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Plant extracts as biocontrol agents against *Aspergillus carbonarius* growth and ochratoxin A production in grapes

Wiem Chtioui [a,b], Sandrina Heleno [b,c,*], Quirico Migheli [a,d], Paula Rodrigues [b,c,*]

[a] *Dipartimento di Agraria, Università degli Studi di Sassari, Via E. De Nicola 9, 07100, Sassari, Italy*
[b] *Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253, Bragança, Portugal*
[c] *Laboratório Associado para a Sustentabilidade e Tecnologia em Regiões de Montanha (SusTEC), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253, Bragança, Portugal*
[d] *Nucleo di Ricerca sulla Desertificazione, Università degli Studi di Sassari, Via E. De Nicola 9, 07100, Sassari, Italy*

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ABSTRACT

Aspergillus carbonarius (Bainier) Thom. is an important pathogen and ochratoxin A (OTA) producer in grapes that can be controlled by adopting sustainable approaches. Here we evaluate the application of natural plant extracts as an alternative to synthetic fungicides to reduce OTA contamination and to prevent infection of grapes by two isolates of *A. carbonarius*. In a preliminary screening, natural extracts of chestnut flower, cistus, eucalyptus, fennel, and orange peel were evaluated for their antifungal and anti-mycotoxigenic efficiency in a grape-based medium at concentrations of 10 and 20 mg/mL. Cistus and orange peel extracts demonstrated the best anti-fungal activity at both concentrations. Although the eucalyptus extract demonstrated no significant effect on *Aspergillus* vegetative growth, it significantly reduced OTA by up to 85.75 % at 10 mg/mL compared to the control. Chestnut flower, cistus, eucalyptus, and orange peel extracts were then tested at the lowest concentration (10 mg/mL) for their antifungal activity in artificially inoculated grape berries. The cistus and orange peel extracts demonstrated the greatest antifungal activity and significantly reduced mold symptoms in grapes. Moreover, all tested natural extracts were able to reduce OTA content in grape berries (17.7 ± 8.3 % - 82.3 ± 3.85 % inhibition), although not always significantly. Eucalyptus extract was particularly efficient, inhibiting OTA production by both strains of *A. carbonarius* by up to >80 % with no effects on fungal growth. The use of natural eucalyptus extract represents a feasible strategy to reduce OTA formation without disrupting fungal growth, apparently maintaining the natural microbial balance, while cistus and orange peel extracts appear promising as inhibitors of *A. carbonarius* mycelial growth. Our findings suggest that plant extracts may be useful sources of bioactive chemicals for preventing *A. carbonarius* contamination and OTA production. Nonetheless, it will be necessary to evaluate their effect on the organoleptic properties of the grapes.

1. Introduction

Ochratoxin A (OTA) is ranked among the five most common and harmful mycotoxins in agriculture (Malir et al., 2016). *Aspergillus* and *Penicillium* species are the main producers of OTA (Wang et al., 2016) and contaminate a number of foodstuffs including grapes and their derivatives (Gil-Serna et al., 2018; Mondani et al., 2020; Ortiz-Villeda et al., 2021; Zimmerli and Dick, 1996). Grapes contribute significantly to human nutrition and are valued for their sensory properties as well as for the vitamins and bioactive compounds (*e.g.*, flavonoids) they contain (FAO-OIV, 2016; Sabra et al., 2021). However, wine and grape juice are ranked after cereals as the second greatest sources of dietary exposure to

OTA (Kizis et al., 2021; Li et al., 2021).

Mycotoxin contamination of grapes typically begins in the vineyard (Tini et al., 2020). *Aspergillus carbonarius* (Bainier) Thom. (and *Aspergillus niger* Tiegh., which produces less OTA) is a major source of OTA contamination in grapes grown in Mediterranean countries due to the ability to grow effectively and to produce significant quantities of toxins at high temperatures (Battilani et al., 2006; Bellí et al., 2006; Cabañes et al., 2002; Lasram et al., 2007). In the current context of climate change, it is predicted that OTA contamination in grapes will increase due to the interaction between temperature and high levels of atmospheric CO_2 (Cervini et al., 2021).

OTA exposure is a major health concern (Bui-Klimke and Wu, 2015;

Stoev, 2022). Data from the European Food Safety Authority (EFSA) most recent risk assessment indicated OTA as a cause of kidney cancer and damage to DNA (Panel on Contaminants in the Food Chain et al., 2020). Based on toxicological and exposure data, the European Commission has set maximum thresholds for the presence of OTA in wine, fruit wine, grape juice, nectar, and must for human consumption (2 µg/kg), and in dried vine fruits (10 µg/kg) (European Commission, 2006).

Fungicides have long been used to reduce fungal proliferation and mycotoxin production, and viticulture is one of the agricultural systems that most frequently uses spraying (Bouagga et al., 2019; Komárek et al., 2010). However, the European Commission is increasing the restrictions on the number of pesticide applications (European Commission, 2009), and on the maximum amount of copper-based fungicides in organic farming (European Commission, 2018). In this context, there is an urgent need for sustainable approaches to manage toxigenic fungi at pre- and postharvest phases that can replace or supplement synthetic fungicides (Ponsone et al., 2012).

Plant extracts are a promising tool for controlling fungal contamination in food commodities (Chen et al., 2019; Makhuvele et al., 2020). In addition to being naturally abundant, they are easily biodegradable and have no negative environmental impacts (da Cruz Cabral et al., 2013). Unlike pure molecules, plant extracts include a wide variety of phenolic compounds and terpenes with distinct modes of action that may trigger a synergistic or additive effect (Badr et al., 2022; Chtioui et al., 2022). Another advantage of plant extracts is that a blend of active compounds with diverse physiological targets can prevent fungal resistance to treatment (Fuentefria et al., 2018; Vaou et al., 2021).

Mediterranean flora provides a rich source of secondary metabolites, especially terpenoids and phenylpropanoids with antibacterial, antifungal and antioxidant characteristics (Alonso-Esteban et al., 2022; Barros et al., 2009; Xavier et al., 2021; Zalegh et al., 2021). For the last 20 years, a comprehensive chemical characterization of the Mediterranean flora, in particular from the northeastern region of Portugal, Trás-os-Montes, has been developed (http://sites.esa.ipb.pt/biochemcore/index.php/list-plants, accessed 17 september 2023). Numerous plant extracts have been tested and explored for their potential against human, animal and agricultural diseases, as well as for their potential as food additives. As a result of the enormous amount of data obtained, several plants or plant organs have been highlighted for their richness in bioactive compounds as well as for their availability as bioresidues from agriculture or food industries. Among these, European chestnut (*Castanea sativa* Mill.) male flowers, eucalyptus (*Eucalyptus globulus* Labill.) leaves, orange (*Citrus aurantium* var. *sinensis* L.) peels, rockrose (*Cistus ladanifer* L.) and fennel (*Foeniculum vulgare* Mill.) leaves have been selected for further studies based on their particularly high contents in bioactive compounds such as flavonoids, phenolic acids, tannins and organic acids, as well as their high antibacterial and antifungal potential as determined by *in vitro* assays (*e.g.* Alaya et al., 2021; Barros et al., 2013a, 2013b; Carocho et al., 2014; Caleja et al., 2015, 2019; Fernandes et al., 2022; Gomes et al., 2018; Martins et al., 2015; Pinho et al., 2014). Moreover, all these plants or plant parts are widely available in the region as bioresidues or as natural or cultivated plants, and their incorporation in the value chain is of high significance for the region from both an environmental and an economic point of view.

The goal of this study was to explore the properties of aqueous extracts from a range of selected Mediterranean plants when used as biofungicides and OTA inhibitors. Extracts of chestnut flowers, cistus, eucalyptus, fennel, and orange peel were prepared at ambient temperature using an inexpensive and eco-friendly extraction procedure. This work was strongly motivated by concerns related to the environment and circular economy.

2. Methodology

2.1. Biological material

2.1.1. Plant material and preparation of extracts

This study used five natural sources from wild and farmed plants, namely: male flowers of European chestnut, rockflower, orange peel, eucalyptus, and fennel. The origin and botanical data of the plant species used are summarized in Fig. 1 and Table 1.

All the selected matrices were previously characterized by our research group and chosen based on their chemical composition, namely the richness in bioactive compounds with strong antimicrobial properties: i) *E. globulus* leaves are sources of phenolic compounds (173 ± 4 mg/g extract, dry weight), highlighting the presence of digalloyl-glucoside (30.5 ± 1.2 mg/g extract), 5-*O*-caffeoylquinic acid (22.3 ± 0.3 mg/g extract), trigalloyl-glucoside (12 ± 1 mg/g extract), and eucaglobulin/Globulusin B (13.9 ± 0.4 mg/g extract) (Gomes et al., 2018); ii) *C. sativa* male flowers are also rich in phenolic compounds (68.10 ± 1.52 mg/g), namely trigalloyl-HHDP-glucose (28.73 ± 1.34 mg/g), pedunculagin isomer (bis-HHDP-glucose) (7.68 ± 0.11 mg/g) (Carocho et al., 2014); iii) *C. sinensis* peels, is a bioresidue with high contents in citric acid (64 ± 2 mg/g) (Fernandes et al., 2022); iv) *F. vulgare* leaves are sources of phenolics (29.76 ± 0.73 mg/g), being quercetin-3-*O*-glucuronide, 1,5-Di-*O*-caffeoylquinic acid, malonyl di-*O*-caffeoylquinic acid, and 5-*O*-caffeoylquinic acid the major compounds (8.81 ± 0.07, 3.84 ± 0.08, 2.48 ± 0.14, 4.54 ± 0.15 mg/g, respectively) (Caleja et a., 2015); v) *C. ladanifer* leaves present punicalagins as the major compounds in the phenolic extract: punicalagin isomer 1, punicalagin gallate 1, punicalagin isomer 2, punicalagin gallate 2 (5.90 ± 0.15, 7.89 ± 0.29, 7.90 ± 0.19, 8.10 ± 0.31 mg/g, respectively) (Barros et al., 2013b).

Aqueous extracts were prepared from the various plant materials. Plants were collected or purchased fresh, and shade-dried at room temperature. They were powdered using a kitchen mill (Moulinex). The powder from each plant material (1 g) was extracted by swirling with 30 mL of distilled water at 150 rpm for 1 h at room temperature. Subsequently, it was filtered through Whatman no. 4 filter paper. Then, 30 mL of water was added to re-extract the residue. The final extracts were frozen and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA; collector chamber at −50 °C and 0.012 Torr).

Aqueous extracts were prepared at concentrations of 10 mg/mL and 20 mg/mL for subsequent assays by dissolving the lyophilized extracts in 5 % of dimethyl sulfoxide (DMSO) (Merck KGaA, Germany), followed by dilution in water to obtain the final concentration. Test concentrations were established after a preliminary microdilution susceptibility test (Svobodova et al., 2017) against *A. carbonarius* MUM 04.46 and MUM 04.52 (see below). *Aspergillus fumigatus* Fresen. (ATCC 204305) and *Aspergillus brasiliensis* Varga, Frisvad & Samson (ATCC 16404) were used as reference fungi (data not shown).

2.1.2. Fungal isolates

Two strains of *A. carbonarius*, MUM 04.46 and MUM 04.52, respectively coded Ac46 and Ac52 in this study, were provided by the fungal culture collection "Micoteca da Universidade do Minho (MUM)", Braga, Portugal. These were originally isolated from Portuguese grapes and confirmed as OTA producers (Serra et al., 2003). The fungi were kept at −20 °C in 20 % glycerol and cultivated on potato dextrose agar (PDA; Biolife, Italy). Whenever needed, the isolates were cultivated in PDA for 5 to 7 d at 25 °C in the dark. Immediately before the assays, spore suspensions of each strain were obtained by scraping the top of a 5- to 7-day-old fungal culture, then diluted in 3 mL of sterile water containing 0.05 % Tween 80. The spore concentrations were adjusted as needed using a Neubauer counting chamber.
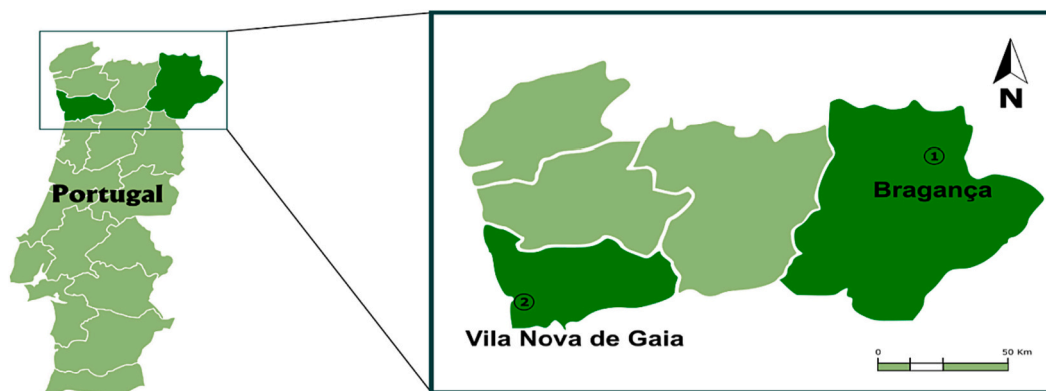
Fig. 1. Sampling area of the plants used in the study: 1) Bragança (chestnut flower, cistus, eucalyptus, and orange); 2) Vila Nova de Gaia (fennel).

Table 1
Plant material used.

Scientific name (Family)	English common name	Source	Plant organ
Castanea sativa Mill. (cv. Judia) (Fagaceae)	Sweet chestnut or European chestnut	Samil, Bragança	Male flowers
Cistus ladanifer L. (Cistaceae)	Gum rockrose, laudanum, labdanum	Orchards, Bragança	Leaves
Citrus aurantium var. sinensis L. (Rutaceae)	Orange	Local market, Bragança	Peel
Eucalyptus globulus Labill. (Myrtaceae)	Eucalyptus or Tasmanian blue gum	Campus of the Polytechnic Institute of Bragança	Leaves
Foeniculum vulgare Mill.	Fennel	Company "Cantinho das Aromáticas", Vila Nova de Gaia	Leaves

2.2. Screening the in vitro effect of the extracts on A. carbonarius growth and OTA production in a grape-based medium

2.2.1. Preparation of the grape-based medium

To simulate the grape matrix, a grape-based medium was prepared from cv. Touriga Franca (red wine cultivar) berries from an orchard in Vila Real, northern Portugal. The medium was created by rinsing berries with 1 % sodium hypochlorite for 1 min, washing them twice with distilled water, and then blending at a low speed to obtain a homogeneous juice. Next, the pH was adjusted to 3.5 with 10 % tartaric acid to ensure proper solidification, and agar was added to achieve a final concentration of 2 %. The medium was autoclaved at 121 °C for 15 min. The cooled medium was then combined with an equal volume of the autoclaved (10 min, 110 °C) aqueous plant extracts (final grape concentration: 50 %, *v*/v) to obtain two final concentrations: 10 mg/mL and 20 mg/mL. Grape juice amended with sterile water was used as a negative control. Additionally, a fungicide control consisted of a mixture containing the commercial formulate Teldor® (active substance fenhexamid, 50 % a.i. (*w*/w); Bayer CropScience, Portugal), which is generally applied as a field fungicide to grapevine at the recommended concentration of 1.5 g/L (fenhexamid 0.75 g/L). The fungicide was added to the medium at the concentration of 0.75 mg/mL of a.i., to reproduce the concentrations recommended in field applications.

The mixtures were then homogenized, and 3 mL of each mixture was pipetted into 12-well plates, in triplicate.

2.2.2. Inoculation and incubation

Spore suspensions of Ac46 and Ac52 at 10^5 spores/mL were used as inoculum. Two μL of inoculum were pipetted into the center of each well containing the different media, and the plates were then incubated in the dark at 25 °C. The fungal colonies were examined after 5 and 10 d of growth to assess the efficiency of each treatment. Three replicates of each treatment were evaluated.

2.2.3. Observation of growth

This assay evaluated fungal growth qualitatively according to inhibition activity (++ = high inhibiting activity, + = inhibiting activity, − = no inhibiting activity) after incubation for 5 and 10 d, compared with the negative control (grape medium without plant extracts).

2.2.4. OTA analysis

After 10 d of incubation, OTA extraction was performed on all cultures. Fungal mycelium and grape medium from the three replications of each treatment were removed from the wells and weighed. OTA was extracted with methanol for 60 min in the dark by mixing every 15 min. The extract was cleaned using an OTA Immunoaffinity column (IAC; Ochratest™, VICAM, Milford, USA) following the manufacturer's instructions, and filtered with a 0.22 μm polytetra-fluorethylene (PTFE) membrane (FiltraTech, Saint Jean de Braye, France). OTA was quantified using the HPLC methodology described below.

2.3. Effect of selected extracts on fungal growth in 9 cm petri dishes

Quantitative growth assessment assays were carried out in 9 cm Petri dishes containing 20 mL of grape-based medium prepared as described above. For this assay, only the four extracts and extract concentrations that showed the best results in the *in vitro* screening assay were selected and tested. Thus, the plant extracts of chestnut flower, eucalyptus, cistus, and orange peel were tested at a concentration of 10 mg/mL. Similarly, fenhexamid (0.75 mg/mL) was used as a positive control, while Petri dishes containing a water-added medium were used as a negative control. Subsequently, 2 μL of 10^5 spores/mL of conidial suspension of Ac46 and Ac52 was deposited in the center of each Petri dish and incubated in the dark at 25 °C. All tests were run in triplicate. The colony diameter was measured daily in two perpendicular directions to determine the maximum growth rate (μ*m*, in cm of radius/day), which was obtained as the slope of the line of the linear regression of colony radii plotted against the incubation time.

2.4. Effects of the selected extracts on fungal growth and OTA production in grapes

2.4.1. Preparation of grape berries

Healthy mature grapes (cv. Touriga Franca) of similar size and showing no signs of mechanical or fungal damage were selected. Berries were surface disinfected with 1 % sodium hypochlorite for 1 min, rinsed twice with sterile distilled water, and air-dried on a laminar flow bench. Subsequently, a single wound (3 mm deep) was made using a sterile needle in the equatorial region of each berry. Thereafter, berries were

immersed for 3 min in the selected extracts: chestnut flower, cistus, eucalyptus, and orange peel extracts (10 mg/mL), and in fenhexamid (0.75 mg/mL) as a control. The four extracts and the concentration used in this assay were selected based on the best results obtained in the *in vitro* screening.

After 2 h, the wound on each berry was inoculated with 10 μL of spore suspension (10^5 spores/mL) of each *A. carbonarius* strain. Wounded berries immersed in sterile water and inoculated after 2 h with a conidial suspension of *A. carbonarius* were used as negative controls. Each treatment consisted of 10 berries, and each experiment was repeated three times. Berries were air-dried and then placed in plastic holders (60 cm × 40 cm × 15 cm, one layer), wrapped in transparent polyethylene foil to avoid evaporation, and incubated for 10 days at 25 °C in the dark.

2.4.2. Fungal growth and disease symptoms in grape berries

Following incubation, fungal growth, and fruit rot were evaluated for each berry by estimating the percentage of its surface area presenting signs of fungus growth and symptoms of spoilage. A score from 0 to 4 was assigned to the percentage of berry area presenting symptoms: 0 = 0 % with symptoms, 1 = 1–25 %, 2 = 26–50 %, 3 = 51–75 %, and 4 = 76–100 %. Subsequently, the infection severity index (McKinney's index), which incorporates the incidence and the severity of the disease, was calculated according to the following formula:

I = [Σ (d × f)/(N × D)] × 100.

where d is the category of the disease intensity scored for the grape bunches, f is the disease frequency, N is the total number of berries examined, and D is the highest category of disease intensity that occurred on the empirical scale (McKinney, 1923).

2.4.3. Quantification of OTA production in grape berries

OTA was extracted from grapes according to Serra et al. (2004). After 10 days of fungal growth, 10 g of previously homogenized berry tissue from each treatment was transferred into a 50 mL Falcon tube and brought up to 30 mL using a solution of 5 % $NaHCO_3$ and 1 % PEG 8000. The mixture was vortexed every 15 min for 1 h and then centrifuged at 8500 rpm for 10 min at 4 °C. The supernatant was filtered through a glass microfiber filter (1.5 μm pore size, Whatman), and 10 mL of this filtrate was passed through the Ochratest IAC for cleaning. OTA was then eluted with methanol and passed through a 0.22 μm PTFE syringe filter before HPLC analysis.

2.5. OTA analysis by HPLC

OTA was analyzed using a High-Performance Liquid Chromatography (HPLC) Smartline Pump 1000 (Knauer, Berlin, Germany) coupled with a fluorescence detector FP-2020 (Jasco, USA). A C18 reverse-phase column PLRP-S 300 Å (250 × 4.6 mm, 8 μm, Polymer Laboratories, Church Stretton, UK) was used at 35 °C. The mobile phase consisted of water: acetonitrile: acetic acid (29.5:70:0.5), and was pumped in an isocratic mode at 0.8 mL/min. The injection volume was 20 μL. OTA was detected at 330 nm (excitation) and 463 nm (emission), with a run time of 15 min. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as follows:

LOD = 3 × (sa/b).

LOQ = 10 × (sa/b).

where *sa* is the standard deviation of the regression line obtained from the calibration curve and *b* is the slope of the line (Taverniers et al., 2004). The LOD and LOQ were 3 and 9 ng/mL, respectively.

2.6. Statistical analysis

Data were analyzed using R software (R Core Team, 2020). The investigated dependent variables (fungal growth, OTA production, McKinney index) were analyzed using the linear regression model below:

Yij = μ + Ti + Fj + eij.

where μ is the overall mean, Ti is the fixed effect of the treatment, Fj is the fixed effect of the fungi and eij is the random residual/error.

The results obtained from OTA production, fungal growth and the McKinney index were tested for normality using the Shapiro test. Since all dependent variables were not normally distributed, a non-parametric Kruskal- Wallis and a *post hoc* Dunn test were also performed. Statistical significance was declared when $p \leq 0.05$.

3. Results

3.1. Screening the in vitro effect of plant extracts on A. carbonarius growth and OTA production in the grape-based medium

The five natural extracts (*i.e.*, chestnut male flower, cistus, eucalyptus, fennel, and orange peel) were tested at concentrations of 10 and 20 mg/mL for their antifungal activity in a grape-based medium. Fig. 2 shows fungal growth for Ac52 as affected by the antifungal preparations.

After 5–10 d of incubation, neither chestnut flower nor eucalyptus or fennel inhibited mycelial growth for either *A. carbonarius* strain when compared to the untreated control. On the other hand, orange peel and cistus extracts at both 10 and 20 mg/mL inhibited mycelial growth compared to the untreated control. When the extract concentration was increased, little to no variability was observed in rates of mycelial growth inhibition. Fenhexamid (0.75 mg/mL) also inhibited fungal growth after 5 days of growth, but not at day 10.

The amendment of the grape-based medium with the different extracts caused a macroscopic change in the *A. carbonarius* morphology when compared to the control. The extracts that inhibited radial growth also resulted in lower sporulation. Moreover, a difference in mycelial pattern was observed between Ac46 and Ac52 for the chestnut flower, cistus, eucalyptus, and fennel extracts.

3.2. Effects of selected extracts on OTA production in the grape-based medium

The two tested *A. carbonarius* strains differed significantly in their ability to produce OTA (Table 2). For Ac46, OTA was reduced significantly from 71.9 ± 9.2 ng/mL of medium (control) to 10.3 ± 2.7 ng/mL (86 % reduction) when the grape medium was amended with eucalyptus at 10 mg/mL and to 14.8 ± 1.0 ng/mL (80 % reduction) when the grape medium was amended with eucalyptus at 20 mg/mL. Similarly, amendment of the medium with 10 mg/mL of chestnut flower significantly reduced OTA to 13.0 ± 3.8 ng/mL (82 % reduction). In contrast, the addition of cistus and orange peel extracts at 20 mg/mL activated OTA accumulation for Ac46 compared to the non-treated control (Table 2).

Regarding Ac52, of the natural plant extracts, cistus and eucalyptus reduced the OTA rate by up to 57.58 % when used at 10 mg/mL and up to 62 % at 20 mg/mL. However, the reduction in OTA was not significant in either case. In contrast, fennel (10 mg/mL and 20 mg/mL) treatments and orange peel (20 mg/mL) significantly activated OTA accumulation in the grape medium.

3.3. Growth assessment

Fig. 3 shows the effect of the different treatments (plant extracts at 10 mg/mL and fenhexamid at 0.75 mg/mL) on Ac46 and Ac52 with regard to growth assessment, compared to the control (water). Concerning the growth rate (μm), the natural extracts of cistus, and orange peel significantly slowed both Ac46 and Ac52 values. Similarly, fenhexamid significantly reduced μm, whereas no significant effects on fungal growth were observed for chestnut flower and eucalyptus extracts.
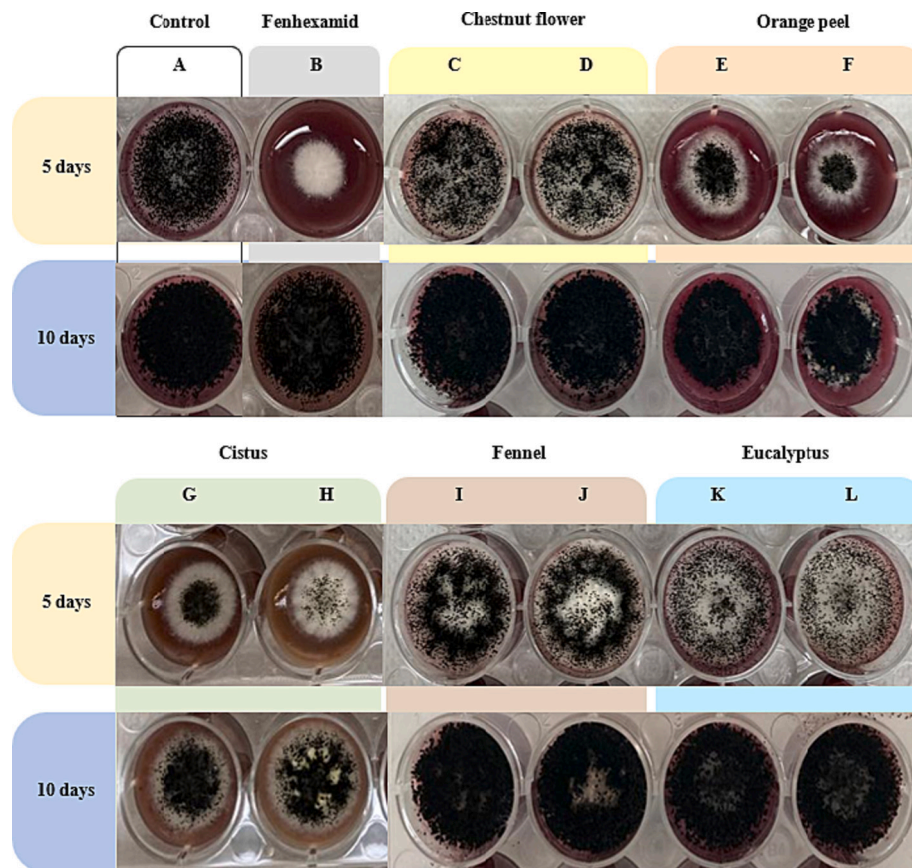
Fig. 2. Morphological aspect of *A. carbonarius* MUM 04.52 in the grape-based medium at 25 °C after 5 (top) and 10 (bottom) days of growth: (A) control; (B) fenhexamid (0.75 mg/mL), (C) male chestnut flower, 10 mg/mL; (D) male chestnut flower, 20 mg/mL; (E) orange peel, 10 mg/mL; (F) orange peel, 20 mg/mL; (G) cistus, 10 mg/mL; (H) cistus, 20 mg/mL; (I) fennel extract, 10 mg/mL; (J) fennel, 20 mg/mL; (K) eucalyptus, 10 mg/mL; (L) eucalyptus, 20 mg/mL.

3.4. Effect of plant extracts on fungal growth in grapes

Based on the *in vitro* results, the natural extracts of chestnut flower, cistus, eucalyptus, and orange peel were selected for evaluation of their ability to modulate *Aspergillus* symptoms in grape berries. In this assay, all plant extracts were tested at 10 mg/mL.

The relative levels of rot severity of the berries treated with the different plant extracts and with the fungicide, as determined by the McKinney index, are shown in Fig. 4, and expressed as percentages in comparison to the non-treated control. The McKinney index of the negative control was 69 % ± 1 % for Ac46 and 68 % ± 9 % for Ac52. No significant difference was detected between the two MUM strains. The orange peel and cistus treatments significantly reduced rot symptoms for Ac46 and Ac52 compared to the untreated grapes, with inhibition levels that ranged between 24 % and 33 %. Chestnut flower extract reduced *Aspergillus* in grape berries significantly (by 33 %) when applied for Ac52 but had no significant effect on Ac46. Eucalyptus extract had no significant effect on berry infection by Ac46 or Ac52 (Fig. 4).

3.5. Effect of plant extracts on OTA production in grape berries

After 10 days of incubation, no significant difference was found between the two *A. carbonarius* strains grown on artificially inoculated berries ($p = 0.1$) and OTA contamination observed on the non-treated grape berries (117.3 ± 60.4 ng/g for Ac46 and 63.5 ± 19.5 ng/g for Ac52).

All treatments reduced the OTA production for both *Aspergillus* strains. Eucalyptus, orange peel, and cistus extracts caused a significant decrease in OTA for Ac46, with inhibition percentages that ranged from 67.7 % to 82.3 %. In contrast, no natural treatment had a statistically

significant effect on OTA production by Ac52, and no significant difference was found between treatments ($p = 0.3$). Eucalyptus was found to give the highest inhibition rate for both *Aspergillus* strains (Fig. 5).

4. Discussion

Plant extracts, essential oils, and phenolic compounds are of interest to researchers as possible control agents against a variety of fungi. The aim of this study was to evaluate the potential of natural plant extracts to reduce OTA contamination and *Aspergillus* infection in grapes using an environmentally friendly extraction procedure.

A grape-based culture medium was used for the *in vitro* screening assay to simulate the growth and OTA production of two strains of *A. carbonarius* in grapes. Culture media prepared from food matrices have been reported as good model systems for the *in vitro* evaluation of fungal growth and mycotoxin production (Pardo et al., 2005), and they have been frequently used in studies involving major OTA-producing species, such as *A. niger* (Astoreca et al., 2009), *A. carbonarius* (Cervini et al., 2021), *Aspergillus ochraceus* G. Wilh. (Pardo et al., 2005), *Aspergillus westerdijkiae* Frisvad & Samson (Álvarez et al., 2023; Meftah et al., 2018; Vipotnik et al., 2017), and *Penicillium nordicum* Dragoni & Marino (Meftah et al., 2018; Vipotnik et al., 2017).

When tested *in vitro*, both orange peel and cistus extracts showed an antifungal effect on *A. carbonarius* at both 10 and 20 mg/mL, but when tested in the fruit at 10 mg/mL, orange peel was the most promising extract for its capacity to inhibit *A. carbonarius* growth. The antifungal or antibacterial activity of extracts obtained from citrus plants has been frequently highlighted. Viuda-Martos et al. (2008) found that lemon, orange, mandarin, and grapefruit essential oils, obtained by cold-pressing the peel, reduced the growth of *A. niger* and *Aspergillus flavus*

Table 2

Ochratoxin A production by *A. carbonarius* MUM 04.46 and *A. carbonarius* MUM 04.52 in grape medium supplemented with natural extracts (chestnut flower, cistus, eucalyptus, fennel, and orange peel) and fenhexamid, expressed as a percentage compared with the control (mean ± standard deviation, $n = 3$).

Treatment	*A. carbonarius* MUM 04.46		*A. carbonarius* MUM 04.52	
	OTA concentration (ng/mL)	OTA variation (%)	OTA concentration (ng/mL)	OTA variation (%)
Control (water)	71.9 ± 9.2		790.9 ± 56.2	
Chestnut flower				
10 mg/mL	13.0 ± 3.8	−82 % **	810.6 ± 14.1	+9.1 %
20 mg/mL	64.6 ± 32.6	−10 %	1056.5 ± 122.7	+33.6 %
Cistus				
10 mg/mL	77.6 ± 6.9	+7.8 %	1024.6 ± 100.4	+29.6 %
20 mg/mL	141.1 ± 13.5	+96.2 %	300.2 ± 14.0	−62.0 %
Eucalyptus				
10 mg/mL	10.3 ± 2.7	−85.7 % **	469.1 ± 169.2	−40.7 %
20 mg/mL	14.8 ± 1.0	−79.5 % *	741.4 ± 156.1	−16.1 %
Fennel				
10 mg/mL	190.6 ± 6.1	+165.1 %	1907.0 ± 185.6	+141.1 % *
20 mg/mL	949.6 ± 88.7	+1220.2 %	2015.2 ± 407.6	+154.8 % *
Orange peel				
10 mg/mL	79.6 ± 2.5	−10.7 %	1238.5 ± 301.3	+56.6 %
20 mg/mL	3977.8 ± 326.3	+5430.2 %	2422.6 ± 353.8	+206.3 % *
Fenhexamid (0.75 mg/mL)	15.2 ± 1.2	−78.9 %	128.3 ± 5.3	−95.4 % ± 3 %

Significance (Kruskal-Wallis + *post hoc* Dunn test), significant differences with respect to the control were declared as following *($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$).

Link. Of these, orange essential oil was found to be the most effective inhibitor of *A. niger*, and mandarin was the most effective inhibitor of *A. flavus*. Here we report for the first time the antifungal activity of orange peel extract against *A. carbonarius*. Velázquez-Nuñez et al. (2013) attributed the antifungal properties of *Citrus sinensis* peel essential oil against *A. flavus* to the presence of limonene as the most important compound (96.62 %) of orange peel, followed by other molecules such as *β*-pinene, *β*-myrcene, *α*-pinene, and citral (*Z* and *E*). Limonene in the monoterpene form was also observed to have antifungal activities

against *A. niger* (Jing et al., 2014). The antifungal activity of orange polyphenolic extracts was also reported on *Monilinia fructicola* (G. Winter) Honey, *Botrytis cinerea* Pers. and *Alternaria alternata* (Fr.) Keissl. At a concentration of 1.5 mg/mL, orange extracts totally inhibited the mycelial growth and conidial germination of these fungi (Hernández et al., 2021).

Among the extracts studied in the present work, cistus was highly effective *in vitro* at 10 mg/mL and also reduced fungal growth *in vivo*. Our results agree well with the existing literature on cistus. Kalli et al. (2018) reported that the hydro-methanolic extract of cistus inhibited the growth of *Aspergillus parasiticus* Speare. by 46 % when applied at a concentration of 0.2 mg/mL. Barros et al. (2013b) described the antifungal activity of cistus phenolic extract against *Candida* species when used at 0.625 mg/mL; they attributed this activity to the presence of phenolic acids and derivatives, ellagic acid derivatives, and flavonoids, specially catechins, flavonols, and flavones.

Overall, aqueous plant extracts can be powerful antifungals against several molds, as reported in literature. For example, aqueous extracts prepared from chestnut flowers have been studied for their capacity to inhibit the growth of *A. parasiticus* in a nutraceutical formulation, due to the presence of phenolic compounds, namely hydrolisable tannins (Fernandes et al., 2020). The same was recorded for methanolic extracts of fennel seeds, observed to inhibit *Candida albicans* (C.P. Robin) Berkhout. and *Aspergillus clavatus* Desm. at 25 μg/mL (Agarwal et al., 2017). Eucalyptus hydromethanolic extracts were described as being powerful antifungal agents against *Candida* species at minimum inhibitory concentrations (MICs) ranging from $MIC_{50} = 0.1875$ mg/mL to 1.5 mg/mL (Martins et al., 2015). However, plant extracts can have a variable effect, depending on the matrix, fungal species and the extraction solvent (Akullo et al., 2022; García-Díaz et al., 2020; Lira-De León et al., 2014). It is also important to note that factors such as harvesting time, storage, and modification processes, among others, may have a substantial impact on the phytochemical content of plant extracts and, as a result, on their antifungal activity (Ali et al., 2018; ElNaker et al., 2021; Mandim et al., 2021; Shao et al., 2022).

With regard to OTA production, while conditions were the same for both *A. carbonarius* strains, Ac52 produced higher levels of mycotoxin *in vitro* than Ac46. The difference in mycotoxin production between different strains of the same fungal species grown in similar conditions is inherent to *Aspergillus* species and has been observed by other studies (Astoreca et al., 2009; Freire et al., 2018; Vipotnik et al., 2017).

The natural extracts showed a strain-dependent effect on OTA production: eucalyptus at 10 mg/mL was highly effective in controlling
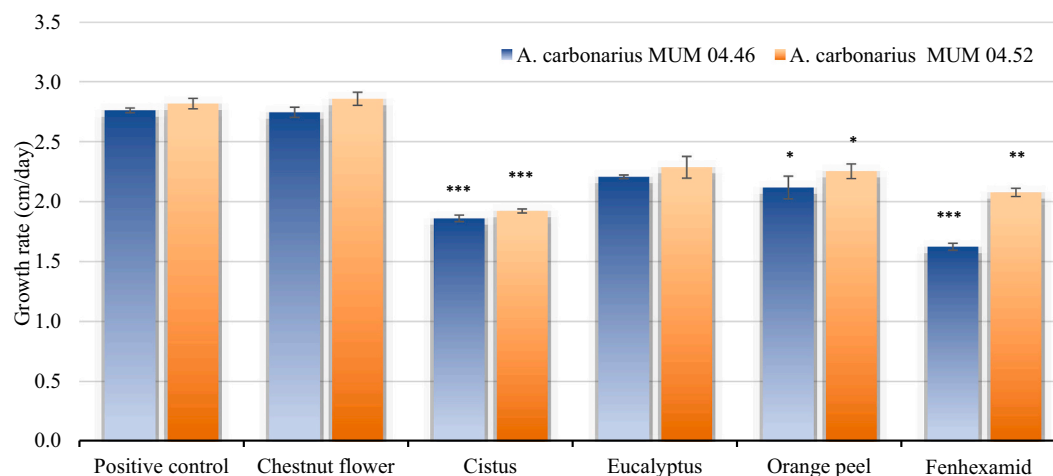
Fig. 3. Maximum growth rate (μm, cm/day) of *A. carbonarius* MUM 04.46 and *A. carbonarius* MUM 04.52 grown for 6 days at 25 °C in a grape-based agar medium with the addition of different natural extracts (chestnut flower, cistus, eucalyptus, and orange peel) at 10 mg/mL, and a commercial antifungal preparation (fenhexamid) at 0.75 mg/mL (mean of three replicas, error bars represent standard deviation). Statistical differences between the non-treated control and treatments are indicated as following *($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$).
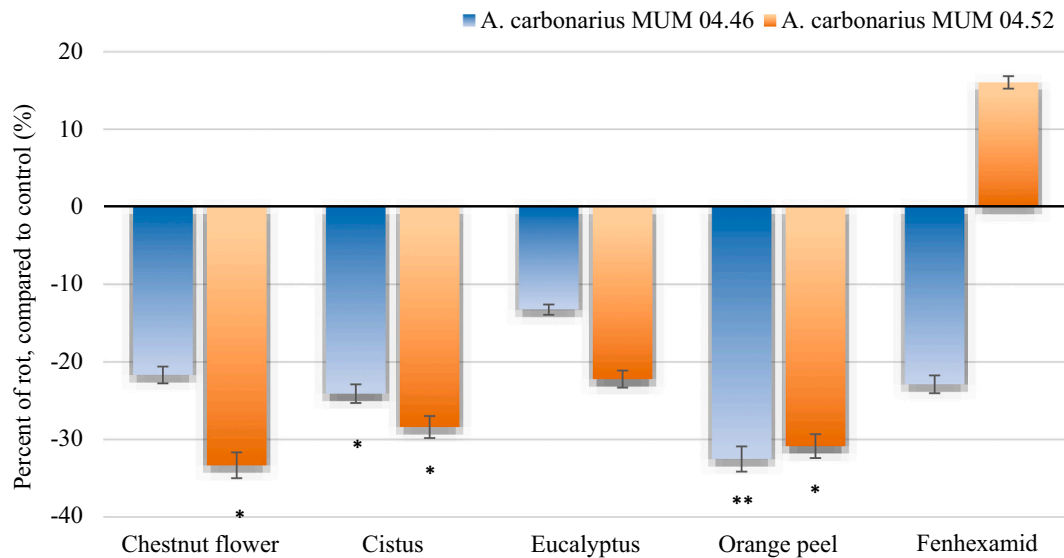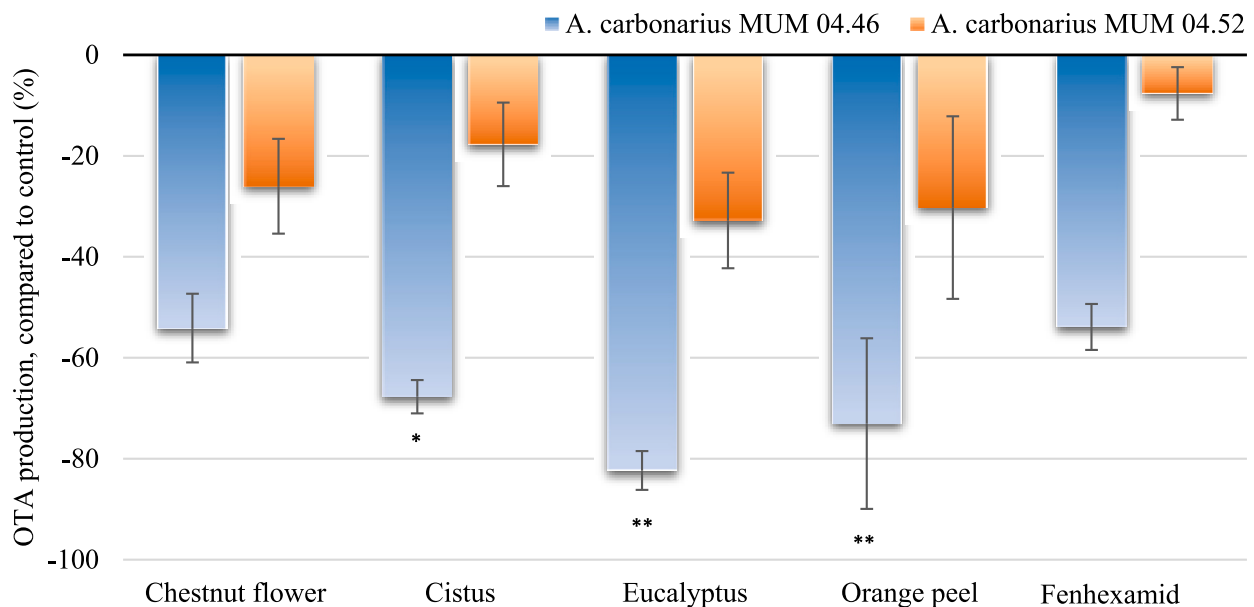
Fig. 4. Rot severity (McKinney Index) caused by *Aspergillus carbonarius* MUM 04.46 and *A. carbonarius* MUM 04.52 in grape berries (cv. Touriga Franca) immersed in natural extracts (chestnut flower, cistus, eucalyptus, and orange peel) at 10 mg/mL, and in a synthetic fungicide (fenhexamid 0.75 mg/mL), expressed as percentage compared to the control (mean of three replicas, error bars represent standard deviation). Statistical differences between the non-treated control and treatments are indicated as following *($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$).

Fig. 5. OTA production by *Aspergillus carbonarius* MUM 04.46 and *A. carbonarius* MUM 04.52 in grape berries (cv. Touriga Franca) immersed in natural extracts (chestnut flower, cistus, eucalyptus, and orange peel) at 10 mg/mL and in a synthetic fungicide (fenhexamid 0.75 mg/mL), expressed as a percentage compared to the control (mean of three replicas, error bars represent standard deviation). Statistical differences between the non-treated control and treatments are indicated as following *($p \leq 0.05$), **($p \leq 0.01$), ***($p \leq 0.001$).

OTA for Ac46, followed in order of decreasing effectiveness by chestnut flower, at both tested concentrations, whereas the most effective extracts for Ac52 were cistus, followed by eucalyptus.

The extracts obtained in the present study with inhibitory capacity against *A. carbonarius* are rich in phenolic compounds, which have been reported as the main bioactive agents in the obtained extracts acting through different mechanisms. For instance, phenolic compounds are able to enter the microbial membrane and get into the cell, causing a significant decrease in the synthesis of essential components such as ergosterol (the main component present in fungal membranes), glucosamine (a growth indicator), proteins, chitin, among others (Brul

and Klis, 1999). There are several mechanisms of action attributed to natural agents depending on their chemical class/chemical structure and include: i) the inhibition of cell wall formation or disruption of cell wall structures, both by inhibiting the synthesis of chitin, glucans or ergosterol; ii) interference in the mitochondria electron transport causing reduction in the membrane potential; iii) inhibiting the synthesis of RNA/DNA synthesis; iv) inhibition of efflux pumps (Lagrouh et al., 2017). The most solid mechanism of action attributed to phenolic compounds is effectively through the disruption of the fungal cell membrane in a concentration-dependent manner (Tian et al., 2012; Wu et al., 2008).

The capacity of plant extracts to modulate mycotoxin synthesis *in vitro* has long been documented. Ahmed et al. (2015) found that fenugreek seed extract inhibited OTA production significantly in grapes by *A. carbonarius*. Similarly, El EL Khoury et al. (2017) used *Salvia officinalis* L. and *Melissa officinalis* L. at 5 μL/mL (essential oil) against *A. carbonarius* in a grape-based medium, and observed OTA reductions of 25 % and 80 %, respectively. However, despite evidence of the potential effectiveness of plant extracts in reducing OTA rate, the *in vitro* effect of natural extracts on mycotoxin accumulation appears largely dependent on the fungal strain, even within the same species.

For example, cistus, fennel, and orange peel extracts activated OTA production by Ac46 in our *in vitro* experiments, while chestnut flower, fennel, and orange peel extracts activated OTA production by Ac52. Several studies and review papers have reported that, under certain conditions and at specific concentrations, phenolic compounds (Boonmee et al., 2020; Chtioui et al., 2022; Etzerodt et al., 2015; Gauthier et al., 2016; Ponts et al., 2011), essential oils (Dammak et al., 2019; Lorán et al., 2022; Prakash et al., 2010) or even plant extracts (Garcia et al., 2011) can activate mycotoxin production.

However, in our study this was observed only in the *in vitro* trial, while all the extracts reduced mycotoxin production in grape berries, regardless of the strain. Although synthetic media are considered a good representation of food matrices (Pardo et al., 2005), they present a uniform distribution of nutrients that makes them easily accessible to fungi. Consequently, the interaction of the fungus with the synthetic media may differ from its behavior in grape berries. The differences in nutrient distribution and water availability between *in vivo* and *in vitro* conditions may also cause differences in mycotoxin production by *Aspergillus* (Maor et al., 2021). What may be an even more important factor is that fresh fruits continue their physiological activity after harvest, so post-harvest treatments can still activate fruit defense mechanisms to combat infection, namely by increasing defense-related enzymes and metabolites (Li et al., 2019; Li et al., 2022; Zixun et al., 2020). This might mean that *in vitro* models are less representative of the *in vivo* conditions for fresh fruits than for other types of food products, especially processed products.

Our findings demonstrate that all extracts reduced OTA production in grapes when applied at 10 mg/mL, with eucalyptus being particularly efficient. The reduction of OTA by orange peel extract may be correlated to the reduction of mycelial growth in grape berries. On the contrary, the eucalyptus extracts reduced OTA production by up to 82.3 % without significantly affecting fungal growth, which suggests that the mechanism of OTA inhibition could be distinct from that of mycelial inhibition. Similar results were reported by Bluma et al. (2008), who found that eucalyptus essential oil had no effects on mycelial growth or on spore germination in *Aspergillus* section *Flavi,* although it significantly reduced aflatoxin production. Our results are also in line with the study of Vilela et al. (2009), who reported an anti-aflatoxigenic effect of eucalyptus on *A. flavus* and *A. parasiticus*. However, to the best of our knowledge, ours is the first report demonstrating the anti-OTA activity of *E. globulus* extract on *A. carbonarius* in grapes and in a grape-based medium.

Teixeira et al. (2019) characterized the phenolic composition of eucalyptus aqueous extract collected from the same sampling area in Bragança, Portugal. They identified seven flavonoids (quercetin, isorhamnetin, and myricetin derivatives), three phenolic acids (chlorogenic acid and ellagic acid derivatives), and eight gallotannin derivatives. While quercetin is a powerful antimicrobial reported to alleviate OTA toxicity (Yang et al., 2020), chlorogenic acid is involved in the mechanism of cereal resistance to *Fusarium* and its deoxynivalenol detoxification (Atanasova-Penichon et al., 2012; Gauthier et al., 2016), and ellagic acid, together with ascorbic acid and α-tocopherol, is regarded as a major antioxidant molecule in plants (Ratnam et al., 2006; Sharifi-Rad et al., 2022). However, regardless of the plant extract and phenolic compound, the precise mechanisms behind ochratoxin inhibition are not fully unveiled. One hypothesis is that inhibition is linked to the disruption of the fungal membrane *via* a modification in its charge, hydrophobicity or porosity. Another hypothesis is that the antioxidant activity of the extract and its phenolic compounds reduces oxidative stress and therefore OTA production. Finally, plant extract and phenolic compounds might induce a downregulation in the key genes involved in OTA biosynthesis (Boonmee et al., 2020).

Our main findings are that orange peel and cistus can be efficient antifungals against *A. carbonarius* strains, given their contents in organic acids (citric acid) and phenolic compounds (punicalagins) described in these matrices. Their ability to modulate fungal growth is of environmental interest. Orange peel is considered an agricultural waste (Farhat et al., 2011) and cistus is a Mediterranean shrub highly tolerant to drought and to poor soils (Zalegh et al., 2021). Their reuse as a natural fungicide could promote a circular economy and sustainability.

On the other hand, eucalyptus is an efficient botanical extract to use for OTA reduction. This is extremely important for preventing OTA contamination in vineyards and in harvested grapes. Therefore, the use of eucalyptus extract could provide a low-cost and environmentally friendly means of limiting OTA formation without disrupting fungal growth, apparently preserving the natural fungal balance.

It is, however, possible that using eucalyptus on wine grapes may affect their organoleptic properties. To this regard, a winery-scale study by González-Rompinelli et al. (2013) tested eucalyptus as an alternative to sulfur dioxide for use during the aging of white wines in oak barrels, and found that the addition of eucalyptus phenolic extracts had no effect on the wine's organoleptic properties. Also, the use of chestnut flowers has been patented as a natural substitute of sulfites in wines (Patent No. WO2017212351A1). According to the results of their study, chestnut flowers had no negative effect on the wine's organoleptic properties, but actually increased the wine's flavor, which is usually affected by the presence of sulfites (Ferreira et al., 2016).

Nonetheless, further research will be required to investigate whether the selected plant extracts have any effect on the organoleptic properties of grapes.

5. Conclusions

The current study represents progress toward the production of environmentally acceptable plant-based fungicides to control *A. carbonarius* and OTA contamination in vineyards. Orange peel and cistus showed high efficiency as antifungals against *A. carbonarius* growth, while eucalyptus was the botanical extract with higher potential for OTA reduction. This is extremely important for preventing OTA contamination in vineyards and in harvested grapes. In particular, the use of eucalyptus extract could provide a low-cost and environmentally friendly means of limiting OTA formation without disrupting fungal growth, thereby preserving the beneficial mycoflora. More and more readily available natural bioresidues capable of being produced as marketable formulation are being sought as alternatives to current synthetic fungicides, and aqueous eucalyptus extract showed to be a strong candidate.

Further research will elucidate the identity of the active compounds responsible for the reported antifungal and anti-mycotoxin effects of these extracts and produce a better understanding of their mode of action. This should enable the enhancement of the plant extracts' efficacy.

CRediT authorship contribution statement

Wiem Chtioui: Investigation, Conceptualization, Formal analysis, Methodology, Validation, Data curation, Software, Visualization, Writing – original draft, Writing – review & editing. **Sandrina Heleno:** Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Quirico Migheli:** Writing – review & editing, Supervision. **Paula Rodrigues:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Data availability

Data will be made available on request.

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