Metabolomic characterization of plant exudates and their correlation with plant defense systems

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Chapter 6

Chemical differentiation of plant latex and their anti-herbivory activity against thrips

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ABSTRACT

Latexes possess a variety of tissue-specific metabolites that differentiate them from other plant tissues. From this, two intriguing questions arise: Why latexes developed their chemical specialization? And what factors influence this specialization? To tackle these questions, latexes and their bearing tissues from diverse species were profiled by ^{1}H NMR-based metabolomics and GC-MS target analysis. To investigate anti-herbivory roles, the extracts were tested against thrips (*Frankliniella occidentalis*). The multivariate data analysis showed clear separation between latexes and bearing tissues. There was higher similarity between latexes from different species, that is, tissues effects are stronger than species effects in latex metabolomes. This differentiation was reflected on latexes' higher mortality index (80.40 % ± 7.5) against thrips when compared with their bearing tissues (55.5 % ± 14.9). The metabolites correlated to the anti-herbivore activity of latexes are triterpenoids and steroids. However, the activity of latexes could not be attributed to any of them when individually tested against thrips. This discrepancy and the reduction of the latex activity after fractionation suggested a synergistic effect of the compound mixture. In addition to terpenoids, many latexes show high concentrations of certain organic acids, even some new acids were identified. These metabolites were proved to play a modulatory role in the latex coagulation, which could be part of the latex-borne defense. This study showed the potential role of latex specialized metabolites to enhance the plant mechanical defense. Thus, latexes are an example of how plants handle an unlimited number of enemies using a limited number of metabolites.

Key words: Latex; triterpenes; steroids; organic acids; multifunctional; constitutive defense.

Chapter 6

1. Introduction

Plant latexes are generally milky saps stored in specialized cells called laticifers. They are immediately exuded after herbivory or mechanical wounding at damaged points (Konno, 2011). Its occurrence seems to be a selective adaptation from plant families that have been especially highly exposed to herbivory (Agrawal and Konno, 2009; Konno, 2011). Moreover, their distribution in the plant kingdom correspond to 8 - 10 % of all angiosperms and landing plants (Agrawal et al., 2008; Agrawal and Konno, 2009; Konno, 2011; Huber et al., 2015). Some plant families evolved very chemo-specific latex compositions such as Asclepiadaceae, Sapotaceae, Apocynaceae and Euphorbiaceae (Ramos et al., 2010).

Their physical and chemical characteristics confer them two distinguished mechanisms, mechanical and biochemical defenses via coagulation, toxicity or anti-feeding effects (Bauer et al., 2014; Konno, 2011). These characters are provided by three major fractions, which include proteins, rubber, and a wide range of small molecules. The small molecules, so-called metabolites, determine most of the chemical diversity of latexes through plant species. These complex mixtures include terpenes, alkaloids, cardenolides, coumarins, and non-protein amino acids (Agrawal and Konno, 2009; Pandey, 2001; Taira et al., 2005). It is generally assumed that these chemicals are mainly associated to anti-herbivores defense. As example, the local concentrations of specialized metabolites increase, even within minutes, specifically at points of herbivory damage (Seiber et al., 1982; Groeneveld et al., 1990; Groeneveld et al., 1991; Sessa et al., 2000; Konno et al., 2004; Konno et al., 2006). Moreover, it has been hypothetically proposed that latexes could be a reservoir of species-specific bioactive metabolites like phytoalexins (Chezem and Clay, 2016).

Besides the species-specific defense involving specialized metabolites, latexes also possess a non-species-specific defense mechanism, which involves constitutive particles like rubber. This procces is known as coagulation, and it is present in the latex-borne defense regardless of the latex bearing species. Furthermore, a certain number of other latex metabolites common through species could suggest that latexes might conserve chemical

features associated with basic functions during plant defense. Consequently, the chemical variation of constitutive latexes would be less determined by environmental factors. On the other hand, chemical differences over induced latexes might be more affected by environmental factors, for example, during plant-specialist herbivore interactions across geographical regions.

Although the studies about the roles of induced specialized metabolites in latexes are increasing, the general roles of latexes given by their constitutive metabolites still remain unclear. If any, the sealing and trapping effects from rubber after coagulation. Therefore, to uncover possible roles of common- or constitutive latex metabolites, a multi-species chemical profile comparison of latexes is required. Profiling overall chemical variation of the latexes from diverse sources, instead of a targeted analysis would be the first approach to uncover their metabolic features. This differentiation should also be reflected on a well distinguished biological activity degree to that of their bearing tissues.

Among numerous profiling techniques, nuclear magnetic resonance (NMR)- and mass spectrometry (MS)-based methods are the most popular platforms considering the essential requirements of analytical platforms such as identification capability, reproducibility and/or sensitivity (Nicholson et al., 2002; Ellis et al., 2012; Kim et al., 2010; Gromski et al., 2015; Markley et al., 2017). In this study, twelve different plant species were collected to get their latexes and leaves. Two analytical platforms, ^{1}H NMR for general metabolome discrimination, and GC-MS for target analysis were employed in a first stage. After the chemical characterization latexes and their bearing tissues were compared by multivariate data analysis. Then, the biological activities of the collected tissues were measured and compared with each other to deduce the roles of latex metabolites. For this, a generalist herbivore, the western flower thrips (*Frankliniella occidentalis*), was selected because of its reliability as a model for testing anti-herbivory properties of plant metabolites (Kirk and Terry, 2003; Liu et al., 2017).

Based on the chemical and biological data of latexes from this study, the types of conserved constitutive latex metabolites through species were identified. Moreover, the possible roles of such metabolites associated to anti-herbivory effects were deduced. Aditionally, the chemical effects of

some metabolites were correlated for the first time to the enhancement of the mechanical character of the latex-borne defense, which could be associated to a primary plant defense response.

2. Results and discussion

Proton nuclear magnetic resonace (^{1}H NMR) was used as a macro-scale metabolic profiling tool to determine the chemical classes specifically accumulated in the latexes. To accomplish the comparison, 12 plant species were collected to get their latexes (9 rubbery latexes and 3 clear latexes) and leaves. The visual inspection of the ^{1}H NMR spectra of the MeOH extracts measured in CH_3OH-d_4 indicated that in general the level of terpenes was much higher in latexes rather than in their bearing tissues. This was confirmed by the comparing characteristic terpenoidal methyl resonances in the range δ 0.7 – δ 1.8. In addition to these methyl signals, some latexes showed two more distinguished resonances at δ 0.35 (d, J = 4.1 Hz) and δ 0.55 (d, J = 4.1 Hz), which were assigned to the H-19*endo* and H-19*exo* in the cyclopropane ring of 24-methylenecycloartanol. Thus, based on the visual inspection of the ^{1}H NMR spectra, latex metabolomes were clearly distinguished by their high concentration of triterpenes while fatty acids, phenolic and sugars were found to be more accumulated in leaves, and their variation was different through species.

The ^{1}H NMR dataset was further analyzed by multivariate data analysis (MVDA). The principal component analysis (PCA) of all of the methanol extracts showed a separation in two clusters along the PC1 (tissue) (Fig. 1A). Interestingly, most of the latexes obtained from the diverse species were well clustered although they were collected from completely difference species. The metabolic variation of latexes was much smaller than the one of leaves. This was more evident in rubbery latexes such as *A. scholaris*, *A. oblonguifolia*, *Allamanda* spp., *D. bengaliensis*, *P. rubra*, *E. myrsinites*, *E. tirucalli*, *C. rosea*, and *F. triangularis*. This tight clustering might be caused by conserved constitutive metabolites of latexes such as triterpenoids. These metabolites were observed in the visual inspection of the ^{1}H NMR analysis and confirmed by the PCA loading plot. The further analysis of the data set

by orthogonal projection to latent structures-discriminant analysis (OPLS-DA) corroborated (Q^2 = 0.86, p < 0.01) the differentiation between latexes and leaves due to terpnoids like compounds (Fig. 1B). To further confirm and scrutinize the terpenoidal and other metabolic groups of both tissues, the collected samples were liquid-liquid partitioned to yield dichloromethane, ethyl acetate and water fractions. Each fraction was measured by ^{1}H NMR and analyzed by orthogonal partial least square-discriminant analysis (OPLS-DA) with two classes: latex and leaves. Most of the terpenoids possess a lipophilic character, thus the dichloromethane fraction was firstly analyzed. Its model was highly validated with a Q^2 = 0.85 in the permutation test, and p < 0.01 in the CV-ANOVA test (Fig. 1C).

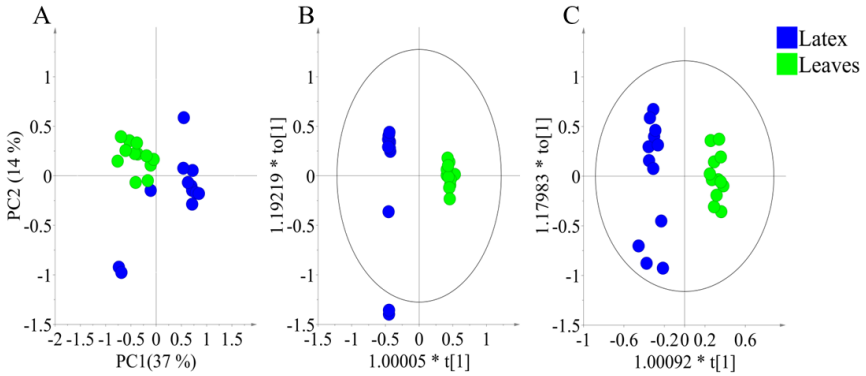

Fig. 1. Multivariate data analysis of the methanol extracts of latexes and leaves from 12 plant species. (A) Principal component analysis (PCA) of the methanol extracts of latexes and leaves. (B) Orthogonal partial least square discriminant analysis (OPLS-DA) of the methanol extracts of latexes and leaves. (C) OPLS-DA model of the dichloromethane fractions of latexes and leaves.

From the S-plot of the OPLS-DA model, the chemical shifts associated to leaves were determined to be in the range of fatty acids. These metabolites included palmitic, stearic, linoleic and oleic acids, which were further confirmed by GC-MS analysis. The higher concentration of fatty acids in leaves might be associated with a specific defense mechanism of this tissue. These acids can function not only as membrane components but as defensive molecules after plant damage. After hydroperoxidation, fatty acids turn into fatty acid hydroperoxides that can be further degraded to highly reactive

epoxides, aldehydes, γ-ketols or even reactive oxygen species (Maffei et al. 2007; Bruinsma et al. 2009). These chemical species cause protein agglutination and chemical modifications of essential amino acids, which results in their deficient assimilation by herbivores (Maffei et al. 2007). Additionally, the end products of peroxidized lipids confer antixenosis or antibiosis effects against herbivores (Hare 2011; War et al. 2012). These characteristics could favour the location and accumulation of fatty acids in leaves as the most external layer of plant defense.

On the other hand, the loadings associated to latexes were two singlets in the range of δ 1.8 – δ 2.0 assigned to acetyl protons from lupeol acetate, cycloartenol acetate and *β*-amyrin acetate. Moreover, other methyl signals of terpenes in δ 0.8 – δ 1.0 were also associated to latexes. The identification ofthese resonances was further determined by GC-MS analysis. Differently from leaves, latexes were supposed to play a role as one of the first barriers to protect plants from pathogens and herbivores based on a generalist mechanism. As shown in chapter IV, latexes play a basic role in plant defense by broad and general physical and chemical retardation of natural enemies, which consequently provide time to the plant to set up a sophisticated defense system based on induced metabolites.

These results were in line with the previous chapter finding as the common metabolite class in latexes triterpenoids. However, there were also species-specific terpenoids in latexes. Some characteristic signals that were assigned in the dichloromethane fractions of the latex of *Akokanthera oblonguifolia* were methyl protons of cycloartenol and *β*-amyrin resonating in the range δ 0.6 - δ 1.6. Moreover, two triplets at δ 5.04 and δ 5.28 were assigned to the H-24 and H-12 of cycloartenol and *β*-amyrin respectively. Two typical double doublets at δ 3.06 (*J* = 11.4, 4.4 Hz,) and δ 3.15 (*J* = 11.4, 4.4 Hz) were assigned to the H-3 also from these triterpenoids. In the dichloromethane fraction of *Alstonia scholaris* and *Dischidia bengaliensis*, diagnostic signals for protons in the side chain at the C19 of lupeol acetate were detected as two broad singlets at δ 4.66 and δ 4.59 corresponding to H-29a and H-29b, respectively. These resonances were complemented by a singlet at δ 1.62 assigned to H-30 in the same side chain. Aditionally, a

resonance at δ 3.15 (dd, *J* = 11.4, 4.4 Hz) corresponded to H-3 next to an ester bond with an acetyl group, which was observed as a singlet between δ 1.95 - δ 1.97.

Furthermore, in the latex of *A. scholaris*, typical signals for cycloeucalenyl acetate were detected. Two extremely up-field shifted doublets at δ 0.11 (d, *J* = 4.0 Hz) and δ 0.35 (d, *J* = 4.0 Hz) were assigned to the H-19b and H-19a in the cyclopropane ring of the triterpenoid, respectively. The acetyl protons were detected as a singlet at the range δ 1.95 – δ 1.97 together with other acethyl signals. Also, in the latex of this species, a triplet at δ 5.13 (H-12, *J* = 3.5 Hz) was assigned to an olefinic proton in the ring C of *β*-amyrin acetate. The acetyl protons were observed as a singlet resonating at the range δ 1.95 – δ 1.97 (Fig. 2).

In the latexes of *C. rosea*, *E. myrsisnites*, and *E. tirucalli* typical methyl proton signals from a lanostan skeleton were detected. These signals were singlets at δ 0.73 (H-28, H-29), δ 0.84 (H-30), δ 0.91 (H-18), δ 0.92 (H-19), δ 1.54 (H-27), and a small doublet with germinal coupling (*J* = 0.8 Hz) at δ 1.61 that was assigned to H-26. The last two resonances are a typical pattern of a terminal isopropylidene group in the side chain of euphol like compounds. Further, a doublet at δ 0.81(H-21, *J* = 6.3 Hz) was assigned to a secondary methyl group in C21 in the same side chain (Fig. 2). The identification of triterpenoids by ^{1}H NMR data was supported and by additional GC-MS analysis and all the detected metabolites in latexes by ^{1}H NMR and GC-MS are listed in Table 1.

Table 1. Compounds detected by 1H NMR and GC-MS in the dichloromethane fraction of latexes from 12 plant species.

Compounds	AS	AO	Al	DB	PR	ADO	CR	FT	ET	EM	JC	JM
dasycarpidan-1-methanol, acetate (ester)	–	–	–	–	GC	–	–	–	–	–	–	–
α-linoleic acid	–	–	–	–	–	–	–	–	–	–	–	GC
(*cis*)-6-octadecenoic acid	–	–	–	–	–	–	–	–	GC	–	–	GC
cis-13-eicosenoic acid	–	–	–	–	GC	–	–	–	–	–	–	–
oleic acid	–	–	GC	–	–	–	–	–	–	–	–	–
octadecanoid acid*	GC	–	GC	–	–	GC	–	–	GC	–	GC	–
hexanedioic acid*	GC	–	GC	GC	–	GC	–	–	GC	–	GC	GC
Squalene	–	–	–	–	–	–	–	^{1}H/GC	–	–	–	–
β-amyrin*	^{1}H/GC	^{1}H/GC	–	–	–	–	–	–	GC	–	–	–
α-amyrin*	GC	GC	–	–	–	–	–	–	–	–	–	–
cycloeucalenyl acetate	^{1}H/GC	–	–	^{1}H/GC	–	–	–	–	–	–	–	–
Cycloartenol	^{1}H/GC	^{1}H/GC	–		–	–	–	–	–	–	–	–
lanosterol/euphol	–	^{1}H/GC	–	^{1}H/GC	–	–	^{1}H/GC	^{1}H/GC	^{1}H/GC	^{1}H/GC	–	–
9,19-cyclo-(9*β*)-lanost-24-en-3*β*-ol	–	–	–		–	–	–	–	–	GC	–	–
(3*β*)- 24-methylenecycloartan-3-ol	–	–	–		–	–	–	–	–	–	–	–
Lanosterone	–	–	–		–	–	–	GC	–	–	–	–
lupeol acetate†	^{1}H/GC	^{1}H/GC	–	^{1}H/GC	^{1}H/GC	–	–	–	–	–	–	–
lupenone*	–	–	–			–	–	GC	–	–	–	–

3-acetoxy-7,8-Epoxylanostan-11-ol†	–	–	–		GC	–	–	–	–	–	–	–
β-amyrin acetate	^{1}H/GC	–	–	^{1}H/GC	–	–	–	–	–	–	–	–
24-methylenecycloartanol*	^{1}H/GC	^{1}H/GC	–	^{1}H/GC	–	–	–	–	–	–	–	–
stigmasterol*	–	–	–	GC	–	–	–	GC	–	–	–	–
β-sitosterol*	–	–	GC	–	–	–	–	–	–	–	–	–
24-methylenelophenol	–	–	GC	GC	–	–	–	–	–	–	–	–
Friedelin	–	–	–	GC	–	–	–	–	–	–	–	–
betulin*	–	–	–	–	–	–	–	GC	GC	–	–	–
(3*β*)-3-(acetyloxy)-urs-12-en-28-al	–	–	–	–	GC	–	–	–	–	–	–	–
cycloartenol acetate	–	–	–	–	–	–	–	GC	–	–	–	–

AS, Alstonia scholaris, AO, Akokanthera oblonguifolia, Al, Allamanda spp., DB, Dischidia bengalensis, PR, Plumeria rubra, ADO, Adenium obesum, CR, Clusia rosea, FT, Ficus triangularis, ET, Euphorbia tirucalli, EM, Euphorbia myrsinites, JC, Jatropha curcas, JM, Jatropha multifida.

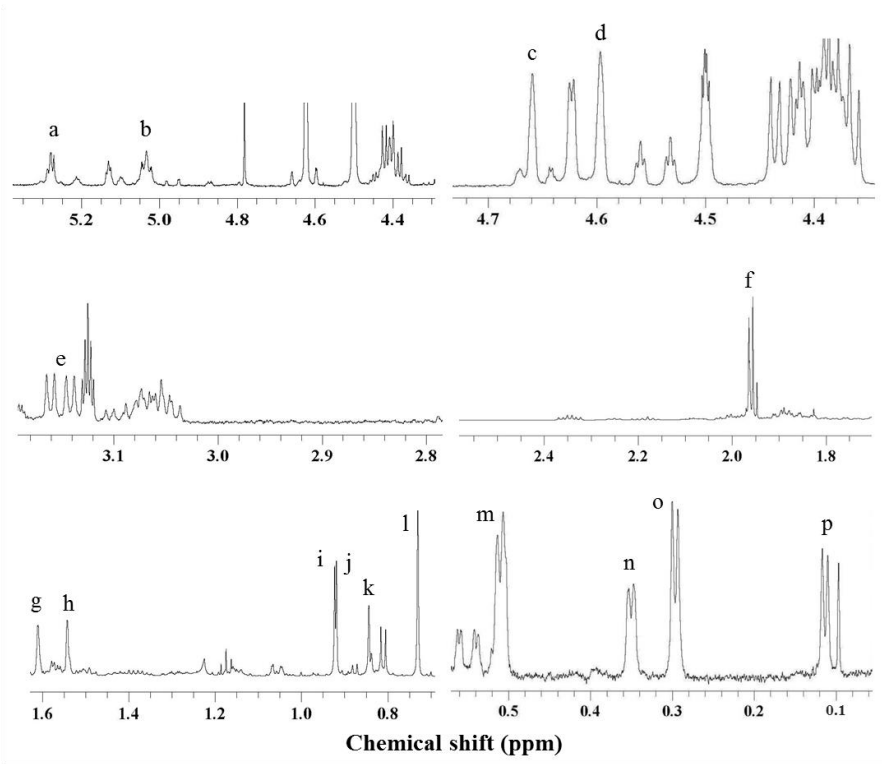

Fig. 2. Diagnostic signals of specific terpenoids in latexes. a, H-12 of *β*-amyrin, b, H-24 cycloartenol, c, H-29a of lupeol acetate, d, H-29b of lupeol acetate, e, H-3 of *β*-amyrin, f, acetyl protons of lupeol acetate and *β*-amyrin acetate, (g), H-26, h, H-27, i, H-19, j, H-18, k, H-30, l, H-28 and H-29 of a lanostan skeleton, m, H-19*exo* of 24-methylenecycloartanol, n, H-19*exo* of cycloeucalenyl acetate, o, H-19*endo* of 24-methylenecycloartanol, p, H-19*endo* of cycloeucalenyl acetate.

After the analysis of lipophilic metabolites in the dichloromethane fraction, the aqueous fractions were analyzed again by ^{1}H NMR. In general, the water fractions of latexes were found to be rich in primary metabolites such as sugars and organic acid derivatives. There was a separation in two clusters as in dichloromethane fractions analyzed by OPLS-DA. However, the model was not validated either by the Q^2 value ($Q^2 < 0.40$) of the permutation test nor the the *p*-value from the CV-ANOVA ($p > 0.01$). This unseparation might be due to a species specific character of the latex-discriminating metabolites in this fractions. That is, these metabolites could

consist of rather slightly different analogues, which could consequently cause the undiscrimination between the two tissues in the model.

However, interestingly some of the latex water fractions showed very simple spectra with a few major organic acids, i.e. *A. scholaris*, *E. myrsinites*, and *P. rubra* (Fig. 3) (Table 2). The major hydrophilic latex metabolites were found to be cerberic acid B in *P. rubra*, 2-methoxy succinic acid in *A. scholaris*, and three isomers of citramaloyl shikimic acid in *E. myrsinites*. The concentrations of all the acids were over 90% of the dry weight of the water fractions of latexes. Of the compounds, 2-methoxysuccinic acid is been reported for the first time in nature. The detailed assignments of ^{1}H resonances of these acids were listed in Table 2. Other metabolites found in latexes water fractions were chlorogenic acid, quinic acid, sucrose and formic acid.

The ethyl acetate fractions resulted in a mixture of compounds found in the dichloromethane and water fractions. Their OPLS-DA model resulted in no separated samples in the score plot and unvalidated Q^2 and *p*-values as in the water fractions case. These results reinforce the fact of terpenoids like triterpenoids and sterols being the distinctive metabolites of latexes.

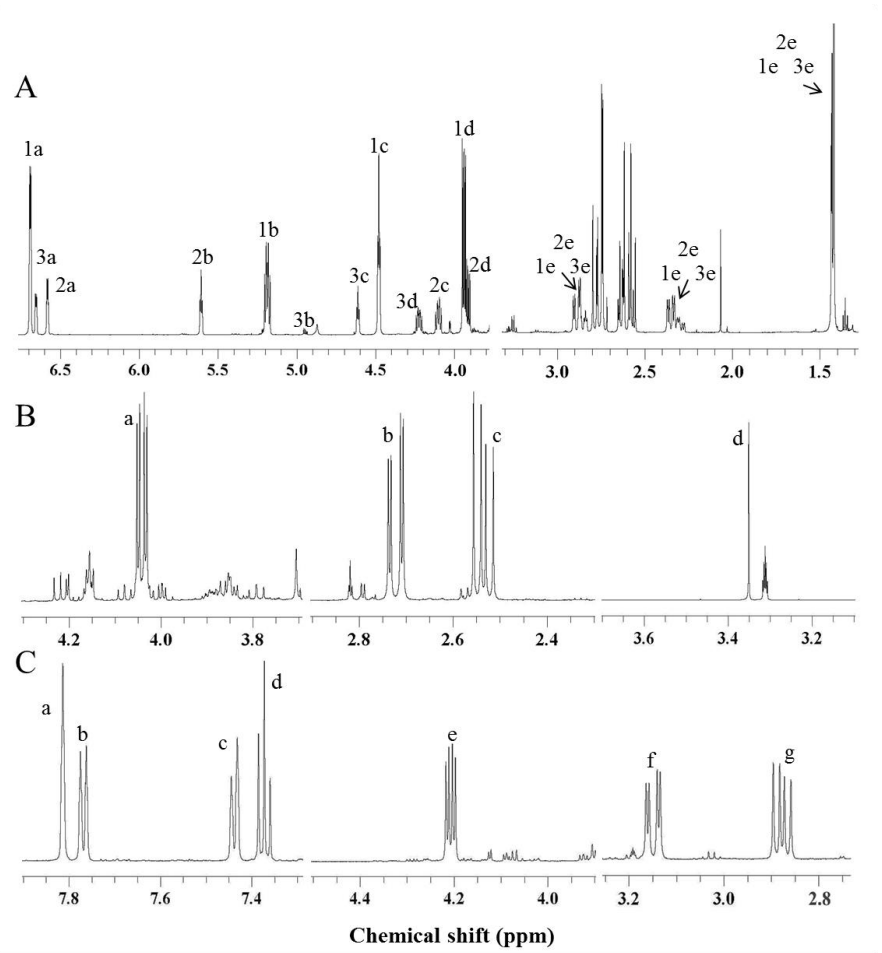

Fig. 3. ^{1}H NMR spectra of water fractions from the latexes of *Euphorbia myrsinites*, *Alstonia scholaris*, and *Plumeria rubra*. (A), Isomer mixture of citramaloyl shkimic acid in *E. myrsinies*; 1a, H-2, 1b, H-5, 1c, H-3, 1d, H-4, 1e, H-6B, and 1f, H-6a protons of 5- citramaloyl shkimic acid. 2a, H-2, 2b, H-5, 2c, H-3, 2d, H-4, 2e, H-6B, and 2f, H-6a protons of 3- citramaloyl shkimic acid. 3a, H-2, 3b, H-5, 3c, H-3, 3d, H-4, 3e, H-6B, and 3f, H-6a protons of 4- citramaloyl shkimic acid. (B) 2-methoxysuccinic acid in *A. scholaris*; a, H-2, b, H-3a, c, H-3b, and d, methoxy protons. (C) Cerberic acid B in *P. rubra*; a, H-2, b, H-4, c, H-6 d, H-5, e, H-8, f, H-7a, and g, H-7b.

Table 2. [1]H chemical shifts of 5-citramaloyl shikimic acid, 4-citramaloyl shikimic acid, 3-citramaloyl shikimic acid, 2-methoxysuccinic acid, and cerberic acid B.

Position	[1]H chemical shifts (ppm); multiplicity; coupling constants (Hz)				
	Compound 1[a]	Compound 2[b]	Compound 3[c]	Compound 4[d]	Compound 5[e]
2	6.69 m	6.65 m	6.58 m	4.04 dd (9.5, 3.5)	7.81 brs
3	4.48 t (4.1)	4.61 t (4.3)	5.61 t (4.2)	–	–
3a	–	–	–	2.72 dd (15.6, 9.5)	–
3b	–	–	–	2.53 dd (15.6, 3.5)	–
4	3.94 dd (8.6, 4.2)	4.94 dd (8.9, 4.3)	3.91 dd (8.6, 4.2)	–	7.76 d (7.7)
5	5.19 m	4.22 m	4.10 m	–	7.37 t (7.7)
6				–	7.44 d (7.7)
6a	2.35 dd (18.4, 6.7)	2.32 dd (16.1, 6.7)	2.29 dd (17.1, 6.9)	–	–
6b	2.82 dd (18.0, 5.9)	2.80 dd (16.1, 4.2)	2.78 dd (18.6, 5.4)	–	–
7a	–	–	–	–	3.15 dd (14.1, 3.9)
7b	–	–	–	–	2.88 dd (14.0, 8.3)
8	–	–	–	–	4.21 dd (8.3, 3.9)
2’CH_3	1.42 s	1.43 s	1.43 s	–	
2OCH_3	–	–	–	3.35 s	–

[a]5-citramaloyl shikimic acid, [b]4-citramaloyl shikimic acid, [c]3-citramaloyl shikimic acid, [d]2-methoxysuccinic acid, and [e]cerberic acid B.

The extremely high concentration of specific metabolites in latexes, terpenoids and organic acids, propel the interest on their biological roles, which might be associated to plant defense. To prove this hypothesis, the methanol extracts of latexes and leaves were tested against *Frankinella occidentalis* (Thrips). Latex based diets generally displayed a significantly higher mortality index of thrips when compared with those of leaf-based diets (Fig. 4A). The average mortality rate of all the latex extracts was 80.42 % ± 1.74 while that of leaves was 55.53 % ± 3.56. The mortality of the extracts was not significantly changed by species but mainly influenced by the tissues effects ($p < 0.0001$). This result demonstrated that the conserved chemical feature of latexes is well distinguished from their bearing tissues and could be related to their biological functions like anti-herbivory.

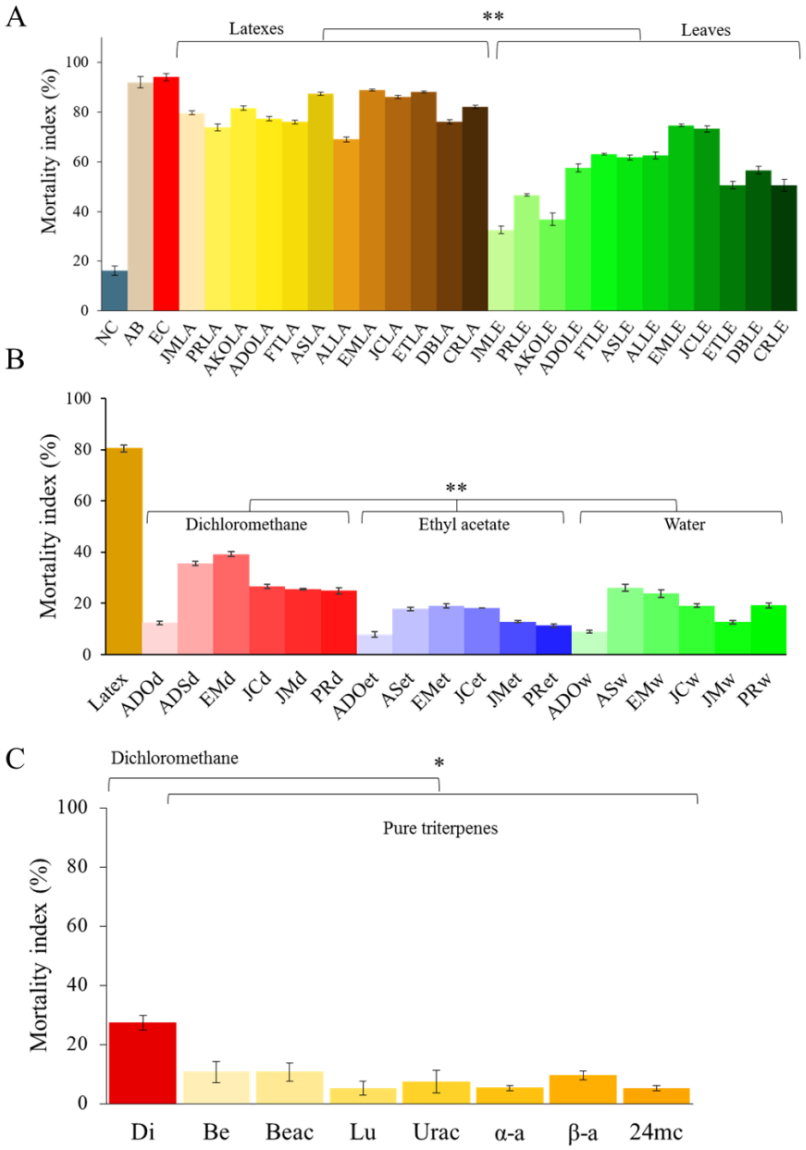

Fig. 4. Anti-herbivore activity of (A), methanol extracts of latexes and leaves of 12 latex bearing species; NC, negative control, AB, abamectine, EC, empty wells, JMLA, *Jathropha multifida*, PRLA, *Plumeria rubra*, AKOLA, *Akokanthera oblonguifolia*, ADO, *Adenium obesum*, FT, *Ficus triangularis*, AS, *Alstonia scholaris*, AL, *Allamanda spp*., EM, *Euphorbia myrsinites*, JC, *Jathropha curcas*, ET, *Euphorbia tirucalli*, DB, *Dischidia bengalensis*, CR, *Clusia rosea*. Species names with LA at the end correspond to latex and LE

to leaves extracts. (B) Dichloromethane, ethyl actetate, and water fractions of latexes from six plant species. Species with "d" at the end correspond to dichloromethane fractions, "et" to ethyl acteate fraction, and "w" to water fraction. (C) Individual triterpenoids; Di, dichloromethane fraction, Be, betulin, Beac, betulinic acid, Lu, lupeol, Urac, ursolic acid, α-a, *α*-amyrin, β-a, *β*-amyrin, and 24mc, 24-methylenecycloartanol.

In fact, a partial least square (PLS) model of the activity and the intensity of ^{1}H NMR resonances of the MeOH extracts was well validated (Q^2 = 0.41, $p < 0.05$) (Fig. 5). The PLS loadings associated to the anti-herbivore activity were signals in the range δ 0.3 – δ 1.7, which in fact corresponded to methyl resonances of terpenoids like triterpenes.

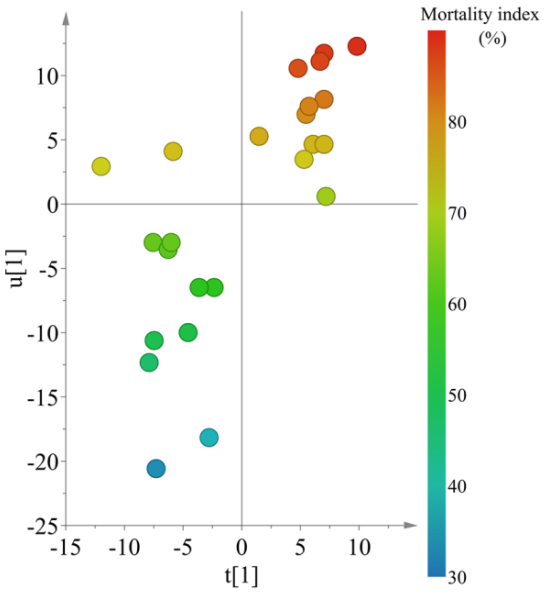

Fig. 5. Partial least square analysis between the anti-herbivory activity *against Frankinella occidentalis* and the ^{1}H NMR data set of the MeOH extracts of latex and leaves.

As a next step to observe the detailed correlation between the mortality index and latex metabolites, some latexes were selected for further study. Based on mortality indexes, six species with the highest mortality index differences between latex and their bearing tissues were selected among the 12 tested species. Three species from the Euphorbiacae family (*J. Multifida*,

J. Curcas and *E. Myrsinites*) and the others from the Apocynacae (*A. Scholaris*¸ *A. Obesdum* and *P. Rubra*). The latexes of the selected species were fractioned with dichloromethane, ethyl acetate and water, and they were tested against the same herbivore. All the three fractions showed certain degree of activity; however their activity was much lower than the methanol extracts with reduction of around 50 % of the mortality effect. Nonetheless, the dichloromethane fraction displayed the highest activity against thrips in all the tested species when compared with the other two fractions ($p < 0.001$). This result is in line with the fact that triterpenes were the compounds correlated to the anti-herbivore activity in the PLS analysis of the methanol extracts. Thus, due to their non-polar character, triterpenes were more concentrated in the dichloromethane fractions resulting in their higher activity (Fig. 4B). The activity decrease after fractionation might be a proof that strongly suggests a synergistic effect between the metabolites from all the fractions although the terpene mixture in latexes contributes to most of their activity.

To continue, several typical triterpenoids such as betulin, betulinic acid, lupeol, ursolic acid, α-amyrin and β-amyrin, and 24-methylenecycloartanol, were tested with the concentration of 37.5 µg mL^{-1}, which was determined by the quantitation results of ^{1}H-NMR of the dichloromethane fractions. All the tested individual triterpenoids showed activity in some degree but the values were much lower than that of the dichloromethane fractions and the total methanol extracts (Fig. 4C). Thus, the results support a possible synergism that none of the compounds were solely responsible for the bioactivity against thrips in the dichloromethane fraction. In the context, when a plant defense arises from co-acting features, a positive covariation between specific subgroups of defensive traits would result in more efficient defense syndromes (Mason et al. 2016). These types of synergisms were often seen in many plant species (Mason et al. 2016; Agrawal and Fishbein, 2006; Agrawal, 2007).

In addition to the insecticidal properties of latexes' triterpenoids, other potential functions could be deduced from their multifunctional characteristics that were previously reported. One of the roles is to function as deterrents instead of showing a direct insecticidal activity. Some

terpenoids and steroids can confer bitter taste to plant tissues, which make them unpalatable for insect-herbivores (González-Coloma et al. 2011). Furthermore, terpenoids can also provide growth retardation effects or even work as chemo-sterilizers against insects, which can affect their reproduction resulting in a reduced production of new offspring. Thus, this effect would be not reflected in their mortality index. This effect was previously shown in the case of *β*-amyrin derivatives (Shankaranarayana et al. 1980), and this could also partially explain why some pure metabolites did not show direct insecticidal effects. This type of supplementary and non-insecticidal mechanisms might help avoid or delay herbivores' resistance against plant defense. A similar result was reported on other exudates such as oleoresins, in these exudates even if their single components possess their own activity, their effect when mixed was found to be much higher (Michelozzi, 1999).

Another possibility of the roles of triterpenoid is to improve membrane permeability. Many sterols are reported to regulate the channels of cell membranes by lowering the motion of fatty acid chains in the cell membrane (Lewis and Elvin-Lewis, 1977). In this way, varying the type, ratio and quantities of diverse membrane sterols they can even influence plant-microorganisms interactions (Griebel and Zeier, 2010; Sharfman et al. 2014).

On the other side, compared with the lipophilic components of latexes, aqueous fractions showed very simple patterns, e.g. a few characteristic organic acids in high concentrations were detected in the water fractions of several samples. The roles of the organic acids in latexes have been relatively less studied than those of triterpenoids or steroids. However, in the past, the role of organic acids in plant-herbivore interactions has been linked to herbivore preference caused by the acid-bitter taste they confer to the tissues that contain them (Cipollini and stiles, 1992). Also, an interesting role of malic acid was recently reported to recruit beneficious bacterial involved in their biofilm formation for their host-plant protection (Rudrappa et al. 2008). In the case of *A. scholaris*, one new analogue of succinic acid was almost a unique component in the water fraction of this species detected by ^{1}H NMR. However, when tested against thrips, the compound did not show any significant activity, even when combined with terpenoids. This could suggest

an indirect complementation effect of this type of chemical components in the latex-borne defense.

There might be another role of these highly concentrated organic acids. During the process of latex exudation in plants, it was reported that the latex coagulation improves under acidic conditions (Gidrol et al. 1994). Therefore, the highly concentrated organic acid might serve as a buffer or regulator medium modulating the latex coagulation speed. In this context, Gidrol and colleagues (1994) found that the binding efficacy of coagulation proteins was pH dependant working better at low pH. Moreover, Oliveira-Reis and co-workers (2015) studied the effect of the rubber particle size and pH on the kinetics of natural rubber gelation. They found that rubber fractions higher than 10^{-2} particles per millilitre with pH close to 4 resulted in the full gelation of the rubber suspensions. However, the specificity of the organic acid is still doubtful. The effects of acids could enhance the mechanical defense of latexes by speeding up the coagulation process for example, by entrapping more insect-herbivores, as well as providing the optimum pH for gelation (Dussourd 1995; Oliveira-Reis et al. 2015).

To test the specificity of organic acids, freeze-dried powders of the latexes of *A. scholaris* (rubbery latex), *A. obesum*, *J. multifida* and *J. curcas* (clear latexes) were re-suspended in pure water and supplemented by 6.7 mM of 2-methoxy succinic acid. Interestingly, in the case of the latex of *A. scholaris* (source of the tested organic acid) the coalescence process became much faster than the latex without the acid (Fig. 6A). However, adding the same acid in the other latexes did not show the always the same effects. In *A. obesum* the compound also increased the coagulation speed like in *A. scholaris*, whereas there was no effect in *J. multifida*. Moreover, in the case of *J. curcas*, the organic acid showed the opposite effect, that is, the latex suspension was stabilized and the coagulation process was delayed (Fig. 6A). These results demonstrated that 2-methoxy succinic acid is an enhancer of the latex coagulation in *A. scholaris*, and potentially a species-specific coagulation enhancer.

The specificity of organic acids in the coagulation process was further scrutinized by spectrophotometry on the latex of *J. curcas*. Alongside 2-methoxy succinic acid, other common organic acids such as succinic acid,

malic acid, citric acid, citramalic acid, fumaric acid and shikimic acid were tested at the same concentration as treatments (pH 2.5) and spectrophotometrically compared with each other. They displayed different effects, as previously observed 2-methoxy succinic acid showed a lower OD value when compared to that of the negative control (water) indicating a lower coagulation rate. A similar effect was observed from malic acid. However, oppositely, citramalic acid increased the coagulation rate in *J. curcas* latex (Fig. 6B). The rest of the compounds did not show any effect on the coagulation process. The results give a strong clue that the coagulation process is not only pH dependent but also relay on chemically specific interactions. Moreover, this could explain why in water fractions organic acids are generally found. Also it could explain that the species variations in this type of metabolites results from the species-specific interactions with other non-polar metabolites correlated to the coagulation process, which can vary from specie to specie (Bauer et al. 2014).

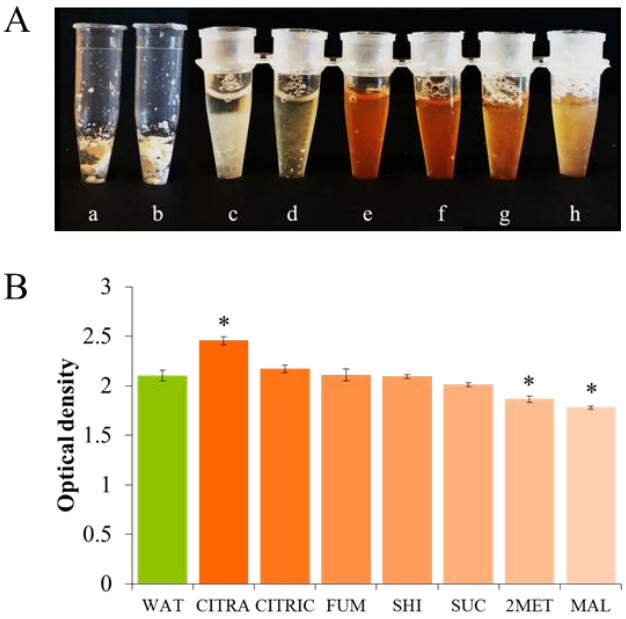

Fig. 6. Coagulation effects of 2-methoxysuccinic acid (2MET) on (A) latex suspensions of *Alstonia scholaris*, *Adenium obesum*, *Jatropha curcas*, and *Jatropha multifida*; a, mixture of *A. scholaris* latex and 2MET, b, *A. scholaris* latex control, c, mixture of *A. obesum* latex and 2MET, d, *A.*

obseum latex control, e, mixture of *J. curcas* latex and 2MET, f, *J. curcas* latex control, g, mixture of *J. multifida* latex and 2MET, h, *J. multifidi* latex control. (B) Effects of organic acids on the latex of *J. multifidi*; WAT, water control, CITRA, citramalic acid, CITRIC, citric acid, FUM, fumaric acid, SHI, shikimic acid, SUC, succinic acid, 2MET, 2-methoxysuccinic acid, MAL, malic acid. The data represent average values (n = 4) ± standard error. Bars with an * indicate significant differences as compared with the water control in a Dunnett test ($p < 0.05$).

3. Conclusions

Latexes possess distinctive metabolomes from their bearing tissues, which denote the biological specialization of their specific-specialized metabolites. The singular chemical signature of latexes consisted mainly of a semi-conserved chemical constitution, but at compound class level rather than at specific metabolites level. This metabolic distinction is reflected in their higher anti-herbivore effect against thrips, which confirms a specialized chemical selection for the dedicated roles of these exudates. Among diverse metabolite classes, triterpenes and steroids were associated not only to their differentiation but to the general defense function of latexes against thrips. However, these type of terpenoids seems to be involved in a synergistic system together with other chemical components of latexes. Furthermore, the specific metabolites of latexes are not limited to non-polar components, but also consist of water-soluble organic acids. These acids did not show activity against thrips, but they are correlated to the regulation of the coagulation process in a chemo-specific way. Thus, the effect of these metabolites could improve the latex-borne defense by speeding up the coagulation process, in other words improving the mechanical defense of latexes. By combining diverse and complementary metabolites, latexes get chemical and physical characteristics that could not be achieved by their individual metabolites. In this context, latexes are an example from nature of a chemo-systematic tissue efficiently used for plant defense. Additionally, they are also an example of how plants using a limited number of metabolites are able to handle an unlimited number of natural enemies.

Chapter 6

4. Experimental

4.1. Plant material and sampling

Diverse latexes and their host leaves were collected from four major latex-producing plant families: The Apocynaceae (*Alstonia scholaris*, *Adenium obesum*, *Allamanda* ssp., *Plumeria rubra*, and *Akokanthera obloguifolia*), Euphorbiaceae (*Jatropha multifida*, *Jatropha curcas*, *Euphorbia tirucalli*, and *Euphorbia myrsinites*), Moraceae (*Ficus triangularis*) and Clusiaseae (*Clusia rosea*). From these samples, the latex of *J. curcas*, *J. multifidi*, and *A. obesum* were non-rubbery latexes, and the other species produced rubbery latexes. Sample collection was made in February 2017 at the Botanical garden of Leiden University (Hortus Botanicus, Leiden, The Netherlands). Latex samples were taken by slicing the tree trunk or plant stems with a sterile razor blade, allowing the latex to flow out. Subsequently, the latex was taken into a 2 mL micro-tube within three minutes. The samples were flash frozen in liquid nitrogen and stored at -80 °C until they were freeze-dried. The leaf samples were manually collected from the plant. The leaves were left to drain as much as possible latex and placed into plastic hermetic bags and frozen in liquid nitrogen. Afterwards, leaf samples were grounded to a fine powder, while still frozen, and placed in 50 mL conical tubes. These samples were also stored at -80 °C until they were freeze-dried.

4.2. Sample extraction and fractionation

Methanol extracts were prepared by extracting 100 mg of dry latex or leaf material with 20 mL of methanol under ultra-sonication for 20 min. The methanol extracts were filtered and reduced under vacuum in a rotary evaporator. The remaining extracts were transferred into 1.5 mL glass vials and taken to total dryness in a speed vacuum device. To obtain three fractions from latexes, the freeze-dried samples were re-suspended in 20 mL of deionized water and ultra-sonicated 20 min. Then, latex suspensions were liquid-liquid extracted three times with 20 mL of organic solvents as follow: dichloromethane, ethyl acetate, and the remaining water fraction. When the

latex was not completely dissolved in the water, the suspension tube was washed with the first extraction solvent. When there was a non-breakable emulsion, the emulsion went to the aqueous phase. The organic fractions were dried under anhydrous sodium sulphate, filtered and reduced under vacuum in a rotary evaporator. The reduced fractions were transferred to 1.5 mL glass vials and further reduced to total dryness in a speed vacuum device. For leaves samples, dry methanol extracts were re-suspended into 20 mL of deionized water and the same fractionation procedure for latexes was followed.

4.3. NMR

For ^{1}H NMR profiling, the dried methanol extracts, dichloromethane and ethyl acetate fractions from latexes and leaves were re-dissolved in 500 µL of methanol-d4 containing 3.93 mM hexamethyldisiloxane (HMDSO). Water fractions were dissolved in a mixture of CH_3OH-*d4*:KH_2PO_4 buffer in D_2O (1:1, v/v, pH 6.0) containing 0.005% (w/w) of trimethylsilylpropionic acid sodium salt (TMSP). The extracts were ultra-sonicated for 20 min, centrifuged at 13000 rpm for 10 min, and transferred into 3 mm-NMR tubes. The 1D-^{1}H NMR, 2D-J-resolved and ^{1}H-^{1}H homonuclear correlation experiments were performed using a 600-MHz Bruker spectrometer (Bruker, Karlsruhe, Germany), operating at proton NMR frequency of 600.13 MHz. For internal locking, methanol-d4 was used. All ^{1}H NMR analyses consisted of 128 scans, requiring an acquisition time of 10 min and 26 sec. Acquisition parameters were: 0.16Hz/point, pulse width (PW) = 30° (11.3 µs), and the relaxation time was 1.5 sec. A pre-saturation sequence was used to suppress the residual H_2O signal using low power selective irradiation at H_2O frequency during the recycle delay. The FIDs were Fourier transformed with exponential line broadening of 0.3 Hz. The resulting spectrums were manually phased and baseline corrected, and calibrated to TMSP and HMDSO at 0.00 and 0.06 ppm respectively for latexes and leaves samples using TOPSPIN V. 3.0 (Bruker). The J-resolved experiment was performed using 16 scans for each increment in F1. A pre-saturation used during the relaxation delay (1.5 s) with an effective field of $\gamma B1 = 50$ Hz were also

applied. Before a magnitude mode Fourier transformation, with a sine shape window functions in both dimensions, the data set was zero-filled to 512 x 32,768 points. The data was then tilted to 45° and symmetrized along the F2 dimension and referenced to the internal TMSP signal. From the 2D-J-resolved spectra 1D-projection along the F2 dimension were extracted using the build-in positive projection routine in Topspin (version 2.1, Bruker Biospin). For ^{1}H-^{1}H correlated spectroscopy (COSY) experiments, the spectra were also adquired at 600.13 MHz, with 6361 Hz of spectral width in both dimesions and 1.0 sec of relaxation time. The window function for COSY spectra was sine-bell (SSB = 0).

4.4. Gas chromatography coupled to mass spectrometry (GC-MS)

To identify fatty acids and terpenoids signals detected by ^{1}H NMR, GC-MS analyses were performed. For sample derivatization, 100 µL of dichloromethane fractions solution (2 mg/mL) were mixed with 200 µL of pyridine and 100 µL of BSTFA mixed with 1% of TMCS (SUPELCO). The solutions were incubated at 80 °C for 50 min and cooled down to room temperature. For chromatographic separation, an Agilent technologies 7890A gas chromatograph equipped with an auto-sampler (7693), and coupled to an Agilent technologies 5975C mass detector was used. The GC-MS system was equipped with a column DB5/50 m x 0.20 mm and 0.33 µm thickness as a stationary phase. Helium (99.9% purity) was used as carrier gas at a flow rate of 1 mL/min. For *A. obesum* latex and leaves dichloromethane fractions analysis, the oven temperature started at 200 °C then increased 4 °C/min up to 280 °C and then increased 0.5 °C/min up to 290 °C and kept for three min. For *E. myrsinites*, *E. tirucalli*, *A. oblonguifolia*, *P. rubra*, *D. bengalensis*, *A. scholaris*, and *C. rosea* latex and leaf dichloromethane fractions, and just the *F. triangularis* dichloromethane fraction from latex, the oven temperature started at 200 °C. The temperature increased 10 °C/min up to 280 °C and subsequently increased 0.5 °C/min up to 290 °C and kept it for 3 min. Finally, for *F. triangularis* leaves, *Allamanda* sp., *J. curcas* and *J. multifida* latex and leaf dichloromethane fractions the oven temperature started at 150 °C then increased 5 °C/min up to 280 °C and then further increased 0.5

°C/min up to 290 °C and held it for three min. The nozzle was set to split mode (10:1) at 280 °C, and the interface temperature was also 280 °C. For the mass detector on SCAN mode, the ion source temperature was 230 °C and 150 °C at the quadrupole. The ionization energy was 70 eV and the injection volume was 1 µL. Peak identification was done by comparing the ion spectra obtained from samples to ion spectra in the NIST V.2008 library, or by comparison of their retention time with standard compounds.

4.5. Anti-herbivory activity

Thrips (*Frankliniella occidentalis*) were reared in a climate chamber on chrysanthemum plants, from which they were able to feed. The thrips were contained inside plastic cages under standard thrips rearing conditions (L:D, 12:12, 25 °C). For the bioactivity assays, adult thrips were taken from these cages and placed inside jars containing string beans for sustenance. To obtain thrips larvae, the jars were left for seven days under the same rearing conditions. From the new offspring, second instar larvae were used for the bioassays. The larvae were placed into cap strips. One larva was placed per cap, subsequently the caps were sealed with a parafilm layer. Besides, 96 wells plates were filled with 55 µL of control and treatments diet solutions. Finally, the cap strip bands containing the larvae were placed on top of the 96 well plates and they were placed upside down, allowing the thrips to feed through the parafilm. After 5 days, the larvae survival index was recorded under a stereo-microscope. Three independent replicates were done for all of the experiments (n = 32).

4.5.1. Controls and treatments

For the first experimental stage, the treatments solutions consisted of latex and leaf methanol extracts dissolved in 40 mM phosphate buffer (pH 7) containing 10 % of fructose. All solutions were at a final concentration of 150 µg/mL and 3% methanol in the well. For the second experimental stage, dichloromethane, ethyl acetate and water fractions of six latexes from Euphorbiacae (*J. Multifida*, *J. Curcas* and *E. Myrsinites*), and Apocynacae

(*A. Scholaris*¸ *A. Obesum* and *P. Rubra*) families were also tested against thrips. The fractions were re-dissolved in DMSO, and diluted in 40 mM phosphate buffer (pH 7) with 10 % fructose. Again, all solutions were at final concentration of 150 µg/mL and 1 % DMSO in the well. On the third experimental stage, the treatments consisted of betulin, betulinic acid, α-amyrin, β-amyrin, ursolic acid, lupeol, and 24-methylenecycloartanol solutions at final concentration of 37.5 µg/mL dissolved in the same buffer used for fractions treatments.

Negative controls for all bioactivity assays consisted of solutions of 40 mM phosphate buffer (pH 7) containing 10% of fructose, and 3% of methanol as negative control for methanol extracts, and 1% of DMSO for fractions treatments and pure compounds. As positive control, abamectin at final concentration of 150 µg/mL dissolved in the previous solutions was used. Another positive control were empty wells, these wells were used to prove that thrips larvae cannot survive without feeding.

4.6. Coagulation experiments

To test the effect of 2-methoxy succinic acid on the coagulation process of the latex of *A. scholaris* and other latexes, an aqueous solution of this compound at final concentration of 6.7 mM was used as a treatment. Five mg of freeze-dried latex of *A. scholaris* were re-suspended in 100 µL of the treatment solution in 500 µL plastic micro-tubes. For latexes from *A. obesum*, *J. mutifida* and *J. curcas*, 3 mg of freeze-dried latex were re-suspended in 150 µL of the treatment solution. The negative controls consisted of the same latexes re-suspended just in milli*Q* water. The effects of the treatment were visually inspected after 20 min to open air conditions. The amount of solution was adjusted to get a similar consistence to that of the natural flowing latexes.

4.6.1. Spectrophotometric assays

Three milligrams of freeze-dried latex of *J. curcas* were individually placed in a 96 swallow well plate. To the latex solution, 150 µL of aqueous

solutions of 2-methoxy succinic acid, succinic acid, malic acid, citric acid, citramalic acid, fumaric acid and shikimic acid (6.7 mM] were added to each well. The latexes were dissolved in their corresponding wells and to avoid optical density reading interferences by bubbles and foam from the latex solutions, 100 µL of these solutions were subsequently softly transferred to the next row of wells. The coagulation control consisted of latex re-dissolved just in deionized water. The blanks solutions consisted of 6.7 mM aqueous solutions of each organic acid and just water for the coagulation control. The spectrophotometric measurements were carried out in a 680 XR multi-well plate reader (BIO-RAD, Osaka, Japan) at 600 nm after 20 min of incubation at room temperature at (22 °C) in open air conditions (without lid). The results were expressed in OD values ± standard deviation (n = 4).

4.7. Data processing and statistical analysis

Spectral data was bucketed using AMIX program, bucket size was 0.04 ppm and the areas were normalised to total intensity. Intervals for methanol and water signals were deleted from the bucket data. Multivariate data analysis was performed using SIMCA P (ver. 15.1, Umetrics, Umeå, Sweden). Principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) were performed for NMR data. PCA and OPLS-DA analysis data was scaled by Pareto scaling method.

To calculate mortality indexes, the mortality ratio of the methanol and DMSO extracts for both latex and leaves were corrected for the survival of its corresponding negative control. To evaluate differences in bioactivity between latex and leaf across all species, a generalized linear mixed model for binomial data was constructed. In this model, the survival rate of the corresponding negative control was again used to correct for the survival of the negative control for each species. Therefore, an offset was constructed with the negative control of each species. The data for each species was also compared to determine whether there was a difference in bioactivity between different species regarding the latex and leaf extracts. The effect of species was taken as a random effect in the offset of the model. The same model was used to compare fractions treatments. To compare individual compounds'

anti-herbivore effects with latex mean value, a two tailed T-test for samples with the same variance was performed for each comparison. For coagulation experiments, the OD average of each treatment (n = 4) was compared with that of the coagulation control by a Dunnett test ($\alpha = 0.05$).

5. References

Agrawal, A.A., 2007. Macroevolution of plant defense strategies. Trends Ecol. Evol. 22:103-109.

Agrawal, A.A., Fishbein, M., 2006. Plant defense syndromes. Ecology 87:S132-S149.

Agrawal, A.A., Konno, K., 2009. Latex: A model for understanding mechanisms, ecology, and evolution of plant defense against herbivory. Annu. Rev. Ecol. Evol. Syst. 40, 311-331.

Agrawal, A.A., Lajeunesse, M.J., Fishbein, M., 2008. Evolution of latex and its constituent defensive chemistry in milkweeds (Asclepias): a phylogenetic test of plant defense escalation. Entomol. Exp. Appl. 128, 126-138.

Bauer, G., Gorb, S.N., Klein, M.C., Nellesen, A., von Tapavicza, M., Speck, T., 2014. Comparative study on plant latex particles and latex coagulation in Ficus benjamina, Campanula glomerata and three Euphorbia species. PLOS ONE. 9(11), e113336.

Bruinsma, M., Posthumus, M.A., Mumm, R., Mueller, M.J., van Loon, J.J.A., Dicke, M., 2009. Jasmonic acid-induced volatiles of Brassica oleracea attract parasitoids: effects of time and dose, and comparison with induction by herbivores. J. Exp. Bot. 60, 2575-2587.

Chezem, W.R., Clay, N.K., 2016. Regulation of plant secondary metabolism and associated specialized cell development by MYBs and bHLHs. Phytochemistry. 131, 26-43.

Cipollini, M.L., Stiles, E.W., 1992. Antifungal activity of ripe ericaceous fruits: phenolic-acid interactions and palatability for dispersers. Biochem. Syst. Ecol. 20(6), 501-514.

De Oliveira-Reis, G., Menut, P., Bonfils, F., Vaysse, L., Hemar, Y., Sanche, C., 2015. Acid-induced aggregation and gelation of natural rubber latex particles. Colloid Surf. A Physicochem. Eng. Asp. 482, 9-17.

Dussourd, D.E., 1995. Entrapment of aphids and whiteflies in lettuce latex. Ann. Entomol. Soc. Am. 88(2), 163-172.

Ellis, D.I., Brewster, V.L., Dunn, W.B., Allwood, J.W., Golovanov, A.P., Goodacre, R., 2012. Fingerprinting food: current technologies for the detection of food adulteration and contamination. Chem. Soc. Rev. 41, 5706-5727.

Gidrol, X., Chrestin, H., Tan, H.L., Kush, A., 1994. Hevein, a lectin-like protein from Hevea brasiliensis (Rubber Tree) is involved in the coagulation of latex. J. Biol. Chem. 269, 9278-9283.

González-Coloma, A., López-Balboa, C., Santana, O., Reina, M., Fraga, B.M., 2011. Triterpene-based plant defenses. Phytochem. Rev. 10, 245-260.

Griebel, T., Zeier, J., 2010. A role for β-sitosterol to stigmasterol conversion in plant-pathogen interactions. Plant. J. 63, 254-268.

Groeneveld, H.V., Binnekamp, A., Seykens, D., 1991. Cardenolide biosynthesis from acetate in Asclepias curassavica. Phytochemistry. 30, 2577-2585.

Groeneveld, H.V., van den Berg, B., Elings, J.C., Seykens, D., 1990. Cardenolide biosynthesis from malonate in Asclepias curassavica. Phytochemistry. 29, 3479-3486.

Gromski, P.S., Muhamadali, H., Ellis, D.I., Xu, Y., Correa, E., Turner, M.L., Goodacre, R., 2015. A tutorial review: Metabolomics and partial least squares-discriminant analysis - a marriage of convenience or a shotgun wedding. Anal. Chim. Acta. 879, 10-23.

Hare, J.D., 2011. Ecological role of volatiles produced by plants in response to damage by herbivorous insects. Annu. Rev. Entomol. 56, 161-80.

Huber, M., Triebwasser-Freese, D., Reichelt, M., Heiling, S., Paetz, C., Chandran, J.N., Bartram, S., Schneider, B., Gershenzon, J., Erb, M., 2015. Identification, quantification, spatiotemporal distribution and genetic variation of major latex secondary metabolites in the common dandelion (Taraxacum officinale agg.). Phytochemistry. 115, 89-98.

Kim, H.K., Choi, Y.H., Verporrte, R., 2010. NMR-based metabolomic analysis of plants. Nat. Protoc. 5, 536-549.

Kirk, W.D.J., Terry, L.I., 2003. The spread of the western flower thrips Frankliniella occidentalis (Pergande). Agric. For. Entomol. 5, 301-310.

Konno, K., 2011. Plant latex and other exudates as plant defense systems: roles of various defense chemicals and proteins contained therein. Phytochemistry. 72, 1510-30.

Konno, K., Hirayama, C., Nakamura, M., Tateishi, K., Tamura, Y., et al. 2004. Papain protects papaya trees from herbivorous insects: role of cysteine protease in latex. Plant. J. 37, 370-78.

Konno, K., Ono, M., Nakamura, M., Tateishi, K., Hirayama, C., et al. 2006. Mulberry latex rich in antidiabetic sugar-mimic alkaloids forces dieting on caterpillars. Proc. Natl. Acad. Sci. USA. 103, 1337-41.

Lewis, W.H., Elvin-Lewis, M.P.F., 1977. Medical Botany: plants affecting mans health. Wiley, New York.

Liu, X., Klinkhamer, P.G.L., Vrieling, K., 2017. The effect of structurally related metabolites on insect herbivores: A case study on pyrrolizidine alkaloids and western flower thrips. Phytochemistry. 138, 93-103.

Maffei, M.E., Mithöfer, A., Boland, W., 2007. Insects feeding on plants: rapid signals and responses preceding the induction of phytochemical release. Phytochemistry. 68, 2946-2959.

Markley, J.L., Brüschweiler, R., Edison, A.S., Eghbalnia, H.R., Powers, R., Raftery, D., Wishart, D.S., 2017. The future of NMR-based metabolomics. Curr. Opin. Biotechnol. 43, 34-40.

Mason, C.M., Bowsher, A.W., Crowell, B.L., Celoy, R.M., Tsai, C.J., Donovan, L.A., 2016. Macroevolution of leaf defenses and secondary metabolites across the genus Helianthus. New Phytol. 209, 1720-1733.

Michelozzi, M. 1999. Defensive roles of terpenoid mixtures in conifers. Acta Bot. Gallica. 146(1), 73-84.

Nicholson, J.K., Connelly, J., Lindon, J.C., Holmes, E., 2002. Metabonomics: A platform for studying drug toxicity and gene function. Nat. Rev. Drug. Disc. 1, 153-161.

Pandey BP (2001) Plant Anatomy. S. Chand and Company, New Delhi

Ramos, M.V., Grangeiro, T.B., Freire, E.A., Sales, M.P., Souza, D.P., Araújo, E.S., Freitas, C.D.T., 2010. The defensive role of latex in plants: detrimental effects on insects. Arthropod-Plant. Interact. 4, 57-67.

Rudrappa, T., Czymmek, K.J., Paré, P.W., Bais, H.P., 2008. Root-Secreted Malic Acid Recruits Beneficial Soil Bacteria1. Plant Physiol. 148, 1547-1556.

Seiber, J.N., Nelson, C.J., Lee, S.M., 1982. Cardenolides in the latex and leaves of seven Asclepias species and Calotropis procera. Phytochemsitry. 21, 2343-48.

Sessa, R., Benett, M.H., Lewin, M.J., Mansfirls, J.W., Beale, M.H., 2000. Metabolite profiling of sesquiterpene lactones from Lactuca species. J. Biol. Chem. 275, 26877-26884.

Shankaranarayana, K.H., Ayyar, K.S., Rao, G.S.K., 1980. Insect growth inhibitor from the bark of Santalum album. Phytochemistry. 19, 1239-1240.

Sharfman, M., Bar, M., Schuster, S., Leibman, M., Avni, A., 2014. Sterol-dependent induction of plant defense responses by a microbe-associated molecular pattern from Trichoderma viride. Plant Physiol. 164, 819-827.

Taira, T., Ohdomar, A., Nakama, N., Shimoji, M., Ishihara, M., 2005. Characterization and antifungal activity of Gazyumaru (Ficus microcarpa) latex chitinases: Both the chitin-binding and the antifungal activities of class I chitinase are reinforced with increasing ionic strength. Biosci. Biotechnol. Biochem. 69, 811-818.

War, A.R., Paulraj, M.G., Ahmad, T., Buhroo, A.A., Hussain, B., Ignacimuthu, S., Sharma, H.C., 2012. Mechanisms of Plant Defense against Insect Herbivores. Plant Signal Behav. 7(10), 1306-1320.