

**IDENTIFICATION OF NOVEL BIOMARKER FOR THE MEDICINALLY IMPORTANT
SPECIES OF SELAGINELLA USING MALDI TOF MS ANALYSIS**

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ABSTRACT

MALDI profiling has become a good tool for the discovery and validation of biomarkers. In the present study, MALDI-TOF MS has been applied as a tool to identify the biochemical (protein) similarities and variations among the selected Selaginella species viz., Selaginella intermedia (Bl.) Spring, Selaginella inaequalifolia (Hook. et Grev.) Spring, Selaginella involvens (Sw.) Spring, Selaginella tenera (Hook & Grev.) Spring, Selaginella wightii Hieron., Selaginella brachystachya (Hook. & Grev.) Spring, Selaginella repanda (Desv.) Spring, Selaginella radicata (Hook. & Grev.) Spring, Selaginella bryopteris (L.) Bak and Selaginella delicatula (Desv.) Alston from their natural habitats of South India. MALDI-TOF MS characterization of Selaginella species collected from various localities of South India expressed different ion peaks ranged from 0 - 1, 00,000 kDa. A total of 556 ranging from 491 to 99939 m/z values MALDI-TOF MS spectral peaks were observed. Among the spectral profile of ten Selaginella species, S. involvens displayed maximum number (84) of spectral peaks ranged from 527 to 99215 m/z values and S. radicata depicted minimum number (21) of spectral peaks varied from 694 to 91754 m/z values. Totally, 139 spectral peaks showed the inter-species resemblance among the studied medicinally important Selaginella species. The cladogram constructed based on the unique spectral values displayed both monophyletic group and paraphyletic taxon. The distinguished character of S. tenera, S. involvens, S. wightii, S. brachystachya, S. repanda and S. delicatula is confirmed in the cladogram by the presence of unique m/z values. The unique m/z values can be act as a biochemical marker to identify the specific Selaginella species in chemosystematics and pharmaceutical industries.

KEYWORDS: MALDI-TOF MS, Selaginella, monophyletic, paraphyletic, chemosystematics.

INTRODUCTION

Protein profiling using MALDI-TOF MS is an important tool for the fast and cost effective identification and characterization of diverse biological systems. MALDI-TOF-MS analysis paves a pathway for the bacterial identification at the species level by measuring molecular masses of proteins (Wang et al., 2012). In recent days, proteome analysis is increasingly being applied to cancer biomarker discovery (Kikkawa et al., 2012). However, only very few reports were available for the applications of MALDI-TOF MS in plant systematics. Matharu et al., (2010) used the MALDI-TOF technique to differentiate between plant parts of *Hemidesmus indicus*. But MALDI-TOF analysis is applied to differentiate the proteomic profile of different developmental stages of plants, to identify the stress tolerant and sensitive protein and to study the effects of nitric oxide on alleviating Cd induced toxicity in rice (Castillejo et al., 2008; Zhao et al., 2012; Datta et al., 2013). Selaginella Pal. Beauv., a

perennial herbaceous plant (spike moss) is the only surviving genus within the Selaginellaceae family. Selaginella is a unique terrestrial heterosporous pteridophyte and is represented by about 700-750 species widely distributed around the globe (Little et al., 2007). These xerophytic species are able to withstand desiccation for months and expands when it is moistened. Due to the drought-tolerance capacities, these species have different bioactive chemical compounds which are medicinally important (Irudayaraj and Johnson, 2013). Before the evolution of electrophoresis and molecular analysis the authentication of medicinal parts / plant is performed by the conventional methodology viz., morphological, anatomical and chemical profiling. Due to the application limitation of the conventional tools, they failed to classify or distinguish the closely related species and adulterants. Therefore, it is necessary to develop an alternate method to differentiate the medicinally important and

morphologically similar species. Although a number of phytochemical and pharmacological studies were carried out in the genus *Selaginella* (Suganya *et al.*, 2011; Sivaraman *et al.*, 2013; Weng and Noel, 2013), only very few reports were available on the metabolic profile of *Selaginella* species (Yobi *et al.*, 2012; Hanna *et al.*, 2012; Narayani and Johnson, 2013). But there is no report on the biochemical and molecular variation studies on *Selaginella* from India. With this knowledge, the present study was carried out to identify novel biomarker for the medicinally important species of *Selaginella* using MALDI TOF MS analysis.

MATERIALS AND METHODS

Collection of plant materials

The young leaves of selected ten *Selaginella* species viz., *Selaginella intermedia* (Bl.) Spring, *Selaginella inaequalifolia* (Hook. et Grev.) Spring, *Selaginella involvens* (Sw.) Spring, *Selaginella tenera* (Hook & Grev.) Spring, *Selaginella wightii* Hieron., *Selaginella brachystachya* (Hook. & Grev.) Spring, *Selaginella repanda* (Desv.) Spring, *Selaginella radicata* (Hook. & Grev.) Spring, *Selaginella bryopteris* (L.) Bak and *Selaginella delicatula* (Desv.) Alston from their natural habitats of South India includes Kaakachi hills (Tirunelveli, Tamil Nadu), Keeriparai and Marunthuvazh Malai (Kanyakumari, Tamil Nadu), Shenbaganur and Eettipallum (Dindigul, Tamil Nadu), Ponnudi (Kerala), Nilgiris (Tamil Nadu) and Thenmalai (Kerala), and performed the genetic polymorphism of the selected species. The specimens were identified by Dr. V. Irudayaraj, Assistant Professor, Department of Botany, St. Xavier's College (Autonomous), Palayamkottai, India. The voucher specimens were deposited in the Xavier's College Herbarium (XCH 25425 - 25435) attached to Centre for Biodiversity and Biotechnology, St. Xavier's College (Autonomous), Palayamkottai, India.

Protein isolation

Fresh and young leaves of selected ten species were collected from various localities of South India. For protein analysis, the collected young leaves of selected *Selaginella* species were washed once in deionized water and mashed in a pre-chilled mortar using 500 µl of phosphate buffer (pH 7.0). The resultant slurry was centrifuged at 10,000 rpm for 10 min at 4°C in an Eppendorf centrifuge and the supernatant was stored at 4°C before use.

MALDI-TOF MS analysis

MALDI spectrum of *Selaginella* species were recorded using Applied Biosystems MALDI-TOF Voyager De-Pro spectrometer. The MALDI sample was prepared by mixing 1 µL of protein sample solution and sinapic acid matrix solution (5 mg/mL sinapic acid in 50% ACN/0.1% TFA). 0.75 µL of the resulting mixture was spotted onto a freshly cleaned stainless 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 to 1, 00, 000 kDa, using a nitrogen laser (337 nm). The acceleration voltages applied for MS was 25 kV. For the systematic studies the peaks with intensity of 20 and above m/z values were selected. The similarity and variation between the bands were estimated by NTS sys software analysis and the cladogram was constructed. For the inter-specific relationship studies, the MALDI-TOF spectral peaks were converted into a "1" and "0" matrix, to indicate the presence or absence of the m/z values, respectively. Genetic similarities (GS) were estimated according to Nei and Li, 1979. To demonstrate the interspecific relationship, a cladogram was constructed by UPGMA using NTSYSpc-2.0 software.

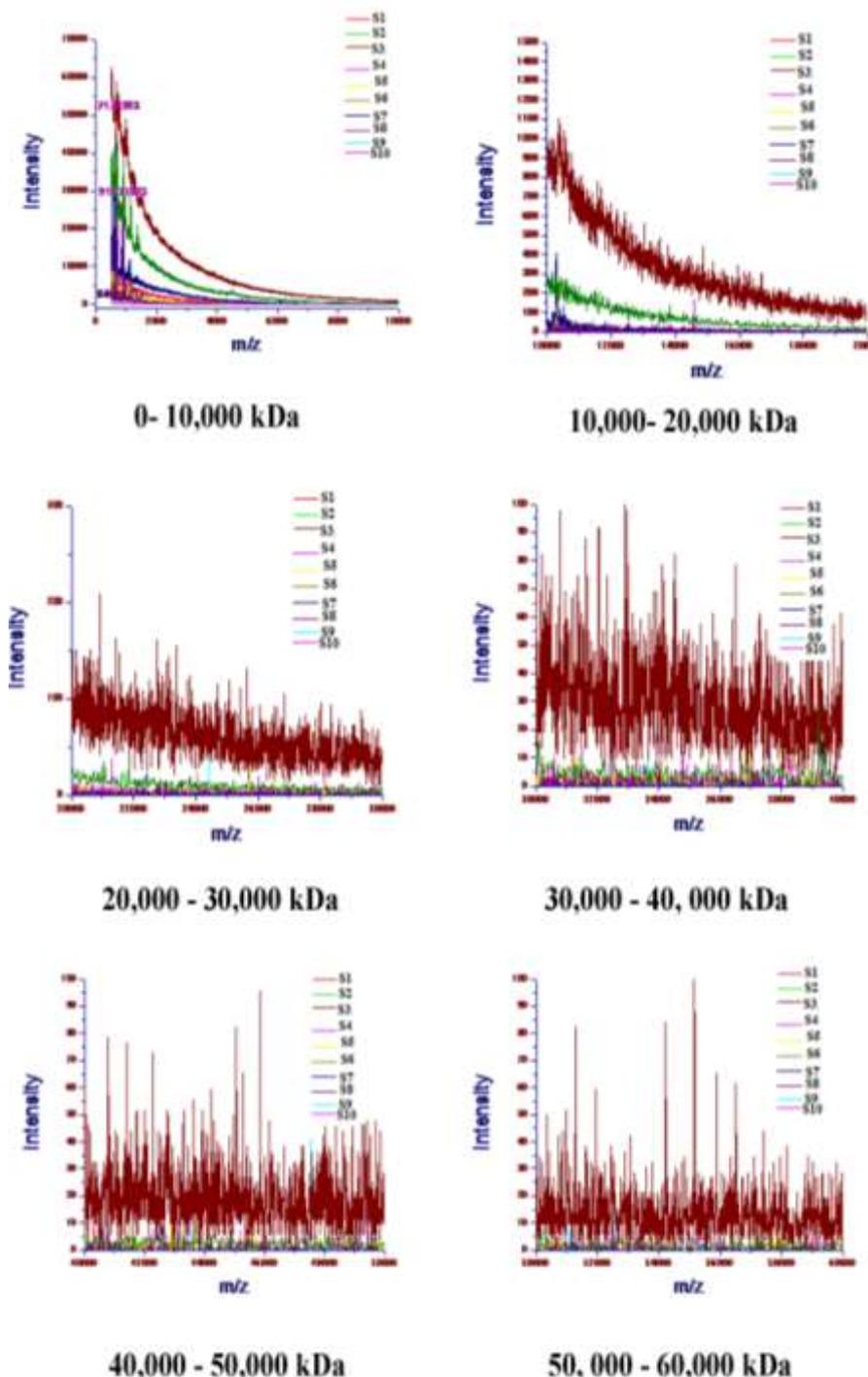
RESULTS

MALDI-TOF MS characterization of *Selaginella* species collected from various localities of South India expressed different ion peaks ranged from 0 - 1, 00,000 kDa (**Fig. 1 and 2; Table 1**). To identify novel biomarker for the medicinally important species of *Selaginella* the positive peaks were selected. A total of 556 MALDI-TOF MS spectral peaks were observed and they ranged from 491 to 99939 m/z values (**Table 1**). Among the spectral profile of ten *Selaginella* species, *S. involvens* displayed maximum number (84) of spectral peaks ranged from 527 to 99215 m/z values. Next to that, *S. tenera* represented 77 spectral peaks with m/z values 719 to 95316. *S. radicata* depicted minimum number (21) of spectral peaks varied from 694 to 91754 m/z values. Among the 556 spectral peak values of the studied ten *Selaginella* species, 139 spectral peaks showed the inter-species resemblance of the studied medicinally important *Selaginella* species (**Table 1**).

Based on the MALDI-TOF mass spectral peaks of studied *Selaginella* species, the similarity indices were calculated and the cladogram was constructed (**Fig. 3**). The cladogram displayed two clades C₁ and C₂. The clade C₁ is made of monophyletic taxon group viz., *S. intermedia*, *S. inaequalifolia*, *S. wightii*, *S. involvens* and a paraphyletic taxon *S. tenera*. Similarly, the clade C₂ consist of monophyletic taxon group viz., *S. brachystachya*, *S. radicata*, *S. repanda*, *S. bryopteris* and a paraphyletic taxon *S. delicatula*. The clade C₁ is divided into two nodes, C₁N¹ and C₁N². The node C₁N² showed the unique existence in *S. tenera*. The node C₁N¹ is branched into C₁N¹B₁ and C₁N¹B₂. The branch C₁N¹B₁ is further divided into two small branches C₁N¹B₁b₁ and C₁N¹B₁b₂, whereas the branch C₁N¹B₂ expressed the exclusive character of *S. involvens*. The small branch C₁N¹B₁b₁ displayed the similarity between *S. intermedia* and *S. inaequalifolia*. The small branch C₁N¹B₁b₂ depicted the distinct character of *S. wightii*. The clade C₂ is divided into two nodes C₂N¹ and C₂N². The C₂N¹ is branched into C₂N¹B₁ and C₂N¹B₂. The branch C₂N¹B₁ is again divided into two small branches C₂N¹B₁b₁ and C₂N¹B₁b₂. The branch C₂N¹B₂ illustrated the unique

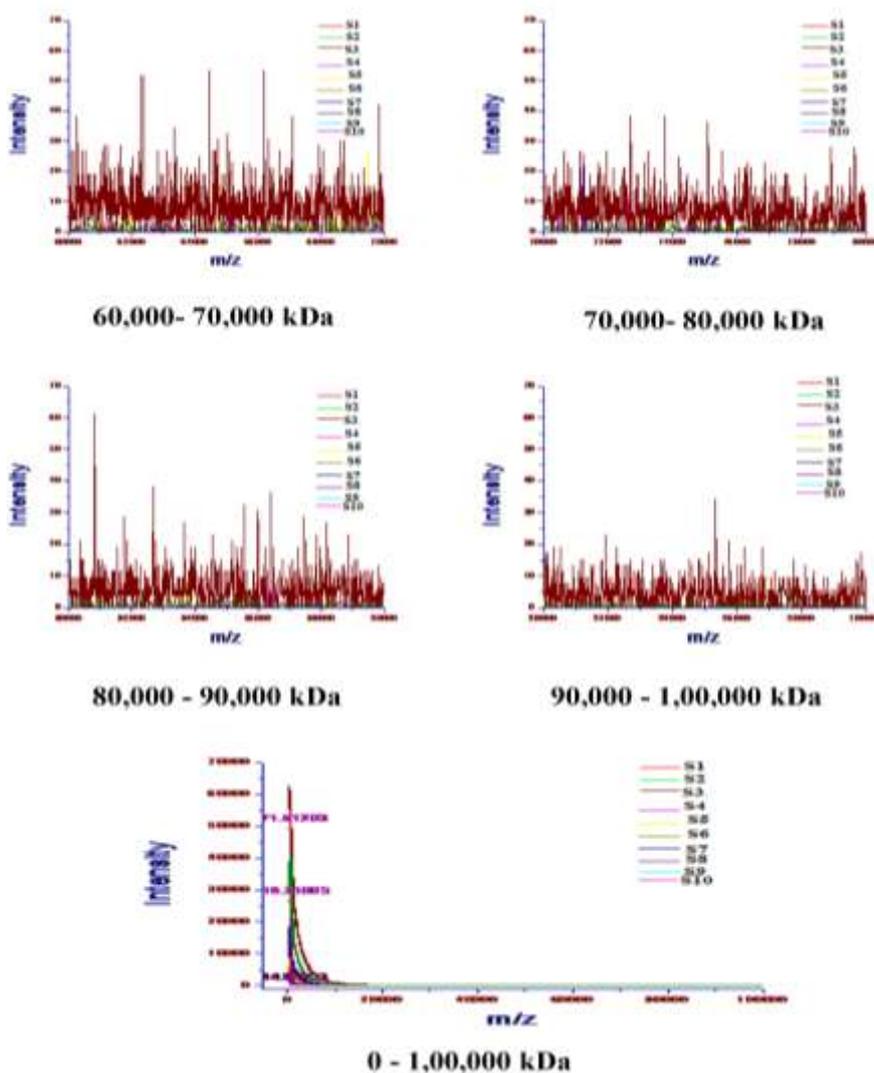
occurrence in *S. bryopteris*. The small branch $C_2N^1B_1b_1$ explained the similarity between *S. brachystachya* ($C_2N^1B_1b_1A$) and *S. radicata* ($C_2N^1B_1b_1B$). The small branch $C_2N^1B_1b_2$ displayed the restricted distribution of *S. repanda*. The clade C_2N^2 showed the distinct nature *S. delicatula*. The distinguished character of *S. tenera*, *S.*

invovens, *S. wightii*, *S. brachystachya*, *S. repanda* and *S. delicatula* is confirmed in the cladogram by the presence of unique *m/z* values (**Fig. 3**). These specific *m/z* values can be act as a biochemical marker to identify the specific *Selaginella* species in chemosystematics and pharmaceutical industries.



S1- *S. intermedia*; S2-*S. inaequalifolia*; S3-*S. involvens*; S4-*S. tenera*;
S5-*S. wightii*; S6- *S. brachystachya*; S7-*S. repanda*; S8-*S. radicata*;
S9-*S. bryopteris*; S10-*S. delicatula*.

Fig. 1: MALDI-TOF MS Spectrum of *Selaginella* species (0-60000 kDa)



S1- *S. intermedia*; S2-*S. inaequalifolia*; S3-*S. involvens*; S4-*S. tenera*;
 S5-*S. wightii*; S6- *S. brachystachya*; S7-*S. repanda*; S8-*S.radicata*;
 S9-*S. bryopteris*; S10-*S. delicatula*.

Fig. 2: MALDI-TOF MS Spectrum of Selaginella species (60000 – 100000 kDa)

Table 1: MALDI-TOF MS analysis of studied Selaginella species

S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
491	501	527	719	522	515	536	694	594	511
522	506	667	1020	594	536	555	11424	677	555
527	522	10045	10750	610	599	669	16000	694	599
636	625	10135	11061	667	694	694	16145	822	694
651	667	10190	11610	916	698	918	19545	11269	698
667	693	10335	13041	920	711	15937	20879	11487	876
688	833	10356	13549	921	798	16145	21460	11830	992
693	905	10362	13787	1165	3675	16238	21481	13280	2257
729	10356	10574	14887	1170	5325	16382	23596	15512	2588
895	10362	10615	20920	10149	10367	16725	24425	16145	9369
916	11874	11460	21065	10252	15678	16995	27785	16694	10554
920	12579	11466	21459	10278	16145	17710	31357	16974	10875
10360	12616	11874	21832	10398	16766	17897	33637	16995	11258
13200	13223	12331	22387	10424	17046	19898	37618	17202	11849
20111	13435	12336	22809	10563	18602	20299	42933	17472	11923

20112	14114	12616	23906	10589	21481	20610	43492	18405	12410
21729	15794	13435	25648	10817	23596	23036	43533	18892	12783
22062	19962	13611	26850	11025	31046	23596	47586	19473	14491
22081	20112	13871	27928	11522	31357	25379	50257	21294	14566
25815	20380	14114	28011	11652	31979	25628	51108	22393	15429
25835	21251	14316	28364	11668	32870	25649	91754	22870	15771
31189	21729	14814	29318	11895	34301	26582		23596	16145
31232	22435	20112	29815	12435	37162	28116		24052	16818
39193	23388	21749	30174	13829	38240	31357		24301	16995
54052	23887	21792	30754	13887	40092	31439		24985	17046
66271	23906	22062	31189	15794	41543	33824		27723	17181
68469	24455	22435	31624	18469	43492	34239		28116	17482
72579	25419	23906	32040	20380	44529	36561		29256	18405
91294	25815	24611	32910	21045	45919	36955		29692	19048
93699	26083	24776	32994	21066	48365	37972		30382	19136
93741	27121	27182	34134	21251	49589	42933		31357	20610
94052	27765	27888	34570	21832	61916	50133		32282	20651
96374	28157	27928	36103	21895	71936	53637		32517	20879
96685	29775	28054	36498	22062	72435	54031		34798	21066
96748	29796	28115	38841	22081	72953	54301		38240	21771
96955	31543	28281	39982	22413	73181	57773		40092	22268
	32579	29796	40796	22435	74322	58013		40506	23761
	33011	29815	41438	23802	77183	59941		42538	25649
	34537	30071	42247	25835	79775	61916		43471	26209
	40837	32742	45087	39193	81428	61979		46312	26707
	40859	33233	45835	39215	81801	64715		54301	29423
	41314	34735	48364	96955	84786	69485		62808	32062
	41688	36103	49234		86737	71066		66250	33326
	43057	36167	50339		89640	75886		69381	33575
	47080	37059	51002		91090	83709		70962	33637
	48260	37182	51272		91754	87110		72601	36561
	48261	37888	51956		92003	88147		74591	38635
	49464	39610	54217		95237	92003		75337	41460
	53264	40257	55212		95279	95528		78770	42890
	54052	40286	55897		97436	99939		86473	42933
	58824	46602	56239		98389			86488	45358
	59179	47370	57390		99633			88685	50962
	66416	47390	58157					92400	51190
	66912	54154	60256					95279	51895
	66913	54177	62310						59672
	67661	57173	64445						62284
	68261	57742	66208						63450
	73368	60984	68633						65379
	73866	62103	69877						70382
	74135	63347	70629						74715
	77723	63512	71127						77411
	79173	65472	72662						79235
	80299	67121	73781						80224
	84446	67370	75067						82341
	84840	69340	75503						83045
	87245	71418	78903						85159
	90610	74612	79630						88187
	92435	75234	80816						90799
	93699	76539	82683						94366
	95025	76561	85523						
	95047	79091	85979						
	95370	84465	86394						
	96748	84466	87482						

		87722	88198						
		87743	88903						
		90610	91956						
		91709	95316						
		91810							
		96748							
		96955							
		97265							
		97687							
		99152							
		99215							

Note: S1 - *S. intermedia*, S2 - *S. inaequalifolia*, S3 - *S. involvens*, S4 - *S. tenera*, S5 - *S. wightii*, S6 - *S. brachystachya*, S7 - *S. repanda*, S8 - *S. radicata*, S9 - *S. bryopteris*, S10 - *S. delicatula*

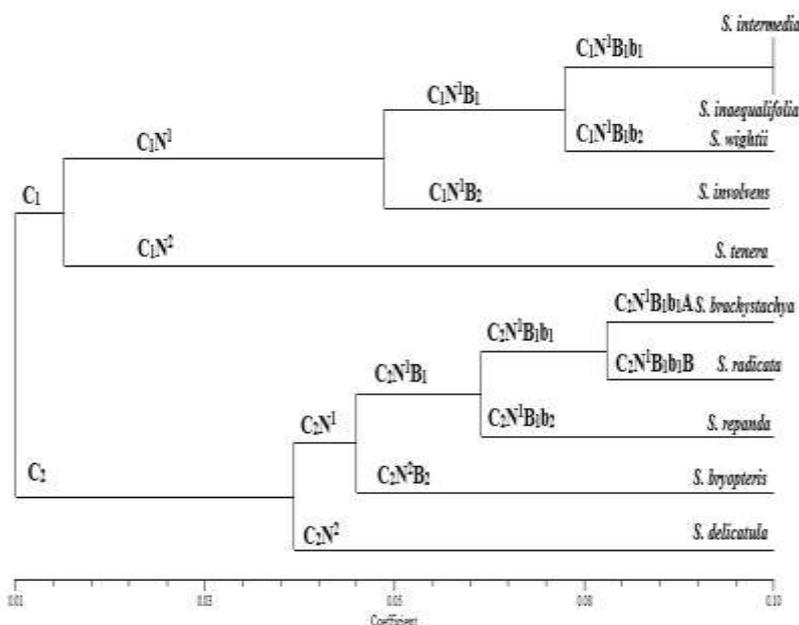


Fig. 3: Cladogram of Selaginella species based on MALDI-TOF MS analysis

DISCUSSION

MALDI profiling offers exceptionally high-throughput and has therefore become an attractive tool for the discovery and validation of biomarkers. In addition, the method must be sufficiently precise to observe relative differences between distinct populations. Profiling has now been applied in thousands of studies includes the quantification of amino acids, lipids, natural products, drugs, polymers, herbicides, metabolites, toxins, oligonucleotides, carbohydrates, peptides and proteins (Duncan *et al.*, 2008). Analyzing proteins with MALDI-TOF MS has several advantages. MALDI-TOF MS is fast; ionization, separation by size and detection of proteins takes milliseconds to complete. By contrast, conventional electrophoretic methods for separating and detecting proteins can take hours to complete. The results are absolute, being based on the intrinsic property of the mass-to-charge ratio (*m/z*). This is inherently more accurate than electrophoresis-based or hybridization array based methods, which are both susceptible to complications from secondary-structure formation in proteins (Park, 2004). Furthermore, protein biomarker development is a complex and challenging task. The

criteria and approach applied for developing each individual biomarker can vary, depending on the purpose of the biomarker and the performance requirement for its application which is now possible by MALDI-TOF MS (Hartwell *et al.*, 2006).

MALDI-TOF MS analysis in proteomic research is advanced in animals, bacteria and fungi (Kersten *et al.*, 2002). In plants, MALDI-TOF MS was carried out to reveal the protein profile of spanish (Yamaguchi and Subramanian, 2000). Castillejo *et al.*, (2008) used MALDI-TOF analysis to differentiate sunflower genotypes corresponded to phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase. Two-dimensional gel electrophoresis (2-DE) coupled with MALDI-TOF MS/MS analysis has been used to identify differentially expressed proteins and 63 spots have been identified successfully (Datta *et al.*, 2013). They compared the proteomic profiling of SA treated leaves versus control leaves demonstrated the changes of many defence related proteins like pathogenesis related protein 10a (PR10a), disease-resistance-like protein, putative late blight-resistance protein, WRKY4, MYB4, etc (Datta *et*

al., 2013). Zhao and Cheng, (2012) studied the comparative proteins profile of the effects of nitric oxide on alleviating Cd induced toxicity in rice. Koller et al., (2002) studied the proteomic survey of metabolic pathways. Kumar and Kumari, (2009) used the MALDI-TOF analysis to distinguish the mother plants and in vitro grown plants of *Artemisia vulgaris*. Kamal et al., (2010) studied the functional proteome analysis of wheat and classified the abiotic stress responsive proteins of wheat. Hong and Nose, (2012) investigated and identified mitochondrial protein expression between two CAM species *Ananas cosmos* and *Kalanchoe pinnata* using two-dimensional electrophoresis (2D-GE), coupled with matrix-assisted desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Lee et al., (2013) explained the importance of MALDI-TOF analysis plant physiology and molecular biology.

In the present study, MALDI-TOF MS analysis of ten *Selaginella* species collected from different localities of South India expressed different ion peaks ranged from 0-1,00,000 kDa. The results showed totally 556 spectral peak values (m/z values) and of which, 139 spectral peaks explained the similarities of the studied *Selaginella* species and the remaining unique peaks shows the variations among the studied medicinally important *Selaginella* species.

CONCLUSION

MALDI-TOF MS are fit for their intended purpose and take advantage of the unique feature of MALDI ionization, especially its exceptional throughput when it is combined with TOF analysis. In the present study, the expression of related and unique spectral peaks of MALDI-TOF MS analysis acted as a spectroscopic tool to distinguish the genetic similarities and variations among the studied *Selaginella* species.

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