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Promising assets of Southern Africa?

Cyclopia genistoides and Hoodia gordonii

Summary of Ph.D. Thesis

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MATERIALS AND METHODS

For the comparison of a *Hoodia* product (Hoodia spray) with authentic plant material, the extract of *H. gordonii* was prepared for high-performance liquid chromatography (HPLC) and high performance thin-layer chromatography (HPTLC) analyses from fresh ground plant material with AcNi, using ultrasonic bath. The Hoodia spray was used directly for these studies. For the investigation of falsification of this product, the Hoodia spray itself was used for thin-layer chromatography (TLC) and mass-spectroscopy (MS) analyses. The Hoodia spray was evaporated to dryness, and then the residue was dissolved with physiological saline-DMSO (95:5), and after filtered to perform the organ baths studies.

The dried fermented and non-fermented herbs of *C. genistoides* were extracted via ultrasonication with methanol. The extract was concentrated *in vacuo*, then diluted with H₂O, and liquid-liquid partition was performed, affording *n*-hexane, dichloromethane, ethyl-acetate, the remnant aqueous layers and insoluble part.

The compounds were isolated by multistep chromatographic methods, including column chromatography (CC), vacuum-liquid chromatography (VLC), rotation planar chromatography (RPC), medium-pressure liquid chromatography (MPLC), preparative layer chromatography (PLC), gel filtration (OCC-SPh) and HPLC. Normal- or reversed-phase SiO₂ (NP/RP), polyamide or Sephadex LH-20 were applied as stationary phases.

The isolated compounds were characterized and their structures were elucidated by means of different spectroscopic methods (NMR, HRESIMS, ESIMS, APCIMS and UV).

For HPLC quantitative determination of compounds, the dried fermented and non-fermented plant materials of *C. genistoides* were extracted with boiling tap water ("cup of tea"). For comparison a methanolic extract was also prepared.

In the course of the bacterial fermentation study, aqueous extracts of the fermented and non-fermented *C. genistoides* were prepared. After lyophilisation, the dry residues were dissolved in DMSO and physiologic saline solution and mixture of probiotic bacteria was added to each solution. These mixtures were mixed at 37 °C for 50 hours, and then objected to HPLC analyses.

The phytoestrogenic effects of the CH₂Cl₂ and the EtOAc extracts from the fermented and non-fermented *C. genistoides* and the isolated compounds thereof were assessed *in vitro* in the transgenic pER8:GUS system.

The compounds of *C. genistoides* were tested for antiproliferative activity *in vitro* against human tumour cell lines (A2780 (ovarian carcinoma), and T47D (breast carcinoma)).

The xanthine-oxidase inhibitor activity of the isolated compounds was also analysed.

RESULTS AND DISCUSSION

Sympathomimetic activity of *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. 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). Hence, as a first step, the comparison of the product with authentic plant material was performed alongside with testing for possible adulterants with potential for causing cardiovascular side effects (e.g. sibutramine, amphetamine derivatives). 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.

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

First, P57, a characteristic compound of *Hoodia*, was chosen for mass spectrometric identification using multiple reaction monitoring (MRM) mode. 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.

The similarities of chromatograms of the plant extract and the product confirmed that the commercial product was based on *Hoodia* extract. Sibutramine, amphetamine, methamphetamine and ephedrine, were not detected in the product.

Beta-adrenergic receptor agonist activity

β -adrenergic receptor agonists are reported to decrease food intake and exhibit anorectic properties. 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. Both α - and β -adrenergic receptors play crucial roles in the motor activity of myometrial smooth muscle, eliciting contraction and relaxation, respectively. In a 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. All experiments were performed with and without

nonselective β -antagonist propranolol (10 μ M). 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. The relaxing effect of the spray was substantially less pronounced on uteri from late-pregnant animals, but propranolol significantly modified it.

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. 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.

Based on the propranolol-sensitive component of the uterine action of the product, a sympathomimetic effect with substantial β -receptor-mediated contribution is proposed. The results support the cardiovascular side effects reported in a human clinical trial (increase in blood pressure, pulse, heart rate). Since the tested *Hoodia* commercial product possesses sympathomimetic effects, its use can cause both appetite suppression and increased thermogenesis, resulting in weight loss.

The recorded sympathomimetic effect with substantial β -receptor-mediated contribution is similar to the mechanism of action of several already withdrawn weight loss medications. Thus, if a *Hoodia* product consumption results in weight-loss, the reported side effects most likely will manifest.

Isolation of compounds

The purification of the CH_2Cl_2 -soluble phases of the fermented and non-fermented *C. genistoides* were performed by CC on polyamide and afforded 14 and 12 main fractions (P1-P14 and PP1-PP12). The EtOAc layer from the non-fermented *C. genistoides* was subjected to VLC on silica gel, and twelve combined fractions (V1-V12) were yielded. Several fractions showed either oestrogenic or xanthine oxidase inhibitory or antiproliferative activity or interesting chemical composition (according to TLC monitoring). Since the fractions demonstrated chemical complexity, they were subjected to a multistep chromatographic procedure (MPLC, HPLC, PLC,

RPC, CC) in order to isolate the compounds (CG1-CG17), with the use of silica gel (RP/NP) or Sephadex LH-20 and different solvent systems (**Figure 1**).

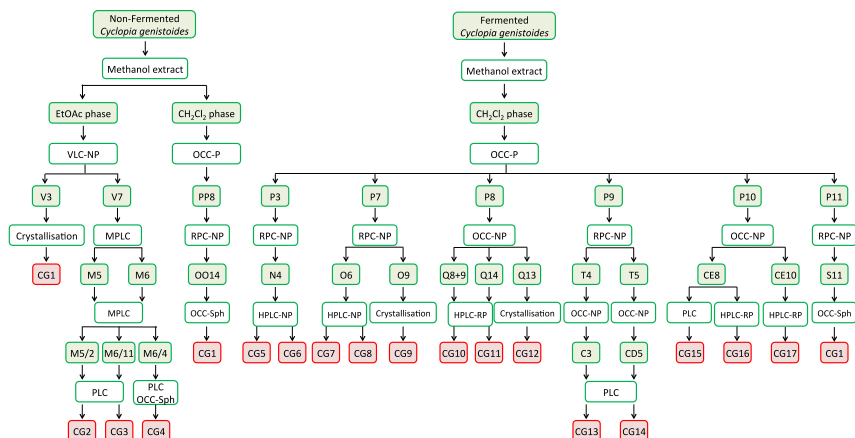


Figure 1. Isolation of compounds from *C. genistoides*

Characterisation and structure elucidation of the isolated compounds

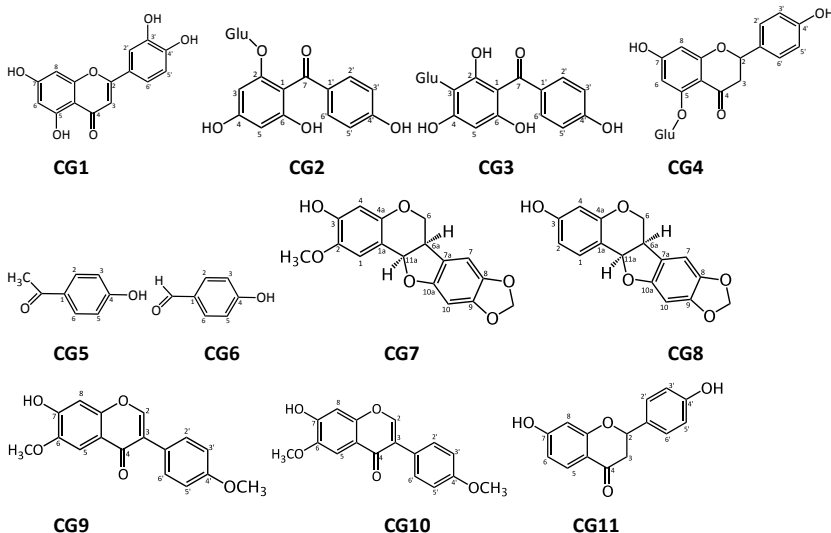
The structures of the isolated compounds were determined by means of spectroscopic methods. High-resolution mass spectrometric measurements allowed the determination of the exact molecular weight and molecular composition of the compounds. The most useful methods in the structure elucidation were 1D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

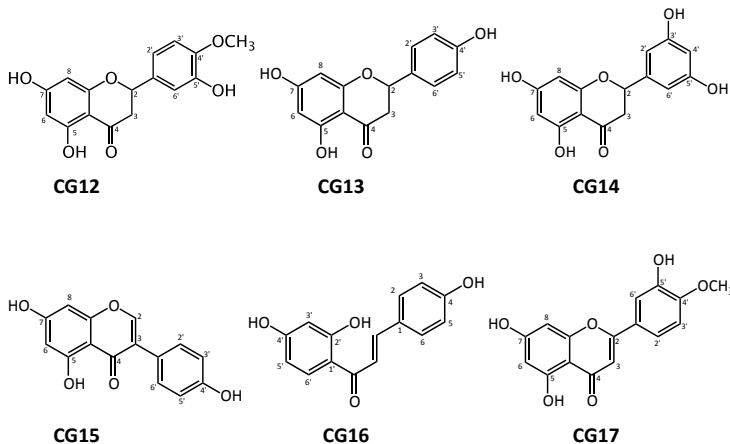
CG2 and **CG3** were identified as benzophenone derivatives based on their spectral characteristics. **CG2** was identified as iriflophenone 2-O- β -glucopyranoside by comparing its spectral data with those reported in the literature. **CG3** proved to be identical with iriflophenone 3-C- β -glucopyranoside, isolated earlier from *C. genistoides* and *C. subternata*.

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 and **CG7** as (6aR,11aR)-(-)-2-methoxymaackiain. Two-dimensional NMR investigations, including $^1\text{H-}^1\text{H}$ COSY, NOESY, HSQC and HMBC experiments, permitted unpublished ^1H and ^{13}C assignments for both compounds. This is the first isolation of 2-methoxymaackiain and maackiain from the *Cyclopia* genus; previously these compounds were published only from *Ulex* and other Fabaceae species.

The further compounds were identified by comparing of their physical and spectroscopic data with reported data as luteolin (**CG1**), naringenin 5-O- β -glucoside (= helichrysin B) (**CG4**), afrormozin (**CG9**), formononetin (**CG10**), liquiritigenin (**CG11**), naringenin (**CG13**), 5,7,3',5'-tetrahydroxyflavanone (**CG14**), genistein (**CG15**), isoliquiritigenin (**CG16**) and diosmetin (**CG17**). **CG5** was identified as piceol (= 4-hydroxyacetophenone) and **CG6** as 4-hydroxybenzaldehyde based on their ^1H -, ^{13}C -NMR and MS data. The ^1H - and ^{13}C -NMR data of hesperetin (**CG12**) were in good agreement with literature data, but in DMSO-d_6 are reported for the first time.

Fractionation (mainly bioactivity guided) of *Cyclopia genistoides* led to the isolation of 17 compounds. Fourteen compounds were first isolated from the species [(iriflophenone 2-O- β -glucopyranoside (**CG2**), helichrysin B (**CG4**), piceol (**CG5**), 4-hydroxybenzaldehyd (**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**)] and ten compounds from the genus *Cyclopia* [iriflophenone 2-O- β -glucopyranoside (**CG2**), helichrysin B (**CG4**), piceol (**CG5**), 4-hydroxybenzaldehyd (**CG6**), (-)-2-methoxymaackiain (**CG7**), (-)-maackiain (**CG8**), liquiritigenin (**CG11**), 5,7,3',5'-tetrahydroxyflavanone (**CG14**), genistein (**CG15**) and isoliquiritigenin (**CG16**)]. 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. Genistein – usually used as positive reference in phytoestrogenic studies – was also found in the herbal substance.





Oestrogen-like activity

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, has the ability to detect both ER agonists and antagonists and can be used to quantify the bioactivity of phytoestrogens. The system contains an oestrogen receptor-based transactivator vector (XVE) as an activator unit and the GUS (β -glucuronidase) gene as a reporter. The XVE activator comprises the regulation region of the ER- α , hence only determines ER- α interactions. It is strictly regulated by oestradiol; in case of the presence of oestrogen active compounds the activator stimulates the expression of GUS transcription. It is a visible system, primary results can be readily observed visually, because the 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 $\mu\text{g}/\text{ml}$. 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 PLC. From the CH_2Cl_2 fraction of the fermented plant material four out of fourteen subfractions, yielded via polyamide column chromatography, exhibited oestrogen-like effects (P8-P11, MAC \leq 200 $\mu\text{g}/\text{ml}$). P9 yielded CG13, CG14, fraction P10 afforded CG15, CG16 and P11

furnished CG1 (**Figure 1**). Fractions P8 and P10 were also analysed by HPLC and CG13 and CG14 were also detected from these fractions, respectively. 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 \leq 200 $\mu\text{g}/\text{ml}$). The active constituent CG1 and CG4 were isolated from active fractions V3 and V7, respectively (**Figure 1**). Active fractions V2 and V3 were also chromatographed by HPLC. CG14 was detected in both fractions, while CG13 only in V2..

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 least potent compound was helichrysin B with a minimum active concentration (MAC) of 115 μM (**Figure 2**). 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 and 86.5 μM . The minimum active concentration of the control, oestradiol (E_2) was found to be 2.5 nM.

Luteolin, genistein, isoliquiritigenin and naringenin are widely known phytoestrogens. Genistein (54 mg/day) was found to have positive effects on bone mineral density on osteopenic postmenopausal women. Isoliquiritigenin is also a promising agent for bone destructive diseases. Genistein and luteolin *in vitro* suppressed the induction of the proliferation-stimulating activity of environmental oestrogens, suggesting anti-oestrogenic and anti-cancer effect; and naringenin attenuated many of the metabolic disturbances associated with ovariectomy in female mice. 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 (selective ER- β antagonist), and formononetin (reported higher affinity towards ER- β). Hesperetin was also isolated, which was reported to be one of the most potent natural aromatase inhibitors.

The presence of these phytoestrogens gives a rationale to the traditional use of honeybush tea for menopausal symptoms. 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.

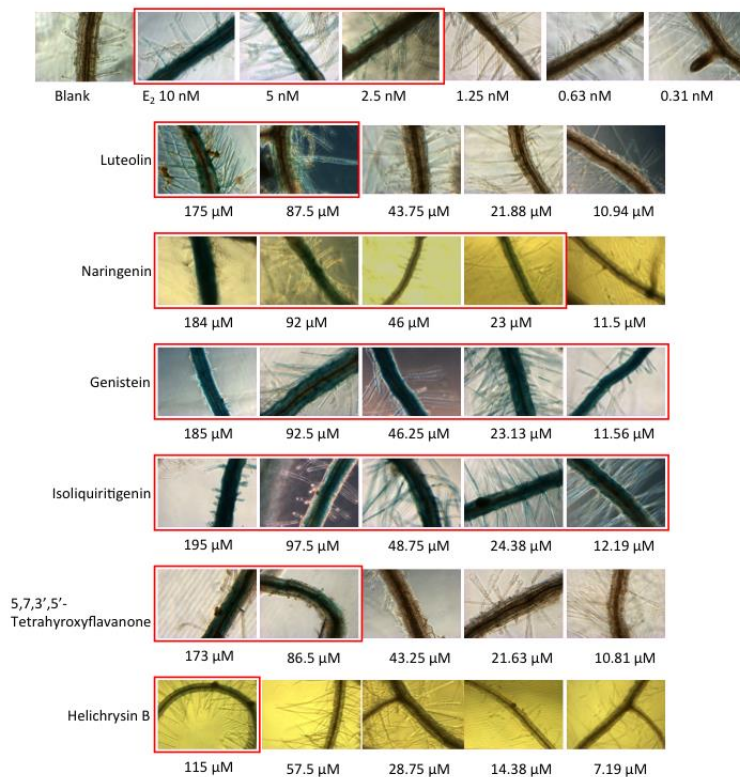


Figure 2. Oestrogenic MAC of the active isolated compounds in the histochemical assay. The concentrations where the blue colour was detectable, are surrounded with red boxes, indicating oestrogenic activity. The last/only concentration where the blue colour was still detectable was considered the minimum active concentration. E₂: oestradiol

HPLC quantification of compounds with oestrogen-like activity and the rationale of fermentation

The quantitative comparison of the six compounds with oestrogen-like activity between the fermented and non-fermented *C. genistoides* was performed by RP-HPLC. 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. 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 the 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.

Fermentation reduces the total phenolic content of *Cyclopia* spp., yet traditionally the fermented tea is consumed. The degradation of flavonoid-glycosides during the fermentation process could explain the difference in the measured amounts of the active compounds between the samples. On one hand, although our experiments reported potent and well-known phytoestrogens to be comprised by *C. genistoides*, the low concentrations of the tested compounds are questioning the potential phytoestrogenic activity of the traditionally used infusion of honeybush.

On the other hand, aqueous extracts of non-fermented or fermented *C. genistoides* and *C. subternata* were able to significantly displace 1 nM [³H]-E₂ 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 noted, that the oestrogenic activity of *Cyclopia* extracts is the result of a fine balance between different polyphenols present in varying amounts with varying phytoestrogenic potential.

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. Naringenin and luteolin were quantified by HPLC before and after bacterial fermentation of the aqueous extracts. While both the processed and unprocessed plants 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.

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.

Antiproliferative activity

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.

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, 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.

Xanthine oxidase inhibitory activity

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. Fractions PP8 from the non-fermented and P10 from the fermented herbal substance were amongst the fractions with the strongest inhibition of xanthine oxidase. 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 μ M (95% confidence interval 0.80 to 0.91 μ M) and 0.53 μ M (95% confidence interval 0.40 to 0.80 μ M), respectively. The inhibitory activity of both compounds significantly exceeded that of allopurinol ($IC_{50} = 7.49 \pm 0.29 \mu$ M), 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 ($IC_{50} = 55.20 \mu$ M (95% confidence interval 41.40 to 73.51 μ M) and 120.55 μ M (95% confidence interval 101.71 to 142.86 μ M), respectively). The rest of the isolated compounds showed no xanthine oxidase inhibition ($IC_{50} > 150 \mu$ M).

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