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

O. Roza a, W.-C. Lai b, I. Zupkó c, J. Hohmann a, N. Jedlinszki a, F.-R. Chang b, D. Csupor a,⁎, J.N. Eloff d,⁎

a Department of Pharmacognosy, University of Szeged, Eötvös u. 6, H-6720, Szeged, Hungary
b Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, 80708, Kaohsiung, Taiwan, ROC
c Department of Pharmacognosy, University of Szeged, Eötvös u. 6, H-6720, Szeged, Hungary
d Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110, Pretoria, South Africa

⁎ Corresponding authors.
E-mail addresses: csupor.dezso@pharmacognosy.hu (D. Csupor), kobus.eloff@up.ac.za (J.N. Eloff).

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.

© 2016 SAAB. Published by Elsevier B.V. All rights reserved.

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 also been questioned. 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 17β-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.
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, fujikinetin, pseudobaptigen), xanthones (eriodictyol, hesperidin and luteolin (Kokotkiewicz et al., 2013). 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 μm, 100 Å, 150 × 4.6 mm) column (Phenomenex, Torrance, USA). For the preoperative reversed-phase HPLC, a Merck Hibar Purospher STAR C18 (5 μm, 250 × 10.0 mm) semi-preparative 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).

Bioactivity-guided fractionation led to the isolation of six compounds, which were each identified 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 ERα/β ratio of the cells, the presence of E2, ERα/β antagonism/agonism 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 μm, 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 (MLPC) was performed by a Büchi apparatus (Büchi Labortechnik AG, Flawil) using a 40 × 150 mm RP18ec column (40–63 μm, Büchi).

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 iriflorone 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 estrogen/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 ERα 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).

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.bergong.com].

Please cite this article as: Roza, O., et al., Bioactivity guided isolation of phytoestrogenic compounds from Cyclopia genistoides by the pER8:GUS reporter system, South African Journal of Botany (2016), http://dx.doi.org/10.1016/j.sajb.2016.06.001
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 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 (168 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 CDS 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 0:1). 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).

---


Please cite this article as: Roza, O., et al., Bioactivity guided isolation of phytoestrogenic compounds from Cyclopia genistoides by the pER8:GUS reporter system, South African Journal of Botany (2016), http://dx.doi.org/10.1016/j.sajb.2016.06.001
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 17β-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 μm, 100 Å, 150 × 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 standard.

DMSO containing the tested substances (at 10 or 30 μg/mL) was prepared with DMSO. 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'-tetratetrahydroxyflavone, 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'-tetratetrahydroxyflavone also had estrogen-like activity with MACs of 87.5, 23 and 86.5 μM. The minimum active concentration of the control, 17β-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 hERα, 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 ovariecotomy 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>
<thead>
<tr>
<th>Substance</th>
<th>Concentration (μg/mL)</th>
<th>Growth inhibition (%) ± SEM</th>
<th>A2780</th>
<th>T47D</th>
</tr>
</thead>
<tbody>
<tr>
<td>V3</td>
<td>10</td>
<td>83.83 ± 0.78</td>
<td>50.90 ± 0.98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>88.35 ± 0.28</td>
<td>69.95 ± 0.57</td>
<td></td>
</tr>
<tr>
<td>V4</td>
<td>10</td>
<td>-a</td>
<td>11.05 ± 1.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>16.16 ± 2.96</td>
<td>33.76 ± 1.57</td>
<td></td>
</tr>
<tr>
<td>P7</td>
<td>10</td>
<td>-a</td>
<td>12.54 ± 2.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>42.83 ± 1.27</td>
<td>40.07 ± 1.59</td>
<td></td>
</tr>
<tr>
<td>P8</td>
<td>10</td>
<td>22.27 ± 2.34</td>
<td>40.81 ± 2.38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>43.44 ± 1.02</td>
<td>48.73 ± 0.97</td>
<td></td>
</tr>
<tr>
<td>P10</td>
<td>10</td>
<td>26.42 ± 1.97</td>
<td>44.97 ± 2.67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>39.90 ± 0.90</td>
<td>50.16 ± 2.29</td>
<td></td>
</tr>
<tr>
<td>P11</td>
<td>10</td>
<td>22.06 ± 2.58</td>
<td>32.90 ± 2.67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>51.97 ± 1.01</td>
<td>44.42 ± 1.76</td>
<td></td>
</tr>
<tr>
<td>Luteolin</td>
<td>10</td>
<td>53.43 ± 0.82</td>
<td>37.43 ± 2.35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>91.60 ± 0.61</td>
<td>65.10 ± 1.17</td>
<td></td>
</tr>
<tr>
<td>Genistein</td>
<td>10</td>
<td>39.40 ± 1.33</td>
<td>14.98 ± 1.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>84.79 ± 0.59</td>
<td>30.92 ± 1.30</td>
<td></td>
</tr>
<tr>
<td>Naringenin</td>
<td>10</td>
<td>15.95 ± 0.97</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Isoliquiritigenin</td>
<td>10</td>
<td>41.42 ± 2.19</td>
<td>22.64 ± 1.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>71.13 ± 0.64</td>
<td>36.50 ± 2.11</td>
<td></td>
</tr>
</tbody>
</table>

* 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.
assay P8–11, V2, 3, 6, and 7 showed estrogenic activity; in the antipro-
liferative tests, P8, 10, 11 and V3 demonstrated inhibition greater than
30% in either cell-lines. Taking into consideration, that these fractions
may have exerted antiproliferative activity.
Except for helichrysin B and 5,7,3′-tetrahydroxyflavanone, all active
compounds (naringenin, luteolin, isoliquiritigenin, genistein) ex-
hibited 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 substan-
tial 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 honey-
bush was more than 30 and 10 folds, respectively (Table 2). On the
other hand, the unfermented Cyclopia had higher quantities of the least effec-
tive naringenin-glycoside. Considering, that flavonoid-glycosides may
degrade during the fermentation process, this might explain the differ-
ence 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 pro-
cess, the low concentrations of the tested compounds are questioning the
potential phytoestrogenic activity of the traditionally used honeybush tea.
Estrogenic isoavonoids, 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 ERβ. 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 indepen-
dent 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 ma-
terial, 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
possibilities, regardless of antagonism or agonism, 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).

Acknowledgments

This research was supported by the European Union and the State of
Hungary, co-financed by the European Social Fund in the framework of

Table 2
The quantitative determination of the active compounds.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MeOH extract Fermented (mg/g dried plant material)</th>
<th>MeOH extract Unfermented (mg/g dried plant material)</th>
<th>Aqueous extract Fermented (mg/g dried plant material)</th>
<th>Aqueous extract Unfermented (mg/g dried plant material)</th>
<th>LOD (μg)</th>
<th>LOQ (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luteolin</td>
<td>0.0439 ± 0.00622</td>
<td>0.0511 ± 0.01439</td>
<td>0.0152 ± 0.00123</td>
<td>0.0174 ± 0.00622</td>
<td>0.2260</td>
<td>0.7533</td>
</tr>
<tr>
<td>Naringenin</td>
<td>0.1334 ± 0.01224</td>
<td>0.0037 ± 0.00047</td>
<td>0.3931 ± 0.00160</td>
<td>0.0207 ± 0.00003</td>
<td>0.1880</td>
<td>0.6268</td>
</tr>
<tr>
<td>Genistein</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.2578</td>
<td>0.8594</td>
</tr>
<tr>
<td>Isoliquiritigenin</td>
<td>0.0038 ± 0.00061</td>
<td>0.0026 ± 0.00007</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.3125</td>
<td>1.0416</td>
</tr>
<tr>
<td>5,7,3′-5′-Tetrahydroxyflavanone</td>
<td>0.0862 ± 0.03007</td>
<td>0.0079 ± 0.00235</td>
<td>0.0489 ± 0.00762</td>
<td>n.d.</td>
<td>0.2570</td>
<td>0.9167</td>
</tr>
<tr>
<td>Helichrysin B</td>
<td>0.1623 ± 0.04625</td>
<td>0.2375 ± 0.05298</td>
<td>0.3279  ± 0.02610</td>
<td>0.3376 ± 0.05396</td>
<td>0.3290</td>
<td>1.0967</td>
</tr>
</tbody>
</table>

n.d. (not detected), LOD limit of detection, and LOQ limit of quantification. Values are given in ±SD.
for phytoestrogens from Caesalpinia sappan sapon. Journal of Natural Products 74, 1098–1108.


