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Use of citrus flavanones to prevent aflatoxin contamination using response surface methodology

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ABSTRACT

The aim of this study was to analyze the possible utilization of flavanones obtained as by-products of the citrus industry, naringin (NAR), hesperidin (HES) and neohesperidin (NEO), to inhibit the production of aflatoxins (AFs) from *Aspergillus flavus*. Response Surface Methodology (RSM) was applied to optimize experimental conditions in terms of the different flavanones concentrations used. Through this methodology these optimal combinations were calculated: HES-NAR: 0.206–0.037 mM, HES-NEO: 0.156–0.283 mM and NAR-NEO: 0.035–0.195 mM. The theoretical concentrations obtained by RSM were assayed, achieving total inhibition of AFB_1 and AFB_2 production. Moreover, the use of these flavanones, obtained at low cost from the residues of citric industry, presents an interesting option for improving the profitability of these industries.

1. Introduction

Mycotoxins are secondary metabolites produced by fungi that are found in food and fodder. They are an object of interest due to the important economic losses that its effects imply on the health of people, the animal's productivity and the national and international trade. Aflatoxins can be found in a variety of agricultural commodities, but the most recognized contamination has been encountered in maize, peanuts, cottonseed, and tree nuts (Klich, 2007). Aflatoxins are biologically active polyketide-derived secondary metabolites produced by certain strains of *Aspergillus* such as *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nominus* (Alpsoy, 2010; Cotty, Bayman, Egel, & Elias, 1994). There are more than 20 aflatoxins described, being B_1 (AFB1), B_2 (AFB2), G_1 (AFG1), G_2 (AFG2), M_1 (AFM1) and M_2 (AFM2) frequently found. AFM_1 is the major metabolite in the milk of animals that have consumed feed contaminated with AFB_1 (JECFA, 2007). These mycotoxins are mutagenic, carcinogenic, hepatotoxic, teratogenic and immunosuppressive as well as inhibitors of several metabolic systems. Among all aflatoxins, AFB_1 presents preeminent toxic effects at lower concentrations and it has been classified as Group 1 (carcinogenic to humans) by the International Agency for Research on Cancer (IARC, 2012). Frequently used regulations for AFs in cereals and other foods vary between 2 and 12 μg/kg for AFB_1, 4 and 15 μg/kg for AFB_2, 4 and 15 μg/kg for total AFs, and 0.05 and 0.5 μg/kg for AFM_1 in milk (EC, 2010; FAO, 2004; MERCOSUR, 2002).

Flavonoids are widespread secondary plant metabolites being their backbone structure composed by two aromatic rings, which are connected through a pyrone or hydropyrone ring (Gattuso, Barreca, Gargiulli, Leuzzi, & Caristi, 2007). They are classified in groups according to the level of oxidation of its central nucleus. Four types of flavonoids occur in *Citrus* sp. and can be classified into these groups: flavanones, flavones, flavanols and anthocyanins (Benavente-García & Castillo, 2008). In particular, flavanones are found in citrus as glycosides (Khan, Zill-E-Huma, & Dangles, 2014). The most common citrus flavanone glycosides are HES or 3′,5,7-trihydroxy-4′-methoxyflavanone-7-6-O-α-L -rhamnopyranosyl-D-

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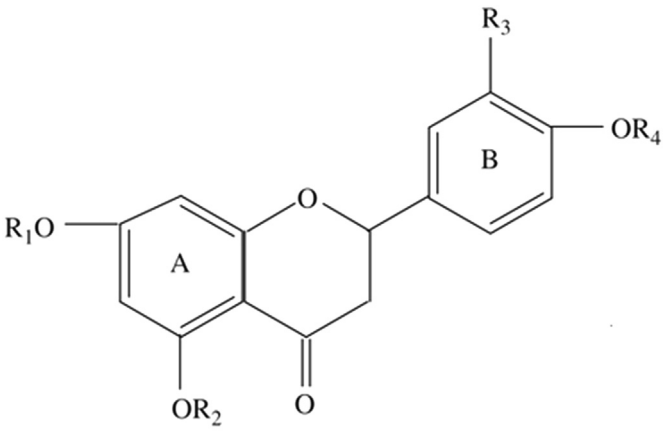
glucopyranoside, which is found in oranges, lemons and other citrus; NAR or 4′,5,7-trihydroxyflavanone 7-β-L-rhamnoglucoside-(1,2)-α-D-glucopyranoside in grapefruits and sour oranges and NEO or (S) 4′-methoxy-3′,5,7-trihydroxyflavanone-7-[2-O-(α-L-rhamnopyranosyl)-β-D-glucopyranoside in sour oranges. Fig. 1 shows the chemical structure of the mentioned flavanones.

The increasing concern of consumers towards food safety has pushed the industries to the elimination of synthetic additives. Their replacement by natural additives is seen as a benefit from the point of view of the quality as well as food safety (Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvareza, 2008).

Some flavonoids have been obtained as by-products of the citrus industry at low cost, particularly flavanones that could present antifungal and antitoxigenic properties (Russo, Bonaccorsi, Inferrera, Dugo, & Mondello, 2015; Salas, Reynoso, Céliz, Daz, & Resnik, 2012). Previous work pointed out that the extract of some *Citrus* sp., that could have some of the flavanones studied in this work, had inhibitory activity against *A. flavus* and aflatoxin accumulation. For example, Bejarano Rodriguez and Centeno Briceño (2009) demonstrated that 20 μl/g of *Citrus limon* extract in poultry feedstuffs initially contaminated with 45 ug/kg of aflatoxin, achieved almost 74% of reduction after 1 h of treatment. Dos Santos Oliveira and Badiale Furlong (2008) found in vitro essays that the phenolic extracts from orange peel inhibited the production of aflatoxin B_1 and B_2 at a concentration of 250 μg/ml. Essential oils from *Citrus reticulata* and *Cymbopogon citratus* completely inhibited aflatoxin B_1 production at 750 and 500 ppm, respectively (Singh et al., 2010). Velázquez-Nuñez, Avila-Sosa, Palou, and López-Malo (2013) found that the minimum inhibitory concentration for *A. flavus* growth was 16 000 mg/l of orange (*Citrus sinensis* var. Valencia) peel essential oil, when applied by direct addition in the culture media.

Factorial designs are efficient methodologies to study the joint effect of two or more factors on a response (Grum & Slabe, 2004). RSM is useful for the modeling and analysis of situations in which a response of interest is influenced by several variables (Ibrahim & Elkhidir, 2011) with much less effort than the classical approaches, resulting in less laborious and time-consuming assays (Esbensen, 2009).

The aim of this study was to analyze the utilization of 3 flavanones obtained from the citrus industry, NAR, HES and NEO applying RSM to find the optimal concentrations to totally inhibit the production of AFB_1 and AFB_2 by *A. flavus*.



NAR R_1: -Rhamnose, R_2: -H, R_3: -H, R_4: -OH
NEO R_1: -Rhamnose, R_2: -H, R_3: -OH, R_4: -OCH_3
HES R_1: -Rutinose, R_2: -H, R_3: -OH, R_4: -OCH_3

Fig. 1. Chemical structure of NEO, NAR and HES.

2. Materials and methods

2.1. Reagents and chemicals

Organic solvents were HPLC grade. Acetonitrile (ACN), ethanol and methanol (MeOH) were purchased from Sintorgan (Buenos Aires, Argentina); hexane from J.T. Baker (Estado de México, México); trifluoroacetic acid (TFA) from Tedia Company Inc. (OH, USA). HPLC quality water was prepared with a Waters Milli-Q system (Waters Associated, MA, USA).

AFs standards were from Sigma (MO, USA) and were prepared according to "Method 990.33" (AOAC, 2012, chap. 49).

The culture medium used for growing the moulds, Malt Extract Agar (MEA), and the Tween 80 were purchased from Biokar (Beauvais, France).

2.2. Flavanones

The flavanones NAR, HES and NEO were obtained in the "Instituto de Investigaciones para la Industria Química", Universidad Nacional de Salta, Salta, Argentina from residues of citric industries according to previous published works (Geronazzo, Robin, Blanco, Cuevas, & Ellenrieder, 2000; Macoritto, Geronazzo, & Ellenrieder, 2001; Macoritto, Robin, Blanco, & Geronazzo, 2004). Briefly, the flavanones obtaining procedure consists of grinding the discarded fruits or the residues of juice process, to an average size of 2 mm in diameter and, after that, performing an extraction in fixed bed column. To obtain NAR, the extraction was done with distilled water as solvent at 80 °C, for HES aqueous solution at pH 10.0–10.5 as extracting solvent at 70 °C was used, and NEO was obtained with ethanol:water (25:50 v/v) at 25 °C. In all three processes the extract obtained was cooled, leading this to the crystallization of the flavanones. The precipitate was then filtered and washed and finally the solid was dried in an oven at 50 °C. This process is considered simple and inexpensive.

2.3. Fungal strains

The *A. flavus* CIM 30456 used was isolated from blueberries (Munitz et al., 2013, 2014) and is kept in the Type Culture Collection of the Natural Science Faculty, University of Buenos Aires (Buenos Aires). This mould was previously cultivated in strains containing Malt Extract Agar (MEA) during 7 days. Then, 10 ml of Tween 80 (0.02%) were added and the tubes were shaken for 1 min in a vortex to separate the conidia from the rest of the medium. The concentration of conidia in suspension was 1.6 10^8 conidia/ml. It was determinated using a Neubauer counting chamber.

2.4. Preparation of flavanone solutions

The studied flavanones were dissolved in ethanol:water (5:95, v/v) solutions at 40 C at 2 different concentrations alone (0.15 and 0.3 mM) and binary mixtures of them (NAR-NEO, NAR-HES, NEO-HES: 0.15–0.15; 0.30–0.30 mM). The studies were carried out in Petri dishes, which had 10 g each of MEA medium with different solutions of flavanones added after sterilization and thoroughly mixed before solidification. Each dish was inoculated centrally with 10 μl of the conidia suspension of *A. flavus* prepared as it was explained in 2.3 and incubated at 25 °C, during 21 days.