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Safety assessment of mushrooms in dietary supplements by combining analytical data with *in silico* toxicology evaluation

Karen M. VanderMolen [a, b, *], Jason G. Little [a, c], Vincent P. Sica [d, e], Tamam El-Elimat [d, f], Huzefa A. Raja [d], Nicholas H. Oberlies [d], Timothy R. Baker [e], Catherine Mahony [g]

[a] Product Safety and Regulatory Affairs, New Chapter, Inc., 90 Technology Dr, Brattleboro, VT 05301, United States
[b] Central Product Safety, The Procter and Gamble Company, Winton Hill Business Center, 6100 Center Hill Ave, Cincinnati, OH 45232, United States
[c] Personal Health Care, The Procter and Gamble Company, Mason Business Center, 8700 Mason-Montogomery Rd, Mason, OH 45040, United States
[d] Department of Chemistry and Biochemistry, University of North Carolina at Greensboro, Greensboro NC 27402, United States
[e] Research and Development, Corporate Functions Analytical, The Procter & Gamble Company, Mason Business Center, 8700 Mason-Montogomery Rd, Mason, OH 45040, United States
[f] Department of Medicinal Chemistry and Pharmacognosy, Faculty of Pharmacy, Jordan University of Science and Technology, Irbid 22110, Jordan
[g] Central Product Safety, Procter and Gamble Company Technical Centres Ltd, Egham, Surrey TW20 9NW, United Kingdom

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ABSTRACT

Despite growing popularity in dietary supplements, many medicinal mushrooms have not been evaluated for their safe human consumption using modern techniques. The multifaceted approach described here relies on five key principles to evaluate the safety of non-culinary fungi for human use: (1) identification by sequencing the nuclear ribosomal internal transcribed spacer (ITS) region (commonly referred to as ITS barcoding), (2) screening an extract of each fungal raw material against a database of known fungal metabolites, (3) comparison of these extracts to those prepared from grocery store-bought culinary mushrooms using UHPLCPDA-ELS-HRMS, (4) review of the toxicological and chemical literature for each fungus, and (5) evaluation of data establishing presence in-market. This weight-of-evidence approach was used to evaluate seven fungal raw materials and determine safe human use for each. Such an approach may provide an effective alternative to conventional toxicological animal studies (or more efficiently identifies when studies are necessary) for the safety assessment of fungal dietary ingredients.

1. Introduction

As a source of diverse secondary metabolites, fungi have enjoyed a long history of use in both culinary and medicinal applications (Das et al., 2010; Kalaivani et al., 2010; Paterson, 2006; Rios, 2011; Xu, 2012). While some of the chemically interesting and/or biologically active constituents are shared across all fungi (e.g, β-glucans (Dalonso et al., 2015)), others are exclusive to specific species or genera (Patakova, 2013; Xu, 2012), and some constituents may only be produced under distinct growing conditions (Wang et al., 2014). In general, many medicinal mushrooms have not been evaluated for their safe human use using modern analytical approaches. While history of use data should be carefully considered, and can provide a foundation for establishing safe use, some toxicological endpoints may be more opaque. Obtaining safety data for developmental and reproductive toxicity (DART), genotoxicity, and chronic endpoints can prove particularly difficult. Complicating the evaluation of such fungi, modern cultivation practices and preparations are rarely consistent with traditional medicinal uses. While fruiting bodies are most often the portion of the organism used in traditional Chinese medicine (TCM) (Bensky et al., 2004), commercial raw materials typically consist of the fungi's mycelium, which grows more quickly and is therefore less expensive to produce. In addition, the growing conditions can impact the secondary metabolite profile of fungi (Bills et al., 2008; Bode et al., 2002; Fiedurek et al., 1996; Miao et al., 2006; Mohanty

* Corresponding author. Central Product Safety, The Procter and Gamble Company, Winton Hill Business Center, 6100 Center Hill Ave, Cincinnati, OH 45232, United States.
E-mail addresses: vandermolen.k@pg.com (K.M. VanderMolen), little.j.6@pg.com (J.G. Little), sica.vp@pg.com (V.P. Sica), telimat@just.edu.jo (T. El-Elimat), haraja@uncg.edu (H.A. Raja), nicholas_oberlies@uncg.edu (N.H. Oberlies), baker.tr@pg.com (T.R. Baker), mahony.c@pg.com (C. Mahony).

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and Prakash, 2009; VanderMolen et al., 2013), which impacts profoundly the ability of investigators to bridge data generated from different raw materials or different suppliers, even of the same species.

Given these challenges, a multifaceted approach was developed to establish safe human use of fungi as dietary ingredients. The approach described here relies on five key principles to evaluate the safety of non-culinary fungi for human consumption. (1) A critical starting point in the safety review of fungal raw material is confirmation of identity. There exist numerous challenges associated with macro- and microscopic identification of fungi, not least of which is the subjectivity of the analysis. Morphological differences due to sexual and asexual states of the same species confuses the conventional taxonomical nomenclature, causing multiple names to be assigned to genetically identical species of fungi (termed 'pleomorphy') (Sugiyama, 1987). With advances in the speed and cost of genetic analysis, the issue of fungal identity can now be more adequately addressed by DNA identification (Raja et al., 2017). All fungal raw materials evaluated in this study were, as a first step, identified by sequencing the nuclear ribosomal internal transcribed spacer (ITS) region (commonly referred to as ITS barcoding) (Raja et al., 2017; Schoch et al., 2012). (2) The next step was to screen an extract of each fungal raw material against a database of known fungal metabolites, as a way to quickly identify known toxins. (3) This was followed by a comparison of these extracts to those prepared from store-bought culinary mushrooms. Mushrooms that are widely consumed and well established as food in broad and diverse populations can provide a relatively innocuous baseline with which to compare common constituents, and exclude shared components that should not raise a safety concern. (4) Available toxicological and chemical literature for each fungus was reviewed for available safety data. (5) Marketplace data were used to establish presence in the market.

This weight-of-evidence approach was used to evaluate seven fungal raw materials and determine safe human use for each; two of the raw materials are used as in-detail examples, while the results of the remaining raw materials are summarized. We believe that this comprehensive approach will provide an effective alternative to conventional toxicological animal studies (or identify when studies are necessary) for the safety assessment of fungal dietary ingredients.

2. Methods

2.1. Approach to safety evaluation

The assessment of each fungal raw material's safe use as a dietary ingredient was performed using a weight-of-evidence approach, with the following criteria: (1) DNA identification, (2) screening fungal raw material extracts against a database of known fungal metabolites (dereplication), (3) similarity to grocery store (culinary) mushrooms as determined by UHPLC-PDA-ELS-HRMS (ultrahigh–performance liquid chromatography–photodiode array–evaporative light scattering–high resolution mass spectrometry), (4) expert opinion and literature review, and (5) marketplace data. Detailed descriptions of each of these criteria are outlined below. Based on the strength and totality of evidence, conclusions were drawn as to the supportable use of the raw material as a dietary ingredient. A decision tree outlines the general approach used to evaluate the available evidence (Fig. 1).

2.2. Materials

Seven fungal raw materials were purchased from a commercial supplier, and were identified by the supplier as Chaga (*Inonotus obliquus*), Wild-Crafted Chaga (*Inonotus obliquus*), Turkey Tail (*Trametes versicolor*), Shiitake (*Lentinula edodes*), Maitake (*Grifola frondosa*), Reishi (*Ganoderma lucidum*) fruiting bodies and mycelium, and Cordyceps (*Cordyceps sinensis*). The Chaga, Turkey Tail, Shiitake, Maitake, and Cordyceps were grown on brown rice and consisted primarily of mycelium. The Wild-Crafted Chaga was harvested from its natural habitat by the supplier. The Reishi was composed 80:20 of two subcomponents: 80% was grown on brown rice and consisted primarily of mycelium, and 20% was fruiting bodies grown on sawdust (no sawdust is incorporated into the raw material). These subcomponents were obtained as separate materials, allowing us to analyze them individually. The supplier also provided a sample of the brown rice that was used to grow the fungi. A selection of dried culinary mushrooms (including Shiitake, Black Trumpet, Chanterelle, Crimini, and Porcini) were purchased from a local grocery store to use during the comparison of the fungal raw materials to store-bought mushrooms.

2.3. DNA identification of fungi samples

Methods for the identification of these fungal samples (including DNA extraction, PCR amplification, and Sanger sequencing of dry powdered mycelium and/or fruiting body) have been described in detail previously (Raja et al., 2017).

2.4. Extractions

All samples were extracted by adding 60 mL of 1:1 methanol-chloroform (MeOH–$CHCl_3$) to a 15 g aliquot of each powdered material and shaking overnight (~16 h) at 100 rpm at RT. The sample was filtered using vacuum, and the remaining residues were washed with 10 mL of 1:1 MeOH–$CHCl_3$. To the filtrate, 90 mL of $CHCl_3$ and 150 mL of H_2O were added; the mixture was stirred for 1 h and then transferred into a separatory funnel. The bottom (organic) and upper (aqueous) layers were drawn off and evaporated to dryness. The dried organic extract was re-constituted in 50 mL of 1:1 MeOH–CH_3CN and 50 mL of hexanes. The biphasic solution was stirred for 0.5 h and then transferred to a separatory funnel. The MeOH–CH_3CN layer was drawn off and evaporated to dryness under vacuum (see Fig. 2).

The extraction scheme was developed based on previous experience of the authors and consideration of the relevant literature, especially an exhaustive study by researchers with the National Cancer Institute (McCloud, 2010). Essentially, the polarity of 1:1 MeOH–$CHCl_3$ is such that most 'organic' soluble molecules are extracted efficiently.

2.5. UHPLC–PDA–ELS–HRMS analysis

Analysis was performed using an ultrahigh–performance liquid chromatography–photodiode array–evaporative light scattering–high resolution mass spectrometry (UHPLC–PDA–ELS–HRMS) method. A splitter was incorporated post–PDA to allow for simultaneous analysis by both the ELS detector (ELSD) and mass spectrometer. HRMS was performed on a Thermo LTQ Orbitrap XL mass spectrometer (ThermoFisher, San Jose, CA) equipped with an electrospray ionization (ESI) source. The source conditions were set to aquire (m/z 100–2000) positive-ionization mode with a capillary temperature of 300 °C, a source voltage of 4.0 kV, a capillary voltage of 20 V, and the tube lens set to 100 V. Dereplicated compounds were confirmed by performing tandem mass spectrometry (MS/MS) with a collision induced dissociation set to 30%. UHPLC was carried out on a Waters Acquity system [using a BEH C18 (2.1 × 50 mm, 1.7 μm) column (Waters Corp., Massachusetts, USA) equilibrated at 40 °C]. Negative-ionization mode was