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Removal of Furanocoumarins in Grapefruit Juice by Edible Fungi

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Furanocoumarins (FCs) in the human diet irreversibly inhibit human cytochrome P450 3A4 (CYP 3A4) and are responsible for the "grapefruit/drug" interaction phenomenon. Previously, we reported that FCs in grapefruit juice (GFJ) bind to autoclaved *Aspergillus niger*, and this binding reduced the GFJ inhibition of CYP 3A4. However, *A. niger* is not an edible fungus, and thus, potentially similar binding by edible fungi was also characterized. In this study, autoclaved *Morchella esculenta*, an edible ascomycete, removed much of the FC content in GFJ, resulting in decreased inhibition of CYP 3A4 activity by the GFJ. Three other edible fungi, *Monascus purpureus*, *Pleurotus sapidus*, and *Agaricus bisporus*, were evaluated for their binding with two of the major FCs in GFJ, 6′,7′-dihydroxybergamottin (DHB) and bergamottin (BM). These autoclaved edible fungi removed these FCs from GFJ, similar to *M. esculenta*, indicating that binding is a general, passive interaction between FCs and fungal hyphae. The removal of FCs was independent of pH in GFJ. Dried fungal material of *M. esculenta* was also effective in removing FCs from GFJ and occurred with GFJ samples prepared from both fresh grapefruit and GFJ concentrate.

KEYWORDS: Edible fungi; furanocoumarins; food binding; adsorption; bergamottin; 6′,7′-dihydroxybergamottin; grapefruit juice; drug interaction

INTRODUCTION

Coumarins and furanocoumarins (FCs) are a class of phenolic compounds produced in certain varieties of citrus, including grapefruit, pummelo, limes, lemons, and others (*1, 2*). Grapefruits are particularly rich in the FCs, where two of the major FCs are 6′,7′-dihydroxybergamottin (DHB) and bergamottin (BM) (*1, 3*). Significantly, most of the FCs in grapefruit juice (GFJ) are powerful inhibitors of human cytochrome P450 (CYP) enzymes 3A4, 2B6, 3A5, 2D6, and 2C9, responsible for the metabolism of many widely prescribed medications (*4*−*7*). This inhibition can possibly influence the bioavailability of these affected medications, which potentially creates a risk of adverse events (*8*). Moreover, this "grapefruit/drug" interaction has adversely affected the citrus industry for years, even though grapefruit possesses antioxidant activity and numerous beneficial health phytochemicals and putatively acts as a protector against cancer and cardiovascular diseases (*9, 10*).

To reduce these interactions, there have been recent attempts to remove FCs from GFJ using chemical, physical, and microbiological methods (*11*−*14*). The method developed by Paine et al. (*12*) used a series of chemical extractions and reconstitutions of compounds in GFJ to produce a FC-free GFJ, while Uesawa and Mohri (*13, 14*) used ultraviolet (UV) radiation and heat to degrade the FCs in GFJ. Meanwhile, the potential use of autoclaved fungus to adsorb and remove FCs in GFJ has also been explored (*11*).

Many edible fungi are rich in vitamins, minerals, and folates and are regarded as nutritive foods, nutraceuticals, and antitumor agents (*15*−*18*). The edible fungi are in the two major fungal groups, ascomycetes and basidiomycetes, differentiated by the formation of their sexual spores, where a diverse array of fruiting bodies are produced (*19*). The facts that adsorption of FCs has recently been described in the ascomycete *Aspergillus niger* (*11*) and that ascomycetes can be differentiated from basidiomycetes by features such as morphology and chemical composition (*20*) raised a question whether basidiomycetes can similarly adsorb the FCs in GFJ. The objectives of this study were to examine whether edible fungi, including two ascomycetes, *Morchella esculenta* and *Monascus purpureus*, and two basidiomycetes, *Pleurotus sapidus* and *Agaricus bisporus*, can remove FCs in GFJ and to evaluate whether the removal can be affected by different factors, i.e., pH of GFJ, sources of GFJ (fresh versus concentrate), and fungal materials (wet versus dry).

MATERIALS AND METHODS

Fungal Organisms and Cultures. A culture of *M. esculenta* Dill. ex Pers. (FP-140146) was obtained from the United States Department of Agriculture (USDA) Forest Service, Center for Forest Mycology Research in Madison, WI. *M. purpureus* Tiegh. was obtained from the Agricultural Research Service (ARS) Culture Collection (NRRL 1596)

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at the National Center for Agricultural Utilization Research. *P. sapidus* (Fr.) P. Krumm. nom. cons. was obtained from the American Type Culture Collection (24987). A culture of *A. bisporus* L. was prepared from an isolation made from the trama tissue of the pileus from a fresh basidiocarp.

The cultures of *M. esculenta* and *M. purpureus* were placed on potato dextrose agar (PDA) (BD/Difco Sparks, MD), and these cultures were maintained to provide initial inoculum by placing fungal hyphae (0.5 g) into 100 mL flasks of YM broth (10.0 g of glucose, 5.0 g of peptone, 3.0 g of yeast extract, and 3.0 g of malt extract in 1 L water) and placing them on an orbital shaker at 100 rpm and 23 °C for 1 week. Mycelial pellets from the cultures were broken apart by placing them into a sterile 1 L Waring stainless-steel blender cup and macerating the tissue for 2 min, and aliquots of the resulting suspension were equally divided into six 2 L flasks containing 1 L of YM broth. The flasks were shaken on a large orbital shaker at 140 rpm and 20 °C until there was enough mycelial tissue (≈20 g). *P. sapidus* and *A. bisporus* were maintained on YM agar containing thiamine (100 μL L^{-1}) and inoculated on YM broth as described above, with the exception that YM broth contained thiamine.

The fully grown fungal tissues in the flasks were harvested and autoclaved for 20 min at 121 °C to kill the fungus. After cooling, the contents (broth + dead mycelium) of the flasks were vacuum-filtered and the remaining mycelial mass was immediately used as an adsorbent. The wet mass of *M. esculenta* was further vacuum-dried at 40 °C for 24 h and ground into fine powder.

Interaction of GFJ with Fungal Materials. The GFJ samples were prepared from either fresh grapefruit or GFJ concentrate. The GFJ from fresh grapefruit was prepared by manually squeezing white Marsh grapefruit (*Citrus paradisi*). The GFJ samples from GFJ concentrate were prepared by mixing 1.5 L water with GFJ concentrate (355 mL), which was purchased at a local store. The pH of GFJ samples was further adjusted to 5.0 to minimize degradation of GFJ FCs.

The macerated *M. esculenta* samples (1.5, 3.0, and 9.0 g) were added to 50 mL of GFJ in 250 mL flasks and mixed for 4 h at 250 rpm and 25 °C to determine optimal amounts of biomass for the removal of GFJ FCs. For additional experiments using *M. esculenta*, *M. purpureus*, *P. sapidus*, and *A. bisporus*, 2.0, 4.0, and 6.0 g of the autoclaved wet fungal hyphae were mixed with GFJ at the same conditions as described above. For experiments using dry *M. esculenta*, 0.26, 0.52, and 0.78 g corresponding to their wet weights (2, 4, and 6 g) were added to GFJ samples and mixed as described above. Dry weights of *M. esculenta* were determined by weighing 10 g of samples before and after vacuum drying at 40 °C for 24 h. After 4 h, the untreated and fungal-treated GFJ samples were vacuum-filtered through a Whatman no. 1 filter, and the resulting filtrates and fungal material on the filter were collected.

Analysis of FCs in GFJ. Both filtrates and fungal materials were extracted with 100 mL of ethyl acetate 3 times. The extracts were dried using a rotary evaporator; the resulting pellets were dissolved in 10 mL of acetone, and 40 μL aliquots were subjected to high-performance liquid chromatography (HPLC) analysis as previously described (*21*). The FCs obtained from control (no fungi added) and treated (various amounts of edible fungal biomass added) GFJ samples were identified by comparing their elution times, UV absorbance at 320 nm, and mass spectrometry (MS) data to authentic FCs and analyzed as previously described (*21*). Concentrations (means ± standard deviations) of DHB and BM in GFJ samples prepared from fresh grapefruit were 5.32 ± 0.46 and 3.34 ± 0.37 ppm, respectively, while those of DHB and BM in GFJ samples prepared from GFJ concentrate were 4.11 ± 0.59 and 1.88 ± 0.53 ppm, respectively.

CYP 3A4 P450 Enzyme Inhibition Assay. Inhibition assay of CYP 3A4 enzyme activity was carried out according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA). The 2.5 μL of orange juice, untreated GFJ, or *Morchella*-treated GFJ was used for the inhibition assays. For treated GFJ, 6.0 g (wet weight) of autoclaved *M. esculenta* was mixed with 50 mL of GFJ for 4 h, the GFJ was filtered through a Whatman no. 1 filter, and the resulting GFJ filtrates were used for the inhibition assays. The control activity (100% activity) using water was determined as 1.086 nmol min^{-1} (nmol of CYP 3A4)$^{-1}$.

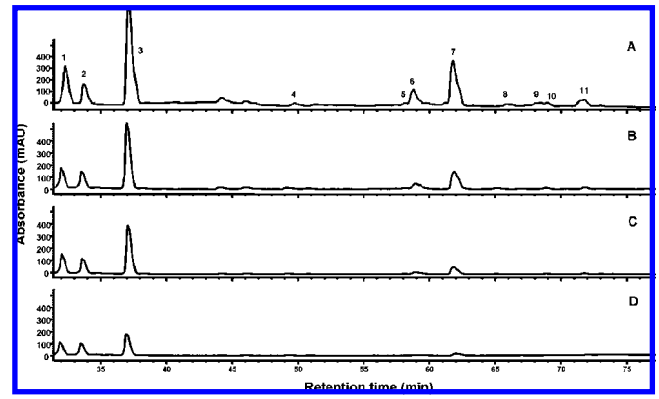

Figure 1. Profiles of FCs in GFJ. GFJs were incubated with or without autoclaved *M. esculenta*: (A) without *M. esculenta*, (B) 1.5 g of *M. esculenta*, (C) 3.0 g of *M. esculenta*, and (D) 9.0 g of *M. esculenta*. The peak numbers (**1**−**11**) and their corresponding compounds are **1**, FCU-1; **2**, FCU-2; **3**, DHB; **4**, 6′,7′-epoxybergamottin; **5**, 726 dimer-1; **6**, 726 dimer-2; **7**, BM; **8**, 708 dimer-1; **9**, 708 dimer-2; **10**, 708 dimer-3; and **11**, 708 dimer-4.

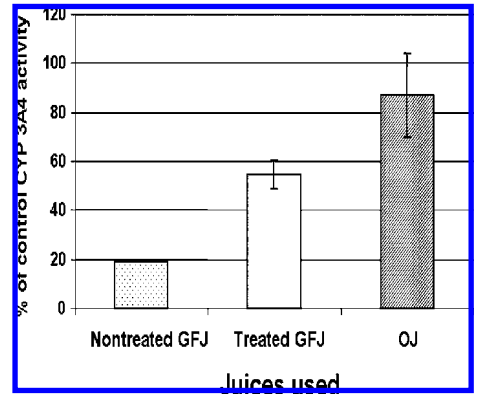

Figure 2. Inhibition of CYP 3A4 activity by autoclaved *M. esculenta*-nontreated or -treated GFJ and orange juice (OJ). For treated GFJ, 6.0 g (wet weight) of autoclaved *M. esculenta* was mixed with 50 mL of GFJ. The 2.5 μL of nontreated or *Morchella*-treated GFJ and OJ was used for the inhibition assays. The control activity (100% activity) using water was 1.086 nmol min^{-1} (nmol of CYP 3A4)$^{-1}$. Bars represent standard deviations of means of triplicates.

Statistical Procedures. All experiments in this study were conducted with three replicates. Student *t* test was used to compare the differences in changes between controls and treatments if applicable. Two-tailed *p* values were calculated to report significant differences in the mean values.

RESULTS

Removal of FCs in GFJ by Autoclaved *M. esculenta*. The removal of 11 GFJ FCs by autoclaved *M. esculenta* was analyzed by HPLC-MS, as previously described (*11*). These compounds included two FC unknowns (FCU)-1 (**1**), FCU-2 (**2**), and a series of FC dimers, exhibiting protonated molecular ions $(M + H)^{+1}$ of 726 *m/z* (**5** and **6**) and 708 *m/z* (**8**−**11**), respectively. Three known compounds, DHB (**3**), 6′,7′-epoxybergamottin (**4**), and BM (**7**) were also monitored. Levels of these FCs were compared in untreated GFJ and in *M. esculenta*-treated GFJ (**Figure 1**). When 1.5 g of autoclaved *M. esculenta* tissue was mixed with 50 mL of GFJ for 4 h, the hydrophobic FCs, **4**−**11**, were largely removed from GFJ (**Figure 1B**). More complete removal in GFJ was achieved with the addition of 3 g of the fungal material (**Figure 1C**), while with 9 g of the fungal material, most of FCs in GFJ were removed, with the

Table 1. Removal of Two Major FCs, DHB and BM, in GFJ by Autoclaved Edible Fungi, *M. purpureus*, *P. sapidus*, and *A. bisporus*[a]

	percent removal of FCs in GFJ by fungi								
	M. purpureus			*P. sapidus*			*A. bisporus*		
fungal material used (g)	2	4	6	2	4	6	2	4	6
DHB	24.8 ± 8.9	39.1 ± 9.1	58.9 ± 4.1	15.1 ± 1.6	48.6 ± 1.8	55.1 ± 1.6	15.2 ± 2.6	25.2 ± 8.2	44.6 ± 4.3
BM	70.9 ± 9.0	93.6 ± 2.0	97.2 ± 0.6	46.3 ± 2.8	82.2 ± 5.3	96.2 ± 2.2	26.8 ± 7.8	44.6 ± 6.4	75.7 ± 4.9

[a] GFJ (50 mL) was mixed with three different amounts (2, 4, and 6 g, wet weight) of autoclaved fungi, and DHB and BM in GFJ were analyzed. Data represent means ± standard deviations of means of triplicates.

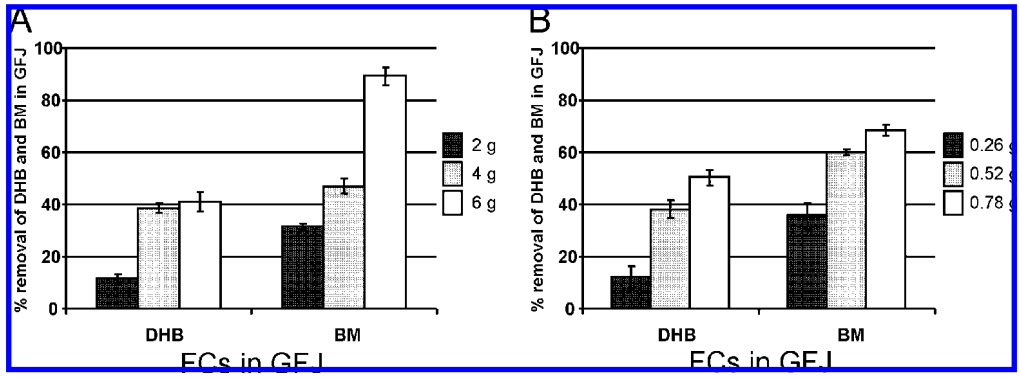

Figure 3. Removal of two major FCs, DHB and BM, in GFJ by autoclaved *M. esculenta*. (A) Autoclaved *M. esculenta* was vacuum-filtered, and the resulting wet fungal biomass (2, 4, and 6 g) was mixed with 50 mL of GFJ for 4 h. (B) Wet biomass was further vacuum-dried, and the resulting dry powder (0.26, 0.52, and 0.78 g) corresponding to wet biomass was mixed with GFJ. Bars represent standard deviations of means of triplicates.

exception of the polar FCs (**1**−**3**) and BM (**7**) (**Figure 1D**). The FCs removed from GFJ were almost fully (>95% for all of the FCs) recovered by acetone extractions of the fungal materials. The pH of the GFJ (originally 3.3) was adjusted to 5.0 to minimize FC hydrolytic degradation, and the binding efficiencies of the FCs to the fungal hyphae were subsequently compared at the two pH conditions. No difference in the FC binding to the fungal hyphae was observed at these two pH values (data not shown), enabling the use of GFJ at pH 5.0 with minimal FC degradation.

To determine whether the removal of FCs in GFJ decreases the inhibition of CYP 3A4 enzyme, 2.5 μL of GFJ treated with 6 g of *M. esculenta* was subjected to an *in vitro* enzymatic assay and compared to nontreated GFJ and orange juice (**Figure 2**). The orange juice is thought to be inactive in *in vivo* inhibition of CYP 3A4 enzyme (*10*), although a small amount of inhibition of CYP 3A4 by orange juice was detected *in vitro* (**Figure 2**). As expected, GFJ treated with *M. esculenta* showed a decreased inhibition (40%) of the enzymatic activity compared to nontreated GFJ.

Removal of FCs in GFJ by Autoclaved *M. purpureus*, *P. sapidus*, and *A. bisporus*. To further determine whether other edible fungi can bind and remove FCs from GFJ, *M. purpureus*, *P. sapidus*, and *A. bisporus* were mixed with GFJ for 4 h and two of the major FCs, DHB and BM, in the GFJ were analyzed (**Table 1**). A total of 2 g of *M. purpureus* removed 24.8% of DHB and 70.9% of BM, and the removal of both FCs was proportionally increased with the amount of fungal biomass added (**Table 1**). The removal of DHB and BM in GFJ was also shown with two other fungi, *P. sapidus* and *A. bisporus*, where approximately 55 and 45% of DHB and 96 and 76% of BM, respectively, were removed from GFJ by 6 g of these fungi. The removal of DHB and BM by 6 g of fungal hyphae did not significantly differ among fungi ($p > 0.05$), except for the less removal of DHB and BM by *A. bisporus* ($p < 0.05$).

Removal of FCs in GFJ by Dry *M. esculenta*. To test whether dried fungal biomass can adsorb FCs in GFJ, autoclaved *M. esculenta* was vacuum-dried and mixed with GFJ. A total of 2, 4, and 6 g of wet fungal biomass removed 11.4, 38.6, and 41.0% of DHB from GFJ, respectively, while the corresponding 0.26, 0.57, and 0.78 g of dry fungal biomass removed 12.2, 38.1, and 50.3% of DHB, respectively (**Figure 3**). Removal of DHB by wet fungal biomass did not differ from that of dry fungal biomass ($p > 0.05$). Meanwhile, 31.7, 47.0, and 89.4% of BM were removed from GFJ when 2, 4, and 6 g of wet fungal biomass were used, while 35.7, 60.0, and 68.6% of BM were removed when 0.26, 0.57, and 0.78 g of dry fungal biomass was used. Similar to the removal of DHB, removal of BM by wet fungal biomass did not differ from that of dry fungal biomass ($p > 0.05$), except for the difference between 6 g of wet biomass and 0.78 g of dry biomass ($p < 0.05$). It is noted that the removal of DHB and BM by the wet and dry biomass was also observed from GFJ samples prepared from commercial GFJ concentrates (data not shown) and the removal of both FCs by wet biomass did not differ from that of dry biomass ($p > 0.05$).

DISCUSSION

The edible *M. esculenta* efficiently adsorbed most of the nonpolar FCs in GFJ, where only a trace amount of BM (**7**) remained in the GFJ treated with 9 g of the autoclaved fungal material (**Figure 1**). The removal of FCs in GFJ by the fungus was comparable to that of FCs by *A. niger*, as previously reported (*11*). Polar FCs, FCU-1, FCU-2, and DHB (**1**−**3**, **Figure 1**), still remained in GFJ treated with 9 g of *M. esculenta*, which was similar to the findings of the same study observed with *A. niger* (*11*). The similarity in removal efficiency of FCs by *M. esculenta* and *A. niger* suggests that these fungi contain a comparable amount of components responsible for the binding within tissue. In addition, 40% of the CYP 3A4 inhibition remained in the fungal-treated GFJ (**Figure 2**), suggesting that the residual activity was likely due to incomplete removal of FCs, i.e. FCU-1, FCU-

2, and DHB (**1**−**3**, **Figure 1**). Nonetheless, together with the results shown in **Figures 1** and **2**, our data demonstrate that *M. esculenta* can remove FCs in GFJ, leading to a decreased inhibition of CYP 3A4 enzymatic activity.

Because basidiomycetes can be differentiated from ascomycetes by chemical composition (*20*), the removal of FCs by *M. esculenta* led us to examine a possible removal of FCs by other edible fungi. Interestingly, removal of DHB and BM by *M. purpureus*, *P. sapidus*, and *A. bisporus* also occurred as shown by *M. esculenta* (**Table 1** and **Figure 3**). The results show that the binding may occur with any fungus, regardless of ascomycetes or basidiomycetes, suggesting that the binding is a general interaction, where FCs passively bind to fungal hyphae. It is likely that the contents of the components responsible for the binding may not differ greatly among these fungi, as evidenced by the removal of DHB and BM from GFJ by these fungi (**Table 1** and **Figure 3**).

The DHB and BM are distributed in the "cloud" of GFJ, defined as the portion of suspended particles retained in suspension after centrifugation at 360*g* for 10 min (*22*). Even though precise localization of FCs in the cloud has not been characterized, given that lipophilic compounds, such as flavonoids and alkanes, are found in endoplasmic reticulum (ER)-derived vesicles in plant cells (*23*) and that the cloud is rich in lipids derived from juice sacs (*24*), it can be postulated that the lipophilic FCs are localized in lipid vesicles protected from an aqueous environment in the GFJ cloud. Moreover, with hypothesized hydrophobic interaction between lipids and FCs (*11, 25*) and little effect of GFJ pH on the binding phenomenon (data not shown), the binding of FCs to fungal biomass further suggests that lipophilic components in fungi sequester FCs, so that nonpolar FCs, particularly BM and FC dimers, associate with hydrophobic sectors in fungal material. In addition, the removal by the wet and dry fungal biomass was observed from GFJ samples prepared from commercial GFJ concentrates (data not shown), suggesting that chemical stability of FCs in GFJ concentrates has been maintained through industrial GFJ processing. Furthermore, the efficient removal of DHB and BM by wet and dry biomass (**Figure 3**) indicates that the drying process does not affect the abundance and binding or physical property of components responsible for the removal. With regard to this, other dried biomaterials efficiently sequestered FCs in GFJ (our unpublished results).

In summary, FCs in GFJ were removed by an edible fungus, *M. esculenta*, and the removal of FCs from GFJ reduced an inhibition of CYP 3A4. The removal of DHB and BM was also observed from other edible fungi, *M. purpureus*, *P. sapidus*, and *A. bisporus*, suggesting that the binding of FCs to fungal hyphae is a passive interaction and these fungi contain components responsible for the binding. The removal of FCs in GFJ was also observed with dry *M. esculenta*, making it possible to use the dry material for the removal of FCs in GFJ and further characterize constituents binding to the FCs.

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