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Bioactivity guided isolation of phytoestrogenic compounds from *Cyclopia genistoides* by the pER8:GUS reporter system

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ABSTRACT

The popular South African herbal tea, honeybush, is made from several *Cyclopia* species (family: *Fabaceae*), amongst them *Cyclopia genistoides*. Phytoestrogenic potential of *C. genistoides* has been recently reported, however bioactivity-guided isolation of compounds with estrogenic activity has not yet been performed.

A transgenic plant system, *Arabidopsis thaliana* pER8:GUS, was used to assay the estrogen-like activity of *C. genistoides*. The quantitative determination of the active compounds in the fermented and non-fermented plant material was performed by HPLC. Subsequent bioactivity-guided fractionation led to the isolation of genistein, naringenin, isoliquiritigenin, luteolin, helichrysin B and 5,7,3',5'-tetrahydroxyflavanone, four of them first reported in the genus.

Helichrysin B, naringenin and 5,7,3',5'-tetrahydroxyflavanone differed in quantity in the fermented and unfermented herbs, the fermented plant material contained two compounds with substantial estrogenic-like activity in higher concentration (naringenin and 5,7,3',5'-tetrahydroxyflavanone), whereas the less active helichrysin B was more abundant in the unfermented herb. The fractions as well as compounds inhibited the growth of human cancer cell lines A2780 and T47D.

These results underline the phytoestrogenic activity of *C. genistoides* and support the rationale to the fermentation process.

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1. Introduction

Current hormone replacement therapy (HRT), using conjugated equine estrogen alone (CEE) for women who had undergone hysterectomy or in combination with progestin (CEE + P) for women with intact uterus, proved to lack overall benefit in chronic disease prevention (osteoporosis, heart disease) and menopausal symptom alleviation (Anderson et al., 2004; Rossouw et al., 2002). Moreover, CEE + P increases the risk of stroke, coronary heart disease, venous thromboembolic disease and breast cancer, while CEE alone does not affect the risk of heart disease, but increases the risk of stroke (Anderson et al., 2004; Rossouw et al., 2002). Alternative solutions, such as selective estrogen receptor modulators (SERMs) have been also questioned.

Abbreviations: HRT, hormone replacement therapy; CEE, conjugated equine estrogen; CEE + P, conjugated equine estrogen in combination with progestin; SERM, selective estrogen receptor modulators; ER, α and β estrogen receptors; E₂, 17- β -estradiol; XVE, estrogen receptor-based transactivator vector; GUS, β -glucuronidase; MAC, minimum active concentration.

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However, the well-known SERMs, raloxifene and tamoxifen, have been reported to decrease the risk of breast cancer and increase bone mineral density, but they have also been associated with the stimulation of endometrial growth, the occurrence of hot flashes and an increased risk of venous thromboembolism (Barrett-Connor et al., 2006; Cranney and Adachi, 2005; Delmas et al., 1997; MacGregor and Jordan, 1998; Vosse et al., 2002; Zidan et al., 2004).

Phytoestrogens might serve as a viable alternative for HRT, given their differentiated effect on α and β estrogen receptors (ERs). They may be able to bind to both ER subtypes, acting as either agonist or antagonist, but unlike 17- β -estradiol (E₂) they generally bind to the ER with a much lower affinity, yet have a higher affinity for ER- β than for ER- α , which is believed to protect against excessive cell proliferation mediated by ER- α (Lindberg et al., 2003; Morito et al., 2001).

Most of the studies concerning phytoestrogens have focused on soybean and one of its isoflavones, genistein, due to epidemiological evidence suggesting that Asian diet rich in soy is protective against hormone-induced cancers such as breast and prostate cancer (Morton et al., 2002). Furthermore, phytoestrogens may be beneficial to alleviate menopausal symptoms and to protect postmenopausal women against cardiovascular disease and osteoporosis, without the risks associated

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with HRT (Tham et al., 1998; Wei et al., 2012). Despite several promising studies, their effect on menopausal symptoms, such as hot flushes, is inconclusive, and the phytoestrogen treatment seems to be less effective than traditional HRT (Glazier, 2001; Lethaby et al., 2013). Yet, the risks of HRT and the increasing popularity of natural products provide a rationale to search for phytoestrogens with selective affinity for ERs.

One of the potential sources of phytoestrogens is the *Cyclopia* genus. The popular caffeine free herbal tea, honeybush, comprises *Cyclopia* species (family: *Fabaceae*), amongst them *Cyclopia genistoides* (L.) Vent., which is native to the western cape province of South Africa. Honeybush is traditionally used as a restorative or expectorant, but anecdotal evidence also exists about its consumption in order to stimulate milk production in breast-feeding women and to alleviate menopausal symptoms (Joubert et al., 2008; Verhoog et al., 2007b). Methanol extracts from *C. genistoides* was also reported to consistently have the highest binding affinity for both ER subtypes in whole-cell competitive receptor binding assays, when comparing four *Cyclopia* species (Verhoog et al., 2007b). Recently, the phytoestrogenic potential of extracts from different *Cyclopia* species was reported, as well as some compounds, present in *Cyclopia* were also tested (Louw et al., 2013; Verhoog et al., 2007a, 2007b; Visser et al., 2013). However bioactivity-guided isolation was reported from *Cyclopia subternata*, but not from *C. genistoides*, which species also displayed significant phyto-estrogenic activity (Mortimer et al., 2015; Verhoog et al., 2007b).

Comprehensive phytochemical investigations of *Cyclopia* species have focused on the polyphenolic composition of three out of the six commercially important species, *Cyclopia intermedia*, *C. subternata* and *C. genistoides*. The aerial parts of *Cyclopia* contain mainly flavones (luteolin, scolymoside, diosmetin), flavanones (naringenin, eriodictyol, hesperitin, narirutin), isoflavones (formononetin, wistin, calycosin, orobol, afrormosin, fujikineticin, pseudobaptigen), xanthones (mangiferin, isomangiferin), coumestans (medicagol, flemmichapparin, sophoracoumestan), catechins (epigallocatechin-3-O-gallate), benzaldehyde derivatives and phenylethanolderivates (Ferreira et al., 1998; Joubert et al., 2008; Joubert et al., 2011; Kamara et al., 2004; Sprent et al., 2010).

A comprehensive phenolic profiling of *C. genistoides* by the means of LC-DAD-MS and -MS/MS has been recently performed. Ten compounds were identified based on comparison with reference standards (iriflophenone-3-C-glucoside, eriocitrin, narirutin, vicenin-2, diosmin, etc), thirty constituents were tentatively identified (e.g. tetrahydroxyxanthone-C-hexoside dimers, naringenin derivatives, eriodictyol glycosides, phloretin-3',5'-di-C-glucoside, glycosidated phenolic acids) (Beelders et al., 2014).

Also recently, a fast and efficient method for the isolation of the C-glucosidated xanthones mangiferin and isomangiferin from *C. genistoides* was developed and additionally, two benzophenone derivatives: 3-C- β -glucosides of maclurin and iriflophenone were isolated together with hesperidin and luteolin (Kokotkiewicz et al., 2013).

In the present study, the methanol extracts from fermented and unfermented *C. genistoides* were assayed with a highly efficient and convenient transgenic plant system, *Arabidopsis thaliana* pER8:GUS line, in order to detect estrogenic/antiestrogenic activity. The transgenic plant pER8:GUS, with the GUS gene as a gene fusion marker for the analysis of gene expression, expresses high estrogenic sensitivity and can be used to quantify the bioactivity of phytoestrogens (Lai et al., 2011). Moreover, it is a visible system, and primary results can be readily observed visually, without the need of special instrumentation. The system contains an estrogen receptor-based transactivator vector (XVE) as an activator unit and the GUS (β -glucuronidase) gene as a reporter (Brand et al., 2006). The XVE system is an estrogen receptor-based chemical-inducible system, which was developed by Zuo et al. in 2000. It comprises a DNA binding domain of the bacterial repressor LexA (X), an acidic transactivating domain of VP16, and the regulation region of the ER- α . The XVE activator is strictly regulated by estradiol; in the case of the presence of estrogen active compounds the activator

stimulates expression of GUS transcription (Brand et al., 2006). GUS protein containing transgenic plants gives blue color, after adding a glucopyranosiduronic acid containing dye.

The cost-effectiveness, tolerance toward higher doses of cytotoxic compounds, the ability to detect both ER agonists and antagonists and high efficiency and versatility made pER8:GUS a convenient screening system for testing estrogen-like effects. However, the pER8:GUS system as used in the current study only screened for ER α agonists not antagonists, despite the fact that theoretically the system may be used to investigate antagonism if the test compounds are administered together with E2. Limitations of this transgenic plant assay may be its relative lower sensitivity and that it only determines ER- α interactions. However, phytoestrogens usually bind both ER- α and ER- β (with higher affinity toward ER- β), hence this model is suitable for natural product screening (Brahmachari, 2015; Brand et al., 2006; Lai et al., 2013; Lai et al., 2011).

Bioactivity-guided fractionation led to the isolation of six compounds, which were quantified by the means of HPLC.

With regard to the reported antiestrogenic and estrogenic activity of *Cyclopia* extracts, fractions and compounds, they can induce and/or inhibit cell-proliferation, depending on their amount, structure, the ER α / β ratio of the cells, the presence of E₂, ER α / β antagonism/antagonism or ER-independent antiproliferative effect of the compounds and their ratio in an extract (Pons et al., 2014; Verhoog et al., 2007a; Visser et al., 2013). In order to measure the antiproliferative effect of the isolated compounds, antiproliferative testing was conducted on T47D and A2780 cells.

2. Materials and methods

2.1. General

Vacuum liquid chromatography (VLC) was carried out on silica gel G (15 μ m, Merck); column chromatography (CC) on polyamide (ICN), silica gel (160–200 mesh, Qingdao Marine Chemical Co., Qingdao, China) and Sephadex LH-20 (Sigma); preparative thin-layer chromatography (preparative TLC) on silica gel 60 F₂₅₄ and 60 RP-18 F₂₅₄ plates (Merck); and rotation planar chromatography (RPC) on silica gel 60 F₂₅₄ (Merck) using a Chromatotron instrument (Model 8924, Harrison Research). Medium pressure liquid chromatography (MPLC) was performed by a Büchi apparatus (Büchi Labortechnik AG, Flawil) using a 40 \times 150 mm RP18ec column (40–63 μ m, Büchi).

The instrumentation for normal-phase HPLC (NP-HPLC) consisted of a Waters Alliance 2695 separations module connected to a Waters 2998 photodiode array (PDA) detector (190–800 nm), (Waters Associates, Milford, MA, USA). The separation was carried out on a Kinetex C18 (5 μ m, 100 Å, 150 \times 4.6 mm) column (Phenomenex, Torrance, USA).

For the preparative reversed-phase HPLC, a Merck Hibar Purospher STAR C18 (5 μ m, 250 \times 10.0 mm) semipreparative column (Merck KGaA, Darmstadt, Germany) was used, and HPLC equipment consisted of two JASCO PU-2080 HPLC pumps connected to a JASCO MD-2010 Plus multi-wavelength detector (JASCO Inc., Tokyo, Japan).

¹H NMR (500 MHz), ¹³C NMR (125 MHz) and 2D NMR were recorded in CD₃OD or CDCl₃ or DMSO using a Bruker Avance DRX 500 spectrometer or a JEOL ECS 400 MHz FT-NMR spectrometer. The signals of the deuterated solvents were taken as reference. Two-dimensional (2D) experiments were performed with standard Bruker software. MS spectra were recorded on a API 2000 Triple Quad mass spectrometer with APCI ion source using positive polarity.

2.2. Plant material

Fermented and non-fermented *C. genistoides* (L.) Vent. were a gift from Van Zyl and Mona Joubert owners of Agulhas Honeybush Tea, on their farm near Bredasdorp in South Africa. Botanical identifications were performed by Dr. Hannes de Lange. Fermentation was carried out according to the traditional method for this material [<http://www>].

agulhashoneybushtea.co.za/art-tea/]. Voucher specimens (no. 825-F and 826-nF) for both the fermented and the unfermented plants have been deposited at the herbarium of the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

2.3. Extraction and isolation

The dried fermented and unfermented plant materials (1.7 and 1.3 kg, respectively) were extracted by ultrasonication with methanol (12 L and 10 L) at room temperature for 30 min. The solvent was evaporated under reduced pressure to yield 228.2 g and 237.6 g of crude MeOH extracts, respectively. These extracts were subjected to solvent–solvent partition, affording *n*-hexane (fermented: 15.7 g, unfermented: 13.2 g), dichloromethane (14.8 g and 6.4 g), ethyl-acetate fractions (29.7 g and 23.35 g), the remnant aqueous layers (128.7 g and 121.4 g) and insoluble part. The layers were assayed for estrogen-like activity using the transgenic plant pER8:GUS reporter system at 100 and 200 µg/mL. Estrogenic activity of the extracts was detected via a histochemical assay for GUS activity. The EtOAc and CH₂Cl₂ layers from both the fermented and unfermented plant materials had estrogen-like activities and thus were subjected to further chromatography. For the schematic detailing of the fractionation process see Fig. 1.

The ¹H proton spectra of the CH₂Cl₂ layers from the unfermented and fermented *C. genistoides* were similar, thus only the CH₂Cl₂ layer from the fermented plant material was further examined. It was separated into fourteen fractions by polyamide CC eluting with MeOH–H₂O (2:3 to 1:0). Fractions P8–P11 had significant estrogenic activity (minimum active concentration (MAC) ≤200 µg/mL).

Fraction P9 (1.68 g) was chromatographed by RPC on silica gel and eluted with cyclohexane–acetone (1:0 to 0:1) to give seven subfractions. Subfractions T4 and T5 (410 mg, 250 mg, respectively) were subjected to silica gel CC, eluted with *n*-hexane–acetone (2:1 to 0:1 and 5:1 to 0:1, respectively) to yield eleven (C1–C11) and eight (CD1–CD8) subfractions, respectively. C3 and CD5 were purified by preparative TLC to provide compounds **1** (16.8 mg) and **2** (7.4 mg), respectively.

Fraction P10 (475.5 mg) was subjected to silica gel CC, eluted with *n*-hexane–acetone (3:1 to 0:1) to yield thirteen (CE1–CE13) subfractions. CE8 was purified by RP-HPLC (Hibar, Rp-18e, 5 µm, MeOH–H₂O, 3:2, flow rate 2 mL/min) and also by preparative TLC (CH₂Cl₂–MeOH 10:0.15) to yield compound **4** (1.65 mg) and compound **3** (1.4 mg) respectively.

Fraction P11 (245 mg) was chromatographed by RPC on silica gel and eluted with cyclohexane–acetone (1:0 to 0:1) to give fifteen subfractions. Subfraction S11 was further purified by Sephadex LH-20 to provide compound **5** (5.6 mg).

The ¹H NMR spectra of the EtOAc layers from the unfermented and fermented *C. genistoides* were similar, thus only the EtOAc layer from the unfermented plant material was further examined. It was separated into twelve fractions by VLC eluting with EtOAc–MeOH (1:0 to 0:1). Fractions V2, V3, V6 and V7 had significant estrogenic activity (MAC ≤200 µg/mL). Fraction V7 was separated by MPLC with EtOAc–MeOH–H₂O (20:1:1 to 0:1:0) to yield 21 subfractions, M1 to M21.

Fraction M6 (777.5 mg) was separated into 12 subfractions by silica gel MPLC eluting with MeOH–H₂O (2:8 to 1:0). Subfraction M6/4 (55.3 mg) was further purified by normal-phase preparative TLC eluting with EtOAc–MeOH–H₂O (100:16:14) and finally by gel filtration chromatography to provide compound **6** (3.3 mg).

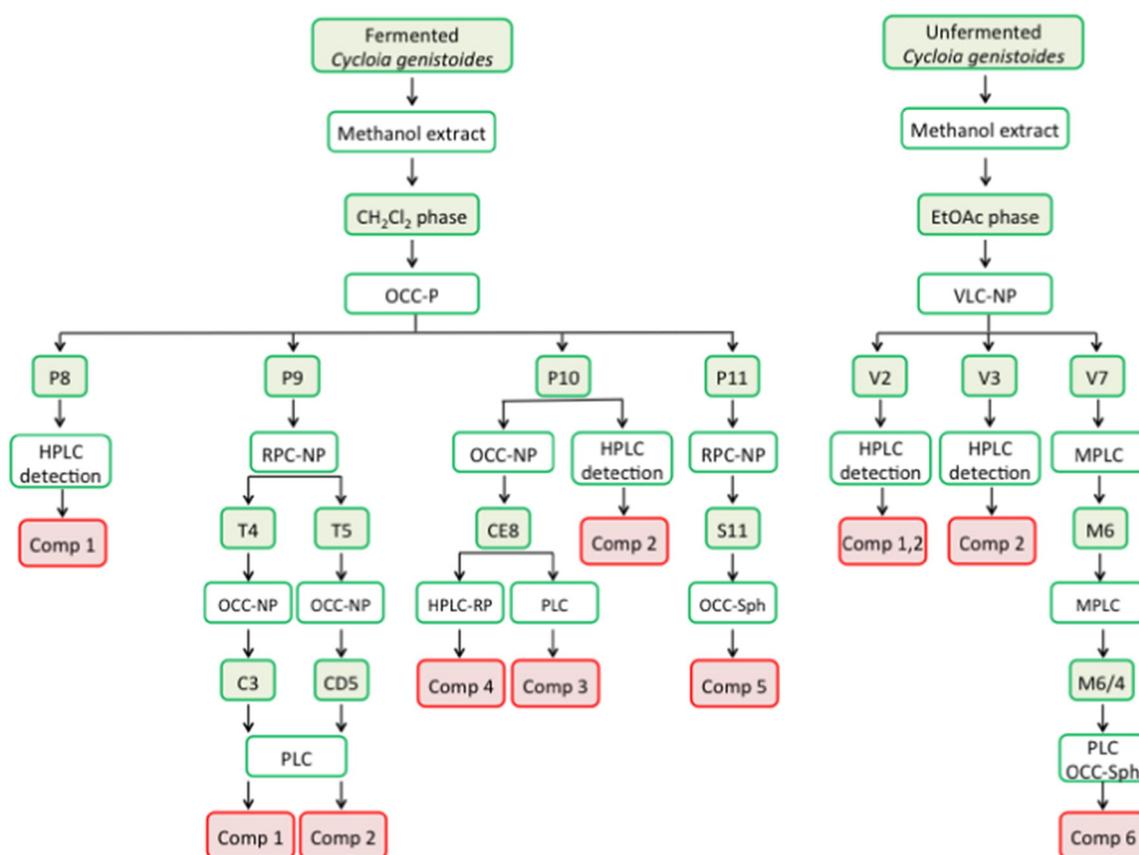


Fig. 1. Bioactivity guided fractionation of the methanolic extracts of fermented and unfermented *Cyclopa genistoides*. Comp 1: compound **1**, naringenin. Comp 2: compound **2**, 5,7,3',5'-tetrahydroxyflavanone. Comp 3: compound **3**, genistein. Comp 4: compound **4**, isoliquiritigenin. Comp 5: compound **5**, luteolin. Comp 6: compound **6**, helichrysin B. OCC-P: Open column chromatography – polyamide. OCC-NP: open column chromatography – normal phase silica gel. RPC-NP: rotation planar chromatography – normal phase silica gel. PLC: preparative thin layer chromatography. OCC-Sph: open column chromatography – Sephadex LH-20. VLC-NP: vacuum liquid chromatography – normal phase silica gel.

2.4. Transgenic plant material and estrogen-like reporter assay

pER8:GUS seeds were grown in the dark for 24–36 h at 4 °C on medium (1/2 MS, 1% sucrose, 0.8% phytoagar) for vernalization and then germinated under white light for 72 h at 24 °C. The plants were transferred to a 24-well microtiter plate in the presence or absence of test samples and incubated at 24 °C for 48 h. 3–5 transgenic plants were added to each well, in order to evaluate estrogenic activity. Plants cultured with 0.31–10 nM 17 β -estradiol were taken as a positive control.

2.5. Histochemical assay

After incubation in the presence or absence of test samples, transgenic plants were soaked in 0.2 mL per well of the GUS assay solution [50 mM Na₃PO₄ buffer (pH 7.0), 10 mM EDTA (pH 8.0), 2 mM X-Gluc, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, and 0.1% Triton X-100] in a 24-well plate and incubated for 3 h or overnight at 37 °C. Then after

washing, 70% aqueous EtOH was used to remove chlorophyll. Using a ZEISS Axiovert 200 inverse microscope, samples were examined for GUS staining and photographed with a digital camera. The minimum active concentration (MAC) of each sample was recorded upon the disappearance of the insoluble blue dye (5,5'-dibromo-4,4'-dichloro-indigo). The last concentration in the series, where the blue color was still detectable was considered the minimum active concentration. The parallel experiments were in accordance, hence no SEM/SD were calculated.

2.6. HPLC quantitative determination

Chromatographic analyses of the aqueous “cup of tea” (100 mL boiling tap water + 4 g plant material, 10 min) and methanolic (10 mL MeOH + 1 g plant material, 10 min, ultrasonication) extracts were performed on the Waters HPLC module. The separation was carried out on a Kinetex C18 column (5 μ m, 100 Å, 150 \times 4.6 mm, Phenomenex, Torrance, USA), operated at 20 °C. Chromatographic elution was

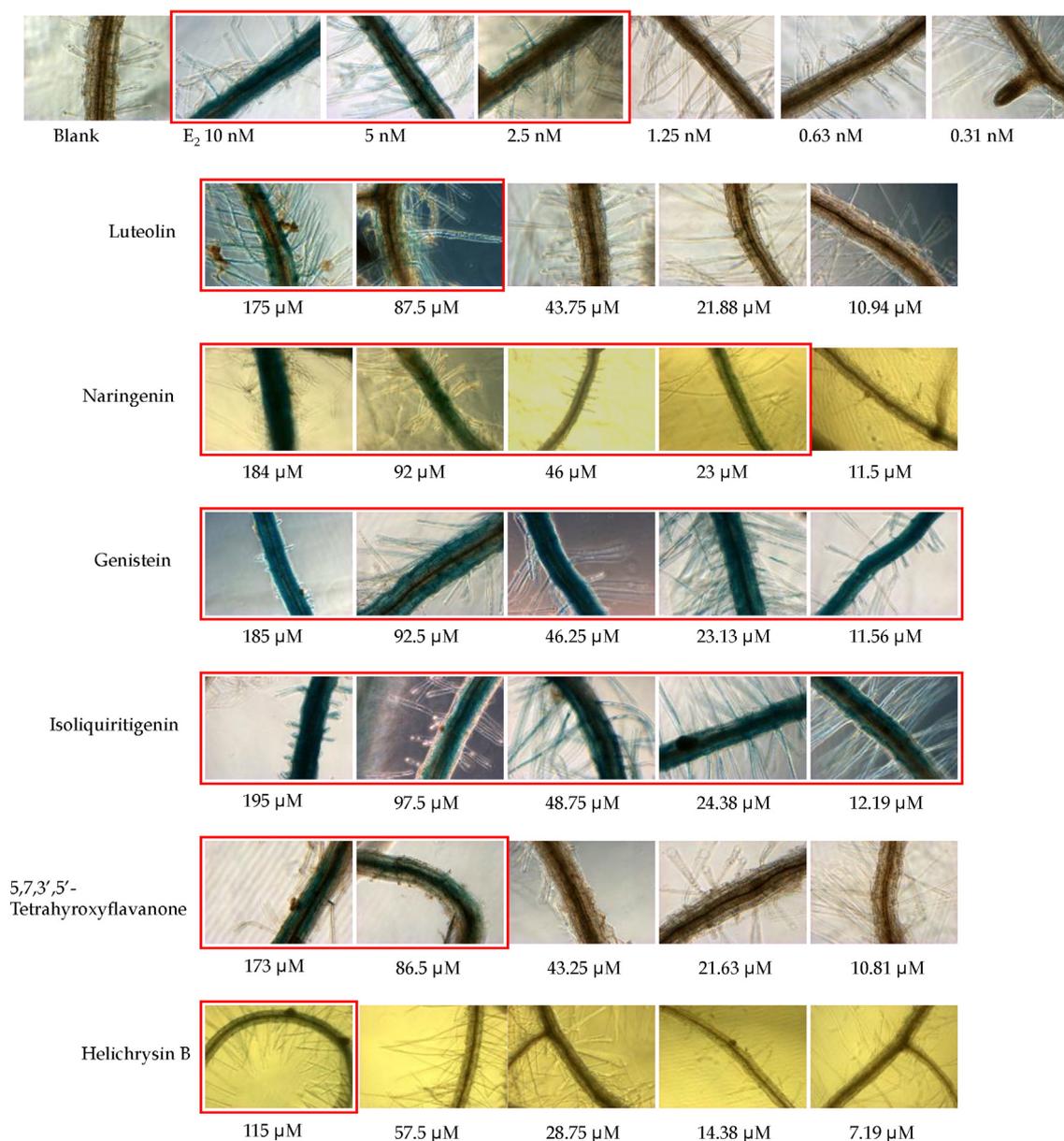


Fig. 2. Estrogenic MAC of the isolated active compounds in the histochemical assay. The concentrations where the blue color was detectable, indicating estrogenic activity, are surrounded with red boxes. The last/only concentration where the blue color was still detectable was considered the minimum active concentration. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.)

accomplished by gradient solvent system consisting of MeOH and acidified H₂O (0.1% H₃PO₄); injection volume was 20 µl. The gradient consisted three steps, for 21 min the % of the acidified water decreased from 80% to 24% then in 1 min it reached 80% again, then for 6 min this ratio was maintained. Peaks were identified by comparison of retention times and UV–vis spectra (PDA detector) with those of the isolated compounds.

2.7. Antiproliferative assay

The antiproliferative properties of the prepared extracts and natural products were determined on two human cancerous cell lines (purchased from ECACC, Salisbury, UK) by using the MTT assay. A2780 and T47D cells (isolated from ovarian and breast carcinoma, respectively), were cultivated in minimal essential medium supplemented with 10% fetal bovine serum, 1% non-essential amino acids and an antibiotic–antimycotic mixture. All media and supplements were obtained from PAA Laboratories GmbH, Pasching, Austria. Near-confluent cancer cells were seeded onto a 96-well microplate (5000/well) and attached to the bottom of the well overnight. On the second day, 200 µL of new medium containing the tested substances (at 10 or 30 µg/mL) was added. After incubation for 72 h at 37 °C in humidified air with 5% CO₂, the living cells were assayed by the addition of 20 µL of 5 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution. MTT was converted by intact mitochondrial reductase and precipitated as blue crystals during a 4 h contact period. The medium was then removed and the precipitated crystals were dissolved in 100 µL of DMSO during a 60 min period of shaking at 25 °C. Finally, the reduced MTT was assayed at 545 nm, using a microplate reader; wells with untreated cells were used as controls (Mosmann, 1983). All experiments were carried out on two microplates with at least five parallel wells. Stock solutions of the tested substances (10 mg/mL) were prepared with DMSO. The highest DMSO content of the medium (0.3%) did not have any substantial effect on the cell proliferation. Cisplatin was used as reference agent which inhibited the proliferation of A2780 and T47D cells with IC₅₀ values of 1.30 and 9.78 µM, respectively. Statistical evaluation of the results was performed by one-way analysis of variance followed by the Dunnett posttest, using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Histochemical assay

The CH₂Cl₂ and EtOAc extracts of the fermented and unfermented *C. genistoides* were active (MAC 200 µg/mL) and were selected for bioactivity-guided fractionation, by using HPLC, MPLC, RPC, CC and preparative TLC. From the CH₂Cl₂ fraction of the fermented plant material four out of fourteen subfractions, yielded via polyamide column chromatography, had estrogenic like effects (P8–P11, MAC 200 µg/mL). P8 and P11 contained one, P9 two and P10 three of the isolated active compounds. From the EtOAc fraction of the unfermented *C. genistoides* four out of twelve VLC subfractions were active (200 µg/mL) in the estrogen-like reporter assay (V2, V3, V6, V7). One, two and two active constituents were found in V7, V2 and V3 subfractions, respectively.

Bioassay-directed chromatographic fractionation led to six flavonoids with estrogenic activity. Compounds **1**, **2**, **3**, **4**, **5** and **6** proved to be naringenin, 5,7,3',5'-tetrahydroxyflavanone, genistein, isoliquiritigenin, luteolin and helichrysin B (naringenin-5-O-glucoside), respectively. The compounds were identified by comparing their physical and spectroscopic data with reported data and by APCIMS/MS (Andrade et al., 2010; Nessa et al., 2004; Patora and Klimek, 2002; Zhao et al., 2011). Fractions P8 and P10 were analyzed by HPLC, compounds **1** and **2** were detected from these fractions as well, respectively. In fractions V2 compounds **1** and **2** and in fraction V2 compound **2** was also detected.

The least potent compound was the helichrysin B with a MAC of 115 µM (Fig. 2). Two compounds, which have not yet been isolated from *C. genistoides*, genistein and isoliquiritigenin had substantial activity with a MAC of <11.56 and 12.19 µM. Luteolin, naringenin and 5,7,3',5'-tetrahydroxyflavanone also had estrogen-like activity with MACs of 87.5, 23 and 86.5 µM. The minimum active concentration of the control, 17-β-estradiol (E2) was 2.5 nM. In a previous study, using the same transgenic plant system pER8:GUS, the minimum active concentration of E2 was found to be lower, 0.62–1.25 nM (Lai et al., 2011).

The constituents luteolin, genistein, isoliquiritigenin and naringenin are well-known phytoestrogens. Naringenin, luteolin and genistein were able to displace 70%, 92% and 95% of the ³H-E₂ from hERβ, exerting the highest displacements when 10 phytoestrogens were compared (Verhoog et al., 2007b). Phytoestrogens have the potential to maintain bone health and delay or prevent osteoporosis, one of the postmenopausal symptoms. Genistein was found to have positive effects on bone mineral density on osteopenic postmenopausal women (Marini et al., 2007). Isoliquiritigenin is also a promising agent for bone destructive diseases (Liu et al., 2016). Next to their effect on the bone they also possess other activities, potentially important in the treatment of postmenopausal symptoms. Genistein and luteolin suppressed the induction of the proliferation-stimulating activity of environmental estrogens, suggesting anti-estrogenic and anti-cancer effect (Han et al., 2002); and naringenin attenuated many of the metabolic disturbances associated with ovariectomy in female mice (Ke et al., 2015). Genistein was also associated with favorable effects on both glycemic control and some cardiovascular risk markers (Atteritano et al., 2007). Regular grapefruit juice (contains high amounts of naringenin) consumption by middle-aged, healthy postmenopausal women was found to be beneficial for arterial stiffness.

Their presence gives a rationale to the traditional use of honeybush tea. Although, in the literature different extracts from different *Cyclopia* species exerted varying phytoestrogenic activity, even between harvestings, questioning the real potential of the infusion in medicinal use.

3.2. Antiproliferative assays

In Table 1, fractions and compounds with inhibition values above 30% either in A2780 or T47D cells are displayed. While in the pER8:GUS

Table 1

Fractions and compounds exhibiting substantial (above 30) antiproliferative activity against either A2780 or T47D cells.

Substance	Concentration (µg/mL)	Growth inhibition (%) ± SEM	
		A2780	T47D
V3	10	83.83 ± 0.78	50.90 ± 0.98
	30	88.35 ± 0.28	69.95 ± 0.57
V4	10	– ^a	11.06 ± 1.20
	30	16.16 ± 2.96	33.76 ± 1.57
P7	10	–	12.54 ± 2.90
	30	42.83 ± 1.27	40.07 ± 1.59
P8	10	22.27 ± 2.34	40.81 ± 2.38
	30	43.44 ± 1.02	48.73 ± 0.97
P10	10	26.42 ± 1.97	44.97 ± 2.67
	30	39.90 ± 0.90	50.16 ± 2.29
P11	10	22.06 ± 2.58	32.90 ± 2.67
	30	51.97 ± 1.01	44.42 ± 1.76
Luteolin	10	53.43 ± 0.82	37.43 ± 2.35
	30	91.60 ± 0.61	65.10 ± 1.17
Genistein	10	39.40 ± 1.33	14.98 ± 1.50
	30	84.79 ± 0.59	39.02 ± 1.30
Naringenin	10	15.95 ± 0.97	–
	30	41.42 ± 2.19	22.64 ± 1.30
Isoliquiritigenin	10	19.53 ± 1.86	–
	30	71.13 ± 0.64	36.50 ± 2.11

^a Conditions exerting inhibition less than 10% are considered ineffective and the exact values are not presented for clarity. All the presented results are statistically different (p < 0.05) from the untreated control cells.

Table 2
The quantitative determination of the active compounds.

Compound	MeOH extract		Aqueous extract		LOD (µg)	LOQ (µg)
	Fermented (mg/g dried plant material)	Unfermented (mg/g dried plant material)	Fermented (mg/g dried plant material)	Unfermented (mg/g dried plant material)		
Luteolin	0.0439 ± 0.00622	0.0511 ± 0.01439	0.0152 ± 0.00123	0.0174 ± 0.00622	0.2260	0.7533
Naringenin	0.1334 ± 0.01224	0.0037 ± 0.00047	0.0391 ± 0.00160	0.0027 ± 0.00003	0.1880	0.6268
Genistein	n.d.	n.d.	n.d.	n.d.	0.2578	0.8594
Isoliquiritigenin	0.0038 ± 0.00061	0.0026 ± 0.00007	n.d.	n.d.	0.3125	1.0416
5,7,3',5'-Tetrahydroxy-flavanone	0.0842 ± 0.03007	0.0079 ± 0.00235	0.0489 ± 0.00762	n.d.	0.2750	0.9167
Helichrysin B	0.1623 ± 0.04625	0.2375 ± 0.09298	0.3279 ± 0.02610	0.3376 ± 0.05396	0.3290	1.0967

n.d. (not detected), LOD limit of detection, and LOQ limit of quantification. Values are given in ±SD.

assay P8–11, V2, 3, 6, and 7 showed estrogenic activity; in the antiproliferative tests, P8, 10, 11 and V3 demonstrated inhibition greater than 30% in either cell-lines. Taking into consideration, that these fractions are complex mixtures, other compounds than the active constituents may have exerted antiproliferative activity.

Except for helichrysin B and 5,7,3',5'-tetrahydroxyflavanone, all active compounds (naringenin, luteolin, isoliquiritigenin, genistein) exhibited substantial antiproliferative activity against the tested cell lines. All four of them had a greater inhibition toward the ER negative A2780, which may suggest an ER independent inhibition of cell-proliferation, or possibly the induction of cell proliferation in the ER positive T47D cell-line; underlining their estrogenic potential. The well-documented ER-mediated actions of these flavonoids cannot be excluded as a component of their antiproliferative properties, however, in our current experimental conditions the cell culture medium contained a substantial amount of natural estrogens, as components of fetal bovine serum, and therefore the obtained results do not support a direct relationship between the two determined activities.

3.3. HPLC quantification

The quantitative comparison of the six active compounds between the fermented and unfermented *C. genistoides* was performed by RP-HPLC. While both the processed and unprocessed plants contained similar amounts of luteolin and isoliquiritigenin, the naringenin and 5,7,3',5'-tetrahydroxyflavanone content in the fermented honeybush was more than 30 and 10 folds, respectively (Table 2). On the other hand, the unfermented *Cyclopia* had higher quantities of the least effective naringenin-glycoside. Considering, that flavonoid-glycosides may degrade during the fermentation process, this might explain the difference in the amounts. 5,7,3',5'-Tetrahydroxyflavanone and naringenin – compounds more abundant in the fermented plant material – displayed stronger estrogen-like activity than helichrysin B, providing a rationale to the fermentation process. The quantitative comparison of the extract used for the bioactivity guided isolation (methanolic extract) and the traditionally used aqueous extract (“cup of tea extract”) was also performed. The “cup of tea” extracts, prepared with boiling tap water, had much lower concentrations of the active compounds. Isoliquiritigenin was below the detection limit in aqueous extracts whereas 5,7,3',5'-tetrahydroxyflavanone was undetectable in the water extract of the unfermented sample. Genistein was not detected in any of the extracts.

On one hand, although our experiments reported potent and well-known phytoestrogens to be comprised by *C. genistoides* and the HPLC quantification underpinned the possible importance of fermentation process, the low concentrations of the tested compounds are questioning the potential phytoestrogenic activity of the traditionally used honeybush tea. Estrogenic isoflavones, such as formononetin and calycosin shown to be present in another *Cyclopia* species, *C. subternata*, but they were also not observed in quantifiable amounts (Louw et al., 2013). Furthermore, in the literature different extracts from different *Cyclopia* species exerted varying phytoestrogenic activity, even between harvestings, adding to the debate of the real potential of the infusion in medicinal use.

On the other hand, according to Verhoog et al. the aqueous extracts of unfermented or fermented *C. genistoides* and *C. subternata* were able to significantly to displace 1 nM 3H-E₂ from hERβ. Although, this effect was not observed in all tested harvestings, it did shown the possibility of an aqueous extract to be estrogenic. It also has to be taken into account, although that the isolated flavonoids are present in small quantity, the estrogenic activity of *Cyclopia* extracts is the result of a fine balance between different polyphenols present in varying amounts with varying phytoestrogenic potential.

4. Conclusion

This is the first bioactivity guided isolation of compounds with estrogenic activity from fermented and unfermented *C. genistoides* samples, which provided six compounds, amongst them genistein, 5,7,3',5'-tetrahydroxyflavanone, helichrysin B and isoliquiritigenin, which have not yet been reported from *Cyclopia* species. Antiproliferative MTT assays were also performed, on A2780 and T47D cell-lines. The results suggested that estrogen induced cell-proliferation or estrogen independent antiproliferative effect might have played a role. The quantitative determination of these compounds showed that two out of the five active flavonoid aglycons are more abundant in the fermented plant material, another two are presented in similar amounts in the two kinds of honeybush and one could not be detected with our method. The least active flavonoid-glycoside helichrysin B was more concentrated in the unfermented *C. genistoides*.

Although, the quantitative comparison of fermented and unfermented honeybush implies, that the fermented tea has a higher amount of these phytoestrogens except the least active compound, the measured low amounts question the biological activity of the traditionally used infusion. However, it does not exclude the possibility that synergism or antagonism of multiple polyphenols targeting multiple ER isoforms, can result in the phytoestrogenic effect of different extracts, even if the individual compounds are small in quantity.

There are plenty methods available for the evaluation of estrogenic potential, yet the complexity of the mechanisms of action of phytoestrogens and phytoestrogen containing herbal preparations trigger divergent outcomes, depending on the method used, for example in the case of transactivation, *Cyclopia* extracts displayed ERα antagonism and ERβ agonism when ER subtypes were expressed separately, however, when co-expressed only agonism was observed (Louw et al., 2013; Visser et al., 2013). Considering that the pER8:GUS assay can identify all compounds which are able to bind to ER-α (regardless of agonism or antagonism), it is an ideal model for the preliminary investigation of plants with proposed estrogen-like activities. Furthermore, while cytotoxicity is a limiting factor of in vitro mammalian cell-based models, the transgenic plant system expressed tolerance toward higher doses of cytotoxic compounds (Lai et al., 2011).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.sajb.2016.06.001>.

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