Screening of Panamanian plant extracts for agricultural and cosmetic activities, and metabolomic study of *Isatis tinctoria* accessions

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List of abbreviations

ABTS	2,2‘-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)
AGROCOS	Agricultural chemistry and cosmetics (FP7 project)
BASF	Badische Anilin- & Soda-Fabrik (German chemical company)
Calcein-AM	*Calcein* acetomethoxy
CA	Canonical analysis
COSY	Correlation spectroscopy (NMR technique)
DAD	Diode array detector
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-pikrylhydrazyl
ECD	Electronic circular dichroism
FP7	Framework Program 7
GABA	*gamma*-aminobutyric acid
HMBC	Heteronuclear multiple bond correlation (NMR technique)
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum correlation (NMR technique)
HTS	High throughput screening
IL	Interleukin
k-NN	k-nearest neighbor concept
MANOVA	Multivariate analysis of variance
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nAChR	Nicotinic acetylcholine receptor
NMR	Nuclear magnetic resonance
OPLS	Orthogonal partial least squares regression
OPLS-DA	Orthogonal partial least squares discriminant analysis
PCA	Principal component analysis
PCA/CA/k-NN	Combination of PCA, CA, and k-NN
PLE	Pressurized liquid extraction
PLS	Partial least squares regression
PLS-DA	Partial least squares discriminant analysis
STOCSY	Statistical total correlation spectroscopy (statistical method)
TLC	Thin layer chromatography
TOCSY	Total correlation spectroscopy (NMR technique)
TOF	Time of flight
TXI	Triple resonance inverse (NMR probe)
UV	Ultraviolet light

Summary

The FP7 project AGROCOS aimed at the discovery of new natural product scaffolds for possible uses in agrochemistry and as cosmetic ingredients. In addition, novel tools for metabolomics and multivariate statistics were applied to *Isatis tinctoria* as a model plant.

Nature provides compounds that are not only important for pharmaceutical industry, but also for agrochemistry and cosmetics. Extracts and pure natural compounds are widely used in cosmetics. For agrochemistry, natural products rather play a role for lead discovery, i.e. the discovery of a model compound that is further developed to a synthetic agrochemical. In the first part of the project, a library of 600 Panamanian plant extracts was screened for agrochemical and cosmetic properties. Selected extracts were submitted to HPLC-based activity profiling whereby five extracts showed activity in the agrochemistry screens, and two extracts in the cosmetic screens.

For the agrochemical part of the project, extracts and microfractions were screened for fungicidal, insecticidal, and herbicidal properties. Compounds from *Bocconia frutescens*, *Miconia affinis*, *Myrcia splendens*, *Combretum affinis laxum*, and *Erythroxylum macrophyllum* were isolated, identified, and submitted to screening for the corresponding indication. Benzophenanthridine alkaloids and arjunolic acid from *B. frutescens* and *M. affinis*, respectively, showed moderate to good fungicidal activity. Myricetin-3-O-(6"-O-galloyl)-β-galactopyranoside from *M. splendens* showed moderate insecticidal activity. From the two plants selected for herbicidal activity of their extracts, no herbicidal compounds could be identified.

For the cosmetic part, phenolic compounds and flavonoids were isolated from methanolic extracts of leaves from *Mosquitoxylum jamaicense* and *Combretum cacoucia*. All isolated compounds showed either activity in the DPPH assay, in the UV-B protection assay, or in both. Gallic acid derivatives were the most active in the DPPH assay (IC_{50} < 10 µg/mL), while protocatechuic acid and isoquercitrin showed the best activity in the UV-B protection assay (< 10% UV-B-induced cell death).

Metabolomics of plant extracts combined with multivariate statistics is increasingly used for the differentiation of plant samples and assessment of quality traits. Metabolomics provides a metabolic fingerprint, in contrast to the classical quantitative analysis of single compounds, or a single class of compounds. The second part of the AGROCOS project comprised a metabolomic study on *Isatis tinctoria* of five different accessions, which were grown under identical and controlled conditions on experimental field plots. Leaves were harvested at six time points during the vegetation period, whereby sampling was done, on the one hand, by collection of single leaves from intact plants, and by repeated mowing of leaf rosettes, on the other hand. The latter regimen was to simulate repeated harvesting of annual plants. Extracts were prepared by PLE with ethyl acetate and 70% aqueous methanol, submitted to ^{1}H NMR spectroscopy, and analyzed by the PCA/CA/k-NN classification method. PCA/CA/k-NN analysis of accessions and time points of harvesting showed differences in both cases. Using pairwise 1D-STOCSY, compounds such as unsaturated fatty acids, porphyrins, carbohydrates, indole derivatives, isoprenoids, phenylpropanoids, and minor aromatic compounds could be identified as metabolites contributing to differences of accessions and harvesting dates. In addition, an effect of repeated harvesting could be observed from the PCA/CA/k-NN plot, and

the 1D-STOCSY plot revealed a decrease of 1,5-anhydroglucitol, sucrose, unsaturated fatty acids, porphyrins, isoprenoids, and a flavonoid upon repeated harvesting.

Zusammenfassung

Im Rahmen des FP7-Projektes AGROCOS wurde die Erforschung von neuen Naturstoff-Leitstrukturen für eine mögliche Anwendung in der Agrochemie und als Kosmetikbestandteil angestrebt. Zusätzlich wurden neue Methoden der Metabolomik und multivariaten Statistik auf *Isatis tinctoria* als Modellpflanze angewendet.

Die Natur ist reich an Verbindungen, die nicht nur in der pharmazeutischen, sondern auch in der agrochemischen und kosmetischen Industrie wichtig sind. Extrakte und reine Naturstoffe werden in der Kosmetik umfangreich genutzt. In der Agrochemie spielen Naturstoffe eher in der Erforschung von neuen Leitstrukturen eine grosse Rolle, d.h. das Auffinden einer Modellverbindung, welches zu einer synthetischen Agrochemikalie weiterentwickelt wird. Im ersten Teil des Projektes wurde eine Extraktbibliothek bestehend aus 600 panamaischen Pflanzenextrakten nach agrochemischen und kosmetischen Eigenschaften getestet. Ausgewählte Extrakte wurden mittels HPLC-basiertem Aktivitäts-Profiling fraktioniert, wobei fünf Extrakte im agrochemischen Screening und zwei Extrakte im kosmetischen Screening aktiv waren.

Für den agrochemischen Teil des Projektes wurden Extrakte und Mikro-Fraktionen nach fungiziden, insektiziden und herbiziden Eigenschaften getestet. Verbindungen aus *Bocconia frutescens*, *Miconia affinis*, *Myrcia splendens*, *Combretum affinis laxum* und *Erythroxylum macrophyllum* wurden isoliert, identifiziert und entsprechend der Indikation getestet. Benzophenanthridin-Alkaloide aus *B. frutescens* und Arjunolsäure aus *M. affinis* zeigten moderate bis gute fungizide Aktivität. Myricetin-3-O-(6''-O-galloyl)-β-galactopyranosid aus *M. splendens* wies eine moderate insektizide Aktivität auf. Aus den zwei Pflanzen, welche für die herbizide Wirkung ausgewählt wurden, konnte keine herbizide Verbindung identifiziert werden.

Für den kosmetischen Teil wurden phenolische Verbindungen und Flavonoide aus methanolischen Extrakten aus den Blättern von *Mosquitoxylum jamaicense* und *Combretum cacoucia* isoliert. Jede isolierte Verbindung war entweder im DPPH-Assay, im UV-B-Schutz-Assay, oder in beiden aktiv. Gallussäure-Derivate waren die Aktivsten im DPPH-Assay (IC_{50} < 10 µg/mL), während Protocatechusäure und Isoquercitrin die höchste Aktivität im UV-B-Schutz-Versuch aufwiesen (< 10% UV-B-induzierter Zelltod).

Metabolomik mit Pflanzenextrakten kombiniert mit multivariater Statistik wird immer mehr für die Unterscheidung verschiedener Proben und für die Untersuchung von Qualitätsmerkmalen genutzt. Metabolomik stellt einen metabolischen Fingerabdruck verschiedener Proben zur Verfügung, im Gegensatz zu der quantitativen Analyse von Einzelverbindungen oder einzelnen Verbindungsklassen. Der zweite Teil des AGROCOS-Projektes bestand aus einer metabolomischen Studie an *Isatis tinctoria* unterschiedlicher Sorten, welche unter identischen und kontrollierten Bedingungen auf experimentellen Feldern angebaut wurden. Die Blätter wurden an sechs Zeitpunkten während der Vegetationsperiode geerntet, wobei zum Einen einzelne Blätter von intakten Pflanzen und zum Anderen Blatt-Rosetten wiederholt mit einer Mähmaschine geerntet wurden. Die letzte Erntemethode diente dazu, das wiederholte Ernten einjähriger Pflanzen zu simulieren. Extrakte wurden mittels PLE mit Ethylacetat und 70% wässrigem Methanol hergestellt, und mittels ^{1}H-NMR-Spektroskopie und der PCA/CA/k-NN-Klassifizierungsmethode analysiert. PCA/CA/k-NN-

Analyse der Sorten und Erntezeitpunkte zeigte in beiden Fällen Unterschiede. Durch die Verwendung eines paarweisen 1D-STOCSY konnten Verbindungen wie ungesättigte Fettsäuren, Porphyrine, Kohlenhydrate, Indolderivate, Isoprenoide, Phenylpropanoide und geringefügig aromatische Verbindungen als verantwortliche Metaboliten identifiziert werden. Des weiteren konnte ein Effekt des wiederholten Erntens aus dem PCA/CA/k-NN-Graph festgestellt werden und der 1D-STOCSY-Graph zeigte eine Abnahme an 1,5-Anhydroglucitol, Saccharose, ungesättigte Fettsäuren, Porphyrine, Isoprenoide und einem Flavonoid bei wiederholtem Ernten.

1. Aim of the work

Natural products and their sources play a crucial role for pharmaceutical, cosmetic, and agrochemical industries. Nature provides a variety of scaffolds for the development of new leads. An additional field in phytochemistry is the development of methods for the investigation of complex extract mixtures in order to find new leads, or to conduct quality control of herbal medicines. A FP7 collaboration project denoted as AGROCOS (acronym for agrochemistry and cosmetics) aims at the discovery of new leads for agrochemistry and cosmetics, as well as the development of analytical tools for the analysis of complex mixtures, including the creation of a database of extracts and pure natural product spectra.

In the first part of the AGROCOS project, extracts from several biodiversity hotspots were screened for agrochemical and cosmetic properties. Agrochemical screening included fungicidal, insecticidal, and herbicidal activity tested on different fungal strains, or species of pests and weeds. Assays for the cosmetic part included DPPH and UV-induced cell death. Our contribution included the follow-up of promising samples from a library of 600 Panamanian plant extracts that was screened by BASF for agrochemical activities and by Korres and Demokritos for cosmetic properties. Extracts fulfilling previously defined activity criteria were submitted to HPLC-based activity profiling in order to track the bioactivity in the extracts. Obtained fractions were submitted to corresponding screenings, and activity profiles were created based on the screening results of the fractions. Eventually, five extracts for the agrochemical and two extracts for the cosmetic part were selected for further investigation. Major peaks in the active time window were isolated, identified, and submitted to agrochemical and cosmetic screening. In order to perform structure-activity estimations, additional major compounds outside the active time window were isolated and identified.

In the second part of the AGROCOS project, metabolomic studies were conducted on *Isatis tinctoria* (Brassicaceae). The plant, *I. tinctoria,* is an ancient dye and a medicinal plant that has been used to treat inflammatory disorders, the activity of which could be confirmed and the active compounds identified in previous studies [1-8]. For previously conducted comparative studies on the content of indigo precursors, tryptanthrin, and glucosinolates [9-11], plants of different accessions were grown on experimental field plots under identical and controlled conditions in the agricultural field station of Thuringia in Dornburg, Germany. Plants were harvested at six different time points during the year 2003. In addition, one part of the individual plants was single and another part repeatedly harvested in order to study the effect of repeated harvesting. Such comprehensive sets of samples are rather unique, and *Isatis* was selected as a model for a metabolomic study with a uniquely well defined set of samples. Differences in the metabolic profile between different accessions, harvesting dates, as well as between single and repeated harvested leaves were investigated using NMR spectroscopy and multivariate data analysis. Due to the high complexity and multiclass properties of the samples, a combination of advanced statistical methods such as CA, MANOVA, k-NN, etc. was used among PCA. 1D-STOCSY was the tool to identify metabolites contributing to the differences. With these methods, differences in the metabolic fingerprints (i.e. the metabolome) of different samples can be investigated in contrast to the classical qualitative and quantitative analysis of single compounds.

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2. Introduction

2.1 Discovery of natural products as lead compounds

Screening and activity profiling of plant extracts

For centuries, plants and extracts from plants have been used for medicinal purposes. Many drugs on the market are natural or semisynthetic products that have been derived from plants and microorganisms. Prominent naturally derived drugs still in use today are morphine from *Papaver somniferum*, digitoxin and digoxin from *Digitalis* species, and taxanes from *Taxus brevifolia*. Examples of semisynthetic products are artemether derived from artemisinin (*Artemisia annua*) and etoposide derived from epipodophyllotoxin (*Podophyllum peltatum*) [1].

The first step in the discovery of natural products that are active against a given biological target is the selection of plant or microbial material, based on either ethnobotanical information, or (chemo-)taxonomically diverse species [2]. Based on these selections, extract libraries are created for screening of the samples. The extraction of the samples for the establishment of the library can be accelerated by automated processes such as pressurized liquid extraction (PLE) using solvents of different polarity. An alternative approach is the screening of pre-purified or fractionated extracts, or of pure compound libraries.

For decades, bioactivity was tracked by a classical approach: an active extract was fractionated, and fractions were submitted to rescreening after every purification step. This is a time-consuming, costly, and risky process with respect to the fact that bioactivity can get lost during the fractionation procedure. Today, there are several approaches to track bioactivity at an early stage. Among them is an HPLC-based activity profiling approach, which combines on-line and off-line detection methods with biological information (**Fig. 1**). The extract is separated by HPLC, collected into 96-well plates in a time-based manner, and submitted for screening. Among others, the major advantage of this method is to decide at an early stage, if an extract is promising and should be further pursued. For example tannin-containing extracts can be excluded if the activity is distributed over the whole hump in the chromatogram, caused by the false-positive results of tannins. Furthermore, compounds can be identified already prior to isolation in the early stages using HPLC-DAD-TOF-MS to get the molecular formula, combined with database and/or literature research. If the compound is well known and commercially available, preparative isolation can be skipped after confirmation by spiking [3, 4]. Many active compounds have already been successfully identified in the early stages by HPLC-based activity profiling in the fields of tropical diseases [5, 6] and GABA receptor modulators [7, 8].

Isolation and structure elucidation of natural products

Depending on the complexity of the extract, isolation of compounds from a complex mixture requires different purification methods and steps. In some cases, pre-purification is necessary (e.g. filtration through polyamide, alkaloid extraction). Afterwards, a combination of different chromatographic methods is used, such as open column chromatography using several stationary phases, HPLC, countercurrent chromatography, etc. [9, 10].

The isolated pure compounds are thereafter elucidated by NMR spectroscopy with the help of spectral information from UV and MS. Using 1mm-TXI probes or cryoprobes, structure elucidation can be achieved with submilligram amounts of a compound. 2D-NMR techniques

such as COSY, HSQC, HMBC, etc. considerably facilitate the structure elucidation of natural products. For chiral molecules, the absolute configuration can be established with electronic circular dichroism (ECD) [11] or by X-ray crystallography.

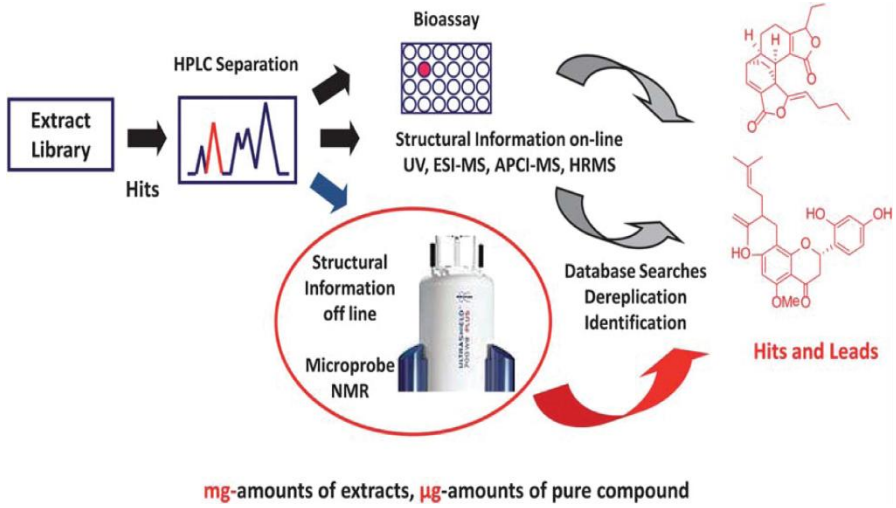

Figure 1. HPLC-based activity profiling of extracts [3, 4].

2.2 Screening of compounds for agricultural chemistry

Natural products as leads for agricultural chemistry

Natural products play a crucial role in agricultural chemistry and have a long history. Already 4000 years ago, poisonous plants were used as insecticides [12]. Today, several natural products and semisynthetic derivatives are on the market, which have been isolated from bacteria, fungi, and plants. Natural products derived from bacteria, specifically actinobacteria, include bactericides (e.g. streptomycin), fungicides (e.g. mildiomycin, polyoxins), one herbicide (bilanafos), and insecticides (e.g. avermectins, spinosad). Bilanafos (**Fig. 2)** is the only naturally derived, pure compound that is used as a herbicide. Its metabolite phosphinotricin inhibits the enzyme glutamine synthetase in plants. Spinosad (**Fig. 2)** is a mixture of spynosins A and D, which are allosteric modulators of invertebrate nicotinic acetylcholine receptors (nAChR). From fungi, one fungicide and one nematicide are in use today, namely, the extract hydrolysates of *Saccharomyces cervisiae* and the dried mycelium of *Myrothecium verrucaria*, respectively. However, the composition and the mode of action are unknown. Plants provide several fungicides and herbicides. Examples of plant derived fungicides are cinnamaldehyde, laminarine, and the extract of *Maceaya cordata*. The latter two products stimulate a self-defense mechanism of the host plant against plant-pathogenic fungi. Herbicides derived from plants include non-selective essential oils and fatty acids (e.g. pelargonic acid). Most of plant derived pesticides act as insecticides and acaricides. They include plant oils or acids, plant powders or extracts, pure natural products, and synthetic analogues. Clove oil and citric acid are commonly used insecticides showing nonspecific irritant and repellant activity. Extracts from *Ryania sp.* contain ryanodine (**Fig. 2)** and related alkaloids, which act as muscle toxins by binding to calcium channels of the sarcoplasmic reticulum. Sabadilla is a seed powder from *Schoenocaulon officinale* containing veratrin, a mixture of steroid-like alkaloids, which act on voltage-gated sodium channels of the insect. However, Sabadilla is not widely used as an insecticide except by indigenous people. Botanically derived pure compounds like azadirachtin, karanjin, rotenone, nicotine, and pyrethrins have been or are still used as insecticides (**Fig. 2)**. Azadirachtin from seeds of *Azadirachta indica* and karanjin from seeds of *Derris indica* are insect growth regulators acting on the ecdysteroid pathway. Rotenone from *Derris*, *Lonchocarpus*, and *Tephrosia* species inhibits the electron transfer complexes in the mitochondria. Pyrethrins from *Tanacetum cinerariaefolium* and nicotine from *Nicotiana sp.* are neurotoxins acting on voltage-gated sodium channels and nAChR, respectively [13]. Both compounds are leads for synthetic derivatives (**Fig. 2**).

Synthetic pyrethroids derived from pyrethrines (e.g. pyrethrin I) have been mainly developed due to the low photostability of pyrethrines. Many analogues were developed mostly based on structure-activity relationship studies. The first developed pyrethroid was bioallethrin, followed by bioresmethrin and other derivatives still containing the cyclopropanecarboxylate ester moiety. Synthetic analogues showed different advantages over the natural product. Some have higher activity but reduced toxicity to mammalians, while others have higher stability, or both. A further strategy for a higher metabolic stability was the replacement of the ester function, which gave etofenprox and derivatives lacking the cyclopropane moiety [14].

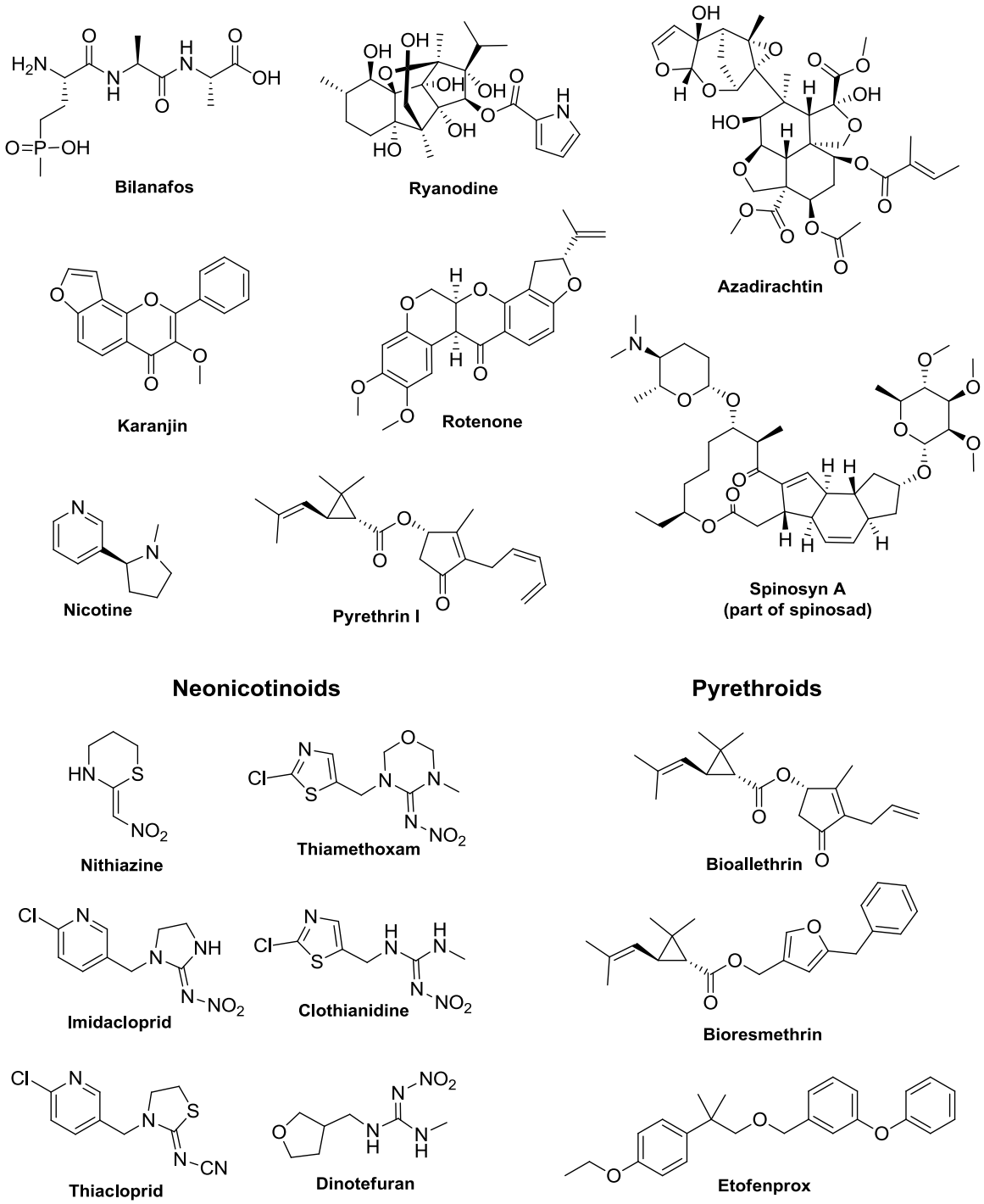

Figure 2 Structure of naturally derived pesticides including synthetic analogues of nicotine and pyrethrins.

Synthetic analogues of nicotine, the neonicotinoids, were developed due to the low effectiveness and non-selective toxicity of nicotine. Nithiazine (**Fig. 2**) was the first lead of neonicotinoids, whose lead pharmacophore is the nitromethylene group. However, it has never been commercialized due to its limited efficacy and low chemical stability under hydrolytic and photolytic conditions. Further development from nithiazine yielded the first commercialized neonicotinoid, imidacloprid. It contains a 6-chloropyridin-2-ylmethyl residue, and the unstable nitromethylene group was replaced by the stable nitroimine group. This compound is effective and very selective towards the insect nAChR due to the nitroimino group that undergoes an additional hydrogen bond with the insect nAChR. Subsequently,

thiacloprid was developed; where, among others, the nitro group was replaced by a nitrile group. After development of further derivatives with ring opening of the guanidine moiety, the 6-chloropyridin-2-ylmethyl moiety was replaced by a 2-chlorothiazol-5-ylmethyl moiety. The guanidine moiety was modified to a cyclic, six-membered (thiamethoxam) or an acyclic guanidine (clothianidine). Eventually, dinotefuran, which is closely related to the other neonicotinoids, was developed using acetylcholine as a lead compound. All these neonicotinoids have similar beneficial properties as imidacloprid. Due to the success of the neonicotinoids, the nAChR is a very interesting and important target for new insecticides. The development of new neonicotinoids is ongoing, and different strategies are being used for that. One strategy is the search for allosteric modulators, where the natural products spinosad and stemafoline are model compounds. Spinetoram is a semi-synthetic derivative of spinosad that is already commercialized. Concerning synthetic analogues of stemafoline, lead optimization studies are ongoing [15, 16].

Methods for agrochemical screening of compounds

The development of agrochemicals is similar to the drug discovery and development process in the pharmaceutical industry. In lead discovery, combinatorial libraries were replaced with diversity-oriented and fragment-based libraries allowing the discovery of new biologically active scaffolds. Thereafter, combinatorial libraries that are based on the biologically active scaffold can be established for lead optimization. Alternative to in-house synthesis, compounds are also purchased from companies or universities that synthesize and possess a large number of diverse compounds. A very helpful tool prior to screening is *in silico* or virtual screening. Virtual docking assays on a well known target help to reduce the number of compounds tested in a bioassay and thus, reduce the costs. Furthermore, the library size can be reduced, and the quality increased. *In silico* approaches are also helpful to find agro-like leads in the early stages, i.e. leads with properties that are applicable in crop protection.

The screening of compounds applied on plants grown in the greenhouse has a long practice. However, high throughput screening (HTS) is the method used by the agrochemical industry today, whereby compounds are tested in automated and miniaturized systems. HTS is performed mechanism based (*in vitro*) and/or on the whole organism (*in vivo*). For *in vitro* screens, compounds are tested on a target that can be a receptor, enzyme, or ion channel. Hits are validated by the characterization of a hit (e.g. assess reactivity) and, if possible, using orthogonal assays, i.e. assays that are closer to the physiological conditions. There is a high interest in finding novel targets and mode of actions. This is achieved by a combination of genomics and target identification by known bioactive compounds. *In vivo* assays have been the primary basis for agrochemical research, and further development towards HTS could be achieved. Since *in vivo* assays are also miniaturized as compared to the pharmaceutical assays, *in vitro* assays can be even skipped. This has additionally the advantage that pharmacokinetic and selectivity hurdles can be included in the early stages. *In vivo* HTS screening requires model organisms that are easy to handle, but also need to be the target organisms essential as crop pest. These organisms are tested in microtiter plates, on leaf discs, or on whole plantlets, depending on what organism is used. In summary, *in vitro* assays provide the mechanism of action, while *in vivo* assays are closer to reality. Therefore, both are still in use in HTS [17].

2.3 Screening of compounds for cosmetics

Cosmetics represent an important area for natural product research. Traditionally, most of the cosmetics consisted of plant extracts and oils. Compared to pure compounds, they contain additional active principles amongst the main active compounds, enabling synergy of the desired effect. In cosmetics, natural products are used, among others, as moisteners, astringents, anti-irritants, anti-inflammatory agents, free radical scavengers, antioxidants, anti-aging agents, and skin-lighteners [18, 19]. Numerous screening designs are available in every cosmetic field.

For radical-scavenging and antioxidant activity, there are several simple assays, of which the DPPH-scavenging assay is the best known. This assay is also used in TLC bioautography, where a plant extract is separated by TLC, and radical scavenging compounds subsequently detected by spraying of the plate with the DPPH radical. With this method, radical scavengers can be tracked in an extract at an early stage [3]. Alternatives to the DPPH assay include the ABTS, superoxide radical, nitric oxide, hydroxyl radical, and hydrogen peroxide assay [20].

UV-induced damage of cells and DNA is an essential issue related to skin cancer and aging. Hence, there are many assays for testing the UV protective properties of compounds. The comet assay is an easy method to detect the breakage of DNA strands. After treatment of fibroblasts or keratinocytes with a test compound and UV-light, DNA fragments can be detected using gel electrophoresis. The protein p53 is a marker of UV induced DNA and cell damage, which can be detected by Western blotting [21]. Other assays to assess UV-induced cell death include MTT, neutral red, calcein-AM, ^{35}S-methionin incorporation, and IL1-α assays [22]. Hyperpigmentation or hypermelanosis is a skin disorder referring to a darkening of an area of the skin caused by an excess of the skin colorant, melanin. A first step for the discovery of skin-lightening products is the tyrosinase inhibition assay. Tyrosinase is an enzyme essential for melanin synthesis, whose inhibition is detectable by a decreased production of dopachrome, which can be measured using spectrophotometry. The following steps are assays on melanocytes and whole epidermis cell cultures using colorimetric or photographic readouts [23].

2.4 Metabolomics and multivariate statistics

Multivariate statistics: A mathematical tool for extracting essential information

Metabolomics refers to the analysis of all metabolites in an organism or specified biological sample. This term is mostly used in plant science and also refers to metabolic fingerprinting of different samples (e.g. plant cultivars), which focuses on the metabolites causing these differences. A similar term called metabonomics is described as the change of the metabolite profile as a response to pathophysiological stimuli or genetic modifications. This is a term mostly used in biomedicine [24, 25].

Usually, the samples are analyzed by HPLC-MS and NMR spectroscopy. Both, metabolomics (i.e. metabolic fingerprinting) and metabonomics use the concept of analyzing multiple metabolites simultaneously, which is a challenging task considering the high number of metabolites in a mixture. Multivariate statistics (also called chemometrics) is a tool that enables the handling of complex data by extracting the essential information by dimensional reduction. There are several methods to do this, of which principal component analysis (PCA) is the basic and simplest multivariate statistical method (**Fig. 3**). The samples (plant extract, urine, blood) are defined as observations, while the measured factors (mass-to-charge ratio, NMR chemical shift buckets) are defined as variables. From this, the data matrix X is created containing the measured, scaled values (e.g. scaled peak integrals). "Scaled" means that the values are centered (subtraction of the values by the mean), followed for instance by division of the values by the square root of the standard deviation (Pareto scaling). This reduces the noise, increases the quality, and gives every value a similar weight. From the data matrix X, the observations are then plotted in a multidimensional space, in which the variables represent the coordinate axes. Additionally, from the data matrix X, the covariance matrix is calculated. This is a squared matrix containing covariance values from all possible combination of data pairs; and hence, an overview of the differences of all samples between each other is obtained. The covariance matrix is the basis to calculate the eigenvectors, which are the principal components t_1-t_n that show the directions from the highest to the lowest overall differences. Eigenvectors are orthogonal to each other and are needed for a proper projection of the ellipsoidal, multidimensional data cloud to the plane, in order to avoid a distortive projection of the data. Finally, the multidimensional space is reduced to two dimensions by projecting along all the eigenvectors, except the two with the highest eigenvalues, accordingly the two that show the direction of the highest variance (i.e. differences). These residual two eigenvectors are the principal components t_1 and t_2, which are the longest eigenvectors and consequently, have the highest eigenvalues [26, 27]. The method described here uses the eigenvalue decomposition method. Alternatively, the principal components can be directly calculated from the scaled, native data matrix using the singular value decomposition method [26].

The concept of the PCA projection can be explained by projecting a shadow of a coffee cup. To unambiguously recognize the coffee cup in the shadow, it has to be positioned upright with the handle perpendicular to the direction of the projection. The axes going along the handle and the height of the cup are the main characteristics, i.e. the essential information defined by the eigenvectors t_1 and t_2 (**Fig. 4**). The thickness of the cup, represented by the third eigenvector t_3 (i.e. the horizontal axis perpendicular to the axis with the handle), is not

relevant and therefore redundant information for this purpose. So the cup is projected along the third eigenvector. A projection along any vector of the cup would cause a distorted shadow, in which the cup could be probably recognizable, but would not represent its real shape and dimensions.

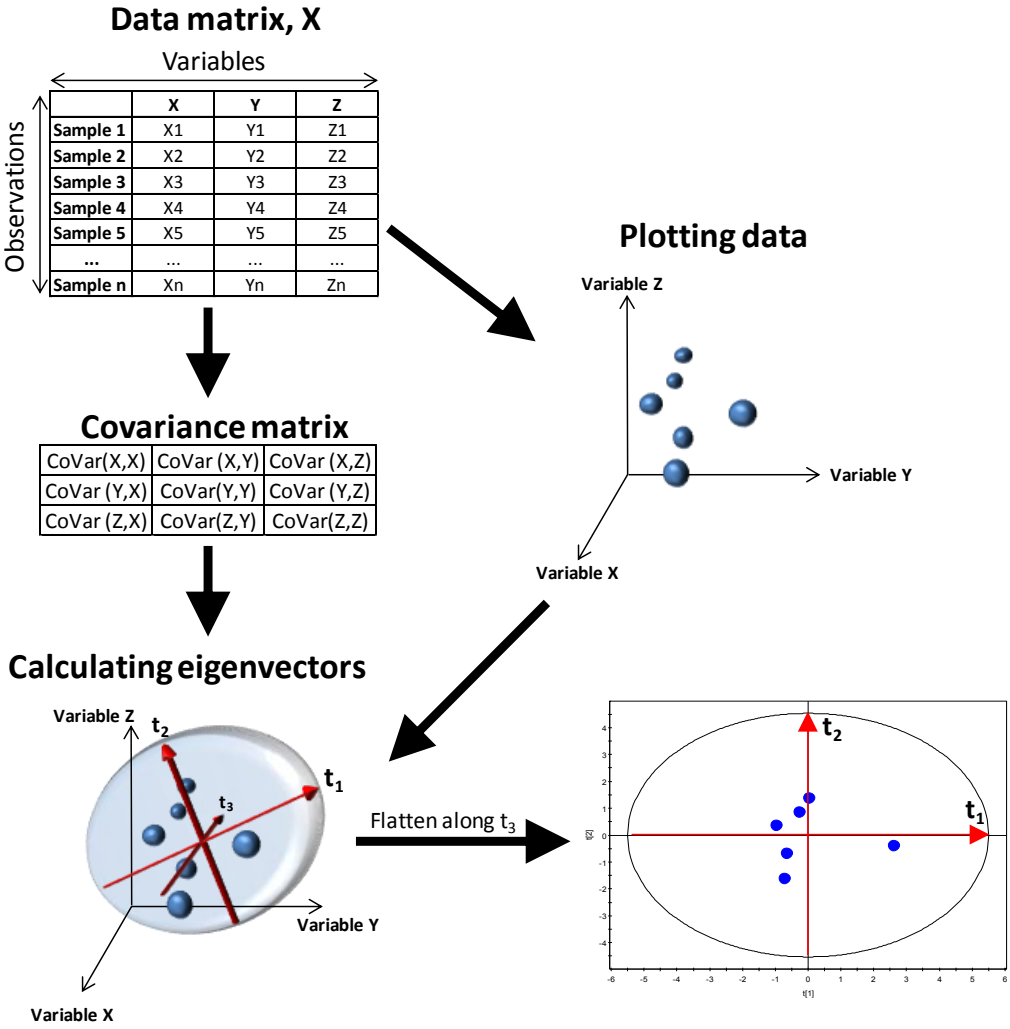

Figure 3 Workflow for the generation of a PCA plot with three variables using the eigenvalue decomposition method. The data matrix consists of the observations (rows) and the variables (columns), containing the measured, scaled value (e.g. peak integral). From this, the data are plotted, and a covariance matrix is calculated. With the covariance matrix, the principal components t_1-t_3 are calculated and integrated into the plot. The longest vector t_1 shows the direction of the highest variance, while the shortest vector t_3 shows the direction of the lowest variance. Projection (or flattening) along the eigenvector t_3 gives the PCA plot, by maintaining the eigenvectors t_1 and t_2, the two most essential information. For a better visualization, the data points in the plots are shown here not centered.

The principal components t_1 and t_2 generate a PCA score plot, which is a scatter plot that shows if and to what extent the samples are different. Groups far away from each other are different, while groups close to each other are similar. Complementary to the PCA score plot, a loading scatter plot shows what variables are responsible for these differences. This is created in the same way as the score plot with the difference that the variables are plotted, and the observations represent the axes. Hence, the loading plot shows which variables change to what extent their values among the observations. Variables in the loading plot migrating in the same direction as a certain observation or group in the score plot means that the variables

have a high value (e.g. integral) in this group, which makes this group different from the others [27]. In **Figure 5**, an example of PCA scatter plots from the comparison of six selected cities of Switzerland is displayed. The cities are the observations, and the factors "area", "habitant", and "altitude" are the variables. In the score plot, Zurich is far away from the other cities meaning high difference, while the residual cities are closer to each other meaning higher similarity. In the loading plot, the variables "area" and "habitants" together migrate to the same direction as Zurich. This means that Zurich is by area and habitant the largest city of Switzerland. In terms of habitants (only considering the "habitant" variable), Zurich, Geneva, and Basel are the largest cities. For the variable "altitude", the readout can be done vertically. Berne is the highest, while Basel is the lowest situated city in Switzerland.

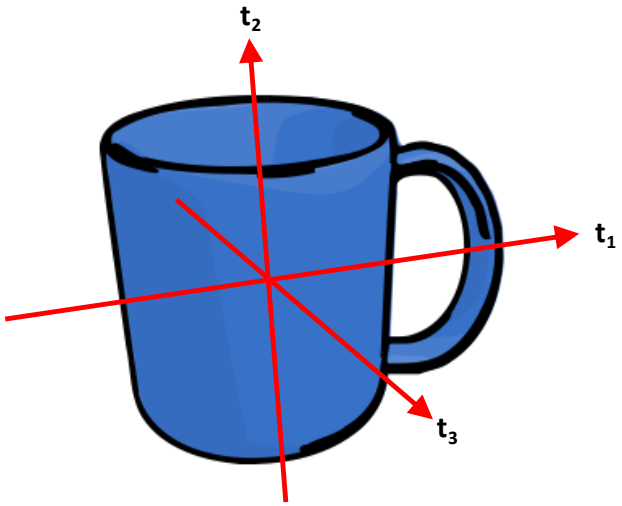

Figure 4 The principal components (eigenvectors) of a coffee cup. The principal components t_1 and t_2 contain the essential information of the cup, i.e. the handle and the height. In contrast, t_3 contains redundant information. Therefore, illuminating the cup in the direction of t_3 gives a representative shadow of the cup, maintaining its true shape and dimension.

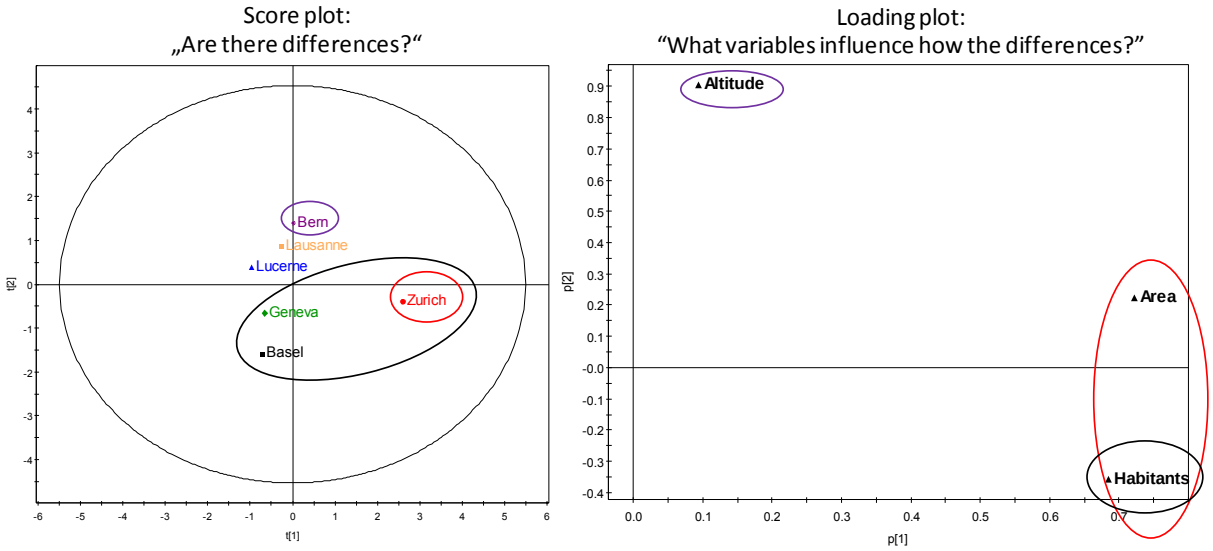

Figure 5 PCA score and loading plots for six selected cities of Switzerland. The score plot shows the differences, and the loading plot shows what caused these differences.

PCA is an unsupervised projection method that projects the data according to the highest differences in the overall point cloud without taking into account the so-called latent variables, which are additional properties or a category. Partial least square regression (PLS) includes the latent variables into an additional matrix Y. This can be now used to identify a possible correlation of the variance of the data to the latent variables. Latent variables are either quantitative (e.g. body weight of the study subject), or categorical (e.g. classes, groups, or gender). For the latter case, the PLS method is extended to partial least square discriminant analysis (PLS-DA), where a binary code is used to categorize the classes. According to this, it can be simply said that while PCA searches for the maximal overall variances, PLS-DA searches for the maximal variance between the classes. The drawback of PLS or PLS-DA is that it can only cope with simple data. If the systematic variation in the X matrix, which is not correlated to the Y matrix, is high (e.g. high differences within one class), the results are affected and can be distorted to an extent that the model will be misinterpreted. Furthermore, the loadings describing the two classes are mixed with the loadings correlated to other differences, which makes the readout from the loading scatter plot problematic. These problems can be overcome by using orthogonal partial least square regression (OPLS) analysis. The variations in the data matrix X are split into two variations: a variation that correlates to the Y matrix and a variation that does not correlate or is orthogonal to Y. This gives a new projection with two new principal components: the first component separating the groups correlated to Y (Y-predictive component $t_{1,p}$), as well as a second component separating observations not correlated to Y (Y-orthogonal component $t_{2,o}$). Analogous to PLS, OPLS can be extended to orthogonal partial least square regression discriminant analysis (OPLS-DA), where the Y matrix consists of categorizing values (e.g. class, gender, etc.). In the case of OPLS-DA, it can easily be said that this model separates the between-class variance from the within-class variance, so that the classes are well separated (**Fig. 6)**. The responsible factors that only describe the between-class differences are easily identifiable from the loading scatter plot, even with a high within-class variation [27].
Additional advanced projection methods include canonical analysis (CA), multivariate analysis of variance (MANOVA), and k-nearest neighbor (k-NN) concept, which were as a combination successfully applied for the differentiation of individuals from the metabolite profile of urine [28, 29].

Application of NMR and multivariate statistics to pharmacognosy

Metabolomics is defined as the analysis of the whole metabolome of an organism (e.g. plants). However, the metabolome of an organism is highly complex, especially in plants. Therefore, only a fraction of the whole metabolome can be identified. Spectral analysis of the whole metabolite profile at once, combined with multivariate statistics, provides a metabolic fingerprint of a sample and enables the classification of different samples which have different metabolic fingerprints. In the latter case, biomarkers can be identified which characterize the classes. While NMR spectroscopy was historically the leading method, mass spectrometry tends to be the first choice in plant metabolomics. This is mainly due to the higher sensitivity of the latter method enabling the additional detection of minor metabolites. In contrast, NMR spectroscopy has the advantage of relative quantitative detection and specific identification of compounds [30]. In this section, only examples that focus on NMR spectroscopy will be discussed.

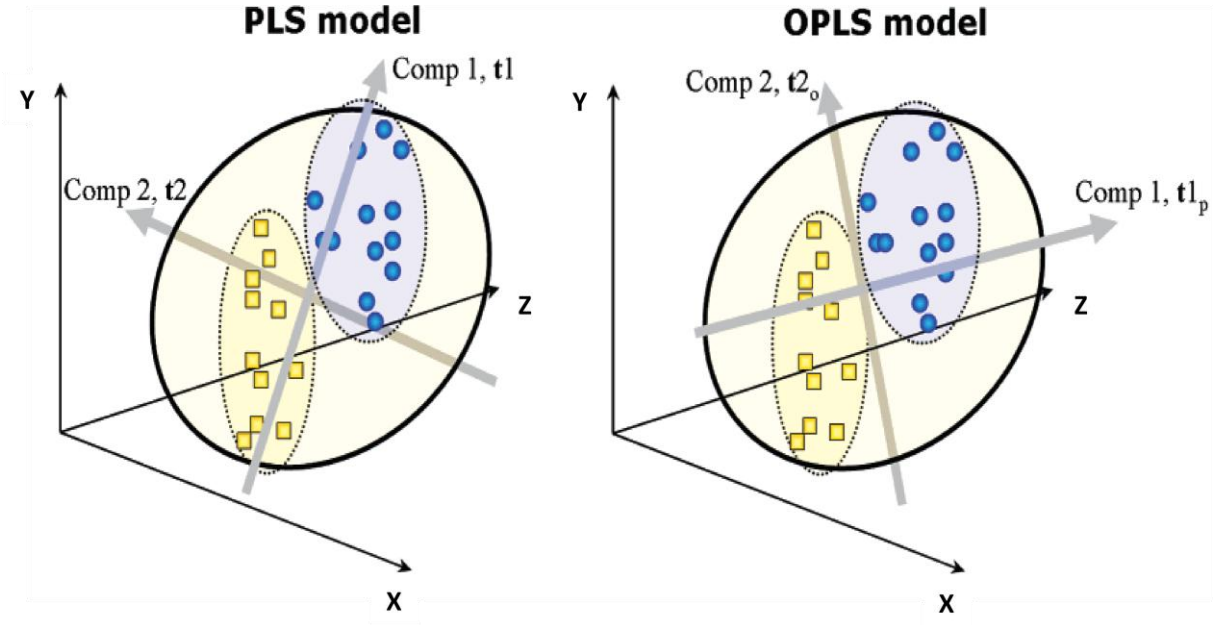

Figure 6 Comparison of PLS(-DA) and OPLS(-DA). Adapted from [27].

Metabolomics using NMR and multivariate statistics can be used to find biomarkers, or to perform metabolic fingerprinting. This is helpful for quality control of different plant cultivars or species. One example of tracking biomarkers is metabolomics on different species, cultivars, and commercial products of ginseng roots. Two species (*Panax ginseng* and *Panax quinquefolius*) were compared, including two geographic origins of *P. ginseng*. Of the cultivars and commercial products, four different samples of each were compared. In all cases, differences could be observed, as well as potential biomarkers identified that belong to amino acids, carbohydrates, and organic acids. In a concluding step, metabolites were quantified to detect the most promising biomarkers, i.e. biomarkers whose concentrations were substantially different [31]. The possibility to perform quality control using metabolite fingerprinting was demonstrated with the chamomile flower (*Matricaria recutita*). Differences between extracts of the chamomile flower from Egypt, Hungary, and Slovakia were investigated. In addition, the change in the metabolite profile, by adding different percentages of chamomile stalks, was examined. Differences were observed between the origins and after addition of the stalks (**Fig. 7**). While the origins were separated along the principal component t1, the differences, after addition of stalks, were separated along t2, with a higher distance from the pure flower sample with increasing percentage of stalks. Biomarkers were glutamine, glutamate, and carbohydrates. In the final step, extraction procedures were also compared resulting in an influence on the metabolic profile [32].

Another application of metabolomics includes the identification of a resistance mechanism, which in turn, identifies resistance biomarkers. A study on grapevine (*Vitis vinifera*) included two susceptible cultivars and four resistant cultivars towards downy and powdery mildew. The PCA score plot showed some differences between the cultivars in general, and the loading plot revealed primary metabolites (amino acids, carbohydrates) together with quercetin-3-O-glucoside and caffeic acid to be responsible for these differences. For the differentiation of susceptible and resistant cultivars, PLS-DA was performed, where the "susceptible" and "resistant" classes were included into the Y matrix. The two classes were well separated along t1, so that the loadings could be alternatively displayed as a column plot

showing the contributing metabolites along t1 (**Fig. 8**). Among others, metabolites such as quercetin-3-O-glucoside and a *trans*-feruloyl derivative were predominantly present in the resistant cultivars [33].

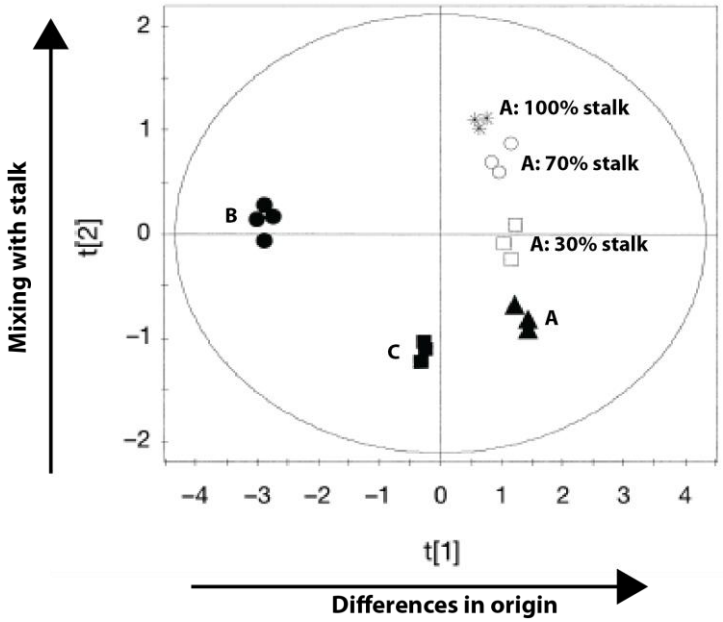

Figure 7 PCA plot of the origins A, B, and C of *Matricaria recutita*. The origins were encoded by the authors. Along t1, origins are separated. Along t2, samples of origin A containing different percentages of stalks are separated; away from the pure flower sample with increasing percentage. Adapted from [32].

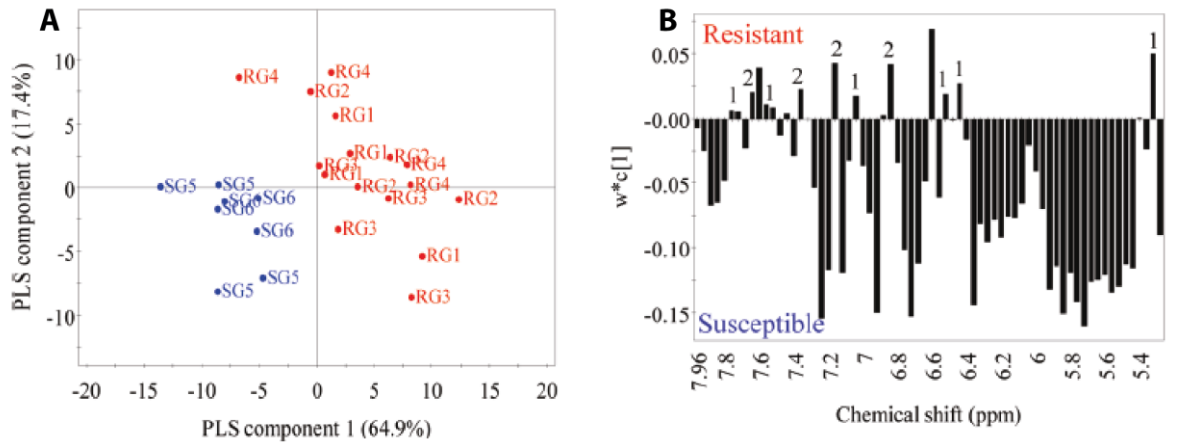

Figure 8 PLS-DA of susceptible (SG, blue) and resistant (RG, red) vine cultivars. **A**: PLS-DA score scatter plot. Numbers indicate the cultivars. **B**: PLS-DA loading column plot. Among others, the two metabolites quercetin-3-O-glucoside (1) and a *trans*-feruloyl derivative (2) are present in a high amount in the resistant cultivars. Adapted from [33].

Alternatively to HPLC-based activity profiling, active compounds can be identified by metabolomics. A study on the Mexican plant *Galphimia glauca* compared six different geographic accessions in Mexico, and correlated them to biological activity. *Galphimia glauca* is a traditional medicinal plant with sedative and anxiolytic activity containing

galphimine derivatives as the active constituents. Extracts of all accessions were screened for anxioloytic and sedative activity, whereby two accessions were found to be active. PCA plots showed the general differences with galphimine derivatives and other metabolites as contributing factors. For PLS-DA, accessions were classified as active and non-active based on the screening results. It could be confirmed that the two active accessions contain galphimine derivatives in contrast to the inactive accessions. In addition, other compounds are thought to contribute to the activity [34]. Four years later, the study was repeated, which again lead to similar results. These findings are also a basis for quality control of the herbal medicine, whose activity depends on the accession [35].

A recently published study identified active constituents from crude extracts of marine sponges. Different species of sponges, of one taxonomic class, were tested for activity against adenosine A1 receptor, and a NMR metabolite profile of all samples recorded. After PCA as an overview, the data were submitted to PLS by inclusion of the screening results into the Y matrix (**Fig. 9**). Since the sponges vary substantially in their metabolite profile, OPLS analysis was subsequently performed, in order to unambiguously identify the metabolites responsible for activity (**Fig. 9**). Sesterterpene derivatives were predominant in the highly active sponges. This led to targeted isolation of the sesterterpenes, which were submitted to bioassays confirming the high activity against the adenosine A1 receptor [36].

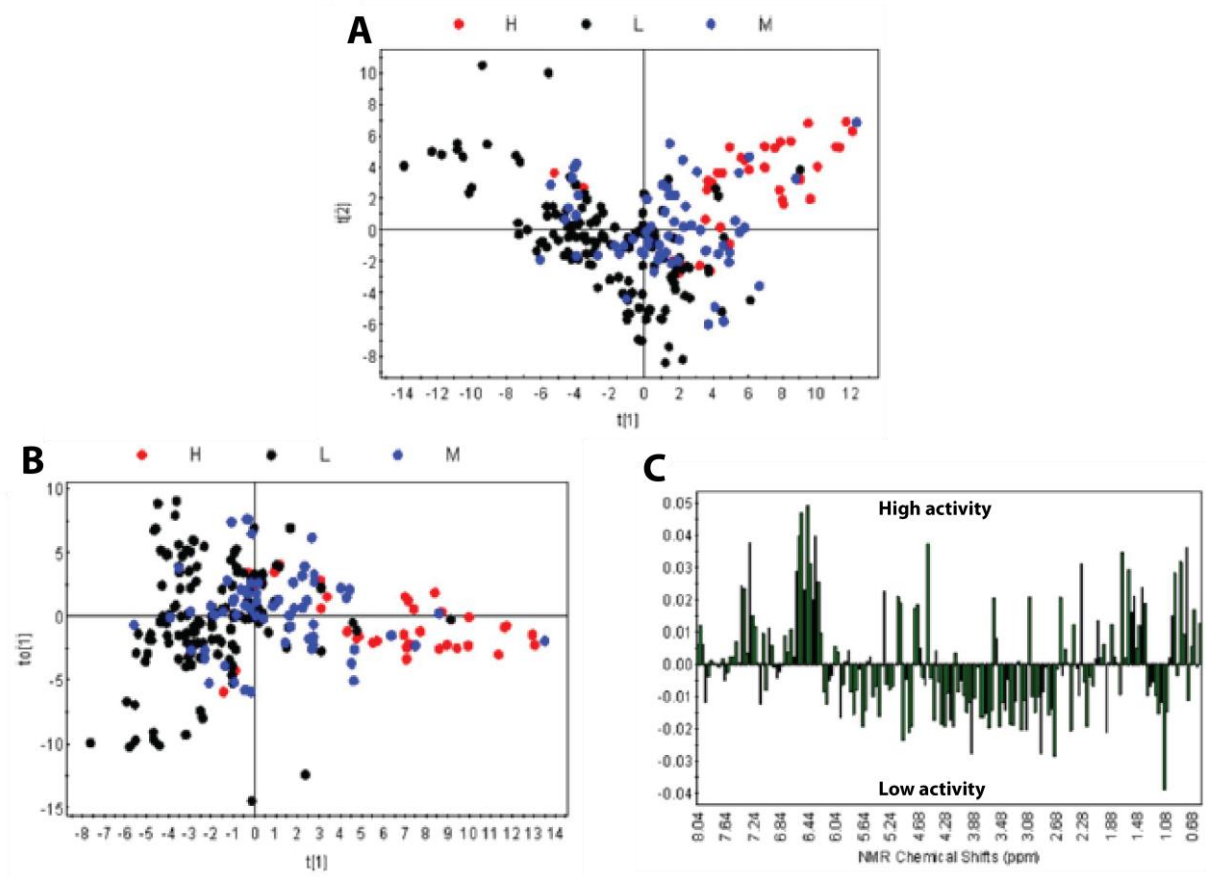

Figure 9 PLS and OPLS plots of the different sponge species. For a convenient visualization, the screening results were divided into high (H, red), medium (M, blue), and low (L, black) activity. **A**: PLS score plot, **B**: OPLS score plot, **C**: OPLS loading column plot along t1. Adapted from [36].

STOCSY as a tool for structure elucidation of compounds in complex mixtures

NMR analysis of complex mixtures is a challenging task due to the high quantity of signals and overlapping peaks. In addition, it is unknown which signals belong to the same molecule. The latter problem can be solved by statistical total correlation spectroscopy (STOCSY). Although the name denotes a spectroscopic analysis, it is actually a statistical, post-spectroscopic data analysis method. The term STOCSY derives from the TOCSY experiments, which detect correlations of protons of one spin system; while STOCSY can detect all protons of a whole molecule, independent of the spin system. The method is based on the Pearson correlation coefficient matrix. Of all variables (i.e. chemical shift buckets), a Pearson correlation coefficient is calculated in all possible combinations and organized into a squared matrix, similar to the covariance matrix. The idea behind STOCSY is that a change in the concentration of a molecule by a certain factor causes a change in the corresponding NMR peak integrals by the same factor, since the stoichiometry is constant. This is calculated by the correlation matrix, where peaks that belong to the same molecule have a theoretical correlation coefficient of 1; while peaks belonging to another molecule have a value below 1, or even negative. In reality, correlating peaks have a value between 0.9 and 1 due to noise and peaks that overlap in the NMR spectrum. The correlation matrix displayed as a 2D contour plot can be analyzed in the same way as a COSY or TOCSY spectrum (**Fig. 10A**). Alternatively, the ^{1}H NMR spectrum can be displayed, whereby the peaks are color coded according to the correlation coefficient between a selected and the residual peaks (**Fig. 10B**) [37].

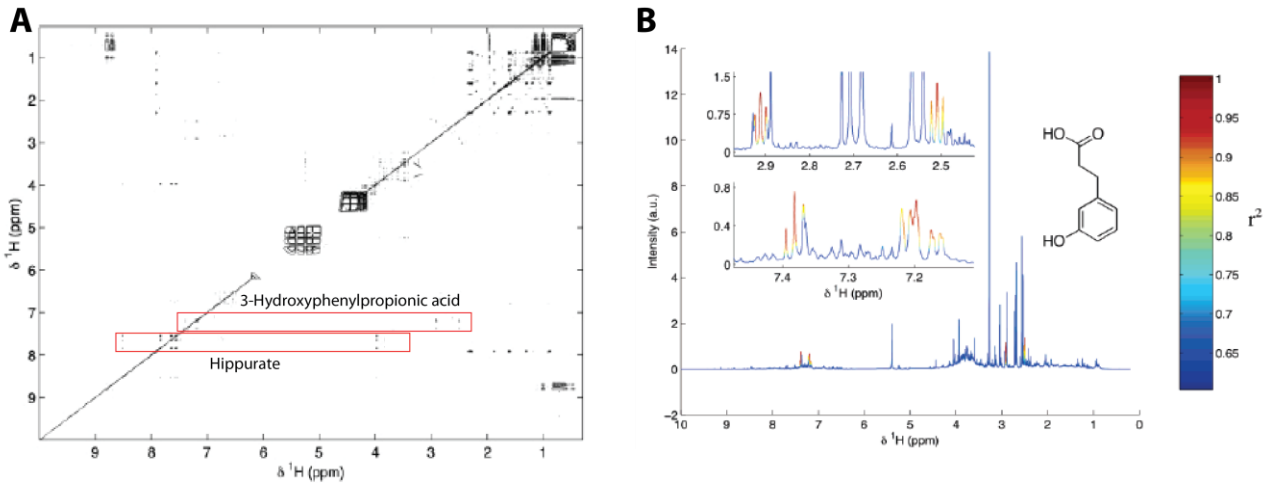

Figure 10 STOCSY calculated from NMR spectra of urine from different diabetic mice strains. **A**: 2D-STOCSY highlighting hippurate and 3-hydroxyphenylpropionic acid as examples. **B**: Color coded ^{1}H NMR spectrum with the selected variable δ_H 2.51 ppm of 3-hydroxyphenylpropionate. Here, the squared Pearson correlation coefficient is calculated. Adapted from [37].

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3. Results and discussion

3.1 Screening of Panamanian plant extracts for pesticidal properties, and HPLC-based identification of active compounds

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A library of 600 Panamanian plant extracts was screened for fungicidal, insecticidal, and herbicidal properties. A total of 19 active extracts were submitted to HPLC-based activity profiling, from which five extracts were selected for isolation of active compounds. Compounds were identified by HPLC-PDA-ESI-MS and 1D and 2D NMR spectroscopy. Benzophenanthridine alkaloids and arjunolic acid showed moderate to good fungicidal activity, while myricetin-3-O-(6"-O-galloyl)-β-galactopyranoside showed moderate insecticidal activity. However, no herbicidal compound was identified.

Selection and provision of plant material was by Mahabir Gupta. Screening of extracts, microfractionation, and pure compounds was the contribution of Tobias Seiser, Christine Wiebe, Joachim Dickhaut, Rüdiger Reingruber, and Oliver Sorgenfrei. HPLC-based activity profiling (except assays), extraction of plant material, isolation of compounds, interpretation of spectroscopic data for structure elucidation (HPLC-PDA-ESI-MS and 1D and 2D NMR), writing of the manuscript draft, and preparation of figures were my contributions to this publication.

Niels Guldbrandsen

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Screening of Panamanian plant extracts for pesticidal properties, and HPLC-based identification of active compounds

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Abstract

A library of 600 taxonomically diverse Panamanian plant extracts was screened for fungicidal, insecticidal, and herbicidal activities. A total of 19 active extracts were submitted to HPLC-based activity profiling, and extracts of *Bocconia frutescens*, *Miconia affinis*, *Myrcia splendens*, *Combretum aff. laxum*, and *Erythroxylum macrophyllum* were selected for the isolation of compounds. Chelerythrine (**2**), macarpine (**3**), dihydrosanguinarine (**5**), and arjunolic acid (**8**) showed moderate to good fungicidal activity. Myricetin-3-O-(6''-O-galloyl)-β-galactopyranoside (**13**) showed moderate insecticidal activity, but no compound with herbicidal activity was identified.

Keywords

Panamanian plant extracts • HPLC-based activity profiling • Fungicide • Insecticide • Herbicide

Introduction

Plants and its extracts are used for a long time for crop protection. They are a promising source for pesticides due to the fact that many plants produce secondary metabolites to defend against pest. However, after evolvement of chemical synthesis of pesticides, the importance of botanical sources decreased [1]. But still, botanical sources play an important role especially in developing countries, where there is a rich indigenous knowledge of using plants and plant extracts for crop protection [2].

Alternatively to ethnobotanical sources, investigation of taxonomically highly diverse and unique plants has been applied successfully in drug discovery [3]. Globally, some 25 so-called biodiversity hotspots are identified combining high diversity with a high degree of endemism. The ranking is based on the number of species per 100,000 km^2 [4]. Panama is one of the biodiversity hotspots with a highly divers flora. Panama and its environment possess the highest diversity of plant species in the world and belong to the 25 most plant-rich countries ranking in the fourth place in the North American continent [5, 6]. Despite the small surface area, its flora comprises 9893 vascular plant species including 1327 (13.4%) endemic plants [7, 8]. Gupta and collaborators have shown in three reviews that the flora of Panama is extremely rich in bioactive compounds and still represents an untapped source of novel compounds for pharmaceutical, agrochemical, and cosmetic industries [9-11].

In a FP7 framework project aiming at discovering new agrochemical compounds, we screened 600 Panamanian plant extracts for fungicidal, insecticidal, and herbicidal properties. Their agrochemical potential was evaluated at BASF. A primary, highly automated screening in 96 wells plates at a concentration of 2500 ppm was done in the three screens. In these assays the fungicidal activity is tested on four plant pathogenic fungi and a ratio of growth rate to standard is estimated by optical density measurement. The herbicidal activity is evaluated on three plants in post and pre-emergence, while the insecticidal activity is assessed on five different insects from four families. These screening systems are highly miniaturized and automated to provide high-throughput evaluations. Whole plants are substituted by leaf fragments and insect eggs or small larvae are used as models for real life pests. As a result these assays are very sensitive in order to not miss an interesting activity. Follow up tests with bigger plants in pots are then used to further characterize these initial hits to identify compounds with market potential. Selected extracts from the primary screens were submitted to a process called HPLC-based activity profiling, which combines physicochemical data recorded online with biological information in parallel to time-based HPLC fractionation [12, 13]. Most of the active constituents were isolated, characterized, and screened for pesticidal activity.

Results and Discussion

A library of 600 extracts prepared from Panamanian plants was screened for fungicidal, insecticidal, and herbicidal activity. A total of 19 extracts fulfilled previously defined activity criteria, which were: a ratio of ≥ 0.75 for fungicidal, ≥ 50% activity against larvae and adult

insects for insecticidal, and ≥ 50% (*Agrostis stolonifera* and *Poa annua*) or ≥ 80% (*Matricaria inodora*) for herbicidal activity (**Tab. 1S**, Supporting Information). A flow chart illustrating the further progression of samples is shown in **Fig. 1**. Active extracts were submitted to HPLC-based activity profiling [12, 13], and collected micro-fractions were submitted to screening in the respective assays. Based on the above activity criteria, 12 extracts were prioritized. With the aid of chromatographic and activity profiles, five extracts were then selected for a detailed investigation. Among these, two extracts were chosen for their fungicidal (**Fig. 2**), one extract for its insecticidal (**Fig. 3**), and two extracts for herbicidal activity (**Fig. 4**).

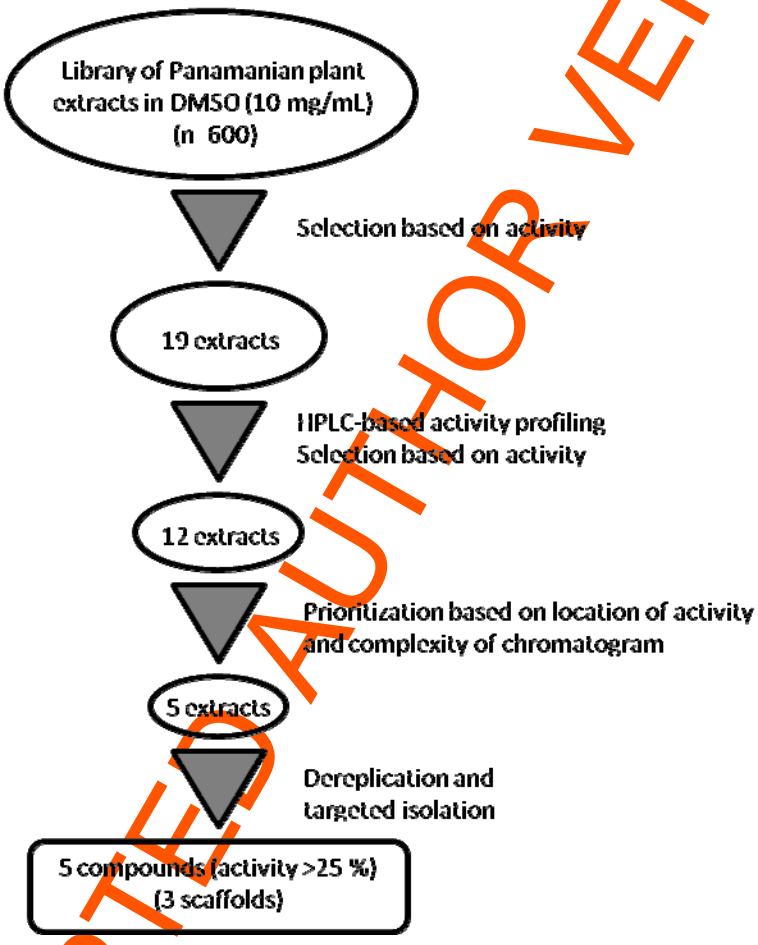

Fig. 1. Workflow for the discovery of agrochemicals from Panamanian plant extracts.

The methanolic extract of *Bocconia frutescens* (Papaveraceae) showed fungicidal activity against *Magnaporte oryzae* in time-windows corresponding to major UV absorbing peaks (**Fig. 2A**). Two of the active fractions, and one additional fraction also showed activity against other fungal strains (**Fig 1S**, Supporting Information). The two early eluting main peaks were identified as sanguinarine (**1**) [14] and chelerythrine (**2**) [14] (**Fig. 5**). Compound **2** showed moderate activity against *Botryotinia fuckeliana*, *M. oryzae*,

Phytophtora infestans, and *Septoria tritici*. The late eluting active peaks were identified as oxysanguinarine (**4**) [15] and dihydrosanguinarine (**5**) [14]. Compound **4** showed no fungicidal activity, while **5** was active against *M. oryzae, P. infestans, and S. tritici*. Macarpine (**3**) [16] was in a microfraction active against *P. infestans* (**Fig 1S B**, Supporting Information). The purified compound showed good fungicidal activity against *P. infestans* and *M. oryzae*. With exception of **4**, the compounds had been previously reported from *B. frutescens* [17, 18].

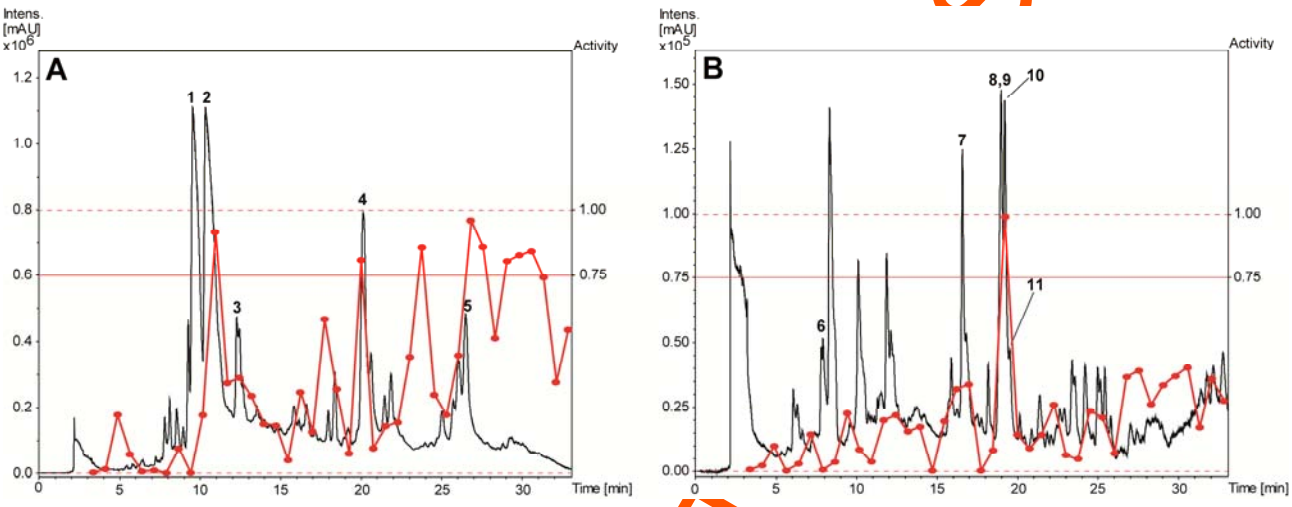

Fig. 2. HPLC-based activity profiling of selected plant extracts for fungicidal activity against *M. oryzae*. SunFire C_{18} column (150 x 10 mm i.d., 5 µm); 5-100% MeCN/0.1% aqueous formic acid in 30 min (A), and 50-100% MeCN/0.1% aqueous formic acid in 30 min (B); 4 mL/min; detection: 200-500 nm, maxplot. (A) *Bocconia frutescens* (MeOH stem extract). (B) *Miconia affinis* (EtOAc stem extract). Activity of microfractions are shown as red curve.

The profile of the ethyl acetate extract of *Miconia affinis* (Melastomataceae) showed one fraction active against *M. oryzae* (**Fig. 2B**) and *S. tritici*. This fraction consisted of three strong UV-absorbing peaks (**9**-**11**) and one non-UV active compound (**8**) (**Fig. 5**). Peak **10** was purified and identified as 3',4',5'-tri-O-methyl-3,4-O,O-methyleneflavellagic acid [19]. UV and MS data of the other two UV absorbing peaks were indicative of 3,4:3',4'-bis(O-O-methylene) ellagic acid (**9**) [20] and 3',4'-di-O-methyl-3,4-O,O-methyleneellagic acid (**11**) [19], and were not further pursued. Arjunolic acid (**8**) [21] was purified by normal phase flash chromatography, and its presence in the active fraction confirmed by HPLC-DAD-ELSD. Compound **8** was active against *M. oryzae* and *S. tritici*. In previous studies [22, 23], fungicidal activity of arjunolic acid (**8**) in a mixture with asiatic acid was reported, while in the current study the activity of purified **8** was confirmed. Two additional compounds outside of the active time window were also isolated and identified as β-hydroxypropiovanillone (**6**) [24] and 3'-O-methyl-3,4-O,O-methyleneellagic acid (**7**) [25]. All compounds are reported for the first time from *M. affinis*, since no phytochemical studies have been conducted on this species before.

A broad hump in the chromatogram of the methanolic extract of *Myrcia splendens* (Myrtaceae) indicated the presence of tannins (**Fig. 3**). However, two distinct windows of insecticidal activity against *Ceratitis capitata* were seen between t_R 7-10 min. After large scale extraction, peaks **a** and **b** depleted, and **c** even disappeared, while peak **15** was extremely enriched in the crude extract. Prior to HPLC purification, the extract was separated over polyamide yielding five tannin-depleted fractions (**Fig 2S**, Supporting Information). From the first active time-window, compound **13** was isolated and identified as myricetin-3-O-(6''-O-galloyl)-β-galactopyranoside [26] (**Fig. 5**). The compound showed weak activity against *C. capitata* at 2500 ppm. From the second active time window, inactive myricitrin (**15**) [27] and quercitrin (**16**) [28] were isolated. Additional compounds isolated from fractions outside of the active time windows were gallic acid (**12**), myricetin-3-O-β-galactopyranoside (**14**) [29], and myricetin (**17**) [30]. Compound **15** had been previously reported from *M. splendens* [31], while the other compounds were new for the species.

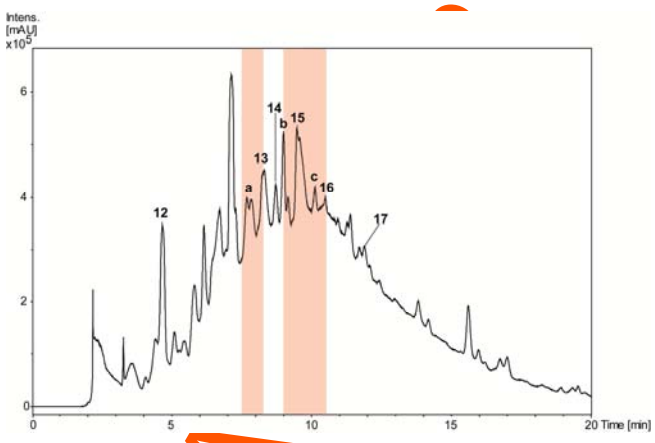

Fig. 3. HPLC-based activity profiling of a MeOH leaf extract of *Myrcia splendens* for insecticidal activity against *Ceratitis capitata*. SunFire C_{18} column (150 x 10 mm i.d., 5 µm); 5-100% MeCN/0.1% aqueous formic acid in 30min; 4 mL/min; time-based fractionation; detection: 200-500nm, maxplot. Windows of insecticidal activity are highlighted in red.

The methanolic leaf extract of *Combretum affinis laxum* (Combretaceae) showed herbicidal activity against pre-emergent *Agrostis stolonifera* (**Fig 4A**), and post-emergent *Poa annua* in the time range of peak **21**. Tannins in the extract were removed by filtration over polyamide, and 2''-O-galloylmyricitrin (**20**) [32], 3''-O-galloylmyricitrin (**21**) [32], 2''-O-galloylquercitrin (**22**) [33], and 3''-O-galloylquercitrin (**23**) [34, 35] were isolated by HPLC from fractions PA4 and PA5 (**Fig 3S**, Supporting Information), Compound **21** showed no significant herbicidal activity. Ellagic acid (**18**) was obtained from PA5 and confirmed by spiking with a commercial sample. In addition, inactive compounds **12**, **15**, **16** were isolated, together with mearnsitrin (**19**) [36]. All compounds were new for *C. aff. laxum*, since no phytochemical data have been previously reported on this species.

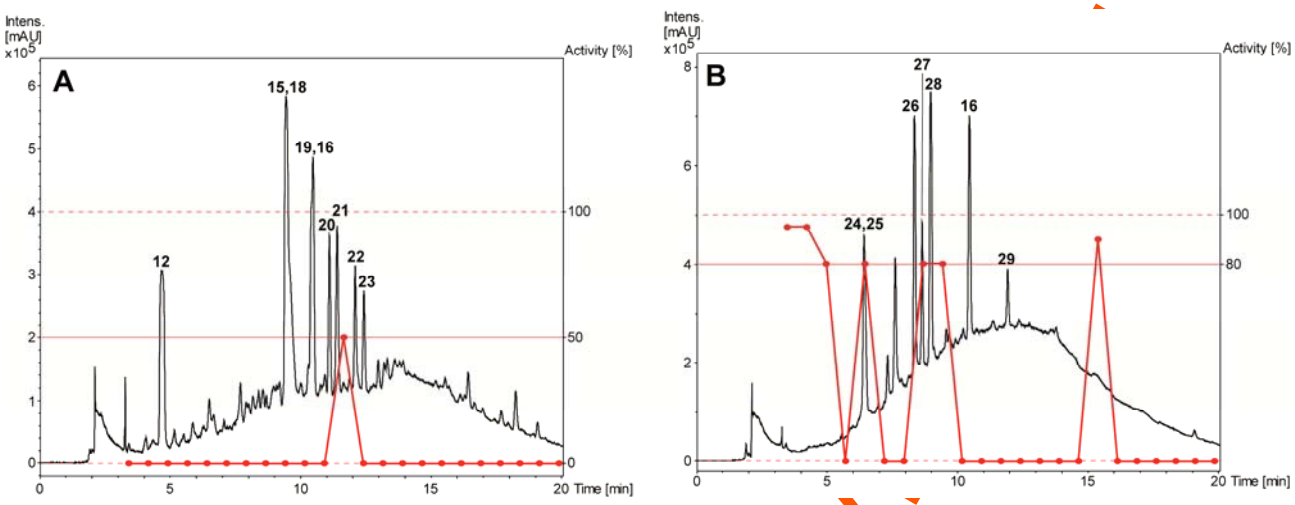

Fig. 4. HPLC-based activity profiling of selected plant extracts for herbicidal activity. SunFire C_{18} column (150 x 10 mm i.d., 5 µm); 5-100% MeCN/0.1% aqueous formic acid in 30min ; 4 mL/min; time-based fractionation; detection: 200-500nm, maxplot. (A) *Combretum aff. laxum* (MeOH leaf extract) against pre-emergent *Agrostis stolonifera*. (B) *Erythroxylum macrophyllum* (MeOH leaf extract) against post-emergent *Matricaria inodora*. Activity of microfractions is shown as red curves.

The extract of *Erythroxylum macrophyllum* (Erythroxylaceae) showed distinct activity against post-emergent *M. inodora*, even though the broad hump in the HPLC chromatogram was indicative of tannins (**Fig 4B**). In time windows t_R 3-5 min and t_R 15-16 min, the activity could not be correlated to a peak in the UV or MS traces. The extract was filtered over polyamide, and five tannin-depleted fractions were obtained (**Fig 4S**, Supporting Information). Compounds in the active time windows were purified by HPLC, and identified as neochlorogenic acid (**24**) [37, 38], protocatechuic acid (**25**) [39], quercetin-3,7-O-α-dirhamnopyranoside (**26**) [40], 5-O-β-glucopyranosylombuin-3-O-β-rutinoside (**27**) [41], and rutin (**28**) [42]. However, none of the flavonoids showed activity in the herbicidal assay when tested as pure compounds. In addition, **16** and ombuin-3-O-β-rutinoside (**29**) [41] were isolated. All compounds are reported here for the first time from *E. macrophyllum*.

In total, four fungicidal and one weakly insecticidal natural product were discovered by means of HPLC-based activity profiling. In contrast, none of the compounds purified from active time windows of *C. aff. laxum* and *E. macrophyllum* showed herbicidal activity. The activity in these time windows may have been, at least in part, due to the presence of tannins. This might have been confirmed by a retest for activity of tannin-depleted extracts. The example of fungicidal compounds showed that the profiling approach could be efficiently used for discovery of bioactive compounds of possible agrochemical interest.

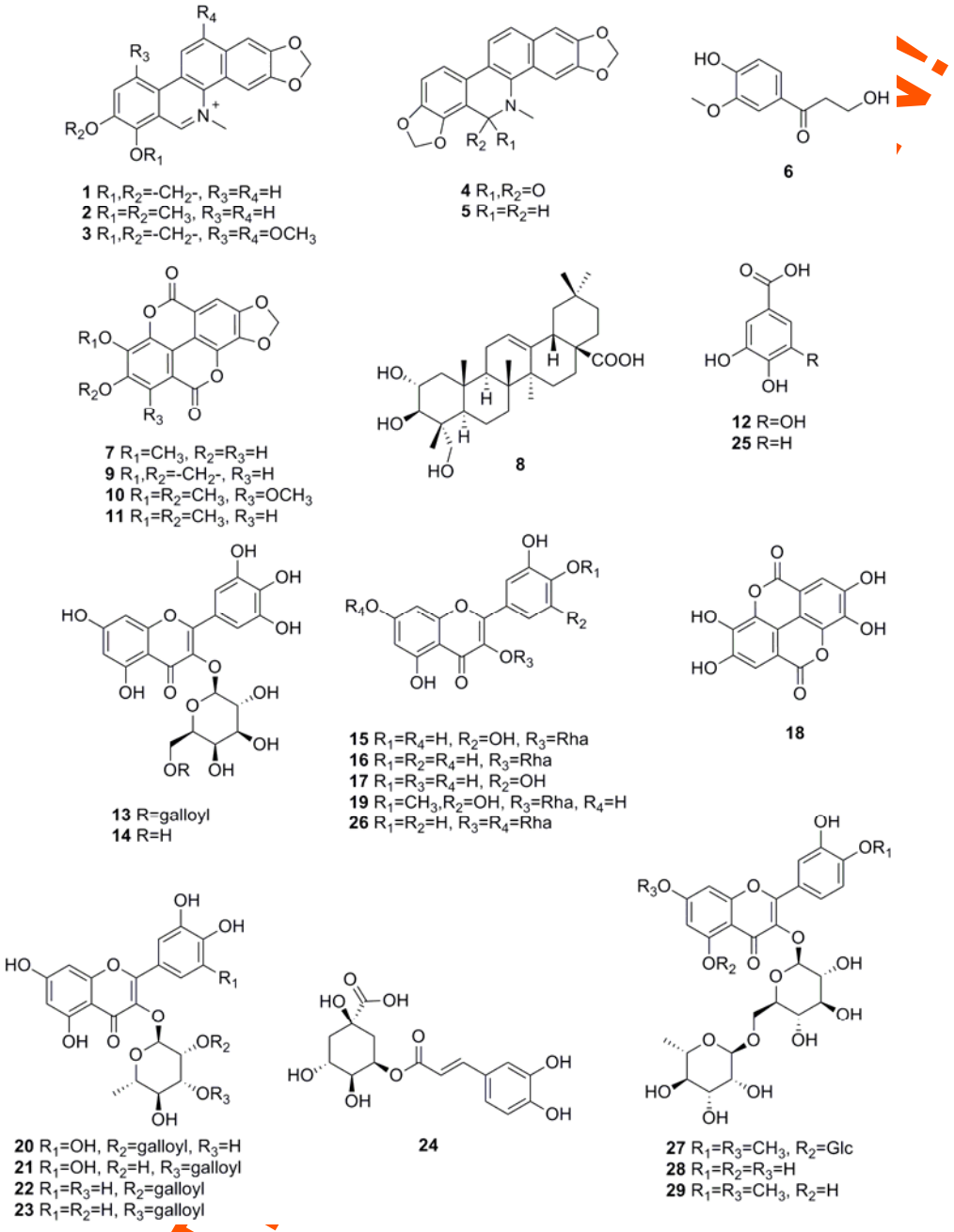

Fig. 5. Structures of identified compounds: sanguinarine (**1**), chelerythrine (**2**), macarpine (**3**), oxysanguinarine (**4**), dihydrosanguinarine (**5**), β-hydroxypropiovanillone (**6**), 3'-O-methyl-3,4-O,O-methyleneellagic acid (**7**), arjunolic acid (**8**), 3,4:3',4'-bis(O-O-methylene)ellagic acid (**9**), 3',4',5'-tri-O-methyl-3,4-O,O-methyleneflavellagic acid (**10**), 3'4'-di-O-methyl-3,4-O,O-methyleneellagic acid (**11**), gallic acid (**12**), myricetin-3-O-(6''-O-galloyl)-β-galactopyranoside (**13**), myricetin-3-O-β-galactopyranoside (**14**), myricitrin (**15**), quercitrin (**16**), myricetin (**17**), ellagic acid (**18**), mearnsitrin (**19**), 2''-O-galloylmyricitrin (**20**), 3''-O-galloylmyricitrin (**21**), 2''-O-galloylquercitrin (**22**), 3''-O-galloylquercitrin (**23**), neochlorogenic acid (**24**), protocatechuic acid (**25**), quercitrin-7-O-α-rhamnopyranoside (**26**), 5-O-β-glucopyranosylombuin-3-O-β-rutinoside (**27**), rutin (**28**), and ombuin-3-O-β-rutinoside (**29**)

Tab. 1. Activity of isolated and tested compounds

Compound	Indication	Activity[a]
Chelerythrine (**2**)	Fungicide	+
Macarpine (**3**)	Fungicide	++
Oxysanguinarine (**4**)	Fungicide	-
Dihydrosanguinarine (**5**)	Fungicide	++
Arjunolic acid (**8**)	Fungicide	++
Myricetin-3-O-(6''-O-galloyl)-β-galactopyranoside (**13**)	Insecticide	+
Myricitrin (**15**)	Insecticide	-
Quercitrin (**16**)	Insecticide	-
3''-O-Galloylmyricitrin (**21**)	Herbicide	-
Neochlorogenic acid (**24**)	Herbicide	-
Protocatechuic acid (**25**)	Herbicide	-
Quercitrin-7-O-α-rhamnopyranoside (**26**)	Herbicide	-
5-O-β-Glucopyranosylombuin-3-O-β-rutinoside (**27**)	Herbicide	-
Rutin (**28**)	Herbicide	-

[a] Data indicated as 0-25% (-), 25-50% (+), 50-75% (++), and 75-100% (+++) activity

Experimental

General experimental procedures

Quercitrin (**16**, ≥98%) and polyamide (particle size: 0.05-0.16 mm) were purchased from Carl Roth. Rutin (**27**, ≥94%) was from Sigma-Aldrich. HPLC-grade acetonitrile and methanol (Reuss Chemie AG), and distilled water were used for HPLC separations. Preparative HPLC was carried out on a LC 8A preparative liquid chromatograph equipped with a SPD-M10A VP PDA detector (all Shimadzu). A SunFire C_{18} column (150 x 30 mm i.d., 5 µm; Waters) connected to a pre-column (10 x 30 mm) was used, at a flow rate of 20 mL/min. HPLC-based activity profiling was performed on an Agilent 1100 system equipped with a PDA detector. A SunFire C_{18} column (150 x 10 mm i.d., 5 µm; Waters) connected to a pre-column (10 x 10 mm) was used; at a flow rate of 4 mL/min. Time-based fractions were collected with a Gilson FC204 fraction collector. Analytical HPLC-DAD-ELSD chromatography was performed on a Waters 2690 Alliance system equipped with a 996 PDA detector and an Alltech ELSD 2000ES. A SunFire C_{18} column (150 x 3 mm i.d., 3.5 µm; Waters) connected to a pre-column (10 x 3 mm) was used, at a flow rate of 0.4 mL/min. Silica gel flash chromatography was performed on an Interchim Puri Flash 4100 system. ESI-MS spectra were obtained on an Esquire 3000 plus ion trap mass spectrometer (Bruker Daltonics). ESI-TOF-MS spectra were recorded in positive mode on a Bruker microTOF ESI-MS system. Mass calibration was done with a reference solution of 0.1% sodium formate in 2-propanol/water (1:1) containing 5 mM NaOH. NMR spectra were recorded on an Avance III 500 MHz spectrometer (Bruker BioSpin) equipped with a 1-mm TXI microprobe and a 5-mm BBO probe.

Plant material

Stems of *Bocconia frutescens L.* were collected in August 2012 in El Valle de Antón, La Mesa, Coclé, Panama. Stems of *Miconia affinis DC.* were collected in October 2007 in Parque Nacional Chagres, section Cerro Azul, Panama. Leaves of *Myrcia splendens (SW.) DC.* were collected in June 2012 in Parque Nacional Altos de Campana, Panama. Stems of *Combretum affinis laxum Jacq.* were collected in November 1996 in Punta Muñiz, Parque Nacional Coiba, Panama. Leaves of *Erythroxylum macrophyllum Kunth* were collected in January 1993 in Parque Nacional Altos de Campana, Panama. The plant material was identified by Alex Espinosa, and vouchers specimens have been deposited at the Herbarium of the University of Panama (PMA). Also, vouchers are kept at the Division of Pharmaceutical Biology, University of Basel: Nr. 844 (*B. frutescens*), Nr. 845 (*M. affinis*), Nr. 901 (*M. splendens*), Nr. 866 (*C. aff. laxum*), and Nr. 867 (*E. lucidum*).

HPLC-based activity profiling

Extracts dissolved in DMSO (50 mg/mL) were separated by semi-preparative HPLC. Two aliquots of 200 µL corresponding to 10 mg extract were injected. For two ethyl acetate extracts (stems of *Bocconia frutescens* and leaves of *Clusia uvitana*), a gradient of 50-100% MeCN in 30 min in 0.1% aqueous formic acid was used. For the other extracts, a gradient of 5-100% MeCN in 30 min in 0.1% aqueous formic acid was employed. Fractions of 0.75 min were collected from t = 3 min to t = 33 min. Fractions were transferred into 96-deepwell plates, evaporated, and submitted to screening.

Extraction and isolation

Powdered stems of *B. frutescens* (1002 g) were percolated with 15 L MeOH to afford 45.9 g extract. A portion (10.0 g) of the extract was submitted to silica gel flash chromatography using CH_2Cl_2 for 30 min, followed by a gradient of 0-5% in 60 min, 5% over 30 min, 5-10% in 30 min, and 10% MeOH in CH_2Cl_2 over 60 min, at a flow rate of 50 mL/min. Eight fractions (Fr. 1-8) were combined on the basis of TLC patterns. Fr. 1 (409.3 mg) was separated by preparative HPLC (80% aqueous MeCN with 0.1% formic acid) to afford dihydrosanguinarine (**5**, 231.7 mg, t_R 11.4 min). A portion (501.9 mg) of Fr. 7 (1620.1 mg) was separated by flash chromatography on silica gel using hexane (solvent A) and ethyl acetate (solvent B) at a flow rate of 10 mL/min. A gradient of 0-19% B in 19 min, 19% B over 5 min, 19-40% B in 21 min, and 40-100% B in 41 min, followed by 100% B over 81 min yielded six fractions (Fr. 7a-7f). Colorless crystals precipitated from Fr. 7d-7f, and were recrystallized from ethyl acetate/CH_2Cl_2 to afford oxysanguinarine (**4**, 18.6 mg). Fractions 7b-7d were submitted to preparative HPLC (aqueous MeCN with 0.025% TFA). Sanguinarine (**1**, 67.0 mg, t_R 8.1 min) was obtained from Fr. 7b (123.3 mg) using 32% MeCN. For Fr. 7c (163.4 mg) and 7d (123.2 mg), 35% MeCN was used to afford chelerythrine (**2**, 105.1 mg, t_R 7.6 min) and macarpine (**3**, 9.5 mg, t_R 13.6 min).

Powdered stems of *M. affinis* (1001.2 g) were percolated with ethyl acetate (11 L) to afford 4.5 g extract. A portion (2.9 g) of the extract was submitted to silica gel flash chromatography using CH_2Cl_2 (solvent A) and ethyl acetate (solvent B). A gradient of 0-

100% B in 120 min, followed by 100% B over 30min was used, at a flow rate of 40 mL/min to afford 12 fractions (Fr. 1-12). Fr. 5 (370.1 mg) was separated by preparative HPLC using 45% aqueous MeCN to give 3'-O-methyl-3,4-O,O-methyleneellagic acid (**7**, 1.8 mg, t_R 12.8 min), 3',4',5'-tri-O-methyl-3,4-O,O-methyleneflavellagic acid (**10**, 4.2 mg, t_R 23.9 min), and a mixture of 3'4'-di-O-methyl-3,4-O,O-methyleneellagic acid (**11**, t_R 24.9 min) and **10**. From Fr. 8 (102.5 mg), a mixture containing 3,4:3',4'-bis(O,O-methylene)ellagic acid (**9**) precipitated after dissolution in DMSO. The supernatant of Fr. 8 was submitted to preparative HPLC (15% aqueous MeCN) to afford β-hydroxypropiovanillone (**6**, 1.4 mg, t_R 12.9 min). Fr. 12 (396.7 mg) was separated by flash chromatography on silica gel, using CH_2Cl_2 (Solvent A) and MeOH (Solvent B) as mobile phase. A gradient of 0-6% B in 8 min, 6% B over 22 min, and 6-100% B in 10 min afforded arjunolic acid (**8**, 241.1 mg).

Powdered leaves of *M. splendens* (801.8 g) were percolated with MeOH (12 L) to afford 217.0 g extract. A portion (10.2 g) of the extract was re-dissolved in 200 mL MeOH and separated on a polyamide column (50-160 µm, 200 g; Roth) with MeOH as eluent. Three fractions (PA1-PA3) of 1 L each, one fraction (PA4) of 3 L, and one fraction (PA5) of 5 L were collected. Fraction PA2 (682.4 mg) was separated by preparative HPLC using 25% aqueous MeCN, to afford myricitrin (**15**, t_R 7.0 min) and quercitrin (**16**, 5.0 mg, t_R 10.2 min). Final purification of **15** was achieved with 20% aqueous MeCN (134.5 mg, 13.7 min). Preparative HPLC of fraction PA3 (19% aqueous MeCN) yielded myricetin-3-O-β-galactopyranoside (**14**, t_R 10.2 min) and **15** (7.8 mg, t_R 15.9 min). **14** was finally purified by semipreparative HPLC using 17% MeCN in 0.05% aqueous formic acid (4.2 mg, t_R 7.6 min). Fraction PA5 was separated by preparative HPLC with a gradient of MeCN in 0.05% aqueous formic acid (5-40% over 15 min). Gallic acid (**12**, 8.9 mg, t_R 7.6 min), myricetin-3-O-(6''-O-galloyl)-β-galactopyranoside (**13**, t_R 12.2 min), and myricetin (**17**, 4.1 mg, t_R 17.3 min) were obtained. Final purification of **13** by semipreparative HPLC was with 15% MeCN in 0.05% aqueous formic acid (6.9 mg, t_R 9.5 min).

Powdered leaves of *C. aff. laxum* (197.7 g) were percolated with MeOH (5 L) to afford 13.2 g extract. A portion (10.1 g) of the extract was re-dissolved in 200 mL MeOH and submitted to polyamide (200 g) filtration. Four fractions (PA1-PA4) of 1 L each, and one fraction of 3 L (PA5) were collected. Fractions PA2, PA4, and PA5 were submitted to preparative HPLC. A portion (500.0 mg) of fraction PA2 (1036.4 mg) was separated with 25% MeCN in 0.05% aqueous formic acid to afford myricitrin (**15**, 54.0 mg, t_R 6.8 min), mearnsitrin (**19**, 0.64 mg, t_R 9.5 min), and quercitrin (**16**, 8.1 mg, 9.8 min). Fraction PA4 (160.7 mg) was separated with 30% MeCN in 0.05% aqueous formic acid to give 2''-O-galloylmyricitrin (**20**, 10.5 mg, t_R 7.4 min), 3''-O-galloylmyricitrin (**21**, 5.3 mg, t_R 8.3 min), 2''-O-galloylquercitrin (**22**, 6.9 mg, t_R 10.5 min), and 3''-O-galloylquercitrin (**23**, 5.0 mg, 11.9 min). Fraction PA5 (785.6 mg) was separated with a gradient of 21-41% MeCN in 0.05% aqueous formic acid over 30min to afford ellagic acid (**18**, 1.3 mg, t_R 9.5 min), **20** (10.0 mg, t_R 14.3 min), **21** (12.6 mg, 15.2 min), **22** (4.9 mg, 18.0 min), and **23** (6.0 mg, 19.1 min).

Powdered leaves of *E. lucidum* (601.5 g) were percolated with MeOH (11 L) to give 77.3 g extract. A portion (20.3 g) of the extract was re-dissolved in 200 mL MeOH and submitted to polyamide (200 g) filtration. Four fractions (PA1-PA4) of 250 mL each, and one fraction

(PA5) of 5 L were collected. A portion (700.1 mg) of fraction PA3 (2183.2 mg) was submitted to preparative HPLC (gradient of 10-55% MeCN in 0.05% aqueous formic acid over 20min) to afford quercitrin-7-O-α-rhamnopyranoside (**26**, 20.1 mg, t_R 10.2 min), rutin (**28**, 22.9 mg, t_R 11.0 min), quercitrin (**16**, 13.1 mg, t_R 13.2 min), and ombuin-3-O-β-rutinoside (**29**, 6.6 mg, t_R 15.1 min). Two mixed fractions (t_R 8.4 min and t_R 10.5 min) were submitted to final purification by preparative HPLC to afford neochlorogenic acid (**24**, 6.2 mg, t_R 5.6 min), protocatechuic acid (**25**, 2.0 mg, t_R 6.2 min), **26** (2.1 mg, t_R 6.5 min), and 5-O-β-glucopyranosylombuin-3-O-β-rutinoside (**27**, 8.2 mg, t_R 7.5 min), respectively.
Compounds were identified with the aid of ^{1}H and 2D NMR, and ESI-MS spectroscopy, and by comparison with literature data. Purity of isolated compounds was >95% as determined by NMR, except for compounds **3** (90%), **4** (80%), **25** (90%), and **29** (80%).

Fungicidal assay

The activity against phytopathogenic fungi (*Botryotinia fuckeliana*, *Magnaporthe oryzae*, *Phytophtora infestans*, and *Septoria tritici*) could be demonstrated by the treatment of fungal spore suspensions and analysis of the growth in microplates using a robot system.
The tests were done in 96 well microtiter plates. Compounds were transferred as DMSO solutions into empty plates, followed by addition of a spore suspension of the fungus of interest in a nutrient solution. Compounds were tested either in a single concentration, or as serial dilutions at 10 concentrations. Each plate contained 8 solvent control wells and 8 reference wells containing a known fungicide. The plates were incubated at 23 °C and 90 % relative humidity. Fungal growth was assessed by measuring the optical density at 620 nm, immediately after treatment, and 10 times in intervals of 15 hours. In order to calculate the activity of a compound at a given concentration, the optical density values of each measurement of a compound was compared with those of the control and the reference, giving results from 0 to 1, whereby higher values indicated higher activity. ED_{50} values were calculated with the aid of the dilution series. A compound having an activity ratio ≥ 0.75, or an ED_{50} ≤ 10 mg/l was considered as active.

Insecticidal assay

Tested insect species were *Anthonomus grandis*, *Heliothis virescens*, *Ceratitis capitata*, *Megoura viciae*, and *Myzus persicae*. Insecticidal activity, either as contact or systemic insecticide, against piercing/sucking insects (adults and offspring) was assessed in a test unit consisting of 24-well-microtiter plates containing broad bean leaf disks. The compounds were formulated using a solution containing 75% v/v water and 25% v/v DMSO. Different concentrations of formulated compounds were sprayed onto the leaf disks at 2.5 µl, using a custom built micro-atomizer. Two replicates were prepared. After application, leaf disks were air-dried, and 5 – 8 adult insects were placed onto the leaf disks placed into wells of a microtiter plate. Insects were then allowed to suck on the treated leaf disks, and were incubated at about 23 ± 1°C and about 50 ± 5 % relative humidity for 5 days. Mortality was visually assessed.

Activity against biting insects (larvae) was evaluated in a test unit consisting of 24-well-microtiter plates containing an insect diet and 20-30 insect eggs. Test compounds were formulated using a solution containing 75% v/v water and 25% v/v DMSO. Aliquots (20 µl) of different concentrations of formulated compounds were sprayed onto the insect diet using a custom built micro-atomizer. Two replicates were used. After application, microtiter plates were incubated for 5 days at 23 ± 1°C and 50 ± 5 % relative humidity. Egg and larval mortality was then visually assessed. Compounds with ≥ 50% mortality in adult insects and larvae were considered as active.

Herbicidal assay

Herbicidal activity was assessed on pre- and post-emergent *Matricaria inodora*, *Agrostis stolonifera*, and *Poa annua*. The culture containers used were plastic 96 well-plates containing peat substrate. For the post-emergence treatment, the test plants once they reached a height of 1-3 cm (depending on the plant species), were sprayed via a spray nozzle with the test compounds in 1000 ppm DMSO solution. The application rate corresponded to 2 kg/ha, with an application volume of 200 L/ha. Plants were kept at 20–35°C. The test period extended over 7 days. During this time, the plants were tended, and their response to the individual treatments was evaluated visually. The cutoff for herbicidal activity was ≥ 50% inhibition of growth (or 80% in the case of *Matricaria inodora*) of the treated weed, either pre- or post-emergence.

Acknowledgement

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Supporting Information

A list of the 19 active extracts, additional activity profiles for *B. frutescens*, polyamide profiles of *M. splendens*, *C. aff. laxum*, and *E. macrophyllum*, and NMR data of compounds **1**-**8**, **10**, **12**-**17**, and **19**-**29** are available in the online version (Type: xxx, Size: ca. xxx): http://dx.doi.org/10.3797/scipharm.xxx

Authors' Statement

Competing Interests

The authors declare no conflict of interest

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Running Title Head

Screening of Panamanian plant extracts

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Supporting Information

Screening of Panamanian plant extracts for pesticidal properties, and HPLC-based identification of active compounds

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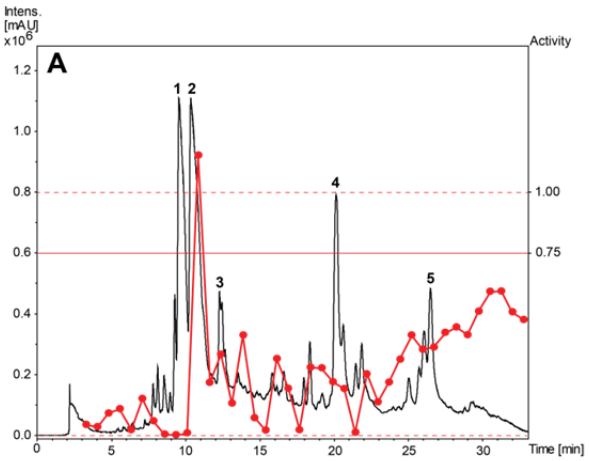
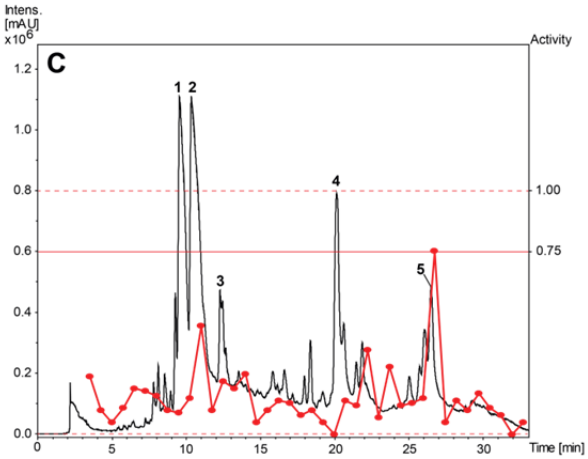
Table 1S List of the 19 active extracts from the extract screening

Plant name	**Extract**	**Indication**
Bocconia frutescens	MeOH (stem)	Fungicide
Miconia affinis	EtOAc (stem)	Fungicide
Bocconia frutescens	EtOAc (stem)	Fungicide
Miconia ligulata	EtOAc (leaves)	Fungicide
Piper gatunense	EtOAc (stem)	Fungicide
Clusia uvitana	EtOAc (leaves)	Fungicide
Salvia alvajaca	MeOH (root)	Insecticide
Simaba cedron	MeOH (stem)	Insecticide
Myrcia splendens	MeOH (leaves)	Insecticide
Psychotria erecta	MeOH (stem)	Insecticide
Psychotria suerrensis	MeOH (root)	Insecticide
Rollinia pittieri	EtOAc (leaves)	Insecticide
Rollinia mucosa	EtOAc (leaves)	Insecticide
Myrcia splendens	MeOH (branch)	Insecticide
Ocotea glaucosericea	EtOAc (stem)	Insecticide
Trichilia hirta	MeOH (stem)	Herbicide
Combretum aff. laxum	MeOH (leaves)	Herbicide
Erythroxylum macrophyllum	MeOH (leaves)	Herbicide
Picramnia antidesma	MeOH (root)	Herbicide

Figure 1S Profiles of the MeOH stem extracts of *Bocconia frutescens* for the plant pathogenic fungi *Botryotinia fuckeliana* (A), *Phytophthora infestans* (B), and *Septoria tritici* (C)





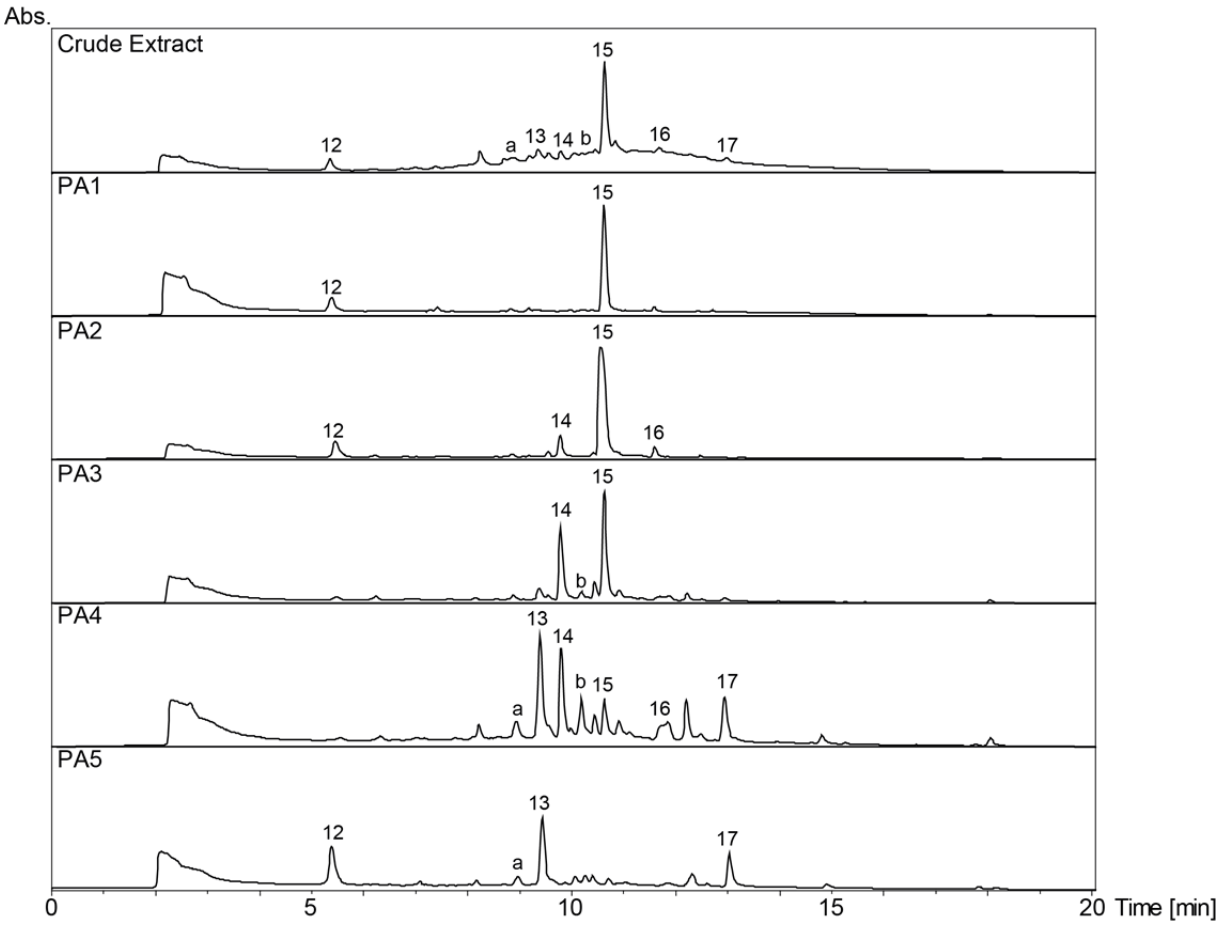


Figure 2S HPLC-DAD chromatograms of the crude extract and its polyamide fractions (PA1-PA5) of *Myrcia splendens*. SunFire C18 column (150 x 3 mm i.d., 3.5 µm); 5-100% MeCN/0.1% aqueous formic acid in 30min, 0.4 mL/min; detection: 210-700nm, maxplot.

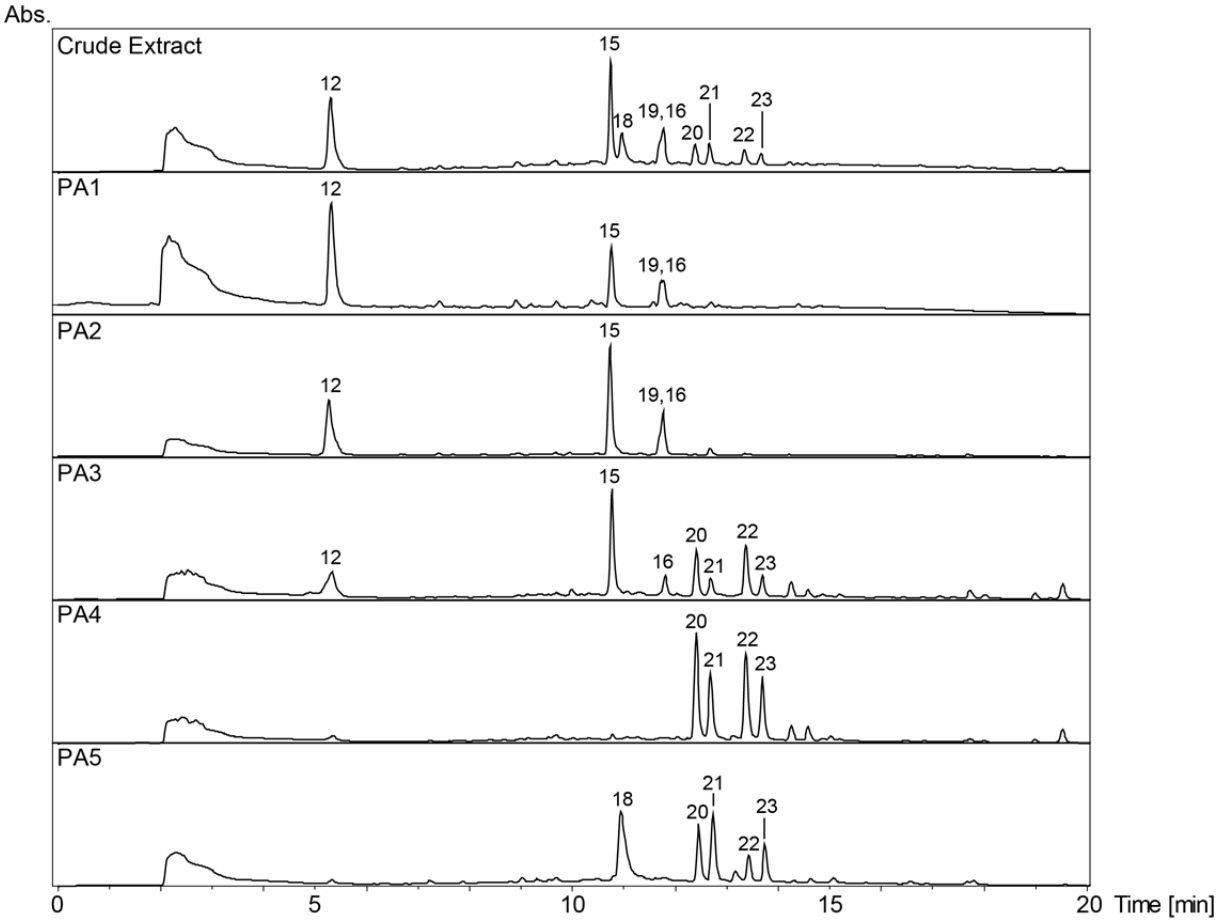


Figure 3S HPLC-DAD chromatograms of the crude extract and its polyamide fractions (PA1-PA5) of *Combretum aff. laxum*. SunFire C18 column (150 x 3 mm i.d., 3.5 µm); 5-100% MeCN/0.1% aqueous formic acid in 30min, 0.4 mL/min; detection: 210-700nm, maxplot.

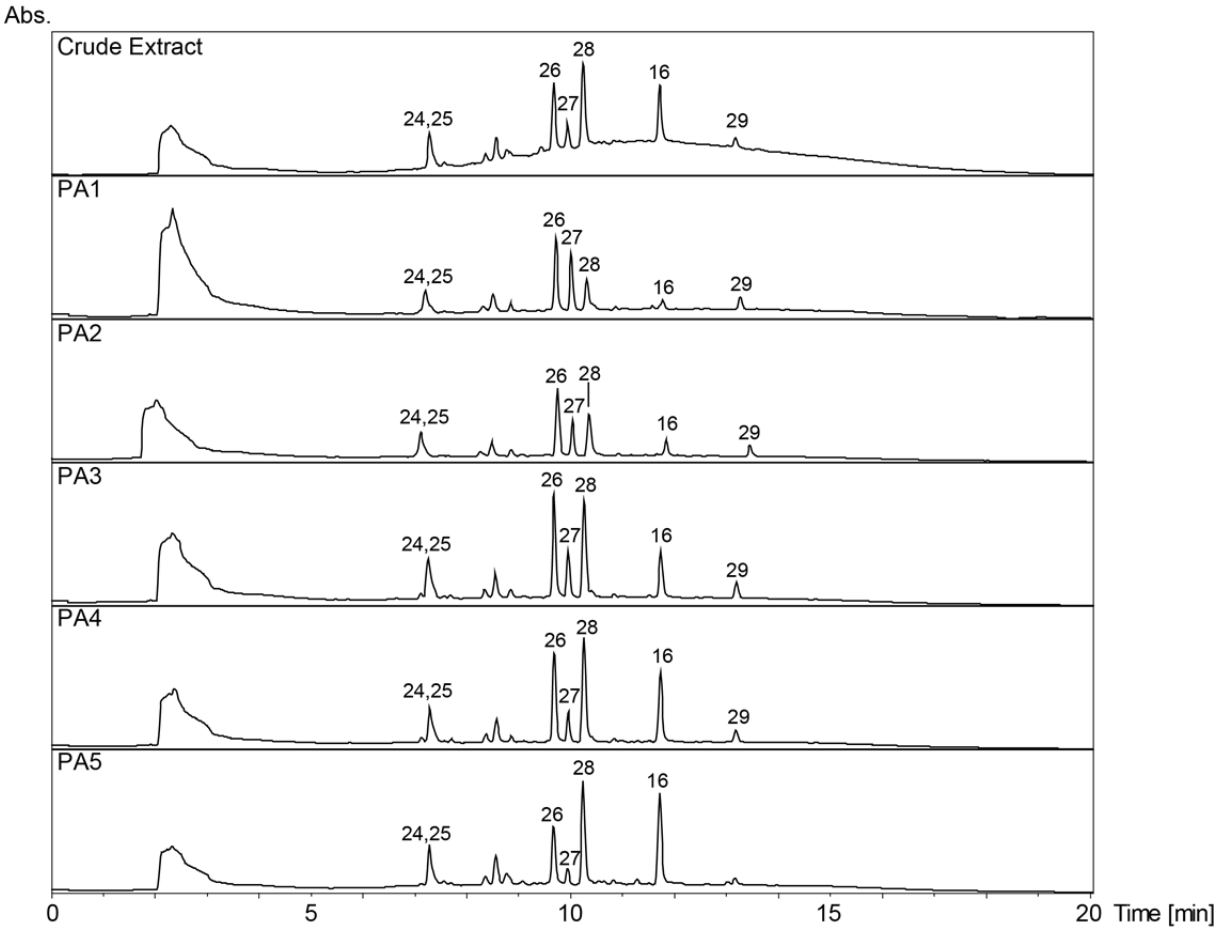


Figure 4S HPLC-DAD chromatograms of the crude extract and its polyamide fractions (PA1-PA5) of *Erythroxylum macrophyllum*. SunFire C18 column (150 x 3 mm i.d., 3.5 µm); 5-100% MeCN/0.1% aqueous formic acid in 30min, 0.4 mL/min; detection: 210-700nm, maxplot.

Table 2S ^{1}H and ^{13}C NMR[a] data (500 MHz) of compounds **1-5**

	1[b]		**2**[b]		**3**[c]		**4**[d]		**5**[c]	
Position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	7.51 (s)	106.9	7.43 (s)	106.7	7.61 (s)	98.8	7.14 (s)	104.5	7.31 (s)	104.0
2	-	150.6	-	150.6	-	148.6	-	147.8	-	147.2
3	-	150.6	-	150.6	-	148.6	-	147.8	-	147.8
4	8.11 (s)	104.8	8.07 (s)	104.6	8.06 (s)	104.0	7.55 (s)	102.2	7.53 (s)	99.4
4a	-	121.4	-	121.5	-	120.7	-	118.9	-	124.9
4b	-	132.8	-	132.9	-	126.2	-	136.2	-	141.0
6	9.90 (s)	150.3	9.85 (s)	151.1	9.72 (s)	145.7	-	162.8	4.16 (s)	47.7
6a	-	110.9	-	120.6	-	109.8	-	111.1	-	112.3
7	-	147.8	-	147.1	-	139.0	-	146.6	-	144.2
8	-	149.2	-	151.6	-	147.5	-	146.6	-	146.6
9	7.93 (d, 8.8)	121.0	8.12 (d, 9.1)	127.2	7.83 (s)	103.8	7.22 (d, 8.6)	113.0	6.95 (d, 8.1)	107.1
10	8.47 (d, 8.8)	118.1	8.52 (d, 9.1)	119.4	-	152.8	7.74 (d, 8.6)	115.2	7.40 (d, 8.1)	116.1
10a	-	128.7	-	129.8	-	113.9	-	133.6	-	126.2
10b	-	127.3	-	126.6	-	123.5	-	115.9	-	123.7
11	8.56 (d, 8.9)	119.4	8.48 (d, 9.0)	119.1	8.71 (s)	99.6	7.96 (d, 8.6)	118.4	7.77 (d, 8.6)	120.0
12	8.16 (d, 8.9)	132.7	8.08 (d, 9.0)	132.2	-	155.0	7.51 (d, 8.6)	123.5	7.57 (d, 8.6)	123.9
12a	-	133.5	-	134.0	-	123.0	-	129.8	-	130.2
2,3-OCH_2O	6.28 (s)	104.0	6.24 (s)	103.8	6.32 (s)	102.3	6.08 (s)	101.1	6.14 (s)	100.9
5-NCH_3	4.93 (s)	52.7	4.94 (s)	52.8	4.78 (s)	51.5	3.89 (s)	40.7	2.53 (s)	41.1
7,8-OCH_2O	6.53 (s)	106.2	-	-	6.51 (s)	103.6	6.25 (s)	102.7	6.10 (s)	101.1
7-OCH_3	-	-	4.27 (s)	62.6	-	-	-	-	-	-
8-OCH_3	-	-	4.11 (s)	57.4	-	-	-	-	-	-
10-OCH_3	-	-	-	-	4.17 (s)	57.5	-	-	-	-
12-OCH_3	-	-	-	-	4.12 (s)	55.7	-	-	-	-

[a] ^{13}C NMR data derived from HSQC and HMBC experiments; [b] recorded in CD_3OD; [c] recorded in DMSO-d_6; [d] recorded in $CDCl_3$

Table 3S ^{1}H and ^{13}C NMR[a] data (500 MHz) of compound **6** in DMSO-d_6

	6	
Position	δ_H	δ_C
1	-	196.7
2	3.04 (t, 6.4)	40.6
3	3.76 (t, 6.4)	57.0
1'	-	127.6
2'	7.43 (d, 1.7)	110.9
3'	-	147.5
4'	-	152.6
5'	6.84 (d, 8.3)	114.8
6'	7.49 (dd, 8.3, 1.7)	123.1
3'-OCH_3	3.81 (s)	55.3

[a] ^{13}C NMR data derived from HSQC and HMBC experiments

Table 4S ^{1}H and ^{13}C NMR[a] data (500 MHz) of compounds **12** and **25**

	12[b]		**25**[c]	
Position	δ_H	δ_C[a]	δ_H	δ_C
1	-	119.4	-	123.2
2	7.09 (s)	107.8	7.36 (s)	116.6
3	-	143.9	-	144.6
4	-	136.7	-	149.1
5	-	143.9	6.77 (d, 8.1)	114.8
6	7.09 (s)	107.8	7.28 (d, 8.1	121.4
7	-	167.4	-	168.3

[a] ^{13}C NMR data derived from HSQC and HMBC experiments; [b]recorded in CD_3OD; [c]recorded in DMSO-d_6

Table 5S ^{1}H and ^{13}C NMR[a] (500 MHz) of compound **7** and **10**

	7[b]		**10**[c]	
Position	δ_H	δ_C[a]	δ_H	δ_C
1	-	n.d.	-	114.5
2	-	n.d.	-	132.4
3	-	138.3	-	138.8
4	-	150.2	-	151.3
5	7.44 (s)	103.3	7.57 (s)	105.0
6	-	n.d.	-	115.7
7	-	n.d.	-	158.6
1'	-	108.5	-	113.0
2'	-	n.d.	-	137.9
3'	-	141.4	-	147.6
4'	-	n.d.	-	148.6
5'	7.47 (s)	113.2	-	154.3
6'	-	n.d.	-	105.9
7'	-	n.d.	-	154.9
3,4-OCH_2O	6.34 (s)	103.6	6.27 (s)	104.2
3'-OCH_3	4.04 (s)	60.2	4.25 (s)	62.3
4'-OCH_3	-	-	4.00 (s)	62.3
5'-OCH_3	-	-	4.02 (s)	62.6

[a] ^{13}C NMR data derived from HSQC and HMBC experiments; [b]recorded in DMSO-d_6; [c]recorded in $CDCl_3$; n.d.: not detected

Table 6S ^{1}H and ^{13}C NMR data (500 MHz) of compound **8** in DMSO-d_6

	8	
Position	δ_H	δ_C
1a	0.73 (m)	46.7
1b	1.75 (dd, 12.5, 4.4)	
2	3.49 (ddd, 11.0, 9.3, 4.4)	67.4
3	3.18 (d, 9.3)	75.5
4	-	42.5
5	1.22 (m)	46.0
6a	1.24 (m)	17.5
6b	1.41 (m)	
7a	1.19 (m)	31.9
7b	1.44 (m)	
8	-	38.9
9	1.55 (t, 8.8)	47.1
10	-	37.4
11	1.84 (m)	23.0
12	5.18 (t, 3.3)	121.5
13	-	143.9
14	-	41.4
15a	0.99 (m)	27.2
15b	1.68 (m)	
16a	1.49 (m)	22.6
16b	1.91 (td, 13.5, 3.7)	
17	-	45.4
18	2.76 (dd, 13.7, 3.9)	40.8
19a	1.07 (m)	45.7
19b	1.62 (m)	
20	-	30.4
21a	1.14 (m)	33.3
21b	1.32 (td, 13.6, 3.6)	
22a	1.44 (m)	32.1
22b	1.63 (m)	
23a	3.06 (d, 10.6)	63.9
23b	3.32 (d, 10.6)	
24	0.56 (s)	13.7
25	0.93 (s)	16.8
26	0.72 (s)	16.9
27	1.10 (s)	25.7
28	-	178.6
29	0.88 (s)	32.9
30	0.88 (s)	23.4

Table 7S ^{1}H and ^{13}C NMR[a] (500 MHz) of compounds **13** and **14** in CD_3OD

	13		**14**	
Position	δ_H	δ_C	δ_H	δ_C
2	-	158.8	-	n.d.
3	-	135.9	-	135.8
4	-	n.d.	-	n.d.
5	-	n.d.	-	162.7
6	6.17 (d, 1.8)	99.8	6.17 (s)	99.5
7	-	n.d.	-	165.7
8	6.35 (d, 1.8)	94.6	6.35 (s)	94.3
9	-	n.d.	-	158.3
10	-	n.d.	-	105.5
1'	-	121.5	-	121.3
2'	7.34 (s)	110.0	7.37 (s)	109.7
3'	-	146.3	-	145.8
4'	-	139.8	-	137.8
5'	-	146.3	-	145.8
6'	7.34 (s)	110.0	7.37 (s)	109.7
1''	5.15 (d, 7.9)	105.2	5.13 (d, 7.8)	105.2
2''	3.87 (dd, 9.5, 7.9)	73.0	3.85 (dd, 9.5, 7.8)	72.9
3''	3.62 (dd, 9.5, 2.9)	74.8	3.61 (m)	74.6
4''	3.91 (d, 2.9)	69.9	3.89 (d, 2.5)	69.6
5''	3.81 (t, 6.4)	74.3	3.52 (t, 6.1)	76.6
6''a	4.25 (dd, 11.1, 5.9)	63.6	3.61 (m)	61.6
6''b	4.33 (dd, 11.1, 6.9)		3.67 (dd, 11.2, 5.9)	
1'''	-	121.1	-	-
2'''	6.91	110.0	-	-
3'''	-	146.3	-	-
4'''	-	138.2	-	-
5'''	-	146.3	-	-
6'''	6.91	110.0	-	-
7'''	-	167.8	-	-

[a] ^{13}C NMR data derived from HSQC and HMBC experiments; n.d.: not detected

Table 8S ^{1}H and ^{13}C NMR[a] (500 MHz) of compounds **15**-**17** in CD_3OD

	15		**16**		**17**	
Position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2	-	158.9	-	158.9	-	n.d.
3	-	135.9	-	135.8	-	n.d.
4	-	179.2	-	n.d.	-	n.d.
5	-	163.2	-	163.6	-	n.d.
6	6.20 (d, 2.0)	99.6	6.20 (d, 1.7)	99.7	6.18 (s)	99.2
7	-	165.5	-	165.4	-	n.d.
8	6.36 (d, 2.0)	94.5	6.36 (d, 1.7)	94.6	6.37 (s)	94.2
9	-	158.0	-	158.2	-	n.d.
10	-	105.4	-	105.6	-	n.d.
1'	-	121.5	-	n.d.	-	123.3
2'	6.95 (s)	109.3	7.34 (d, 2.1)	116.9	7.35 (s)	108.5
3'	-	146.4	-	146.2	-	147.5
4'	-	137.4	-	149.1	-	137.1
5'	-	146.4	6.92 (d, 6.3)	116.2	-	147.5
6'	6.95 (s)	109.3	7.30 (dd, 6.3, 2.1)	122.7	7.35 (s)	108.5
1''	5.32 (d, 1.5)	103.4	5.36 (d, 1.3)	103.3	-	-
2''	4.23 (dd, 3.3, 1.5)	71.7	4.23 (dd, 3.1, 1.3)	71.7	-	-
3''	3.79 (dd, 9.5, 3.3)	71.9	3.76 (dd, 9.3, 3.1)	72.1	-	-
4''	3.35 (t, 9.6)	73.2	3.35 (9.4)	73.2	-	-
5''	3.52 (dq, 9.7, 6.2)	71.8	3.43 (dq, 9.6, 6.1)	71.8	-	-
6''	0.97 (d, 6.2)	17.6	0.95 (d, 6.1)	17.5	-	-

[a] ^{13}C NMR data derived from HSQC and HMBC experiments; n.d.: not detected

Table 9S ^{1}H and ^{13}C NMR[a] (500 MHz) of compounds **19** and **26** in DMSO-d_6

	19		**26**	
Position	δ_H	δ_C	δ_H	δ_C
2	-	157.1	-	n.d
3	-	n.d.	-	135.0
4	-	n.d.	-	n.d
5	-	n.d.	-	n.d
6	6.24 (d, 1.2)	98.5	6.44 (d, 1.6)	99.1
7	-	n.d.	-	n.d
8	6.44 (d, 1.2)	93.2	6.75 (d, 1.6)	94.1
9	-	n.d.	-	n.d
10	-	104.2	-	105.6
1'	-	n.d.	-	n.d
2'	6.86 (s)	107.8	7.36 (d, 1.8)	115.5
3'	-	150.7	-	145.0
4'	-	137.9	-	148.1
5'	-	150.7	6.89 (d, 8.2)	115.2
6'	6.86 (s)	107.8	7.30 (dd, 8.2, 1.8)	120.9
1''	5.16 (d, 1.0)	101.8	5.28 (s)	101.6
2''	4.00 (m)	69.7	4.00 (bs)	69.8
3''	3.56 (m)	70.1	3.55 (dd, 9.2, 3.1)	70.2
4''	3.16 (m)	71.0	3.18 (m)	71.0
5''	3.22 (m)	70.0	3.28 (m)	70.2
6''	0.83 (d, 6.1)	17.1	0.85 (d, 6.1)	17.3
1'''	-	-	5.55 (s)	98.2
2'''	-	-	3.86 (bs)	69.5
3'''	-	-	3.65 (dd, 9.2, 3.0)	70.0
4'''	-	-	3.32 (m)	71.4
5'''	-	-	3.46 (m)	69.8
6'''	-	-	1.14 (d, 6.1)	17.6
4'-OCH_3	3.75 (s)	59.3	-	-

[a] ^{13}C NMR data derived from HSQC and HMBC experiments; n.d.: not detected

Table 10S ^{1}H and ^{13}C NMR[a] (500 MHz) of compounds **20**-**23** in CD_3OD

	20		**21**		**22**		**23**	
Position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2	-	158.7	-	158.9	-	158.9	-	159.3
3	-	135.1	-	135.7	-	135.2	-	135.9
4	-	178.9	-	n.d.	-	178.7	-	n.d.
5	-	163.3	-	163.6	-	163.4	-	163.9
6	6.18 (d, 1.8)	99.3	6.21 (s)	99.3	6.19 (d, 1.9)	99.6	6.21 (s)	99.8
7	-	165.5	-	165.3	-	165.2	-	165.8
8	6.35 (d, 1.8)	94.2	6.37 (s)	94.2	6.36 (d, 1.9)	94.4	6.38 (s)	94.7
9	-	157.9	-	157.8	-	158.0	-	158.3
10	-	105.4	-	105.6	-	105.4	-	106.0
1'	-	121.6	-	121.7	-	n.d.	-	n.d.
2'	7.00 (s)	109.3	7.02 (s)	109.3	7.37 (d, 2.0)	116.7	7.40 (m)	116.9
3'	-	146.2	-	146.4	-	146.0	-	146.5
4'	-	137.2	-	137.5	-	149.3	-	149.6
5'	-	146.2	-	146.4	6.94 (d, 8.3)	116.2	6.96 (d, 8.7)	116.5
6'	7.00 (s)	109.3	7.02 (s)	109.3	7.33 (dd, 8.3, 2.0)	122.6	7.40 (m)	123.0
1''	5.51 (d, 1.1)	100.0	5.34 (d, 1.1)	103.0	5.50 (d, 1.5)	100.2	5.41 (bs)	103.3
2''	5.64 (dd, 3.1, 1.1)	73.0	4.51 (dd, 2.8, 1.1)	69.5	5.64 (dd, 3.2, 1.5)	73.3	4.49 (m)	69.9
3''	4.08 (dd, 8.9, 3.1)	70.2	5.27 (dd, 8.9, 2.8)	75.0	4.04 (dd, 8.9, 3.2)	70.5	5.24 (9.7, 3.0)	75.3
4''	3.50 (m)	73.4	3.70 (m)	70.5	3.47 (m)	73.5	3.69 (t, 9.7)	70.9
5''	3.53 (m)	71.7	3.70 (m)	71.8	3.47 (m)	71.9	3.59 (m)	72.2
6''	1.05 (d, 5.6)	17.2	1.02 (d, 4.5)	17.1	1.04 (d, 5.4)	17.4	1.01 (d, 6.1)	17.6
1'''	-	120.8	-	121.2	-	121.0	-	121.7
2'''	7.09 (s)	110.0	7.19 (s)	110.1	7.09 (s)	110.2	7.19 (s)	110.5
3'''	-	145.6	-	145.8	-	146.0	-	146.3
4'''	-	139.3	-	139.5	-	139.4	-	139.9
5'''	-	145.6	-	145.8	-	146.0	-	146.3
6'''	7.09 (s)	110.0	7.19 (s)	110.1	7.09 (s)	110.2	7.19 (s)	110.5
7'''	-	166.9	-	168.2	-	167.1	-	168.4

[a] ^{13}C NMR data derived from HSQC and HMBC experiments; n.d.: not detected

Table 11S ^{1}H and ^{13}C NMR[a] (500 MHz) of compounds **24** in DMSO-d_6

	24	
Position	δ_H	δ_C
1	-	n.d.
2a	1.71 (d, 12.8)	37.2
2b	1.89 (m)	
3	3.76 (m)	68.1
4	3.63 (m)	70.6
5	5.26 (bs)	70.4
6a	1.85 (m)	35.6
6b	1.93 (m)	
1'	-	165.6
2'	6.20 (d, 15.9)	114.6
3'	7.47 (d, 15.9)	144.1
4'	-	125.2
5'	7.04 (s)	114.4
6'	-	145.3
7'	-	147.6
8'	6.78 (d, 7.9)	115.6
9'	6.96 (d, 7.9)	120.7
1-COOH	-	n.d.

[a] ^{13}C NMR data derived from HSQC and HMBC experiments; n.d.: not detected

Table 12S ^{1}H and ^{13}C NMR (500 MHz) of compounds **27**-**29** in DMSO-d_6

	27		**28**		**29**	
Position	δ_H	δ_C	δ_H	δ_C [a]	δ_H	δ_C [a]
2	-	154.7	-	156.2	-	n.d.
3	-	136.0	-	n.d.	-	n.d.
4	-	173.2	-	n.d.	-	n.d.
5	-	158.4	-	160.9	-	n.d.
6	6.86 (s)	102.4	6.19 (s)	98.5	6.35 (s)	97.5
7	-	163.8	-	163.9	-	164.6
8	6.91 (s)	95.7	6.39 (s)	93.3	6.67 (s)	91.6
9	-	157.7	-	156.0	-	n.d.
10	-	109.0	-	103.5	-	n.d.
1'	-	122.5	-	120.9	-	121.8
2'	7.57 (s)	115.8	7.54 (m)	116.0	7.56 (s)	115.3
3'	-	145.9	-	144.4	-	145.5
4'	-	150.0	-	147.6	-	149.7
5'	7.03 (d, 8.5)	111.3	6.85 (d, 7.8)	114.9	7.04 (d, 8.7)	110.9
6'	7.74 (d, 8.5)	121.5	7.54 (m)	121.2	7.72 (d, 8.7)	120.9
1''	5.25 (d, 6.9)	101.4	5.34 (bd)	101.0	5.39 (d, 6.6)	100.6
2''	3.26 (m)	74.1	3.25 (m)	73.8	3.22 (m)	73.5
3''	3.27 (m)	75.7	3.24 (m)	76.1	3.23 (m)	75.8
4''	3.12 (m)	69.8	3.30 (m)	70.3	3.10 (m)	69.4
5''	3.28 (m)	76.5	3.26 (m)	75.6	3.25 (m)	75.4
6''a	3.34 (m)	66.9	3.30 (m)	66.8	3.31 (m)	66.4
6''b	3.71 (d, 11.1)		3.72 (d, 10.8)		3.69 (m)	
1'''	4.42 (bs)	100.8	4.40 (s)	100.4	4.39 (s)	100.3
2'''	3.42 (m)	70.4	3.42 (m)	70.1	3.41 (m)	69.9
3'''	3.33 (m)	70.7	3.08 (m)	69.8	3.29 (m)	70.1
4'''	3.10 (m)	71.9	3.09 (m)	71.6	3.08 (m)	71.3
5'''	3.30 (m)	68.3	3.29 (m)	67.8	3.27 (m)	67.8
6'''	0.99 (d, 5.9)	17.9	1.00 (d, 4.4)	17.5	0.97 (d, 6.0)	17.4
1''''	4.84 (d, 7.4)	103.7				
2''''	3.42 (m)	73.6				
3''''	3.34 (m)	75.9				
4''''	3.21 (m)	69.9				
5''''	3.41 (m)	77.7				
6''''a	3.53 (dd, 11.1, 6.0)	60.9				
6''''b	3.77 (d, 11.1)					
7-OCH_3	3.89 (s)	56.2	-	-	3.86 (s)	55.3
4'-OCH_3	3.86 (s)	55.7	-	-	3.86 (s)	55.3

[a] ^{13}C NMR data derived from HSQC and HMBC experiments; n.d.: not detected

3.2 Screening of Panamanian plants for cosmetic properties, and HPLC-based identification of constituents with antioxidant and UV-B protecting activities

Niels Guldbrandsen, Maria De Mieri, Mahabir Gupta, Eleni Liakou, Harris Pratsinis, Dimitris Kletsas, Eliza Chaita, Nektarios Aligiannis, Alexios-Leandros Skaltsounis, Matthias Hamburger

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A library of 600 Panamanian plant extracts was screened for cosmetic properties. Assays assessed DPPH-scavenging properties, and protection against UV-B-induced cell death. Three active extracts were submitted to HPLC-based activity profiling, from which two extracts were selected for isolation of active compounds. Compounds were identified by HPLC-PDA-ESI-MS and NMR spectroscopy, and submitted to cosmetic screening. All isolated compounds were known phenolic compounds and flavonoids, and showed activity either in the DPPH assay, in the UV-B protection assay, or in both. Gallic and digallic acid derivatives were the most radical scavenging compounds (IC_{50} < 10 μg/mL), while protocatechuic acid and isoquercitrin were the most active in the UV-B protection assay (< 10% UV-induced cell death).

Selection and provision of plant material was done by Mahabir Gupta. Screening of extracts, microfractions, and pure compounds was the contribution of Eliza Chaita, Eleni Liakou, Harris Pratsinis, Dimitris Kletsas, and Nektarios Aligiannis. HPLC-based activity profiling (except assays), extraction of plant material, isolation of compounds, interpretation of spectroscopic data for structure elucidation (HPLC-PDA-ESI-MS and 1D and 2D NMR), writing of the manuscript draft, and preparation of figures were my contributions to this publication.

Niels Guldbrandsen

Research article Open Access

Screening of Panamanian Plants for Cosmetic Properties, and HPLC-Based Identification of Constituents with Antioxidant and UV-B Protecting Activities

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Abstract

A library of 600 taxonomically diverse Panamanian plant extracts was screened for DPPH scavenging and UV-B protective activities, and the methanolic extracts of *Mosquitoxylum jamaicense*, *Combretum cacoucia*, and *Casearia commersionia* were submitted to HPLC-based activity profiling. The compounds located in the active time windows were isolated and identified as gallic acid derivatives and flavonoids. Gallic acid methyl ester (**3**) and digallic acid derivatives (**2**, **6**) showed the highest DPPH scavenging activity (<10 µg/mL), while protocatechuic acid (**7**) and isoquercitrin (**10**) exhibited the highest UV-B protective properties.

Keywords

Panamanian plant extract • HPLC-based activity profiling • DPPH scavenging • UV-B protection

Introduction

The skin is the largest organ of the human body, functioning as an effective barrier against the harmful effects of the environment [1]. Several factors affect skin health and promote skin aging, such as ionizing radiation, severe physical and psychological stress, alcohol intake, poor nutrition, overeating, environmental pollution, and exposure to UV radiation. The latter is believed to contribute up to 80% of extrinsic skin damage [2].

In cosmetics, natural products play a major role as active ingredients given that they are considered by many as safer alternatives to synthetic products and, therefore, possess higher consumer acceptance. Numerous cosmetic products for dry skin, skin protection (ROS, radicals, and UV light), prevention or alleviation of skin inflammation, hyper-pigmentation, and anti-aging products are commercially available [3–5].

Free radical formation can induce skin damage through a series of mechanisms leading to cell death and ultimately, to skin aging. In a search for new active ingredients for skin care products, compounds and extracts of natural origin are of significant interest [6]. The potential of purified plant compounds in skin protection is generally recognized, but plant extracts also show significant potential due to their complex composition [7].

In the field of cosmetic ingredients, relatively few studies on novel plant extracts or pure natural products have been published in recent years, and the majority of these studies were linked to ethnobotanical sources [3]. Screening of taxonomically diverse and unique plant collections is an alternative strategy to an ethnobotany-driven approach, and it has been successfully applied in the drug discovery field [8]. A diversity-oriented approach is the most successful if plants from regions of high biodiversity can be accessed. Panama is located in one of the 25 biodiversity hotspots worldwide. Despite the small surface of the country, its flora comprises 9,893 vascular plant species including 1,327 (13.4%) endemic plants [9–11]. The flora of Panama is a rich source of bioactive molecules and represents a largely untapped source for new compounds with promising activities for pharmaceutical, agrochemical, and cosmetic industries [12–14].

In an FP7 framework project aiming at the discovery of new natural products for cosmetic use, we screened a library of 600 extracts generated from a set of taxonomically diverse Panamanian plants. The focus was on the identification of plants with promising UV-protective and anti-aging properties. The best extracts were submitted to a process termed HPLC-based activity profiling [15], whereby physicochemical data recorded online are combined with bioassay data of HPLC microfractions.

A broad range of assays have been reported for the analysis of radical scavenging and antioxidant activities of natural products, and for the assessment of UV-protective properties. Free radical scavenging properties are frequently detected with the stable radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH), due to the simplicity of the assay which is easily amenable to screening large numbers of samples [16]. UV protection can be readily

assessed based on the capability of the test products to reverse UV-induced cell death by using the widely accepted MTT methodology [17–19].

Results and Discussion

A library of 600 extracts prepared from Panamanian plants was screened for antioxidant capacity and the ability to protect human skin fibroblasts against UV-B-induced cell death. The screening results of the selected extracts are given in Table 1, and a flow chart for the further progression of samples is shown in Fig. 1. A total of 19 extracts were found to possess considerable radical scavenging activity, i.e. $IC_{50} \leq 30$ µg/ml in the DPPH assay. These extracts were screened for their ability to protect human skin fibroblasts against UV-B-induced cytotoxicity, and three extracts were found to reduce UV-B-induced cell death to ≤15% of the control.

Tab. 1. Activity data of selected extracts in DPPH and UV-B protection assays

Species	**Family**	**Organ**	**Extract**	**DPPH scav-enging**	**UV-induced cell death**
				IC_{50} (µg/ml)	**% of control**
Casearia commersoniana Camb.	Salic.	Stem	MeOH	25	0
Combretum cacoucia Exell ex Sandw.	Combret.	Leaves	MeOH	28	0
Mosquitoxylum jamaicense Krug. & Urb.	Anacardi.	Leaves	MeOH	17	15
Terminalia oblonga (R. & P.) Steud.	Combret.	Branch	MeOH	30	28
Eugenia sp.	Myrt.	Inflorescens	MeOH	26	29
Terminalia amazonia (J.F. Gmel.) Exell	Combret.	Branch	MeOH	29	39
Spondias purpurea L.	Anacardi.	Bark (Stem)	MeOH	14	42
Cordia megalantha Blake	Boragin.	Bark (Stem)	MeOH	29	44
Terminalia lucida Hoffmanns ex. Mart.	Combret.	Stem	MeOH	19	48
Terminalia lucida Hoffmanns ex. Mart.	Combret.	Leaves	MeOH	20	71
Caesalpinia coriaria (Jacq.) Willd.	Fab.-Caesalpin.	Stem	MeOH	23	78
Mosquitoxylum jamaicense Krug. & Urb.	Anacardi.	Stem	EtOAc	20	89
Psychotria chagrensis Standl.	Rubi.	Stem	EtOAc	24	97
Mosquitoxylum jamaicense Krug. & Urb.	Anacardi.	Stem	MeOH	10	99
Miconia nervosa (Sm.) Triana	Melastomat.	Leaves	MeOH	17	>100
Terminalia oblonga (R. & P.) Steud.	Combret.	Fruit	MeOH	25	>100
Mosquitoxylum jamaicense Krug. & Urb.	Anacardi.	Leaves	EtOAc	25	>100
Psychotria chagrensis Standl.	Rubi.	Leaves	EtOAc	28	>100
Psychotria horizontalis Sw.	Rubi.	Stem	EtOAc	29	>100

Active extracts were then submitted to HPLC-based activity profiling [15] in order to track the active constituents in the extract. Time-based microfractions were collected and submitted to screens. HPLC traces and activity profiles are shown in Fig. 2. Extracts were then prioritized on the basis of HPLC traces and activity profiles. In the case of the MeOH extract of *Casearia commersionia* (Salicaceae) (Fig. 2A), a broad window of activity

corresponded to a broad hump in the baseline of the HPLC chromatogram. This was a strong indicator for the presence of tannins, and the extract was therefore excluded from the follow-up. In contrast, for the MeOH extracts of *Mosquitoxylum jamaicense* (Anacardiaceae) (Fig. 2B) and *Combretum cacoucia* (Combretaceae) (Fig. 2C), the activity profile correlated with discrete peaks in the chromatograms, even though broad humps in the baseline were also indicative of tannins. These two extracts were selected for characterization of the active constituents.

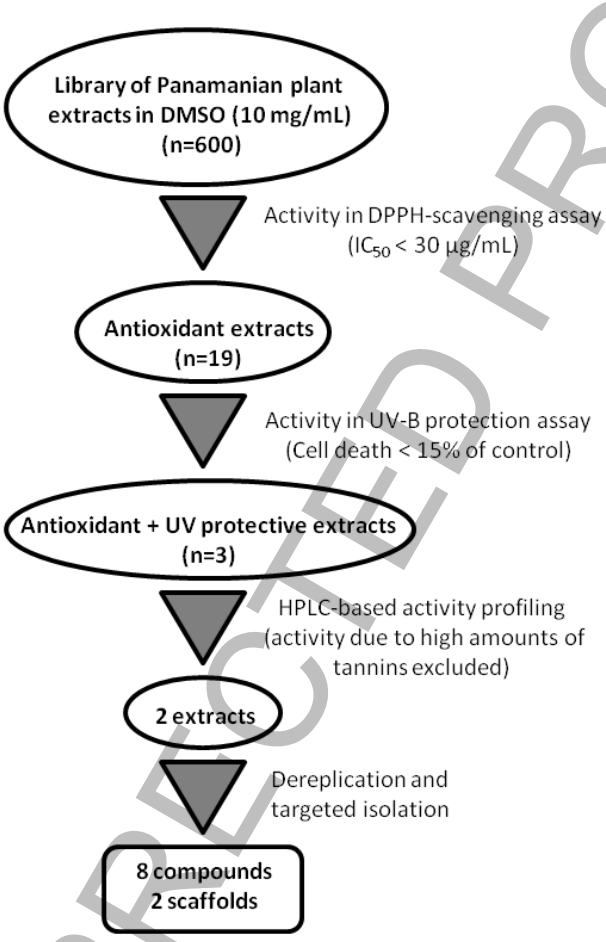

Fig. 1. Workflow for the discovery of cosmetics from Panamanian plant extracts

The tannins in the two extracts were removed by filtration over polyamide (Figs 1S and 2S, Supporting Information). The MeOH leaf extract of *Mosquitoxylum jamaicense* showed activity in time windows corresponding to UV-absorbing peaks in the HPLC chromatogram (Fig. 2B). The tannin-depleted fractions from polyamide (Fig 1S, Supporting Information) were submitted to further purification by HPLC. Peak **1** was identified as gallic acid (Fig. 3), by spiking with a commercial reference and by NMR spectroscopy. Given that the radical scavenging and antioxidant properties of gallic acid are known [20], the compound was not pursued further. The other two early-eluting peaks were identified as a 7:3-mixture of meta- and para-digallic acid (**2**) [21] and a gallic acid methyl ester (**3**) [22]. Both compounds were found to possess good radical scavenging activity (Table 2), which was in accordance with the well-known radical scavenging properties of gallic acid [20]. In addition, compounds **2** and **3** showed protective capacity against UV-B radiation.

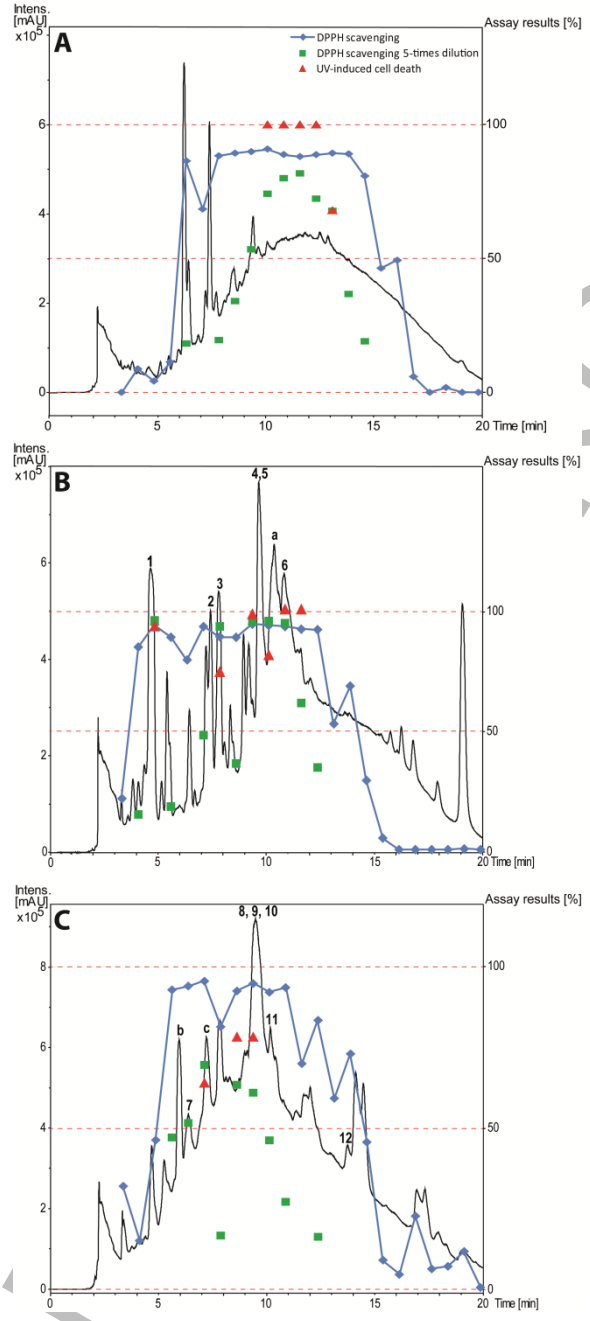

Fig. 2. HPLC-based activity profiling of the three selected plant extracts in the active time window (0–20 min). SunFire C_{18} column (150 x 10 mm i.d., 5 µm); 5–100% MeCN/0.1% aqueous formic acid for 30 min and 100% MeCN/0.1% aqueous formic acid for 5 min, 4 mL/min; time-based fractionation; detection: 200–500 nm, maxplot.
A *Casearia commersoniana* (stems) MeOH extract.
B *Mosquitoxylum jamaicense* (leaves) MeOH extract.
C *Combretum cacoucia* (leaves) MeOH extract.
The assay results are expressed as the radical scavenging capacity of the microfractions in the DPPH assay, compared to gallic acid as the positive control, and as cell death in the UV-B protection assay, as compared to the UV-B irradiated cells without the addition of fractions.

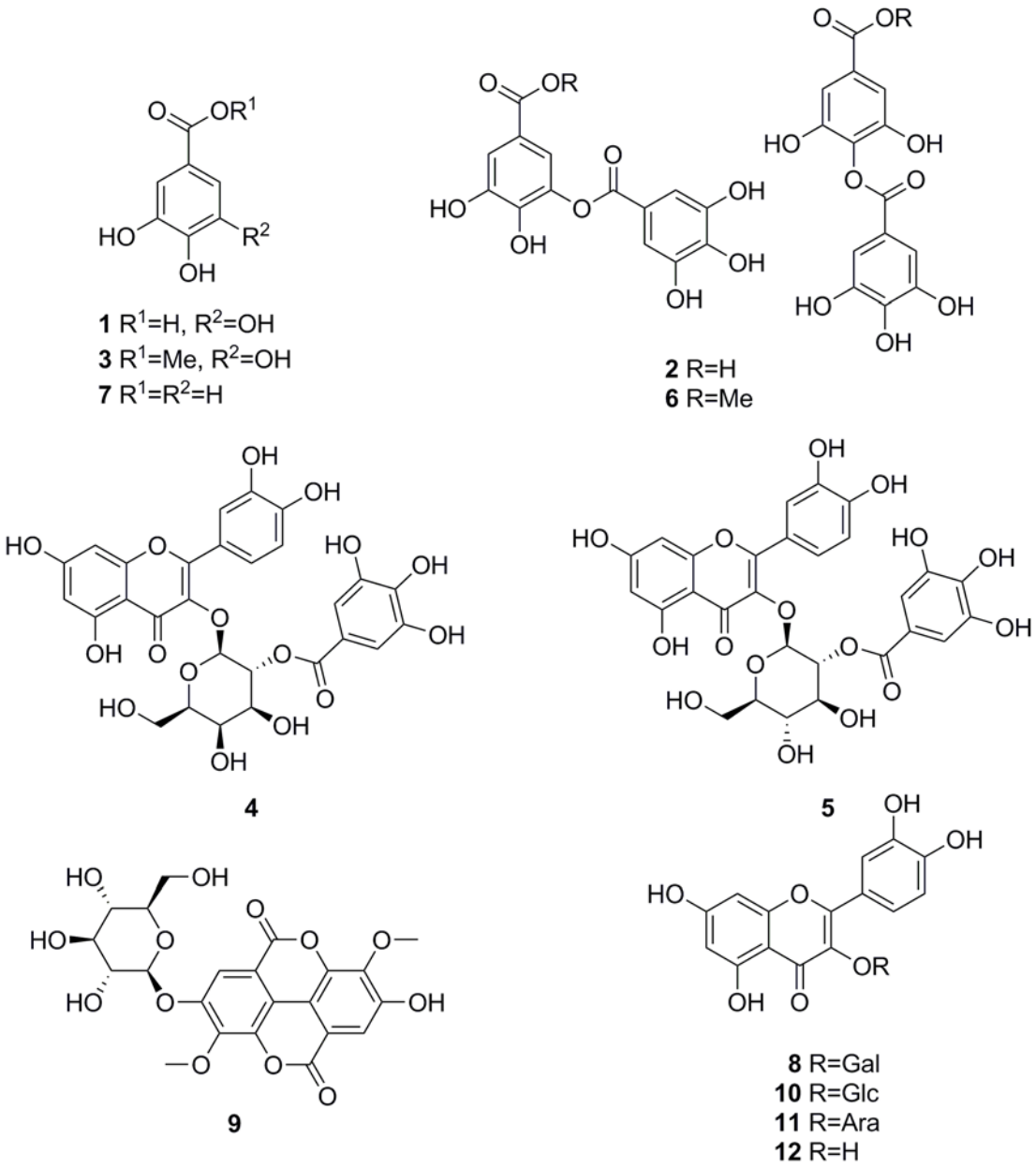

Fig. 3. Structures of identified compounds, gallic acid (**1**), 7:3-mixture of meta- and para-digallic acid (**2**), gallic acid methyl ester (**3**), quercetin-3-*O*-(2''-*O*-galloyl)-β-galactopyranoside (**4**), quercetin-3-*O*-(2''-*O*-galloyl)-β-glucopyranoside (**5**), 7:3-mixture of meta- and para-digallic acid methyl ester (**6**), protocatechuic acid (**7**), hyperoside (**8**), 3,3'-dimethylellagic acid 4-*O*-β-glucopyranoside (**9**), isoquercitrin (**10**), guaijaverin (**11**), and quercetin (**12**)

The major peak at t_R 10 min in the HPLC chromatogram consisted of two co-eluting flavonol glycosides **4** and **5**. Compound **4** was purified and identified as quercetin-3-*O*-(2''-O-galloyl)-β-galactopyranoside [23], while quercetin-3-O-(2''-O-galloyl)-β-glucopyranoside (**5**) [24] was identified from a fraction containing **4** and **5**. Compound **4** was found to be a slightly weaker antioxidant and photoprotectant than **2** and **3** (Table 2). Peak **a** consisted of several co-eluting compounds and was not pursued further. Peak **6** was enriched by

filtration over polyamide, and HPLC purification afforded a 7:3-mixture of meta- and para-digallic acid methyl ester (**6**) [25]. Compound **6** showed radical scavenging activity comparable to **2** and **3,** but no protective capacity against UV-B (Table 2).

Tab. 2. Activity data of pure compounds

Compound	**IC_{50} in DPPH-scavenging assay (µg/ml)**	**Cell death in UV-B protection assay (% of control)**
Meta and para digallic acid (7:3 mixture) (**2**)	9.0 (± 0.4)	14.0 (± 2.9)[a]
Gallic acid methyl ester (**3**)	7.3 (± 0.8)	10.5 (± 1.0)
Quercetin-3-*O*-(2''-*O*-galloyl)-β-galactopyranoside (**4**)	20.4 (± 1.7)	17.4 (± 1.6)
Meta and para digallic acid methyl ester (7:3 mixture) (**6**)	8.7 (± 0.4)	96.8 (± 5.7)[a]
Protocatechuic acid (**7**)	60.1 (± 1.0)	6.1 (± 2.1)
Hyperoside (**8**)	24.2 (± 1.0)	14.4 (± 0.3)
Isoquercitrin (**10**)	27.8 (± 0.4)	3.3 (± 0.8)
Guaijaverin (**11**)	28.0 (± 0.9)	28.9 (± 3.1)[a]
Quercetin (**12**)	12.6 (± 1.2)	72.8 (± 26.9)[a]

[a] compounds tested at 20 µg/ml in the UV-B protection assay, due to cytotoxicity at 100 µg/ml.

The activity profile recorded for the methanolic leaf extract of *Combretum cacoucia* showed a zone of radical scavenging capacity between t_R 5 and 15 min (Fig. 2C). Filtration over polyamide afforded five tannin-depleted fractions (Fig. 2S, Supporting Information) from which peaks **b** and **c** had disappeared. The main peak at t_R 10 min consisted of three compounds which were further purified and identified as hyperoside (**8**) [26], 3,3'-di-methylellagic acid 4-*O*-β-glucopyranoside (**9**) [27], and isoquercitrin (**10**) [26]. Compound **9** could not be obtained at a purity of ≥95% required for further testing. Compounds **8** and **10** were found to possess DPPH scavenging activity and the capacity to protect human skin fibroblasts from UV-B radiation (Table 2). Isoquercitrin (**10**) almost completely blocked cell death. Three minor peaks (**7**, **11**, and **12**) in the active time window were enriched in fraction PA2, and were identified as protocatechuic acid (**7**) [28], guaijaverin (**11**) [29], and quercetin (**12**) [30]. Compound **7** could only be obtained at 90% purity, with traces of phenolic glycosides as contaminants. The compound was nevertheless tested and exhibited weak antioxidant activity, but very good UV-B protection. Quercetin (**12**) and its glycosides **8**, **10**, and **11** showed significant free radical scavenging properties as previously reported [31–33], but only the glycosides **8**, **10** and **11** showed significant UV-protective activity (Table 2). However, the lower test concentrations for compounds **11** and **12** had to be taken into account.

The screening of a taxonomically diverse library of Panamanian plant extracts followed by an activity-driven identification of radical scavenging and UV-B protecting properties led to the identification of a series of known polyphenols. The example shows that the profiling approach can be efficiently used not only for the discovery of bioactive compounds of pharmaceutical, but also of cosmetic interest.

Experimental

Chemicals and General Experimental Procedures

Quercetin (**12**, >98%) was purchased from Sigma-Aldrich. Gallic acid (**1**, >98%), hyperoside (**8**, >95%), isoquercitrin (**10**, >95%), and polyamide (particle size: 0.05–0.16 mm) were from Carl Roth. HPLC-grade acetonitrile and methanol (Reuss Chemie AG), and distilled water were used for HPLC separations.

Preparative HPLC was carried out on an LC 8A preparative liquid chromatograph equipped with a SPD-M10A VP PDA detector (all Shimadzu). A SunFire C_{18} column (150 x 30 mm i.d., 5 µm; Waters) connected to a pre-column (10 x 10 mm) was used, at a flow rate of 20 mL/min. HPLC-based activity profiling was performed on an Agilent 1100 system equipped with a PDA detector. A SunFire C_{18} column (150 x 10 mm i.d., 5 µm; Waters) connected to a pre-column (10 x 10 mm) was used. The flow rate was 4 mL/min. Time-based fractions were collected with a Gilson FC204 fraction collector. ESI-MS spectra were obtained on an Esquire 3000 Plus ion trap mass spectrometer (Bruker Daltonics). NMR spectra were recorded on an Avance III 500 MHz spectrometer (Bruker BioSpin) equipped with a 1-mm TXI microprobe.

Plant Material

The leaves of *Mosquitoxylum jamaicense* were collected in May 2000 in Parque Nacional Soberanía, Camino del Oleoducto, Km 17, Panama. The leaves of *Combretum cacoucia* were collected in April 1995 in Costa Arriba, San Antonio, Colón, Panama. *Casearia commersoniana* was collected in Peninsula Gigante, Chorrera in June, 1995. The plant material was identified by Alex Espinosa and voucher specimens have been deposited at the Herbarium of the University of Panama (PMA). Also, vouchers were kept at the Division of Pharmaceutical Biology, University of Basel: Nr. 857 (*M. jamaicense*), Nr. 859 (*C. cacoucia*), and 903 (*C. commersoniana*).

HPLC-Based Activity Profiling

Extract solutions dissolved in DMSO (50 mg/mL) were separated by semi-preparative HPLC. Two aliquots of 200 µL corresponding to 10 mg of the extract were injected. A gradient of 5–100% MeCN in 30 min in 0.1% aqueous formic acid, followed by 100% MeCN over 5 min was used. Fractions of 0.75 min were collected from t = 3 min to t = 33 min. Fractions were transferred into 96-deepwell plates, evaporated, and submitted to screening.

Extraction and Isolation

Powdered leaves of *M. jamaicense* (704.6 g) were percolated with 12 L MeOH to afford 198.5 g of the extract. A portion (20.2 g) of the extract was re-dissolved in 200 mL MeOH and submitted to filtration over a polyamide (200 g) column. Four fractions (PA1-PA4) of 500 mL each, and one fraction (PA5) of 3 L were collected. A portion (1.01 g) of PA1 (4.99 g) was separated by preparative HPLC (16% MeCN in 0.1% aqueous formic acid) to afford gallic acid methyl ester (**3**, 14.3 mg, t_R 11.3 min). Preparative HPLC of fraction PA3 (338.9 mg) (50–80% MeOH in 0.1% aqueous formic acid over 15 min) yielded a 7:3-mixture of meta- and para-digallic acid methyl ester (**6**, 24.5 mg, t_R 12.5 min). Fraction PA5 (629.2 mg) was separated on a Sephadex LH-20 column (5 x 75 cm i.d.) and eluted with MeOH

to give 17 fractions (Fr. 1-17). Preparative HPLC (25% aqueous MeOH with 0.1% formic acid) of Fr. 11 (136.3 mg) afforded a 7:3-mixture of meta- and para-digallic acid (**2**, 67.8 mg, t_R 12.1 min). From Fr. 13 (69.8 mg), quercetin-3-*O*-(2''-*O*-galloyl)-β-galactopyranoside (**4**, 8.2 mg, t_R 11.3 min) and a mixture of quercetin-3-*O*-(2''-*O*-galloyl)-β-glucopyranoside (**5**, t_R 11.5 min) and **4** were obtained by preparative HPLC (44% aqueous MeOH with 0.1% formic acid).

Powdered leaves of *C. cacoucia* (125.2 g) were percolated with MeOH (3 L) to afford 14.1 g of the extract. A portion (10.1 g) of the extract was re-dissolved in 200 mL MeOH and filtered over a polyamide (200 g) column. Two fractions (PA1-PA2) of 500 mL each, two fractions (PA3-PA4) of 1 L each, and one fraction (PA5) of 3 L were collected. Polyamide fractions were submitted to preparative HPLC. A portion (1.07 g) of fraction PA1 (3.79 g) was separated with a gradient of MeCN in 0.05% aqueous formic acid (5–40% over 15 min) to afford protocatechuic acid (**7**, 8.5 mg, t_R 10.1 min). From PA2 (473.4 mg), 3,3'-dimethylellagic acid 4-*O*-β-glucopyranoside (**9**, 7.8 mg, t_R 6.8 min) and isoquercitrin (**10**, 20.2 mg, t_R 7.8 min) were isolated using 50% MeOH in 0.1% aqueous formic acid. Final purification of **10** was with 20% MeCN in 0.1% aqueous formic acid (1.6 mg, t_R 18.6 min). Fraction PA3 (283.8 mg) was separated with a gradient of MeCN in 0.05% aqueous formic acid (20–60%, 20 min) to afford a mixture of hyperoside (**8**) and isoquercitrin (**10**) (35.5 mg, t_R 7.9 min), and guaijaverin (**11**, 14.9 mg, t_R 8.9 min). Quercetin (**12**, 16.7 mg, t_R 11.1 min) was isolated from fraction PA4 (161.8 mg) using a gradient of 30-60% MeCN in 0.05% aqueous formic acid over 20 min.

Compounds were identified with the aid of ^{1}H- and 2D-NMR, and ESI-MS spectroscopy, and by comparison with the literature data. The purity of the isolated compounds was >95% as determined by NMR except for compounds **7** (90%) and **9** (<70%).

DPPH Radical Scavenging Assay

The antioxidant potential of the test samples was monitored by the change in optical density of the DPPH radical. A stock solution of 0.314 mM DPPH in EtOH was prepared. This stock solution was prepared fresh every day. Extracts were initially tested at 200 µg/ml. Samples that exhibited a strong DPPH scavenging activity, i.e > 80% scavenging, were further evaluated at lower concentrations.

Dry microfractions of the selected extracts in 96-deepwell plates were dissolved in DMSO and tested directly against DPPH scavenging. When a large number of active microfractions appeared for one extract, the most active fractions were tested at a 5-fold dilution. In a 96-well plate, 10 µl of the sample (extract/ fraction/ compound) in DMSO and 190 µl of DPPH solution were mixed and incubated in the dark for 30 min at ambient temperature. Absorbance was measured at 517 nm using an Infinite M200Pro plate reader (Tecan, Männedorf, Switzerland). Measurements were done in triplicate. Blanks for every sample without DPPH were also measured. Gallic acid was used as the positive control. The percentage of DPPH scavenging was estimated by the following equation:

{[(A-B)-(C-D)]/(A-B)}x100, where A: Control (without sample), B: Blank (without sample, without DPPH), C: Sample, D: Blank sample (without DPPH). IC_{50} values, which are defined as the amount of sample necessary to decrease the initial free radical

concentration by 50%, were estimated for the isolated compounds and most active extracts.

Cell Protection Against UV-B Irradiation

A human skin fibroblast cell line (AG01523; Coriell Institute for Medical Research, Camden, NJ, USA) was used for the assessment. Cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with antibiotics (100 IU/ml penicillin; 100 µg/ml streptomycin) and 15% Fetal Bovine Serum (FBS) in an environment of 5% CO_2, 85% humidity, at 37°C, and subcultured once a week at a 1:2 split ratio, using a trypsin–citrate solution (0.25%–0.3%, respectively). Cell counting after trypsinization was performed using a Coulter counter.

For assessing the possible cytotoxicity of the samples (extracts, fractions, or isolated compounds), cells were plated in flat-bottom, tissue culture-treated 96-well plates at a density of 5,000 cells/well. After 48 hours of growth, the medium was changed to serum-free, phenol red-free DMEM, and serial dilutions of the test samples were added. The corresponding dilutions of dimethylsulfoxide (DMSO) served as negative controls. Following incubation with the test samples for 72 hours, the medium was changed to serum-free, phenol red-free DMEM containing 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described by Kostakis et al. [34]. After incubation with MTT for 4 hours, the medium was discarded, and the MTT-formazan crystals were dissolved in isopropanol. Absorbance was measured at 550 nm (reference wavelength; 690 nm) in an Infinite M200 microplate reader (Tecan) using Magellan^{TM} software.

The highest non-cytotoxic concentration of each sample (extract, fraction, or isolated compound) was tested for the ability to protect human skin fibroblasts against toxicity of UV-B irradiation. Cells were plated in 96-well-plates and left to grow as described above. Then samples were added at the test concentrations determined as described above, along with serum-free, phenol red-free DMEM. After incubation for 18 hours, cells were subjected to UV-B irradiation for 10 min (corresponding to 726 mJ/cm^2) using a black box equipped with a closely spaced array of four Sankyo Denki UV-B lamps (Zhe Jiang, China) emitting between 280 nm and 360 nm (peak at 306 nm). Following further incubation for 72 hours, cytotoxicity was estimated using the MTT-method, as described in the previous paragraph. The plates treated in an identical manner, except for the UV-B irradiation, were used as the controls. The UV-B-protective capacity of the samples was calculated using the following equation: % Cell death = [1-(D/C)/1-(B/A)]x100 where A = absorbance (DMSO untreated), B = absorbance (DMSO UV-B-treated), C = absorbance (test sample untreated), and D = absorbance (test sample UV-B-treated). A value of 100 indicated the absence of protection, and 0 indicated the maximum protective capacity against UV-B.

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Supporting Information

The HPLC profiles of the polyamide fractions and NMR data of compounds **2**–**12** are available in the online version (Type: PDF, Size: ca. 0.3 MB): http://dx.doi.org/10.3797/scipharm.1409-12

Authors' Statement

Competing Interests

The authors declare no conflict of interest.

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Supporting Information to

Screening of Panamanian Plants for Cosmetic Properties, and HPLC-Based Identification of Constituents with Antioxidant and UV-B Protecting Activities

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Fig. 1S. HPLC-DAD chromatograms of the crude extract and its polyamide fractions (PA1-PA5) of *Mosquitoxylum jamaicense*. SunFire C18 column (150 x 3 mm i.d., 3.5 µm); 5–100% MeCN/0.1% aqeous formic acid in 30min, 0.4 mL/min; detection: 210–700nm, maxplot.

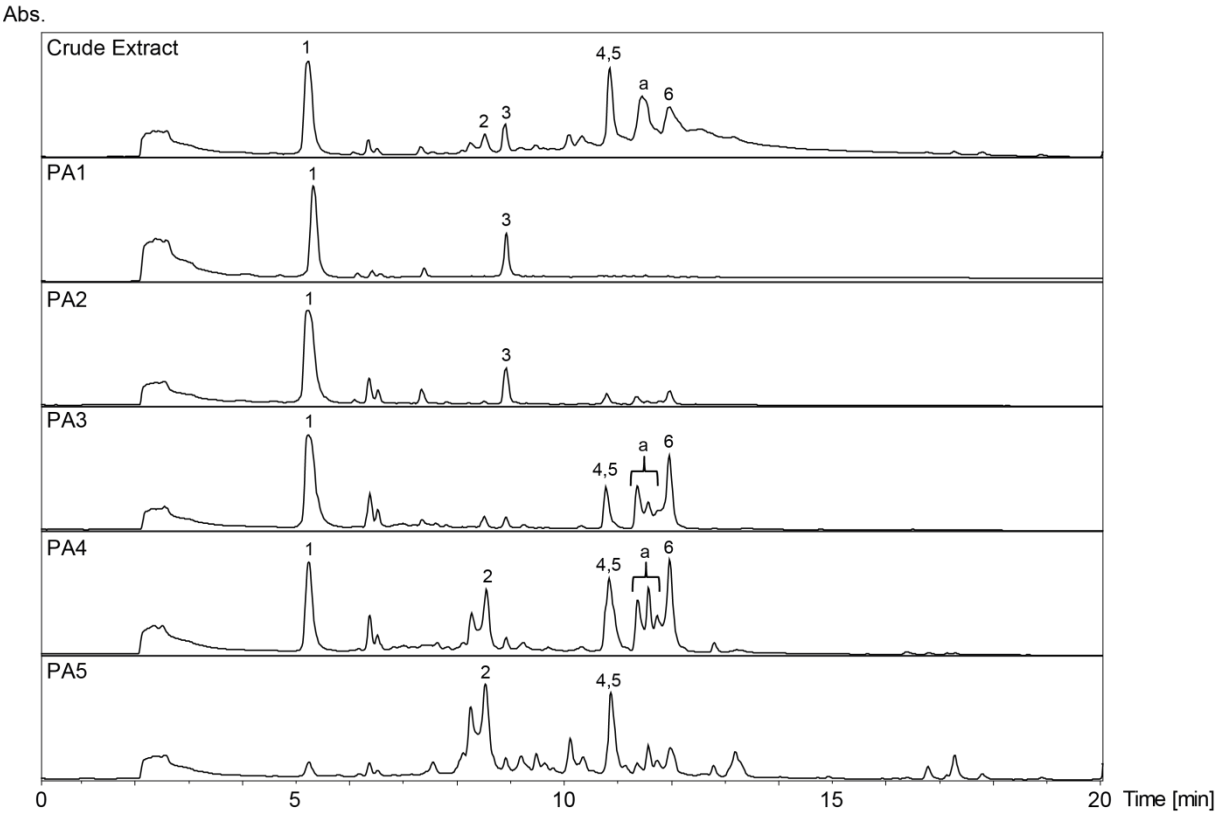
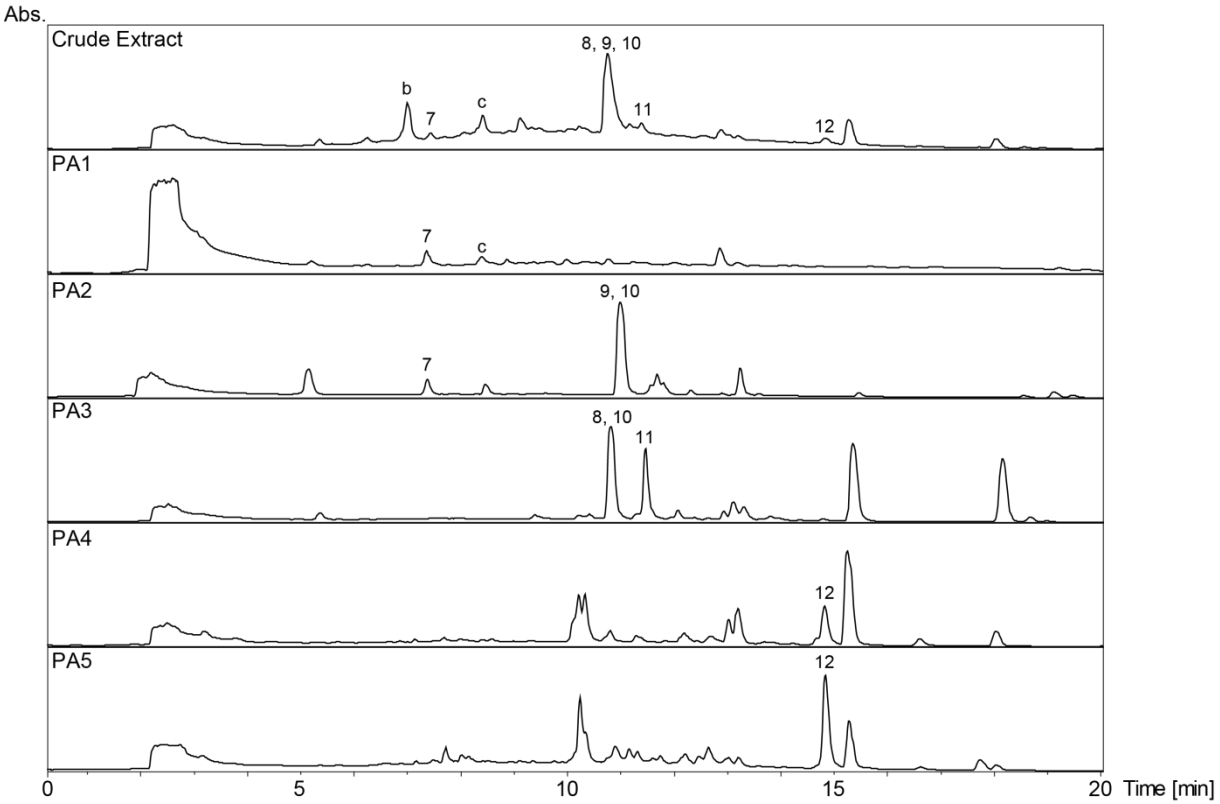


Fig. 2S. HPLC-DAD chromatograms of the crude extract and its polyamide fractions (PA1-PA5) of *Combretum cacoucia*. SunFire C_{18} column (150 x 3 mm i.d., 3.5 µm); 5–100% MeCN/0.1% aqeous formic acid in 30min, 0.4 mL/min; detection: 210–700nm, maxplot.



Tab.1S. ^{1}H and ^{13}C NMR[a] data (500 MHz) of compounds **3** and **7**

	3[b]		**7**[c]	
Position	**δ_H**	**δ_C**	**δ_H**	**δ_C**
1	–	121.0	–	n.d.
2	7.06 (s)	109.9	7.34 (d, 1.8)	116.5
3	–	146.0	–	144.6
4	–	139.4	–	149.7
5	–	146.0	6.79 (d, 8.2)	114.9
6	7.06 (s)	109.9	7.28 (dd, 8.2, 1.8)	121.6
7	–	168.6	–	167.1
7-OMe	3.80 (s)	52.0	–	–

[a] ^{13}C NMR data derived from HSQC and HMBC experiments;
[b] recorded in CD_3OD; [c] recorded in DMSO-d_6; n.d.: not detected

Tab. 2S. ^{1}H and ^{13}C NMR[a] data (500 MHz) of compounds **4** and **5** in CD_3OD

	4		**5**	
Position	**δ_H**	**δ_C**	**δ_H**	**δ_C**
2	–	158.1	-	n.d.
3	–	135.5	-	n.d.
4	–	n.d.	-	n.d.
5	–	163.8	-	163.5
6	6.16 (br s)	99.5	6.16 (s)	99.3
7	–	165.5	-	164.9
8	6.31 (br s)	94.4	6.32 (s)	94.1
9	–	158.0	-	157.7
10	–	105.7	-	105.5
1'	–	n.d.	-	n.d.
2'	7.62 (d, 1.9)	117.0	7.56 (d, 1.4)	116.7
3'	–	145.8	-	145.5
4'	–	149.5	-	149.1
5'	6.78 (d, 8.5)	116.0	6.82 (d, 8.4)	115.7
6'	7.47 (dd, 8.5, 1.9)	122.9	7.50 (d, 8.4, 1.4)	122.7
1''	5.65 (d, 7.9)	101.0	5.70 (d, 8.0)	100.3
2''	5.43 (dd, 9.8, 7.9)	74.5	5.13 (t, 8.7)	75.6
3''	3.83 (dd, 9.8, 2.9)	73.3	3.68 (m)	76.0
4''	3.95 (br s)	70.4	3.49 (t, 9.3)	71.2
5''	3.62 (t, 5.6)	77.1	3.36 (m)	78.1
6''a 6''b	3.72 (d, 5.6)	62.0	3.65 (m) 3.81 (m)	62.1
1'''	–	121.5	-	121.1
2'''	7.13 (s)	110.6	7.13 (s)	110.3
3'''	–	146.3	-	145.8
4'''	–	139.9	-	139.3
5'''	–	146.3	-	145.8
6'''	7.13 (s)	110.6	7.13 (s)	110.3
7'''	–	168.0	-	167.3

[a] ^{13}C NMR data derived from HSQC and HMBC experiments;
n.d.: not detected

Tab. 3S. ^{1}H and ^{13}C NMR[a] data (500 MHz) of compounds **2** and **6** in CD_3OD

	2 (meta)		**2** (para)		**6** (meta)		**6** (para)	
Position	**δ_H**	**δ_C**	**δ_H**	**δ_C**	**δ_H**	**δ_C**	**δ_H**	**δ_C**
1	–	122.2	–	129.4	–	121.2	–	128.7
2	7.29 (d, 1.5)	117.3	7.14 (s)	110.0	7.28 (d, 1.9)	117.0	7.12 (s)	109.5
3	–	143.5	–	151.5	–	140.0	–	151.2
4	–	143.9	–	132.6	–	143.8	–	132.5
5	–	147.1	–	151.5	–	147.0	–	151.2
6	7.42 (d, 1.5)	115.0	7.14 (s)	110.0	7.40 (d, 1.9)	114.4	7.12 (s)	109.5
7	–	169.7	–	169.7	–	167.9	–	167.9
1'	–	120.6	–	120.6	–	120.2	–	120.2
2'	7.25 (s)	110.8	7.26 (s)	110.8	7.25 (s)	110.5	7.26 (s)	110.5
3'	–	146.4	–	146.4	–	146.1	–	146.1
4'	–	140.3	–	140.3	–	140.0	–	140.0
5'	–	146.4	–	146.4	–	146.1	–	146.1
6'	7.25 (s)	110.8	7.26 (s)	110.8	7.25 (s)	110.5	7.26 (s)	110.5
7'	–	166.5	–	166.1	–	166.3	–	165.8
7-OMe	–	–	–	–	3.83 (s)	52.0	3.85 (s)	52.3

[a] ^{13}C NMR data derived from HSQC and HMBC experiments

Tab. 4S. ^{1}H and ^{13}C NMR[a] data (500 MHz) of compounds **8**, **10**, **11**, and **12** in DMSO-d_6

	8		**10**		**11**		**12**	
Pos.	**δ_H**	**δ_C**	**δ_H**	**δ_C**	**δ_H**	**δ_C**	**δ_H**	**δ_C**
2	–	156.0	–	n.d.	–	155.0	–	n.d.
3	–	133.4	–	n.d.	–	133.4	–	n.d.
4	–	177.1	–	n.d.	–	n.d.	–	n.d.
5	–	161.1	–	161.2	–	161.0	–	160.6
6	6.21 (d, 2.0)	98.5	6.19 (d, 1.5)	98.5	6.20 (s)	98.6	6.19 (s)	98.1
7	–	163.8	–	163.9	–	164.3	–	163.2
8	6.41 (d, 2.0)	93.3	6.39 (d, 1.5)	93.4	6.40 (s)	93.4	6.41 (s)	93.2
9	–	155.9	–	156.3	–	156.1	–	155.7
10	–	103.6	–	103.8	–	103.7	–	102.8
1'	–	120.9	–	121.0	–	120.8	–	121.6
2'	7.56 (d, 2.2)	115.9	7.58 (d, 2.0)	116.0	7.54 (d, 1.7)	115.6	7.68 (s)	114.8
3'	–	144.6	–	144.7	–	144.8	–	144.7
4'	–	147.9	–	148.0	–	148.1	–	146.9
5'	6.83 (d, 8.5)	115.0	6.85 (d, 8.3)	115.1	6.86 (d, 8.5)	115.1	6.89 (d, 8.4)	115.3
6'	7.66 (dd, 8.5, 2.2)	121.7	7.57 (dd, 8.3, 2.0)	121.2	7.63 (dd, 8.5, 1.7)	121.6	7.55 (d, 8.4)	119.7
1''	5.36 (7.7)	101.8	5.43 (d, 7.1)	100.9	5.27 (d, 5.2)	101.3	–	-
2''	3.59 (t, 8.3)	71.1	3.26 (m)	73.9	3.78 (dd, 6.6, 5.2)	70.5	–	-
3''	3.40 (m)	73.1	3.25 (m)	76.4	3.54 (dd, 6.6, 3.0)	71.5	–	-
4''	3.68 (d, 2.7)	67.8	3.12 (m)	69.8	3.68 (m)	65.8	–	-
5''a	3.36 (m)	75.6	3.11 (m)	77.2	3.24 (dd, 11.5, 1.7)	64.0	–	-
5''b	–	–	–	–	3.63 (dd, 11.5, 5.1)		–	-
6''a	3.33 (m)		3.35 (m)		–		–	
6''b	3.49 (dd, 9.7, 5.3)	59.9	3.58 (d, 11.5)	60.8	–	–	–	-

[a] ^{13}C NMR data derived from HSQC and HMBC experiments; n.d.: not detected

Tab. 5S. ^{1}H and ^{13}C NMR data (500 MHz) of compound **9** in DMSO-d_6

Position	δ_H	δ_C
1	–	114.2
2	–	141.0
3	–	141.8
4	–	151.6
5	7.81 (s)	111.9
6	–	112.0
7	–	158.5
1'	–	111.2
2'	–	141.7
3'	–	140.2
4'	–	153.0
5'	7.61 (s)	111.7
6'	–	112.8
7'	–	158.5
1''	5.14 (d, 7.2)	101.4
2''	3.36 (m)	73.4
3''	3.36 (m)	76.5
4''	3.23 (m)	69.5
5''	3.42 (m)	77.3
6''a	3.52 (dd, 11.9, 5.3)	60.6
6''b	3.69 (dd, 11.9, 1.8)	
3-OMe	4.10 (s)	61.8
3'-OMe	4.05 (s)	61.1

3.3 Metabolomic studies on *Isatis tinctoria* – Comparison of different accessions, harvesting dates, and the effect of repeated harvesting

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NMR based metabolomics with multivariate statistics was applied on ethyl acetate and 70% aqueous methanol leaf extracts of *Isatis tinctoria*, which was grown under identical and controlled conditions on experimental field plots. Different accessions and harvesting dates were compared, and the effect of repeated harvesting was studied. Differences in the accessions and harvesting dates could be observed, and metabolites such as fatty acids, porphyrins, carbohydrates, indole derivatives, isoprenoids, phenylpropanoids, and minor aromatic compounds could be identified as the contributing factors. Also an effect of repeated harvesting could be observed yielding to a decrease of 1,5-anhydroglucitol, sucrose, unsaturated fatty acids, porphyrins, isoprenoids, and a flavonoid.

Preparation of the PCA/CA/k-NN and STOCSY plots was the contribution of Hartmut Schäfer from Bruker BioSpin. Cryomilling, extraction of plant material, sample preparation for NMR, recording of NMR spectra (major contribution by Sarantos Kostidis and Maria De Mieri), interpretation of PCA/CA/k-NN and STOCSY plots, structure elucidation, and writing of the manuscript draft (except NMR parameters, which were written by Sarantos Kostidis and Maria De Mieri) were my contribution to this publication.

Niels Guldbrandsen

Metabolomic studies on *Isatis tinctoria*– Comparison of different accessions, harvesting dates, and the effect of repeated harvesting

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ABSTRACT: *Isatis tinctoria* (Brassicaceae) is an ancient dye and medicinal plant with potent anti-inflammatory and anti-allergic properties. We investigated metabolic differences by NMR spectroscopy of accessions from different origins that were grown under identical conditions on experimental plots. For these accessions, metabolite profiles at different harvesting dates were analyzed, and single and repeatedly harvested plants were compared. Leaf samples were shock-frozen in liquid N_2 immediately after harvest, freeze-dried, and cryomilled prior to extraction. Extracts were prepared by pressurized liquid extraction (PLE) with ethyl acetate and 70% aqueous methanol. NMR spectra were analyzed using a combination of different methods of multivariate data analysis such as principal component analysis (PCA), canonical analysis (CA), and k-nearest neighbor concept (k-NN). Accessions and harvesting dates were well separated in the PCA/CA/k-NN analysis in both extracts. Pairwise statistical total correlation spectroscopy (STOCSY) revealed unsaturated fatty acids, porphyrins, carbohydrates, indole derivatives, isoprenoids, phenylpropanoids, and minor aromatic compounds as cause of these differences. In addition, the metabolite profile was affected by the repeated harvest regime causing a decrease of 1,5-anhydroglucitol, sucrose, unsaturated fatty acids, porphyrins, isoprenoids, and a flavonoid.

Woad (*Isatis tinctoria,* Brassicaceae) has been used as an indigo dye and medicinal plant for the treatment of inflammatory diseases in central Europe since antiquity.[1] In previous studies, the anti-inflammatory potential of lipophilic woad extracts was demonstrated *in vitro*, and in animal and human studies. With the aid of HPLC-based activity profiling,[2, 3] compounds such as tryptanthrin, γ-linolenic acid, and (*E*)-3-(3',5'-dimethoxy-4'-hydroxybenzylidene)-2-indolinone (indolinone) were identified as inhibitors of cyclooxigenase-2, 5-lipoxigenase, expression of inducible nitric oxide synthase, human neutrophil elastase, and of histamine release from mast cells.[4-8] The unique molecular modes of action of tryptanthrin and indolinone were investigated in more detail.[9, 10] *In vivo* studies with a lipophilic woad extract in models of acute and chronic inflammation, contact allergy, and rheumatoid arthritis, and a pilot study in experimentally induced skin erythema in human volunteers, substantiated the potential of *Isatis* as an anti-inflammatory herbal drug.[11-13]

In parallel to these pharmacological studies, a comprehensive phytochemical characterization of woad was conducted. Flavone glycosides and novel indigo precursors were identified.[14, 15] It was shown that the metabolite profile in leaves underwent profound changes during the drying process, whereby indigo precursors and glucosinolates disappeared and were converted into indigo, and pharmacologically active compounds such as tryptanthrin and indirubin.[16-18] Eventually, a comprehensive metabolite profiling was performed using HPLC coupled to several detectors such as UV, ESI-MS, APCI-MS, and ELSD.[19] Overall, 65 compounds were unambiguously identified, and tentative structural assignments was achieved for a series of additional compounds.

In recent years, NMR based metabolomics with multivariate statistics such as principal component analysis (PCA) and advanced projection methods have been successfully used for phytochemical analysis. In contrast to a classical qualitative and quantitative analysis of single compounds, NMR based approaches provide a non-discriminatory fingerprint of the entire

metabolome. NMR based metabolomics has been used, for example, to differentiate between geographic origins of plants, to detect pharmacologically active compounds, or to identify specific traits of plants. Typical examples include the study of different accessions of *Matricaria recutita* flowers,[20] the study of cultivars of *Cannabis sativa*,[21] the differentiation of *Vitis vinifera* cultivars susceptible or resistant to downy mildew,[22] and the identification of pharmacologically active constituents in the anxiolytic and sedative plant *Galphimia glauca*.[23]

In order to develop a tool for quality control of *I. tinctoria*, we investigated the differences in the metabolite profiles of different accessions grown on experimental field plots under identical and controlled conditions. In addition, seasonal changes in the metabolite profile, and possible effects of different harvesting regimes on metabolite profiles were studied. Analyses were conducted with ethyl acetate and 70% aqueous methanol extracts obtained by pressurized liquid extraction (PLE)[24, 25] from leaf samples that had been shock-frozen immediately after harvest and subsequently freeze-dried. These measures were taken in order to maintain the phytochemical profile of leaves, considering that conventional drying of leaves led to profound compositional changes.[16, 17] PLE was used to ensure a rapid and exhaustive extraction and, hence, reproducible composition of extracts. Since we investigated a variety of parameters such as accession, harvesting dates, and repeated harvesting, we used a supervised combination of different multivariate statistical methods, which had been successfully applied in the characterization of individuals from their metabolic fingerprint of urine.[26, 27]

RESULTS AND DISCUSSION

^{1}H NMR spectroscopic analysis of *I. tinctoria* leaf extracts. The ^{1}H NMR spectra of ethyl acetate extracts (**Fig. S1,** Supporting Information) showed abundant resonances attributed to fatty acids and their glycerol esters, as was to be expected for a moderately lipophilic extract.

In addition, resonances attributable to porphyrins, and signals in the carbohydrate region were detected. To a lower extent, resonances of aromatic compounds such as indole derivatives were present.

The ^{1}H NMR spectrum of the 70% aqueous methanol extracts (**Fig. S2**, Supporting Information) was dominated by resonances attributed to unsaturated fatty acids and carbohydrates. To a higher extent than in the ethyl acetate extract, signals of aromatic compounds such as indoles and flavonoids were present in the low field range. The extraction using 70% aqueous methanol followed a protocol that had been developed for the investigation of glucosinolates in woad.[28] In a publication on the quantitative analysis of glucosinolates in woad,[18] high concentrations of indolicglucosinolates (e.g. glucobrassicin) as compared to aliphatic glucosinolates (e.g. progoitrin) were reported. However, resonances characteristic for the CH_2 bridge between the indole and iminosulfate moieties (δ_H ~4.1 ppm and δ_C ~29 ppm)[29] could not be detected in the NMR spectra. Also, characteristic signals of the anomeric center of thioglucosyl moieties in glucosinolates (δ_H~5 ppm and δ_C~82 ppm)[30] were present in traces only, or even absent. Given that glucosinolates had been previously shown to be exhaustively extracted by 70% aqueous methanol,[28] the compounds apparently were only poorly soluble in DMSO-d_6/D_2O 9:1. This was corroborated by the observation of an insoluble residue when preparing samples for NMR analysis.

Comparison of metabolite profiles of different accessions. The raw data from the NMR analysis were first submitted to principal component analysis (PCA) for dimensional reduction. For a maximal class separation and suppression of artifacts, canonical analysis (CA) combined with multivariate analysis of variance (MANOVA) was performed within PCA subspace. A classification rule was introduced using the k-nearest neighbor (k-NN) concept applied within the relevant PCA/CA subspace defined by maximum class separation.

All models were validated using test set validation (TSV), cross validation (CV), and Monte-Carlo embedded cross validation (MCCV).

Comparison of ethyl acetate extracts from the different accessions by PCA/CA/k-NN (**Fig. 1A**) revealed differences, whereby "Thüringer Waid" and "Swiss woad" were well isolated, while the other accessions were similar. For a better visualization, only samples from single harvests were compared. The confusion matrix from the MCCV showed good prediction rate with an average of 97.3 % (**Fig. 1B**). While "Thüringer Waid" and "Swiss woad" alone were predicted with a 100% hit rate, the other accessions were, to a low extent, confused with each other. This was indicative of significant differences between "Thüringer Waid" and "Swiss woad", and the other accessions. To identify metabolites responsible for the differences, a pairwise statistical total correlation spectroscopy (STOCSY) approach was performed using a covariance and correlation matrix. **Fig. 2** shows STOCSY plots of the region δ_H 0-5 ppm from a pairwise comparison of accessions, whereby the intensity represented the covariance, i.e. the change of metabolite concentration, and the color code represented the correlation coefficient, in order to identify covariances that correlate to the covariance of the accessions. As expected, changes in the metabolite profile were observed in pairwise comparison of "Thüringer Waid" and "Swiss woad" with the other accessions. "French woad", "Kieler Waid", and "Jenaer Waid" showed higher concentrations of pheophytin a (or a phaeophytin derivative) with characteristic STOCSY correlations at δ_H 0.77-0.85 ppm (phytol part) and at the four singlets at δ_H 3.24, 3.41, 3.68, and 3.90 ppm (**Fig. 2**). In addition, correlations were seen in the low field region of the STOCSY (δ_H 6-10 ppm, **Fig. 3S**, Supporting Information) in the three peaks at δ_H 8.58, 9.38, and 9.53 ppm, which corresponded to the protons of the porphyrin π-system. Comparison of "Thüringer Waid" to "French woad" showed negative correlation at δ_H 3.21 ppm and indicated a higher amount of 1,5-anhydroglucitol in "Thüringer Waid". The polyol 1,5-anhydroglucitol is believed to be formed in plants by degradation of starch.[31] When

compared to “Thüringer Waid” and “Swiss woad”, “Kieler Waid” showed high positive correlations at e.g. δ_H 2.81 ppm, which indicated higher concentration of unsaturated fatty acids in the latter. “ThüringerWaid” and “Swiss woad” contained higher concentrations of a second, minor porphyrin derivative based on pheophytin a. It was identified by a signal pattern that was comparable to pheophytin a but was slightly shifted compared to the latter. Pairwise STOCSY of “Thüringer Waid” and “Swiss woad” showed high covariances, but moderate correlations at δ_H 0.89 and 1.25 ppm, indicative of higher concentration of saturated fatty acids or other aliphatic compounds in “Thüringer Waid”.

A comparison of 70% methanolic extracts of the different accessions gave a good separation in the PCA/CA/k-NN plot (Fig. 3A). Only samples from single harvest were compared. “Thüringer Waid” was well separated from the other accessions, while the others were similar to each other. Also the confusion matrix from the Monte-Carlo embedded cross-validation (MCCV) showed a 100% hit rate for “Thüringer Waid” (**Fig 3B**), reflecting its difference to the other accessions. Pairwise STOCSY from δ_H 0-5 ppm (**Fig. 4**) of “Thüringer Waid” and other accessions showed strong negative correlations at δ_H 2.04 and 2.78 ppm, with exception of “Swiss woad”. This indicated higher concentrations of unsaturated fatty acids in “Thüringer Waid”. In contrast, “Kieler Waid” dominated over “Thüringer Waid” with respect to unsaturated fatty acids in the ethyl acetate extract. Considering that fatty acids can be either free or part of more complex lipids, the seemingly conflicting results were likely due to fatty acids in different types of lipids. Another abundant metabolite in “Thüringer Waid” is indole-3-acetonitrile, which can be observed from the correlations at δ_H 4.03 ppm (CH_2CN group) and in the low field STOCSY (δ_H 6-10 ppm, **Fig. 4S**, Supporting Information) at e.g. δ_H 7.84 ppm (aromatic proton). Indole-3-acetonitrile is a well known degradation product of glucobrassicin and related glucosinolates.[32] Pairwise STOCSY of “Thüringer Waid” and “Swiss woad” revealed higher concentration of 1,5-anhydroglucitol (δ_H 3.01-3.03 ppm) and

sucrose (δ_H 3.91 ppm) in “Thüringer Waid”, and higher concentration of glucose (δ_H 2.93 and 4.31 ppm) in “Swiss woad”. Similar differences in 1,5-anhydroglucitol and sucrose were also observed in the comparison of “ThüringerWaid” and “French woad” (**Fig. 4**).

Comparison of metabolite profiles of different harvesting dates. Data were treated in the same way as for comparison of accessions, with the difference that harvesting dates were classified instead. Ethyl acetate extracts differed with respect to harvesting date (**Fig. 5A**), and the confusion matrix showed a nearly perfect prediction rate of 99.6 % (**Fig. 5B**). The samples from July 29 and August 21 were very similar, while samples harvested on September 15 were completely isolated and, hence, highly different. Changes in the metabolite profile during the season were visualized with the aid of pairwise STOCSY (**Fig. 6**). From June 11 to June 23, a decrease of indirubin was seen from a negative STOCSY correlation at δ_H 6.94 and 7.27 ppm. A positive correlation at δ_H 3.21 ppm indicated an increase of 1,5-anhydroglucitol. Towards July 16, only few increases of minor peaks at δ_H 6.69, 6.76 and 6.81 ppm were observed, whereby the latter two signals were from protons of two phenylpropanoid derivatives. In previous studies on *I. tinctoria*, phenylpropanoids such as sinapic acid and ferulic acid had been reported.[19] The signal at δ_H 6.69 ppm likely belonged to a monophenolic compound, which subsequently drops together with another phenolic compound (6.83 ppm) towards July 29. In addition, a decrease of minor aliphatic compounds (δ_H 0.86, 0.88, and 0.91 ppm) was observed. On the other hand, 1,5-anhydroglucitol (δ_H 3.21 ppm) concentrations were increased on July 29. In August 21, very strong positive STOCSY correlations were observed at δ_H 1.69 and 2.03 ppm, suggesting an increase of isoprenoid concentrations. In contrast a signal at δ_H 9.99 ppm that was indicative of a porphyrin derived from chlorophyll b was decreased.Towards the end of the vegetative season on September 15, a strong negative correlation was observed at δ_H 1.69 and 2.03 ppm which was indicative of a decrease of previously mentioned isoprenoids. Also, 1,5-anhydroglucitol (δ_H 3.21 ppm) was

decreased. Concentrations of phenylpropanoids (δ_H 6.76 and 6.81 ppm), in contrast, increased towards the end of the growth season.

The PCA/CA/k-NN plot of 70% aqueous methanolic extracts comparing the harvesting dates also showed changes in the metabolic fingerprint during the growth season (**Fig. 7A**), with a mean prediction rate of 98.6% (**Fig. 7B**). Leaves harvested in June 11 and 23 were similar to each other, while the other harvesting dates showed more differences. Leaves harvested on September 15 were the most separated from other, and the same was also true for the corresponding ethyl acetate extracts. The pairwise STOCSY (**Fig. 8**) showed an increase of sucrose and α- and β-glucose towards June 23 [positive correlation at δ_H 3.92 ppm (sucrose), δ_H 4.31 ppm (β-glucose) and at δ_H 4.94 ppm (α-glucose)]. A negative correlation in the aromatic region (e.g. δ_H 8.78 ppm) was due to a minor indole derivative, most likely indirubin. The negative correlations at δ_H 0.86, 2.28, 4.15, and 4.35 ppm indicated a decrease of saturated acylglycerols, most likely phospholipids. In July 16, strong positive correlations at δ_H 4.05 ppm and in the aromatic region (e.g. δ_H 7.84 ppm) indicated a substantial increase of indole-3-acetonitril, a major degradation product of indole glucosinolates.[32] Also on July 16, an increase of 1,5-anhydroglucitol (δ_H 3.03 ppm) was observed, while sucrose (δ_H 3.92 ppm) and glucose (δ_H 4.31 and 4.94 ppm) were decreased. In the case of the latter three compounds, the same changes happen towards July 29 with a lower positive correlation for 1,5-anhydroglucitol (δ_H 3,03 ppm) (**Fig. 8**). In August 21, there was a distinct increase of 1,5-anhydroglucitol (δ_H 3.03 ppm) and sucrose (δ_H 3.92 ppm), followed by a decrease towards the end of the growth season as represented by the samples harvested on September 15.

Effect of repeated harvesting. For accessions “Thüringer Waid”, “French woad”, and “Jenaer Waid”, the effect of repeated harvesting was investigated in comparison to leaf samples from single harvest. For that purpose, plants had been left untouched on the one half of the experimental plots, and only small samples of leaves were taken for analysis (single

harvest). On the other half of the plot, leaf rosettes were harvested with a mowing machine on June 24 and July 31, in order to simulate a typical harvest regime. Of the re-grown leaf rosettes, small samples were taken for analysis at the indicated dates (repeated harvest).

PCA/CA/k-NN analysis of the ethyl acetate extracts revealed differences between single and repeated harvest (**Fig. 9A**), with a mean prediction rate of 98.1 % (**Fig. 9B**). The pairwise STOCSY for repeatedly harvested leaves (**Fig. 9C**) showed only negative correlations, indicative of a decrease of corresponding metabolites. Strongest correlations were found at δ_H 1.61, 1.69, and 2.03 ppm, suggesting a decrease in isoprenoids. The same correlation but with lower covariance was seen, among others, for signals which were indicative of the π-system of a porphyrin derivative (δ_H 8.21, 9.65, and 9.99 ppm). A correlation at δ_H 11.19 ppm (aldehyde) suggested that porphyrins derived from chlorophyll b decreased upon repeated harvesting.

For the 70% methanolic extracts the PCA/CA/k-NN plot (**Fig. 10A**) also showed an effect of repeated harvesting on the metabolite profile, albeit with a lower mean prediction rate of 93.8 % (**Fig. 10B**). The pairwise STOCSY (**Fig. 10C**) again indicated a decrease of metabolites upon repeated harvesting. The strongest negative covariances and correlations were found for 1,5-anhydroglucitol (e.g. δ_H 3.03 and 3.66 ppm).With similar covariance but lower correlations, a decrease of unsaturated fatty acids (δ_H 2.04, 2.78, and 5.35 ppm) and sucrose (δ_H 5.21 ppm) was also seen. Eventually, to a lower extent, there is a decrease at δ_H 6.99 ppm belonging to a flavonoid. Additional, non-aromatic correlations from the flavonoid at δ_H 3.91 (aromatic methoxy group), 4.33, 4.41, and 4.70 ppm suggested that isoscoparin-3"-O-β-glucopyranoside[14] decreased upon repeated harvesting.

This study showed that both lipophilic and polar metabolites were affected by the harvesting regime. This was in contrast to quantitative studies on indigo precursors and glucosinolates,[16, 19] where repeated harvesting did not noticeably affect their concentrations.

Previously, the effect of repeated harvesting was studied in detail on indole glucosinolates,[33] which are defense molecules, and are up-regulated upon injury of the plant. Up-regulation of indole glucosinolates is only transient, reaching a maximum one day after injury, and dropping to normal levels after two weeks. In the current study, it could be shown that primary and other metabolites can be affected for a longer time after repeated harvesting, which suggests also an altered energy metabolism for regeneration of the plant.

With exception of 1,5-anhydroglucitol, the identity of all metabolites identified here were confirmed by spiking, or by comparison with published NMR data of compounds. NMR data of 1,5-anhydroglucitol were compared with literature,[34] and its presence in the extract confirmed by HPLC-MS-MS.[35]

Cultivation of *I. tinctoria* accessions under identical conditions enabled us to eliminate differences due to environmental influences, and allowed to focus on differences in the metabolic fingerprints that were linked to accessions, dates of harvest, and harvesting regime. The supervised PCA/CA/k-NN analysis enabled us to find differences in metabolite profile of accessions and due to differing harvesting regimes. However, differences in metabolites during the season were much higher, and were predominant in an unsupervised PCA analysis (data not shown).

EXPERIMENTAL SECTION

General experimental procedures. 1D ^{1}H NMR spectra and 2D *J*-resolved spectra of all samples were recorded at 27 °C on a Bruker 600 MHz Avance III NMR spectrometer operating at 600.11 MHz (^{1}H) and 150.89 MHz (^{13}C) equipped with a BBIprobe and Z-gradient system. 2D data of the 70% aqueous methanol extracts were also measured on a Bruker 500 MHz Avance III NMR spectrometer operating at 500.13 (^{1}H) and 125.76 (^{13}C) MHz equipped with a BBI probe and Z-gradient system. Deuterated solvents including

standards were purchased from Sigma Aldrich. Spectra of ethyl acetate extracts were referenced to TMS, and spectra of the 70% methanolic extracts to the doublet at 5.2090 ppm (sucrose). Harvested leaves were lyophilized with a Freezemobile 12XL (Virtis) and powdered with a ZM 1 ultracentrifugal mill (Retsch, with 0.75 mm Conidur sieve). Extraction was carried out with an ASE 200 instrument (Dionex) with attached solvent controller.

Plant material. Leaf material was harvested in 2003 from first year plants (rosette stage) of five defined accessions of *Isatis tinctoria* L. ("Thüringer Waid", "Swiss woad", "French woad", "Kieler Waid", and "Jenaer Waid") at the following dates: 11th and 23rd June, 16th and 29th July, 21st August, and 15th September. All plants were cultivated on experimental plots of the Agricultural Field Station of Thuringia (TLL), Dornburg, Germany. "Thüringer Waid" was a mix of genotypes growing in the state of Thuringia which had been cultivated for many years. The accessions "Swiss woad", "Kieler Waid", and "Jenaer Waid" were from plants grown in the botanical gardens of the University of Lausanne, Switzerland, the University of Kiel, Germany, and the University of Jena, Germany, respectively. "French woad" originated from a collection of wild plants growing at Château de Magrin, near Toulouse, France. The accessions were obtained in 1991/1992 by selection breeding of positive traits grown and multiplied under isolation conditions. Annual elimination of plants which did not correspond to the desired phenotype led to a high degree of phenotypical homogeneity of accessions by the year 2003. Specimens of seeds from the accessions are kept at the TLL, under accession numbers 153/PG 1 (Thüringer Waid), 153/PG 4 (Swiss woad), 153/PG 5 (French woad), 153/PG 9 (Kieler Waid), and 153/PG 10 (Jenaer Waid). Sowing was in early spring at 5 kg seeds/ha, nitrogen fertilization was at 120 kg N/ha at the time of sowing, and 50 kg N/ha each after first and second cuts. A defined treatment of herbicides was applied for weed control. In addition to plants that were left to grow over the entire growth period without being cut, further plants from "Thüringer Waid", "French woad", and "Jenaer Waid" were cultivated

under identical conditions but leaf rosettes were cut with a mowing machine on 24th June and 31st July, and the newly grown leaves were analyzed to study the effect of repeated harvesting. Samples were taken at the same harvest dates as described above, with the exception of 11th June.

Harvests, post-harvest treatments, and extraction. Freshly harvested leaf material was cut into small pieces of 2-3 cm length and immediately shock frozen with liquid nitrogen. Leaves were lyophilized for 48 h, powdered frozen in liquid nitrogen, and kept frozen at below -20 °C until extraction by PLE.

Ethyl acetate extracts were obtained from 0.5 g of powdered leaves, except for the accessions "Swiss woad" and "French woad" harvested on 21st August and 15th September (1 g). All leaf samples were extracted in triplicates. Conditions for extractions were as follows: Extraction cells of 11 mL; extraction solvent ethyl acetate; temperature 50 °C; 2 extraction cycles of 5 min each; preheating time 1 min; flush with 100% of cell volume; purge of 120s with nitrogen; pressure of 120 bar.

For 70% aqueous methanol extracts, 0.3 g of powdered leaves were extracted, except for the accessions "Swiss woad" and "French woad" harvested on 21st August and 15th September (0.5 g), and for repeatedly harvested "French woad" from the dates of 29th July and 21st August (0.1 g). All leaf samples were extracted in triplicates. Conditions for extractions were the same as for the ethyl acetate extracts, with the exception that the extraction solvent was 70% aqueous methanol.

PLE extracts of two extraction cycles were combined, and the solvent evaporated under reduced pressure. Dried extracts were stored at -20 °C under argon until analysis.

NMR sample preparation. Ethyl acetate extracts (9.7-12.2 mg) were dissolved in 0.97-1.22 mL of a mixture of $CDCl_3$/CD_3OD 7:3 containing 0.011 % TMS to give a final

concentration of 10 mg/mL. For analysis, 550 µL of the solution were transferred into 5 mm NMR tubes.

The 70% aqueous methanol extracts (9.7-10.2 mg) were suspended in 0.97-1.02 mL of a mixture of DMSO-d_6/D_2O 9:1 containing 0.012% TSP to give a concentration of 10 mg/mL. The mixture was vortexed, sonicated for 10 min, and centrifuged at 10,000 rpm for 10 min. The supernatant (550 µL) was transferred into 5 mm NMR tubes for analysis.

Data analysis and statistics. Chemical shifts of 1H NMR spectra were referenced to the doublet at 5.209 ppm. Scaling to TSP (-0.07 to 0.07 ppm), and reduction to integrated regions of equal width (0.02 ppm) corresponding to the region of 0.5-11.7 ppm was performed with AMIX (version 3.9.13, Bruker Biospin). Regions of δ_H 2.4-2.7 and 3.7-3.8 ppm were excluded due to residual signals of DMSO-d_6 and H_2O, respectively. Multivariate statistics was performed in MATLAB (The MathWorks, Natrick, MA) using standard procedures provided in MATLAB itself, in the MATLAB STATISTICS TOOLBOX, and by in-house written routines.

For the PCA/CA/k-NN classification, PCA was applied for dimension reduction first. Then, canonical analysis (CA), in combination with MANOVA, was applied to determine the subspace for maximum class separation and its respective dimension. Finally, a classification rule was introduced, via the k-nearest neighbor (k-NN) concept. This gave the PCA/CA/k-NN classification procedure: For classification of a new test sample, it was projected into the PCA-CA subspace first, and k-NN was used to assign its class membership.

All models were validated using test set validation (TSV), cross validation (CV), and Monte-Carlo embedded cross validation (MCCV). TSV divided a bucket table into two parts, one serving as model set, and the other as independent test set. Models were built from the model set only, and test sets objects were classified on basis of model sets. As an extension of TSV, CV separated the initial bucket table into n disjunctive segments, ideally all with equal

number of objects. CV iterated over n TSV, each time taking one of the disjunctive segments as test set, and combining the others into a model set. At the end, each object of the bucket table had been member of a test set once, and such, had been classified independently. In order to avoid any bias from a particular segmentation of the bucket table in the CV, MCCV was introduced to iterate over CV, each time starting from a new random segmentation. As a result, an MCCV with 100 independent segmentation iterations provided 100 classifications for each object of the bucket table. From the MCCV results, the confusion matrix was generated.

STOCSY plots were generated as follows: First, highly resolved bucket tables (8000 buckets within 0.25 – 11.5ppm) were calculated from the spectra under consideration. The covariance and the correlation between group specific category variables (e.g group membership) and the intensities of each bucket were calculated. The resulting bucket specific covariance values were presented as pseudo-spectrum, i.e. covariance as a function of spectral position in ppm. Each point of this function was additionally color coded according to the respective correlation values.

ASSOCIATED CONTENT

Supporting information

Detailed description for NMR measurement, overlaid spectra of both extracts of all harvesting dates of "ThüringerWaid", and the STOCSY plot of the low field region for the accession comparison of both extracts is available free of charge via the internet at http://pubs.acs.org.

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List of Figures

Figure 6. Pairwise STOCSY of the ethyl acetate extracts (all accessions) from harvesting dates June 11 (1), June 23 (2), July 16 (3), July 29 (4), August 21 (5), and September 15 (6). Intensities represent the covariance, and color the correlation. Positive correlation indicates higher concentration in the later date of the respective pair.

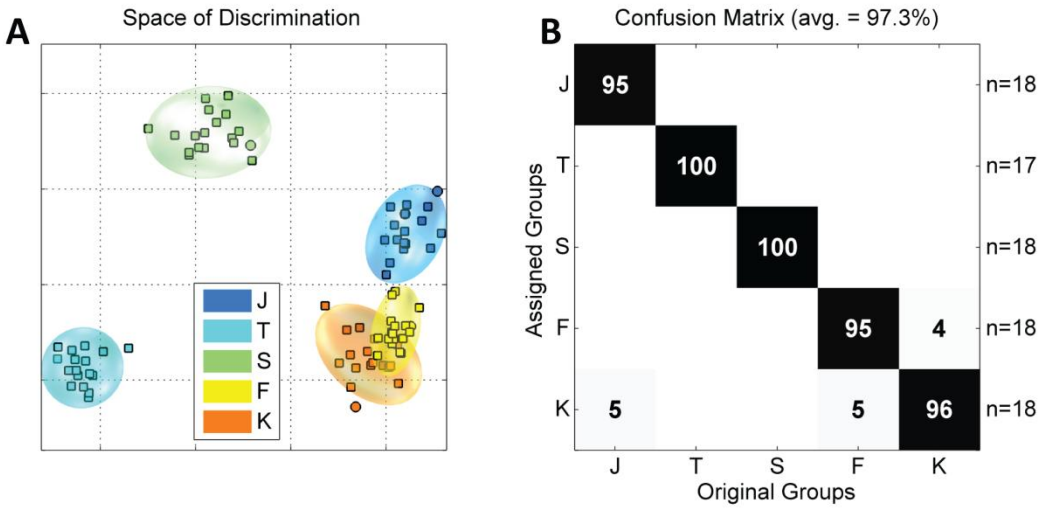
Figure 7. Comparison of different harvesting dates of the 70% methanolic extracts (all accessions). A: PCA/CA/k-NN plot for harvesting dates June 11 (1), June 23 (2), July 16 (3), July 29 (4), August 21 (5), and September 15 (6). B: Confusion matrix from the Monte Carlo embedded cross-validation.

Figure 8. Pairwise STOCSY of the 70% methanolic extracts (all accessions) from harvesting dates June 11 (1), June 23 (2), July 16 (3), July 29 (4), August 21 (5), and September 15 (6). Intensities represent covariance, and colors represent the correlation. Positive correlation indicates higher concentration in the later date of the respective pair.

Figure 9. Repeated harvesting of all *Isatis* accessions (ethyl acetate extracts). A: PCA/CA/k-NN plot comparing single (i) to repeated (r) harvesting. B: Confusion matrix from the Monte Carlo embedded cross-validation. C: Pair wise STOCSY displaying the covariance (intensity) and correlation (color code). Positive correlation indicates higher concentration in repeatedly harvested samples.

Figure 10. Repeated harvesting of all *Isatis* accessions (70% methanolic extracts). A: PCA/CA/k-NN plot comparing single (i) to repeated (r) harvesting. B: Confusion matrix from the Monte Carlo embedded cross-validation. C: Pair wise STOCSY displaying the covariance (intensity) and correlation (color code). Positive correlation indicates higher concentration in repeatedly harvested samples.

Fig. 1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Confusion Matrix (avg. = 97.3%)
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Fig. 2

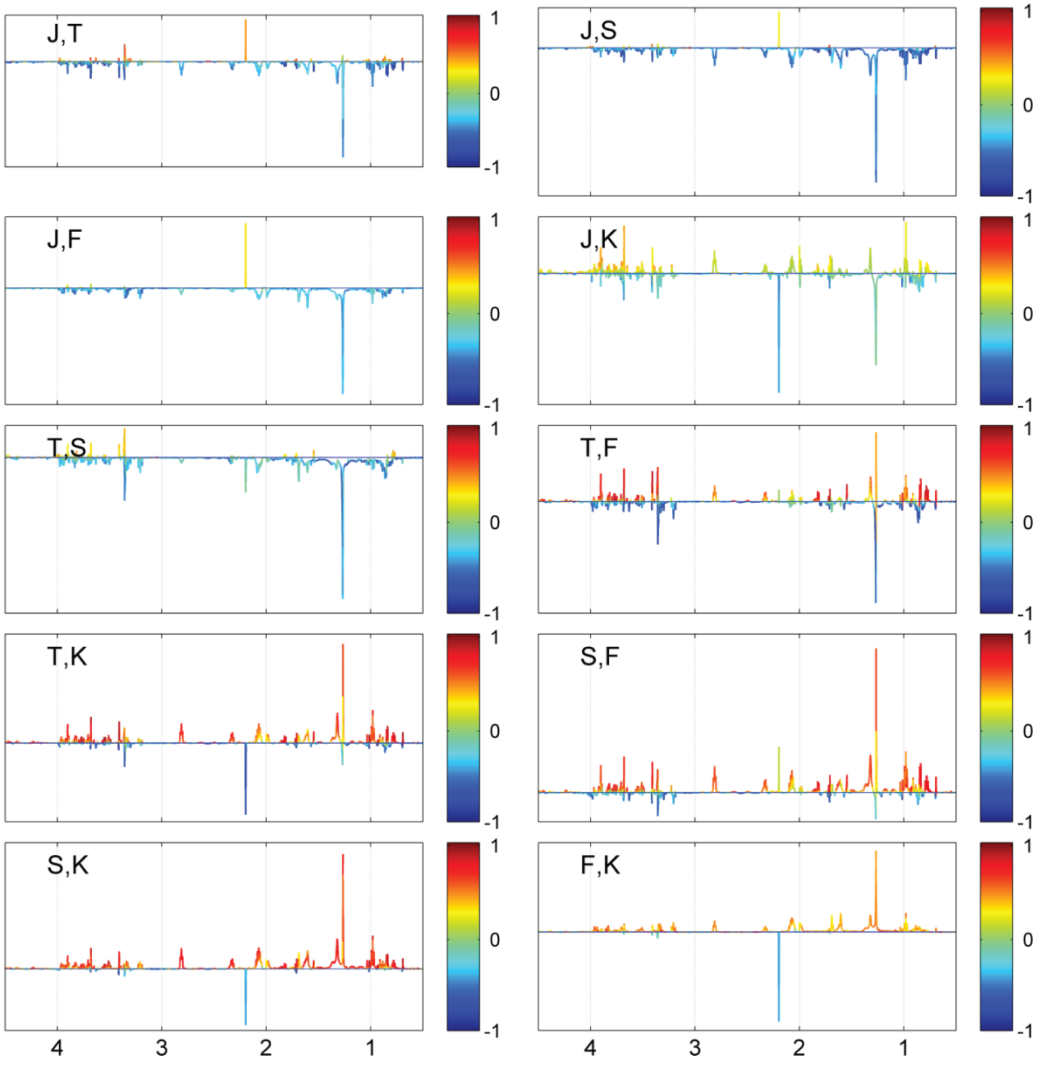
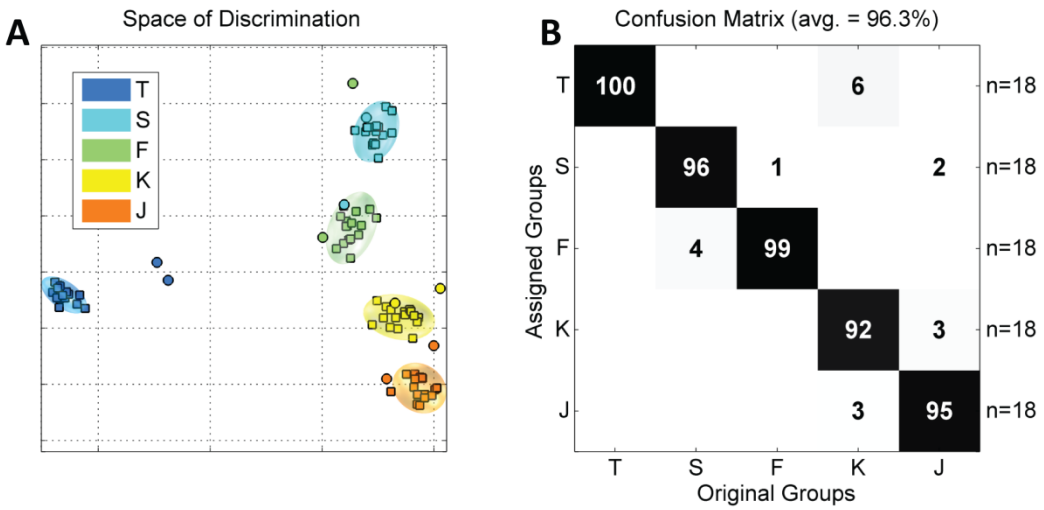


Fig. 3


A
Space of Discrimination
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Confusion Matrix (avg. = 96.3%)
Assigned Groups
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96
1
2
4
99
92
3
3
95
n=18
n=18
n=18
n=18
n=18

Fig. 4

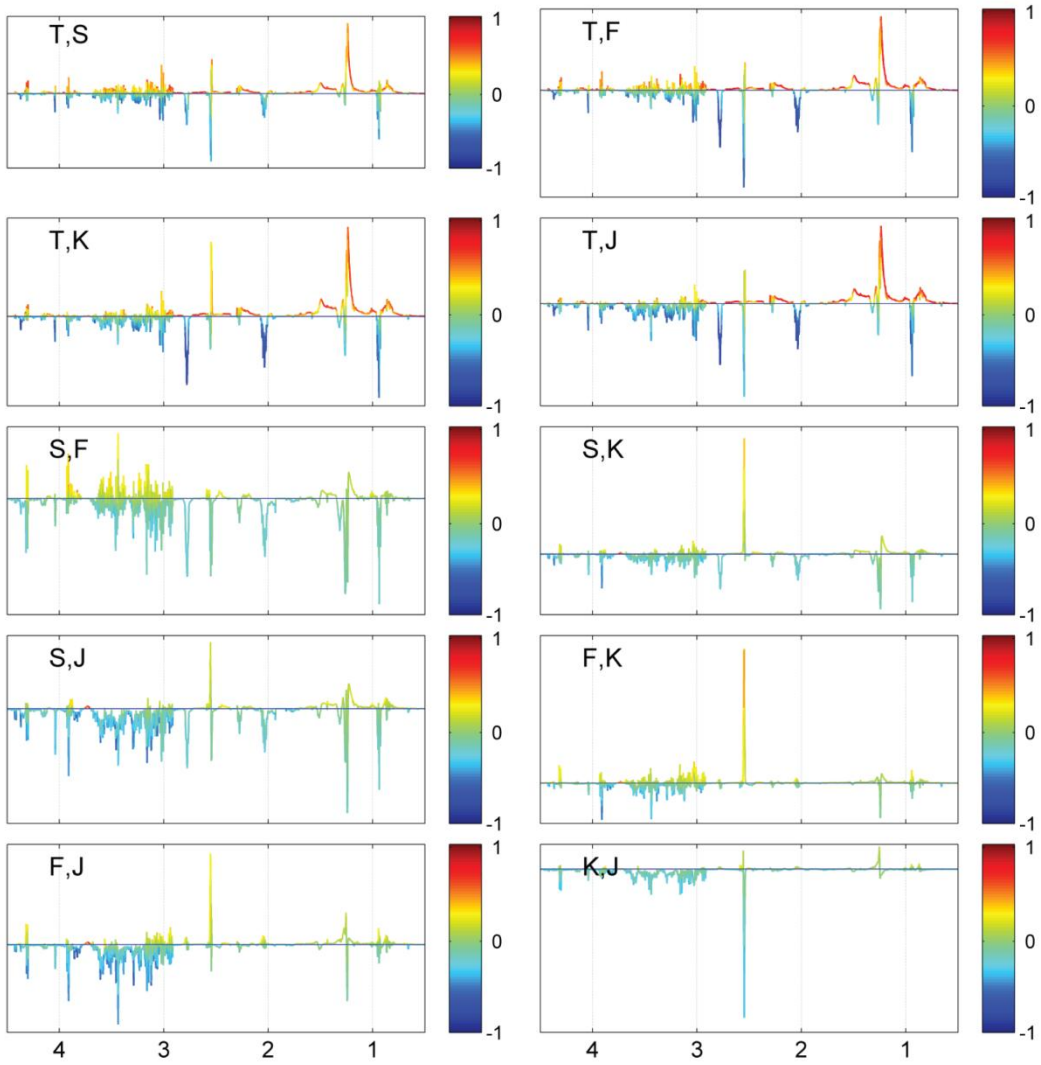


Fig. 5

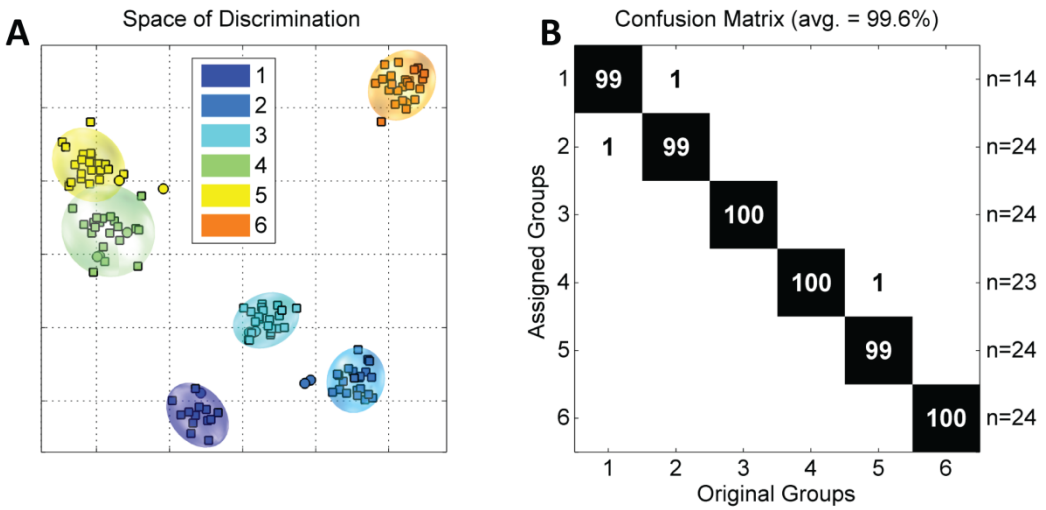

A
Space of Discrimination
1
2
3
4
5
6
B
Confusion Matrix (avg. = 99.6%)
Assigned Groups
Original Groups
99
1
1
99
100
100
1
99
100
n=14
n=24
n=24
n=23
n=24
n=24
1
2
3
4
5
6

Fig. 6

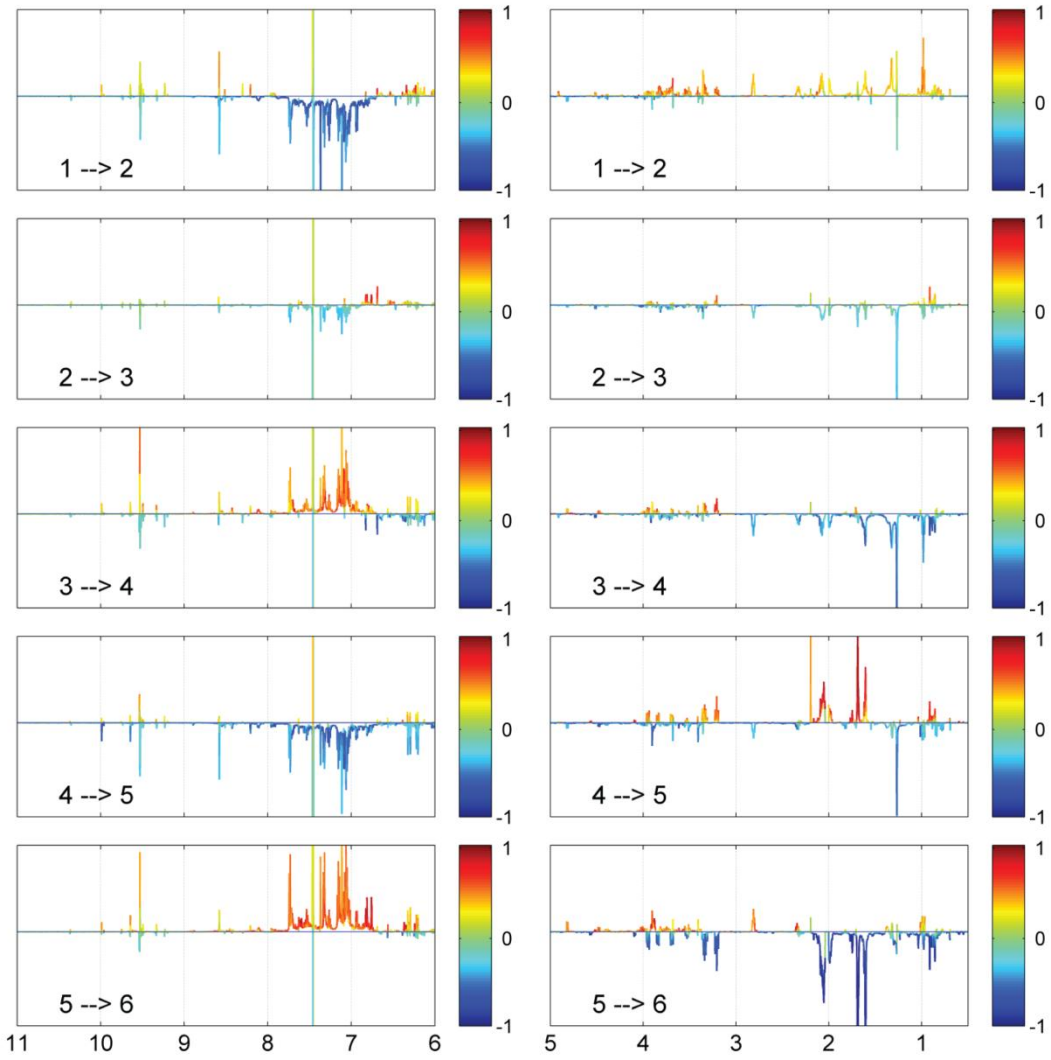


Fig. 7

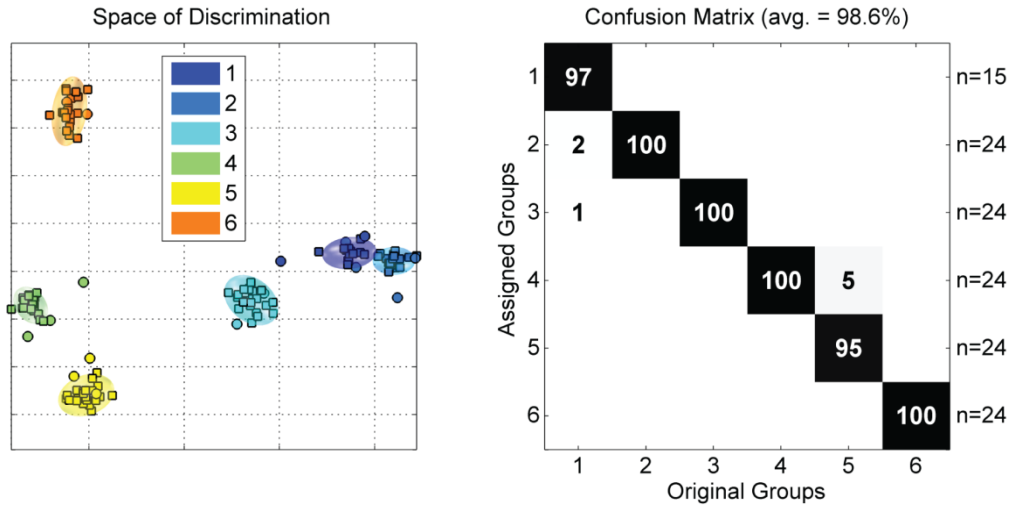

Space of Discrimination
1
2
3
4
5
6
Confusion Matrix (avg. = 98.6%)
Assigned Groups
Original Groups
97
2
100
1
100
100
5
95
100
n=15
n=24
n=24
n=24
n=24
n=24
1
2
3
4
5
6

Fig. 8

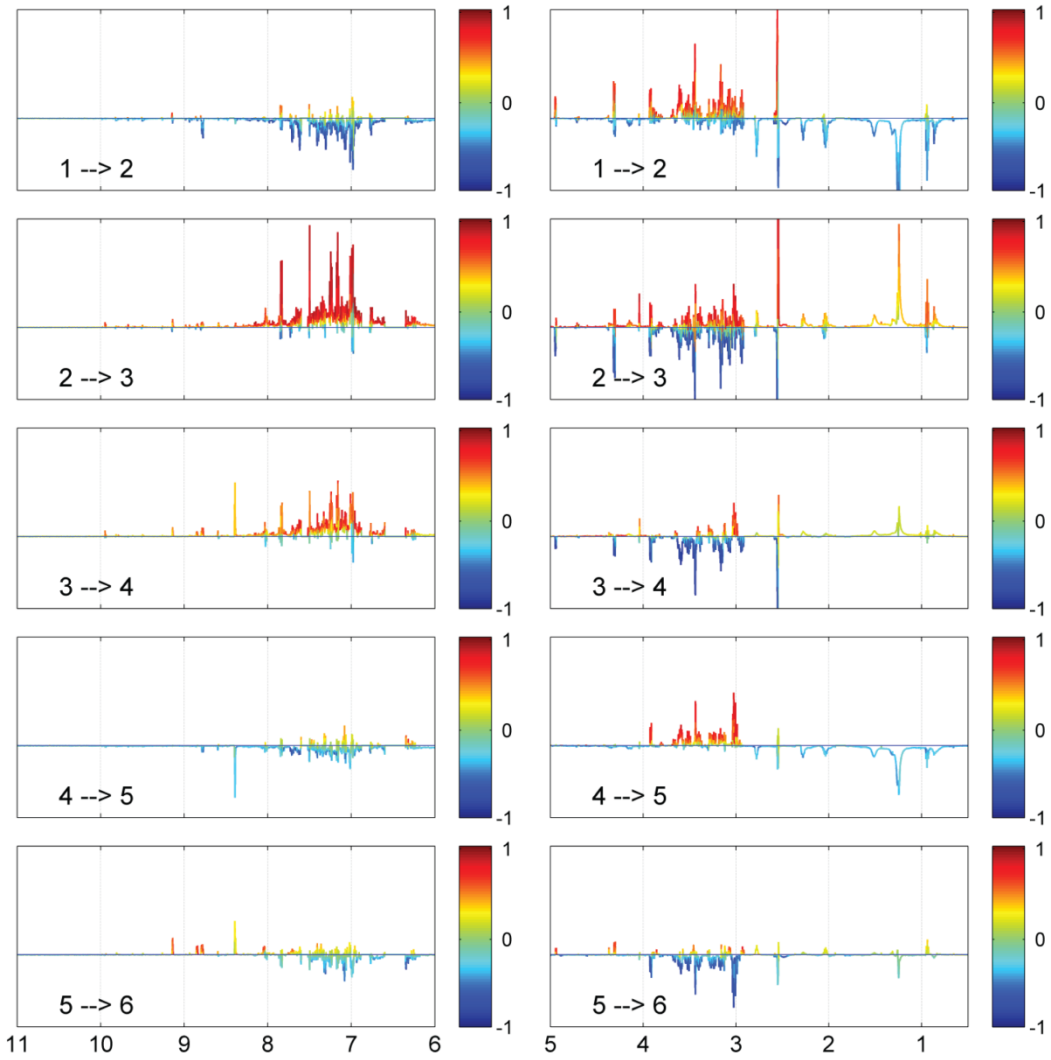


Fig. 9

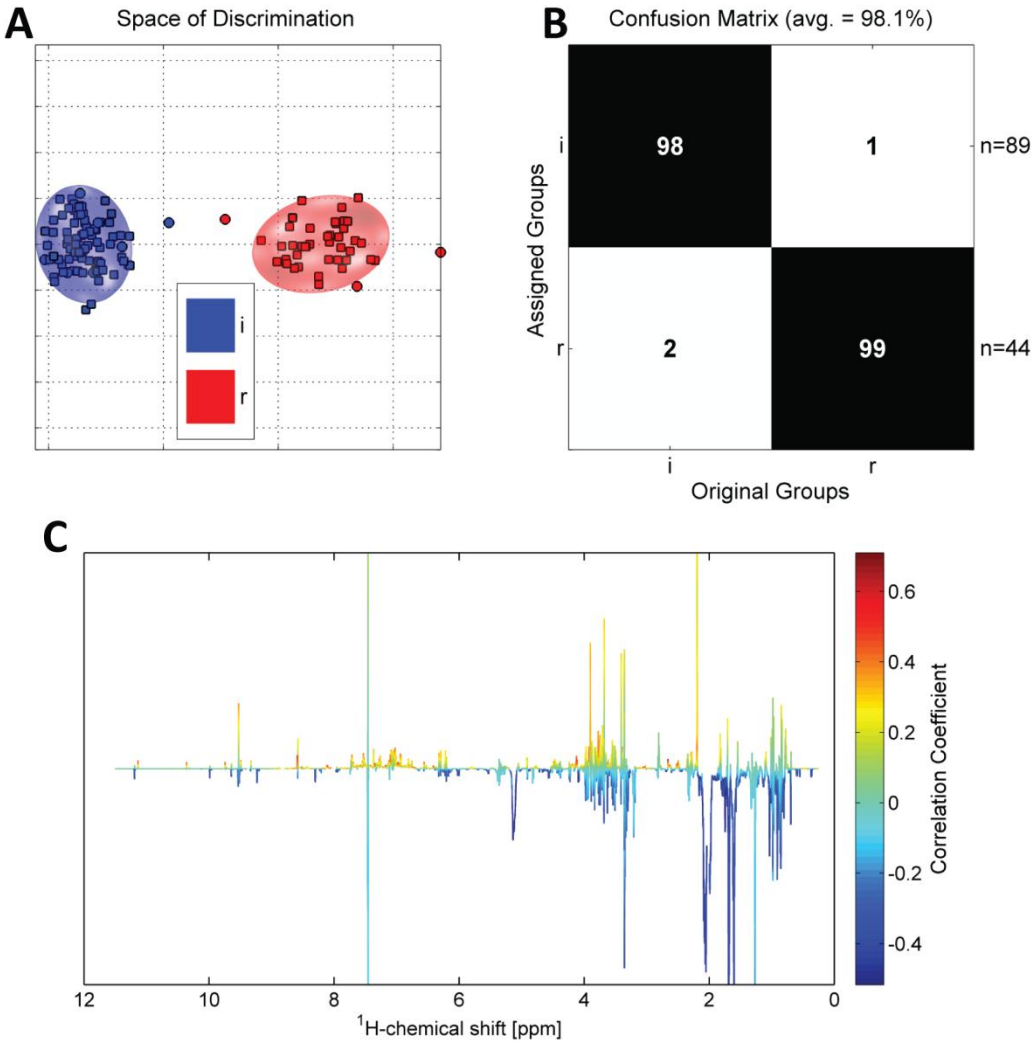
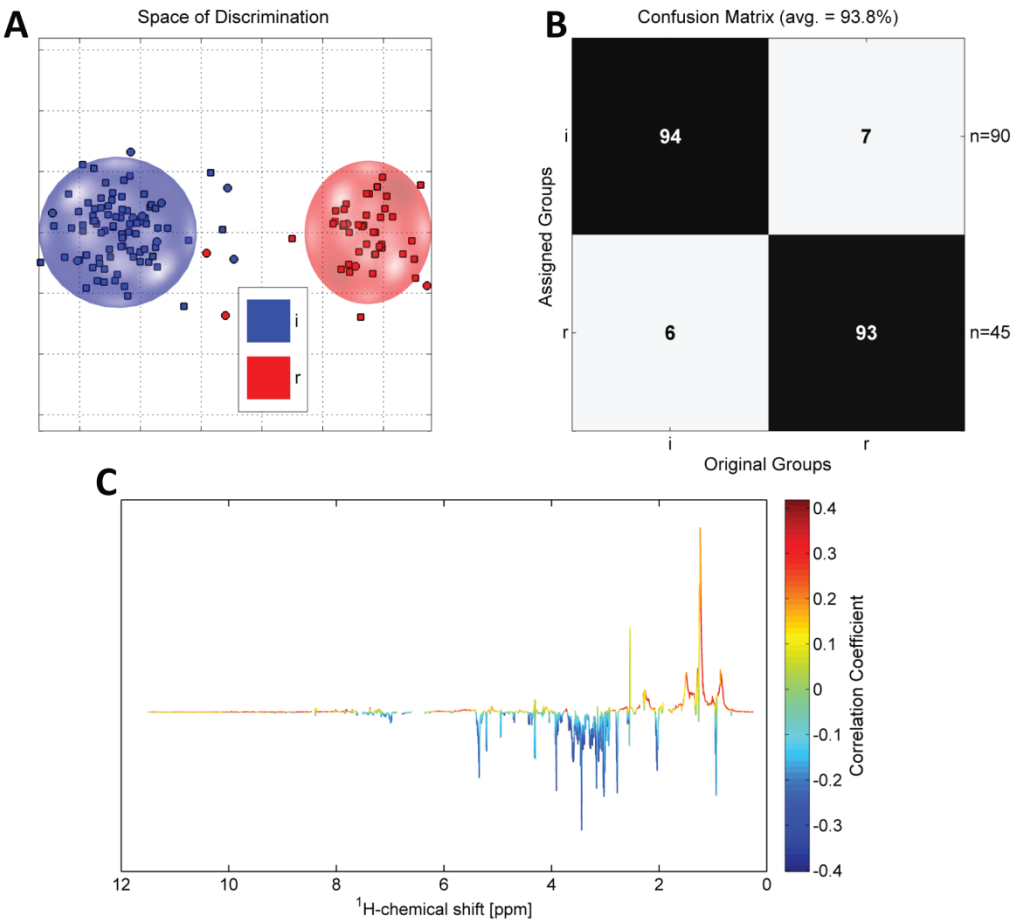

A
Space of Discrimination
i
r
B
Confusion Matrix (avg. = 98.1%)
98
1
2
99
n=89
n=44
Assigned Groups
Original Groups
i
r
C
0.6
0.4
0.2
0
-0.2
-0.4
Correlation Coefficient
12
10
8
6
4
2
0
1H-chemical shift [ppm]

Fig. 10


A
Space of Discrimination
i
r
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93
n=90
n=45
Assigned Groups
Original Groups
i
r
C
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
Correlation Coefficient
12
10
8
6
4
2
0
1H-chemical shift [ppm]

Supporting information

Metabolomic studies on *Isatis tinctoria* – Comparison of different accessions, harvesting dates, and the effect of repeated harvesting

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1. Detailed experimental section for NMR measurements

The samples from both the ethyl acetate and the 70% methanolic extracts were submitted to 1D-^{1}H NMR and 2D-*J*-resolved NMR experiments using a Bruker Avance III 600 MHz NMR spectrometer, operating at 600.11 MHz (^{1}H) and 150.89 MHz (^{13}C). A standard operating procedure was used for all samples including temperature control (27°C), and an automation routine implemented in ICONNMR (Topspin 2.1, Bruker Biospin Gmbh, Germany) for both acquisition (tuning and matching of probe, shimming and 90° pulse calibration) and processing (Fourier transformation, phase and baseline correction) of the NMR data. 1D-^{1}H NMR were recorded using the noesygppr1d pulse sequence (Bruker) with presaturation of γB1= 25 Hz, a relaxation delay of 4 s, and a mixing time of 0.01 s. A total of 65536 data points were recorded with 32 and 64 scans for ethyl acetate and 70% methanolic extracts, respectively, covering a spectral window of 12335.5 Hz. The offset for presaturation was set at 4.27 and 3.76 ppm for ethyl acetate and 70% methanolic extracts, respectively. An exponential window function with a line broadening factor of 0.3 Hz was applied prior to Fourier transformation. 2D-*J*-resolved NMR spectra (jresgpprqf; Bruker pulse program) were acquired with a single scan per increment in F1 recording a matrix of 40 x 8192 data points in a 78 x 10000 Hz spectral window. Presaturation was applied during a relaxation delay of 1 sec with an effective field of γB1 25 Hz using the same offsets as with the 1D-^{1}H experiments. Data were zero-filled to 128 x 8192 points prior to magnitude mode Fourier transformation with a sine shape window function in both dimensions.

A subset of representative samples were selected for 2D homonuclear (^{1}H-^{1}H) correlation and total correlation spectroscopy (COSY and TOCSY, respectively), as well as 2D ^{1}H-^{13}C multiplicity edited heteronuclear single quantum coherence (edited HSQC). The COSY spectra were acquired using a gradient-selected pulse sequence with presaturation of γB1= 25 Hz (cosygpprqf; Bruker) and a relaxation delay of 4 s. A total of 256 x 2048 data points were recorded with 8 scans per increment covering a spectral window of 12019 x 12019 Hz. FIDs

were weighted using a sine-bell apodization function in *t*1 and *t*2 prior to Fourier transformation. The TOCSY spectra were recorded in the phase-sensitive mode using states time proportional phase incrementation (States-TPPI), presaturation of γB1= 25 Hz, relaxation delay of 4 s and DIPSI2 for spin lock over 0.07 s (dipsi2phpr; Bruker). A total of 256 x 4096 data points were collected using 16 scans per increment over a 12019 x 12019 Hz spectral window. Weighting of the data was performed using a sine-bell-square apodization in *t*1 and *t*2 prior to Fourier transformation and phase correction. ^{13}C Multiplicity edited ^{1}H-^{13}C HSQC spectra (hsqcedetgpsisp2.3 Bruker pulse program) were recorded for a data matrix of 256 x 1024 points covering 27163 x 12019 Hz, with 80 scans per increment. INEPT transfer delays optimized for a heteronuclear coupling of 145 Hz and a relaxation delay of 1.5 s were applied. Data were zero filled to 1024 x 1024 data points and weighted by a sine-bell-squared window function prior to 2D Fourier transformation and phase correction. The offsets were set as in the respective 1D NMR experiments.

The 70% methanolic extracts of "Thüringer Waid" from harvesting dates of June 23 and August 21 were submitted to 1D-^{1}H NMR, 2D-*J*-resolved, 2D-heteronuclear single quantum coherence (HSQC), 2D-heteronuclear multiple bond correlation (HMBC), and 2D-heteronuclear single quantum coherence-total correlation spectroscopy (HSQC-TOCSY) experiments using a Bruker AVANCE III 500 MHz spectrometer operating at 500.13 (^{1}H) and 125.76 (^{13}C) MHz, respectively. Measurements were carried out at 23°C in a BBI 5mm probe with Z-gradient. Samples were dissolved in a mixture of dmso-d_6/D_2O (9:1), and dmso-d_6 was used for internal lock and for spectra referencing. 1D-^{1}H NMR spectra were recorded using the noesygppr1d pulse sequence (Bruker) with presaturation during relaxation delay and mixing time, and spoil gradients. 128 Scans with 4 initial dummy scans were recorded where a 90° pulse was applied for excitation with a relaxation delay of 2 s. The offset was optimized at 3.59 ppm by applying a saturation power with an effective field of γB1= 25 Hz. A total of 32.768 data points were recorded covering a spectral window of 10000 Hz. Data were zero-

filled to 65.536 points, and an exponential window function with a line broadening factor of 0.3 Hz was applied prior to Fourier transformation. The resulting spectra were manually phased and baseline corrected. 2D-*J*-resolved NMR spectra (jresgpprqf Bruker pulse program) were acquired with 8 scans for each increment in F1 with a data matrix of 128 x 8192 data points covering 65 x 5155 Hz. Presaturation was applied during a relaxation delay of 2 sec with an effective field of γB1 25 Hz. Data were zero-filled to 512 x 8192 points prior to magnitude mode Fourier transformation with a sine shape window function in both dimensions. HSQC spectra (hsqcedetgpsisp2.3 Bruker pulse program) were recorded for a data matrix of 256 x 1024 points covering 20832x 5263 Hz, with 64 scans for each increment. INEPT transfer delays were optimized for a heteronuclear coupling of 145 Hz, and a relaxation delay of 1.5 s was applied. Data were zero filled to 1024 x 2048 prior to 2D Fourier transformation, and a pure cosine squared sine window function was applied to both dimensions. HMBC spectra (hmbcgplpndqf Bruker pulse program) were recorded with a data matrix of 128 x 4096 points covering 27933 x 5263 Hz were recorded with 128 scans for each increment in F1. An evolution delay of 50 ms, and a coherence transfer delay optimized for a long range coupling of 8 Hz were applied. Data were zero filled to 512 x 8192 prior to 2D Fourier transformation, and non-shifted sine bell window functions were applied to both dimensions. HSQC-TOCSY spectra (hsqcetgpml Bruker pulse program) were recorded with a data matrix of 256 x 1024 points covering 20832 x 5263 Hz using 128 scans for each increment in F1. TOCSY mixing time of 80 ms, and INEPT transfer delays optimized for a heteronuclear coupling of 145 Hz were applied. Data were zero filled to 1024 x 2048 prior to 2D Fourier transformation, and a pure cosine squared sine window function was applied to both dimensions.

2. ^{1}H NMR spectra and additional STOCSY plots

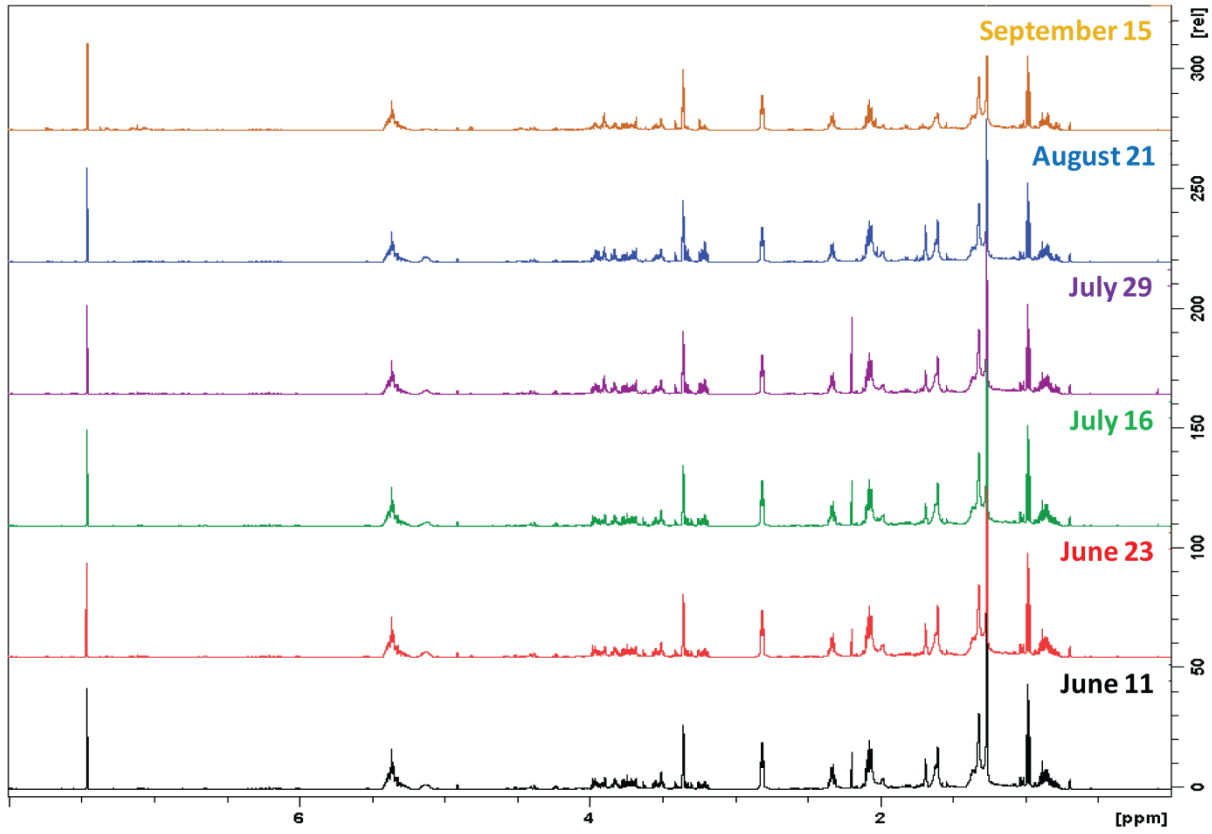

Figure S1. ^{1}H NMR spectra of ethyl acetate extracts of "Thüringer Waid" harvested at dates indicated.

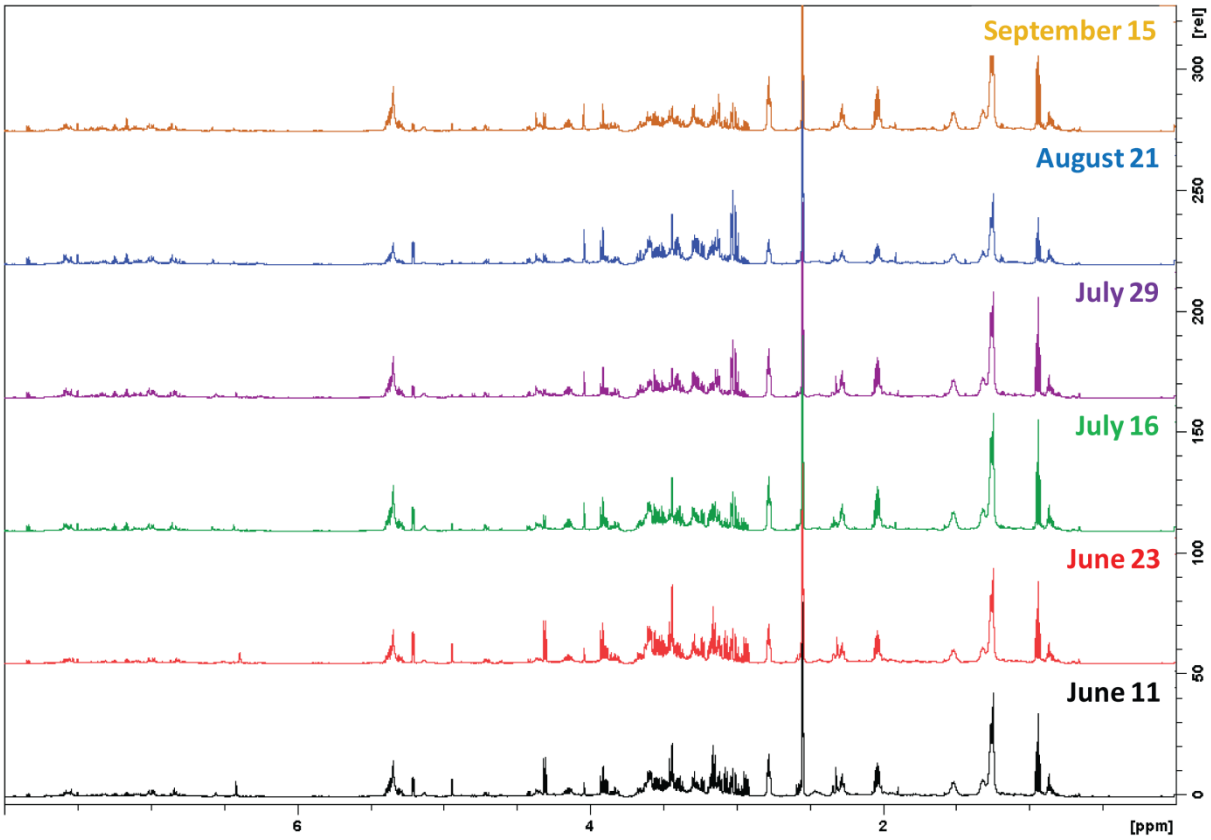

Figure S2. ^{1}H NMR spectra of 70% methanolic extracts of "Thüringer Waid" harvested at dates indicated.

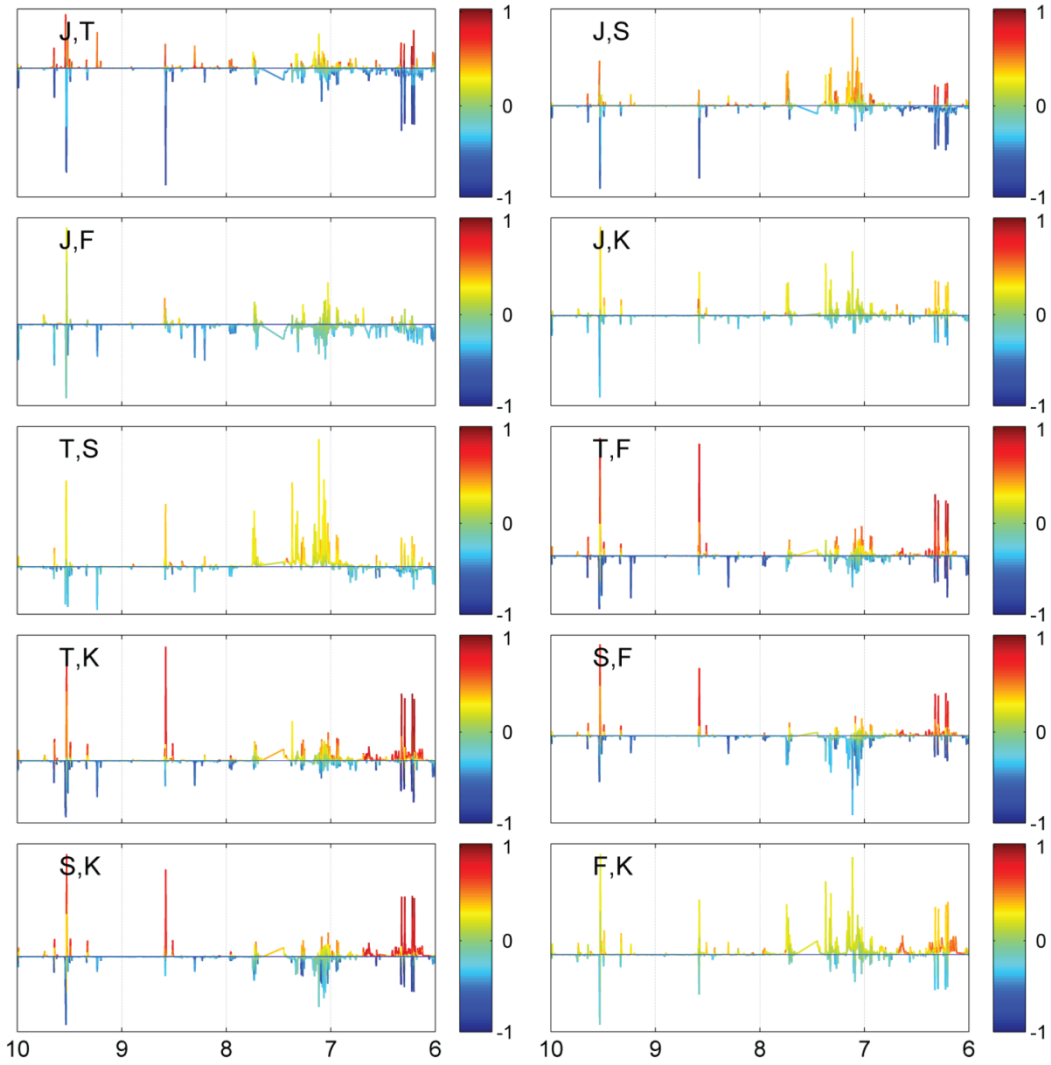

Figure S3. Pairwise STOCSY in the low field region of the ethyl acetate extracts (single harvest, all harvest dates) from accessions "Jenaer Waid" (J), "Thüringer Waid" (T), "Swiss woad" (S), "French woad" (F), and "Kieler Waid" (K). Intensities represent the covariance, and color the correlation. Positive correlation indicates higher concentration in the second accession of the respective pair.

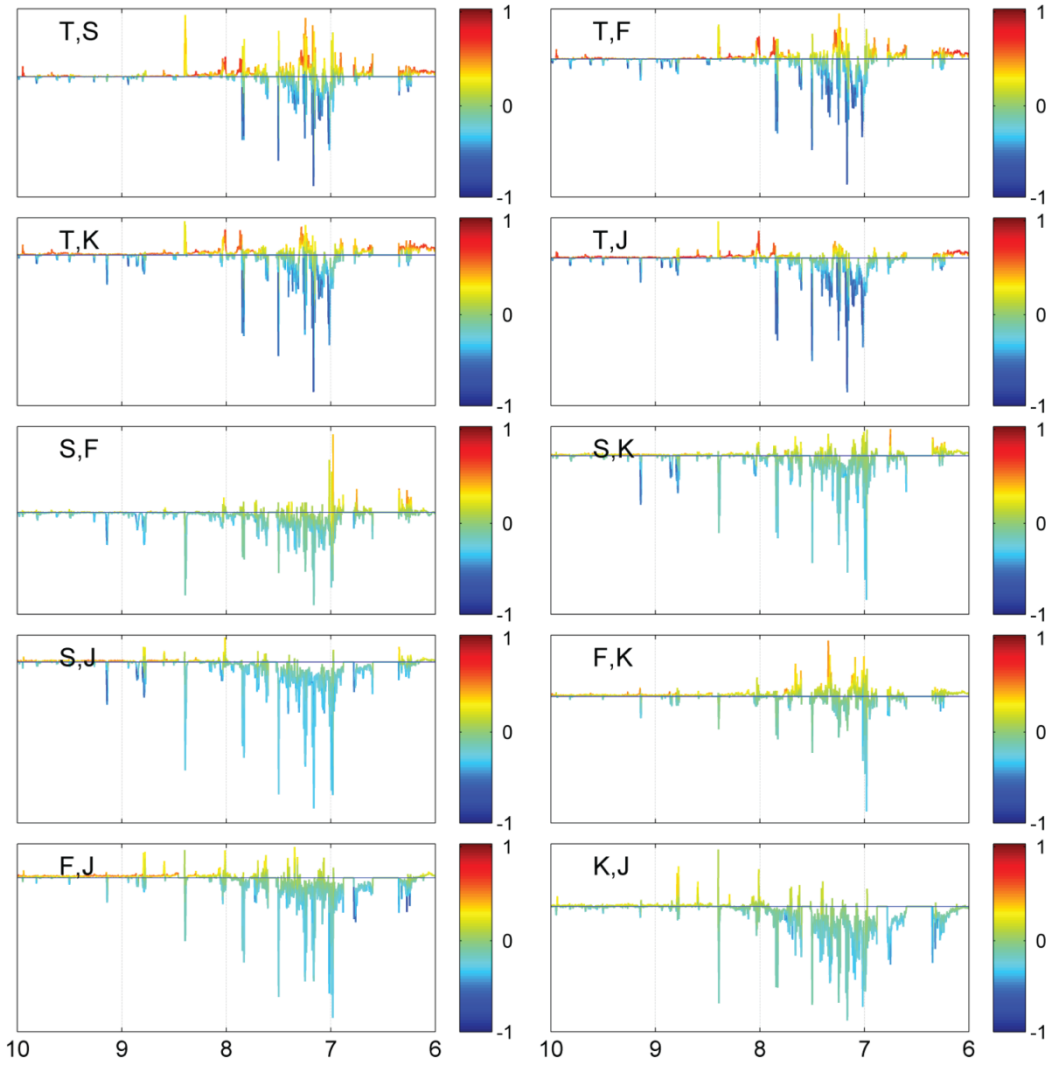

Figure 4. Pairwise STOCSY in the low field region of the 70% methanolic extracts (single harvest, all harvest dates) from accessions "Jenaer Waid" (J), "Thüringer Waid" (T), "Swiss woad" (S), "French woad" (F), and "Kieler Waid" (K). Intensities represent the covariance, and color the correlation. Positive correlation indicates higher concentration in the second accession of the respective pair.

4. Conclusions and outlook

A library consisting of 600 extracts from taxonomically diverse plant species was screened for useful agrochemical and cosmetic properties. Based on previously defined thresholds, active extracts were selected and submitted to HPLC-based activity profiling. In a next step, extracts were prioritized for isolation. Extracts of *Bocconia frutescens* stems, *Miconia affinis* stems, *Myrcia splendens* leaves, *Combretum aff. laxum* leaves, and *Erythroxylum macrophyllum* leaves were pursued for agrochemical activities, while *Combretum cacoucia* leaves and *Mosquitoxylum jamaicense* leaves were selected for cosmetic purposes. All these extracts fulfilled criteria, such as (i) discrete activities in fractions that could be assigned to one or several UV peaks, and (ii) low to medium complexity of the metabolite profile. In addition, extracts for the agrochemical project were further prioritized according to their activity.

In summary, a total of 39 compounds were isolated and characterized. The chemodiversity of compounds (active and inactive) was not as high as expected, although all plants - except for the two *Combretum* species - belonged to different families. Substance classes included benzophenanthridine alkaloids, oleanane-type triterpenes, phenylpropanoids, phenolic acids such as gallic and ellagic acid derivatives, and flavonoids. A majority of compounds (over 50%) belonged to the latter class. It has to be considered that phenolic compounds, in particular flavonoids, occur widely in plants since they are part of defense strategies against environmental stress [1].

The agrochemical screening revealed in total five compounds as active (>25 %) in the screening for pesticidal activity (**Chapter 3.1**). Four fungicidal compounds with two different scaffolds were discovered. The first class were benzophenanthridine alkaloids from *B. frutescens*, whereby three of five isolated compounds were active. Sanguinarine was outside of the active window in the activity profile. Oxysanguinarine showed no activity, while chelerythrine showed moderate, and macarpine and dihydrosanguinarine good activity. Considering the structures (**Fig. 11**), it seemed that methoxy groups at positions 7 and 8 were favorable for activity. Active macarpine and dihydrosanguinarine were also derivatives of sanguinarine bearing two methoxy groups at positions 10 and 12, and a reduced *N*-methyl group, respectively. These two structural features seemed more critical for activity than methoxy groups at positions 7 and 8. The inactive oxysanguinarine suggested that a carbonyl group at position 6 did not increase activity. These preliminary findings should be substantiated by a screening of a structurally more diverse set of derivatives. As to the oleanane triterpene arjunolic acid from *M. affinis*, fungicidal activity had been previously assessed in a mixture with asiatic acid [2, 3]. In the current study, the activity of arjunolic acid alone could be confirmed. Since it was the only active triterpene found in this study, other triterpenes should be screened for fungicidal activity, e.g. including triterpenoids with different scaffolds and decoration patterns. As for insecticidal activity, only myricetin-3-O-(6"-O-galloyl)-β-galactopyranoside from *M. splendens* was moderately active. When comparing to the structure of inactive myricitrin, it seemed that the 6"-O-galloyl-β-galactopyranosyl moiety favored the activity. From the herbicidal screening, no active compound was identified although discrete windows of activity were present in the HPLC-based activity profiles. Considering that the extracts of *C. aff. laxum* and *E. macrophyllum* contained tannins, it is likely that the tannins - at least in part - were responsible for the positive results in the extract screening for herbicidal activity. This could have been corroborated by a retest of extracts after removal of tannins. It remains now to be discovered, what the molecular targets are, and if there are other targets that are influenced by the active

compounds. This can be assessed by target based *in vitro* screenings on already known targets and established assays, combined with virtual screening [4]. This will be helpful to perform structure-activity-relation studies in order to develop synthetic or semi-synthetic pesticides.

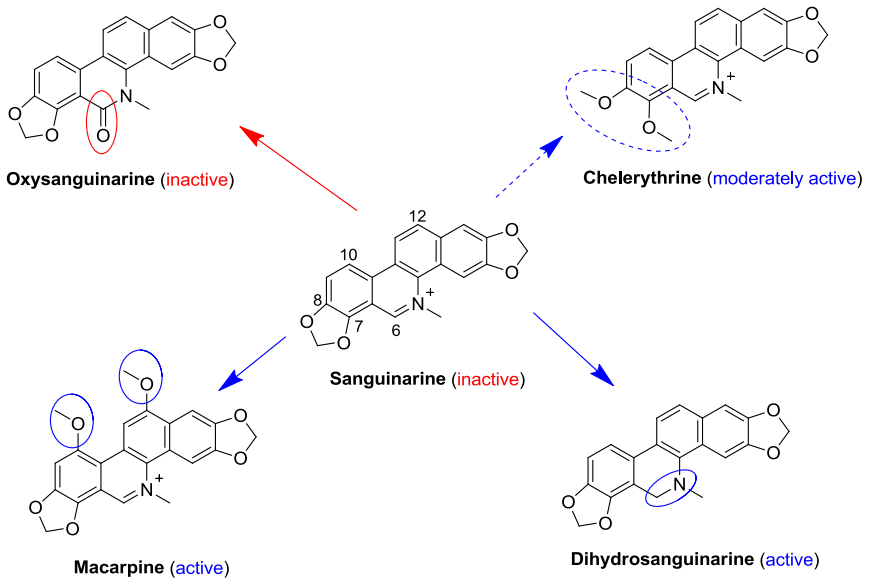

Fig. 11 Structure-activity estimations for benzophenanthridine alkaloids from *B. frutescens*. Features that do not favor activity are highlighted in red, features favoring moderately activity are highlighted in dashed blue, and features favoring significantly activity are highlighted in solid blue.

Eight compounds were found to be active in the cosmetic screens, either in the DPPH assay, the UV-B protection assay, or in both (**Chapter 3.2**). Overall analysis of the structures of compounds revealed that radical scavenging activity was highest for gallic and free digallic acid derivatives, as compared to flavonoids. Protocatechuic acid, which differed from gallic acid by the lack of a hydroxyl group, showed only weak radical scavenging activity. Protocatechuic acid and isoquercitrin showed the highest UV-B protective activity. For UV-B protective properties, data of the quercetin derivatives suggested that the carbohydrate moiety modulated activity. Isoquercitrin (glucopyranoside) was the most active, followed by hyperoside (galactopyranoside), quercetin-3-O-(2''-O-galloyl)-β-galactopyranoside, guaijaverin (arabinopyranoside), and the aglycon quercetin. Additional, and more representative assays such as e.g. calcein-AM, ^{35}S-methionin incorporation, and IL1-α assays [5] will be needed to substantiate results from the MTT assay.

A second part of the project was devoted to the metabolomic analysis of *Isatis tinctoria* (**Chapter 3.3**). The metabolite profiles in leaf extracts of different strains and harvesting dates were compared using NMR spectroscopy and multivariate data analysis. In addition, the effect of repeated harvesting on the metabolite profile was analyzed. Due to the high complexity and multi-class properties of the samples, simple PCA analysis was not sufficient. Combination of advanced methods such as CA, MANOVA, and k-NN enabled us to see differences between accessions, and between single and repeatedly harvested leaves, even though differences between the harvesting dates were predominant. Using pairwise 1D-STOCSY combining covariance and correlation matrices, we identified various metabolites contributing to the differences. All these metabolites were different from those analyzed in previous quantitative studies on freeze-dried leaves of *I. tinctoria* [6, 7]. These data could

serve as a basis for quality control of *I. tinctoria*, whereby identified biomarkers could be used to characterize accessions, date of harvest, and harvest regime. As to accessions, "Thüringer Waid" was easy to characterize, while the other accessions were very similar among each other so that differentiation was difficult, or even impossible. It has to be considered that the range of identified metabolites was limited. This was, among others, due to the low sensitivity and selectivity of NMR spectroscopy. This limitation can be overcome by HPLC-MS, which is also a frequently used method in plant metabolomics [8]. A combination of the strengths of NMR and HPLC-MS will maximize the range of detected metabolites and hence, will enable a better characterization of these accessions. Another problem was the re-dissolution of extracts for NMR measurements, in particular for the 70% methanol extracts. Other methods of sample preparation should be explored. Verpoorte and co-workers, for example, developed an extraction method that uses directly deuterated solvents of different polarities for extraction [9]. This has the advantage that NMR spectra can be recorded directly after extraction, without need of further manipulations. However, maceration in deuterated solvents may not be exhaustive as compared to PLE [10], and only solves the problem concerning re-dissolution for NMR analysis. Hence, development of a protocol for PLE extracts of *I. tinctoria* is necessary, to combine the advantage of exhaustive extraction with complete re-dissolution of the extracts for NMR analysis. This could be achieved by (i) varying PLE extraction solvents in order to identify extracts that are more convenient for re-dissolution (as it was the case with the ethyl acetate extracts), and (ii) by establishing a more extensive set of solvent mixtures for NMR analysis, in order to increase the probability of having an appropriate solvent/solvent mixture for a given type of extracts.

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2008-2010	MSc studies in pharmaceutical sciences at the university of Basel, Switzerland, and master thesis in the group of Prof. Dr. Beat Ernst, Division of Molecular Pharmacy, University of Basel, Switzerland
2005-2008	Bachelor studies in pharmaceutical sciences at the University of Basel, Switzerland

Publications and Awards

Publications:

Guldbrandsen N, De Mieri M, Gupta M, Seiser T, Wiebe C, Dickhaut J, Reingruber R, Sorgenfrei O, Hamburger M. *Screening of Panamanian plant extracts for pesticidal properties, and HPLC-based identification of active compounds*. Scientia Pharmaceutica, in press. doi:10.3797/scipharm.1410-10

Guldbrandsen N, De Mieri M, Gupta M, Liakou E, Pratsinis H, Kletsas D, Chaita E, Aligiannis N, Skaltsounis A-L, Hamburger M. *Screening of Panamanian plants for cosmetic properties, and HPLC-based identification of constituents with antioxidant and UV-B protecting activities*. Scientia Pharmaceutica, in press. doi:10.3797/scipharm.1409-12

Guldbrandsen N, Kostidis S, Schäfer H, De Mieri M, Spraul M, Skaltsounis A-L, Mikros E, Hamburger M. *Metabolomic studies on Isatis tinctoria – Comparison of different accessions, harvesting dates, and the effect of repeated harvesting*. Journal of Natural Products, submitted for publication

Awards:

Poster award at the 4th Annual Research Meeting of the Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland, February 2012 for:

Guldbrandsen N, Kostidis S, Skaltsounis A-L, Mikros E, Hamburger M. *Metabolomic studies on Isatis tinctoria – Comparison of different accessions, harvesting dates, and the effect of repeated harvesting*.