South African Journal of Botany xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

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

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ARTICLE INFO

Article history:
Received 6 November 2015
Received in revised form 1 June 2016
Accepted 5 June 2016
Available online xxxx

Edited by D De Beer

Keywords:
Cyclopia
Phytoestrogen
pER8:GUS
Menopause
Genistein
Luteolin

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.

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. 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 (E_2) 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

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; E_2, 17-β-estradiol; XVE, estrogen receptor-based transactivator vector; GUS, β-glucuronidase; MAC, minimum active concentration.

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http://dx.doi.org/10.1016/j.sajb.2016.06.001
0254-6299/

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 (mangiferin, isomangiferin), coumestans (medicagol, flemmichapparin, sophoracoumestan), catechins (epigallocatechin-3-*O*-gallate), benzaldehyde derivates 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 derivates, eriodyctiol 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 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).

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 ERα/β ratio of the cells, the presence of E_2, ERα/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 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 F_{254} and 60 RP-18 F_{254} plates (Merck); and rotation planar chromatography (RPC) on silica gel 60 F_{254} (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 × 150 mm RP18ec column (40–63 μm, 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 μm, 100 Å, 150 × 4.6 mm) column (Phenomenex, Torrance, USA).

For the preparative reversed-phase HPLC, a Merck Hibar Purospher STAR C18 (5 μm, 250 × 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).

^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz) and 2D NMR were recorded in CD_3OD or $CDCl_3$ 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.

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