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PAPER

Rapid identification of molecular changes in tulsi (*Ocimum sanctum* Linn) upon ageing using leaf spray ionization mass spectrometry†

Depanjan Sarkar, Amitava Srimany and T. Pradeep*

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Tulsi or Holy Basil (*Ocimum sanctum* Linn) is a medicinally important plant. Ursolic acid (UA) and oleanolic acid (OA) are among its major constituents which account for many medicinal activities of the plant. In the present work, we deployed a new ambient ionization method, leaf spray ionization, for rapid detection of UA, OA and their oxidation products from tulsi leaves. Tandem electrospray ionization mass spectrometry (ESI-MS) has been performed on tulsi leaf extracts in methanol to establish the identity of the compounds. We probed changes occurring in the relative amounts of the parent compounds (UA and OA) with their oxidized products and the latter show an increasing trend upon ageing. The findings are verified by ESI-MS analysis of tulsi leaf extracts, which shows the same trend proving the reliability of the leaf spray method.

Introduction

Mass spectrometry is an ever growing field and is probably the most versatile amongst other analytical techniques in terms of instrumentation and methodologies. When hyphenated with other analytical tools such as liquid chromatography (LC) or gas chromatography (GC), its performance and area of applications become much broader. For example, analysis of complex mixtures derived from plant extracts has been conventionally performed by liquid chromatography-mass spectrometry (LC-MS).[1–3] In spite of its usefulness, the main drawback of LC-MS is the time constraint associated with the method. Recently a new class of mass spectrometry, known as ambient ionization mass spectrometry,[4–10] has emerged with the introduction of desorption electrospray ionization mass spectrometry (DESI-MS) in 2004.[11] Subsequently other methods like direct analysis in real time (DART)[12] and many others have been developed in this category, all aiming to achieve ionization from atmospheric pressure with minimal or no sample preparations, decreasing the time required for analysis. Until now, various ambient methods such as DESI,[13–20] DART,[21–23] laser ablation electrospray ionization (LAESI),[24–26] and extractive electrospray ionization (EESI)[27,28] have been successfully used for plant tissue analysis without or with minimal sample preparation. Leaf spray ionization,[29,30] tissue-spray ionization[31] or similar kinds of direct analysis[32] are basically the same in terms of operational principle as they are extended versions of paper spray ionization.[33–36] These are also emerging ambient mass spectrometric tools for rapid identification of molecules from plant tissues.

Tulsi or Holy Basil (*Ocimum sanctum* Linn) is a very common plant (Fig. 1A) seen throughout India. It is also found in other places of the globe such as Malaysia, Australia, West Africa and several Arabian countries. The name tulsi or tulasi came from the ancient Indian language, Sanskrit, which means 'matchless one'.[37] Besides its sacredness in Hindu culture, tulsi is very popular in India owing to its high medicinal activities. For this reason, it has long been used in the traditional Indian medical system called *Ayurveda*, for treatment of various diseases and modern research also suggests its medicinal activities for similar conditions.[38] UA and OA are two of the major constituents of tulsi leaves. They are a triterpenoid class of compounds and structurally almost similar

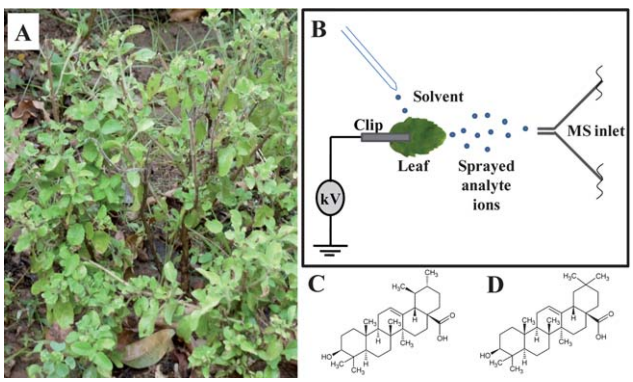

Fig. 1 (A) Photograph of a tulsi plant found in the IIT Madras campus. (B) Schematic diagram of the leaf spray experimental set-up. (C and D) Structures of ursolic acid and oleanolic acid, respectively.

Department of Chemistry, Indian Institute of Technology Madras, Chennai 600036, India. E-mail: pradeep@iitm.ac.in; Fax: +91 44 2257-0545; Tel: +91 44 2257-4208

† Electronic supplementary information (ESI) available: Different categories of tulsi leaves, experimental set-up photograph, tandem mass spectra for *m/z* 455.5, tandem mass spectra for *m/z* 471.5, tandem mass spectra for *m/z* 487.5, expanded view of leaf spray and ESI spectra near the region *m/z* 487.5, and tandem mass spectra for *m/z* 488.3. See DOI: 10.1039/c2an35655d

(Fig. 1C and D), having the same mass. These two compounds are of major importance because of their pharmacological properties like antimicrobial,[39] anti-inflammatory,[39,40] anti-HIV,[41] antiulcer,[42] *etc.* to name a few. Details of their pharmacological properties can be found in a review by Liu.[39]

In the present study, we show the applicability of a newly developed leaf spray method to detect compounds from tulsi leaves. A schematic of the experimental set-up is shown in Fig. 1B. UA and OA, two of the major compounds present in tulsi leaves, and their oxidation products with 16 Da and 32 Da higher mass than the parent compounds have been detected. The identities of these compounds have also been established by tandem ESI-MS. A semi-quantitative approach has been adopted to monitor the changes in the total amount of the above compounds with respect to their oxidized products during ageing of the leaf by the leaf spray ionization method. The results indicate the relative increase in the oxidized product with ageing. The trend is verified by ESI-MS data from the methanol extracts of leaves.

Materials and methods

Tulsi is available everywhere in India and, for the experiments, tulsi leaves were collected from the campus of IIT Madras. In our experiments, leaves were categorized in 5 different classes. They were tender leaf (TL, just coming out from the branch, ~3 to 4 days old), young leaf (YL, after some days of maturation, ~6 to 7 days old), mature leaf (ML, after maximum growth, ~2 weeks old), old leaf (OL, after becoming yellowish, ~6 weeks old) and dried leaf (DL, after drying the mature leaves in the laboratory at 25 °C for 10 days in a Petri dish). The maturation mentioned above may vary depending on the atmospheric conditions where the plant is exposed and the season. Fig. S1† shows a photograph of all the five categories of tulsi leaves. Methanol (HPLC grade) was purchased from Standard Reagents Pvt. Ltd., Hyderabad, India. Deionized water was used for washing.

For all the mass spectrometric measurements, an ion trap LTQ XL mass spectrometer from Thermo Scientific, San Jose, CA was used. For leaf spray experiments, samples were set at a distance of approximately 5 mm from the mass spectrometer inlet in all the cases. The experimental set-up is shown in Fig. S2.† Mass spectra were acquired in negative ion mode in the mass range of *m/z* 150 to 500 under the following conditions: source voltage 7 kV, capillary temperature 275 °C, capillary voltage −35 V and tube lens voltage −100 V. All leaf spray ionization mass spectra presented correspond to an average of 10 scans. For the ESI experiments, methanol extracts of various tulsi leaves were prepared by dipping the leaves for 3 hours in 1.5 mL methanol, taken in a 2 mL vial separately. After the specified time, the solutions were decanted to another vial and centrifuged for 10 minutes at a speed of 10 000 rpm. These extracts were electrosprayed at a flow rate of 5 μL min^{-1} and mass spectra were acquired in negative ion mode in the mass range of *m/z* 150 to 500. The following parameters were used for all the measurements: source voltage 5 kV, sheath gas (nitrogen) flow rate 8 (manufacturer's unit), capillary temperature 275 °C, capillary voltage −35 V and tube lens voltage −100 V. All ESI mass spectra presented correspond to an average of 100 scans. Tandem mass spectrometric experiments have been done with collision induced dissociation (CID).

Results and discussion

Methanol was used as a solvent in leaf spray experiments and 10 μL of it was added during each experiment. A tulsi leaf was cut in a triangular shape and connected with a clip to apply the high voltage. The spectrum of this sample is compared with that from the whole leaf (Fig. 2) which showed that a better quality of data can be obtained from the uncut leaf itself. This is possible because of the sharpness of the tip. Here the *m/z* 455.5 peak is due to UA and OA combined. As both of the compounds have the same mass and are structurally similar, it is difficult to distinguish them even by tandem mass spectrometry. Along with the *m/z* 455.5 peak, other two peaks are formed at *m/z* 471.5 and *m/z* 487.5. But in Fig. 2, the peak shown is at *m/z* 488.3, which will be discussed later. There is no report available in the literature about molecules with *m/z* 471.5 and *m/z* 487.5 present in tulsi. As the peaks are shifted from the peak at *m/z* 455.5 by 16 Da and 32 Da, respectively, it enables us to speculate that the new peaks are the oxidization products of UA and OA. Some other compounds of tulsi like rosmarinic acid and eugenol are also seen at *m/z* 359.3 and 163.2, respectively.

To know the structures of the two oxidized products, a tandem mass spectrometric study was conducted with the methanol extract of the tulsi leaf. ESI was used to get more signal intensity.

Fig. S3–S5† show the tandem mass spectrometry data and possible fragmentation products for *m/z* 455.5, 471.5 and 487.5, respectively. Concise results from this study are shown in Table 1. In the MS^2 spectrum of *m/z* 455.5, the peak is formed at *m/z* 407.5 with a loss of 48 Da due to the loss of neutral fragments, HCHO

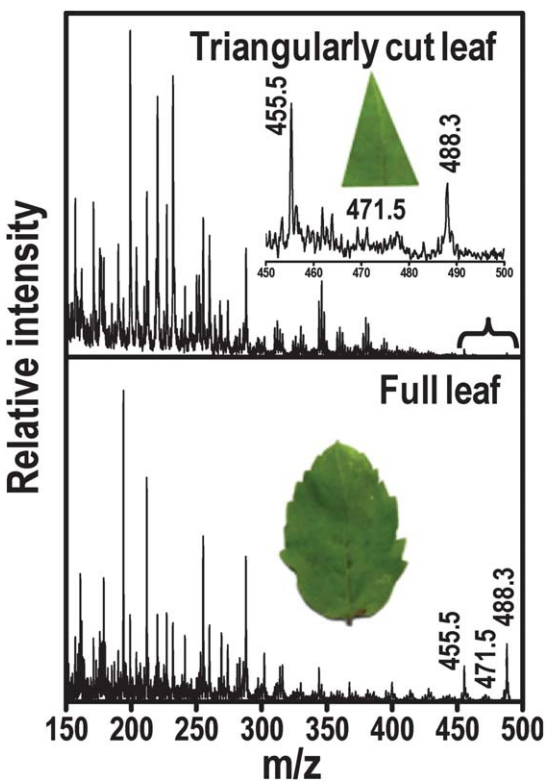

Fig. 2 Leaf spray mass spectra from a triangularly cut tulsi leaf (top) and a whole tulsi leaf (bottom). Inset of the top figure shows the expanded view from *m/z* 450 to 500.

Table 1 Tabulated tandem ESI-MS data from *m/z* 455.5, 471.5 and 487.5

m/z	MS^2	MS^3	MS^4
455.5	407.5	391.5 377.5	—
471.5	453.5	407.5 391.5	391.5 377.5
487.5	469.5	407.5 391.5 377.5	—

(30 Da) and H_2O (18 Da). Upon MS^3 of *m/z* 407.5, two peaks are formed at *m/z* 391.5 and *m/z* 377.5 because of the loss of CH_4 and C_2H_6, respectively. The results are supported by a previous report in the literature.[43] Similar results were obtained from leaf spray data also for *m/z* 455.5 (data not shown). The similarity of the fragmentation peaks confirms our speculation about the new peaks at *m/z* 471.5 and *m/z* 487.5 as oxidation products of UA and OA with one and two oxygen additions, respectively. It is noteworthy to mention at this point that ambient ionization process like DESI can oxidize analyte molecules during analysis. However, it has been on totally unrelated compounds that the same sample which gives oxidized products in DESI does not give oxidized products during ESI analysis.[44] In contrast, both ESI and leaf spray give the oxidized products in the present experiments, which confirms that the observed oxidation does not occur during leaf spray ionization. Upon a close look at the ESI spectrum of tulsi leaf extract, peaks at *m/z* 487.5 and *m/z* 488.3 are resolved properly which are not very clear in the leaf spray data where the peak at *m/z* 488.3 is somewhat broad (Fig. S6†). Again, tandem mass spectra of *m/z* 488.3 (Fig. S7†) do not reveal any similarity with other peaks. These two observations indicate that the ion at *m/z* 488.3 is different from that at *m/z* 487.5.

Five different types of leaves, *i.e.* TL, YL, ML, OL and DL (dried from OL) were collected from tulsi plants. Six sets were made from three different plants; two sets from each plant. After collection, the leaves were washed with deionized water and kept in a small closed container for 2 hours to make them uniformly moistened. The dried leaves were also moistened uniformly using the same methodology. After taking the leaves out of the container, they were wiped gently with tissue paper to remove excess water. Then 10 μL of methanol was spotted on them using a micro-pipette and the whole leaf was attached with the clip. All other experimental parameters were the same as mentioned before. Then averages of 10 scans were taken from each sample and intensities of *m/z* 455.5, 471.5 and 487.5 were noted. The signal duration (time window during which electrospray occurs from the leaf) was approximately one minute in each case. Fig. 3 shows the leaf spray mass spectra of ML and DL showing prominent changes in the intensities. The intensity ratios of *m/z* 471.5/455.5 and *m/z* 487.5/455.5 were calculated and the average values were plotted for all the five types of leaves (Fig. 4). From the plot, it is clear that the combined intensity of UA and OA decreased, compared to their oxidized products, with ageing of the leaf. As the size and shape of each leaf was different, and so were their origins, it is expected that the chemical contents will

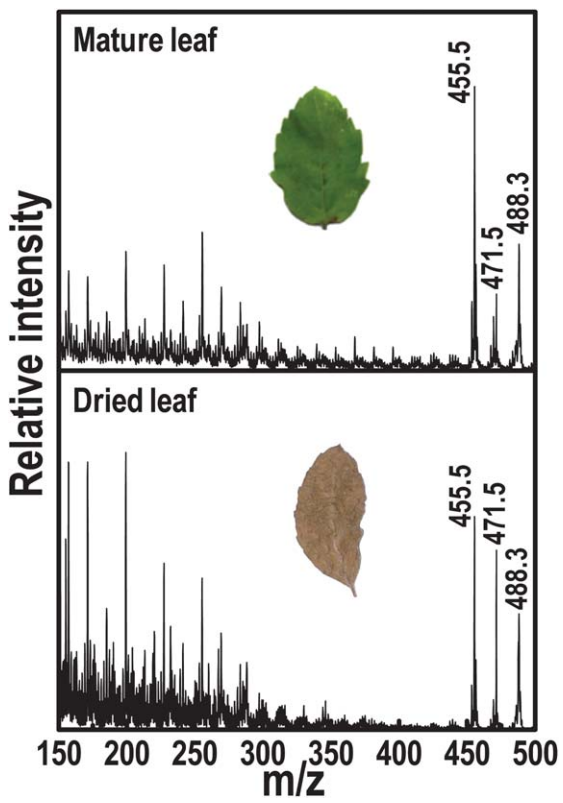

Fig. 3 Leaf spray ionization mass spectra of a mature tulsi leaf (top) and a dried tulsi leaf (bottom) using 10 μL of methanol as a solvent.

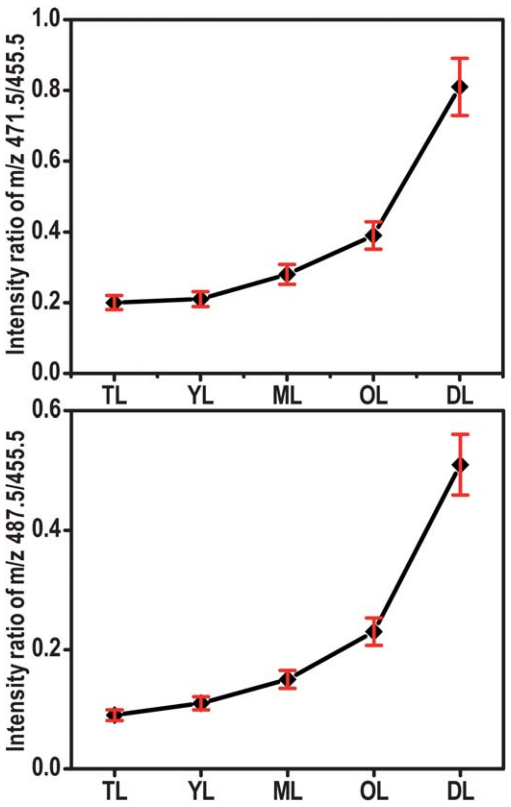

Fig. 4 Changes in intensity ratios, *m/z* 471.5/455.5 (top) and *m/z* 487.5/455.5 (bottom) with ageing of tulsi leaves, from leaf spray experiments. The error bars typically represent 10% error in absolute scale.

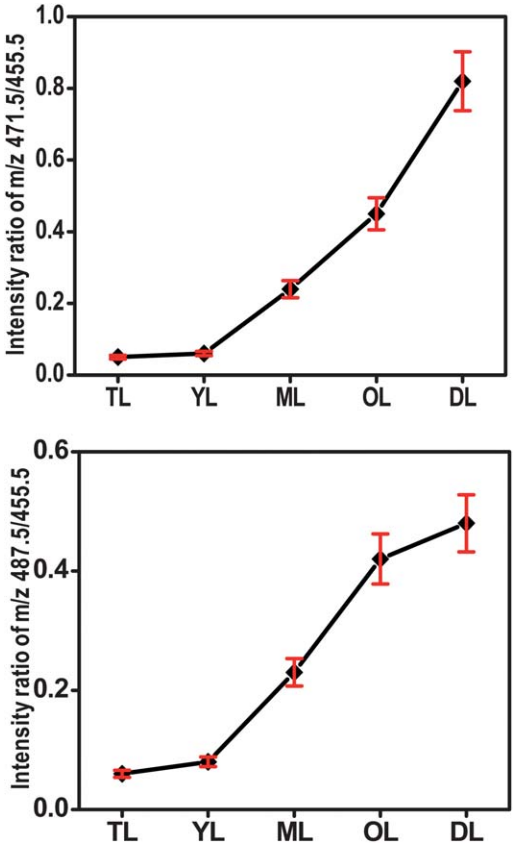

Fig. 5 Changes in intensity ratios, *m*/*z* 471.5/455.5 (top) and *m*/*z* 487.5/455.5 (bottom) with ageing of tulsi leaves from electrospray ionization experiments. The error bars typically represent 10% error in absolute scale.

also vary somewhat. This is the reason for taking the intensity ratios instead of the absolute intensities. All the six sets gave almost the same trends and the average of these is shown in Fig. 4. The same experiments were done without moistening the leaves, but they did not yield consistent results. This is also attributed to the very different nature of each leaf; *i.e.* having different degrees of moisture content. So maintaining a constant moistening condition for all of the leaves is necessary, as analyte transport is achieved by the solvent present, to get concordant datasets in experiments where comparison is needed.

The same experiments were also done with methanol extracts of tulsi leaves using ESI. Six sets of samples were made in a similar way as mentioned previously. The plots obtained from ESI are shown in Fig. 5 and are similar to those obtained from leaf spray experiments. These plots support the reliability of the data obtained from the leaf spray ionization method. In the case of ESI-MS of leaf extracts, the peak at *m*/*z* 359.3 due to rosmarinic acid occurs at high intensity whereas in leaf spray mass spectra it is poor. This enables other peaks of interest like at *m*/*z* 455.5, 471.5 and 487.5 to become prominent in the spectra and makes leaf spray ionization a better choice over ESI for studying UA, OA and their oxidation products.

Conclusions

In this work, fast detection of UA, OA and their oxidation products has been achieved by leaf spray ionization mass spectrometry. The identity has been confirmed by tandem ESI-MS experiments. By the leaf spray ionization technique it has been shown that the total combined amount of UA and OA decreased, compared to their oxidized products, with ageing of tulsi leaves. As the above mentioned medicinally important compounds are getting oxidized, medicinal activity of tulsi leaf is reduced with ageing. It also shows the vulnerability of these compounds towards oxidation. The same trend is observed in ESI experiments. It shows the reliability of the leaf spray ionization method, which can be used for other similar applications. Leaf spray ionization could become important in the field of phytochemical research where it could become a prominent analytical tool in the near future. Additionally, this method can be implemented with handheld mass spectrometers for rapid field studies.

The exact structural identification of oxidized products (position of oxygen addition) requires several spectroscopic studies such as NMR, IR, UV-visible, *etc.* which require a reasonable amount of these compounds in their purest form. The presence of these compounds in trace quantities in tulsi leaves and their similar polarities make it almost impossible to separate them by separation techniques such as HPLC. Extensive work towards method development is underway to determine the exact structures of the oxidized products.

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