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# *Leonurus japonicus*, *Leonurus cardiaca*, *Leonotis leonurus*: A novel HPLC study on the occurrence and content of the pharmacologically active guanidino derivative leonurine

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Leonurine is a prominent pharmacologically active guanidine alkaloid (4-{[amino(imino)methyl]amino}butyl-4-hydroxy-3,5-dimethoxybenzoate), mainly exerting cardiovascular, hypotensive, uterotonic, and neuroprotective effects. It is commonly regarded as the predominant active principle of *Leonurus* and *Leonotis* drugs (subfamily Lamioideae), though its presence has only been unambiguously proven for the aerial parts of *Leonurus japonicus* Houtt. (yimucao/Chin.Ph.,DAB), used in TCM/Kampo for the treatment of various gynaecological and cardiovascular disorders. Although a series of claims concerning the occurrence of leonurine in European *Leonurus cardiaca* L. (Ph.Eur.) can be found describing it as an important active principle, this has never been conclusively demonstrated. The same holds true for the officinal *Leonurus japonicus* fruits (chongweizi/Chin.Ph.) and the closely related South African herb *Leonotis leonurus* (L.) R.Br. Since no reliable HPLC determination and quantification method for leonurine has been published up to now, in the present study, a highly reproducible RP-HPLC method was newly developed using a special octadecyl-bonded stationary phase and an acetonitrile/water gradient (adjusted to pH 2.5 by phosphoric acid) as mobile phase (DAD/277 nm). In particular, this use of reversed phase packing with hydrophilic endcapping clearly contributes to an improved peak shape for leonurine, to our knowledge the first application of this technique on a natural zwitterionic analyte, and clearly enhances the selectivity of separation compared to classical RP-phases. The method was shown to be precise with respect to concentration, exhibiting a linear response in the range of 2.5–12.5 μg/ml leonurine, detection limit well below 0.5 μg/ml, and correlation coefficients constantly higher than 0.99 (5 levels, n = 3) over numerous inter day repetitions, demonstrating the robustness of the newly developed HPLC protocol. Thus, nine samples of *L. japonicus* aerial parts, two of *L. japonicus* fruits, four of *L. cardiaca* aerial parts, as well as one sample each of *L. cardiaca* fruits, and *Leonotis leonurus* aerial parts were examined. No leonurine could be detected in any sample of *L. cardiaca* in contrast to newly published official drug assessments, which consequently have to be revised. *Leonotis leonurus* and surprisingly, seeds of *L. japonicus* did not contain leonurine, either. However, in aerial parts of *L. japonicus* drug samples, obtained from China and Japan, leonurine contents between 0.001 and 0.049% were determined, while *L. japonicus* from domestic cultivation displayed significantly higher amounts of at least 0.1%. Thus, the HPLC method described above could be used for quality control of leonurine contained in TCM/Kampo medicines and in pharmacopeial analytics for the differentiation of *L. japonicus* and *L. cardiaca* samples.

## 1. Introduction

Leonurine (4-{[amino(imino)methyl]amino}butyl-4-hydroxy-3,5-dimethoxybenzoate) was first isolated in 1930 (Kubota and Nakashima) from the aerial parts of *L. japonicus* (yimucao/Chin.Ph.,DAB), which has remained the only plant in which the presence of leonurine has ever been proven beyond doubt. For a detailed review see Kuchta (2012). Essential pharmacological investigations of leonurine demonstrated its stimulating effect on the excised working frog heart (Kubota and Nakashima 1930), uterotonic activity (bioassay guided fractionation) (Kong and Yeung 1974), and most recently protective effects on both central nervous and cardiovascular tissues (Qi et al. 2010; Liu et al. 2009a, 2009b, 2010), especially hinting to a possible $Ca^{2+}$-channel blocking activity (Chen and Kwan 2001). Thus, leonurine could be considered as a possible cardioactive principle in European *L. cardiaca* herb (Ph.Eur.), since an antiarrhythmic refined extract exhibited a profound effect on the L-type $Ca^{2+}$-channel and Dragendorff-positive substances such as leonurine (Ritter et al. 2010). In fact, a comprehensive literature screening yielded a series of claims on the occurrence of leonurine in *L. cardiaca* in current textbooks,

monographs, research papers (Gulabov and Tchervenkova-Veleva 1970; Wichtl 2009; Kartnig et al. 1993; Bradley 1993; Hiller and Melzig 2007; Teuscher et al. 2004; van Wyk et al. 2004; Schantz 2009; Barnes et al. 2007; Shikov et al. 2011), and even most up to date official drug assessments – in the latter with a claimed content of 0.0068 % (Keller 2010). Surprisingly, only one single claim in primary literature (Gulabov and Tchervenkova-Veleva 1970) could be found concerning an isolation of leonurine from *L. cardiaca*: Its identification was performed only by means of classical elementar (CHNO) and IR analyses, and by TLC comparison (mobile phase not mentioned) with an uncharacterised reference sample isolated from *L. japonicus*. These results have, however, never been replicated. Furthermore, claims on the occurrence of leonurine exist for officinal fruits (chongweizi/Chin.Ph.) (e.g. Hiller and Melzig 2007) and the closely related, cardioactive (Burger and Kabatembe 2008) South African *Leonotis leonurus* (L.) R.Br. herb, even in an official governmental drug security assessment (Pichini et al. 2010).

Thus, the present paper aims at the detection and quantification of leonurine, the best-examined pharmacologically active single constituent of *L. japonicus* (Kubota and Nakashima 1930; Kong and Yeung 1974) in seventeen samples of the above-mentioned *Leonurus* and *Leonotis* drugs (additionally *L. cardiaca* fruits) using state of the art HPLC technology. As the only two previously published HPLC methods (Hong et al. 2001; Chao et al. 2004) displayed conceptual deficits and proved not reproducible as discussed later, a novel Pyramid RP-phase with hydrophilic endcapping, especially designed for polar compounds and aqueous eluents (Przybyciel 2003; Lesellier et al. 2006) was applied to a zwitterionic analyte for the first time. In this context, the Chin.Ph. resorts for quality control of yimucao to the photometrical measurement of its overall content in N-containing compounds complexated with Reinecke salt ($NH_4[Cr(SCN)_4(NH_3)_2]$). In contrast, the German Pharmacopoeia (DAB), in which the aerial parts of *L. japonicus* were monographed in 2010 for the first time, prescribes a total flavonoid content of at least 0.3 % of the dry weight, calculated as hyperoside, analogues to the Ph.Eur.-method for *L. cardiaca*.

## 2. Investigations, results and discussions

As there is currently no reproducible HPLC method for routine quantification of leonurine available, the development of such a procedure was defined as the aim of the present research project. Identity and purity (> 99%) of the isolated leonurine standard were determined by $^1$H-/$^{13}$C-NMR- and ESI-MS-data, not yet completely published before (Hong et al. 2001). As for the two HPLC methods for *L. japonicus* published so far, quantitative determination of leonurine by customary RP-HPLC has been described (Hong et al. 2001), however, not a single HPLC-chromatogram of a sample injection is depicted and attempts to reproduce these results in our laboratory resulted in irreproducible retention times varying by several minutes depending on the injected leonurine standard concentration. In the second published method (Chao et al. 2004), instead of a classical RP-column a specialised YMC-Park cyano phase column and a complicated aqueous mobile phase containing 10 % MeOH, 0.00125 mol/l of the ion-pair forming agent SDS, and 0.05 % of reactive and oxidative perchloric acid were applied. In the paper at hand, the use of a CN-phase column – both with and without ion-pair forming agents – could not achieve a baseline separation of leonurine in the extracts as described below. This may be correlated with the fact that only short exerts of HPLC-chromatograms are depicted (Chao et al. 2004). However, the most significant problem with the HPLC method described by Chao et al. (2004) is the inability to inject raw extracts directly. Instead, a special measurement solution analogous to the photometric Chin.Ph. method for measuring the total alkaloid content of yimucao had to be laboriously prepared with each step resulting in losses and thus additional systematic error. For developing a new HPLC method for the reproducible routine determination of leonurine in drug samples, a variety of different HPLC columns such as the customary RP phase 250/4,6 Nucleodur 100-5 C18ec or the CN phase EC 250/4,6 Nucleodur 100-5 CN RP were used and evaluated. Additionally, ion-pair forming reagents such as sodium 1-octanesulfonate monohydrate were added to the preliminary gradients in combination with all tested columns but did not contribute to any improvement of peak shape or separation. Only the use of a reversed phase packing with a polar endcapping, namely a 250/4 Nucleodur C18 Pyramid column with an 8/4 Nucleodur C18 Pyramid precolumn, clearly yields an improved peak shape for leonurine and enhances the selectivity of separation compared to classical RP-phases. This Pyramid RP-phase has been especially designed for the analysis of polar constituents in eluent systems containing up to 100 % water (Przybyciel 2003; Lesellier et al. 2006), and appears particularly suitable for zwitterionic analytes like leonurine, the first natural compound of this class to be analysed in this way. Consequently, solvents and gradients were adjusted in order to obtain a good separation of the peaks and to elute the compounds in a reasonable duration. It could be shown that the retention times achieved with the special pyramid phase remained nearly unchanged between initial injections and restart after the flow had been stopped for ca. 12 h, whilst the performance of the conventional RP-column collapsed already after several injections of pure leonurine standard. In contrast to classical octadecyl-bonded silicas, no unwanted interaction, or so called 'lack in base-deactivation' could be observed, which is regarded as most difficult to achieve (Lesellier et al. 2006). As no further improvement was achieved by the addition of ion-pair forming reagents, these compounds were not included in the final gradient. The completely developed method was shown to be precise with respect to concentration, exhibiting a linear response in the range of 2.5–12.5 μg/ml leonurine, detection limit well below 0.5 μg/ml, and correlation coefficients constantly higher than 0.99 (5 levels, n = 3) over numerous inter day repetitions. Leonurine can now be determined in *L. japonicus* extracts in any basic routine laboratory using this simple, reliable, low cost method.

Subsequently, this new HPLC procedure was used for examining of nine samples of *L. japonici* herba (Chin.Ph.,DAB) demonstrating that all products bought in Beijing as well as all material from cultivation overseas in Germany and Japan contained relatively high amounts of leonurine between 0.020 and 0.104 % of the dried drug weight with the latter, maximal content detected in the drug from German cultivation obtained from Galke. The highest content in native Chinese material was measured in the drug sample from the Xinainian Shiji Dayaofang apothecary in Beijing, which contained 0.049 %. The leonurine content of 0.024 % in the sample harvested and sold in Japan by Nakajima Shouyaku was in the same order of magnitude as the Chinese samples bought in Beijing. On the other hand, two samples of the *L. japonicus* aerial parts bought in apothecaries in southern China as well as the material which was exported officially to Germany via the Sinophyto company contained significantly lower amounts of leonurine with the highest content of 0.007 % in the Sinophyto export drug sample harvested in the province of Jiangsu. As an exception among all examined *L. japonicus* samples, the drug harvested in the province of Guangxi and purchased in Haikou from the Yongjingtang TCM drug shop did only contain not quantifiable traces of leonurine even when the highest possible amount of sample was applied to the HPLC col-

umn. This is however in accordance with the fact that both this sample and the sample bought at the Xinhuairen Yaoju apothecary in Xiamen, which only contained 0.001 % of the alkaloid, did not meet the requirements of Chin.Ph. and consisted almost entirely of lignified trunks hardly containing any leaves of inflorescences. As far as chongweizi is concerned, which share most medical indications with the aerial parts (Anonymous 2000), surprisingly no leonurine could be detected in any of the samples from both Chinese and Japanese origin, in contrast to earlier statements (Chang and But 1987). The fruits of *L. cardiaca* did not contain any leonurine, either.

Astonishingly, no leonurine could be detected in any sample of *L. cardiacae* herba or *Leonotis leonurus* aerial parts, indicating that instead of both their similar use and numerous claims of its presence in these plants (for literature sources see Introduction), leonurine has to be regarded as an unique taxonomic marker of *L. japonicus* and essential active principle of *L. japonici* herba (Chin.Ph.), and can definitively be excluded as an active constituent of *L. cardiacae* herba (Ph.Eur.) and its cardioactive refined extract (Ritter et al. 2010), in which the abovementioned Dragendorff-positive substances could be assigned to N-containing betaine type compounds. Consequently, an actual official EMA drug assessment of *L. cardiaca* mentioning a leonurine content of approximately 0.0068 % (Keller 2010) has to be revised. In this context, the only experimental data were published for a sample of Bulgarian origin without citing any voucher specimen (Gulabov and Tchervenkova-Veleva 1970; cp. Introduction). It could therefore well be that these authors accidentally examined the Balkan species *Leonurus quinquelobatus* Gilib, sometimes regarded as a subspecies of *L. cardiaca* under the name *Leonurus cardiaca* L. var. *villosus* DESF (Keller 2010), known for its use in Bulgaria, Turkey, Romania (Krestovskaja 1992), containing an "unknown alkaloid" (Kozlova 1967) that has not been examined up to now. It further has to be mentioned that the source plant of the two TCM drugs is *Leonurus japonicus* Houtt., including its taxonomic synonyms *Leonurus sibiricus* L., *Leonurus artemisia* (Lour.) S.Y. Hu, and *Leonurus heterophyllus* Sweet (Harley and Paton 2001; Krestovskaja 1992). In the present study, leonurine could not be detected in the South African *Leonotis leonurus*, either. Conflicting reports on the Internet, e.g. in Wikipedia articles, and even in an Italian governmental drug safety report (Pichini et al. 2010) seem to be based on a simple confusion of the species *Leonotis leonurus* with the genus *Leonurus*.

The ever-increasing role of TCM in European health care and the rising demand for *L. japonicus* by German TCM practitioners has already promoted the adoption of yimucao to the current DAB (Anonymous 2010) as one of the very first in a series of further additions of East Asian drugs. In preparation of this step, an international attempt to develop monographs for the pharmaceutical analysis of prominent TCM drugs already yielded an HPLC method for yimucao, which did however not concentrate on the active constituent leonurine but on the phytochemically easier accessible and chromatographically easier separable but more unspecific hyperoside, rutin, and ajugoside, the latter iridoid without reference substance, measured in the more unselective very short wave UV at 205 nm (Bauer et al. 2009). This HPLC method was also applied to *L. cardiac*a using the same analytical markers (Bauer et al. 2009). Accordingly, the new DAB monograph for *L. japonici* herba (Anonymous 2010) does not concentrate on leonurine as the major pharmaceutically active constituent, but includes a conventional spectrophotometrical determination of the total "flavonoid" content, calculated for the flavonol hyperoside. Neither of these phenolic constituents, also typical for numerous other flavonoid drugs, is discussed in the literature as a contributor to the specific pharmacological activity of *L. japonicus*, whereas the present report presents the first reproducible HPLC measurement of leonurine content, facilitating the standardisation of *L. japonicus* preparations directly on its most prominent constituent, whereas the above mentioned HPLC fingerprint analysis (Bauer et al. 2009) seems hardly suitable for a clear analytical differentiation between the two *Leonurus* species. The high leonurine contents in the two drug samples from German cultivation may be especially interesting as it was observed in patients that the effect of dynamisation of the 'xue' according to Chin.Ph. appears clearly more intensive than the effects of Chinese reference samples (Bomme 2008). Accordingly, the study at hand demonstrates that the content of the active principle leonurine in the Bavarian drug is twice as high as the highest measured content in officinal Chinese samples and almost fifteen times as high as in the sample, which was purchased from China via a professional TCM drug import company.

In this context, our findings should have implications concerning practical pharmacy: Since the recent addition of yimucao to DAB, the two morphologically very similar and hardly differentiable herbs of the same botanical genus are present side by side in German pharmacies without a reliable HPL-chromatographic method for their differentiation. For example a commercially purchased drug declared as "herba leonuri" and sold as *L. cardiaca*, was found to be *L. japonicus*, constituting a serious mistake in identity or adulteration, respectively. In consequence, any questionable drug sample containing this alkaloid can clearly be identified as originating from the East Asian species. Taking into consideration the high probability of a mix up of the drugs in everyday pharmaceutical practice, the addition of such a phytochemical approach for their differentiation to the current DAB monograph can be regarded as highly desirable. The observed tremendous variation of leonurine content in the examined yimucao trading samples makes a qualitative and quantitative method of pharmacopeial analytics for this constituent even more pressing. On the basis of typical leonurine contents in samples obtained from well-respected and renowned East Asian drug trading companies (Tongrentang Beijing; Seikoen Hosono Clinic Kyouto) a requirement of a minimal content of 0.020 % of leonurine in *L. japonicus* herbal drug could be proposed for the DAB monograph. The newly developed RP-HPLC-method with hydrophilic endcapping is suitable for its purpose, namely for the quality assurance of leonurine containing herbal medicines, or for the differentiation of *L. japonicus* and *L. cardiaca* samples.

## 3. Experimental

### *3.1. Plant material*

The aerial parts (yimucao) and fruits (chongweizi) of *Leonurus japonicus* (Chin.Ph., DAB) (Anonymous 2000; 2010) as well as *Leonuri cardiacae* herba (Ph.Eur.) were purchased from the sources listed in the Table 1 and controlled for their quality according to the requirements of the Chin.Ph. or Ph.Eur. respectively, regulations which were met by all analysed samples with the exception of samples D and E which almost entirely consisted of lignified trunks and hardly contained any leaves of inflorescences. All examined samples were in complete accordance to coinvestigated authentic voucher specimen, which are deposited in the herbarium of the Institute of Special Botany, Leipzig University under the registration number LZ 203412 for the TCM drug and under the EDV registration number 167244 for the European herb. *Leonotis leonurus* was cultivated at the gardens of botany of Leipzig University. Fresh flowering aerial parts were collected for examination. A voucher specimen of these was deposited in the abovementioned herbarium under the registration number LZ 203520.

### *3.2. Extract preparation*

Pulverised drug material (6.00 g) was extracted with boiling water (120 ml) for 60 min under reflux. The resulting infusion was filtered under vacuum until the residue was dry and the liquid was clear. It was subsequently evaporated to dryness and lyophilised. The resulting dry extract was weighed in

**Table: Identity and leonurine content of the examined drug samples**

| | Drug Name | Bought in | Apothecary | Company | Batch number | Origin | Extract % (w/w) in the drug | Leonurine % (w/w) in the drug (n = 3) |
|---|---|---|---|---|---|---|---|---|
| A | *L. japonici* herba | Beijing | Beijing Tongrentang | Tongrentang | 0128028 | Province of Hebei (PRC) | 14.60 | 0.045 |
| B | *L. japonici* herba | Beijing | Beitaipingzhuang Dayaofang | Beijing Shengshi-long | 0807183 | Province of Hebei (PRC) | 16.62 | 0.020 |
| C | *L. japonici* herba | Beijing | Xinainian Shiji Dayaofang | Xingainian | LY10322007 | PRC | 14.80 | 0.049 |
| D | *L. japonici* herba | Xiamen | Xinhuairen Yaoju | Longrentang | 0810692 | PRC | 14.07 | 0.001 |
| E | *L. japonici* herba | Haikou | Yongjingtang | Xiangbao Zhongyaoy-inpian | 20070402 | Province of Guangxi (PRC) | 10.57 | 0.000 |
| F | *L. japonici* herba | — | — | SinoPhytoMed | 13964K262-4-100 | Province of Jiangsu (PRC) | 11.95 | 0.007 |
| G | *L. japonici* herba | Kyouto | Seikoen Hosono Clinic | Nakajima Shouyaku | 11112A075 | Jap | 13.59 | 0.024 |
| H | *L. japonici* herba | — | — | Kräuterhof Frieß | E7558A | State of Bavaria (Ger) | 24.66 | 0.102 |
| I | *L. japonici* herba | — | — | Galke | 0000011580 | Ger | 23.16 | 0.104 |
| J | *L. japonici* fruits | — | — | SinoPhytoMed | 13963K263-4-100 | Province of Anhui (PRC) | 9.28 | — |
| K | *L. japonici* fruits | Kyouto | Seikoen Hosono Clinic | Uchida Wakanyaku | 7B33213 | Jap | 10.48 | — |
| L | *L. cardiacae* herba | — | — | Galke | 10477 | Ger | 24.17 | — |
| M | *L. cardiacae* herba | — | — | Caesar & Loretz | 32054354 | Ger | 21.96 | — |
| N | *L. cardiacae* herba | — | — | Galke Organic Farming | 0000010957 | Ger | 26.86 | — |
| O | *L. cardiacae* herba | — | — | Botanical Garden | — | Leipzig | 9.53 | — |
| P | *L. cardiacae* fruits | — | — | Botanical Garden | — | Leipzig | 7.53 | — |
| Q | *L. leonuri* herba | —- | — | Botanical Garden | — | Leipzig | 13.45 | — |

In the case of sample E, the detected amount of leonurine was to low for quantification
China = PRC; Japan = Jap; Germany = Ger

order to calculate the drug extract ratio. Finally, all extracts were powdered and stored in sealed glass flasks at −20 °C.

### 3.3. Purification of leonurine from L. japonici herba

For purification of leonurine, the aqueous extract (1.0 g) of sample H (Table) from *L. japonicus* aerial parts was fractioned by preparative RP18 MPLC (Büchi, Flawil, CH; C-615 gradient pumping station; two C-605 pumps; Sepacore C-690 glass column No. 19675; RP-C18 packing by Merck, Darmstadt) with water-acetonitrile gradients as mobile phases at 4 ml/min. An aliquot of the lyophilised enriched leonurine fractions, obtained from the first MPLC eluates, was dissolved in water in a concentration of 50 mg/ml for further purification by preparative silica TLC. Bands with a length of 1 cm, each containing 1.5 mg of the purified extract, divided by 0.5 cm empty space were applied to 20 × 20 cm TLC silica gel 60 $F_{254}$ sheets (Merck, Darmstadt) with 1 cm distance to the rim. These TLC sheets were placed in a development chamber saturated with the mobile phase MeOH:$CH_2Cl_2$:$NH_3$ 25% (8:2:3). The TLC run was allowed to proceed to a total propagation of 18.0 cm. Following careful evaporation of the mobile phase, the TLC plates were inspected under 254 nm UV light, revealing leonurine as an UV quenching zone at $R_f = 0.31$. Spraying of one sample plate with Dragendorff solution led to the development of a striking red spot at this position thus confirming our identification. These quenching zones were cut out of several the TLC sheets, the silica gel scratched of and extracted with a solution of MeOH : $CH_2Cl_2$ (1 : 1) for 3 h under constant shaking. The resulting solution was filtered and subsequently evaporated to dryness to afford about 3 mg of leonurine. This was dissolved in 700 µl of DMSO-$d_6$ and placed in NMR glass tubes for recording $^1$H and $^{13}$C spectra on a BRUKER DRX 400 (Bruker; Billerica, USA) spectrometer, revealing the identity of the isolated molecule with the leonurine reference substance.

### 3.4. Leonurine reference substance

For the HPLC experiments, synthetic leonurine as a reference compound was purchased from Anhui New Star Pharmaceutical Development Co., Hefei, PRC. The purchased pure substance proofed to be identical to the sample isolated from the drug as described above. Their identities were proven by positive ESI-MS determination ($[M + H]^+ = m/z$ 312) (sample dissolved in methanol with $N_2$ spray; Esquire 3000 Plus ion-trap, Bruker, Bremen, Germany) and purity was determined to be at least 99 % according to the subsequently described NMR experiments.

### 3.5. NMR measurements

In NMR glass tubes obtained from Chemotrade (Leipzig, Germany), approximately 15 mg of leonurine were dissolved in 700 µl of DMSO-$d_6$ resp. $CD_3OD$ due to different splitting of NH-signals. The $^1$H spectra of the entire solution were recorded with 16 scans at 400 MHz and the $^{13}$C spectra with 1000 scans at 100 MHz. For HSQC (Heteronuclear Single Quantum Coherence), 4 scans with 256 increments were carried out. A BRUKER DRX 400 (Bruker; Billerica, USA) spectrometer was used for all of these measurements. The same sample was inserted in a VARIAN Mercury 400 plus (Varian; Palo Alto, USA) apparatus for recording $^1$H spectra with 32 scans at 400 MHz, $^{13}$C was measured with 2000 scans at 100 MHz (just as all other $^{13}$C data containing experiments), and APT (Attached Proton Test) with 5000 scans. Furthermore, COSY (Correlation spectroscopy) was recorded using 4 scans with 256 increments, and for HMBC (Heteronuclear

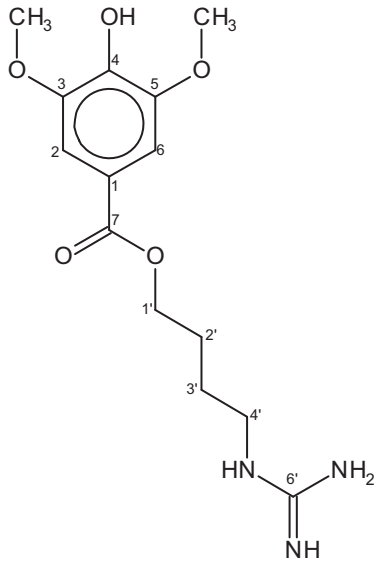


Fig. 1: Chemical structure of leonurine

Multiple Bond Correlation) (8 Hz) 32 scans with 256 increments were used. With the combination of these multiple 2D NMR techniques, every signal in both the $^{1}H$ and $^{13}C$ NMR spectra of leonurine could be assigned to the respective atom in its structural formula (Fig. 1).
$^{1}H$ NMR (DMSO-$d_6$, δ [ppm]): 9.31 (1H, s br, 4-OH); 7.89 (1H, t, $J = 5.6$, 4'-NH); 7.20 (3H, m br, 6'-$NH_2$/NH); 7.19 (2H, s, H-2, H-6); 4.24 (2H, t, $J = 6.4$, H-1'); 3.80 (6H, s, 3-/5-$OCH_3$); 3.16 (2H, m, H-4'); 1.73 (2H, m, H–2'); 1.58 (2H, m, H-3'). $^{13}C$-NMR (DMSO-$d_6$, δ [ppm]): 165.61 (C7); 157.05 (C6'); 147.56 (C3, C5); 140.72 (C4); 119.35 (C1); 106.78 (C2, C6); 63.90 (C1'); 56.10 (C3-/C5-$OCH_3$); 40.29 (C4'); 25.55 (C2'); 25.25 (C3').
$^{1}H$-NMR ($CD_3OD$, δ [ppm]): 7.23 (2H, s, H-2, H-6); 4.31 (2H, t, $J = 6.2$, H-1'); 3.86 (6H, s, 3-/5-$OCH_3$); 3.24 (2H, t, $J = 6.8$, H-4'); 1.81 (2H, m, H-2'); 1.73 (2H, m, H-3'). $^{13}C$-NMR ($CD_3OD$, δ [ppm]): 169.12 (C7); 157.76 (C6'); 148.24 (C3, C5); 140.71 (C4); 121.21 (C1); 107.99 (C2, C6); 66.10 (C1'); 57.09 (C3-/C5-$OCH_3$); 41.73 (C4'); 26.23 (C2'); 25.78 (C3').

### 3.6. HPLC

#### 3.6.1. General and specific chromatographic conditions

The optimal HPLC conditions were determined after investigating several characteristics. All solvents used for HPLC were of gradient grade quality. A first attempt to reproduce the leonurine HPLC-method described by Hong et al. (2001), using a conventional Shiseido ODS column, revealed it to be not reproducible at all, with the retention time of the alkaloid shifting depending on the injected concentration in the scale of several minutes. In further preliminary experiments, several other HPLC columns such as a EC 250/4,6 Nucleodur 100–5 CN RP or a 250/4,6 Nucleodur 100–5 C18 ec column (Macherey-Nagel, Düren) with the respective precolumns were tested, but none of these did yield any reproducible results. However, a 250/4 Nucleodur C18 Pyramid column (5 µm) with an 8/4 Nucleodur C18 Pyramid precolumn (5 µm), (Macherey-Nagel, Düren) was successful. This Pyramid RP-phase has been especially designed for the analysis of polar constituents in eluent systems containing up to 100 % water (Przybyciel 2003; Lesellier et al. 2006). All injections were applied using a Dionex Gina 50 autosam-

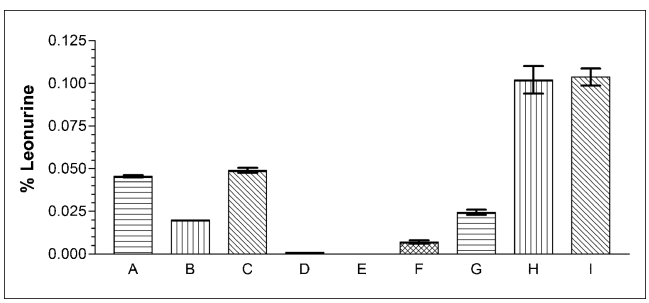


Fig. 2: Leonurine content (w/w) in nine samples of *Leonurus japonicus* herb (Chin.Ph., DAB), determined by HPLC. For identification of the individual samples see Table. All measurements were performed in triplicates. The standard deviation of the raw data is given in the form of error bars

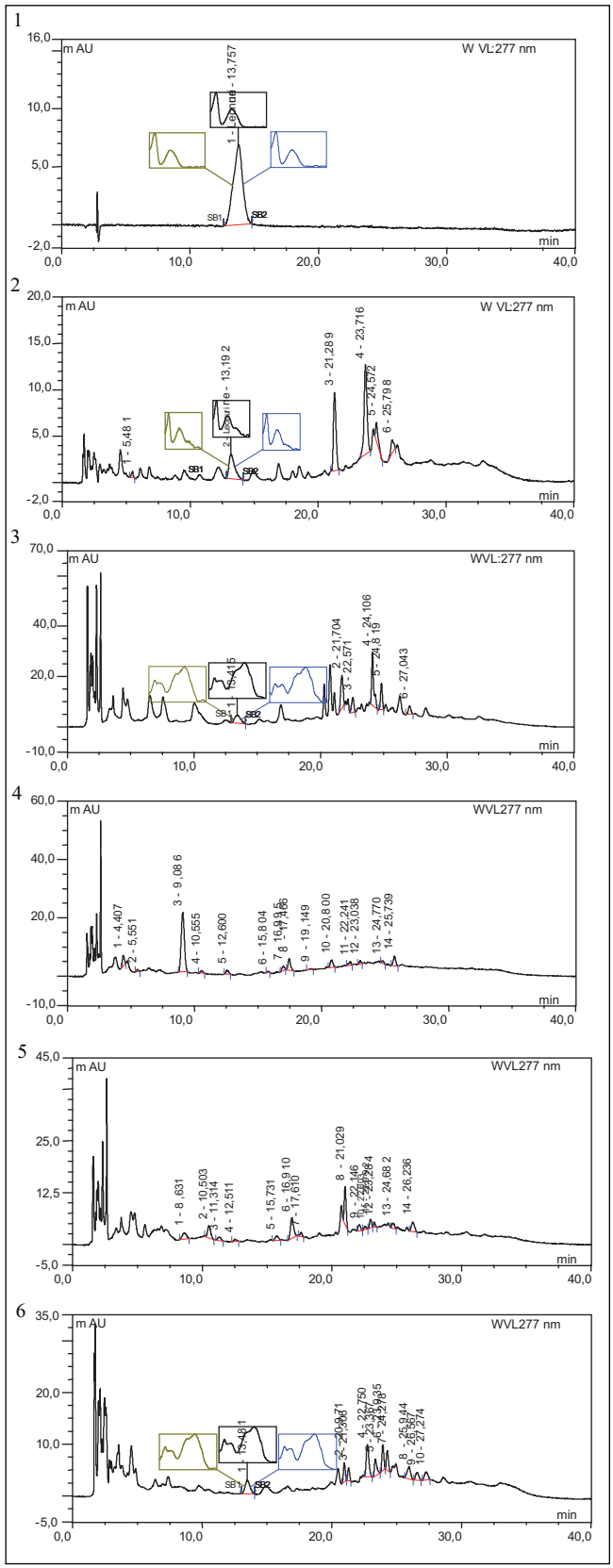


Fig. 3: Typical HPLC-DAD chromatograms of representative injections of 0.0125 mg/ml chemosynthetic leonurine standard (1) as well as crude extract samples of *Leonuri japonici* herba H (2), *Leonuri cardiacae* herba L (3), *Leonuri japonici* semen K (4), *Leonuri cardiacae* semen P (5), and *Leonotis leonurus* aerial parts Q (6). In the case of sample H with the alkaloid actually present, it is observed as the biggest peak in the range of retention times between 5.0 and 20.0 min. The detection wavelength was set at 277 nm. The UV spectra corresponding to the leonurine peaks or other peaks in the area that might be wrongly identified as leonurine without DAD detection are shown at the beginning, at the apex and at the end of the respective peaks. For exact chromatographic conditions see text

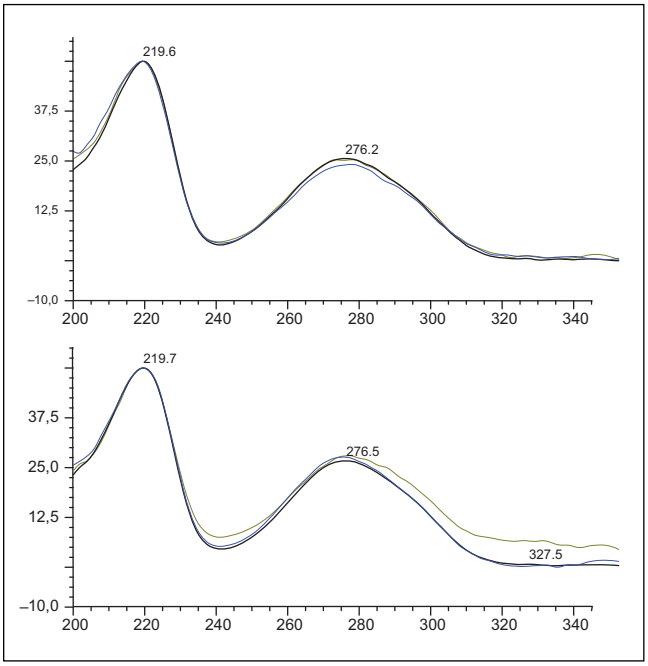


Fig. 4: UV spectra of leonurine measured by the DAD detector in the standard (above) and in the *Leonuri japonici* herba sample H (below) demonstrating the high peak purity achieved with the presented HPLC method

pler. Gradient elution was performed with a Dionex P680 HPLC pump applying a constant flow of 1.00 ml/min. This approach had been followed with some variations also with the other tested column types in the preliminary experiments. These did also include some experiments with ion-pair forming reagents such as sodium 1-octanesulfonate monohydrate (Sigma-Aldrich, St. Louis, USA) in combination with all tested columns, an attempt that proved unnecessary in the case of the 250/4 Nucleodur C18 Pyramid column and futile in all other cases. In order to prevent peak broadening, phosphoric acid was added to the aqueous phase of the gradient. HPLC was carried out with a $H_2O/CH_3CN$ gradient. Eluent A consisted of acetonitrile, eluent B consisted of water (pH 2.5, reached with phosphoric acid). The flow rate was set at 1.00 ml/min and the temperature at 25 °C. After equilibration with 90 % eluent A, the sample was injected. After 10 min, eluent B was increased and reached 20 % after 20 min, only to decrease again after 30 min, thus returning to 10 % at 35 min. The run was terminated after 40 min. Thus in the final 5 min the column was flushed and equilibrated with 90 % eluent A. The column temperature was kept constant at 25 °C using a Dionex STH 585 column oven. A Dionex UVD340U photodiode array detector (DAD) was used for peak identification via 3D-spectra and quantification at 277 nm. This wavelength was chosen as it represents an absorption maximum in the UV spectrum of leonurine in the optimal working range of the detector. All data were analysed using the Chromeleon 6.70 Software (Dionex Corporation, Sunnyvale).

### *3.6.2. Sample preparation*

For all examined plant extracts, several preliminary injections were performed to make an educated guess for their approximate leonurine content so that the final injections for determining its exact concentration could be aimed into the concentration range of the external standards on which the linear calibration equation was based ($y = 0.4649 \times +0.02475$, $r^2 = 0.9994$). The samples were dissolved in $H_2O$ accordingly and filtered using a Chromafil GF/PET-45/25 filter with 1.0/0.45 µm pore size and 25 mm diameter. 20 µl of these mixtures were injected onto the HPLC column for each measurement. The exact extract concentrations were noted for every injection and saved for the later calculation. Both standards and samples were injected in triplicates.

### *3.6.3. Method calibration and validation*

In order to prove the suitability of the method for its intended use, different characteristics can be investigated during validation. In this work, the calibration model of leonurine was checked for its linearity by analysing five concentration levels covering a range of 2.5–12.5 µg/ml. Therefore, leonurine was dissolved in water at the respective concentrations and subsequently treated and injected just as the extract samples. Graphical examination of the regression line and the residual plot proved that the method to be linear for leonurine. The correlation coefficient was approved since it was at least 0.99. The intermediate precision was examined by performing analyses on several days and at different concentration levels. In a dilution series, the detection limit for the guanidino derivative was determined as well below 0.5 µg/ml under the applied HPLC conditions. The peak purity of leonurine in the crude extract was examined using the DAD detector. The UV spectra at the beginning, at the apex and at the end of the peak were inspected and no deviations were observed (Fig. 3.1, 3.2, 5). It was concluded that the method is specific. After investigating these characteristics, it could be concluded that the developed analytical method is suitable, i.e. precise, accurate, and specific, for its intended use.

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