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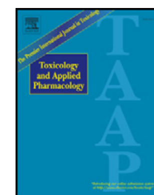

Identification of a compound isolated from German chamomile (*Matricaria chamomilla*) with dermal sensitization potential

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ABSTRACT

German chamomile is one of the most popular herbal ingredients used in cosmetics and personal care products. Allergic skin reactions following topical application of German chamomile have been occasionally reported, although it is not fully understood which of the chemical constituents is responsible for this adverse effect. In the present work, three candidate sensitizers were isolated from German chamomile based on activity-guided fractionation of chamomile extracts tested using the *in vitro* KeratinoSens™ assay. The compounds were identified as the polyacetylene tonghaosu (**1**), and both *trans*- and *cis*-glucomethoxycinnamic acids (**2** and **3**). These three compounds were classified as non- to weakly reactive using *in chemico* methods; however, aged tonghaosu was found to be more reactive when compared to freshly isolated tonghaosu. The polyacetylene (**1**) constituent was determined to be chemically unstable, generating a small electrophilic spirolactone, 1,6-dioxaspiro[4.4]non-3-en-2-one (**4**), upon aging. This small lactone (**4**) was strongly reactive in both *in chemico* HTS- and NMR-DCYA methods and further confirmed as a potential skin sensitizer by Local Lymph Node Assay (LLNA).

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1. Introduction

The Center for Food Safety & Applied Nutrition (CFSAN) at the FDA is aware that a relatively high number of regulated products include, among their ingredients, chamomile plant extracts. As an example, many cosmetic products use these plant extracts as fragrance ingredients and skin conditioning agents. German chamomile (*Matricaria chamomilla* syn. *Matricaria recutita*) and Roman chamomile (*Chamaemelum nobile*) are the two most common chamomile species used in cosmetic and personal care formulations.

Despite its reputation as a medicinal plant, there are numerous reports of allergic reactions in people who topically applied products containing chamomile extracts (Ketel, 1987, 1982; West and Maibach, 1995; Foti et al., 2000; Rudzki et al., 2003). Both German and Roman chamomile have been mentioned in these, and other, reports of adverse effects but there is some uncertainty concerning the allergens involved. The dermal sensitization properties of chamomile have been reported to be associated with sesquiterpene lactones such as matricarin and anthecotulide (Ketel, 1987; Lundh et al., 2006). Another contributing factor may be the cross-reactivity that patients with allergies to plants in the Asteraceae/Compositae/Aster family (*e.g.*, asters, mugwort, chrysanthemums, sunflower) or ragweed pollens may have to chamomile extracts. Finally, impurities (adulterants) in chamomile products are common and may also be the cause of the adverse effects (Brandão et al., 1998).

We previously reported the development and validation of an analytical method using Ultra-High Performance Liquid Chromatography coupled to UV detector and Mass Spectrometer (UHPLC-UV–MS) to quickly produce chemical fingerprints of phenolic compounds in German and Roman chamomile (Avula et al., 2014). This effort in differentiation between the two mentioned species using chemical fingerprinting was followed up with testing for skin sensitizing potential of the corresponding constituents, and the results are reported in this publication.

Testing for skin sensitizing potential involved the KeratinoSens™ assay, fluorescence trap method (HTS-DCYA), NMR-based method (NMR-DCYA) and the Local Lymph Node Assay (LLNA). The KeratinoSens™ assay is a cell-based reporter gene assay, which identifies skin sensitizers by measuring the induction of luciferase under the control of the antioxidant response element (ARE) in a human keratinocyte cell line (HaCaT). The reporter construct allows for the

Abbreviations: ACN, Acetonitrile; DBN, 1,5-Diazabicyclo[4.3.0]non-5-ene; DCYA, Dansyl CYsteamine; DNE, 1,6-Dioxaspiro[4.4]non-3-en-2-one; DMSO, Dimethyl sulfoxide; DPRA, Direct Peptide Reactivity Assay; ESI, Electro spray ionization; HTS, High throughput screening; LLNA, Local Lymph Node Assay; NMR, Nuclear Magnetic Resonance; OECD, Organisation for Economic Co-operation and Development; RI, Reactivity Index; SI, Stimulation index; THF, Tetrahydrofuran.

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detection of activation of the Keap1-Nrf2 signaling pathway, which has previously been shown to be activated during skin sensitization events (Natsch et al., 2011). Both the HTS-DCYA and NMR-DCYA methods are *in chemico* methods recently reported to assess the ability of potential skin sensitizers to covalently bind to a model dansyl thiol (DCYA) (PCT application filed, PCT/US15/38,142). The model thiol serves as a surrogate of skin proteins to investigate the potential of the candidate sensitizer to elicit early haptenation events. The HTS-DCYA method enables the rapid and sensitive detection of electrophilic compounds in a high throughput manner using fluorescence assays in 96-well microplates (Avonto et al., 2015). The fluorescent thiol is incubated with the candidate sensitizer and activated to promote a covalent binding. The unreacted thiol is then selectively scavenged and the fluorescence response of the resulting solution (DCYA-adducts) is quantified using end-point readings. In the NMR-DCYA assay, the degree of electrophile depletion over time is quantified using ^{1}H Nuclear Magnetic Resonance (NMR) techniques (Chittiboyina et al., 2015). This method provides a major advantage compared to other state-of-the art assays in that the quantification is performed by assessing the depletion of the sensitizer (in contrast to currently validated methods which are based on the depletion of the nucleophile and to avoid false positives). In addition to depletion of electrophile, the NMR-DCYA method can provide structural information for the elucidation of reaction mechanisms and to unambiguously identify the site of reaction in the presence of multiple or ambiguous mechanistic domains. Finally, the LLNA was developed in mice and validated as an alternative to guinea pig tests conducted for the causal identification of skin sensitizing chemicals (Basketter et al., 1996). This assay is also informative in regards to the potency of lymphocyte proliferation induced in the draining lymph nodes of mice by the testing article and it has therefore been used for hazard characterization (Kimber et al., 2003).

As a result of this work, a major constituent of German chamomile extracts which may be a causative agent responsible for skin sensitization has been identified. This may have potential implications for manufacturers using German chamomile in their commercial preparations.

2. Materials and methods

2.1. Chemicals and materials

Gravity column chromatography purifications were performed using silica gel (40–63 μm, 60 Å, Sorbtech™), reversed-phase RP-C18 silica (Polarbond, J.T. Baker) or Sephadex LH-20 (Sigma-Aldrich). SNAP cartridges or Flash + HPFC cartridges were used for flash chromatography using a Biotage Isolera Four system (Uppsala, Sweden). Silica gel cartridges were KP-Sil (40–63 μm, 60 Å) or KP-C18-HS (35–70 μm, 90 Å), 18% carbon load (by weight). Standardized buffer solution pH 10 ± 0.02 (cat. # SB116-500), microcentrifuge tubes and polypropylene solvent-resistant 96-well microplates were purchased from Fisher Scientific (Suwanee, GA, USA). Polymer-supported maleimide (SiliaBond®, maleimide load ≥0.64 mmol/g) was purchased from SiliCycle (Quebec City, Quebec, Canada). The fluorescent compounds DCYA and DCYA disulfide were synthesized as described previously (Chittiboyina et al., 2015). Dansyl chloride, cystamine hydrochloride, 1,5-diazabicyclo[4.3.0]non-5-ene (DBN), rose bengal, 2,5-dimethylfuran, lithium aluminum hydride, ethyl 3-(furan-2-yl)propanoate and cinnamaldehyde were purchased from Sigma-Aldrich. HPLC grade acetonitrile was used for the HTS-DCYA assay; certified ACS grade was used for purification purposes (all solvents were purchased from Fisher Scientific (Suwanee, GA, USA). Chloroform-*d* was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA).

2.2. Plant material

Matricaria chamomilla flower heads (voucher n. 3670 and 9365) were supplied by Frontier Natural Products Co-op. (Norway, IA, USA). *Chamaemelum nobile* flower heads (voucher n. 9254) were obtained from the University of Mississippi Medicinal Plant Garden. Both plant materials were authenticated by Dr. Vijayasankar Raman, and sample specimens are available at the National Center for Natural Product Research, University of Mississippi.

2.3. In vitro assessment of skin sensitization potential

The *in vitro* KeratinoSens™ assays were conducted according to the method previously described (Natsch and Emter, 2008) and recently adopted as Organisation for Economic Co-operation and Development (OECD) Guideline 442D (OECD, 2015). Whole extracts as well as chemically fractionated samples of whole extracts were sent blind-coded for testing. Given the test articles were mixtures, hence no defined molecular weight, they were diluted on a μg/mL (w/v) basis rather than based upon molarity. An initial solubility test was performed for the test articles at the highest 100× concentration (40,000 μg/mL) to confirm that they were soluble in DMSO. The final 1× tested concentrations were 400, 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.781, 0.391 and 0.195 μg/mL. Additionally, the cytotoxicity of a test article was determined using cell viability endpoints with Neutral Red Uptake (NRU) and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) vital dyes. The maximal induction (I_{max}), the concentration for maximal gene induction (CI_{max}), the EC1.5 value (the concentration achieving statistically significant induction of luciferase activity above the solvent control) and the IC_{50} (the concentration leading to a 50% reduction in cell viability) were determined for each test article. For each assay, the KeratinoSens™ cells were cultured in quintuplicate plates for 24 h, treated with the test article for 48 h, and assessed for luciferase induction (3 plates) and cytotoxicity (2 plates). Cinnamaldehyde (CAS no. 14371-10-9) was used as the positive control.

2.4. Spectroscopic characterization of isolated chamomile compounds

NMR spectra were recorded using an Agilent 500 MHz and a 400 MHz spectrometer equipped with a Vnmr J4 software. Chemical shifts are reported in ppm relative to the ^{1}H residual signal of the $CDCl_3$ solvent peak (^{1}H 7.26 ppm, ^{13}C 77.16). Structural characterizations were performed by 1- and 2-dimensional NMR experiments. Homonuclear ^{1}H correlations were established by 2D-COSY experiments. Gradient-HMQC experiments were used to determine one-bond heteronuclear ^{1}H–^{13}C connectivities. Two- and three-bond ^{1}H–^{13}C correlations were determined by gradient-HMBC experiments, in which the evolution period for long-range ^{1}H–^{13}C coupling constants was optimized for a $^{2,3}J_{C,H}$ of 8 Hz.

2.5. Extraction and isolation of tonghaosu

Flower heads of *M. chamomilla* (840 g) were air-dried and ground to a fine powder. Methanol (3 L) was added and the extraction was sonicated (30 min, four times). The precipitate was filtered and the solvent was evaporated under vacuum at temperatures below 40 °C. The crude extract thus obtained (171.0 g) was then suspended in water (500 mL)

Table 1
KeratinoSens™ results for German and Roman chamomiles.

Extract/test article	EC 1.5 value (μg/mL)	Mean IC_{50} (μg/mL)	
		MTT	NRU
German chamomile, ethanol extract	21.23	>400	>400
German chamomile, hexane fraction	0.67	168	160
Roman chamomile, ethanol extract	0.72	83.5	80.7
Roman chamomile, hexane fraction	2.66	88.5	81.4
German chamomile ethanol sub-fraction	0.471	>400	>400
Cinnamaldehyde, positive control	10.26 μM	>64 μM	>64 μM

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