**University of Szeged**
**Faculty of Pharmacy**
Department of Pharmacognosy

# PROMISING ASSETS OF SOUTHERN AFRICA?
# *CYCLOPIA GENISTOIDES* AND *HOODIA GORDONII*

**Ph.D. Thesis**

Orsolya Roza Pharm.D.

**Supervisors:**
Prof. Judit Hohmann DSc.
Dezső Csupor PhD.

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Bioactivity-guided isolation of phytoestrogenic compounds from *Cyclopia genistoides* by pER8:GUS reporter system
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Flavonoids from *Cyclopia genistoides* and their xanthine oxidase inhibitory activity
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## ABBREVIATIONS

| | |
|---|---|
| 1D | one-dimensional |
| 2D | two-dimensional |
| AcNi | acetonitrile |
| APCI | atmospheric pressure chemical ionization |
| COSY | correlated spectroscopy |
| DAD | diode-array detection |
| $\delta$ | chemical shift |
| $E_2$ | 17-β-oestradiol |
| ESI-MS | electrospray ionisation mass spectroscopy |
| ER | oestrogen receptor |
| ERE | oestrogen responsive element |
| EtOAc | ethyl acetate |
| F | fermented |
| GUS | β-glucuronidase |
| HMBC | heteronuclear multiple-bond correlation spectroscopy |
| HSQC | heteronuclear single-quantum coherence spectroscopy |
| HPLC | high-performance liquid chromatography |
| HPTLC | high performance thin-layer chromatography |
| HRESIMS | high-resolution electron (spray) ionisation mass spectroscopy |
| JMOD | *J*-modulated spin-echo experiment |
| MAC | minimum active concentration |
| MeOH | methanol |
| MPLC | medium-pressure liquid chromatography |
| MS | mass spectroscopy |
| MS/MS | tandem mass spectroscopy |
| ½ MS | half strength Murashige & Skoog medium |
| nF | non-fermented |
| NMR | nuclear magnetic resonance |
| NOESY | nuclear *Overhauser* effect spectroscopy |
| NP | normal-phase |
| OCC | open-column chromatography |
| pER8:GUS | *Arabidopsis thaliana* transgenic plant system |
| PLC | preparative-layer chromatography |
| RP | reversed-phase |
| RPC | rotation planar chromatography |
| TLC | thin-layer chromatography |
| UPLC | ultra performance liquid chromatography |
| UV | ultraviolet |
| VLC | vacuum-liquid chromatography |

# 1. INTRODUCTION

The Southern African flora is remarkably distinct. Considerable part of its richness lies in the Cape Floristic Region with a surface of 89,957 $km^2$. Despite this small size, it is considered as one of the only six floristic kingdoms of the World, beside e.g. the vast Boreal Floristic Region, comprising North America, Europe, northern and central Asia, and North Africa [1]. The Cape Floristic Region covers less than 4% of Southern Africa, yet includes almost half of its species, 8550, of which 73% are endemic [2].

The Cape flora is exceptionally diverse and evidently the Southern African traditional medicine is rich, using circa 3000 medicinal plants. *Cyclopia genistoides* and *Hoodia gordonii* are amongst those few, about 38, Southern African indigenous species, which have been commercialised to some extent [3]. Since 1995, the end of the cultural and economic isolation of South Africa, they have also gained scientific interest, due to their pharmacological properties, potentially promising in conditions of great importance, such as obesity and menopause, affecting millions of people worldwide.

According to WHO, 65% of the world's population live in countries where overweight and obesity kills more people than underweight. Obesity has become an epidemic, while safe and effective pharmacotherapy is still absent [4].

By 2030 it is projected that approximately 47 million women will go through menopause every year worldwide [5]. As phytoestrogens are compounds derived from plants that could act through at least one of the main isoforms of the oestrogen receptor, they potentially can be used to counterbalance the hormone deficiency in menopause. However, phytoestrogens are also considered to be endocrine disruptors. Hence, the evaluation of the oestrogen-like activity of natural compounds is important both from a safety and efficacy point of view [6].

Tackling chronic diseases is high priority, taken into consideration the size of the affected population. Obesity and menopause are lacking safe and effective treatment; hence new approaches are urgently needed.

The therapeutic potential of *Cyclopia genistoides* and *Hoodia gordonii* is notable based on previous pharmacological studies and their traditional use; however they have poorly examined pharmacology and chemistry. These facts made these two species from the Southern African flora perfect candidates for our investigation.

## 2. AIMS OF THE STUDY

The aim of the present work was to explore the potential of metabolites of two Southern African medicinal plants in the treatment of obesity and menopause.

In order to achieve these aims, the main tasks were to:

- investigate the mechanism of action of *Hoodia gordonii* that is possibly also in the background of reported side effects of a commercial product
- develop analytical method for the authentication of *Hoodia*-based products
- perform bioactivity-guided fractionation of *C. genistoides* (honeybush) in order to identify secondary metabolites with phytoestrogenic activity
- isolate compounds from *C. genistoides* by a combination of various chromatographic methods (OCC, VLC, RPC, MPLC, PLC and HPLC)
- elucidate the structures of the isolated compounds by NMR and MS methods, provide missing NMR data on the already-known constituents
- compare the content of active compounds, isolated from fermented and non-fermented honeybush tea
- examine the effect of probiotic bacteria on flavonoid glycosides isolated from *C. genistoides*, in order to investigate the possible role of intestinal bacterial flora in the metabolism of flavonoid glycosides and bioavailability of aglycones of honeybush tea
- determine the antiproliferative effects of phytoestrogens isolated from *C. genistoides*
- evaluate the xanthine oxidase inhibitory activity of the isolated compounds of *C. genistoides*

## 3. LITERATURE OVERVIEW

### 3.1. Botany of the *Cyclopia* and *Hoodia* genus and the investigated species

#### 3.1.1. The genus *Hoodia* and *H. gordonii*

The *Hoodia* genus belongs to the tribe Ceropegieae of the subfamily Asclepiadoidae in the family Apocynaceae, order Gentianales, subclass Asteridae, class Magnoliopsida and division Magnoliophyta [7-9].

The genus *Hoodia* belongs to the stapeliads, formerly known as Stapeliineae, a group of highly succulent leafless plants with angled stem and clear sap, consisting 31 genera and 328 species. Stapeliads form a monophyletic group, which is united by the derived character of fleshy flowers. Stapeliad flowers are nearly universally fly-pollinated, emitting wide range

of odours from mango or honey-like to dung or urine, but colouration and texture are also part of the deception. *H. gordonii* was reported to mimic the scent of omnivore/carnivore faeces or carcasses [7, 10]. Stapeliineae was merged into the tribe Ceropegieae belonging to the subfamily Asclepiadoidae, commonly known as 'milkweeds', which comprises ~2 700, mainly tropical and subtropical, species, belonging to 222 genera [7, 11].

The already known 13 *Hoodia* species are limited to arid regions, they are found only in southern Africa and the south-western corner of Angola, from 13°S mainly within 100 km of the coast. In Namibia they occur along the western shoreline, in South Africa primarily in the Northern Cape. *H. currorii* subsp. *lugardii* is the only taxon reported from Botswana and Zimbabwe. *H. gordonii* covers almost the whole range of distribution of the genus, but avoids the winter-rainfall areas [7]. All *Hoodia* species are endangered and are in the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) Appendix II, which lists species that are not necessarily threatened now with extinction but may become so, unless their trade is closely controlled.

*H. gordonii* (Masson) Sweet ex Decne. is a many-stemmed, leafless, perennial shrub, usually up to 1 m tall and 0.6 m broad, with spiny greenish cylindrical stems (0.1-1.0 m long, 25-50 mm thick), branching from the base. The tubercles protruding, fused in lower half into 11-17 angles along the stem, tipped with 6-12 mm spines. The flowering of *H. gordonii* is opportunistic; about a month after heavy raining, plants would be covered with flowers, which may vary in colour from intense purple and pale flesh-pink to yellowish, usually with darker venation. The 5-lobed corolla is between 50-100 mm in diameter and the lobes are 15 mm long, but the size decreases as the flowering period advances, the largest appearing often first. The interior of the corolla is glabrous to finely papillate, papillae shortly setose. The corolla tube is usually pentagonal in outline [12-14].

### 3.1.2. **The genus *Cyclopia* and *C. genistoides***

The *Cyclopia* genus belongs to the tribe of Podalyrieae, subfamily of Faboideae, family of Fabaceae, order of Fabales, subclass of Rosidae, class of Magnoliopsida and division of Magnoliophyta [9, 15-17].

The *Cyclopia* genus includes 23 species of endemic fynbos shrubs. They live in the coastal districts of the Cape Province, ranging from Cederberg Mountains (Western Cape) to Cape Peninsula in the south, and Port Elisabeth in Eastern Cape Province. Their habitat mostly sandy, acidic and infertile soils and as many Fabaceae species, they also exhibit nitrogen-fixing abilities. In consequence of the frequent fires in fynbos shrub lands, two

survival strategies have developed within *Cyclopia* species. Resprouters (*C. intermedia, C. genistoides*) have strongly developed underground part, capable of surviving fires and resprouting thereafter. Re-seeders (*C. subternata*), are typically destroyed by fire but afterwards their populations are recovered from soil-stored seeds [18].

Their name probably derives from either the Greek *kyklos* 'circle round ring' and *ops* 'an eye' referring to a blotch on the standard, or according from *kyklos* and *pous* 'foot', indicating the circular base of the calyx [19, 20].

*Cyclopia* plants are many branched, woody, perennial, non-climbing, smooth, broom-like shrubs, characterized by digitately or palmately trifoliate needle-like leaves with narrow or board, hairless or hairy leaflets with rolled under margins. The bracts are paired and fused; the yellow, sweet, honey-like scented pea flowers are solitary or axillary and have a thrust-in calyx base [18-22]. In natuael veld, *Cyclopia* bushes are 1.5-3 m tall, depending on the species.

*Cyclopia genistoides* (L.) Vent. has ribbed branches becoming terete, glabrous, or puberulous, nude at base but leafy above. The leaves are dense; leaflets are ovate or linear, subterete or open, backed with margins incompletely rolled in. The large golden yellow flowers are marked with red at the base of standard. Seeds are kidney shaped [21, 22]

### 3.2. Chemistry of the *Cyclopia* and *Hoodia* genus and the investigated species

#### 3.2.1. Phytochemistry of the genus *Hoodia*

A considerable portion of the secondary metabolites isolated from the subfamily Asclepiadoideae (Apocynaceae) has been characterized as pregnane glycosides [23-26]. The phytochemical studies of the genus *Hoodia*, which is limited to *Hoodia gordonii* have been focused on this class of compounds, since the investigation for the active compound of *Hoodia gordonii*, responsible for its claimed appetite-suppressant activity, led to the isolation of an oxypregnane glycoside P57AS3 (hereinafter referred to as P57). Interestingly, two species, members of the Asclepiadoideae subfamily have been reported with similar effects as *H. gordonii*. One of the most abundant pregnane glycosides of *Cynanchum auriculatum*, wilfoside K1N, which structure resembles to P57 and the extract of *Caralluma fimbriata*, rich in pregnane glycosides, have been described to have appetite suppressant activity [27, 28].

Besides P57, an abundance of pregnane glycosides, comprising five aglycones, were identified as main constituents. Further components belonging to the compound classes of sterols, fatty acids, and alcohols were detected in low concentrations in the saponified methanolic extract of *H. gordonii* [29].

The flower volatiles of *H. gordonii* were also studied, given the fact, that *Hoodia* species are stapeliads, emitting strong fetid scents in order to attract pollinators [10].

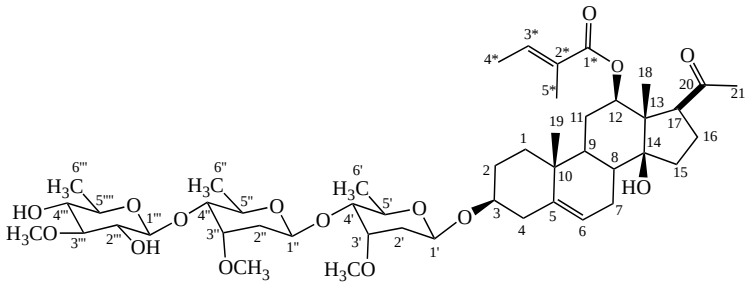
*Pregnane glycosides*

Pregnane glycosides, containing 6-deoxy-, 2,6-dideoxy sugars (β-D-thevetose, β-D-oleandrose, β-D-cymarose, β-D-digitoxose, 3-*O*-methyl-6-deoxy-D-allose) and glucose, can be grouped according to five aglycones: hoodigogenin A, calogenin, isoramanone, hoodistanal and dehidrohoodistanal. One sugar moiety is always attached to C-3, whereas the second moiety, if present, attached to C-20.

**Hoodigogenin A**, the most common aglycone of the pregnane glycosides isolated from *H. gordonii,* is a unique pregnane derivate owing to the tigloyl functionality linked to position C-12 and the cis-fusion of rings C and D of the steroid skeleton [30].

So far 24 hoodigogenin A glycosides were described from *H. gordonii,* amongst them P57, which until recently was the only recognised anti-obesity compound in the species. P57 was also detected in *H. ruschii, H. currorri*, *H. parviflora, Stapelia hirsuta* and *Orbeanthus hardii* [31, 32]. Among four *Hoodia* species, the P57 content was the highest in *H. ruschii* (0.254 mg/100 mg dried material) and the lowest in *H. gordonii* (0.043 mg/100 mg dried material) [33].

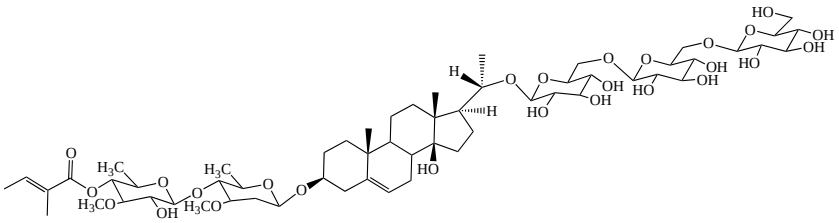
Owing to the parallel work of four research groups, the naming of the hoodigogenin A glycosides is ambiguous. P57, compound 2, hoodigoside A-K, W, gorgonoside A-I, L, formula 6-12 represents only 24 pregnane glycosides, because gorgonoside A, C, F, G, H and Formula 6 are the same as hoodigogenin A, hoodigoside C, formula 9, 10, hoodigoside E and P57 respectively [34-38].



**P57**

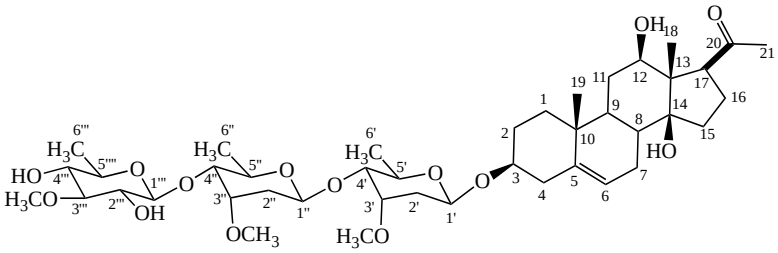
**Calogenin**, the aglycone of thirteen pregnane glycosides, namely hoodigoside L-V, Y, Z, is not specific to the genus *Hoodia*, but is common in the subfamily [37, 39-42]. However, the tigloyl ester substitution at C-4 of the terminal sugar is a characteristic feature of calogenin glycosides derived from *H. gordonii*.

In contrast to hoodigogenin A, calogenin lacks the C-12 tigloyl ester functionality, and the majority of calogenin glycosides are bisdesmosides. The main steroid glycoside present in a $CHCl_3$/MeOH (1:1) extract of *H. gordonii* was identified as hoodigoside L (**2**) [39].



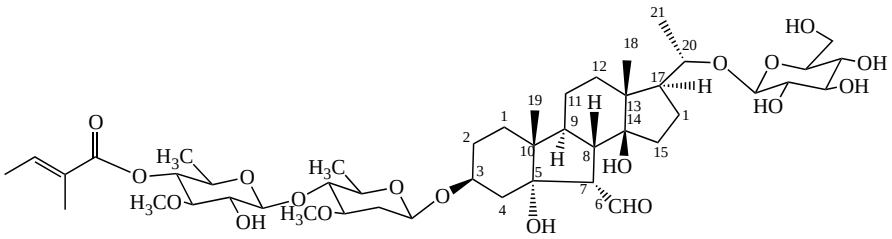
**Hoodigoside L**

**Isoramanone** glycoside, hoodigoside X is the only representative comprising this aglycone in the *Hoodia* genus so far [37]. Isoramanone skeleton is similar to that of hoodigogenin A, except it does not contain the tigloyl ester substitution at C-12.

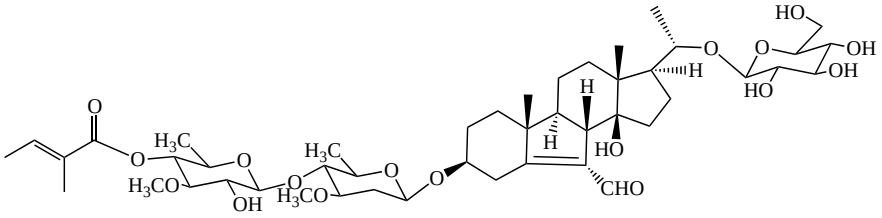


**Hoodigoside X**

**Hoodistanal** and **dehydrohoodistanal** glycosides, hoodistanaloside A and B, respectively, were isolated as the first two naturally occurring glycosides of 5(6→7) abeo-sterol aglycones [37]. In nature, 6-5-6-5 fused ring sterols are exceptionally rare, they have been identified only in two marine sponges (*Stelletta hivasaensis, Svenzea zeai*) and in *Taiwania cryptomerioides* (*Taxodiaceae*) [43-45].



**Hoodistanaloside A**



**Hoodistanaloside B**

*Volatiles*

In the subtribe Stapeliinae (Asclepiadoideae-Ceropegieae) approximately 400 species, among them the members of *Hoodia* genus, form sapromyiophilous flowers, emitting fetid scents, mimicking food sources or oviposition sites in order to attract pollinators [46]. A comparative floral scent analysis of 15 species from 11 genera within Stapeliinae, showed that *H. gordonii* contained the greatest amount of the identified compounds, 94 out of 149. Among the reported benzenoids, monoterpenoids, sesquiterpenoids, fatty acid derivates, nitrogenous compounds and sulphides, the compound with the highest relative amount was dimethyl trisulphide with 11.3%. Although, there was a considerable variation between different individuals [10].

*Adulteration of Hoodia products*

As obesity reaching epidemic proportions, the demand for weight loss products, amongst them *H. gordonii* containing dietary supplements, is growing, yet the plant material is scarce, and the supply cannot meet this pressing demand. Therefore, adulteration of *Hoodia* products has become a major problem, which can occur by using synthetic pharmacons (e.g. sibutramine) as adulterants to intensify the anticipated effect, by substituting plant material with other botanicals or by simply omitting *H. gordonii* from the product [32, 33, 47-49]. The development of efficient and reliable analytical procedures for qualitative and quantitative analysis of *Hoodia* plant material as well as products claiming *Hoodia* content is crucial to authenticate commercial products undergoing scientific evaluation.

In the last 8 years several methods have been published: HPLC-UV for chemical fingerprinting of three *Hoodia* species, HPLC-UV-Q-TOFMS, HPLC-ESI-TOFMS, ESI-MS/MS, UPLC-UV-ESI-MS, HPLC-ESI-MS/MS techniques for the identification, characterisation or quantification of one or more pregnane glycosides in *H. gordonii* extracts, plant materials or products. HPLC-MS/MS identification was also established and validated for P57 in human, rat, mouse and rabbit plasma in accordance with FDA guidelines [31, 50-53]. Simpler and faster methods for chemical fingerprinting of *Hoodia* species were also developed by the means of HPTLC [48, 49, 54]. Recently DNA barcoding for identification of *H. gordonii* was reported, which has been shown to be more accurate and sensitive to detect *Hoodia* than chemical profiling [32].

### 3.2.2. **Phytochemistry of the genus *Cyclopia***

Tea derived from the leaves of *Camellia sinensis* in the forms of green, oolong, and black tea is the most consumed beverage in the world aside from water [55]. However, the popularity of the two South African herbal teas, rooibos (*Aspalathus linearis* (Burm.f.) R. Dahlgren) and honeybush (*Cyclopia*) is increasing as health beverages [56]. Green, black tea, rooibos and also honeybush are rich in polyphenolic compounds, but their profiles greatly differ. The major monomeric phenolic compounds in *Camellia* are flavanoles and their oxidational products; in *Aspalathus* the flavonoid aspalathin, a dihydrochalcone and its flavone analogues; in *Cyclopia* species the xanthones mangiferin and isomangiferin and the flavanone hesperidin [57]. These three compounds are present in all *Cyclopia* species analysed to date, but in different quantities [56]. According to Joubert *et al. C. genistoides* contains the highest concentrations of mangiferin (3.61 g/100 g) and isomangiferin (0.54 g/100 g) and *C. intermedia* the highest concentration of hesperidin (1.74 g/100 g), when four species were compared, including *C. sessiliflora* and *C. maculata* [58].

Comprehensive phytochemical investigations of *Cyclopia* species have focused on the polyphenolic composition of three out of the six commercially important species, *C. intermedia*, *C. subternata* and *C. genistoides*. The aerial parts of *Cyclopia* species contain mainly flavones (luteolin, scolymoside, diosmetin), flavanones (naringenin, eriodictyol, hesperetin, narirutin, eriocitrin), isoflavones (formononetin, wistin, calycosin, orobol, afrormosin, fujikinetin, pseudobaptigen), xanthones (mangiferin, isomangiferin), benzophenones (iriflophenone-3-*C*-β-glucoside), coumestans (medicagol, flemmichapparin, sophoracoumestan), catechins (epigallocatechin-3-*O*-gallate), benzaldehyde derivates, phenylethanolderivates, but in contrast to *Camellia sinensis*, they do not contain caffeine and have low tannin content (proanthocyanidins, 4.34% of the hot water soluble solids of fermented honeybush) [56, 59-63]. The chemical uniqueness of *Cyclopia* is owed to the presence of mangiferin, hesperetin and isosakuranetin, as these compounds are absent in other genera of the *Podalyrieae* and *Liparieae*; and the absence of alkaloids, which accumulate in large amounts within the two tribes [64].

There is a great inter- and intraspecies variation of polyphenols, resulting in different pharmacological properties [65]. Harvesting time, environmental conditions and fermentation greatly influence the phenolic content and thus the biological effects. Harvesting during summer, due to the higher level of stress (high temperature, water deficit, higher solar radiation) resulted in the highest levels of mangiferin, isomangiferin and iriflophenone-3-*C*-glucoside [66]. Fermentation reduced the concentrations of total polyphenolic and individual

phenolic content (eriocitrin, narirutin, hesperidin, mangiferin, isomangiferin, hesperetin, hesperidin) in *Cyclopia* species, but *C. genistoides* was the least affected by it (77% retention) when compared to the other three, commercially important *Cyclopia* species [67-69]. Yet, traditionally fermented plant material is used for the preparation of infusion/decoction. Lately non-fermented honeybush is also available on the market.

*Cyclopia genistoides*

A comprehensive phenolic profiling of the hot water extracts of fermented and non-fermented *C. genistoides* by the means of HPLC-DAD-ESI-MS and MS/MS detection has been recently performed. Ten compounds were identified based on comparison with reference standards and thirty constituents were tentatively identified (e.g. tetrahydroxyxanthone-*C*-hexoside dimers, naringenin derivates, eriodyctiol glycosides, dihydrochalcones, glycosylated phenolic acids) [70].

Also recently, a fast and efficient method for the isolation of the *C*-glucosylated 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 [71].

The compounds isolated/identified until today from *C. genistoides* are displayed in (**Table 1**).

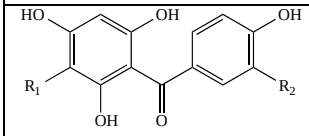
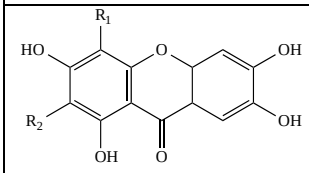
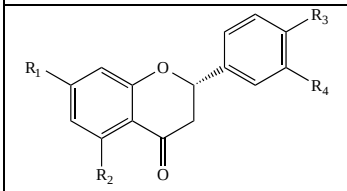
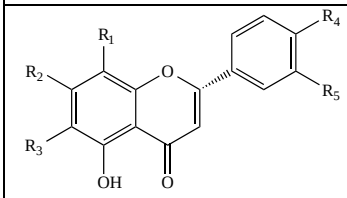
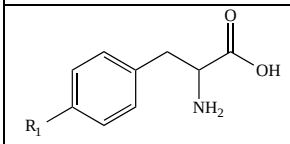
| General structure | Substituents | Compound name | Reference |
|---|---|---|---|
| *Benzophenone* | | | |
|  | $R_1$ = *C*-β-D-glucosyl<br>$R_2$ =H | iriflophenone-3-*C*-glucoside | [70, 71] |
| | $R_1$ = *C*-β-D-glucosyl<br>$R_2$ = OH | maclurin-3-*C*-glucoside | [71] |
| *Xanthones* | | | |
|  | $R_1$ = H<br>$R_2$ = *C*-β-D-glucosyl | mangiferin | [60, 65, 67, 68, 70, 72-74] |
| | $R_1$ = *C*-β-D-glucosyl<br>$R_2$ = H | isomangiferin | [60, 65, 67, 68, 70, 72-74] |
| *Flavanones* | | | |
|  | $R_1$ = *O*-rutinosyl, $R_2$ = $R_3$ = $R_4$ = OH | eriocitrin | [60, 65, 68, 70, 73] |
| | $R_1$ = $R_2$ = $R_3$ = $R_4$ = OH | eriodyctiol | [73] |
| | $R_1$ = *O*-rutinosyl, $R_2$ = $R_3$ = OH, $R_4$ = H | narirutin | [65, 67, 70] |
| | $R_1$ = *O*-rutinosyl, $R_2$ = OH, $R_3$ = $OCH_3$, $R_4$ = OH | hesperidin | [60, 65, 67, 70-73] |
| | $R_1$ = $R_2$ = OH, $R_3$ = $OCH_3$, $R_4$ = OH | hesperetin | [65, 73] |
| *Flavones* | | | |
|  | $R_1$ = *C*-glucosyl, $R_2$ = OH, $R_3$ = *C*-glucosyl, $R_4$ = OH, $R_5$ = H | vicenin-2 | [70] |
| | $R_1$ = H, $R_2$ = *O*-rutinosyl, $R_3$ = OH, $R_4$ = $OCH_3$, $R_5$ = OH | diosmin | [70] |
| | $R_1$ = H, $R_2$ = OH, $R_3$ = H, $R_4$=$R_5$ = OH | luteolin | [67, 71-74] |
| | $R_1$ = H, $R_2$ = *O*-rutinosyl, $R_3$ = H, $R_4$ = $R_5$ = OH | scolymoside | [60] |
| *Amino acids* | | | |
|  | $R_1$ = OH | tyrosine | [70] |
| | $R_1$ = H | phenylalanine | [70] |

**Table 1.** Constituents isolated from *C. genistoides*

*C. genistoides* has two chemotypes, the Overberg type with higher mangiferin and lesser isomangiferin content than the West coast type, but hesperidin was more prominent in the West Coast type in a study performed by Joubert *et al* [75]. The mangiferin content of both types decreased with harvesting date ($p < 0.05$).

There are limited data on other, non-polyphenolic constituents of the genus. The volatile fraction of non-fermented and fermented *C. genistoides* contains numerous saturated and unsaturated alcohols, aldehydes, methyl ketones and terpenoids. The non-fermented plant material had lower relative concentration of terpenoids; 6-methyl-5-hepten-2-one was its major constituent (54%), while in the fermented plant material, linalool was the most abundant compound with 36% [76].

## 3.3. Ethnomedicinal use of the investigated species

### 3.3.1. *Hoodia gordonii*

The modern World first recorded encounter with *Hoodia* was in 1774, when Francis Masson and Carl P. Thunberg gathered *Hoodia pilifera* and documented, that the stems of *Hoodia pilifera* were eaten by the "Hottentots" in order to suppress appetite and to quench thirst. In 1932 a pharmacist, R. Marloth experienced these effects after consumption of *H. pilifera* [7, 77].

Shepherds and hunters primarily of the Khoisan people of the Kalahari, consume *Hoodia* species, and they use the term 'veldkos' to refer to them, which means food from the veld [11, 77]. *H. flava, H. officinalis* and *H. pilifera* are commonly known as 'ghaap' and often consumed for their edible stems. After cutting or breaking the stems off, the spines are removed by rubbing them on a stone, and then the stems are cut into strips and eaten. They have a peculiar persistent sweet taste and are said to quench thirst and suppress appetite. Some species are also used for various diseases, such as *H. officinalis* for pulmonary tuberculosis and haemorrhoids, *H. currori* for diabetes, honey obtained from flowers of *H. gordonii* for cancer, or several *Hoodia* species as stomachic [7, 11, 78, 79].

The large, hard-spined species like *H. alstonii, H. currorii* and *H. gordonii* are less often eaten. *Hoodia gordonii*, 'bitterghaap' in Afrikaans, is used as an appetite suppressant, but paradoxically, it can also be consumed in order to stimulate the appetite; and it is also eaten for abdominal pain, indicating peptic ulceration and tuberculosis [79, 80]. Since *H. gordonii* has a bitter taste, in some areas it is considered worthless as food or medication. Yet, in the Kalahari it is consumed raw or cooked, directly after good rains, which ameliorate the bitterness [11, 81].

The documented use of *Hoodia* species as a food and water substitute in colonial botanical accounts led to its inclusion to a project in 1963. This research programme by CSIR (Council for Scientific and Industrial Research) aimed at determining the nutritional and possible toxic properties of edible wild plants of the region, the "foods from the veld". As a result, *Hoodia gordonii*, in particular, P57 was patented in 1995 as an appetite-suppressant, which triggered scientific interest.

### 3.3.2. *Cyclopia genistoides*

In the 17$^{th}$ century, Dutch colonists began to discover the local herbs of South Africa probably from local tribes such as the Khoisan. They learned that honeybush was a pleasant tea, and a treatment for coughs, other respiratory conditions (expectorant), but was also used

as a restorative, to stimulate milk production in breast-feeding women and to alleviate menopausal symptoms [19, 56, 65, 82]. It used to be common practice in some rural districts that a kettle of honeybush tea was kept infusing on the stove ready for drinking while scenting the whole house [83]. The first appearance of *Cyclopia* in botanical literature was in 1705; an illustration was published with the name of *Genista rosmarinifolio triphyllos* Promont, Bonae Spei. In the following years, it was cited under various synonyms, then eventually in 1825 it was named as *Cyclopia genistoides* [21].

Honeybush is not one distinct species, and trade in the tea varies according to region [83]. *Cyclopia genistoides* is the original honeybush tea, but today other species are harvested commercially [20]. At present *Cyclopia intermedia, subternata, genistoides* and to a lesser extent *sessiliflora* are used for honeybush tea production [18].

## 3.4. Pharmacology of the investigated species

### 3.4.1. ***Hoodia gordonii***

Since obesity is reaching epidemic proportions and effective and safe treatment is lacking, the development of weight-loss medications is a great priority in western countries. In the history of anti-obesity drugs, several effective compounds were discovered and used, but their serious side effects – usually as a result of sympathomimetic action – made the risk/benefit ratio quite unfavourable. Hence, after the patent – which revealed the appetite-suppressant activity of *H. gordonii* – was published, the majority of the performed *in vivo* and *in vitro* studies were conducted in order to enlighten its mechanism of action. Also pharmacokinetic properties of *Hoodia* were of great interest, because understanding the pharmacokinetic profile is fundamental for an accurate pharmacodynamic hypothesis.

*Pharmacokinetics*

P57, the primary active constituent of *Hoodia gordonii* was reported to have a moderate, 47.5% bioavailability after oral administration (enriched *H. gordonii* extract equivalent to 25 mg/kg P57) in mice [84]. The moderate bioavailability may be due to its degradation in stimulated gastric and intestinal fluids (SGF and SIF) and to efflux mechanisms, showed by *in vitro* experiments. Parallel to the degradation of P57 in SGF, its aglycone, hoodigogenin A appeared, highly stable in biological fluids. The transport of P57 across CaCo2 cell monolayers was concentration dependant, linear with time and the involvement of efflux mechanism via P-gp and MRP was indicated. The transport of hoodigogenin A, using the same model was bidirectional too, but unlike P57, its aglycone showed high intestinal permeability and passive transport [85, 86]. After rapid absorption, the concentration of P57

was the highest in the intestines, lower in the kidney and the lowest in the liver and it could not be detected in the brain. Following iv. administration (25 mg/kg) P57 concentration in tissues was much higher and detectable in the brain [84]. When P57 and hoodigogenin A were incubated with human liver microsomes, P57 showed high, its aglycone exhibited low stability against Phase-I oxidative metabolism, but inhibited ($IC_{50}$ = 45 and 3 μM, respectively) the catalytic activity of CYP3A4. The activity of CYP 2C9 and 1A2 were also inhibited ($IC_{50}$ = 40 and 50 μM) but only by the aglycone [85, 86]. P57 elimination was complete after 24 hours of the oral administration, with a half-life of 2.81 hours in mice [85]. Hoodigogenin A displayed high protein binding, which taken together with its extensive metabolism may limit the oral bioavailability. P57 also has unfavourable pharmacokinetics, as it proved to be unstable in stimulated gastric fluids and its absorption is bounded by efflux mechanisms. However, it was reported that the transport of P57 from crude extract was significantly higher than the transport of the pure P57, across fresh porcine buccal and intestinal tissue. The moderate bioavailability underlines the rationale of the traditional use of the plant, as it is consumed as food, in substantial quantities [87].

*Pharmacodynamics*

Although many claims and non-reviewed data are available in several patent applications, but *in vivo* investigations in peer-reviewed articles are scarce.

The first scientific report from the appetite suppressant activity of *Hoodia* was included in a patent application in 1995, which data was later published. Van Heerden *et al.* have reported, that *H. gordonii* (6.25-50 mg/kg) orally administered for three consecutive days has significantly decreased food intake in rats (n = 3-6/group) when compared to placebo. The effects were monitored for 8 days and the effect of *Hoodia* supplementation was comparable with the also effective anti-obesity drug, fenfluramin; the body mass gain was even lower in the *Hoodia* group [36]. Yet, because of the small population and short duration, this study cannot be considered as a definitive scientific proof.

In 2004 MacLean and Luo reported an increase ($p < 0.05$) in the ATP content of the hypothalamus after intracerebroventricular (i.c.v.) injections of P57 (40 nM) in rats, maintained on hypocaloric or normal diet. A reduction in food intake by 50-60% was also observed in the 24 hours following the i.c.v. administration of P57 (0.4-40 nM) in rats (n = 4-6/group), but the aglycone, hoodigogenin A had no effect. It was hypothesized, that it might trigger a signal of satiety and consequently suppresses appetite. In two whole cell systems, P57 (5 μM) failed to demonstrate the inhibition of $Na/K^+$-ATPase or the binding of the

agonist ouabain, yet it caused a likely functional antagonism of ouabain and increased the ATP content in hypothalamic cultures, following 30 minutes incubation *in vitro* [88]. However, this mechanism of action was questioned based on the pharmacokinetic studies. P57 was undetectable in the brain after oral administration of P57 enriched methanolic *H. gordonii* extract in mice in the tested concentrations, thus the central nervous system does not likely to have a pivotal role in the suggested anorexigenic effect of P57 [84].

Peripheral targets were also proposed. P57 (0.5 mM) was reported to elicit a significant cholecystokinin (CCK) secretion in a rat *ex vivo* duodenal tissue preparations and in HuTu-80 human duodenal cell line, supposedly mediated via TAS2R14 bitter taste receptors [89]. CCK release was associated with induced satiety with rodents and in humans [90, 91].

The findings, published by Jain *et al.* were in accordance with these results. The authors concluded that *H. gordonii* could modulate hunger, because it altered metabolic responses to calorie restriction. Calorie restricted (25%) rats (n = 12/group) supplemented with *H. gordonii* extract ($CH_2Cl_2$:MeOH 1:1, 100 mg/kg) displayed a significant ($p < 0.05$) decline in the ghrelin and an increase in CCK levels, when compared to the calorie restricted group without supplementation [92]. This research group conducted a study in rats (n = 6/group) with the same extract at doses 50, 100 and 150 mg/kg body weight, given orally for five days. Dose-dependent reduction in food intake (12–26%) was observed at the two higher doses ($p < 0.05$) and there was an increase in liver glycogen stores, activity of mitochondrial CPT-1, thyroid hormones, CCK, leptin and a decrease in NPY and IGF-1 levels. There was no change in blood glucose; and insulin levels were not affected significantly [93].

In another study, pregnane-glycosides enriched extract of *H. gordonii* (4-100 μg/ml) exhibited an inhibitory effect on forskolin-stimulated and basal steroid release ($p < 0.01$) of H295R cells in a dose-dependent manner, but it is not clear how it relates to the appetite suppressant activity of *H. gordonii* [94].

Recently, a G-protein coupled receptor, GPR119 was described as a potential target for the steroid glycosides of *Hoodia*. Its activation in the pancreatic β cells and intestinal L cells can stimulate insulin secretion and GLP-1 release - an insulin-releasing, appetite-suppressing hormone -, respectively. This was the first study that reported gorgonoside F, not P57 as the active constituent. From six compounds tested, only gorgonoside F activated GPR119-mediated signal transduction *in vitro*; and also demonstrated insulinotropic effect on wild type isolated islets, but not on GPR119 knockout islets. *In vivo* gorgonosid F (200

mg/kg) and a *Hoodia* extract (1000 mg/kg) significantly improved oral glucose tolerance; increased insulin and GLP-1 level in the wild type, but not in the GPR119-deficient mice. During a one-day *in vivo* study both gorgonosid F (100-400 mg/kg) and a *Hoodia gordonii* extract (500-2000 mg/kg) was able to reduce the food intake in mice (n = 8/group). Interestingly, the extract and the compound at higher concentrations had appetite suppressive effect also on knockout mice but it was significantly lower than in the wild type subjects; implying the existence of mechanisms other than GPR119 activation [95].

Smith *et al.* also reported anti-obesity properties, but the treatment did not have any effect on blood glucose levels. In lean and obese rats, administered with high and low dose (80 and 160 mg/kg twice daily, n = 4/group) of a *H. gordonii* hydromethanolic extract, significant body mass reduction was observed, but in contrast to the lean subjects, obese rats responded significantly ($p < 0.01$) only to the higher dose of treatment. After supplementation (14 days) the adipose cell size decreased ($p < 0.01$) in both obese and lean subjects, which was in accordance with the previously reported decreased fat pad size by 40% in broiler chickens treated (12 days, n = 10/group) with *H. gordonii* (300 mg/day) [96]. However, muscle atrophy and left ventricular hypertrophy were also registered in the treatment groups. Losses in muscle mass were greater in lean rats (35%, both doses) than in obese animals (20 and 30%), while ventricular hypertrophy, which can imply sympathomimetic activity, was present in all animals supplemented with the extract independent of dose [97].

This cardiovascular adverse effect aligns with the observations detected in humans. In a 15-day study, *H. gordonii* purified extract (2 × 1100 mg) showed no reduction in energy intake in healthy, overweight women (n = 25); however, blood pressure, pulse, heart rate, bilirubin and alkaline-phosphatase levels showed significant increases ($p < 0.05$) compared to placebo (n = 24) [98]. These side effects correspond with other weight loss medications, like sibutramine or amphetamine derivatives, already withdrawn from the market because of their serious side effects, including increased blood pressure and elevated pulse rate.

There were other investigations performed on humans, but companies, that markets *Hoodia* products, conducted two of them; while another two, open-label observational studies were performed by two physicians. They administered their patients (7 and 8 patients) with *Hoodia* supplements. All four surveys reported weight–loss effect, yet only one of them, testing a *H. parviflora* product, was published in a peer-reviewed journal and the conflict of interest is presumable [99, 100].

Considering that *H. gordonii* is widely commercialized, safety studies were also conducted. A *H. gordonii* purified extract proved to be non-genotoxic in three independent assays, two *in vitro,* and one *in vivo* (mice), doses up to 400 mg/kg. Above 350 mg/kg clinical signs of toxicity were observed, such as swollen abdomen or increased mortality [101]. Prenatal developmental investigations were also performed. Both in rabbit (0, 3, 6, 12 mg/kg, 25 days) and in mice (0, 5, 15, 50 mg/kg, 12 days) models a *H. gordonii* purified methanolic extract caused dose related reduction in feed intake and body weight gain, but in mice at the highest dose it also delayed foetal development; in rabbit at 16 mg/kg thickened stomach content was observed [102, 103].

Activities beyond appetite-suppression were also investigated, such as the antioxidant and antiproliferative properties of hoodigoside A-K and P57; gastric acid reductive, gastroprotective and antidiabetic effect of P57 or the antidepressant-like effect of a commercial dried purified extract of *H. gordonii* [34], [104-106]

Although in animal studies the appetite-suppressive effect of different *H. gordonii* preparations is unanimously shown, in the only clinical trial it was not proven. In conclusion, given the severity of the observed side effects and the lack of knowledge of the mechanisms of these events, further investigations are desirable. The mechanism of action of *H. gordonii* is still in dispute, but it is likely, that the observed effects are the result of a fine balance between different peripheral and/or central mechanisms, caused by several compounds.

### 3.4.2. ***Cyclopia genistoides***

There have been no clinical trials performed with any of the *Cyclopia* species yet*;* only pre-clinical data are available. According to the *in vivo, ex vivo* and *in vitro* laboratory experiments, the different extracts of *Cyclopia* spp. possess pleiotrop effects (antimutagenic/chemopreventive, phytoestrogenic and antioxidant). Based on these studies, the investigated effects are most likely due to the polyphenolic content of honeybush. The exhibited pharmacological effects had intra- and interspecies variation, possibly because of quantitative variation of chemical composition within and between species [56, 57, 67, 107-113].

In many cases fermentation could reduce the observed effects of *Cyclopia* spp., but not that of *C. genistoides* in case of phytoestrogenic and antioxidant activity [56, 65, 67, 114]. A systematic review of all of these studies will not be attempted here; rather a selection with emphasis on studies investigating the phytoestrogenic effect of *Cyclopia* species.

Phytoestrogens (plant-derived non-steroidal, polyphenolic secondary metabolites with structural and functional similarity to oestrogen) might serve as viable alternatives for hormone replacement therapy (HRT), given their differentiated effect on α and β oestrogen receptors (ER). They may be able to bind to both ER subtypes, acting as either agonist or antagonist, but unlike 17β-oestradiol ($E_2$) 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 [115, 116]. Furthermore, phytoestrogens may be beneficial to alleviate menopausal symptoms and to protect postmenopausal women against cardiovascular diseases and osteoporosis, without the risks associated with HRT [117, 118].

HRT, using conjugated equine oestrogen alone (CEE) or in combination with progestin (CEE + P), proved to lack overall benefit in chronic disease prevention (osteoporosis, heart disease) and menopausal symptom alleviation, due to increased risks of stroke (CEE and CEE + P), coronary heart disease, venous thromboembolic disease and breast cancer (CEE + P) [119, 120]. Alternative solutions, such as selective oestrogen receptor modulators (raloxifene and tamoxifen) have also been questioned because of their side-effects, such as the stimulation of endometrial growth, the occurrence of hot flashes and an increased risk of venous thromboembolism [121-126].

However, despite several promising studies, phytoestrogen treatment seems to be less effective than traditional HRT, and the effect of phytoestrogens on menopausal symptoms, such as hot flushes, is inconclusive [127, 128]. Yet, the risks of HRT and the increasing popularity of natural products give a rationale to the investigations seeking phytoestrogens with selective affinity on ERs.

*Phytoestrogenic activity*

Initial screening for phytoestrogenicity has focused on *in vitro* oestrogen receptor binding of water and methanol extracts (1.5 μg/ml) of three harvestings of four *Cyclopia* species (*sessiliflora, subternata, intemerdia, genistoides*). *C. subternata* and *genistoides* displayed significant ($p < 0.01$) phytoestrogenic activity and methanol extracts from non-fermented plant material generally displayed greater activity. Interestingly, fermentation did not affect the ER binding of *C. genistoides,* but *C. subternata.* A great variation of ER binding within species has also been reported. Depending on harvestings tested *C. genistoides* binded to hERβ, either subtypes or neither. *C. genistoides* (methanolic extract) demonstrated the strongest oestrogen receptor binding with the highest consistency to hERβ amongst the

species tested [65]. In a later study it was shown by the means of different assays (ERα/β binding, transactivation of an ERE-containing promoter reporter, proliferation of MCF-7-BUS and MDA-MB-231 breast cancer cells and binding to SHBG), that *C. genistoides* methanol extracts (three different harvestings were tested) displayed phytoestrogenic activity and acted predominantly via ERβ (transactivated only through ERβ despite binding to both subtypes). HPLC-MS analysis suggested that the observed phytoestrogenic activity cannot only be ascribed to polyphenols known to be present in *Cyclopia* spp. [65, 72]. Mfenyana *et al.* reported positive correlation between luteolin content and efficacy and between an unknown flavanone and potency of *C. genistoides* and *subternata* extracts according to alkaline phosphatase assay and E-screen, respectively. The cup-of-tea extracts from these species did not only displayed oestrogenic activity, but had comparable efficacy and potency to commercial products containing soy isoflavones, black cohosh or red clover [73]. In an exhaustive investigation, *C. subternata* and *C. genistoides* extracts displayed ERα antagonism and ERβ agonism when expressed separately, but when co-expressed only agonism was observed. Breast cancer cell proliferation assays indicated that extracts antagonized cell proliferation in the presence of oestrogen, at lower concentrations than that required for proliferation. Also, lack of uterine growth and delayed vaginal opening were reported in immature rat uterothropic model (2000 mg/kg *C. subternata* extracts/day, n = 10/group), implying ERα antagonism, supporting the potential of *Cyclopia* extracts as a source of oestrogen analogous with a reduced risk profile [74].

*Cyclopia* extracts from specific harvestings seem promising, but blanket claim for their oestrogenicity cannot be made, as the observed oestrogenic potency has great inter- and intraspecies variability [65, 72-74]. Moreover, in a recent study, the activity guided fractionation of an extract from *C. subternata*, which displayed three desirable oestrogenic attributes (ERα antagonism, ERβ agonism, antagonism of $E_2$-induced breast cancer cell proliferation) showed, that the retention of all these attributes in one fraction is not an attainable goal [63].

# 4. MATERIALS AND METHODS

## 4.1. Plant material

The aerial parts of *Hoodia gordonii* were obtained from a local succulent plant nursery (707.7 g) in 2013 January Szeged, Hungary, and authenticated by the author (O. Roza).

Fermented and non-fermented *Cyclopia genistoides* (L.) Vent. (1.7 and 1.3 kg, respectively) were a gift from Van Zyl and Mona Joubert owners of Agulhas Honeybush Tea, from their farm near Bredasdorp in South Africa. Botanical identifications were performed by Dr. Hannes de Lange, an expert in taxonomy and commercializing of honeybush. Fermentation was carried out according to the traditional method for this material [129].

The plant materials of *C. genistoides* were stored at room temperature until preparation, while the aerial parts of *H. gordonii* were freshly grounded and extracted. Voucher specimens (no. 812 - *H. gordonii*, 825 - F *C. genistoides* and 826 - nF *C. genistoides*, respectively) have been deposited at the herbarium of the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

Fytofontana Dietceutical *Hoodia* spray (distributed by Herb-Pharma Hu Ltd., Budapest, Hungary), a medical device registered in Hungary, was bought in a local pharmacy in Szeged, Hungary. The composition of the product displayed on the package is the following: *Hoodia gordonii* extract (corresponding to 2.2 g plant material /2 ml solution), peppermint, alcohol.

The commercial probiotic bacteria mixture ProBio 6 capsule (Béres Pharmaceuticals Ltd., Budapest, Hungary) is claimed to contain 5 billion bacteria (*Lactobacillus helveticus, Lactococcus lactis, Lactobacillus rhamnosus, Bifidobacterium longum, Bifidobacterium breve, Bifidobacterium bifidum*) per 515 mg.

## 4.2. Extraction of plant materials

The extract of authentic *H. gordonii* for HPLC and HPTLC analyses was prepared from 7 g fresh, ground plant material with 3 × 25 ml of AcNi, using ultrasonic bath (15 min). After filtration, the solution was evaporated to dryness under reduced pressure. The residue was dissolved in 2 ml of MeOH, filtered and then evaluated.

Twenty-five ml of Hoodia spray was evaporated to dryness, and then the residue was dissolved to the original volume with physiological saline-DMSO (95:5), and after filtered to perform the organ baths studies. The Hoodia spray itself was used for TLC, MS, HPLC and HPTLC analyses.

The dried fermented and non-fermented herbs of *C. genistoides* were extracted via ultrasonication with methanol (12 l and 10 l) at room temperature for 30 minutes. The extract was concentrated *in vacuo* and then diluted with $H_2O$, and liquid-liquid partition was performed (5 × 500 ml), affording *n*-hexane, dichloromethane, ethyl-acetate, the remnant aqueous layers and insoluble part.

The aqueous "cup of tea" extracts were prepared for the HPLC quantitative determination. 5 g plant material was extracted with 100 ml boiling tap water for 10 minutes. For comparison a methanolic extract was also prepared (10 ml MeOH + 1 g plant material, 10 minutes, ultrasonication).

Aqueous extracts of the fermented and non-fermented honeybush tea were prepared for the testing of bacterial fermentation by adding freshly boiled tap water to the dried plant material (200 ml boiling tap water + 10 g drug). The teas were allowed to steep for 30 minutes at room temperature, and then filtered. After lyophilisation, 200 mg of the dry residues were dissolved in 10 g DMSO and 189.98 g of 37 °C physiologic saline solution and 3 g ProBio 6 was added to each solution. Blank experiments were also performed, which did not contain the probiotic bacteria. The mixtures were gently mixed at 37 °C for 50 hours. After lyophilisation of the mixtures, they were re-dissolved in 5 ml MeOH and filtered through 0.45 μm syringe filter (Labex Ltd., FilterBio®, PTFE-L syringe filter). Measurements were carried out in triplicate, luteolin and naringenin contents were also measured in the teas before bacterial fermentation. Luteolin and naringenin were used as standards, previously isolated in our lab (wavelength of detection 348 nm and 288 nm, respectively) [130].

### 4.3. Comparison of the *Hoodia* product with authentic plant material and investigation of its falsification

*HPLC:* HPLC was carried out on a Kinetex XB-C18 (2.6 μm, 100 Å, 100 × 4.6 mm) column (Phenomenex, Torrance, USA), operated at 40 °C, using:

Instrument 1: Waters Alliance 2695 separations module with Empower software (Waters Associates, Milford, MA), Waters 600 controller and pump, 2487 dual absorbance detector

Method 1: Injection volume of 20 μl, gradient solvent system consisting methanol, acetonitrile and $H_2O$, was according to the method Janssen *et al.* [51].

*TLC*: thin layer chromatographic analyses were carried out on aluminium sheets (20 × 20 cm), coated with silica gel 60 $F_{254}$ (Merck KgaA, Darmstadt, Germany). For each test 10 µl of *Hoodia* spray and standard solutions were applied.

Mobile phases, visualisation:

**TLC-A**: MeOH (95:5), Dragendorff reagent

**TLC-B**: MeOH - cc. $NH_3$ (100:1.5), Marquis reagent (cc. $H_2SO_4$ - formalin 20:1.5), heating at 110 °C for 2 minutes, detection under UV light (366 nm) [131]

**TLC-C**: $CH_2Cl_2$ - cc. $NH_3$ - 2-propanol (5:15:80), ninhydrin reagent, heating at 110 °C for 5 minutes

*HPTLC:* HPTLC comparison of the product and authentic *H. gordonii* extract was carried out according to the method described by Rumalla *et al.,* using silica gel coated 60 $F_{254}$ plates (10 × 20 cm) (Merck KgaA, Darmstadt, Germany) [48].

Mobile phases, visualisation:

**HPTLC-D**: $CHCl_3$ - MeOH - $H_2O$ (70:30:3), anisaldehyde, heating at 110 °C for 5 minutes

*Mass spectrometry:* analyses were performed on an API 2000 triple quadrupole tandem mass spectrometer (AB Sciex Instruments, Foster, CA.). Data integration was performed with Analyst 1.5.2 software version (AB Sciex Instruments). Electrospray ionisation interface was used with a source temperature of 250 °C, operating in positive mode using multiple reaction monitoring (MRM). The following conditions were used: capillary voltage 5500, curtain gas pressure 10 psi, collusion gas pressure 6 psi, nebulizer gas pressure 50 psi and auxiliary gas pressure 20 psi.

### 4.4. *In vitro* organ bath studies

Uteri were removed from non-pregnant rats in the oestrus phase and from pregnant rats on day 22 of gestation. The tension of the myometrial rings was measured with a gauge transducer (SG-02; Experimetria Ltd., Budapest, Hungary) and recorded with SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd., Budapest, Hungary) [132]. The uterus-relaxant effect of Hoodia spray was investigated on spontaneous and 25 mM KCl-induced contractions alone and in the presence of 10 µM propranolol. All experiments were carried out on at least 6 animals (female Sprague-Dawley rats). Unpaired t-test was used for statistical evaluation of the presence of propranolol. All calculations and statistical analyses

were performed with Prism 5.0 computer software (Graph Pad Software Inc, San Diego, CA, USA). For further details please see Appendix I.

### 4.5. Isolation, purification and quantification of compounds from *C. genistoides*

*Open-column chromatography:* OCC was performed on polyamide for column chromatography (ICN), on silica gel (160–200 mesh, Qingdao Marine Chemical Co., Qingdao, China) and on Sephadex LH-20 (Sigma). The extracts, after dissolution, were adsorbed on stationary phase, corresponding to double the amount of their dried weight. After drying, the yielded powders were placed on the stationary phase (fourfold amount of the powder).

**OCC-P**: eluent: mixtures of MeOH - $H_2O$ (2:3, 3:2, 4:1, 1:0); volume of collected fractions: 100 ml; sorbent: polyamide

**OCC-NP-1**: eluent: *n*-hexane - acetone (2:1 to 0:1); volume of collected fractions: 20 ml; sorbent: silica gel

**OCC-NP-2**: eluent: *n*-hexane - acetone (3:1 to 0:1); volume of collected fractions: 20 ml; sorbent: silica gel

**OCC-NP-3**: eluent: *n*-hexane - acetone (5:1 to 0:1); volume of collected fractions: 20 ml; sorbent: silica gel

**OCC-Sph**: eluent: methanol; volume of collected fractions: 1 ml; sorbent: Sephadex LH-20

*Vacuum-liquid chromatography:* The extracts, after dissolution, were adsorbed on silica gel 60 $GF_{254}$ (15 $\mu$m, Merck), corresponding to double the amount of their dried weight. After drying, the yielded powders were placed on the stationary phase (fourfold amount of the powder) in a filter funnel. The VLC columns were developed under gentle vacuum, provided by a water pump.

**VLC-NP**: eluent: stepwise gradient, EtOAc - MeOH (1:0 to 0:1); volume of collected fractions: 30 ml; sorbent: silica gel

*Rotation planar chromatography:* RPC was carried on a Harrison Model 8924 Chromatotron instrument (Harrison Research). The stationary phase for RPC was silica gel 60 $GF_{254}$ (Merck), manually coated on the rotor as a 1 or 2 or 4 mm layer. The flow rates were 4 to 8 ml/min. The separation was achieved with gradient elution in 5 or 6 steps with cyclohexane - acetone (1:0 to 0:1). This method is herein after referred to as RPC-NP.

*Preparative layer chromatography:* PLC was performed on 10 × 20 cm silica plates (silica gel 60 $F_{254}$, Merck and 60 RP-18 $F_{254}$s). The plates were developed by an ascending

technique in a glass chamber at room temperature. Separation was monitored in UV light at 254 nm. Compounds were eluted from the scraped adsorbent with $CHCl_3$ - MeOH (5:1).

Mobile phases: PLC-E: MeOH - $H_2O$ (4:6)

PLC-F: EtOAc - MeOH - $H_2O$ (100:16:14)

PLC-G: Acetone - *n*-hexane (1:2)

PLC-H: $CH_2Cl_2$ - MeOH (10:0.15)

*HPLC:* The following instruments and methods were used:

Instrument 1: Waters Alliance 2695 separations module with Empower software (Waters Associates, Milford, MA), Waters 600 controller and pump, 2487 dual absorbance detector

Instrument 2: Waters Alliance 2695 separations module with Empower software (Waters Associates, Milford, MA), Waters 600 controller and pump, Waters Photodiode Array Detector 2998 (190–800 nm), Injector Rheodine 7725i

Instrument 3: two JASCO PU-2080 HPLC pumps, JASCO MD-2010 Plus multi-wavelength detector (JASCO Inc., Tokyo, Japan)

Instrument 4: dual Shimadzu LC-10AT pumps, Shimadzu SPD-10A UV-vis detector

Method 2: eluent: gradient solvent system consisting of MeOH and acidified (0.1% $H_3PO_4$) water; flow rate: 1 ml/min; column: Kinetex C18 (5µm, 100 Å, 150 × 4.6 mm) column (Phenomenex, Torrance, USA), operated at 20 °C. Injection volume was 20 µl. The gradient consisted of three steps: for 21 minutes the % of the acified water decreased from 80% to 24% then in one minute it reached 80 % again then for six minutes this ratio was maintained.

Method 3: eluent: MeOH - $H_2O$, (3:2); flow rate 2 ml/min; column: Hibar Purospher STAR RP-18e (5 µm, 250 × 10.0 mm) semipreparative column (Merck KGaA, Darmstadt, Germany)

Method 4: eluent: *n*-hexane - $CH_2Cl_2$ - MeOH (4:8:0.015); flow rate: 2 ml/min; column: Luna CN (5 µm, 250 × 10.0 mm) semipreparative column (Phenomenex Inc., Torrance, USA)

Method 5: eluent: AcNi - $H_2O$ (3.5:10); flow rate 0.75 ml/min; column: LiChroCART RP-18e (5 µm, 250 × 4 mm)

Chromatographic separations were monitored at a wavelength range of 190-400 nm.

*Medium-performance liquid chromatography*: MPLC was performed by a Büchi apparatus (Büchi Labortechnik AG, Flawil) using a 40 × 150 mm RP18ec column (40-63 μm, Büchi).

MPLC-1: EtOAc - MeOH - $H_2O$ (20:1:1 to 0:1:0); flow rate: 18 ml/min

MPLC-2: MeOH - $H_2O$ (2:8 to 1:0); flow rate: 35 ml/min

### 4.6. Characterisation and structure determination of the isolated compounds

$^1$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 and chemical shifts were given in δ (ppm) value relative to tetramethylsilane (TMS) as internal standard. The signals of the deuterated solvents were taken as reference. Two-dimensional (2D) experiments were performed with standard Bruker software. In the COSY, HSQC and HMBC experiments, gradient-enhanced versions were used.

MS spectra were recorded on an API 2000 Triple Quad mass spectrometer with APCI or ESI ion source using positive or negative of polarity.

The HRESIMS data were recorded, on a Shimadzu IT-TOF spectrometer, equipped with electrospray source.

### 4.7. Oestrogen-like reporter assay

pER8:GUS seeds were grown in the dark for 24-36 hours at 4 °C on medium (½ MS, 1% sucrose, 0.8% phytoagar) for vernalisation and then germinated under white light for 72 hours 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 hours. Transgenic plants (3-5) were added to each well, in order to evaluate oestrogenic activity. Plants cultured with 0.31-10 nM $E_2$ were taken as a positive control.

### 4.8. 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 $Na_3PO_4$ buffer (pH 7.0), 10 mM EDTA (pH 8.0), 2 mM X-Gluc, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, and 0.1% Triton X-100] in a 24-well plate and incubated for 3 hours or overnight at 37 °C. 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 colour was still detectable was considered the minimum active concentration. The parallel experiments were in accordance, hence no SEM/SD were calculated.

### 4.9. Antiproliferative assay

The antiproliferative properties of the prepared extracts and natural products were determined on A2780 and T47D cancer cell lines (isolated from ovarian and breast carcinoma, respectively; purchased from ECACC, Salisbury, UK) by means of the MTT assay [133]. All *in vitro* experiments were carried out on 2 microplates (96-well) with at least 5 parallel wells. Stock solutions of 10 mg/ml of the tested materials 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 $IC_{50}$ values of 1.30 and 9.78 μM, respectively.

Statistical evaluation of the results was performed by one-way analysis of variance followed by Dunnett posttest, using GraphPad Prism 4 (GraphPad Software San Diego, CA, USA). For further details please see Appendix II.

### 4.10. Xanthine oxidase inhibitor assay

Inhibition of xanthine oxidase activity was measured using the protocol recommended by Sigma-Aldrich, re-adapted to an assay volume of 300 μl, and published in detail before [134, 135]. Briefly, the enzyme activity at pH 7.5 was determined by the production of uric acid from xanthine. Uric acid was measured at 290 nm for 3 minutes in 96-well plate, using the plate reader FluoSTAR OPTIMA (BMG LABTECH, Germany). The enzyme inhibitory effect was determined by the decreased production of uric acid. Xanthine oxidase (XO), isolated from bovine milk (lyophilized powder), and xanthine powder were purchased from Sigma-Aldrich. Allopurinol (Sigma-Aldrich, ≥99%), a well-known inhibitor of XO was used as positive control. Each compound or fraction was dissolved in DMSO. The final concentration of DMSO in the assay did not exceed 3.3% of the total volume. After the addition of all other reagents, the reaction was initiated by automatic addition of XO solution. All XO activity measurements were made in triplicate.

Fifty percent inhibitory concentrations ($IC_{50}$) were calculated using non-linear regression curve fitting of log(inhibitor) *vs.* normalized response of GraphPad Prism 5.04 software (GraphPad Software Inc., La Jolla, CA, USA).

# 5. RESULTS

## 5.1. Sympathomimetic activity of a *Hoodia* product

The only available human clinical study on *Hoodia gordonii* reported no change in body weight or energy expenditure; however, administration of a purified *H. gordonii* extract was associated with significant increase in blood pressure and pulse rate [18]. Similar side effects have been reported to the Department of Pharmacognosy, University of Szeged, by consumers of a product available on the Hungarian market (Hoodia spray). As a first step, the comparison of the product with authenthic plant material was performed alongside with testing for possible adulterants with potential for causing cardiovascular side effects (e.g. sibutramine, amphetamine). Then the effect of the Hoodia spray on β-adrenergic receptors in rat uterus was measured to explore the potential role of β-adrenergic receptor agonist activity in the possible cardiovascular adverse effects of the plant.

### 5.1.1. Comparison of the *Hoodia* product with authentic plant material and investigation of its falsification

First, the Hoodia spray was tested for the presence of P57 by mass spectrometry in multiple reaction monitoring (MRM) mode. In our experiments, P57, a characteristic compound of *Hoodia* species, was chosen for mass spectrometric identification. $LiCl_3$ was added to the sample, to enhance sensitivity. Both *m/z* 311.3 and *m/z* 785.5 product ions were detected with the precursor ion *m/z* 885.5, indicating that P57 is present in the Hoodia spray. The aglycone was identified too, with the product ions of *m/z* 319.3 and *m/z* 337.3. All of these transitions have been reported in the literature for P57 [27].

The HPLC chromatogram of the plant extract and the product showed similarity (**Instrument 1, Method 1**), having similar fingerprints between 16 and 36 minutes at 220 nm (**Figure 1A**). Differences in peak heights and areas can be explained with the dissimilarity of extraction methods (in the product's leaflet there was no reference to the extraction method applied). In the chromatogram of the product, few other peaks occurred, possibly due to additives present in the spray, such as *Mentha piperita*.

Regarding the colours and retention factors of the detected spots, HPTLC analysis (**HPTLC-D**) also confirmed the similarity of the product and the plant extract. On **Figure 1B** the HPTLC chromatograms of the Hoodia spray and *H. gordonii* extract can be seen, together with the HPTLC chromatograms of four *Hoodia* species found in literature, using the same eluent system [48]. As other *Hoodia* species can contain several of the same constituents, but

in different ratios, the chance that *Hoodia* species other than *H. gordonii* being used in the preparation cannot be excluded [25].

Using TLC comparison with reference standards, sibutramine (**TLC-A**), amphetamine, methamphetamine (**TLC-B**) and ephedrine (**TLC-C**), were not detected in the product.

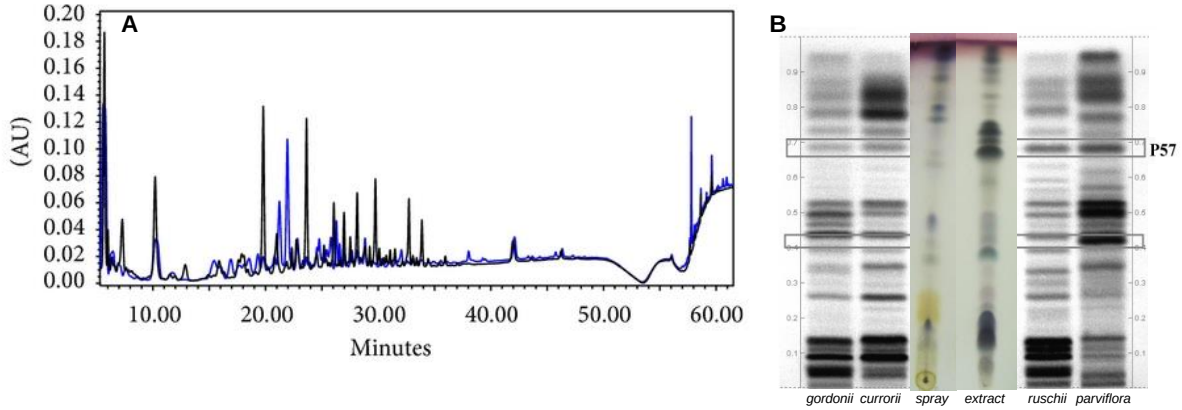



**Figure 1**. **A** HPLC fingerprint chromatograms of *Hoodia* extract (blue) and Hoodia spray (black)
**B** HPTLC fingerprint chromatograms of different *Hoodia* species, Hoodia spray and extract

### 5.1.2. **Beta-adrenergic receptor agonist activity**

β-adrenergic receptor agonists are reported to decrease food intake and exhibit anorectic properties [28]. Stimulation of β-adrenoceptors also results in different cardiovascular symptoms. Since cardiovascular side effects have been reported for a *H. gordonii* extract, the stimulation of β-adrenergic receptors seemed to be a rational explanation for the mechanism of its anorectic action [18]. Both α- and β-adrenergic receptors play crucial roles in the motor activity of myometrial smooth muscle, eliciting contraction and relaxation, respectively; rendering uterine preparations ideal for investigations of drugs with actions on the sympathetic system. In the presented set of *in vitro* organ bath experiments, the myometrial effects of the *H. gordonii* extract-containing product were monitored against spontaneous and KCl-stimulated contractions of uterine rings from nonpregnant and late-pregnant (day 22) rats; the extraction and sample preparation were carried out as described in section 4.2. In order to separate the actions mediated through α- and β-adrenoceptors all experiments were performed with and without nonselective β-antagonist propranolol (10 μM). The product elicited a marked and concentration-dependent relaxation against both spontaneous and stimulated contractions. The inhibition of spontaneous contractility was significantly decreased in the presence of propranolol (**Figure 2**). The relaxation of KCl-stimulated uteri was not modified in the presence of propranolol. The relaxing effect of the spray was substantially less pronounced on uteri from late-pregnant animals, but propranolol significantly modified it.

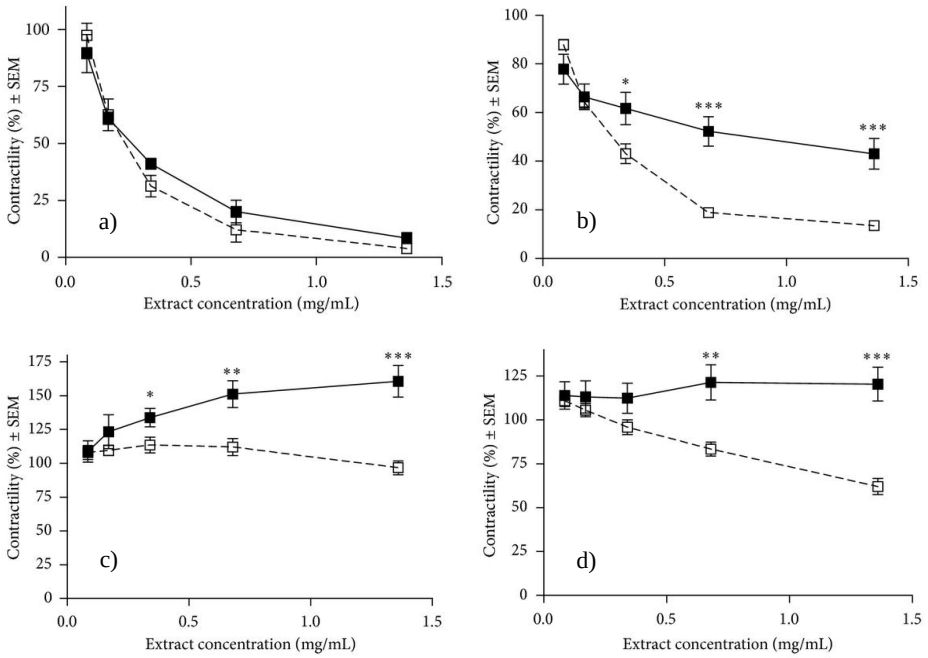



**Figure 2**. Effects of Hoodia spray on spontaneous ((a) and (c)) and KCl-stimulated ((b) and (d)) contractility of uterine rings from nonpregnant ((a) and (b)) and late-pregnant ((c) and (d)) animals. Experiments were performed in the presence (■) and absence (□) of propranolol. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

## 5.2. Flavonoids from *C. genistoides*

### 5.2.1. Isolation of compounds

The dried fermented and non-fermented plant materials (1.7 and 1.3 kg, respectively) were extracted via ultrasonication with methanol (12 l and 10 l) at room temperature. 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 (F-15.7 g, nF-13.2 g), dichloromethane (F-14.8 g, nF-6.4 g), ethyl-acetate (F-29.7 g, nF-23.35 g), the remnant aqueous layers (F-128.7 g, nF-121.4 g) and insoluble part.

The $^{1}$H NMR spectra of the EtOAc layers from the non-fermented and fermented *C genistoides* were similar, thus only the EtOAc layer (23.35 g) from the non-fermented drug was further examined. It was subjected to VLC (**VLC-NP**), using a stepwise gradient of EtOAc - MeOH affording twelve combined fractions (V1-V12), after TLC monitoring.

Fraction V3 was crystallised with $CHCl_3$ - MeOH to yield **CG1**.

Fraction V7 was separated by **MPLC-1** with EtOAc - MeOH - $H_2O$ to give 21 subfractions (M1-M21). Among these subfractions, M5 and M6 were subjected to further chromatography. Fractions M6 (777.5 mg) and M5 (65.5 mg) were separated into twelve (M6/1-12) and six (M5/1-6) subfractions by **MPLC-2,** using mixtures of MeOH - $H_2O$. Subfractions M5/2 (11.2 mg) and M6/11 (21.5 mg) were purified by reverse-phase preparative TLC (**PLC-E**) to provide **CG2** (1.8 mg) and **CG3** (4 mg), respectively.

Subfraction M6/4 (55.3 mg) was further purified by normal-phase preparative TLC (**PLC-F**) and finally by gel filtration chromatography (**OCC-Sph**) to provide **CG4** (3.3 mg).

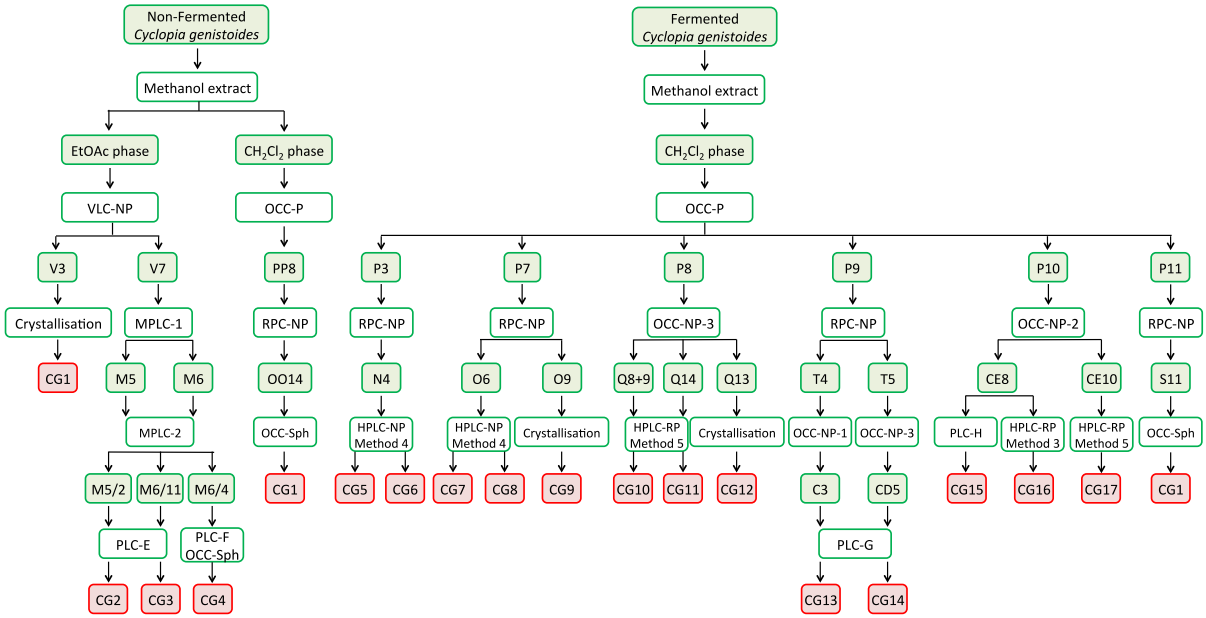



**Figure 3**. Isolation of compounds from *C. genistoides*

The concentrated $CH_2Cl_2$ phases (F-14.8 g, nF-6.4 g) were chromatographed on a polyamide column (**OCC-P**) with mixtures of MeOH and $H_2O$ as eluents. The **OCC-P** fractions were combined into 14 (F: P1-P14) and 12 fractions (nF: PP1-PP12) according to the TLC monitoring (**Figure 3**).

Fraction PP8 (136.8 mg) was separated on **RPC-NP**, eluted with a gradient system of cyclohexane - acetone. The RPC-NP fractions were combined after TLC monitoring into 14 fractions (OO1-OO14). Subfraction OO14 (8.9 mg) was further purified by gel filtration chromatography (**OCC-Sph**) to provide **CG1** (5.6 mg), isolated previously from the EtOAc phase of the non-fermented plant material.

Fraction P3 (570 mg) was chromatographed by **RPC-NP**, using a gradient system of cyclohexane - acetone. The RPC-NP fractions were combined into 15 fractions (N1-N15). Then subfraction N4 (38.5 mg) was separated on normal phase HPLC (**Method 4, Instrument 4**), to yield **CG5** (2.3 mg) and **CG6** (2.8 mg).

Fraction P7 (300 mg) was also subjected to **RPC-NP**, using the same gradient system to yield seventeen subfractions (O1-O17), from which O6 (11 mg) was further separated by normal-phase HPLC (**Method 4, Instrument 4**) to provide **CG7** (1.7 mg) and **CG8** (1.8 mg), whereas the crystallisation of O9 with $CHCl_3$-MeOH provided **CG9** (7.6 mg).

Fraction P8 (750 mg) was separated on **OCC-NP-3** with an *n*-hexane - acetone gradient system to yield twenty-two subfractions (Q1-Q22). The combined subfractions Q8 + 9 and Q14 were chromatographed by reverse-phase HPLC (**Method 5, Instrument 1**) to provide **CG10** (1.45 mg) and **CG11** (1.7 mg), respectively. Crystallisation of Q13 with $CHCl_3$-MeOH provided **CG12** (16.2 mg).

Fraction P9 (1.68 g) was chromatographed by **RPC-NP** with a gradient system of cyclohexane - acetone to give seven subfractions (T1-T7). Subfraction T4 and T5 (410 mg, 250 mg, respectively) were subjected to **OCC-NP-1** and **OCC-NP-3**, respectively, to yield eleven (C1-C11) and eight (CD1-CD8) subfractions, respectively. C3 and CD5 were further purified by preparative TLC (**PLC-G**) to provide **CG13** (16.8 mg) and **CG14** (7.4 mg), respectively.

Fraction P10 (475.5 mg) was subjected to silica gel CC, eluted with *n*-hexane – acetone (**OCC-NP-2**) to yield thirteen (CE1-CE13) subfractions. CE8 (7.5 mg) was purified by preparative TLC (**PLC-H**) to yield **CG15** (1.4 mg). CE8 was also purified alongside with CE10 (5.1 mg) by RP-HPLC with **Method 3 on Instrument 3** and with **Method 5 on Instrument 1**, respectively, to provide **CG16** (1.65 mg) and **CG17** (1.6 mg), respectively.

Fraction P11 (245 mg) was chromatographed by **RPC-NP** and eluted with cyclohexane - acetone to give fifteen subfractions. Subfraction 11 (S11) was further purified by gel filtration chromatography (**OCC-Sph**) to provide **CG1** (5.6 mg), isolated previously from the non-fermented plant material.

### 5.2.2. **Characterisation and structure elucidation of the isolated compounds**

The structures of the isolated compounds were determined by means of spectroscopic methods. High-resolution mass spectrometric measurements allowed the determination of the exact molecular weight and molecular composition of the compounds. The most useful methods in the structure elucidation were 1D and 2D NMR experiments including $^{1}H$, $^{13}C$, JMOD, $^{1}H$-$^{1}H$ COSY, HSQC, HMBC and NOESY measurements.

Iriflophenone 2-*O*-β-glucopyranoside (**CG2**): amorphous solid; $\alpha]_D^{25}$ –28 (*c* 0.1, MeOH); APCI-MS positive *m/z* 409 $[M+H]^+$, 247 $[(M+H)–C_6H_{10}O_5]^+$, 153 $[C_7H_5O_2+MeOH]^+$, 121 $[C_7H_5O_2]^+$; HRESIMS: *m/z* 431.0940 $[M+Na]^+$ (calcd for $C_{19}H_{20}O_{10}Na$ 431.0954); $^{1}H$- and $^{13}C$-NMR data are identical with published data [136].

Iriflophenone 3-*C*-β-glucopyranoside (**CG3**): amorphous powder; APCI-MS *m/z* 409 $[M+H]^+$, 231, 219, 195; $^{1}H$ NMR (500 MHz, $CD_3OD$) δ (ppm): 7.62 (2H, d, *J* = 8.7 Hz, H-2',6'), 6.79 (2H, d, *J* = 8.7 Hz, H-3',5'), 5.98 (1H, s, H-5), 4.87 (1H, d, *J* = 9.6 Hz, H-1"),

3.88 (2H, m, H-2",6a"), 3.75 (1H, dd, *J* = 12.0, 5.1 Hz, H-6b"), 3.48 (2H, m, H-3",H-5"), 3.42 (1H, m, H-4"). $^{1}$H-NMR data are in agreement with those published earlier for DMSO-$d_6$ solution [137].

(6a*R*,11a*R*)-(−)-Maackiain (**CG8**): white powder; $[\alpha]_D^{25}$ −177 (*c* 0.1, $CHCl_3$); $^{1}$H-NMR (500 MHz, DMSO-$d_6$) δ (ppm): 9.61 (1H, s, OH), 7.23 (1H, d, *J* = 8.4 Hz, H-1), 6.96 (1H, s, H-7), 6.53 (1H, d, *J* = 1.8 Hz, H-4), 6.52 (1H, s, H-10), 6.46 (1H, dd, *J* = 8.4, 1.9 Hz, H-2), 5.94 and 5.91 (2x1H, 2xs, -$OCH_2O$-), 5.50 (1H, d, *J* = 6.9 Hz, H-11a), 4.22 (1H, dd, *J* = 10.1, 3.8, H-6), 3.58 (1H, m, H-6), 3.54 (1H, m, H-6a); $^{13}$C-NMR (125 MHz, DMSO-$d_6$) δ (ppm): 158.7 (C-3), 156.3 (C-4a), 153.7 (C-10a), 14.4 (C-9), 141.0 (C-8), 132.0 (C-1), 118.4 (C-7a), 111.3 (C-1a), 109.7 (C-2), 105.3 (C-7), 101.0 (-$OCH_2O$-), 102.8 (C-4), 93.2 (C-10), 77.9 (C-11a), 65.8 (C-6), 39.0 (C-6a).

(6a*R*,11a*R*)-(−)-2-Methoxymaackiain (**CG7**): white powder; $[\alpha]_D^{25}$ −331 (*c* 0.1, $CHCl_3$); APCI-MS positive *m/z* 315 $[M+H]^+$; $^{1}$H- and $^{13}$C-NMR data in $CDCl_3$ were in good agreement with literature data [138, 139]. NMR data in DMSO-$d_6$ are reported for the first time: $^{1}$H-NMR (500 MHz, DMSO-$d_6$) δ (ppm): 9.30 (1H, s, OH), 6.96 (1H, s, H-1), 6.93 (1H, s, H-7), 6.53 (1H, s, H-4), 6.32 (1H, s, H-10), 5.49 (1H, d, *J* = 6.7 Hz, H-11a), 5.94 and 5.91 (2x1H, 2xs, -$OCH_2O$-), 4.19 (1H, m, H-6), 3.53 (2H, m, H-6, H-6a), 3.74 (3H, s, $OCH_3$); $^{13}$C-NMR (125 MHz, DMSO-$d_6$) δ (ppm): 153.7 (C-10a), 149.6 (C-4a), 148.2 (C-9), 147.4 (C-3), 143.0 (C-2), 141.0 (C-8), 118.5 (C-7a), 113.8 (C-1), 110.1 (C-1a), 105.4 (C-7), 103.8 (C-4), 101.0 (-$OCH_2O$-), 93.2 (C-10), 78.2 (C-11a), 65.9 (C-6), 56.2 ($OCH_3$), 40.0 (C-6a).

Hesperetin (**CG12**): APCI-MS positive *m/z* 303 $[M+H]^+$, 176, 153; $^{1}$H- and $^{13}$C-NMR data were in good agreement with literature data [140], but in DMSO-$d_6$ are reported for the first time: $^{1}$H-NMR (500 MHz, DMSO-$d_6$) δ (ppm): 12.10 (1H, brs, OH), 6.93 (1H, d, *J* = 8.5 Hz, H-5'), 6.92 (1H, d, *J* = 1.7 Hz, H-2'), 6.86 (1H, dd, *J* = 8.4, 1.7 Hz, H-6'), 5.88 and 5.86 (2x1H, 2xd, *J* = 1.9 Hz, H-6, H-8), 5.42 (1H, dd, *J* = 12.3, 2.9 Hz, H-2), 3.77 (3H, s, OCH3), 3.18 (1H, dd, *J* = 17.1, 12.5, H-3a), 2.69 (1H, dd, *J* = 17.1, 3.0, H-3b); $^{13}$C-NMR (125 MHz, DMSO-$d_6$) δ (ppm): 196.0 (C-4), 167.2 (C-7), 163.5 (C-5), 162.8 (C-9), 147.9 (C-4'), 146.5 (C-3'), 131.2 (C-1'), 117.7 (C-6'), 114.1 (C-5'), 112.0 (C-2'), 101.6 (C-10), 95.9 (C-6), 95.1 (C-8), 78.2 (C-2), 55.7 ($OCH_3$), 42.1 (C-3).

The further compounds were identified by comparing of their physical and spectroscopic data with reported data as luteolin (**CG1**) [141, 142], naringenin 5-*O*-β-glucoside (= helichrysin B) (**CG4**) [143], afrormozin (**CG9**) [144], formononetin (**CG10**) [145], liquiritigenin (**CG11**) [137, 140], naringenin (**CG13**) [137, 140], 5,7,3',5'-

tetrahydroxyflavanone (**CG14**) [142], genistein (**CG15**) [146], isoliquiritigenin (**CG16**) [147] and diosmetin (**CG17**) [140]. **CG5** was identified as piceol (= 4-hydroxyacetophenone) and **CG6** as 4-hydroxybenzaldehide based on their $^{1}$H-, $^{13}$C-NMR and MS data.

### 5.2.3. **Oestrogen-like activity**

Recently, the phytoestrogenic potential of extracts from different *Cyclopia* species was reported, as well as some compounds, present in *Cyclopia* were also tested [6, 65, 72, 74]. However bioactivity-guided isolation was reported from *C. subternata*, but not from *C. genistoides*, which species also displayed significant phytoestrogenic activity in previous studies [63, 65].

The methanol extracts from fermented and non-fermented *C. genistoides* were assayed with a highly efficient and convenient transgenic plant system, *Arabidopsis thaliana* pER8:GUS line, in order to detect oestrogenic/anti-oestrogenic activity. The transgenic plant pER8:GUS, with the GUS gene as a gene fusion marker for the analysis of gene expression, can be used to quantify the bioactivity of phytoestrogens [148]. Moreover, it is a visible system, primary results can be readily observed visually, without the need of special instrumentation. The system contains an oestrogen receptor-based transactivator vector (XVE) as an activator unit and the GUS (β-glucuronidase) gene as a reporter [149].

The XVE system is an oestrogen 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 oestradiol; in case of the presence of oestrogen active compounds the activator stimulates the expression of GUS transcription [149]. GUS protein containing transgenic plants give blue colour, after adding a glucopyranosiduronic acid containing dye.

After the solvent-solvent partition of the methanolic extracts of the fermented and non-fermented herb of *Cyclopia genistoides*, the *n*-hexane, EtOAc, $CH_2Cl_2$ and aqueous MeOH layers were screened by the transgenic plant pER8:GUS reporter system at 100 and 200 μg/ml. Oestrogenic activity of the extracts was detected via a histochemical assay for GUS activity. The $CH_2Cl_2$ and EtOAc layers of the fermented and non-fermented *C. genistoides* were proved to be active, thus were selected for bioactivity-guided fractionation, by the means of HPLC, MPLC, VLC, RPC, CC and preparative TLC. From the $CH_2Cl_2$ fraction of the fermented plant material four out of fourteen subfractions, yielded via polyamide column chromatography, exhibited oestrogenic like effects (P8-P11, MAC ≤ 200

μg/ml). P8 and P11 contained one, P9 two and P10 three of the isolated compounds with oestrogen-like activity. From the EtOAc fraction of the non-fermented *C. genistoides* four out of twelve VLC subfractions were active in the oestrogen-like reporter assay (V2, V3, V6, V7, MAC ≤ 200 μg/ml). One, two and two active constituents were found in V7, V2 and V3 subfractions, respectively.

Bioassay-directed chromatographic fractionation led to the isolation of six flavonoids with oestrogenic activity: CG1, CG4, CG13, CG14, CG15, and CG16, luteolin, helichrysin B (naringenin-5-*O*-β-glucoside), naringenin, 5,7,3',5'-tetrahydroxyflavanone, genistein and isoliquiritigenin, respectively. The compounds were identified by comparing their physical and spectroscopic data with reported data and by APCIMS/MS [145, 150-152]. From the fractions of the $CH_2Cl_2$ layer exhibiting oestrogenic activity, P9 yielded CG13, CG14, fraction P10 afforded CG15, CG16 and P11 furnished CG1 (**Figure 3**). Fractions P8 and P10 were also analysed by HPLC (**Method 2, Instrument 2**) and CG13 and CG14 were also detected from these fractions, respectively. From the EtOAc layer, the active fractions V2 and V3 were also chromatographed by HPLC (**Method 2, Instrument 2**). CG14 was detected in both fractions, while CG13 only in V2. The active constituent CG1 and CG4 were isolated from active fractions V3 and V7, respectively (**Figure 3**).

The least potent compound was helichrysin B with a MAC of 115 μM (**Figure 4**). Two compounds, which have not yet been isolated from *C. genistoides*, genistein and isoliquiritigenin exhibited substantial effect with a MAC of < 11.56 and 12.19 μM. Luteolin, naringenin and 5,7,3',5'-tetrahydroxyflavanone possessed oestrogen-like activity too, with a MAC of 87.5, 23.0 and 86.5 μM. The minimum active concentration of the control, $E_2$ was found to be 2.5 nM.

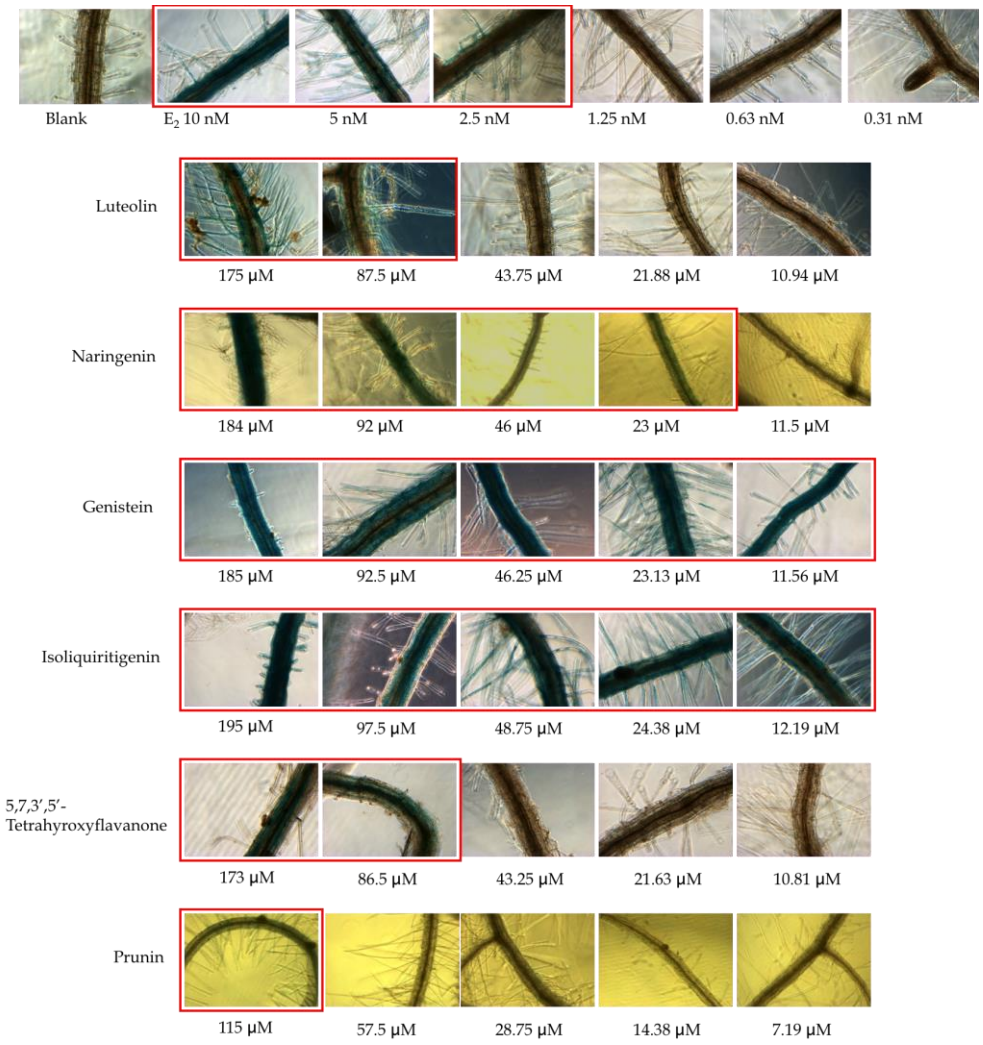



**Figure 4.** Oestrogenic MAC of the active isolated compounds in the histochemical assay
The concentrations where the blue colour was detectable are surrounded with red boxes, indicating oestrogenic activity. The last/only concentration where the blue colour was still detectable was considered the minimum active concentration.
$E_2$: 17-β-oestradiol

### 5.2.4. **HPLC quantification of compounds with oestrogen-like activity**

The quantitative comparison of the six active compounds between the fermented and non-fermented *C. genistoides* was performed by RP-HPLC (**Method 2, Instrument 2**). Peaks were identified by comparison of retention times and UV-Vis spectra (PDA detector) with those of the isolated compounds.

While both the processed and unprocessed plant 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 fold, respectively (**Table 2**). On the other hand, the non-fermented *Cyclopia* had higher quantities of the naringenin-glycoside, which showed the weakest oestrogen-like activity amongst the six active compounds.

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 non-fermented sample. Genistein was not detected in any of the extracts.

| **Compound** | **MeOH extract** | | **Aqueous extract** | | LOD (µg) | LOQ (µg) |
|---|---|---|---|---|---|---|
| | Fermented (mg/g dried plant material) | Non-fermented (mg/g dried plant material) | Fermented (mg/g dried plant material) | Non-fermented (mg/g dried plant material) | | |
| Luteolin | 0.0439 ± 0.0062 | 0.0511 ± 0.0144 | 0.0152 ± 0.0012 | 0.0174 ± 0.0062 | 0.2260 | 0.7533 |
| Naringenin | 0.1334 ± 0.0122 | 0.0037 ± 0.0005 | 0.0391 ± 0.0016 | 0.0027 ± 0.00003 | 0.1880 | 0.6268 |
| Genistein | n.d. | n.d. | n.d. | n.d. | 0.2578 | 0.8594 |
| Isoliquiritigenin | 0.0038 ± 0.0006 | 0.0026 ± 0.0001 | n.d. | n.d. | 0.3125 | 1.0416 |
| 5,7,3',5'-tetrahydroxy-flavanone | 0.0842 ± 0.0301 | 0.0079 ± 0.0024 | 0.0489 ± 0.0076 | n.d. | 0.2750 | 0.9167 |
| Helichrysin B | 0.1623 ± 0.0463 | 0.2375 ± 0.0930 | 0.3279 ± 0.0261 | 0.3376 ± 0.0540 | 0.3290 | 1.0967 |

**Table 2**. The quantitative determination of the active compounds
n.d. (not detected), LOD limit of detection, LOQ limit of quantification, values are given in ±SD

In another experiment the possible effect of the intestinal flora on aqueous extracts of fermented and non-fermented *C. genistoides* using commercially available mixture of probiotic bacteria was assessed. Two bioactive constituents (naringenin, luteolin) were quantified by HLPC (**Instrument 2, Method 2** – with an injection volume of 45 µl) before and after bacterial fermentation of the aqueous extracts. While both the processed and unprocessed plant contained similar amounts of luteolin, the naringenin content in the fermented honeybush was higher. However, after 50 hours of bacterial fermentation most likely due to the loss of sugar moiety, the naringenin and luteolin content was approximately similar in both the fermented and non-fermented honeybush (**Annex 2**). The concentration of luteolin and naringenin were higher but similar in both processed and unprocessed herbs after glycosidic cleavage by bacteria.

### 5.2.5. **Antiproliferative activity**

Fractions and compounds *of Cyclopia* extracts, were also reported to be able to 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 [72, 74, 153]. In order to measure the

antiproliferative effect of the isolated compounds, antiproliferative testing was conducted on T47D (ER positive) and A2780 (ER negative) human cancer cells.

In **Table 3**, fractions and compounds with inhibition values above 30% either in A2780 or T47D cells are displayed. While in the pER8:GUS assay P8-11, V2, V3, V6, V7 showed oestrogenic activity; in the antiproliferative tests, P8, P10, P11 and V3 demonstrated inhibition greater than 30% in either cell-lines. Except of helichrysin B and 5,7,3',5'-tetrahydroxyflavanone, all compounds with oestrogen-like activity (naringenin, luteolin, isoliquiritigenin, genistein) exhibited substantial antiproliferative activity against the tested cell lines. All four of them had a greater inhibition towards the ER negative A2780.

| Substance / fraction | 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 | –* | 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 |

**Table 3.** Fractions and compounds exhibiting substantial (above 30%) antiproliferative activity against either A2780 or T47D cells.
*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.

### 5.2.6. **Xanthine oxidase inhibitory activity**

*Cyclopia* species are valuable sources of bioactive compounds, due their content of vast range of polyphenolic constituents. They are likely to be responsible for the studied oestrogen-like, antimutagen, chemopreventive, pancreatic β-cell protective and antioxidant activities [6, 67, 110, 154]. Considering the phytochemistry and bioactivities of *Cyclopia* species, testing the extracts and compounds for xanthine oxidase inhibitory activity seemed plausible.

Both dichloromethane layers derived from the methanolic extract of the fermented and non-fermented plant material exerted xanthine oxidase inhibitory activity thus were subjected

to further chromatography. The $CH_2Cl_2$ layers of the fermented and non-fermented plant material were separated into fourteen and twelve fractions by a polyamide column (**OCC-P**) with mixtures of MeOH and $H_2O$ as eluents. Fractions PP8 from the non-fermented and P10 from the fermented herbal substance were amongst the fractions with the strongest inhibition of xanthine oxidase (**Figure 5**). Further purification of these fractions led to the isolation of luteolin and diosmetin (**CG1** and **CG17)** exerting remarkable XO inhibitory effect with $IC_{50}$ values of 0.84 μM (95% confidence interval 0.80 to 0.91 μM) and 0.53 μM (95% confidence interval 0.40 to 0.80 μM), respectively. The inhibitory activity of both compounds significantly exceeded that of allopurinol ($IC_{50}$ = 7.49 ± 0.29 μM), which was used as a positive control.

Alongside with the bioactivity-guided isolation, all other isolated compounds were tested. From the 15 already isolated constituents only two structurally close flavanones, hesperetin (**CG12**) and 5,7,3',5'-tetrahydroxyflavone (**CG14**) exhibited a weak inhibition ($IC_{50}$ = 55.20 μM (95% confidence interval 41.40 to 73.51 μM) and 120.55 μM (95% confidence interval 101.71 to 142.86 μM), respectively). The rest of the isolated compounds showed no xanthine oxidase inhibition ($IC_{50}$ > 150 μM).

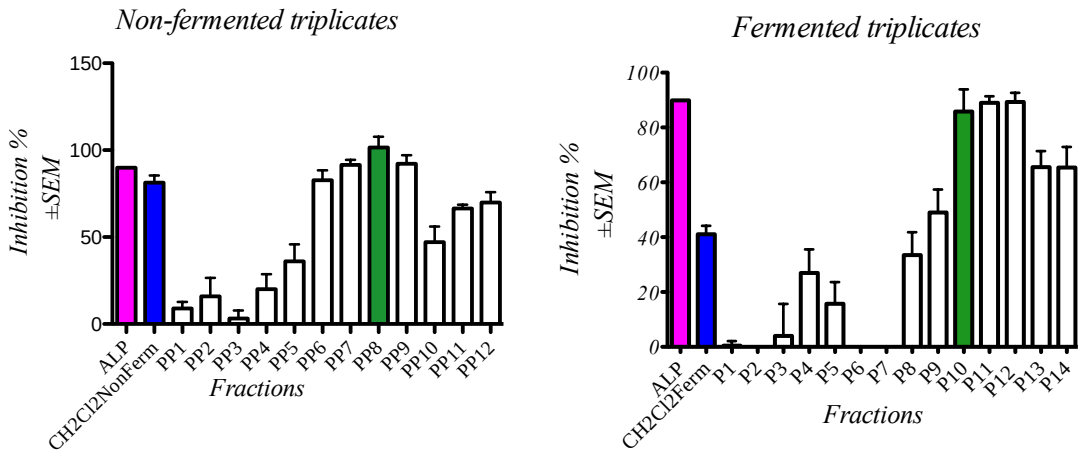



**Figure 5**. Xanthine oxidase inhibition by the fractions yielded from the $CH_2Cl_2$ layers of the fermented (P1-P14) and non-fermented (PP1-PP12) plant material. ALP - allopurinol. $CH_2Cl_2$Ferm - $CH_2Cl_2$ layer of the fermented honeybush. $CH_2Cl_2$NonFerm - $CH_2Cl_2$ layer of the non-fermented honeybush.

# 6. DISCUSSION

## 6.1. Sympathomimetic activity of *H. gordonii*

In the course of the investigation of the mechanism of action of the *Hoodia gordonii* extract containing product, it showed a propranolol-sensitive and concentration-dependent relaxation of uterine rings, against both spontaneous and stimulated contractions. Hence a sympathomimetic effect with substantial β-receptor-mediated contribution is proposed. The results support the cardiovascular side effects reported in a human clinical trial [98].

The gestation-dependent myometrial effect (weaker relaxing effect on uteri from late-pregnant animals) of substances acting on the sympathetic system could be explained by the changes in the receptor function during pregnancy. The nonpregnant uterus of the rat exhibits limited α-adrenoceptor-mediated contraction but efficiently relaxed by β-adrenergic stimulation. Late-pregnant myometrium is sensitive to both α- and β-adrenergic stimulation, and therefore, the overall response is determined by the receptor preference of the tested substance [135]. It is plausible that the limited relaxation of late-pregnant myometrium, induced by the product, is a consequence of a balanced α- and β-adrenoceptor stimulation that can be shifted towards increased contractility by masking the action, mediated through β-receptors.

The *Hoodia* product contained alcohol, and since ethanol may alter β-adrenergic receptor binding affinity [155], the sample of Hoodia spray was evaporated to dryness, and then the residue was dissolved to the original volume with physiological saline - DMSO (95:5). Since the tested *Hoodia* commercial product possesses sympathomimetic effects, its use may cause both appetite suppression and increased thermogenesis, resulting in weight loss [156, 157]. Moreover, in the experiments performed in the US patent application, increased water consumption was recorded during feeding studies, which is a well-known effect of sympathomimetic agents. The side effects (increase in blood pressure, pulse, heart rate), reported in the human clinical trial, is also in line with a possible sympathomimetic action [98].

Chemical composition of *Hoodia* has not been fully mapped yet. The investigations performed with *H. gordonii* primarily focussed on the pregnane glycosides; however, other potentially active constituents with sympathomimetic activity may also be present in the plant.

In an animal study a *H. gordonii* commercial dried extract interacted with the noradrenergic, dopaminergic and serotonergic systems [106]. Although, this study examined

the anti-depressant effect of *Hoodia*, the mechanism of action showed similarity with other anti-obesity drugs; for example, a popular combination of two weight loss medication fenfluramin and phentermin (fen-phen), acting on the noradrenergic and serotonergic signaling pathways. However, fen-phen, was withdrawn due to its association with cardiac valvular heart disease and pulmonary hypertension [158]. Sympathomimetic agents as amphetamine derivatives or sibutramine are effective in weight management, but they pose serious risk to public health due to their serious cardiovascular side effects. In the case of *H. gordonii*, cardiovascular side effects and proposed anorectic property are reported, raising the questions whether these effects are caused by the same constituents; and if they are, whether the responsible compound is truly P57. Our results are indicating that *Hoodia* acts similarily to amphetamine derivates, thus if a *Hoodia* product consumption results in weight-loss, the reported side effects most likely will manifest. Hence, the inclusion of *Hoodia species* to the food-supplement area may be dangerous.

### 6.2. Structure elucidation

Fractionation (mainly bioactivity-guided) of *Cyclopia genistoides* led to the isolation of 17 compounds from which fourteen were first isolated from this species, ten from the genus.

The isolated compounds are amorphous solids or crystals. The structures of the isolated compounds were elucidated by means of spectroscopic methods. From HRESIMS measurements, the molecular compositions were determined. The most useful data concerning the chemical structures were furnished by 1D and 2D NMR spectroscopy.

Multistep chromatographic separation and purification procedure, including CC, VLC, RPC, PLC, MPLC and HPLC, resulted in the isolation of pure compounds **CG1-17**. **CG2** and **CG3** were identified as benzophenone derivatives based on their spectral characteristics. **CG2** was identified as iriflophenone 2-*O*-β-glucopyranoside by comparing its spectral data with those reported in the literature [136]. **CG3** proved to be identical with iriflophenone 3-*C*-β-glucopyranoside, isolated earlier from *C. genistoides* [70], and *C. subternata* [159].

**CG7** and **CG8** were found to have a pterocarpan nucleus, substituted with methylenedioxy, hydroxy and methoxy groups. After detailed MS and NMR studies **CG8** could be identified as (6aR,11aR)-(−)-maackiain [160] and **CG7** as (6aR,11aR)-(−)-2-methoxymaackiain [138, 139]. Two-dimensional NMR investigations, including $^{1}$H-$^{1}$H COSY, NOESY, HSQC and HMBC experiments, permitted unpublished $^{1}$H and $^{13}$C assignments for both compounds. This is the first isolation of 2-methoxymaackiain (**CG7**)

and maackiain (**CG8)** from the *Cyclopia* genus; previously these compounds were published only from *Ulex* and other Fabaceae species [161-163].

Fourteen compounds [(iriflophenone 2-*O*-β-glucopyranoside (**CG2**), helichrysin B (**CG4**), piceol (**CG5**), 4-hydroxybenzaldehid (**CG6**), (−)-2-methoxymaackiain (**CG7**), (−)-maackiain (**CG8**), afrormozin (**CG9**), formononetin (**CG10**), liquiritigenin (**CG11**), naringenin (**CG13**), 5,7,3',5'-tetrahydroxyflavanone (**CG14**), genistein (**CG15**), isoliquiritigenin (**CG16**), diosmetin (**CG17**)] were first isolated from the species and ten [iriflophenone 2-*O*-β-glucopyranoside (**CG2**), helichrysin B (**CG4**), piceol (**CG5**), 4-hydroxybenzaldehid (**CG6**), (−)-2-methoxymaackiain (**CG7**), (−)-maackiain (**CG8**), liquiritigenin (**CG11**), 5,7,3',5'-tetrahydroxyflavanone (**CG14**), genistein (**CG15**) and isoliquiritigenin (**CG16**)] from the genus *Cyclopia*.

### 6.3. Oestrogen-like activity of *C. genistoides*

There are plenty available methods for the investigation of oestrogenic potential, yet the complexity of the mechanisms of action of phytoestrogens and phytoestrogen containing herbal preparations triggers divergent outcomes, depending on the method used. 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 an ideal screening system for the preliminary investigation of plants with proposed oestrogen-like activities. Furthermore, while cytotoxicity is a limiting factor of *in vitro* mammalian cell-based models, the transgenic plant system expressed tolerance towards higher doses of cytotoxic compounds [164]. pER8:GUS cannot distinguish between ER agonists and antagonists, however, theoretically it may be possible, if the test compounds are administered together with $E_2$. 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 towards ER-β), hence this model is suitable for natural product screening [148, 149, 165, 166].

Mainly, the pharmacological studies, testing phytoestrogenic potential of *Cyclopia* species have focused on different extracts. Bioactivity-guided isolation made it possible to identify six compounds responsible for the action, four of them first reported in the genus *Cyclopia*. Interestingly naringenin was present in the plant in high quantities, despite the fact that in two studies it was declared absent according to HPLC determination [65, 72]. Genistein – usually used as positive reference in phytoestrogenic studies – was also found in the herbal substance. During the later preparative work, further well-known phytoestrogens were

isolated from the $CH_2Cl_2$ layer of the methanolic extract of the fermented plant material: diosmetin, afrormosin, liquiritigenin (a selective ER-β antagonist) and formononetin (reported higher affinity towards ER-β) [72, 167, 168]. Hesperetin was also isolated, which was reported to be one of the most potent natural aromatase inhibitors [169]. Luteolin, genistein, isoliquiritigenin and naringenin are widely known phytoestrogens. Naringenin, luteolin and genistein were able to displace 70%, 92% and 95% of the [$^3$H]-$E_2$ from *h*ERβ, respectively, exerting the highest displacements when 10 phytoestrogens were compared [65]. Phytoestrogens have the potential to maintain bone health and delay or prevent osteoporosis, one of the postmenopausal symptoms. Genistein (54 mg/day) was found to have positive effects on bone mineral density on osteopenic postmenopausal women [170]. Isoliquiritigenin is also a promising agent for bone destructive diseases [171]. Next to their effect on the bone they also possess other activities, potentially important in the treatment of postmenopausal symptoms. Genistein and luteolin *in vitro* suppressed the induction of the proliferation-stimulating activity of environmental oestrogens, suggesting anti-estrogenic and anti-cancer effect [172]; and naringenin attenuated many of the metabolic disturbances associated with ovariectomy in female mice [173]. Genistein was also associated with favourable effects on both glycaemic control and some cardiovascular risk markers in postmenopausal women [174]. Regular grapefruit juice (contains high amounts of naringenin) consumption by middle-aged, healthy postmenopausal women was found to be beneficial for arterial stiffness [175].

The presence of these phytoestrogens 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 medicinal use of honeybush [65, 72-74].

### 6.4. HPLC quantification of compounds with oestrogen-like activity

The effect of the fermentation on honeybush tea and the plausibility of the tradition of fermentation was an interesting aspect to explore. Fermentation reduces the total phenolic content, yet traditionally the fermented tea is consumed. It is also important to note that it was shown that the total phenolic content of *C. genistoides* - the first *Cyclopia* species to be used as tea - was the least affected by fermentation (77% retention) when compared to the other three, commercially important *Cyclopia* species [67]. The degradation of flavonoid-glycosides during the fermentation process could explain the difference in the measured amounts of the active compounds between the samples. From the six compounds with

oestrogen-like activity, three differed in quantity between the fermented and non-fermented herbs. Compounds more abundant in the fermented plant material (5,7,3',5'-tetrahydroxyflavanone, naringenin) displayed stronger oestrogen-like activity than helichrysin B (higher quantities in the non-fermented honeybush) providing a rationale to the fermentation process.

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 infusion of honeybush. Oestrogenic 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 [6]. 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 non-fermented or fermented *C. genistoides* and *C. subternata* were able to significantly displace 1 nM [$^3$H]-$E_2$ from hERβ. Although, this effect was not observed in all tested harvestings, it did show the possibility of an aqueous extract to be oestrogenic. It also has to be taken into account, that although the isolated flavonoids are present in small quantities, the oestrogenic activity of *Cyclopia* extracts is the result of a fine balance between different polyphenols present in varying amounts with varying phytoestrogenic potential. A good example is a recently performed study, where the activity guided fractionation of an extract from *C. subternata*, which displayed three desirable oestrogenic attributes, showed, that the retention of all these attributes in one fraction is not an attainable goal [63].

Although, the HPLC quantification underpinned the tradition of fermentation, but taking into account the conversion of polyphenolic glycosides by intestinal flora, eventually the difference between the two forms of honeybush may not be substantial. The traditional fermentation process (high temperature oxidation) reduces the total polyphenolic content of *Cyclopia* spp., but according to our results, bacterial fermentation may increase the content of aglycones, which usually possess more pronounced bioactivities than their corresponding glycosides, and their bioavailability is remarkably higher than those of the respective glycosides [67, 176-180].

The naringenin and luteolin content was measured in processed and unprocessed *C. genistoides*, before and after bacterial fermentation. Both bioactive aglycones are present

in the plant alongside with their glycosides, making them perfect candidates for studying glycosidic cleavage. While the naringenin content was higher in the traditionally used, processed plant material, the bacterial fermentation equalised this difference. Hence, the difference in the concentration of other bioactive aglycones between processed and unprocessed herbal substance may also decrease or disappear after bacterial fermentation. However, it has to be noted, that several studies on the phenolic composition of honeybush have demonstrated that large variation exists between different batches of plant material from the same plantation. Hence, a direct comparison of the processed and unprocessed *C. genistoides* would be more ideal when the plant material is processed on lab-scale and not on factory-scale, which could not been performed in our study.

In our experiment the concentration of luteolin and naringenin were higher in both processed and unprocessed herbs after glycosidic cleavage by bacteria, which is important regarding, that glycosides cannot be absorbed in their native form in the small intestine, but must be hydrolysed by before absorption [178]. In unprocessed plant materials glycosides are usually present in higher quantities. For example, soybean contains negligible amounts of isoflavone aglycones unless it is fermented [177].

The majority of the commercially available honeybush teas are fermented products, similar to the traditionally consumed teas, however, recently the non-fermented form has also been commercialised. In some advertisements it is claimed that e.g. “the unfermented “green” honeybush is also popular and contains even more anti-oxidants” [181]. This notion may be based on *in vitro* experiments, where the traditional fermentation process reduced the total polyphenol content and consequently the antioxidant activity of honeybush [67]. However, taking into account the conversion of polyphenolic glycosides by intestinal flora, the difference between the two forms of honeybush practically may not be substantial.

### 6.5. Antiproliferative activity of *C. genistoides*

In the MTT assay, naringenin, luteolin, isoliquiritigenin and genistein exhibited substantial antiproliferative activity against the tested cell lines. All four of them had a greater inhibition towards the ER negative A2780 cell line, 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 oestrogenic 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 oestrogens, as components of foetal bovine serum, and

therefore the obtained results do not support a direct relationship between the two determined activities.

### 6.6. Xanthine oxidase inhibitory activity of *Cyclopia genistoides*

Gout is the most prevalent form of inflammatory arthropathy with the precondition of elevated serum urate levels; thus urate-lowering xanthine oxidase inhibitors are the cornerstones of a successful long-term gout management [182]. The first-line treatment in the urate-lowering therapy (ULT) of gout is the xanthine oxidase inhibitor allopurinol, which needs to be gradually increased to achieve the therapeutic target, and one of its adverse reactions is the rare but potentially lethal allopurinol hypersensitivity syndrome. Of the two agents available for ULT, febuxostat is more expensive which may in part limit its use, and rarely associated with hypersensitivity vasculitis. Hence new xanthine oxidase inhibitors are needed in gout therapy, but beside gout, hyperuricaemia may also be an independent risk factor in cardiovascular and renal diseases, which underlines the importance of this class of compounds [183].

The bioactivity-guided isolation of the fermented and non-fermented plant materials led to the isolation of diosmetin and luteolin with an inhibitory activity exceeding that of allopurinol, currently used in therapy.

## 7. SUMMARY

*H. gordonii* and *C. genistoides* attracted considerable interest because of their potential therapeutic use.

Although, previous pharmacological studies have shown that *H. gordonii* could have different mechanism of action than the already withdrawn sympathomimetic medicines, thus a possibly better risk/benefit ratio than the former antiobesity drugs. Yet, our research indicated otherwise. The recorded sympathomimetic effect with substantial β-receptor-mediated contribution is similar to the mechanism of action of several already withdrawn weight loss medications. As this result is in accordance with the reported side effects, the use of *Hoodia gordonii* as a possible anti-obesity drug is questionable.

We linked the phytoestrogenic activity of *C. genistoides* extracts to compounds, isolated via bioactivity-guided fractionation, using the transgenic plant system pER8:GUS. The extracts of the plant materials were subjected to multiple step chromatographic separation (including CC, VLC, MPLC, HPLC, PLC, RPC) resulting in the isolation of 17 compounds from which 14 were first isolated from this species and 10 from the genus.

Antiproliferative MTT assays were also performed on A2780 and T47D human cancer cell lines. The results suggested, that oestrogen induced cell-proliferation or oestrogen independent antiproliferative effect might have played a role.

The recorded xanthine oxidase inhibitory activity of two compounds isolated from the dichloromethane layers, added to the long list of bioactivities of *Cyclopia* species.

The effect of fermentation on the phytochemical content was also investigated with the means of HPLC. The results supported the tradition, as the fermented honeybush tea was rich in compounds with stronger phytoestrogenic activity, while the non-fermented tea contained larger amounts from the less active glycoside. However, bacterial fermentation levelled the difference in the concentration of naringenin between the aqueous extracts from fermented and non-fermented plant materials.

Although, the quantitative comparison of fermented and non-fermented honeybush implies, that the fermented tea has 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 different ER isoforms can result in phytoestrogenic effect of different extracts, even if the individual compounds are small in quantity.

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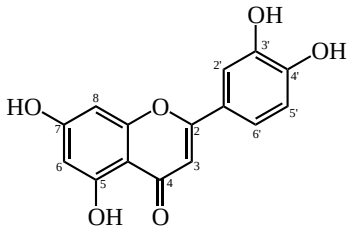
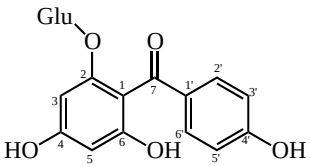
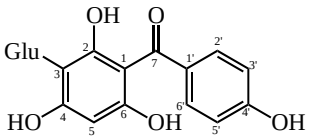
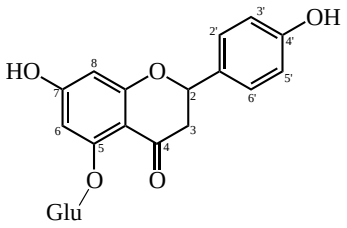
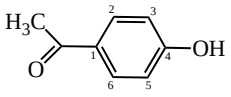
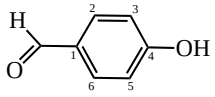
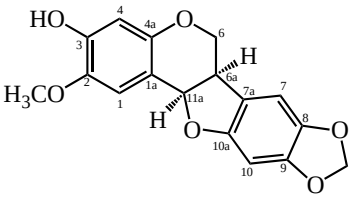
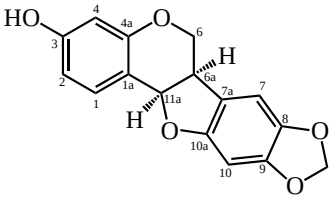
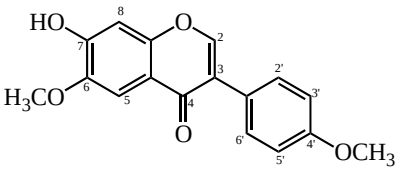
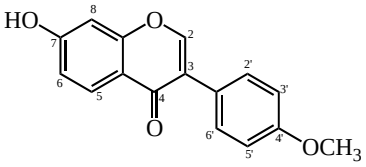
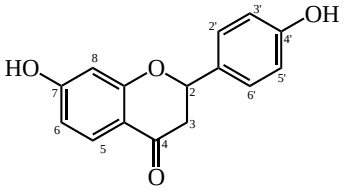
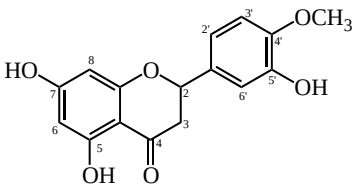
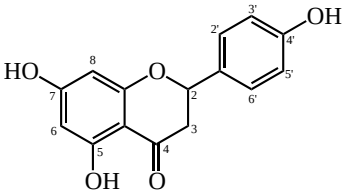
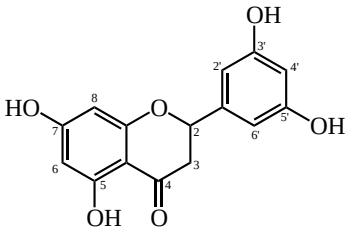
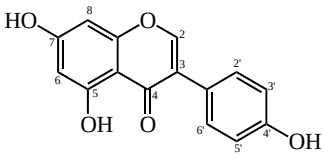
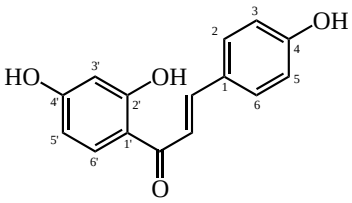
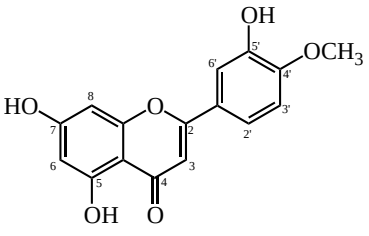
**Annex 1.** Isolated compounds from *Cyclopia genistoides*

| | | |
|---|---|---|
|  |  |  |
| Luteolin | Iriflophenone 2-*O*-β-glucoside | Iriflophenone-3-*C*-β-glucoside |
|  |  |  |
| Helichrysin B | Piceol | 4-hydroxybenzaldehid |
|  |  |  |
| (-)-2-methoxymaackiain | (-)-maackiain | Afformosin |
|  |  |  |
| Formononetin | Liquiritigenin | Hesperetin |
|  |  |  |
| Naringenin | 5,7,3’,5’ tetrahydroxyflavanone | Genistein |
|  |  | |
| Isoliquiritgenin | Diosmetin | |

**Annex 2.** Naringenin and luteolin content of fermented and non-fermented *Cyclopia genistoides* before and after 50 hours of bacterial fermentation

| | Luteolin mg/gram | | Naringenin mg/gram | |
|---|---|---|---|---|
| | 0 hour | 50 hours | 0 hour | 50 hours |
| Fermented plant material | 0.0092 ± 0.006 | 0.0101 ± 0.0008 | 0.0233 ± 0.0012 | 0.0243 ± 0.0002 |
| Non-fermented plant material | 0.0081 ± 0.0006 | 0.0111 ± 0.0007 | 0.0185 ± 0.0004 | 0.0254 ± 0.0001 |

## APPENDICES

The thesis is based on the following publications:

I. **Roza O**, Lovász N, Zupkó I, Hohmann J, Csupor D
Sympathomimetic activity of a *Hoodia gordonii* product: a possible mechanism of cardiovascular side effects
*Biomed Res Int* 2013; **Paper ID 171059**: 6 pages

II. **Roza O**, Lai W-C, Zupkó I, Hohmann J, Eloff JN, Chang F-R, Csupor D
Bioactivity-guided isolation of phytoestrogenic compounds from *Cyclopia genistoides* by pER8:GUS reporter system
*South African Journal of Botany* 2016; *In Press*.

III. **Roza O**, Martins A, Hohmann J, Lai W-C, Eloff JN, Chang F-R, Csupor D
Flavonoids from *Cyclopia genistoides* and their xanthine oxidase activity
*Planta Medica* 2016; **82**:1274-1278.