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Efficacy of flavanones obtained from citrus residues to prevent patulin contamination

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ABSTRACT

Patulin is a mycotoxin produced by different types of molds from the genera *Penicillium*, *Apergillus* and *Byssochlamys* among others. These fungi are the principal reason for the deterioration of apples, pears and its industrial products, generating economic losses. 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), neohesperidin (NEO), prunin (P), hesperetin glucoside (HG), and glucoside esters of these flavanones, prunin 6″-O-butyryl ester (PB), prunin 6″-O-decanoyl ester (PD), prunin 6″-O-lauroyl ester (PL), prunin 6″-O-stearoyl ester (PS) and hesperetin glucoside 6″-O-lauroyl ester (HGL) to inhibit the production of patulin from *Penicillium expansum*, *Aspergillus terreus* and *Byssochlamys fulva*.

All flavanones tested reduced patulin accumulation in at least 95% compared to the control. No efficacy differences were found between the flavanones tested for each mold. In the case of *P. expansum*, the patulin accumulation presented a 98% of reduction in average comparing to the control. For *B. fulva*, also the flavanones exhibited an inhibitory effect greater than 99% and for *A. terrus*, all these compounds completely inhibited patulin accumulation.

These promising results lead to think that different concentrations or mixtures of flavanones could reach total inhibition of patulin production, so further researches, both in vitro and different food matrixes, are necessary.

1. Introduction

Mycotoxins are secondary metabolites produced by fungi that are found in food and fodder. Effects on population health and economic losses due to reduction in livestock production make mycotoxins a matter of concern. Patulin, 4-hydroxy-4*H*-furo [3,2-*c*]pyran-2(6*H*)-one (Fig. 1), it is a mycotoxin produced by certain types of molds from the genera *Aspergillus*, *Penicillium* and *Byssochlamys*, among others. It is colorless, crystalline compound with melting point of 110 °C (González-Osnaya, Soriano, Moltó, & Mañez, 2007) and stable in water solution at 105–125 °C in a range of pH of 3.5–5.5, increasing its degradation by the increment of the pH's solution (Lovett & Peeler, 1974). At pH 6 and 100 °C, 50% of patulin (PAT) is degraded (Collin, Bodart, Badot, Bouseta, & Nizet, 2008). Therefore, heat treatment (pasteurization) cannot completely inactivate PAT in products like apple juice or ciders.

Penicillium expansum, the mold commonly found in apples, is thought to be the major producer of PAT (McKinley & Carlton, 1991). This fungus can also be found in a wide variety of fruits as pears, berries, grapes, apricots and peaches (Cheraghali et al., 2005), but is known to invade not only fruit but also other foodstuff such as vegetables, bread and meat products (Ritieni, 2003; Rychlik & Schieberle, 2001). In apple juice, PAT has been detected in various countries around the world such as Argentina, Finland, Australia, Chile and Spain (Anderson de Souza, Rosenthal, & Rodriguez de Massaguer, 2008; Funes & Resnik, 2009). *Codex Alimentarius* recommends a maximum level of 50 μg/kg in apple products (Food & Agriculture Organization, 2004).

Flavonoids are one of the most widespread groups of secondary plant metabolites and their backbone structure is composed of two aromatic rings (A, B), which are connected through a pyrone or hydropyrone ring (C), the flavones or flavanones, respectively (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. Fig. 2 shows the basic chemical structure of a flavanone. The most common citrus flavanone glycosides are

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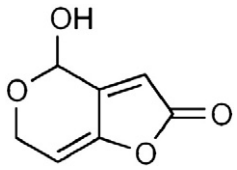

Fig. 1. Chemical structure of PAT.

hesperidin (7-β-D-α-L-rhamnosyl(1→6)-β-D-glucosyl-3′,5,7-trihydroxi-4′-methoxyflavanone) which is found in oranges, lemons and other citrus, naringin (7-β-D-α-L-rhamnosyl(1→2)-β-D-glucosyl-4′,5,7-trihydroxyflavanone) in grapefruits and sour oranges and neohesperidin (7-β-D-α-L-rhamnosyl(1→2)-β-D-glucosyl-3′,5,7–trihydroxy-4′-methoxyflavanone) in sour oranges.

There are many factors that affect food safety such as global trade, socio-economic and technological development, urbanization and agricultural land use. Climate change and variability are among the multiple factors that can provoke changes in the nature and occurrence of food safety hazards (Tirado, Clarke, Jaykus, McQuatters-Gollop, & Frank, 2010). In particular, mycotoxins depend on climate, plant and storage associated problems and non-infectious factors such as bioavailability of nutrients, insect damage, and pest attacks (Paterson & Lima, 2010, 2011). Imposing limits on mycotoxins content in commodities, producing predictive models of plant pathogens and seasonal mycotoxin contamination, have been some of the approaches carried on to prevent exposure to mycotoxins (Tirado et al., 2010).

At the same time, the increasing concern of the consumers towards food safety has pushed the industries to the elimination of synthetic additives and 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). Therefore it is necessary to develop alternative treatments to replace synthetic additives (Phillips, Laird, & Allen, 2011).

Some investigations have been studying the effect that flavonoids can present against fungi (Rauha et al., 2000; Salas, Celiz, Daz, Geronazzo & Resnik, 2010; Silva, Weidenbörner, & Cavaleiro, 1998; Wächter, Hoffmann, Furbacher, Blake & Timmermann, 1999; Weidenbörner, Hindorf, Jha, & Tsotsonos, 1990).

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 mycotoxins. For example, lemongrass oil at 500 μg/g maize grain had significant inhibitory effect against Fumonisin B_1 at 30 °C according to Velluti, Sanchis, Ramos, Egido and Marin (2003). Bejarano Rodriguez and Centeno Briceño (2009) demonstrated that 20 μl/g of *Citrus lemon* extract in poultry feedstuffs initially contaminated with 45 μg/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. Romero, Alberto, Manca de Nadra, and Vaamonde (2009) demonstrated in vitro essays that rutin and quercetin, flavonols commonly found in grapes and some *Citrus sp*., reduced at 250 mg/l more than 50% the contamination with ochratoxin A produced by *Aspergillus carbonarius*. Mallozzi, Correa, Haraguchi and Neto (1996) worked with quercetin and found that this flavonol reduced 55% the accumulation of aflatoxin B_1 at 25 ppm, and that naringenin, the aglycone of the flavanone naringin, also reduced aflatoxin B_1 in almost 41% at the same concentration.

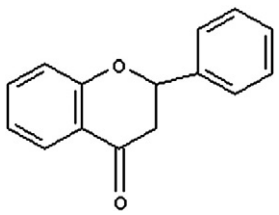

Fig. 2. Basic chemical structure of a flavanone.

Nevertheless, no study has been found in bibliography about the individual effect of *Citrus* flavanones related to production or accumulation of PAT.

The aim of this study was to analyze the possible efficacy of these citric by-products to inhibit the accumulation of PAT from *P. expansum*, *Aspergillus terreus* and *Byssochlamys fulva*.

2. Materials and methods

2.1. Flavanones

The flavanones naringin (NAR), hesperidin (HES) and neohesperidin (NEO) were obtained at low cost from residues of citric industries according to previous works already done by Instituto de Investigaciones para la Industria Química, Universidad Nacional de Salta, Salta, Argentina (INIQUI) (Geronazzo, Robin, Blanco, Cuevas, & Ellenrieder, 2000; Macoritto, Geronazzo, & Ellenrieder, 2001). We also included in the study some modificated flavanones in order to analyze the chemical structure on PAT accumulation. These flavanones were: Prunin (P), hesperetin glucoside (HG), prunin 6″-O-butyryl ester (PB), prunin 6″-O-decanoyl ester (PD), prunin 6″-O-lauroyl ester (PL), prunin 6″-O-stearoyl ester (PS) and hesperetin glucoside 6″-O-lauroyl ester (HGL). Prunin (P) or 7-β-D-glucosyl-4′,5,7-trihydroxiflavanone and hesperetin glucoside (HG) or 7-β-D-glucosyl-3′,5,7-trihydroxi-4′-methoxyflavanone were obtained by enzymatic hydrolysis of supersaturated solution of naringin and neohesperidin according to the methodology described by Ellenrieder, Blanco, and Daz (1998), Ellenrieder (2004) and Soria and Ellenrieder (2002). The synthesis of the other flavonoid glucoside esters was carried out by enzymatic catalysis as described by Céliz and Daz (2010).

2.2. Reagents and chemicals

Acetic acid, acetonitrile and perchloric acid HPLC grade were from Carlo Erba. Dimethylformamide (DMF) and glacial acetic acid A.C.S grade were ordered from J. T. Baker (US), methanol (MeOH) HPLC grade was from Tedia (US), ethyl acetate was from Mallinckrodt UltimAR (US), and nitrogen was purchased from Oxígeno Centre (Argentina). Water HPLC grade was obtained from water purification systems NANO pure Diamond (Barnstead International, US). PAT standard was ordered from Sigma-Aldrich (US). The culture medium used for growing the molds, Malt Extract Agar (MEA), and the Tween 80 were purchased from Biokar (France).

2.3. Fungal strains

Several molds were isolated from apples and tested for their toxigenic capacity in order to see their ability to produce PAT. Each of the isolated fungi was incubated for 7 days and the production of PAT was measured according to the procedure described in Sections 2.6 and 2.7. The molds that were found to be toxic were included in the Type Culture Collection of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (BACF). The flavanones were individually tested against *A. terreus* (BACF 701) *P. expansum* (BACF 3560) and *B. fulva* (BACF 3561).

2.4. Preparation of flavanone solutions

The studied flavanones were dissolved in DMF in order to achieve a final concentration of DMF of 1% (v/v) at the growth media (MEA, 40 g/l) and 0.25 mM of each flavanone. For that purpose, each flavanone was weighted and dissolved in 2.5 ml of DMF, added to 250 ml of culture medium at 42–45 °C and homogenized. Then 10 ml was

poured into disposable Petri dishes (90×15 mm, Massobact). Before molds were inoculated, the dishes were left to dry out two days.

2.5. Inocula

The molds were previously cultivated in strains containing MEA during 7 days at 25 °C ± 1 °C. Then, 10 ml of Tween 80 0.02% was added and the tubes were shaken 1 min in a vortex to separate the conidia from the rest of the medium. The concentration of conidia was measured with a Neubauer chamber. The values obtained were for *A. terreus* 1.6×10^5 conidia/ml (standard deviation SD = 0.1×10^5 conidia/ml), *B. fulva* 8.2×10^8 conidia/ml (SD = 0.8×10^8 conidia/ml) and *P. expansum* 1.6×10^8 conidia/ml (SD = 0.3×10^8 conidia/ml).

MEA plates without flavanones (control), MEA plates with DMF and MEA plates with the flavanones solutions were inoculated with the same inoculum for each mold by quintuplet. In each case the sowing volume in the Petri dishes was 10 μl (10 ± 0.2 μl). The dishes were incubated at 25 °C ± 1 °C in the darkness during 7 days.

2.6. Extraction of patulin

After 7 days of incubation, 10 ml of ethyl acetate was poured in a Falcon tube (Biologix Research Company Shawnee, US) with the culture media and the grown mold. They were sonicated with a Bransonic Sonicator (Branson Ultrasonic Corporation, US) during 20 min, centrifuged at 3000 rpm (Rolco, Argentina) and then filtered through a filter paper (Whatman No. 4). This procedure was repeated two more times to assure a correct extraction of PAT from the culture media. After that, 5 ml of the filtered was taken and evaporated to 2 ml in a gentle stream of nitrogen where 150 μl of glacial acetic acid was added until the remaining filtrate was completely evaporated to dryness. Finally, the residue was resuspended in 300 μl of 0.1% acetic acid.

2.7. Chromatographic conditions

The quantification of PAT was carried out by High-Performance Liquid Chromatography (HPLC) as previously described by Funes and Resnik (2009). An HPLC from Waters Alliance (US) was used. This chromatographer has two modules, a Separation Module (Waters Alliance 2695) with thermostat for samples and columns, room for five individual sample carousels, quaternary pump and automatic injector, and a Photodiode Array detector (Waters 2998) spectrophotometer UV/visible, with diode array of 512 photodiodes. The analytical column used was a Thermo BDS Hypersil C 18.5 μm, 50 mm×4.6 mm (Thermo Electron Corporation, UK) with guard column BDS-Hypersil - C 18.5 μm 10×4 mm (Termo Scientific, UK). The wavelength for UV detection was set at 275 nm. The chromatographic conditions were: column temperature, 40 °C; sample temperature, 10 °C; injection volume, 50 μl, flow rate, 0.85 ml/min; mobile phase: acetonitrile—0.01% perchloric acid (A solvent); water—0.01% perchloric acid (B solvent). The elution gradient used started at t = 0 min and t = 0.25 min with 4% of solvent A and 96% of solvent B. At t = 10 min the composition of the mobile phase turned to 2% of solvent A and 98% of solvent B. From t = 11 min to t = 12 min the composition was 60% of solvent A and 40% of solvent B, and at t = 16 min it was 4% of solvent A and 96% of solvent B. The retention time for PAT resulted in 9.7 min.

For recovery studies, uncontaminated Petri dishes with MEA were spiked with PAT solutions at level equivalent to 100 μg PAT/kg of culture media by triplicate and stored 24 h at −20 °C prior to extraction.

The standard solutions preparation was made diluting PAT with a solution of 0.1% acetic acid. The calibration curve was determined in triplicate using independent dilutions. Linearity of the method was determined by analyzing nine calibrations standards in the range of 1–2255 μg/kg. The detection (LOD) and quantification limits (LOQ) were calculated with spiked samples with the most diluted PAT standard level of the calibration curve. Noise was calculated with HP Chemstation software as six times the standard deviation of the lineal regression of the drift at the selected time range. The LOD was set at three times the noise and LOD at five times.

2.8. Statistical analysis

Analysis of variance (ANOVA) was applied to determine differences ($p<0.05$). To ascertain significant differences between levels of the main factor, Bonferroni's test was applied between means. ANOVA was used to determine the influence of the different flavonoids among the percentage of PAT accumulation. The analysis was conducted using the program R (Statistical Software).

3. Results and discussion

The recovery of PAT from MEA dishes at 100 μg PAT/kg was: 102 + 17.9%. The correlation coefficient fulfills the requirements for a linear method (R2 = 0.998) and the calculated LOD and LOQ were 0.15 and 0.5 μg/kg respectively. This described method is adequate for the purpose of this study.

The concentrations most commonly used to prove the antifungal activity of flavonoids varied from 0.2 to 0.8 mM (Carrillo, Gómez Molina & Benitez Ahrendts, 1999; Weidenbörner, Hindorf, Jha, Tsotsonos, & Egge, 1990; Weidenbörner & Jha, 1994, 1997). In this study it was chosen to work with a concentration near the inferior limit, 0.25 mM as a first approach. This study was conducted in a solid medium to resemble solid surfaces of food.

A. terreus produced much less quantity of PAT (7 days control, 1.2×10^3 mg/kg of PAT) than the big producers *P. expansum* (7 days control $1.0*10^6$ mg/kg of PAT) and *B. fulva* (7 days control $1.9*10^7$ mg/kg of PAT).

The plates with 1% DMF presented an average inhibition in PAT accumulation of 26.4% (SD = 5.3).

Fig. 3a shows a chromatogram of the extract of *P. expansum* without any flavanone (control) and Fig. 3b a chromatogram of the extract of the same mold with NAR, where a decrease of the peak area can clearly be seen (different retention time but confirmed by UV spectra).

All 10 flavonoids completely inhibited PAT accumulation for *A. terreus*. The results of PAT inhibition in the case of *P. expansum* and *B. fulva* are shown in Table 1. No significant differences were found among the flavanones for each mold according to Bonferroni's multiple range test ($p>0.05$).

For *P. expansum*, the percentage of PAT reduction reached an average of 98%. In the case of *B. fulva*, it was observed that all the flavanones were more active against PAT accumulation than for *P. expansum*, reaching in all the cases more than 99% of reduction, except for P, PL and HGL.

Data found in the bibliography shows that complete inhibition of PAT formation was attained using 0.2% of lemon oil in glucose-Czapek's apple medium, as it was reported by Hasan (2000). Sanzani et al. (2009) found that PAT accumulation was reduced by the use of quercetin and umbelliferone, a flavonol and a coumarin that can be found in *Citrus sp*. In this study PAT accumulation was reduced almost 50% at 17.8 mM/l and 30.8 mM/l respectively. In our case, concentrations of 0.25 mM/l, less than 0.02% of flavanones, showed activity against PAT accumulation, reducing it at least 95% compared to the control. Some authors suggest that phenolic compounds could influence secondary metabolites biosynthesis (Sanzani et al., 2009), but there is no information in literature regarding the effect of these flavanones on PAT biosynthesis.

4. Conclusions

This work was an approach to study the individual potential efficacy of these flavanones as natural compounds that effectively control PAT

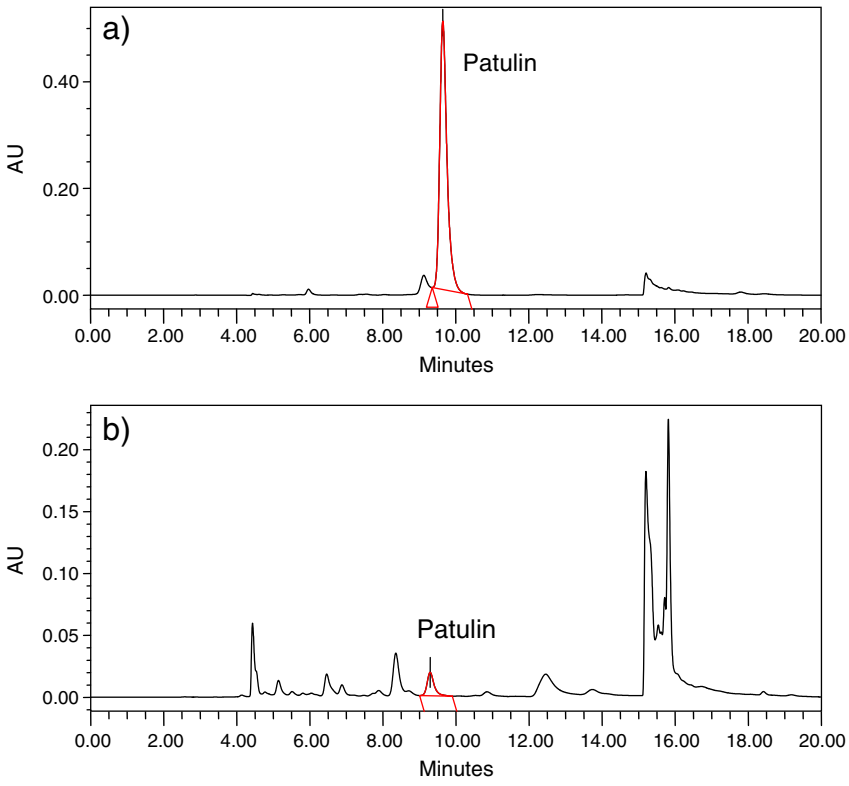

Fig. 3. Chromatograms of *P. expansum*, a) control, and b) with NAR.

Table 1
Percentage of reduction in PAT accumulation with different flavanones against *P. expansum* and *B. fulva*.

	% of inhibition ± SD	
Flavanone	*P. expansum*	*B. fulva*
HES	95 ± 8.5	99 ± 7.3
PS	98 ± 3.7	99 ± 4.6
P	97 ± 5.6	98 ± 11.7
NEO	99 ± 0.5	99 ± 10.3
HG	99 ± 0.2	99 ± 7.5
HGL	99 ± 1.0	98 ± 4.5
PL	99 ± 0.6	98 ± 9.4
PD	99 ± 2.0	99 ± 5.8
PB	97 ± 5.5	99 ± 12.9
NAR	96 ± 6.6	99 ± 4.3

Percentage of reduction PAT accumulation against control without any flavanone; SD: standard deviation. All values are not significantly different ($p > 0.05$) according to Bonferroni's multiple range test. HES (hesperidin) PS (prunin 6″-O-stearoyl ester), P (prunin), NEO (neohesperidin), HG (hesperetin glucoside), HGL (hesperetin glucoside 6″-O-lauroyl ester), PL (prunin 6″-O-lauroyl ester), PD (prunin 6″-O-decanoyl ester), PB (prunin 6″-O-butyryl ester), NAR (naringin).

accumulation. As there were no significant differences between the flavanones or tested molds, no further discussion could be made about the structure of these substances or fungal strain variation on the PAT inhibition. This possible activity against PAT of these substances opens an interesting option for the citrus industries. As it was shown, there is a high possibility that working with mixtures and higher concentrations of these substances could totally inhibit PAT. So, further experiments should be continued in order to evaluate whether the conditions tested adapt to different food matrices.

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