

DOCTORAL THESIS

Pharmacognostic studies on Herba Oldenlandiae

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Date of Award:
2007

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Pharmacognostic Studies on Herba Oldenlandiae

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A thesis submitted in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Principal Supervisor: Prof. ZHAO Zhongzhen

Hong Kong Baptist University

August 2007

ABSTRACT

Drinking herbal tea is more and more popular in China and Southeast Asian Nations. However, study on the species identification and quality evaluation of the commonly used herbal tea materials is insufficient.

Herba Oldenlandiae, herbal tea material commonly used for the treatment of typhlitis, snakebites and fibroids, is derived from the dried herb of *Oldenlandia diffusa* (Willd.) Roxb. according to the Chinese pharmacopoeia. It also is a commonly used Chinese Materia Medica (CMM), which has been widely used since 1960s. A systematic herbal market investigation on Herba Oldenlandiae was conducted, and the results indicated that two other species, namely, *O. corymbosa* (L.) Lam and *O. tenelliflora* Bl., from the *Oldenlandia* genus are being used as Herba Oldenlandiae. According to a review of textual research, chemical constituents, pharmacological studies and quality control of Herba Oldenlandiae and its substitutes, previous phytochemical and pharmacological studies mainly focused on the species *O. diffusa*. Comparative studies on the constituents of Herbal Oldenlandiae and its substitutes were scarce. The confusion with *O. corymbosa* and *O. tenelliflora* in the herbal market has led to a growing concern about their safety and efficacy. Safe and effective use of Herba Oldenlandiae depends on proper authentication of the source material. Moreover, no specific marker components for quality evaluation of Herbal Oldenlandiae have been found. Therefore, this thesis focuses on species identification and quality evaluation of Herba Oldenlandiae and its substitutes.

In this study, the first step was to collect multiple samples of Herba Oldenlandiae and its substitutes from different growing areas. The second step was to establish the characteristics by which the three species of *Oldenlandia* under investigation could be distinguished. On the basis of the identified results by taxonomy identification, the techniques of fluorescence microscopy and DNA molecular marker were applied to authenticate Herba Oldenlandiae and its substitutes. Fluorescence microscopy revealed important distinguishing characteristics. When examined by fluorescence microscopy, some tissues of the three herbs were observed to emit autofluorescence. Specifically, the endoderm cell walls of *O. diffusa* and *O. tenelliflora* could emit autofluorescence, while similar tissues of *O. corymbosa* did not. The shape of transverse sections of *O. diffusa* and *O. tenelliflora* were distinctly different. Thus, fluorescence microscopy usefully differentiated the three species

Sequences of internal transcribed spacer (ITS) were also used to identify Herba Oldenlandiae and its substitutes. According to the alignment of those sequences, thirteen position-specific nucleotides were found in the ITS sequences of *O. diffusa* which could be used for identification Herba Oldenlandiae and its substitutes. In addition, the phylogenetic tree based on the tested ITS sequences was reconstructed. The results showed that *O. diffusa* had a close relationship to *O. tenelliflora* but not to

O. corymbosa.

The antiproliferative effect of *O. diffusa* and its substitutes, *O. corymbosa* and *O. tenelliflora*, on human colon carcinoma CaCo2 cells and hepatoma HepG2 cells was analyzed. The SRB assay on human hepatoma HepG2 cells and human colon carcinoma CaCo2 cells was used for this purpose. The results showed that there was almost no antiproliferative effect of the methanol extracts from *O. diffusa*, *O. corymbosa* and *O. tenelliflora*, while the chloroform extracts from *O. diffusa* and *O. corymbosa* exhibited slightly antiproliferative effects. Further investigation on the antiproliferative constituents of *O. diffusa* led to the isolation of a new compound, *E-6-O-p-coumaroyl scandoside methyl ester-10-O-methyl ether* (**54**), together with six known compounds-- asperuloside (**1**), *E-6-O-p-coumaroyl scandoside methyl ester* (**3**), oleanolic acid (**22**), ursolic acid (**23**), β -sitosterol glucoside (**51**) and betulin (**53**). Among the above compounds, compound **53** was firstly isolated from this herb, and compound **54** was a new iridoid glucoside. The results also indicated that the chloroform fraction of *O. diffusa* contained a great quantity of ursolic acid. Therefore, ursolic acid was considered to be the main antiproliferative constituent.

The differences in the constituents of Herba Oldenlandiae and its substitutes were analyzed by HPLC fingerprints and LC-HR-ESI-MS methods. Five compounds-- **1**, **3**, **22**, **23** and **54**-- were used as chemical markers. The results showed that the chromatograms of seventeen samples of *O. diffusa* varied greatly. Among the seventeen chromatograms, the chromatographic peak of compound **54** could be found in eleven samples. Moreover, a special group of chromatographic peaks, appeared in three chromatograms of *O. diffusa* samples detected at 238 nm. Despite the variety of chromatograms of *O. diffusa*, eleven common chromatographic peaks were found. On the basis of chemical markers, on-line HR-ESI-MS data, UV spectra and literature investigations, eight chromatographic peaks were identified as asperuloside (**1**), *E-6-O-p-coumaroyl scandoside methyl ester* (**3**), *6-O-p-feruloyl scandoside methyl ester*, *Z-6-O-p-coumaroyl scandoside methyl ester* (**2**), *E-6-O-p-coumaroyl scandoside methyl ester-10-O-methyl ether* (**54**), oleanolic acid (**22**), ursolic acid (**23**) and stigmaterol, respectively. Comparing the chromatograms of *O. corymbosa* and *O. tenelliflora* obtained under the same chromatographic conditions, the presence/absence and contents of compounds **1**, **3** and **54** were variable in *O. diffusa*, *O. corymbosa* and *O. tenelliflora*. Furthermore, the compounds **1**, **3** and **54** were abundant in most *O. diffusa* samples. Therefore, the three compounds were recommended to be used as special chemical markers for quality evaluation of Herba Oldenlandiae.

As oleanolic acid and ursolic acid are the anti-tumor components of *O. diffusa* reported in the literatures and as there is no systematic study comparing the contents of these constituents in multiple samples of the three species used as Herba Oldenlandiae, the parameters were attempted to establish by which these two components could be used to evaluate the quality of Herba Oldenlandiae and its substitutes. A convenient method with good resolution was developed. The results showed that the contents of

oleanolic acid and ursolic acid in *O. diffusa* were generally lower by almost two times than in *O. corymbosa*. Therefore, quantifying these constituents could readily and reliably distinguish *O. diffusa* from *O. corymbosa*.—but not from *O. tenelliflora* because the contents of these two compounds in this species were similar to those of *O. diffusa*.

The quality of Herba Oldenlandiae and its substitutes was also evaluated by determination of the contents of compounds **1**, **3** and **54**. Firstly, a convenient and reliable method was developed for the first time by optimizing the extraction efficiency of these three compounds. The results showed that all samples of *O. tenelliflora* contained compound **1** but not containing compounds **3** and **54**. Few samples of *O. corymbosa* contained compounds **3** and **54** but most samples of *O. diffusa* contained them. Such findings were reported for the first time. In tested samples, the contents of compounds **1** and **3** of *O. diffusa* were higher than those of *O. corymbosa*. Therefore, the relative occurrence of compounds **1**, **3** and **54** could be used as specific chemical markers for the quality evaluation of Herba Oldenlandiae and its substitutes.

Six batches of dry and fresh samples of *O. diffusa* and *O. corymbosa* were analyzed using the above developed methods. The results showed that there were minor differences of the contents of oleanolic acid (**22**) and ursolic acid (**23**) between the fresh samples and dry samples. Conversely, the level of compounds **1**, **3** and **54** in fresh sample was higher than those of dry samples.

In conclusion, the techniques of fluorescence microscopy and DNA molecular markers were used to distinguish Herba Oldenlandiae and its substitutes. The chemicals of *O. diffusa*, *O. corymbosa* and *O. tenelliflora* were quite different based on the above analysis. All the results indicated that the three herbs should not be used interchangeably. Moreover, the level of iridoid glucosides in the dry and fresh herbal samples was significantly different. These studies successfully addressed the problem of species identification and quality evaluation of Herba Oldenlandiae.

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