Investigating the parts to understand the whole: Contribution of phenolic compounds to the desired estrogenic activity of SM6Met fractions from *Cyclopia subternata*

by
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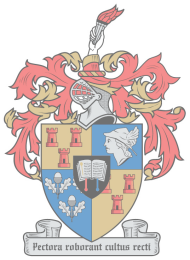

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Declaration

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Summary

Decreasing estrogen levels, associated with menopause, may cause several unpleasant symptoms. Hormone replacement therapy (HRT) relieves these symptoms, but increases the risk of developing breast cancer. As estrogens promote estrogen-receptor positive (ER^+) breast cancer, women are seeking alternative forms of hormone therapy and are turning to plant-based products high in phytoestrogens, since they are considered to be a more natural, healthier and potentially safer alternative to HRT. Another alluring aspect of plant-based products are their ability to exert their effect through multiple target mechanisms, which is potentially due to the complex mixture of different bio-active and supporting compounds working together. The current study forms part of a larger ongoing study, which focuses on *Cyclopia,* an indigenous South African fynbos plant, as a potential nutraceutical to reduce menopausal symptoms, while decreasing the risk of breast cancer. The sequential methanol extract of *Cyclopia subternata* (SM6Met) displayed promising results in different *in vitro* and *in vivo* breast cancer experimental systems, with the desired estrogenic characteristics of ER subtype selectivity, specifically ERβ agonism and ERα antagonism (which is important in ER^+ breast cancer since ERα is known to induce proliferation, whereas ERβ antagonises ERα), and the inhibition of estrogen-induced breast cancer cell proliferation. However, the compounds responsible for the desired estrogenic characteristics have not been identified and thus, as a starting point, we focused on the 7 major phenolic compounds quantified in a fraction, F3, obtained from SM6Met. F3 previously displayed robust ERβ agonism, and the aim of the current study was thus to evaluate the combinatorial effect of these 7 major phenolic compounds to the favourable estrogenic profile of F3. Firstly, an experimental system sensitive enough to detect small drug induced changes was successfully established, after which the phytoestrogenic activity of the 7 major phenolic compounds were tested individually and in combination. Interestingly, the reconstituted faction

(F3R), in contrast to F3, displayed no significant estrogenic activity when the 7 major phenolic compounds were reconstituted at a concentration equivalent to that which is present in F3. However, when F3R was reconstituted at 100-fold its concentration, a significant increase in activity was observed via both ER subtypes. Mangiferin and luteolin were the only two phenolic compounds that displayed significant and dose-dependent l estrogenic activity when tested alone. Since, at this stage of the study, not much was known regarding the estrogenicity of mangiferin, a more in detailed investigation revealed activity via ERα and also, as a novel finding, via ERβ. The current study concluded that F3's robust ERβ agonist activity could not be recreated using only the 7 major phenolic compounds at their concentration in F3 but that the minor phenolic compounds play a crucial supporting role in the desired estrogenic profile of this fraction. Thus, the contribution of the 7 major phenolic compounds to F3's activity, and subsequently the multi-target activity of SM6Met, should not be dismissed, since other compounds present in F3, but not in F3R, may act synergistically with the major phenolic compounds to produce the robust ERβ activity of F3.

Opsomming

Die vermindering van estrogeenvlakke, wat met menopouse gepaard gaan, kan verskeie onaangename simptome veroorsaak. Hormoon-vervangingsterapie (HVT) verlig hierdie simptome, maar verhoog die risiko om borskanker te ontwikkel. Aangesien estrogeen-reseptor positiewe (ER^+) borskanker deur estrogeen bevorder word, soek vrouens alternatiewe vorme van hormoonterapie en wend hulle na plantgebaseerde produkte wat hoog is in fitoestrogene, aangesien fitoestrogene beskou word as 'n meer natuurlike, gesonder en potensieel veiliger alternatief teenoor HVT. Nog 'n aanloklike aspek van plantgebaseerde produkte is hul vermoë om hul effek uit te oefen deur verskeie teiken meganismes, wat moontlik te wyte is aan die komplekse mengsel van verskillende bio-aktiewe en ondersteunende verbindings wat saamwerk. Die huidige studie vorm deel van 'n groter deurlopende studie wat fokus op *Cyclopia*, 'n inheemse Suid-Afrikaanse fynbosplant, as 'n potensiële nutraceutiese middel om menopousale simptome te verminder, terwyl die risiko van borskanker verlaag word. Die sekwensiële metanol ekstrak van *Cyclopia subternata* (SM6Met) het belowende resultate in verskeie *in vitro* en *in vivo* borskanker eksperimentele stelsels getoon, met die gewensde estrogeen eienskappe van ER subtypeselektiwiteit, spesifiek ERβ-agonisme en ERα-antagonisme (wat belangrik is in ER^+ borskanker, aangesien ERα bekend is om proliferasie te bewerkstellig, terwyl ERβ vir ERα antagoniseer) en die inhibisie van estrogeen-geïnduseerde borskanker proliferasie. Die verbindings wat verantwoordelik is vir die gewensde estrogeen eienskappe is egter nog nie geïdentifiseer nie, en dus as ‘n begin punt, word daar gefokus op die 7 hoof fenoliese verbindings wat gekwantifiseer is in 'n fraksie, F3, verkry uit SM6Met. F3 het voorheen robuuste ERβ-agonisme getoon, en die doel van die huidige studie was dus om die kombinatoriese effek van hierdie 7 hoof fenoliese verbindings te evalueer op die gunstige estrogeenprofiel van F3. Eerstens was 'n eksperimentele stelsel daargestel wat sensitief genoeg is om klein middel-

geïnduceerde veranderings suksesvol op te tel, waarna die fitoestrogeen aktiwiteit van die 7 hoof fenoliese verbindings individueel en in kombinasie getoets is. Interessant genoeg, het die hersaamgestelde fraksie (F3R), in teenstelling met F3, geen beduidende estrogeen aktiwiteit getoon nie toe die 7 hoof fenoliese verbindings gekombineer is by 'n konsentrasie gelykstaande aan wat in F3 teenwoordig is. Toe F3R egter teen 100-voudige konsentrasie hersaamgestel is, is 'n beduidende toename in aktiwiteit waargeneem via beide ER subtipes. Mangiferin en luteolin was die enigste twee fenoliese verbindings wat beduidende en dosis afhanklike estrogeen aktiwiteit getoon het toe hulle alleen getoets is. Aangesien daar op hierdie stadium van die studie nie veel bekend was omtrent die estrogenisiteit van mangiferin nie, het 'n meer gedetailleerde ondersoek aktiwiteit via ERα, en ook as 'n nuwe bevinding via ERβ, aan die lig gebring. Die huidige studie het tot die gevolgtrekking gekom dat F3 se robuuste ERβ-agonistiese aktiwiteit nie herskep kon word deur slegs die 7 hoof fenoliese verbindings by hul konsentrasie in F3 te gebruik nie, maar dat die ander fenoliese verbindings 'n belangrike ondersteunings rol speel in die gewenste estrogeenprofiel van hierdie fraksie. Dus, moet die bydrae van die 7 hoof fenoliese verbindings tot F3 se aktiwiteit, en die daaropvolgende multi-doelaktiwiteit van SM6Met, nie gering geskat word nie, aangesien ander verbindings teenwoordig in F3, maar nie in F3R, sinergisties mag optree met die hoof fenoliese verbindings om die robuuste ERβ aktiwiteit van F3 te produseer.

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List of Abbreviations

^{3}H-E_2	Tritiated 17-β estradiol
2-AAF	2-acetylaminofluorene
ABTS•+	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid
AD	anno Domini
AF-1	Activation function-1
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of variance
AOM	Azoxymethane
BC	Before Christ
BRCA	Breast Cancer
BSA	Bovine serum albumin
CAF	Central Analytical Facilities
CAM	Complementary/alternative medicine
cAMP	Cyclic adenosine monophosphate
CANSA	Cancer Association of South Africa
CPM	Counts per minute
DBD	DNA binding domain
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

E_1	Estrone
E_2	17-β Estradiol
E_3	Estriol
EAc	Ethyl acetate
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ER^+	Estrogen receptor positive
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERE	Estrogen response element
ERK	Extracellular signal-regulated kinase
ERm	Membrane-bound estrogen receptor
ESI	Electrospray ionization
Eth/EtOH	Ethanol
F1	Fraction 1
F2	Fraction 2
F3	Fraction 3
F3R	Reconstituted Fraction 3
FCS	Fetal calf serum

FRAP	Ferric Reducing Ability of Plasma
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GF	Growth factor
GSH	Glutathione
GSSG	Glutathione disulfide
HIV	Human immunodeficiency virus
HPCCC	High performance counter-current chromatography
HRP	Horseradish peroxidase
HRT	Hormone replacement therapy
ICI	fulvestrant (ICI 182,780)
ICR	Institute of Cancer Research
IGF-1R	Insulin-like growth factor 1 receptor
Iri	Iriflophenone
Isoman	Isomangiferin
kDa	Kilo Dalton
LB	lysogeny broth
LBD	Ligand binding domain
Luc	Luciferase
Lut	Luteolin
Man	Mangiferin
MAP	Mitogen-activated protein

Met	Methanol
MNU	*N*-Methyl-*N*-nitrosourea
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MS	Mass spectrometry
N	Non-sequential extracts
NMR	Nuclear magnetic resonance
NPF	Non-polar fraction
NSB	Non-specific binding
ORAC	Oxygen Radical Absorbance Capacity
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
p-Cou	*p*-Coumaric acid
PDA	Photo diode array
PF	Polar fraction
PKA	Protein kinase A
PRSCF	Polyphenol rich seed coat fraction
Proto	Protocatechuic acid
PTEN/MMAC1	Phosphatase, tensin homologue/mutated in multiple advanced cancers
QTOF	Quadrupole time-of-flight
RLU	Relative light unit
ROS	Reactive oxygen species

S	Sequential extracts
SB	Specific binding
Scoly	Scolymoside
SDS	Sodium dodecyl sulphate
SE	Standard error
SED	Standard error of a difference
SERM	Selective estrogen receptor modulator
SM6Met	Sequential methanol extracts of *Cyclopia subternata* harvesting M6
STZ	Streptozotocin
TB	Total binding
TBS	Tris-buffered saline
TBST	Tris-buffered saline tween-20
TF	Transcription factor
TCM	Traditional Chinese medicine
TEAC	Trolox Equivalent Antioxidant Capacity
UPLC	Ultra-performance liquid chromatography
Wat/dH_2O	Distilled water
WHO	World Health Orginisation

To

My mother and father, Estelle and Herman Claassen, thank you for all your love, support and encouragement. I am eternal grateful for the sacrifices you made for me.

Table of Contents

Chapter 1: Introduction

Estrogens are steroid hormones that play an important role in the development, functioning and maintenance of various systems including the reproductive, immune, musculoskeletal, cardiovascular, and central nervous system [1–6]. The different endogenous estrogens: estrone (E_1), estradiol (E_2) and estriol (E_3), have tissue specific roles and their concentrations in the female body vary during different stages of a woman's life (puberty, menstrual cycle, pregnancy and menopause), with E_2 being the most abundant and potent estrogen [3,7–15]. Therefore, a decrease in endogenous estrogens, associated with the onset of menopause, causes many unpleasant vasomotor symptoms such as hot flashes and night sweats, and could also lead to the development of osteoporosis, cardiovascular diseases, mental diseases such as Schizophrenia and Alzheimer's disease, and strokes [4,15–21].

Hormone replacement therapy (HRT) was for many years considered to be the best treatment for menopausal symptoms and related diseases, until numerous clinical studies showed that the intake of exogenous or synthetic estrogens have various risks and side effects, including, but not limited to, weight gain, irregular bleeding, headaches, breast tenderness and pain, depression, and the promotion of estrogen-related cancers such as breast cancer [15,22–29].

Breast cancer is the most common occurring cancer in women, and although breast cancer statistics vary between different population groups, ethnic groups and ages, this epidemic has a social and economic impact on both developed and developing countries [25,30–40]. South Africa is no exception, with breast cancer ranking top of the list of cancers among South African women [41,42]. According to the Cancer Association of South Africa (CANSA), the lifetime risk of developing breast cancer for South African women of all races average 1 in every 29 [43]. Estrogen receptor (ER) positive breast cancer is the most common type of breast cancer, and many of the risk factors associated with ER positive breast cancer (obesity, early menarche, the use of hormone contraceptives, late birth of first child, late menopause) are linked to estrogens [30,44–47]. Also, with a higher occurrence of breast cancer after the

age of 50, women are seeking safer alternatives for the treatment of menopausal symptoms and related diseases, with reduced risk of developing breast cancers [30,32,48–51].

Estrogens (endogenous and exogenous) mediate their intracellular effects through the two ER's, ERα and ERβ [6,52,53]. The amino acid sequence difference in the structural (A to F region) and functional (DNA binding, ligand binding, AF-1 and AF-2) domains between ERα and ERβ indicate that these two receptors have different physiological functions [52–55]. ERα is known to induce cell proliferation, whereas ERβ has shown anti-proliferative activity by antagonising ERα [55–58]. Most therapeutic targets for estrogen related diseases, such as breast cancer, focus on compounds that mediate their effects directly or indirectly through ERα [52,54,59,60]. Specifically, for breast cancer, the current hormonal therapy for ER positive breast cancer focuses on tamoxifen (a selective estrogen receptor modulators (SERM)) as 1st line of therapy, and fulvestrant (a full ER antagonist) as a follow up therapy when tamoxifen fails [60–65]. However, the current therapies for breast cancer have side effects and often result in therapy resistance. Thus, new therapeutic targets are needed [65–68]. ERβ is considered an eligible molecular target due to the 'yin yang' relationship that ERα and ERβ display and, therefore, compounds that mediate their effects via ERβ (potentially inhibiting ERα), are of great interest in novel ER related therapy development [57,68–70].

Plant-based products high in phytoestrogens (e.g. soy or red clover) are considered to be a more natural, healthier and potentially safer alternative to HRT [71]. Phytoestrogens are structurally similar to endogenous estrogen, and therefore, can exert an estrogenic and/or anti-estrogenic effect directly or indirectly via the ERs [72–75]. Many phytoestrogens have been shown to mediate their effect through ERβ, making these compounds desirable as novel phytoestrogenic nutraceuticals [68,74,76,77]. Various studies have also linked plant extracts and plant-based diets to lower prevalence of cancer, including breast cancer, and they have

been shown to have protective properties against osteoporosis, type II diabetes, cardiovascular-, immune-, and neurological diseases [71,78–87].

Combination therapies using different phytocompounds have also been shown to be more effective in treating complex diseases, such as cancer, compared to single active phytocompounds, suggesting that the mixture of compounds interact in an additive or synergistic manner to elicit a multi-target mode of action [88–94]. The higher effectiveness of medicinal plants and plant extracts is thought to be due to the complex mixture of different (active and supporting) phytocompounds, with the supporting compounds contributing to the stability, solubility, bioavailability, absorption and/or potency of the active compounds [87,92,95,96]. However, plant extracts and plant products still need to be used with caution, since the phytochemical interactions and the molecular mechanisms of all the different phytocompounds are not as well studied as single active compounds or synthetic drugs [97,98].

The current study forms part of a larger ongoing study, which focuses on *Cyclopia*, an indigenous South African fynbos plant that has displayed nutraceutical potential with phytoestrogenic, anti-mutagenic, anti-inflammatory and anti-diabetic activity [99–105]. A decade of *Cyclopia* work has been conducted within our research group, initiated by a study done by Verhoog *et al.* [106,74,100], who investigated the phytoestrogenic activity of different *Cyclopia* species: *C. intermedia*, *C. sessiliflora*, *C. genistoides* and *C. subternata*, with the latter two species displaying the highest phytoestrogenic activity. In addition, Mfenyana *et al.* [107], investigated different harvestings of *C. genistoides* and *C. subternata*. The air-dried dichloromethane plant extracts were prepared in three different ways: sequential extracts (S), non-sequential extracts (N) and cup-of-tea extracts, and five solvents were used based on polarity differences: ethyl acetate (EAc), ethanol (Eth), methanol (Met), 50% methanol–distilled water (Hlf) and distilled water (Wat). The sequential methanol

extract from *C. subternata* harvesting M6 (SM6Met) produced both high yield and high phytoestrogenic potency in the different experimental systems (promoter-reporter assay, whole cell ER-binding, alkaline phosphatase activity and E-screen) used in the study [107,108]. Furthermore, in a study by Visser *et al.*, SM6Met displayed ERα antagonism, ERβ agonism, and inhibition of E_2-induced breast cancer cell proliferation *in vitro*, as well as ERα antagonism *in vivo* by delaying ERα induced uterine growth in an immature rat uterotrophic model [109,105,81]. These favourable estrogenic attributes of SM6Met led to an *in vivo* study by Visser *et al*. where SM6Met reduced tumour frequency, mass and volume in a *N*-Methyl-*N*-nitrosourea (MNU)-induced rat mammary gland carcinogenesis model [105].

To fully evaluate SM6Met's multi-target mode of action, the extract was fractionated and the contribution of all the fractions of SM6Met to its favourable estrogenic attributes was investigated. Specifically, SM6Met was fractionated by Mortimer *et al.* [90,110], using liquid-liquid fractionation to obtain a polar fraction (PF) and a non-polar fraction (NPF), with the NPF retaining all the favourable estrogenic attributes of SM6Met. The NPF was then fractionated into 3 fractions (F1, F2 and F3) using high performance counter-current chromatography (HPCCC) and the phenolic content of these fractions were identified and quantified with liquid chromatography tandem mass spectrometry (LC-MS/MS) and quantitative HPLC, respectively. SM6Met's favourable estrogenic profile was, however, divided between the fractions with F1 and F2 displaying ERα antagonism and F3 displaying robust ERβ agonism. Further research is needed to understand how the different phenolic compounds in each fraction contribute to that fraction's favourable estrogenic profile.

The current study continues on from Mortimer's findings and focusses on F3, since compounds that mediate their activity via ERβ, and subsequently inhibit ERα activity, are of great interest in novel treatment development for estrogen-related diseases [56,57,68,76]. Mortimer identified 15 phenolic compounds in F3, however, only 7 of these compounds were

quantified [90]. The main focus of the current study is to investigate the combinatorial or possible synergistic contribution of these 7 major phenolic compounds in F3 to the favourable estrogenic profile of F3.

The Aims of the current study therefore are to:

1. Develop an estrogenic assay with increased sensitivity to detect compound-induced changes, and subsequently establish higher accuracy when investigating the interaction of compounds and possible synergism.
2. Reconstitute F3 (F3R) using only the 7 major phenolic compounds (mangiferin, isomangiferin, luteolin, scolymoside, iriflophenone-3-*C*-glucoside, protocatechuic acid and *p*-coumaric acid) as a starting point to investigate their contribution to F3's activity.
3. Investigate the estrogenic activity of the individual phenolic compounds in F3R to establish which of the compounds are the active estrogenic compounds.
4. Use addition and subtraction studies to investigate the supporting role of the non-estrogenic phenolic compounds in the estrogenic activity of F3R.
5. Investigate combinatorial or synergistic activity of the estrogenic phenolic compounds using a fixed ratio isobologam.

The remainder of the dissertation consists of five chapters. The literature review **(Chapter 2)** will touch on the history and evolution of traditional and modern medicine, the limitations of modern medicine in combating complex multifaceted diseases, such as cancer, and the future of phytomedicine and intelligent drug mixtures. Furthermore, an overview of the molecular mechanisms and related pathways of phytocompounds, specifically phytoestrogens, will be discussed. The literature review will also include a section on *Cyclopia* and related phytocompounds used in the current study. In addition, the literature review will elaborate on the concept of synergism, including the advantages of synergism in therapy development and

methods to determine combinatorial or synergistic drug interactions. **Chapter 3** contains the materials and methods used in the current study. **Chapter 4** addresses Aim 1 and focusses on the optimisation and validation of two *in vitro* experimental systems, the cell proliferation assay in MCF-7 BUS cells and promoter reporter studies in HEK293 cells, with the purpose of establishing an experimental system with increased sensitivity, which is especially important when testing drug interactions and possible synergism. **Chapter 5** addresses Aims 2-5, and discusses the limitations of the current study including the susceptibility of phenolic compounds and tissue culture systems to external changes. The last chapter **(Chapter 6)** contextualise the results from the current study into the bigger scope of phytomedicine and addresses future work.

With the current findings being but a small piece of a larger puzzle, investigating the contribution of the 7 major phenolic compounds in F3 will provide insight into the desired ERβ agonist activity of this fraction and reveal the complexity of the combinatorial activity of plant extracts.

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Chapter 2: Literature Review

2.1. Introduction

Plants have for centuries played a vital role in human health and wellbeing, not only as a source of shelter and food, but also for the treatment of various ailments and diseases [1–5]. Ancient civilisations developed phytomedical systems by observing nature and using a trial and error approach, through which they determined whether a plant was beneficial or toxic [1–3]. Some of these findings were documented and the knowledge passed on from generation to generation [6–11]. Ancient phytomedicine* has formed the foundation upon which modern medicine is built, contributing to the discovery and development of numerous drugs [1–3,5,12–14].

The last few decades, medical and scientific research have made great strides in understanding the dynamics of health and the mechanisms of disease [15–18]. This increase in biological knowledge revealed the complexity of the human body, the fragility of its various intricate systems, and identified the multiple contributing factors which builds and shapes our physiological composition as humans [15–20].

As drug development progressed, the main focus shifted from using whole plant extracts towards identifying active ingredients, investigating their mechanisms of action, and using these active ingredients to our advantage in the fight against complex diseases, such as cancer [13,21,22]. But with new found information and techniques, came new challenges such as drug resistance, dangerous side effects and decreased drug efficacy, and this method of reductionism (as it is referred to in an interesting review article by Corning [16]), was revealed not to be the ideal approach in treating complex diseases [23–27].

* Phytomedicine is also known as botanic medicine, herbal medicine or ethnomedicine [56,167,168]

The current study falls within the bigger scope of re-discovering the medicinal power of plants and whole plant extracts, since combination therapies, inspired by the unique mixture of phytocompounds in medicinal plants, have been shown to be more effective than mono-therapies in combating complex or multifaceted diseases [28–33]. To narrow it down, the current study will focus on one specific plant of interest, *Cyclopia*, and its medicinal potential for combating estrogen related diseases, such as breast cancer. This indigenous South Africa plant has shown a variety of disease fighting properties, but the main interest of the current study is the phytoestrogenic activity of *Cyclopia*.

This literature review is divided into 5 sections: (1) the history and evolution of phytomedicine, (2) the concept of synergism (with specific reference to phytomedicine), (3) *Cyclopia*, (4) mechanism of action of phytoestrogens (with specific reference to the estrogen signalling pathway) and lastly (5) the major phenolic compounds in the *Cyclopia* SM6Met fraction.

2.2. History and evolution of phytomedicine

2.2.1. Ancient traditional medicine

The history of medicine is as old as mankind itself, and the earliest form of medicine was derived mainly from plants [3,4,9,34]. During pre-historical times, not much was known about the human body, and pre-historical civilisations relied strongly on nature, as well as their different religious/belief systems, for the treatment of various ailments and diseases [5,34]. Some cultures believed that diseases were caused by higher powers or spirits, and plants were not only used for treating physical ailments, but also in spiritual healing rituals [3,9,35–37].

Some of the oldest documentations of medicinal plants stem from ancient Egyptian, Mesopotamian and Greek traditions dating back to the 1600 BC (Figure 2.1) [9,37]. Clay tablets, scrolls and papyri gave insight into the herbal medicine used by these ancient groups, along with their discoveries regarding pathophysiology and the human anatomy [35,38,39]. A more rational approach to medicine arose and medical practices focused on the observation of symptoms and visible abnormalities, such as examining body fluids, listening to respiratory function and observing skin discoloration [35,38–40]. The Mesopotamians had two types of medical practitioners, the *ashipu*, who was responsible for diagnosis of ailments, and the *asu*, who was the specialist in herbal remedies [35]. The Egyptians were also considered to be excellent herbalists and the Ebers papyrus (dating from 1500 BC) contains 877 recipes and prescriptions for internal medicine, as well as skin and eye problems [3,39–41].

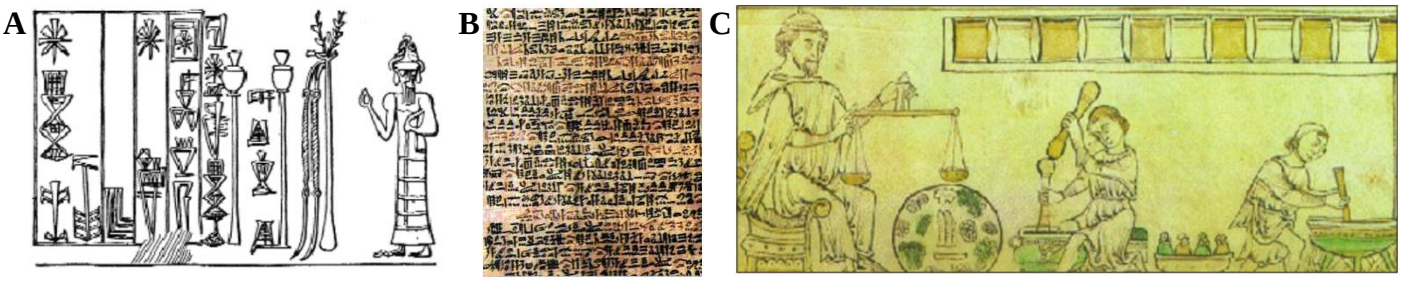

Figure 2.1: Demonstrations and documentations of ancient medicinal practices. (A) representation of a Mesopotamian medical practitioner, (B) fragment of Ebers Papyrus, and (C) monks processing and weighing herbs [35,37].

Furthermore, other traditional medical systems that arose independently in different parts of the world also had medicinal plants forming the centre of their medical practices. Some of these traditional medical systems include: traditional Chinese medicine (TCM) used in parts of Asia, Ayurveda used in parts of India, Native American traditional medicine used in North America, and different local systems in Africa (which strongly depends on demographic and different cultural beliefs) [2,5,8,41,42].

Ancient Chinese and Indian medicines had a more holistic approach to health and disease than the Egyptian, Mesopotamian and Greek systems, but were also known for their

extensive use of plant-based products [40,43]. They believed that disease was a result of an imbalance between the mind, body and spirit, and in order to restore balance and harmony, they used herbal remedies combined with physical and spiritual practices such as yoga, meditation and acupuncture [2,43]. The ancient texts of *Veda* describe the earliest use of medicinal plants in Ayurvedic medicine [2]. Likewise, the Chinese medicinal book, *Wu Shi Er Bing Fang*, consists of a list of approximately 250 natural agents and 150 combinatorial formulations for disease treatment [40]. Although ancient African traditional medicine (excluding traditional Egyptian medicine) is not as well documented compared to ancient phytomedicine from Europe and Asia, it is considered to be the most diverse of all medicine systems with influences from a variety of different cultures within Africa [2,44].

Since ancient times, various phytomedicinal books and manuscripts were published, describing and categorising the medicinal use of certain plants [35,37,38]. Dioscorides wrote the book, *De Materia Medica* (circa 77AD), wherein he categorised plants according to their physical appearance, locality, method of preparation, and medicinal properties [9,37,41]. His work provided the foundation for pharmacognosy, the study of drugs originating from plants or natural products [9,41]. Other examples of phytomedical books written during the middle ages included *Avicenna* (980-1037), *Herbarium Apuleius* (480-1050), *The Leech Book of Bald* (925), *Physicians of Myddvai* (1250), *Herbal* (Brunfels), *Herbal* (Brock) and *Herbal* (Mattioli) (1500-1577) [35,37]. There was, however, still a gap in knowledge regarding the plant's active components, mode of actions and the potential side effects the use of medicinal plants may have over time.

2.2.2. Introduction of science and development of modern medicine

The 17th and 18th century provided the stepping stones for modern medicine with the discovery of various physiological structures and functions (e.g. circulation of blood), the

increase in theoretical knowledge regarding diseases such as scurvy, rabies and smallpox, the introduction of opium and digitalis (from poppy seeds and foxgloves, respectively), and the invention of the microscope [3,5,35,38,45]. The field of pharmacognosy was also established during this period, since the demand for compound drugs increased [9,35,41]. Since the 18th century, phytomedicine moved in a more scientific systematic direction in order to identify active compounds and avoid past problem such as toxicity [40].

The most significant developments of modern medicine occurred during the 19th and 20th century. Progress during this period include the introduction of diagnostic techniques and new medical tools (X-ray, stethoscope, thermometer, ophthalmoscope and laryngoscope), better understanding of and novel detection methods for diseases such as cholera, tuberculosis, diphtheria and typhoid, isolation of alkaloids and glycosides from plants, as well as discovery of active substances such as tannins, vitamins, saponosides and etheric oils [38,41,46,47].

As medicine and related techniques evolved, so did the use of medicinal plants, and the focus shifted from using whole plant extracts to identifying and isolating active compounds in medicinal plants. Past challenges and gaps in phytomedicine knowledge opened up research opportunities, and evidence based research became the pioneers of drug development [45]. The 20th century was also marked as the beginning of the modern pharmaceutical industry, with the introduction of various synthetic versions of natural products [3,38,45].

The continued progress of modern medicine in the 21st century was reinforced by the improvements of high-tech screening and analytical methods such as high-performance liquid chromatography (HPLC), mass spectrometry (MS), and nuclear magnetic resonance (NMR), which, together with collaborations from various scientific fields including genomics, proteomics, metabolomics and bioinformatics, accelerated the identification, characterisation

and isolation of active compounds from medicinal plants [48–50]. However, in the beginning of the 21st century, most pharmaceutical companies moved away from natural products in favour of combinatorial and synthetic chemistry, which were able to reduce the development time of drugs as well as the complexity of the drugs developed, and provide a more comprehensive understanding of drug interactions [50,51].

Despite the scientific advances made in drug discovery, 25% of current modern drugs still originate directly or indirectly from medicinal plants, showing that plants continue to play an important role in the development of modern medicine [49,52]. In addition, during the last decade a gradual shift was observed from mono-drug therapies to multi-target therapies and multi-drug combinations, with an increased use of traditional and non-conventional medicine incorporated into modern medical practices [1,53–56]. A review article by Li *et al.*[57], refers to this transition as the Easternization of Western medicine, and proposes that this shift may help discover novel drug interactions and bridge the gap between traditional and modern medicine.

2.2.3. Traditional medicine in the 21st century

Looking back on how medicine has changed and evolved over time, it is apparent that medicinal plants and traditional knowledge have formed the foundation of modern medicine, and that even with the strides made in the medical and pharmaceutical industry, 65-80% of rural communities worldwide (predominantly in India, China, Africa and parts of South America) still rely on traditional medicine and medicinal plants as their primary source of health care [44,50,51,57–59]. The use of traditional medicine practices in the 21st century is fuelled by poverty, lack of pharmaceutical manufacturing capacity, and limited access to modern medicine [52,60,61]. Other factors like accessibility/availability, cultural influences, lack of effective treatments, and the use of phytomedicine for the relief of side effects caused

by modern medicine (e.g. chemotherapy, antiretroviral drugs, and malaria treatments), also play a role [44,51,59].

Scientific studies on the medicinal potential of plants are limited, with only 10-14% of all plant species being used for medicinal purposes [59,62]. However, some countries (such as Nigeria, China, India, and the USA), have made an effort to invest in phytomedical research, and have sparked a renewed interest in medicinal plants used in traditional medicine [62,63]. A paradigm shift is also occurring towards investigating whole plant extracts or phytomixtures, instead of focussing on single bioactive phytocompounds, which have, until recently, formed the majority of publications in the field of phytomedicine [54–56,63].

An article by Gwynn *et al*. [64], asks the very important question: "How can future opportunities be derived from traditional medicine?' Although there are many hurdles ahead for the integration of phytomedicine into modern medicine, combining traditional knowledge and modern techniques, could perhaps be the answer to our challenges in modern medicine regarding decreases in effectiveness, drug resistance, toxicity, and combating complex diseases such as AIDS and cancer [14,62,64,65]. In an age of advance communication and technology, integrating the knowledge of medicinal plants obtained from traditional practitioners, may potentially help identify medicinal plants for investigation faster, which in turn may help reduce the selecting and screening process [62,64]. Some traditional practitioners also have extensive experience in combining different phytocompounds or herbs in order to enhance the desired medicinal effects (also referred to as synergism) or fight disease on multiple levels [42,62].

2.3. The concept (theory and practice) of synergism

The higher effectiveness of whole plant extracts and multi-compound combinations, in comparison to single plant derived active compounds, is thought to be due to the synergistic effects of the mixture of different bio-active and supporting constituents [56,66–68] The supporting compounds themselves could be pharmacologically inactive, but contribute to the stability, solubility and/or absorption rate of the active compounds [68–70]. In addition, the multi-target potential of medicinal plant extracts may also be due to the complex mixture of different bio-active and supporting phytocompounds [56,68].

Among the first to define the concept of synergy, specifically pharmacological synergy, was Berenbaum in the 1980's [71]. He developed the isobole method, which is considered to be one of the easier methods to test for synergism, since it is a demonstrative method that it is also independent of the mechanisms of action [68,71,72]. The dose of the two individual compounds being compared that gives half maximal response (EC_{50}) is plotted separately on respectively the x and y axes of a graph. If the EC_{50} of combinations of the two compounds are plotted and connected on the graph and form a straight line, it will represent a combined or additive effect, meaning that there is no interaction between the two compounds, but that the combined effect is the sum of the two separate effects. If, however, there is an interaction between the two compounds, the "iso-effect" curve will be observed (Figure 2.3). Synergism is represented by a concave curve, with the combined effect of the two compounds being greater than the sum of the two separate effects. Antagonism is represented by a convex curve and the effect of the combination is less than the sum of the two compounds.

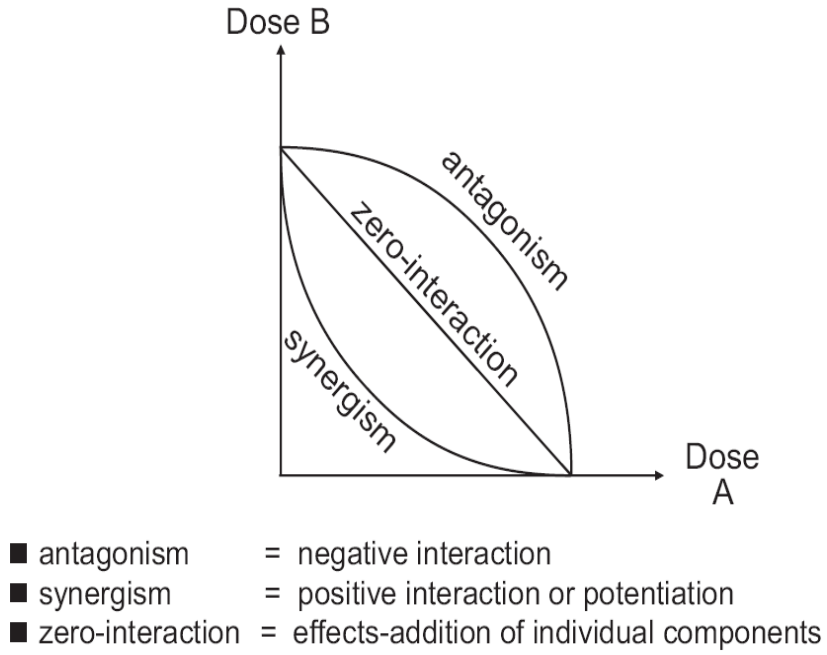

Figure 2.3: Isoboles for antagonism, synergism and zero-interaction as obtained from Wagner *et al.*, [73].

A different and less labour intensive approach to Berenbaums' isobole method would be using fixed ratio combinations. In a study done by Tallarida *et al.* [74], investigating synergism between cocaine and buprenorphine, two fixed-ratio combinations (1:1 and 1:3) were used to test for synergy. Similar to the isobole method, the EC_{50} concentrations of the two individual drugs being compared were plotted separately on the x and y axes of the graph and a line drawn though the two points. This line represents the theoretically additive combination doses. The additive EC_{50} values (z_{add}) are calculated from the individual drug EC_{50} concentrations according to the fixed-ratio combination used and plotted on the straight line, as can be observed in Figure 2.4 as point A (ratio of 1:1) and B (ratio of 1:3). Equation 1 and 2 (below) are used to calculate the additive values (z_{add}) and standard error (SE) for the 1:3 ratio. These equations may just be adjusted to apply to any ratio. The two drugs were then combined according to the fixed-ratios and the EC_{50} values of the combinations were determined. The individual drug concentrations in the combinations, at the EC_{50} value, were then plotted on the graph as seen in Figure 2.4 at point C (ratio of 1:1) and D (ratio of 1:3). The effect of the combined drug concentrations were less than the expected additive

concentrations, and therefore represent a synergistic effect. To determine synergism, it is therefore not necessary to determine multiple drug combination EC_{50}s to draw the curve as seen in Figure 2.3, [74].

$$z_{add} = (0.25 \times 10) + (0.75 \times 0.0175) = 2.51 \quad [1]$$

$$SE\ (z_{add}) = (0.25 \times 1.14)2 + (0.75 \times 0.00438)2 = 0.285 \quad [2]$$

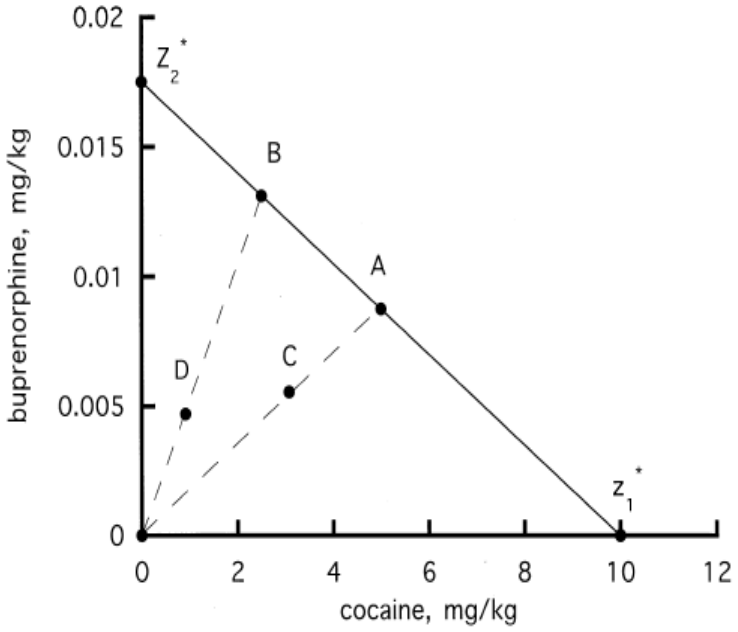

Figure 2.4: Isobologram showing synergism for the two fixed ratio mixtures of cocaine: buprenorphine as demonstrated in Tallarida *et al*, [74]. The EC_{50} of cocaine is 10 mg/kg (plotted on x-axis) and the EC_{50} of burprenorephine is 0.0175 mg/kg (plotted on y-axis). At point A, the drug ratio is 1:1 (5 mg/kg of cocaine: 0.00875 mg/kg of burprenorephine) and at point B the drug ratio is 1:3 (2.5 mg/kg of cocaine: 0.013125 mg/kg of burprenorephine). Point C and D represent the synergistic effect of the combined drugs as a ratio of 1:1 and 1:3, respectively. See text for further details.

Some of the advantages of synergy in phytotherapy or drug development include the multi-target effect, due to the presence of various active and supporting compounds, which in turn, also inhibits drug resistance [55,56,69,73,75,76]. Subsequently, a decrease in toxicity and fewer side effects are also reported, since lower concentrations are needed to exert an effect [56,69,73,75]. The focus of some drug combination studies are not only to inhibit the mechanisms contributing to a specific disease, but also to activate protective and repair mechanisms to promote self-healing and prevent recurrence of the specific disease [54,76]. In

a review article by Wagner *et al.* [73], the authors provide various examples of successful phytopharmaceutical drug combinations, including Iberogast® which is comprised of nine plant extracts and is able to combat functional dysplasia and motility-related intestinal disorders on multiple levels, the effectiveness of *Hypericum* compounds (Hypericin together with Epicatechin, Procyanidin, Hyperosid and/or Rutin) as an antidepressant compared to synthetic psychopharmacological drugs such as Imipramin, Flumazenil, Fluoxetin and Amitriptylin, and the efficacy of combination of the antimicrobial Amphotericin B together with a grape seed extract (*Vitis vinifera*) to double the survival rate of *Candida albicans*-infected mice compare a to Amphotericin B alone.

Within the scope of the current study, combinations of different phytoestrogens, at concentrations that may be reached physiologically, have previously been shown to enhance anticancer effects by exerting their effects through many different molecular mechanisms [69]. For example, Kumar *et al.* [30] investigated the synergistic effects of three phytoestrogens (genistein, quercetin and biochanin A) and found that these phytoestrogens in a select combination (1:1:1 at 8.33µM each) had a more potent inhibitory effect on androgen-responsive prostate cancer cell growth *in vitro* that the higher concentration (25µM) of the individual phytoestrogens on their own.

2.4. *Cyclopia* (Honeybush)

The genus *Cyclopia* (family: Fabaceae, tribe: Podalyrieae), more commonly known as honeybush due to its sweet honey-scented aroma, forms part of the indigenous South African fynbos biome and is predominantly found in the Eastern and Western Cape provinces (Figure 2.2) [77–80]. There are approximately 24 species of *Cyclopia* in the wild of which a few are commercially cultivated as herbal tea including, but not limited to, ‘kustee’ (*C. genistoides*), ‘vleitee’ (*C. subternata* & *C. maculata*), ‘bergtee’ (*C. intermedia*), and ‘Heidelbergtee’ (*C.*

sessiliflora) [77,78,81–86]. This woody leguminous scrub consists of small trifoliate leaves, with the leaf shape and size differing between species, and distinctive bright yellow flowers (Figure 2.5) [78,80,81]. The stem, leaves and flowers of the plant are harvested for the manufacture of fermented[†] and unfermented (green) honeybush tea [78,81,83,84,86,87]. Apart from its sweet aroma and taste, honeybush tea is also low in tannins and contains no caffeine, contributing to its increased popularity as a healthy beverage [77,78,83,88].

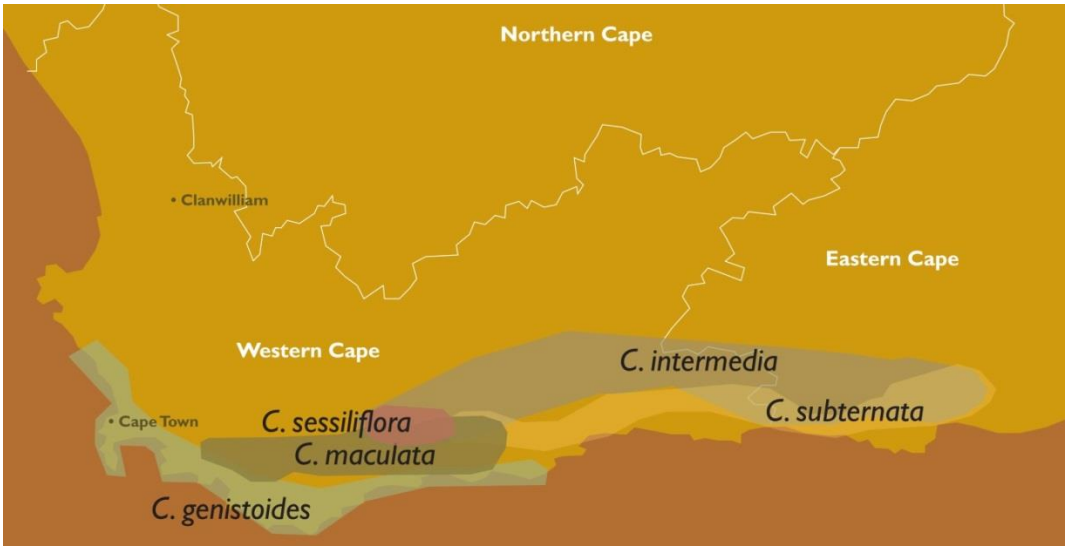

Figure 2.2: Distribution of some *Cyclopia* species in Western and Eastern Cape region [89].

The use of *Cyclopia*, together with *Aspalathus linearis* (rooibos) and *Agathosma betulina* (buchu) by humans has a long history, and anecdotal evidence suggest that these indigenous South African plants were used by native inhabitants, such as the Khoi-San, as traditional medicinal teas to treat various ailments including digestive disorders (loss of appetite, nausea, spastic colon, colic), respiratory infections and disorders (asthma, chronic catarrh, pulmonary tuberculosis), and to help ease arthritis pain, and stimulate milk production in lactating women [77,80,85,90–92]. Gradually, the medicinal role of *Cyclopia* became less important while interest in the agriculture and agro-processing industry grew [80,93]. The honeybush industry is still young and in its developmental stage, but it has established itself in the herbal

[†] Fermentation of tea entails temperature-controlled oxidation of the plant material to enhance flavour and contribute to the renowned golden brown colour of classical tea [78,81,169].

tea market [77,80]. Currently, 100-175 tons of *Cyclopia* is exported annually, mainly to the United States and Europe (the United Kingdom, Germany, the Netherlands and Poland) [86,94,95].

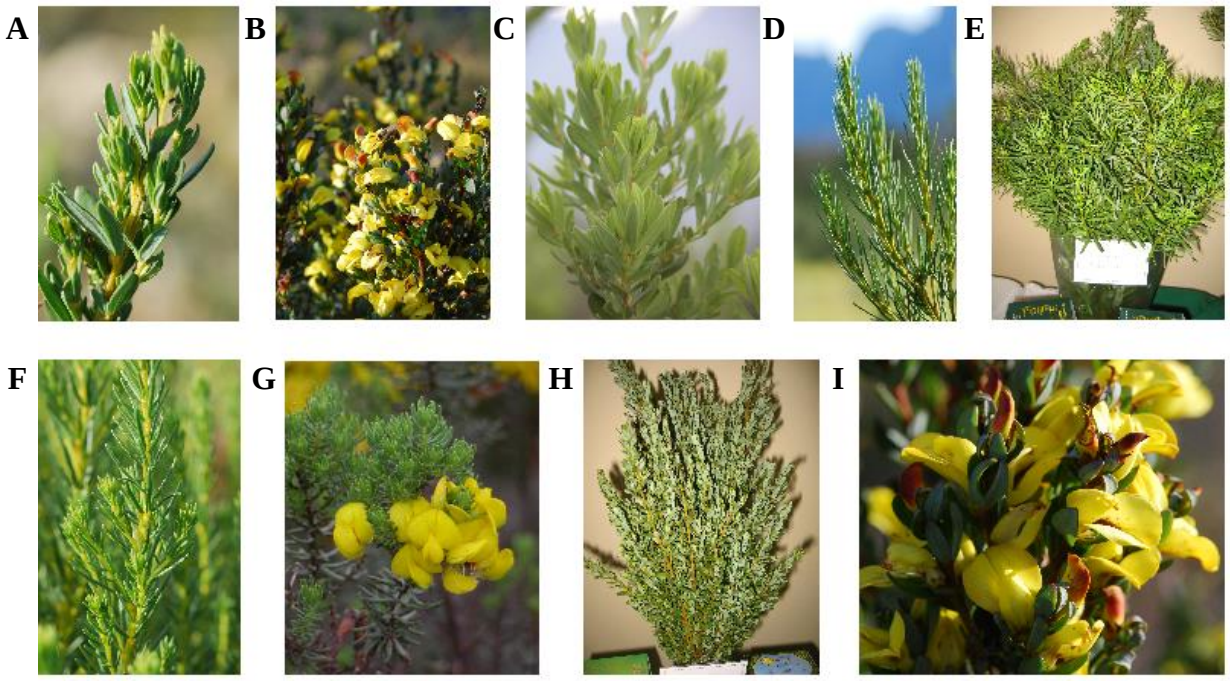

Figure 2.5: The leaves and flowers of different *Cyclopia* species. (A) *Cyclopia subternata* leaves, (B) *Cyclopia subternata* flowers, (C) *Cyclopia intermedia* leaves, (D) *Cyclopia maculata* leaves, (E) *Cyclopia longifolia* leaves, (F) *Cyclopia genistoides* leaves, (G) *Cyclopia genistoidses* flowers, (H) *Cyclopia sessilifora* leaves and (I) *Cyclopia sessilifora* flowers [89].

A global shift in health-conscious lifestyle and food choices have resulted in a renewed interest in the potential health benefits and medicinal properties of *Cyclopia* [77,90,96,97]. The 2015 Nielsen Global Health and Wellness survey showed that 88% of consumers worldwide (Latin America (94%), Asia-Pacific (93%), Africa/Middle East (92%), Europe (79%) and North America (80%)) say they are willing to pay more for food that have proven health attributes [98]. This health-conscious trend, together with growing scientific research regarding health benefits of herbal teas, could create a possible new market for *Cyclopia* as a source of nutraceutical and potential pharmaceutical phytocompounds, especially since this indigenous plant has shown a vast variety of health promoting and disease fighting properties including, but not limited to, antioxidant, anti-inflammatory, -diabetic and -mutagenic properties as well as chemo-preventive or anti-cancer activity [28,77,78,93,99–105].

2.4.1. Health benefits of *Cyclopia*

The bioactivity of the *Cyclopia* species has been investigated and demonstrated in various research papers, and *Cyclopia* extracts have shown promising results in *in vitro*, *in vivo* and *ex vivo* studies.

One of *Cyclopia*'s most prominent beneficial traits is its proven antioxidant activity. In a study by Dube *et al.* [106], the antioxidant capacity of different *Cyclopia* extracts were investigated using three methods: Oxygen Radical Absorbance Capacity (ORAC) assay, Trolox Equivalent Antioxidant Capacity (TEAC) assay and Ferric Reducing Ability of Plasma (FRAP) assay, and all the *Cyclopia* extracts, to some extent, displayed antioxidant behaviour by either scavenging free radicals, intercepting oxidation of oxidizable molecules, or preventing formation of reactive oxygen species (ROS). Similar antioxidant results were found in various other studies where different *Cyclopia* extracts and related phenolic compounds were able to inhibit Fe^{2+}-induced microsomal lipid peroxidation, capture free 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) radicals, and decrease oxidised glutathione (GSSG) in rat livers, thereby increasing the GSH/GSSG ratio, thus resulting in more effective cell resistance against oxidative stress [84,93,94,99,107].

Cyclopia's antioxidant properties go hand in hand with its anti-mutagenic and anti-cancer activity, since oxidative stress and free radicals have been linked to DNA damage and cancer progression [108]. Therefore, it is no surprise that *Cyclopia* extracts also displayed protective activity against the pro-carcinogens, aflatoxin B1 and 2-acetylaminofluorene (2-AAF), in the Salmonella mutagenicity assay, inhibited chemically induced skin tumour formation in ICR (Institute of Cancer Research) mice, and reduced azoxymethane (AOM) induced cell proliferation in the colonic mucosa of rats [93,99,107,109,110].

In addition to *Cyclopia*'s antioxidant, anti-mutagenic and anti-cancer activity, this indigenous plant also displayed anti-diabetic activity [78,107,111,112]. In a study by Muller *et al.* [111], *Cyclopia intermedia* extracts effectively reduced plasma glucose levels in Streptozotocin (STZ)-induced diabetic rats, as well as normalizing blood glucose levels and reducing plasma cholesterol levels in hyperglycaemic, high fat diet-induced OBIR rats.

Another key characteristic of this multi-faceted plant, which forms the basis of the current study and the focus of our research group, is *Cyclopia*'s phytoestrogenic activity. Verhoog *et al.* [113–115], was one of the first to describe the phytoestrogenic activity of different *Cyclopia* species *(C. intermedia, C. sessiliflora, C. genistoides* and *C. subternata*), followed by Mfenyana *et al.* [116,117], Visser *et al.* [102,105,118], and Mortimer *et al.* [28,119], who showed estrogen receptor (ER) subtype selectivity of *Cyclopia* fractions and related phytocompounds, inhibition of E_2-induced breast cancer cell proliferation *in vitro*, delayed ERα induced uterine growth in an immature rat uterotrophic model, and reduced tumour formation in a *N*-Methyl-*N*-nitrosourea (MNU)-induced rat mammary gland carcinogenesis model (as discussed in Chapter 1).

To fully understand the importance of phytoestrogens, the next section will cover the mechanism of actions of phytoestrogens, as well as give a brief overview of the estrogen receptor signalling pathway.

2.5. Mechanisms of action of phytoestrogens

Phytoestrogens are plant derived non-steroidal compounds with structural similarity to 17-β estradiol (Figure 2.5.1), and therefore can cause estrogenic, anti-estrogenic or selective estrogen receptor modulator (SERM)-like effects in animals and humans [120–122]. They are also referred to as dietary estrogens, since they are present in a variety of fruits, vegetables, grains, and seeds [121,123]. There are different classes of phytoestrogens including **isoflavones** (legumes and soybean products), **coumestans** (bean sprouts and fodder crops), **stilbenoids** (fruits such as grapes) and **lignans** (whole grains, fruits such as berries, pears and apples, and sunflower seeds) [121,124–127].

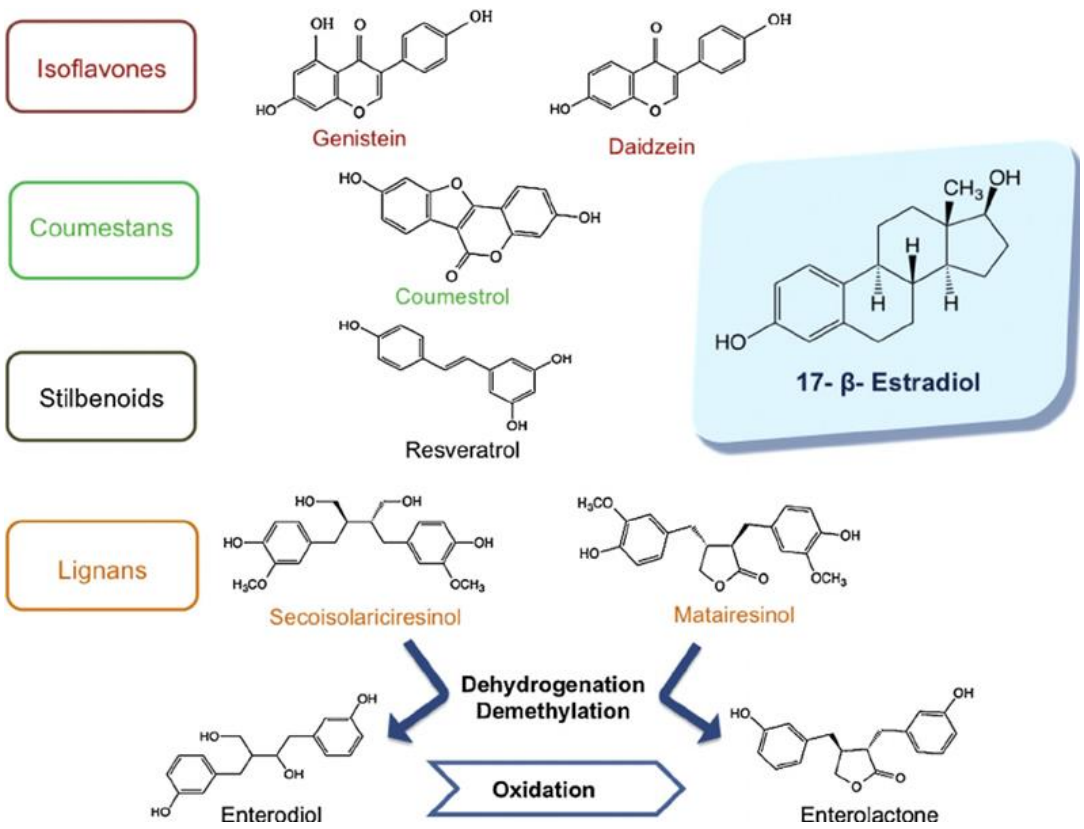

Figure 2.5. Chemical structure similarity between the different classes of phytoestrogens (isoflavones, coumertans, stilbenoids, and lignans) and the endogenous female estrogen (17-β-estradiol). Figure obtained from Moreira *et al*. [127].

The health potential of phytoestrogen-rich food is supported by epidemiology, in vitro and in vivo studies [120]. The occurrence of, for example, breast cancer in Asian and Eastern European countries, which predominantly consume a plant-based diet high in phytoestrogens, is lower than in Western countries, which consumes a diet high in animal based products [120,126,128]. Specifically, some of the phytoestrogens in soy, like genistein, have in various studies displayed anti-proliferative activity in both ER negative breast cancer cell lines (MDA 231 and MDA 435) and in ER positive MCF-7 cells [120,129].

Due to the favourable outcome of several studies, phytoestrogens are considered to be a safer and more natural alternative to synthetic estrogens used in HRT, with the same benefits as HRT (relief of menopausal symptoms, prevention of cardiovascular disease and osteoporosis) but with a lower risk of developing estrogen related cancers [120,126,130]. These potentially beneficial effects of phytoestrogens have resulted in a global increase in the consumption of phytoestrogen rich food and products [126]. However, the risks or side effects of phytoestrogens have not yet been clearly established. A review article by Patisaul *et al*. [131], describing the pros and cons of phytoestrogens, reveals the two-faced characteristic of some phytoestrogens. Genistein is an excellent examples of this, since this phytocompound has shown a biphasic response with agonist, instead of antagonist, effects at low concentrations and consequently, causing, rather than preventing, proliferation of cancer cells [124,129,132,133]. This example emphasizes the importance of fully understanding how phytocompounds exert their effects [124,132,133].

The main structural feature of phytoestrogens are their phenolic rings together with hydroxyl groups, which is a prerequisite for compounds to bind to the ERs [126,127,134]. Phytoestrogens are, therefore, able to mimic endogenous estrogens and mediate their effects though the same estrogen signalling pathway [120]. Apart from the ability of phytoestrogens to bind to the ERs, they can also activate other receptors, including serotoninergic-, IGF-1-,

aryl hydrocarbon-, and peroxisome proliferator-activated receptors, act like antioxidants by binding to free radicals, and affect signalling pathways important for cell survival and apoptosis including, but not limited to, tyrosine kinase, cAMP/protein kinase A, and MAP (ERK1,2, p38) kinase pathways [30,123,135]. The multifaceted characteristics of phytocompounds make them alluring candidates for cancer therapy development. For the purpose of the current study, however, we focus specifically on the estrogen signalling pathway.

2.5.1. Estrogen signalling pathway

The biological effects of estrogenic molecules are mediated through two intracellular receptors, ERα and ERβ [136–138]. These receptors are ligand-dependent transcriptional factors which are expressed in specific target tissues (Figure 2.6) [136–139]. The structural (A to F region) and functional (DNA binding, ligand binding, activation demains: AF-1 and AF-2) domains between ERα and ERβ differ (Figure 2.7), which results in these subtypes having different physiological functions [136,140–143]. In terms of breast cancer, ERα is known to induce cell proliferation, whereas ERβ has shown anti-proliferative activity by antagonising ERα, thus creating a balance between opposite forces (as described by Heldring *et al*.) [137,143–146].

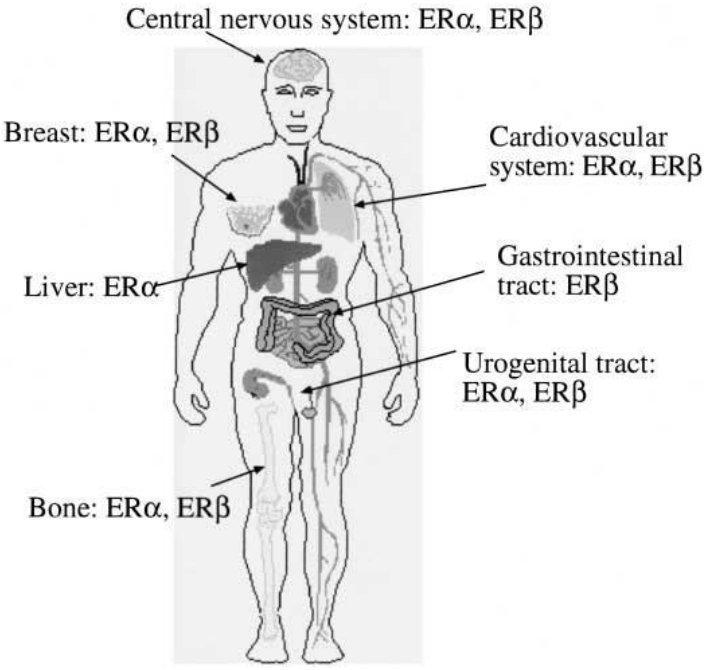

Figure 2.6: Distribution of ERα and ERβ in the human body, as demonstrated by Gustafsson [139].

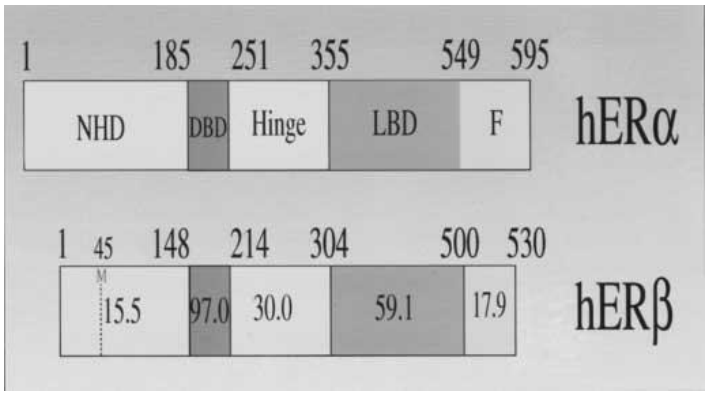

Figure 2.7: The structure representation of human ERα (top) and ERβ (bottom). Number 1 (left) is the N-terminal, the numbers above the receptor illustration indicates the number of amino acids, and the numbers within the receptor illustration (ERβ) indicates the % homology between the respective domains of the two ERs [139].

There are four main ER signalling pathways including the (1) classical ligand-dependent, (2) ligand-independent, (3) estrogen response element (ERE)-independent, and (4) nongenomic (cell-surface signalling) pathways (Figure 2.8) [138]. When estrogenic molecules reach their target cells, they diffuse across the cellular membrane and bind to the ER in the cytoplasm, forming a ligand-ER complex, thereby activating and causing a conformational change of the ER [136–138]. Pathway 1, 3 and 4 are ligand-dependent, and will be discussed first.

Pathway 1: In the classical ligand-dependent pathway, the ligand-ER complex binds to the EREs in the promoter region of target genes, regulating the transcription of these genes [136–138,147].

Pathway 3: The ERE-independent, also referred to as the tethered ligand dependent pathway, involves indirect binding of the ligand-ER complex to the promoter region of genes that do not contain EREs, by tethering to other transcription factors, such as Fos/Jun, and thereby influencing the regulation of these gene [136–138,147].

Pathway 4: In the non-genomic pathway, the estrogenic molecule binds to a membrane-bound ER, activating enzymes, such as phosphatases and kinases. These affect membrane permeability, which this in turn results in rapid physiological changes [136–138,148].

Pathway 2: In contracts to the other signalling pathways, which are all ligand dependent, the ligand-independent pathway involves the phosphorylation of the ER by kinases, activating the ER via other signalling pathways (e.g. growth factor signalling) [136–138,148,149].

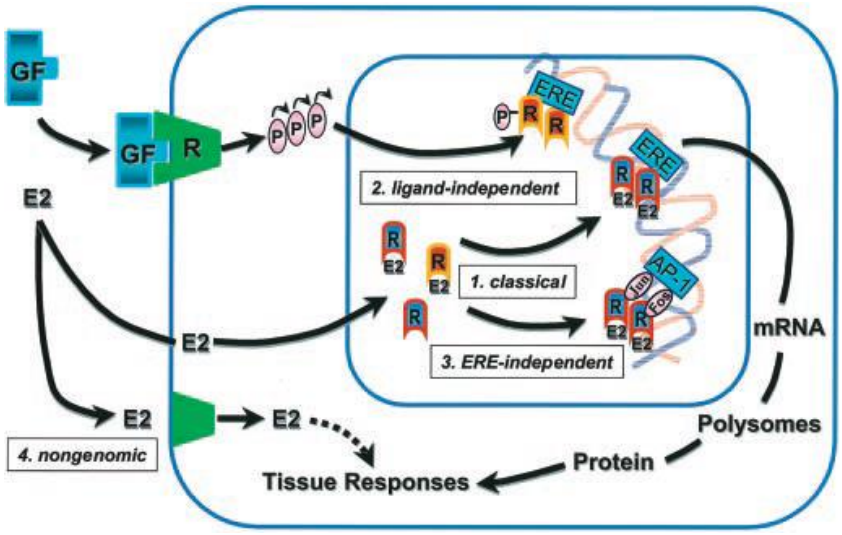

Figure 2.8: The four main ER signalling pathways including the (1) classical ligand-dependent, (2) ligand independent, (3) estrogen response element (ERE)-independent, and (4) nongenomic (cell-surface) signalling pathways, as obtained from Hall *et al*. [138].

2.6. Major phenolic compounds in *Cyclopia* SM6Met fraction

As discussed in Chapter 1, the current study is built on the foundation of the findings from Verhoog *et al*. [113–115], Mfenyana *et al*. [116,117], Visser *et al*. [102,105,118] and Mortimers *et al*. [28,119]. *Cyclopia*, specifically the SM6Met extract and F3 fraction, like most other plants, consists of a variety of phytocompounds, of which the major phenolic compounds quantified are shown in (Table 1). However, the current study only focusses on the 7 major phenolic compound identified and quantified in the ER beta agonist fraction (F3), of SM6Met. The phenolic compounds used in the current study were, however, purchased, and not isolated from *Cyclopia*.

Table 1: Major phenolic compounds quantified in the *Cyclopia* SM6Met extract [28,119].

Polyphenols	Compound class	SM6Met	F3
Mangiferin	Xanthone	1.899	11.565
Isomangiferin	Xanthone	0.645	4.016
p-Coumaric acid	Hydroxycinnamic acid	co-elution[b]	0.570
Luteolin-7-O-rutinoside (scolymoside)	Flavone	1.289	7.669
Luteolin	Flavone	0.040	0.205
Protocatechuic acid	Hydroxybenzoic acid	0.113	0.647
Iriflophenone-glc	Benzophenone	0.669	0.092
Aspalathin-glc	Flavone	0.700	nd[c]
Eriodictyol-7-O-rutinoside (eriocitrin)	Flavanone	0.846	nd[c]
Phloretin-diglc	Dihydrochalcone	1.278	nd[c]
Hesperetin-7-O-rutinoside (hesperidin)	Flavanone	2.049	nd[c]

[a] Gram polyphenols per 100 gram dried plant extract
[b] *p*-Coumaric acid co-elutes with aspalathin-glc where present; this means that the concentration cannot be determined accurately
[c] Not detected

It has been shown in literature that all 7 major phenolic compounds in F3 possess potential pharmaceutical characteristics including, but not limited to, antioxidant, anticancer, antibacterial and/or antidiabetic activity [100,150–160]. Of these 7 compounds, only 2

(luteolin and mangiferin) have phytoestrogenic activity [158,161–163]. Even so, the contribution of the other 5 compounds should not be dismissed, since, as discussed previously, the inactive (non-estrogenic) compounds might support the active (estrogenic) compounds by contributing to the stability, solubility and/or absorption rate of the active compounds [68–70].

2.7. Conclusion

It is apparent from reviewing the current literature that medicinal plant and related phytocompounds, have provided us with an endless source of inspiration, information and opportunities [8,50,164–166]. The title from an article by Pandey *et al*. [8]: ” Phytomedicine: An ancient approach turning into future potential source of therapeutics”, perfectly describes the historical transition of phytomedicine, since even though medicine has evolved dramatically over the decades with the introduction and development of evidence-based science and related techniques, traditional medicine and medicinal plants still remain a vital source of treatment in same parts of the world and may herald the new pharmaceuticals of the 21st century [44,50,51,57–59].

Cyclopia, an indigenous South Africa plant used in the past as traditional medicine by the Khoi-San, has regained popularity in the phytomedical and nutraceutical industry, due to its multifaceted medicinal properties. *Cyclopia* extracts have shown promising properties in *in vitro*, *in vivo* and *ex vivo* studies, which includes phytoestrogenic, antioxidant, anti-diabetic, anti-inflammatory and anti-mutagenic activity [86,93,100,103–105,115]. It is apparent from these findings, that *Cyclopia* mediates it desired effects via multi-target mechanisms, potentially due to the complex composition of different phytocompounds in the plant. It has recently been shown in our research group that the ERβ-specific activity of SM6Met cannot be attributed to any of the individual major phenolic compounds identified and quantified in

F3, and therefore the current study aims to investigate the possible combinatorial effects of these compounds [28].

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Chapter 3: Material and Methods

3.1 Characterisation of cell lines used in study

In this study, three different cell lines were used based on specific characteristics. Human embryonic kidney (HEK293) cells and African green monkey kidney fibroblast (COS-1) cells do not contain any endogenous estrogen receptors (ERs) and were used to investigate ER subtype specific activity, whereas MCF-7 BUS cells are an ER positive human breast cancer cell line and contain both ERα and ERβ. Medium used for cell maintenance and experimental procedures were supplemented as indicated in Table 3.1**.**

3.1.1 Cell culture

Human embryonic kidney (HEK293) cells (a kind gift from R. Louw, Stellenbosch University, ATCC (USA) cat# CRL-1573, passage 33), human breast cancer (MCF-7 BUS) cells (a kind gift from A. Soto, Tufts University, Boston, Massachusetts, USA, passage 118) and African green monkey kidney fibroblast (COS-1) cells (ATCC, USA cat# CCL-70, passage 4) were maintained in T75 flasks (Bio-Smart Scientific, South Africa), in **supplemented phenol-red DMEM** (Table 3.1), in a humidified cell incubator (95% humidity, 5% CO_2 at 37˚C). All the cells were tested for mycoplasma infections using Hoechst stain [1] and only mycoplasma free cells were used.

Table 3.1: Types of Dulbecco's Modified Eagle's medium (DMEM) used for cell culture maintenance and experimental procedures.

	Supplemented phenol-red DMEM	**Un-supplemented phenol-red free DMEM**	**Supplemented phenol-red free DMEM**	**Supplemented DMEM:HamF-12 (1:1) medium**
Type of medium	**High glucose** (4.5 g/L) phenol-red DMEM [a]	**Low glucose** (1 g/L) phenol-red free DMEM [a]	**Low glucose** (1 g/L) phenol-red free DMEM [a]	**High glucose** (3.2 g/L) phenol red DMEM F-12 Nutrient mixture (HAM) [b]
BUFFER Sodium bicarbonate (1.5 g/L) [a]	☑	☑	☑	**(contains 1.2 g/L)**
L-Glucose (3.5 g/L) [a]	-	☑	☑	-
Sodium pyruvate (0.11 g/L) [a]	☑	**(contains 0.11 g/L)**	**(contains 0.11 g/L)**	**(contains 0.055 g/L)**
1% (v/v) antibiotic mixture [c, d]	☑	-	☑	☑
10% (v/v) Charcoal stripped fetal calf serum [a, e]	-	-	☑	-
10% (v/v) Fetal calf serum [e] (not stripped)	☑	-	-	☑
Purpose of medium	**Maintaining cells Replating COS-1 & MCF7 cells**	**Serum starving cells**	**Inducing cells with compounds**	**Replating HEK293 cells**

[a] Sigma-Aldrich®, South Africa

[b] Gibco® by Life Technologies, USA

[c] 100IU/ml penicillin and 100µg/ml streptomycin

[d] Invitrogen, South Africa

[e] Merck, South Africa

3.1.2 Western blot analysis

Western blot analysis was conducted to characterise cell lines (HEK293 and MCF-7) used in this study in terms of ER subtypes. Un-transfected COS-1/HEK293 cells were used as negative control and COS-1/HEK293 cells transfected with the ER subtypes respectively, were used as positive controls. The presence/absence of ERα/β was identified with specific primary and secondary antibodies from Santa Cruz Biotechnology, Inc. and from Abcam, England (as indicated in results figure legends) at appropriate concentrations (Table 3.2).

Table 3.2: Antibodies used in Western blot analysis.

Protein Size	Primary antibody	Dilution	Secondary antibody	Dilution
ERα (66kDa)	ERα (MC-20): sc-542 rabbit polyclonal antibody	1:500	goat anti-rabbit IgG - HRP sc-2030	1:1000
	ERα (E115): ab32063 rabbit monoclonal antibody	1:400		
ERβ (56kDa)	ERβ (H-150): sc-8974 rabbit polyclonal antibody	1:1000	goat anti-rabbit IgG - HRP sc-2030	1:1000
	ERβ (EPR3777): ab92306 rabbit monoclonal antibody	1:400		
GAPDH (37kDa)	GAPDH (0411): sc-47724 mouse monoclonal antibody	1:1000	goat anti-mouse IgG - HRP Sc-2005	1:5000

3.1.2.1 Transfection of cells

HEK293 and COS-1 cells, transfected with either ERα or ERβ, where used as positive control to investigate the presence or absence of the ERs in cell lines used in this study.

HEK293 cells were seeded at a cell density of 4 x 10^6 cells per sterile 10cm cell binding plate (Corning®, USA) in **supplemented DMEM: HamF-12 (1:1)** medium (Table 3.1) and COS-1 cells were seeded at a cell density of 1.5 x 10^6 cells per sterile 10 cm plate in **supplemented**

phenol-red DMEM. After 24 hours, the medium was changed to **supplemented phenol-red free DMEM** (Table 3.1) and the cells were transfected with 150ng of either ERα (pSG5-hERα) or ERβ (pSG5-hERβ) expression vectors (see section 3.2.4.1) using FugeneXtreme Transfection Reagent (Roche®, South Africa) according to manufacturer's specifications (3µl FugeneXtreme:µg DNA). The next day the cells were re-plated at a cell density of 2.5 x 10^5 cells per well in a sterile 6 well plate in **supplemented phenol-red free DMEM** and again allowed to settle overnight.

3.1.2.2 Preparations for Western blot analyses

The following day the transfected cells were rinsed with 2ml ice cold PBS/well and lysed with 150µl lysis buffer A (10 mM KCl, 0.1% NP-40, 10 mM, Hepes pH 7.5, 1.5 mM $MgCl_2$) and Complete Mini protease inhibitor cocktail (Roche®, South Africa)) was added. Plates were shaken on ice for 15 - 20 minutes and frozen overnight at -20°C to ensure complete cell lysis. The cells were then thawed and scraped loose from the plate using a cell scraper after which the lysates were transferred to a 1.5ml tube. Cell lysates were centrifuged at 12 000 x g for 10 minute at 4°C and the clear lysates were then transferred to a new 1.5ml tube and stored at -20°C.

Untransfected MCF-7, HEK293 and COS-1 cells were seeded at a cell density of 2.5 x 10^5 cells per sterile 6 well plates. After 24 hours, the medium was changed to **supplemented phenol-red free DMEM** and after 48 hours the cells were lysed as described above.

3.1.2.3 Western blot

The cleared cell lysates (15µl) were separated with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using a 10% SDS-PAGE gel (70V for 15 minutes and

100V for 90 minutes using a BIO-RAD PowerPac™ HC and BIO-RAD Model Mini Protean®), and transferred to a Hybond-ECL nitrocellulose membrane using an ECL Semi-dry Blotter (Amersham Biosciences, USA) at 0.18A for 90 minutes. The membrane was then incubated in 10% (w/v) fat free milk powder (ERβ and GAPDH) or 5% (w/v) casein powder (ERα) dissolved in Tris-buffered saline (TBS) for 120 minutes on an orbital shaker. Thereafter the membrane was rinsed once with TBST (tris-buffered saline and 0.1% (v/v) Tween20), the specific primary antibody (see Table 3.2) was added to the membrane and incubated overnight at 4°C on an orbital shaker. Following incubation the membrane was washed with TBST (once for 20 minutes and twice for 5 minutes) after which the specific secondary antibody (which contains horseradish peroxidase [2]), see Table 3.2, was added and incubated at room temperature for 120 minutes on an orbital shaker. Thereafter, the membranes were washed again with TBST (same as before) and once with TBS after which the membrane was incubated with a chemiluminescent substrate (Clarity ^{TM} Western ECL Substrate from BIO-RAD) for 5 minutes and visualized either on medical x-ray film (Axim) or using the MyECL Imager (Thermo Fisher Scientific, Inc.), as indicated in results figure legends. The developed x-ray film was scanned in and the image adjusted in Microsoft Word 2010, whereas the images from MyECL Imager was analysed and adjusted on MyECL Imager software version 2.0. A colour protein ladder (Color Protein Standard Broad Range ladder, Biolabs New England) was used to determine protein sizes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein loading control to evaluate the relative amount of total protein in each well.

3.2 Assays to test estrogenic activity

3.2.1 Test compounds

17β-Estradiol (E_2) and fulvestrant (ICI 182,780), obtained from Sigma-Aldrich®, were used as ER agonist and antagonist standards, respectively. *Cyclopia* extracts and fractions used in this study, previously prepared by Mortimer *et al*. [3], include the *Cyclopia subternata* (harvesting M6) sequential methanol (SM6Met) extract, the non-polar fraction (NP) of SM6Met and fraction 3 (F3) of the NP fraction. *Cyclopia* extracts and fractions were stored in a vacuum-sealed desiccator at room temperature in the dark, so as to prevent/decrease oxidation of samples [4–7]. Phenolic compounds investigated include: mangiferin, luteolin, luteolin 7-rutinoside (scolymoside), and iriflophenone-3-C-glucoside all from Sigma-Aldrich® (South Africa), protocatechuic acid and *p*-coumaric acid from Fluka™ Analytical, Sigma-Aldrich®, and isomangiferin from Chemos GmbH® (Germany). Phenolic compounds were stored as recommended by manufacturers/suppliers.

3.2.2 Preparation of compounds

Stock solutions of compounds/extracts/fractions used in the study were prepared in one of three ways: (1) dissolved in dimethyl sulfoxide (DMSO), (2) dissolved in ethanol (EtOH), which was then evaporated or (3) dissolved directly in medium. Compounds prepared in DMSO were diluted 1000x in medium to achieve a 0.1% (v/v) DMSO concentration. Stock Solutions were stored at -20 ºC.

17β-Estradiol (E_2), fulvestrant (ICI 182,780), and the SM6Met extract, NP fraction and fraction 3 were prepared in DMSO from Sigma-Aldrich® (South Africa). For re-constitution experiments, phenolic compounds, excluding mangiferin, were prepared in ethanol (EtOH).

The EtOH was evaporated using nitrogen gas (Techne Sample Concentrator) in order to concentrate the compound mixtures at desired concentrations (Table 3.1), avoid oxidation of polyphenols and, in turn, also avoid the use of high levels of DMSO in tissue culture. Medium was added to the evaporated mixtures. Mangiferin did not dissolve in EtOH at high concentrations, but did dissolve in water and was consequently added directly to the medium at desired concentrations (as indicated in results figure legends).

Table 3.1: Phenolic content in SM6Met F3 as identified in Mortimer *et al*. [3]**, which was used in reconstitution experiments.**

Concentrations	Mangiferin	Isomangiferin	p-Coumaric acid	Scolymoside	Luteolin	Protocatechuic acid	Iriflophenone-glc
mg/9.8mg [a]	1.1334	0.3936	0.0559	0.7515	0.0201	0.0634	0.0089
1x M[b]	2.684×10^{-6}	0.932×10^{-6}	3.404×10^{-7}	1.264×10^{-6}	4.735×10^{-8}	4.111×10^{-7}	2.204×10^{-8}
10x M	2.684×10^{-5}	0.932×10^{-5}	3.404×10^{-6}	1.264×10^{-5}	4.735×10^{-7}	4.111×10^{-6}	2.204×10^{-7}
100x M	2.684×10^{-4}	0.932×10^{-4}	3.404×10^{-5}	1.264×10^{-4}	4.735×10^{-6}	4.111×10^{-5}	2.204×10^{-6}

[a] mg polyphenol in 9.8 mg SM6Met F3

[b] molar concentration of polyphenols corresponding to 9.8mg/ml SM6Met F3

3.2.3 Proliferation assay

Methylthiszol Tetrazolium (MTT) [8] was used to investigate the proliferation activity of the test compounds/extracts/fractions in MCF-7 BUS cells (Figure 3.1). Live cells are able to reduce MTT to formazan, which can be measured with a spectrophotometer. Therefore, an increase in live cells signifies cell proliferation. The MTT assay was optimised from a previous lab protocol (see pre-optimisation protocol) to obtain optimal proliferation activity. Agonist (in the absence of E_2) and antagonist (in the presence of E_2 $10^{-11}/10^{-12}$M) mode was tested.

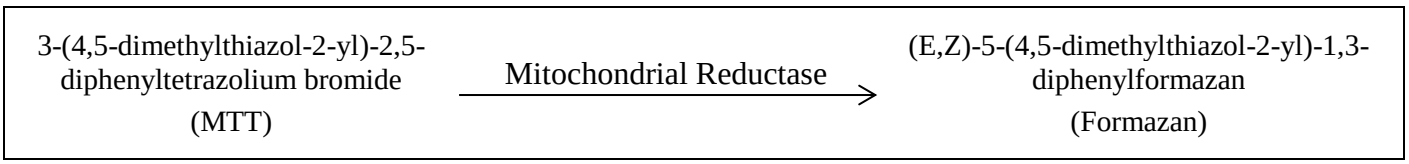

Figure 3.1: Conversion of MTT to formazan via mitochondrial reductase in live cells.

3.2.3.1 Pre-optimisation protocol (previous lab protocol)

MCF-7 BUS cells (withdrawn from pen-strep for seven days), were seeded at a density of 1 x 10^4 cells per well in a sterile 96 well plate in **supplemented phenol-red DMEM** and allowed to settle overnight. The cells were then rinsed with 200µl/well pre-heated (37˚C) PBS after which they were steroid and serum starved with 200µl/well **un-supplemented phenol red-free DMEM** (Table 3.1) for 24 hours. After starvation, the cells were treated with test compounds/extracts/fractions (as indicated in results figure legends) in **supplemented phenol-red free DMEM** for 24 hours (day 1), and re-induced after 48 (day 2) and 72 hours (day 3). Thereafter the MTT assay was performed. MTT powder (thiazolyl blue tetrazolium bromide from Sigma-Aldrich®) was dissolved in PBS (5 mg/ml) and filtered using a syringe and a cellulose acetate 0.2 µM 25mm syringe filter (Lasec, South Africa). After incubation with the compounds/extracts/fractions, cells were rinsed with sterile pre-heated (37˚C) PBS and subsequently incubated for 4 hours in MTT solution and **un-supplemented phenol red free DMEM** at a ratio of 1:4 (v/v). After the 4 hours, the medium containing the MTT solution was aspirated and 200µl/well DMSO was added to dissolve the formazan crystals and obtain a purple solution. Absorbance was measured at 550 nm on a spectrophotometer (BioTek® Gen5TM). All results were expressed as fold relative to solvent (set at 1).

3.2.3.2 Optimisation

As dose response curves were required and optimal potency (EC_{50}) and efficacy (fold induction) could not be obtained with the pre-optimization proliferation assay protocol,

further optimisation was therefore needed. Optimization included changes in cell density, type of tissue culture plates and induction times (as indicated in results figure legends). Different cell densities were investigated in different size tissue culture plates: MCF-7 cells were seeded in sterile 96 well plates (1 x 10^4 cells/well), 24 well plates (5 x 10^4 cells/well) and 6 well plates (1 x 10^5 and 2.5 x 10^5 cells/well, respectively) in **supplemented phenol red DMEM**. Cells were also treated with a range of different concentrations of E_2 (as indicated in results figure legends) to investigate optimal proliferative activity. In addition, induction time periods were optimised according to Karmaker *et al*. [9], which suggested an induction time period of 120 hours (5 days) with test compounds.

3.2.3.3 Post-optimisation protocol

Optimisation of the MTT assay revealed that Karmaker's protocol provided the best cell proliferation results. Cell density, plate type and induction time period were changed accordingly.

MCF-7 BUS cells were seeded at a cell density of 1 x 10^5 cells per well in a sterile 6 well plate in **supplemented phenol-red DMEM** and allowed to settle overnight. The next day the cells were rinsed with 2ml/well pre-heated (37˚C) PBS and the medium was changed to 2ml/well un-**supplemented phenol red free DMEM**. The cells were steroid and serum starved for 24 hours. After cell starvation (day 0), the cells were treated with test compounds/extracts/fractions (as indicated in results figure legends) prepared in **supplemented phenol-red free DMEM** for 72 hours (day 0 - 3), and re-induced for another 48 hours (day 3 – 5). After inducing the cells with the compounds/extracts/fractions for a total of 120 hours (5 days), the MTT assay was conducted as described in the pre-optimisation protocol. After 4 hours the medium containing the MTT solution was aspirated and 2ml of DMSO was added to each well and mixed until a clear purple solution was

observed. The purple DMSO cell mixture was then re-plated into a 96 well plate and the absorbance measured at 550 nm with a spectrophotometer (BioTek® Gen5TM). All results were expressed as fold relative to solvent (set at 1).

3.2.4 Promoter Reporter Studies

3.2.4.1 Plasmids and plasmid preparation

The following plasmids were used in promoter reporter studies:

- The estrogen receptor (ER) subtype expression vectors for human ERα (pSG5-hERα, 5860 bp) and human ERβ (pSG5-hERβ, 5663 bp) were obtained from S. Denger and F. Gannon [10].
- Two estrogen response element (ERE) containing promoter reporter plasmids, one containing the ERE from the vitellogenin gene (ERE.vit2.TATA.luc, ±5730bp) was obtained from K. Karach [11] and the second containing the ERE from the human PS2 gene (pGL3-2ERE.PS2.luc, ±5614bp) was obtained from B. Belandia [12]. Both plasmids contain a coding region for luciferase.
- The pGL2 basic empty vector (5598 bp), obtained from Promega (USA), contains no eukaryotic promoter or enhancer sequences and was use as a filler plasmid.

Glycerol stocks of transformed DH5α *Escherichia coli* cells (made competent using the calcium chloride method and transformed using the heat shock method [13]) containing the relevant plasmids, were grown overnight at 37° C in 200ml lysogeny broth (LB) medium (0.024 M NaCl, 5 g/L Yeast Extract (Merck, South Africa), and 10 g/L Tryptone (Merck, South Africa)) in the presence of 50 µg/ml ampicillin until the bacterial cells reached an optical density ($O.D._{600}$) of 2–4.

The PureYield™ Plasmid Maxiprep System (Promega, USA) was used according to the manufacture's specifications to isolate plasmid DNA. The concentration and purity of DNA was determined using the NanoDrop® ND-100 spectrophotometer (Thermo Fisher Scientific Inc.). The DNA concentration was calculated by the NanoDrop® 2000 software from the absorbance determined at 260 nm, while the purity was calculated using the absorbance ratio of 260/280 nm, since pure DNA at this wavelength would give a ratio of ~1.8. Restriction enzyme digestion of the plasmid DNA was conducted using the restriction enzyme HindIII (5U/µg) obtained from Fermentas (Thermo Fisher Scientific Inc.) for all the plasmid constructs. The plasmid DNA (0.2µg DNA/well, digested and undigested) was separated using agarose gel electrophoresis (100V for 30 - 60 minutes) on a 1% agarose gel. Plasmid DNA size and integrity was subsequently analysed and evaluated using a GeneRuler™ 1kb DNA Ladder (Inqaba, South Africa) and E-Capt Vilber Lourmat Software version 12.9.

3.2.4.2 Promoter reporter assay

Promoter reporter assays [14] were used to evaluate ER subtype specific agonist and antagonist activity of the test compounds/extracts/fractions in HEK293 cells. These cells were transfected with either an ERα expression vector (pSG5-hERα) or an ERβ expression vector (pSG5-hERβ), as well as an ERE-containing promoter reporter plasmid (ERE.vit2.luc/ERE.PS2.luc) and a pGL2 basic empty vector. Fulvestrant (ICI), SM6Met, NP and F3 were tested in agonist (in the absence of E_2) and antagonist (in the presence of E_2 10^{-11}M) mode for both ER subtypes. Promoter reporter assays were also used to (1) conduct dose response analysis (3.2.4.2.1), to obtain potency (EC_{50}) and efficacy (fold inductions) of E_2 and selected polyphenols (Table 3.1), as well as for (2) addition and subtraction studies (3.2.4.2.2) and (3) to investigate synergism (3.2.4.2.3). These three studies were only conducted in agonist mode (in the absence of E_2) for both ER subtypes.

HEK293 cells were seeded at a density of 4 x 10^6 cells per sterile 10cm cell-binding plate (Corning®, USA) in **supplemented DMEM:HamF-12 (1:1)** medium, and allowed to settle overnight. The next day the plates were rinsed with pre-heated (37˚C) PBS and the medium was changed to **supplemented phenol red free DMEM**. Cells were then transfected with a total of 6150ng plasmid DNA (150ng ER, 1500ng ERE and 4500ng pGL2basic) per 10cm plate using FugeneXtreme Transfection Reagent (Roche®, South Africa) according to manufacturer's specifications. 24 Hours after transfection, cells were re-plated at a cell density of 5 x 10^4 cells per well in sterile 24 well binding plates (Corning®, USA). The following day the cells were treated with selected test compounds/extracts/fractions (at concentrations as indicated in results figure legends) for another 24 hours. Thereafter, the cells were rinsed with 500µl/well ice cold PBS and lysed with 50µl/well passive lysis buffer (10% (v/v) glycerol, 0.2% (v/v) Triton, 2.8% (v/v) Tris-phosphate EDTA and 1.44 mM EDTA) after which the cell lysates were frozen overnight at -20˚C. Luciferase activity of cell lysates were measured in relative light units (RLU) using the luciferase assay system (Figure 3.2) and a Veritas microplate luminometer. Luciferase assay reagent (beetle luciferin) [Promega, USA] was added to cell lysates at a ratio of 5:1. RLU values were normalised using protein concentrations of lysates (5µl) determined with the Bradford method [15], measured at 595 nm with a BioTek® Gen5™ spectrophotometer. This was done to ensure that any increase in luciferase activity was due to transactivation of the promoter reporter by the ligand activated receptor, and not due to plating error or an increase in cell number. Results were expressed as fold relative to solvent (set at 1).

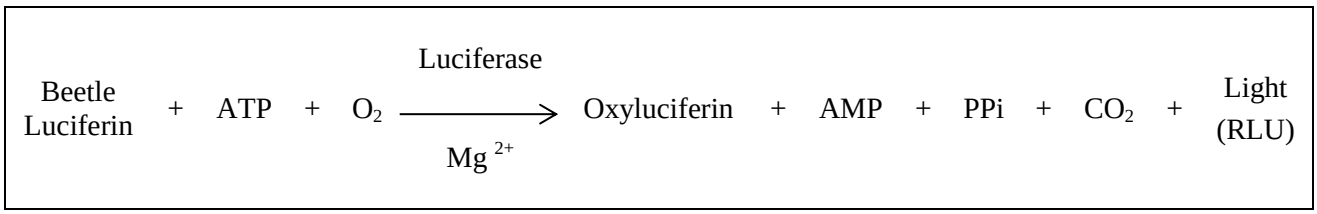

Figure 3.2: Mechanism of luciferase activity

3.2.4.2.1 Dose Response protocol

Dose response promoter reporter analysis was conducted with increasing concentrations of test compounds (individually and in fixed-ratio combinations). The effects were plotted using a XY line graph in Graphpad where the logarithm of the increasing concentrations of the tests compounds are plotted on the X axis and the response in RLU/mg protein on the Y axis. Data was analysed using a nonlinear regression curve fit with log (agonist) vs. response (three parameters). From the dose response curve, the potency (EC_{50} values) and efficacy (fold induction) of the test compounds (individually and in fixed-ratio combinations) were obtained.

3.2.4.2.2 Addition and Subtraction protocol

F3 was reconstituted using the 7 major phenolic compounds identified in this F3 **(**Table 3.1) [3]. For the addition protocol, the concentrations of one of the seven compounds was individually increased to 10-fold (10x) or 100-fold (100x) the concentration in F3, respectively, whereas for the subtraction protocol, six of the seven phenolic compounds were prepared at 100-fold (100x) the concentration of these compounds in F3 with the concentration of only one compound per experiment decreased (subtracted) to the concentration in F3 (1x).

3.2.4.2.3 Synergism protocol

A well-known protocol to determine synergism is the isobologram method as demonstrated in Tallarida *et al*. [16]. In this protocol the EC_{50} values, obtained from dose response curves (3.2.4.2.1), of the individual compounds to be evaluated would be plotted on the y and x – axes, respectively, with a line drawn between the two points. The EC_{50} values of the individual compounds would then be combined in a fixed ratio manner, were the combination is increased and decreased exponentially at the fixed ratio, to determine the EC_{50} of the combination. If the EC_{50} value of the combination fell upon this line, the effected is considered to be additive. Values below the line are considered to be synergistic, while those above the line are considered antagonistic. For a more detailed description of the isobologram method, see section 2.3 in the literature review. In this study, mangiferin and luteolin were chosen to be investigated for synergism. Since the EC_{50} of mangiferin could not be obtained, increasing concentrations of mangiferin was combined with the EC_{50} of luteolin.

3.2.5 Whole cell binding

COS-1 cells were seeded at a cell density of 1.5 x 10^6 cells in **supplemented phenol-red DMEM** in sterile 10cm plates and allowed to settle overnight. The next day the medium was changed to **supplemented phenol-red free DMEM** and the cells were transfected with 150ng of either ERα (pSG5-hERα) or ERβ (pSG5-hERβ) expression vectors (see section 3.2.4.1) using FugeneXtreme Transfection Reagent according to manufacturer's specifications (3µl FugeneXtreme:1µg DNA). After 24 hours, the cells were re-plated in **supplemented phenol-red free DMEM** at a cell density of 5 x 10^4 cells per well in sterile 24 well plates and again allowed to settle overnight. Thereafter the cells were rinsed three times with sterile pre-heated (37°C) PBS and incubated for 4 hours in **un-supplemented phenol-red free DMEM** with 20 nM radio-labeled estradiol (81Ci/mmol 2,4,6,7-^{3}H-17-β-etradiol

from Amersham Biosciences®) alone (total binding) or in combination with 10^{-5}M of unlabelled estradiol (non-specific binding) to evaluate specific binding of mangiferin and luteolin at 100x their concentration in F3 (Table 3.1). After 4 hours, the cells were placed on ice and rinsed (three times for 15 minutes, respectively) with ice cold 0.2% PBS-BSA (bovine serum albumin from Sigma-Aldrich®). Cells were lysed with 100µl/well passive lysis buffer (10% (v/v) glycerol, 0.2% (v/v) Triton, 2.8% (v/v) Tris-phosphate EDTA and 1.44 mM EDTA) and shaken for 20 minutes at room temperature after which the cell lysates were frozen at -20˚C.

Protein concentration of lysates (5µl) was determined using the Bradford method [15]. The remaining lysates were transferred to scintillation vials to which 1ml scintillation fluid per vial (Quickszint FLOW 2, Zinsser Analytic, South Africa) was added. Counts per minute (CPM) were determined using the Beckman LS3801 Beta-scintillation counter (Beckman®, South Africa) and results (CPM) were normalised to protein (mg/ml) values. Data was presented as specific binding which was determined by subtracting nonspecific binding from total binding (Specific Binding = Total Binding – Non-Specific Binding).

3.3 LC-MS/MS analysis

LC-MS/MS analyses were conducted at the Central Analytical Facilities (CAF) of Stellenbosch University on a Waters Acquity® ultra-performance liquid chromatography (UPLC) system connected to a Waters Synapt G2 quadrupole time-of-flight (QTOF) mass spectrometer (Milford, MA, USA). The UPLC system comprised a binary pump, an in-line degasser, column compartment and an Acquity photo diode array (PDA) detector. The mass spectrometer was equipped with an electrospray ionization (ESI) source and samples were ionized as described in Albrecht *et al.* [17]. A Waters UPLC BEH C18 column (2.1 × 50 mm,

1.7 µm particle size) was used for separation and 1 µl of each sample was injected. The gradient program used was as indicated in Table 3.1.

Table 3.1: LC-MS/MS gradient program

Time (min)	Flow (ml/min)	% Solvent A H_2O (0.1% formic acid)	% Solvent B Acetonitrile (0.1% formic acid)
Initial	0.350	90.0	10.0
0.20	0.350	90.0	10.0
14.00	0.350	66.0	43.0
15.00	0.350	40.0	60.0
15.10	0.350	90.0	10.0
17.00	0.350	90.0	10.0

3.4 NMR analysis

^{1}H NMR spectra were recorded at the NMR unit in the Central Analytical Facilities (CAF) of Stellenbosch University. The samples were dried under vacuum, dissolved in DMSO-d6 or pyridin-d5, respectively, and the proton spectra acquired on a 600MHz Agilent Unity Inova spectrometer utilizing an Inverse Detection Pulsed Field Gradient, 5mm probe and utilizing the default proton acquisition parameters of the VnmrJ 4.2 instrument software. The spectra were obtained at room temperature and the recorded data further processed and referenced against the residual DMSO signal set at 2.5ppm, using the Mestrenova 11 software package. Electrospray ionization mass spectrometry (ESI-MS) was used to ddetermine the accurate mass of the molecular ion (M+1) in the positive ionization mode, thereby further confirming each structure molecular weight and molecular formula.

3.5 Statistical analysis

The Graph Pad Prism® v5.0.3.477 software was used to manipulate, graphically visualize and statistically analyse the data obtained from all assays. Bar graphs were statistically analysed

using One-way ANOVA and Dunnett's multiple comparison's test as post-test. Dose response graphs were fitted using a nonlinear regression curve fit with log (agonist) vs. response (three parameters) to determine potency (EC_{50}) and efficacy (fold induction). *P*-values were represented as follow: $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***), as indicated in results figure legends. Average ± SEM is indicative of (n) independent experiments, as indicated in results figure legends.

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Chapter 4: Optimisation and Validation of Experimental Systems

4.1. Introduction

Recently, the concept of combinatorial or synergistic drug interactions has received considerable attention due to the discovery and understanding of novel phytoestrogenic nutraceuticals for both the treatment of menopausal symptoms as well as the prevention of estrogen-induced breast cancer [1–3]. Due to the increased risk of developing breast cancer associated with hormone replacement therapy, women are seeking alternatives such as plant extracts containing high levels of phytoestrogens, that have been shown to alleviate menopausal symptoms with possible fewer side effects and risks [1,4–6]. However, more research is required to fully understand how the phytoestrogens in complex plant extracts interact to exert their favourable combinatorial or synergistic effects.

Currently, the most common method to investigate drug interactions and to determine possible synergism is the isobole method [3,7–10]. This method requires establishing the potencies of the individual drugs involved, as well as the potencies of fixed drug combinations. To achieve accurate drug potencies, various factors need to be considered since optimal and accurate results are not only dependent on the concentration and effect of the drug, but also on the sensitivity of the experimental system (cell type, target e.g. receptors or response elements, and assay) [11–14].

The current project investigates F3, one of the fractions of the phytoestrogenic nutraceutical, SM6Met [15]. Specifically, as the current project aims to evaluate the interaction and possible synergistic contribution of the 7 individual major phenolic compounds identified in F3 to the favourable estrogenic profile of this fraction, optimisation of the experimental model was required. For this reason, the cell lines used in the experimental model were characterised and optimisation of the experimental protocols were investigated to increase

sensitivity. Following optimisation, the experimental protocol was validated using SM6Met and fractions thereof [15].

4.2. Results and Discussion

4.2.1. Characterisation of cell lines

The two cell lines used, human embryonic kidney cells (HEK293) and human breast cancer cells (MCF-7 BUS), were chosen based on specific characteristics needed to aid in investigating the estrogenicity of compounds/extracts/fractions. The HEK293 cells do not express any endogenous ERα or ERβ [16], and for this reason was used to investigate ER subtype specific activity through separate transfections with expression vectors for ERα and ERβ, respectively. Since this cell line is of human origin [17], it is thought to be a more physiologically relevant system for investigation of the expression of human ER. On the other hand, the MCF-7 BUS cells endogenously expresses both ERα and ERβ [18], and is therefore a physiologically relevant system for the investigation of the effect of the compounds/extracts/fractions on breast cancer cell proliferation.

Western blot analysis was performed to validate the ER status of the HEK293 and MCF-7 BUS cells. As expected, the HEK293 cells did not express any endogenous ERα or ERβ (Figure 4.1 A & B, lane 3), whereas the MCF-7 BUS cells expressed both ER subtypes (Figure 4.1 C & D, lane 4). Furthermore, it was observed that the intensity of the ERα (66kDa) protein band (Figure 4.1 C, lane 4) was more prominent than the intensity of the ERβ (56kDa) protein band (Figure 4.1 D, lane 4), suggesting higher expression of ERα than ERβ in the MCF-7 BUS cell line. However, since different antibodies were used for the ERα and β subtypes, albeit from the same company, affinity of the antibodies for the respected

epitopes should not be assumed to be identical. Nonetheless, literature has also shown higher expression of ERα in the MCF-7 breast cancer cell line [18].

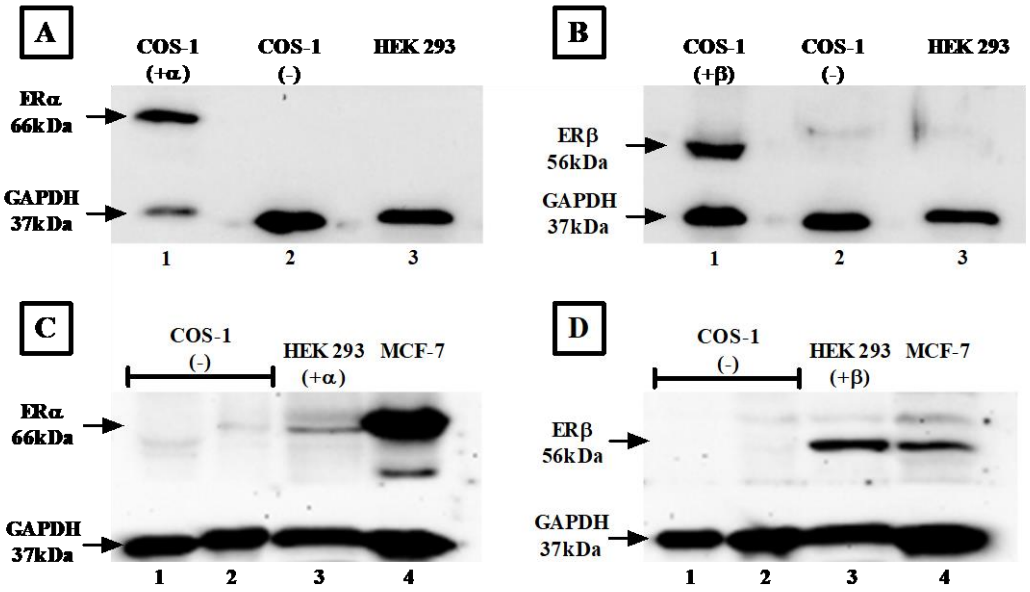

Figure 4.1: Characterization of the human embryonic kidney (HEK293) and the human breast cancer (MCF-7 BUS) cell lines. A & B: Western blot analysis confirms the absence of the estrogen receptors subtypes (ERα and ERβ) in the HEK293 cell line. Un-transfected COS-1 cells, indicated as COS-1 (-), were used as negative control and COS-1 cells, transfected with either pSG5-hERα or pSG5-hERβ, indicated as COS-1 (+α) and COS-1 (+β), respectively, were used as positive controls. **(A)** ERα (MC-20):sc-542 was diluted 1:500 and **(B)** ERβ (H-150):sc-8974 was diluted 1:1000. Results were visualised on x-ray film. **C & D:** Western blot analysis confirms the presence of both estrogen receptor subtypes (ERα and ERβ) in the MCF-7 BUS cell line. Un-transfected COS-1 cells, indicated as COS-1 (-), were used as negative control and HEK293 cells transfected with either pSG5-hERα or pSG5-hERβ, indicated as HEK (+α) and HEK (+β), respectively, were used as positive controls. **(C)** ERα (ab75635) and **(D)** ERβ (ab3576) were both diluted 1:400. Results were visualised on a MyECL Imager. **A, B, C & D:** Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a protein loading control.

From the Western blot results we may conclude that the HEK293 cells do not contain the ER subtypes and is therefore an ideal cell line to investigate and compare the transactivational activity of the phytoestrogenic extracts and fractions via the respective ER subtypes. In addition, this cell line is generally considered a good model for transactivation studies [19]. In contrast to the HEK293 cell line, the MCF-7 BUS cells endogenously expresses both ERα and ERβ, and are thus an ideal system for investigating the effects of the phytoestrogenic extracts and fractions in a physiological relevant cell environment.

4.2.2. Optimisations of assays used to test estrogenic activity

Promoter reporter and cell proliferation assays were previously used to evaluate the favourable estrogenic profile of SM6Met. Specifically, previous studies demonstrated that SM6Met displayed ERα antagonism and ERβ agonism in both COS-1 and HEK293 cells, and inhibited estrogen induced MCF-7 BUS breast cancer cell proliferation [15,20]. Therefore, following from the SM6Met studies, the same assays were used to investigate a fraction (F3) of SM6Met, which displayed augmented ERβ agonist activity [15]. However, no estrogenic activity was observed with any of the individual major phenolic compounds in F3 [15], even though some of these compounds displayed estrogenic activity in other studies [21–23].

To determine interaction and possible synergism of the phenolic compounds identified in SM6Met F3, a robust system, with high sensitivity, is required to detect small changes in the inflection point of a dose response curve. Consequently, the assays used in the current study, cell proliferation in MCF-7 BUS cells and promoter reporter assay in HEK293 cells, required optimisation to increase sensitivity, specifically by obtaining optimal efficacies (fold induction) and accurate potencies (EC_{50}). Estradiol (E_2), an endogenous ER ligand that binds to both ER subypes with high affinity [24] and promotes proliferation of breast cancer cell lines [25], was used as positive control for the optimisation of both assays.

4.2.2.1. Optimisation of cell proliferation assay protocol: investigating different cell numbers, tissue culture plate sizes and various induction times

The proliferation assay was optimised from a previous cell proliferation assay laboratory protocol [15], which entailed using 96 well plates with a cell density of 1 x 10^4 cells/well. The MCF-7 BUS cells were induced with increasing concentrations of E_2 for a total of 72 hours (3 days), and produced an efficacy of only 1.54 ± 0.04 fold (Figure 4.2 A). An experimental system with a higher efficacy would ultimately be more sensitive to small drug

induced changes, and subsequently produce a more accurate potency. This increase in sensitivity is essential when investigating interaction and possible synergism of complex mixtures such as in SM6Met and its fractions. Therefore, a literature search for other protocols with increased sensitivity was conducted. One of the highest efficacies found during the literature search was in a study by Karmakar *et al.* [26], where the authors achieved a 5-fold induction with 10^{-9} M E_2 using 6 well plates (1 x 10^5 cells/well) and inducing for a total of 120 hours (5 days). Since the differences between the two protocols were: (1) total induction time, (2) tissue culture plate sizes and (3) cell number per surface area, these three conditions (Table 4.1) were investigated in order to achieve increased efficacy in our cell proliferation assay.

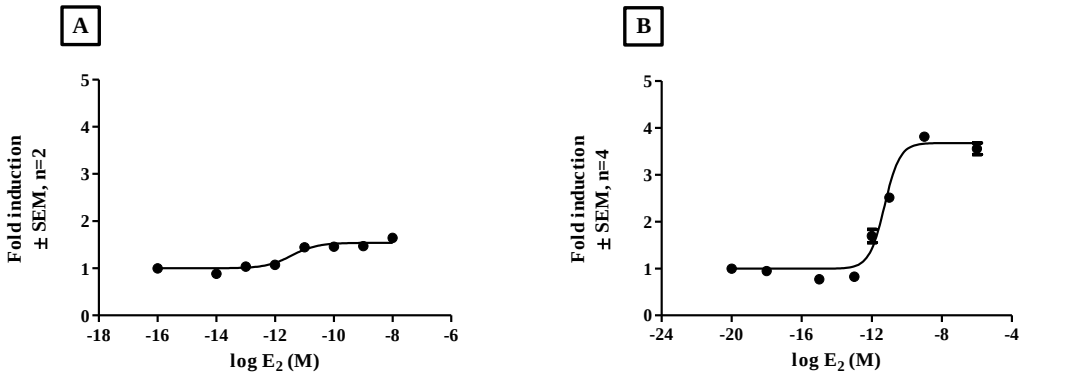

Figure 4.2: Cell proliferation studies comparing potency (EC_{50}) and efficacy (fold induction) of (A) a previously used laboratory protocol and (B) a new post-optimisation protocol. (A) MCF-7 BUS cells were plated at a cell density of 1 x 10^4 cell/well in 96 well plates and induced with increasing concentrations of E_2 for a total of 3 days. (**B)** MCF-7 BUS cells were plated at a cell density of 1 x 10^5 cell/well in a 6 well plate and induced with increasing concentrations of E_2 for a total of 5 days. Curve fitting: nonlinear regression with log (agonist) vs. response (three parameters). Statistical analysis: unpaired t-test to compare efficacy and log EC_{50} of (A) a previously used laboratory protocol and (B) a new post-optimisation protocol,*P <0.05, **P< 0.001, ***P<0.0001 and ^{ns}no significant difference.

Cells were either induced for a total of 72 hours, from previous laboratory protocol (Figure 4.3 A, C, E & G), or 120 hours, from the Karmakar protocol (Figure 4.3 B, D & F). Various cell densities were also investigated with different tissue culture plate sizes: 96 well plates with 1 x 10^4 cells/well, from previous laboratory protocol (Figure 4.3 A & B), 24 well plates

with 5 x 10^4 cells/well (Figure 4.3 C & D) and 6 well plates with either 1 x 10^5 cells/well, from the Karmakar protocol (Figure 4.3 E & F), or 2.5 x 10^5 cells/well (Figure 4.3 G). Increasing concentrations of E_2 (10^{-11}, 10^{-9} and 10^{-6} M) was used as a positive control. Since 10^{-9} M E_2 was the point where the curve plateaued in Figure 4.2 A, 10^{-9} M E_2 fold induction for the three conditions where summarised in Table 4.1.

Table 4.1: Comparison of E_2 (10^{-9} M) fold induction using different optimisation conditions (total induction time, plate size and cell density).

Plate size	Cells/well	10^{-9} M E_2 fold induction after 72 hours (3 days)	10^{-9}M E_2 fold induction after 120 hours (5 days)
96 well	1 x 10^4	1.07 ± 0.03	1.61 ± 0.02 ***
24 well	5 x 10^4	1.23 ± 0.02 ###	1.45 ± 0.01 ***,###
6 well	1 x 10^5	1.49 ± 0.10 ###	3.06 ± 0.05 ***,###
6 well	2.5 x 10^5	1.46 ± 0.03 ns	-

Statistical analysis: unpaired t-test, 72 hour (3 day) protocol vs 120 hour (5 day) protocol *P <0.05, **P< 0.001, ***P<0.0001, 96well vs 24 well vs 6 well plates #P<0.05, ##P<0.001, ###P<0.0001 and nsno significant difference.

The results from Table 4.1 and Figure 4.3 suggest that the longer induction time period of 120 hours (Figure 4.3 B, D & F) achieved significantly (P<0.0001) higher fold induction throughout compared to the short induction time period of 72 hours (Figure 4.3 A, C, E & G). Furthermore, the different plate sizes with their allocated cell densities were compared within each of the induction time periods (72 hours and 120 hours). Within the 72 hours protocol, there was a significant increase (P<0.0001) in fold induction associated with the increase in surface area of the cell culture plates (Figure 4.3 A, C & E). There was, however, no significant difference in fold induction between the different cell densities (1 x 10^5 cells/well and 2.5 x 10^5 cells/well) in the 6 well plates (Table 4.1). Within the 120 hour protocol, there was a significant (P<0.0001) decrease in fold induction between the 96 well and 24 well plates, and a significant (P<0.0001) increase in fold induction when comparing the 6 well plate to the 96 well and 24 well plates. The highest cell proliferation induction, 3.06 ± 0.05 fold, was observed with the 6 well plates (1 x 10^5 cells/well) using the longer induction time

period (Table 4.1; Figure 4.3 F). Accurate results for the 6 well plates (2.5 x 10^5 cells/well) using the longer induction time period could not be obtained, since the cells were already 100% confluent on day 3 and began to lift from the plate surface (results not shown in Figure 4.3).

Although the 3.06-fold induction of proliferation obtained from 10^{-9} M E_2 (Figure 4.3 F) was lower than that of Karmakar *et al*. [26], it was a significant improvement compared to the efficacy (1.07-fold) achieved with the previous laboratory protocol (Figure 4.3 A) and the other optimisation conditions tested (Figure 4.3 B, C, D, E & G). Cell density, plate size and induction time period were thus changed accordingly and a dose response curve was produced for the post-optimised protocol to compare potency (EC_{50}) and efficacy (fold induction) to that of the pre-optimised protocol (Figure 4.2). Although the potencies for the pre- and post-optimised protocols were very similar and not significantly different (3.71 x 10^{-12} M and 5.37 x 10^{-12} M, respectively), the efficacy obtained with the post-optimised protocol (Figure 4.2 B), was more than double and significantly different ($P<0.0001$) from the efficacy obtained with the pre-optimised protocol (Figure 4.2 A) at 3.67 ± 0.08 fold vs 1.54 ± 0.04 fold, respectively.

As optimisation of the cell proliferation assay revealed that the Karmakar protocol [26] provided a significant overall improvement in cell proliferation efficacy, compared to the previous protocol, cell density, plate size and total induction time was changed accordingly for the validation study (4.2.3.1). The post-optimised protocol produced an efficacy twice that of the pre-optimised protocol, resulting in a more sensitive system, which, as stated previously, is important when investigating drug interactions and possible synergism.

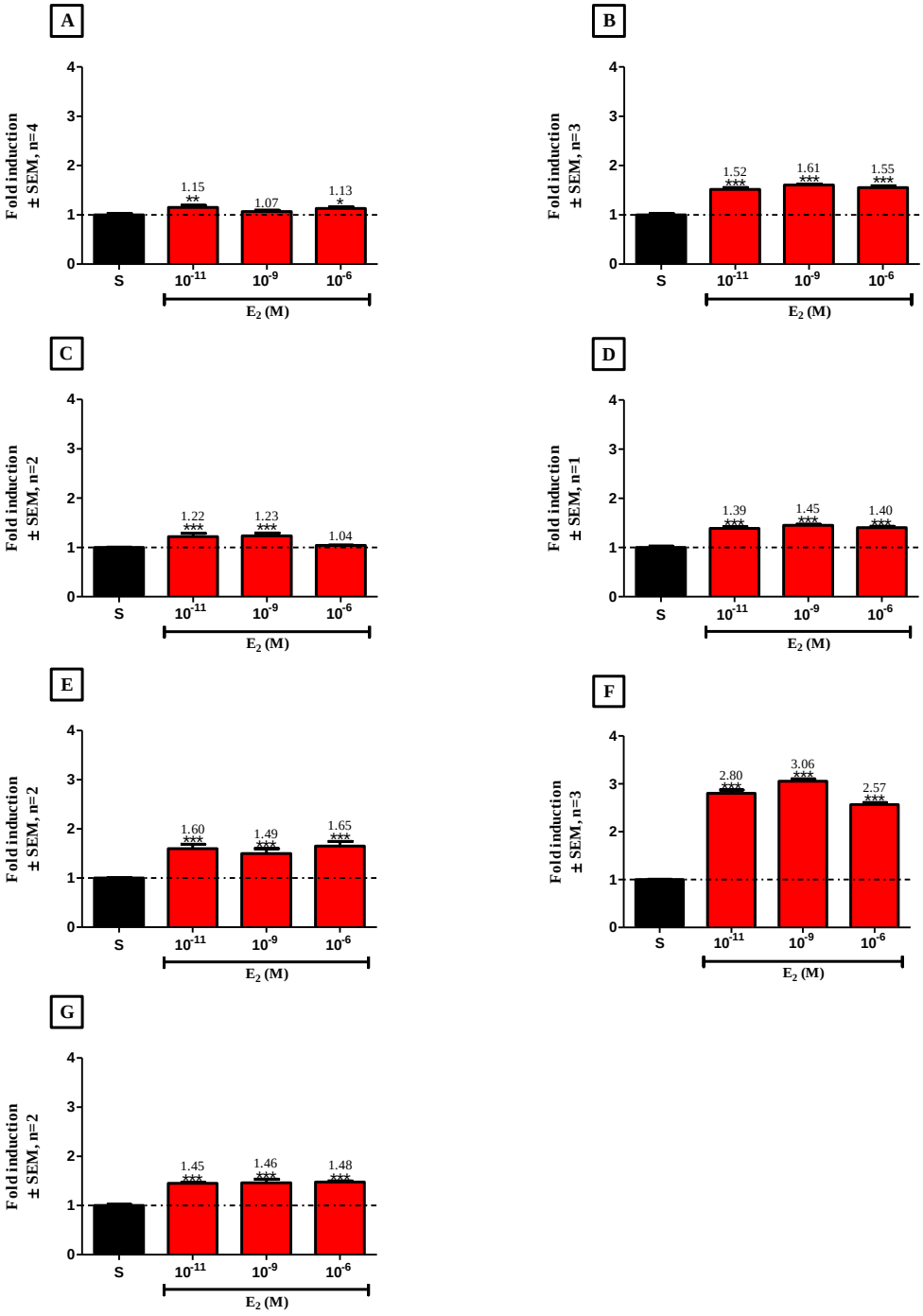

Figure 4.3: Optimisation of cell proliferation studies. Different cell densities, plate sizes and induction time periods were investigated with increasing concentrations of E_2 (10^{-11}, 10^{-9} and 10^{-6} M) to determine optimal MCF-7 BUS cell proliferation conditions. **A & B:** 96 well plates (1 x 10^4 cells/well) with an induction time of 3 days **(A)** or 5 days **(B)**. **C & D:** 24 well plates (5 x 10^4 cells/well) with an induction time of 3 days **(C)** or 5 days **(D)**. **E & F:** 6 well plates (1 x 10^5 cells/well) with an induction time of 3 days **(E)** or 5 days **(F)**. **G:** 6 well plates (2.5 x 10^5 cells/well) with an induction time of 3 days. Statistical analysis: One-way ANOVA with Dunnett's Multiple Comparisons Test as post-test, all columns compared to solvent control set to 1 (black bar),* P <0.05, ** P< 0.001, *** P<0.0001.

4.2.2.2. Optimisation of Promotor Reporter Assay

Previous studies in our group investigated the estrogenicity of *Cyclopia* extracts and phenolic compounds using promoter reporter assays in COS-1 cells, an African green monkey kidney cell line [20,27]. In addition, Mortimer *et al.* [16], focused on the optimisation of a promoter-reporter assay for determining the estrogenicity of the SM6Met extract and phenolic compounds in HEK293 cells, a human embryonic kidney cell line. This cell line is thought to be a more physiologically relevant model for human related studies, since it is a human cell line whereas the COS-1 cell line is of monkey origin. Mortimer's protocol entailed transfecting HEK293 cells with either hERα or hERβ, and an ERE.vit2.luc promoter reporter construct, which contained two ERE sequences obtained from the vitellogenin gene expressed in ovipare species [16,28,29]. Results obtained from Mortimer's study demonstrated the ability of E_2 (10^{-9} M) treatment to induce the promoter-reporter construct (ERE.vit2.luc) by 3.1-fold and 6.3-fold via ERα and ERβ, respectively [15,16].

As mentioned before, a higher efficacy would ideally result in a more sensitive system to investigate drug interaction and determine possible synergism. Therefore, in the current study, a second ERE-containing promoter reporter construct (ERE.PS2.luc) was investigated. This ERE-containing reporter construct consists of an ERE sequence obtained from the human PS2 gene expressed in breast cancer cells [12][30], and previously demonstrated good transactivation activity via the ER subtypes (predominantly ERα), however, the results varied between different cell types [11–14,31]. For example, 10^{-9} M E_2-induced transactivation of the PS2-ERE via ERα resulted in a 2-fold induction in Cho-K1 cells [11], an approximate 5-fold induction in COS-1 cells [12], and around 12-fold induction in Hela cells [31]. Thus, in the current project, the E_2-induced transactivation of ERE.vit2.luc and ERE.PS2.luc via ERα, as well as ERβ, was investigated and the obtained efficacies and potencies, specifically in the HEK293 cells, were compared.

The two EREs showed similar results for ERα (Figure 4.4 A & C), with no significant difference between the efficacies (6.02 ± 0.22 fold for ERE.vit2.luc and 6.22 ± 0.14 fold for ERE.PS2.luc) or potencies (8.14 x 10^{-12} M and 1.21 x 10^{-11} M for ERE.vit2.luc and ERE.PS2.luc, respectively). Moreover, ERβ (Figure 4.4 B & D) displayed a small but significant ($P<0.05$) difference between the potencies (8.04 x 10^{-10} M for ERE.vit2.luc and 1.62 x 10^{-10} M for ERE.PS2.luc). The efficacy of ERE.PS2.luc induction via ERβ, on the other hand, was a significantly ($P<0.0001$) 9 times higher than that of ERE.vit2.luc (77.36 ± 3.37 fold vs. 8.52 ± 0.45 fold, respectively).

It is apparent from these results in HEK293 cells that the sensitivity of E_2 transactivation of the ERE.PS2.luc system via ERβ was more sensitive when compared to other studies, which used different cell lines, as well as when compared to the study of Mortimer *et al*. [15], which used the same cell line but a different ERE [11–13,31]. These results further emphasise how important it is to take various factors, like cell types or response elements, into account when testing drug interactions, and to not only focus on drug concentrations. Furthermore, in the current study the ERE.PS2.luc system using HEK293 cells will be an ideal system to investigate the interaction and contribution of the phenolic compounds to the estrogenic activity of F3, which was shown to display robust ERβ transactivation [15].

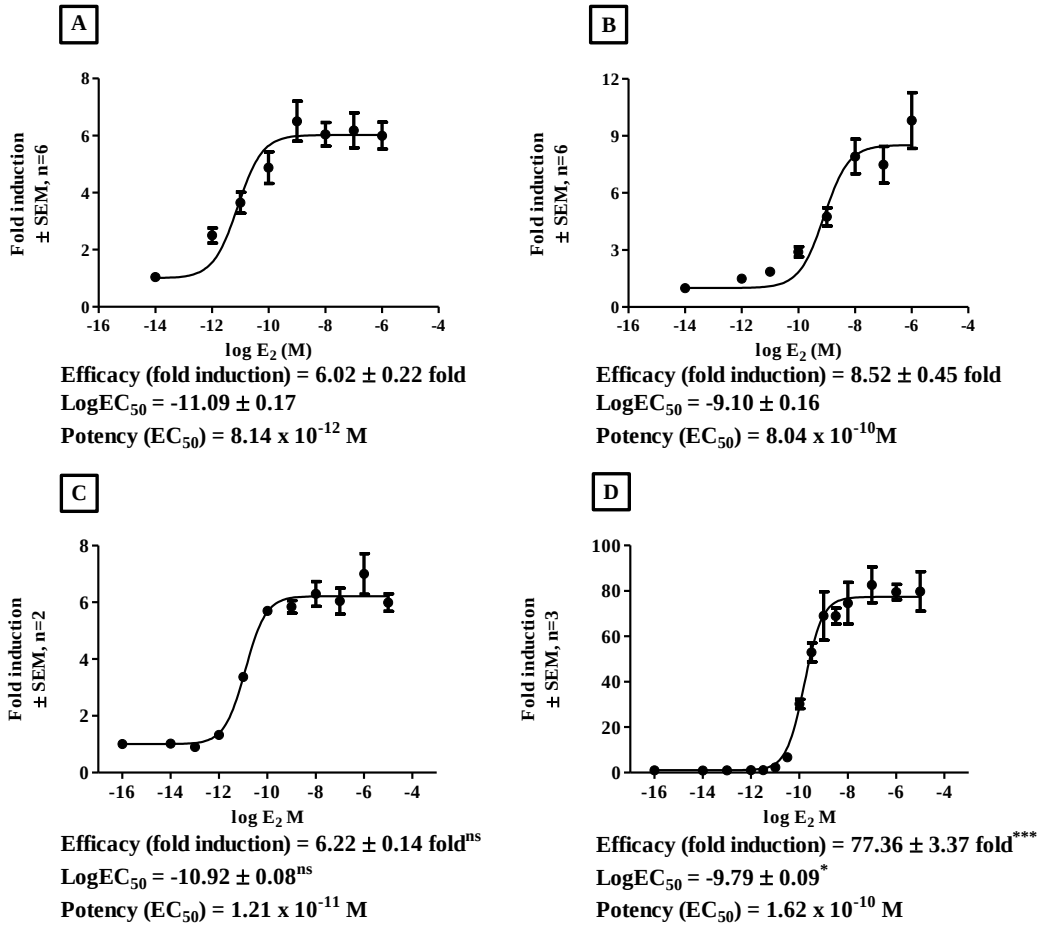

Figure 4.4: Promoter reporter studies investigating ERα and ERβ dose response curves. A & B (pre-optimised protocol): HEK293 cells were transfected with pSG5-hERα **(A)** or pSG5-hERβ **(B)**, and **vit2.ERE.luc** and a pGL2 basic empty vector, and induced with increasing concentrations of E_2 (10^{-12} – 10^{-6} M). **C & D (post-optimised protocol):** HEK293 cells were transfected with pSG5-hERα **(C)** or pSG5-hERβ **(D)**, and **PS2.ERE.luc** and a pGL2 basic empty vector, and induced with increasing concentrations of E_2 (10^{-14} – 10^{-5} M). **Curve fitting:** nonlinear regression with log (agonist) vs. response (three parameters). **Statistical analysis**: unpaired t-test, pre-optimised protocol vs post-

4.2.3. Validation of optimised experimental protocols

In previous studies, Mfenyana *et al*. [15] and Visser *et al.* [20], SM6Met was shown to be an ERβ agonist, an ERα antagonist and an inhibitor of E_2-induced breast cancer cell proliferation. This extract (SM6Met) was subsequently separated into two fractions, namely, a polar (PF) and a non-polar fraction (NPF) [15]. Furthermore, the NPF, displaying the highest phytoestrogenic activity, was further fractionated into three fractions with fraction 3 (F3) displaying the highest estrogenic activity via ERβ, when compared to the other NPF fractions [15].

Whilst the main focus of the current study is to investigate the contribution of the 7 major phenolic compounds previously identified in F3 to the estrogenic activity of F3, it forms part of a larger, ongoing investigation to identify the active components of the *Cyclopia* extracts. Therefore, to validate the protocols optimised in the current study, results from the optimised protocols were compared to that of previous laboratory protocols (pre-optimised protocols) used to obtain results in previous studies [15,20]. The SM6Met extract, the NPF and F3 were thus tested using the pre- and post-optimised protocols for both the proliferation assay and the promoter reporter assay. E_2 was used as an agonist standard whereas fulvestrant (ICI 182,780) was used as antagonist standard.

4.2.3.1. Validation of optimised proliferation protocol using SM6Met, NPF and F3

The SM6Met extract and the NPF have previously been shown to antagonise E_2-induced cell proliferation of MCF-7 BUS cells, whereas F3 displayed no antagonistic activity. Furthermore, only F3, but not SM6Met and the NPF, displayed significant agonist activity [15]. To validate the optimised experimental protocol for MCF-7 BUS cell proliferation, the results obtained were compared to that of previous studies.

The optimised cell proliferation protocol elicited a 3.23 ± 0.08 fold induction with 10^{-9} M E_2 (Figure 4.5 B) in comparison to the 1.74 ± 0.07 fold obtained with the pre-optimised protocol (Figure 4.5 A), which supports the results obtained during the optimisation procedure (Figure 4.3 F). In the absence of E_2, both the pre- and post-optimised protocols showed significant agonist activity for SM6Met, the NPF and F3. However, the activity of the extracts/fractions, in both the pre- and post-optimised protocol were not significantly different from that of 10^{-11} M E_2, with the exception of SM6Met in the post-optimised protocol, which displayed a significantly ($P<0.0001$) lower activity than 10^{-11} M E_2. As expected, fulvestrant (ICI 182,780) showed no agonist activity.

In antagonist mode, none of the extracts or fractions displayed antagonism of 10^{-11}M E_2, with either (pre-and post-optimised) protocol (Figure 4.5 C & D). Fulvestrant (ICI 182,780), however, did display significant (P<0.05) antagonism.

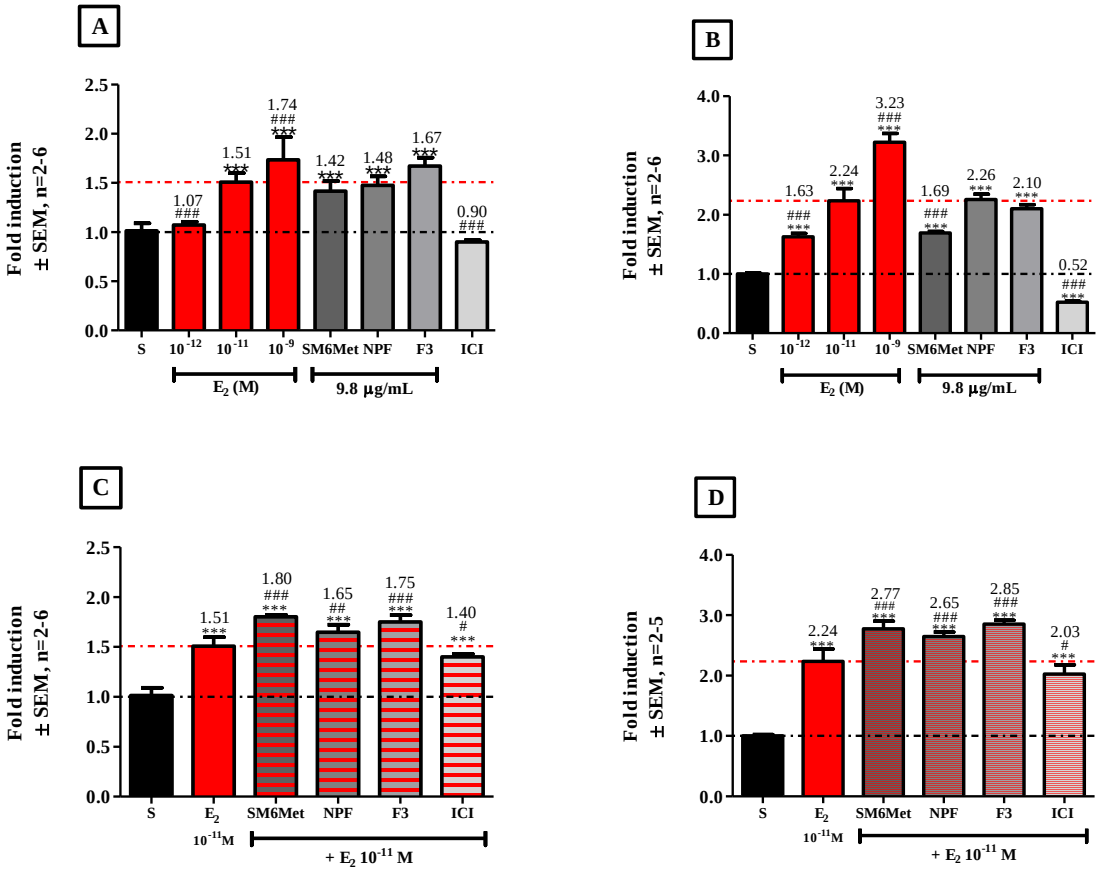

Figure 4.5: Cell proliferation studies investigating the agonist (A & B) and antagonist (C & D) activity of SM6Met, NPF, F3 & fulvestrant (ICI 182,780). Cells were induced with solvent (S), increasing concentrations of E_2 (10^{-12}, 10^{-11} & 10^{-9} M) as agonist standard, SM6Met, NPF and F3 (9.8µg/ml), and ICI 182,780 (10^{-10} M) as antagonist standard, both in **(A & B)** agonist (absence of E_2) and **(C & D)** antagonist (presence of E_2 10^{-11} M) mode. Both the pre-optimised laboratory protocol **(A & C)** and the post-optimised laboratory protocol **(B & D)** were investigated. Statistical analysis: One-way ANOVA with Dunnett's Multiple Comparisons Test as post-test, all columns compared to solvent control set to 1 (black dotted line), *P<0.05, **P<0.001, ***P<0.0001, and extracts/fractions/compounds compared to E_2 10^{-11} M (red dotted line) $^{\#}$P<0.05, $^{\#\#}$P<0.001, $^{\#\#\#}$P<0.0001.

Validation of the optimised cell proliferation assay indicated that this system was still not sensitive enough to obtain accurate results, since even after optimisation, F3 only displayed a induction of 2-fold. In addition, the favourable estrogenic profile of SM6Met previously reported was also not present, since neither SM6Met nor NPF displayed antagonism of E_2-

induced cell proliferation [15,20]. Consequently, the results were not comparable to previous studies.

Furthermore, in the current study proliferative activity (measured with the MTT assay) was also compared to cell viability (measured with viable cell counting), since polyphenols have been shown to influence mitochondrial activity and, as MTT is metabolised via mitochondrial enzymes, may interfere with the MTT assay [32,33]. No difference between proliferative activity and cell viability (Figure 4.6), upon SM6Met induction was observed, which corresponds with similar results from Mortimer's study [16].

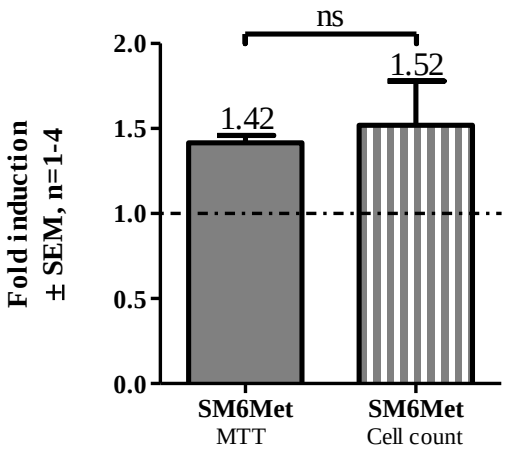

Figure 4.6: Comparison of cell proliferation vs cell viability. MCF-7 BUS cells were induced with 9.8µg/ml SM6Met to investigate and compare the effect of the extract on proliferative activity (MTT assay) and cell viability (cell count). Statistical analysis: t-test, solvent control set to 1 (black dotted line),*P<0.05, **P<0.001, ***P<0.0001, ^{ns}no significant difference.

4.2.3.2. Validation of optimised promoter reporter protocol using SM6Met, NPF and F3

Following optimisation of the promoter reporter assay, a more sensitive system was established for ERβ by using the ERE.PS2.luc promoter reporter construct. To ensure that the favourable F3 characteristic of robust ERβ agonism, previously identified by Mortimer *et al.* [15], was maintained with the post-optimised protocol, F3 activity was compared in both the pre- and post-optimised protocols using both ER subtypes. Furthermore, in order to compare results from the current study to that of previous studies, SM6Met and the NPF were also

included to validate the pre- and post-optimised protocols in both agonist (Figure 4.7) and antagonist mode (Figure 4.8).

Due to the significant ($P<0.0001$) difference in efficacy observed between ERα and ERβ for the post-optimised protocol (Figure 4.4 C & D), the activity of the compounds/extracts/ fractions were compared to increasing concentrations of E_2 (10^{-11}, 10^{-10} & 10^{-9} M) within each ER subtype protocol.

The pre-optimised protocol, using ERE.vit2.luc, presented similar results compared to a previous study done by Mortimer *et al*. [15], with F3 displaying robust and significant ($P<0.0001$) ERβ agonist activity at 2.64 ± 0.13 fold induction (Figure 4.7 B) and no significant ERα activity (Figure 4.7 A).

The post-optimised protocol, using ERE.PS2.luc, however, provided a significantly ($P<0.0001$) 10.5-fold increase in sensitivity via ERβ, with 10^{-9} M E_2 producing a 62.10 ± 2.05 fold induction (Figure 4.7 D) compared to the pre-optimised protocol, which only produced a 5.92 ± 0.26 fold induction (Figure 4.7 B). Similarly, the post-optimised protocol via ERα also displayed a significant ($P<0.001$) 1.8-fold increase in sensitivity with 10^{-9} M E_2.

In the post-optimised protocol, F3 also showed a significantly ($P<0.0001$) more robust ERβ agonist activity compared to the pre-optimised protocol. This increase in sensitivity in the post-optimised protocol is not only apparent when comparing absolute fold induction (21.13 ± 3.62 vs 2.64 ± 0.13 fold induction), but also when comparing F3 to 10^{-11} M E_2 (10 vs 1.5 fold induction). Despite the increase in sensitivity in the post-optimised protocol, the rank order of F3 activation via ERβ remains similar, with F3 displaying comparable induction to that of 10^{-10} M E_2 with both protocols (Figure 4.7 B & D).

The increased sensitivity with ERα observed in the post-optimised protocol has, however, now resulted in F3 displaying significant ($P<0.0001$) ERα agonist activity, in contrast to what was observed in the pre-optimised protocol and the Mortimer study [15]. Nonetheless, when comparing F3 fold induction to 10^{-11} M E_2, it was significantly ($P<0.05$) lower in the post-optimised protocol. In addition, in both protocols F3 induction is around 0.6-fold that of 10^{-11} M E_2 (Figure 4.7 A & C).

SM6Met and the NPF, on the other hand, displayed no significant agonist activity with either ER subtype for the pre-optimised protocol when compared to respective solvent treatments. These results did not correspond with the findings of Mortimer *et al*. [15], which showed significant ERα and β agonist activity with SM6Met and significant ERβ agonist activity with the NPF. With the post-optimised protocol, SM6Met displayed no significant agonist activity with either ER subtype, while the NPF only displayed significant ERα agonist activity ($P<0.05$). However, when conducting statistical analysis (unpaired t-test) to compare only the NPF to 10^{-11} E_2 M, the NPF displayed significant ($P<0.001$) ERβ agonist activity via the post-optimised protocol.

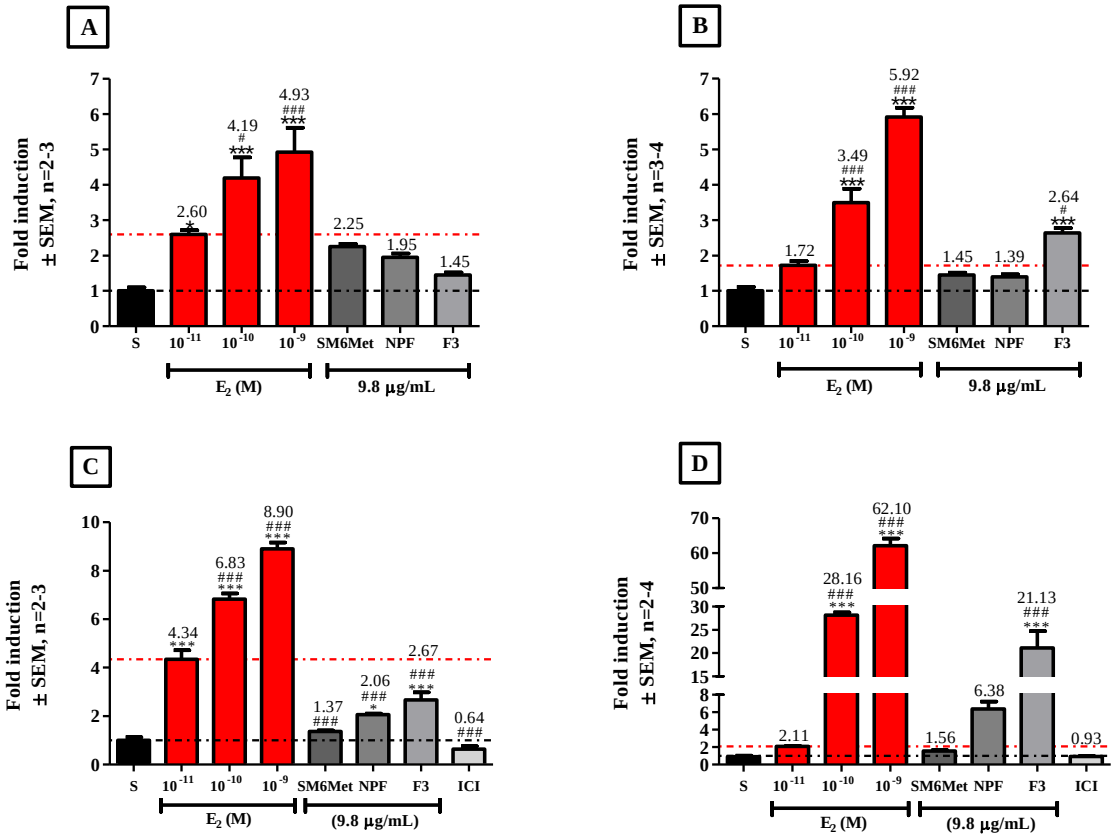

Figure 4.7: Promotor reporter studies investigating the effect of E_2, SM6Met extract, NPF and F3 on ERα (A & C) and ERβ (B & D) transactivation via vit2.ERE.luc (A & B) and PS2.ERE.luc (C & D) in against mode. HEK293 cells were transfected with expression vectors pSG5-hERα/pSG5-hERβ, vit2.ERE.luc/PS2.ERE.luc and a pGL2 basic empty vector, and induced with increasing concentrations of E2 (10^{-11} – 10^{-9} M) as agonist standards, SM6Met, NPF and F3 (9.8µg/ml), and ICI (10^{-10} M) as ER antagonist standard. Statistical analysis: One-way ANOVA with Dunnett's Multiple Comparisons Test as post-test, all columns compared to solvent control set to 1 (black bar),* P <0.05, ** P< 0.001, *** P<0.0001, or compared to E_2 10^{-11} M (red bar) $^{\#}$ P<0.05, $^{\#\#}$ P<0.001, $^{\#\#\#}$ P<0.0001.

Antagonist mode was investigated in the presence of 10^{-11} M E_2 with the induction of extract and fractions expressed relative to E_2, which was set as 1. F3 displayed no antagonist activity but rather strong (P<0.0001) ERβ agonist activity, at 2.06 ± 0.38 fold induction (Figure 4.8 B), with the pre-optimised protocol, thus appearing to have an additive effect via the ERβ subtype. F3, however, did display significant (P<0.001) levels of ERα antagonism with the pre-optimised protocol (Figure 4.8 A). This is in contrast to the results of Mortimer *et al.* [15], where no significant antagonism of F3 for either ER subtype was observed.

In the post-optimised protocol, F3 displayed no antagonistic activity for either ER subtype, in accordance with results from Mortimer *et al* [15]. F3 did, however, display a significant

($P<0.0001$) 9.48-fold higher ERβ agonist activity (Figure 4.8 D) in the presence of E_2, which further substantiates F3's strong additive ERβ agonist activity.

In the pre-optimised protocol, neither SM6Met nor the NPF displayed significant antagonist activity in the presence of 10^{-11} M E_2 via either ER subtype, which contradicts the ERα antagonistic profile of SM6Met and NPF previously identified by Mortimer *et al* [15].

SM6Met did, however, present with significant ($P<0.05$) ERα antagonist activity (Figure 4.8 C) in the post-optimised protocol, which corroborates previous studies [15,20]. The increased 10^{-11} M E_2 activity via ERα observed with the post-optimised protocol (Figure 4.7 C) could have resulted in the recovered ERα antagonist activity of SM6Met (Figure 4.8 C), which was not detected in the pre-optimised protocol (Figure 4.8 A). The increased sensitivity observed in the post-optimised protocol has, however, not resulted in the NPF displaying significant ERα antagonist activity.

To summarise the validation of the promoter reporter protocol, F3 demonstrated strong and significant ERβ agonist activity in both the pre- and post-optimised protocol. However, the post-optimised protocol with ERE.PS2.Luc is a more sensitive protocol with F3 displaying a 10-fold increase in ERβ agonist activity relative to 10^{-11} M E_2 versus a 1.5-fold increase via ERE.Vit2.luc in the pre-optimised protocol. Therefore, for the current study, the post-optimised protocol would be ideal to investigate the interaction and possible synergism of the 7 major phenolic compounds identified in F3 in terms of ERβ activity [15].

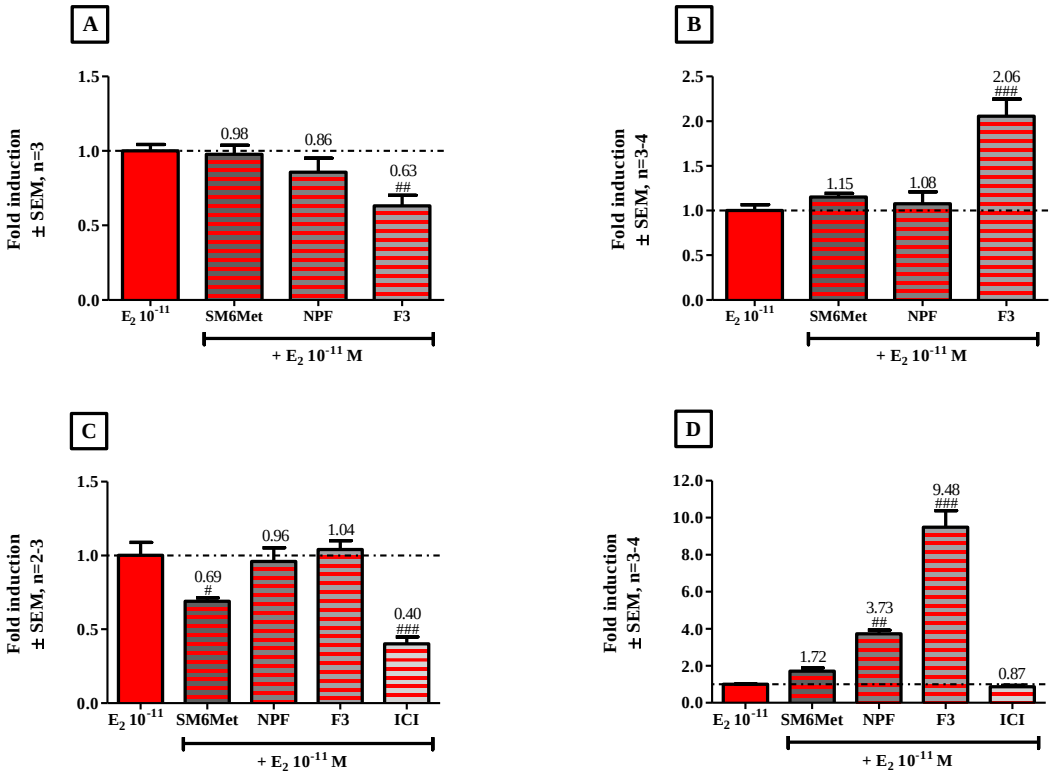

Figure 4.8: Promotor reporter studies investigating the effect of E_2, SM6Met extract, NPF, F3 and fulvestrant (ICI) on ERα (A & C) and ERβ (B & D) transactivation via vit2.ERE.luc (A & B) and PS2.ERE.luc (C & D) in antagainst mode. HEK293 cells were transfected with expression vectors pSG5-hERα/pSG5-hERβ, vit2.ERE.luc/PS2.ERE.luc and a pGL2 basic empty vector, and induced with E_2 10^{-11} M, as well as SM6Met, NP and F3 (9.8µg/ml), and ICI (10^{-10} M) as ER antagonist standard, in the presence of E_2 10^{-11} M. Statistical analysis: One-way ANOVA with Dunnett's Multiple Comparisons Test as post-test, all columns compared to E_2 10^{-11}M set to 1 (black dotted line), $^{\#}$ P<0.05, $^{\#\#}$ P<0.001, $^{\#\#\#}$ P<0.0001.

4.3. Conclusion

As mentioned frequently throughout this chapter, a sensitive system is essential when testing drug interactions and possible synergism. This is especially important when testing phytocompounds since the interactions of the plant phenolic mixtures are complex and not fully understood. For example, it has been shown that compounds that do not display individual activity, could contribute to the bioavailability of active compounds within a mixture [7,34].

Therefore, in the current study, various factors were investigated (including cell types, ER subtypes and ERE promoter reporter constructs, and optimisation of experimental

procedures) in order to obtain an optimised protocol which is sensitive enough to detect small changes, produce accurate results and determine combinatory or possible synergistic effects.

From the optimisation of the experimental protocols (cell proliferation assay and promoter reporter assay), it is clear that the promoter reporter assay is a more sensitive experimental system to test phytocompound interactions, since the efficacies of E_2 via the promoter reporter assay (Figure 4.4) at 77.36 ± 3.37 and 6.22 ± 0.14 fold for ERβ and α, respectively, were higher than that of the cell proliferation assay (Figure 4.2) at 3.67 ± 0.08 fold.

The promoter reporter assay also produced results similar to previous work done on F3, the focus of the current study, with robust ERβ agonism (10-fold compared to 10^{-11} M E_2) and weak ERα agonism (0.6-fold compared to 10^{-11} M E_2). Furthermore, since F3 also displayed no antagonistic activity via either ER subtype using the post-optimised protocol as shown in Mortimer's study (Table 4.2), it was decided that further investigation would only be conducted in agonist mode.

In addition, the agonist and antagonist activity of SM6Met and the NPF in the post-optimised promoter reporter assay, were also compared to that of Mortimer *et al*. [15], (Table 4.2). SM6Met only displayed comparable results to that of Mortimer *et al*., in antagonism mode via both ER subtypes, while the NPF results differed in all cases, except for ERβ antagonism. Furthermore, the antagonist results obtained for SM6Met and NPF via the MTT assay were also different from previous studies by Mortimer *et al*. [15], and Visser *et al*. [20]. These studies, however, used a higher concentration of E_2 (10^{-9} M) to investigate antagonism, whereas the current study used 10^{-11} M E_2, since this concentration of E_2 is situated on the inflection point line (Figure 4.2 B). It could be that 10^{-11} M E_2 did not display sufficiently strong agonist activity in order to detect the counter effects of the antagonist compounds. It could also be that degradation of phytoestrogens or important phenolic compounds in the

extract/fraction [35,36], some already identified [15,22,27], some yet to be discovered, could play a role. Combined these factors make it difficult to establish the possible causes of the change in SM6Met and the NPF's activity.

Table 4.2: Summary of validation outcomes of promoter reporter and proliferation (MTT) assays using SM6Met, the NPF and F3 which either display significant agonist (Ag) or antagonist (Antag) activity. Green represents results similar to whereas red represents results opposite to that of Mortimer *et al* [15].

		ERα			ERβ			MTT		
		Pre[a]	Post[b]	Mort[c]	Pre	Post	Mort	Pre[d]	Post[e]	Mort
Agonism	SM6Met	-[f]	-	Ag	-	-	Ag	Ag	Ag	-
	NPF	-	Ag	-	-	-	Ag	Ag	Ag	-
	F3	-	Ag	Ag	Ag	Ag	Ag	Ag	Ag	Ag
Antagonism	SM6Met	-[g]	Antag	Antag	-	-	-	-	-	Antag
	NPF	-	-	Antag	-	-	-	-	-	Antag
	F3	Antag	-	-	-	-	-	-	-	-

[a] summary from pre-optimised protocol (ERE.vit2.luc) – Figure 4.7 A & B, Figure 4.8 A & B
[b] summary from post-optimised protocol (ERE.PS2.luc) – Figure 4.7 A & B, Figure 4.8 A & B
[c] summary from Mortminer *et al.* [15]
[d] summary from pre-optimised protocol (MTT) – Figure 4.5 A & C
[e] summary from post-optimised protocol (MTT) – Figure 4.5 B & D
[f] no agonist activity
[g] no antagonist activity

In summary, it was decided that the cell proliferation assay would not be used for further investigations. The promoter reporter assay, on the other hand, provided a more sensitive system and F3 results comparable to that of previous studies, and will therefore be used to further investigate the interaction and possible synergistic activity of the phenolic compounds previously compounds identified in F3 [15].

4.4. References

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Chapter 5: Investigating the contribution of the major phenolic compounds identified in F3, to the robust ERβ activity of F3.

5.1. Introduction

Plants form part of our daily diet, and with increased evidence linking plant based food to improved health, as well as disease prevention, plants are not only considered a source of macronutrients (proteins, carbohydrates and fats), but also micronutrients (vitamins, minerals, dietary fibre, antioxidants and polyphenols) that have potential anti-inflammatory, anti-diabetic and anti-carcinogenic properties [1–8].

For many years plants have played a role in the healing rituals of indigenous and traditional populations such as in India, China and Africa [9–13]. The main focus of traditional phytomedicine is on using combination therapy to treat a patient as a whole while combating disease on multiple levels, instead of using a single well characterised active compound that is target specific [12,14]. This holistic approach of traditional medicine has transformed modern drug development and changed the way therapies for complex diseases, such as cancer, is approached [9,15].

The higher effectiveness of plant extracts in comparison to single active plant derived compounds is thought to be due to the combinatorial and/or synergistic effects of the mixture of different active and supporting constituents [7,16–18]. The supporting constituents themselves could be pharmacologically inactive, but contribute to the stability, solubility and/or absorption rate of the active compounds [19]. The advantages of combinatorial or synergy approaches in cancer drug development are the possible multi-target effects that could result in the prevention of DNA damage, down regulation of oncogenes, up regulation of tumour-suppressor genes, and inhibition of pathways involved in the proliferation of defective cells [8,12,20–22].

One plant of particular interest for the current study regarding possible novel nutraceutical development, that contains high levels of phenolic compounds and has displayed

phytoestrogenic, anti-diabetic, anti-inflammatory and anti-mutagenic activity, is *Cyclopia*, which is more commonly known as Honeybush [6,23–28]. *Cyclopia* forms part of the indigenous South African fynbos and is commercially available as a herbal tea [29,30]. In studies conducted by Mfenyana *et al*. [31] and Visser *et al*. [32], the sequential methanol extract from *Cyclopia subternata* harvesting M6 (SM6Met) was shown to be an ERβ agonist, an ERα antagonist and an inhibitor of E_2-induced breast cancer cell proliferation. The current study, as mentioned in previous Chapters, focuses on F3, one of the fractions isolated from SM6Met [33].

In Chapter 4, F3 was shown to display robust ERβ agonism, with a 10-fold increase in activity when compared to 10^{-11} M E_2 (the physiological relevant concentrations of E_2 in healthy women) [34]. Additionally, F3 displayed 1.6-fold lower activity than 10^{-11} M E_2 via ERα. This demonstrates that F3 exhibits ER subtype selective activity, and highlights its potential as a nutraceutical in the treatment of estrogen-related cancer, especially considering that ERβ has been shown to inhibit the proliferative effects of ERα [35–38].

The phytoestrogenic activity of some of the individual phenolic compounds in *Cyclopia*, and subsequently in F3, was previously established [23,30]. However, the behaviour of these compounds in combination is still unknown. Therefore, the current study focuses on combining the 7 individual major phenolic compounds, identified and quantified in F3 [33], in order to determine their combinatorial or possible synergistic contribution to the favourable estrogenic profile of this fraction. The activity of the individual phytoestrogenic compounds was also investigated, in an attempt to determine their contribution to the estrogenic profile of F3.

Following on from the optimisation and validation of estrogenic bioassays (Chapter 4), the promoter reporter assay was established as the more sensitive system to test phytocompound

interactions and possible synergism, and therefore, the optimised protocol, in agonist mode, was used to investigate the phytoestrogenic activity of the phenolic compounds in the current Chapter.

5.2. Results and discussion

5.2.1. Evaluation of the estrogenic activity of reconstituted F3 (F3R)

To evaluate and fully understand the nutraceutical potential of plant extracts, it is important to first understand the combinatorial role of all their components. This is, however, a difficult task when the activity and role of all the individual compounds are not yet known, as in the case of F3. Mortimer *et al*. [33], identified 15 phenolic compounds in F3 using LCMS-MS, and quantified 7 of these phenolic compounds using qHPLC. Mangiferin, isomangiferin, scolymoside, *p*-coumaric acid and protochatechuic acid were identified as the major phenolic compounds present at the highest concentrations in F3. In his study, Mortimer also included two minor compounds in terms of mass, iriflophenone-3-*C*-glucoside and luteolin, since they were commercially available and could be detected in 9.8mg/ml[*] of F3. These 7 phenolic compounds[†] were then classified by Mortimer as the major phenolic compounds in F3, an appellation we also use throughout the current study [33,39].

The ideal approach to understand how F3 mediates its favourable ERβ agonist effect, would be to investigate all the major and minor phenolic compounds previously identified in F3 [39]. However, as a starting point, only the 7 major phenolic compounds identified and quantified by Mortimer were used to reconstitute F3 (F3R), and to investigate their contribution to the robust ERβ agonist activity of F3, as demonstrated in Chapter 4.

[*] 9.8mg/ml is a concentration used throughout our research group based on a study by Verhoog *et al.* [23][127].
[†] The 7 phenolic compounds were purchased from different suppliers (see section 3.2.1).

F3's activity was validated prior to reconstituting the fraction. F3 displayed significant ($P<0.001$) ERβ agonist activity at 28.00 ± 2.78 fold induction (Figure 5.1 B) and no significant ERα activity at 1.23 ± 0.07 fold induction (Figure 5.1 A). Furthermore, when comparing F3 to the E_2 standards (10^{-11}, 10^{-10} & 10^{-9} M E_2), it displayed a significant 8.8-fold increase in activity via ERβ compared to 10^{-11} M E_2, and no significant difference when compared to 10^{-10} M E_2, whereas via ERα, F3 displayed a 1.12-fold decrease in activity compared 10^{-11} M E_2. This is consistent with the findings from Chapter 4 and Mortimer *et al.*, [33].

The reconstituted F3 (F3R) was assembled according to the concentrations in Table 5.1, and the phytoestrogenic activity compared to the original F3, to determine if the major phenolic compounds are responsible for F3's robust ERβ mediated activity. F3R (1x), showed no estrogenic activity via ERβ compared to solvent and was a significant ($P<0.001$) 28-fold lower than F3 (Figure 5.1 B). F3R (1x) also showed no significant ERα activity, as expected, since F3 also showed little ERα activity (1.23 ± 0.07 fold induction).

Table 5.1: Phenolic content in F3 as identified in Mortimer *et al.* [33]**, which was used in reconstitution studies.**

Concentrations	Mangiferin	Isomangiferin	*p*-Coumaric acid	Scolymoside	Luteolin	Protocatechuic acid	Iriflophenone-3-*C*-glc
µg/9.8µg [a]	1.133	0.394	0.056	0.752	0.020	0.063	0.009
1x F3R (M[b])	2.684×10^{-6}	0.932×10^{-6}	3.404×10^{-7}	1.264×10^{-6}	4.735×10^{-8}	4.111×10^{-7}	2.204×10^{-8}
10x F3R (M)	2.684×10^{-5}	0.932×10^{-5}	3.404×10^{-6}	1.264×10^{-5}	4.735×10^{-7}	4.111×10^{-6}	2.204×10^{-7}
100x F3R (M)	2.684×10^{-4}	0.932×10^{-4}	3.404×10^{-5}	1.264×10^{-4}	4.735×10^{-6}	4.111×10^{-5}	2.204×10^{-6}

[a] µg polyphenol in 9.8µg/ml F3

[b] molar concentration of polyphenols corresponding to µg in 9.8µg/ml F3, as used in cell culture experiments

To ensure that the lack of phytoestrogenic activity observed with F3R (1x) was not due to inaccurate sample preparation, LC-MS was performed and the phenolic content of F3R was compared to that in F3 (Figure S.1 & S.2). Data was processed using MassLynx V4.1

software (Waters, USA) and the major phenolic compounds in F3R displayed similar peak areas and retention times to the compounds in F3, indicating that F3R was made up correctly and that the lack of phytoestrogenic activity was not due to sample preparation errors.

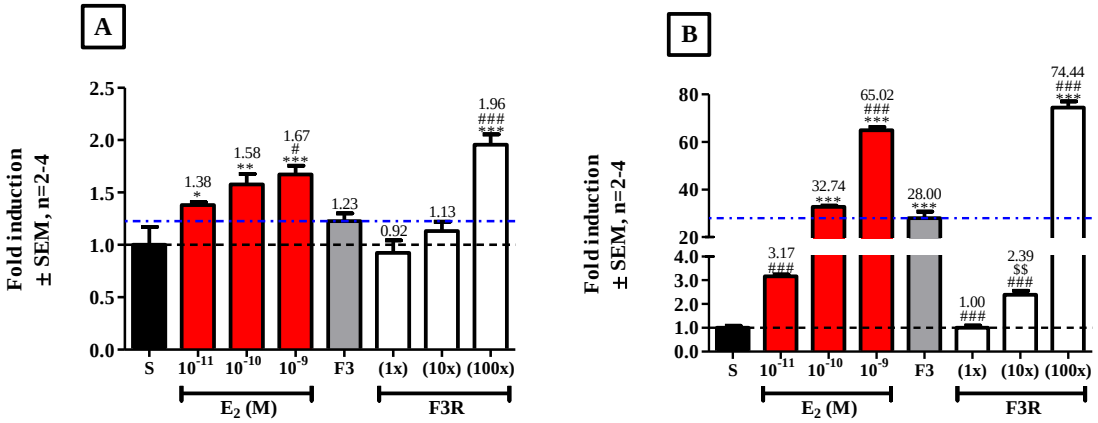

Figure 5.1: Promotor reporter studies investigating the effect of E_2, F3 and F3R on ERα (A) and ERβ (B) transactivation in agonist mode. HEK293 cells were transfected with pSG5-hERα/pSG5-hERβ, ERE.PS2.luc and a pGL2 basic empty vector, and induced with E_2 (10^{-11} – 10^{-9} M), F3 (9.8µg/ml) and F3R reconstituted at 1x, 10x and 100x the concentrations of major phenolic compounds as identified in F3 (9.8µg/ml). Statistical analysis: One-way ANOVA with Dunnett's Multiple Comparisons Test as post-test, all columns compared to solvent control set to 1 (black bar), *P<0.05, **P<0.01, ***P<0.001, and to F3 (grey bar) $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001. Unpaired t-test, solvent vs F3R (10x): $^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001.

In addition, F3R was reconstituted at higher concentrations, as some of the phenolic compounds, like luteolin, have previously been shown to display phytoestrogenic activity [23,30,32,40,41]. F3R reconstituted at 10-fold (10x) its concentration in F3, F3R (10x), showed no significant activity via ERα and a 2.39-fold increase in activity via ERβ, although still not significant when compared to solvent. Conducting an unpaired t-test analysis, comparing only solvent to F3R (10x), a significant (P<0.01) induction was observed via ERβ, but not via ERα. The ERβ activity was, however, still a significant 11.72-fold lower than F3.

Only at 100-fold (100x) the original concentration in F3 did F3R display significantly higher (P<0.001) fold induction via both ERα and ERβ, at 1.96 ± 0.09 and 74.44 ± 2.65 fold induction, respectively. A significant (P<0.001) 1.59-fold increase in activity was observed via ERα when compared to F3, and no significant difference when compared to 10^{-9} M E_2.

With ERβ, F3R (100x) displayed a significant ($P<0.001$) 2.66-fold increase in activity compared to F3, and a significant ($P<0.01$) 1.15-fold increase compared to 10^{-9} M E_2.

From these results, we can conclude that the 7 major phenolic compounds reconstituted at their original concentration as quantified in F3, do not recapitulate the activity of F3. This further accentuates the importance of the fraction as a whole and of including minor phenolic compounds. Only at 100x the concentration did F3R display significantly higher ($P<0.001$) agonist activity than F3 via both ER subtypes. It could be that the minor phenolic compounds not included in F3R play a supporting role (e.g. by influencing absorption, stability or export) in the phytoestrogenic activity of the major phenolic compounds in F3, and that without these minor compounds the major compounds in F3R are not present in the cells at a sufficient concentration to induce an estrogenic response. However, at higher concentrations, varying between 10^{-6} M and 10^{-4} M (Table 5.1), the major phenolic compounds are able to elicit an estrogenic response as seen with F3R (100x). These higher concentrations (Table 5.1) also corresponded to the concentration (10^{-5} M) of individual phenolic compounds, such as luteolin, previously shown to display phytoestrogenic activity within our research group [42].

To determine which of the 7 major phenolic compounds in F3R contribute to the activity observed at higher concentrations (10x F3R and 100x F3R), a further investigation was conducted regarding the individual activity of these compounds.

5.2.2. Estrogenic activity of the phenolic compounds in F3R

5.2.2.1. Estrogenic activity of the individual phenolic compounds in F3R

Various societies globally are dependent on traditional medicinal plant sources for disease treatment due to poverty, lack of pharmaceutical manufacturing capacity or limited access to modern medicine [13,43]. Plant extracts consist of a variety of compounds that display

complex interactions, and the adverse effects of traditional phytomedicine are not as well studied as that of synthetic drugs [9,19,44–47]. Therefore, to understand how plant extracts exert their effects as a whole, it is not only important to investigate the combinatorial effect of the constituent parts, but also the individual activity of all the compounds in order to establish safety and efficacy [19,44].

F3R did not display any phytoestrogenic activity when reconstituted at the same concentrations of major phenolic compounds as quantified in F3 (Table 5.1). At higher concentrations, however, a significant increase in agonist activity was observed with F3R. As mentioned before, luteolin has previously been shown to have phytoestrogenic activity, but at concentrations higher than that of luteolin in F3 (1×10^{-5} M vs 4.7×10^{-8} M, respectively) [23,42,48]. Therefore, to determine the individual activity profile and to discover which of the phenolic compounds could possibly contribute to F3R's estrogenic activity at higher concentrations, the individual compounds were also investigated at increasing concentrations (1x, 10x, 100x).

A decrease in sensitivity of the test system (especially via ERβ) was observed at this stage of the study, and various aspects were investigated including cell passage number, growth medium, DNA plasmid quality, as well as incubator functionality, but no deviations or decrease in quality was noticed. Despite the decrease in sensitivity and ongoing search for the possible cause, the rank order of F3 activity compared to the E_2 controls remained similar. Specifically as seen in Figure 5.2, F3 elicited a response that was not significantly different than 10^{-11} M via ERα (as also seen in Figure 5.1 A), and retained its position between 10^{-10} M and 10^{-9} M E_2 via ERβ.

None of the 7 phenolic compounds displayed significant estrogenic activity at 1x their concentration in F3 (Figure 5.2 A – J & Figure 5.3 A – D). This concurs with findings from

Mortimer's study [33] and was expected since F3R (1x) displaced no activity (Figure 5.1). In addition, isomangiferin, scolymoside, iriflophenone-3-*C*-glucoside, *p*-coumaric acid and protocatechuic acid also did not display any significant phytoestrogenic activity at increased (10x and 100x) concentrations (Figure 5.2 A – J). Although these phenolic compounds did not display phytoestrogenic activity in our system, some of them (protocatechuic acid and *p*-coumaric acid) have been shown to possess anti-carcinogenic and chemo-preventative properties in breast cancer cell lines [49–52].

Mangiferin and luteolin were the only two phenolic compounds that demonstrated phytoestrogenic activity in our test system at increased concentrations (Figure 5.3 A – D). At the time, mangiferin's phytoestrogenic activity was a novel finding [30,33,42].

Mangiferin (10x) displayed a significant ($P<0.01$) increase in activity via both ERα (Figure 5.3 A) and ERβ (Figure 5.3B), at 1.26 ± 0.03 and 3.44 ± 0.11 fold induction, respectively. Even so, mangiferin (10x) still displayed a significantly ($P<0.001$) lower activity when compared to that of F3 via ERβ (Figure 5.3 B), but no significant difference via ERα (Figure 5.3 A). Luteolin, on the other hand, displayed no significant estrogenic activity at 10x its concentration in F3 via either ER subtype (Figure 5.3 C & D).

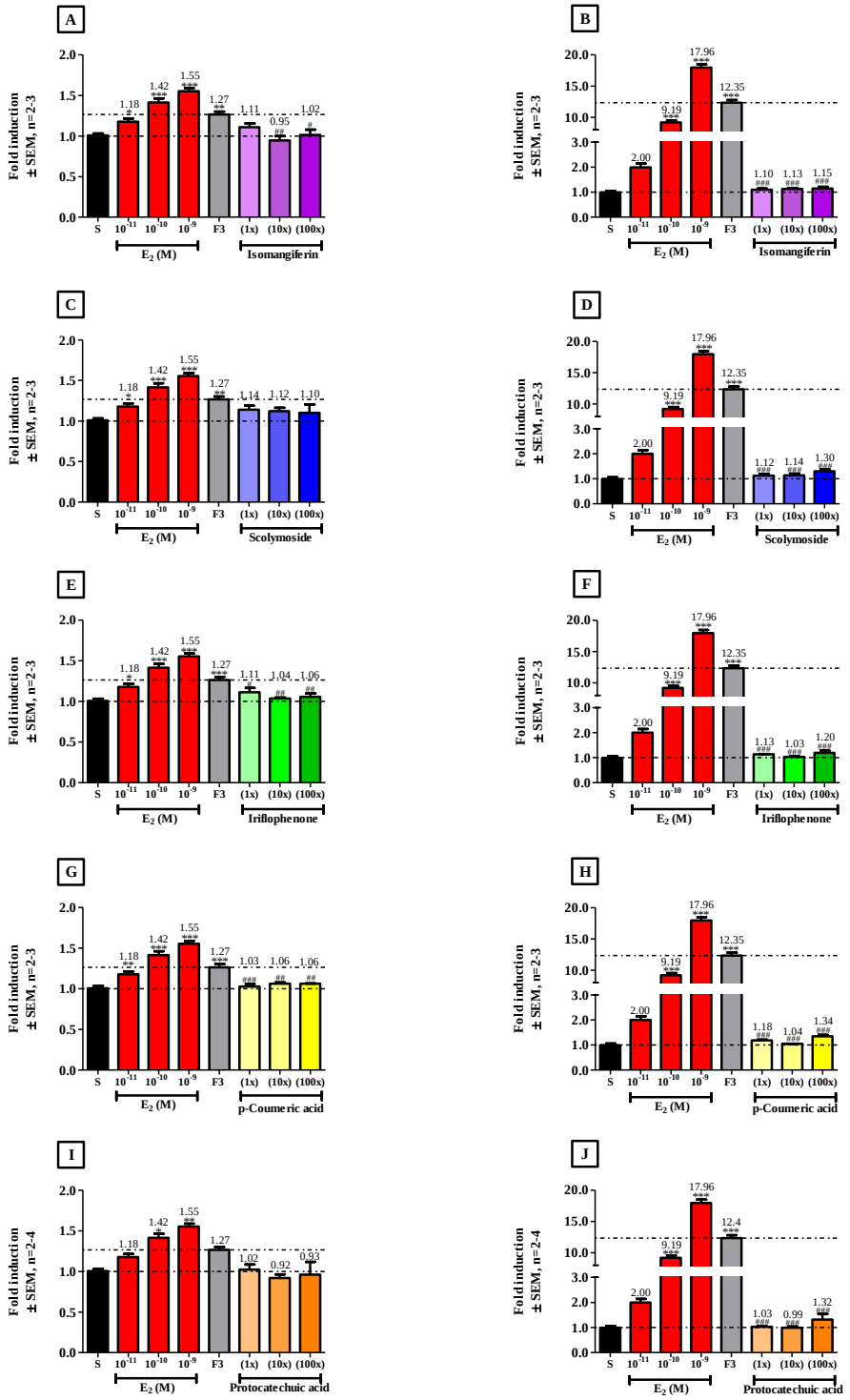

Figure 5.2: Promotor reporter studies investigating the effect of E_2, F3 and the individual major phenolic compounds identified in F3 via ERα (A, C, E, G & I) and ERβ (B, D, F, H & J) transactivation in agonist mode. HEK293 cells were transfected with pSG5-hERα/pSG5-hERβ), ERE.PS2.luc and a pGL2 basic empty vector, and induced with E_2 (10^{-11} – 10^{-9}M), F3 (9.8µg/ml) and individual phenolic compounds at 1x, 10x and 100x their concentrations as identified in 9.8µg/ml F3 (Table 5.1): isomangiferin (A & B), scolymoside (C & D), iriflophenone-3-*C*-glucoside (E & F), *p*-coumaric acid (G & H) & protocatechuic acid (I & J). Statistical analysis: One-way ANOVA with Dunnett's Multiple Comparisons Test as post-test, all columns compared to solvent control set to 1 (black bar), *P<0.05, **P<0.01, ***P<0.001, and individual phenolic compounds compared to F3 (Grey bar), $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.

Mangiferin and luteolin, at 100x their concentration in F3, however, both presented a significant (P<0.001) increase in activity via both the ER subtypes. Compared to F3, mangiferin displayed a small, yet significant (P<0.05), 1.16-fold increase in activity via ERα, and a significant (P<0.05) 1.16-fold lower activity via ERβ. Luteolin (100x) demonstrated robust ER agonist activity with a significant (P<0.001) 1.43- and 1.44-fold increase in activity when compared to F3, via ERα and ERβ, respectively.

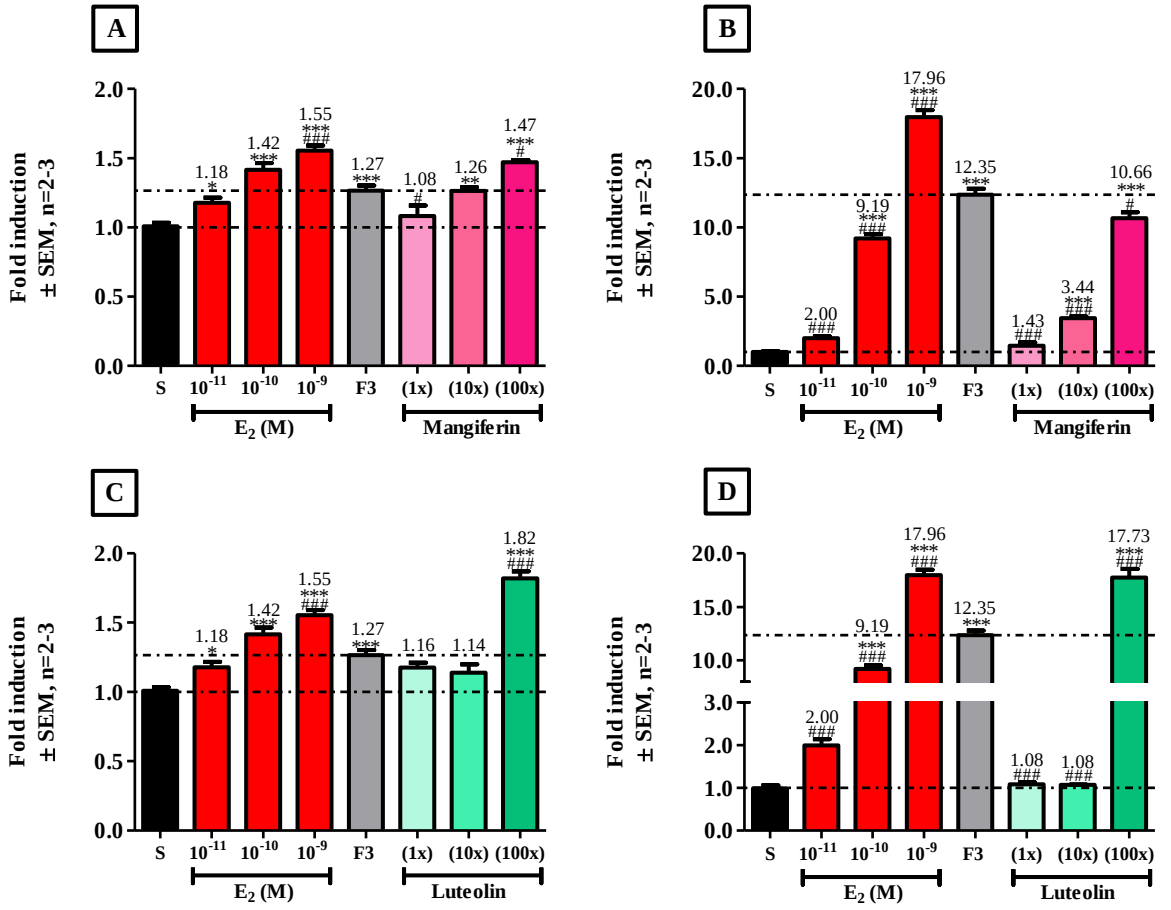

Figure 5.3: Promotor reporter studies investigating the effect of E_2, F3 and the individual phenolic compounds, mangiferin and luteolin, identified in F3 via ERα (A & C) and ERβ (B & D) transactivation in agonist mode. HEK293 cells were transfected with pSG5-hERα/pSG5-hERβ), ERE.PS2.luc and a pGL2 basic empty vector, and induced with E_2 (10^{-11} – 10^{-9}M), F3 (9.8µg/ml) and individual phenolic compounds at 1x, 10x and 100x their concentrations as identified in 9.8µg/ml F3 (Table 5.1): mangiferin (A & B) and luteolin (C & D). Statistical analysis: One-way ANOVA with Dunnett's Multiple Comparisons Test as post-test, all columns compared to solvent control set to 1 (black bar), *P<0.05, **P<0.01, ***P<0.001, and individual phenolic compounds compared to F3 (grey bar) $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.

To conclude, none of the 7 individual major phenolic compounds displayed significant estrogenic activity at their concentration in 9.8µg/ml F3R. These results correlates with the findings of Mortimer *et al.* [33]. In addition, mangiferin and luteolin were the only two phenolic compounds that displayed phytoestrogenic activity at higher concentrations. The estrogenic activity of mangiferin was a novel discovery at this stage of the current study, as no articles or papers, to our knowledge then, had been published regarding mangiferin's estrogenicity, in contrast to luteolin, which had shown estrogenic activity via both ER subtypes in various experimental systems [23,30,42,53–55].

5.2.2.2. Detailed investigation of estrogenic activity of the estrogenic compounds, mangiferin and luteolin

In the literature, mangiferin and luteolin display a variety of health benefits including decreasing the prevalence of metabolic disorders, cardiovascular protective properties, and combating various cancers on multiple levels [56–62]. In breast cancer studies, luteolin has been shown to act like an aromatase inhibitor, by down regulating aromatase transcription and enzyme activity, to suppress ERα expression, and to inhibit MCF-7 breast cancer cell growth [40,55,57,62]. Mangiferin has been shown to decrease cell proliferation and increase apoptosis, which consequently resulted in the decrease in cell viability of ER-positive and ER-negative breast cancer cell lines [63–65].

ER signalling may be either ligand-dependent or ligand-independent, and estrogenic compounds may mediate their effect directly through the ER, or by binding to other receptors and influencing other signalling pathways [47,55,66,67]. Therefore, to determine mangiferin and luteolin's receptor-mediated mode of action, an ER binding assay was performed to establish whether these phenolic compounds would compete with radio-labelled 17β-estradiol (^{3}H-E_2) for binding to the ER subtypes.

The specific binding[‡] of unlabelled 10^{-5} M E_2, mangiferin (100x) and luteolin (100x) were determined, and the specific binding of the phenolic compounds were expressed relative to E_2, set to 100% (Figure 5.4). Mangiferin and luteolin were able to displace 20 nM ^{3}H-E_2 from both ER subtypes, however, not to the same extent as 10^{-5} M E_2.

Luteolin's displacing of ^{3}H-E_2, compared to E_2, was significantly ($P<0.001$) lower for both ERα and ERβ, at 47% and 31%, respectively. In a study conducted by Verhoog *et al.* [42], luteolin displaced more than 50% of ^{3}H-E_2 from both ER subtypes with a higher displacement percentage for ERβ. The concentration of luteolin in Verhoog's study was, however, double the concentration used in the current study. The concentration difference could therefore be the cause of luteolin's lower binding capacity observed with ERβ in the current study [42].

Mangiferin's ability to displace ^{3}H-E_2 from ERα and ERβ was also significantly ($P<0.01$) lower than E_2, yet, significantly ($P<0.05$) higher than luteolin via ERα. The only previous indication of mangiferin's ability to bind to the ER, is in a study by Kitalong *et al.* [68], were 10^{-3} M mangiferin (a concentration approximately 4-fold higher than used in the current study) exhibited 50% displacement of fluorescence-labelled E_2 from ERα. Although the experimental system is different in the current study, mangiferin has, to our knowledge, never before been shown to display binding activity via ERβ [30,42], and is therefore this is a novel finding.

[‡] Specific binding = total binding – non-specific binding [128]

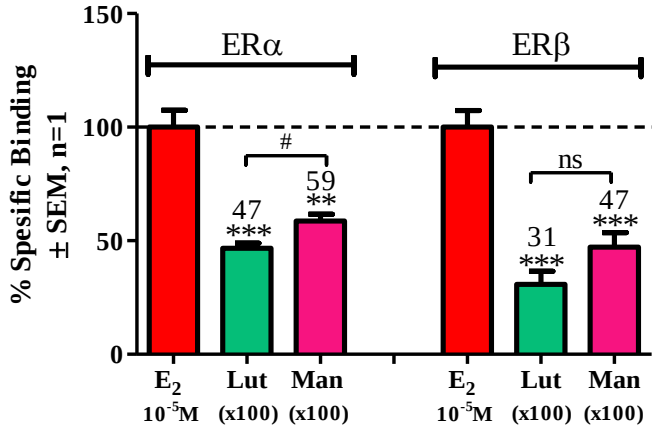

Figure 5.4: Whole cell binding studies investigating the specific binding activity of E_2, luteolin and mangiferin. COS-1 cells were transfected with pSG5-hERα or pSG5-hERβ), and incubated with unlabelled E_2 (10^{-5} M), 2.68 x 10^{-4} M mangiferin (100x) or 4.74 x 10^{-6} M luteolin (100x), in the presence of tritiated E_2 (20 nM). Statistical analysis: One-way ANOVA with Dunnett's Multiple Comparisons Test as post-test, phenolic compounds compared to E_2 control set to 100% (red bar), *P<0.05, **P<0.01, ***P<0.001, and individual phenolic compounds compared to each other, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.

The ability of a ligand to bind to the ER is not necessarily related to that ligand's ability to mediate an estrogenic response [30,55]. From the previous result, it is clear that both mangiferin and luteolin bind to the ERs, however, to determine if these phytoestrogenic compounds mediated their transcriptional effect through the ER subtypes, fulvestrant (a full ER antagonist also known as ICI 182,780 [69,70]) was added in combination with mangiferin (100x) or luteolin (100x).

A small, yet significant (P<0.05), decrease in activity was observed for both mangiferin (Figure 5.5 A) and luteolin (Figure 5.5 C) via ERα when fulvestrant (ICI) was added. With ERβ, on the other hand, addition of fulvestrant resulted in a significant (P<0.001) 2.76- and 2.32-fold decrease in mangiferin and luteolin's activity, respectively (Figure 5.5 B & D). Even though the concentration of ICI (10^{-10} M) was relatively low compared to the concentrations of mangiferin (2.68 x 10^{-4} M) and luteolin (4.74 x 10^{-6} M), ICI was still able to decrease the estrogenic activity of these two phenolic compounds (by downregulating the ERs [70]), indicating that mangiferin and luteolin mediate their estrogenic effect by binding to the ERs.

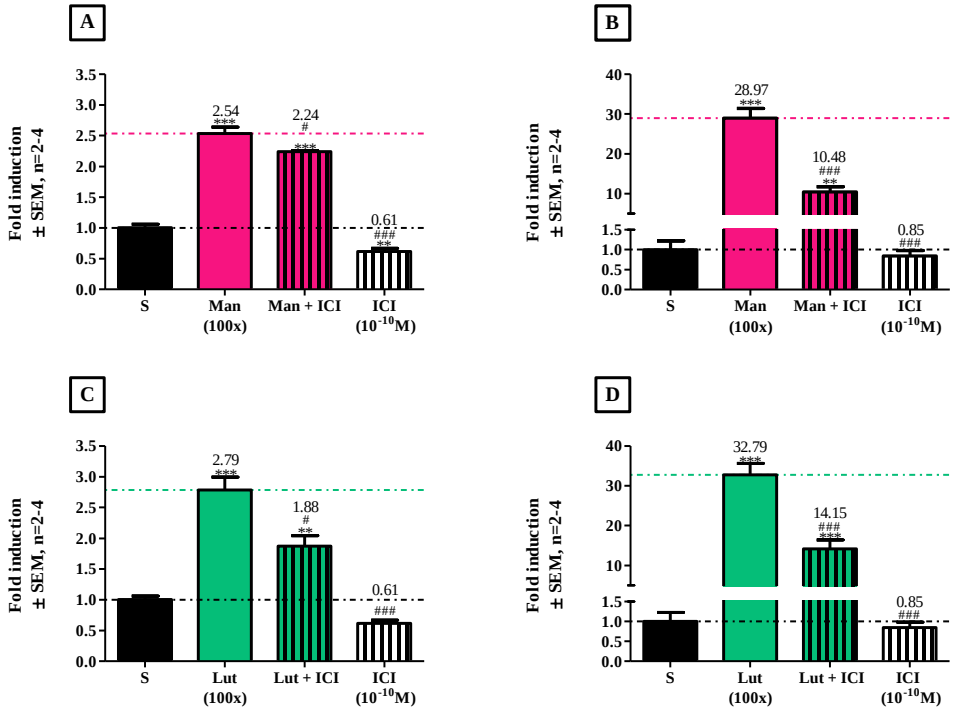

Figure 5.5: Promotor reporter studies investigating the effect of the estrogenic phenolic compounds (mangiferin and luteolin) via ERα (A & C) and ERβ (B & D) transactivation in the absence and presence of an ER antagonist. HEK293 cells were transfected with pSG5-hERα/pSG5-hERβ, ERE.PS2.luc and a pGL2 basic empty vector, and induced with 2.68 x 10^{-4} M mangiferin (100x) **(A & B),** and 4.74 x 10^{-6} M luteolin (100x) **(C & D)**, in the absence and presence of 10^{-10} M fulvestrant (ICI 182,780). Statistical analysis: One-way ANOVA with Dunnett's Multiple Comparisons Test as post-test, all columns compared to solvent control set to 1 (black bar), *P<0.05, **P<0.01, ***P<0.001, and individual phenolic compounds (Man/Lut) compared to Lut/Man + ICI and ICI, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.

In addition, the efficacy and potency of mangiferin and luteolin were also investigated as a prelude to determining the potential combinatorial or synergistic activity of these estrogenic phenolic compounds. Unfortunately, the potency and efficacy of mangiferin could not be obtained (Figure 5.6 A & B), since the compound did not attain maximal response at the concentrations used, and even though higher concentrations were investigated, the solubility of mangiferin reached saturation in the medium.

Luteolin, on the other hand, displayed a biphasic response (a phenomenon also observed with estrogen [71]) with a dose dependent increase in activity up till 140x its concentration in F3R (6.63 x 10^{-6} M), after which a decrease in activity was observed (Figure 5.6 C & D). Although there is a significant (P<0.001) 17-fold difference in efficacy between ERα and

ERβ, no significant difference was observed between their potencies at 4.36 x 10^{-6} M and 4.46 x 10^{-6} M, respectively (Figure 5.6 C & D). Similar results were obtained from another study by Verhoog *et al*. [23], which established a potency of 6.78 x 10^{-6} M via ERα and 1.23 x 10^{-5} M via ERβ for luteoin, within their experimental model. Relative to E_2, luteolin acts as a partial ER agonist with reduced potency, since luteolin displayed significantly (P<0.05) lower efficacy and potency than E_2 (Figure 5.6 E & F) via both ERs.

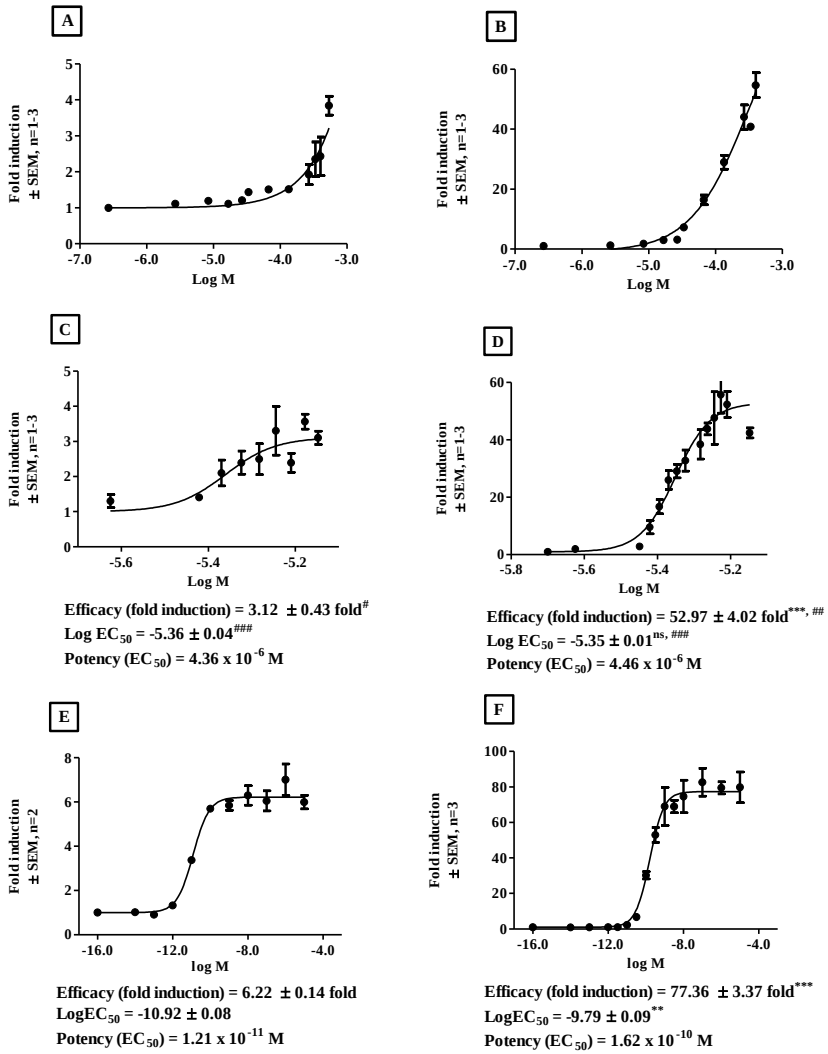

Figure 5.6: Promoter reporter studies investigating the efficacy (maximal fold induction) and potency (EC_{50}) of mangiferin, luteolin and E_2 via ERα (A, C & E) and ERβ (B, D & F). HEK293 cells were transfected with pSG5-hERα or pSG5-hERβ, and PS2.ERE.luc and a pGL2 basic empty vector, and induced with increasing concentrations of mangiferin: 2.68 x 10^{-6} – 5.36 x 10^{-4} M **(A & B)**, luteolin: 2.37 x 10^{-6} – 7.10 x 10^{-6} M **(C & D)** and E_2: 10^{-14} – 10^{-5} M **(E & F)**. Curve fitting: nonlinear regression with log (agonist) vs. response (three parameters). Statistical analysis of efficacy and potency: unpaired t-test, ERα vs ERβ, *P<0.05, **P<0.01, ***P<0.001 and ^{ns}no significant difference, and E_2 vs lut, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.

Luteolin has been shown to display estrogenic and anti-estrogenic activity in different experimental systems including luciferase based ERE-containing promoter reporter assays, ER binding assays and cell proliferation assays [23,30,42,57]. Luteolin has also been shown to inhibit other receptors including epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF-1R) and related signalling pathways, which indirectly affects ERα mediated activity [54,62,66,72]. The current study only investigated luteolin's ability to bind to the ER's and to mediate an estrogenic response via the promoter reporter assay. To fully understand luteolin's potential and contribution to the estrogenic profile of F3, more research is needed.

In contrast, little is known about mangiferin's estrogenic mode of action and many studies have claimed that mangiferin demonstrates no estrogenic activity in their experimental systems [23,30,32,42,68]. However, in a recent study by Wilkinson *et al.* [73], mangiferin (at 3×10^{-4} M, a concertation similar to mangiferin (100x) in the current study) displayed ERα mediated estrogenic activity but no ERβ activity in COS-1 cells transfected with either pRST7-ERα or pRST7-ERβ, and a pGL2-TATA-Inr-Luc-3XERETATALuc reporter construct. These findings contradict the strong ERβ mediated estrogenic activity of mangiferin (2.68×10^{-4} M) observed in the current study.

5.2.2.3. Identifying the phenolic compounds contributing to the activity of 100x F3R

Investigation of the individual phytoestrogenic activity of the 7 major phenolic compounds confirmed that mangiferin and luteolin were the only two phenolic compounds in F3R that displayed individual estrogenicity at higher concentrations. Thus, to determine mangiferin and luteolin's contribution to the robust ERα and β agonist activity of F3R (100x), F3R was reconstituted with these estrogenic phenolic compounds at 100x their concentration, respectively, while the remaining phenolic compounds were maintained at 1x their

concentration in F3. Thus, when mangiferin was added at 100x, luteolin would only be added at 1x its concentration together with the other phenolic compounds, and vice versa. The activity of the newly constructed fractions, F3R Man 100x (Figure 5.7 A & C) and F3R Lut 100x (Figure 5.7 B & D), were subsequently compared to F3R as well as to their respective phenolic compounds at increasing concentrations.

Prior to comparing the newly reconstituted fractions, F3R was initially compared to the respective phenolic compounds, mangiferin and luteolin, alone at increasing concentrations. F3R (1x), mangiferin (1x) (Figure 5.7 A & B) and luteolin (1x) (Figure 5.7 C & D) displayed no significant agonist activity via either ER subtype. Although, F3R (10x), mangiferin (10x) and luteolin (10x) also did not display significant activity via either ER subtype, via ERβ, both F3R (10x) and mangiferin (10x) presented with a circa 2-fold increase in activity (Figure 5.7 B). Statistical analysis (not shown) in the absence of F3R (100x), F3R Man (100x) and mangiferin (100x), showed a significant difference ($P<0.001$) between 1x and 10x F3R. Furthermore, a significant difference ($P<0.01$) was then also observed between 1x and 10x mangiferin, with no significant difference between F3R (10x) and mangiferin (10x), indicating that mangiferin (10x) was potentially responsible for the 2.39 ± 0.16 fold induction observed with F3R (10x) via ERβ.

In addition, in a separate experiment, F3R was also reconstituted with each of the 7 major phenolic compounds at 10x their concentration, respectively, while the remaining phenolic compounds were maintained at 1x their concentration in F3 (Table 5.1 & Figure S.3). Only F3R Man (10x) displayed comparable results to F3R (10x) via ERβ at 3.80 ± 0.12 and 3.96 ± 0.17 fold induction, respectively, confirming mangiferin's (10x) role in F3R (10x) induction via ERβ. Via ERα, F3R (10x) and F3R Man (10x) were also the only two reconstituted mixtures that displayed significant ($P<0.05$) estrogenic activity, however, the overall fold induction for ERα was very low and there was no significant difference between any of the 7

newly reconstructed F3Rs (10x) and F3R (10x). Since the phenolic compounds are very expensive, it was decided at this point in the study to only focus on the phenolic compounds that contributed to the estrogenic activity observed with F3R (100x).

Comparing mangiferin (100x) with F3R (100x), a significant ($P<0.001$) 1.61- and 1.99-fold decrease in activity was observed via ERα (2.14 ± 0.08 vs 1.33 ± 0.04 fold induction) and ERβ (74.44 ± 2.65 vs 37.47 ± 2.20 fold induction) (Figure 5.7 A & B), respectively. Luteolin (100x) also displayed lower activity than F3R (100x), with a significant ($P<0.05$) 1.20- and 1.70-fold difference via ERα and ERβ, respectively (Figure 5.7 C & D). Suggesting that neither mangiferin (100x) nor luteolin (100x) alone could recapitulate the activity of F3R (100x).

Subsequently, the activity of the newly reconstituted fraction, F3R Man (100x), was compared to F3R (100x) and mangiferin (100x). Via ERα, F3R Man (100x), like Man (x100), displayed no significant phytoestrogenic activity, which was significantly ($P<0.01$) lower than that of F3R (100x). However, in contrast, via ERβ there was no significant difference observed between F3R Man (100x) and F3R (100x). Mangiferin (100x) on its own, on the other hand, displayed significantly ($P<0.01$) lower ERβ activity compared to F3R Man (100x).

Comparing the response of the other newly reconstituted fraction (F3R Lut (100x)) to that of F3R (100x), a significant ($P<0.01$) difference was observed via ERα and no significant difference via ERβ. There was also no significant difference observed between luteolin (100x) and F3R Lut (100x) via either ER subtype, although Lut (100x) was significantly ($P<0.05$) lower than F3R (100x) via both ERβ and ERα.

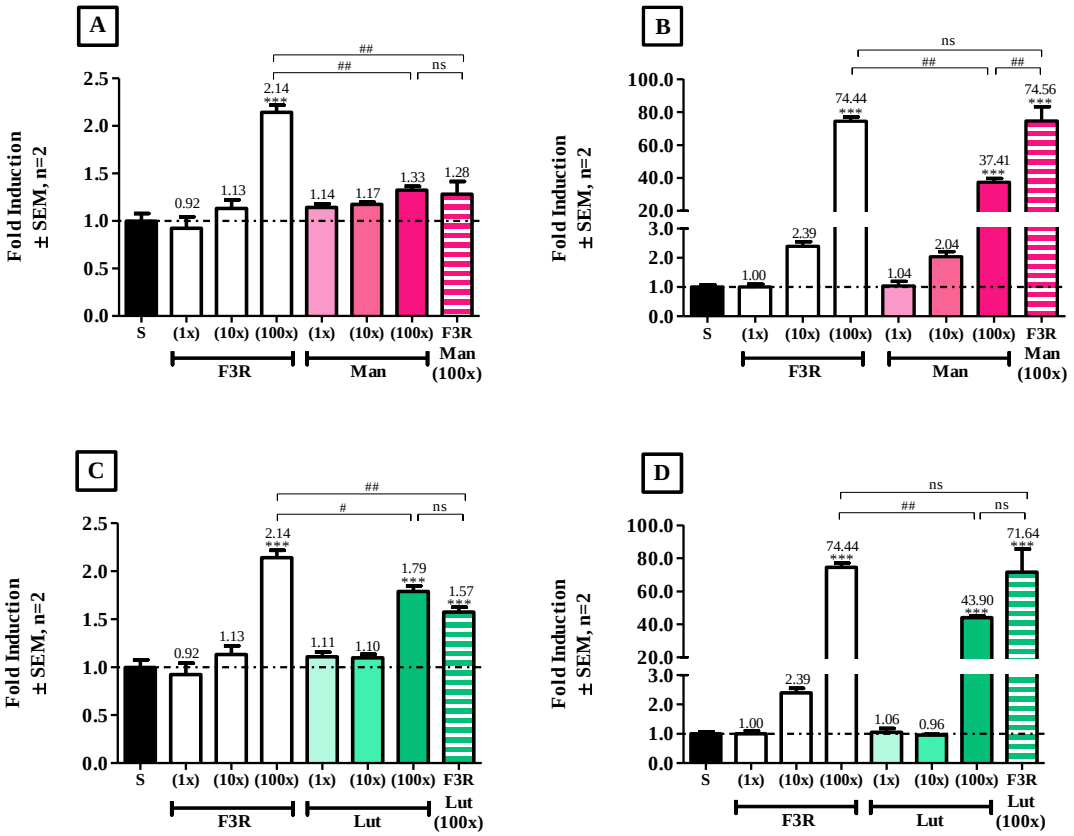

Figure 5.7: Promotor reporter studies comparing the activity of F3R, the newly reconstructed fractions, F3R Lut 100x and F3R Man 100x, and the estrogenic phenolic compounds, magiferin and luteolin, via ERα (A & C) and ERβ (B & D) transactivation in agonist mode. HEK293 cells were transfected with pSG5-hERα/pSG5-hERβ), ERE.PS2.luc and a pGL2 basic empty vector, and induced with F3R (1x, 10x and 100x), mangiferin (1x, 10x & 100x) and F3R Man 100x (A & B), as well as luteolin (1x, 10x & 100x) and F3R Lut 100x (C & D). Statistical analysis: One-way ANOVA with Dunnett's Multiple Comparisons Test as post-test, all columns compared to solvent control set to 1 (black bar), $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, or the activity of F3R (100x), newly reconstituted fractions and phenolic compounds (100x) compared to each other, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$.

Assessing the results for ERα, it is evident that neither mangiferin nor luteolin at 100x their concentration in F3R, individually or combined with other polyphenols (F3R Man 100x & F3R Lut 100x), are potentially responsible for the ERα agonist activity observed with F3R (100x). Mangiferin (100x) and F3R Man 100x displayed no significant estrogenic activity, and their responses were significantly ($P<0.01$) lower than F3R (100x). Luteolin (100x) and F3R Lut 100x, on the other hand, displayed significant ($P<0.001$) agonist activity with no significant difference observed between them, however, the activity of luteolin (100x) and F3R Lut 100x was significantly ($P<0.05$) lower than that of F3R (100x). These results suggests that, even though luteolin appears to be the main estrogenic contributor, one or more

of the other major phenolic compounds, at 100x their concentrations, are also contributing to F3R (100x)'s agonist activity via ERα.

For ERβ, it is apparent that mangiferin (10x) is responsible for the activity observed with F3R (10x). However, at 100x their concentration in F3R, the two individual phytoestrogenic phenolic compounds (luteolin and mangiferin) displayed significantly ($P<0.01$) lower activity than observed with F3R (100x). Yet, when combined with the rest of the major phenolic compounds, F3R Lut 100x and F3R Man 100x displayed no significant difference compared to F3R (100x). This suggests that either both mangiferin and luteolin contribute to F3R (100x)'s robust agonist activity in an additive or possibly synergistic manner, or that the non-estrogenic compounds in F3R (isomangiferin, scolymoside, iriflophenone-3-*C*-glucoside, *p*-coumaric acid and protocatechuic acid) are enhancing the phytoestrogenic activity of mangiferin and luteolin.

Consequently, F3R was again reconstituted with the 5 non-estrogenic phenolic compounds at 1x their concentrations in the F3 fraction, and with both mangiferin and luteolin at 100x their concentration, to examine if an increase in activity is observed when both the estrogenic phenolic compounds are present at 100x their concentration (Figure 5.8 A & B).

F3R (Man + Lut) 100x, like F3R Man 100x and F3R Lut 100x, displayed significantly ($P<0.05$) lower ERα activity compared to F3R (100x) (Figure 5.8 A). F3R Man 100x was also a significantly ($P<0.05$) 1.34-fold lower than F3R (Man + Lut) 100x, whereas F3R Lut 100x displayed no significant difference from F3R (Man + Lut) 100x, again demonstrating that, in contrast to luteolin, mangiferin is not the main contributor to the estrogenic activity of F3R (Man + Lut) 100x via ERα. Furthermore, the newly reconstructed fractions (F3R (Man + Lut) 100x, F3R Man 100x and F3R Lut 100x) and F3R (100x) displayed no significant

difference in activity via ERβ, indicating that mangiferin and luteolin's role in F3R's activity via ERβ may be interchangeable (Figure 5.8 B).

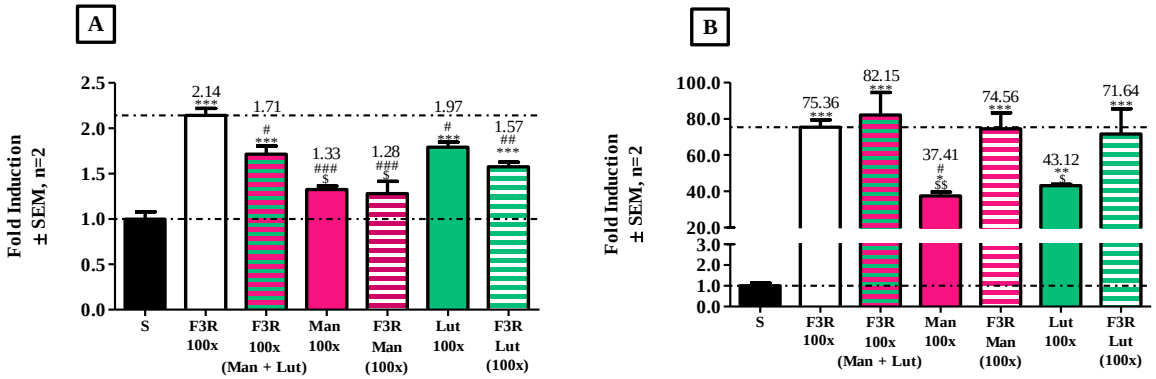

Figure 5.8: Promotor reporter studies comparing the activity of F3R, the newly reconstructed fractions, F3R (Man + Lut) 100x, F3R Lut 100x and F3R Man 100x via ERα (A) and ERβ (B) transactivation in agonist mode. HEK293 cells were transfected with pSG5-hERα/pSG5-hERβ), ERE.PS2.luc and a pGL2 basic empty vector, and induced with F3R (100x), F3R (Man + Lut) 100x, F3R Lut 100x and F3R Man 100x, as well as individual estrogenic phenolic compounds, mangiferin (100x) and luteolin (100x). Statistical analysis: One-way ANOVA with Dunnett's Multiple Comparisons Test as post-test, all columns compared to solvent control set to 1 (black bar), *P<0.05, **P<0.01, ***P<0.001. Activity of F3R (100x) compared to the newly reconstituted fractions or individual phenolic compounds (100x), $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, and F3R (Man + Lut) 100x compared to F3R Lut 100x and F3R Man 100x, $^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.001.

In addition, subtraction of the individual phenolic compounds from 100x F3R were conducted to further validate mangiferin and luteolin's involvement in the activity observed in 100x F3R, and to investigate the role of the non-estrogenic phenolic compounds (Figure S.4). Since the compounds are expensive and limited, this study was only conducted via ERβ. Unfortunately, mangiferin lost its ER agonist activity, and the activity observed in the 100x F3R was only due to 100x luteolin (Figure S.4).

One of the limitations of cell culture experimental systems is the cell's vulnerability to external changes, such as nutrition, humidity, temperature, CO_2 and pH, which could influence the cell's uptake of nutrients and hormones [74–77]. Apart from the fragility of the cells, phenolic compounds are also susceptible to external factors, such as storage conditions, temperature, exposure to light and oxygen, and the composition and quality of the solvent or medium. These various factors could cause deprotonation, oxidation or degradation of the

compounds and could results in the loss of function or desired activity [78–85]. Investigating the possible causes for the loss of mangiferin's once robust ERβ agonist activity, the composition of the cell culture medium and the structural integrity of the compound was investigated.

Within our research group, it was noted that the pre-made phenol red medium transitioned colour from red to a pink-red colour during storage, indicating that the pH of the medium shifted from neutral to alkaline [86,87]. The pH of the medium was always adjusted to 7.2 during preparation, but after being stored for a few weeks (2 weeks and longer), the pH of the pre-made medium tested at ≥9. The general protocol for medium preparation was adapted (by changing the sodium bicarbonate concentration), with the purpose of ensuring more stable medium pH levels during storage and cell culture maintenance. At the time, this change in protocol was not thought to be a contributing factor to the observed change in phytoactivity of mangiferin, since the activity of the E_2 controls did not change. However, recent publications have indicated the importance of pH in the bio-activity of mangiferin [85]. The ramifications of pH drifting will be further addressed in the conclusion.

Another possible cause for mangiferin's loss of activity was thought to be due to the break-down of the compound during storage. LC-MS (Figure S.5) and NMR (Figure S.6) were used to investigate this theory [88–91]. There was, however, no structural degradation observed between any of the mangiferin stocks (26/09/14, 25/10/14, 07/04/15 and 16/06/15)[§], since they all displayed the expected molecular mass of 422g/mol and molecular formula of $C_{19}H_{18}O_{11}$, while the 1H-NMR signals correlated with previous studies [90,92–94]. Interestingly, an additional structure was observed in the mangiferin sample with a molecular mass of 436g/mol and molecular formula of $C_{20}H_{20}O_{11}$ (Figure S.5). NMR results (Figure S.7), and findings from literature, confirm that the second compound was homomangiferin

[§] Mangiferin stocks were labelled according to the date the compounds were received from the manufacturer.

[88,89,91]. In addition to testing the bought mangiferin stocks, mangiferin recently isolated from *Cyclopia* (Gerderblom *et al.* 2016, unpublished) was also included in the NMR study. Homomangiferin was, surprisingly, not detected in Gelderblom's sample. Like mangiferin, not much is known about homomangiferin's possible phytoestrogenic activity.

Findings from this section highlight the importance of all the compounds in F3R, and subsequently F3. Even though mangiferin and the non-estrogenic phenolic compounds did not show a contribution to F3R's activity in the subtraction study (Figure S.4), their contribution to F3's activity should not be dismissed.

5.2.3. Combination of the estrogenic compounds, mangiferin and luteolin, to investigate possible interaction

There are different methods to investigate possible combinatorial activity or synergism including the median effect analysis, response surface analysis, combination index, and the isobole method of Berenbaum [18,95–98]. The latter is one of the easier methods to test for additive/synergistic activity since it is a demonstrative method and it is also independent of the mechanisms of action [18].

The isobologram, by Tallarida *et al*. [96], is a more simplified approach to the isobole method and uses fixed ratio combinations of the drug EC_{50}'s instead of a range of drug EC_{50} combinations. This is a more cost effective approach, since less of the compounds are used. It was, however, not possible to combine mangiferin and luteolin at a fixed ratio manner (as demonstated by Tallarida *et al*. [96]), due to the loss of mangiferin's once robust phytoestrogenic activity. A different approach to this complication was to use increasing concentrations of mangiferin combined with the EC_{50} concentration (4×10^{-6} M) of luteolin, which is similar to luteolin (100x) (Figure 5.6 C & D), with the hope of mangiferin regaining activity in the presence of luteolin.

Luteolin (100x) displayed phytoestrogenic activity via both ERα and ERβ, at 8.83 ± 0.39 and 24.10 ± 1.05 fold induction, respectively (Figure 5.9 A & B). Mangiferin (100x), on the other hand, displayed no estrogenic activity. The activity observed with the combination of the two polyphenols, displayed no significant difference when compared to luteolin (100x) alone, suggesting that luteolin is the only polyphenol out of the 7 major phenolic compounds that displayed phytoestrogenic activity at this stage of the study, validating previous results (Figure S.4).

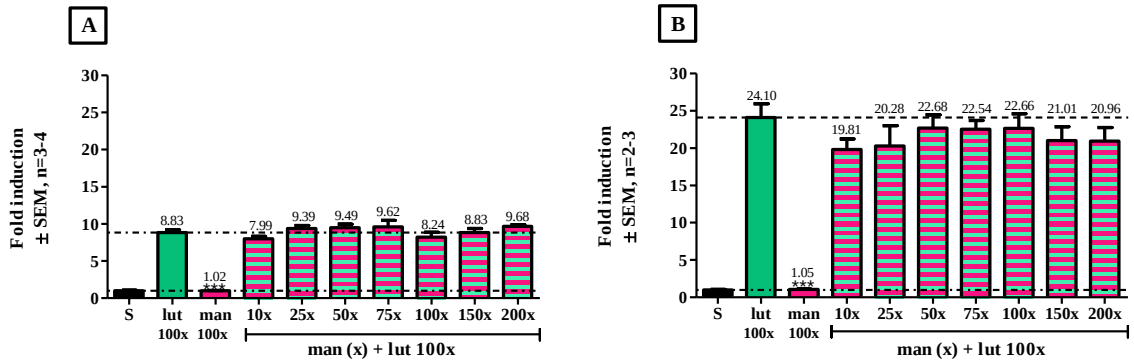

Figure 5.9: Promoter reporter studies investigating the combined effect of mangiferin and luteolin on ERα (A) and ERβ (B) transactivation in agonist mode: HEK293 cells were transfected with pSG5-hERα/pSG5-hERβ, ERE.PS2.luc and a pGL2 basic empty vector, and induced with 4.74 x 10^{-6} M luteolin (100x) combined with increasing concentrations of mangiferin, 2.68 x 10^{-5} – 5.36 x 10^{-4} M (10x – 200x). All compounds normalised to solvent, set as 1. Statistical analysis: One-way ANOVA with Dunnett's Multiple Comparisons Test as post-test, all columns compared to lut (100x) (turquoise bar), *P<0.05, **P<0.01, ***P<0.001.

Even though the estrogenic activity of mangiferin could not be restored in the presence of luteolin, mangiferin should not be dismissed as a possible candidate to F3R's (and subsequently F3's) agonist activity as recent studies have now demonstrated that mangiferin has phytoestrogenic potential [68,73]. Furthermore, to fully demonstrate the possible combinatorial or synergism potential of F3R, the non-estrogenic phenolic compounds should also be considered in future studies, since the estrogenic phenolic compounds (mangiferin and luteolin), on their own, previously displayed lower activity compared to F3R in the current study (Figure 5.8).

5.3. Conclusion

Traditional medicine, which gained knowledge through observing nature, formed the foundation upon which modern medicine is built [99,100]. The development and improvement of chemical and analytical techniques provided insight into the phytochemical composition of medicinal plants, and created a platform for the identification, isolation and synthesis of single, well characterised, active compounds that are target specific [14,15,101]. However, as modern medicine evolved, new challenges arose (drug resistance, dangerous side effects and decreased drug efficacy) and a renewed approach to therapy development is needed to overcome these problems [102–106].

Medicinal plants consist of various phytochemicals that interact in complex molecular networks to achieve stability, as well as to enhance the bioavailability and potency of the active compounds [12,19,107]. Whole plant extracts and combination therapies have been shown to be more effective than mono-therapies (which only focus on single active or target specific compounds in plant extracts) in treating multifaceted diseases, such as cancer [33,62,95,108–110]. Nevertheless, medicinal plants need to be used with caution, since often their mechanism of action is not as well studied compared to mono-therapies and synthetic drugs [45,46,111]. Perhaps, combining the knowledge of traditional plant medicine with the support of scientific methods would produce intelligent drug mixtures capable of overcoming the current limitations of modern medicine [9,112].

Cyclopia, indigenous to South Africa's fynbos biome and traditionally used as a herbal tea, has gained growing interest for its health benefits, and various studies have demonstrated it antioxidant, anti-inflammatory, -diabetic and -mutagenic properties as well as chemo-preventive activity [6,24–28,32,33]. This potential multi-action activity of *Cyclopia* makes it an ideal candidate for nutraceutical, and possible pharmaceutical, therapy development. The

current study forms part of a larger, ongoing investigation to identify the active and contributing components of the *Cyclopia* extracts and fractions previously isolated [23,31–33,42], with the purpose of creating the ideal nutraceutical to combat estrogen-related diseases, and avoid drug resistance, toxicity and side effects.

Cyclopia extracts and fractions, like most plant material, consist of a vast variety of phenolic compounds. Some of these compounds have previously been identified in our research group [23,32,33] and by other groups [113–116], however, their combined activity and interactions have not yet been established. The main interest of the current study, therefore, was to investigate the combinatorial activity of some phenolic compounds identified and quantified in F3, a fraction isolated from the *Cyclopia subternata* extract, SM6Met [30,33]. What distinguished F3 from the other SM6Met fractions was its robust ERβ agonist activity [33]. ERα is known to induce cell proliferation, whereas ERβ has shown anti-proliferative activity by antagonising ERα, and therefore, compounds that mediate their response via ERβ is of great interest in the development of treatments for estrogen-related diseases [35–38].

As mentioned previously, only the 7 major phenolic compounds were used as a starting point, to investigate their contribution to F3's robust ERβ agonist activity [33]. Some of these phenolic compounds, such as luteolin, have previously shown phytoestrogenic activity in different experimental systems [30,42,53,73]. Interestingly, however, when F3 was reconstituted using the 7 major phenolic compounds at their concentrations in F3, no significant ER activity was observed (Figure 5.1). Possible reasons could be: (1) the minor phenolic compounds, alone or in combination, are responsible for F3's estrogenic activity, (2) the minor compounds create a supportive environment for the active major compounds to elicit a response, or (3) there are still unidentified major phenolic compounds in F3 responsible for its phytoestrogenic activity.

However, when F3 was reconstituted at 100x its concentration (F3R (100x)), a significant ($P<0.001$) increase in activity was observed via both ER subtypes. In addition, F3R (100x)'s activity was a significant 1.59- and 2.66-fold higher than that of F3, via ERα and ERβ, respectively (Figure 5.1). Luteolin was thought to be the cause of this increase in activity, since a literature search at the time indicated that it was the only compound to have previously been shown to display estrogenic activity via both ER subtypes [23,30,42,54,55]. However, when the 7 individual phenolic compounds were tested at increasing concentrations (10x and 100x their concentration in F3), both mangiferin and luteolin displayed significant ($P<0.001$) ERβ agonist activity at higher concentrations (100x). In fact mangiferin already display significant activity at 10x its concentration (Figure 5.3).

At this stage of the study, not much was known about mangiferin's phytoestrogenic activity, and the ER mediated mode of action of both estrogenic phenolic compounds was investigated further. Both mangiferin and luteolin displayed binding activity to both ER subtypes and their transactivation via both ER subtypes was significantly antagonised by ICI 182,780 (fulvestrant), indicating that their estrogenic activity is mediated via the ER (Figure 5.4 & 5.5). To our knowledge, mangiferin's ERβ mediated activity is a novel finding, with literature only presenting ERα related activity [68,73].

To understand the contribution of mangiferin and luteolin to the ERβ activity of F3R (100x), both 'addition' and 'subtraction' studies were conducted. Addition studies entailed reconstituting mangiferin and luteolin at 100x their concentration in F3R, separately (Figure 5.7) as well as combined (Figure 5.8), and with or without the other phenolic compounds at 1x their concentration in F3R. From these results it became clear that, even though luteolin and mangiferin were the only two estrogenic compounds in F3R, they were not the only contributing factor to the ERβ mediated activity of F3R (100x), since their individual activities were significantly lower than that of the reconstituted fraction, F3R (100x). For

example, mangiferin (100x) was a significant ($P<0.01$) 2-fold lower than F3R Man (100x) and F3R (100x) via ERβ (Figure 5.8 B). F3R 100x (Man & Lut), on the other hand, displayed no significant difference compared to F3R (100x), F3R Man (100x) or F3R Lut (100x), indicating that the concentration of the two estrogenic phenolic compounds in F3R (100x) are interchangeable, and that either one at 100x their concentration, in combination with the rest of the phenolic compounds, will produce the same response as F3R (100x).

With subtraction studies (Figure S.4), each phenolic compound was "subtracted" from F3R (100x) by individually adding them at 1x their concentration, while the rest of the phenolic compounds were reconstituted at 100x their concentration in F3R. By doing this, we had expected to identify which of the non-estrogenic phenolic compounds in F3R contributed to the activity of the estrogenic phenolic compounds. Unfortunately, at this stage of the study, mangiferin lost its estrogenic activity, and luteolin appeared to be the only remaining active and contributing component in F3R, with no significant difference between luteolin (100x) and F3R (100x) (Figure S.4). Possible synergistic activity could, therefore, not be determined (Figure 5.9).

Different stocks of mangiferin (26/09/14, 25/10/14, 07/04/15 and 16/06/15) were tested to determine if degradation of the compound had occurred, however, no structural change was detected via LC-MS (Figure S.5) and NMR (Figure S.6) studies. On the other hand, homomangiferin was also detected in the mangiferin stocks (Figure S.5 & Figure S.7). Plant sources that are used to isolate mangiferin, for example *Mangifera indica* or *Rhizoma anemarrhenae*, also contains other xanthone glucosides such as isomangiferin, homomangiferin and neomangiferin [60,88–90,117,118]. Furthermore, mangiferin also produces more than 20 possible metabolites, which begs the question if there were other undetected metabolites in the mangiferin sample [73,88,119]. In a recent study by Wilkinson *et al.* [73], for example, norathyriol (a putative metabolite and aglycone derivative of

mangiferin [119,120]) showed strong phytoestrogenic activity via both ERα and ERβ, and was able to reduce MCF-7 cell viability, in contrast to mangiferin, which was only able to act via ERα.

Furthermore, the modification in the medium preparation protocol should also be considered as a possible factor involved in the change observed in mangiferin (Figure 5.9) and consequently F3R's activity (Figure S.4). Medium preparation instructions and suggested sodium bicarbonate concentration were obtained from the manufacture's protocol. Although the pH of the medium was adjusted to 7.2 during preparation, it came to our attention that long storage of pre-made medium has resulted in a pH increase (pH≥9). Upon further investigation, it became apparent that the buffer capacity of the suggested sodium bicarbonate concentration is ideal for a 10% CO_2 incubator (although not specifically stated by the manufacturer) and not for a 5% CO_2 incubator, as used in our facilities. The buffer capacity could also not compensate for the lower atmospheric CO_2 (0.04%) captured in the bottle when sealed, resulting in a gradual pH shift during storage. The sodium bicarbonate concentration was since adapted to compensate and prevent this pH increase during storage, especially since cells are sensitive to the cell culture environment and external factors such as pH [74–77,121].

In addition to affecting cell behaviour, variation in pH levels can also affect phytocompounds, particularly phenolic acids [76,81,82,122–124]. Phenolic acids are renowned for their role in food quality and colour, and have beneficial antioxidant properties, but have been shown to be less stable in alkaline environments and maybe converted from an antioxidant to a pro-oxidant [49,81,82,123–126]. In the current study, two of the 7 major phenolic compounds in F3R are phenolic acids, namely protochatechuic acid and *p*-coumaric acid. In a study conducted by Chethan *et al.* [124], the phenolic acid content of their polyphenol rich seed coat fraction (PRSCF), which also included protocatechuic acid and *p*-

coumaric acid, decreased with increasing pH levels. Furthermore, a pH-induced shift in the ultraviolet absorption spectra of phenolic acids were noticed, which is an indication of compound instability and is influenced by the nature of the compound and it surroundings [81,124]. pH drifting could also affect hydroxyl groups in other phenolic compounds, such as mangiferin, which contains four aromatic hydroxyl groups [63,81,85]. For example, a recent study by Mendoza-Sarmiento *et al.* [85], demonstrated the deprotonation of mangiferin's four hydroxyl groups between pH 7 and 9.6, which consequently increased mangiferin's peroxyl radical scavenging activity.

To conclude, the findings from the current study, as well as from other studies, reveals the complexity of plant extracts and further supports the argument that taking F3 apart may not be the ideal approach to investigating its possible synergistic mechanisms of action [7,16–19]. It is evident that the minor phenolic compounds play a crucial role in F3 and F3R's estrogenic activity, either by directly contributing to the estrogenic activity or more probably by creating a supportive environment for the estrogenic phenolic compounds. Further research is, however, needed regarding the influence of pH on F3R, the contributions of the minor phenolic compounds, and if mangiferin's activity is influenced by deprotonation.

5.4. References

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5.5. Supplementary Data

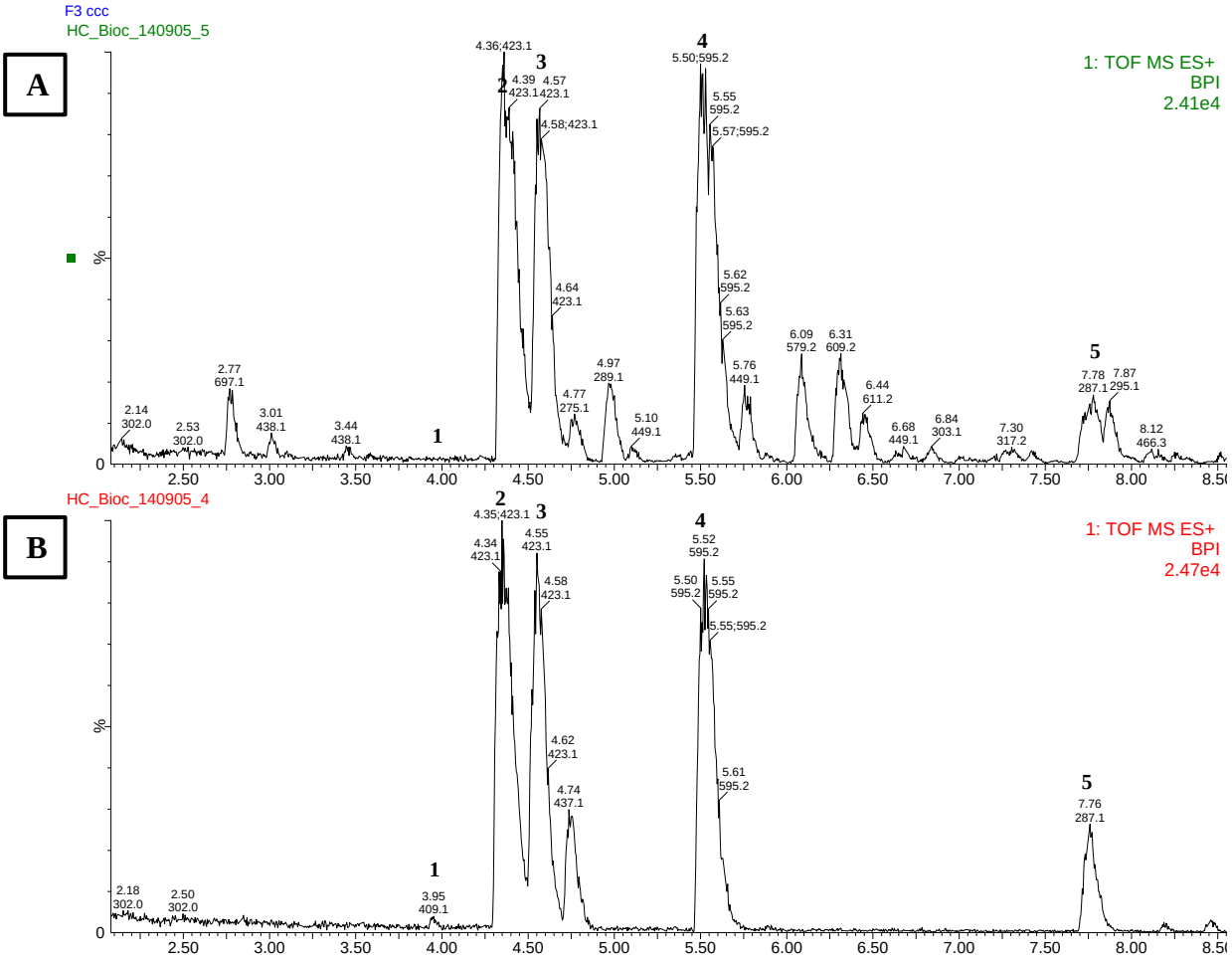
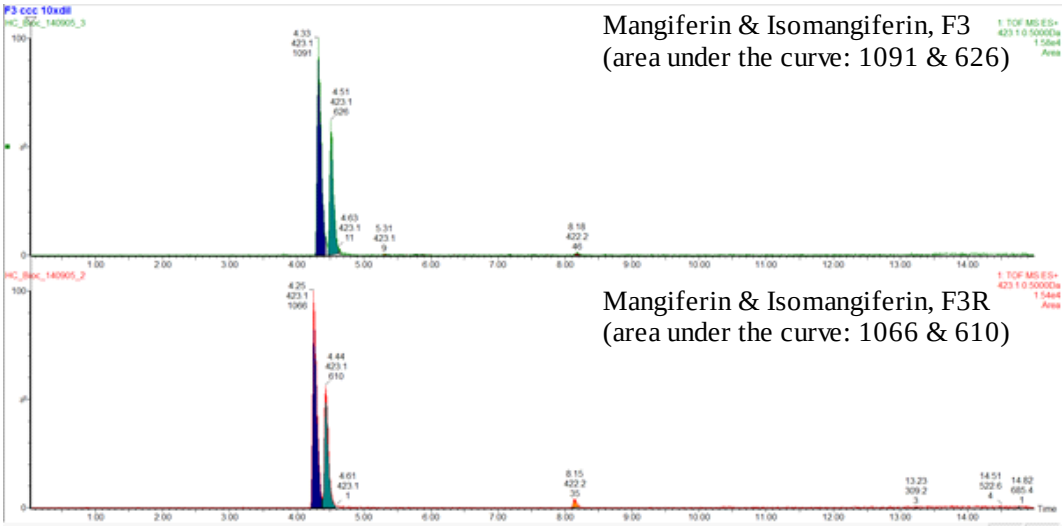


Figure S.1: LC-ESI-MS chromatogram of F3 (A) and F3R (1x) (B) in positive ion mode. Comparing peaks of phenolic content in F3 and F3R (1x). F3R (1x) was reconstituted using 5 major phenolic compounds: mangiferin (2), isomangiferin (3), scolymoside (4), *p*-coumaric acid (elutes with other compounds) & protocatechuic acid, and 2 minor phenolic compounds: iriflophenone-3-*C*-glc (1) & luteolin (5).

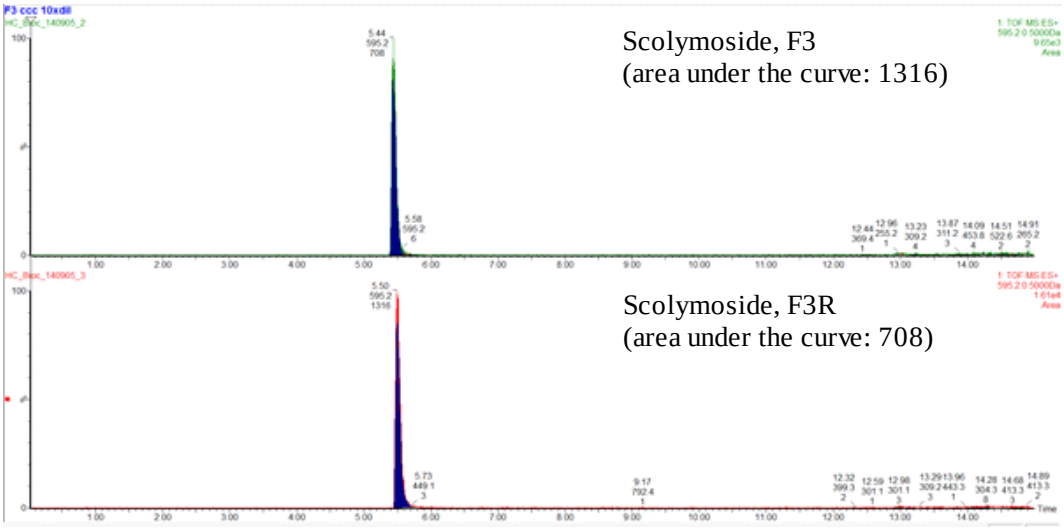
A



Mangiferin & Isomangiferin, F3
(area under the curve: 1091 & 626)

Mangiferin & Isomangiferin, F3R
(area under the curve: 1066 & 610)

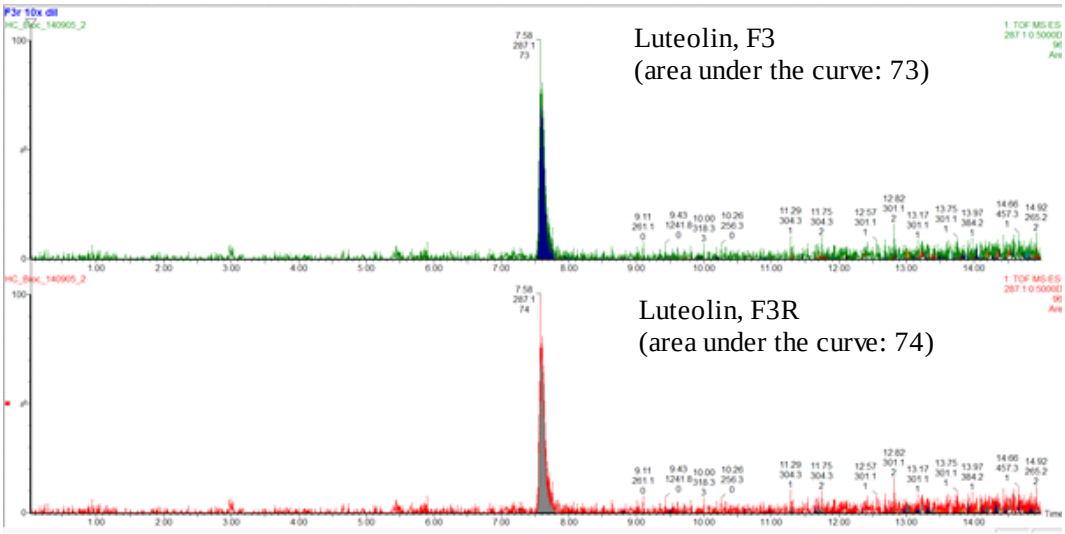
B



Scolymoside, F3
(area under the curve: 1316)

Scolymoside, F3R
(area under the curve: 708)

C



Luteolin, F3
(area under the curve: 73)

Luteolin, F3R
(area under the curve: 74)

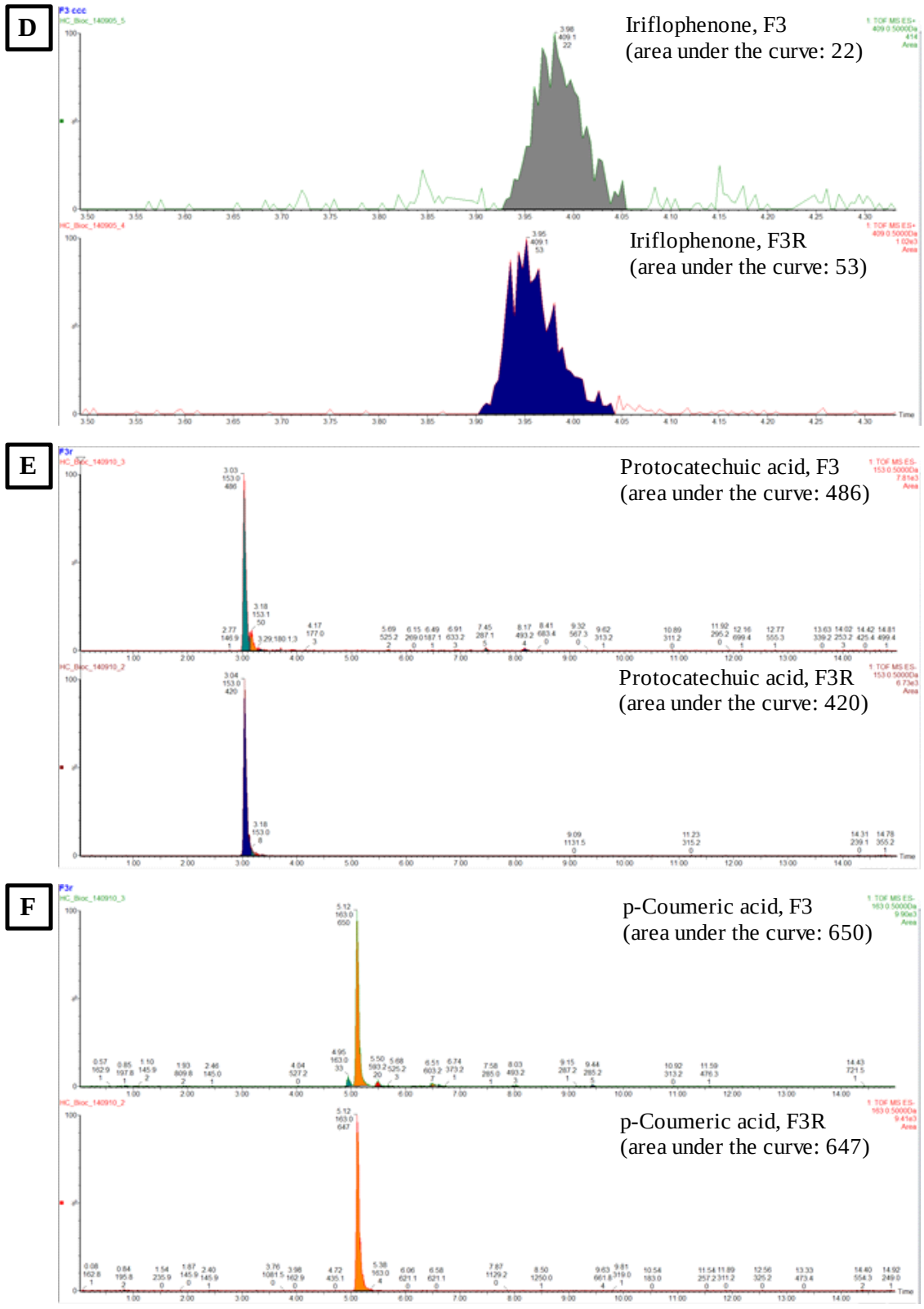


Figure S.2: LC-ESI-MS chromatogram of F3 (top) and F3R (1x) (bottom) in positive/negative ion mode. Comparing peaks of individual phenolic compounds in F3 and F3R: (A) mangiferin & isomangiferin, (B) scolymoside[**], (C) luteolin, (D) iriflophenone-3-*C*-glc[§], (E) protocatechuic acid & (F) *p*-coumaric acid.

[**] The concentrations of the phenolic compounds were previously determined by Mortimer *et al.* [33][39], however, at the time there were no authentic standards for scolymoside and iriflophenone-3-*C*-glc, and these compounds were quantified relative to luteolin and iriflophenone-3-*C*-glucoside (isolated from *Cyclopia genistoides*), respectively.

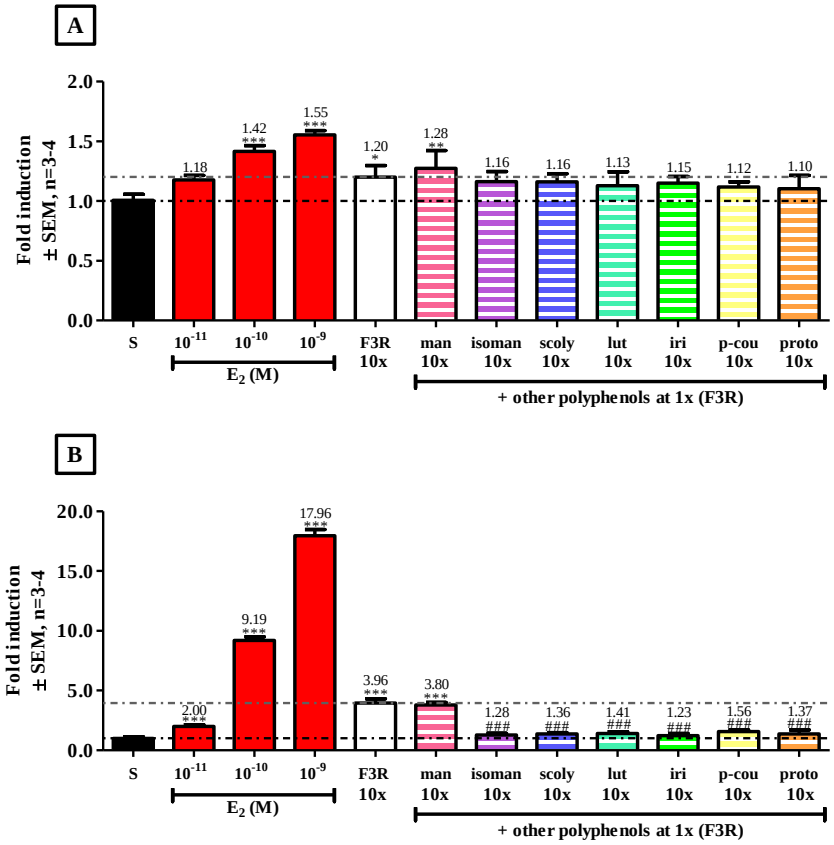


Figure S.3: Promotor reporter studies comparing the activity of F3R (10x) to the newly reconstructed 10x fractions, via ERα (A) and ERβ (B) transactivation in agonist mode. HEK293 cells were transfected with pSG5-hERα/pSG5-hERβ), ERE.PS2.luc and a pGL2 basic empty vector, and induced with E_2 (10^{-11} – 10^{-9} M), F3R (10x), F3R mangiferin 10x, F3R isomangiferin 10x, F3R scolymoside 10x, F3R luteolin 10x, F3R iriflophenone-3-*C*-glucoside 10x, F3R *p*-coumaric acid 10x and F3R protocatechuic acid 10x. Statistical analysis: One-way ANOVA with Dunnett's Multiple Comparisons Test as post-test, all columns compared to solvent control set to 1 (black bar),*P<0.05, **P<0.01, ***P<0.001, or newly reconstituted 10x fractions compared to F3R (10x), $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001.

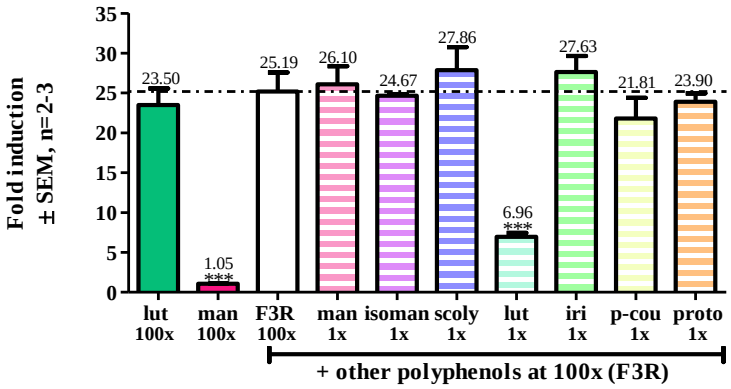
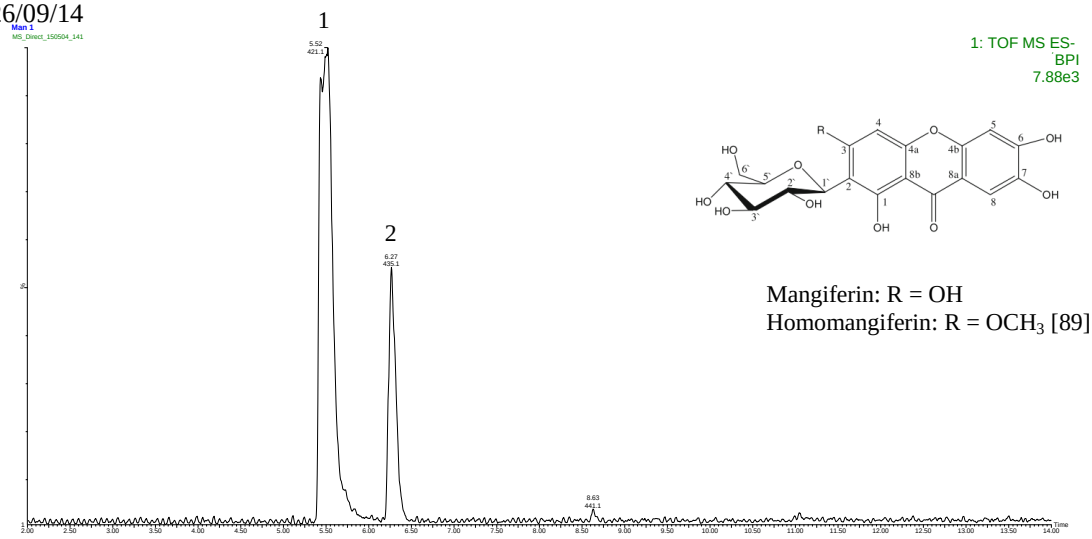


Figure S.4: Promoter reporter studies investigating the effect of subtraction of phenolic compounds from F3R (100x) via ERβ transactivation in agonist mode: HEK293 cells were transfected with pSG5-hERβ, ERE.PS2.luc and a pGL2 basic empty vector, and induced with luteolin (100x), F3R (100x) and subtracted F3R (100x)'s, which consist of 6 phenolic compounds at 100x their concentration and one compound at 1x its concentration, respectively. Statistical analysis: One-way ANOVA with Dunnett's Multiple Comparisons Test as post-test, all columns compared to F3R (100x) (white bar), *P<0.05, **P<0.01, ***P<0.001.

A

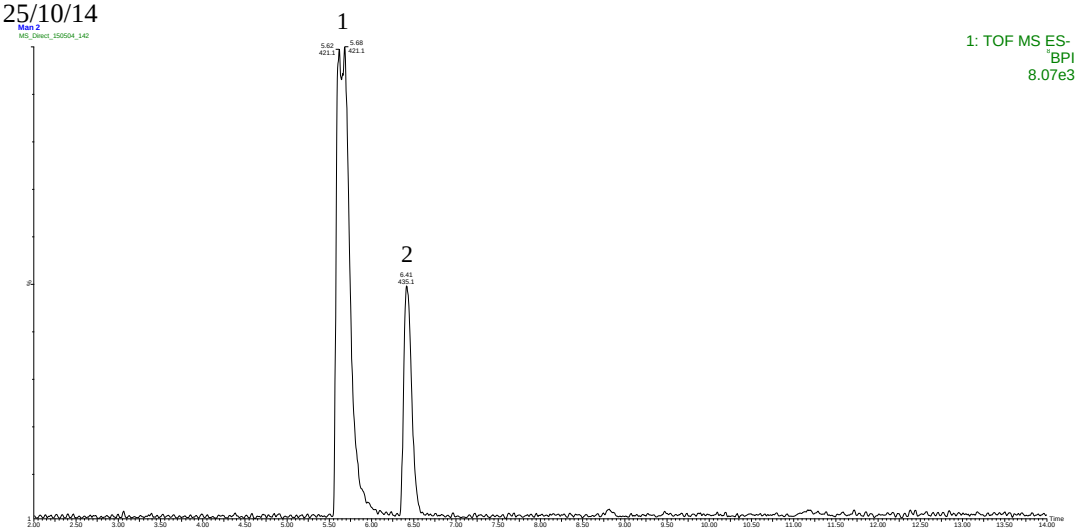
26/09/14



Mangiferin: R = OH
Homomangiferin: R = OCH_3 [89]

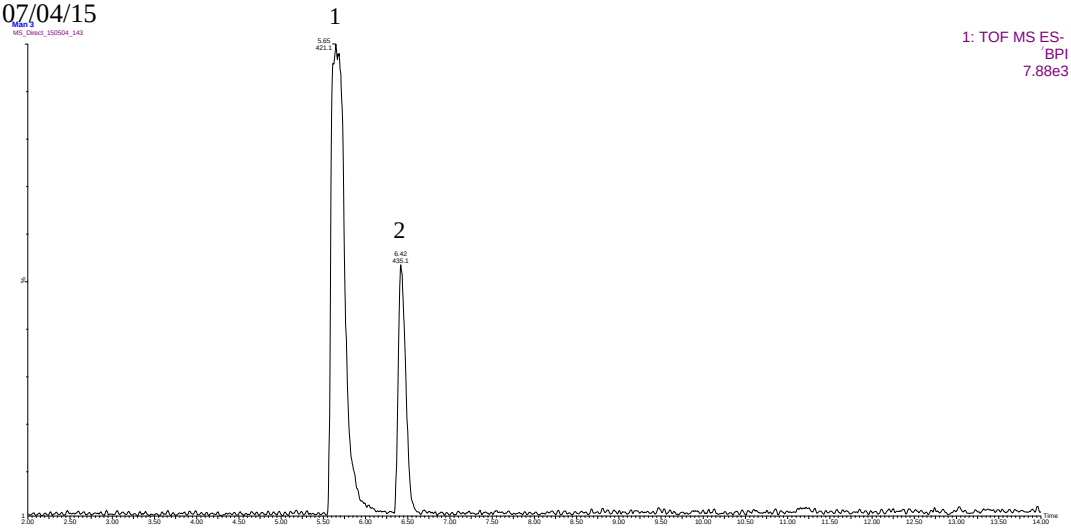
B

25/10/14



C

07/04/15



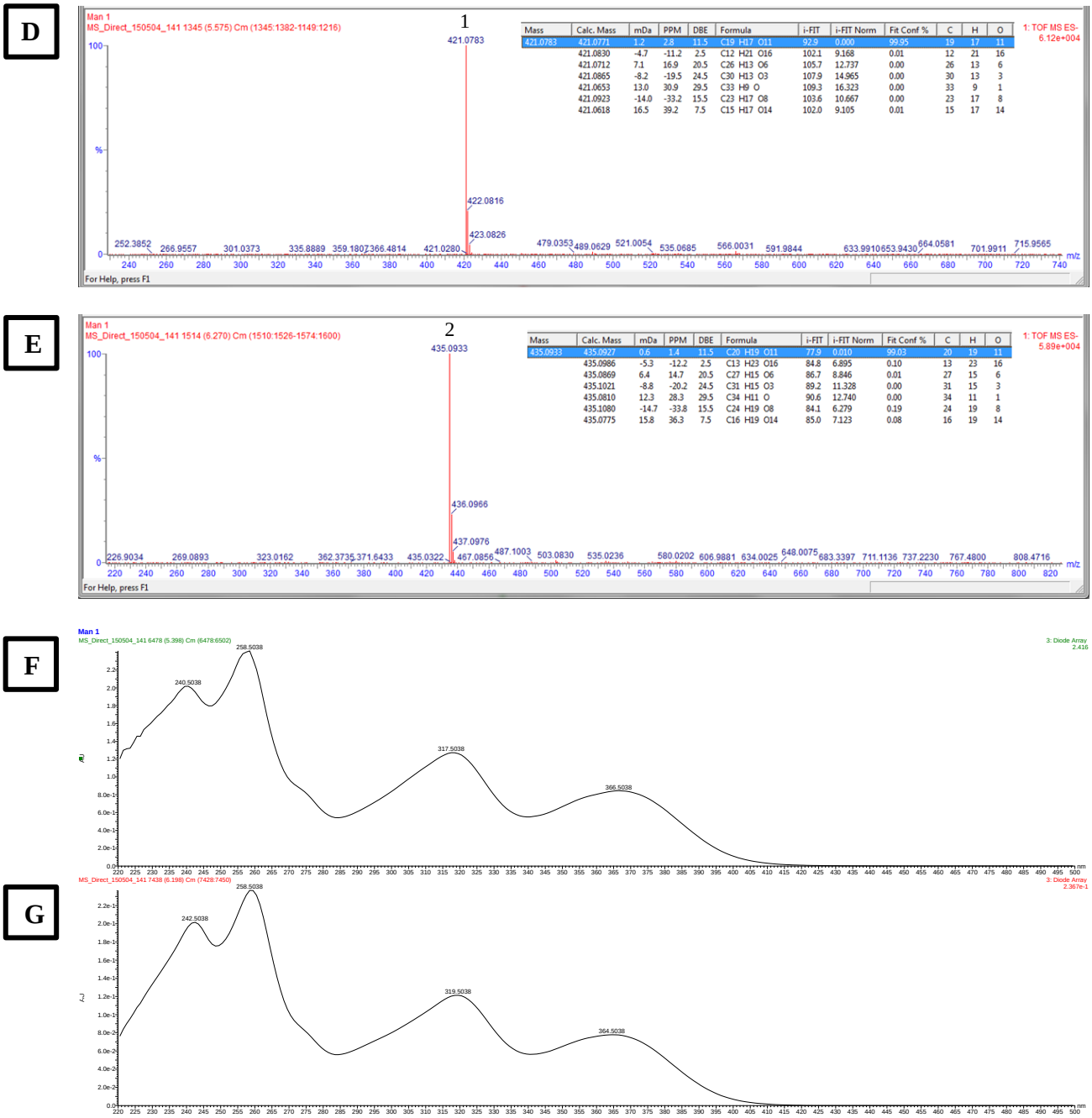


Figure S.5: LC-ESI-MS chromatogram in negative ion mode (A - C), TOFMS spectra (D & E) and UV spectra (F & G) of mangiferin analytical standard stocks. Mangiferin stocks were labelled according to receiving date: (A) 26/09/14, (B) 25/10/14 & (C - G) 07/04/15, and both mangiferin (1) and homomangiferin (2) were detected in all the mangiferin stocks tested. TOFMS analysis of mangiferin stock (07/04/15) validates the presence of both (D) mangiferin (Mr 422,33g/mol, $C_{19}H_{18}O_{11}$) and (E) homomangiferin (Mr 436,37g/mol, $C_{20}H_{20}O_{11}$). UV-visible absorption data of (F) mangiferin and (G) homomangiferin correspond with findings from Qin *et al.* [89].

A

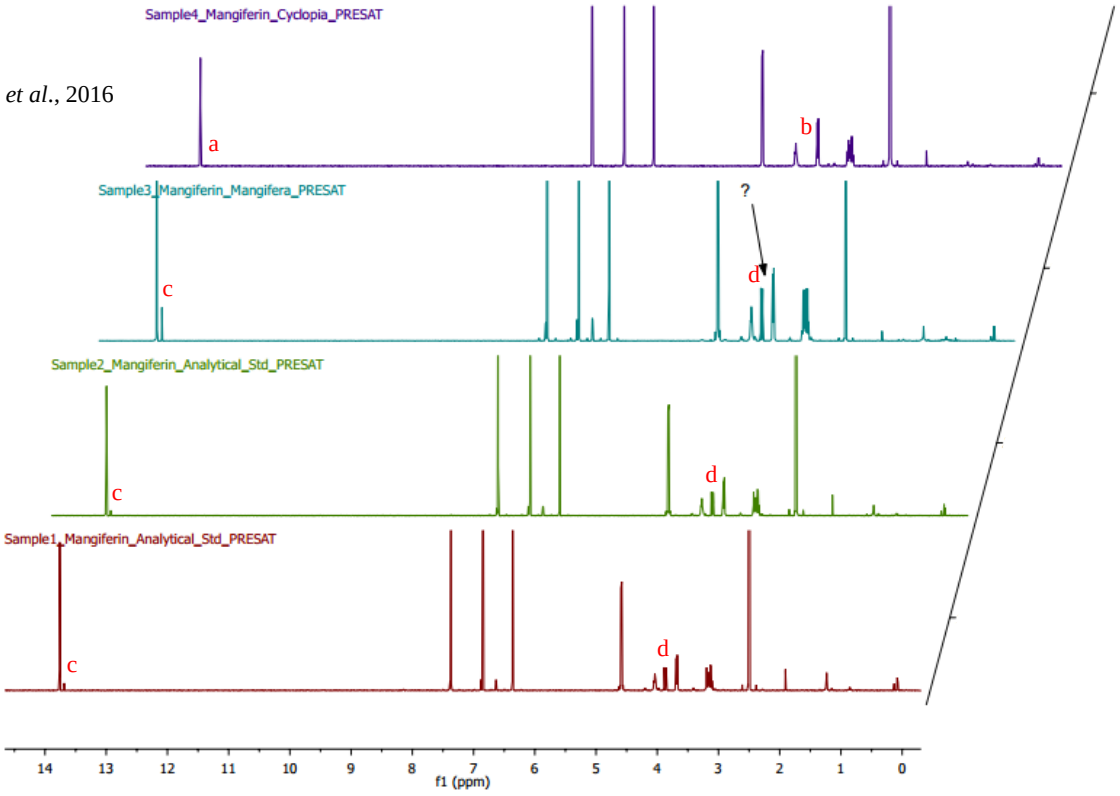
Mangiferin

(1) Gelderblom *et al.*, 2016

(2) 16/06/15*

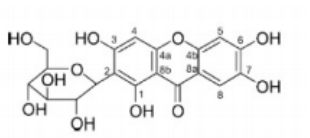
(3) 16/06/15

(4) 07/04/15



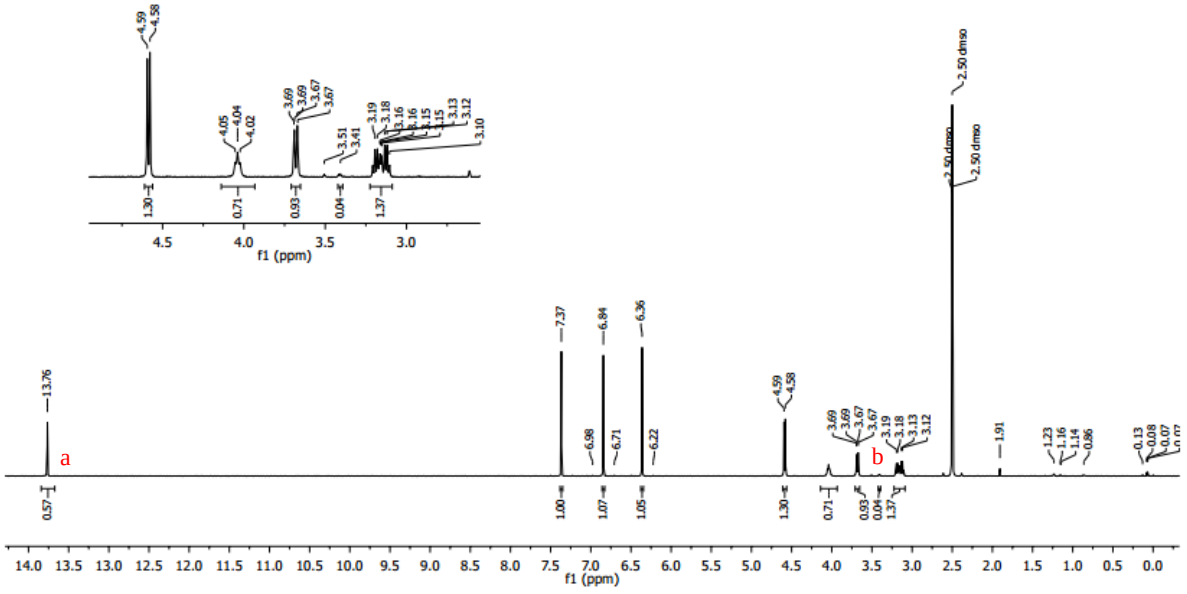
B

Sample4_Mangiferin_Cyclopia_PRESAT



Mangiferin: ESI-MS m/z: 421 (M-H)2;. negative ion mode. 1H NMR (DMSO-d6, 400 MHz): d 13.77 (1H, s, 1-OH), 7.37 (1H, s, H-8), 6.85 (1H, s, H-5), 6.37 (1H, s, H-4), 4.58 (1H, d, J = 9.8 Hz, H-1'), 4.04 (1H, t-like, J = 9.0 Hz, H-2'), 3.68 (1H, d, J = 11.3 Hz, H-6'a), 3.41 (1H, dd, J = 11.3, 5.7 Hz, H-6'b) 3.20 (1H, m, H-3'), 3.16 (1H, m, H-5'), 3.14 (1H, m, H-4').

(1) Gelderblom *et al.*, 2016

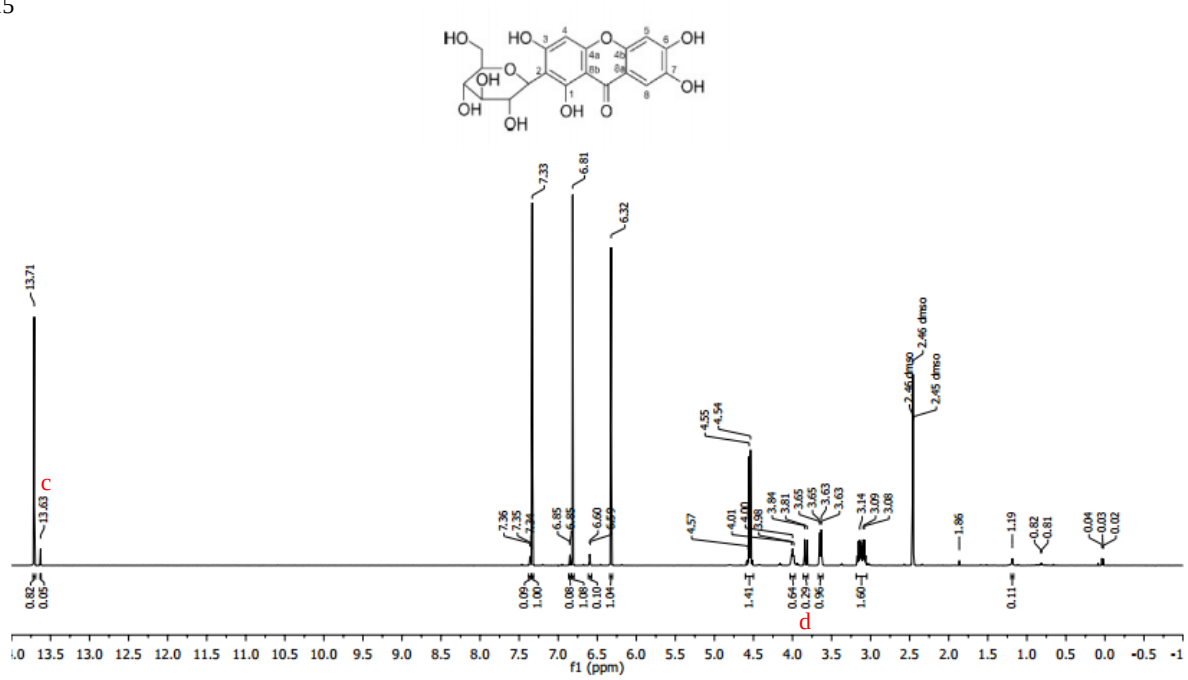


C

Sample3_Mangiferin_Mangifera_PRESAT

Mangiferin: ESI-MS m/z: 421 (M-H)2;. negative ion mode. 1H NMR (DMSO-d6, 400 MHz): d 13.77 (1H, s, 1-OH), 7.37 (1H, s, H-8), 6.85 (1H, s, H-5), 6.37 (1H, s, H-4), 4.58 (1H, d, J = 9.8 Hz, H-1'), 4.04 (1H, t-like, J = 9.0 Hz, H-2'), 3.68 (1H, d, J = 11.3 Hz, H-6'a), 3.41 (1H, dd, J = 11.3, 5.7 Hz, H-6'b) 3.20 (1H, m, H-3'), 3.16 (1H, m, H-5'), 3.14 (1H, m, H-4').

(2) 16/06/15*

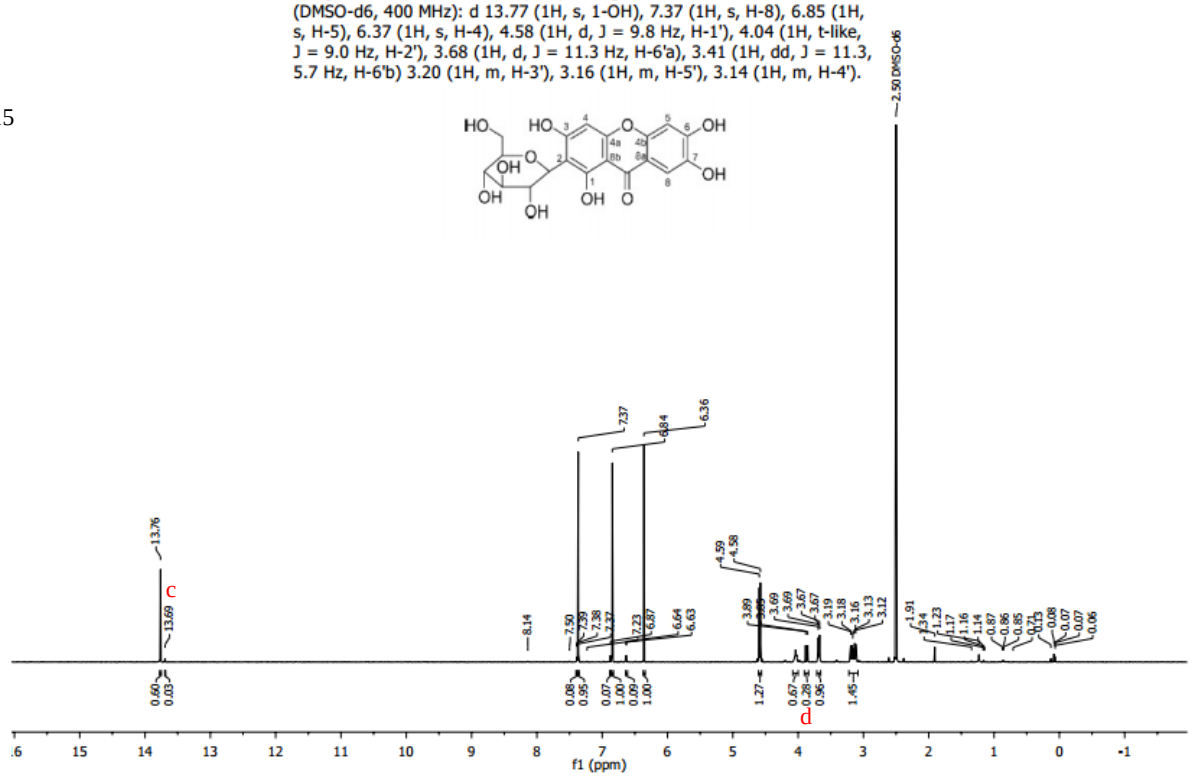


D

Sample2_Mangiferin_Analytical_Std_PRESAT

Mangiferin: ESI-MS m/z: 421 (M-H)2;. negative ion mode. 1H NMR (DMSO-d6, 400 MHz): d 13.77 (1H, s, 1-OH), 7.37 (1H, s, H-8), 6.85 (1H, s, H-5), 6.37 (1H, s, H-4), 4.58 (1H, d, J = 9.8 Hz, H-1'), 4.04 (1H, t-like, J = 9.0 Hz, H-2'), 3.68 (1H, d, J = 11.3 Hz, H-6'a), 3.41 (1H, dd, J = 11.3, 5.7 Hz, H-6'b) 3.20 (1H, m, H-3'), 3.16 (1H, m, H-5'), 3.14 (1H, m, H-4').

(3) 16/06/15



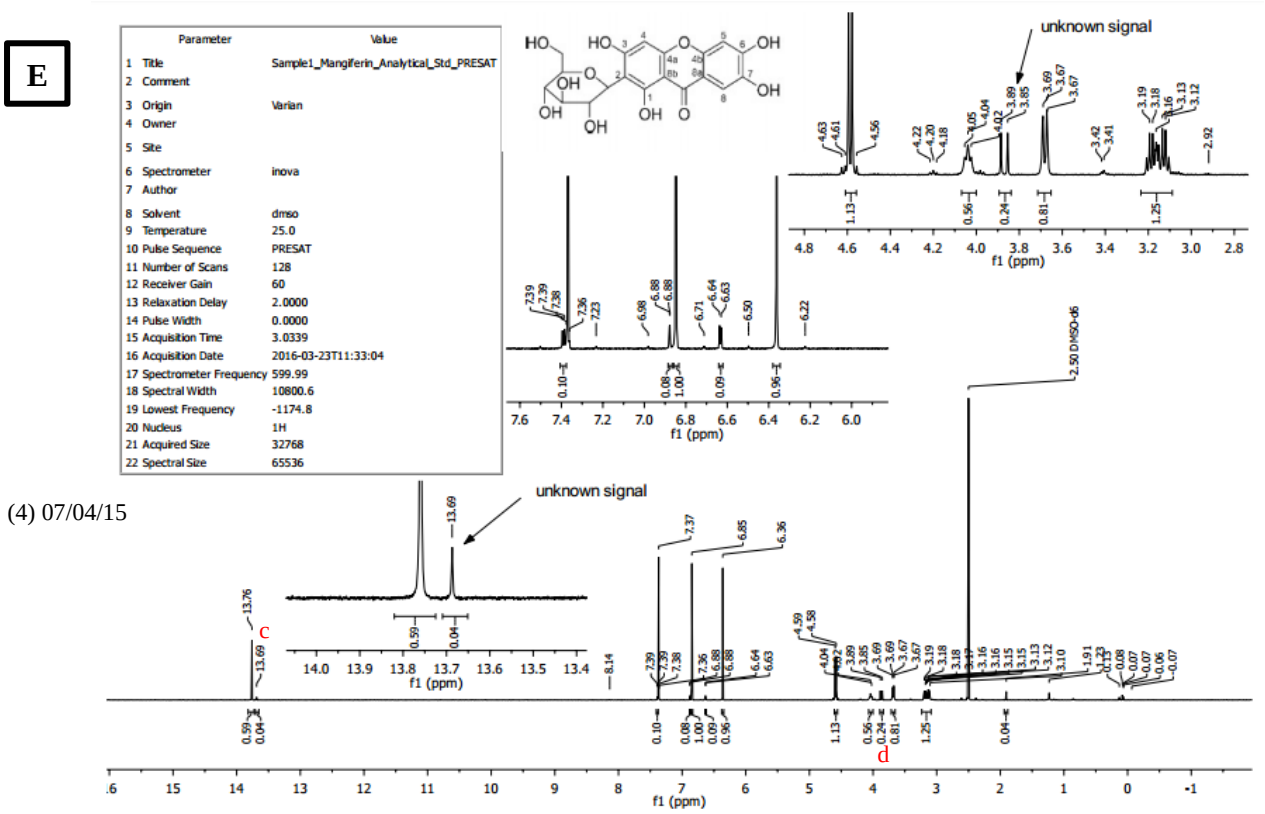
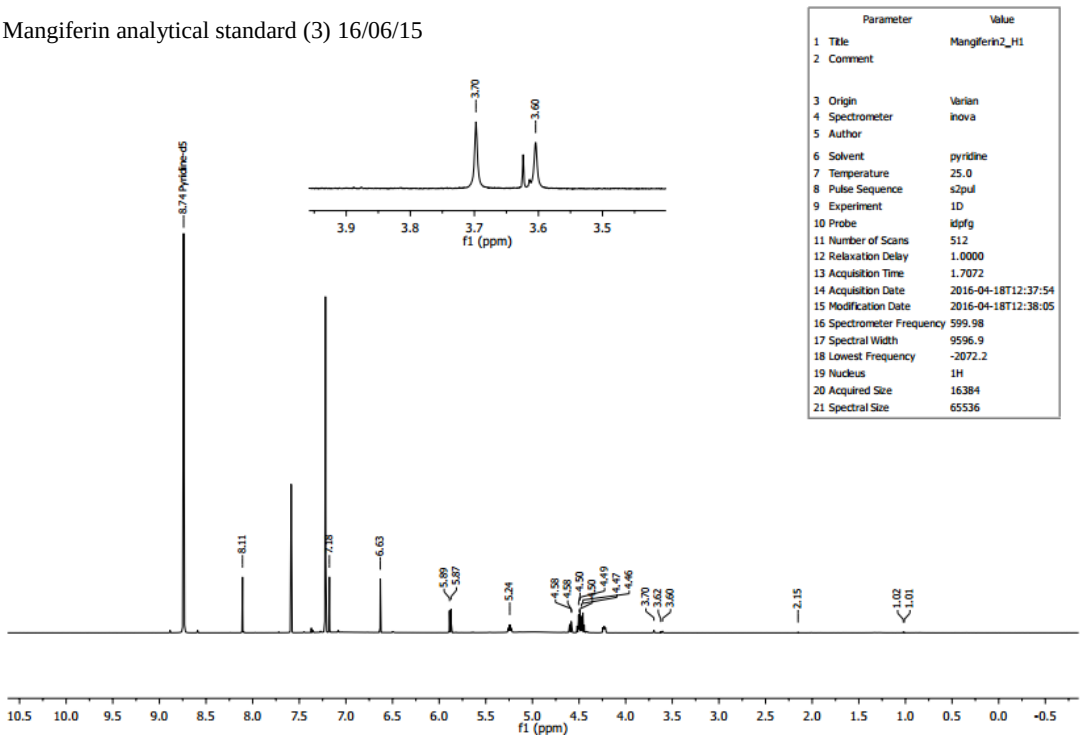


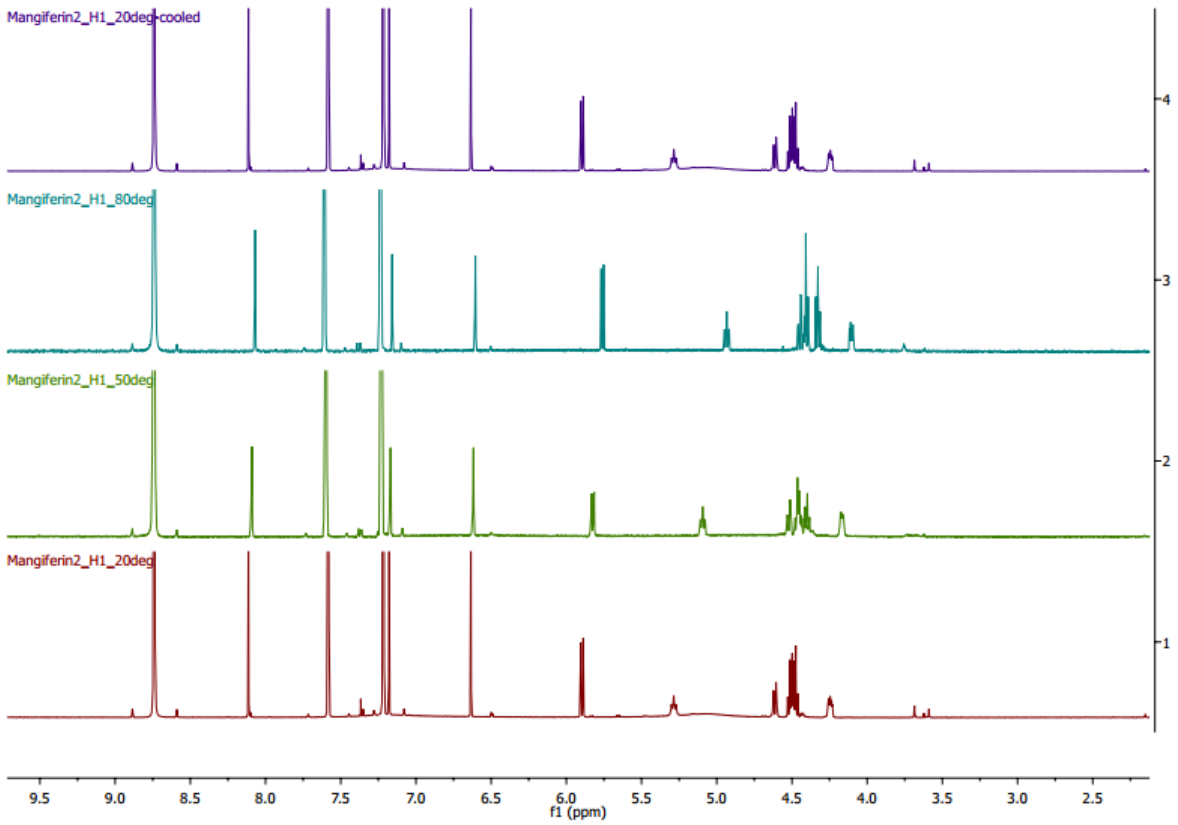
Figure S.6: 400 MHz ^{1}H-NMR spectra (25 °C, DMSO-d6,) of mangiferin stocks. (A) NMR spectra of different stocks of mangiferin labelled according to receiving date: **(1)** Geldeblom *et al.*, 2016, **(2)** 16/06/15, **(3)** 16/06/15*, and **(4)** 07/04/15. Unknown signals detected at (c) 13.69 ppm and (d) 3.87ppm in samples 2 - 4. **(B)** Sample 1: mangiferin isolated and purified from *Cyclopia* by Gelderblom *et al.*, 2016 (unpublished). No signals observed at position (a) 13.69 ppm and (b) 3.87ppm in sample 1. **(C)** Sample 2: mangiferin from *Mangifera indica* (16/6/15)*. **(D)** Sample 3: mangiferin analytical standard (16/6/15). **(E)** Sample 4: mangiferin analytical standard (7/4/15). Unknown signals were subsequently identified as an OH (c) and OMe (d) signal from homomangiferin.

A

Mangiferin analytical standard (3) 16/06/15



B



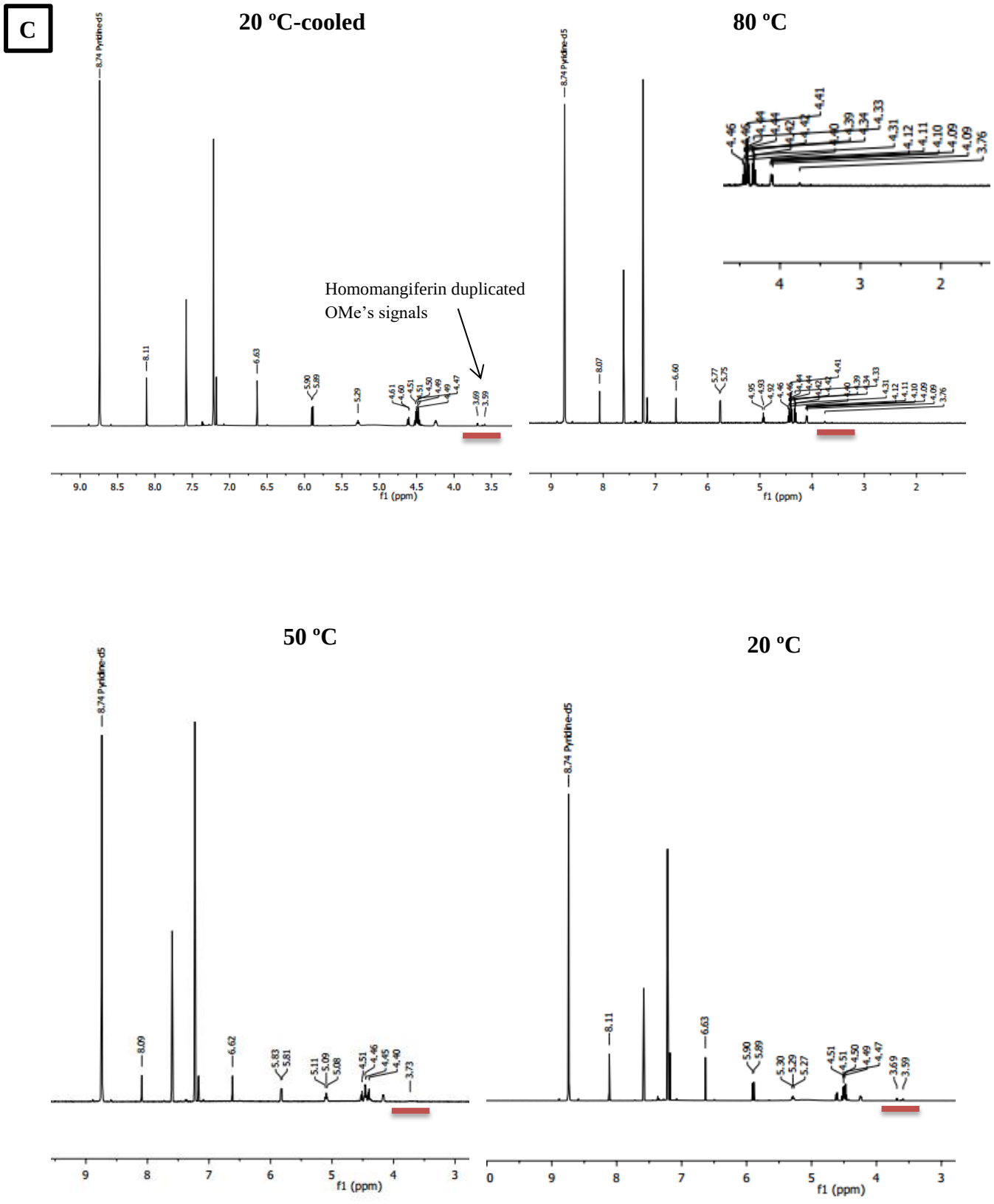


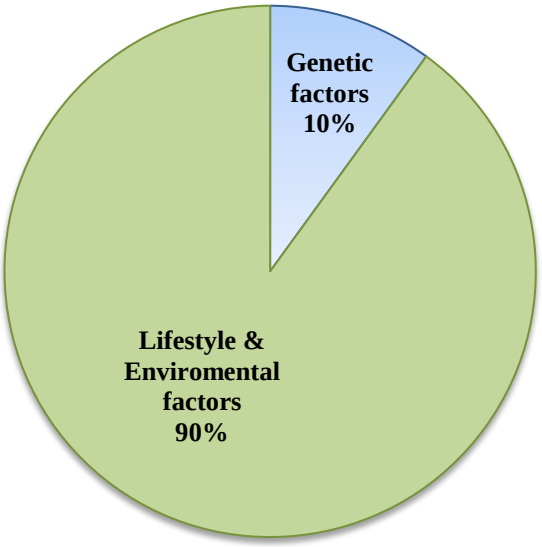
Figure S.7: 400 MHz ^{1}H-NMR spectra (pyridine-d5) of homomangiferin. (A) NMR spectra of homomangiferin in sample 3, mangiferin analytical standard (16/6/15). Signals detected at 3.7 ppm and 3.6 ppm was identical to the methoxyl (OMe) chemical shift found in Wu *et al.* [88]. **(B)** NMR temperature ramp of sample 2 at 80, 50 & 20ºC. **(C)** Temperature ramp shows coalescence of duplicated OMe signals (3.7 & 3.6 ppm) at 80 ºC, confirming the presence of homomangiferin.

Chapter 6: Final Discussion and Conclusion

Cancer is a multifaceted disease characterised by abnormal and uncontrolled cell growth and division due to genomic mutations, which results in ineffective cellular repair and/or delayed apoptosis of defaulted cells [1,2]. Cancer accounts for approximately 13% of all deaths worldwide, and has a socio-economic impact on both developed and developing countries [2–11]. Despite considerable progress made in cancer research (understanding cancer biology, identifying risk factors, early detection, accurate diagnosis and improved or novel therapies), there are still various limitations such as drug resistance, toxicity, and high costs associated with cancer therapies, with no definite cure for this disease [9,12,13]. The fight against cancer is complicated by the fragility of cells and the complex molecular network they form part of [14–16]. Furthermore, each cancer is unique to the host as a result of genetic and epigenetic changes caused by lifestyle and environmental factors [1,17–20].

There are various risk factors that contribute to cancer incidence and mortality. Interestingly, only 10% of these risk factors are due to inherited genetic defects, whereas 90% of cancer-causing risk factors are lifestyle choices and environmental factors (Figure 6.1), including but not limited to tobacco smoking, alcohol consumption, diet low in nutrients, obesity, environmental pollution, oncogenic viruses, and exposure to UV, radiation and/or carcinogenic compounds [9,16,17,21–25]. Therefore, 30-40% of most cancer types can be prevented with a healthy lifestyle and dietary modifications [23–29]. Various epidemiological studies have linked diets high in plant based foods*, which are rich in dietary fibre, vitamins, minerals, antioxidants and phenolic compounds, to disease prevention and improved health [27–38].

* Plant based foods include fruit, vegetables, whole grains, nuts, legumes and herbs [30,31,38].



Lifestyle & environmental risk factors	Percentage[a]
Tobacco	25-30%
Diet	30-35%
Infections	15-20%
Obesity	10-20%
Alcohol	4-6%
Others	10-15%

[a] % of individual risk factors contributing to the 90% lifestyle & environmental risk factors

Figure 6.1: Cancer-related risk factors as adapted from Anand *et al.*, [23].

In addition to using plants as a source of health promoting macro–and micronutrients, various cultures and societies are also dependant on plants for medicinal purposes [38–42]. Some of the poorer or lower–income countries lack proper health care systems, medical facilities and pharmaceutical manufacturing capacity to provide safe and affordable medical care [43–45]. Consequently, the native communities rely predominantly on alternative phytomedicine and traditional practices [40,42,46–48]. It has been estimated that 70-80% of African countries use some form of traditional or complementary/alternative medicine (CAM)[†], either as primary source of treatment or to alleviate side effects associated with some conventional therapies, such as in the case of HIV/AIDS treatments [43,49,50].

Various pharmaceutical and nutraceutical products come from medicinal plants and plant extracts. Generally, the plants are selected for specific favourable properties and the main active compounds are isolated, characterised and synthesized for target specific drug therapies [39,51,52]. Examples of such pharmaceutical drugs originating from plant sources

[†] Complementary and alternative medicine (CAM) is a term used for medicinal products or practices that are not part of conventional medicine, but are often used together with (complementary) or instead of (alternative) conventional medicine [124].

include: aspirin (*Filipendula ulmaria*), codeine and morphine (*Papaver somniferum*), which are used as analgesics, artemisinin (*Artemisia annua*), which is used as an anti-malaria drug, and galantamine (*Galanthus nivalis*), which is used for the treatment of Alzheimer's disease [41,53–56]. Although single well-characterised active compounds work effectively in treating particular diseases, they show less effectiveness in the treatment of multifaceted diseases, such as cancer, and in some case result in drug resistance [57,58].

Whole plant extracts and combination phytotherapies, on the other hand, consist of multiple constituents (active and supporting) and can therefore display multiple actions [59]. In the plant, the mixture of different phytocompounds works together in a combinatorial or synergistic manner to create a supporting environment for the active compounds, as well as enhancing their bioactivity [31,59–62]. Furthermore, plant extracts have the potential to interact with multiple biological targets and systems, for example, *Salacia oblonga* extracts, which are used in Ayurvedic medicine for the prevention and treatment of type 2 diabetes, also displays anti-proliferative activity in MCA-MB-231 breast cancer cells [59,63–65]. In addition, within each biological system the phytocompounds may direct their activity at various intracellular targets and intervene with different cell signalling processes [63,66,67]. This multi-target action of medicinal plants and their extracts provides renewed insight into combatting complex diseases and could perhaps supply an intelligent drug mixture able to combat cancer on multiple levels [62,68]. However, much is yet to be learned about the complex networks and synergistic interactions within plant extracts.

Our research group focus specifically on breast cancer, the most common diagnosed cancer amongst women [69–71]. Genetic factors account for 5-10% of all breast cancer cases with mutations in genes such as BRCA1, BRCA2, P53, ATM, PTEN/MMAC1 and LKB1 resulting in greater genetic susceptibility for breast cancer [72–75]. Other risk factors associated with breast cancer include alcohol consumption, obesity, diet, and exposure to

radiation, in addition to hormone related risk factors (early menarche, the use of hormone contraceptives, late pregnancy of first child, and late menopause), which increases the risk of estrogen receptor (ER) positive breast cancer, the most common type of breast cancer [9,22,23,26,69,73,76].

Estrogens (endogenous and exogenous) are the main driving force behind ER positive breast cancer, however, estrogens also play a vital role in the development and function of the female reproductive system as well as in bone maintenance, cardiovascular protection and cognitive functioning [77–82]. Therefore, the use of exogenous estrogens, as in the case of menopausal hormone replacement therapy, provides both a protective role (prevention of osteoporosis, cardiovascular diseases and mental diseases) and a harmful role (increased risk of estrogen related cancers, such as breast cancer), thus creating a double edged sword scenario where woman are at risk for one group of diseases if they do not take hormone replacement therapy, and at risk for another group of diseases if they do [77–85]. For this reason, women are seeking alternative forms of hormone therapies and are turning to plant extracts high in phytoestrogens, since they are deemed as safer and more natural sources of estrogens [86–90]. These plant extracts should, however, still be used with caution, since the combination of all the different constituents, including the phytoestrogens, are not as well studied as conventional hormone therapies [91].

The goal of our research group is to develop a potential nutraceutical that reduces menopausal symptoms and related diseases, while decreasing the risk of developing estrogen related cancers, specifically breast cancer. One plant of particular interest for us regarding possible novel nutraceutical or pharmaceutical development is *Cyclopia*, an indigenous South African fynbos plant more commonly known as honeybush [92–95]. *Cyclopia* contains high levels of phenolic compounds and has, in various studies, displayed phytoestrogenic, anti-

diabetic, anti-inflammatory and anti-mutagenic activity [94–100]. Unlocking the pharmaceutical potential of *Cyclopia* could also be beneficial to South Africa's economy.

The sequential methanol extract from *Cyclopia subternata* (SM6Met) has shown promising *in vitro* and *in vivo* results in different breast cancer experimental systems and models [100–102]. The desired estrogenic characteristics of this extract include ERα antagonism and ERβ agonism (which is important in ER positive breast cancer therapies since ERα is known to induce cell proliferation, whereas ERβ has shown to antagonise ERα mediated activity), and inhibition of E_2-induced breast cancer cell proliferation [100–106]. To evaluate the multi-target potential of SM6Met, Mortimer *et al.* fractionated the extract and found that the phenolic content as well as favourable estrogenic profile of SM6Met was divided between the three fractions, with F1 and F2 displaying ERα antagonism and F3 displaying robust ERβ agonism, thus reaffirming the importance of the whole extract [107]. Mortimer also identified some of the phenolic compounds in the different fractions, however, the combinatorial or possible synergistic activity of all the different phytocompounds has never before been investigated. The current study continued on from Mortimer's work, and as a starting point, focused specifically on F3 and the 7 major phenolic compounds quantified in this fraction from amongst the 15 phenolic compounds identified by Mortimer *et al.* [107].

The first aim of the current study was to establish an experimental system sensitive enough to detect small drug induced changes. Following on from Mortimer's study, the same *in vitro* experimental systems were used (promoter reporter assay using HEK 293 cells and proliferation assay using MCF-7 BUS cells). Various factors were investigated including cell types, the ER subtypes combined with different ERE promoter reporter constructs, and the optimisation of the experimental procedures, after which is was established that the promoter reporter assay (post-optimisation), is a more sensitive experimental system to test phytocompound interactions. Specifically, optimisation resulted in a significant ($P<0.001$) 9-

fold increase in the efficacy of E_2 via ERβ, with no significant increase via ERα. Despite the difference in efficacy between ERα and ERβ, the increase in sensitivity via ERβ is a more desired outcome since F3 is characterised as a robust ERβ agonist.

The second aim entailed the reconstitution of F3 (F3R) using the 7 major phenolic compounds (mangiferin, isomangiferin, scolymoside, luteolin, iriflophenone, *p*-coumeric acid and protocatechuic acid)[‡] as identified and quantified by Mortimer *et al.* [107], to investigate their contribution to the robust ERβ activity of F3. Interestingly, F3R displayed no significant estrogenic activity with the 7 major phenolic compounds at 1-fold their concentration in F3. Possible reasons for the lack of estrogenic activity could be that the excluded phenolic compounds in F3 (alone or combined) are responsible for F3's estrogenic activity, or that they create a supporting environment for the active compounds to elicit a response. However, when F3R was reconstituted at 100-fold (F3R (100x)) its concentration, a significant ($P<0.001$) increase in activity was observed via both ERα and ERβ, indicating that some of the phenolic compounds in F3R do possess estrogenic activity. It was initially though that luteolin was the cause of the increase in estrogenic activity observed with F3R (100x), since, at this stage of the study, it was the only phenolic compound out of the 7 that had in previous studies displayed phytoestrogenic activity via both ER's [95,96,108–110].

The next step (Aim 3) was to investigate the estrogenic activity of the individual phenolic compounds in F3R, to establish which of the compounds are, in fact, the active estrogenic compounds. As expected, luteolin displayed estrogenic activity at increased concentrations via both ERα and ERβ, mangiferin, however, also displayed estrogenic activity at increased concentrations, via both ER subtypes. A detailed investigation of the estrogenic activity of these two estrogenic compounds, confirmed mangiferin and luteolin's ER mediated mode of action. To our knowledge, mangiferin's ERβ mediated activity is a novel finding, with

[‡] Phenolic compounds were purchased. See section 3.2.1.

literature only recently presenting ERα related activity in different experimental systems [111,112].

After establishing that mangiferin and luteolin were the only two estrogenic phenolic compounds in F3R, addition and subtraction studies (Aim 4) were conducted to investigate the supporting role of the 5 non-estrogenic phenolic compounds (in combination with the 2 estrogenic phenolic compounds) in the estrogenic activity of F3R (100x). This was necessitated as mangiferin and luteolin's individual estrogenic activity (at 100x their concertation in F3R) was still significantly lower than that of F3R (100x). In the addition studies, mangiferin and luteolin displayed interchangeable activity in F3R (100x) via ERβ, with no significant difference between F3R (100x) and the newly reconstructed fractions (F3R Man (100x), F3R Lut (100x) & F3R 100x (Man + Lut)). This further accentuates the importance of the non-estrogenic phenolic compounds in F3R (100x), and suggests that the non-estrogenic phenolic compounds, even at 1x their concentration in F3R, enhance the activity of the estrogenic phenolic compounds. Via ERα, these newly reconstructed fractions, however, displayed significantly lower activity compared to F3R (100x), indicating that one or more of the non-estrogenic phenolic compounds are needed, at 100x their concertation, in order to recreate F3R (100x)'s activity. Aim 4, however, could only be completed in part, since, with the subtraction studies, mangiferin lost its estrogenic activity and luteolin appeared to be the only contributing factor to the activity of F3R (100x). Consequently, the last aim could also not be completed and the combinatorial or possible synergism activity of the 7 major phenolic compounds could not be determined.

An additional investigation was conducted to determine the possible cause for the sudden loss of mangiferin's activity. Various mangiferin stocks were tested and no structural changes or degradation of the phenolic compound was detected via LC-MS and NMR studies, however, homomangiferin was detected in all the mangiferin stocks used for experimental work. Like

mangiferin, not much is known about homomangiferin's estrogenic mode of action. Another possible cause was speculated to be external factors such as pH, since pH drifting has been shown to be responsible for deprotonation of hydroxyl groups in phenolic compounds, including mangiferin [113–115]. Further investigation is, however, needed regarding the influence of pH drifting on F3R activity.

To summarise the findings from the current study, F3's robust ERβ agonist activity could not be recreated using only the 7 major phenolic compounds quantified in this fraction, and it is apparent that the minor phenolic compounds play a crucial role in the desired estrogenic profile of this fraction. Even though the major phenolic compounds showed no estrogenic activity (individual and combined) at their concentrations in F3 (F3R (1x)), their contribution to this fraction's activity, and subsequently the multi-target activity of SM6Met, should not be dismissed. In a review article by Gertsch [62], the author describes the possibility of weaker or non-active secondary metabolites in plant extracts to still exert an effect in the absence of the bioactive compounds due to biochemical synergism or network pharmacology. When F3 was reconstitution with the 7 major phenolic compounds at 100-fold their concentration in F3, a significant ($P<0.001$) increase in activity was observed via both ER subtypes. Upon further investigation, it became clear that the two estrogenic phenolic compounds, mangiferin and luteolin, were not the only contributing factors to F3R (100x)'s robust estrogenic activity, and the non-estrogenic compounds potentially played a supporting role in the bioactivity of this fraction. Various studies have presented similar results where the isolated active phytocompounds showed less effectiveness or a decrease in bioactivity compared to the plant extract [62,68,116–119]. In addition, mangiferin's ERβ mediated activity was a novel finding, unfortunately, mangiferin lost this activity during the final stages of the current study. Future work should investigate the contribution of the minor phenolic compounds in F3 to the favourable attributes of this fraction, in order to establish which phytocompounds to target for the investigation of combinatorial or possible synergistic

interactions. *In silico* (computational) methods could also aid in identifying potential synergistic phytocompounds, by means of virtual screening of natural chemical libraries such as DrugBank, Asinex, ZINC databases, in combination with drug synergy software such as Compusyn and Combenefit [66,120–123].

Plants may provide us with future intelligent drug mixtures, with the possibility of overcoming the limitations of current therapies, however, a great deal is yet to be discovered regarding the complex interactions of phytocompounds. Like the game of Jenga, removing vital phytocompounds from the mixture creates instability, and eventually results in the collapse of the complex multi-target system.

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