Fungal profiles in various milk thistle botanicals from US retail

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A B S T R A C T

Milk thistle (MT) dietary supplements are widely consumed due to their possible beneficial effect on liver health. As botanicals, they can be contaminated with a variety of fungi and their secondary metabolites, mycotoxins. This study was conducted in an effort to determine the mycological quality of various MT botanical supplements from the US market. Conventional plating methods were used for the isolation and enumeration of fungi, while conventional microscopy as well as molecular methods were employed for the speciation of the isolated strains. Results showed that a high percentage of the MT samples tested were contaminated with fungi. Total counts ranged between <2.00 and 5.60 log10 colony forming units per gram (cfu/g). MT whole seeds carried the highest fungal levels followed by MT cut herb. No live fungi were recovered from MT seed tea bags, liquid extracts, capsules or soft gels. Potentially toxigenic molds from the Aspergillus sections Flavi and Nigri as well as Eurotium, Penicillium, Fusarium and Alternaria species were isolated from MT supplements. The predominant molds were Eurotium [E. repens, E. amstelodami and E. rubram], A. flavus, A. tubingenensis, A. Niger and A. candidus. To our knowledge, this is the first study reporting on fungal contamination profiles of MT botanicals.

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1. Introduction

Various milk thistle (MT) derivatives have been used as health-promoting remedies for centuries; these products were consumed as far back as ancient Greece and China. The edible part of thistle was named Silybum by the ancient Greek physician, Dioscorides. In recent years, the use of MT botanical supplements by consumers in the Western Societies has increased markedly due to a trend for use of natural therapies. In the US the marketing and consumption of various dietary supplements including milk thistle derivatives have greatly increased after the enactment of the Dietary Supplement Health and Education Act (DSHEA) (USFDA, 1994). The main health-promoting property of MT supplements is the support of liver health due to their high content of silymarin (a complex of flavonolignans and polyphenols containing silibin, isosilibin, silichristin and silidianin) (Morazzoni and Bombardelli, 1995; Saller et al., 2001). Other potentially-beneficial attributes of MT botanicals include anti-hypercholesterolemic and anti-oxidant properties, and chemoprotective effects against lung and prostate cancers (Wellington and Jarvis, 2001; Skottova and Krecman, 1998; Singh et al., 2006, 2008).

Contamination of various botanical supplements with potentially toxigenic fungi has been occasionally reported in the literature. Truckssess and Scott (2008) mentioned the presence of penicillia, Aspergillus flavus and A. parasiticus in botanicals, while Rizzo et al. (2004) documented the occurrence of Fusarium, A. flavus and A. parasiticus in Argentinean medicinal plants. Sato et al. (1992) recovered several molds including Alternaria alternata and Fusarium spp. from medicinal plants, while Raman et al. (2004) reported that several botanical supplements from the US market contained molds and bacteria. Mold secondary metabolites, mycotoxins (e.g. aflatoxins, fumonisins, ochratoxin A, etc.) have also been reported in past studies. Aflatoxins were found in traditional Chinese herbal medicines, medicinal plants and in kava kava (Yang et al., 2005; Selim et al., 1996; Weaver and Truckssess, 2010). Fumonisins have been detected in black tea and in medicinal plants (Martins et al., 2001; Omurtag and Yazicioglu, 2004), while the presence of ochratoxin A in medicinal plant materials from Tilia grandifolia was reported by Halt (1998).

A study on the occurrence of aflatoxins (AFs) in MT supplements revealed the presence of these toxins at low levels (0.04–2.00 ng/g) in MT seeds and oil-based liquid seed extracts (Tournas et al., 2012). Further studies by the same investigators showed that certain A. flavus strains could produce high levels of AFs in artificially-infected MT seed powder and lesser amounts in MT herb powders. The mycological quality of MT supplements, however, has not been investigated. Preliminary tests had shown that about 25% of the MT samples tested contained potential aflatoxin-producing Aspergillus section Flavi isolates (sometimes at levels exceeding 4.50 log10 cfu/g). Other toxin producing molds with similar growth requirements (environmental and nutritional) could also be present in these commodities. Therefore, we conducted this study to investigate the presence and levels of potentially-toxigenic molds in MT botanical supplements.
2. Materials and methods

2.1. MT sample preparation

Organic MT supplements (whole and powdered seed, 113.5 or 454 g/sample; cut and powdered herb, 113.5 or 454 g/sample; ground seed in tea bags, 30 g/sample; oil-based liquid seed extracts, 30 ml/sample; alcohol-based seed extracts, 120 ml/sample; capsules, 100 units/sample; and soft gels, 60 units/sample) were purchased from commercial sources. Samples were mixed well in their sealed containers before the portions for analysis were taken out as follows: liquid samples were mixed in a vortex mixer for 1 min; seed and herb powders, whole seeds and minced herb were manually shaken 10 times in a 180° angle.

2.2. Chemicals, reagents and other supplies

PCR reagents and DNA ladder were purchased from Fisher Scientific (Houston, TX, USA); DNA extraction kits were obtained from Norgen Biotek Corp. (Thorold, ON, Canada); and primers were purchased from Integrated DNA Technologies (IDT) (Coraville, IA, USA). Agarose gels were obtained from Bio-Rad (Hercules, CA, USA), PD broth, PBS and all the mycological media utilized for the isolation and conventional plating identification of fungal specimens were prepared in-house according to procedures and formulas described in the Bacteriological Analytical Manual (BAM online, 2001) and in Fungi and Food Spoilage (Pitt and Hocking, 2009).

2.3. Fungal isolation and enumeration

Isolation and enumeration of viable fungi were accomplished following the method described in BAM online (2001) as follows: twenty-five gram-portions from each MT seed and herb sample were aseptically transferred into sterile blender jars and 225 ml of 0.1% peptone water was added to each jar. Ten-gram portions from each MT extract, capsule, soft gel and tea bag sample (+ 90 ml 0.1% peptone) were analyzed. The samples were blended for 45 s to achieve complete homogenization. Serial dilutions were prepared by adding 1 ml of homogenate to 9 ml of 0.1% peptone water. Subsequently, 0.1 ml portions were inoculated with sterile bent glass rods and the plates were incubated upright at 30 °C for 24 h. After the incubation period, samples were centrifuged for 10 min at 10,000 rpm to pellet and the supernatants were discarded. Subsequently, 10 ml of PBS buffer were added to each tube and the pellets were homogenized using a vortex. The tubes were centrifuged again under the same as above conditions and the supernatants were discarded. Then, 1 ml of PBS buffer was added to each sample and the tubes were vortexed. Subsequently, 50 μl were removed from each tube and transferred to 2.0-ml microcentrifuge tubes to proceed with DNA extraction. DNA extraction was facilitated using the Norgen Biotek Fungi/Yeast Genomic DNA Isolation Kit according to manufacturer’s instructions.

2.5.2. PCR

The primers encoding the β-tubulin gene, Bt2a (5′-GGTAACCAATCAG GTGCCTTTCT-3′) and Bt2b (5′-ACCATGATGTACGACCTTTC-3′) (Glass and Donaldson, 1995) were used and a slight modification of the method described by Asefa et al. (2009) was utilized as follows: PCR was performed in reaction tubes with a final volume of 50 μl consisting of 25 μl Promega GoTaq Hot Start colorless master mix, 21.5 μl Promega nuclease-free water, 1.5 μl DNA template, and 1 μl of each primer. The mixture was spun and the PCR reaction was run in an Eppendorf 2231 thermocycler (Eppendorf North America, Hauppauge, NY, USA) programmed with the following conditions: denaturation at 95 °C for 10 min, 38 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 7 min.

2.5.3. Gel electrophoresis

The PCR products were subjected to electrophoresis in 1% agarose gels (Bio-Rad Ready Agarose ethidium bromide precast gels) to determine the band sizes and to confirm amplification of the DNA. Gels were run using Tris–borate–EDTA (TBE) buffer, at 60 V for 70 min and subsequently, were visualized and photographed under UV light in an Alpha Imager (Alpha Innotech Corp., Santa Clara, CA, USA).

2.5.4. Sequencing and species identification

Purification and sequencing of the PCR products were performed by MCLAB (South San Francisco, CA, USA) and the resulting sequences were trimmed and edited using FinchTV 1.4.0 software. Subsequently, sequence comparisons and speciation were done by utilizing the basic local alignment search tool (BLAST) in Genbank (www.ncbi.nlm.nih.gov/BLAST).

2.3. Yeast and mold (YM) levels

A total of 223 fungal strains (214 molds and 9 yeasts) were isolated from the MT supplements tested in this study. Sixty percent of the MT samples tested here were contaminated with fungi. Eighty-eight percent of whole seed, 100% of seed powder, 57% of cut herb and 100% of herb powder samples had YM levels higher than 2.00 log10 cfu/g, the limit recommended by the US Pharmacopeia (USP, 2011). The highest levels were observed in whole seed samples (up to 5.60 log10 cfu/g) followed by cut herb (up to 4.59 log10 cfu/g). The powdered samples had somewhat lower YM counts reaching as high as 4.34 and 4.36 log10 cfu/g for seed and herb powders, respectively. All MT herb powder samples, however, carried higher than 4.00 log10 cfu/g. No molds or yeasts were recovered from MT tea bags, liquid seed extracts, capsules or soft gels (Table 1).

3.2. Potentially-toxigenic molds

Potential-toxigenic molds from the Aspergillus sections Flavi and Nigri as well as Eurotium, Penicillium, Fusarium and Alternaria spp. were isolated from MT supplements. The predominant molds were

Species

Aspergillus

Fusarium

Penicillium

Alternaria

Eurotium

Species

Species

Species

Species

Species
Table 2
Fungal species and levels recovered from various MTa botanical supplements.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Contamination levels (log10 cfu/g-range) in MT supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole seeds (n = 34)a</td>
</tr>
<tr>
<td>Alternaria spp.</td>
<td>2.30–4.30 (15)</td>
</tr>
<tr>
<td>Aspergillus spp.</td>
<td>2.00–2.30 (6)</td>
</tr>
<tr>
<td>Aspergillus awamori</td>
<td>2.0 (1)</td>
</tr>
<tr>
<td>Aspergillus candidus</td>
<td>2.74–4.96 (15)</td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>2.00–5.51 (29)</td>
</tr>
<tr>
<td>Aspergillus foetidus</td>
<td>2.00–5.23 (6)</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>2.00–4.54 (12)</td>
</tr>
<tr>
<td>Aspergillus oryaeus</td>
<td>ND</td>
</tr>
<tr>
<td>Aspergillus paraisotus</td>
<td>ND</td>
</tr>
<tr>
<td>Aspergillus penicillioides</td>
<td>2.40–5.04 (15)</td>
</tr>
<tr>
<td>Aspergillus sydowii</td>
<td>3.08 (3)</td>
</tr>
<tr>
<td>Aspergillus tamari</td>
<td>3.30–3.72 (6)</td>
</tr>
<tr>
<td>Aspergillus tritici</td>
<td>4.11–4.41 (6)</td>
</tr>
<tr>
<td>Aspergillus tubingenesis</td>
<td>2.65 (3)</td>
</tr>
<tr>
<td>Aspergillus versicolor</td>
<td>2.30–5.08 (15)</td>
</tr>
<tr>
<td>Cladosporium spp.</td>
<td>ND</td>
</tr>
<tr>
<td>Eurotium spp.</td>
<td>3.00–4.00 (9)</td>
</tr>
<tr>
<td>Eurotium amstelodami</td>
<td>2.00–5.08 (12)</td>
</tr>
<tr>
<td>Eurotium chevalieri</td>
<td>ND</td>
</tr>
<tr>
<td>Eurotium repens</td>
<td>2.30–5.40 (29)</td>
</tr>
<tr>
<td>Eurotium rubrum</td>
<td>2.30–4.63 (26)</td>
</tr>
<tr>
<td>Fusarium spp.</td>
<td>2.30–4.32 (18)</td>
</tr>
<tr>
<td>Fusarium proliferatum</td>
<td>2.95–4.48 (6)</td>
</tr>
<tr>
<td>Fusarium subglutinans</td>
<td>ND</td>
</tr>
<tr>
<td>Fusarium verticillioides</td>
<td>2.00 (3)</td>
</tr>
<tr>
<td>Penicillium spp.</td>
<td>2.48–3.00 (6)</td>
</tr>
<tr>
<td>Penicillium brevicompactum</td>
<td>ND</td>
</tr>
<tr>
<td>Penicillium chrysogenum</td>
<td>2.00–4.95 (21)</td>
</tr>
<tr>
<td>Penicillium dierckxii</td>
<td>ND</td>
</tr>
<tr>
<td>Penicillium polonicum</td>
<td>2.00 (3)</td>
</tr>
<tr>
<td>Rhizopus spp.</td>
<td>2.00–3.78 (15)</td>
</tr>
<tr>
<td>Yeasts</td>
<td>2.65–4.38 (6)</td>
</tr>
<tr>
<td>No growth</td>
<td>(12)</td>
</tr>
</tbody>
</table>

Aspergilli and eurotia were identified using PCR; all other species were identified by conventional plating methods.

a MT = milk thistle.
b cfu = colony forming units.
c n = number of samples tested.
d Numbers in parentheses indicate % samples contaminated with respective organisms.
e ND = none detected.
whole seeds. This organism also has the potential of producing the mutagenic toxins, sterigmatocystin, and versicolorin (Engelhart et al., 2002; Frisvad et al., 2006; Jakišić et al., 2012) and its presence in food and feed commodities should be monitored and controlled. Black aspergilli (A. niger, A. foetidus and A. tubingensis) were isolated from both MT seed and herb samples. The highest levels of A. niger (4.54 log10 cfu/g) and A. foetidus (5.23 log10 cfu/g) were found in whole seeds, whereas the highest concentration of A. tubingensis (4.38 log10 cfu/g) was encountered in cut herb. Some black aspergilli have the ability to produce the mycotoxin, ochratoxin A, which was linked to kidney disease in humans (Perrone et al., 2006). Therefore, their presence at high levels in consumer products is a cause of concern.

Some eutoria (i.e. E. amstelodami, E. rubrum and E. repens) were found in high concentrations in MT seeds. The most abundant was E. rubrum found in 52% of the MT herb and 27% of the seed samples at levels ranging between 2.00 and 4.63 log10 cfu/g. E. repens was also quite common, found in 27% of the seed and in 38% of the herb samples. One whole seed had E. repens counts of 5.40 log10 cfu/g. E. amstelodami was encountered in 19% of the seed and 14% of the herb samples. The highest E. amstelodami counts (5.08 log10 cfu/g) were observed in a whole seed sample. This organism was found to exert toxic effects on chicken embryos (Frisvad and Samson, 1991).

Most of the Penicillia recovered during the course of this study were encountered at low levels and they were probably random contaminants. Two whole seed samples, however, had very high levels (above 4.25 log10 cfu/g) of P. chrysogenum. This organism has the ability of producing antibiotics and some isolates could also elaborate the mycotoxin, citrinin (Devi et al., 2009).

Fusarium spp. were isolated from a few MT seed and herb samples at relatively low levels, but one whole seed sample had 4.48 log10 cfu/g of F. proliferatum. This level is quite high and considering the fact that F. proliferatum has the potential of producing mycotoxins such as fumonisins (Desjardins et al., 2007), its presence in MT supplements is a cause of concern. Alternaria species were recovered only from 15% of the MT whole seed and 14% of the seed powder samples, but two whole seed samples contained high numbers of this organism (≥4.00 log10 cfu/g). Such levels are troublesome because some Alternaria species could produce a variety of mycotoxins (e.g. alternariol, alternariol methyl ether, altenuene, tenuazonic acid, etc.) which cause a wide spectrum of adverse effects on humans and animals (Tournas and Stack, 2001).

The counts of potentially-toxicogenic molds such as A. flavus, A. versicolor, A. candidus and Fusarium spp. recovered from some samples were above 4.0 log10 cfu/g; this fact suggests that the organisms were growing on the product at some stage of production or marketing. Although there are no reports on the mycological quality of MT supplements, Aspergillus, Penicillium and Fusarium species were previously isolated from dried chamomile (herb and flowers), a member of the same family as milk thistle and perhaps of similar biochemical composition as the MT supplements analyzed in this study (Tournas and Katsoudas, 2008). A. flavus, A. parasiticus, A. niger, F. oxysporum and various Penicillia were also isolated from chamomile and other medicinal plants by Aziz et al. (1998). The mold incidence reported in this study was high, sometimes occurring in 100% of the samples tested.

Most of the molds recovered during the course of our study thrive under reduced water activity (a_w) conditions. Since the predominant species were xerophilic, it appears that growth took place when the products were incompletely dried or came in contact with and acquired some moisture after complete drying, during storage. Therefore, in order to avoid fungal growth and possible mycotoxin production, care should be taken to quickly dry the MT botanicals after harvest and store them under such conditions that will prevent increase of a_w. Since some of the contaminants could have been added to the products during and after harvest (during cleaning, washing, drying, transport, packing, etc.), adherence to good manufacturing practices (GMPs) at all postharvest stages of production would contribute to minimizing microbial contamination and substantially improving product quality.

3.3. Yeasts

Overall, yeasts were recovered from 10% of the tested samples. Yeast counts reached as high as 4.57 log10 cfu/g. Relatively high levels of viable yeasts (≥3.0 log10 cfu/g) were recovered from 14% of herb powder, cut herb and seed powder samples, and from 3% of the whole seed samples. Some of the yeasts isolated in the present study probably originated from the personnel handling the commodity during harvesting, processing and packaging and could indicate lack of strict adherence to GMPs; such yeast species could be opportunistic human pathogens and should be eliminated from the final product (Hazen, 1995). Other species are part of the natural plant microbiota, residing in various plant parts (during plant growth) where they can attach and acquire nutrients from the plant without causing disease.

4. Conclusions

A high percentage of the tested MT samples contained potentially-toxicogenic molds (i.e. A. flavus, A. parasiticus, A. niger, A. versicolor and various penicillia and fusaria) at levels above 3.0 log10 cfu/g. Most of the isolated species grew well at low a_w; this perhaps indicates that mold growth took place after harvest when the products were partially dried but they still retained enough moisture to support growth of xerophilic species. Therefore, these botanicals should be cleaned and dried quickly after harvest and packaged and stored under such conditions that would not allow increase of a_w above 0.65 in order to prevent mold growth and mycotoxin production.

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References


