The study of functional groups and matrix optimization for MALDI-TOF analysis of proteins in *Jatropha curcas* L. latex

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Abstract

Jatropha curcas L., a drought resistant plant has been classified into two varieties based on the presence of phorbol ester including toxic and non-toxic varieties. The plant contains latex (produced by laticifers) where complex mixtures of terpenoids, phenolics, proteins and alkaloids are localized. Several secondary metabolites in latex have been informed to have medicinal properties. However, functional group and protein fingerprint are not well defined in toxic and non-toxic *J. curcas* L. latex. The study aims to determine functional group using Fourier Transform Infrared (FTIR), and also optimize matrix for protein analysis in those two varieties by MALDI-TOF MS. The results from FTIR analysis of latex extracted from toxic and non-toxic *J. curcas* trees showed that the pattern of the infrared (IR) spectra of toxic was similar to non-toxic, and the functional group of those two varieties was nearly identical to cis-1,4-polyisoprene references. Proteins characterization using MALDI-TOF MS incorporated PCA software revealed that peptides patterns of the toxic were different from non-toxic latex proteins. Comparison of matrixes revealed that SA matrix gave the best result for protein homogeneity compared to CHCA and DHBA matrixes, which can be considered as a suitable matrix for protein identification and differentiation in Jatropha latex. These results provided novel insight into latex components and will benefit the understanding of latex proteins in those two varieties.

Keywords: *Jatropha curcas*, latex, Fourier Transform Infrared (FTIR), MALDI-TOF MS, Sinapinic acid (SA) matrix

Introduction

Jatropha curcas Linn. (hereafter referred to Jatropha) is a member of the family Euphorbiaceae (Ovando-Medina et al., 2011). Two varieties were found based on the presence of phorbol ester including toxic and non-toxic varieties (Makkar et al., 1998). The plant exudes milky fluid upon damaged tissues, known as latex (Konno, 2011). The plant latex is a natural plant polymer, which composed of 320 to 35,000 isoprene molecules (Kang et al., 2000), and it is stored in specialized cells called laticifer (Upadhyay, 2011). Latex lean to be phytochemically diverse that contains complex mixtures of terpenoids, phenolics, proteins and alkaloids (Agrawal and Konno, 2009). Although, all parts of the plant have been used for medicinal purposes (Duke, 1985; Gubitz et al., 1999), it is toxic to humans and animals also. Comprehensive literature studied on the plant latex revealed that latex contains wide diversities of defense chemicals and defense proteins (Konno, 2011), which displayed different biological activities (Kitajima et al., 2011). Moreover, some compound with wound-healing properties was reportedly found in latex (Gubitz et al., 1999). Although, the plant latex has been extensively studied but functional groups or protein fingerprint of Jatropha latex have not been well documented. In the present work, we attempted to analyze and compare functional groups using FTIR and also study proteins by optimizing MALDI-TOF

matrix, in toxic and non-toxic Jatropha latex.

Methodology

Extraction of Jatropha latex for FTIR analysis

The latex was collected directly from 3-5 year old non-toxic (Mexico accession) and toxic (Chiang Mai accession) *J.curcas* trees in 1.5 mL microcentrifuge tubes. The latex was extracted from Jatropha tree by acetone-benzene extraction method as described elsewhere (Stipanovic et al., 1980; Ji et al., 1993; Kang et al., 2000). The latex samples were centrifuged at 12,000 rpm to remove particles. The supernatant was transferred into a new 1.5 mL microcentrifuge tube, and then extracted using acetone in the ratio of 1:1 and homogenized. The homogenized sample was centrifuged at 6,000 rpm for 10 min and the acetone supernatant was discarded. The pellet was extracted once more with acetone. The pellet was then extracted by homogenizing it with followed by centrifugation at 7,000 rpm for 10 min (repeat twice). After centrifugation, the benzene fraction was subjected to heat box (50°C) to remove the solvent.

FTIR spectroscopy

The dried latex extract was mixed with powdered potassium bromide for preparing the pressed discs. The mixture was ground thoroughly in a smooth agate mortar and pressed under a pressure of 10,000 to 15,000 psi to make a thin film. IR spectra were collected in the mid region from 4,000 to 370 cm^{-1}, at a resolution of 2 cm^{-1} over 4 averaged scans, using a FTIR spectrometer (PerkinElmer).

Extraction of protein from Jatropha latex for MALDI-TOF MS analysis

The latex was collected directly from 3-5 year old non-toxic (Mexico accession) and toxic (Chiang Mai accession) *J.curcas* trees in 1.5 mL microcentrifuge tubes. The latex samples were centrifuged at 12,000 rpm to remove particles. The supernatant was transferred into a new 1.5mL microcentrifuge tube, and then precipitated using acetone in the ratio of 2:1. The precipitates were harvested by centrifugation at 12,000 rpm for 10 min. After centrifugation, the supernatant was discarded and the excessive amount of acetone was removed by evaporation. The precipitate was dissolved in 0.5% SDS and assayed for protein concentration.

Determination of protein concentration by Lowry's method

Protein concentration (obtained from acetone precipitation) was determined by Lowry's method (Lowry et al., 1951). Protein concentration was measured at 750 nm using the Lowry's method and bovine serum albumin was used as the standard protein.

Matrix optimization for MALDI-TOF MS analysis of proteins in toxic and non-toxic Jatropha latex

Protein samples were prepared for MALDI-TOF MS analysis, 0.1 ug/uL of protein samples were prepared and also 10 mg of matrix mix with 1 mL of 0.1% Trifluoroacetic acid (TFA) contain 50% acetonitril (ratio 1:1). Then, the protein samples were mixed with matrix solution ratio 1:1 on MTP AnchorChip 600/384 (MALDI plate, Bruker GmbH) as dried droplet method. 2,5-dihydroxybenzoic acid (DHBA) and α-cyano-4 hydroxycinnamic acid (CHCA) were used as matrixes. DHBA and CHCA were compared to the sinapinic acid (SA) matrix, from the previous study (Gruneck et al., 2012). The samples were analyzed by using Ultraflex III TOF/TOF (Bruker, GmbH).

MALDI-TOF/TOF data analysis
The peptide mass was prepared by smooth subtract (mass of peptides will be started at zero), and the mass was listed to show the peptide pattern. Flex analysis 3.0 (Build 92) was the software that used for data analysis. The peptides were categorized by using ClinProTools software 2.2 (Build 78) and grouped by using Principle Component Analysis (PCA) (feature of ClinProTools) to show homogeneity of peptide in different matrix.

Results

Fourier Transform Infrared (FTIR) analysis.

Latex was extracted from toxic and non-toxic Jatropha trees with acetone and benzene, and the benzene extracts were analyzed by FTIR. The IR spectra revealed that the functional group of toxic was similar to non-toxic (Figure 1), and nearly identical to *cis*-1,4-polyisoprene references (rubber extracted from *Euphorbia characias* latex) with the absorption ranges of 1664 and 835 cm^{-1} (C=C stretching and C-H bending respectively).

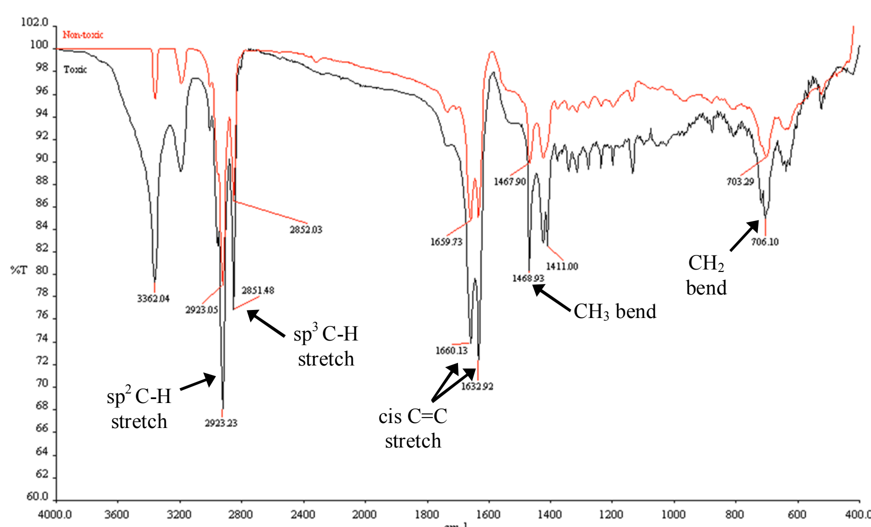

Figure 1: FTIR spectra of the latex extracted from toxic (black spectra) and non-toxic (red spectra) *J. curcas* trees in potassium bromide disc. Four scans were summed in the spectral range of 4,000 to 370 cm^{-1} at a resolution of 2 cm^{-1}.

From the IR spectrum of latex showed that the functional groups were C-H stretch of an aliphatic compound, where we observe the asymmetric C-H stretch of the methyl group (2923 cm^{-1}) occurring at higher frequency than symmetric vibration (2852 cm^{-1} for methylene). Akenyl C=C stretch of an olefinic group was found at 1660 cm^{-1}, which links as the backbone, producing the skeletal vibrations. C-H bend of the aliphatic compound was detected in the fingerprint region (1468 cm-1 for methylene). C-H bend of alkene was observed at the absorption range of 706 cm^{-1} which indicating of a *cis* isomer. The latex from *J. curcas* showed the FTIR band characteristic of *cis*-1,4 polyisoprene at 1660 cm^{-1} and 706 cm^{-1} which were due to C=C stretching and C-H bending, respectively.

Table 1 The band frequencies of various functional groups in toxic and non-toxic Jatropha latex

Functional groups	Frequency (cm^{-1})		
	Toxic (n=4)	Non-toxic (n=4)	*P*-value
CH_2 asymmetric stretching	2922.97 ± 0.08	2923.26 ± 0.23	0.730
CH_3 symmetric stretching	2851.96 ± 0.46	2852.15 ± 0.41	0.773
C=C stretching (alkene)	1667.13 ± 0.20	1659.87 ± 0.37	0.393
C=C stretching (alkene)	1633.00 ± 0.34	1633.40 ± 1.25	0.480
CH_3 bending	1466.49 ± 0.80	1467.30 ± 1.06	0.121
CH_3 bending (cis)	1423.69 ± 1.62	1423.30 ± 0.18	0.670
CH_2 bending (cis)	703.74 ± 1.02	704.93 ± 1.07	0.242

*Significant level at 0.05 ($P < 0.05$)

Toxic and non-toxic Jatropha latex had a very similar FTIR spectrum (Figure 1), indicating that those two varieties contain similar functional groups. The average spectra in the region of 400-4,000 cm^{-1} illustrated that non-toxic latex have a higher frequency of the CH_2 asymmetric stretching at 2923 cm^{-1}, CH_3 bending at 1467 cm^{-1} and CH_2 bending at 705 cm^{-1} than those in toxic latex. In contrast, the frequency of C=C stretching at 1660 cm^{-1} is lower than that of toxic latex. However, there was no significant difference between the frequency values of the four main functional groups of the two varieties. As the pattern of the infrared (IR) spectra of toxic and non-toxic were not different, we further analyzed proteins or peptides in latex using MALDI-TOF MS.

Sample preparation and protein concentration

To further investigated protein fingerprint in latex, the crude latex of Jatropha was collected from 5 years of toxic and non-toxic *J. curcas* germplasm, which located at Mae Fah Luang University. The latex samples were precipitated by acetone in ratio of 2:1 (three replicates for toxic non-toxic). and protein concentration of which precipitated by acetone were determined by Lowry’s method, absorbance at 750nm. Protein concentration of toxic was 4.5 µg/µL and non-toxic was 8 µg/µL. Total protein concentration in toxic latex was 2.25 mg and non-toxic latex was 4 mg. High amount of protein were founded in non-toxic than toxic latex

Matrix optimization for MALDI-TOF MS analysis

To investigate the proteins in toxic and non-toxic latex, protein samples extracted from latex were prepared by mixing with two matrixes including CHCA and DHBA, in order to compare between different matrixes. Peptide pattern was observed in those two varities and the ranges of proteins detected were between 600-6000 Da (MALDI-TOF MS parameter). The protein samples which dissolved in 0.5% SDS were analyzed by MALDI-TOF MS using two different matrices including CHCA (α-cyano-4-hydroxycinnamic acid) and DHBA (2,5-dihydroxybenzoic acid) and also compared to SA (sinapinic acid) from the previous study (Gruneck et al., 2012).

CHCA (α-cyano-4-hydroxycinnamic acid) (Hoyer et al., 2007)	DHBA (2,5-dihydroxybenzoic acid) (Montaudo et al., 2006)	SA (Sinapinic acid) (Hoyer et al., 2007)

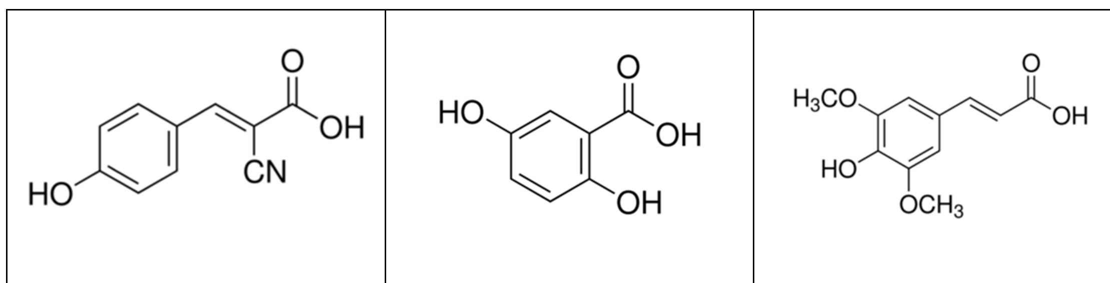

Figure 2: Chemical structure of CHCA (α-cyano-4-hydroxycinnamic acid), DHBA (2,5-dihydroxybenzoic acid) and SA (sinapinic acid).

Comparing CHCA and DHBA with SA (reference) matrices, all of three matrices gave a very similar peptide pattern both in toxic and non-toxic varieties (Figure 3). From the signal of peptides could not show which matrix was the best for homogeneity of protein samples.

	Jatropha curcas varieties	
Matrices	Non-toxic	Toxic
CHCA (α-cyano-4-hydroxycinnamic acid)	1390.054 1083.857 1257.966 1654.108 A1	1084.986 A2
DHBA (2,5-dihydroxybenzoic acid)	1083.629 1367.806 1220.711 B1	1084.961 B2
SA (Sinapinic acid) (Gruneck et al., 2012)	1367.799 1083.680 1140.234 1235.671 1654.002 C1	1154.324 1252.149 C2

Figure 3: Peptide patterns analyzed by different matrices including; CHCA (α-cyano-4-hydroxycinnamic acid), DHBA (2,5-dihydroxybenzoic acid) and SA (Sinapinic acid) (Gruneck et al., 2012), showed similar peptide pattern both in toxic (A2, B2 and C2) and non-toxic (A1, B1 and C1). However, the signal (peaks) presented the difference between peptide patterns of toxic and non-toxic varieties.

Using principle component analysis (PCA) could help to show the homogeneity of protein in different matrices. Since it is a multivariate method (statistical method) that used for differentiation, the spectra (each sample) with similar characteristics can be clustered together and the differences between samples can be distinguished (Cho et al., 2012). Figure 4 represented plots of the PCA results used for differentiating mass spectra (A1, B1 and C1) from toxic and non-toxic latex. The size of peptides both in toxic and non-toxic latex was less than 1,500 Da.

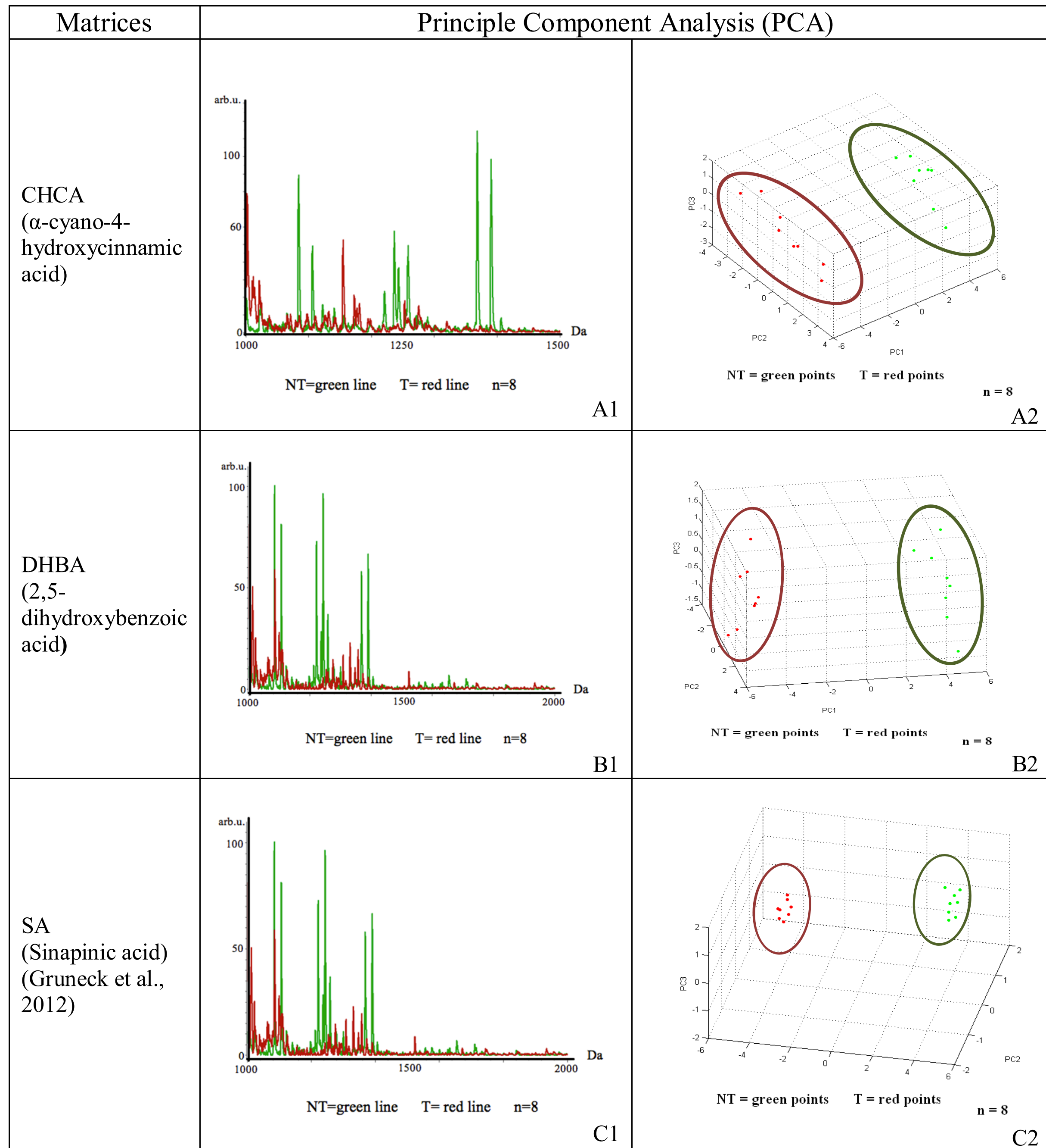

Figure 4: Principle component analysis (PCA) showed differentiation of peptides derived from toxic and non-toxic varieties and homogeneity of peptides analyzed by different

matrices; (A2) CHCA (α-cyano-4-hydroxycinnamic acid), (B2) DHBA (2,5-dihydroxybenzoic acid) and (C2) SA (sinapinic acid) (reference). Protein samples were spotted 8 replications (green color represented non-toxic and red color represented toxic).

Figure 4 showed that peptides derived from toxic and non-toxic were different since red and green spots presented in different position of PCA plots (A2, B2 and C2) and there was no overlapping peptide. When considering the distribution area of the cluster of the spots on PCA plots, the model illustrated that SA matrix (reference) gave the best result, which the distribution area of spots on SA matrix (reference) were less than CHCA and DHBA, indicating that protein samples prepared with SA were more homogeneous than those prepared with CHCA and DHBA matrixes.

Discussion

FTIR anlaysis of toxic and non-toxic Jatropha latex.

FTIR spectrum of toxic and non-toxic latex were similar and almost identical to that of *cis*-1,4 polyisoprene references (Kang et al., 2000; Spano et al., 2012). The results from FTIR analysis indicated that Jatropha latex contains a natural rubber which the structure of this rubber can be identified as *cis*-1,4 polyisoprene (Spano et al., 2012). Moreover, functional groups were studied in two different species of medical plants (*Tephrosia tinctoria and Atylosia albicans*) and the results indicated that both *T. tinctoria* and *A. albicans* are rich in phenolic compounds, and also contain different biomolecule concentration in different plant parts (Kumar and Prasad, 2011). The results suggested that FTIR could help to identify the functional groups in plants of the same species and/or different species, and also concentration of the components based on the intensity of the absorption (Simonescu, 2012).

Matrix optimization for MALDI-TOF MS analysis of proteins in toxic and non-toxic Jatropha latex.

Protein precipitation with acetone showed that protein content in non-toxic was approximately 2 times higher than that of toxic latex. Although, high protein yield was founded in non-toxic latex, the previous reports have illustrated that latex of milkweeds, lettuce and papaya contains higher concentration (50-1000 times) of defense chemicals or protein than those found in leaves (Seiber et al., 1982; Sessa et al., 2000; Konno et al., 2004; Agrawal and Konno, 2009). This suggested that the two varieties might contain diverse defensive chemicals and various types of proteins in latex. The proteins obtained from acetone extraction were then analyzed by MALDI-TOF MS. The results achieved by MALDI-TOF MS were compared with that obtained from the previous work. For matrices comparison, CHCA and DHBA showed similar peptide pattern and was not different from SA matrix (Gruneck et al., 2012). From the principle component analysis (PCA), the difference of signals would indicate different peptide presented in two types of Jatropha latex. The sizes of peptides present in toxic and non-toxic latex were less than 1,500 Da. Although, in this study, CHCA and DHBA were uses as matrices for protein analysis, but SA gave a better result for protein homogeneity than those two matrices, from the cluster of spots (Gruneck et al., 2012). Since the chemical structure of SA contains both hydroxyl OH (hydrophilic group) and methoxy OCH_3 (hydrophobic group), which are able to combine with hydrophilic and hydrophobic peptides respectively, while those CHCA and DHBA, contain hydroxyl groups that sensitize to hydrophilic peptides (as shown in Figure 2). These unique spots (SA matrix) for each variety offer a strong proof in differentiating the two

varieties (toxic and non-toxic) of Jatropha. Nevertheless various studies (the comparison of different matrix) indicated that SA is a good matrix for peptide mass above 2.5 kDa (Lewis et al., 2000), CHCA, is a suitable matrix for peptides with mass ions below 2.5 kDa (Cohen and Chait, 1996; Jensen et al., 1997; Gonnet et al., 2003) and DHBA is recommended for glycopeptides, glycoproteins, small proteins, and oligonucleotides less than 10 bases (Lewis et al., 2000).

Conclusion

In this report, we determine the functional group and compare protein fingerprint in toxic latex to that in non-toxic latex. The obtained results illustrated that FTIR spectra revealed the same functional groups in toxic and non-toxic latex whereas MALDI-TOF MS incorporated PCA software could differentiate those two varieties (differences in the protein fingerprint). The results suggested that SA matrix (Gruneck et al., 2012) was the best matrix for protein homogeneity, which was able to distinguish proteins between toxic and non-toxic Jatropha latex compared to CHCA and DHBA matrixes. To our knowledge, it is the first record on the functional groups and protein fingerprint of toxic and non-toxic Jatropha latex. Accordingly, SA can be considered as a suitable matrix for protein identification and differentiation in Jatropha latex. From the differences in protein fingerprint between toxic and non-toxic latex could possibly be used for biomarker development or breeding to obtain varieties with desirable traits.

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