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Chapter 6

The neglected side of interactions among plant metabolites: a case study of mutagenicity of plant extracts containing pyrrolizidine alkaloids

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Abstract

To determine the correlation between the bioactivity of a given plant and its metabolites, these are often isolated and tested as pure metabolites. If these metabolites lack the targeted bioactivity it is tempting to assume that they are not responsible for the bioactivity of the plant. However, the selected metabolites are obviously not alone in the plant. They co-exist with many other metabolites producing a chemically diverse array of bioactive metabolites. Although the mechanisms are not always known, it is highly likely that the bioactivity of a given metabolite is potentiated or reduced by other metabolites in the plant.

We tested five different shoot fractions and five root fractions derived from a methanol extract of *Jacobaea* plants. When the chloroform shoot fraction and the *n*-butanol root fraction of *Jacobaea* plants were investigated by the Ames test TA100 strain, significant mutagenic activity was found. Upon further fractionation the mutagenicity was largely lost in the sub-fractions, while the level of mutagenicity was restored when the sub-fractions were combined again, showing that the metabolites responsible for the mutagenic activity had not been degraded or were lost in the fractionation process. This suggests the presence of synergistic interactions between metabolites that were separated in the fractionation process. We then tested the mutagenicity of individual pyrrolizidine alkaloids (retrorsine and its N-oxide). We subsequently combined retrorsine with five shoot fractions, which naturally contain small amounts of retrorsine and other PAs. Retrorsine combined with chloroform and ethyl acetate fractions showed significant synergistic effects on mutagenicity while retrorsine showed equal or even lower mutagenicity when combined with the hexane, *n*-butanol and aqueous fractions. The mutagenic index (MI) of the combination of retrorsine and chloroform fraction was 2.7 times higher than the expected MI (4.09) calculated from the MIs of the retrorsine and the chloroform fraction alone.

This study used two methods to study mixtures for synergistic or antagonistic interactions. Specifically in the case of *Jacobaea* plants, it was shown that the weak or moderate mutagenicity for individual PAs was greatly potentiated when these metabolites were combined with chloroform and ethyl acetate fractions of the plant itself, indicative of synergistic interactions. The growing body of evidence on the importance of interactions between metabolites has major implications for the design of bioactivity studies in fields as toxicity, mutagenicity, health and plant protection.

**Keywords:** Natural background, Synergy, Antagonism, Ames test, Fractionation
Introduction

When searching for bioactive components, many phytochemical studies focus on assessing the bioactivity of individual metabolites (Hadacek 2002). These metabolites often have less activity than the plant material from which the metabolite was isolated (Herrera and Amor 2011). Plants contain a complex mixture of chemically highly diverse metabolites. It is well known that the bioactivity and metabolism of one metabolite may be affected by the presence of other metabolites through many mechanisms. Such mechanisms include for example, an increase in membrane permeability (Keukens et al. 1995), modification of metabolic processes (Keukens et al. 1995), the formation of a new complex, like the π-molecular complex between caffeine and CGA (Mösli Waldhauer and Baumann 1996), and blocking or disturbing membrane-bound receptor functions in insects, like the binding of alkaloids to insect alpha2, serotonin and nicotinic acetylcholine receptors (Wink et al. 1998; Liu et al. 2008). Saponins are well known to modify the cell membrane and thus facilitate the uptake of chemicals from the midgut (Gee et al. 1996; Herrmann and Wink 2011). Monoterpenes also can interact with the lipophilic side chain of phospholipids or cholesterol of the membrane and thus increase the membrane fluidity and permeability (Berenbaum and Zangerl 1993). In the black swallowtail Papilio polyxenes, combinations of furanocoumarins significantly reduced larval growth since individual furanocoumarins interfered with cytochrome P450-mediated metabolism of other furanocoumarins and prevented further metabolism (Berenbaum and Zangerl 1993; Nelson and Kursar 1998). These examples show that the occurrence of numerous metabolites in plants can result in many possible interactions to enhance or decrease bioactivity (Houghton 2000).

In nature, plants can benefit from the interactions between various metabolites. In combating natural enemies, synergistic interactions can increase plant fitness by producing a greater bioactivity at a lower cost. Interactions also have a wide range of applications, for instance in herbal medicine (Tallarida 2001; Williamson et al. 2001; Ma et al. 2009). Few studies address the interactions between plant metabolites and the effects of such interactions on the bioactivity of individual metabolites, especially the less active or inactive metabolites. In part this is due to the difficulty of detecting and analyzing interactions between plant metabolites in a proper manner (Nelson and Kursar 1999).

There are several ways to study the importance of interactions in plant extracts. One way to find prove for such interactions is to further sub-fractionate an active fraction. If the activity is based on interactions between metabolites, separating the interacting metabolites into different sub-fractions may result in the loss of the activity which can be restored again if the sub-fractions are recombined in their original proportions. Another approach is to test the effect of plant metabolites not only individually but also when added to the fraction or sub-fraction of plant extracts. In this paper we used both methods showing how the low or
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moderate mutagenic activity of individual metabolites was potentiated when they were added to fractionated plant material they were derived from.

Pyrrolizidine alkaloids (PAs) are a group of plant metabolites that occur naturally in *Jacobaea* plants. These PAs are notorious contaminants of animal fodder but also enter the human food chain e.g. via honey and tea (Mathon et al. 2014). Several PAs have been shown to be carcinogenic in rats (European Food Safety Authority 2011) and genotoxic in various assays, causing DNA binding and effects like DNA cross-linking, DNA-protein cross-linking, and mutagenicity (Fu et al. 2002). An important indicator of bioactivity is the Ames test for mutagenicity. Previous studies have measured the mutagenicity of individual PAs, demonstrating that clivorine, heliotrine, lasiocarpine, senkirkine, retrorsine, seneciphylline and riddelline were mutagenic in the Ames test using *Salmonella typhimurium* TA100 (Yamanaka et al. 1979; Frei et al. 1992; Rubiolo et al. 1992; Fu et al. 2001). However, in a recent study we showed that individual PAs were only weakly mutagenic, while extracts prepared from *S. jacobaea* were strongly mutagenic to *S. typhimurium* TA98 (Bovee et al. 2015). In this study, we investigated the mutagenicity of *Jacobaea* plant fractions, sub-fractions and the recombined sub-fractions. We also studied the effect on the mutagenicity of combining retrorsine with the different plant fractions. We specifically addressed the following questions: are fractions of *Jacobaea* plant extracts mutagenic? Is mutagenicity maintained after sub-fractionation of the active fractions? If not is mutagenicity restored if sub-fractions are put together again? Is the effect of retrorsine potentiated or mitigated when added to plant fractions?

**Material and Methods**

**Plant materials and chemicals**

One hundred *Jacobaea* genotypes were planted in a garden in Lisse (52° 25’ 12” N, 4° 54’10” E, The Netherlands) and grown for 17 months and harvested in March 2013. All plants were separated into shoots and roots, and were dried in an oven at 50°C, milled to a fine powder, then stored in an air-tight container and kept at room temperature until further use. All plants were separated into shoots and roots. The shoots of all plants were pooled and the same was done for the roots. The plant materials were extracted in April 2013 and the two most active fractions were sub-fractionated in August of 2013 (see below).

Retrorsine (R-0382, Lot 70K3450) and retrorsine N-oxide (R-0507, Lot 31K1407) were purchased from Sigma Aldrich (St. Louis, MO, USA). Quercetin, 2-amino-anthracene, biotin and L-histidine were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). Na₂HPO₄·2H₂O, NaH₂PO₄, MgSO₄·7H₂O, citric acid monohydrate, K₂HPO₄, NaNH₄HPO₄·4H₂O and glucose monohydrate were obtained from Merck (Darmstadt, Germany). NADP disodium salt and glucose-6-phosphate were obtained from Roche.
Diagnostics (Almere, The Netherlands), S9 rat liver mix from Trinova Biochem (Giessen, Germany), nutrient broth Nr 2 from Thermo Scientific (Landsmeer, The Netherlands) and agarose from Becton Dickinson (Breda, The Netherlands). The TA 100 strain used for the mutagenicity test was obtained from Prof. Bruce Ames (Berkeley, CA, USA).

**Extraction of *Jacobaea* shoots and roots and sub-fractionation**

Two separate extracts were made from the same batch of dried ground shoot and root plant material that was stored at -80°C. The first methanol extract of shoots and of roots was used to obtain five shoot fractions and five root fractions. These ten fractions were used in the Ames test.

Fifty grams of powdered *Jacobaea* shoot and root material were 3 times extracted for one hour with 80% methanol containing 0.1% formic acid (3×3 L) using a speed extractor (Büchi E-916, Büchi Labortechnik, Flawil, Switzerland) at 30°C and 50 bar. The three crude methanolic extracts were combined, evaporated under reduced pressure and redissolved in 100 mL of water. The aqueous extract was successively extracted with hexane (3×100 mL), chloroform (CHCl₃) (3×100 mL), ethyl acetate (EtOAc) (3×100 mL) and n-butanol (n-BuOH) (3×100 mL) (Supplementary material Figure S1). Removal of the solvents under reduced pressure yielded the hexane, CHCl₃, EtOAc, n-BuOH fractions and the residual H₂O fraction. Residues of these five fractions were each re-dissolved in 3.57 mL DMSO, from which 1.5 mL was used for testing a fraction in the Ames test, 199.4 μL was used for testing the fractions with the addition of retrorsine and 0.5 mL was evaporated and stored for PA analysis. The PAs in these root and shoot fractions were analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) as described below.

**Sub-fractionation of the chloroform shoot fraction and of the n-butanol root fraction**

The 2nd batch of dried ground plant material (50 g) was extracted and fractionated as indicated above. Because the CHCl₃ shoot fraction and the n-BuOH root fraction showed the strongest mutagenic effects (see below), they were used for a further sub-fractionation.

The complete CHCl₃ shoot fraction was dissolved in 1 mL CHCl₃ (384 mg corresponding to 50 g dry shoot) and was fractionated on a silica gel column (4 g, Büchi Labortechnik AG, Flawil, Switzerland), using a step-wise gradient elution of CHCl₃ and MeOH in the following compositions (v/v; 14 mL/increment): 100/0, 99.5/0.5, 99/1, 98.5/1.5, 97/3, 95/5, 90/10, 80/20, 70/30, 60/40, 50/50, 30/70 and 10/90. The profiles of most natural compounds e.g. phenols, sugars, steroids, and terpenes in these sub-fractions were visualized by thin-layer chromatography (TLC) on silica gel 60 F₂₅₄ (Merck, Darmstadt, Germany) with two different mobile phases: MeOH:CHCl₃ (1:1) and MeOH:CHCl₃ (1:4).
Spots were visualized with UV (ultraviolet) light (366 nm) and by spraying with an anisaldehyde reagent. The anisaldehyde reagent was a freshly prepared solution of 0.5 mL p-anisaldehyde in 50 mL glacial acetic acid and 1 mL sulfuric acid. Based on the TLC results, the 13 sub-fractions were pooled into 7 sub-fractions. The amounts of these 7 sub-fractions are given in the supplementary material (Figure S2).

The complete n-BuOH root fraction (556 mg) was dissolved in 2 mL 5% methanol in water and was fractionated on a C-18 column (10 g, 60 mL, Phenomenex, Torrance, CA, USA) and eluted using a step-wise gradient (60 mL/increment) of MeOH and H2O, starting at 5% MeOH with 5%-increments to 50% MeOH followed with 10%-increments to 100% MeOH. A total of 15 sub-fractions were collected and analyzed by TLC on silica gel (Si 60 F254) plates as above and eluted with a mobile phase of EtOAc:HCOOH:AcOH-H2O (100:11:11:27) and CHCl3-MeOH-H2O (6:5:1). Spots were visualized with UV light (366 nm) and by spraying with an anisaldehyde reagent. Based on the TLC results, the 15 sub-fractions were pooled into 6 sub-fractions. The amounts of these sub-fractions are given in Supplementary material Figure S2.

Each of the 7 CHCl3 shoot sub-fractions were dissolved in 5.95 mL dimethylsulfoxide (DMSO), from which 1.19 mL solutions (equal to 10 g plant material) were tested for the mutagenicity of individual sub-fractions. For testing the mutagenicity of the re-combined fractions, 1.19 mL of CHCl3 shoot sub-fraction was evaporated by freeze drying to dryness and re-dissolved in 170 μL DMSO (58.8 g plant material/mL). And another 0.5 mL solution was used for PA analysis by LC-MS/MS.

Each of the 6 n-BuOH root sub-fractions were dissolved in 5.95 mL DMSO, from which 1.19 mL solutions were tested for the mutagenicity of individual sub-fractions. For testing the mutagenicity of the re-combined fractions, 1.19 mL of n-BuOH root sub-fraction was evaporated to dryness and re-dissolved in 198 μL DMSO (58.8 g plant material/mL). And another 0.5 mL solution was used for PA analysis by LC-MS/MS.

Our previous results revealed that the mutagenic effects of methanol/water and acetone extracts of a mixture of Jacobaea vulgaris and S. inaequidens could be attributed to the flavonoid quercetin (Bovee et al. 2015). Therefore the shoot and root fractions of Jacobaea were analysed for quercetin by LC-MS/MS (see Bovee et al. 2015 for experimental details) but no quercetin was detected above the limit of detection (1 µg/g dry plant material).

**Analysis of pyrrolizidine alkaloids**

The LC-MS/MS analyses of PAs of the plant fractions and sub-fractions were conducted based on the protocol described in Cheng et al. (2011). Prior to analysis, 10 µL of each fraction or sub-fraction in DMSO was diluted with 1 mL of water and transferred to an
HPLC vial. Analysis was conducted on an Acquity UPLC system coupled to a Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA) operated in positive electrospray mode. Separation of the PAs was accomplished on a BEH C18 150 x 2.1 mm, 1.7 µm, UPLC column (Waters) by using an acetonitrile/water/6.5 mM ammonia gradient, from 0 to 50% acetonitrile in 12 min. Column temperature was set at 50°C and the flow at 400 µL/min. The PAs were quantified using external standard calibration prepared from a blank extract spiked with PA standards (range: 0-500 ng/mL). The limit of quantification for individual PAs in the fractions was approx. 0.5 µg/g dry plant material.

The Salmonella mutagenicity test (Ames test)

The Salmonella/microsome mutagenicity test, also known as the Ames test, is a short-term in vitro mutation assay designed to investigate whether chemical substances can cause gene mutations (Ames et al. 1975; McCann et al. 1975). Based on pilot studies we used Salmonella tester strain TA100, which is histidine dependent due to a mutation in the histidine operon (Maron and Ames 1983). The mutation is reverted to the wild-type state by mutagens that cause base-pair substitutions (point mutations) (Maron and Ames 1983).

We also measured the mutagenicity with metabolic activation using Arochlor induced rat S9 (for details of the method and the obtained results see the Supplementary File).

The mutagenicity assay was performed using a pre-incubation assay, by adding 50 µL of sample extract in DMSO to 500 µL of sodium phosphate buffer (0.2 M, pH 7.4), or to 500 µL of S9 mix, subsequently mixed with 100 µL of an overnight culture of S. typhimurium TA100, and then pre-incubated at 37°C for 30 min. Subsequently, 2 mL of molten top agar supplemented with 200 µL solution of L-histidine (0.5 mM) and D-biotin (0.5 mM) at 48°C was added to the mixture. After vortexing, the mixture (2.85 mL in total) was poured onto minimal glucose agar plates. The plates were incubated at 37°C for 3 days after which the revertant colonies were counted. An aqueous solution of 1.7% DMSO was applied as a negative (solvent) control and 1.0 mg/mL quercetin served as the positive control (Bjeldanes and Chang 1977; Czeczot et al. 1990; Resende et al. 2012). All fractions and sub-fractions were tested on triplicate plates.

Mutagenicity testing of Jacobaea plant fractions

The five root and five shoot fractions of the first batch prepared from Jacobaea plants were dissolved in 3.57 mL DMSO, corresponding to 14.0 g plant material/mL DMSO (See supplementary Figure S2). From these stock solutions further dilutions in DMSO with an end volume of 180 µL were prepared corresponding to 0.08, 0.14, 0.80, 1.4, 2.8, 8.4 and 14 g plant material/mL DMSO. Of each solution 50 µL was added to the plates, corresponding to: 3.99, 6.84, 39.90, 68.40, 139.65, 418.95 and 698.25 mg plant material/plate. The root
and shoot fractions were assayed on TA 100 without and with S9. Assuming an average water content of 90% in *Jacobaea* plants, this corresponds to 0.015x, 0.025x, 0.15x, 0.25x, 0.5x, 1.5x and 2.5x the average *Jacobaea* fresh weight concentration in a plant.

**Mutagenicity testing of the CHCl₃ and n-BuOH sub-fractions**

The sub-fractions each were dissolved in 5.95 mL DMSO (see above). The concentration of these stock solutions corresponded to 8.4 g plant material/mL (418.95 mg/plate) based on the amounts of sub-fractions (Supplementary material Figure S2). From these stock solutions further dilutions in DMSO with an end volume of 180 μL were prepared corresponding to 0.08, 0.14, 0.80, 1.4, 2.8 and 8.4 g plant material/mL DMSO. Note that the highest concentration of 14 g plant material/mL DMSO was not tested for the sub-fractions. Of each solution 50 μL was added to the plates, corresponding to: 3.99, 6.84, 39.90, 68.40, 139.65 and 418.95 mg plant material/plate.

**Mutagenicity testing of recombined sub-fractions**

The seven CHCl₃ shoot sub-fractions and six n-BuOH root sub-fractions were recombined in the original proportions to reconstitute the original CHCl₃ and n-BuOH fractions. These combined sub-fractions were only tested at a concentration of 8.4 g/mL plant to test whether the original observed mutagenicity could be restored. To avoid a dilution effect during the combining process, 1.19 mL of the 7 shoot CHCl₃ sub-fractions in DMSO were evaporated and re-dissolved in 170 μL DMSO (see above). The concentration of these solutions is exactly 7 times higher than the original concentration, so that on combining those solutions in equal amounts the final concentration is equal to the undiluted CHCl₃ fraction. In practice, 60 μL solution from each of the sub-fractions was added together to obtain the re-combined fraction.

To avoid a dilution effect during the combining process, 1.19 mL of the 6 root n-BuOH sub-fractions in DMSO were evaporated and re-dissolved in 198 μL DMSO (as explained above). The concentration of these solutions is exactly 6 times higher than the original concentration, so that on combining those solutions in equal amounts the final concentration is equal to the undiluted n-BuOH fraction. In practice, 60 μL solution from each of the sub-fractions was added together to obtain the re-combined fraction.

**Mutagenicity testing of PA standards**

Retrorsine and retrorsine N-oxide (174.5 mg) were dissolved in 1.33 mL of DMSO, respectively, to prepare two stock solutions with a concentration of 131.2 mg/mL. The stock solutions were diluted with DMSO to a series of dilutions with a concentration of 5.8, 11.6, 28.4, 79.8 and 131.2 mg/mL DMSO. Of each solution 50 μL was added to the plates, corresponding to: 0.29, 0.57, 1.42, 3.99 and 6.56 mg retrorsine or retrorsine N-oxide/plate.
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The concentrations were equivalent to approximately 0.2x, 0.4x, 1.0x, 2.8x and 4.6x, respectively, of the total PA concentration in fresh weight *Jacobaea* plants.

**Adding retrorsine to fractions of *Jacobaea* shoots**

This test was carried out with retrorsine only because it gave a higher mutagenicity than retrorsine N-oxide (see results). To test for interactions between PAs and *Jacobaea* fractions the combination of retrorsine and five shoot fractions was used. Retrorsine (316.8 mg) was dissolved in 1.2 mL DMSO to provide a stock solution of 264 mg/mL. 199.4 μL aliquots of the stock solutions of the five fractions (50 g shoots in 3.57 mL DMSO, see above) were evaporated by freeze drying to dryness and re-dissolved in 100 μL DMSO corresponding to 27.93 g shoot/mL. From this stock solution further dilutions in DMSO with an end volume of 100 μL were prepared corresponding to 0.279, 2.79 and 27.93 g shoot material/mL DMSO. On each plate, a mixture of 25 μL retrorsine stock solution and 25 μL fraction stock solution was added. Consequently, the final concentration was 6.56 mg/plate of retrorsine and three different concentrations (6.84, 68.40 and 698.25 mg shoot/plate) of the five shoot fractions.

The five shoot fractions of the first batch prepared from *Jacobaea* plants used in testing shoot and root fractions from above were used a control. Of each solution 50 μL was added to the plates, corresponding to: 6.84, 68.40, and 698.25 mg plant material/plate. We also included again retrorsine as control using the stock as indicated above. Of the solution 50 μL was added to the plates, corresponding to 6.56 mg retrosine/plate.

**Statistical analysis**

*Colony numbers and mutagenic index (MI)*

The colony numbers counted per plate were averaged over the three replicates and expressed as the mean number of revertant colonies per plate ± standard error of the mean (SE). To determine whether there was a dose-related increase or decrease in the colony numbers, the colony numbers per plate were regressed against PA concentrations.

A commonly used measure is the mutagenic index (MI) (Bjeldanes and Chang 1977; Maron and Ames 1983; Czeczot et al. 1990; Resende et al. 2012). For all treatments, the mutagenic index (MI) was calculated as the average number of revertant colonies per plate divided by the average number of revertants per plate of the negative (solvent) control. The 95% confidence intervals (CIs) of MIs were estimated by equation (1) and were used to compare the effects of different PAs. MI therefore represents the mean ± 95% CIs of three replicates (n = 3 in each group).
95% CIs = $\frac{\text{Var}}{\sqrt{n}} \times 1.96$  \hspace{1cm} (1)

With $\text{Var}$ being the variance of the MIs of the three replicates in each group and $n$ being the sample size of each group. If two MIs have non-overlapping 95% CIs they are assumed to be significantly different at the $p < 0.05$ level.

The mutagenic effects of retrorsine and retrorsine N-oxide were compared by a two-way analysis of variance (ANOVA) with PAs and PA concentrations as fixed factors and MI as dependent variable.

**Testing on mutagenicity on two or more metabolites or fractions**

To evaluate interactions, one typically constructs an expected “null interaction” model that predicts the effect of metabolites in the absence of an interaction. We constructed a null model under the assumption that there is no interaction between two metabolites. Since the probability of a revertant colony (circa 300) is very small compared to the initial number of cells (circa $10^5$–$10^6$) the effects of different metabolites can be considered additive if there is no interaction.

The expected combined effect of metabolites A and B ($N_{A,B}$) is the sum of the effect of metabolite A ($N_A$) and the effect of metabolite B ($N_B$) if these metabolites do not interact.

$$N_{A,B} = N_A + N_B \hspace{1cm} (2)$$

In the Ames test, the final colony number ($N$) on plates is counted. The observed number of colonies results from spontaneous mutations and from the mutagenic effect of the tested metabolite. Therefore the total colony number ($N_{\text{obs},X}$) found after testing metabolite X can be written as:

$$N_{\text{obs},X} = N_X + N_{\text{NCX}} \hspace{1cm} \text{and hence:} \hspace{1cm} N_X = N_{\text{obs},X} - N_{\text{NCX}} \hspace{1cm} (3)$$

With $N_X$ being the number of colonies due to metabolite X and $N_{\text{NCX}}$ being the number of spontaneous colonies in the negative control.

Assuming there is not interaction, for a mixture of two metabolites equations (2) and (3) can be combined to obtain:

$$(N_{\text{obs},A,B} - N_{\text{NCA,B}}) = (N_{\text{obs},A} - N_{\text{NCA}}) + (N_{\text{obs},B} - N_{\text{NCB}}) \hspace{1cm} (4)$$

With $N_{\text{obs},A,B}$ being the observed colony number in the treatment with metabolites A and B together, $N_{\text{NCA,B}}, N_{\text{NCA}}$ and $N_{\text{NCB}}$ being the number of spontaneous colonies in the negative control (DMSO only). $N_{\text{obs},A}$ being the observed number of colonies for the treatment with
metabolite A and \(N_{\text{obs,B}}\) being the observed number of colonies for the treatment with metabolite B. As each treatment has its own negative control, we weighed the above equation by dividing the colony numbers by their own negative controls, because the number of the revertant colonies of negative controls reflects the density of the bacterial suspension in the experiment:

\[
\frac{(N_{\text{obs,A,B}} - N_{\text{NCA,B}})}{N_{\text{NCA,B}}} = \frac{(N_{\text{obs,A}} - N_{\text{NCA}})}{N_{\text{NCA}}} + \frac{(N_{\text{obs,B}} - N_{\text{NCB}})}{N_{\text{NCB}}} \quad (5)
\]

\[
\frac{N_{\text{obs,A,B}}}{N_{\text{NCA,B}}} - 1 = \frac{N_{\text{obs,A}}}{N_{\text{NCA}}} - 1 + \frac{N_{\text{obs,B}}}{N_{\text{NCB}}} - 1 \quad (6)
\]

Because the colony number of a treatment group divided by the colony number of its negative control is the mutagenic index (MI), assuming no interaction between metabolites A and B equation (6) can be expressed as follows:

\[
\text{MI}_{\text{obs,A,B}} = \text{MI}_{\text{obsA}} + \text{MI}_{\text{obsB}} - 1 \quad (7)
\]

With \(\text{MI}_{\text{obs,A,B}}\) being the observed MI in treatment with metabolites A and B, \(\text{MI}_{\text{obsA}}\) and \(\text{MI}_{\text{obsB}}\) being the MI of the treatment of metabolite A and B, respectively. When the equation of formula 7 is not met i.e. \(\text{MI}_{\text{obsA}} + \text{MI}_{\text{obsB}} - \text{MI}_{\text{obs,A,B}} \neq 1\) it can concluded that an interaction is present.

For the combination of n metabolites/fractions we get:

\[
\text{MI}_{\text{obs,1,2,...n}} = \text{MI}_{\text{obs1}} + \text{MI}_{\text{obs2}} + \text{MI}_{\text{obs3}} + \cdots + \text{MI}_{\text{obsn}} - (n-1) \quad (8)
\]

Again, when \(\text{MI}_{\text{obs1}} + \text{MI}_{\text{obs2}} + \text{MI}_{\text{obs3}} + \cdots + \text{MI}_{\text{obsn}} - \text{MI}_{\text{obs,1,2,...n}} \neq n-1\), it can concluded that an interaction is present.

The variance of \(\text{MI}_{\text{obs1}} + \text{MI}_{\text{obs2}} + \text{MI}_{\text{obs3}} + \cdots + \text{MI}_{\text{obsn}} - \text{MI}_{\text{obs,1,2,...n}}\) can be estimated with:

\[
\text{Var (MI}_{\text{obs1}}) + \text{Var (MI}_{\text{obs2}}) + \cdots + \text{Var (MI}_{\text{obsn}}) + \text{Var (MI}_{\text{obs,1,2,...n}}) \quad (9)
\]

The 95% CIs of \(\text{MI}_{\text{obs1}} + \text{MI}_{\text{obs2}} + \text{MI}_{\text{obs3}} + \cdots + \text{MI}_{\text{obsn}} - \text{MI}_{\text{obs,1,2,...n}}\) is then estimated by equation (9) multiplied by 1.96. This 95% confidence limit was used to estimate if \(\text{MI}_{\text{obs1}} + \text{MI}_{\text{obs2}} + \text{MI}_{\text{obs3}} + \cdots + \text{MI}_{\text{obsn}} - \text{MI}_{\text{obs,1,2,...n}}\) is significantly deviating from n-1.

Synergistic interactions are present if the experimentally observed MI is significantly higher than n-1 and antagonistic interactions are present if the observed MI is significantly lower than n-1.

All statistical analysis were performed using SPSS software for Windows (version 21.0; SPSS Inc., Chicago, IL).
Chapter 6

Results

Mutagenicity of *Jacobaea* plant fractions

In both cases of shoots and roots, with the exception of the hexane fraction, all the other fractions resulted in a concentration-dependent increase in revertant colony numbers of TA100 (Figure 1). Of the ten fractions, only the CHCl₃ fraction of shoots and the *n*-BuOH fraction of roots exhibited MIs above 2.

![Figure 1](image)

*Figure 1. Number of revertant colonies of *Salmonella typhimurium* TA100 exposed to 7 different concentrations of five fractions of *Jacobaea* shoots (A) and roots (B) and a control (without metabolic activation).* Data are presented as the mean number of revertant colonies per plate ± standard error of the mean (SE) of three replicates. The dashed lines shows MI = 2. Arrows indicate a significant positive correlation between plant shoot/root concentration and colony number. Levels indicated are the amount of original plant material per plate.

With metabolic activator S9, the hexane and the EtOAc fractions of shoots had no significant effects on TA100 strain while the CHCl₃, *n*-BuOH and H₂O fractions of shoots...
induced a concentration-dependent increase in revertant colony numbers in TA100 (Figure S3A). All root fractions, except the hexane fraction induced a concentration dependent increase of revertant colony numbers of TA100 with S9 (Figure S3B). With S9, the MIs of the CHCl₃ shoot fraction and the n-BuOH root fraction were above 2 (Figure S3).

Of the five shoot fractions, the highest content of the total PA was detected in the H₂O fraction (0.60 mg/plate), followed by the n-BuOH fraction (0.48 mg/plate) and the CHCl₃ fraction (0.34 mg/plate) (Figure 2A). Although the total PA content in the n-BuOH fraction was 1.5 times higher than that in the CHCl₃ fraction, the latter caused the highest mutagenicity (MI = 4.10) among the five shoot fractions. For retrorsine N-oxide at a concentration similar to that of the total PA’s in the CHCl₃ fraction we found MI=1.21. Among the five root fractions, the n-BuOH fraction contained the highest content of the total PA content (1.06 mg/plate), followed by the H₂O fraction (0.64 mg/plate) and the CHCl₃ fraction (0.59 mg/plate) (Figure 2B). Containing the highest total PA content, the n-BuOH root fraction also caused the strongest mutagenicity (MI = 2.51). For retrorsine N-oxide at a concentration similar to that of the total PA’s in the n-BuOH fraction we found MI = 1.46. Altogether, the results of the PA analysis of the fractions suggest that natural occurring PA’s were not the main cause of mutagenicity.

The two most active fractions, the shoot CHCl₃ fraction and the root n-BuOH fraction, were subjected to further fractionation.

![Figure 2](image-url)

**Figure 2.** The total amount of naturally occurring PAs per Ames test plate for the five shoot fractions (A) and the five root fractions (B), for the highest tested dose of *Jacobaea* plant shoot (698 mg/plate).

**Synergistic effects between the sub-fractions of the CHCl₃ shoot and n-BuOH root fractions**

The 1ˢᵗ, 2ⁿᵈ, 4ᵗʰ, and 7ᵗʰ sub-fractions of the CHCl₃ shoot fraction induced a concentration-related increase of revertant colony numbers (Figure 3A). However, the MIs of all sub-
fractions were below 2 (Figure 3A), despite the fact that the original CHCl₃ fraction resulted in MI values above 3.3 at the same concentration. Similar results were observed for the sub-fractions of the \textit{n}-BuOH fraction. The MIs of all sub-fractions were below 2 (Figure 3B). Thus, in both cases, sub-fractionation led to a loss of most of the mutagenic activity. In spite of this, PAs are still present in some of the sub-fractions, and to a different extent. Of the seven sub-fractions of the CHCl₃ shoot fraction, SFr.S7 contained the highest amount of PAs (0.08 mg/plate) while SFr.S6 contained 0.02 mg/plate (Figure 4A). For sub-fractions of the \textit{n}-BuOH root fraction, the total PA content in SFr.R2, SFr.R3 and SFr.R4 on one plate was 0.04, 0.17, and 0.19 mg (Figure 4B). This suggests that PAs themselves may not be related to the observed MIs but they may play a role in combination with other compounds.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Number of revertant colonies (mean ± S.E., n=3) of \textit{Salmonella typhimurium} TA100 for seven sub-fractions from \textit{Jacobaea} CHCl₃ shoot extract (SFr.S1-SFr.S7) at 6 different concentrations and a control (A) and six sub-fractions from \textit{Jacobaea} \textit{n}-BuOH root extract (SFr.R1-SFr.R7) (B) \textit{(without metabolic activation)}. The dashed lines shows MI = 2. Arrows indicate a significant positive correlation between plant shoot/root concentration and colony numbers. Levels indicated correspond to the original amount of plant material extracted.}
\end{figure}
Figure 4. The total amount of naturally occurring PAs per Ames test plate for the 7 sub-fractions of the CHCl₃ shoot fraction (A) and the 6 sub-fractions of the n-BuOH root fraction (B) for the highest tested dose of Jacobaea plant shoot (698 mg/plate).

The reconstitution of the CHCl₃ shoot fraction by combining the sub-fractions in their original mass ratios restored 85% of the MI value of the original fraction (Figure 5). Reconstitution of the n-BuOH root sub-fractions fully restored the MI value of the original fraction (Figure 5). The MI of the restored CHCl₃ fraction was significantly higher than the expected MI (2.38 ± 0.29) (Figure 5) showing a synergistic interaction between the sub-fractions. Similarly, the MI of the restored n-BuOH fraction was significantly higher than the expected MI of 1.76 (± 0.04) (Figure 5).

Figure 5. Observed and expected mutagenic index (MI) of the CHCl₃ fraction and the expected and observed MI of the recombined seven sub-fractions of the CHCl₃ fraction (SFr.S1-SFr.S7) and the six sub-fractions of the n-BuOH fraction (SFr.R1-SFr.R6) (TA100 without metabolic activation) at 418.95 mg plant material/plate. The open bar indicates the expected MI based on the MIs of the individual sub-fractions assuming no interaction (see M&M for the calculation). The solid bar gives the MI of the restored fraction obtained by recombination of the sub-fractions. The hatched bar gives the MI of the original fraction. Data are presented as means ± 95% confidence intervals (CIs) of three replicates. Different letters indicate significant differences between the three columns.
Chapter 6

Mutagenicity of individual PAs

Overall, retrorsine and retrorsine N-oxide caused mutagenicity in a dose dependent manner (Figure 6). The effect of concentration depended on the PA tested as indicated by a significant interaction between PA and concentration, which resulted in a significant interaction in the ANOVA (Table 1).

Table 1. Two-way ANOVA with PA (retrorsine and retrorsine N-oxide) and PA concentration as fixed factors with mutagenic index (MI) without metabolic activator S9 of the Ames test as a dependent variable.

<table>
<thead>
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<th>Factors</th>
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<tbody>
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<td>PA* Concentration</td>
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<td>8</td>
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</table>

Interaction between retrorsine and *Jacobaea* shoots fractions

The model to test for interactions that we described above assumes that the relationship between the concentration of a component and its activity is linear. Inspection of (Figure S4) showed that this was only true for the two lowest concentrations of the shoot fractions. With higher concentrations the mutagenicity levels off. Consequently, if we would calculate significant interactions at the highest concentration of the fractions, such an estimate would most likely be an under estimation for synergistic interaction and an overestimation of antagonistic effects.

Retrorsine showed a MI of 2.82 (± 0.04) at 6.56 mg/plate whereas the MI for all three concentrations of the hexane shoot fractions were below 1 (Figure 7A). The combinations...
of hexane fractions at 6.84 and 68.4 mg/plate with 6.56 mg/plate of retrorsine, gave an MI that was below the expected MI assuming no interaction. This significant reduction in MI compared to the expected MI indicates that an antagonistic interaction is present. The combinations of the n-butanol fraction and the aqueous fraction with retrorsine also showed significant antagonistic interactions (Figure 7D, 7E).

In contrast, we found synergistic effects when retrorsine was added to the CHCl₃ and ethyl acetate shoot fractions (Figure 7B, 7C). Therefore, the combined treatments of Jacobaea shoot fractions and retrorsine showed both synergistic and antagonistic interactions.
Figure 7. Mutagenic index (MI) ± 95% confidence intervals of retrorsine, 5 shoot fractions, and retrorsine added to these shoot fractions (Salmonella typhimurium TA100, without metabolic activation). Results represent the mean mutagenic index (MI) of three replicates. The solid bars give the observed MIs of retrorsine and fractions alone. The open bars give the expected MIs based on the MIs of retrorsine and the individual fractions assuming no interaction (see Equation 8). The hatched bars give the observed MIs of retrorsine added to plant fractions. A. the hexane fraction; B. the CHCl₃ fraction; C. the ethyl acetate fraction; D. the n-butanol fraction; E. the aqueous fraction. * indicates a significant difference between expected and observed MI.

Discussion

In this paper, we showed interacting effects between metabolites co-occurring in the same plant on a given bioactivity. We used two different methods. In the first place, we showed that sub-fractionation led to loss of activity. When the sub-fractions were recombined again the activity was restored, demonstrating synergistic interactions among plant metabolites. Secondly, we showed that adding individual metabolites to plant fractions, that already contained low amounts of that metabolite, resulted in a greater or lower level of activity than expected under the assumption that there is no interaction. This implies the presence of synergistic and antagonistic interactions, respectively.

Jacobaea fractions caused base-pair substitution mutations according to the Ames test with tester strain TA100. After screening all the fractions derived from the methanol plant
The neglected side of interactions among plant metabolites

extracts, we selected the chloroform shoot fraction and the n-butanol root fraction for further sub-fractionation because they showed the strongest mutagenicity.

As the highest mutagenicity was observed without S9 we tested the sub-fractions only on the TA100 strain without S9. After sub-fractionation the sum of the mutagenic effects of the individual sub-fractions was much lower than that of the total fractions. Further sub-fractionation could even have resulted in a stronger decline in mutagenic activity resulting in the failure to isolate the active ingredient(s) responsible for the mutagenic activity of the fractions (Stermitz et al. 2000; Liu et al. 2003). Because the mutagenicity was restored after recombining the sub-fractions, this weaker mutagenicity cannot be attributed to a loss or a decrease in the concentration of a certain metabolites as a result of the fractionation process. We compared the contents of the total PA in fractions and sub-fractions and found that the natural occurring PAs can only partly explain the observed mutagenicity.

When attempting to identify the metabolites that are responsible for a certain bioactivity in a plant, it is possible to overlook the contribution of a metabolite because it shows a low or perhaps even no activity when tested as a pure metabolite. Such a result may be misleading if the metabolite is only active in the presence of other metabolites that are present in the plant material. Here, in 11 out of 15 cases studied, significant interactions were detected, showing that interactions between metabolites are more the rule than the exception. To study the importance of these interactions, we investigated the mutagenicity of individual metabolites in absence and presence of their natural background by adding retrorsine to plant fractions that also contain this PAs and related PAs naturally. Retrorsine is known to cause base-pair substitution mutations in the Ames test (Rubiolo et al. 1992). In general, we found a significant dose-dependent relationship between revertant colony numbers and retrorsine.

When we added retrorsine to the shoot CHCl₃ and ethyl acetate fractions of Jacobaea plants, they showed a significant synergistic interaction with retrorsine. The hexane fraction was non-mutagenic at all the concentrations that were tested. However, in combination with retrorsine, the mutagenicity of the combination was lower than that of retrorsine alone at all the concentrations, demonstrating clear antagonistic effects. For the combination of n-BuOH fraction and retrorsine and the combination of H₂O fraction and retrorsine, we found antagonistic effect at all concentrations. The strongest antagonistic effect was observed at the highest concentration. However, we should be careful about the apparent effect at the highest concentration, which could be overestimated because the dose-response curves for the two fractions were non-linear. This demonstrates that most likely the natural occurring PAs in fractions were not causing these interactions between retrorsine and fractions because the CHCl₃ fraction exhibited synergistic interactions whereas the n-BuOH fraction exhibited antagonistic interactions, even though a higher amount of naturally occurring total PA was detected in the n-BuOH fraction than in the CHCl₃ fraction (Figure 2).
The co-existence of numerous metabolites in plants suggests an enormous potential for interactions between plant metabolites. If it is the interaction between certain metabolites rather than a particular metabolite that contributes to a certain bioactivity of a plant, the traditional fractionation approach searching for bioactive metabolites may fail. On top of this, a central issue is how to detect the influence of interactions between plant metabolites on bioactivity given the infinite number of possible combinations of plant metabolites. Consequently, it is extremely difficult to detected interactions from regression studies without prior knowledge about the metabolites involved. The same is true for experimental studies which cannot cover all combination arrays of plant metabolites. In this regard, a natural phytochemical background, e.g. plant extract or fractions of extracts, provides a natural combination treatment and therefore offers an effective strategy to measure the potential interactions of plant metabolites.

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References

European Food Safety Authority. 2011. Scientific opinion on pyrrolizidine alkaloids in food and feed. EFSA Journal. Parma, Italy. 9:2406
The neglected side of interactions among plant metabolites


Herrmann F, Wink M. 2011. Synergistic interactions of saponins and monoterpenes in HeLa cells, Cos7 cells and in erythrocytes. Phytomedicine 18:1191-1196


Liu RH. 2003. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am J Clin Nutr 78:517s-520s


Mösli Waldhauer SS, Baumann TW. 1996. Compartmentation of caffeine and related purine alkaloids depends exclusively on the physical chemistry of their vacuolar complex formation with chlorogenic acids. Phytochemistry 42:985-996


Supplementary Materials:

Preparation of metabolic activator S9

Within organisms such as herbivores, interactions may occur not only between the original metabolites that are eaten but also between metabolites that are formed after the original compounds are metabolized. Many substances are broken down in the liver. However, in some cases the breakdown of metabolites in the liver leads to (unwanted) metabolic activation. For instance, some carcinogenic chemicals, such as aromatic amines, are biologically inactive unless they are metabolized to active forms. In humans and animals, the cytochrome-P450 metabolic system, which is present mainly in the liver, is capable of metabolizing a large number of chemicals to DNA-reactive, electrophilic forms. Since bacteria do not have this metabolic capability, an exogenous mammalian organ activation system needs to be added together with the test chemical and the bacteria in order to assess the activity after metabolization. Therefore we used a commonly used metabolic activator, lyophilized S9 from the liver of Aroclor 1254-induced male Sprague-Dawley rats (Molecular Toxicology, Lot. no. 3015, Boone, NC, USA). The S9 fraction was reconstituted in water and added to the culture medium that contained the cofactors for the generation of NADPH. The final composition of the S9 mixture was as follows: MgCl2–KCl salt solution (1.65 M KCl + 0.4 M MgCl2) (0.6 mL), 0.2 M sodium phosphate buffer pH 7.4 (15 mL), 0.1 M NADP (1.2 mL), 1 M glucose-6-phosphate (0.15 mL), S9 (2.1 mL), sterile distilled water (10 mL). The S9 mixture was freshly prepared and stored on ice for each experiment.
Figure S1. Extraction flowchart of the 1st batch of *Jacobaea* shoots and roots and absolute yield in g and the yield (%) in relation to the amount of starting material.
Figure S2. Extraction and fractionation flowchart of the 2nd batch of *Jacobaea* shoots and roots and absolute yield in g and the yield (%) in relation to the amount of starting material.
Figure S3. Number of revertant colonies (mean ± S.E.) of *Salmonella typhimurium* TA100 exposed to a control and five fractions at 7 different concentrations of *jacobaea* shoots (A) and roots (B) (with metabolic activation). Dashed lines show MI = 2. Arrows indicate a significant positive correlation between plant shoot/root concentration and colony numbers. Levels indicate the amount of original plant material per plate.
Figure S4. Colony numbers of five fractions of *Jacobaea* shoots (A) and retrorsine (B) against the concentrations (mg/plate). Concentrations that were tested for interaction effects are indicated by arrows. Note that the lowest concentration tested is very close to the origin.