

RESEARCH ARTICLE

In chemico skin sensitization risk assessment of botanical ingredients

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Abstract

Skin sensitization risk assessment of botanical ingredients is necessary for consumers' protection and occupational hazard identification. There are currently very few available alternative methods that can assist in the evaluation of complex mixtures. Chemical methods can provide essential information in a timely manner and thus help to reduce the need for in vivo testing, and they can complement and facilitate targeted in vitro assays. In the present work, the applicability of the high-throughput screening with dansyl cysteamine (DCYA) method for the systematic evaluation of skin sensitization of complex botanicals was explored. Botanical ingredients of four unrelated plant species were obtained and tested with the high-throughput fluorescence method at three concentrations. To illustrate the minimal matrix effects of the tested extracts on the developed method, the least DCYA-reactive extract (*Rosa canina*) was spiked with known sensitizers at different concentrations. The data obtained from the four plant extracts and the spiking experiments with known sensitizers, suggest that the high-throughput screening–DCYA method can be successfully applied for estimating the skin sensitization potential of complex botanical matrices. This is the first report of an attempt to develop a versatile in chemico method for the rapid detection of reactive skin sensitizers in complex botanical extracts, which could complement the battery of existing validated, non-animal methods.

KEYWORDS

alternative methods, botanicals, HTS-DCYA, in chemico methods, Skin sensitization

1 | INTRODUCTION

Botanical ingredients are added to a widespread assortment of consumer products, including aromatherapy, cosmetics, detergents, household and perfumes products. The term botanical ingredients includes a variety of preparations, from solvent extracts to distilled essential oils, expressed juices, tinctures, waxes, vegetable oils, lipids, plant carbohydrates and purified plant components. Safety assessment of botanical ingredients is therefore necessary for consumers' protection.

As many of these formulations are intended for external topical use, allergic contact dermatitis is one of the most prevalent adverse effects associated with formulations containing botanical ingredients

(Thomson & Wilkinson, 2000; Uter et al., 2010). In vivo animal models, such as the local lymph node assay, have been considered for decades to be the gold standard in dermato-toxicology and the only stand-alone method for evaluating the skin sensitization risk of complex mixtures. However, there has been a growing trend towards a "reduce, replace and refine" approach to minimize progressively the use of animals for toxicological testing of cosmetic products (Roberts, 2015). Non-animal in vitro and in chemico approaches are gaining momentum as more ethical, faster and economical alternatives for predictive toxicology. In this perspective, in chemico methods for skin sensitization can represent a powerful tool at pre-screening stages by providing information about the likelihood of a molecule to bind covalently to skin proteins (OECD, 2015).

While animal methods are available for screening of botanical ingredients, the literature concerning the application of non-animal in vitro models is scarce, and in chemico approaches are practically non-existent. Only the in vitro KeratinoSens™ assay has been tested

Abbreviations: ACN, acetonitrile; DCYA, dansyl cysteamine; HTS, high-throughput screening; SL, sesquiterpene lactone; TLC, thin-layer chromatography; TRI, total reactive index

in a pilot study to assess the capacity for detecting skin sensitizers in plant extracts, with some limitations (Andres et al., 2013). Development of methods for testing botanical ingredients is challenging because plant material can contain an eclectic variety of ubiquitous metabolites, including (but not limited to) flavonoids, polyphenols, chlorophylls, sugar derivatives and tannins. Some of these constituents are known to interfere with a variety of in vitro assays due to non-specific interactions (Baell, 2016; Bruggisser, von Daeniken, Jundt, Schaffner, & Tullberg-Reinert, 2002).

The in chemico method Direct Peptide Reactivity Assay (DPRA), has been recently tested with binary mixtures (Lang, Giménez-Arnau, & Lepoittevin, 2017), but its optimization for the assessment of more complex formulations is still at early developmental stages. Yet, the applicability of chemical methods for predictive screening of mixtures is desirable and certainly needed to provide complementary information to aid the prioritization of more extensive toxicological studies.

The in chemico high-throughput screening method using dansyl cysteamine (HTS-DCYA) is suitable for the rapid evaluation of chemical reactivity toward a model fluorescent nucleophile, which is used as a surrogate of skin proteins. In contrast to the majority of state-of-the-art methods, the HTS-DCYA method enables reactivity classification based on the fluorescence emission of the resulting DCYA-adducts. The designed workflow along with the specificity of the detection mode has several advantages because no chromatographic separation of the reaction mixture is necessary. The HTS-DCYA method can therefore be applied to investigate the presence of thio-reactive compounds in complex mixtures such as botanical ingredients.

In the current study, the potential applicability of the HTS-DCYA method to identify rapidly the potential skin sensitizers in botanical extracts was explored for the first time. The samples were obtained from four plants with reported clinical histories of allergic contact dermatitis. Each extract was tested at three concentrations (5, 10 and 20 mg mL⁻¹) to account for sensitizers present at various concentrations and their degree of chemical reactivity. Because plant ingredients (in particular, solvent extracts) are very complex matrices, it is necessary to test the robustness of the method in the presence of common non-specific metabolites, which could negatively affect the outcome of the method. The results herein reported suggest that the HTS-DCYA in chemico method may have application for estimating the skin sensitization potential of complex botanical mixtures and complement the battery of existing validated, non-animal methods.

2 | MATERIAL AND METHODS

2.1 | Plant material

Four plants were chosen based on the clinical evidence of reported skin sensitization. Specimens of all plant samples are deposited at the botanical repository at the National Center for Natural Products Research (NCNPR, The University of Mississippi, USA) and documented with NCNPR accession codes. *Magnolia grandiflora* leaves (NCNPR no. 17054) were collected in Oxford, MS (USA). *Rosa canina* (Rosaceae) fruits (no. 3652) and *Calendula officinalis* flowers (no. 3668) were obtained from Mountain Rose Herbs (Eugene, OR, USA).

Cinnamomum verum barks (no. 5502) were collected at the Missouri Botanical Garden (St. Louis, MO, USA).

2.2 | Chemicals and reagents

The fluorescent compounds DCYA and DCYA disulfide were synthesized as previously described. (Chittiboyina, Avonto, Rua, & Khan, 2015). Parthenolide (CAS no. 20554-84-1), cinnamaldehyde (no. 14371-10-9) and coumarin (no. 91-64-5) were purchased by Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) and standardized pH 10 ± 0.02 buffer (cat. no. SB116-500) were purchased from Fisher Scientific (Suwanee, GA, USA). Silica gel 60 (175–225 µm) thin-layer chromatography (TLC) plates (cat. no. 105553) were purchased from Merck (Darmstadt, Germany). Polymer-supported maleimide (SiliaBond® Maleimide, ≥0.64 mmol g⁻¹) was purchased from SiliCycle (Quebec City, Quebec, Canada).

2.3 | Optimization of solvent extraction

M. grandiflora leaves were used for the optimization of solvent extraction. This plant was chosen based on its content of sesquiterpene lactones (SLs) including the known sensitizers, parthenolide and costunolide. In addition to SLs, leaves extract of *M. grandiflora* contains several non-specific components (e.g., chlorophylls, pigment, etc.) and the presence of such components was exploited to assess the robustness and limitations of the HTS-DCYA method. The fresh leaves of *M. grandiflora* were air-dried and ground to a fine powder. Methanol, acetone, diethyl ether, ethyl acetate, cyclohexane and hexane were evaluated in the solvent optimization test. One gram of powdered material was accurately weighed and placed in a 15 ml centrifuge tube, then extracted with 5 ml of the corresponding solvent and sonicated for 1 hour. The samples were centrifuged at 1900g (4000 rpm) for 15 minutes and filtered using a syringe equipped with 0.45 PES Whatman™ Puradisc filters (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Each solvent extraction was evaluated qualitatively on silica gel TLC plates using various solvent systems. The plates were treated with *p*-anisaldehyde stain reagent for visualization purposes. The best extraction solvent was qualitatively evaluated by spotting 15 µl of each sample on multiple TLC plate; each plate was eluted in solvent systems with different polarity. The extraction efficiency was visually determined against a known concentration of parthenolide standard.

The obtained extracts of *M. grandiflora* (50 µl) were incubated with 50 µl of DCYA in ACN (1 or 5 mg mL⁻¹) to detect the presence of reactive compounds. Twenty microliters of standardized pH 10 buffer solution was added and the reaction was incubated for 22 ± 3 minutes at room temperature. The samples were then checked on a silica gel TLC plate using hexane/EtOAc binary solvent systems as the mobile phase. High-performance liquid chromatography (HPLC) analyses were performed on an Agilent 1290 Infinity ultra-HPLC/ultraviolet (UV) system equipped with an Agilent 6120 quadrupole mass detector (Agilent Technologies, Santa Clara, CA, USA) and an ultra-HPLC BEH C18 column (1.7 µm × 2.1 mm × 100 mm; Waters Corporation, Milford, MA, USA). Column temperature was 30°C. The carrier solvent used was water (A) and ACN (B), both containing 0.05% formic acid. An eluent gradient was performed as follows: 15%–80% B (0.250 mL min⁻¹) in 20 minutes, then to 100% B in 2 minutes and held for 3 minutes. UV

absorption was monitored at 254 and 280 nm. Fluorescence emission was monitored at 520 nm (excitation wavelength 350 nm). Positive and negative electrospray ionization scans were recorded in the range m/z 100–1000.

2.4 | Extraction of *Cinnamomum verum* and *Calendula officinalis*

The barks of *C. verum* and flowers of *C. officinalis* were ground to a fine powder. These two botanicals were selected due to presence of the moderate sensitizer cinnamaldehyde in cinnamon bark, and non-allergenic components in *C. officinalis*. One gram of powdered material was accurately weighed and placed in a 15 ml centrifuge tube, then extracted with 5 ml of methanol (four times) with sonication for 1 hour. The samples were centrifuged at 1900g for 15 minutes and filtered using a syringe equipped with 0.45 PES Whatman™ Puradisc filters. The solvent was removed under nitrogen to obtain 262.2 and 141.3 mg of dry extract, respectively.

2.5 | Extraction of *R. canina* flowers

Similarly to *C. officinalis*, the *R. canina* flowers extract was selected to assess the selectivity of the HTS-DCYA method with a botanical considered a non-sensitizer. The dry flowers of *R. canina* (2.115 g) were finely powdered and extracted with 8 ml of methanol in a centrifuge tube with sonication (1 hour). The resulting suspension was then centrifuged at 1900g. The extraction process was repeated three additional times and the supernatants were combined and concentrated under nitrogen. The concentrated solution was then filtered with a syringe equipped with 0.45 PES filter (Puradisc; Whatman) in a vial and dried completely in a stream of nitrogen. The obtained dry extract (724 mg) was used for experiments including spiking experiments.

2.6 | Preparation of spiked plant extracts

To determine the sensitivity and specificity of the fluorescence response in the presence of complex matrices, the non-reactive plant extract (obtained from *R. canina*) was spiked with three known allergens, namely, strong (parthenolide), moderate (cinnamaldehyde) and non-sensitizers (coumarin). The *R. canina* methanol extract was accurately weighed (602.9 ± 0.1 mg) and dissolved to 40 mg ml^{-1} in ACN: water 85:15 (v/v). The solution was sonicated for 15 minutes and centrifuged at 1900g for 20 minutes. The clear solution thus obtained was used for the spiking experiments. Parthenolide, cinnamaldehyde or coumarin stock solutions (20 mM) were prepared in ACN. Each stock solution of chemical sensitizer to be spiked was diluted to obtain three concentrations and mixed with a 40 mg ml^{-1} solution of *R. canina* plant extract to obtain spiked samples of plant extract (20 mg ml^{-1}) containing final concentrations of either 2.5, 5 or 10 mM of each of the three sensitizers. The final concentration of compound to be spiked was chosen to cover from 50 to 150% of the optimum concentration (5 mM) for pure compounds. Each spiked sample was then tested in the HTS-DCYA assay as described in Section 2.8.

2.7 | General sample preparation procedure

Twenty milligrams of each plant extract was accurately weighed ($20 \text{ mg} \pm 0.1 \text{ mg}$) and dissolved to a final concentration of 20 mg ml^{-1} using ACN. Up to 20% water in ACN was used if significant precipitate or insolubles were observed. The samples were sonicated for about 20 minutes then centrifuged at 1900g for 20 minutes and the precipitate was discarded. The soluble fraction was further diluted to obtain two additional concentrations (5 and 10 mg ml^{-1}) in ACN.

2.8 | High-throughput screening–dansyl cysteamine

A modified version of the protocol previously described was used (Avonto, Chittiboyina, Rua, & Khan, 2015). The method enables the detection and quantification of thio-reacting compounds as fluorescent adducts upon reaction with DCYA, which is used as model nucleophile. The reactivity of DCYA with potential sensitizers in the mixture is reported as the total reactive index (TRI). Each plant extract was tested at three concentrations (5, 10 and 20 mg ml^{-1} , respectively). Briefly, a solution of DCYA (2.5 mM in ACN) was mixed with an equal volume of plant extract. The DCYA-extract solution was then split into two microcentrifuge tubes ($150 \mu\text{M}$ each). Thirty microliters of aqueous pH 10 buffer was added to the reaction vials (R) and to the negative controls (NC), ACN was added in place of the buffer to blank controls (BI) and to positive controls (PC). Solvent (ACN) was added in place of the test article in both positive controls and negative controls. After 20 minutes incubation, the unreacted DCYA was removed by addition of SiliaBond® resin (30 mg) and the obtained suspension was incubated for 60 minutes at room temperature. Sixty microliters of supernatant was diluted to a final volume of 1 ml with ACN. Two hundred microliters was then transferred into a solvent-resistant 96-well microplate (in triplicate) and each plate was read three times using fluorescence end-point readings. The fluorescence readings were performed on a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Data were acquired and processed using SoftMax Pro 5 (Molecular Devices). The readings were recorded at $\lambda_{520 \text{ nm}}$ (excitation $\lambda_{350 \text{ nm}}$, cut-off 420 nm), at 23 °C. The TRI was calculated based on the average of nine readings per sample (three readings per well in triplicate) as follows:

$$TRI = 100 \times (1 - (BI - R) / (PC - NC) - (PC - BI) / PC)$$

3 | RESULTS

Four plant extracts with different chemical compositions were tested to determine if a generic extraction/screening procedure using the HTS-DCYA method might have applicability to the systematic screening of botanical ingredients.

The HTS-DCYA assay relies on the quantification of the fluorescent emission of the dansyl moiety. Various classes of botanical constituents may interfere with the excitation/emission mechanism of the fluorophore; it is thus important to test the robustness, sensitivity and specificity of the DCYA method in the presence of a variety of matrices and other potential fluorescence interferences.

3.1 | Optimization of sample extraction

To determine optimal parameters for a standard extraction protocol, the leaves of Southern magnolia (*M. grandiflora* L.) were used. Southern magnolia is a widespread ornamental tree native of the Southeast United States. This plant is well known for its content of allergenic SLs, predominantly germacranes, such as parthenolide and costunolide (El-Feraly & Chan, 1978; Guin, Schosser, & Rosenberg, 1990). Both parthenolide and costunolide are electrophilic compounds known to be strong skin sensitizers (Paulsen, Andersen, & Hausen, 2001) and have been previously determined to be highly reactive with DCYA (Avonto et al., 2015). The leaves of *M. grandiflora* also contain a variety of metabolites, including a significant amount of chlorophylls and pigments, which make this plant a good choice to test the robustness of the HTS-DCYA method.

To determine the best extraction solvent, small-scale extractions of the ground leaves were prepared with various solvents, including polar protic, polar aprotic and non-polar solvents. Volatile solvents were preferred due to the nature of many natural sensitizers, which are known to be small, lipophilic compounds, often unstable and with low boiling points. Based on the TLC profiles (in Supporting information, Figure S11), acetone and methanol were found to be the most effective extractive solvents, followed by EtOAc, Et₂O, cyclohexane and hexane. The presence of parthenolide and costunolide in all of the extracts was qualitatively confirmed by TLC comparison with pure reference standards. Methanol resulted in a more exhaustive extraction (which also included a higher percentage of unwanted compounds), while a higher selectivity was achieved with hexane.

Small aliquots of the methanol and hexane extracts from *M. grandiflora* were then incubated with a stock solution of DCYA and pH 10 buffer; any electrophilic components in the extracts may form fluorescent DCYA-adducts in these conditions. At the end of the incubation time, the samples were evaluated by TLC for the presence of fluorescent DCYA-adducts. In both the methanol and hexane extracts, several fluorescent spots were observed by TLC analyses (Figures S12 and S13). Both extracts clearly contained multiple reactive species, with parthenolide as the major reactive constituent. An almost complete depletion of parthenolide was found in all the reaction mixtures (monitored by TLC), along with the formation of a corresponding major fluorescent adduct. Costunolide also reacted with DCYA, along with a number of uncharacterized fluorescent spots (Figure S13), which were allegedly related to DCYA-adducts of other minor SLs known to be present in magnolia extracts (Castaneda-Acosta, Cain, Fischer, & Knopf, 1995; El-Feraly & Chan, 1978; El-Feraly, Chan, & Benigni, 1979; El-Feraly, Chan, Capiton, Daskotch, & Fairchild, 1979; Wu et al., 2001). The formation of multiple DCYA-adducts was further confirmed with HPLC-UV-mass spectrometry coupled with a fluorescence detector (Figure S14). The *m/z* values of these newly formed adducts were found to be 543, 559, 575 and 591 (Figure S15) corresponding to DCYA covalent adducts with SLs masses of 232, 248, 264 and 280 (Table S11). Numerous SLs having compatible mass were reported as constituents of *M. grandiflora* leaves (Table S12). Both adducts of parthenolide and costunolide were confirmed with reference standards. The HPLC-UV-mass spectrometry data corroborated the selectivity of DCYA as model nucleophile for trapping electrophilic

species in complex mixtures. In the adopted conditions, the reaction was also sensitive enough to enable the indirect identification (as DCYA-adducts) of numerous minor compounds, along with the major components parthenolide and costunolide.

Although both methanol and hexane extracts of *M. grandiflora* contained multiple reactive species, differences in the degree of reactivity were observed. No residual DCYA was noticed in the methanol extract, while a significant amount of unreacted DCYA was still present in the hexane extract after the reaction. Hence, methanol was selected as the preferred solvent to maximize the extraction yield.

3.2 | Reactivity of model extracts in high-throughput screening-dansyl cysteamine assay

In the attempt to determine the effects of different types of plant matrices on the final outcome, three additional methanol extracts were prepared from cinnamon (*C. verum*) barks, marigold (*C. officinalis*) and *R. canina* flowers, along with *M. grandiflora* leaves extract. These botanical sources were chosen based on the presence of evidence (particularly from human patch tests and clinical reports) of each plant as potential concern for skin sensitization. Cinnamon barks were chosen because of their content in cinnamaldehyde (a moderately electrophilic sensitizer) as one of the major constituents (Avula, Smillie, Wang, Zweigenbaum, & Khan, 2015). Marigold (*C. officinalis*) and *R. canina* are considered rare or non-allergic plants (Reider, Komericki, Hausen, Fritsch, & Aberer, 2001; Thomson & Wilkinson, 2000) and were chosen as negative controls for comparison purposes to determine the selectivity of the quantification method.

The four extracts were dried at room temperature under nitrogen to minimize the oxidation and/or loss of volatile organic compounds. Neither the content, concentration nor reactivity of each individual component of the extracts was known a priori. Strong electrophiles (such as SLs) are known to react promptly with the DCYA even at low concentrations. It was thus expected that these compounds would result in a positive TRI quantification even in diluted solutions. Other moderate and weak sensitizers typically result in partial thiol depletion under optimum conditions. In highly diluted solutions, the fluorescence response may be too low and result in minimal or even negative quantification. Because of the unknown composition and concentration of reactive compounds of the four extracts, each plant extract was tested at three concentrations (5, 10 and 20 mg mL⁻¹) to ensure that less reactive compounds could also be detected. The reactivity results of the four extracts are summarized in Figure 1. A significant increase of fluorescent emission at increasing concentration of tested extract was quantified for magnolia and cinnamon extracts. The increase of fluorescence response for *M. grandiflora* was estimated to be 2.0 and 2.6 times (for 10 and 20 mg mL⁻¹, respectively) higher compared to the minimum concentration tested (5 mg mL⁻¹). The increase of fluorescence response for cinnamon samples at 10 and 20 mg mL⁻¹ was only 1.7 and 2.0 times the 5 mg mL⁻¹ concentration. The marigold extract was minimally reactive. In the case of *R. canina* extract, a negative reactivity trend was observed at increasing concentrations.

A partial decrease of the absolute fluorescence emission values was occasionally observed with extracts at higher concentrations.

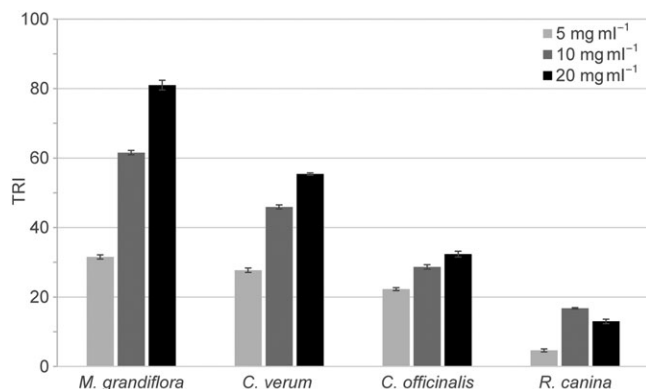


FIGURE 1 Reactivity of the methanol extracts of four plants at three concentrations in the high-throughput screening–dansyl cysteamine assay. TRI, total reactive index

Blanks samples of calendula and *R. canina* >10 mg ml⁻¹ and magnolia (20 mg ml⁻¹) resulted in decreases in fluorescence emission while no significant interference was observed for cinnamon samples at any concentration. This fluorescence loss could be due to the presence of potential matrix interferences, which can lead to fluorescence quenching. The overall reactivity was corrected for such interferences (Section 2.8) thus net TRI and overall outcome were minimally affected.

3.3 | Spiking experiments

To determine the sensitivity and selectivity of the HTS-DCYA method with botanical mixtures, the extract (*R. canina*) with negligible reactivity was spiked with three known sensitizers at three variable concentrations. Parthenolide, cinnamaldehyde and coumarin were chosen as representatives of strong, moderate and non-sensitizers, respectively (Avonto et al., 2015). The HTS-DCYA method was originally validated for concentrations of pure compound at 5 mM (2:1 equiv. compared to the concentration of thiol). At such a concentration, clear differences in the range of reactivity with DCYA can be observed. The plant material was thus spiked with three concentrations ranging from 50% to 150% of the optimum 5 mM concentration.

The methanol extract of *R. canina* spiked with parthenolide resulted in measurable DCYA depletion (TRI increase) at any tested

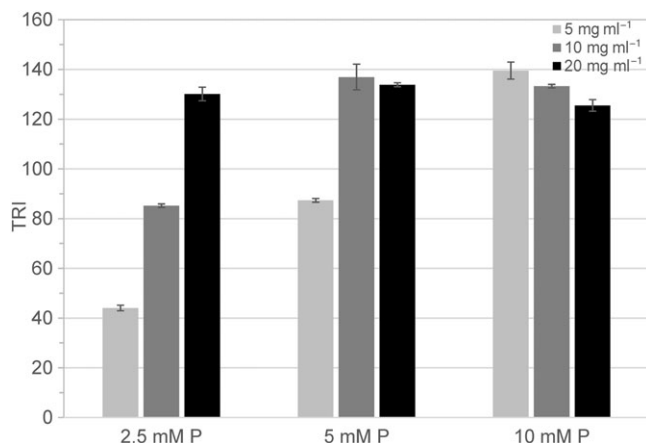


FIGURE 2 Reactivity of *Rosa canina* methanol extract spiked with three concentrations of parthenolide. P, parthenolide; TRI, total reactive index

concentration (Figure 2). When the plant material was spiked with 1 equiv. of parthenolide, concentration-dependent reactivity was evident from 5 to 20 mg ml⁻¹ of total extract. A saturated response was observed with ratios parthenolide/DCYA above 2:1, with complete DCYA depletion at any concentration of the plant extract.

Samples of *R. canina* spiked with the moderate sensitizer cinnamaldehyde generated a concentration-dependent fluorescence response, although only a partial reactivity with DCYA was observed at any tested concentration (Figure 3). In the presence of coumarin (as non-reactive electrophile), no concentration-dependent reactivity was observed (Figure 4).

4 | DISCUSSION

Identification of skin sensitization adverse effects related to the use of botanical ingredients in topical formulations has important implications for consumers. As a result of the paucity of in vitro safety data on the sensitization potential of botanical extracts, there is a great need for rapid methods to screen potential sensitizers found in complex mixtures, such as botanical extracts, which are widely used in a variety of formulations to be applied on the skin. In the present work,

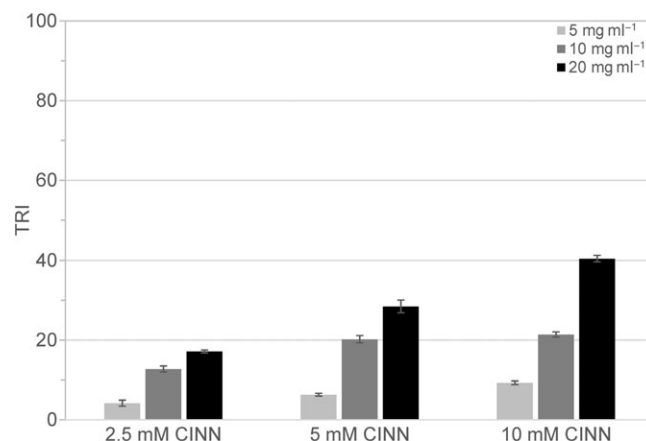


FIGURE 3 Reactivity of *Rosa canina* extract spiked with three concentrations of cinnamaldehyde. CINN, cinnamaldehyde; TRI, total reactive index

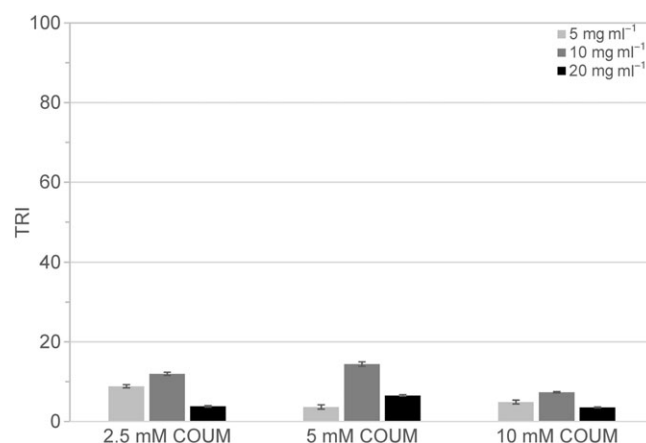


FIGURE 4 Reactivity of *Rosa canina* extract spiked with three concentrations of coumarin. COUM, coumarin; TRI, total reactive index

a modified HTS-DCYA assay was used to evaluate the chemical reactivity of four botanical extracts to determine if this rapid screening method can be an alternative for the safety assessment of complex botanical ingredients. The HTS-DCYA method is based on the quantification of thio-adducts formed upon reaction of potential skin sensitizers with a fluorescent probe (DCYA). Assuming the DCYA unit of the covalent adducts is the only contributor to the fluorescence emission (if no quenching phenomena occur), then the fluorescence emission (calculated as a TRI) can be extrapolated as the total number of DCYA-adducts in solution, independently from the type of generated adduct. In other words, the TRI can be regarded as a parameter of reactivity of the overall mixture even when the contributions of individual components is unknown (Avonto et al., 2016; Chittiboyina, Avonto, & Khan, 2016).

We have attempted to extend this approach to the design of a general strategy for the rapid evaluation of diverse complex mixtures such as plant extracts. The safety evaluation of botanical ingredients can be a daunting task because plant tissues can sometimes contain mixtures of hundreds of chemical components. In many instances, a systematic isolation and safety assessment of each individual component may be laborious if even feasible. The applicability of in vitro methods is limited to non-cytotoxic mixtures, thus orthogonal approaches are desirable to minimize the need of animal experiments. Testing botanicals using in chemico methods could provide valuable information for more focused evaluations. The application of sensitive high-throughput methods could help to prioritize and expedite the identification and targeted isolation of compounds of concern for further testing using more expensive/time consuming in vitro/in vivo methods. This is particularly valuable in the presence of non-characterized mixtures or unstable compounds (Avonto, Rua, Lasonkar, Chittiboyina, & Khan, 2017).

In the examples reported, the plant selection covered various types of botanical extracts. In the case of magnolia extract, the TRI response was related primarily to the reactivity of the SL components. SLs have been previously classified in HTS-DCYA as strong/extremely reactive, with average depletion of >80% of DCYA (Avonto et al., 2015). When the non-reactive *R. canina* extract was spiked with parthenolide, a strong TRI response comparable to magnolia extract was obtained even at concentrations of parthenolide as low as 0.156 mg mL⁻¹. It was thus estimated that the HTS-DCYA method would be sensitive enough to enable the detection of highly reactive compounds present in a total concentration of 2–3% w/w. Similarly, moderately reactive compounds such as cinnamaldehyde were easily detected at concentrations above 10 mM, while for concentrations below the 5 mM increase in TRI was still noticeable but to a minor extent.

In conclusion, our results suggest that the HTS-DCYA method may be useful to rapidly identify the potential compounds of concern in botanicals and mixtures. This could be extremely valuable not only for safety assessment, but also as a screening tool for the development of minimally sensitizing formulations of cosmetic products containing botanical ingredients.

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CONFLICT OF INTEREST

The authors did not report any conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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***In chemico* skin sensitization risk assessment of botanical ingredients.**

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Supporting Information

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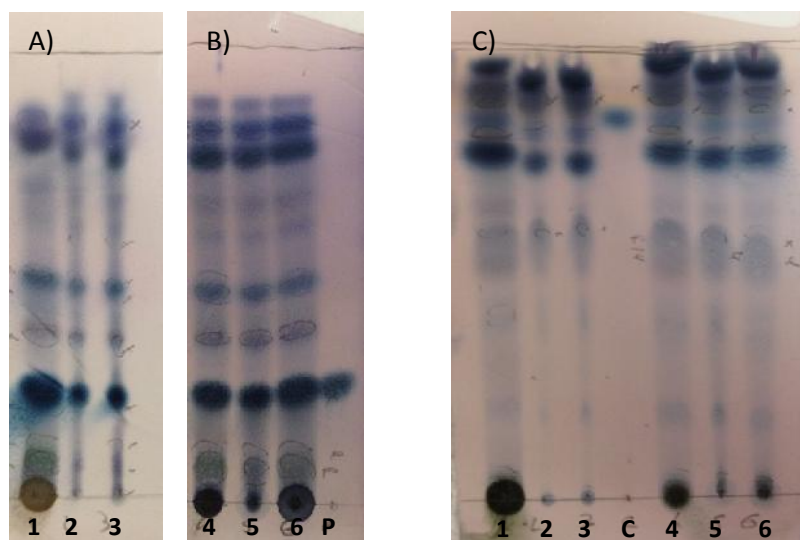


Figure SI1. Chemical profile of *M. grandiflora* leaves extracted with different solvents. 1= MeOH; 2= hexane; 3= cyclohexane; 4) acetone; 5) diethyl ether; 6) ethyl acetate; C= costunolide standard; P = parthenolide standard. TLC in silica gel after staining with *p*-anisaldehyde stain solution. TLC conditions: A-B) 30% ethyl acetate in hexane; C) 80% ethyl acetate in hexane.

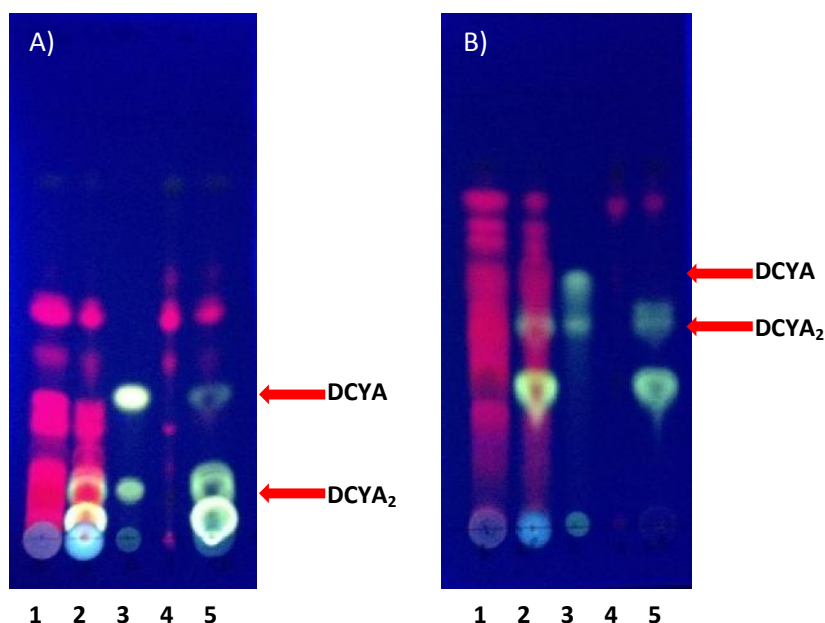


Figure SI2. Chemical profile of *M. grandiflora* leaves after reaction with 1 mg/mL DCYA (in acetonitrile). TLC in silica gel (UV 365 nm). TLC conditions: A) 30% ethyl acetate in hexane; B) 40% ethyl acetate in hexane. 1= standard MeOH extract; 2 = reaction of MeOH extract; 3 = DCYA + DCYA disulfide standards; 4 = standard hexane extract; 5 = reaction of hexane extract.

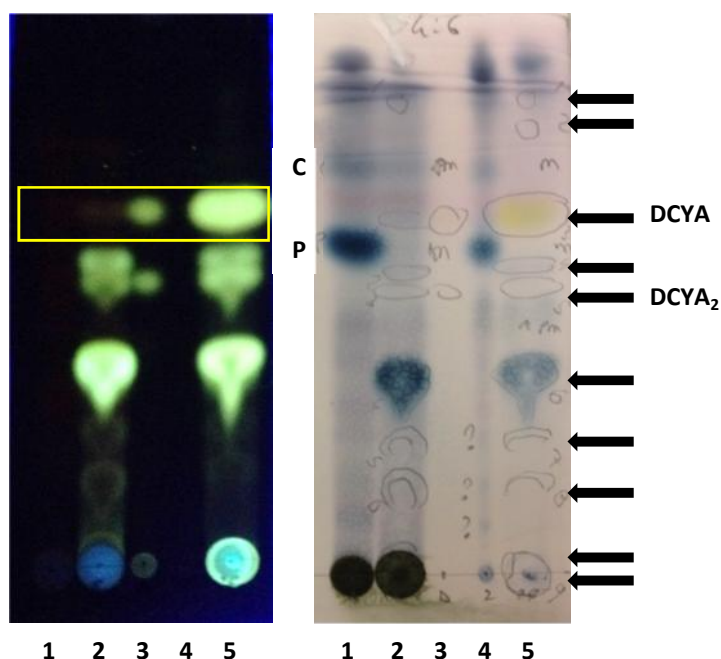


Figure SI3. Chemical profile of *M. grandiflora* leaves after reaction with 5 mg/mL DCYA (in acetonitrile). TLC in silica gel (40% ethyl acetate in hexane). TLC conditions: A) UV profile at 254 nm; B) same TLC after staining with *p*-anisaldehyde solution. 1= standard MeOH extract; 2 = reaction of MeOH extract; 3 = DCYA + DCYA disulfide standards; 4 = standard hexane extract; 5 = reaction of hexane extract. Fluorescent spots are indicated by the arrows.

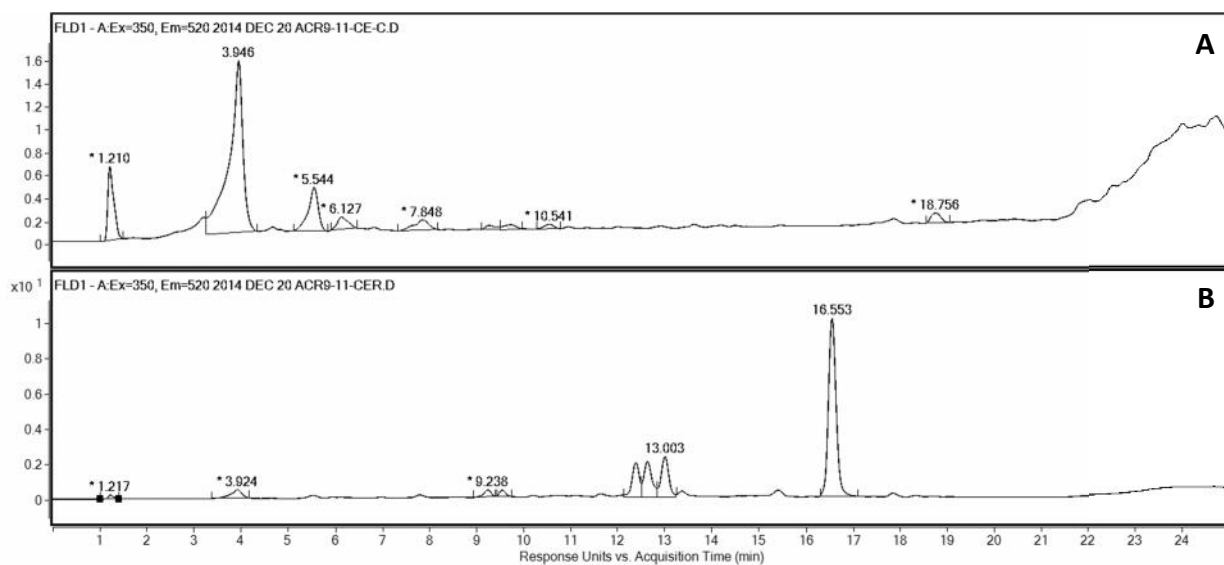


Figure SI4. LC chromatograms of the crude extract of *M. grandiflora* before and after reaction with DCYA. Detection mode: fluorescent detector (Ex 350 nm, Em 520 nm).

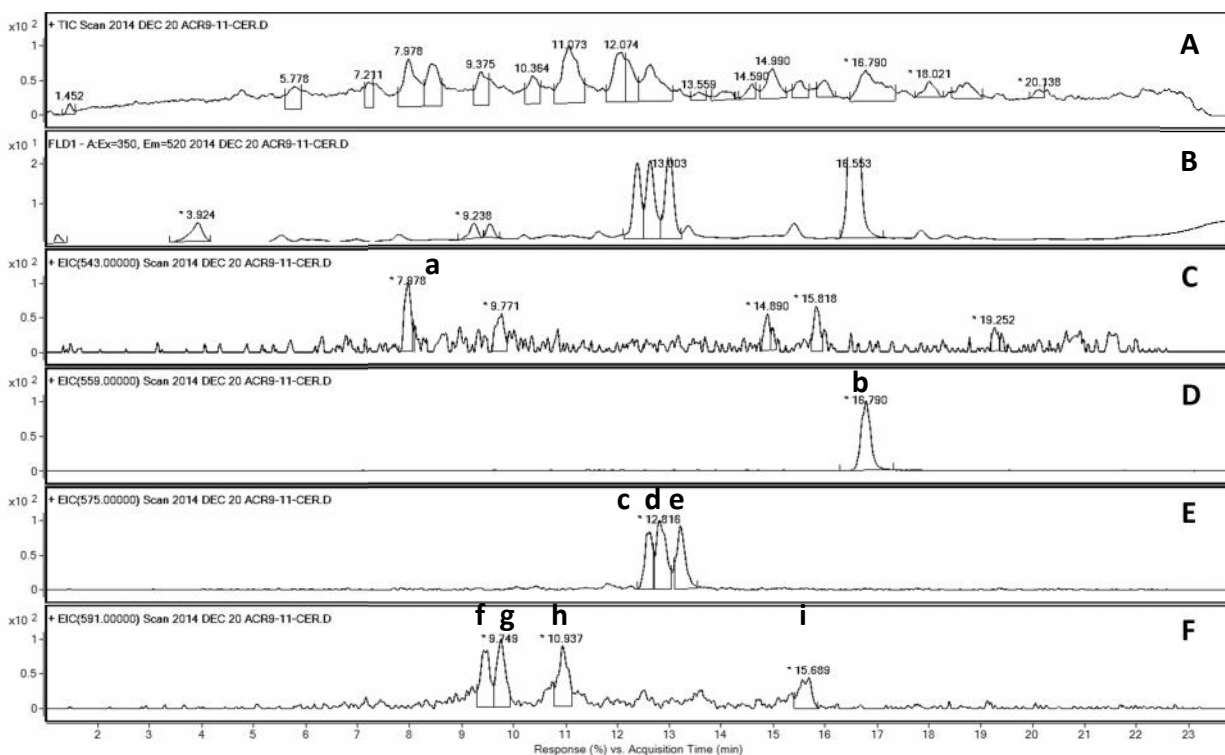


Figure SI 5. LC-MS profile of putative DCYA adducts in extract of *M. grandiflora* after reaction with DCYA: a) +ESI TIC profile b) fluorescent emission (Ex 350 nm, Em 520 nm); c-f) extracted ion chromatogram (EIC) profile for m/z 543, 559, 575 and 591, respectively.

Table SI 1. Calculated MW for the fluorescent DCYA adducts found in *M. grandiflora* leaves extract.

Peak	$[M+DCYA+H]^+$	Adduct MW $[M + DCYA]$	MW [M]
a	543	542	232
b	559	558	248
c,d,e	575	574	264
f,g,h,i	591	590	280

Table SI 2. Known sesquiterpene lactones previously reported in *M. grandiflora* leaves.

Compound	Name	CAS N.	MW	Reference
1	Costunolide	553-21-9	232.2	1
2	Parthenolide	20554-84-1	248.1	1
3	Reynosin	28254-53-7	248.1	1
4	Santamarine	4290-13-5	248.1	1
5	Magnolialide	72145-13-2	248.1	2
6	11,13- Dehydrocompressanolide	126621-30-5	248.1	
7	Michelenolide	66392-96-9	264.1	1
8	Verlotorin	31105-79-0	264.1	4
9	Melampomagnolide A	93930-16-6	264.1	5
10	Melampomagnolide B	93930-15-5	264.1	5
11	Magnograndiolide	92618-98-9	266.2	3
12	Peroxyparthenolide	64845-91-6	280.1	4

1) El-Feraly, F. S. and Chan Y.-M., 1978. *J. Pharm. Sci.* **67**, 347-350; 2) El-Feraly, F. S. *et al.*, 1979. *Phytochemistry*, **18**, 881-882; 3) Halim, A. F., *et al.*, 1984. *Phytochemistry* **23**, 914-915; 4) El-Feraly, F. S. *et al.*, 1977. *Tetrahedron Lett.*, **23**, 1973-6; 5) El-Feraly, 1984. *Phytochemistry*, **23**, 2372-4; 6) Yang, M. H. *et al.*, 1994. *Planta med.*, **60**, 390-390.

Figure SI 6. Structures of known sesquiterpene lactones from *M. grandiflora* leaves

