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ANALYSIS OF *LIGUSTRUM LUCIDUM AIT* LEAVES USING LEAF SPRAY MASS SPECTROMETRY

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ABSTRACT

Extracts from *Ligustrum lucidum Ait* leaves have been used in medicine for centuries, a fast and effective method to detect phytochemicals in *Ligustrum lucidum Ait* leaves has important significance for pharmaceutical research. In this study, leaf spray-MS was employed to directly fingerprint the phytochemicals in *Ligustrum lucidum Ait* leaves. By adding 10 μL methanol on the surface of the V-shaped sample, charged droplets containing a variety of phytochemicals extracted from the leaf tissue such as sugars, alkaloids and flavonoids were produced with high voltage applied on the sample. The metabolism information during the growth of *Ligustrum lucidum Ait* leaves were preliminary investigated by comparing the phytochemicals patterns of the half-growth and the full-growth leaves. Our experimental results provide useful information for the pharmaceutical research and clinical applications of *Ligustrum lucidum Ait* leaf.

KEYWORDS

Ligustrum Lucidum Ait leaf, Ambient ionization, Mass spectrometry and Leaf spray.

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INTRODUCTION

Ligustrum lucidum Ait has been used in traditional Chinese medicine for over 1000 years because of its anti-tumor, antimutagenic, antidiabetic, and hepatoprotective properties function[1]. Extracts from different parts (fruits and leaves) of *Ligustrum lucidum Ait* have certain medicinal efficacy, the fruits are known as “nv zhen zi” in traditional Chinese medicine and are believed to prevent and treat of several diseases, such as diabetes[2] and

coronary heart disease[3]. However, little is known about the specific medicinal value of the *Ligustrum lucidum Ait* leaves. A broad pharmacological potential (e.g., anti-inflammatory, detumescence, etc.) of *Ligustrum lucidum Ait* leaf was recorded in the Dictionary of Medicinal Plant. Thus, the study of medicinal constituents of *Ligustrum lucidum Ait* leaves has important guiding significance in pharmaceutical research and clinical application[4]. Secondary metabolites, the main source of medicinal composition of *Ligustrum lucidum Ait* leaves, are a consequence of the interaction between plants and environments in the long process of plant evolution[5,6]. Secondary metabolites are various in different growth stages, and the differences of secondary metabolites in plant tissue is closely related to the medicinal value of the plant. Therefore, the accurate identification of secondary metabolites in different growth stages' *Ligustrum lucidum Ait* leaves to evaluate its medicinal value is of important significance.

In previous study, the analysis of the leach liquor of *Ligustrum lucidum Ait* leaves used photometric method[7], gas chromatography-mass spectrometer (GC-MS)[8], high performance liquid chromatography (HPLC)[9] or liquid chromatography-mass spectrometry (LC-MS)[10]. However, these conventional methods are usually involved a time- and solvent-consuming sample pretreatment, such as comminution, maceration and extraction[11], unable to realize direct and rapid analysis of the chemical composition in plant tissues. Recently, ambient ionization mass spectrometry has been developed for the direct analysis of complex matrix samples without sample pretreatment, provides a reliable way to realize the simple and rapid analysis of complex matrix samples[12,14]. For example, laser ablation electrospray ionization mass spectrometry (LAESI-MS) is applicable to plant surface analysis[15,16], and internal extractive electrospray ionization mass spectrometry (iEESI-MS) has been applied for direct profiling of the phytochemicals inherent in various native plant tissues, including leaves, roots and fruits[17,18].

Leaf spray mass spectrometry is a new ambient ionization method applicable to the direct analysis of living plants, for example, plant leaves. Chemicals are directly ionized from leaves or thin plant tissues with minimum sample treatment[19,21]. In this paper, *Ligustrum lucidum Ait* leaves were directly analyzed using leaf spray mass spectrometry. A preliminary comparison of chemical composition in *Ligustrum lucidum Ait* leaves with different growth stages were investigated, providing a scientific basis to further reveal the growth and metabolism of *Ligustrum lucidum Ait* leaves for pharmaceutical research.

MATERIAL AND METHODS

Reagents and chemicals

High-purity water was obtained with a D11911 NANO pure ® Diamond ™ water-purification system (resistivity of 18.2 MΩ . cm). Methanol (A.R. grade) was bought from Chinese Chemical Reagent Co. Ltd. (Shanghai, China). *Ligustrum lucidum Ait* leaves (10 centimeters long and 5 centimeters broad) were collected in the campus, washed by high-purity water and dried at room temperature, according to the morphological features of *Ligustrum lucidum Ait* leaves, the leaves were divided into both half-growth leaves and full-growth leaves two groups, each group contains 9 pieces of similar size and shape leaves, each leaf as a separate samples to perform analysis.

Instrumentation

Experiments were carried out using a commercial linear trap quadruple (LTQ) mass spectrometer (LTQ-XL, Thermo Scientific, U.S.). The LTQ mass spectrometer was set to work in positive ion detection mode. The mass spectra were acquired in the mass range of *m/z* 50-1000, recorded with an average recording time of 100 ms and background subtracted. The voltage (+4.5 kV) was applied to the tissue, with a spray solvent of methanol, to generate a spray of charged droplets carrying endogenous chemicals toward the ion inlet of the mass spectrometer. The temperature of ion transport tube was 150 ℃. The collision induced dissociation (CID) experiments were performed by applying 18-

30 % collision energy (CE) to the precursor ions of interest, which were isolated using a mass-to-charge window width of 1.0 Da.

Leaf spray ionization

Leaf spray mass spectrometry was employed to directly analysis the phytochemicals in *Ligustrum lucidum Ait* leaves. The leaves were washed with high-purity water and dried at room temperature and then cut into a "V" shape (small triangle), the size with a side length of ca. 15 mm and sharp corner of 30°. The apex of the plant tissue was pointed to inlet to the mass spectrometer with a distance of 10 mm (Figure No.1). By adding 10 μL methanol on the surface of the "V" shape sample, charged droplets containing a variety of phytochemicals extracted from the leaf tissue were produced when high voltage (4.5 kV) was applied on the sample. The charged droplets containing analytes were carried into the mass spectrometer analysis.

RESULTS AND DISCUSSION

Phytochemicals analysis of *Ligustrum lucidum Ait* leaf

Ligustrum lucidum Ait, a member of the oleaceae family distributed worldwide, is often used as an ornamental tree. The *Ligustrum lucidum Ait* have been used in traditional Chinese and Japanese medicine for centuries, are believed to nourish liver and kidney and brighten eyes and hair. Leaf spray mass spectrometry was performed as a fast and simple way for direct analysis of a V-shaped half-growth *Ligustrum lucidum Ait* leaf tissue in this paper. The mass spectrum was shown in Figure No.2. Phytochemicals in the *Ligustrum lucidum Ait* leaf was extracted for the ionization and a large number of cationic molecules peak, such as $[M]^+$, $[M+H]^+$, $[M+K]^+$ was acquired. Potassium ions was identified in the form of $[CH_3OH + K]^+$ with a high relative abundance at mass spectrum peak *m/z* 71. The potassium can lower the risk of high blood pressure, stroke and heart disease. Hypokalemia contributes to the pathogenesis of cardiovascular disease, and many cardiovascular disorders and drugs aggravate hypokalemia[22-24]. Choline was identified in the form of a cationic $[M]^+$ at the mass spectrum signal peak of *m/z* 104. Sugars such as glucose or sucrose (such as *m/z* 219 $[Glucose + K]^+$, 381 $[Sucrose + K]^+$) were identified by mass measurement in *Ligustrum lucidum Ait* leaf tissue. The results were consistent with the literatures[20], showing that *Ligustrum lucidum Ait* leaf contains rich compositions of potassium and sugars.

Three groups fragment ions of *Ligustrum lucidum Ait* leaves were recorded, including *m/z* 401, 417, 433, 449, 465; *m/z* 563, 579, 595, 611, 627 and *m/z* 778, 794, 810, 826, 842, 858, 874 were obtained, corresponding to the *m/z* ratio increasing Δ=16 u $[M + 16]^+$ in each group and those corresponding to the between-group loss of $[M-146]^+$ and $[M-162]^+$, respectively. These findings lead to the hypothesis that flavonoids (such as apigenin glycoside, kaempferol glycoside and quercetin glycoside) may be detected in the *Ligustrum lucidum Ait* leaf tissue[10]. Flavonoids are used in the prevention of cancer, dementia, atherosclerosis and coronary heart disease[25-27]. Flavonoids may occur in various modified forms in plants corresponding to additional hydroxylation, methylation and most importantly, glycosylation[28]. These *m/z* ratio differences of the three groups' mass spectrometry signal peaks are most likely due to the differences of molecular structures as well as sugar residues losing. The molecular structures of flavonoids, such as precursors of apigenin (Mw 270), kaempferol (Mw 286) and quercetin (Mw 302) are similar, the differences of the three phytochemicals is directly related to the number and the position of -OH, corresponded to the adduct with oxygen $[M+O]^+$, this is a factor of Δ=16 u difference between the molecular structures.

The losing of sugar residues, such as glycosidase (162 u) or rhamnose (146 u, natural deoxidized sugar), may cause the between-group differences. So the three regularity and complex mass spectrum signal peak groups may be the ionization of flavonoids glycoside material in the *Ligustrum lucidum Ait* leaf. Based on the above speculation, main peaks at *m/z* 778-874 (shown in Figure No.2) (such as 778, 794, 810, 826, 842, 858, 874) increasing Δ=16 u may be obtained by the

ionization of biflavone glycosides in *Ligustrum lucidum Ait* leaf. The above experimental results show that the metabolism of total flavonoids in *Ligustrum lucidum Ait* leaf is a very complex process, it is difficult to separate and purify a single structure of flavonoids because of structures' diversity and similarity of *Ligustrum lucidum Ait* leaf.

Analysis of different growth stages leaves

The major phytochemicals in the *Ligustrum lucidum Ait* leaves across different growth stage were identified by leaf spray mass spectrometry, providing the necessary scientific basis for the pharmaceutical research and the medicinal development of *Ligustrum lucidum Ait* leaves. There were significant differences in spectra of half-growth leaf tissue (Figure No.3a) and full-growth leaf tissue (Figure No.3b). The relative abundance of mass spectrum peaks at *m/z* 417, 579 and 595 of full-growth leaf are lower than those of young-growth leaves, but the relative abundance of main peak between *m/z* 778-874 which increasing Δ=16 u in the full-growth leaf is higher. In positive ion detection mode, the mass spectral fingerprint data at the range of *m/z* 50-1000 of the full-growth leaves and half-growth leaves were observed in the principal component analysis (PCA), which was performed using the Matlab (version 7.8.0). Figure No.3c shows the PCA score plots obtained using leaf spray-MS with 9 separate samples of each growth stage leaves. The PCA score plots showed fairly clear differences between the half-growth leaves and full-growth leaves tissues. Three coordinates represent the principal component PC1, PC2, PC3 variance of the total contribution rate is 66.2%. The loading plots shown in Figure No.3d clearly indicated that the contribution rate of the mass spectrum characteristic peak *m/z* 104, 417, 433, 449, 579, 627, 794, 810, 826 to distinguish the different growth stages of *Ligustrum lucidum Ait* leaves is higher, demonstrating that the selected ions play an important role in distinguishing those organization samples. The results illustrated that the species and components of flavonoids in half-growth leaves is obviously different from full-growth leaves. Therefore, it is speculated that flavonoids intermoleculars occurred polymerization reaction from monoflavone glycosides converting into biflavone glycosides with the growth of *Ligustrum lucidum Ait* leaves. Flavonoids are important antioxidants, and provide several beneficial effects, such as Anti-viral, Anti-cancer, Anti-inflammatory and Anti-allergic. Therefore, *Ligustrum lucidum Ait* leaves may provide a new source of drugs for prevention and treatment of diseases such as myocarditis.

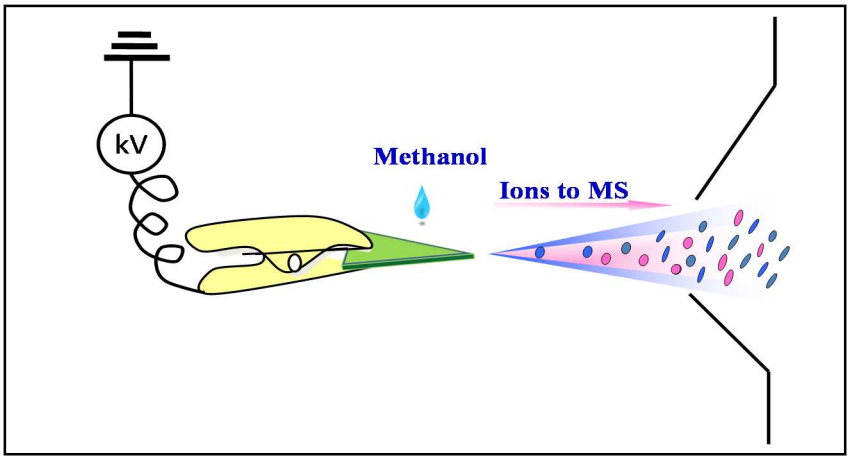

Figure No.1: Schematic diagram of Leaf spray ionization mass spectrometry

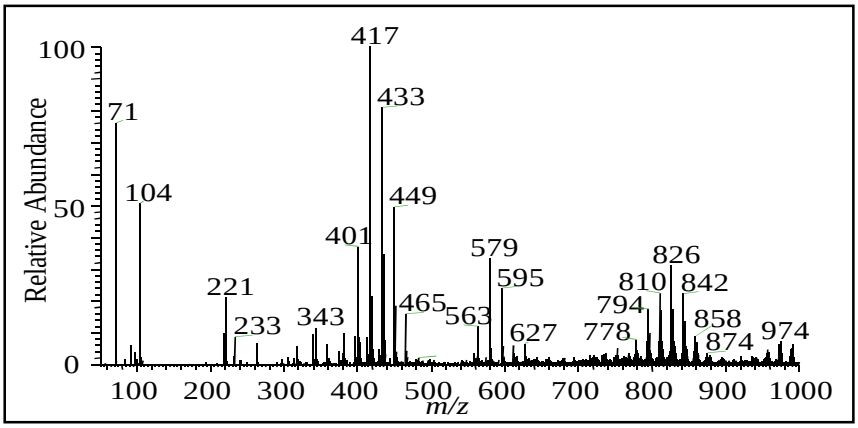

Figure No.2: Spectra of *Ligustrum lucidum Ait* leaf acquired in positive ion detection mode

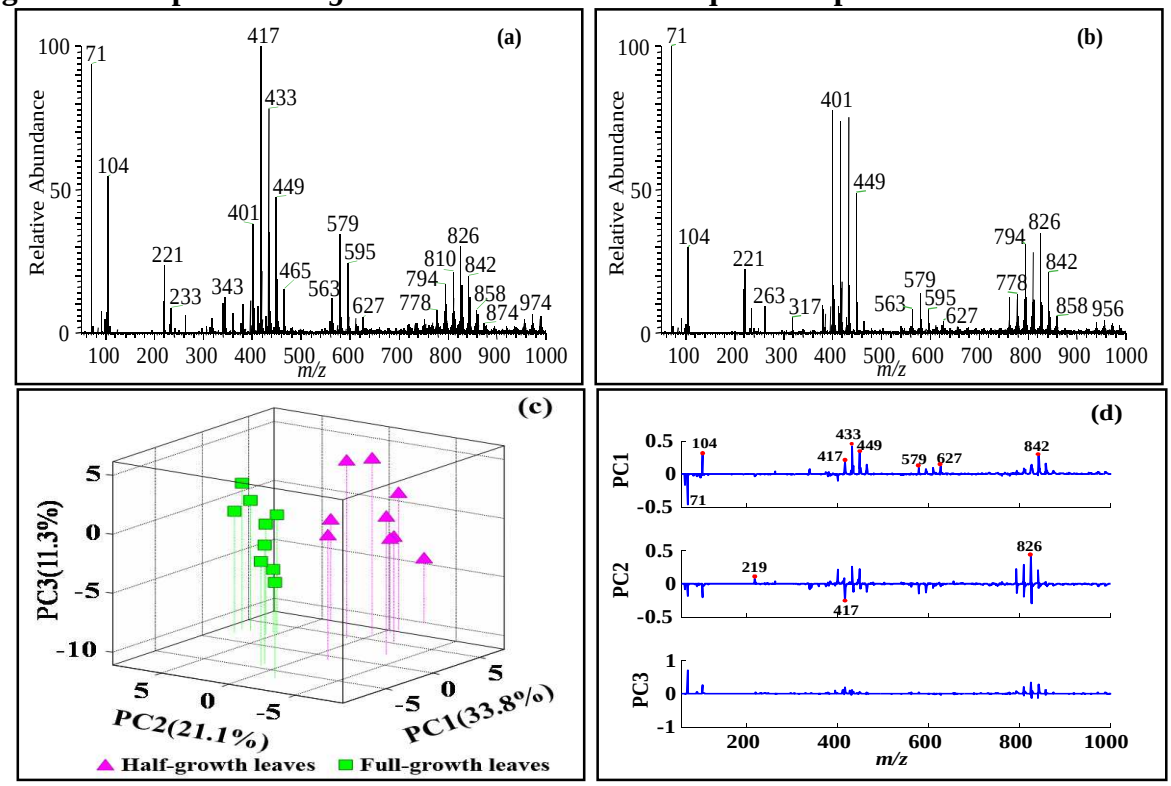

Figure No.3: Leaf spray-MS analysis of *Ligustrum lucidum Ait* leaves of different growth stage

(a) half-growth leaves; (b) full-growth leaves; (c) 3D-PCA score plots of leaf spray-MS fingerprints of the two growth stages set of *Ligustrum lucidum Ait* leaf; (d) PCA loading plots.

CONCLUSION

A leaf spray mass spectrometry method has been used for the detection of phytochemicals from different growth period *Ligustrum lucidum Ait* leaves, the phytochemicals such as potassium, flavonoids, sugars and other beneficial ingredients were sensitively detected, providing a new analysis approach for the development and utilization of medicinal plant resources of *Ligustrum lucidum Ait* leaves. Fast identification of the chemical composition information in different growth stages of *Ligustrum lucidum Ait* leaves has been successfully achieved by processing the leaf spray mass spectral fingerprint data with principal component analysis (PCA). Our experimental results further demonstrate that the established leaf

spray-MS approach is potentially useful for direct phytochemicals studies of different growth period leaves of *Ligustrum lucidum Ait* without pretreatment, allowing the direct analysis of plant tissue samples with the advantage of high speed, high throughput and simplicity.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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