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function of protein fragments. Thus, the present m/z values of MALDI-TOF MS analysis acted as a spectroscopic tool to recognise the inter-specific variation among the selected *Plumbago* species.

Conflict of interest statement

We declare that we have no conflict of interest.

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these proteins could be optimal biomarkers at the inter-specific and intra-specific level. The hypothesis of (C.A. Rebuffo *et al*, 2006) correlated with the present study demonstrating the MALDI-TOF MS analysis as a biomarker to differentiate the *Plumbago* species collected from various accessions of South India.

Conclusion

MALDI-TOF MS are an effective strategy for determining the protein domains. The similarity and variation among the selected *Plumbago* species were observed through MALDI-TOF MS analysis and Swiss prot database. While comparing the sequence coverage in MASCOT search value the lowest average sequence coverage was identified in the protein samples of *P. rosea* 39% compared to other two *Plumbago* species, the peptides detected are confined to a specific region of the protein, such as the protein N- or C-terminal. This information could easily be incorporated into protein identification tables. Regional coverage information is not readily available from either MS analysis. Some of the protein fragments correspond to chains produced by known cellular processing and activation pathways were detected and compared. Others have been detected as functional and structural domains (Fig.1). By using tools that allow both protein identification and measurement of molecular weight, we can assess the abundance and distribution of protein fragments. Correlation of these results with targeted functional studies on specific proteins will elucidate the biological

MALDI-TOF MS analysis and the single linkage cluster analysis was used to study the phylogenetic variation. The results were compared using ITS and tef1 sequences. Dominating peaks were observed in two different mass ranges. The most important one was situated between m/z 6,000 and 8,000. In the present study, MALDI-TOF MS analysis was carried out among the selected *Plumbago* species collected from different accessions of South India. The resulting peak lists of individual samples were submitted to NTSYs software to produce a taxonomic tree to reveal the inter-specific and intra-specific variation. Among the three *Plumbago* species, *Plumbago zeylanica* represented maximum number (21) of m/z peaks ranged from 1242 to 39429 m/z values. Next to that, the *Plumbago auriculata* demonstrated nine m/z peaks ranged from 1422 – 92970. Similarly, *Plumbago rosea* depicted two distinct spectral values 822 and 4058 respectively. From the research it was reinforced that, MALDI-TOF MS analysis was used as a taxonomic tool to study the similarity among the selected species and is a quick and reliable tool for species identification which can be a valid alternative to gene sequencing for species diagnosis. The primary advantage of MALDI-TOF MS is the speed by which identification can be made. The MALDI-TOF MS analysis can be completed in a few minutes as opposed to two or more days required for DNA sequence analysis (I.S. Druzhinina *et al.*, 2008, M.R. Hermosa *et al.*, 2004, G.J. Samuels and A. Ismaiel, 2006). Neuhof *et al.*, (2007) proposed the hypothesis that

shows the mass value as 17273 kDa with the top score sequence coverage as 39 % revealed the Putative defense protein 2. The MASCOT results were displayed in Fig.1.

Discussion

MALDI-TOF MS analysis accurately reflected the phylogenetic classification, in most cases, species identified by DNA sequence analysis clustered together by MALDI-TOF MS. The resolution of MALDI-TOF MS was performed roughly equivalent to ITS rDNA. The MALDI-TOF MS technique analyzes peptides and represents a rough equivalent to sequencing, thus representing a valid alternative for taxanomic identification of plants (Respinis *et al.*, (2010). Chuntaratin, (2006) studied the plumbagin protein conjugation by glutaraldehyde reaction using MALDI-TOF MS. By means of MALDI-TOF MS analysis it was confirmed that the molecular weight of the proteins of plumbagin-protein conjugate band was higher than the normal protein showing the molecular weight of 72817.704 and 66564.232. The present research showed a proximate spectra of varied ion peaks (m/z) ranged from 0 - 1, 00, 000 in correspondence to varied intensities were recorded in the peptide mass finger printing profile of individual *Plumbago* species.

Respinis *et al.*, (2010) investigated the peptide mass finger printing profile of 129 morphologically similar strains of *Hypocrea* and *Trichoderma* species using

unique m/z peaks compared to other accessions [Fig. 2]. These MALDI-TOF MS spectroscopic profile can act as a biological spectroscopic tool to study the inter-specific variation and similarity of selected *Plumbago* species collected from South India.

Fig. 2: Cladogram based on MALDI-TOF MS m/z values of *Plumbago* species

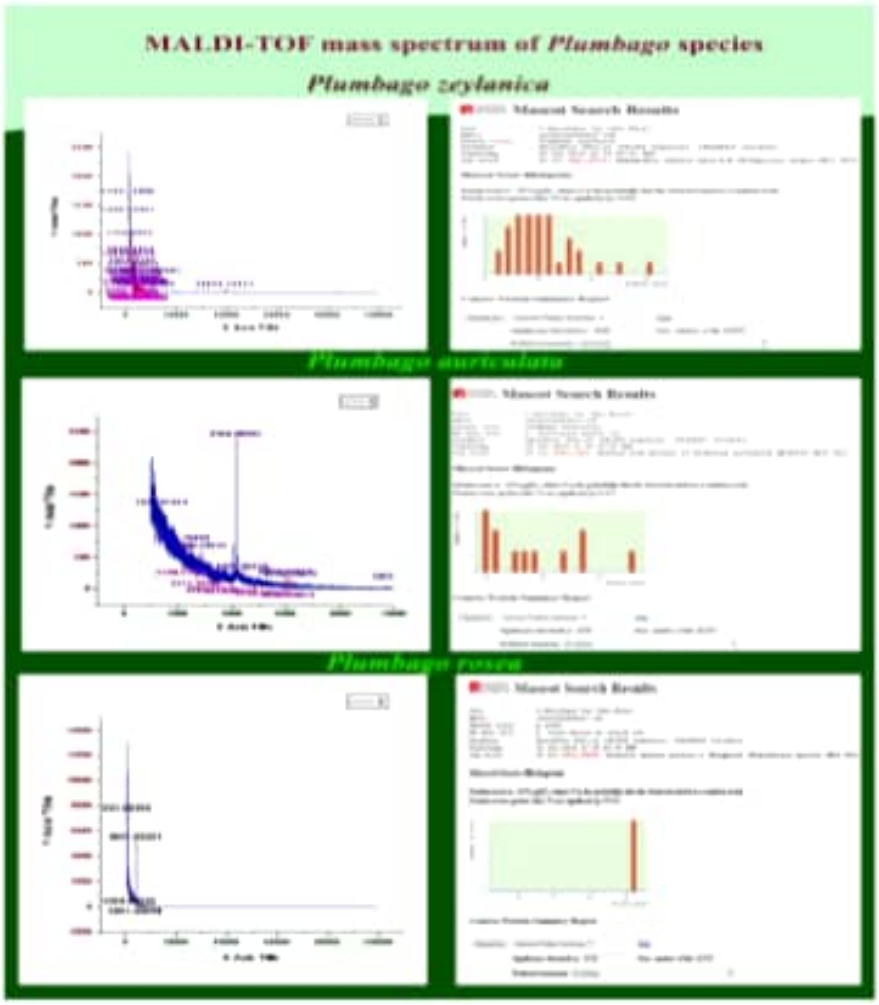
Protein Identification

In the MALDI-TOF MS analysis, the protein was identified with significant hits [$p<0.05$] in MASCOT probability analysis. The MASCOT peptide mass fingerprint search represented varied proteins related to the m/z values among these the top score value was selected for the protein identification. The MASCOT peptide mass fingerprint search for *P. zeylanica* shows the mass value as 16251 kDa with the top score sequence coverage as 45% revealed the Hemoglobin subunit beta-A/B protein. The MASCOT peptide mass fingerprint search for *P. auriculata* shows the mass value as 14325 kDa with the top score sequence coverage as 43 % revealed the Proline-rich protein. The MASCOT peptide mass fingerprint search for *P. rosea*

Table - 1: Mass spectral (m/z) values of *Plumbago* species

Plumbago species m/z peak values		
P. zeylanica	*P. auriculata*	*P. rosea*
1242		822
1454	**1422**	
1724		
2047		
2446	2246	
2829	**2883**	
3111		
3367		
3602		
4124	**4167**	4058
4365		
4785		
5293		
5708		
6254	**6198**	
6821		
7479	10254	
9062	56644	
10441	82260	
39429	92970	

The cladogram constructed based on the MALDI TOF-MS analysis and the results revealed the inter-specific variations and similarities among the *Plumbago* species collected from South India viz., *Plumbago zeylanica, Plumbago auriculata* and *Plumbago rosea.* The cladogram distinguished two clades viz., C_1 and C_2 based on the m/z peak values. Clade 1 (C_1) was shared by *P. zeylanica* and *P. auriculata*. Clade 2 (C_2) showed the individual presence of the *P. rosea*. The exclusive presence of the *P. rosea* in a separate clade$_2$ represented the presence of some



showed a proximate spectra of varied ion peaks m/z ranged from 0 - 1,00,000 kDa. The results of MALDI-TOF MS analysis showed both positive and negative peaks; to reveal the inter-specific similarity and variation between the *Plumbago* species, the positive peaks were selected. The obtained spectral profiles were further screened for the presence of recurring peaks or biomarker ions specific for all the species. Based on the unique spectral values the cladogram was constructed. Totally 31 spectral peak values / m/z values were selected and summarized in Table-1. Among the three *Plumbago* species, *Plumbago zeylanica* represented maximum number (21) of m/z peaks ranged from 1242 to 39429 m/z values; of which sixteen specific peaks were observed only in the *P. zeylanica*. Next to that, the *Plumbago auriculata* demonstrated nine m/z peaks ranged from 1422 - 92970 respectively. Out of nine peaks, only five unique peaks were observed in *P. auriculata*. Similarly, *Plumbago rosea* depicted two distinct spectral values 822 and 4058 respectively. These ionic unique spectral peaks of the *Plumbago* species collected from various accessions offer a strong proof in differentiating the selected accession and paved a way to study the similarity and variation among the accession using MALDI-TOF MS analysis [Fig.1].

Fig.1 MALDITOF- MS of *Plumbago* species

The aerial parts of *P. zeylanica*, *P. auriculata* and *P. rosea* were collected from different parts of Tamil Nadu, South India. *P. zeylanica* were collected from Papanasam (Tamil Nadu), *P. auriculata* were collected from Tenkasi (Tamil Nadu) and *P. rosea* were collected from Dana (Tamil Nadu) respectively. The collected species of *Plumbago* were identified by taxonomist from St. Xavier's college, Palayamkottai and the Herbarium specimens were deposited in the St. Xavier's College Herbarium (XCH), Palayamkottai (*P. zeylanica* – XCH 28089; *P. auriculata* – XCH 28093 and *P. rosea* – XCH 28101).

MALDI –TOF MS Analysis

MALDI spectrum of *Plumbago* species were recorded using Applied Biosystems MALDI-TOF Voyager De-Pro spectrometer. The MALDI sample was prepared by mixing 1µL of protein sample solution and sinapic acid matrix solution (5 mg/mL sinapic acid in 50% ACN/0.1% TFA). 0.75µL of the resulting mixture was spotted onto a freshly cleaned stain less steel MALDI target plate. After air drying, the crystallized spots were processed with a MALDI-TOF mass spectrometer (Voyager DE PRO) (Applied Biosystem). MS was recorded in the positive and negative mode within a mass range from 0 - 1, 00, 000 kDa, using a nitrogen laser (337 nm). The acceleration voltages applied for MS was 25 kV.

Result

MALDI-TOF MS characterization of *Plumbago* species collected from Karnataka

pharmacological activities such as anti-malarial (N. Didry *et al.*, 1994), antioxidant activity (G. Nahak and Sahu R.K. 2011) anticancer, cardiotonic, antifertility action, antibiotic and antineoplastic (K.R. Kiritkar and B.D. Basu, 1975, M. Krishnaswamy and K.K. Purushottamam, 1980, N.G.K. Pillai *et al.*, 1981). Its other constituents in roots are chitranone, zeylanone, dihydrosterone, 2- methyl naphthaquin, plumbazeylanone and terpenoids, lupeol and teraxesterol. The plant also contains alkaloids, glycosides, tannin, saponins and steroids (R.R. Chakraborty and A.T. Patil, 1997). The roots are used extensively in China and other Asian countries for the treatment of cancer, rheumatoid arthritis, dysmenorrhoea, and contusion of extremities (Atta-ur-Rahman, 1988). Extract of the root is given internally or applied to the *Ostium uteri*, causes abortion (P. Premakumari et al., 1977, S.K. Bharghava, 1984). Matrix-assisted laser desorption/ionization TOF mass spectrometry (MALDI-TOF MS) is an important proteomic technology. The protein biomarkers of the selected *Plumbago* species have been indicated by using MALDI-TOF MS to analyze the variation and similarity among them. The goal of this study was to screen for protein biomarkers in the selected *Plumbago* species using MALDI-TOF MS combined with magnetic beads and pattern recognition software.

MATERIALS AND METHODS

Collection of plant materials

tolerance to non-volatile buffers and impurities (F. Hillenkamp *et al*, 1991: J. Hardouin, 2007). The samples for MALDI are typically applied to solid supports and used off-line from liquid or gel separations (K.L. Walker *et al*, 1995, T. Rejtar *et al*, 2002). The MALDI-TOF MS technique analyzes peptides and represents a rough equivalent to sequencing, making this method a useful adjunct for determination of species limits. It also allows simple, reliable, and quick species identification, thus representing a valid alternative to gene sequencing for species diagnosis of bacterial and plant taxa (Sophie De Respinis *et al*, 2010). Using this technology it has been estimated that up to 99% of species tested are correctly identified when comparing with commercial phenotypic identification panels or gene sequencing (C.A. Chen *et al*, 2009, A. Bizzini *et al*, 2010, A. Cherkaoui *et al*, 2010). Chuntaratin, (2006) studied the plumbagin protein conjugated glutaraldehyde reaction in *P. indica* using MALDI-TOF MS analysis. The plant species *Plumbago zeylanica* (Plumbaginaceae), known vernacularly as Chitraka and popularly as Ceylon Leadwort, is distributed as a weed throughout the tropical and subtropical countries of the world. The genus *Plumbago* includes 3 species viz., *Plumbago indica* L. (P. *rosea* L.), *P. auriculata* L., and *P. zeylanica* L., which are distributed in several parts of India (K.M. Chetty *et al*, 2006). The roots of the *Plumbago* species contain an alkaloid called plumbagin, a natural napthaquinone (5-hydroxy-2-methyl-1, 4- naphthoquinone), possessing various

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presence of recurring peaks or biomarker ions specific for all the species. Based on the unique spectral values the cladogram was constructed. Database MASCOT search provides a assumption of the related protein in the particular kDa. These ionic unique spectral peaks obtained from the three different *Plumbago* species offer a strong proof in differentiating the selected *Plumbago* species and paved a way to study the similarity and variation among the species using MALDI-TOF MS analysis. The mass spectral values are higher in *P. zeylanica* species compared to other two species. This is the first report on protein identification and variation among *Plumbago* species.

Keywords: *Plumbago* species, MALDI-TOF MS, proteins, mass spectral values, cladogram

Introduction

Proteomics has become an important research tool to study complex biological systems in the post-genomics era, and the large-scale, systematic analysis of tissue and organelle specific proteins provides a more direct view of cellular processes not avail- able through the measurement of DNA. Proteomics can provide insight on the specialized biochemistry of distinct tissues, protein localization, protein–protein interactions, enzymatic complexes, protein-metabolite complexes, post-translational modifications, and cellular signaling (B. Kersten *et al*., 2002: S. Baginsky, 2009). MALDI is fast and efficient and has a high

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Abstract

Matrix-Assisted Laser Desorption/Ionisation (MALDI) mass spectrometry uses the power of high mass resolution time of flight (TOF) mass spectrometry coupled to the raster of lasers shots across the cut surface of tissues to provide new insights into the spatial distribution of bio-molecules within biological tissues. In the present study, Protein biomarker identification from MALDI-TOF MS analysis was used as a efficient spectroscopic and taxonomic tool to differentiate the protein expression of three *Plumbago* species viz., *Plumbago zeylanica* Linn., *Plumbago auriculata* L., *Plumbago rosea* L. collected from Karnataka. Following a simple protein isolation procedure, plant proteins were fingerprinted by analysing biomarker cellular proteins ranged from 0 - 1, 00, 000 kDa or m/z values using Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) mass spectrometry. Totally 31 spectral peak values / m/z values were selected. The obtained spectral profiles were further screened for the

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Chapter–XVIII

18

MALDI-TOF MASS SPECTROMETRY AS A RELIABLE SPECTROSCOPIC TOOL TO DIFFERENTIATE *PLUMBAGO* SPECIES

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