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**Title:** Phytochemical background matters for bioactivity of plant metabolites: a case study with pyrrolizidine alkaloids
**Issue Date:** 2016-12-22
Chapter 5

The influence of the natural background on the bioactivity of individual plant metabolites: a case study with fractions of *Jacobaea* extracts and pyrrolizidine alkaloids

Xiaojie Liu, Klaas Vrieling, Peter G. L. Klinkhamer
Abstract

Plants produce an extremely diverse array of chemical metabolites that mediate many aspects of plant-environment interactions. In plants, metabolites co-occur with each other. This co-occurrence presents the natural background for individual metabolites. Given that herbivores always encounter a mixture of metabolites, the natural background is of ecological significance because interactions of metabolites will occur and that may positively or negatively affect the bioactivity of the metabolites. In the context of plant-herbivore interactions, it is as yet poorly understood how natural backgrounds shape the bioactivity of individual metabolites. We tested the effects of a methanol extract of Jacobaea plants and five fractions derived from this extract, and of two pyrrolizidine alkaloids (PAs) retrorsine and retrorsine N-oxide on survival of the western flower thrips (Franklinella occidentalis). We subsequently added the two PAs to the most and to the least active fraction to investigate the influence of the natural background on the bioactivity of PAs. When tested alone, retrorsine showed a stronger effect on thrips survival than retrorsine N-oxide. The methanol extract resulted in lower thrips survival than any of the five fractions derived from it. In addition, the effect of the methanol extract was higher than would be expected on basis of the combined effects of the fractions if there would be no interactions. The latter suggests that synergistic interactions between metabolites predominate in the extract. The five fractions differed in their effects on thrips survival. The n-BuOH fraction showed the highest effect on thrips while the CHCl₃ fraction was the least active against thrips. The latter fraction contained the majority of the PAs. Both PAs interacted synergistically with both these fractions in their activity against thrips. These finding strongly suggests that the activity of PAs against thrips is potentiated by other metabolites present in the plant. This study supports a commonly held notion that plant chemical defence is dependent on a variety of metabolites, which together shape the outcome of the defensive efficacy. The approach that we put forward here, to determine the associations between the bioactivity of a given plant and its metabolites, suggests a starting point for studying the effects of interactions between metabolites on their bioactivity. It also shows that the assessment of bioactivity cannot be decoupled from the natural phytochemical background in which these metabolites occur.

Keywords: Natural backgrounds, Metabolite interaction, Pyrrolizidine alkaloids, Synergy, Plant defence
**Introduction**

Plant metabolites play an important role in aiding the plant to survive various biotic and abiotic stresses (Fraenkel 1959; Wink 1988; Kliebenstein 2014). When attempting to identify the metabolites that are responsible for a certain bioactivity in a plant, the most common way of testing is to isolate single metabolites and test them in bioassays (Hadacek 2002). However, plants metabolites occur together with probably thousands of other metabolites with which they can interact (Williamson 2001; Wink 2003). The bioactivity of such mixtures of metabolites will be different from the sum of the effects of the individual metabolites because most likely many chemical and biological interactions will occur.

The co-occurrence of plant metabolites provides a strong likelihood of interactions between them. From a plant’s perspective, metabolite interactions are of vital importance. Plants can benefit from synergism between metabolites if they increase bioactivity at a lower cost (Berenbaum and Zangerl 1993; Nelson and Kursar 1999). For a single metabolite, the mode(s) of action in concert with other metabolites may differ from that as a single compound. In the context of plant-insect associations, only few studies have demonstrated the interaction effects of plant metabolites on insects. Previous studies mostly focused on the combinations of two or more known metabolites of the same chemical class (Diawara et al. 1993; Smith et al. 2001; Dyer et al. 2003; Richards et al. 2010; Richards et al. 2012; Whitehead and Bowers 2014; Chapter 2 of this thesis). Less studied are interactions between metabolites of different classes (Berenbaum and Neal 1985; Neal 1989; Nelson and Kursar 1999; Guillet et al. 1998; Nuringtyas, PhD thesis, 2014; Chapters 3 and 4). However, it is worthwhile to put more emphasis on interactions between metabolites from different chemical classes as well. Before metabolites become bioactive they have to be taken up, pass membranes, should be protected against metabolization and secretion and reach the target site (Wu and Baldwin 2010). Many of these processes can, to different extents, be influenced by metabolites from different classes. For instance, monoterpenes inhibited cytochrome P450 enzymes involved in α-terthienyl (a terthiophene) degradation, thereby increasing the effect of α-terthienyl on the reduction of relative growth rate of the European corn borer *Ostrinia nubilalis* larvae (Guillet et al. 1998).

Interactions between plant metabolites present a great challenge to researchers in terms of the number of metabolites and even more so in terms of the number of potential combinations. Given the enormous number of metabolites in a single plant, it is impossible to evaluate all combination of metabolites. In addition, interactions may occur among unidentified or even unknown metabolites, which represent a great part of the total amount of plant metabolites (Trethewey 2004). In view of these facts, it is of concern how to measure the interactions between plant metabolites without prior knowledge about which specific metabolites are involved and what their potential mode of action is. A way forward would be to start with combinations of classes of metabolites of which we know that the
potential of interactions is high such as saponins together with metabolites that not easily pass membranes (Gee et al. 1996; Herrmann and Wink 2011).

Here we took the first step of a different approach. We studied the bioactivity of pyrrolizidine alkaloids (PAs) when they are added to (part of) their natural phytochemical background. This can be seen as a top-down approach. When the bioactivity of a compound against a particular target is increased when it occurs in extracts or fractions of extracts this would point to the importance of interactions among plant compounds for the plants defence system. We found that the mutagenicity of retrorsine, a pyryolzidine alkaloid, towards *Salmonella typhimurium* bacterial strains was significantly enhanced by the CHCl₃ and the EtOAc fractions of *Jacobaea* shoots while it was decreased by the n-BuOH and the H₂O fractions (Chapter 6). In this chapter we will study the bioactivity of retrorsine and retrorsine N-oxide against the western flower thrips (*Franklinella occidentalis*) in combination with two fractions derived from a methanol extract of shoots from *Jacobaea vulgaris*.

*Jacobaea* species are characterized by their PAs, which serve an important function in plant defence. Negative effects on mammalian herbivores (EFSA 2007; Fu et al. 2001 and 2002; Trigo 2011 and references therein), generalist insect herbivores (Dreyer et al. 1985; de Boer 1999; Reina et al. 2001; Siciliano et al. 2005; Dominguez et al. 2008; Macel 2011 and references therein) and pathogens (Rubiolo et al. 1992; Joosten and van Veen 2011 and references therein; Bovee et al. 2015; Jing et al. 2015) have been reported. As single metabolites, the PA N-oxides are less active against insect herbivores than the corresponding free bases (Dreyer et al. 1985; van Dam et al. 1995; Macel et al. 2005; Nuringtyas et al. 2014; Chapter 2). In plants, PAs occur mostly as N-oxide (Hartmann et al. 1989) with some jacobine-like PAs occurring up to 50% as free base in *J. vulgaris* (Joosten et al. 2011). However, the bioactivity of the two forms against thrips was reversed when PAs were added to artificial diets together with chlorogenic acid (CGA) (Chapters 3 and 4). Evidence for the role of PAs in the plant’s defence system mostly comes from correlative studies (Vrielings et al. 1991; Leiss et al. 2009; Cheng et al. 2011; Kostenko et al. 2013; Wei et al. 2015; see review by Trigo 2011 and Macel 2011) and to a lesser extent from bioassays with single PAs (but see Macel et al. 2005). The question how the bioactivity of PAs is increased or decreased in concert with other co-occurring metabolites has as yet not been addressed (but see Chapters 3 and 4).

The purpose of this chapter is threefold. First, we tested the effect of, respectively, retrorsine and retrorsine N-oxide on western flower thrips (*Franklinella occidentalis*). Secondly, we tested the effect of the methanol extract and five fractions from *Jacobaea* shoots on thrips survival. And finally we investigated whether the combination of plant fractions and retrorsine or retrorsine N-oxide showed synergistic or antagonistic effects on thrips survival.
Materials and Methods

Chemicals and plant materials

Retrorsine and retrorsine N-oxide were purchased from Sigma (St. Louis, MO, USA). As the starting material, we used the same dried ground plant material that was kept in -80°C freezer and that was used in Chapter 6. Details of extraction and fractionation have been described in Chapter 6. Briefly, a total methanol extract was obtained from dried and ground *Jacobaea* shoots. Subsequently, the methanol extract was fractionated by extraction with different polar solvents, yielding the hexane, CHCl₃, EtOAc, *n*-BuOH and the residual H₂O fractions (Chapter 6).

Retrorsine and retrorsine N-oxide were dissolved in 30 µL methanol (MeOH) to prepare stock solutions (14 mM). From these stock solutions dilutions in MeOH were prepared with a concentration of 2.8 mM. These solutions were used to prepare test solutions for the thrips bioassay.

Thrips bioassay

Full details about the bioassay and the rearing conditions of thrips were described in Chapters 2 and 3 of this thesis. Briefly: per two 96-well plates there are 24 columns (1-24) of 8 wells. A column received the same treatment and therefore consisted of 8 replicates. Of the 24 columns, 16 columns were filled with 16 different treatments and 8 columns were left empty. The 16 treatments included one negative control (55 µL of a phosphate buffered medium (Na₂HPO₄ and NaH₂PO₄, 40 mM, pH 7) with 10% fructose and 3% MeOH), 13 treatment groups (4 concentrations of a plant fraction, 3 concentrations of a PA and 6 concentrations of the combination of a plant fraction and a PA) and two positive controls (empty wells and abamectin). Four sets of two 96 well plates were carried out simultaneously yielding 32 replicates for all treatments. The complete bioassay was repeated at a different time so that we obtained two independent estimates of 32 replicates each. Experiments were conducted twice to have two independent estimates of thrips survival for analysis of variance.

Experiments of *Jacobaea* extract and five fractions on thrips

A solution was made containing 10% fructose in sodium phosphate buffer (40 mM, pH 7). The total methanol extract and five fractions were dissolved in 60 µL of methanol and 1.94 mL buffer solution was added so that the final concentration of methanol was always 3%. The concentrations of the extract and fractions were expressed as the equivalent amount of dried plant shoot material from which they were derived: 0.02, 0.04, 0.06 and 0.08 g plant
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shoot mass/mL. One g of plant shoot mass yielded 3.0, 5.7, 7.4, 22.8 and 128.9 mg of hexane, CHCl₃, EtOAc, n-BuOH and H₂O fraction, respectively.

Adding retrorsine and retrorsine N-oxide to fractions of Jacobaea shoot extract

After screening the five fractions, the most active fraction and the least active fraction were chosen to be combined with the two PAs. Three doses (0, 1.4 and 7.0 mM) of retrorsine and retrorsine N-oxide were combined with four doses (i.e. 0, 0.01, 0.05, and 0.09 g plant dry shoot mass/ml) of the CHCl₃ and the n-BuOH fractions, yielding 12 combinations including the tests with single PAs or fractions. The doses of retrosine and retrosine N-oxide represented approximately 1.0 and 5.0 times the total PA concentration of fresh mass of shoots of J. vulgaris plants.

For retrorsine and retrorsine N-oxide alone, we obtained four independent survival data at each concentration, from the two bioassays with two replicates each in which we tested the effect of the single compounds and fractions and their combinations.

Pyrrolizidine alkaloid content of the fractions

It was assumed that the PA content of each of the plant fractions was the same as determined for the plant fractions isolated in Chapter 6. No new analysis was therefore performed. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses of PAs of the plant fractions were conducted based on the protocol described in Cheng et al. (2011) and detailed in Chapter 6.

Statistical analysis

Construction of an interaction model and correcting for survival in the negative control

Details have been described in Chapter 3. In brief, in order to examine the effect of combinations, we constructed a multiplicative null model, in which the survival after the application of a combination is the product of the survival after application of its constituents, assuming no interaction effects. Furthermore this model assumes that the relationship between log survival and the concentration tested is linear.

In a similar manner, we corrected for survival in the negative control. The survival resulting from the application of the metabolite Sₓ can be calculated as:

\[
S_x = \frac{S_{x+NC}}{S_{NC}}
\]

(1)

Sₓ+NC is the observed experimental survival while S NC is the survival when the control solvent is added to the artificial medium.

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Testing the interaction effects of the combination on survival

The survival of thrips for the combination of a PA and a fraction ($S_{PA+F}$) results from the survival after application of the PA ($S_{PA}$), the survival after application of the fraction ($S_F$), and their interaction ($S_{PA*F}$). Consequently, the effect of the interaction on survival can be calculated by:

$$S_{PA*F} = S_{PA+F} / (S_{PA} * S_F)$$  (2)

In which $S_{PA+F}$ is the observed survival in experiments with combinations while $S_{PA}$ and $S_F$ are the observed thrips survival in experiments with a single PA and fraction respectively while $S_{PA*F}$ denotes the interaction effect. The interaction effect thus denotes the effect of the interaction between the PA and on survival fraction of thrips. The interaction effect can be synergistic ($S_{PA*F} < 1$) or antagonistic ($S_{PA*F} > 1$). To avoid confusion with statistical interaction terms, we will always refer to the value of the interaction between the metabolites as the “interaction effect $S_{PA*F}$”. The interaction effect $S_{PA*F}$ was calculated for all combinations and was expressed as mean value ± standard error of the mean (SE). As each experiment is repeated twice two independent estimates of the interaction effect are obtained.

Four-way analysis of variance (ANOVA) was performed with two PAs (retrorsine and retrorsine N-oxide), PA concentration, two fractions (the CHCl$_3$ fraction and the n-BuOH fraction) and fraction concentration as factors with the interaction effect $S_{PA*F}$ as a dependent variable.

Comparison of the effects of the crude shoot extract and its five fractions

Thrips survival was log-transformed to obtain linear relationships with the tested concentrations. Log-transformed survival was regressed against concentrations to test whether there was a dose-dependent effect. Moreover, we calculated the slopes of the regression lines and their 95% confidence intervals (CIs) to estimate and compare the effects of the extract and different fractions. If two slopes have non-overlapping 95% confidence intervals they are assumed to be significantly different at the $P < 0.05$ level.

Further, we calculated the expected thrips survival when hypothetically combining the five fractions assuming no interaction, by extending formula (1) to the following formula:

$$S_{expected} = S_{hexane} * S_{chloroform} * S_{EtOAc} * S_{n-BuOH} * S_{aqueous}$$  (3)

Thereafter, according to Formula (2), the interaction effect was calculated as the observed survival of the methanol extract divided by the expected survival of the combined five fractions assuming that no interactions occurred (Formula 4).
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\[ S_{F*F} = S_{MeOH} / S_{expected} \]  (4)

All statistical analysis were performed using SPSS software for Windows (version 21.0; SPSS Inc., Chicago, IL).

Results

Thrips survival in the negative control was on average 0.86 ± 0.01. The positive control with the insecticide abamectine (50 μg/mL) solution showed an average thrips survival of 0.12 ± 0.02. The control with empty wells, to verify that thrips cannot survive without feeding, had an average survival of 0.06 ± 0.01.

Effect of the total extract and fractions of *Jacobaea* shoots on thrips survival

With increasing concentrations of the methanol extract, thrips survival decreased (Figure 1). After fractionation, the effect of any of the individual fractions was significantly smaller than that of the methanol extract. The methanol extract resulted in the strongest slope (average ± 95% CIs: -13 ± 3.2) between log-transformed thrips survival and concentration (Figure 1). All five fractions also reduced thrips survival but to different extents. The CHCl₃, EtOAc and *n*-BuOH fractions resulted in a significant concentration-dependent decrease of log-transformed thrips survival while the hexane and H₂O fractions did not (Figure 1). Of the first three fractions, the rank of the slopes (± 95% CIs) was *n*-BuOH (-4.4 ± 1.8), EtOAc (-1.4 ± 1.0) and CHCl₃ fraction (-0.9 ± 0.6) (Figure 1). Based on the 95% CIs of the slopes, log-transformed survival significantly differed between the *n*-BuOH fraction and EtOAc/CHCl₃ fractions.

![Shoot methanol extract]( Shoot methanol extract (Slope: -13 ± 3.2, \[ R^2 = 0.98, F_{1,4}=168, P<0.001 \) )

![Shoot hexane fraction]( Shoot hexane fraction (\[ R^2 =0.52, F_{1,8}=2.1, P =0.28 \) )}
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Figure 1. Log-transformed survival of 2nd instar western flower thrips (*Frankliniella occidentalis*) against the amount of *J. vulgaris* plant shoot mass of a methanol extract and five fractions of the methanol extract. Thrips larvae were put on an artificial diet at five concentrations for 5 days. Survival was corrected for differences among negative controls. For significant regressions, lines are shown.

At all four tested concentrations, the expected survival if there was no interaction between the five fractions was higher than that of the original methanol extract from which they were derived. This means that the interaction effect between the combined five fractions was lower than one (Figure 2A), indicating that overall synergistic interactions prevailed. Taking the highest concentration as an example, the individual effects of the five fractions were compared with the interaction effect among the five fractions. The fraction survival as a result from the interaction (SF×F) was 0.6. The effect of the interaction on thrips survival was stronger than the effect of four of the five fractions while being only weaker than the effect of the *n*-BuOH fraction (0.4, Figure 2B).
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Figure 2. The effect of the interaction among the five fractions (A), and thrips survival for five fractions (S_f) and the effect of the interaction among the five fractions (S_f*F) at the concentration of 0.08 plant shoot mass (g/mL) (B). The expected survival for the combination of the five fractions if no interaction occurs was calculated by Equation 3. The interaction effect was calculated as the observed survival from the methanol extract divided by this expected survival for the combination of the five fractions (Equation 4).

PA levels and composition in fractions and the identification of CGA

In Chapter 6 we found that of the five shoot fractions, the majority of the PAs were present in the CHCl₃ fraction (67 %) (Figure 3). The n-BuOH fraction contained 24 %, the aqueous fraction 5 % and the ethyl acetate fraction 3 % and the hexane fraction 0.2 % of the total PA content (Figure 3). The identification of CGA was based on the analysis of NMR experiments, together with the comparison of reference compounds and previously reported data (Choi et al. 2006). In brief, the signals from the protons of CGA (H-8’ at δ 6.34, H-5’ at δ 6.84, H-6’ at δ 7.03, H-2’8 at δ 7.13, H-7’at δ 7.58) were clearly identified in the fractions. Peak areas were used for comparative qualitative analysis of CGA in different fractions.

Effect of individual PAs on thrips survival

Retrorsine and retrorsine N-oxide showed a significant dose-dependent effect on thrips survival (Figure 4). At a concentration of 1.4 mM, the thrips survival was not significantly different for retrorsine and retrorsine N-oxide, while at 7 mM, retrorsine showed a significantly lower survival than retrorsine N-oxide (Figure 4).
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Figure 3. The total PA concentration in the five fractions of *Jacobaea* shoots derived from the methanol extract (mg/g). Data are based on the LC-MS/MS analysis of the fractions in Chapter 6.

Figure 4. Log-transformed survival of 2nd instar western flower thrips (*Frankliniella occidentalis*) against the concentrations of retrorsine (solid dots) and retrorsine N-oxide (open dots). Dots in the graph are presented as average ± SE (n=4). The regression lines shown are based on the 12 individual measurements. The slopes (± 95% confidence intervals) are based on 4 independent replicates of bioassays (see Figure 5).
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Figure 5. Left: Fraction mortality (1 - fraction survival) of 2nd instar larvae of thrips (Frankliniella occidentalis) against the concentration of J. vulgaris plant shoot mass from which the CHCl₃ fraction was derived from the methanol extract and the concentration of retrorsine (A) and retrorsine N-oxide (B), and against the concentration of J. vulgaris plant shoot mass from which the n-BuOH fraction was derived with retrorsine (C) and retrorsine N-oxide (D). Right: Fraction survival (mean ± 95% confidence intervals) of thrips caused by the pyrrolizidine alkaloid alone (SPA) (white bars), plant fractions alone at three concentrations (SF, hatched bars), and the interaction effects (SPA*F, see Equation 2) between PAs and the plant fraction (grey bars). In the 3D figures, fraction mortality was plotted to increase the readability of the figure. In the right figures, dashed lines represent an interaction effect on thrips survival of one. * In one replicate the fraction survival of thrips in the combination of n-BuOH and retrorsine in one case was zero while the other replicate had a value of 0.038.
Effect of the combination of PAs and *Jacobaea* fractions on thrips survival

Compared to the survival in the presence of retrorsine alone, the combination with the CHCl$_3$ and the *n*-BuOH fractions decreased the effect of retrorsine and retrorsine N-oxide on thrips survival (Figure 5). Thrips survival for the different combinations of the two fractions and the two PAs was lower than expected assuming there was no interaction between them (Figure 5). With the exception of the combination of the CHCl$_3$ fraction and retrorsine (Figure 5A), the other 3 combinations showed significant synergistic effects, as the interactions effects $S_{PA*F}$ of all these combinations were significantly lower than 1 (Table 1 and Figure 5). For the combination of the CHCl$_3$ fraction and retrorsine the intercept was not significant, however the main effect, retrorsine concentration, was significant. The interaction effect $S_{PA*F}$ showed synergism present at 7 mM while antagonistic effects were present at 1.4 mM (Figure 5A).

Table 1. Two-way analyses of variance (ANOVAs) with fraction concentration and pyrrolizidine alkaloid (PA) concentration as fixed factors and the interaction effect $S_{F*PA}$ (Equation 2) minus one as a dependent variable. Survival was corrected for differences among the negative controls (Equation 1). A significant intercept or main effects indicate that synergistic or antagonistic interactions occur.

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<th>df</th>
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NS = Not significant

The synergistic effect is fraction-specific. Combined with PAs, the *n*-BuOH fraction showed a significantly stronger synergistic effect than the CHCl$_3$ fraction, irrespective of the PA type. The strength of the synergistic effect increased with PA concentration but was independent from the concentration of the fractions (Table 2). The interaction effect depended only marginally on the combination of the PA and the fraction (P = 0.06, Table 2).
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The ranking of the strength of the interaction effect $S_{PA*F}$ (mean ± S.E.) was: retrorsine + $n$-BuOH (0.57 ± 0.08), retrorsine N-oxide + $n$-BuOH (0.63 ± 0.09), retrorsine N-oxide + CHCl₃ (0.72 ± 0.04) and retrorsine + CHCl₃ (0.89 ± 0.07). The effects of the interaction on thrips survival were in all cases stronger or at least similar to that of PAs or fractions alone. As an example, the interaction effects between retrorsine N-oxide and the CHCl₃ fraction on thrips survival were significantly stronger than that of the CHCl₃ fraction at 0.01 and 0.09 g/mL (Figure 5B). For the combination of retrorsine N-oxide and the $n$-BuOH fractions, the interaction effects on thrips survival were significantly stronger than that of $n$-BuOH fractions at 0.01 and 0.09 g/mL (Table 2). The strongest effect was found for the interaction between retrorsine N-oxide and the $n$-BuOH fraction at 0.09 g/mL which lead to a survival of 0.27 (Figure 5D).

Table 2. Four-way analysis of variance (ANOVA) with PAs, fractions, PA concentration and fraction concentration as factors with the interaction effect $S_{PA*F}$ (Equation 2) as a dependent variable. PAs tested are retrorsine and retrorsine N-oxide. The fractions are the CHCl₃ and the $n$-BuOH fractions of a methanol extract from Jacobaea shoots. Each combination was tested in two independent bioassays. Survival was corrected for differences among the negative controls.

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<tr>
<td>PAs * Fractions * PA concentration * Fraction concentration</td>
<td>2, 46</td>
<td>1.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS = Not significant

Discussion

Are plant extracts and fractions of Jacobaea shoots toxic to thrips?

The methanol extract of Jacobaea shoots significantly decreased thrips survival compared to the negative control, even at concentrations that were much lower than the original concentration in the plant. Upon fractionation, the effects of individual fractions on thrips
survival were significantly smaller than that of the total methanol extract. We also theoretically combined the fractions and calculated the expected survival assuming no interaction. The magnitude of the interaction effect between the five fractions calculated in this way was significantly smaller than one, indicating an overall synergistic interaction. We should be careful however with the interpretation of the data because the loss of activity may be due to the loss of active constituents during the fractionation. The extract and fractions that were tested for the thrips bioassays from this chapter came from the same batch of ground plant material as for the Ames test (Chapter 6). As described in Chapter 6, we lost about 10% of the material during sub-fractionation. We therefore assume that the loss of activity upon sub-fractionation in the thrips bioassay was not caused by a loss of active constituents. We suggest that fractionation disrupted or even eliminated the interactions between metabolites. Our results show that using single metabolites to study their bioactivity would often not be a fruitful strategy if interactions are present. Similar conclusions were reached in a number of phytochemical studies that also often reported a loss of bioactivity upon fractionation, however without providing specific reasons for the observed loss of bioactivity (Williamson 2001; Herrera and Amor 2011; Labuschagne et al. 2012; Inui et al. 2012).

**Do plant fractions of *Jacobaea* shoots differ in their effects on thrips?**

The five shoot fractions of the methanol extract differed in their effects on thrips survival. The order of the PA content of the fractions was not consistent with the order of activity against thrips, suggesting that besides PAs, other metabolites also contributed directly or indirectly, through interacting effects, to the overall activity of the fractions. By using solvents with increasing polarity in the fractionation process, the fractions will contain different types of metabolites (Sasidharan et al. 2011). For instance, metabolites with low polarity (e.g. essential oils) will be extracted by the solvent with the lowest polarity, hexane (polarity index of 0.1). Moderately polar solvents such as CHCl$_3$ (polarity index of 2.7) and EtOAc (polarity index of 4.1) mainly will extract steroids, alkaloids, etc. Polar components like phenolic compounds, e.g. flavonoids and glycosides, are concentrated in the $n$-BuOH fraction (polarity index of 6.0). Water, the most polar solvent (polarity index of 10.2) is effective in extracting the metabolites with higher molecular weights such as proteins, glycans, etc. (Cos et al. 2006; Anupam et al. 2012). PAs were mainly present in the CHCl$_3$ fraction while the $n$-BuOH fraction showed the largest peak area for chlorogenic acid (CGA). Identification and characterization of chemical components especially those of concern will be a part of further work.

**How natural backgrounds influence the effects of individual PAs on thrips?**

To study possible interactions and to address the importance of natural backgrounds, we investigated the effects of individual metabolites in absence and presence of their natural
background by adding individual PAs to the most active fraction (the \( n \)-BuOH fraction) and the least active fraction (the CHCl\(_3\) fraction). When tested alone, retrorsine showed a stronger effect on thrips than retrorsine N-oxide. Both the CHCl\(_3\) and the \( n \)-BuOH fractions increased the effect of retrorsine and retrorsine N-oxide on thrips survival, showing synergistic interactions. In Chapters 3 and 4, we studied the interacting effects of PAs and CGA on thrips. We found antagonistic effects between retrorsine and CGA on thrips survival (Chapter 3) while we found synergistic effects between retrorsine N-oxide and CGA (Chapter 4). The synergistic effect between retrorsine N-oxide and CGA on thrips is in line with the fact that we found the strongest interactions between retoresine N-oxide and the \( n \)-BuOH fraction, because the \( n \)-BuOH fraction had the highest concentration of CGA (NMR data). However, in contrast to our expectation based on Chapter 3, we also found synergistic effects between retrorsine and the \( n \)-BuOH fraction. Apparently, there were effects of other metabolites that masked or even override the antagonistic effects between CGA and retrorsine. Further sub-fractionation and recombining sub-fractions can be used to narrow down the candidate metabolites that are involved in these interactions.

Following the whole-mixture analysis, a component-interaction analysis will provide more detailed information. The synergistic effects are fraction-specific. Specifically, the strength of the interaction of the \( n \)-BuOH fraction with PAs was stronger than that of the CHCl\(_3\) fraction. The PA concentration in the CHCl\(_3\) fraction was about three times higher than in the \( n \)-BuOH fraction. These results suggest that interactions between PAs and metabolites of other classes may dominate the overall synergistic effects between fractions and PAs on thrips.

**How important are natural backgrounds for the bioactivity of individual SMs?**

Because insect herbivores always encounter mixtures of metabolites in nature, the natural phytochemical background is of ecological relevance to both insects and plants. Despite this, for plant-insect associations, we know little about how natural backgrounds shape the bioactivity of individual metabolites. The effect of the interactions was similar or stronger than the effects of the metabolites alone (Figure 5), demonstrating the importance of interactions between these metabolites. Potential interactions may be even more interesting for compounds that are only weakly active or inactive on their own.

The result that the strength of the interaction depended on the fractions suggests that the effects of plant metabolites may vary depending on the phytochemical background in where they are. In a broad sense, this could explain, to some extent, that a single metabolite is active in one species while it is less active or even inactive in another species. For instance, CGA in *Chrysanthemum* was negatively correlated with the feeding damage of thrips (Leiss et al. 2009), while no effect of CGA on thrips was detected in tomato *Solanum lycopersicum* (Mirnezhad 2011).
A top-down approach to interactions between plant metabolites

In this chapter, we took a first step of a top-down approach to study the effect of the interactions between plant metabolites in their bioactivity. Results from this initial step gave us a general impression of the interactions between metabolites from a whole-metabolome point of view. Further progress demands to determine which metabolites are most likely to be involved in these interactions. This can be achieved by sub-fractionation in combination with e.g. NMR. One challenge in the following component-interaction analysis is to measure the effects of the infinite number of possible combinations due to the enormous number of metabolites in a plant. This would be an impossible task with the bioassays that are now used to study plant-insect interactions. In this light, high-throughput screening is essential for assaying the bioactivity of a large number of potential candidates against a chosen target. One of potential useful approaches to screen for anti-herbivore activities is the use of insect cell lines. However, for thrips cell lines are not available yet. Still, we can use cell lines of other insects, e.g. the beet armyworm, *S. exigua*. This has been used in our group (Nuringtyas et al. 2014). We found similar results regarding the interactions between PAs and CGA. On the other hand, bioassays on cell lines does not account for the digestive track on the toxicity of the metabolites. However, the cell lines can still be employed as the first step of the screening system to select candidate compounds or combinations of interest to be used in bioassays with the living organisms.

Acknowledgements

Xiaojie Liu thanks the China Scholarship Council of the Ministry of Education for financial support. We are grateful to Xianqin Wei for supplying plant material of *Jacobaea species*. We also thank Young Hae Choi for helpful suggestions on the extraction.

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The influence of the natural background


Chapter 5


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