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Puerariae lobatae root extracts and the regulation of brown fat activity

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Vorgelegt von:

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Meiner Familie, meinen Lehrern und allen, die an der Studie mitgewirkt haben.

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Original Article

Puerariae lobatae root extracts and the regulation of brown fat activity

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ABSTRACT

Background: Obesity is one of the major health problems worldwide. The induction of brown adipocyte formation and activity represents a promising therapeutic option by increasing energy expenditure. Asian herbs have the potential to treat obesity, however, pharmacological effects should be well documented at the molecular level first. *Hypothesis:* A novel hypothesis-driven screening approach identified the root of *Pueraria montana* var. *lobata* (Willd.) Sanjappa & Pradeep (PLR) to have potential effects on obesity by stimulating brown adipocytes. Study design: This study explored the metabolic effects of PLR water extract (PLRE) in a high-fat diet-induced obesity mouse model and characterized its secondary metabolite composition. *Methods:* Animals were orally treated daily for two weeks and the bioactivity of PLRE evaluated by measuring various parameters including body weight, circulating metabolites, energy expenditure and insulin sensitivity. The chemical composition of the mains components was obtained by HPLC–MS–ELSD–PDA. Based on the dereplication results and semi-quantitative estimation, pure molecules were selected for tests on adipocytes *in vitro. Results:* PLRE induces brown adipocyte activity and triggers the formation of brown-like cells in inguinal fat tissue, weight loss, and improved glucose metabolism. These effects are primarily caused by cell-autonomous activation of brown adipocytes and not by autonomic nervous system regulation. Even though the analysis of PLRE revealed puerarin as the most abundant secondary metabolite, it showed no effect on brown adipocyte formation and function. Brown adipocyte activity was induced dose-dependently by two other isoflavones, daidzein, and genistein. Daidzein is present in a very small amount in PLRE, but various glycosidic isoflavones, including puerarin, may release daidzein after metabolism. *Conclusion:* This approach demonstrated the positive effects of PLRE on a diet-induced obesity mouse model and provided clues on the mode of action of PLRE at the molecular level.

Introduction

According to the World Health Organization (WHO, 2018) global obesity has nearly tripled since 1975. Hence, novel long-term treatments for obesity and the associated metabolic complications are urgently needed. Adipose tissue is subdivided into two distinct types, white and brown adipose tissue. Contrary to white adipocytes, brown adipocytes (BAs) dissipate energy in the form of heat (thermogenesis) through a BAs specific protein, uncoupling protein 1 (UCP1)

(Lowell et al., 1993). Targeted ablation of BAs results in diet-induced obesity, diabetes, and hyperlipidemia in mice (Lowell et al., 1993), indicating that BAs may represent important checkpoints in whole-body energy homeostasis.

Traditionally used Asian herbs have drawn the attention regarding their healing potential (Tilburt and Kaptchuk, 2008). To select potential herbs successfully, literature has to be systematically reviewed in combination with the traditional medicinal knowledge and with existing data of bioactive compounds (Friedemann et al., 2015).

Furthermore, primary screenings in selected *in vivo* and *in vitro* models, and efficacy assays, followed by downstream analyses to develop mode-of-action hypotheses have to be carried out (Wang et al., 2013). It is also important to document the composition of the selected herbal preparation in a qualitative and possibly semi-quantitative manner. State-of-the-art metabolite profiling methods, such as ultra-high performance liquid chromatography (UHPLC) coupled to high-resolution mass spectrometry (HRMS), ultraviolet photodiode array (PDA) and evaporative light scattering detection (ELSD) are used to identify secondary metabolites in a process called dereplication (Wolfender et al., 2019).

Plants from the genus Puerariae have been reported to regulate metabolism. Investigations in high-fat diet mice and human studies show an anti-obesity effect of Puerariae (Kamiya et al., 2012b, c). Twenty different accepted species and 95 synonyms of the Puerariae genus exist (theplantlist.org, 2017). Since 2005, the roots of two different varieties, *Puerariae lobatae* (PLR) and *Puerariae thomsonii* (PTR) are listed separately in the Chinese Pharmacopoeia, although they are still commonly used interchangeably (Wong et al., 2015). The accepted botanical name in the case of PLR is *Pueraria montana* var. *lobata* (Willd.) Sanjappa & Pradeep and of PTR, *Pueraria montana* var. *chinensis* (Ohwi) Sanjappa & Pradeep. Both varieties contain mainly isoflavonoids while the isoflavonoid content of PLR is distinctly higher than of PTR. This study investigates the metabolic effects of the well-tolerated PLR concerning its effect on basal metabolic rate and BAs. Besides, we aimed to identify active components from PLR by *in vitro* experiments with the most promising isoflavonoids (Zhang et al., 2013). The already reported beneficial effects of the isoflavonoids on metabolism and obesity (van der Velpen et al., 2014) and the measured levels in PLR substantiate the exploration of puerarin, daidzein, and genistein.

Material and methods

Herb material, extraction, and dosage for animal experiments

Two herbs were used, PLR for examination and Ephedra herba (EH) as the positive control (PC). Both herbs were acquired as dried herbs from Herba Sinica (PLR: Ch.B. 130301H083, EH: Ch.B 100201H059; D-91,126 Rednitzhembach). PLR and EH were tested for identity and purity, including that selected pesticides/pesticide residues, micro-biologicals, heavy metals were not present above acceptable limits by Dr. Uwe Gasser, (Sebastian Kneipp research laboratory, D-86,825 Bad Wörishofen). The extracts were prepared by aqueous extraction, which corresponds to the traditional application form (Zhang et al., 2013).

Therefore, the powdered herb was boiled 40 min in distilled deionized water at a ratio of 1 g per 10 ml of water for each, PLR and EH. After cooling to 60 °C, the solution was filtered and then the residue was directly extracted a second time with the same volume of water. The two filtrates were combined and freeze-dried for 48 h (−80 °C, 0.01 bar, Christ Alpha 2-4 LD plus, Osterode am Harz, Germany), resulting in a yield of 50.2% (w/w) of extraction. The freeze-dried extract was stored at −20 °C for the duration of the study.

Cell culture

Immortalized brown pre-adipocytes were handled as previously described (Perdikari et al., 2017). Details on preadipocyte differentiation and cell treatments are listed in the suppl.data A.1.

Western blot analysis

Western Blot analyses were performed as reported previously (Perdikari et al., 2017). Homogenized tissues were lysed in RIPA buffer and protein concentrations were determined by DC Protein Assay (Bio-Rad). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis, transferred to a PVDF membrane (Amersham Biosciences) and blotted for UCP1 (Antibody 1:1000, Thermo Scientific.

RNA extraction and RT-qPCR

Animal tissues were snap-frozen in liquid nitrogen after harvesting, stored at −80°C and processed as described previously (Perdikari et al., 2017). Total RNA was isolated by TRIzol Reagent (Invitrogen). cDNA was synthesized from 0.75 μg total RNA using the High Capacity cDNA kit (Applied Biosystems, Thermo Fisher Scientific, Vilnius, Lithuania). Intron-exon spanning primers were obtained from the Roche Universal ProbeLibrary to assess changes in brown marker genes (e.g. Ucp1, Cox8b, Pparγ1, Pparγ2, Cidea, Pparα). qRT-PCR assays were performed with Viia7 (Applied Biosystems). 36b4 or Tbp were used as the housekeeping gene. Relative quantification was done using the ΔΔCT method. Gene expression was considered not detectable (n.d.) when Ct ≥ 34.

Animal studies

All animal experiments were performed following the recommendations in the Animal Welfare Ordinance (TSchV 455.1) of the Swiss Federal Food Safety and Veterinary Office. The experiments were prior approved by the veterinary office of Zurich, Switzerland (ZH40/16).

We employed a diet-induced obesity model in which 4 weeks old mice were pretreated for 12 weeks with a high-fat diet (35% fat, 23.9% protein, 4.9% fibres, 5% ashes, Provimi Kliba SA) to induce obesity and insulin resistance. Four groups of five mice per group were subsequently treated by oral gavage. Two different doses groups of PLRE with 0.8 g/kg body weight (BW) (PLRE1) and 0.4 g/kg BW (PLRE2) per day for two weeks were employed. As a negative control, we applied water and as positive control an extract of EH (0.27 g/kg BW/day). EH induces brown fat activity and mass as well as weight loss (Dulloo et al., 1991). Details concerning animal housing, metabolic phenotyping, and blood measurements are found in the suppl.data (A.2.-3.).

Plant material and extraction for chromatographic analyses

Standards of puerarin, daidzin, genistin, daidzein, and genistein were purchased from Chengdu Biopurify Phytochemicals (Chengdu, China). For profiling, PLRE was prepared at a concentration of 10 mg/ml for UHPLC-PDA-ELSD and of 2 mg/ml for UHPLC−HRMS/MS in water and methanol (85/15 v/v) and filtered (syringe filter PVDF, 0.45 μm, BGB Analytik AG, Geneva, Switzerland). The positive control EH was prepared at a concentration of 1 mg/ml and analysed by UHPLC−HRMS as described in the suppl.data (A.10.) (Eugster et al., 2014).

Metabolite profiling of PLRE and quantification of selected isoflavones

Metabolite profiling was performed on two complementary UHPLC systems, the first equipped with PDA and ELSD detections and the second with an Orbitrap Q-Exactive Plus mass spectrometer. Generic long profiling chromatographic conditions were set as previously described (Eugster et al., 2014); details are found in the suppl.data (A.4.-5.).

For dereplication, the ThermoRAW MS data were converted to mzXML using ProteoWizzard (Kessner et al., 2008). MZmine 2.14.2 (open source) was used for data processing (Pluskal et al., 2010). Deconvolution was performed with the algorithm GridMass (Trevino et al., 2015). For identification in MZmine, custom databases were compiled (DNP®; Buskingham, 2010; Zhou et al., 2013). Molecular formulas were extracted and validated after heuristic filtering for molecular ions and related fragments (suppl.data A.6.).

Rapid quantification of puerarin, daidzin, genistin, daidzein and
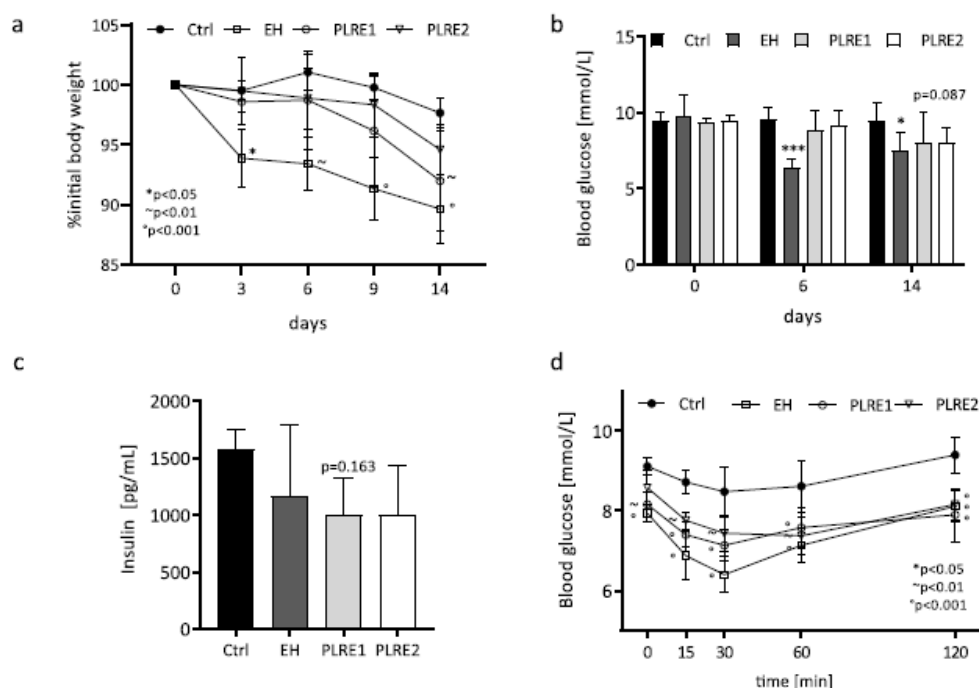
Fig. 1. PLR treatment leads to weight loss and improved glucose metabolism. Feeding Puerariae lobatae radix extract (PLRE) resulted in reduced (a) body weight, (b) plasma insulin levels, (c) blood glucose levels and improved (d) insulin sensitivity in mice treated for 2 weeks. First dosage group with 0.8 g/kg body weight (BW)/day PLRE (PLRE1), second dosage group with 0.4 g/kg BW/day (PLRE2) and 0.27 g/kg BW/day for the positive control Ephedrae herba (EH). (Mean ± SD, $n = 5$). One-way or two-way ANOVA compared to control group with post hoc tests. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

genistein were performed by multiple reaction monitoring (MRM) on a UHPLC–MS-triple quadrupole (TQ) (suppl.data A.7.). Besides, complementary ^{1}H-Nuclear Magnetic Resonance (^{1}H NMR) profiling enabled the quantification of all puerarin related metabolites (suppl.data A.8.).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (versions 7 and 8) using one-way or two-way ANOVA compared to control animals with Dunnett´s or Tukey's post hoc tests. Data are expressed as mean ± SD. For comparisons between groups, analyses were performed using unpaired *t*-tests on all test items. Significance was set at $p < 0,05$ vs control.

Results and discussion

As seen in Fig. 1a, mice treated with PLRE lost weight in a dose-dependent manner ($p < 0.01$ for PLR1), while weight loss in PC treated mice was even more profound. This loss in BW was accompanied by a strong but not statistically significant drop in fasting insulin levels for PLRE, while insulin levels from PC treated mice showed large variability between animals with a trend towards reduction (Fig. 1b). Furthermore, we observed a significant reduction in fasted blood glucose levels for PC treated mice and lower levels for both PLRE doses (Fig. 1c). These slight alterations in glucose homeostasis were paralleled by a profound increment in insulin sensitivity as measured with an insulin tolerance test (Fig. 1d).

To quantify changes in energy expenditure, mice were subjected to indirect calorimetry. Interestingly, mice exhibited increased VO_2 consumption and VCO_2 production in the light and the dark phase

indicating a higher metabolic rate (Fig. 2a/b) while the respiratory exchange ratio (RER, VCO_2/VO_2) remained unaltered (Fig. 2c). Similarly, in the PLRE groups, we did not observe any changes in spontaneous movements (Fig. 2d), food or water intake (Fig. 2e) indicating that the enhanced energy expenditure is due to a systemic increase of metabolism.

In line with the changes in energy expenditure and the fact that triacylglycerols (TAG) are a major energy source for BAs (Bartelt et al., 2011) we observed a significant decrease in circulating TAG in animals treated with PLRE1 and PLRE2 (Fig. 2f). In contrast, plasma free fatty acid (FFA) and cholesterol concentrations remained unchanged (Fig 2g–h). This is consistent with the findings for Pueraria thomsonii flower (Kamiya et al., 2012a) and might base on too weak estrogenic activity of PLRE. Taken together these data demonstrate that PLRE supplementation leads to increased energy expenditure concomitant with lower triglyceride levels.

To elucidate the basis of these changes we focused on activity growth of BAs as a source for increased energy expenditure. Mice were sacrificed after two weeks of treatment and inguinal WAT (ingWAT), as well as intrascapular brown adipose tissue (BAT), were analyzed. While the weight of the intrascapular BAT depot remained unchanged (data not shown), UCP1 mRNA levels in PLRE treated mice increased 1,5fold (Fig. 3a) compared to the control. A similar trend was also observed for protein levels of UCP1 (Fig. 3b and c) as a sign of enhanced BAs activity. In contrast, we did not observe any changes in other markers of brown fat in these mice (Fig. 3d–h).

Since ingWAT can form beige adipocytes (BAs in WAT) we subsequently examined whether PLRE or PC treatment could induce the formation of these cells in mice. We first analyzed the expression of UCP1 and other beige/brown specific genes. As shown in Fig. 4a, we observed an induction of UCP1 mRNA with PLRE1 treatment and with
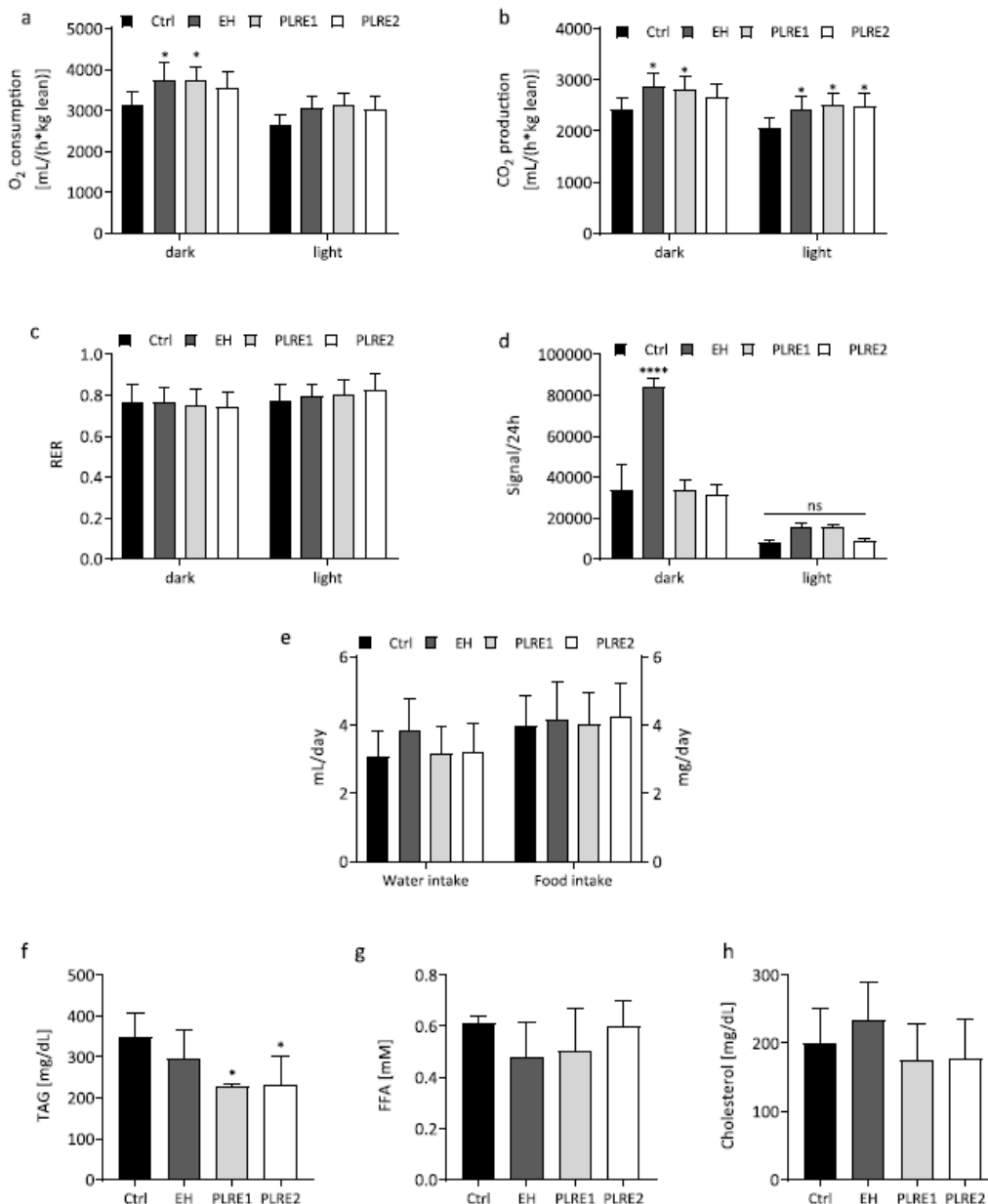
Fig. 2. (a–g). PLR intake increases energy expenditure and reduces plasma lipids in mice. Puerariae lobatae radix extract (PLRE) treatment raises (a) oxygen consumption (VO_2) and (b) CO_2 production (VCO_2), while the (c) RER (respiratory exchange ratio, VCO_2/VO_2), as well as the (d) spontaneous movements and (e) the water and food intake remained unchanged. PLRE feeding reduces (f) plasma triglyceride (TAG) levels dose-dependently. Trends toward reduced plasma lipid levels are shown for (g) plasma free fatty acids (FFA) in the high dose and for (h) plasma cholesterol. First dosage group with 0.8 g/kg body weight (BW)/day PLRE (PLRE1), second dosage group with 0.4 g/kg BW/day (PLRE2) and 0.27 g/kg BW/day for the positive control Ephedrae herba (EH). (Mean ± SD, ($n = 5$). One-way or two-way ANOVA compared to control group with post hoc tests. Statistical significance is denoted with* $p < 0.05$, ** $p < 0.01$.

PC. Similarly, the expression of other markers of brown fat activity was increased such as PPARγ1, PPARγ2 (Fig. 4c/d), PPARα (Fig. 4e) and showed a tendency for Coxb8 (Fig. 4b) and CIDEA (Fig. 4f). On the contrary, classical white markers such as adiponectin remained unchanged (data not shown). Since only low amounts of beige cells are formed, we were not able to quantify UCP1 protein levels in ingWAT.

These data demonstrate that PLRE and PC treatment leads to an upregulation of brown fat markers in ingWAT.

For comprehensive metabolite profiling, the composition of PLRE was analysed by UHPLC-ELSD-PDA-HRMS/MS (Fig. 5). Except for very polar compounds not retained in reversed-phase (Fig. 5b), the most strongly component detected by UHPLC-ELSD was identified as
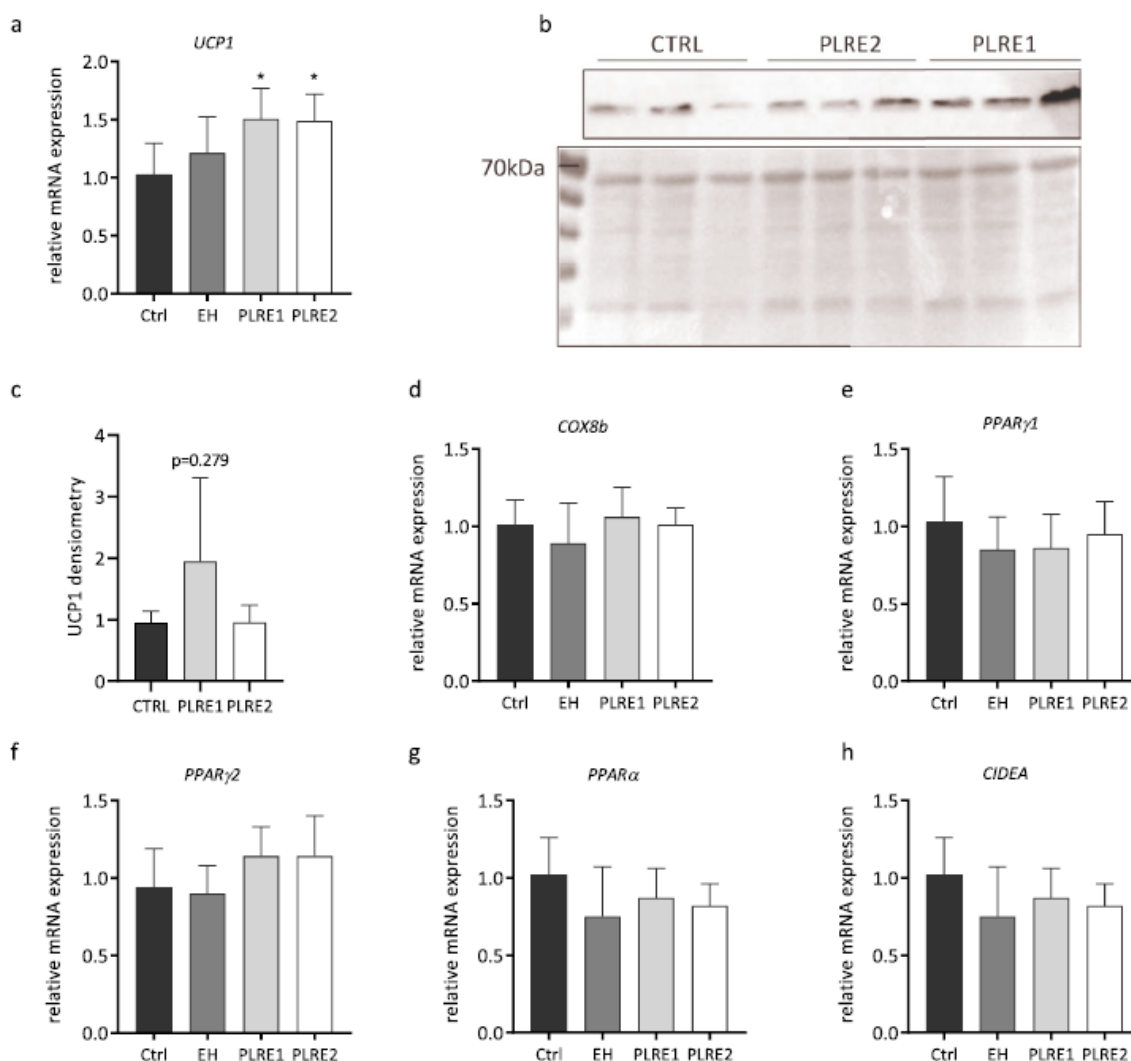
Fig. 3. (a–g) PLRE supply conducts a mild activation of intrascapular brown adipose tissue. Increased (a) UCP1 mRNA expression levels and (b-c) UCP1 protein levels in the Puerariae lobatae radix extract (PLRE) groups can be observed, while (d-h) all other brown fat markers remained unchanged. First dosage group with 0.8 g/kg body weight (BW)/day (PLRE1), second dosage group with 0.4 g/kg BW/day (PLRE2) and 0.27 g/kg BW/day for the positive control Ephedrae herba (EH). (Mean ± SD, $n = 5$). One-way ANOVA compared to control with post hoc tests. Statistical significance is denoted with*$p < 0.05$, **$p < 0.01$).

puerarin (**4**) (Fig. 5d). This identification based on spectral data and comparison with a pure standard (Wong et al., 2015). Most of the other metabolites detectable in UHPLC–ELSD (Fig. 5a, **1-13, 15**) were dereplicated (Fig. 5a–c, h, suppl.data Tab.A.9.) and were found to be isoflavonoids except for a puerol glycoside (**14**). The identification of these compounds, directly in the PLRE, was based on the molecular formulas deduced from HRMS data and cross-search with literature. The identities were further confirmed by HRMS/MS fragmentation and PDA spectra.

Two different types of isoflavones were distinguished, C-glycosides (**1, 3–7**) (including puerarin (**4**)) and O-glycosides (**2, 8–13**) (including daidzin (**8**)). Daidzein (**15**), the common aglycone of puerarin (**4**) and daidzin (**8**) as well as (**2, 10, 12–13**) was also detected. Concerning the C-glycosides, three isoflavones differed from puerarin (**4**) on their sugar moiety (**1, 5–6**), while two other presented different aglycone substitutions (3′-hydroxypuerarin (**3**) and 3′-methoxypuerarin (**7**)).

Similarly, concerning O-glycosides isoflavones, four were related to daidzin (**8**) only with sugar moiety variations (**2, 10, 12–13**), while two other presented distinct aglycones (genistin (**11**) and methoxydaidzein (**9**)). Genistein (**16**), the aglycone of genistin (**11**), was also identified (Fig. 5a–c, h, suppl.data Tab.A.9.). Genistin (**11**), daidzin (**8**), and their related aglycones, daidzein (**15**) and genistein (**16**) were unambiguously identified by spiking experiments with pure standards.

The choice of daidzein, genistein and puerarin for further *in vitro* investigation was justified by the data related to the metabolism of the isoflavones and by the high level of puerarin in PLRE. Concerning the O-glycosides isoflavones, their small intestinal metabolism into daidzein was already described (Rowland et al., 2003). This metabolism results from enzymatic cleavage of the O-glycosidic bonds. Regarding the C-glycosides, they are reported to be mainly observed in the blood circulation, while their unabsorbed fractions which reach the colon undergo further microbial metabolism into their aglycones (Qiao et al.,
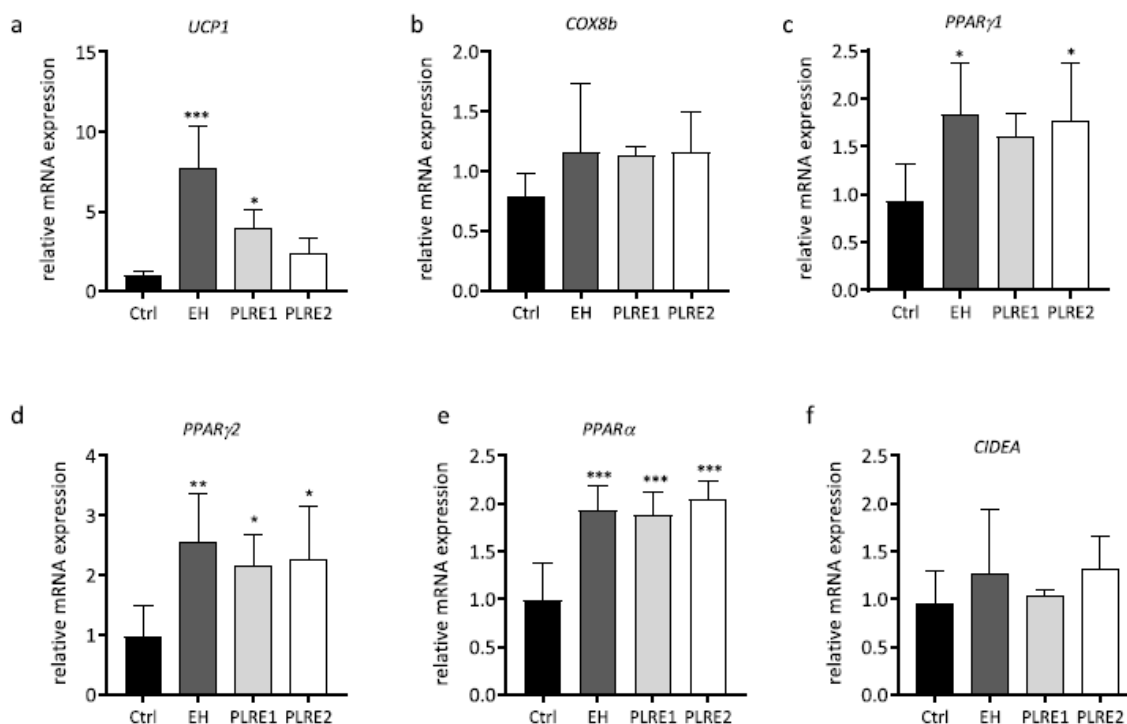
Fig. 4. (a–f) Puerariae lobatae radix extract (PLRE) causes an upregulation of brown fat markers in inguinal white adipose tissue (ingWAT). mRNA expression levels in ingWAT increased significantly in (a) UCP1, (c) PPARγ1, (d) PPARγ2, (e) PPARα. (b) COX8b and (f) CIDEA only exhibit a trend toward higher mRNA expression levels. First dosage group with 0.8 g/kg body weight (BW)/day PLRE (PLRE1), second dosage group with 0.4 g/kg BW/day (PLRE2) and 0.27 g/kg BW/day for the positive control Ephedrae herba (EH). (Mean $\pm$ SD, $n = 5$). One-way ANOA compared to control group with post hoc test. Statistical significance is denoted with * $p < 0.05$, ** $p < 0.01$ and ***$p < 0.001$.

2016). Consequently, both types of isoflavone glycosides in PLRE are a potential source of daidzein before their phase II metabolism (Qiao et al., 2016). Thus, the dereplicated isoflavones (**1-2, 5-6, 10, 12, 13**) may contribute to the pool of daidzein in addition to puerarin (**4**) and daidzin (**8**). Only genistin (**15**) contributes to the level of genistein (**16**) after metabolism. The residual aglycones (**3, 7, 9**) may have to be further investigated.

In quantitative terms, based on ELSD, puerarin (**4**) accounted for more than 69% of the signals of the secondary metabolites, 19% were due to the other C-glycosides isoflavones (**1, 3, 5-7**) and the remaining 12% were related to the O-glycosides (**2, 8-13**). ^{1}H NMR indicated that total isoflavones content accounted for 6.6% (w/w).

Additionally, UHPLC-TQ-MRM was used for the precise quantification of puerarin (4), daidzin (8), genistin (11), daidzein (15) and genistein (16) in PLRE with reference to standards. The observed w/w concentrations were 5.44% (CV 0.95%) for puerarin, 1.10% (w/w, CV 1.52%) for daidzin, 0.1% (w/w, CV 1.39%) for genistin, 0.05% (w/w, CV 1.45%) for daidzein and 0.004% (w/w, CV 5.39%) for genistein.

Thus, the two dosages of PLRE given to mice (0.4 and 0.8 g/kg BW) corresponded to an intake of isoflavones of 22 and 44 mg/kg BW of puerarin, 4.4 and 8.8 mg/kg BW of daidzin and 0.4 and 0.8 mg/kg BW of genistin, 0.2 and 0.4 mg/kg BW of daidzein, and 0.016 and 0.032 mg/kg BW of genistein. This indicates, that the doses for puerarin in our experiments were in the low range (Wu et al., 2013; Zhang et al., 2010), and the other ingested metabolites were present in smaller amounts (Lai et al., 2011; Lephart et al., 2004; Ulbricht et al., 2015; van der Velpen et al., 2014).

Since the dosages in this study (5 and 10 g/day, respectively) were low in comparison to human dosage (up to 21 g/day; (Bensky et al., 2015)), higher doses might enhance the positive effects observed on weight loss and energy expenditure through the activation of brown fat.

Since puerarin is the main constituent of PLRE, we subsequently analyzed in vitro whether the effect of PLRE is due to direct regulation of brown adipocyte formation or activity by puerarin and/or the aglycones daidzein and genistein. Representative pictures of the developed brown fat cells are shown in Fig. 6a. Daidzein was only employed at concentrations of 40 and 200 μM and genistein at 40 μM, respectively since higher amounts induced cytotoxic effects in our assay. Interestingly, we observed that PLRE, as well as puerarin itself, significantly increased nuclear numbers, indicative for enhanced proliferation, when administered during the first two days of differentiation (Fig. 6b). Contrary, daidzein did not affect cell proliferation. Furthermore, we observed a dose-dependent induction of adipocyte differentiation when cells were treated with PLRE, puerarin and 200 μM of daidzein (Fig. 6c) as measured by lipid droplet formation. A significant increase in UCP1 intensity, which indicates enhanced BA activity, was only observed with PRLE and genistein (Fig. 6d), with a trend for daidzein at 200 μM.

As PLRE might directly regulate brown adipocyte activity, we measured the induction of brown fat activity in a brown adipocyte reporter cell line, which expresses luciferase under the control of the UCP1 promoter. As indicated in Fig. 6e-f we observed a dose-dependent luciferase increase, which was initiated by daidzein and genistein but not by puerarin.

The novel key message of this investigation states that PLRE can induce brown adipocyte activity and to a minor extent brown adipocyte formation in vitro in a cell-autonomous manner. The two isoflavones, daidzein and genistein appear as direct active mediators, but not the most abundant flavonoid puerarin. Given the bioavailability of the
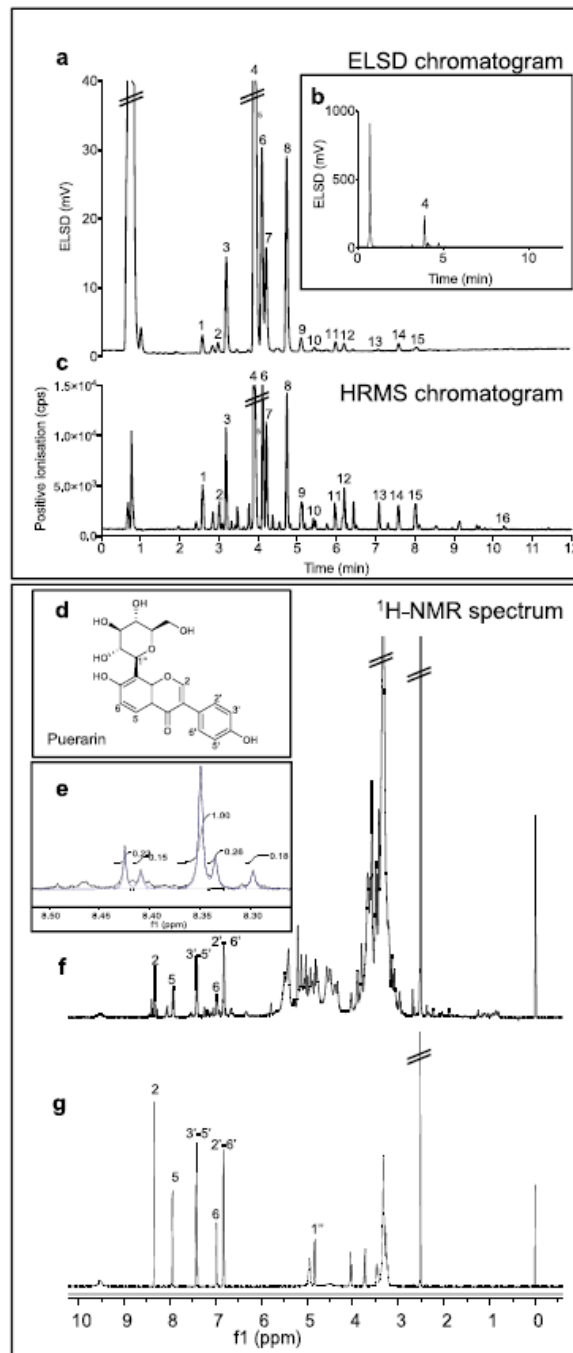
Fig. 5. (a–f) Analyses of PLRE: (a) UHPLC-ELSD profile with intensity set at 40 mV, (b) UHPLC-ELSD profile, (c) UHPLC–HRMS profile (positive ionization), (d) chemical structure of puerarin, (e) and (f) ^{1}H NMR spectrum of Puerariae lobatae radix (PLR), (g) ^{1}H NMR spectrum of standard puerarin. (h) Chemical structures, classified according to their type of glycosides and aglycones.

Puerariae isoflavones, puerarin could be hypothesized to cause an in-direct UCP1-dependent mitochondrial uncoupling in white (Aziz et al., 2017; Crespillo et al., 2011) and brown fat tissue.

The underlying pathway of daidzein and genistein on brown/beige fat induction is unclear. Both isoflavones are reported to bind directly to the intracellular receptors PPARγ and PPARα, which were significantly upregulated in ingWAT but not in BAT in our murine experiments and play key roles in the UCP1 thermogenesis (Mezei et al., 2003; Villarroya et al., 2007).

Regarding prospective human BAT studies, the induction of inguinal

Puerarin C-glycoside derivatives (78.2%*)

puerarin (4) (68.9%)

daidzein (15) (0.2%)

-puerarin-6″-O-glucoside
-puerarin-4′-glucoside (1) (0.8%)

mirificin (6) (8.5%)

6″-xylopuerarin (5) (with 4)

Puerarin aglycone derivatives (9%)

3′-hydroxypuerarin (3)
(4.2%)

3′-methoxypuerarin (7)
(4.8%)

* Semi-quantitative estimation from ELSD area [%]
  relative to all isoflavonoid signals.
** nd: non-detectable in ELSD trace.

Daidzin O-glycoside derivatives (10.0%)

daidzin (8) (8.5%)

daidzein-4′-glucoside (10) (0.5%)

daidzin-4″-O-glucoside (2) (0.5%)

6″-malonyldaidzin (12) (0.4%)

6″-acetyldaidzin (13) (0.1%)

Daidzin aglycone derivatives (1.1%)

methoxydaidzin (9) (0.7%)

genistin (11) (0.4%)

genistein (16) (nd**)

Fig. 5. (continued)

13

e

(caption on next page)

14

Fig. 6. (a) Representative confocal images of developed brown fat cells. Incubation of immortalized brown pre-adipocytes and in the 2nd examination of fully mature brown adipocytes with (a) negative (DMSO) and positive control (isoproterenol), Puerariae lobatae radix extract (PLRE), puerarin, daidzein and genistein. Green represents UCP1, blue staining the nuclei. (b–d) Proliferation, differentiation and activation of brown adipocytes. Puerariae lobatae radix extract (PLRE) and puerarin induce dose-dependent cell proliferation, while PLRE, puerarin and daidzein at 200 μM promote cell differentiation. PLRE at 1 mM, daidzein at 200 μM and genistein at 40 μM increase UCP1 activity. (Mean ± SD, $n = 3$). Two-way ANOA compared to control group with post hoc test. Statistical significance is denoted with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (e-f). Direct induction of brown adipocyte activity in a brown adipocyte reporter cell line. Puerariae lobatae radix extract (PLRE) at the highest dose, daidzein at 40 and 200 μM and genistein at 40 μM concentrations induce significant UCP1 expression, while higher isoflavone concentrations had cytotoxic effects. (Mean ± SD, $n = 8$). Two-way ANOA compared to control group with post hoc test. Statistical significance is denoted with ** $p < 0.01$, *** $p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

beige cells is promising. In particular, as human BAT is genetically more similar to the murine inguinal beige cells than to the classical BAs (Wu et al., 2012).
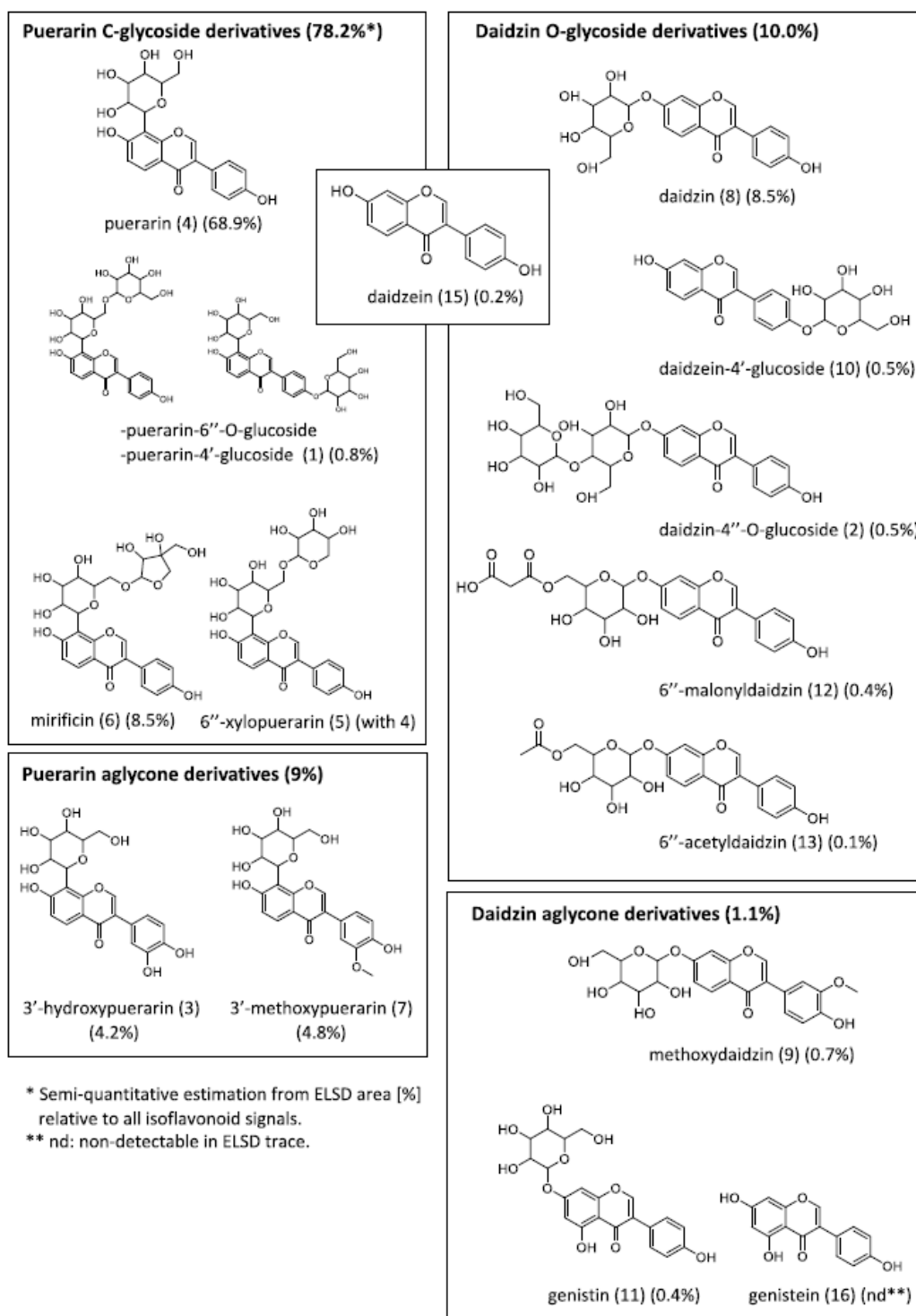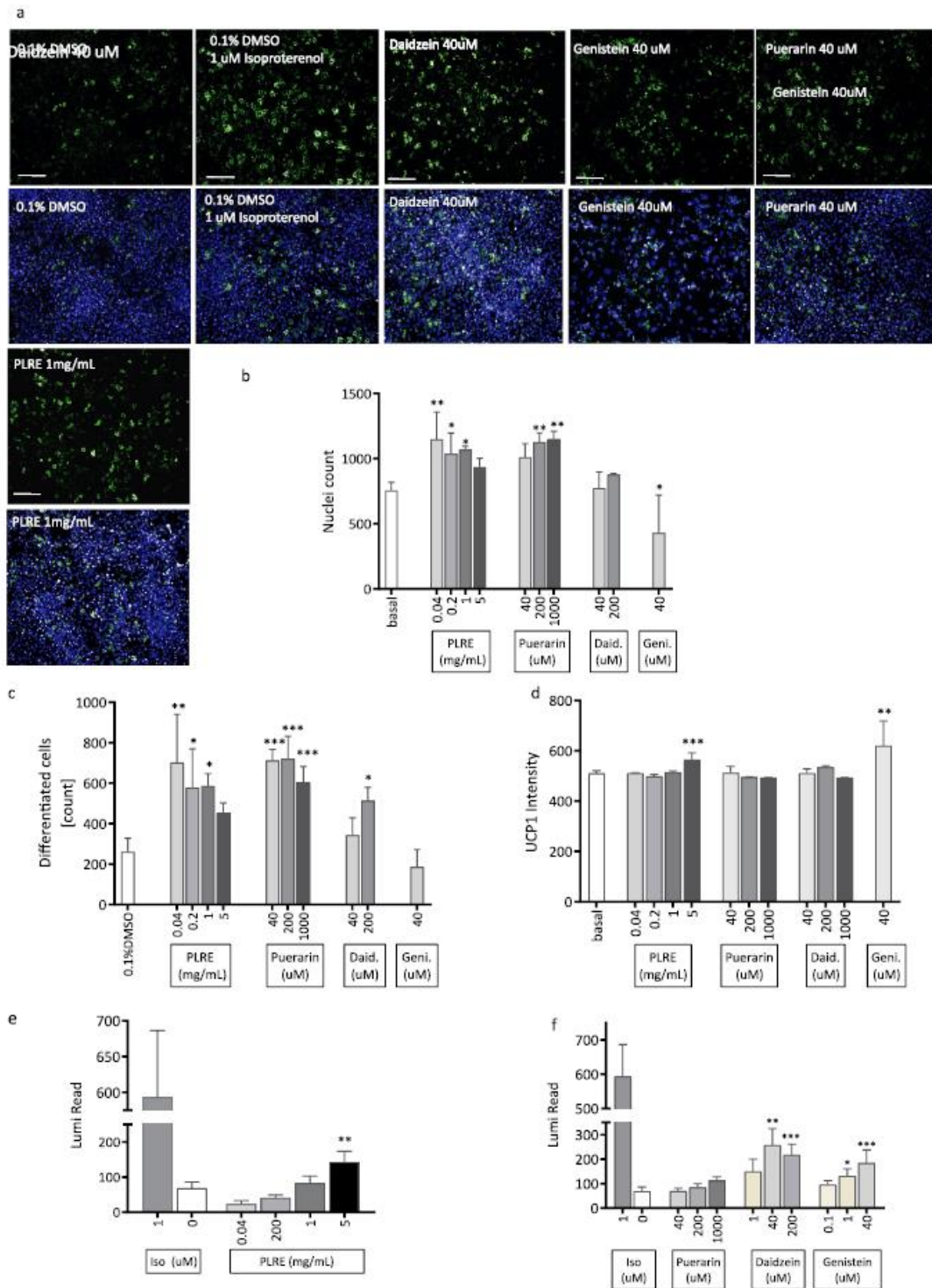
Conclusion

This study investigated the weight lowering metabolic effects of PLRE in mice models. The results suggested that the observed energy expenditure in HFD-obese mice is probably a combined effect (synergistic or/and additive) of different isoflavones of PLRE. These findings open an objective for further research, namely to clarify the effectiveness in humans, furthermore to answer the question whether the main constituent of PLRE, puerarin leads indirectly to noteworthy anti-obesity effects by metabolism to daidzein in the colon and last, whether the crude plant, an isoflavone fraction, a new isoflavone combination or a mono-isoflavone preparation is the most effective remedy in adiposity therapy due to PLRE treatment.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. The HanseMerkur Krankenversicherung AG as the sponsor of the HanseMerkur Center for Traditional Chinese Medicine at the University Medical Center Hamburg Eppendorf has provided a doctoral student allowance for Elisabeth Buhlmann.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2019.153075.

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1.2. Graphical Abstract:
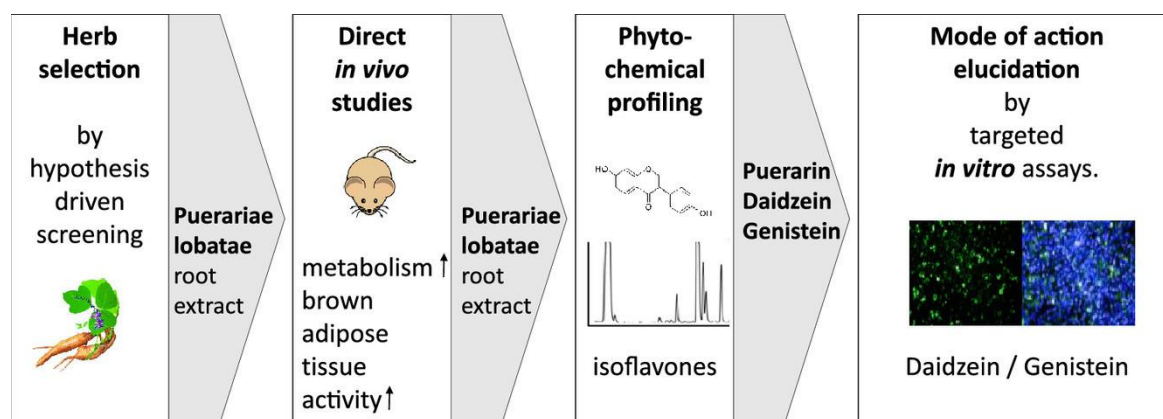
1.3. Appendix A.1.-10.: Supplementary data and table

A.1. Brown preadipocyte differentiation protocol and cell treatment regimes

Cells were cultured in high glucose DMEM with 10% FBS and 1% Pen/Strep (Gibco, ThermoFisher) and seeded on collagen-coated plates for experiments. Mature adipocytes were obtained by treating the cells with an induction cocktail for 2 days (5 mM IBMX, 20 nM insulin, 25 mM indomethacin, 1 uM T3 and10 mM dexamethasone days), followed by maintenance media (20 nM insulin, 1 uM T3 and 1uM 4-hydroxytamoxifen only for the reporter line)). Besides the C-glycoside puerarin, we used the aglycone forms of the O-glycosides daidzin and genistin for cell culture experiments, because they get hydrolyzed to daidzein and genistein **(Rowland et al., 2003)**. An immortalized brown adipocyte reporter cell line, which expresses luciferase under the control of the UCP1 promoter was analyzed using a luciferase assay system (Promega, Madison, U.S.). Mature adipocytes were treated with the indicated extract or compounds for 3 days (day6 to 9 of differentiation). Preadipocytes were treated for 2 days prior to induction for the differentiation and proliferation analyses. Automated analysis of adipocyte differentiation and lipid droplet size were analyzed as described previously (Meissburger et al., 2011).

A.2. General housing conditions and measurements

Twenty male C57BL/6 mice were kept on a 12/12 h reversed light cycle in a pathogen-free animal facility on an ad libitum diet (standard chow diet, 18% proteins, 4.5% fat, 4.5% fibers, 6.3% ashes, Provimi Kliba SA). Housing temperature was set to 23°C for all experiments. 4-5 animals were housed in a cage and cages were enriched with houses, embedding and tissues. No running wheels were included.

During the study, body weight was measured every 3rd day and prior to euthanasia (day 14). Animals were euthanized by CO_2 overdose at the end of the study. Random fed blood glucose levels were measured after day0 and day6. Fasting blood glucose levels were measured at day 14 after an overnight fast. Two separate cohorts were used in this study. A first cohort of mice was used for the insulin tolerance test (performed at day 11) and a second cohort for the metabolic cage experiments (performed days 10-12).

A.3. Metabolic phenotyping

Indirect calorimetry: Indirect calorimetry was performed with the metabolic cage system Phenomaster from TSE Systems according to the manufracture's instructions. Animals were single caged for adaptation two days before the metabolic recording started and the animals were checked two times daily during the measurement. O2 and CO2 levels were measured every 13 min for 60 seconds. Activity was registered with specific frames around the cages equipped with infra-red light beams. Monitoring of the interruptions of these light beams allows the estimation of total activity (X, Y movements plus rearing). RER was calculated form the ratio of CO2 production and O2 consumption rates.

Insulin tolerance test: Insulin tolerance test was performed in unrestrained animals after 6 hours fast. Fasted blood glucose levels were measured and insulin administrated intraperitoneally at a dose of (0.75 U/kg body weight, Actrapid human insulin, Novo Nordisk). Blood glucose concentrations levels were then monitored using a glucometer (ACCU-CHEK Aviva, Roche) from a blood droplet obtained through a small incision in the tail.

Blood parameters: Circulating levels of cholesterol, insulin, free fatty acids and triglycerides were measured at the end of the 14 days treatment in blood plasma samples collected after 6 hours fast. Animals were euthanized using CO2 and blood collected by cardiac puncture. Whole blood samples were spun at 8000g for 8 min to collect the blood plasma. The following commercial kits were used for subsequent analysis: cholesterol (11491458 216, Roche/Hitachi), triglyceride (*11877771 216, Roche/Hitachi)*, insulin (K152BZC-2, MSD) and free fatty acids (436-91995 and 434-91795, FUJIFILM Wako Chemicals).

A.4.   UHPLC-PDA-ELSD metabolite profiling of the PLR extract

UHPLC measurements were performed using the Acquity UHPLC system (Waters), which included a binary pumping system, an auto-sampler (set at 10°C), a column manager with a pre-column heater (set at 40°C), a PDA detector and an evaporative light scattering detector (ELSD) (Sedex 85, Sedere LT-ELSD). The system was controlled using Empower 3 Software (Waters). PDA detection was performed from 210 to 500 nm (1.2 nm resolution). The binary system was using two mobile phases: water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) (ULC/MS grade, Biosolve Chimie SARL). For the high-resolution metabolite profiling the separation was achieved on the Acquity UPLC® BEH C18 column (1.7 µm, 2.1 x 150 mm; Waters). A linear gradient was applied from 95% of A and 5% of B over a period of 30 min, followed by an isocratic step during 10 min with 95% of B and a re-equilibration step of 10 min. Injection volume was set at 2 µL and the flow rate at 0.46 mL/min. A solution containing standards (rutin (Fluka AG) and glycyrrhetinic acid (Carl Roth) at 500 µg/mL) was injected before the analyses to check the reliability of the measured retention times and to allow the comparison with the other chromatographic devices.

A.5.   UHPLC-MS/MS metabolite profiling of the PLR extract

Chromatographic separation was performed on a Thermo Dionex Ultimate 3000 UHPLC system interfaced to an Orbitrap Q-Exactive Plus mass spectrometer (Thermo Scientific), using a heated electrospray ionization (HESI-II) source. The chromatographic conditions were similar to those used for the UHPLC-PDA-ELSD metabolite profiling. A solution containing standards (rutin (20 µg/mL), puerarin (20 µg/mL) and glycyrrhetinic acid (10 µg/mL)) was injected before the analyses to check the reliability of the measured retention times and to allow the comparison with the other chromatographic devices. The full MS analyses were performed in positive and negative mode with a mass range of 150-1300 at a resolution of 70000 full width at half maximum (FWHM) (at m/z 200). The ion injection time used was 200 ms. In positive mode, diisooctyl phthalate $C_{24}H_{38}O_4$ $[M+H]^+$ ion (m/z 391.28429) was used as internal lock mass. The optimized HESI-II parameters were the following: source voltage: 4.0 kV (pos), 2.5 kV (neg); sheath gas flow rate (N_2): 51 units (pos), 48 units (neg); auxiliary gas flow rate: 13 units (pos), 11 units (neg); spare gas flow rate: 2.6 (pos), 2.3 (neg); capillary temperature: 266.25°C (pos), 256.25°C (neg); S-Lens RF Level: 50. The mass analyser was calibrated according to the manufacturer's directions using a mixture of caffeine, methionine-arginine-phenylalanine-alanine-acetate (MRFA), sodium dodecyl sulfate, sodium taurocholate and Ultramark 1621 in an acetonitrile/methanol/water solution containing 1% acetic acid by direct injection. The data-dependent MS/MS events were performed on the 5 most intense ions detected in full scan MS (Top5 experiment). The MS/MS isolation window width was 1

m/z and the normalized collision energy (NCE) was 35 units. In data-dependent MS/MS experiment, full scans were acquired at a resolution of 35000 FWHM with a maximum injection time of 50 ms and an AGC target of $1e^6$. MS/MS scans were acquired at 17500 FWHM, with a maximum injection time of 50 ms and an AGC target of $1e^5$. After being acquired in MS/MS scan, parent ions were placed in a dynamic exclusion list for 5.0 second.

A.6.   HRMS/MS data processing and dereplication

The MS raw data obtained from the Orbitrap (ThermoRAW) were converted to mzXML using ProteoWizzard (Kessner et al., 2008). The open-source software MZMine 2.14.2 was used as a toolbox for data processing (Pluskal et al., 2010). The algorithm GridMass was employed for the 2D detection of the features (Trevino et al., 2015). The peak lists were deisotoped, filtered to remove potential duplication and subject to adduct search. A first custom database was compiled with the secondary metabolites referenced in the Dictionary of Natural Product (DNP®) (Buskingham 2010) for PLR and additional ones described in (Zhou et al., 2013) and used in MZMine. Molecular formulas were extracted and validated after heuristic filtering within MZMine(Pluskal et al., 2012) for both molecular ions and related fragments.

A.7.   UHPLC-TQ-MRM quantification

For the rapid quantification of puerarin, daidzin, genistin, daidzein and genistein, the experiments were performed with the Acquity UPLC system (Waters) composed of a binary solvent manager (Class I), a sample manager FL (Class I) and a column manager. This UHPLC system was connected with a triple quadrupole (TQ) mass spectrometer (Waters Xevo TQ-S Micro) equipped with Z-spray® electrospray ionization source (ESI) operating in positive mode. Masslynx V4.1 software was employed for data acquisition, data handling and instrument control. The nebulizing gas was high purity nitrogen and the collision one was high purity argon. The capillary voltage of ESI was set at 3.8 kV, desolvation gas temperature at 350°C and its gas flow at 650 L/h. Multiple reaction monitoring (MRM) were performed for selective detection and quantification. Cone voltage and collision energies were optimized for each compound by using the embedded module Intellistart. MRM with the following precursor → product(s) *m/z* and cone and collision voltages were the following: puerarin, 417 → 267 + 297, cone voltage 62 V and collision one at 28 V; daidzin, 417 → 91 + 199 + 255 cone voltage 44 V and collision ones at respectively 70, 44 and 16 V; genistin, 433 → 91 +153 +271, cone voltage 40 V and collision ones at respectively 66, 48, 28 V; daidzein, 255 → 91 + 137 +199, cone voltage 68 V and collision ones at respectively 30, 24, 22 V; genistein, 271 → 91, 153, 215, cone voltage 70 V and collision ones at respectively 36, 28, 22 V.

Samples were injected (2 µL) into the Acquity UPLC® BEH C18 column (1.7 µm, 2.1 x 100 mm; Waters) and eluted (0.5 mL/min, 40°C) with water (A) and acetonitrile (B) both with 0.1%

formic acid. The following gradient was used: from 10 to 17% of B from 0 to 5 min, 17 to 75% from 5 to 11 min, 75 to 98% from 11 to 12 min, an isocratic step at 98 % for 2 minutes and a re-equilibration step of 2 min. The limit of quantification (LOQ) was measured according to the international conference of harmonization (ICH Q2(R1)), standard deviation of the response and the slope method). The LOQ were the following: 0.11 nM for puerarin, 0.08 nM for daidzin, 0.64 nM for genistin, 2.4 nM for daidzein and 4.5 nM for genistein. A calibration curve was established for each compound with 6 different concentrations (1, 4, 8, 10, 20 and 50 μM for puerarin, 0.7, 1, 4, 8, 10 and 20 μM for daidzin, 80, 100, 200, 400, 700 nM and 1 μM for genistin and daidzein, 5, 10, 50, 80, 100 and 200 nM for genistein. The following linear regression equations were obtained: for puerarin $y = 98824x + 135668$ (r^2 0.99), for daidzin $y = 401414x + 128785$ (r^2 0.99), for genistin $y = 303815x - 787$ (r^2 1.00), for daidzein $y = 153071 + 891$ (r^2 0.99) and for genistein $y = 52840x - 331$ (r^2 0.99).

PLR extract was diluted in water/methanol (70/30 v/v) at 100 μg/mL except for puerarin for which an extract concentration of 50 μg/mL was used to avoid signal saturation. Three analytical replicates were acquired.

A.8. ^{1}H-Nuclear Magnetic Resonance (H^1-NMR)

Data were recorded on an Agilent Varian Inova 500 MHz spectrometer (499.87 MHz). Chemical shifts are reported in parts per million (δ) using the TSP (sodium trimethylsilyl propionate) as internal reference and coupling constants (J) are reported in Hz. For the quantification of puerarin the ^{1}H-NMR spectra were recorded in 600 μL of deuterated DMSO containing 483.7 μM of TSP, which was used as internal standard. In these conditions, the chemical shifts of the puerarin were the following: δ 4.83 (1H, d, J=9.82 Hz, H-1''), 6.82 (2H, m, H-3'-5'), 6.98 (1H, d(d), J=8.82 Hz, H-6), 7.41 (2H, m, H-2'-5'), 7.94 (1H, d(d), J=8.81), H-5) and 8.34 (1H, s, H-2).
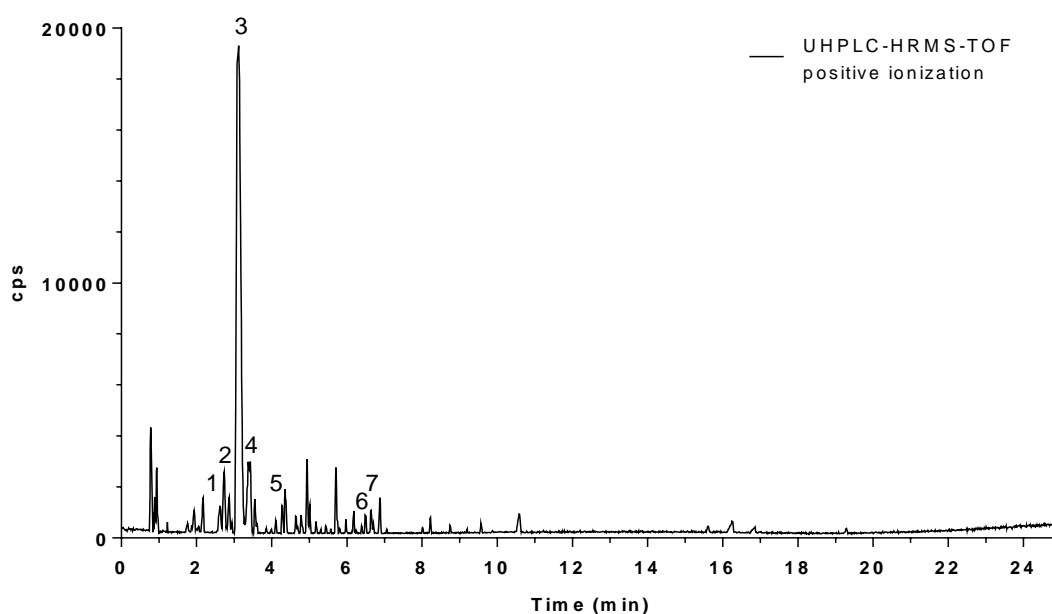
A.9. Table of dereplication

N°	ELSD Relative Area [%]	m/z [M+H]$^+$	Retention time [min]	Molecular formula	Common name	Mass accuracy [ppm]	Isotope pattern Score [%]	Fragments m/z [M+H]$^+$	Molecular formula	Mass accuracy [ppm]	PDA UV max [nm]
1	0.2	579.17108	3.16	$C_{27}H_{30}O_{14}$	-puerarin-4'-O-glucoside -puerarin-6''-O-glucoside	-0.5	91.1	297.0759	$C_{17}H_{12}O_5$	-1.4	249-304
								267.0651	$C_{16}H_{10}O_4$	-2.3	
								399.1078	$C_{21}H_{18}O_8$	-0.5	
2	0.1	579.1711	3.62	$C_{27}H_{30}O_{14}$	daidzin-4''-glucoside	-0.5	90.7	255.0652	$C_{15}H_{10}O_4$	-2.0	248-299
3	1.0	433.1131	3.79	$C_{21}H_{20}O_{10}$	3'-hydroxypuerarin	-0.9	98.7	313.0708	$C_{17}H_{12}O_6$	-1.3	220(sh)-249-291
								283.0602	$C_{16}H_{10}O_5$	-1.6	
4	16.4	417.1181	4.58	$C_{21}H_{20}O_9$	Puerarin**	-1.1	98.6	297.0762	$C_{17}H_{12}O_5$	-0.4	251-305
								267.0656	$C_{16}H_{10}O_4$	-0.5	
5		549.1605	4.64	$C_{26}H_{28}O_{13}$	6''-xylo-puerarin	-0.5	91.9	297.0760	$C_{17}H_{12}O_5$	-1.1	-
6	2.0	549.1606	4.82	$C_{26}H_{28}O_{13}$	mirificin	-0.3	98.3	297.0758	$C_{17}H_{12}O_5$	-1.8	250-305
7	1.1	447.1288	4.91	$C_{22}H_{22}O_{10}$	3'-methoxypuerarin	-0.7	98.4	327.0865	$C_{18}H_{14}O_6$	-1.1	220(sh)-250-300
								297.0761	$C_{17}H_{12}O_5$	-0.8	
8	2.0	417.1181	5.44	$C_{21}H_{20}O_9$	daidzin**	-1.1	99.1	255.0653	$C_{15}H_{10}O_4$	-1.6	249-305
9	0.2	447.1288	5.87	$C_{22}H_{22}O_{10}$	3'-methoxydaidzin	-0.8	93.7	285.0757	$C_{16}H_{12}O_5$	-2.1	-
10	0.1	417.1181	6.22	$C_{21}H_{20}O_9$	daidzein-4'-glucoside	-1.1	/	255.0652	$C_{15}H_{10}O_4$	-2.0	-
11	0.1	433.1131	6.75	$C_{21}H_{20}O_{10}$	genistin**	-0.9	94.6	271.0602	$C_{15}H_{10}O_5$	-1.7	-
12	0.1	503.1186	7.01	$C_{24}H_{22}O_{12}$	6''-malonyl-daidzin	-0.9	92.8	255.0652	$C_{15}H_{10}O_4$	-2.0	-
13	<0.1	459.1287	7.94	$C_{23}H_{22}O_{10}$	6''-O-acetyldaidzin	-0.9	93.3	255.0652	$C_{15}H_{10}O_4$	-2.0	-
14	0.1	475.1602	8.45	$C_{24}H_{26}O_{10}$	-sophoraside A -puerol A-4''-methelether -4'O-glucoside	-0.5	93.1	107.0496	C_7H_6O	-0.8	-
								276.1016	$C_{17}H_{14}O_3$	-1.9	
								295.0966	$C_{18}H_{14}O_4$	-1.5	
15	<0.1	255.0652	8.89	$C_{15}H_{10}O_4$	Daidzein**	-1.9	96.9	199.0754	$C_{13}H_{10}O_2$	-2.5	-
16	<0.1	271.0601	10.71	$C_{15}H_{10}O_5$	Genistein**	-0.1	/	215.0704	$C_{13}H_{10}O_3$	0.5	-

*All m/z were also validated in negative ionization mode. **Formally identified by comparison with standards.

A.10. Chromatogram and dereplication table of positive control Ephedra herba

The MS raw data obtained from the UHPLC-HRMS-TOF (MasslynxRAW) were converted to CDF format using Databridge (Masslynx 4.1, Waters).The open-source software MZMine 2.38 was used as a toolbox for data processing (Pluskal et al., 2010). The algorithm ADAP was employed for the detection of the features (Myers et al., 2017). The peak lists were deisotoped and subjected to adduct search. A custom database was compiled with the secondary metabolites referenced in the Dictionary of Natural Product (DNP®) (Buckingham, 2010) for the family Ephedraceae and used in MZMine. Molecular formulas were extracted and validated after heuristic filtering within MZMine (Pluskal et al., 2012). Results of dereplication are summarized in the table S2.

Figure S1: UHPLC-HRMS-TOF chromatogram (positive mode) of Ephedra Herba with the numbers of dereplicated metabolites.

Table S2: dereplication of Ephedra herba

N°	m/z	Feature	Retention time (min)	Common name	Molecular Formula	Mass accuracy [ppm]
1	152.10538	[M+H]$^+$	2.62	norephedrine	$C_9H_{13}NO$	-10.7
2	166.12183	[M+H]$^+$	2.87	pseudoephedrine	$C_{10}H_{15}NO$	-5.2
3	**166.12180**	**[M+H]$^+$**	**3.08**	**ephedrine**	**$C_{10}H_{15}NO$**	**-5.2**
4	180.13782	[M+H]$^+$	3.41	methylephedrine	$C_{11}H_{17}NO$	-2.90
5	220.06093	[M+H]$^+$	4.27	6-Methoxykynurenic acid	$C_{11}H_9NO_4$	2.00
6	611.15784	[M+H]$^+$	6.39	Herbacetin 7-O-neohesperidoside	$C_{27}H_{30}O_{16}$	-4.70
7	317.06522	[M+H]$^+$	6.49	Pollenitin	$C_{16}H_{12}O_7$	-1.30

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2. Darstellung der Publikation mit Literaturverzeichnis

2.1. Introduction

2.1.1. Brown fat induction and implication in obesity control

As the World Health Organization (WHO) newsletter, 2021, indicates, obesity has become a global epidemic in recent decades, with at least 2.8 million people dying yearly from being overweight or obese. Once associated with high-income countries, obesity is now also prevalent in low- and middle-income so most of the world's population lives in countries where overweight/obesity kills more people than underweight. These facts show the urgency of available effective obesity-reducing treatments with good tolerability in long-term use and improvement of metabolic parameters.

Adipose tissue can be subdivided into two distinct types of fat cells, namely white and brown (Feldmann et al., 2009). White adipocytes (WAs) are specialized for storing of chemical energy, such as triglycerides. However, in recent years they have also been shown to play a central role as an endocrine organ and for inflammatory cell signalling (Richard et al., 2000). Under obese conditions, WAs represent a dysfunctional tissue (Tran et al., 2008), characterized by tissue inflammation, energy overload, fibrosis and insulin resistance (Lin et al., 2016). In addition, WAs change their distribution focus from subcutaneous to visceral depots during ageing in parallel to chronic metabolic diseases. In contrast, brown adipocytes (BAs) dissipate energy in the form of heat (thermogenesis) through uncoupling protein one (Ucp1) (Feldmann et al., 2009, Gesta et al., 2007). In newborn humans, infantile BAs are found to decrease shortly after birth up to the age of 20 (Heaton, 1972). They have traditionally been considered to be insignificant for health concerns in adults (Cannon and Nedergaard, 2004). Interestingly, targeted ablation of BAs results in diet-induced obesity, diabetes, and hyperlipidemia in mice (Lowell et al., 1993). Also, Ucp1-deficient rodents exhibit increased susceptibility to age- and diet-related obesity (Kontani et al., 2005), indicating that brown adipocytes may represent important checkpoints in whole-body energy homeostasis (Almind et al., 2007). Lastly, a recent retrospective study in humans has demonstrated substantial protection from dysfunctional metabolism in people with active brown adipose tissue (Becher et al., 2021).

Almost two decades ago, studies using [18F]-2-fluoro-D-2-deoxy-D-glucose positron emission tomography (PET) brought a complete paradigm shift by demonstrating that metabolically active BAs are not restricted to smaller mammals and human infants but can also be found in discrete areas of adult humans, including cervical, supraclavicular, axillary, and paravertebral regions (Nedergaard et al., 2007, Enerback, 2010, Frontini and Cinti, 2010). In addition to brown adipocytes, UCP1-positive cells also lie scattered in certain white adipose tissue (WAT) depots. The development of these so-called peripheral, inducible brown adipocytes, brite (brown in white) or beige cells (Rosenwald and Wolfrum, 2014, Petrovic et al., 2010, Lazar, 2008) can be stimulated by a process called 'browning' (Bartelt and Heeren, 2014, Wu et al., 2012, Ishibashi and Seale, 2010). Both brown and beige adipocytes are characterized by a large number of mitochondria and numerous small lipid droplets and both cell types are functionally thermogenic (Shabalina et al., 2013). All different fat cells (white, brown and brite) have high plasticity, reflecting a brisk and continuous environment-dependent adaptation of this tissue (Scheele and Wolfrum, 2020), while the origin of the different fat cells or their possibility of interconversion is still discussed (Lee et al., 2014, Sarjeant and Stephens, 2012, Wang and Seale, 2016, Frontini et al., 2013, Sun et al., 2021).

It was previously assumed that UCP-1-dependent thermogenesis could only be induced by ß3-receptor adrenergic stimulation (Cinti, 2005) or by sustained cold stimulus (Huttunen et al., 1981), batokine-mediated pathways have since become known (Scheele and Wolfrum, 2020, Singh et al., 2021). Batokines, produced and secreted from mature BAs, are involved in stimulating angiogenesis

/vascularization and neurite outgrowth within brown adipose tissue (BAT) (Pellegrinelli et al., 2018). Herein lies a promising option for an adrenergic-independent activation of BAs to reduce obesity and improve metabolic disorders through a targeted and well-tolerated pharmacological treatment. Comparable lipid accumulation of rodent and human BAs, and thus similar metabolism, can be obtained by housing mice in a thermoneutral environment with a high-fat diet, a so-called "humanized BAT mouse" for study purposes (de Jong et al., 2019).

2.1.2. Hypothesis driven screening and secondary metabolite profiling

In the last decades, traditionally used Asian herbs have drawn attention worldwide regarding their treatment potential (Tilburt and Kaptchuk, 2008). An essential part of effective drugs comes from natural sources, but the yield of drugs available on the market is limited (Verpoorte et al., 2005). However, the search for plant-derived isolated compounds acting on single-target reductionist bio-activity-guided approach (Verpoorte et al., 2005) based on high-throughput screening campaigns without any regard to the pre-existing therapeutic knowledge, was found problematic (Normile, 2003). This might be due to the fact that different concepts of traditional Asian and western medicine have not been considered in the screening process for herbal candidates and since pathophysiological features in both systems are often described by different terminologies. Therefore, selecting effective herbal candidates and developing the optimal experimental design requires an interdisciplinary and intercultural validation process taking modern scientific data as well as traditional empirical knowledge into account. At first, candidate herbs have to be reviewed systematically in the scientific and medical literature for their *in vitro*, *in vivo*, and clinical actions. These data should be combined with traditional medicinal knowledge and usage, and with existing data of bioactive compounds of a potential herb candidate (Friedemann et al., 2015). This procedure provides the basis for primary screenings in selected *in vivo* and *in vitro* models and efficacy assays, followed by transcriptomic, proteomic, and metabolomic analysis to develop mode-of-action hypotheses (Wang et al., 2013). Mechanistic and clinical studies complete the described process. Such an approach includes the advantage that the selected candidate substances have already been successfully tested in humans and have been defined as hypothesis-driven screening (Friedemann et al., 2015).

The composition of the hypothesis-based selected and prepared plant extracts should be precisely analyzed and documented. For the rapid estimation of the chemical composition of herbal extracts, metabolites profiling methods based on the hyphenation of liquid chromatography to mass spectrometry (LC-MS) are commonly used. Today, metabolite profiles are acquired with state-of-the-art techniques, such as ultrahigh performance liquid chromatography (UHPLC) coupled to high-resolution mass spectrometry (HRMS), ultraviolet photodiode array (PDA) and evaporative light scattering detection (ELSD) (Wolfender et al., 2015). With these methods, the identity of secondary metabolites is inferred from online spectroscopic and spectrometric data (PDA, HRMS, HRMS/MS), in combination with chemotaxonomic information, in a process called dereplication (Allard et al., 2016). Additionally, a semi-quantitative estimation can be obtained by the universal detection of ELSD (Dvorackova et al., 2014).

2.1.3. Asian herbs with potential brown fat regulating effects

The described screening procedure led us to the following weight-lowering herbs/herbal combinations with the potential for BAs activation or formation: *Jiang-Zhi-Ning* (JZN), *Celosiae semen* (C), *Puerariae lobatae radix* (PLR) and Ephedrae herba (EH).

2.1.3.1. Jiang-Zhi-Ning

JZN is a traditional recipe containing *Polygonum multiflorum radix, Nelumbo nucifera folium, Crataegi fructus* and Cassiae s*emen*. JZN has been used traditionally to treat arrhythmia and prevent and cure atherosclerosis and lower lipids for thousands of years (Chen et al., 2011). This composition demonstrated its ability to reduce the levels of blood lipids significantly (Chen et al., 2011, Chen et al., 2012a). Alkaloids from *Nelumbo nucifera folium* have been detected for lipid-lowering and weight-loss-inducing functions (Boustie et al., 1998, Kashiwada et al., 2005). 2-Hydroxy-1-methoxyaporphin (2H1M), an alkaloid from *Nelumbo nucifera* was identified as the constituent showing the major pharmacodynamic effect (Chen et al., 2012b). Four major components of JZN, stilbene glycoside, nuciferine, hyperine, and chrysophanol, showed a close relationship to the process of cholesterol metabolism (Chen et al., 2011, Chen et al., 2012b).

2.1.3.2. *Celosiae semen*

Celosiae semen (C) extracts have shown liver protecting, antioxidant, anti-inflammatory and anti-tumor activities. In a molecular dynamics simulation analysis (http://tcm.cmu.edu.tw), two compounds of C act as Fat-mass-and-obesity-associated- protein - inhibiting ligands (FTO). Interestingly, FTO-negative mice expressed significantly higher UCP1 in gonadal and inguinal adipose depots (Stratigopoulos and Leibel, 2010, Tews et al., 2013). The C compounds(S)-tryptophan-betaxanthin and 3-methoxytyramine-betaxanthin might be competitive FTO inhibitors and therefore induce UCP-1 expression in WAT (Chang et al., 2011).

2.1.3.3. The genus *Pueraria and Puerariae lobatae radix* (PLR)

Different species and plant parts of Puerariae have been extensively used in East Asia in various application forms, especially for the treatment of cardio-cerebrovascular related conditions and metabolic disorders like hypertension and diabetes, with more than 500 clinical trials in China (Zhang et al., 2013). Investigations in high-fat diet mice and human studies show an anti-obesity and anti-fatty liver effect of Puerariae (Kamiya et al., 2012b, Kamiya et al., 2012c). However, most of these studies lack randomized controls and double-blind designs (Zhang et al., 2013). A further handicap for effective study interpretation is the diversity of the existing nomenclature and interchangeably used species or varieties of significantly different Puerariae plants (Wong et al., 2015). Twenty different accepted species and 95 synonyms of the Puerariae genus exist (theplantlist.org, 2017). Since 2005, the roots of two different varieties, Puerariae lobatae (PLR) and Puerariae thomsonii (PTR) are listed separately in the Chinese Pharmacopoeia. However, they are still commonly used interchangeably in clinical practice in China (Wong et al., 2015). Since 2012, the European Pharmacopeia has also incorporated two distinct monographs for these two varieties (2012). The accepted botanical names are, in the case of PLR, Pueraria montana var. lobata (Willd.) Sanjappa & Pradeep and of PTR, Pueraria montana var. chinensis (Ohwi) Sanjappa & Pradeep (theplantlist.org, 2017). Both varieties contain mainly isoflavonoids, with puerarin as the main isoflavone (Wong et al., 2015). Until now, the C-glucoside puerarin has been reported to be absorbed and excreted principally unmodified (Barnes et al., 2011, Houriet et al., 2021). Besides puerarin, several O-glycoside isoflavonoids are described, such as daidzin and genistin (Barnes et al., 2011), which are more extensively metabolized and are hydrolyzed in the enterocytes of the small intestine to the aglycones daidzein and genistein (Barnes et al., 2011, Houriet et al., 2021). Puerarin likely contributes to the systemically relevant daidzein pool through deglycosylation because of observed daidzein in the urine after ingestion of puerarin in in-vivo studies in rats (Yasuda et al., 1995, Prasain et al., 2004). Consistent with this, systemically effective levels of puerarin were measured in blood plasma after oral PLR ingestion in human metabolism studies (Penetar et al., 2006). Furthermore, oral PLR ingestion in another human pharmacokinetic study led to detectable blood plasma concentrations of puerarin and daidzein simultaniously (Jung et al., 2014). These findings could be

confirmed by using porcine jejunum ex vivo to study absorption and biotransformation of PLR isoflavones (Houriet et al., 2021). The most abundant secondary metabolite and potentially active principle puerarin was found to be eleven times higher in PLR than in PTR (Wong et al., 2015) and can also be detected in the flower of PL (PLF), while there is no description that the flower of PT (PTF) contains puerarin. The highest isoflavone concentrations were detected in the outer bark of PLR (Chen, 2012). The major isoflavones of PLF are specified as kakkalide, 3'-hydroxy-puerarin, puerarin and puerarin-7-xyloside (Li et al., 2017) and of PTF 6-hydroxygenistein-6,7-di-O-glucoside, tectorigenin 7-O-xylosylglucoside, tectoridin, genistin and genistein (Hirayama et al., 2011) .

2.1.3.4. *Ephedrae herba*

Especially noteworthy are the proven anti-obesity effects of Ephedrae herba (EH) in rodents via adrenergic stimulation browning WAT. However, the clinical applicability is limited due to its active compound *ephedrine,* an indirect sympathomimetic drug that causes serious adrenergic cardiovascular side effects (Abourashed et al., 2003, Akagiri et al., 2008).

In this work, based on the observed metabolic and anti-obesity effects of the well-tolerated isoflavone-rich Puerariae herbs (Zhang et al., 2013, Kamiya et al., 2012c) the aim was to investigate the weight-lowering metabolic effects of PLR in an diet-induced obesity (DIO) mice model with focus on brown fat activity in white and brown fat tissue and to correlate our findings with its isoflavone content.

2.2. Material and Methods

The following Material and Methods of the screening study, which differ from the publication's, are added.

2.2.1. Herb material

The chosen candidate medicinal herbs analyzed in the screening process for the first test in an animal model are Jiang-zhi-ning (JZN), a traditional herbal recipe, consistent of Reynoutria multiflora (Thunb.) Moldenke radix (botanical name) versus Polygonum multiflorum radix (PMR) (pharmaceutical name), Nelumbo nucifera Gaertn. folium (botanical name) versus Nelumbinis nucifera folium (NNF) (pharmaceutical name), Senna obtusifolia (L.) H.S.Irwin & Barneby semen (botanical name) versus Cassiae semen (CS) (pharmaceutical name) and Crataegus pinnatifida var. major N.E.Br. fructus (Shan Zha) (botanical name) versus Crataegi fructus (CF) (pharmaceutical name) (Chen et al., 2012a, Chen et al., 2012b). Further single candidate herbs were Celosiae argentea L. semen (botanical name) versus Celosiae semen (C) (pharmaceutical name), Pueraria montana var. lobata (Willd.) Sanjappa & Pradeep root (botanical name) versus Pueraria lobata radix (PLR) (pharmaceutical name) and Ephedra sinica Stapf. (Mu Cao Ma Huang)/Ephedra equisetina Bunge. (Mu Zei Ma Huang) (botanical name) Ephedra herba (EH) (pharmaceutical name). The above listed Asian botanicals were all acquired as dried herbs from Herba Sinica (NNS: 121101H207, PMR: 120501H031, CF Ch.B 121101H055, CS: 121201H122, C: 121001H223, PLR: Ch.B. 130301H083, EH: Ch.B 100201H059; D-91126 Rednitzhembach, Germany). They all were tested for identity, purity and for residues and organic traces by Dr Uwe Gasser, Sebastian Kneipp research laboratory, D-86825 Bad Wörishofen, Germany). The accepted pharmaceutical and botanical plant names of all used herbs in this publication were verified with www.theplantlist.org on April 8th 2017, out of a Materica Medica (Chen et al., 2004).

2.2.2. Herb extraction

The Herb extraction method differs from the published method using rotation-vacuum evaporation (60°C, 200 mbar, Büchi, Rotavapor-R) and vacuum centrifugation (Bachofer) for drying.

2.2.3. Herb dosage for in animal study

The chosen doses were for PLR 10 and 5 g/day as a medium and low human dose, respectively (Lukas et al., 2005, Bensky et al., 2015), 6 g/day for EH as a typical human standard dose with a recommended range of 2 to 9 g/day (Bensky et al., 1993) and a standard dose of 10 g/day for C with a given range of 6 to 15 g/day (Chen et al., 2004). Three different treatment groups for JZN were used. The classic recipe was first mentioned in the "*Qian Jin Fang*" (652 bc) and is now recorded in the Volume 13 of Chinese Medicine Prescriptions Standard issued by PRC health ministry. The recommended percentages of the classic recipe of JZN are 4% PMR: 12% NNF: 80% CF: 4% CS with a predefined daily dosage of 90 g/day (Zhang et al., 2021, Chen et al., 2011). This dose was set for the first JZN group (J1-1) though the single dosage for CF is 71 g/day, which is much higher than listed in Chinese Materica Medica (Bensky et al., 2015). Therefore, a second JNZ group (J1-2) with lower concentrated (18 g/day) JZN comparable to low concentrations available in ready-to-use granules with a recommended daily use of 2-3 x 10g JZN (for example, on www.qgyyzs.net/e/products/view1551.htm) was built. The second dosage strengths consist of 14 g/day CF, which represents an average standard dose (Chen et al., 2004). Because of the high ratio of CF up to the 20-fold in this combination and because variations of JZN in Asia are commercially available as ready-to-use remedies with almost the same dosage strengths of the contained single herbs ((脂降宁片)说明书,脂降宁片12片x3板/盒多少钱-药房网商城 (yaofangwang.com)) a third JZN group (J2) with a ratio of 25% PMR: 25% NNF: 25% CF: 25% CS for each component of JZN with a total dose of 30 g/day was provided. The recommended daily doses for the JZN components are for PMR 10 g (range 10-30 g), NNF 6 g (range 6-15 g), CF 10 g (range 10-30 g) and CS 9 g (range 9-30g) (Chen et al., 2011, Bensky et al., 2015, Chen et al., 2004, Hempen, 2009). Using a formula to appropriate the doses for the mouse model (Reagan-Shaw et al., 2008, Reigner and Blesch, 2002), the daily dosages were converted to mice from the mentioned human doses above. The crude-to-extracted drug ratio and conversion into human and mice extract dosages are listed in Table 1 and 2.

2.2.4. Animal studies

In the first round, 40 obese, insulin-resistant mice were treated by daily oral gavage with different extracts of JZN (J1-1, J1-2, J2), PLR (P1 and P2), EH, C and water as a negative control for two weeks in compositions and concentrations listed in 2.2.3. The extracts prepared for the screening trials by the HanseMerkur Center for TCM at the UKE Hamburg and provided for the initial mouse trials at the ETH Zurich were anonymized so that the handlers were blinded to the substances administered. In the second round, DIO mice received the calculated PLR extracts with the daily food of 0,8 and 0,4 g/kg BW per day. Water was applied as s negative control, extract of EH with 0.27 g/kg BW per day as PC, since EH has been demonstrated to induce brown fat activity and mass as well as weight loss (Akagiri et al., 2008).

2.3. Results

2.3.1. Screening results of herbs/herbal combination candidates treatment in the DIO mouse model

2.3.1.1. Effects on metabolism by the different extracts

In order to perform metabolism studies on mice *in vivo*, an extract corresponding to the traditional PLR decoction was prepared at a scale which enabled the whole experiments. This extract was given to mice by gavage once a day over two weeks for the first metabolism study. Mice were monitored for body weight (BW) and circulating blood glucose and insulin levels during the two weeks. Mice treated with EH, PLR, C and JZN lost weight in a dose-dependent manner during treatment time except for JZN in the all-equal-dose composition J2 but significant BW reduction was only seen for EH. The reduced levels in BW on day 14 are due to a 12-hours fast at day 14 of the study (Fig1a). A significant reduction in blood glucose levels over time could only be observed by EH (Fig1b). PLR in the lower dose and JZN in the all-equal-dose composition J2 showed a significant drop in insulin levels after two weeks of herb administration, while insulin levels from EH and the higher dose PLR treated mice showed a clear trend towards reduction of insulin levels (Fig. 1c). In summary, improved metabolism for EH and for PLR was measured over the study period.

Concerning the measured lipid levels a significant decrease in circulating triacylglycerols (TAG) in animals treated with extracts of PLR in both dose strengths (Fig. 2b) and trends for C and JZN in all dosage groups (J1-1, J1-2, J2) towards reduction of TAGs can be observed. For free fatty acid levels (FFA), only trends toward reduction appeared after treatment with all tested extracts in relation to the negative control (Fig. 2c). Cholesterol level changes were not significant (Fig. 2a). Overall, an improved metabolism by supplementation of PLR extracts was obtained over the study period of 14 days.

2.3.1.2. Analysis of adipose tissue browning

To accurately assess the metabolic effects of the candidate herbs on adipose tissue in the DIO mouse model, mice were sacrificed after two weeks of treatment and inguinal (ingWAT) and visceral WAT (viscWAT), as well as intrascapular brown adipose tissue (BAT), were analyzed. The mean adipocyte size of the viscWAT was significantly reduced after C extract intake (Fig.3a), while the mean adipocyte size of the ingWAT was significantly decreased after PLRE intake in both dosage groups (Fig.3b).

Next, the focus turned to adipose tissue browning as a cause for enhanced energy expenditure by measuring the mRNA levels of brown and white fat markers. UCP1 mRNA levels increased in both dosage groups of PLRE (Fig. 4a) in comparison to the control. A similar trend was also observed for PPARγ2 and PLR extracts (Fig. 4b), but no change in any other markers of brown fat in these mice (Fig. 4c-e) was observed. In summary, it could be shown that both dosage strengths of PLRE in obese mice lead to an increase of brown fat markers (UCP1 and PPARγ2) in brown fat.

Since ingWAT has the capacity to form beige adipocytes, it was subsequently examined whether the herb drugs could induce the formation of these cells in our animal model system. To this end, mRNA expression of UCP1 and other beige/brown specific marker expressions in these animals were analyzed first. Strong induction of UCP1 mRNA with PLR treatment in two doses as well with *Ephedrae herba* was observed (Fig 5a). Similarly, the expression of other markers of brown fat activity, such as PPARγ2 (Fig. 5b) and PPARα (Fig. 5c) was increased for the treatment while no clear effect on Coxb8 (Fig. 5e) and Adiponectin (Fig. 5d) could be observed. On the contrary, the other proven substances exhibit no significant effects on the measured mRNA levels. Taken

together, these data demonstrate that PLR and *Ephedrae herba* treatment leads to the formation or activation of beige adipocytes in ingWAT.

2.3.2. Study results of PLR

The results of this preliminary study showed a significant increase in brown fat markers in interscapular brown and inguinal white fat depots accompanied by an improved metabolism in terms of weight loss, glucose metabolism and reduction of serum lipids for PLR. *Ephedrae herba* also achieves similar results in the DIO mouse model, but its brown fat activity-inducing ability is already well known, and therefore it was used further as a positive control (PC). Thus, PLR fulfils the prerequisite for in-depth in vivo and in vitro studies as well as for chemical analyses to identify the active substance(s) and their mechanism of action.

2.4. Discussion

Traditionally used anti-obesity Asian botanicals affect BW, among other pathways, by elevating thermogenesis and energy expenditure by inducing the formation or activity of UCP1-positive cells (U1pc) (Yuliana et al., 2014, Spiegelman and Flier, 2001). Meanwhile, several of these Asian plants or plant-derived substances have proven to effectively acitvate these cells, like isoflavones, capsaicin, berberine, ephedrine (Horvath and Wolfrum, 2020) and formononetin (Nie et al., 2018). For detailed information about dietary phytochemicals for body weight control via energy expenditure look up in the review from Horvath 2020 (Horvath and Wolfrum, 2020). In search for a well-tolerated effective anti-obesity weight loss agent from Asian botanicals by hypothesis-driven screening (Friedemann et al., 2015) led to PLR as a promising candidate herb with high potential to induce and/or activate U1pc.

The findings of this trial demonstrated significant weight loss, improved glucose tolerance, insulin resistance and serum lipids in a dose-depended manner in the PLR- and PC-treated mice. These effects were in line with the observed increase in energy expenditure, which might be due to the observed browning of ingWAT and the induction of BA activity (Kalinovich et al., 2017). *In vitro* experiments on brown adipocytes in this study proved that the isoflavones daidzein and genistein are responsible for the direct induction of UCP1-dependent BA activity and not the most abundant isoflavone of PLR, puerarin.

2.4.1. PLR and its isoflavone content

Puerariae plants are rich in isoflavones, although the isoflavone compositions of the Pueraria species are different (Li et al., 2017, Hirayama et al., 2011). Several isoflavones have been intensively studied, often in the research related to soy diet (*Glycine max* (L.) Merr.), and beneficial effects on metabolism and obesity were reported (van der Velpen et al., 2014, Wang et al., 2020). The doses of isoflavones employed in these previous studies were converted to murine dosages with the formula described above (Reagan-Shaw et al., 2008) in order to compare them with our PLR extract. The range of doses spread for puerarin from 20 to 400 mg/kg BW (Wu et al., 2013, Zhang et al., 2010), for daidzin from 8 to 32 mg/kg BW (van der Velpen et al., 2014, Lephart et al., 2004), for genistin from 3 to 46 mg/kg BW (van der Velpen et al., 2014, Lephart et al., 2004), for daidzein from 20 to 80 mg/kg BW (Ulbricht et al., 2015) and for genistein from 4 to 65 mg/kg BW (Lai et al., 2011). This means that the doses for puerarin in our experiments were in the lower range compared to studies presented in the literature, while the other ingested metabolites were present in smaller

amounts. Considering the fact that we have been working with a converted low and middle daily human dose of 5 and 10 g per day Puerariae radix crude drug while high recommended daily human doses can be increased up to 21 g per day (Chen et al., 2004, Bensky et al., 2015) and at maximum up to 60 g per day (Chen et al., 2004), higher doses may open the opportunity of enhancing our effects on obesity, especially on the formation and activation of brown fat.

2.4.2. Isoflavones and their *in vitro* effects on U1pc

Few *in vivo* studies about the competition of isoflavones to affect brown/beige adipocytes exist. Kamiya et al. analyzed that the isoflavones in PTF increase oxygen consumption, BAT activity and improve metabolism in HFD-obese mice (Kamiya et al., 2012a). In this project, we obtained comparable results with PLR treatment, despite the containing isoflavones of PTF and PLR being very different (Li et al., 2017, Hirayama et al., 2011). Besides hydroxypuerarin, common but not most abundant isoflavones of PTF and PLR are genistin and its aglycone genistein (Wong et al., 2015). Since we could identify genistein and daidzein and exclude the most abundant isoflavone puerarin to be responsible for the upregulation of UCP1 in BAs *in vitro*, genistein could be the common key compound to induce BAs activity in both PLR and PTF. However, there are differences. While a decrease of the visceral WAT weight in the PTF study has been observed, our research detected a mild UCP1 upregulation in BAT but a highly significant one in ingWAT with concomitant reduction of the mean adipocyte size in the inguinal but not in the visceral WAT. Regarding prospective human BAT studies, the induction of inguinal (subcutaneous) beige cells is promising since human adult BAT is known to be genetically and morphology more similar to the murine inguinal beige cells housed under cold conditions or to the classical interscapular BAT of mice housed at thermoneutrality (Wu et al., 2012, de Jong et al., 2019, Sanchez-Gurmaches et al., 2018).

To date, no reports exist about the most abundant ingredients of PLR, puerarin and of PTF, tectorigenin, to influence brown/beige fat tissue. The distinctions concerning the observed thermogenic effects in our study with the species PLR might be caused by other effective isoflavones, like daidzein, which is present in PLR but not in PTF and not by the most abundant and Puerariae species-specific isoflavones. In accordance with our murine *in vivo* findings, daidzein treatment was shown to increase the UCP1 expression in rats (Crespillo et al., 2011). For soy isoflavones, a BAT activation in rats is described (Crespillo et al., 2011), but no similar *in vivo* study for genistein has been performed so far. Our cell assays showed direct brown fat activation as measured by UCP1 upregulation in cultured BAs when incubated with genistein or daidzein. Aziz et al. (Aziz et al., 2017) recently found a browning process in 3T3-L1 adipocytes after genistein administration. This formation of beige cells was directly triggered by genistein and did not follow the classical estrogen receptor (ER) pathway. Daidzein upregulated UCP1 strongly in cultured BAs and promoted BAs differentiation significantly in our study. Cell differentiation was also promoted in 3T3-L1 adipocytes (Cho et al., 2010), while another group observed reduced proliferation in the same cell type (He et al., 2016). In summary, direct BAs activation in white and brown adipocytes is shown to be caused by the well-known soy isoflavones genistein and daidzein and not by puerarin.

2.4.3. The role of puerarin in metabolic regulation

Besides the data, which demonstrates that PLR treatment upregulates UCP1 in ingWAT and BAT, PLR also was shown to reduce obesity by affecting muscle mass by Jung et al (Jung et al., 2017). Consistent metabolic effects of PLR in C57BL/6 mice on our findings have been presented. In that study, puerarin was indicated to improve mitochondrial function and myotube differentiation in skeletal muscle cells *in vitro* (Jung et al., 2017). The anti-obesity effect was supposed to be caused by increased energy metabolism in hypertrophy skeletal muscles by puerarin-induced upregulation of PGC1α levels (Jung et al., 2017). PGC1α acts as an essential mediator of the

mitochondriogenesis in both muscle (Sandri et al., 2006) and UCP1-dependent fat tissue (Barbera et al., 2001). Furthermore, muscle mass is positively linked to secretion of the muscle polypeptide hormone irisin (Kurdiova et al., 2014), which has been reported to induce beige cells in ingWAT (Bostrom et al., 2012). Thus, puerarin might also play an indirect role in the browning process of ingWAT conciliated by irisin. Similar PLR-mediated crosstalk between fat tissue and different organs, mainly the liver, is feasible since puerarin has been shown to promote bile acids metabolism (Yan et al., 2006) and bile acid has been shown to be involved in UCP1 dependent BAs activation in ingWAT and BAT in other studies (Zietak and Kozak, 2016, Worthmann et al., 2017). Whether puerarin unfolds indirect UCP1 expression in mice has to be clarified in further explorations. In our in vitro examinations, puerarin offered strong support of cell differentiation and, to lower extent, cell formation without direct involvement in the UCP1 upregulation in cultured brown adipocytes and is described to promote differentiation in white preadipocytes too (Lee et al., 2010).

2.4.4. Working mechanism of daidzein and genistein on UCP1

The underlaying pathway of daidzein and genistein dependent brown/beige fat induction is not clear. Both isoflavones bind directly to the intracellular receptors PPARγ and PPARα which are supposed to play key roles in the UCP1 thermogenesis (Villarroya et al., 2007, Mezei et al., 2003). Both PPARs were significantly upregulated in ingWAT but not in BAT. This difference is due to the already strong BAT activity. In addition phytoestrogens, daidzein and genistein bind to the ER with estrogen-like effects (Kuiper et al., 1998), however at least genistein was disproven to induce UCP1 expression in cultured white adipocytes via ER (Kurdiova et al., 2014), suggesting that ER might not be the mediator for the observed UCP1 activity induced by genistein and daidzein. In contrast, only the blockage of all three ER (α, β, γ) in BAs ablated UCP1 upregulation *in vitro* (Gantner et al., 2016). Further pathways through PPAR regulated genes and other transcription factors or through an endocannabinoid pathway in the case of daidzein are possible mechanisms by which these two isoflavones might induce BAT function (Barbera et al., 2001).

2.4.5. Possible roles of the other isoflavones

In addition to the quantified isoflavones, the study of the chemical composition of PLR identified other isoflavones by dereplication (Fig.5, suppl. data Tab.1). The choice of daidzein, genistein and puerarin for further *in vitro* investigation was justified on the one hand by the data related to the metabolism of the isoflavones and on the other hand by the high level of puerarin in the PLR extract. Concerning the O-glycosides isoflavones, their small intestinal metabolism into daidzein was already described previously (Rowland et al., 2003, Manach et al., 2005). Indeed, their O-glycosidic bond is cleaved by an enzymatic process. C-glycosides are reported to be mainly observed in the blood circulation, while their unabsorbed fractions which reach the colon undergo further microbial metabolism into their aglycones (Prasain et al., 2007, Qiao et al., 2016). All isoflavones also undergo phase II metabolism (Zhou et al., 2013, Qiao et al., 2016). Consequently, isoflavone glycosides in PLR extracts are a potential source of daidzein before their phase II metabolism. Thus, according to our dereplication (Fig.5, suppl. data Tab.1), the isoflavones (**1-2, 5-6, 10, 12, 13**) may contribute to the pool of daidzein in addition to puerarin (**4**) and daidzin (**8**). Only genistin (**15**) contributes to the level of genistein (**16**) after metabolism. The residual aglycones (**3, 7, 9**) (9.7 % of the isoflavone content estimated by ELSD) may have to be further investigated. Assuming that the whole pool of isoflavones reaches the colon, they could potentially yield daidzein at a maximum of 88 % of the isoflavone content estimated by ELSD. This estimation correlates with the [1]HMR measurement (puerarin at 7 % w/w and other isoflavonoids at 6.6 % w/w, giving a total of 13.6 % w/w), it could be suggested that about 11.9 % w/w of the PLR extract can produce daidzein. Meanwhile, a co-author of our study was able to confirm isoflavone metabolism in a porcine jejunum model (Houriet et al.,

2021). In this study, formononetin was also detected at low levels in PLR. Formononetin acts as a PPARγ agonist and, like daidzein and genestein, activates thermogenesis in adipocytes (Nie et al., 2018).

2.4.6. Human trials

Concerning the investigation of isoflavones, to date, no human clinical trial included energy expenditure or BAT activation as outcome measures (Horvath and Wolfrum, 2020). Clinical studies with the genus Pueraria showed beneficial effects in reducing postmenopausal symptoms and reduced low-density lipoprotein (LDL) cholesterol but enrolled only small number of test subjects (Wang et al., 2020). In the future, it has to be elucidated if the induction of BAs activation, the increased energy expenditure, and the improved metabolism in rodents by PLR are transferable to humans. Study results from animal models are not easily extrapolated to human conditions for various reasons (see this in detail (Horvath and Wolfrum, 2020)). Kamiya et al. (Kamiya et al., 2012c) tested PTF in obese humans and observed weight loss and a decrease in visceral but not in subcutaneous fat. These results are consistent with their previous animal study (Kamiya et al., 2012a, Kamiya et al., 2012b) even if measurements of energy expenditure and BAT activity in humans were missing. However, a recent animal study failed to verify the visceral fat reduction in ovariectomized mice fed a standard diet by adminisering lower doses of PTF isoflavone extracts than in the studies mentioned above (Tousen et al., 2019).

Currently, the detection of BAT thermogenesis in humans is limited to invariably invasive methods like positron emission tomography (PET), single photon emission computerized tomography (SPECT), magnetic resonance imaging (MRI), near-infrared fluorescence imaging (NIRFI) and ultrasound. In future, stable biomarkers, i.e., TSPO, a translocator protein expressed in mitochondria, might improve BAT mass imaging (Yang et al., 2021). Recently, Chen et al. (Chen et al., 2016) identified the exosomal micro RNA miR 92a to represent a specific serum biomarker for UCP1 activity in humans. This innovation might permit simple measurements of the UCP1 activity by blood analysis in large human cohorts in the near future. Two human PET studies with ephedrine, the active compound of our positive control EH, were performed and did not detect any increase in BAT thermogenesis but yet a decrease of human BAT activity by chronic ephedrine treatment (Cypess et al., 2012, Carey et al., 2015). EH is proven to induce browning in rodents via β3-adrenergic stimulation but also to cause serious cardiovascular side effects due to the co-existing affinity to β1-receptor (Akagiri et al., 2008, Baker, 2010). Contrary to rodents, β3-adrenergic receptors are only marginally expressed in human adipocytes (Jaworski et al., 2007). Compared with EH, PLR can be considered a well-tolerated and safe drug if administered orally in an average dose range (Zhang et al., 2013). Only in high dosages of PLR exhibit hepatotoxic and anti-fertility side effects (Wang et al., 2020).

2.4.7. Future perspectives

Several studied Asian herbs are isoflavone-rich and showed beneficial effects on metabolism and obesity by different mechanisms, as specified above. The long-term objective might be to determine the ideal anti-obesity active isoflavone combination with regards to its ingredient effectiveness, bioavailability and side effects. Some seminal examples from drug discovery history underline this vital point. Youyou Tu received the Nobel prize in 2015 for her discovery of a malaria drug out of Artemisia annua herba (Liu and Liu, 2016). She received the key information for a low-temperature extraction method out of a Chinese handbook from 1574 AD. This led to the identification of the non-toxic, effective single active compound arteminisin (Tu, 2011).

In contrast, the Asian herb EH and its active compound ephedrine show serious side effects and high cell toxicity if used in a pulverized extraction form (Lee et al., 2000). Therefore, the FDA banned both in 2004 as supplements to lose weight, while it is still accepted for the medical treatment of respiratory diseases (FDA, 2004). After intensive research of the Chinese literature, the Center for Safety of Chinese Herbal Therapy (CTCA) 2015 in Germany could not elicit those negative effects of the widely used EH if the pure herb was traditionally employed in a classic decoction recipe. Though, the crude extract from Hypericum perforatum, a botanical used worldwide to treat mild depression, was found to be most efficient, if standardized fractions of the plant were extracted compared to the single-isolated constituents (Russo et al., 2014). Therefore, further active compounds have to be identified, the underlying pathways clarified, and metabolomics analysis performed, followed by clinical studies to demonstrate effectiveness in humans. Regarding PLR, we can note that the observed energy expenditure in HFD-obese mice is probably a combined effect (synergistic or/and additive) of different isoflavones of the plant. The aglycones genistein and daidzein have been shown to cause direct UCP1-dependent mitochondrial uncoupling in white (Aziz et al., 2017, Crespillo et al., 2011) and brown adipose tissue. Taking into account the bioavailability of Pueraria isoflavones, it can be assumed that puerarin contributes substantially to the daidzein pool and, by this alone, causes indirectly mediated browning of ingWAT. In line with this, it has been shown that puerarin increases energy expenditure by supporting skeletal muscle metabolism (Jung et al., 2017). These results open an objective for further research, namely, to clarify the effectiveness in humans and to answer the question of whether the crude plant, an isoflavone fraction, a new isoflavone combination or a mono-isoflavone preparation is the most effective remedy in adiposity therapy.

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2.6. Anhang

2.6.1. Table 1

|	PLR (I)	PLR (II)	EH	C	PMR	NNF	CF	CS
Ratio crude to extract	0,47	0,4	022	0,06	0,08	0,43	0,33	0,08

Table 1. Extraction ratio. Ratio between the amount of crude and extracted drug for PLR (Puerariae lobatae radix) for the first (PLR (I) and second (PLR (II)) herb extraction method, EH (Ephedrae herba), C (Celosiae semen), PMR (Polygoni multiflora radix), NNF (Nelumbinis nucifera folium), CF (Crataegi fructus) and CS (Cassiae semen).

2.6.2. Table 2

		PLR1 (I)	PLR2 (I)	PLR1 (II)	PLR2 (II)	EH	C	J1-1	J1-2	J2
A	Human crude dose [g/d]	10	5	10	5	6	10	90	14	30
B	Human extract dose [g/d]	4,6	2,3	4	2	1,3	0,57	29	5,8	6,5
C	Mice extract dose [g/kg/d]	0,94	0,47	0,8	0,4	0,27	0,12	6	1,2	1,3

Table 2. Calculation of the daily extract doses of mice. Conversion of A) daily human crude drug doses of the herbs (g/day) to B) corresponding extract doses for humans in g/day and C) for mice in g/kg body weight (BW) per day (BW 60 kg, body surface area 1.6 m^2). PLR (Puerariae lobatae radix) extract dosage (PLR1 and 2) for the pre- (PLR (I)) and for the main study (PLR (II)), EH (Ephedrae herba) extract, C (Celosiae semen) extract, J1 (4% PMR (Polygoni multiflora radix): 12% NNF (Nelumbinis nucifera folium): 80% CF (Crataegi fructus): 4% CS (Cassiae semen) extract (dosage J1-1 and J1-2) and J2 (25% PMR: 25% NNF: 25% CF: 25% CS) extract.

2.6.3. Figure 1
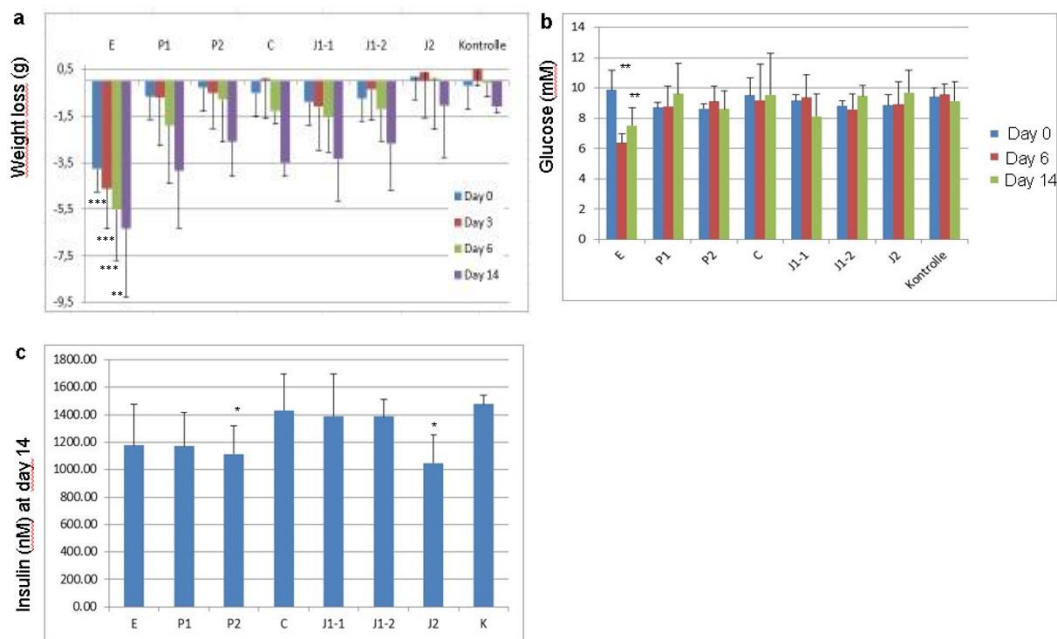
Figure 1a-c. Treatment effects by feeding different herbs/ herbal combinations on weight loss and glucose metabolism. *a) Weight loss was gained predominantly with extracts of Ephedrae herba (E) but as well with Celosiae semen (C) and dose-dependent with extracts of Puerariae lobatae radix (PLR) in both dosage groups P1+P2 and Jiang-zhi-ning (JZN) in the dosage groups J1-1 and J1-2. b) Improved glucose metabolism was mainly achieved with E and c) improved plasma insulin levels with PLR in a lower dosage (P2) and JZN in the all-equal-dose composition J2. Feeding daily per gavage at 4 pm over two weeks. Treatment with extracts of 0.27 g/kg body weight (BW)/day for E, first dosage group with 0,94 g/kg BW/day PLR (P1), second dosage group PLR with 0,47 g/kg BW/day (P2), 0,12 g/kg BW/day for C, 6 g/kg BW/day for the first dosage group of the Jiang-zhi-ning (J1-1) with a mixing ratio of 1:3:20:1 of Polygonum multiflori radix (PM):Nelumbinis folium (N):Crataegi fructus (CF):Cassiae semen (CS) and second group J1-2 with the same ratio and 1,2 g/kg BW/day and third group J2 with a ratio of 1:1:1:1 with 1,3 g/kg BW/day. (Mean +/- SD, n=5). One-way or two-way ANOVA compared to the control group with post hoc tests. * P < 0.05, ** P < 0.01, *** P < 0.001.*

2.6.4. Figure 2
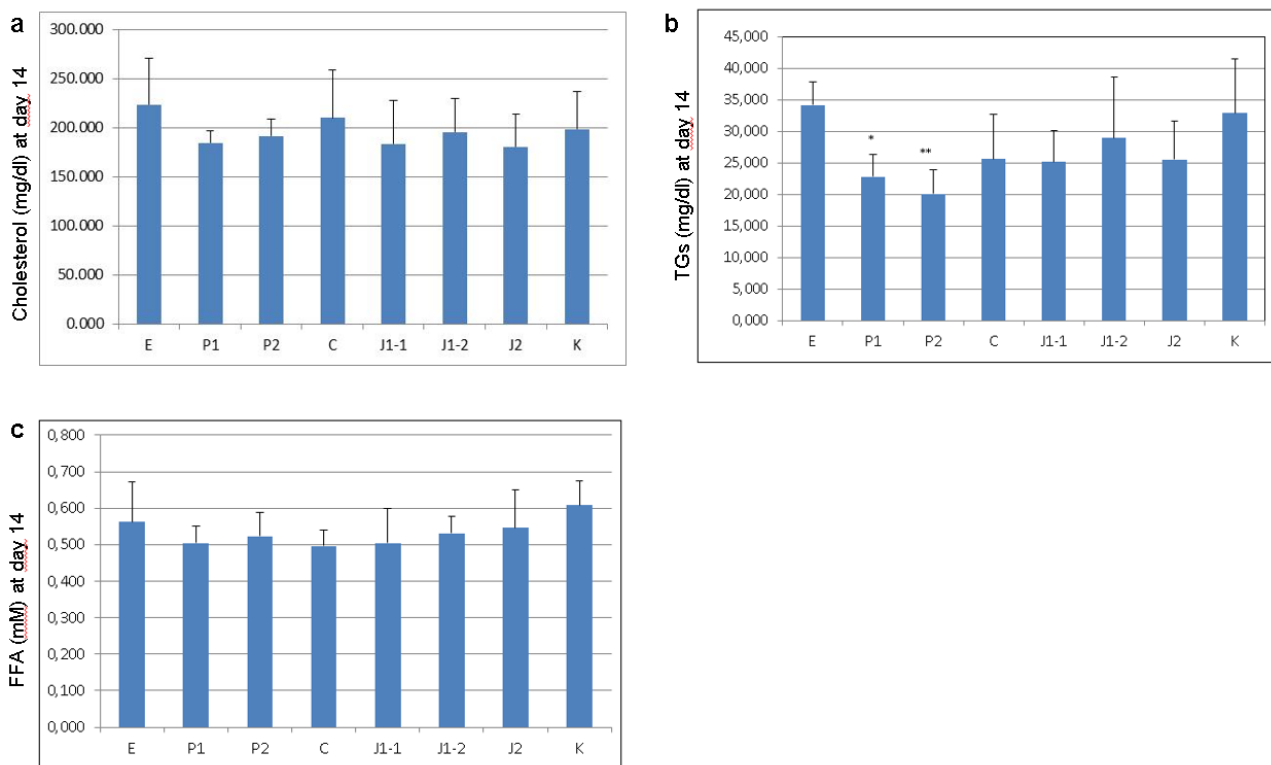
Figure 2a-c. *Puerariae lobatae radix extracts (PLRE) intake reduces plasma lipids in mice.* PLRE treatment significantly decreases b) plasma triglyceride (TG) levels, while trends can be observed for all other treatment groups too, except for Ephedrae herba (E). Only trends of reduced c) free fatty acids (FFA) are demonstrated for all extract groups, while the a) cholesterol levels remained unchanged. Feeding daily per gavage at 4 pm over for two weeks. Treatment with extracts of 0.27 g/kg body weight (BW)/day for E, first dosage group with 0,94 g/kg BW/day PLR (P1), second dosage group PLR with 0,47 g/kg BW/day (P2), 0,12 g/kg BW/day for C, 6 g/kg BW/day for the first dosage group of the Jiang-zhi-ning (J1-1) with a mixing ratio of 1:3:20:1 of Polygonum multiflori radix (PM):Nelumbinis folium (N):Crataegi fructus (CF):Cassiae semen (CS) and second group J1-2 with the same ratio and 1,2 g/kg BW/day and third group J2 with a ratio of 1:1:1:1 with 1,3 g/kg BW/day. (Mean +/- SD, n=5). One-way or two-way ANOVA compared to the control group with post hoc tests. * P < 0.05, ** P < 0.01, *** P < 0.001.

2.6.5. Figure 3
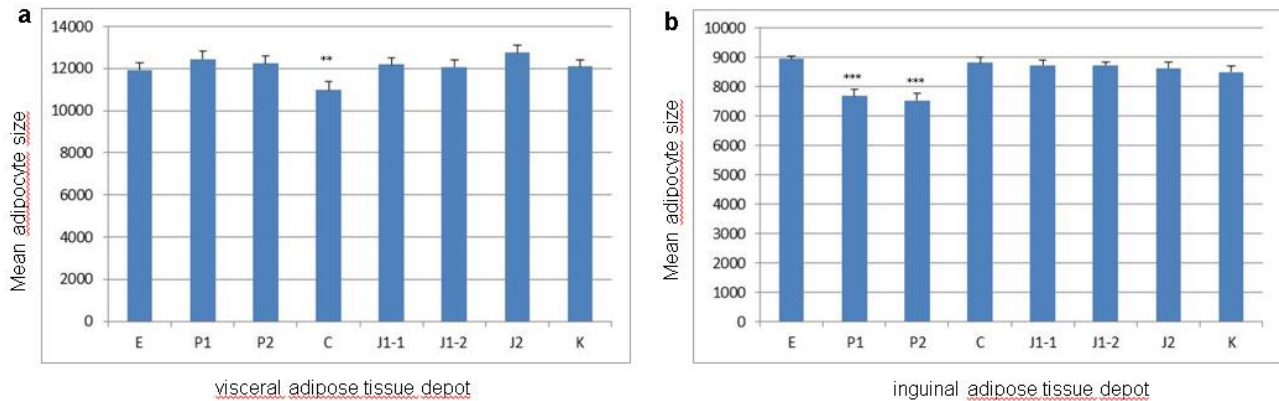
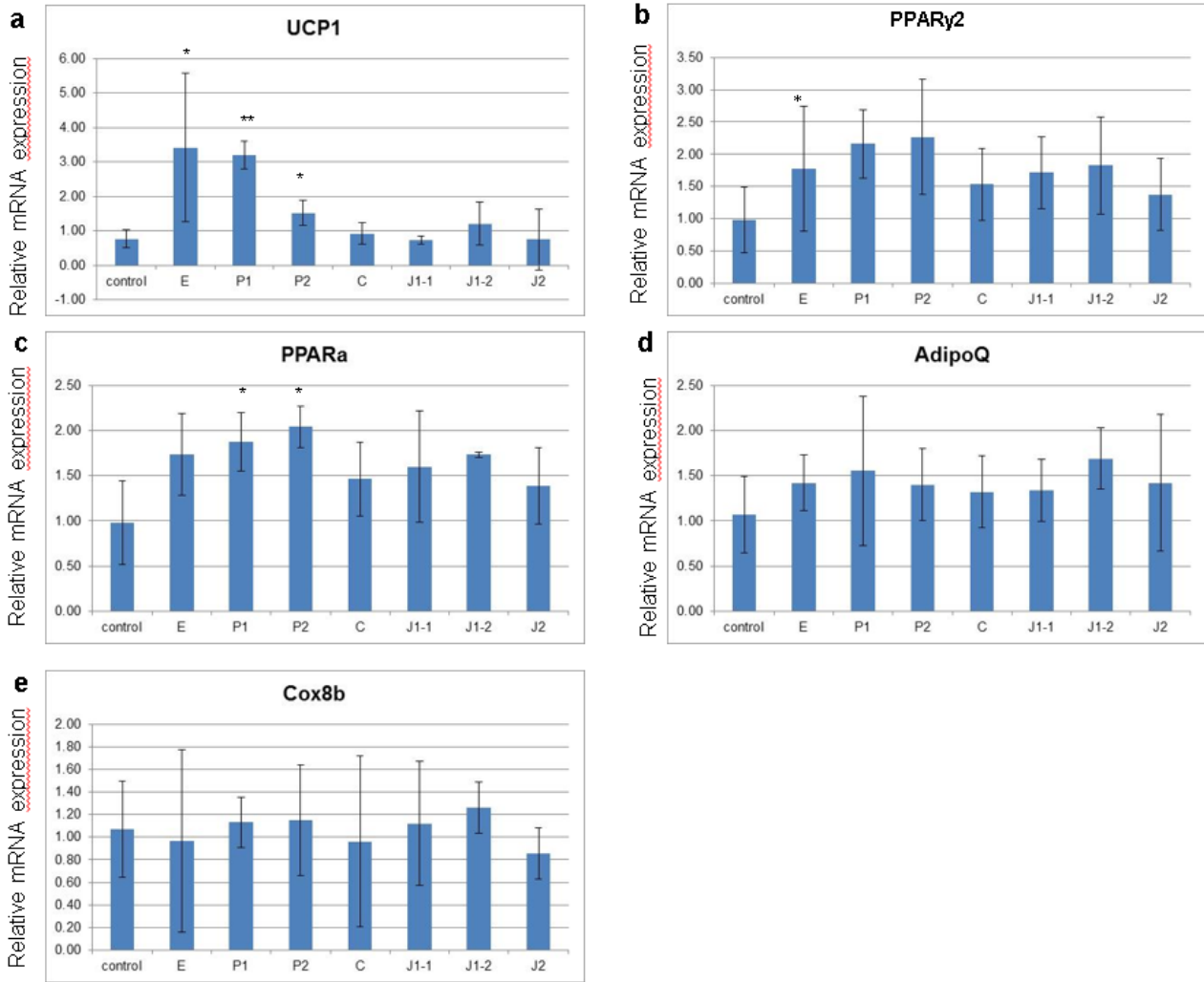***Figure 3a-b Puerariae lobatae radix extracts (PLRE) intake reduces b) the mean adipocyte size in inguinal tissue depot high significantly and a) Celosiae semen (C) the mean adipocyte size in visceral adipose tissue.*** *Feeding daily per gavage at 4 pm over for two weeks. Treatment with extracts of 0.27 g/kg body weight (BW)/day for E, first dosage group with 0,94 g/kg BW/day PLR (P1), second dosage group PLR with 0,47 g/kg BW/day (P2), 0,12 g/kg BW/day for C, 6 g/kg BW/day for the first dosage group of the Jiang-zhi-ning (J1-1) with a mixing ratio of 1:3:20:1 of Polygonum multiflori radix (PM):Nelumbinis folium (N):Crataegi fructus (CF):Cassiae semen (CS) and second group J1-2 with the same ratio and 1,2 g/kg BW/day and third group J2 with a ratio of 1:1:1:1 with 1,3 g/kg BW/day. (Mean +/- SD, n=5). One-way or two-way ANOVA compared to the control group with post hoc tests. * P < 0.05, ** P < 0.01, *** P < 0.001.*

2.6.6. Figure 4
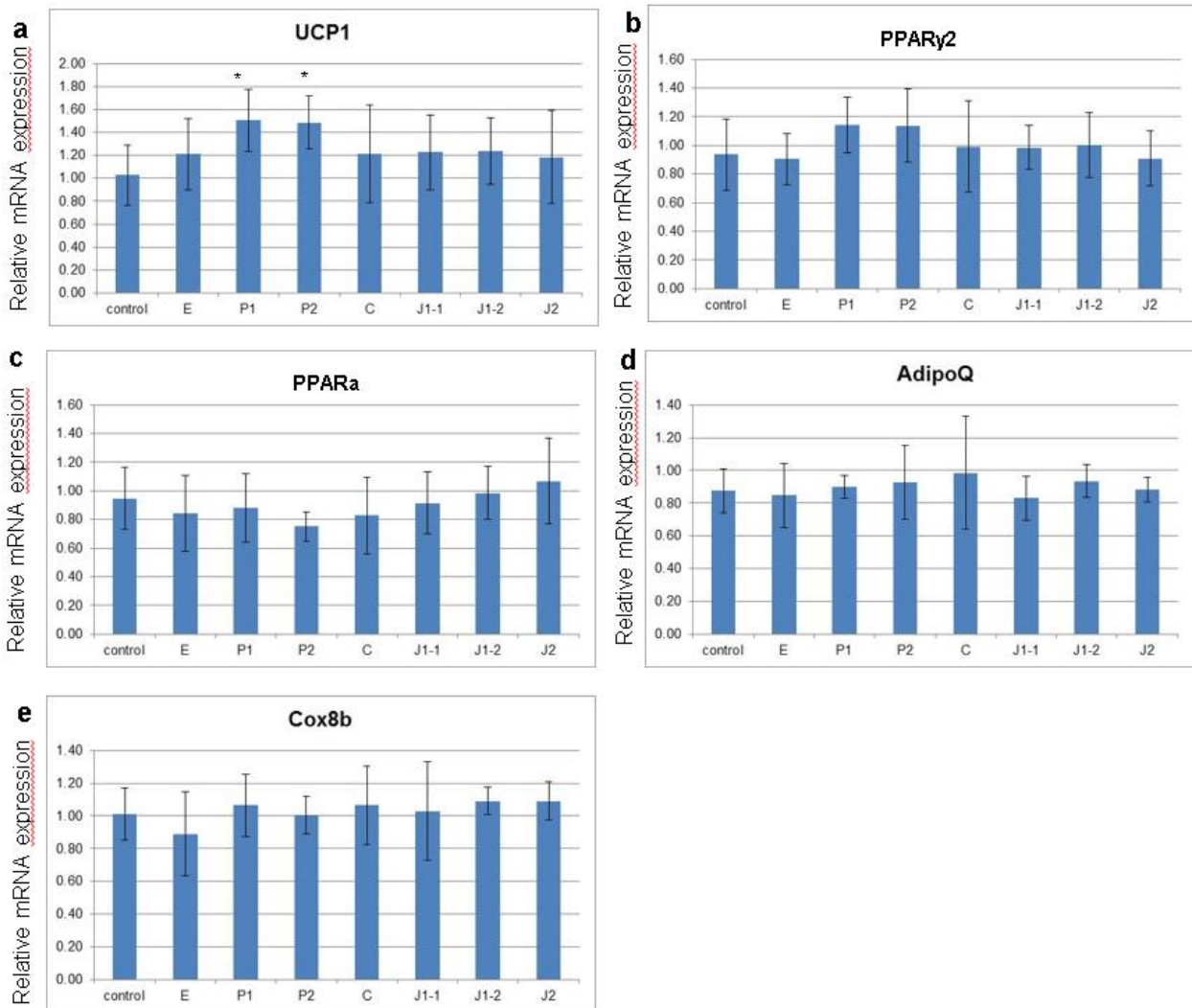
Figure 4a-e. Puerariae lobatae radix extracts (PLRE) supply conducts a significant activation of interscapular brown adipose tissue. *Significantly increased a) UCP1 mRNA expression levels and mildly increased b) PPARγ2, while b-e) all other brown fat markers remained unchanged. Treatment with extracts of 0.27 g/kg body weight (BW)/day for E, first dosage group with 0,94 g/kg BW/day PLR (P1), second dosage group PLR with 0,47 g/kg BW/day (P2), 0,12 g/kg BW/day for C, 6 g/kg BW/day for the first dosage group of the Jiang-zhi-ning (J1-1) with a mixing ratio of 1:3:20:1 of Polygonum multiflori radix (PM):Nelumbinis folium (N):Crataegi fructus (CF):Cassiae semen (CS) and second group J1-2 with the same ratio and 1,2 g/kg BW/day and third group J2 with a ratio of 1:1:1:1 with 1,3 g/kg BW/day. (Mean +/- SD, n=5). One-way or two-way ANOVA compared to the control group with post hoc tests. * P < 0.05, ** P < 0.01, *** P < 0.001.*

2.6.7. Figure 5

Figure 5a-e. Puerariae lobatae radix extract (PLRE) and Ephedrae herba (E) cause an upregulation of brown fat markers in inguinal white adipose tissue (ingWAT). *mRNA expression levels in ingWAT increased significantly in a) UCP1 and c) PPARα and with a trend in b) PPARγ2 with PLRE. Ephedrae herba (E) significantly increased mRNA levels in a) UCP1 and b) PPARγ2. Treatment with extracts of 0.27 g/kg body weight (BW)/day for E, first dosage group with 0,94 g/kg BW/day PLR (P1), second dosage group PLR with 0,47 g/kg BW/day (P2), 0,12 g/kg BW/day for C, 6 g/kg BW/day for the first dosage group of the Jiang-zhi-ning (J1-1) with a mixing ratio of 1:3:20:1 of Polygonum multiflori radix (PM):Nelumbinis folium (N):Crataegi fructus (CF):Cassiae semen (CS) and second group J1-2 with the same ratio and 1,2 g/kg BW/day and third group J2 with a ratio of 1:1:1:1 with 1,3 g/kg BW/day. (Mean +/- SD, n=5). One-way or two-way ANOVA compared to the control group with post hoc tests. * P < 0.05, ** P < 0.01, *** P < 0.001.*

3. Zusammenfassung

In den letzten Jahrzehnten hat sich Übergewicht zu einem der größten Gesundheitsrisiken für die Weltbevölkerung entwickelt. Dauerhaft erfolgreiche Therapiekonzepte zur Gewichtsreduktion stehen bislang nicht zur Verfügung. Die Bildung und Aktivierung brauner Adipozyten mit konsekutiver Erhöhung des Energieverbrauchs könnte sich zukünftig als effektive Therapieoption erweisen. Zahlreiche asiatische Kräuter werden traditionell zur Gewichtsreduktion eingesetzt. Ziel dieser Arbeit war es, asiatische Kräuter auf ihre Fähigkeit zur Bildung oder Aktivierung von braunem Fett zu untersuchen. Potentiell braunes Fett aktivierende asiatische Kräuter wurden mit einer neuartigen Methode, dem Hypothesen-basierten-Screening, ausgewählt und in Mäusen mit diätinduzierter Fettleibigkeit (DIO-Mausmodell) getestet. Wirksam in der Aktivierung brauner Adipozyten erwies sich die Wurzel von Pueraria montana var. lobata (Willd.) Sanjappa & Pradeep (PLR), woraufhin eine umfassende Metaboliten-Profilierung des hydrophilen Extrakts von PLR erfolgte. In-vitro-Experimente wurden in einem nächsten Schritt zur Klärung der Wirkungsweise von PLR auf molekularer Ebene durchgeführt. Die Hauptwirkung von PLR bestand in einem signifikanten Gewichtsverlust der Mäuse und einem verbesserten Glukosestoffwechsel bei gleichzeitiger Bildung und Aktivierung von braunen Adipozyten im inguinalen Fettgewebe. In Zellkulturexperimenten wie dem Luciferase-Assay ließ sich eine zellautonome Aktivierung der braunen Adipozyten nachweisen, die nicht primär durch das autonome Nervensystem gesteuert wird. Der Hauptsekundärmetabolit von PLR, Puerarin, weist keine Wirkung auf die Bildung und Funktion brauner Adipozyten auf, sondern hat lediglich Effekte auf die Zelldifferenzierung. Die Induktion braunen Fetts wird durch zwei weitere Isoflavone des PLR, Daidzein und Genistein, in dosisabhängiger Weise induziert. Daidzein ist zwar nur in sehr geringen Mengen in PLR enthalten, jedoch wurde die in dieser Arbeit aufgestellte Hypothese, dass glykosidische Isoflavone, vor allem Puerarin, metabolisch zum Daidzein-Pool beitragen, inzwischen in aktuellen Studien wissenschaftlich untermauert. Dies erklärt wiederum die starken metabolischen Effekte in unseren in-vivo-Experimenten, trotz der von uns gewählten niedrigen PLR-Dosierung. Inwieweit indirekte, durch das autonome Nervensystem vermittelte Effekte zu den in der Maus erzielten Ergebnissen beitragen, muss in weiteren Untersuchungen geklärt werden. Zusammenfassend hat PLR positive Effekte auf den Stoffwechsel und die Gewichtsabnahme im DIO-Mausmodell gezeigt und Hinweise auf die Wirkungsweise von PLR auf molekularer Ebene geliefert.

Wesentliche Ergebnisse:
- Pueraria lobatae radix (PLR) induzierte die Bildung und Aktivität brauner Adipozyten bei fettleibigen Mäusen.
- Braune Fettaktivität bzw. -bildung wurde nicht durch den Hauptmetaboliten, Puerarin, sondern durch Daidzein, einem gering konzentrierten Metaboliten in PLR induziert.
- Chemische Analysen von PLR zeigten das Vorhandensein von Daidzein Vorstufen.

4. Abstract

In recent decades, obesity has become one of the most significant health risks facing the global population. The success of weight-loss therapy is, up to now, very limited. Induction of brown adipocyte formation and activity represents a promising therapy to ameliorate obesity and metabolic disorders by increasing energy expenditure. Numerous Asian herbs are traditionally used to treat obesity. This work aims to investigate the potential of Asian herbs in treating of obesity through the formation or activation of brown fat. Promising herb candidates were selected using a novel method, a hypothesis-driven screening approach, and tested in an initial diet-induced obesity (DIO) mouse model trial. The root of *Pueraria montana* var. *lobata* (Willd.) Sanjappa & Pradeep (PLR) was identified to be a potential brown adipocyte activator. In the next step, we verified the results of PLR water extract in our DIO mouse model and characterized its secondary metabolite composition, followed by in vitro experiments to clarify the mode of action on a molecular level. The main effects of PLR consisted of significant weight loss in the mice and improved glucose metabolism with concomitant induction of brown adipocyte formation and activity in inguinal fat tissue. Using cell culture experiments such as luciferase assay, we demonstrated cell autonomous activation of brown adipocytes that is not primarily controlled by the autonomic nervous system. Brown adipocyte formation and function were not released by the most abundant secondary metabolite puerarin, which had effects simply on cell differentiation. However, two other isoflavones of PLR, daidzein and genistein induced brown fat cells and activity in a dose-dependent manner. Daidzein is present only in very small amounts in PLR extract but the hypothesis in this paper that various glycosidic isoflavones, including mainly puerarin, release daidzein after metabolism and thus contribute to the systemic daidzein pool has been scientifically confirmed in recent studies. This accounts for the solid metabolic effects in our in vivo experiments despite the selected low dose strength of the PLR extract. The extent to which indirect effects mediated by the autonomic nervous system contribute to our results obtained in vivo must be clarified in further investigations. In summary, this approach demonstrated positive metabolic and weight loss effects of PLR on the DIO mouse model and provided evidence for the mode of action of PLR at the molecular level.

Highlights

- Pueraria lobate radix (PLR) induced brown adipocyte formation and activity in obese mice.
- The most abundant component of PLR, puerarin, showed no effect.
- A lower concentrated component, daidzein, induced brown adipocyte formation and activity.
- The Chemical investigation highlighted the presence of precursors of daidzein in PLR.

5. Erklärung des Eigenanteils an der Publikation

Hiermit versichere ich, Elisabeth Buhlmann, dass ich die folgenden Anteile für die Erstellung der Publikationspromotion: „Puerariae lobatae root extracts and the regulation of brown fat activity." selbständig erarbeitet habe:

- Anwendung eines neuartigen hypothesengesteuerten Screening-Ansatzes zur Identifikation potentieller Screening Kandidaten (Asiatische Phythopharmaka) nach Dr. rer. nat. Thomas Friedemann und PD Dr. med. Sven Schröder [Friedemann et al 2015] und folglicher Identifikation von 5 Screening Kandidaten unter Betreuung von PD Dr. med. Sven Schröder
- Bereitstellung hydrophiler Extrakte für die Tierversuche an der ETH Zürich und der Analytik an der Universität Genf, einschließlich:
  - des Erwerbs der Rohdrogen sowie der Überprüfung der Zertifizierung dieser Substanzen
  - der Festlegung mittlerer humaner Kräuterdosen in Absprache mit PD Dr. med. Sven Schröder.
  - Umrechnung der humanen Dosen auf äquivalente Mausdosen nach Herstellung hydrophiler Extrakte aus den Rohdrogen.
- Auswertung der Screening Ergebnisse aus dem Mausversuch sowie konsekutiver Auswahl einer Verumsubstanz sowie einer Positivkontrolle unter Mithilfe von Prof. Dr. rer. nat. Christian Wolfrum
- Promotionsskizze
- Praktische Versuchsdurchführungen an der ETH Zürich unter Anleitung von Dr. rer. nat. Carla Horváth und Prof. Dr. rer. nat. Christian Wolfrum
  - Betreuung der Versuchstiere im Mauslabor
  - Durchführung von Zellkulturversuchen mit dem Rohdrogenextrakt sowie dessen Hauptwirksubstanzen Puerarin, Genestein und Daidzein
  - Durchführung von PCR Analysen aus den oben genannten Zellkulturversuchen zu Braunen Fettmarkern
  - Durchführung eines Luciferase Assays
- Analyse und Interpretation der Daten in Zusammenarbeit mit den Co-Autoren
- Literaturrecherche und -auswertung
- Deskriptive Datenauswertung (außer chemisch-analytischen Teil).
- Erstentwurf und Bearbeitung des Manuskripts sowie Koordination zwischen den Autoren.

Anteil der Co-Autoren:

- Herstellung hydrophiler Extrakte aus den Rohdrogen am HanseMerkur Zentrum für Trad.Chin.Med. am UKE unter der Leitung von Dr. rer. nat. Janine Radtke.
- Studiendesign sowie fachliche Revision des Manuskriptes durch Prof. Dr. rer. nat. Christian Wolfrum, ETH Zürich und Prof. Dr. rer. nat. Jean-Luc Wolfender Genf, Schweiz
- Durchführung der Analytik an der Universität Genf: Ausarbeitung der Methoden und Datenauswertung durch Dr. rer. nat. Joëlle Houriet sowie Datenerhebung durch Dr. rer. nat. Joëlle Houriet und L. Marcourt
- Durchführung der Screening Versuche im Mausmodell durch Elke Kiehlmann und Prof. Dr. rer. nat. Christian Wolfrum
- Durchführung der Verum Versuche im Mausmodell durch Dr. rer. nat. Carla Horváth und Elke Kiehlmann
- Datenerhebung der Versuche im Mausmodell durch Dr. rer. nat. Carla Horváth
- Statistische Auswertung der Daten sowie Entwurf der Grafiken durch Dr. rer. nat. Carla Horváth
- Planung des Projektes durch Prof. Dr. rer. nat. Christian Wolfrum, PD Dr.Sven Schröder und Prof. Dr. rer. nat. Jean-Luc Wolfender

6. Danksagung

7. Lebenslauf

Entfällt aus datenschutzrechtlichen Gründen

8. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit (entsprechend den aufgeführten Eigenanteilen) selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe. Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe. Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: ....