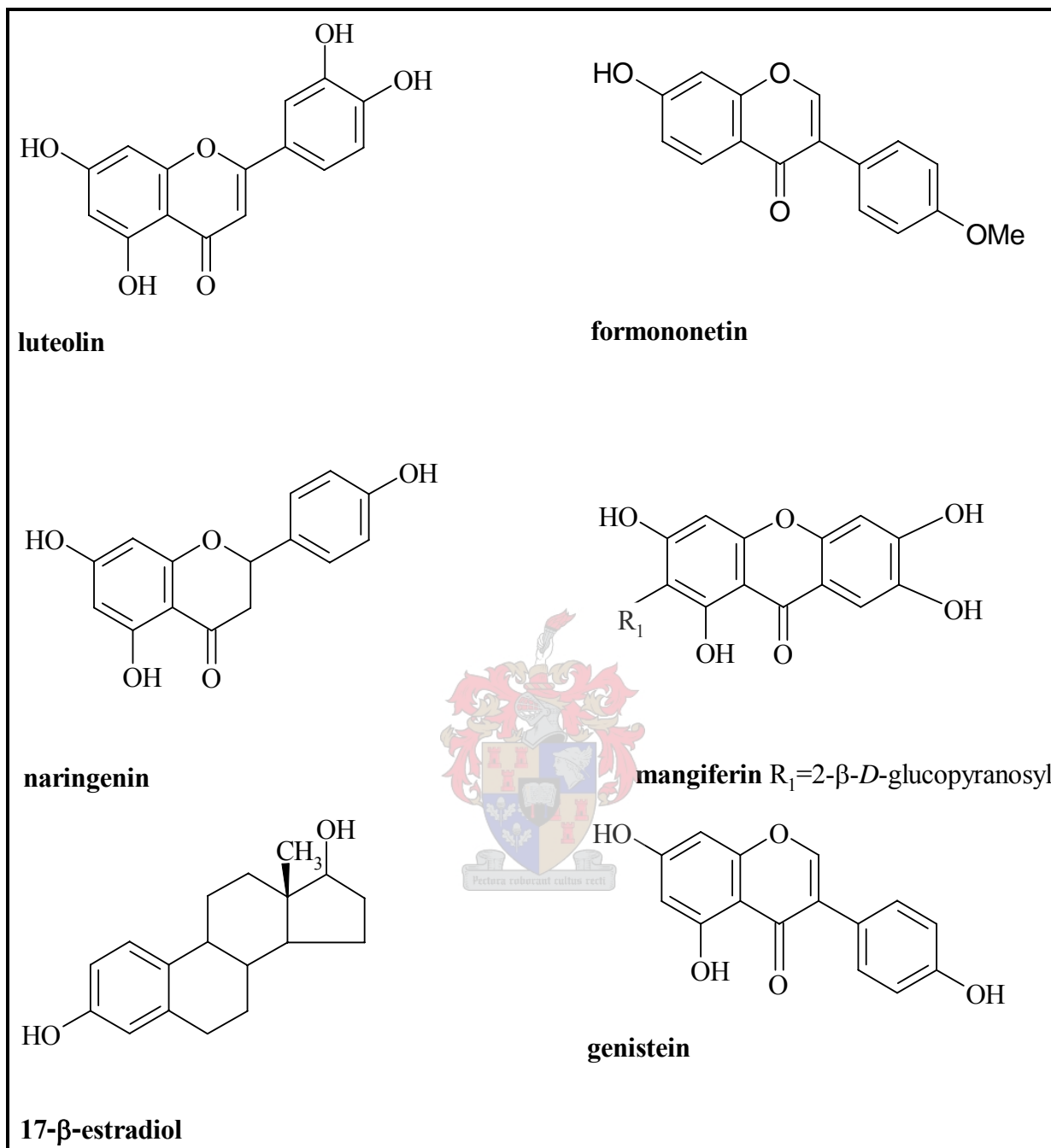


Figure 1: Chemical structures of the plant polyphenols investigated together with that of E₂.

3.3. Materials and methods

Test compounds used

Test compounds used in this study included 17- β -estradiol (1, 3, 5(10)-estratriene), genistein (4', 5, 7-trihydroxyisoflavone), mangiferin (2- β -D-glucopyranosyl-1,3,6,7-tetrahydroxy-9H-xanthene-9-one) and naringenin (5,7,4'-trihydroxyflavanone) that were purchased from Sigma-Aldrich, South Africa. Luteolin (5,7,3',4'-tetrahydroxyflavone) and formononetin (7-hydroxy-4'-methoxyisoflavone) were purchased from Extrasynthese, France. Radiolabelled ligand, ^3H -17- β -estradiol (2,4,6,7- ^3H estradiol, specific activity 87.0 Ci/mmol, counting efficiency 46% in our system) was purchased from Amersham, South Africa.

Plant material

Cyclopia genistoides plants were chosen randomly and several bushes were harvested on each occasion. The harvested plant material (Table 1A) was dried according the standard procedure for unfermented tea,⁴⁵ except that the plant material, comprising stems and leaves, was dried whole at 40°C, where after it was pulverized with a Retsch rotary mill (1 mm sieve) and stored in plastic containers at room temperature.

Dried methanol extract (DME) preparation

Two methanol extracts of unfermented *C. genistoides* were prepared from each of three independent harvestings. The extraction was repeated to compare different methanol extractions of the same plant material (Table 1B). Briefly, dried pulverized unfermented plant material (25 g) was placed in a 250 ml Erlenmeyer flask with 50 ml dichloromethane (UNIV AR, Merck, South Africa). The plant material was allowed to stir continually for 20 hours at room temperature after which it was recovered by filtering through Whatman No. 4 filter paper and the filtrate was discarded. Extraction with dichloromethane was repeated three times after which the plant material was air-dried in a fume cabinet. This preliminary extraction procedure was to remove chlorophyll. Dichloromethane extraction was followed by addition of 50 ml methanol (Pro-analysi GR, Merck, South Africa) to the air-dried plant material and again this was allowed to stir continually for 20 hours. Methanol extraction was performed twice. The methanol extracts

were pooled, a small volume of deionised water was added, and evaporated under vacuum on a rotary evaporator before freeze-drying. Freeze-dried methanol extracts were ground with a mortar and pestle in a darkened room, until a fine homogenous powder was obtained. The methanol extracts were stored in screw cap glass vials, covered with aluminium foil, and placed in vacuum-sealed desiccators in the dark at room temperature. All glassware used during the extraction process was covered with aluminium foil to protect the extracts from light at all times. Yield of extract was determined by calculating the mass (g) of dry methanol extract (DME) per 100 g of initial processed plant material (Table 1B).

Cell culture conditions

African green monkey kidney fibroblast (COS-1) cells (ATCC, United States of America) and estrogen insensitive MDA-MB-231 cells⁴⁶ (a kind gift from G. Haegemann, University of Gent, Belgium) were cultured in 175 cm² culture flasks (Greiner Bio-One International, Austria) in Dulbecco's modified Eagle's medium (DMEM) from Sigma-Aldrich, South Africa, supplemented with 10% (v/v) fetal calf serum (FCS) from Highveld Biologicals, South Africa, and a penicillin (100 IU/ml) and streptomycin (100 µl/ml) mixture (penicillin-streptomycin) from Gibco-BRL Life Technologies, United Kingdom. The ER α and ER β positive MCF-7-BUS cells⁴⁶, (a kind gift from A. Soto, Tufts University, Boston, Massachusetts, United States of America) were maintained in DMEM supplemented with 5% (v/v) heat inactivated FCS, but without antibiotics in 175 cm² culture flasks (Nunc, Denmark). All cells were maintained in a humidified cell incubator set at 97% relative humidity and 5% CO₂ at 37°C.

Transient transfections and whole cell binding assays in COS-1 cells

COS-1 cells were plated at a density of 2×10^6 cells per 10 cm tissue culture dish (Nunc, Denmark) and incubated for 24 hours before transfecting to allow full adherence.

Twenty-four hours after plating COS-1 cells were transiently transfected with expression vectors for the ER subtypes, pcDNA3-hER α (D. Harnish, Womens's Health Research Institute, Wyeth-Ayerst Research, Radnor, United States of America) or pSG5-hER β (F. Gannon, European Molecular Biology Laboratory, Heidelberg, Germany) and a filler vector, pGL2-basic

(Promega Corp., United States of America). Two different transfections methods were used to transfect the ER subtypes. The Fugene6™ (Roche Applied Science, South Africa) transfection reagent was used for the hER α transfections and the DEAE-Dextran transfection method for hER β transfections.

The total DNA transfected for both transfection protocols was 6 μ g/10 cm dish that consisted of 0.72 μ g receptor and 5.28 μ g empty vector.

The Fugene6™ transfection protocol, used for ER α , was per manufacturer's instructions. Briefly, Fugene6™ reagent (12 μ l) was allowed to react with 6 μ g DNA in the presence of 188 μ l DMEM for 30 minutes, which was then added to the cells and incubated for 24 hours.

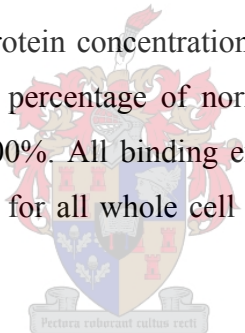
The DEAE-Dextran transfection method was used for hER β transfections: The transfection medium with a final volume of 5 ml per 10 cm dish consisted of DMEM, pre-heated to 37°C, 0.1 mM chloroquine (Sigma-Aldrich, South Africa) (stock solution 100 mM), 6 μ g DNA and finally 0.1 mg/ml DEAE-Dextran (Sigma-Aldrich, South Africa) solution (stock solution 10 mg/ml). The transfection medium was added to the 10 cm dish and cells were incubated for 1 hour at 37°C in a humidified incubator (97% relative humidity) with 5% CO₂. After removal of the transfection medium the cells were shocked with 10 ml pre-heated 10% DMSO-PBS for not less than two minutes, but not more than 10 minutes. Finally, transiently transfected cells were incubated at 37°C overnight in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin mixture.

The following day the transfected COS-1 cells were trypsinized, pooled and counted. The cells were replated into 24-well tissue culture plates (Nunc, Denmark) at a density of 5 x10⁴ cells/well and incubated for 24 hours. The next day the cells were washed three times with 500 μ l PBS/well (pre-heated at 37°C) to remove any endogenous estrogen-like compounds present in the culture medium.

This was followed by a two-hour incubation of the transfected cells with 1 nM radiolabelled estradiol (³H-E₂) and various concentrations of unlabeled competitors, i.e. extracts and polyphenols, in DMEM without phenol red and FCS. All unlabeled competitors were dissolved in DMSO (Merck, South Africa) and added to the culture medium that the final concentrations of DMSO did not exceed 0.1% (v/v). All assays included a total binding point, which was in the presence of 0.1% DMSO only, and E₂ and genistein as positive controls.

After the three-hour incubation period the cells were immediately placed on ice and further work was done at 4°C to ensure that the ligand remained bound to the receptor. Cells were washed three times with 1 ml 0.2% bovine serum albumin-PBS (bovine serum albumin from Roche Applied Science, South Africa) with an interval of 15 minutes between washes to remove free ligand. Cells were then lysed with 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) per well. For effective lysis, plates were placed on a shaker for approximately 15 minutes and thereafter allowed to freeze at -20°C.

On thawing of samples, 5 µl lysate was used for protein determination. Protein concentrations were determined using the Bradford protein assay method.⁴⁷ Another 50 µl of lysis buffer was added to the remaining lysate in the wells and this was transferred to scintillation vials to which 3 ml scintillation fluid (Quickszint FLOW 2; Zinsser Analytic, United Kingdom) was added. Radioactivity of the assay samples was determined by recording the counts per minute (CPM) for each sample using a Beta-scintillation counter (Beckman scintillation counter LS 3801, Beckman, South Africa). The protein concentrations were used to normalise radioactivity readings and results are expressed as percentage of normalised control with total binding (in presence of 0.1% DMSO) taken as 100%. All binding experiments also included a control for ligand depletion. The ligand depletion for all whole cell ER binding experiments was less than 10%.



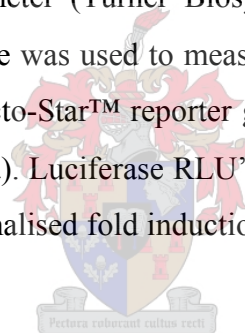
Transient transfections and ERE-containing promoter reporter assays in COS-1 cells

For the ERE-containing promoter reporter assays only hERβ was investigated. COS-1 cells were plated at a density of 2 x10⁶ cells/10 cm dish and were incubated for 24 hours allowing the cells to adhere. The cells were transfected the following day using the Fugene6™ reagent. The total DNA transfected per 10 cm dish was 9.6 µg consisting of 0.8 µg hERβ expression plasmid (pSG5-hERβ), 8 µg ERE-containing promoter reporter construct (ERE.vit2.luc, a kind gift from K. Korach, National Institute of Environmental Health Science, United States of America) and 0.8 µg pCMV-β-galactosidase (Stratagene, United States of America) for normalization of transfection efficiency. The Fugene6™ protocol was as per manufacturer's instructions. Briefly, 19.2 µl of Fugene6™ reagent was allowed to react with 9.6 µg DNA in the

presence of 300.8 μ l DMEM for 30 minutes which was then added to the cells and incubated for 24 hours.

The following day cells were trypsinized, pooled and replated at a density of 5×10^4 cells per well into 24-well tissue culture plates and incubated for 24 hours. The cells were then induced with various concentrations of polyphenol compounds or methanol extracts (dissolved in DMSO) such that the final concentration of DMSO did not exceed 0.1% (v/v). All assays included a negative control, which consisted of 0.1% (v/v) DMSO only, and E₂ and genistein as positive controls. After the 24-hour induction period the medium was aspirated, 50 μ l of lysis buffer (Tropic Inc, United States of America) added and cells frozen at -20°C overnight.

Luciferase assay reagent (Promega Corp., United States of America) was used to quantify luciferase activity in accordance with the manufacturer's instructions. Briefly, 10 μ l cell lysate was allowed to react with 50 μ l luciferase assay reagent. The relative light units (RLU's) were measured using the Veritas luminometer (Turner Biosystems, United States of America). A further 5 μ l cell lysate for each sample was used to measure β -galactosidase activity with the β -galactosidase chemiluminescent Galacto-Star™ reporter gene assay system for mammalian cells (Tropix Inc., United States of America). Luciferase RLU's were normalized with β -galactosidase readings and results expressed as normalised fold induction with negative controls (0.1% DMSO) taken as 1.



MTT cell proliferation assay

A solution of 5 mg/ml MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, South Africa) prepared in PBS was filter sterilized and kept at 4°C in the dark. Metabolically active cells reduce the soluble yellow MTT tetrazolium salt to an insoluble purple formazan precipitate that can be solubilized and quantified spectrophotometrically.

The breast cancer cells, MCF-7 BUS and MDA-MB-231, were plated at a density of 2 500 cells/well in 96 well plates and incubated for 24 hours allowing the cells to adhere. The cells were then washed with 200 μ l PBS, pre-warmed to 37°C, followed by addition of DMEM pre-warmed to 37°C without phenol red, but supplemented with 5% charcoal stripped FCS (Highveld Biologicals, South Africa) and 1% penicillin-streptomycin mixture. The cells were then incubated

72 hours at 37°C and 97% relative humidity in 5% CO₂. This allowed steroid starving of the cells as both FCS and DMEM with phenol red is estrogenic and might influence the assay. On day five the medium was aspirated and cells were induced with increasing concentrations of test compounds or methanol extracts, dissolved in DMSO and prepared in DMEM without phenol red, but supplemented with 5% charcoal stripped FCS and 1% penicillin-streptomycin mixture, such that the final concentration of DMSO did not exceed 0.1% (v/v). Cells were then incubated for 48 hours where after the colorimetric MTT assay was performed. All assays included a negative control, which consisted of 0.1% (v/v) DMSO only, and E₂ and genistein as positive controls.

Co-treatment by both E₂ (10⁻⁹ M) and the polyphenols (10⁻⁵ M except for genistein which was tested at 10⁻⁷ M) or methanol extracts (9.8 µg/ml) was investigated. In addition, induction with test compounds and methanol extracts was investigated in the presence of 1nM ER antagonist, ICI 182,780 (Tocris, United Kingdom).

The MTT assay entails that five hours before the end of the incubation period the assay medium is changed to unsupplemented DMEM without phenol red where after 20 µl of MTT solution (5 mg/ml) is added to each well. Cells were incubated for five hours at 37°C, the medium was then removed and 200 µl solubilization solution (DMSO) was added to each well. The DMSO was pipetted up and down in the well to dissolve crystals until a uniform purple colour had formed. The plate was then placed in a 37°C incubator for 5 min and absorbance read at 540 nm in a micotiter plate reader (Titertek™ Multiskan Plus, Titertek Instruments Inc., United States of America). Results are expressed as fold induction with negative controls (0.1% DMSO) taken as 1.

Competitive SHBG binding assay

Displacement of 20 nM ³H-E₂ by test compounds and methanol extracts from SHBG was determined by the competitive SHBG binding assay as adapted from the method used by Hammond and Lähteenmäki.⁴⁸ Pooled human pregnancy serum with a SHBG concentration of 408.6 nM was diluted (1:100) with dextran coated charcoal (DCC). Briefly, 20 µl pregnancy serum was added to 2 ml (DCC) slurry together with 20 pM cortisol and incubated through shaking at room temperature for 30 min to remove any endogenous steroids. Following

centrifugation (Sigma 113 centrifuge, Sigma-Aldrich, South Africa) at 5000 g at room temperature, the supernatant was collected and 100 μ l diluted serum was added to 100 μ l each of unlabelled E₂, polyphenols, methanol extracts and DMSO vehicle only (negative control) as competitors. This was followed by the addition of 100 μ l PBS containing 60 nM ³H-E₂, which was allowed to incubate for one hour at room temperature followed by 15 minutes incubation in an ice-water bath kept at 4°C. The unbound ³H-E₂ was then removed by incubating with 750 μ l ice cold DCC slurry for 10 minutes followed by centrifugation at 3000 g for 3 minutes at 4°C. The supernatant was quickly decanted, and a constant volume (750 μ l) added to scintillation vials containing 3 ml scintillation fluid (Quickszint FLOW 2; Zinsser Analytic, United Kingdom). Radioactivity (CPM) was read on a Scintillation counter (Beckman scintillation counter LS 3801, Beckman, South Africa).

The DCC slurry was prepared as follows: 1.25 g of activated charcoal Norit CA1 (Sigma-Aldrich, South Africa) was added to 0.125 g of dextran with average molecular weight of 73 090 (Sigma-Aldrich, South Africa) and dissolved in 500 ml of 0.02% gelatin-PBS mixture to prepare the DCC slurry. This mixture was stirred for eight hours at room temperature followed by further stirring at 4°C overnight, where after the DCC was kept at 4°C until used in the assay.

All polyphenols and E₂ were investigated at 10⁻⁵ M and the methanol extracts were investigated at 9.8 μ g/ml. The competitors were dissolved in DMSO and prepared in PBS so that the DMSO concentration did not exceed 0.1% (v/v). Results are expressed as the percentage 20 nM ³H-E₂ displaced from SHBG. The total bound, i.e. in the presence of vehicle (DMSO) only, represents 0% ³H-E₂ displaced from the SHBG.

Data manipulation and statistical analysis

The GraphPad Prism® version 4.00 for Windows (GraphPad Software, San Diego California USA) was used for graphical representations and statistical analysis. One-way ANOVA and Dunnett's multiple comparisons' test as post-test were used for statistical analysis. P-values are represented as follows: P < 0.05 by *, P < 0.01 by **, and P < 0.001 by ***. Non-linear regression and one site competition curve fitting were used to graph the data from the whole cell binding assays and to determine EC₅₀ values. The relative binding affinity (RBA) is expressed relative to that of E₂ (100%) and was calculated as follows: 100 x EC₅₀ (E₂) / EC₅₀ (test

compound). The K_i values were determined from the equation by Cheng and Prusoff.⁴⁹ Non-linear regression and sigmoidal dose response curve fitting were used to graph the data from the ERE-containing promoter reporter and proliferation experiments and to determine fold induction and EC_{50} . Pearson correlations (two-tailed) were done and are expressed as Pearson correlation coefficient (r). For all experiments, unless otherwise indicated, the error bars represent the SEM of three independent experiments, where each point was determined in triplicate.

3.4. Results

3.4.1. Binding to ER subtypes

Whole cell binding assays were used to determine the binding potency (IC_{50}), relative binding affinity (RBA) and affinity (K_i) of the polyphenols, luteolin, formononetin, naringenin and mangiferin (Figure 2 and Table 2) and the IC_{50} and RBA of the duplicate methanol extracts of three independent randomly chosen harvestings of unfermented *C. genistoides* to the ER subtypes, $ER\alpha$ and $ER\beta$ (Figure 3 and Table 3). COS-1 cells, which do not endogenously express the ER subtypes⁵⁰, were transiently transfected with either $hER\alpha$ or $hER\beta$ expression plasmids thereby allowing for discrimination between binding affinities to the two ER subtypes.

The binding assays were done both to compare and evaluate the potency (IC_{50}) of compounds and extracts with that of the two standards, E_2 , the endogenous hormone, and genistein, an example of a well-known phytoestrogen, and to evaluate the binding affinities (K_i) for the two ER subtypes. Both the IC_{50} value, a measure of the competitor's potency in competing for the receptor with $^3H-E_2$, and the RBA, calculated from the IC_{50} values determined such that the affinity of E_2 for both ER subtypes is equated to 100%, were determined. This manner of expressing binding affinities is useful in comparing binding potencies relative to a standard compound, such as E_2 within the same assay. The β/α ratio of the RBAs as a measure of preference for the $ER\beta$ relative to E_2 was also calculated. Because IC_{50} values are determined by three factors, i.e. the affinity of the competitor for the receptor, the concentration of the radioligand, and the affinity of the radioligand for the receptor, it is not a true measure of the ligand's affinity and thus the K_i values or binding equilibrium dissociation constants of the various polyphenols were determined from the K_d values of E_2 for the ER subtypes. The K_i value is an absolute binding affinity value that allows for comparison of binding affinities of ligands to

different ER subtypes. The β/α ratio of the K_i values was also calculated as a measure of the absolute, and not relative to E_2 , preference for the ER β .

3.4.1.1. Competitive binding of various polyphenols and E_2 to the ER subtypes

The polyphenols, luteolin, formononetin, naringenin and mangiferin, together with the known phytoestrogen, genistein, and the natural ligand, E_2 , were investigated for binding to the ER subtypes (Figure 2). All polyphenols were able to bind to both ER subtypes, except for the xanthone, mangiferin.

The natural ligand, E_2 was able to fully displace $^3\text{H-E}_2$ from both hER α and hER β with K_i values of 0.37 nM (CV=5.44%) and 1.17 nM (CV=5.01%), respectively. This is comparable to most K_i values in the literature that are in the nM range^{26;28}, although slightly higher than values obtained from Muthyala⁵¹, which were 0.2 nM and 0.5 nM, respectively, for ER α and ER β . The slightly higher K_d values obtained in the present study could be due to the source of ER expression plasmids for ER subtype used in the present study versus purified hER subtypes used in other studies, or the assay conditions themselves, i.e. whole cell binding assay in the present study versus *in vitro* binding studies in other studies.^{26;51} The present study, like previous studies, also showed that E_2 has a higher binding affinity for hER α than hER β .²⁸

Genistein, luteolin, formononetin and naringenin were unable to fully displace $^3\text{H-E}_2$ from hER α . However, IC_{50} , RBA and K_i values (Table 2) could be determined for all polyphenols investigated, except for mangiferin that did not displace $^3\text{H-E}_2$ from hER α at the concentrations tested. The order of potency for hER α (IC_{50} values) was: E_2 (1.39 nM) \gg formononetin (0.15 μM) $>$ genistein (0.19 μM) $>$ naringenin (1.44 μM) $>$ luteolin (52.5 μM). All IC_{50} values determined for hER α binding were significantly ($P < 0.05$) different from those of E_2 . However, the IC_{50} value for genistein was not significantly ($P > 0.05$) different from that of formononetin and naringenin. This contrasts with a previous study by Branham *et al.*⁵² that defined formononetin as a slight binder as they were unable to determine an IC_{50} value for binding to the rER α .⁵² Luteolin was only able to displace $^3\text{H-E}_2$ at 10^{-5} M and due to solubility could not be tested at a higher concentration. Generally, all polyphenols bound to ER α displayed very weak

binding as compared to E₂ with RBA values ranging from 0.93% for formononetin to 0.003% for luteolin.

In contrast to results with the hER α , genistein was able to fully displace ³H-E₂ from the hER β . Similarly to results with the hER α the polyphenols luteolin, formononetin and naringenin were, however, also not able to fully displace ³H-E₂ from hER β and mangiferin was unable to compete with ³H-E₂ for binding to hER β (Figure 2). Again the IC₅₀, RBA and K_i values were determined (Table 2) with the order of potency (IC₅₀) for hER β being: E₂ (1.97 nM) > genistein (1.99 nM) >> luteolin (0.49 μ M) > formononetin (0.55 μ M) > naringenin (0.56 μ M). All polyphenol IC₅₀ values for binding to hER β , except for genistein, were significantly (P < 0.01) different from E₂. Similarly, all polyphenols were significantly (P < 0.01) less potent than genistein. The IC₅₀ values of 0.45 and 0.56 μ M determined for luteolin and naringenin, respectively, are in a similar range to those previously determined by others.^{26;53} Similarly to results with hER α binding, all polyphenols bound to ER β , but except for genistein, displayed weak binding as compared to E₂ with RBA values ranging from 0.89 for naringenin to 0.26 for luteolin. In the present study, genistein binds to hER β (K_i of 1.01 nM) with almost the same affinity as E₂ (K_i of 1.17 nM), similarly to what was shown by Morito *et al.*²⁷

All the polyphenols that bound, except formononetin, had a higher binding affinity for the hER β , in contrast to E₂ that had a slightly higher affinity for ER α . Genistein, especially, has a very high binding affinity (K_i value = 1.01 nM) for hER β , and showed a strong preference for this subtype (K_i β/α ratio = 42.7), as also previously shown by others.^{26;51;54;55} Luteolin, similarly, greatly preferred binding to hER β (K_i β/α ratio = 31.3), despite having a relatively low binding affinity (K_i value = 0.39 μ M) for the hER β . Naringenin, similarly to genistein and luteolin, displayed a higher binding affinity (K_i) for hER β than hER α (K_i β/α ratio = 2.5) albeit to a lesser extent than that shown by genistein (K_i β/α ratio = 42.7) and luteolin (K_i β/α ratio = 31.3). Formononetin, similarly to E₂, has a slight binding preference (K_i β/α ratio = 0.25) for hER α . This, however, is contrary to what others have shown^{54;55} and differs to what is found for most phytoestrogens.^{26;28;51}

3.4.1.2. Competitive binding of unfermented *C. genistoides* methanol extracts to the ER subtypes

Together with polyphenols present in *Cyclopia* unfermented *C. genistoides* methanol extracts were also investigated for binding to the ER subtypes (Figure 3). Binding by the natural ligand, E₂, and the known phytoestrogen, genistein, was included for comparison. The methanol extracts, even though from the same species, portrayed large variations in binding to the ER subtypes with only the extracts from the P104 harvesting (first and second extracts) able to significantly compete with ³H-E₂ for binding to the ER subtypes (Figure 3). The IC₅₀ values of the new methanol extract from the P104 harvesting (N P104) for the hER α and hER β were 2.08 x 10⁻⁴ mg/ml and 1.27 x 10⁻¹ mg/ml, respectively. The first (older) methanol extract from the P104 harvesting (O P104), although also able to compete with ³H-E₂ for binding to both ER subtypes, displayed a lower potency than N P104 with IC₅₀ values of 5.89 x 10⁻⁴ mg/ml and 2.29 x 10⁻¹ mg/ml for the hER α and hER β , respectively. The binding potencies measured for hER α for *C. genistoides* extracts, N P104, O P104 and genistein is significantly different (P > 0.01) from E₂ with only O P104 significantly different (P > 0.01) from genistein. For binding to hER β (Table 3), the IC₅₀ values calculated for both E₂ and genistein were significantly (P < 0.01) lower than those of *C. genistoides* extracts, N P104 and O P104. Although N P104 in comparison to O P104, has higher potencies for both ER subtypes they were not significantly (P > 0.05) different from each other (statistical data not shown).

In contrast to most of the polyphenols investigated, formononetin and mangiferin excluded, P104 had a higher RBA and a stronger preference (RBA β/α ratio = 0.003 and 0.006 for N P104 and O P104, respectively) for the ER α subtype. Other plant extracts such as red wine, *Ginkgo biloba*, kudzu root and red clover extracts, have been shown to have binding affinity for both ER subtypes, but with a higher binding affinity for ER β .^{55;56;57;58} It was therefore not expected that the *C. genistoides* methanol extracts would preferentially bind to the ER α .

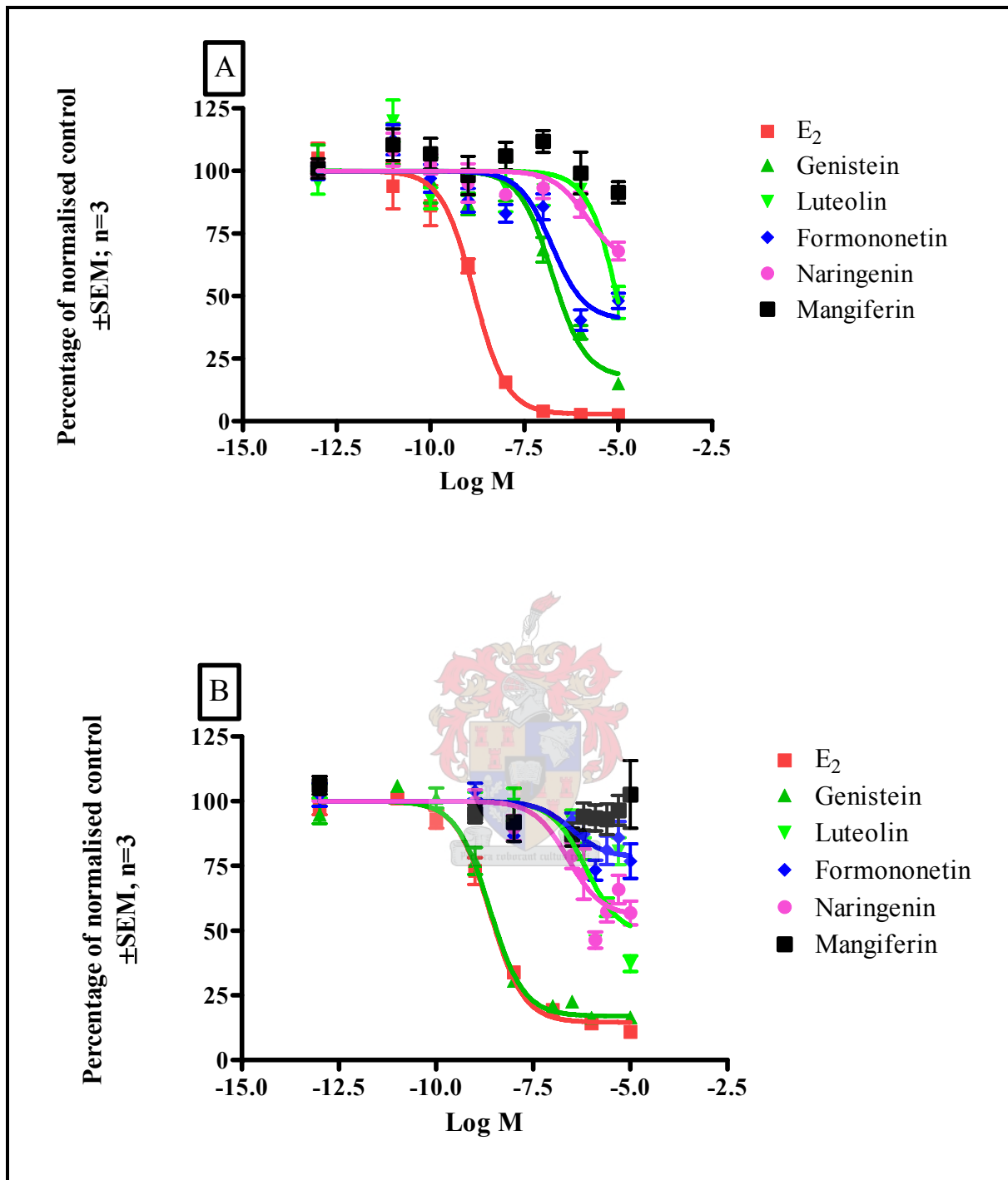


Figure 2: Competitive binding curves of various polyphenols and E₂ with (A) hER α and (B) hER β expressed in COS-1 cells. Transiently transfected COS-1 cells were incubated with 1 nM ³H-E₂ in the absence and presence of increasing concentrations of polyphenols or E₂. Results shown are of at least three independent experiments performed in triplicate.

Table 2: Summary of ER competitive binding results for E₂ and polyphenols.

Test compounds	IC ₅₀ ^a		RBA ^b (%)		β/α of RBA ^c	K _i ^d		β/α of K _i ^c
	hERα	hERβ	hERα	hERβ		hERα	hERβ	
E₂	1.39 nM (2.50) ^{##g}	1.97 nM (3.46)	100	100	1	0.37 nM (5.44)	1.17 nM (5.01)	0.3
Genistein	0.19 μM (3.79) ^{**f}	1.99 nM (3.80)	0.73	98.99	135.6	43.1 nM (8.99)*	1.01 nM (4.98)	42.7
Luteolin	52.5 μM (27.85) ^{** #}	0.49 μM (3.07) ^{** ##}	0.003	0.40	146.67	12.20 μM (25.61) ^{**}	0.39 μM (8.79) ^{**}	31.3
Formononetin	0.15 μM (4.59) ^{**}	0.55 μM (0.45) ^{** ##}	0.93	0.36	0.387	34.51 nM (9.51)*	0.14 μM (7.52) ^{**}	0.25
Naringenin	1.44 μM (8.33) ^{**}	0.56 μM (2.88) ^{** ##}	0.097	0.35	3.61	0.27 μM (0.79) ^{**}	0.11 μM (10.46) ^{**}	2.5
Mangiferin	NB ^e	NB	NB	NB	NB	NB	NB	NB

^aIC₅₀ value is the ligand concentration displacing ³H-E₂ binding by 50% and is derived from non-linear curve fitting, one site competition binding and are given as the mean (CV). The IC₅₀ and CV (coefficient of variation) values are calculated from the log IC₅₀ values from at least three independent experiments.

^bRBA or relative binding affinity is expressed relative to that of E₂ (100%) and was calculated as follows; 100 x EC₅₀ (E₂) / EC₅₀ (test compound).

^cβ/α ratio of RBA or K_i is such that the ratio is > 1 for compounds having a higher affinity for hERβ than hERα, < 1 if compounds have a higher binding affinity for hERα than hERβ and equal to 1 for compounds having a similar affinity for both ER subtypes. The β/α ratio of RBA is calculated by RBA hERβ/RBA hERα and the β/α ratio of K_i is calculated by K_i hERα/ K_i hERβ.

^dK_i values were determined from the K_d of E₂. The K_d values of E₂ for hERα and hERβ were 0.37 nM ± 0.38 and 1.17 nM ± 0.18, respectively

^eNB= non binder; compound was unable to displace ³H-E₂ from the ER.

^fStatistically different from E₂ with * representing P < 0.05, ** representing P < 0.01 and *** representing P < 0.001.

^gStatistically different from Genistein with # representing P < 0.05, ## representing P < 0.01 and ### representing P < 0.001

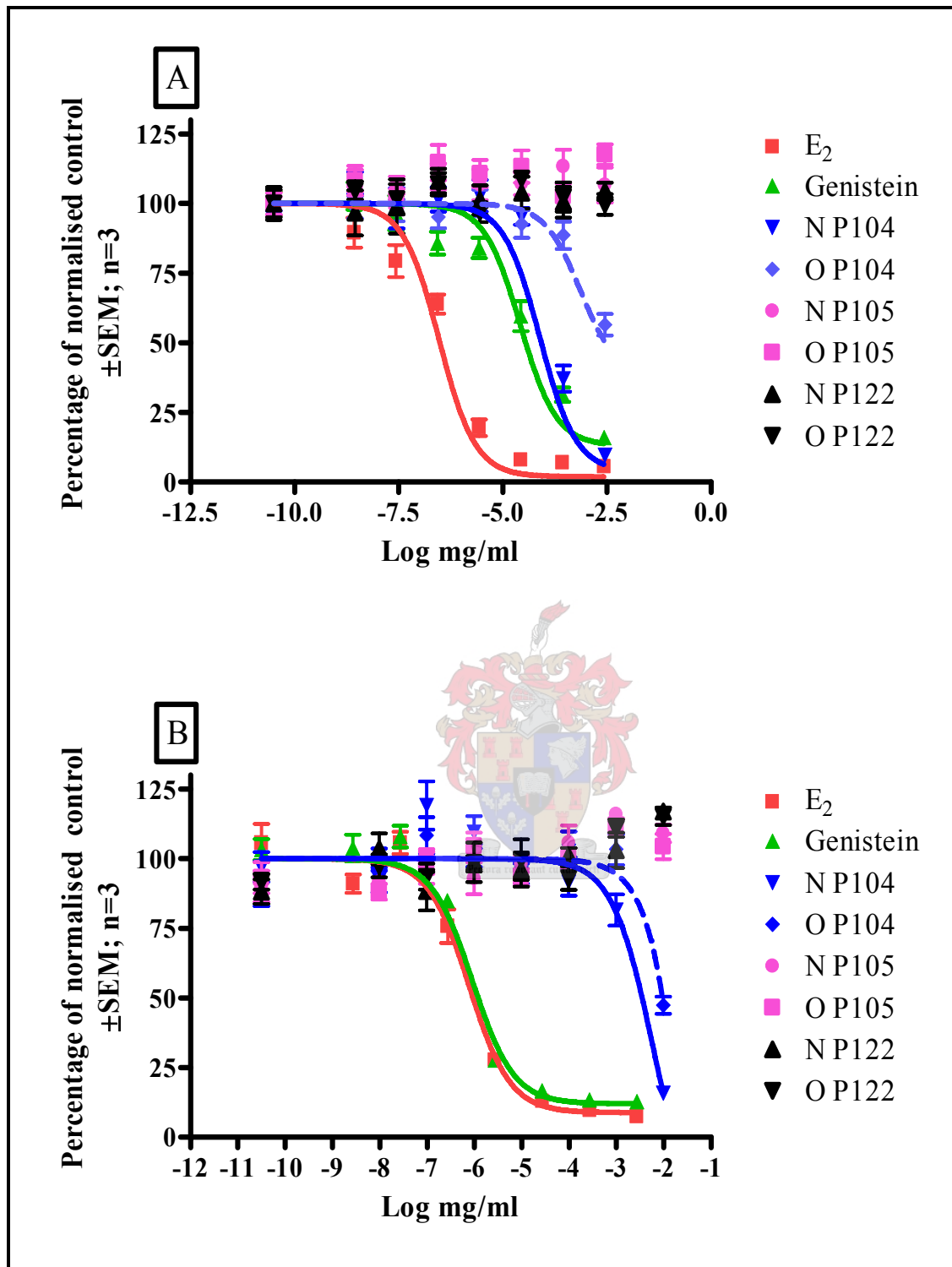


Figure 3: Competitive binding curves of unfermented *C. genistoides* methanol extracts and E₂ with (A) hER α and (B) hER β expressed in COS-1 cells. Transiently transfected COS-1 cells were incubated with 1 nM ³H-E₂ in the absence and presence of increasing concentrations of *C. genistoides* methanol extracts, genistein or E₂. Results shown are of at least three independent experiments performed in triplicate.

Table 3: Summary of ER competitive binding results for E₂, polyphenols and *C. genistoides* DME.

Test compounds and <i>C. genistoides</i> DME	IC ₅₀ (mg/ml) ^a		RBA ^b (%)		β/α of RBA ^c
	hERα	hERβ	hERα	hERβ	
E ₂	3.68 x 10 ⁻⁷ (3.63) ^{###d}	7.29 x 10 ⁻⁷ (4.74)	100	100	1
Genistein	4.23 x 10 ⁻⁵ (37.32)** ^f	9.04 x 10 ⁻⁷ (1.23)	0.73	98.99	136.3
Luteolin	1.50 x 10 ⁻² (4.88)** ^{##}	1.40 x 10 ⁻⁴ (3.07)** ^{##}	0.003	0.40	86.7
Formononetin	4.12 x 10 ⁻⁵ (4.59)**	1.53 x 10 ⁻⁴ (0.45)** ^{##}	0.93	0.36	0.66
Naringenin	3.92 x 10 ⁻⁴ (8.33)**	1.53 x 10 ⁻⁴ (2.88)** ^{##}	0.097	0.35	7.4
Mangiferin	NB ^e	NB	NB	NB	NB
N P104	2.08 x 10 ⁻⁴ (4.88)** ^{##}	1.27 x 10 ⁻¹ (26.28)** ^{##}	0.18	0.0006	0.003
O P104	5.89 x 10 ⁻⁴ (18.07)** ^{##}	2.29 x 10 ⁻¹ (19.24)** ^{##}	0.05	0.0003	0.006
N P105	NB	NB	NB ^f	NB	NB
O P105	NB	NB	NB	NB	NB
N P122	NB	NB	NB	NB	NB
O P122	NB	NB	NB	NB	NB

^aIC₅₀ value is the ligand concentration displacing ³H-E₂ binding by 50% and is derived from non-linear curve fitting one site competition binding and is given as the mean (CV). The IC₅₀ and CV (coefficient of variation) values are calculated from the log IC₅₀ values from at least three independent experiments.

^bRBA or relative binding affinity is expressed relative to that of E₂ (100%) and was calculated as follows; 100 x EC₅₀ (E₂) / EC₅₀ (test compound).

^cβ/α ratio of RBA > 1 for compounds or extracts having a higher affinity for hERβ than hERα, < 1 compounds or extracts have a higher binding affinity for hERα than hERβ and equal to 1 for compounds or extracts having a similar affinity for both ER subtypes. The β/α ratio of RBA is calculated by RBA hERβ/RBA hERα.

^dStatistically different from Genistein with [#] representing P < 0.05, ^{##} representing P < 0.01 and ^{###} representing P < 0.001.

^fStatistically different from E₂ with * representing P < 0.05, ** representing P < 0.01 and *** representing P < 0.001.

^eNB = non-binder polyphenols or extracts were unable to displace ³H-E₂ from ER subtype

3.4.2. Transactivation of an ERE-containing promoter reporter construct *via* the hER β

Binding to the ER subtypes alone cannot determine whether a ligand is an agonist, and thus whether the ER is activated to induce a biological response through binding to the ERE. ERE-containing promoter reporter assays are useful to determine both, (i) whether phytoestrogens are able to transactivate *via* an ERE-containing promoter reporter construct and, (ii) to compare efficacy (fold induction) and potency (EC_{50}) with known estrogens such as E_2 and the phytoestrogen genistein. COS-1 cells were transiently co-transfected with hER β and an ERE-containing promoter reporter construct and treated with increasing concentrations of polyphenols, unfermented *C. genistoides* methanol extracts and E_2 to investigate transactivation.

3.4.2.1. ERE-containing promoter reporter assays for various polyphenols and E_2

All the polyphenols investigated, except mangiferin, were able to induce the ERE-containing promoter reporter gene *via* the hER β (Figure 4). The order of potency of E_2 and the polyphenols was: E_2 (0.51 nM) > genistein (3.92 nM) >> formononetin (0.16 μ M) > naringenin (0.39 μ M) > luteolin (12.33 μ M) (Table 4). The potency of E_2 was significantly ($P < 0.01$) higher than that of the polyphenols, except genistein, while the potency of genistein, however, was significantly different ($P < 0.05$) from that of luteolin and naringenin, but not formononetin.

The transactivational efficacy of the various polyphenols did not differ significantly ($P > 0.05$) from each other or from that of E_2 and genistein (Table 4). The order of efficacy for the various polyphenols and E_2 was as follows: luteolin (3.69 fold) > naringenin (2.99 fold) > genistein (2.76 fold) > E_2 (2.34 fold) > formononetin (2.20 fold).

Although, both E_2 and genistein, in contrast to the *Cyclopia* polyphenols tested, displayed a relatively high potency for both binding and ERE-containing promoter reporter assays *via* hER β , the transactivational efficacy was approximately similar for all polyphenols and E_2 ($P > 0.05$).

3.4.2.2. ERE-containing promoter reporter assay for unfermented *C. genistoides* methanol extracts, genistein and E₂

The unfermented methanol extracts were able to induce the ERE-containing promoter reporter construct *via* the hER β (Figure 5) despite the fact that only the extracts from the P104 harvesting were able to displace ³H-E₂ from ER β (Figure 3). The order of potency (EC₅₀) of E₂, genistein and extracts was as follows: E₂ (2.62 x 10⁻⁷ mg/ml) > genistein (2.12 x 10⁻⁶ mg/ml) > O P122 (2.48 x 10⁻⁶ mg/ml) > N P105 (9.20 x 10⁻⁵ mg/ml) > O P104 (1.18 x 10⁻⁵ mg/ml) > N P104 (1.51 x 10⁻⁵ mg/ml) > O P105 (2.93x 10⁻⁵ mg/ml) > N P122 (6.90 x 10⁻⁵ mg/ml) (Table 5). Potencies of extracts were not significantly (P > 0.05) different from E₂, except for N P104 and O P105, while none of the extracts were significantly different from genistein. The order of efficacy or maximal fold induction was: genistein (2.58 fold) > O P105 (2.53 fold) > N P104 (2.44 fold) > O P104 (2.39 fold) > E₂ (2.22 fold) > O P122 (1.94 fold) > N P122 (1.90 fold) > N P105 (1.63 fold). The efficacy of the extracts was not significantly different (P > 0.05) from genistein and E₂.

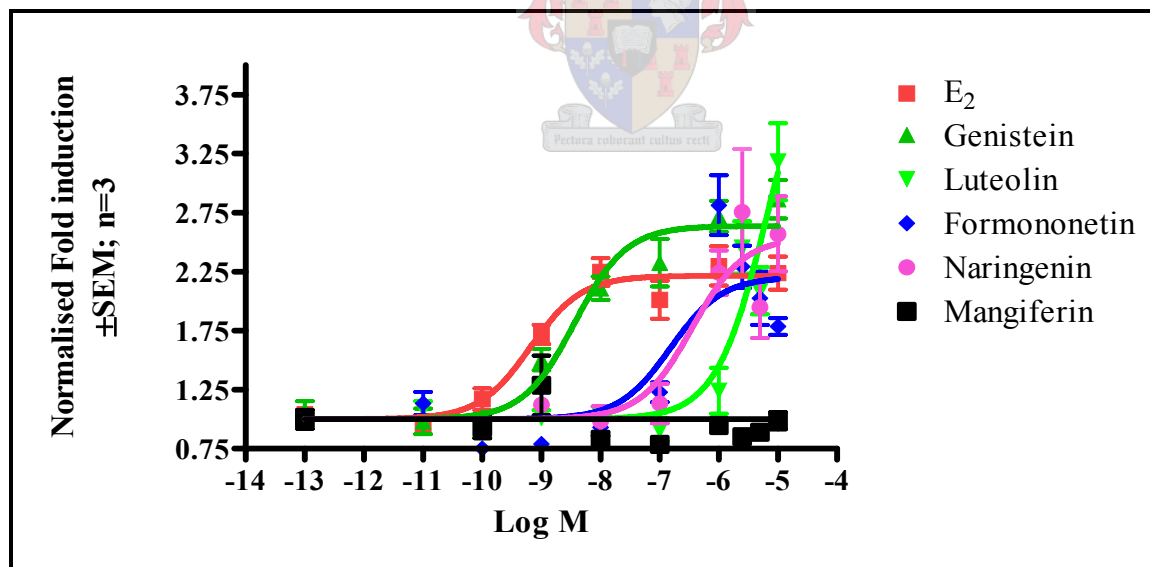


Figure 4: Transactivation of an ERE-containing promoter reporter construct (ERE.vit2.Luc) by hER β in the presence of increasing concentrations of various polyphenols. All experiments were performed with E₂ (natural ligand) and genistein (known phytoestrogen) as positive controls. Both luciferase and β -galactosidase activity were assayed with luciferase relative light units (RLU's) normalised with β -galactosidase RLU's. Results shown are of at least three independent experiments performed in triplicate.

Table 4: Potency (EC_{50}) and efficacy (maximal fold induction) values as determined for various polyphenols and E_2 from transactivation of an ERE-containing promoter reporter gene construct via hER β .

Test compounds	Potency (EC_{50}) ^a	Efficacy (maximal fold induction)
	hER β	
E_2	0.51 nM (4.99) ^b	2.34 (6.48)
Genistein	3.92 nM (4.93)	2.76 (17.18)
Luteolin	12.33 μ M (38.69)** ^c ### ^d	3.69 (48.22)
Formononetin	0.16 μ M (5.53)**	2.20 (18.42)
Naringenin	0.38 μ M (4.68)** #	2.99 (33.94)
Mangiferin	N/A ^e	N/A

^a EC_{50} values calculated from the log EC_{50} values of three independent experiments given as the mean CV.

^bCV (coefficient of variation) calculated from the log EC_{50} of at least three independent experiments performed in triplicate.

^cStatistically different from E_2 where ** represents $P < 0.01$.

^dStatistically different from Genistein with # representing $P < 0.05$, ## representing $P < 0.01$ and ### representing $P < 0.001$

^eN/A - test compound did not induce the ERE-containing promoter reporter gene construct via the hER β .

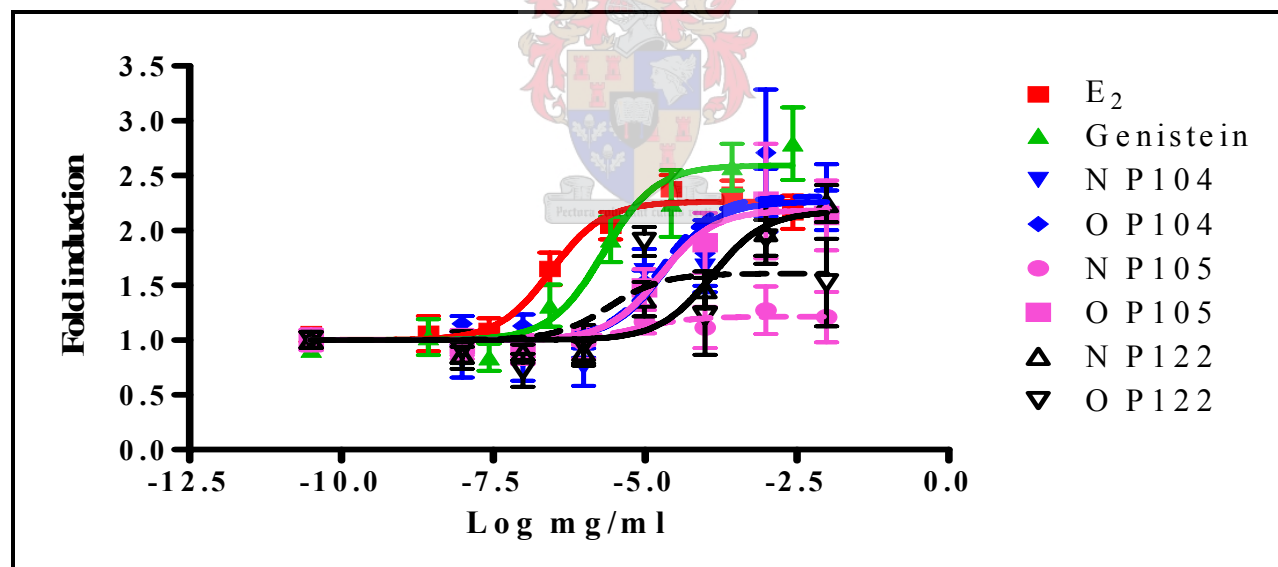


Figure 5: Transactivation of an ERE-containing promoter reporter construct (ERE.vit2.Luc) by hER β in the presence of increasing concentrations of unfermented *C. genistoides* methanol extracts. All experiments were performed with E_2 (natural ligand) and genistein (known phytoestrogen) as positive controls. Both luciferase and β -galactosidase activity were assayed with luciferase relative light units (RLU's) normalised with β -galactosidase RLU's. Results shown are of at least three independent experiments performed in triplicate.

Table 5: Potency (EC_{50}) and efficacy (maximal fold induction) values as determined for unfermented *C. genistoides* methanol extracts, genistein and E_2 from transactivation of an ERE-containing promoter reporter construct via hER β .

Test compounds and extracts	Potency (EC_{50} in mg/ml) ^a	Efficacy (maximal fold induction)
	hER β	
E_2	2.62×10^{-7} (4.35) ^b	2.22 (12.18)
Genistein	2.12×10^{-6} (5.53)	2.58 (24.25)
N P104	1.51×10^{-5} (22.60)* ^c	2.44 (36.78)
O P104	1.18×10^{-5} (21.36)	2.39 (21.11)
N P105	9.20×10^{-5} (12.31)	1.63 (3.25)
O P105	2.93×10^{-5} (22.66)*	2.53 (62.77)
N P122	6.90×10^{-5} (0.21)	1.90 (20.52)
O P122	2.48×10^{-6} (0.59)	1.94 (27.49)

^a EC_{50} values calculated from the log EC_{50} values of three independent experiments given as the mean CV.

^bCV or coefficient of variation calculated from the log EC_{50} of at least three independent experiments performed in triplicate.

^cStatistically different from E_2 where * represents $P < 0.05$.

3.4.3. Proliferation of breast cancer cells

The measurement of cell proliferation is a common method of measuring estrogenic activity and often called the E-screen.⁵⁹ It is, however, a more complex endpoint to measure than transactivation of a reporter gene. Cell proliferation entails an array of signalling pathways, growth regulators, and genes involved in the stimulation of proliferation or the inhibition of apoptosis.⁶⁰ MCF-7 human breast cancer cells respond to estrogen stimulation by proliferating, with the MCF-7-BUS stock being more sensitive to estrogen than other stocks of MCF-7 cells.⁶¹ Whereas MCF-7 cells contain both ER subtypes another breast cancer cell line, MDA-MB-231, contains no ERs.^{62;63}

The various polyphenols and unfermented *C. genistoides* methanol extracts together with genistein and E_2 were investigated to determine whether they were able to induce cell proliferation of MCF-7-BUS and MDA-MB-231. Steroid starved MCF-7-BUS and MDA-MB-231 cells were treated with increasing concentrations of E_2 , polyphenols or extracts for 48 hours, where after the degree of proliferation was determined (colorimetric MTT assay). Polyphenols or extracts were compared in terms of potency (EC_{50}) and efficacy (fold induction) determined from the dose response curves generated. Furthermore, to establish whether induced cell proliferation

was ER dependent, cells were co-treated with an ER antagonist, ICI 182,780. In addition, the effect of the polyphenols or *C. genistoides* extracts on E₂ induced proliferation was investigated. Physiologically more relevant, this would establish how the polyphenols and extracts would react in the presence of the endogenous ligand.

3.4.3.1. Proliferation of MCF-7-BUS and MDA-MB-231 breast cancer cells in the presence of polyphenols and E₂

All polyphenols investigated were able to induce cell proliferation in a dose dependent manner, except mangiferin, which only induced cell proliferation at the highest concentration (10⁻⁵ M) used (Figure 6). The order of potency for the cell proliferation of the MCF-7-BUS cells was as follows: E₂ (0.40 pM) >> naringenin (0.12 nM) > genistein (0.28 nM) > luteolin (8.89 nM) > formononetin (55.08 nM) > mangiferin (0.74 μM) (Table 7). All the potencies of the polyphenols were significantly different (P < 0.05) from that of E₂ (Table 7) when mangiferin was excluded from the statistical analysis. Mangiferin was excluded due to the very high CV. The potency of genistein, however, was not significantly (P > 0.05) different from that of any of the other polyphenols.

The order of efficacy for the cell proliferation of the MCF-7-BUS cells was: E₂ (2.25 fold) > genistein (2.09 fold) > naringenin (2.08 fold) > mangiferin (1.72 fold) > formononetin (1.38 fold) > luteolin (1.26 fold) (Table 6). None of the efficacy values determined for the polyphenols were significantly (P > 0.05) different from that of E₂ or genistein except for luteolin and formononetin (P < 0.01).

The polyphenols acted as weak agonists in the cell proliferation assay with potencies (EC₅₀) 1000 to 10 000 times less than that of E₂, although efficacies (fold induction) were in the same range. The EC₅₀ values determined for E₂ and genistein compared well with that of Bentrem *et al.*⁶⁴, who reported EC₅₀ values of 3 pM and 3 nM, respectively, for the two compounds. However, in another study EC₅₀ values for genistein, naringenin and luteolin were found to be between 100-1000 fold less than the EC₅₀ values determined in the present study.⁶⁵

The literature is not in agreement on the agonist effect of naringenin on cell proliferation as some, like the present study, report proliferation⁶⁶ while others do not.^{67;68} It has, however, been shown to act as a weak estrogen by inducing transactivation of ERE driven genes *in vitro*.⁶⁸

Neither the polyphenols nor E₂ were able to induce significant proliferation of the MDA-MB-231 cells (Figure 7). The MDA-MB-231 cells are well known for their unresponsiveness to cell proliferation when stimulated by estrogens.^{66,69}

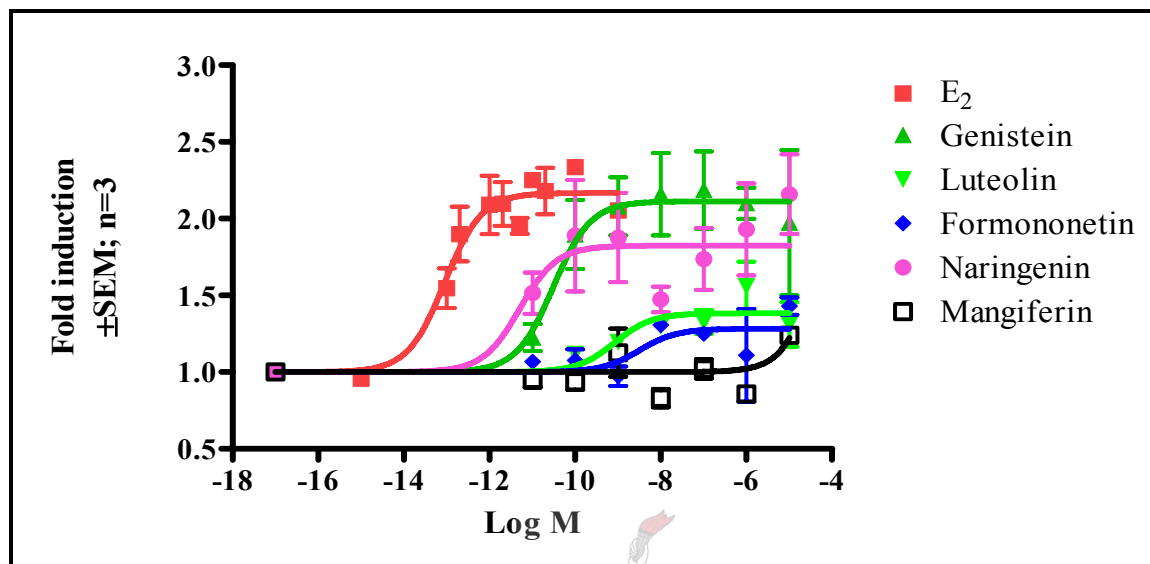


Figure 6: Cell proliferation of MCF-7-BUS cells treated with increasing concentrations of polyphenols for 48 hours after which the colorimetric MTT assay was used to measure cell proliferation. All experiments were performed with E₂ (natural ligand) and genistein (known phytoestrogen) as positive controls. Results shown are representative of one experiment performed in triplicate.

Table 6: Potency (EC₅₀) and efficacy (maximal fold induction) values determined for various polyphenols and E₂ from cell proliferation assays in MCF-7-BUS cells.

Test compounds	Potency (EC ₅₀) ^a	Efficacy (maximal fold induction)
E ₂	0.40 pM (1.71) ^{b#d}	2.25 (6.74)
Genistein	0.28 nM (9.32) ^{*c}	2.09 (4.17)
Luteolin	8.89 nM (15.77) ^{**}	1.26 (2.52) ^{** ##}
Formononetin	55.08 nM (14.90) ^{**}	1.38 (4.15) ^{** ##}
Naringenin	0.12 nM (1.60) [*]	2.08 (4.15)
Mangiferin	0.74 μM (31.07) ^{**}	1.72 (3.44)

^aEC₅₀ values calculated from the log EC₅₀ values of three independent experiments given as the mean CV.

^bCV or coefficient of variation calculated from the log EC₅₀ of at least three independent experiments performed in triplicate.

^cStatistically different from E₂ where * represents P < 0.05 and ** represents P < 0.01. Mangiferin was excluded from statistical analysis of other polyphenols due to its high CV.

^dStatistically different from Genistein with # representing P < 0.05, ## representing P < 0.01 and ### representing P < 0.001

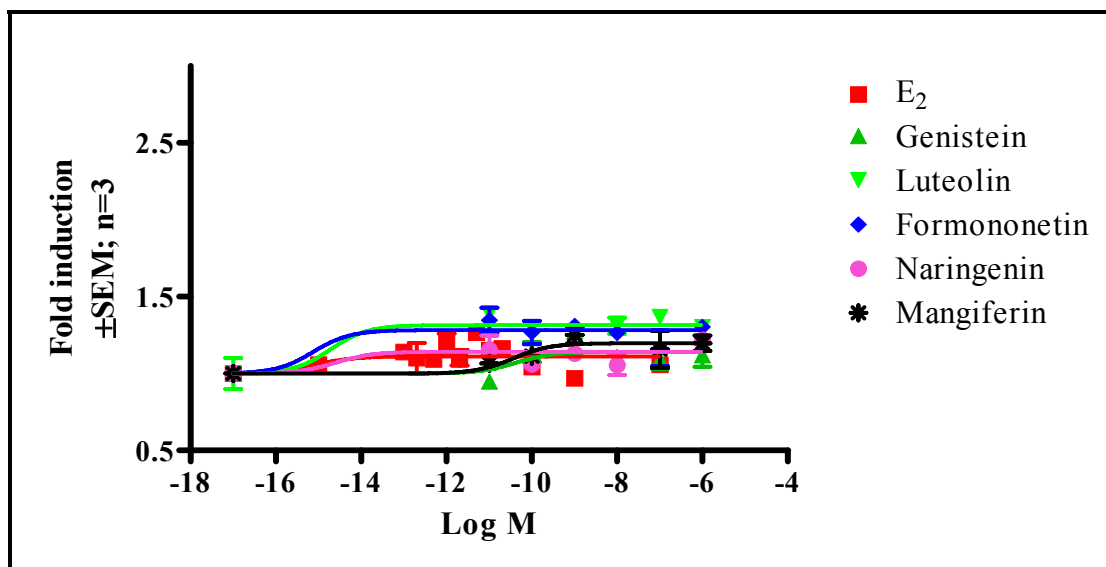


Figure 7: Cell proliferation of MDA-MB-231 cells treated with increasing concentrations of polyphenols for 48 hours after which the colorimetric MTT assay was used to measure cell proliferation. All experiments were performed with E₂ (natural ligand) and genistein (known phytoestrogen) as positive controls. Results shown are representative of one experiment performed in triplicate.

3.4.3.2. Proliferation of MCF-7-BUS and MDA-MB-231 breast cancer cells in the presence of unfermented *C. genistoides* methanol extracts and E₂

Methanol extracts from harvestings P104 and P105 were able to induce cell proliferation of both human breast cancer cells, whereas methanol extracts from harvesting P122 were unable to induce proliferation of either of the two cell lines tested (Figures 8 and 9).

The potency of the extracts in MCF-7-BUS cells (Table 7) were all significantly ($P < 0.01$) lower than that of E₂ with only O P104 and O P105 having a significantly ($P < 0.05$) lower potency than genistein. The rank order of potency (Table 6) was as follows: E₂ (2.79×10^{-10} mg/ml) \gg genistein (1.02×10^{-6} mg/ml) $>$ N P104 (1.98×10^{-6} mg/ml) $>$ N P105 (6.52×10^{-6} mg/ml) $>$ O P104 (1.34×10^{-4} mg/ml) $>$ O P105 (1.47×10^{-4} mg/ml). P122 was unable to induce cell proliferation of MCF-7-BUS cells in a dose dependent manner and thus EC₅₀ values could not be determined (Figure 8). In addition, only the efficacy of the P122 extracts was significantly ($P < 0.05$) lower than that of E₂. The efficacy of genistein, however, was also significantly ($P < 0.05$) different from that of O P104 and O P105. The rank order of efficacy (Table 7) was as follows: genistein (2.35 fold) $>$ O P104 (2.17 fold) $>$ E₂ (2.14 fold) $>$ N P104 (2.07 fold) $>$ N P105 (1.82 fold) $>$ O P105 (1.50 fold) $>$ O P122 (1.15 fold) $>$ N P122 (1.13 fold).

Similar to results with MCF-7-BUS cells P104 and P105 were able to induce, albeit to a lesser extent, cell proliferation of the estrogen insensitive MDA-MB-231 cell line (Figure 9). However, P122, E₂ and genistein were unable to induce proliferation. The rank order of potency (Table 8) was as follows: O P104 (1.39×10^{-10} mg/ml) > N P105 (2.62×10^{-10} mg/ml) > O P105 (2.79×10^{-10} mg/ml) > N P104 (2.47×10^{-9} mg/ml). The potency values calculated for the harvestings are not significantly ($P > 0.05$) different from each other (statistical data not shown). The rank order of efficacy (Table 8) was as follows: N P105 (1.81) > N P104 (1.62) > O P104 (1.59) > O P105 (1.38). None of the efficacies were significantly different from each other.

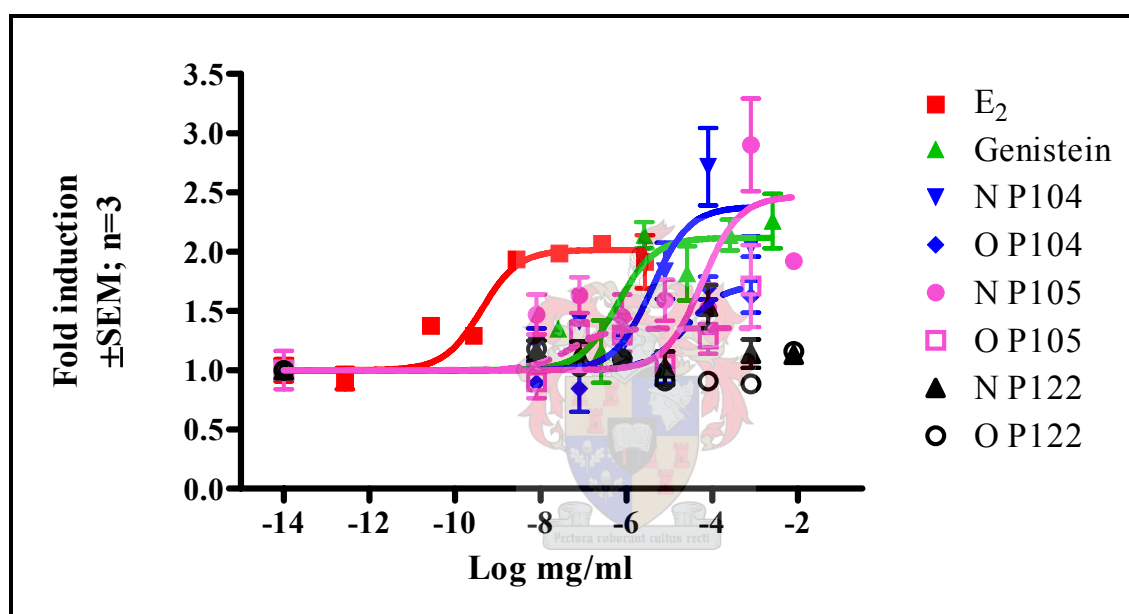


Figure 8: Cell proliferation of MCF-7-BUS cells treated with increasing concentrations of unfermented *C. genistoides* methanol extracts for 48 hours after which the colorimetric MTT assay was used to measure cell proliferation. All experiments were performed with E₂ (natural ligand) and genistein (known phytoestrogen) as positive controls. Results shown are representative of one experiment performed in triplicate.

Table 7: Potency (EC_{50}) and efficacy (maximal fold induction) values determined for unfermented *C. genistoides* methanol extracts, genistein and E_2 from cell proliferation assays in MCF-7-BUS cells.

Test compounds & extracts	Potency (EC_{50} in mg/ml) ^a	Efficacy (maximal fold induction)
E_2	2.79×10^{-10} (2.92) ^{b##c}	2.14 (8.46)
Gen	1.02×10^{-6} (7.56) ^{**d}	2.35 (10.57)
N P104	1.98×10^{-6} (7.34) ^{**}	2.07 (17.05)
O P104	1.34×10^{-4} (17.64) ^{** ##}	2.17 (18.21)
N P105	6.52×10^{-6} (25.71) ^{**}	1.82 (31.08)
O P105	1.47×10^{-4} (2.45) ^{** #}	1.50 (13.37)
N P122	N/A ^e	1.13 (12.19) ^{** ##}
O P122	N/A	1.15 (17.69) ^{* ##}

^a EC_{50} values calculated from the log EC_{50} values of three independent experiments given as the mean CV.

^bCV or coefficient of variation calculated from the log EC_{50} of at least three independent experiments performed in triplicate.

^cStatistically different from Genistein with # representing $P < 0.05$, and ## representing $P < 0.01$.

^dStatistically different from E_2 with * representing $P < 0.05$ and ** representing $P < 0.01$.

^eN/A not applicable as it could not be determined

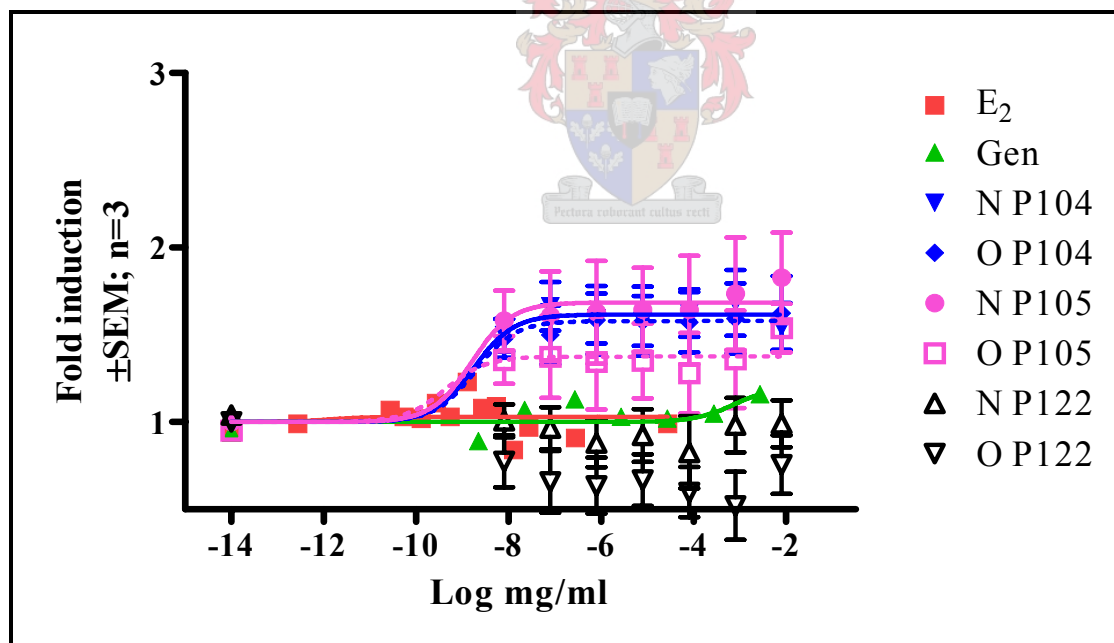


Figure 9: Cell proliferation of MDA-MB-231 cells treated with increasing concentrations of unfermented *C. genistoides* methanol extracts for 48 hours after which the colorimetric MTT assay was used to measure cell proliferation. All experiments were performed with E_2 (natural ligand) and genistein (known phytoestrogen) as positive controls. Results shown are representative of one experiment performed in triplicate.

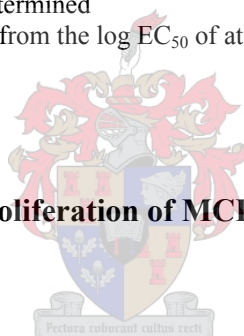
Table 8: Potency (EC₅₀) and efficacy (maximal fold induction) values determined for unfermented *C. genistoides* methanol extracts, genistein and E₂ from cell proliferation assays in MDA-MB-231 cells.

Test compounds and extracts	Potency (EC ₅₀ in mg/ml) ^a	Efficacy (maximal fold induction)
E ₂	N/A ^b	1.07 (10.30)
Gen	N/A	1.08 (2.42)
N P104	2.47 x 10 ⁻⁹ (2.66) ^c	1.62 (19.31)
O P104	1.39 x 10 ⁻¹⁰ (18.87)	1.59 (25.56)
N P105	2.62 x 10 ⁻¹⁰ (16.81)	1.81 (32.24)
O P105	2.79 x 10 ⁻¹⁰ (17.23)	1.38 (35.85)
N P122	N/A	1.24 (17.80)
O P122	N/A	0.92 (29.21)

^aEC₅₀ values calculated from the log EC₅₀ values of three independent experiments given as the mean CV.

^bN/A not applicable as it could not be determined

^cCV (coefficient of variation) calculated from the log EC₅₀ of at least three independent experiments performed in triplicate.



3.3.4.3. Investigating whether cell proliferation of MCF-7-BUS and MDA-MB-231 cells are ER-dependent

The cell proliferation assay measures a response induced by estrogens, but does not directly reveal the mechanism involved, i.e. ER-dependent or ER-independent. Thus by co-treating cells with an ER antagonist one could determine if the response measured is *via* the ER. MCF-7-BUS and MDA-MB-231 cells were co-treated with 1 nM ER antagonist (ICI 182,780) together with 10 μM polyphenols or 9.8 μg/ml methanol extract from unfermented *C. genistoides*, respectively (Figures 10 and 11). Genistein, however, was investigated at 0.1 μM and E₂ at 1nM as they induce maximum response at this concentration.

In MCF-7 BUS cells co-treatment with 1 nM ER antagonist, ICI 182,780, reduced the response induced by all polyphenols (Figure 10A), extracts (Figure 11A) and E₂ to that of the level of the control suggesting that the proliferation response in these cells is ER-dependent as has been previously suggested.^{61,70} Similarly, in the MDA-MB-231 cells, ICI 182,780 reduced the minimal induction by all the polyphenols (Figure 10B) and E₂ to that of the level of the

control. Induction by the extracts in MDA-MB-231 cells, however, was only partially reversed by ICI 182,780 in the case of P104 and P105, while in the case of P122 the antagonist appeared to stimulate induction (Figure 11B). Although the results in MDA-MB-231 cells were not statistically significant, which may simply be due to the very small induction elicited by the compounds and extracts, the consistent pattern of reduction of proliferation in the presence of antagonist as seen for the polyphenols, E₂ and some extracts (P104 and P105) does suggest that the minimal induction may be due in part to low levels of ER. Although MDA-MB-231 cells are generally considered to be estrogen insensitive^{66,69}, some authors have shown the presence of ERβ mRNA⁴⁶, which may be indicative of low levels of protein. The fact that full reversal of induction was not seen with P104 and P105 may in addition, suggest that ER-independent mechanisms contribute to proliferation in these cells. The stimulation of proliferation seen during co-treatment of MDA-MB-231 cells with P122 and the antagonist is difficult to explain but may be indicative of ER-independent mechanisms.

3.4.3.4. Effect of polyphenols and unfermented *C. genistoides* methanol extracts on the E₂ induced proliferation of MCF-7-BUS and MDA-MB-231 cells

E₂ significantly (P < 0.01) induced proliferation in MCF-7-BUS cells. This proliferation was prevented by co-treatment with all the polyphenols, except mangiferin and formononetin (Figure 12A). Genistein, luteolin, and naringenin therefore antagonised E₂ induced proliferation and appear to act as antiestrogens in the presence of 1 nM E₂. The antiestrogenic activity of naringenin and luteolin in the presence of E₂ has also previously been demonstrated by others.^{66,68,71} Both formononetin and mangiferin were unable to significantly (P > 0.05) antagonise E₂ induced cell proliferation. In MDA-MB-231 cells no antagonistic activity of the polyphenols were observed (Figure 12B), which may be ascribed to the low induction by all compounds.

All the unfermented *C. genistoides* methanol extracts, except N P122, were able to significantly (P < 0.05) antagonise E₂ induced cell proliferation of MCF-7-BUS cells (Figure 13A). However, in MDA-MB-231 cells none of the methanol extracts were able to significantly antagonise E₂ induced proliferation, which may once again be ascribed to the low induction of proliferation by E₂ in these cells.

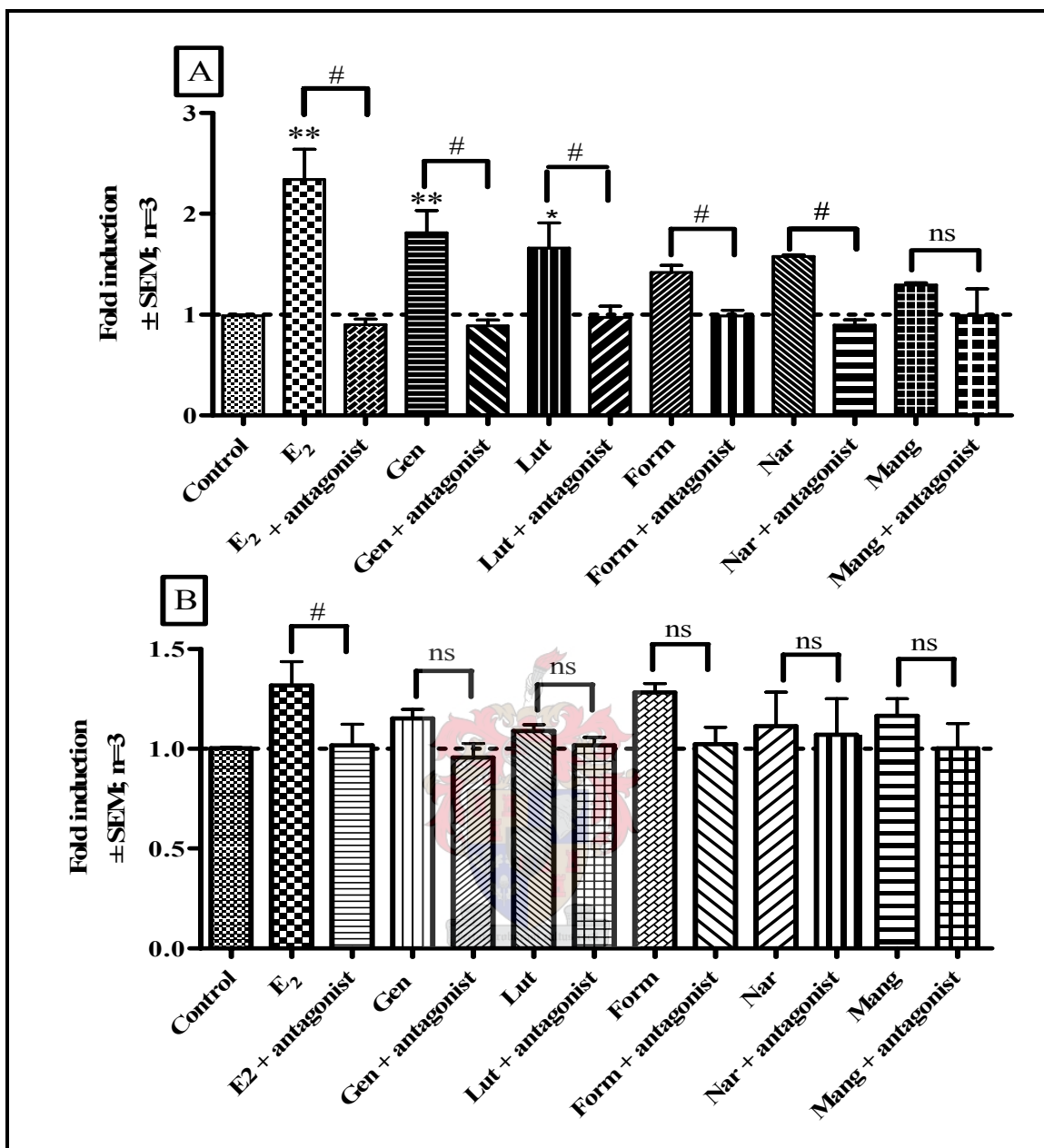


Figure 10: Cell proliferation of (A) MCF-7-BUS and (B) MDA-MB-231 breast cancer cells. Co-treatment with ER antagonist, ICI 182,780, identifies if induced response is ER-dependent. All compounds were tested at 10 μ M except for E₂ and genistein (Gen), which were investigated at 1 nM and 0.1 μ M, respectively. The control represents vehicle (DMSO) only. Statistical analysis (i) compared cell proliferation induced by compounds with that of the control (uninduced) using one-way ANOVA with Dunnet's multiple comparison's post test (* $P < 0.05$; ** $P < 0.01$) or (ii) compared induction by a specific compound in the absence and presence of the ER antagonist using two-tailed t-tests (# = $P < 0.05$; ns = $P > 0.05$ or not significantly different). Abbreviations: genistein (Gen), luteolin (Lut), formononetin (Form), naringenin (Nar), and mangiferin (Mang).

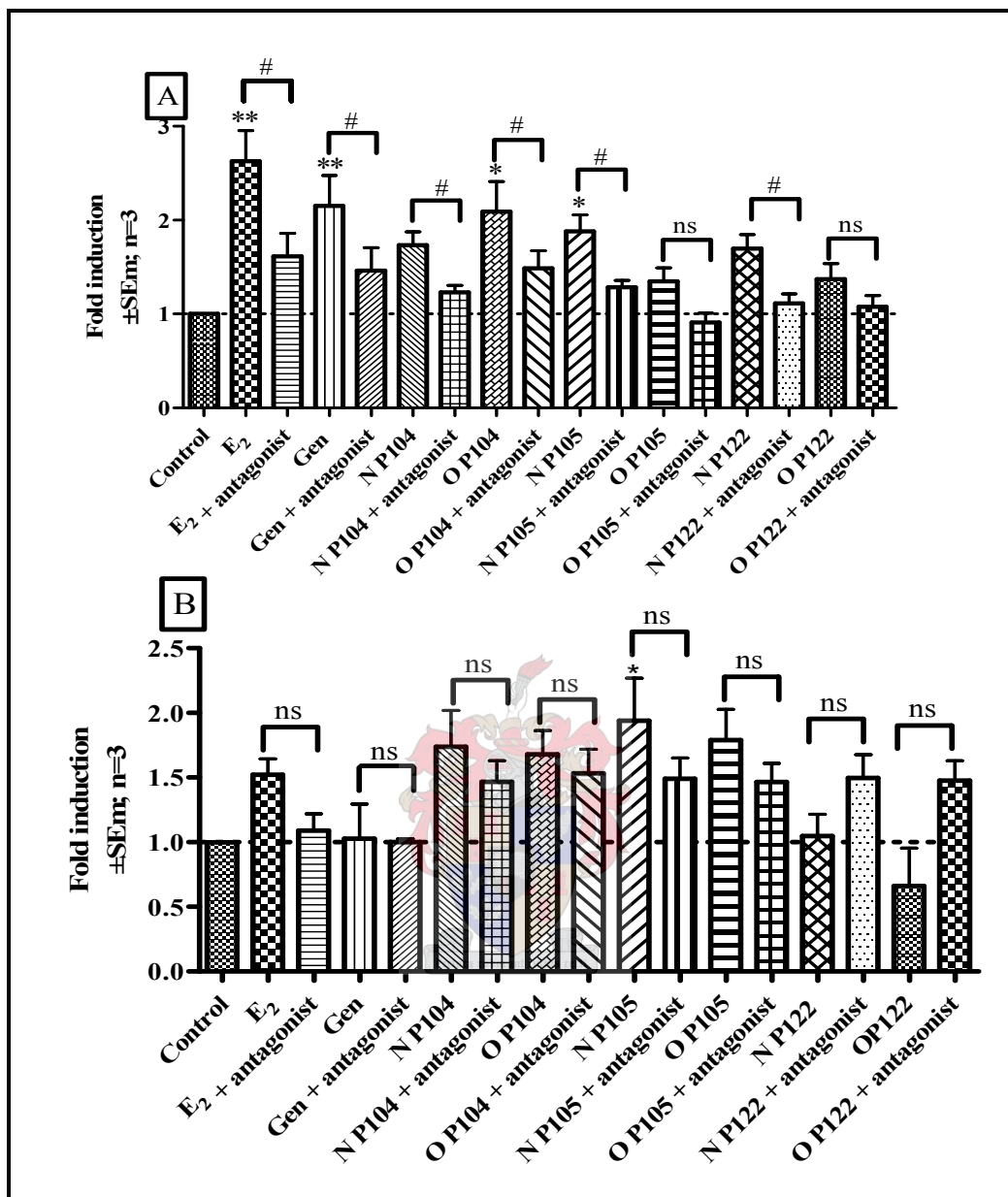


Figure 11: Cell proliferation of (A) MCF-7-BUS and (B) MDA-MB-231 breast cancer cells. Co-treatment with ER antagonist, ICI 182,780, identifies if induced response is ER dependent. All extracts were investigated at 9.8 $\mu\text{g/ml}$ E₂ and genistein (Gen) at 1 nM and 10 μM , respectively. Genistein was included as an example of a phytoestrogen. The control represents vehicle (DMSO) only. Statistical analysis (i) compared cell proliferation induced by compounds with that of the control (uninduced) using one-way ANOVA with Dunnet's multiple comparison's post test (*P < 0.05; ** P < 0.01) or (ii) compared induction by a specific compound in the absence and presence of the ER antagonist using two-tailed t-tests (# = P < 0.05; ns = P > 0.05 or not significantly different). Abbreviations: genistein (Gen)

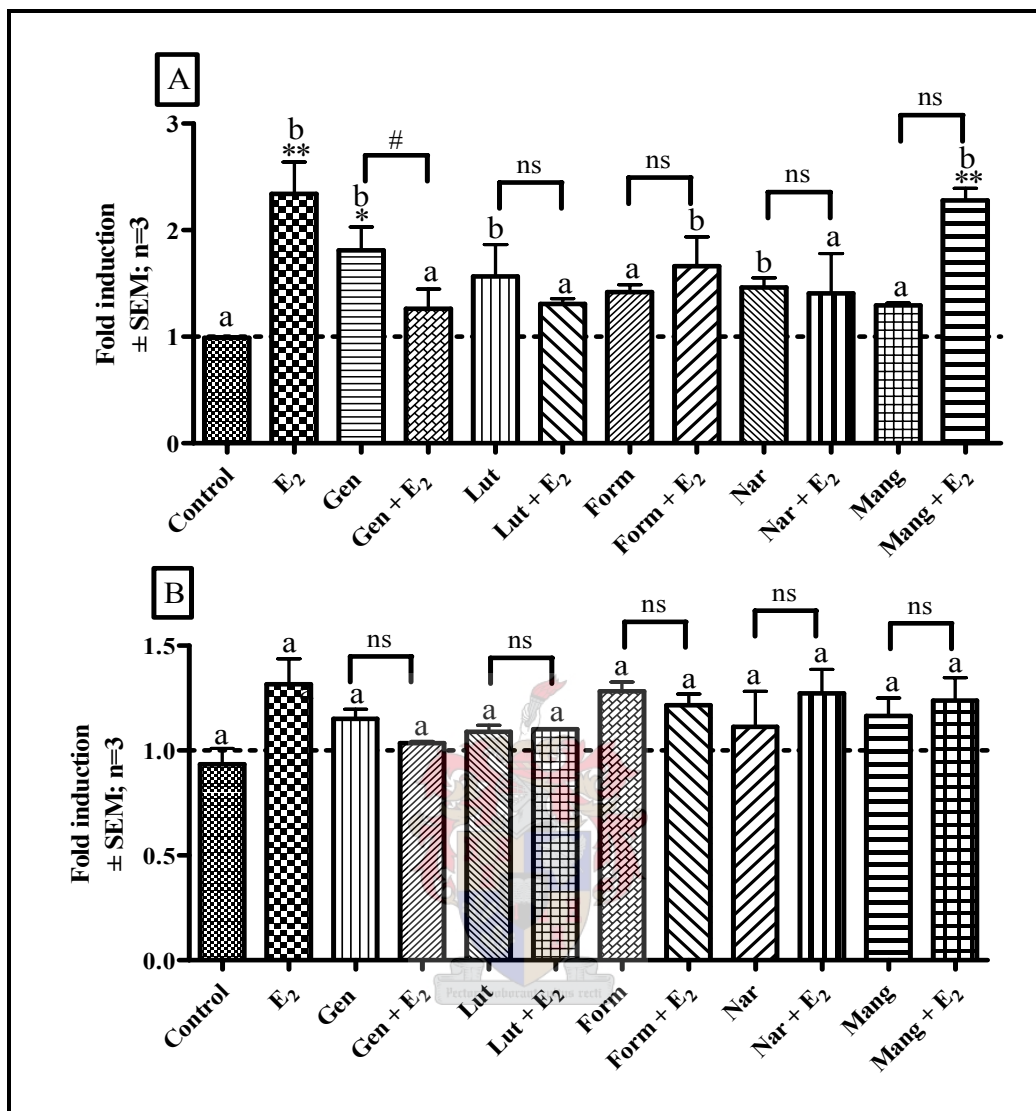


Figure 12: Effect of polyphenols on E₂ (1nM) induced proliferation of (A) MCF-7-BUS and (B) MDA-MB-231 breast cancer cells. All polyphenols were tested at 10 μ M except for genistein, which was investigated at 0.1 μ M. The control represents vehicle (DMSO) only. Statistical analysis (i) compared cell proliferation induced by compounds with that of the control (uninduced) using one-way ANOVA with Dunnet's multiple comparison's post test (*P < 0.05; ** (P < 0.01), (ii) compared induction by a specific compound in the absence and presence of 1 nM E₂ using two-tailed t-tests (# = P < 0.05; ns = P > 0.05 or not significantly different) or (iii) compared all columns with each other using two-tailed t-tests. Columns represented with the same letter are not significantly (P > 0.05) different from each other. Abbreviations: genistein (Gen), luteolin (Lut), formononetin (Form), naringenin (Nar), and mangiferin (Mang).

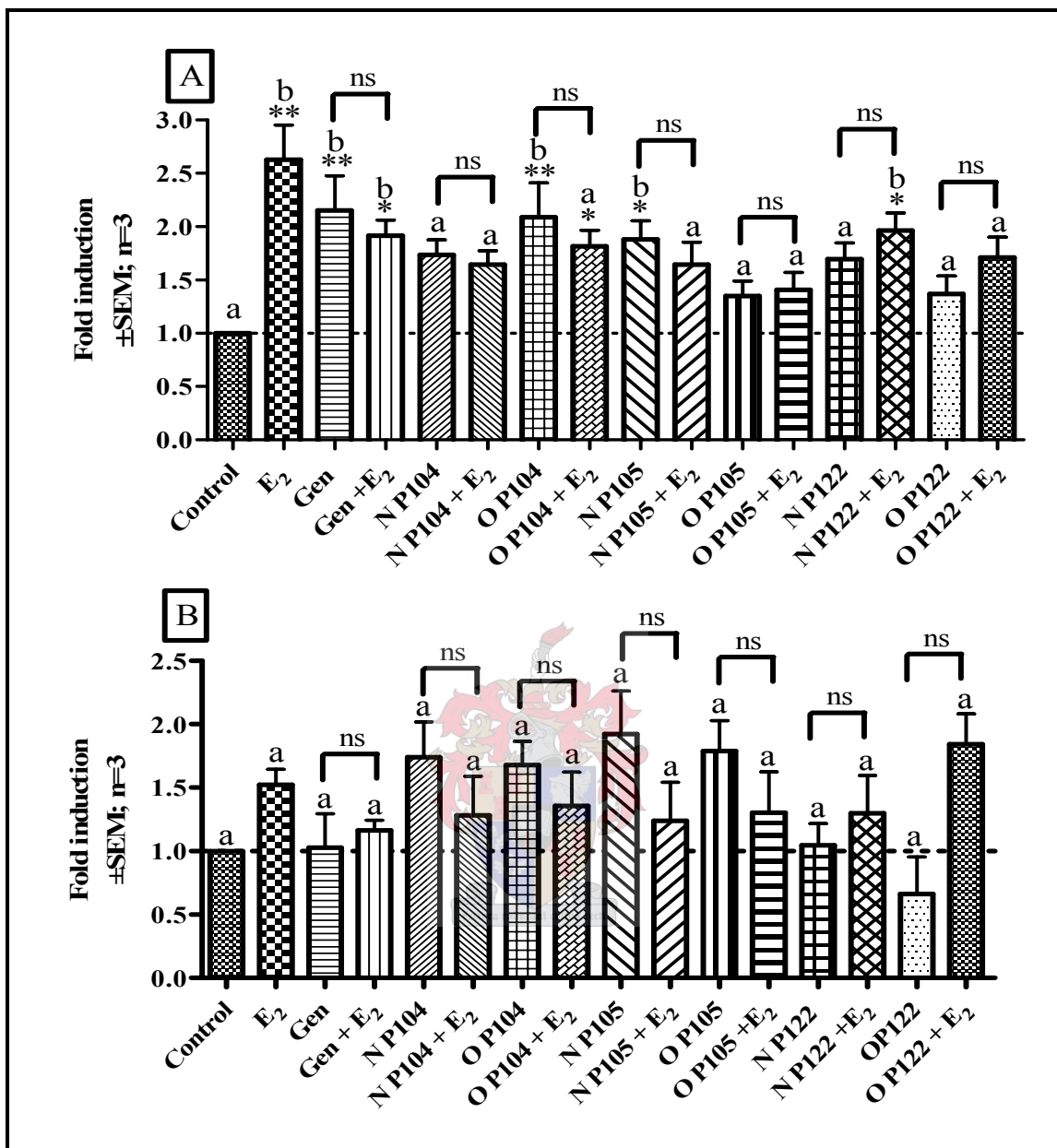


Figure 13: Effect of *C. genistoides* DME on E₂ (1nM) induced proliferation of (A) MCF-7-BUS and (B) MDA-MB-231 cells. All extracts were investigated at 9.8 µg/ml, while E₂ and genistein (Gen) were investigated at 1 nM and 10 µM, respectively. Genistein was included as an example of a phytoestrogen. The control represents vehicle (DMSO) only. Statistical analysis (i) compared cell proliferation induced by DME with that of the control (uninduced) using one-way ANOVA with Dunnet's multiple comparison's post test (*P < 0.05; ** P < 0.01), (ii) compared induction by a specific DME in the absence and presence of 1 nM E₂ using two-tailed t-tests (# = P < 0.05; ns = P > 0.05 or not significantly different) or (iii) compared all columns with each other using two-tailed t-tests. Columns represented with the same letter are not significantly (P > 0.05) different from each other. Abbreviations: genistein (Gen)

3.4.4. Binding to SHBG and displacement of E₂

SHBG is considered important in regulating the amount of biologically available sex-steroids such as E₂ and testosterone.³⁶ Binding of various polyphenols and unfermented *C. genistoides* methanol extracts to SHBG was investigated using human pregnancy serum. Binding to SHBG would imply that phytoestrogens could be transported in the bloodstream to target tissues and would thus also be protected from metabolism and subsequent secretion as was proposed for the endogenous estrogen.⁷² In addition, displacement of endogenous steroids, like E₂, from SHBG would result in an increase in the amount of free steroid, but also an increase in the metabolic clearance of this steroid.^{37;39}

The percentage 20 nM ³H-E₂ displaced from SHBG by the polyphenols (Figure 14A) and unfermented *C. genistoides* methanol extracts (Figure 14B) was significant (P < 0.05), except in the case of mangiferin. The order of displacement for E₂ and the polyphenols was as follows: E₂ (62.56 ± 6.72%) > naringenin (46.24 ± 5.65%) > genistein (38.77 ± 4.25%) > luteolin (35.95 ± 7.16%) > formononetin (35.77 ± 6.94%) > mangiferin (19.74 ± 6.17%). Displacement by naringenin, which was higher than that of genistein, was not significantly different from that of E₂, similar to what was found by others.⁷³

The order of displacement by the extracts was as follows: E₂ (52.19 ± 2.90%) > N P104 (37.06 ± 3.70%) > O P122 (35.60 ± 2.46%) > genistein (33.86 ± 5.69%) > N P105 (32.86 ± 7.11%) > N P122 (30.71 ± 4.09) > O P104 (29.55 ± 0.88%) > O P105 (29.29 ± 6.8%). N P104 and O P122 were as effective as E₂ in competing with ³H-E₂ for binding to the SHBG (statistical data not shown).

These findings would suggest that the polyphenols, mangiferin excluded, and unfermented *C. genistoides* methanol extracts investigated are ligands for SHBG and displace significant amounts of E₂.

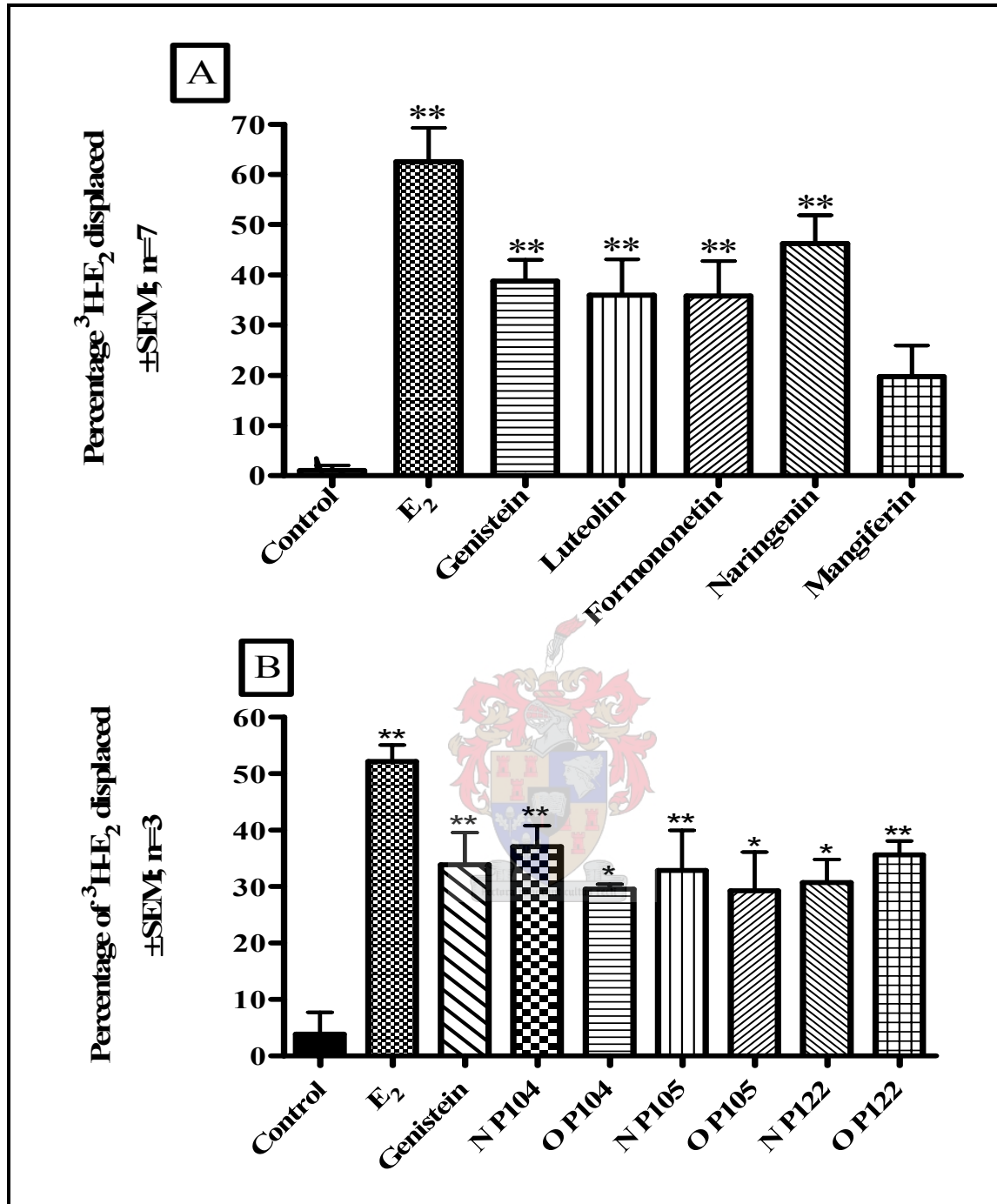


Figure 14: Competitive binding of (A) polyphenols and (B) *C. genistoides* methanol extracts to SHBG in DCC stripped human pregnancy serum incubated with 20 nM $^3\text{H-E}_2$. Polyphenols and E_2 were used at a concentration of 10^{-5} M and the extracts at a concentration of 9.8 $\mu\text{g/ml}$. The control in both represents vehicle (DMSO) only. For statistical analysis one-way ANOVA was used with Dunnet's multiple comparison's post test comparing percentage $^3\text{H-E}_2$ displaced to control. P-values are represented as follows: $P < 0.05$ by *, $P < 0.01$ by **.

3.4.5. Correlation between select parameters measured for polyphenols in the different *in vitro* assays

During this study several *in vitro* assays to measure estrogenicity were performed. To evaluate if binding to the ER receptors would show a relationship with some of the assays measuring biological response we correlated (i) log IC₅₀ values obtained with ERβ in whole cell ER binding assays with log EC₅₀ values from the ERE-containing promoter reporter gene assays *via* the hERβ and (ii) log IC₅₀ values obtained with both ER subtypes in whole cell ER binding assays with log EC₅₀ values from cell proliferation assays with MCF-7-BUS cells. There was a significant correlation (P = 0.0378; Pearson r = 0.899) between the whole cell ERβ binding assays and the ERE-containing promoter reporter gene assays *via* the hERβ (Figure 15A), but interestingly enough no significant correlation between log IC₅₀ values obtained with ER subtypes with log EC₅₀ values from cell proliferation assays with MCF-7-BUS cells (Figure 15B). There was, however, a significantly negative correlation (P = 0.0238; Pearson r = -0.926) between log EC₅₀ of cell proliferation of MCF-7-BUS cells and SHBG binding (Figure 15C). This could have some biological relevance as SHBG receptors are present on the cell surface of MCF-7 breast cancer cells⁷⁴ and have been shown to modulate proliferation.^{75;76} In contrast, there was no significant (P > 0.05) correlation between hERα and hERβ binding (Figure 15D), the ERE-containing promoter reporter assay *via* the hERβ and cell proliferation of MCF-7-BUS (Figure 15E), or SHBG binding and binding to both ER subtypes (Figure 15F).

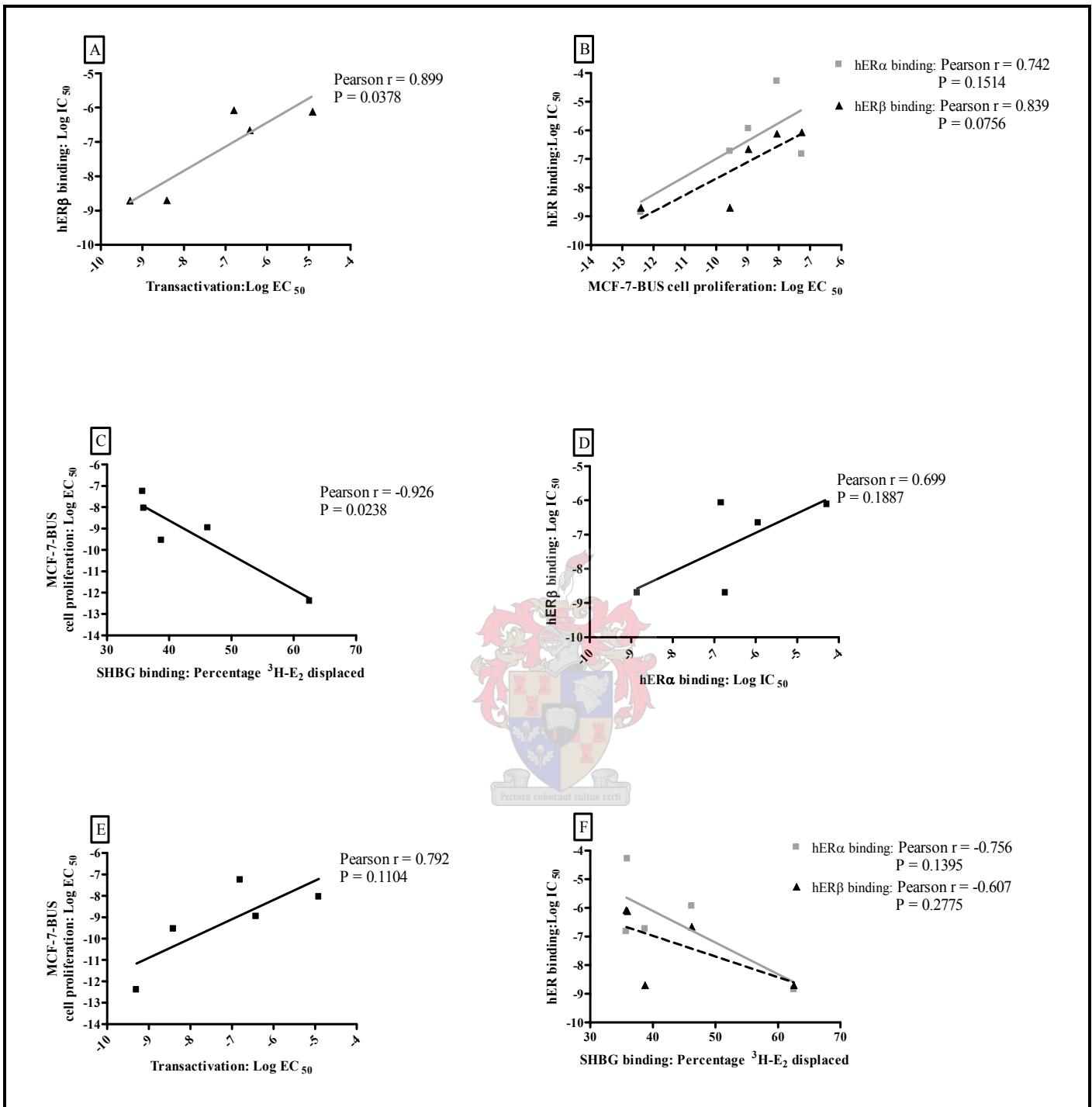


Figure 15: Correlations between select parameters from various *in vitro* assays used to measure phytoestrogenic activity of E₂ and polyphenols. Pearson correlations (two-tailed) were done using GraphPad Prism™.

Table 9: Summary of select parameters determined for polyphenols in assays for measurement of estrogenicity.

Test compounds	Competitive binding assays IC ₅₀ ^a		ERE-containing promoter reporter assays EC ₅₀ ^b	MCF-7-BUS cell proliferation EC ₅₀ ^c	SHBG-binding (% displacement) ^d
	hER α	hER β	hER β		
E₂	1.39 nM ^{##e}	1.97 nM	0.51 nM	0.40 pM [#]	62.56
Genistein	0.19 μ M ^{**f}	1.99 nM	3.92 nM	0.28 nM [*]	38.77
Luteolin	52.5 μ M ^{** ##}	0.49 μ M ^{** ##}	12.33 μ M ^{** ##}	8.89 nM ^{**}	35.95
Formononetin	0.15 μ M ^{**}	0.55 μ M ^{** ##}	0.16 μ M ^{**}	55.08 nM ^{**}	35.77
Naringenin	1.44 μ M ^{**}	0.56 μ M ^{** ##}	0.38 μ M ^{** #}	1.09 nM [*]	46.24
Mangiferin	NB ^g	NB	Did not induce the ERE-containing promoter reporter construct	0.74 μ M ^{**}	19.74

^aRefer to Table 2 for parameters on how calculated

^bRefer to Table 4 for parameters on how calculated

^cRefer to Table 6 form parameters on how calculated

^dRefer to text on how calculated (pg 175)

^eStatistically different from Genistein with # representing P < 0.05, ## representing P < 0.01 and ### representing P < 0.001

^fStatistically different from E₂ with * representing P < 0.05, ** representing P < 0.01 and *** representing P < 0.001.

^gNB = non-binder; polyphenols were unable to displace ³H-E₂ from ER subtype

Table 10: Summary of select parameters determined for polyphenols unfermented *C. genistoides* DME in assays for estrogenicity.

Test compounds and extracts	Competitive binding assays IC ₅₀ (mg/ml) ^a		ERE-containing promoter reporter assays EC ₅₀ (mg/ml) ^b	MCF-7-BUS cell proliferation assays EC ₅₀ (mg/ml) ^c	SHBG-binding (% displacement) ^d
	hER α	hER β	hER β		
E₂	3.68 x 10 ^{-7###c}	7.29 x 10 ⁻⁷	2.62 x 10 ⁻⁷	2.79 x 10 ^{-10##}	52.19
Genistein	4.23 x 10 ^{-5**f}	9.04 x 10 ⁻⁷	2.12 x 10 ⁻⁶	1.02 x 10 ^{-6**}	33.86
Luteolin	1.50 x 10 ^{-2** ##}	1.40 x 10 ^{-4** ##}	3.52 x 10 ^{-3** ##}	2.54 x 10 ^{-6**}	35.95
Formononetin	4.12 x 10 ^{-5**}	1.53 x 10 ^{-4** ##}	4.42 x 10 ^{-5**}	1.52 x 10 ^{-5**}	35.77
Naringenin	3.92 x 10 ^{-4**}	1.53 x 10 ^{-4** ##}	1.05 x 10 ^{-4** #}	3.27 x 10 ^{-8*}	46.24
Mangiferin	NB ^f	NB	Did not induce the ERE-containing promoter reporter construct	3.13 x 10 ^{-4**}	19.74
N P104	2.08 x 10 ^{-4** ##}	1.27 x 10 ^{-1** ##}	1.51 x 10 ^{-5*c}	1.98 x 10 ^{-6**}	37.06
O P104	5.89 x 10 ^{-4** ##}	2.29 x 10 ^{-1** ##}	1.18 x 10 ⁻⁵	1.34 x 10 ^{-4** ##}	29.55
N P105	NB	NB	9.20 x 10 ⁻⁵	6.52 x 10 ^{-6**}	32.86
O P105	NB	NB	2.93 x 10 ^{-5*}	1.47 x 10 ^{-4** #}	29.29
N P122	NB	NB	6.90 x 10 ⁻⁵	Did not cause proliferation	30.71
O P122	NB	NB	2.48 x 10 ⁻⁶	Did not cause proliferation	35.60

^aRefer to Table 2 and Table 3 for parameters on how calculated

^bRefer to Table 4 and Table 5 for parameters on how calculated

^cRefer to Table 6 and Table 7 for parameters on how calculated

^dRefer to text on how calculated (pg175)

^eStatistically different from Genistein with # representing P < 0.05, ## representing P < 0.01 and ### representing P < 0.001

^fStatistically different from E₂ with * representing P < 0.05, ** representing P < 0.01 and *** representing P < 0.001.

^gNB = non-binder; polyphenols or extracts were unable to displace ³H-E₂ from ER subtype

3.5. Discussion

Investigation into phytoestrogens as an alternative to conventional HRT is becoming increasingly popular, particularly research pertaining to the phytoestrogen found in soy, genistein, has increased significantly over the last few years.⁷⁷ Interest has been further motivated by the premature termination of two WHI clinical trials, which found numerous health risks, such as an increased occurrence of breast cancer and strokes, associated with conventional HRT usage.^{20;21} The ideal estrogen for use in relieving menopausal symptoms, both short and long-term, should be potent enough to promote bone formation and protect against cardiovascular disease, but not potent enough to induce estrogen-related cancers.^{5;78;79;80} Epidemiological studies have shown that Asian populations have lower incidences of breast and prostate cancer, which is believed to be related to the Asian diet.^{4;5} A high consumption of soy in eastern countries is thought to mediate the observed protective effects against hormone-induced cancers.^{4;5;6} Genistein, an isoflavone, is thought to be the etiological agent responsible, as it has been shown to act as a mild estrogen through binding to the ER subtypes.^{3;26} Most studies on phytoestrogens have concentrated almost solely on genistein and its mechanism of action despite the fact that numerous other plants contain phenolic compounds similar in structure to the natural hormone E₂.³ The presence of the phytoestrogens, formononetin, naringenin, eriodictyol and luteolin, in *Cyclopia*, coupled to anecdotal evidence of its use for the treatment of menopausal symptoms, has led to the investigation of phytoestrogenic activity in *Cyclopia* as a potential source of phytoestrogens indigenous to South Africa.^{43;44}

A previous study (Chapter 2) identified *C. genistoides*, amongst the four species of *Cyclopia* tested, as the most consistent in demonstrating phytoestrogenic activity through binding to the ER subtypes. Thus *C. genistoides* was chosen for further in-depth study. Similarly, luteolin, formononetin, naringenin and mangiferin were chosen as plant polyphenols previously shown to be present in *Cyclopia* species,^{43;44;81} although only mangiferin is present in detectable quantities in the specific extracts investigated in the present study. All polyphenols, except for mangiferin, were chosen for their ability to bind to both ER subtypes (Chapter 2). Mangiferin was chosen as it is the most abundant polyphenol present in honeybush.^{81;82}

Binding of the ligand to the ER subtypes, ER α and ER β , initiates a cascade of events that transduces an estrogenic response. The ER-ligand complex recognizes EREs in the promoters of estrogen responsive genes, which induce the transcription of such genes. Furthermore, estrogen is

responsible for a complex array of events, some of which are responsible for cell proliferation of certain estrogen sensitive cells.⁶⁰ All these mechanisms or events can be used to identify phytoestrogens. Thus binding to the ER subtypes, transactivation of an ERE, and induction of cell proliferation are all endpoints used to identify and evaluate potential phytoestrogens.^{59;83} In the present study these endpoints were used to establish and evaluate the estrogenicity of selected *Cyclopia* polyphenols and methanol extracts from unfermented *C. genistoides* and to compare their estrogenicity with that of the known phytoestrogen, genistein, and the natural ligand, E₂.

The *C. genistoides* extracts all transactivated an ERE-containing promoter reporter construct in COS-1 cells transfected with ER β , while in proliferation studies in MCF-7 cells (E-screen) all but one harvesting, P122, induced proliferation with a potency similar to that of genistein despite the fact that only one harvesting, P104, bound to the ER (Table 3 & 10). These results established that certain extracts of *C. genistoides* have estrogenic activity. In addition, the results suggest that there may be differences in the sensitivity of the screening assays used with the order of sensitivity being: transactivation of an ERE > MCF-7 cell proliferation > binding to ER subtypes. An examination of the potencies obtained with the different assays, however, suggests that the order of sensitivity may actually be reversed for the first two assays (Table 9 and Table 10). For E₂, for example, the order of potencies is: MCF-7 cell proliferation (0.40 pM) > transactivation of an ERE (0.51 nM) > binding to ER subtypes (1.39 nM for ER α and 1.97 nM for ER β). Although this does not hold for all the compounds and extracts evaluated, generally the MCF-7 cell proliferation assay does appear to be the most sensitive of the assays used.

Two attributes of phytoestrogens, weak estrogenicity and preference for ER β , have been linked to their beneficial health effects. Both attributes are to be discussed here as they pertain to the results obtained with *C. genistoides* extracts. To facilitate evaluation we will also compare activities with that of E₂, the endogenous estrogen linked to both adverse^{84;85} and beneficial health effects⁸⁴, and genistein, a well-studied phytoestrogen.³

It has been suggested that the weak estrogenic potential of phytoestrogens may contribute to health promoting effects such as protecting against the onset of osteoporosis, cardiovascular disease and certain hormone dependent cancers.^{5;77;79;86} The unfermented *C. genistoides* methanol extracts and the polyphenols tested were consistently less potent than E₂ (Table 10). In binding studies to ER subtypes extracts and polyphenols were from 10² to 10⁵ times less potent (IC₅₀ values) than E₂ in binding to ER α and ER β . In the ERE-containing promoter reporter and

proliferation studies, respectively, potencies (EC_{50} values) were up to 10^4 and 10^6 times lower than that of E_2 (Tables 9 and 10).

The $ER\beta$ is believed to be a modulator of $ER\alpha$ activity as it inhibits proliferation of breast cancer cells and immature rat uterus.^{35,87;88} It has been shown, in $ER\alpha$ containing T47D breast cancer cells, that $ER\beta$ inhibits E_2 induced cell proliferation if the cells are transfected with $ER\beta$ to such an extent that the mRNA levels of the two ER subtypes were equal.³⁵ These findings would suggest that either $ER\beta$ has an anti-proliferative effect on breast cancer cells or it quenches $ER\alpha$ activity.^{35;89} Competitive binding with both ER subtypes was investigated as numerous studies have shown that phytoestrogens bind preferentially to the $ER\beta$.^{26;28;51,54} The present study did indeed demonstrate that the phytoestrogens, genistein, luteolin and naringenin, but not formononetin, bind with a higher affinity to the $ER\beta$ subtype (Table 2 & 9) confirming results by others.^{26;27;53} Not only did all polyphenols, except mangiferin, bind to $ER\beta$ they also induced the ERE-containing promoter reporter gene *via* $ER\beta$ and cell proliferation of MCF-7-BUS cells. Of the three harvestings of *C. genistoides* tested, only one, P104, bound to the ER subtypes. Unlike the phytoestrogens, however, it bound, preferentially to the $ER\beta$, like E_2 (Table 3 & 10). Despite binding preferentially to the $ER\alpha$ and binding to the $ER\beta$ with a potency significantly ($P < 0.001$) lower than that of either E_2 or genistein, P104 was able to transactivate an ERE-containing reporter promoter *via* $ER\beta$ with a potency similar to that of E_2 and genistein and to induce MCF-7 cell proliferation with a potency similar to that of genistein but significantly ($P < 0.01$) lower than that of E_2 (Table 8 & 10). In addition, although P105 and P122 were unable to compete with 3H - E_2 for binding to $hER\beta$, both extracts were able to induce the ERE-containing promoter reporter *via* the $hER\beta$, and P105 was able to induce proliferation. These results seem to suggest that the *C. genistoides* extracts are disproportionately effective in activating the $ER\beta$. Further evidence for the activity of the extracts through $ER\beta$ comes from their ability to antagonise E_2 induced cell proliferation of MCF-7-BUS cells (Figure 13A), also seen with the polyphenols, genistein, luteolin and naringenin (Figure 12A). Although definitive proof for differential activation of $ER\alpha$ and $ER\beta$ by extracts is lacking results to date appear to support the hypothesis. The ERE-containing promoter reporter studies *via* the $ER\alpha$ could, however, have nicely complemented the binding of polyphenols and *C. genistoides* extracts to $hER\alpha$ as well as shedding light on the preferential activation of the $ER\beta$, and should be considered. Polyphenols and extracts, which are

able to act preferentially *via* ER β could be of physiological importance as this could play a role in the prevention of excessive cell proliferation which is associated with cancer formation.³¹ Further evaluation and elucidation of mechanism of action of the polyphenols and extracts come from the studies with the ER antagonist, ICI 182,780, the estrogen insensitive cell line, MDA-MB-231, and SHBG binding.

The induction of cell proliferation in MCF-7-BUS cells by all the polyphenols was antagonised by ICI 182,780 (Figure 10A), while none of the polyphenols, like E₂, were able to induce significant cell proliferation of MDA-MB-231 cells (Figure 7). This suggests that the proliferative action of the polyphenols, like that of E₂, is ER-dependent. In contrast, MCF-7 cell proliferation induced by the extracts was only partially, though significantly, reversed by ICI 182,780 (Figure 11A), while all of the extracts, except P122, could induce cell proliferation in the MDA-MB-231 cells (Figure 9), which could not be effectively blocked with the ER antagonist (Figure 11B). This suggests that in addition to an ER-dependent mechanism of action, the extracts may also display an ER-independent mechanism of action. Confirmation of this would, however, require further study and future studies should investigate both ER-dependent and ER-independent cell proliferation in more detail to identify the signal transduction pathways involved.

In addition to measuring and validating phytoestrogenic activity, SHBG binding was also measured. All the polyphenols, except mangiferin, were able to significantly ($P < 0.01$) compete with ³H-E₂ for binding to SHBG. Furthermore, the unfermented *C. genistoides* methanol extracts were able to significantly ($P < 0.05$) displace ³H-E₂ from SHBG. Binding to SHBG would imply that the plant phenolic compounds and extracts can be transported in the bloodstream through binding to SHBG, which would consequently decrease metabolic clearance rate and subsequent excretion as was proposed for endogenous estrogens.^{37;39} For future studies, it would be interesting to investigate whether extracts of *C. genistoides* and relevant polyphenols would increase the secretion of SHBG from liver cells as it has been shown that phytoestrogens can increase the synthesis of SHBG,^{90;91} and an increase in the concentration of SHBG would affect the amount of biologically free steroid.^{37;38}

Investigations into the estrogenic activity of other plant extracts have yielded results similar to those found in the present study. Kudzu root, soybean, red clover and alfalfa sprout, all legumes, like *Cyclopia*, displayed agonist activity through the ERE-containing promoter reporter

assays by activating both ER α and ER β , with preferential activation of ER β observed.⁵⁸ In addition, several plant extracts have been shown to induce cell proliferation of estrogen sensitive MCF-7 cells. *Moghania philippinensis*, which like *Cyclopia* is a Fabaceae, was shown to be active in the cell proliferation assay at concentrations of 1 μ g/ml and 10 μ g/ml.⁹² Kudzu root, red clover, alfalfa sprout and soybean could also induce MCF-7 cell proliferation.⁵⁸ Additionally, *Ginkgo biloba* extracts were shown to induce proliferation of MCF-7 cells that is ER-dependent as the response could be blocked with an ER antagonist.^{57;58} The *Ginkgo biloba* extracts could, however, not induce cell proliferation of MDA-MB-231 cells.⁵⁷ On the other hand, methanol extracts from *M. philippinensis* have previously been shown to antagonise MCF-7 cell proliferation induced by 10⁻⁷ M or 10⁻¹⁰ M E₂.⁹²

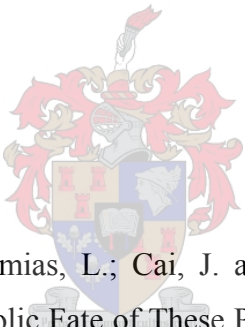
To summarise, the present study showed that the polyphenols, luteolin, formononetin and naringenin, present in *Cyclopia spp.* and some methanol extracts from *C. genistoides* are estrogenic *in vitro* through binding to both ER subtypes, inducing the ERE-containing promoter reporter *via* the ER β subtype, and by inducing cell proliferation of the estrogen sensitive MCF-7-BUS cells (Tables 8 & 10). Proliferation of the estrogen-insensitive MDA-MB-231 cell line was, however, only stimulated by extracts (Figure 9). Mangiferin is one of the major constituents of *Cyclopia spp.*, and although OH-groups are present at positions 7 and 4' (structural requirements for phytoestrogenic activity)⁹³ the xanthone structure does differ from that of the basic flavonoid structure, which would explain the inability of mangiferin to display any significant phytoestrogenic activity. Thus, despite being one of the polyphenols present at high concentrations in *Cyclopia spp.*, mangiferin is not an appropriate compound for enrichment for nutraceutical usage intended for the phytoestrogen market, as it clearly did not portray adequate phytoestrogenic activity. To conclude, although the present study showed that *C. genistoides* is a potential source of phytoestrogens, caution should, however, be exercised as variation within the species does exist. Methanol extracts from only one harvesting (P104) was able to displace ³H-E₂ from the ER subtypes and methanol extracts of only two harvestings (P104 and P105) were able to induce proliferation of the MCF-7-BUS cells, while all three unfermented *C. genistoides* methanol extracts (P104, P105 and P122) portrayed estrogenicity through induction of the ERE-containing promoter reporter *via* ER β . The variations in estrogenicity may be ascribed to polyclonal plant material and stress factors such as temperature and soil requirements.^{94;95}

Therefore each individual batch of plant material available at this stage in the industry would probably have to be screened if it is to be used to prepare a nutraceutical.

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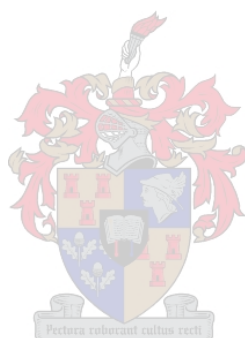
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CHAPTER 4

General discussion and conclusion

The premature termination of two WHI studies sparked a debate on the safety of traditional HRT as numerous risks were shown to be associated with conventional HRT.^{1;2;3;4;5} New alternative therapies are being investigated to replace or complement conventional HRT.^{2;6} Natural or herbal remedies have been used for centuries and are still popular in a modern lifestyle. More importantly, a plant-based diet is more than often associated with a healthier individual. Epidemiological studies commonly compare the low incidence of hormone-dependent cancer in the Asian population to that of their Western counterparts.^{7;8} The soy isoflavone, genistein, which is found in the Asian diet and frequently consumed, has been identified as the plant compound responsible for this phenomenon.⁹ Genistein and structurally similar flavonoids, commonly referred to as phytoestrogens, are able to mimic endogenous estrogen through binding to the ER subtypes.^{10;11} They are also able to compete with endogenous estrogens for binding to the ER subtypes and inhibiting the natural ligand, thereby acting as anti-estrogens.¹¹ Phytoestrogens are pleiotropic in nature and thus capable of acting through numerous mechanisms.^{11;12} These plant compounds are becoming increasingly popular as alternative treatments for menopausal symptoms and already several products are available to the public (Chapter 1). Nutraceuticals is becoming increasingly well accepted amongst consumers, resulting in a lucrative industry with huge potential for herbal remedies.¹³

Cyclopia, more commonly known as honeybush, is enjoyed as a fragrant herbal infusion (beverage) and is available to the consumer as honeybush tea. *Cyclopia* has been shown to have both antioxidant and antimutagenic activity.^{14;15;16;17} However, as indicated by both studies, its antioxidant and antimutagenic activity is minor compared to that of the more popular and well-known rooibos tea.¹⁶ Qualitative analysis of the polyphenols present in *C. intermedia* and *C. subternata*^{18;19} have identified the known phytoestrogens, formononetin, naringenin, eriodictyol and luteolin.^{20;21;22} This together with anecdotal evidence as to the effectiveness of honeybush in alleviating menopausal symptoms may place honeybush in another league, separate from rooibos, with respect to health-promoting claims and economic potential as a nutraceutical. Before this potential beneficial property of *Cyclopia* could be exploited, investigation into its putative phytoestrogenic activity is required. Therefore, the main aim of this thesis was to

identify whether *Cyclopia* has phytoestrogenic activity. Phytoestrogenic activity can be assessed by *in vitro* methods such as ER binding, reporter gene assays and cellular proliferation in comparison to activity of the endogenous ligand E₂ and other well-known phytoestrogens such as genistein. From these methods a rank order of estrogenic potency can be established.

The study was two-tiered in its investigation and will be briefly summarised. Firstly, the four commercially available *Cyclopia* species, *C. genistoides*, *C. sessiliflora*, *C. subternata* and *C. intermedia*, together with nine commercially available phenolic compounds present in *Cyclopia* were examined for estrogenic activity through binding to the two ER subtypes, ER α and ER β (Chapter 2). The purpose of including the known phytoestrogens, formononetin, naringenin, eriodictyol and luteolin was to confirm their estrogenicity in the assay system used, while the other polyphenols were either major compounds, and therefore of importance, if estrogenicity could be established, or minor compounds, but of the flavanone type, suggesting that they could potentially exhibit estrogenic activity and thus be classified as phytoestrogens. In addition, they could serve as marker compounds when *Cyclopia* extracts are investigated for phytoestrogenic activity. The flavanone, naringenin, the isoflavone, formononetin and the flavone, luteolin were the only compounds able to compete with tritiated E₂ for binding to both ER subtypes. Naringenin and formononetin were previously shown to compete with E₂ for binding to both ER subtypes¹⁰, whereas luteolin has not been previously investigated for binding to ER α , but has been shown to compete with E₂ for binding to the ER β subtype.²⁰ The flavanones, eriodictyol, narirutin and eriocitrin, were only able to compete for binding to the hER β . Eriodictyol has been previously shown to be a weak phytoestrogen through cell proliferation and reporter gene assays (ER not specified).^{21;22} Narirutin and eriocitrin, to our knowledge, have not been previously investigated for phytoestrogenic activity and are shown in this study for the first time to bind to the hER β . Both aqueous and methanol extracts were prepared and investigated, which allowed for comparison of extract solvent as extraction selectivity may differ due to solubility of compounds. Additionally, aqueous extracts of both fermented and unfermented plant material were examined. Again this allowed for the comparison of processed plant material (fermented) with that of unprocessed (unfermented) plant material. However, the main aim of investigating ER subtype binding of the four commercially available *Cyclopia* species was to screen for the species with the highest estrogenic activity. This prepared the ground for the second level of the study where the species identified to have the highest

estrogenic activity would be intensively studied and evaluated for estrogenic activity by determining potency and efficacy values (Chapter 3).

The first part of the study identified both *C. genistoides* and *C. subternata* as having significant estrogenic activity with methanol extracts from unfermented plant material having greater activity. It was evident that great variation within a species does exist as was found with *C. genistoides*. Only one harvesting (Gen P104) was able to significantly ($P < 0.001$) compete with tritiated E_2 for binding to both ER subtypes, whereas the other harvestings Gen P105 and Gen P122 were either only able to displace tritiated E_2 from hER β or unable to displace significantly from either ER subtype. Variation in the concentration of secondary metabolites such as flavonoids, which are dependent on environmental factors such as temperature and soil requirements^{23;24;25} in addition to location harvested²⁶, as well as genotype differences may explain the disparity.

Thus, the preliminary screening results for phytoestrogenic activity of the four *Cyclopia* species and the nine selected polyphenols identified methanol extracts of unfermented *C. genistoides* and luteolin, formononetin and naringenin for further in-depth validation studies of phytoestrogenic activity in the second part of this thesis. Additionally, mangiferin was included as it is the polyphenol present in *Cyclopia* at the highest concentration although it displayed no estrogenic activity through binding to the ER subtypes. Further studies were thus required to confirm that mangiferin does not contribute to the phytoestrogenicity of *Cyclopia spp.* Competitive binding to both ER subtypes, transactivation of an ERE-containing promoter reporter construct *via* the hER β , and breast cancer cell proliferation dose response curves were generated and SHBG binding assay conducted to determine efficacy and potency values or to ascertain the biological response relative to vehicle (DMSO) control. Efficacy and potency parameters were compared with those of E_2 and genistein to establish rank order of phytoestrogenicity.

Selected unfermented *C. genistoides* methanol extracts displayed estrogenic activity in binding to both ER subtypes, transactivating an ERE-containing promoter construct *via* the hER β and inducing cell proliferation of estrogen sensitive MCF-7-BUS breast cancer cells. Furthermore, all the polyphenols tested, except for mangiferin, displayed estrogenicity. More specifically, the polyphenols, except mangiferin, and only one *C. genistoides* extract (P104) bound to both human ER subtypes with P104, formononetin and naringenin displaying similar

potencies (IC_{50}) as genistein for the $hER\alpha$, but not for $hER\beta$. Similar to genistein, all the polyphenols that bound, except for formononetin, had a higher affinity (K_i) for the $hER\beta$. In contrast P104 and formononetin, like E_2 , exhibited preference for the $hER\alpha$. This was disconcerting as a higher affinity for $ER\beta$ and preferential induction of this subtype are hypothesized to be responsible for the health promoting effects of phytoestrogens.²⁷ However, despite this all the *C. genistoides* extracts (P104, P105 and P122) did activate the $hER\beta$ to induce transcription. Moreover all harvestings, except P122, were able to significantly induce MCF-7-BUS cell proliferation. Similarly, all the polyphenols that bound to the ER also transactivated *via* $ER\beta$ and induced cell proliferation. Mangiferin, which was chosen solely because of its high concentration in *Cyclopia*, was consistent in displaying no significant estrogenic activity, confirming its unsuitability as a marker compound for estrogenicity despite high concentrations in *Cyclopia*.

Concerning the rank order of estrogenicity, comparison of potencies and efficacies with those of E_2 and genistein revealed that all the extracts transactivated the $hER\beta$ with potencies and efficacies comparable to that of E_2 and genistein. In the case of proliferation in MCF-7-BUS cells, which contain both $ER\alpha$ and $ER\beta$, all the extracts, except P122, displayed efficacies and potencies similar to those of genistein, but potencies significantly ($P < 0.001$) lower than that found for E_2 . This suggests that the *C. genistoides* extracts, despite displaying a preference for $ER\alpha$ in binding assays, like genistein, preferentially induce through $ER\beta$.

Furthermore, acting *via* the ER was substantiated as cell proliferation induced by the polyphenols and extracts was blocked in the presence of the ER antagonist, ICI 182,780. Blocking was complete for the polyphenol-induced proliferation, but only partial for the extracts. This result, coupled to the fact that the extracts, unlike the polyphenols, could induce proliferation in the estrogen insensitive MDA-MB-231 cell line suggests that the phytoestrogens in the extracts may act *via* both ER-dependent and ER-independent mechanisms in inducing cell proliferation. More relevant clinically, in the presence of 1 nM E_2 all the *C. genistoides* extracts were able to antagonise E_2 -induced proliferation of the MCF-7 breast cancer cell line. This phenomenon, also seen with other phytoestrogens^{28;29}, has been ascribed to preferential induction of $ER\beta$, as physiologically, $ER\beta$ negatively modulates $ER\alpha$ action, including its ability to induce proliferation.^{30;31} To further elucidate mechanism of action, binding to SHBG was evaluated. The extracts bound to SHBG resulting in significant displacement of E_2 . This could have implications

both for the biological half-life of the phytoestrogens in the extracts and in affecting the free levels of the endogenous sex steroids.³²

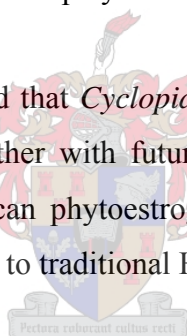
Despite the fact that the results obtained from the three assays used in the in-depth study (Chapter 3) validated the rank order of estrogenicity of the extracts (P104 > P105 > P122) as found in the original screening of the *Cyclopia* species (Chapter 2), some discrepancies between the two studies were observed. Formononetin, for example, displayed a higher binding affinity (K_i) for the ER α subtype (Chapter 3), which does not agree with previous results (Chapter 2), showing that at 10 μ M formononetin competed more strongly with 3 H-E $_2$ for binding to the hER β than hER α . A similar trend was also observed with P104. Additionally, P105 was initially shown to significantly ($P < 0.05$) displace tritiated E $_2$ from the ER β subtype (Chapter 2), a result that could not be reproduced in the subsequent study (Chapter 3). These discrepancies could be due to variations in the receptor levels between the two studies, which could influence the potencies of the test compounds.^{33,34} This highlights the fact that, although ER binding is the initial event of estrogen signalling and is per definition an attribute of phytoestrogens, it may be less sensitive than other screening assays in identifying potential compounds with estrogenic activity and may return more false negatives. Indeed our current study suggests that both transactivation of promoter reporter constructs and proliferation assays are more sensitive in identifying weak estrogens.

However, as clearly indicated and stated in both studies (Chapters 2 and 3), there is substantial variation in the estrogenic potential within species of *Cyclopia*. For example, all *C. genistoides* extracts (P104, P105 and P122) transactivated, two (P104 and P105) induced proliferation, and only one (P104) bound to the ER subtypes. This suggests a rank order of estrogenicity of P104 > P105 > P122 and highlights the variations in responses induced by the three harvestings. This variation within species is supported by the HPLC analysis of the specific extracts used in the preliminary screening study (Chapter 2) that showed no detectable luteolin, formononetin, naringenin or eriodictyol in any of the extracts despite the fact that previous studies identified these polyphenols.^{18,19} The fact that the known phytoestrogens, luteolin, formononetin, naringenin and eriodictyol were not detected in the extracts used in the current study, not only makes using these polyphenols as marker compounds difficult, it also suggests that unknown polyphenolic compounds present in *Cyclopia*, awaiting identification, are responsible for the estrogenic activity displayed. No blanket claims as to the proven estrogenicity

of all preparations of honeybush tea should thus be made and individual batches of harvestings would have to be tested to establish estrogenicity before nutraceutical production from the current plant material available in the industry, is commenced.

Future studies thus would entail activity-guided fractionation of unfermented *C. genistoides* methanol extracts to identify and isolate the estrogenically active compound(s) in the extracts. In addition, plant variability should be studied to investigate factors contributing to variability that may be managed to ensure a standardised product. Standardisation of a potent extract would entail quantification of the active compound(s) within an extract and a routine, high-throughput phytoestrogenic-screening assay would be required. Furthermore, the development of a nutraceutical product from *Cyclopia* would require not only evaluation of its phytoestrogenic potential and health effects, but also assessment of side-effects and toxicity. Additionally, the molecular mechanism of action of the phytoestrogens in *C. genistoides* extracts should be studied further as they seem to display activities that are both similar and different to those of genistein.

The current study has established that *Cyclopia*, specifically *C. genistoides* extracts, has phytoestrogenic potential and this together with future investigations would contribute to the development of a uniquely South African phytoestrogen nutraceutical product from *Cyclopia* aimed at women in search of alternatives to traditional HRT.



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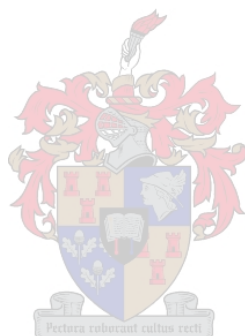
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Addendum A

Method development for investigating transactivation of an ERE containing promoter reporter construct via hER α

Aim: To optimise a transactivation assay whereby an ERE containing promoter reporter construct is activated *via* the hER α for evaluation of ER α mediated transactivation.

In Chapter 3 we evaluated the estrogenicity of unfermented *C. genistoides* methanol extracts and selected polyphenols, by amongst others a transactivation assay in COS-1 cells transiently transfected with ER β . As indicated in the Chapter it would have been informative to compare transactivation with ER α , especially in the light of differential binding to ER α and ER β and the fact that we observed inhibition of E₂ induced proliferation in MCF-7 cells. It has been suggested that E₂ induced proliferation is mediated by ER α while inhibition of proliferation by phytoestrogens is *via* ER β .

We obtained significant transactivation by E₂, the positive control, using hER β (Figure 1). However, when using the same conditions (cell number, amount of total DNA and ratios of different constructs used) with hER α we were unable to get transactivation with E₂ (Figure 2).

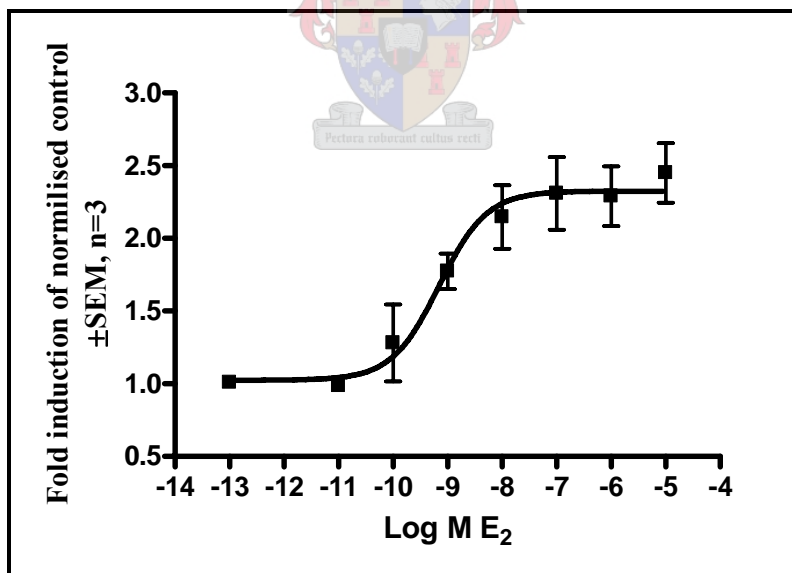


Figure 1: Representative of one experiment of transactivation of ERE.vit2.luc *via* hER β by E₂ in a dose dependent manner. COS-1 cells (2×10^6 cells/10 cm dish) were transfected with 9.6 μ g DNA (0.8 μ g hER β expression plasmid (pSG5-hER β), 8 μ g ERE-containing promoter reporter construct (ERE.vit2.luc) and 0.8 μ g pCMV- β -galactosidase).

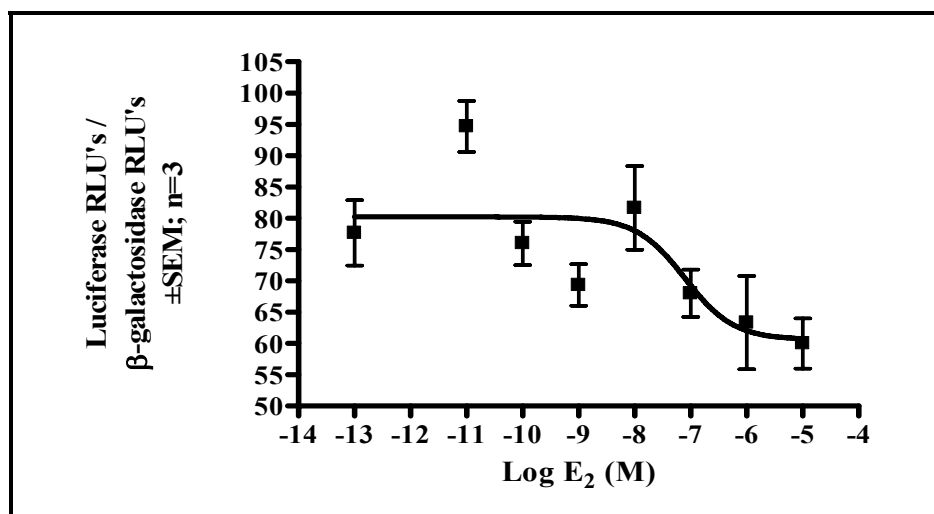


Figure 2: Representative of one experiment of transactivation of ERE.vit2.luc *via* hER α by E₂ in a dose dependent manner. COS-1 cells (2×10^6 cells/10 cm dish) were transfected with 9.6 μ g DNA (0.8 μ g hER β expression plasmid (pcDNA3-hER α), 8 μ g ERE-containing promoter reporter (ERE.vit2.luc) and 0.8 μ g pCMV- β -galactosidase).

Numerous parameters were investigated to obtain a dose response curve similar to that of hER β . The optimization for transactivation *via* the hER α was extensive and not all parameters are showed, i.e. all the different amounts of DNA or ratio's transfected. Parameters that were investigated included (i) total DNA and ratios of reporter to receptor DNA transfected (Figure 3); (ii) types of ERE driven promoters (Figure 4); (iii) cell lines (Figure 5); (iv) expression vectors for the hER α (Figure 6); and (v) transfection medium (phenol red medium *vs* medium without phenol red) (Figure 7). Firstly, if the difference between vehicle control and induction by 10^{-5} M E₂ is compared (Figure 3), the induction of luciferase activity was only minimal at all the different total DNA concentrations and different ratios of reporter to receptor investigated. Similarly, minimal induction was induced, insufficient for obtaining full dose response curves when different promoter reporter constructs (3xERE.tata.luc and 2xERE.tk.luc) were investigated (Figure 4) or when different cells (HepG2) were used (Figure 5). Dose response curves were obtained when COS-1 cells were transfected with a different expression vector for hER α , pSG5-hER α *vs* pcDNA3-hER α (Figure 6) or when transfections were done in medium without phenol red (Figure 7). These results were, however, not reproducible and due to time constraints further efforts to optimise the transactivation assay with hER α had to be terminated.

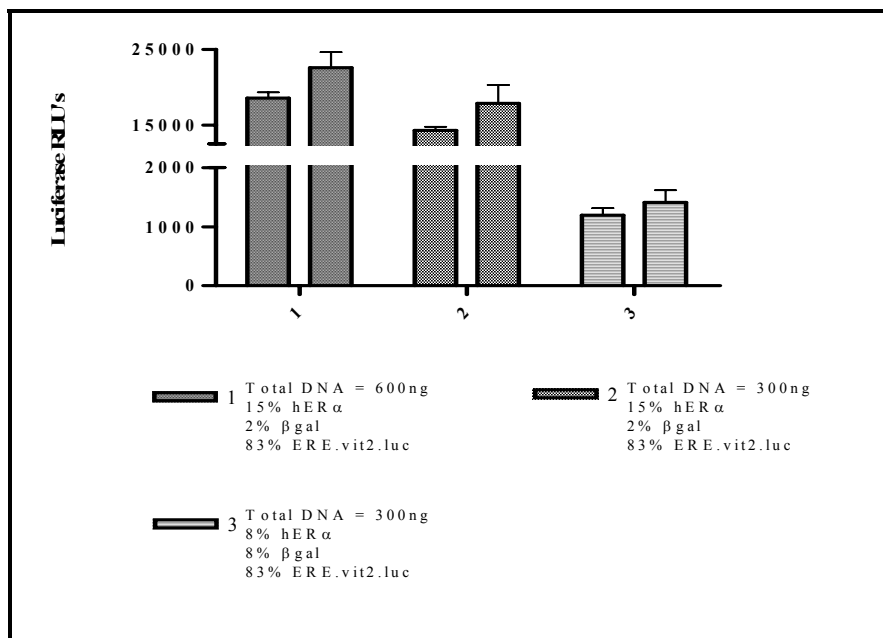


Figure 3: Different ratios of reporter to receptor and amount of total DNA used to transfect COS-1 (5×10^5 cells/well/ in 24 well plates). Control is 0.1% DMSO.

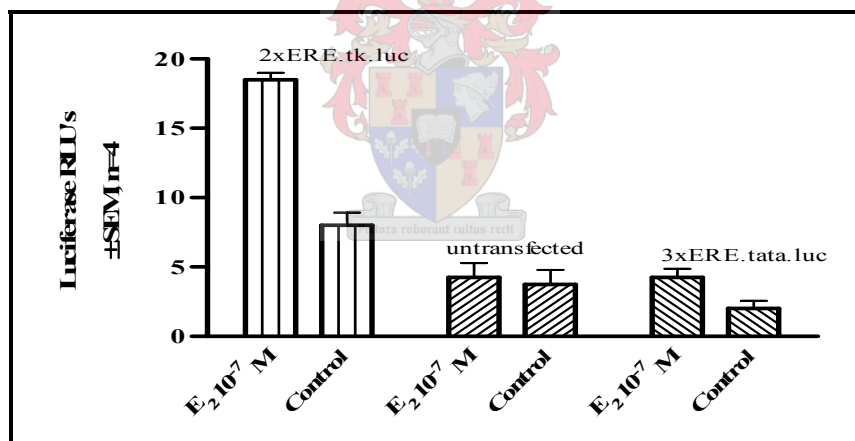


Figure 4: COS-1 cells (5×10^5 cell/well/in 24 well plates) were transfected with different types of ERE-containing promoter reporter constructs at 300 ng total DNA, with 60% promoter reporter construct, 33% hER α (pcDNA3-hER α) and 7% normalized vector (pCMV- β -galactosidase). Control is 0.1% DMSO vehicle.

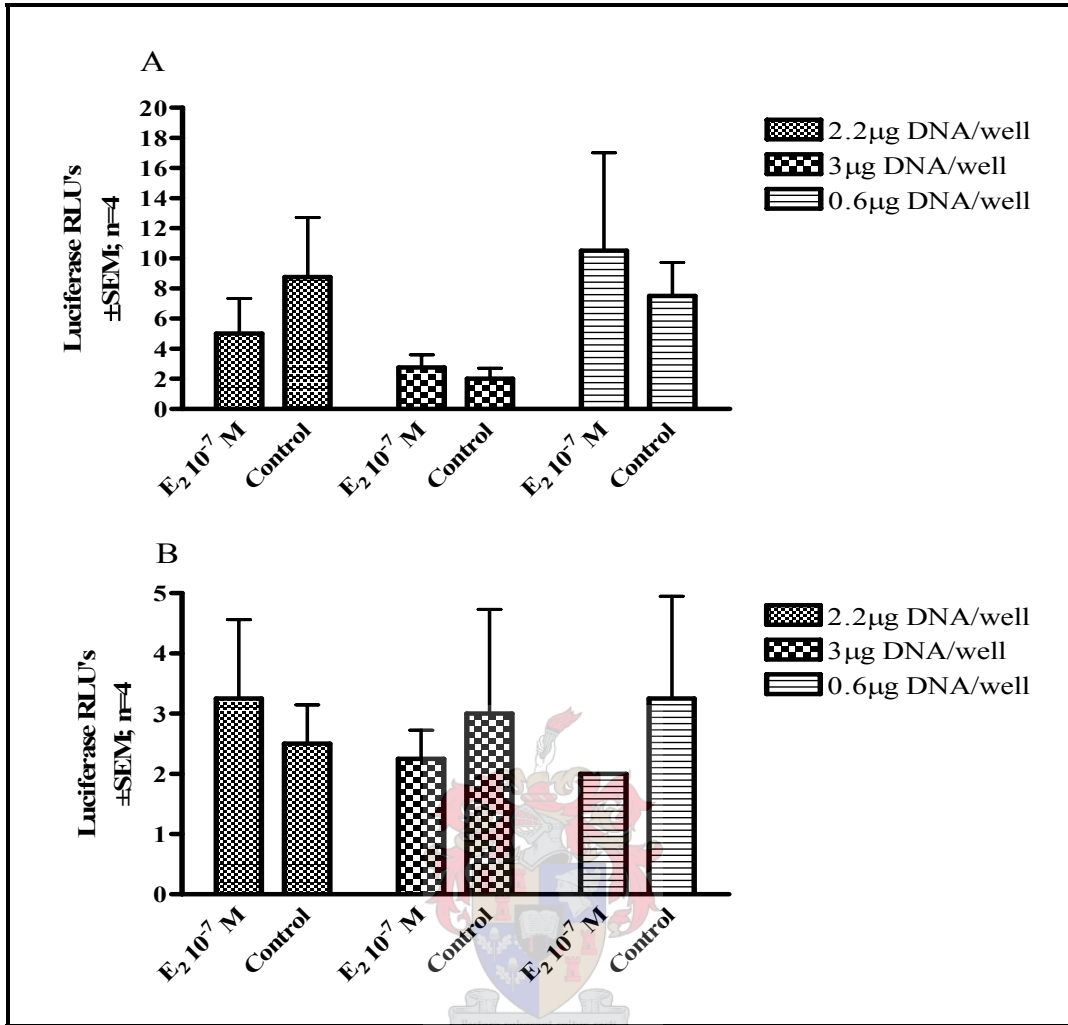


Figure 5: HepG2 cells (5×10^4 M cells/well/in 24 well plates) were transfected with various amounts of total DNA and with different promoter reporter constructs (A) 2xERE.tk.luc (B) 3xERE.tata.luc together with hER α (pcDNA3-hER α) and normalizing vector (pCMV- β -galactosidase). Control is 0.1% DMSO vehicle.

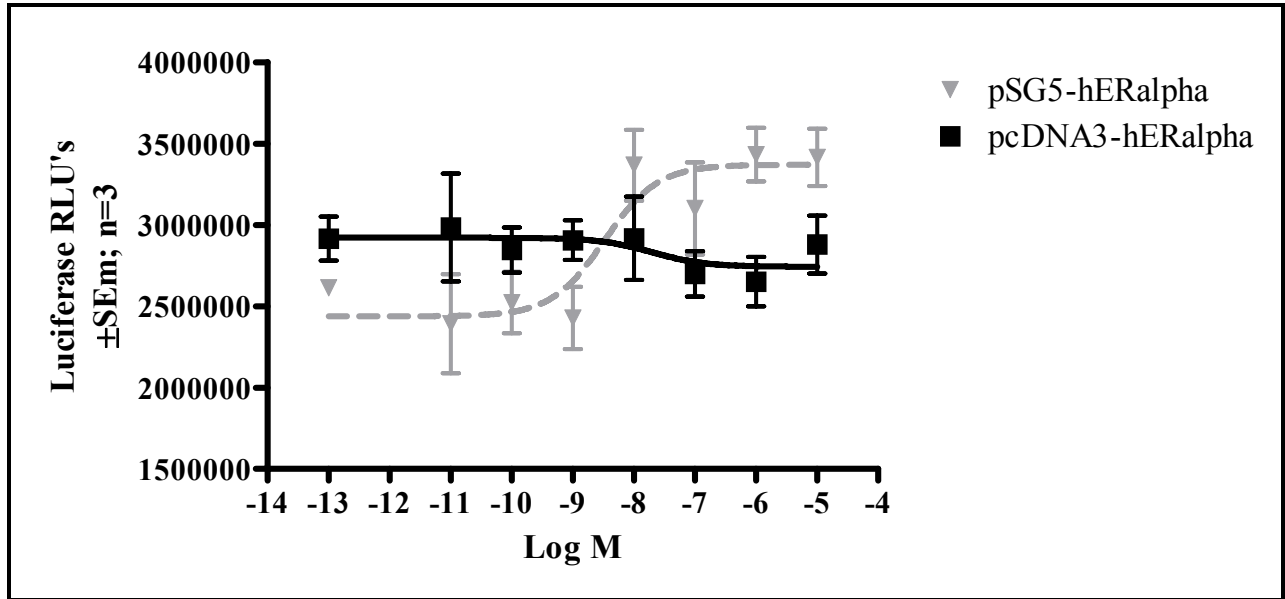


Figure 6: Different expression vectors for hER α . COS-1 cells were transfected with ERE.vit2.luc and either pcDNA3-hER α or pSG5-hER α . COS-1 cells (5×10^5 cells per well) were plated in 24 wells and transiently transfected with 300 ng DNA per well (6 ng ER expressing plasmid, 249 ng ERE.vit2.luc, 9 ng normalising vector and 36 ng filler vector).

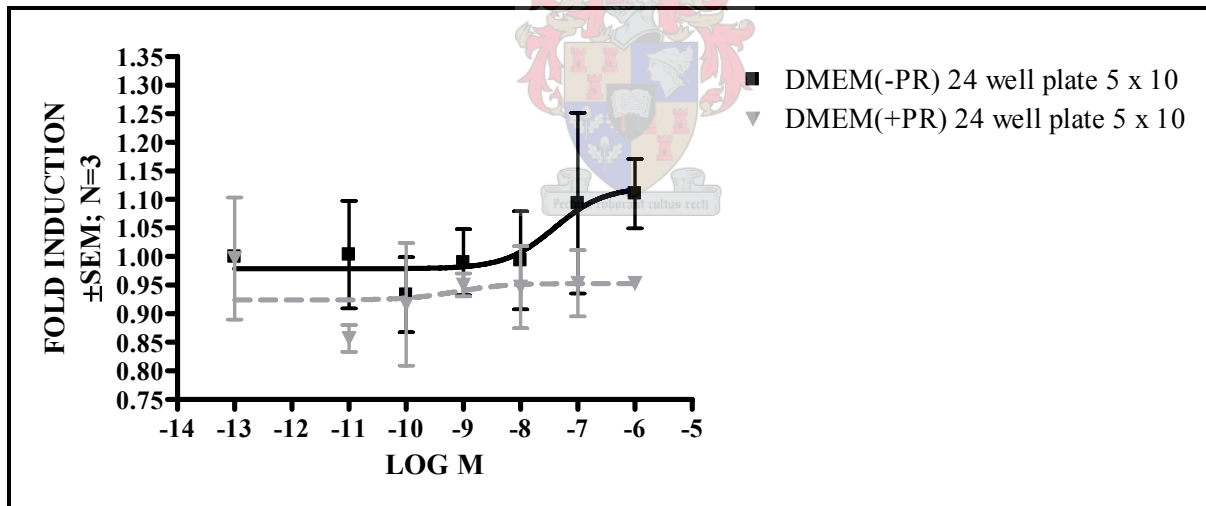


Figure 7: COS-1 cells transfected with ERE.vit2.luc and pSG5-hER α . Transfection occurred either in medium containing phenol red (+ PR) or without phenol red (-PR).

Addendum B

Method development for cell proliferation assay

Aim: To set up and optimise a cell proliferation assay for both MCF-7-BUS and MDA-MB-231 breast cancer cells, which would allow for the evaluation of estrogenicity of various plant polyphenols and unfermented *C. genistoides* methanol extracts.

The MCF-7-BUS cells were a kind gift from A Soto (Tufts University, Boston, Massachusetts, United States of America) who also included a much appreciated detailed protocol. These cells are reported to be extremely sensitive to E_2 ¹, however, when we followed the given protocol this was not evident (Figure 1). None of the days assayed showed any cell proliferation and from about day 3 onwards a decrease in cell number was noticed. It was then decided to steroid starve the MCF-7-BUS cells prior to induction by pre-incubating them for 3 days in medium without phenol red in an attempt to make them more sensitive to the induction by E_2 , with the rationale being that as the cells were maintained in full medium down regulation of ER may occur. In addition, after starving, cells were induced for different times (1-6 days). A full dose response curve was obtained after 2 days incubation with E_2 (Figure 2).

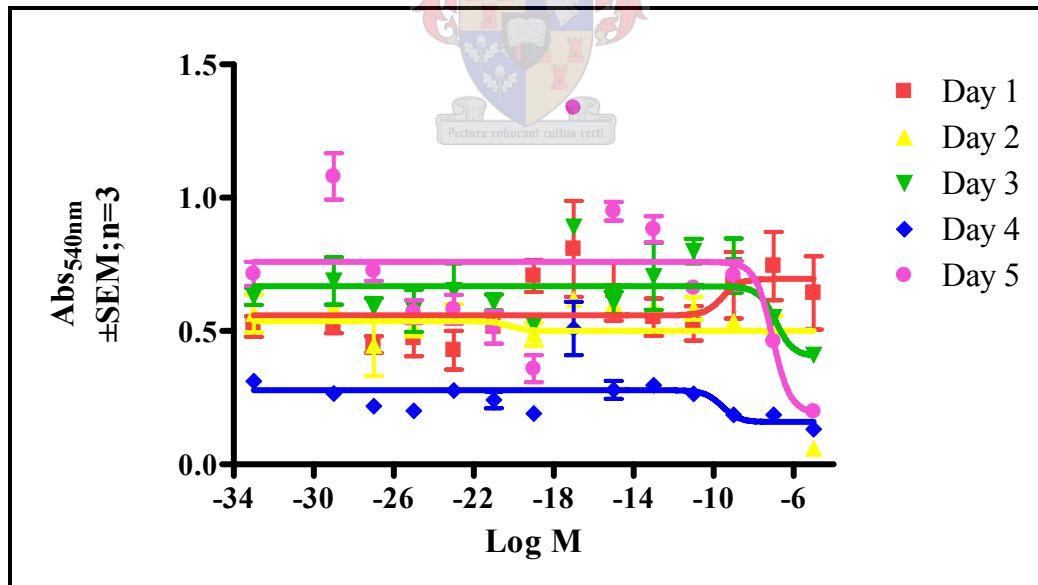


Figure 1: MCF-7-BUS cells were plated at a density of 2 500 cells per well in 96 well plates and the original cell proliferation protocol received from A.Soto was executed on various days as indicated on graph.

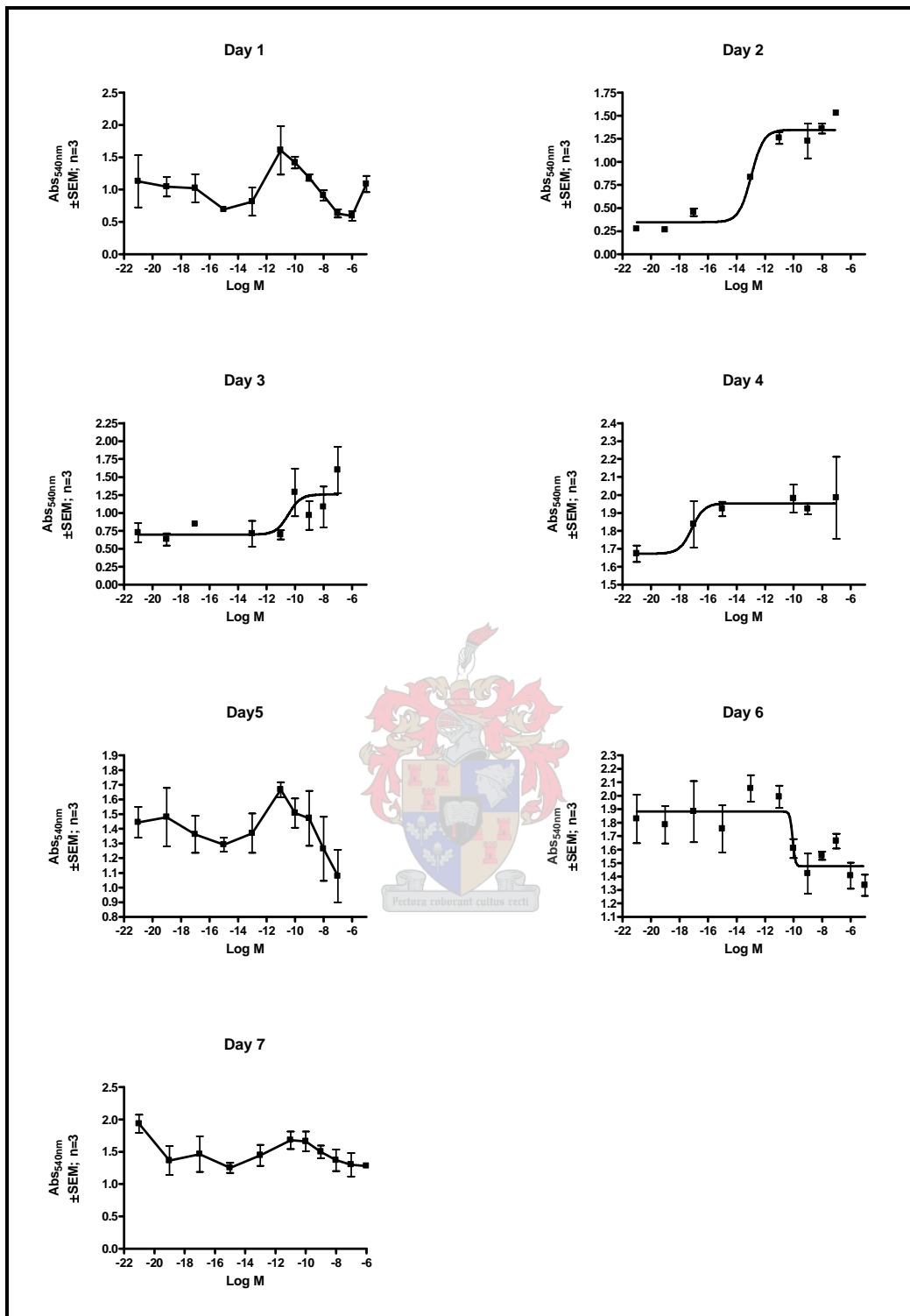


Figure 2: MCF-7-BUS cells were estrogen starved for 3 days followed by induction with E₂ for indicated days.

Cell proliferation may be measured using different techniques. We investigated two: MTT, which measures viable cells and SRB, which assays for total amount of protein. In comparing quantification of proliferation by MTT with that obtained using SRB (Figure 3) it was noted that the MTT assay gave a more reproducible result.

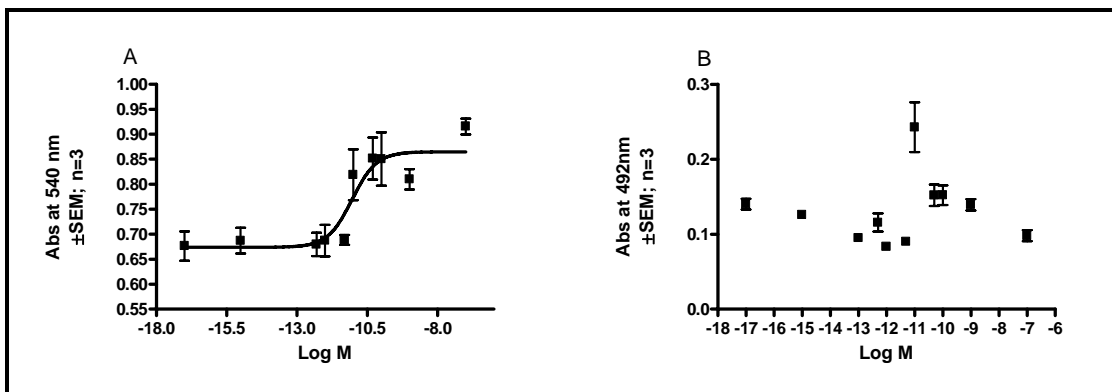
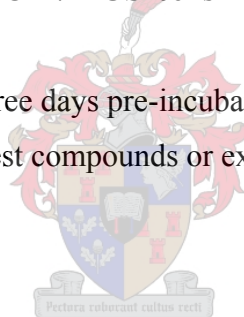


Figure 3: Cell proliferation of MCF-7-BUS cells measured with (A) MTT or (B) SRB.

In conclusion, it was decided on three days pre-incubation in medium without phenol red followed by two days induction with test compounds or extracts and measurement with MTT.



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List of Conference Abstracts

Conference Abstracts (Poster presentations)

N. J. D. Verhoog, E. Joubert, and A. Louw (2003) Estrogenic activity of Honeybush (*Cyclopia*) extracts. 18th Congress of the S.A. Society for Biochemistry and Molecular Biology, University of Pretoria

N. J. D. Verhoog, E. Joubert and A. Louw (2005) Phytoestrogenic activity in *Cyclopia genistoides*, 19th Congress of the S.A. Society for Biochemistry and Molecular Biology. University of Stellenbosch

N. J. D. Verhoog, E. Joubert and A. Louw (2005) Phytoestrogenic activity in *Cyclopia genistoides*. SAAFoST 18th Biennial International congress, University of Stellenbosch

Conference Abstracts (Oral presentation)

Verhoog, N. J. D., Joubert, E., Louw, A. (2004) Evaluation of the phytoestrogen activity of Honeybush (*Cyclopia*). Indigenous Plant Users Forum, Clanwilliam

