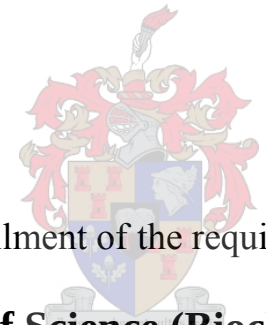
# Evaluation of the phytoestrogenic activity of honeybush (*Cyclopia*)

**Nicolette Jeanette Dorothy Verhoog**



Thesis submitted in fulfillment of the requirements for the Degree of

**Master of Science (Biochemistry)**

at the University of Stellenbosch

**Supervisor: Dr Ann Louw**

**Co-supervisor: Dr Elizabeth Joubert**

**April 2006**

# Declaration

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

Signature:_____

Date: _____

## Summary

The phytoestrogenic activity of *Cyclopia,* used to prepare honeybush tea, was evaluated and compared with that of the endogenous estrogen, 17-β-estradiol ($E_2$) and the known phytoestrogen, genistein. Phytoestrogens are plant polyphenols much in demand in the nutraceutical market as they mediate an estrogenic effect through binding to estrogen receptor (ER) subtypes, ERα and ERβ. Aqueous and methanol extracts of "unfermented" and "fermented" commercially available *Cyclopia spp*., *C. genistoides, C. subternata, C. sessiliflora* and *C. intermedia,* together with known estrogenic polyphenols shown to be present in some or all species, luteolin, formononetin and naringenin, as well as other *Cyclopia* polyphenols, mangiferin, eriodictyol, eriocitrin, narirutin, hesperidin and hesperetin, were initially screened by evaluating ER subtype binding. The results suggest that *C. genistoides* and *C. subternata* extracts display the highest phytoestrogenic activity and that methanol extracts from unfermented plant material generally display greater activity. Of the polyphenols tested, only luteolin, formononetin and naringenin were able to significantly displace tritiated $E_2$ from both ER subtypes. Subsequent in-depth *in vitro* studies evaluated the estrogenic potential of unfermented *C. genistoides* methanol extracts and selected polyphenols by comparing (i) potency ($IC_{50}$) and binding affinity ($K_i$) in whole cell competitive binding assays to both hERα and hERβ, (ii) potency ($EC_{50}$) and efficacy (fold-induction) in ERE-containing promoter reporter studies and proliferation assays in MCF-7-BUS and MDA-MB-231 cells, and (iii) displacement of $^3$H-$E_2$ from human SHBG. Although only one of the three extracts (P104) competed with $E_2$ for binding to both ER subtypes, two extracts (P104 and P105) stimulated cell proliferation of MCF-BUS cells, while all three extracts (P104, P105, and P122) transactivated *via* hERβ. P104, like $E_2$, displayed a higher binding affinity for ERα in contrast with the polyphenols, except for formononitin, that bound with a higher affinity to ERβ. Despite this, all extracts transactivated *via* ERβ with potencies and efficacies similar to that of $E_2$ and genistein and induced MCF-7-BUS cell proliferation with potencies and efficacies similar to that of genistein, but with potencies significantly lower than $E_2$. In addition, all extracts displaced $^3$H-$E_2$ from SHBG to a similar degree as genistein. ER-dependent proliferation was confirmed in MCF-7-BUS cells by use of the ER antagonist, ICI 182,780. Physiologically more relevant, *C. genistoides* extracts antagonised $E_2$ induced MCF-7-BUS cell proliferation. Furthermore, all extracts, except P122, were able to induce cell

proliferation of the estrogen insensitive MDA-MB-231 breast cancer cell line, suggesting that the extracts are able to induce ER-dependent and ER-independent cell proliferation. Together the results presented in this thesis, show that the *C. genistoides* extracts investigated have weak estrogenic activity and are potent activators of ERβ, thus inhibiting $E_2$-induced breast cancer cell proliferation. *Cyclopia* is thus a potential source for phytoestrogen-rich extracts for the nutraceutical industry.

# Samevatting

Die fito-estrogeniese aktiwiteit van *Cyclopia*, wat gebruik word vir heuningbostee produksie, is geëvalueer en vergelyk met die van die endogene estrogeen, 17-β-estradiol ($E_2$) en die bekende fito-estrogeen, genistein. Fito-estrogene is plant polifenole in groot aanvraag in die neutraseutiese bedryf omdat hul 'n estrogeniese effek bemiddel deur aan die estrogeen reseptor (ER) subtipes, ERα en ERβ, te bind. Aanvanklike sifting van water en metanol ekstrakte van "ongefermenteerde" en "gefermenteerde" kommersieël beskikbare *Cyclopia spp*., *C. genistoides, C. subternata, C. sessiliflora en C. Intermedia,* en bekende estrogeniese polifenole teenwoordig in sommige of alle spesies, luteolin, formononetin en naringenin, sowel as ander *Cyclopia* polifenole, mangiferin, eriodictyol, eriocitrin, narirutin, hesperidin en hesperetin, het evaluering van binding aan beide ER subtipes behels. Die resultate dui aan dat *C. genistoides* en *C. subternata* ekstrakte die hoogste fito-estrogeniese aktiwiteit toon en dat metanol ekstrakte van ongefermenteerde plant materiaal deurlopend die hoogste aktiwiteit toon. Luteolin, formononetin and naringenin was die enigste polifenole was betekenisvolle verplasing van $E_2$ vanaf albei ER subtipes getoon het. Daaropvolgende in diepte *in vitro* studies, het die estrogeniese potensiaal van die ongefermenteerde *C. genistoides* metanol ekstrakte en geselekteerde polifenole getoets deur, (i) die sterkte ($IC_{50}$) en bindings affiniteit ($K_i$) in heel sel kompeterende binding studies aan beide ERα en ERβ, (ii) die sterkte ($EC_{50}$) en effektiwiteit (mate van induksie) in transaktiverings studies en proliferasie essais in MCF-7-BUS en MDA-MB-231 selle, en (iii) verplasing van $^3$H-$E_2$ vanaf mens SHBG te vergelyk. Alhoewel net een uit die drie ekstrakte (P104) met $E_2$ gekompeteer het vir binding aan beide ER subtipes, het twee ekstrakte (P104 en P105) MCF-7-BUS selle gestimuleer om te prolifereer, en kon al drie ekstrakte (P104, P105 en P122) deur hERβ transaktiveer. P104, soos $E_2$ en formononetin, het hoër bindings affiniteit getoon vir hERα, in teenstelling met die ander polifenole wat 'n hoër affiniteit getoon het vir hERβ. Nogtans kon al die ekstrakte *via* die hERβ transaktiveer met soortgelyke sterkte en effektiwiteit as $E_2$ en genistein. Die ekstrakte het ook MCF-7-BUS sel proliferasie geïnduseer met 'n sterkte en effektiwiteit soortgelyk aan die van genistein, maar met sterktes betekenisvol minder as die van $E_2$. Alle ekstrakte kon ook $^3$H-$E_2$ verplaas van SHBG tot 'n soortgelyke mate as genistein. ER-afhanklikheid van proliferasie van MCF-7-BUS selle is bevestig deur die gebruik van die ER antagonis, ICI 182,780. Fisiologies meer relevant, *C. genistoides* ekstrakte kon $E_2$ geïnduseerde

MCF-7-BUS sel proliferasie antagoniseer. Verder, kon alle ekstrakte, behalwe P122, die estrogeen onsensitiewe MDA-MB-231 selle induseer om te prolifereer wat aandui dat die ekstrakte proliferasie op beide ’n ER-afhanklike en ER-onafhanklike wyse kan induseer. Tesame dui die resultate voorgele in hierdie tesis daarop dat *C. genistoides* ekstrakte swak estrogeniese aktiwiteit toon en ERβ sterk aktiveer, en dus $E_2$ geïnduseerde, borskanker sel proliferasie inhibeer. *Cyclopia* kan dus as ’n moontlike bron van fito-estrogeen-ryk ekstrakte dien vir die neutraseutiese bedryf.

**I would like to dedicate this thesis to my parents, Olive and Errol Verhoog**

# Acknowledgements

I would greatly like to thank the following people and institutions without whom this study would not have been possible:

- First and foremost, I would like to thank my supervisor, **Dr Ann Louw**, for her remarkable dedication and supervision. I truly learned so much from you. Thank you for allowing me to work independently and inspiring me to give only my best and seeing the good when I only saw the bad. Knowing that your door was always open was very reassuring to me. I will always remember your thoughtfulness, concern and unquestionable kindness through my toughest times both personal and work related. It was an honour doing this project under your supervision.

- I would also like to thank my co-supervisor, **Dr Lizette Joubert,** for the many wonderful opportunities you have given me. They have really enriched my young career as a researcher. Thank you for your positive criticism, advice and encouragement especially concerning the writing of this thesis.

- **Ms Carmen Langeveldt**, our laboratory supervisor, for the smooth running of the lab and the many hours spend in tissue culture with the plating of the cells. I will be for ever indebted to you.

- **Christie Malherbe, for** the HPLC analysis of the methanol extracts.

- The NRF, THRIP, ARC Infruitec-Nietvoorbij, Medical Research Council, National Brands Ltd, THRIP, Western Cape Department of Agriculture and the National Department of Agriculture for the funding of this project and the Department of Biochemistry, NRF, ARC Infruitec-Nietvoorbij and THRIP for bursaries.

- My parents, **Olive and Errol Verhoog**, for all the sacrifices you made, so that I can get an education, and for only wanting the best for me. The love and support you gave pulled me through the most trying times I was faced with in this project. Your faith, encouragement and

love allowed me to complete this. Thank you for the interest you always showed in my work. I am sorry Daddy that this kept me away from you when you needed me the most.

- **Donita Aficander**, thank you for your support throughout my time in the department. I appreciate your encouragement, guidance and support but most of all our friendship. Thank you for motivating me and being there to talk too.

- **Carmen Langeveldt,** I am thankful for our friendship. Thank you for always lending an ear, I valued your advice and appreciate your concern. You and Donita truly kept me sane and made my work so much more pleasurable.

- **To my family and friends,** thank you for your support and love.

  **George Damons and Welma Maart**, for your assistance when needed. Your kindness and goodness always made it a pleasure to be in your company.

- **Woelies, Abbi and Thor,** for loving me unconditionally.

- **The Lord Almighty,** my source of strength, Whom without, this would not have been possible.

**I can do all things through Christ, which strengtheneth me**

***Philippians 4:13***

# List of abbreviations

| | |
|---|---|
| 17β-HSD | -17β-hydroxysteroid dehydrogenase |
| 3β-HSD | -3β-hydroxysteroid dehydrogenase |
| AF-1 | -Transcription activation function -1 |
| AF-2 | -Transcription activation function-2 |
| Akt | -Serine-threonine kinase |
| AP-1 | -Activating protein-1 |
| ARE | -Antioxidant response element |
| cAMP | -cyclic adenosine monophosphate |
| CAT | -chloramphenicol acetyltransferase |
| cDNA | -complementary DNA |
| CHO | -Chinese hamster ovary |
| DAE | -dry aqueous extract |
| DBD | -DNA binding domain |
| D-box | -dimerisation box |
| DCC | -dextran coated charcoal |
| DHEA | -dehydroepiandrosterone |
| DME | -dry methanol extract |
| DMEM | -Dulbecco's modified Eagle's medium |
| DMSO | -Dimethyl sulfoxide |
| DNA | -deoxyribonucleic acid |
| $E_1$ | -estrone |
| $E_2$ | -17-β-estradiol |
| $E_3$ | -estriol |
| $EC_{50}$ | -half maximal effective concentration |
| Efficacy | -maximal response induced |
| EGF | -epidermal growth factor |
| EGF-R | -epidermal growth factor-receptor |
| eNOS | -endothelial nitric oxide synthatase |
| ER | -estrogen receptor |

| | |
|---|---|
| ERE | -estrogen response element |
| ERK | -extracellular regulated kinase |
| ERα | -estrogen receptor alpha |
| ERβ | -estrogen receptor beta |
| FCS | -fetal calf serum |
| FSH | -follicle stimulating hormone |
| GnRH | -gonadotrophin-releasing hormone |
| GPRC | -G-protein coupled receptors |
| GSTs | -glutathione-*S*-transferases |
| hERα | human estrogen receptor alpha |
| hERβ | human estrogen receptor beta |
| HPLC | -high performance liquid chromatography |
| HRT | -hormone replacement therapy |
| IGF-1 | -insulin-like growth factor-1 |
| IGF-R | -insulin-like growth factor receptor |
| IL-6 | -interleukin-6 |
| JNK | -c-Jun N-terminal kinase |
| Kd | - equilibrium dissociation constant |
| $K_i$ | - equilibrium dissociation constant |
| LBD | -ligand binding domain |
| LDL | -low-density lipoprotein |
| LH | -luteinizing hormone |
| MAPK | -mitogen activated protein kinase |
| MTT | -3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NF-κB | -nuclear factor κB |
| NIH | -National Health Institute |
| P-box | -proximal box |
| PCR | -polymerase chain reaction |
| PI3K | -phosphatidylinositol 3-OH kinase |
| PKA | -protein kinase A |
| Potency | -$EC_{50}$ |

| | |
|---|---|
| PR | -progesterone receptor |
| QR | -quinone reductase |
| RBA | -relative binding affinity |
| SBP | -sex binding protein |
| SERM | -selective estrogen receptor modulator |
| SF-1 | -steroidogenic factor 1 |
| SHBG | -sex hormone binding globulin |
| SRB | -sulforhodamine B |
| TeBG | -testosterone-estrogen binding globulin |
| TNF-α | -tumour necrosis factor α |
| TPP | -total polyphenol |
| VEGF | -vascular endothelial growth factor |