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Antimicrobial properties of tannin extracts against the phytopathogenic oomycete *Plasmopara viticola*

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ABSTRACT

Downy mildew (*Plasmopara viticola*) is one of the most devastating diseases for European grapevines (*Vitis vinifera* L.), requiring extensive use of copper-based or synthetic pesticides. Due to the negative effects of these products (*e.g.*, impacts on environment and human health, acquisition of pathogen resistance), there is a crucial need to develop alternative strategies for controlling downy mildew in the vineyard. Tannins are compounds that are directly toxic to diverse microorganisms, but little is known about their antimicrobial activities against grapevine pathogens. The aim of our study was therefore to explore the anti-oomycete activities of tannins extracts originating from by-products of four different botanical sources (nut gall, chestnut wood and, grape skin and seeds) in order to help promote a circular economy. We first characterised these five extracts and found that the chemical composition of the tannin extract depended highly on its botanical source. We then tested their *in vitro* antimicrobial activities against *P. viticola*: two grape seed tannin extracts (a homemade and a commercial extract), showed promising inhibitory activity against downy mildew sporulation at 400 mg/L. The IC_{50} (concentration inhibiting 50 %) of each of these two extracts were thus determined for their inhibition of *P. viticola* sporulation, zoospore motility and zoospore release from sporangia. The results showed that the homemade grape seed extract was more active against *P. viticola* than the commercial one. Finally, the homemade grape seed extract was fractioned based on the polymerisation degree (mDP) of condensed tannins, and the three fractions generated were tested for their capacity to inhibit the sporulation of the oomycete. The fraction containing procyanidins with higher mDP values was noted as the most antimicrobial (96 % inhibition at 400 mg/L). Thus, grape seed extracts rich in procyanidins of high mDP could represent a promising solution for controlling downy mildew.

KEYWORDS: downy mildew, polyphenols, procyanidins, plant by-products, grapevine, degree of polymerisation

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is one of the major crops in the world, which is especially used for the production of grape berries to produce wines (OIV, 2022). Nonetheless, grapevine cultivation is subject to a variety of problems, including diseases, which diminish both grape berry yield and quality. Downy mildew, caused by *Plasmopara viticola*, is one of the most destructive diseases in vineyards, particularly affecting *V. vinifera* cultivars, which can cause losses of up to several millions of euros (Gouveia *et al.,* 2024; Koledenkova *et al.,* 2022). *P. viticola* is an oomycete and an obligate biotrophic agent, which infects all the green organs of grapevine, particularly the leaves and young berries, triggering a cascade of discoloration, necrosis and ultimately defoliation. *P. viticola* is a polycyclic pathogen: mature oospores (sexual spores) germinate and produce macrosporangia which release zoospores (asexual spores) under wet conditions during the spring (Rossi *et al.,* 2008; Rossi & Caffi, 2007). These zoospores swim to stomata and encyst. Then, a germ tube is produced and forms an appressorium to penetrate the intercellular space and uptake nutrients with haustoria (Allègre *et al.,* 2007; Unger *et al.,* 2007). After that, mycelium growth is latent and symptoms of primary infection appear 5 to 18 days later on the adaxial leaf surface (yellow area called "oilspot") (Rossi *et al.,* 2013). With moist conditions and warm temperature (> 25 °C), sporangia emerge from stomata onto the abaxial surface of leaves ("white downy") (Fröbel and Zyprian, 2019; Unger *et al.,* 2007). The cycle of secondary infections starts with the release of zoospores from these sporangia by the action of wind or raindrops (Rossi *et al.,* 2013). At the end of the growing season, oospores are formed in the infected leaves, shoots and berries as a result of the union of male (antheridia) and female (oogonia) structures which have differentiated from mycelial hyphae (Rossi *et al.,* 2013).

Currently, to control downy mildew, the most economical and effective strategies rely on chemicals (Clippinger *et al.,* 2024; Peng *et al.,* 2024). Copper-based (*e.g.*, Bordeaux mixture) and synthetic fungicides are the main chemical products used in viticulture (Koledenkova *et al.,* 2022), and they are subject to regulation by the European Union (Dir. 2009/128/EC and Reg. 1107/2009/EC). However, in addition to the potential harm of these chemicals on humans and the environment, their extensive use has led to soil contamination and the development of fungicide resistance, with the emergence of resistant strains of *P. viticola* (Massi *et al.,* 2021).

It is worth noting that winegrowers often opt for an Integrated Pest Management (IPM) approach, in which control solutions are used that minimise risks towards health and the environment (Pertot *et al.,* 2017). These include specific cultural practices (Clippinger *et al.,* 2024; de Bem *et al.,* 2016), resistant grapevine cultivars (Divilov *et al.,* 2018) and biological control products that comprise biotic agents (*e.g.*, fungi, yeast, bacteria (El-Sharkawy *et al.,* 2018; Zhang *et al.,* 2017)) or natural antimicrobial substances (*e.g.*, plant extracts and essential oils (Dagostin *et al.,* 2010; Gabaston *et al.,* 2017)).

While stilbenes, the main phytoalexins found in grapevine, are well-known for their antimicrobial, and particularly anti-oomycete activities (Gabaston *et al.,* 2017; Pébarthé-Courrouilh *et al.,* 2024; Taillis *et al.,* 2022), other polyphenols such as tannins are also of interest for their antimicrobial properties. Tannins are the fourth most abundant components to be extracted from biomass (Arbenz & Avérous, 2016). They are considered as specialised metabolites in plants, have a molecular weight ranging from 500 to 30000 Da, and are characterised by the presence of one or more aromatic rings. They are grouped into four main classes: hydrolysable tannins, complex tannins, phlorotannins and condensed tannins (Serrano *et al.,* 2009). Hydrolysable tannins are composed of polyesters of a sugar moiety and organic acids. After acid hydrolysis, gallotannins and ellagitannins release gallic acid and ellagic acid, respectively. Phlorotannins are polymers or oligomers of phloroglucinol-derived units. Complex tannins are compounds containing structural elements of hydrolysable tannins and condensed tannins. Condensed tannins (also known as proanthocyanidins) are oligomers or polymers of flavanol units linked through covalent B-type inter-flavanol bonds (de Hoyos-Martínez *et al.,* 2019; Serrano *et al.,* 2009). They are widely distributed throughout the plant kingdom and are synthesised in almost all the different parts of plants (*e.g.*, seeds, roots, twigs, bark, wood, leaves and fruit) (Barbehenn & Constabel, 2011), with different monomeric compositions depending on the part and the plant studied (de Hoyos-Martínez *et al.,* 2019). Grape seed tannins belong to the procyanidin group and are mainly composed of catechin, epicatechin and epicatechin-3-*O*-gallate units, such as flavanol, while skin tannins are mainly prodelphinidins composed of gallocatechin and epigallocatechin flavanol units (Mattivi *et al.,* 2009).

Tannins are also known to act on plant defences and can be either constitutive (phytoanticipins) or inducible (phytoalexins) in response to pathogens and herbivores (insects and animals) (Barbehenn & Constabel, 2011; Sharma, 2019; Treutter, 2006; War *et al.,* 2012). In grapevine, they have been found to accumulate after bacterial (Wallis & Chen, 2012), viral (Gutha *et al.,* 2010) and fungal attacks (Del Río *et al.,* 2004), making them good candidates for grapevine protection. Their role in plant defence is due in large part to their ability to complex and precipitate proteins, such as pectinases, cellulases, amylases, β-galactosidases, lipases, proteases and laccases (Khanbabaee & Ree, 2001; Pizzi, 2019; Sharma, 2019; Vignault *et al.,* 2020). In addition, some tannin extracts (bay laurel, black oak and white oak extracts) have shown antimicrobial activities against oomycetes that have infected plants such as *Phytophthora ramorum* (Stong *et al.,* 2013) and *Aphanomyces cochlioides* (Islam *et al.,* 2002). In light of these characteristics, we can hypothesise that tannins are good biopesticide candidates for protecting vineyards from downy mildew. The tannins present in the co-products (*e.g.*, chestnut wood, nut galls, grape skins and seeds) of many cultivated plants could be

easily recovered and valorised, thereby enhancing their value and promoting a circular economy. In the oenology sector, tannins (gallotannins, ellagitannins and condensed tannins) have largely been used to ensure wine stability, their main sources being nut galls and tara (gallotannins), oak and chestnut (ellagitannins), grape, acacia, quebracho and mimosa (condensed tannins) (Vignault *et al.,* 2018).

The aim of this study was to evaluate the antimicrobial effects of tannins on *Plasmopara viticola*. To this end, we first characterised the chemical composition of five tannin extracts (one from nut gall, one from chestnut wood, one from grape skin and two from grape seeds). Then, we evaluated their capacity to inhibit downy mildew sporulation. The concentrations inhibiting 50 % of downy mildew sporulation, sporangia release and zoospore motility were determined for the two most active extracts. Finally, the homemade crude grape seed tannin extract was fractionated according to the polymerisation degree of condensed tannins. The resulting fractions were tested for their anti-mildew activity.

MATERIALS AND METHODS

1. Chemical and reagents

All solvents were HPLC grade. Acetic acid (HPLC grade) and hydrochloric acid (HPLC grade) were obtained from Fisher Scientific (Illkirch, France). Folin-Ciocalteu reagent, sodium hydroxide, sodium carbonate, ascorbic acid, phloroglucinol, as well as catechin, epicatechin, epigallocatechin, epicatechin-3-*O*-gallate (ECG), vescalagin and ellagic acid were purchased from Sigma-Aldrich (St. Quentin, Fallavier, France). Procyanidins B1-B4 were obtained from Extrasynthèse (Genay, France). Ethanol, used for the biological assays, was purchased from VWR® (Radnor, USA) and Milli-Q water was obtained using a Milli-Q® reference water purification system from Merck Millipore (Burlington, USA).

2. Tannin extracts and chemical characterisation

Five tannin extracts were used in this study: one nut gall commercial extract (Tanin Galalcool® SP), one chestnut wood commercial extract (Kingbrown®), one grape skin commercial extract (Tanin VR Skin®) and two grape seed extracts (one homemade and one commercial (Tanin VR Grape®)). All commercial extracts were provided by Laffort® (Bordeaux, France) and King Tree (Arras, France). The homemade grape seed tannin extract was prepared applying the procedures described in Ćurko *et al*. (2014). Grape seeds were manually separated from Cabernet-Sauvignon grapes collected at maturity from a Bordeaux vineyard. The seeds were frozen under liquid nitrogen and freeze-dried for two days, then ground in a ball grinder. A 6 g portion of the obtained powder was extracted using 55 mL of acetone/water (80:20, v/v) for 8 h, by repeating three extraction cycles on the powder. After centrifugation, the supernatants were combined and evaporated under reduced pressure at 30 °C to remove the organic solvents. Then, the obtained crude tannin extract was dissolved in 200 mL of water/ethanol (95:5, v/v) and extracted three times with 200 mL of chloroform to remove the lipophilic material. The obtained aqueous phase was then evaporated under reduced pressure at 30 °C to remove any traces of organic solvents, and then freeze-dried to obtain a crude grape seed tannin extract.

Each tannin extract was characterised in terms of richness and composition using the analytical methods described below. All the analyses were carried out in triplicate (at a minimum).

The total phenolic content of the tannin extracts was estimated using the Folin-Ciocalteu assay according to the method proposed by Lorrain *et al*. (2012) using 96-well microplates. The tannin richness of the grape skin and seed extracts were determined using a modified Bate-Smith method according to Lagarde *et al*. (2023) and Ribéreau-Gayon and Stonestreet (1966). The monomeric and dimeric flavanol content of the skin and seed tannins extract were determined by HPLC-UV-fluo according to Lagarde *et al*. (2023) and Ćurko *et al*. (2014), while the proanthocyanidins' mean degree of polymerisation (mDP), as well as the monomeric composition, were determined by HPLC-UV after phloroglucinolysis depolymerisation, following the procedure described by Drinkine *et al.* (2007). The content and composition of ellagitannins in the chestnut wood tannin extract were determined by HPLC-UV-MS according to Gancel *et al*. (2023).

3. *Plasmopara viticola* assays

Isolate ANN-01 of the oomycete *Plasmopara viticola* (originating from a vineyard in Charente (France)), was maintained in the laboratory by performing weekly subcultures according to the procedure used by Corio-Costet *et al*. (2011). Briefly, *V. vinifera* cv. Cabernet-Sauvignon leaves collected from two-month-old cuttings (grown in greenhouses located in Villenave-d'Ornon, France) were inoculated with *P. viticola.* After seven-days, sporangia were collected and suspended in cold water. Microdroplets (15 µL) of this suspension (10.10^3 sporangia/mL) were deposited on the abaxial surface of the Cabernet-Sauvignon leaves, which were then placed on Whatman paper in Petri dishes. One-day latter, droplets were removed, and dishes were incubated for six more days. Incubation was carried out in controlled conditions (24 ± 2 °C and 16/8 h light/dark).

3.1. Sporulation assays

Fresh leaves (the third and fourth leaves on the apex) of two-month-old foliar cuttings of *Vitis vinifera* cv. Cabernet-Sauvignon were collected and washed with tap water. Foliar discs that were 22 mm in diameter were deposited on a moistened Whatman paper in 90 mm diameter Petri dishes (3 mL of water, 6 discs per dish).

Tannin extract solutions were prepared extemporaneously in EtOH 0.2 %, and 400 mg/L was sprayed onto the foliar discs until microdroplets formed (32 ± 4 µL per disc, 50-100 µm droplet size). An ethanol concentration up to 1 % did not affect oomycete sporulation (Pezet *et al.,* 2004). After drying the

extract solutions in a laminar flux cabinet, the foliar discs were inoculated with 15 µL droplets (3 per disc) of *P. viticola* in a sporangia solution of 10.10^3 sporangia/mL. The inoculated discs were incubated in Petri dishes for one day in darkness before the sporangia droplets were removed. After that, the dishes were placed in a culture room with a photoperiod of 16/8 h day/night at 24 ± 2 °C. The downy mildew sporulation density was evaluated 7 days after inoculation. It was estimated as the percentage of mildew sporulation. The control condition (EtOH 0.2 %) was set as 0 % sporulation inhibition, corresponding to the maximal sporulation level of the oomycete, and sporulation inhibition values for all other conditions were compared to this control (Corio-Costet *et al.,* 2011). Each condition corresponded to the mean of three biological replicates and the experiments were carried out four times each.

The concentrations that inhibited 50 % of mildew sporulation (IC_{50}) were calculated for the most active extracts (*i.e.*, the homemade and commercial grape seed extracts). To this end, the followed final concentrations were used: 0, 300, 350, 400, 450, 500 and, 550 (for the homemade grape seed extract) and 700 mg/L (for the commercial grape seed extract). Each condition was the mean of three Petri dishes (biological replicates) containing six foliar discs each (technical replicates). Experiments were conducted five and seven times on the homemade and the commercial grape seed extracts, respectively.

3.2. Zoospore motility assays

Fresh sporangia of *P. viticola* were collected in Milli-Q water and the suspension was adjusted to a final concentration of 15.10^4 sporangia/mL. The sporangia solution was kept for 2 h in the dark at ambient temperature to ensure zoospore release (Hong & Scherm, 2020; Krzyzaniak *et al.,* 2018). After that, the zoospore solution was mixed with grape seed extracts (homemade or commercial) or ethanol (0.2 %, considered as control). In the literature, a final concentration of less than 1 % ethanol has been reported to not affect the zoospore motility of *P. viticola* (Pezet *et al.,* 2004). The solutions were then incubated for 5 min in darkness before carrying out the optical microscopic observations (DM750, Leica®, 400-fold magnification), for which 15 µL of solution was deposited on a glass slide and a reading of the motile and immobile zoospores at a defined point was taken for 30 s, according to the procedure described by Pezet *et al.* (2004) and Taillis *et al.* (2022).

The following concentrations of the two grape seed extracts (homemade and commercial) were tested: 0, 25, 50, 75, 100, 125 and 150, as well as 175 mg/L in the case of the commercial grape seed extract. In addition to the IC_{50}, we determined the IC_{100} ; *i.e.*, the lowest tested concentration that showed total inhibitory activity.

At each extract concentration, the mean of three biological replicates (reaction mixtures) was calculated and each biological replicate was analysed in three different microscopic observations (technical replicates). All the experiments were carried out in triplicate.

3.3. Zoospore release assays

Fresh sporangia of *P. viticola* were collected in Milli-Q water and the suspension was adjusted to reach a final concentration of 15.10^4 sporangia/mL. The grape seed extract solutions or EtOH 0.2 % (control condition) were added to the sporangia suspension. The samples were immediately placed in the dark at ambient temperature for 2 h, to enable zoospore release from the sporangia. After that, for each sample, three readings of 30 sporangia (full or empty) were performed under an optical microscope (ML100, VWR®, 400-fold magnification) according to the procedure described by Raveau *et al.* (2024).

The following concentrations of the two grape seed extracts (homemade and commercial) were tested: 0, 5, 10, 20, 40 and 80 mg/L. EtOH 0.2 % did not affect zoospore release from the sporangia (data not shown). The percentage inhibition of zoospore release in the control conditions (EtOH 0.2 %) was set to 0 %, and the zoospore release inhibition percentage in all the other conditions was calculated relative to the control. It was thereby possible to determine the IC_{50} of the two tannin extracts.

At each extract concentration, the mean of three biological replicates (reaction mixtures) was calculated, and each biological replicate was analysed in three different microscopic observations (technical replicates). All the experiments were carried out in triplicate.

4. Homemade grape seed extract fractionation, chemical characterisation and sporulation assays

The homemade grape seed extract was fractionated using the method described by Lorrain *et al*. (2012), that was slightly modified. Briefly, the crude extract was first dissolved in 250 mL of water/ethanol (95:5, v/v) and extracted three times using ether (250 mL) to obtain the monomeric-small oligomeric fraction (Fraction 1) after removing the organic solvent under reduced pressure. Then, the aqueous phase underwent an extraction process three times using ethyl acetate (250 mL) to obtain the oligomeric fraction (Fraction 2) after removing the organic solvent under reduced pressure. The remaining aqueous phase was frozen and freeze-dried to obtain the polymeric fraction (Fraction 3).

Each tannin fraction was characterised in terms of richness and composition using the analytical methods described in Section 2. All the analyses were carried out in, at the minimum, triplicate.

The antimicrobial action on downy mildew sporulation was assessed (at 400 mg/L) as described in Section 3.1. Each condition corresponded to the mean of three biological replicates and each experiment was carried out four times.

5. Statistical analyses

Statistical analyses were performed using R Studio software (2022.07.1). After assessing residue normality (Shapiro test) and homoscedasticity (Levene test), multiple comparison analyses were performed using a one-way ANOVA followed by a post-hoc test (Tukey), or with a non-parametric ANOVA

(Kruskal Wallis) followed by a non-parametric post-hoc test (Pairwise Wilcoxon). For comparison analyses of two groups, we used Student tests (t-tests). Significant differences were set at $p < 0.05$.

Dose response curves for grape seed extracts in all biological assays on *P. viticola* were generated by plotting the relative inhibition values against $\log_{10}$ of the extract concentration tested. The IC_{50} and R^2 were determined from the regression equation generated through the linear part of the sigmoid curve on Excel software (2019 MSO 64 bits), according to Corio-Costet *et al.* (2011). The R^2 is a measure of the correlation between the concentration of the tannin extracts and the inhibition effects.

RESULTS AND DISCUSSION

1. Chemical characterisation of tannin extracts

The five tannin extracts (Figure 1A) were chemically characterised. Total phenolic content values obtained in the Folin-Ciocalteu assay were 55.10, 43.80, 42.89, 37.15 and 36.72 % for nut gall, chestnut wood, commercial grape seed, grape skin and homemade grape seed extracts, respectively; thus, they all had a high content of polyphenols. These total phenolic content values are consistent with the ones reported by Vignault *et al*. (2018). Chestnut wood and nut gall extracts are known to contain high amount of ellagitannins and gallotannins, respectively (Vignault *et al.,* 2018), whereas grape seed and skin extracts have a high content of condensed tannins (Bordiga *et al.,* 2011; Ćurko *et al.,* 2014). We therefore used the Bate-Smith method to determine the condensed tannin richness of the latter two extracts: both the homemade and the commercial grape seed extracts had higher condensed tannin content (99.20 % and 94.70 %, respectively) than the grape skin extract (Table 1).

The mean degree of polymerisation (mDP) and the percentage of galloylation (% Gal) of condensed tannins in the grape extracts were determined. The mDP values were 4.7, 3.1 and 4.9 for grape skin, commercial grape seed and homemade grape seed extracts, respectively. Values for % Gal were 40.3, 7.1 and 28.3 %, for grape skin, commercial grape seed and homemade grape seed extract, respectively. In the literature,

TABLE 1. Chemical characterisation of the five tannin extracts.

		Chestnut wood	Nut gall	Grape skin	Grape seed (homemade)	Grape seed (commercial)
Total phenolic content (%)	Folin-Ciocalteu method	43.80^{a} ± 1.64	55.10^{b} ± 1.23	37.15^{c} ± 0.52	36.72^{c} ± 1.59	42.89^{a} ± 0.48
	Bate-Smith method	-	-	88.40^{a} ± 0.21	99.20^{b} ± 3.90	94.70^{ab} ± 2.77
Proanthocyanindin oligomers	mDP	-	-	4.70^{a} ± 0.10	4.90^{a} ± 0.41	3.10^{b} ± 0.10
	% Gal	-	-	40.30^{a} ± 0.62	28.30^{b} ± 1.13	7.10^{c} ± 0.41
	% P	-	-	44.10 ± 1.11	*nd*	*nd*
Flavanols and procyanidin dimers (mg/g)	Catechin	-	-	*nq*	4.55^{a} ± 0.09	8.02^{b} ± 0.07
	Epicatechin	-	-	*nq*	1.81^{a} ± 0.06	7.22^{b} ± 0.05
	Procyanidin dimer B1	-	-	*nq*	0.12^{a} ± 0.02	1.75^{b} ± 0.02
	Procyanidin dimer B2	-	-	*nq*	0.61^{a} ± 0.11	4.35^{b} ± 0.09
	Procyanidin dimer B3	-	-	*nq*	1.51^{a} ± 0.05	2.09^{b} ± 0.04
	Procyanidin dimer B4	-	-	*nq*	0.16^{a} ± 0.02	1.94^{b} ± 0.02
Ellagitannins and ellagic acid (mg/g)	Vescalagin	2.69 ± 0.44	-	-	-	-
	Castalagin[1]	32.24 ± 3.24	-	-	-	-
	Roburin A[1]	0.65 ± 0.69	-	-	-	-
	Roburin B[1]	1.56 ± 0.25	-	-	-	-
	Roburin C[1]	2.49 ± 0.17	-	-	-	-
	Roburin D[1]	6.05 ± 0.05	-	-	-	-
	Roburin E[1]	2.67 ± 0.26	-	-	-	-
	Grandinin[1]	2.54 ± 0.53	-	-	-	-
	Ellagic acid	19.5 ± 3.33	-	-	-	-

[1] Concentrations are expressed as mg/g of vescalagin. mDP = mean degree of polymerisation; % Gal = percentage of galloylation; % P = percentage of prodelphinidin monomers; *nq* = not quantified; *nd* = not detected. The lowercase letters represent the significant differences between each extract in the different chemical assays.

grape skins have been reported to have generally higher mDP values and lower % Gal than grape seeds (Chira *et al.,* 2009; Rousserie *et al.,* 2019). It is worth noting that, except the homemade grape seed extract, the two other extracts that were found to have a high content of condensed tannins (grape skin and commercial grape seed extracts) are commercial oenological tannins. Since tannins of high molecular weights are very weakly soluble in water, it is highly likely that they were removed from the final product to ensure the solubility of these commercial extracts in wine.

The monomeric composition of the oligomers and polymers of the condensed tannin in the three grape extracts was determined by phloroglucinolysis. The seed tannins did not contain any flavanol monomers with pyrogallol B rings, such as gallocatechin and epigallocatechin (*i.e.*, % P was not detected). By contrast, and as can be expected in skin, % P of the condensed tannins from the skin was high.

Compared to the commercial grape seed extract, the homemade extract was slightly richer in condensed tannins. This may be due to the higher levels of flavanols and procyanidin dimers in the commercial extract (2.5 % of the dry extract) than in the homemade one (0.9 % of the dry extract). This may also explain why the mDP of the homemade extract was higher than the commercial one. Finally, we were unable to quantify the flavanols and procyanidin dimers in the grape skin extract, as they were under the limits of quantification. It is known, however, that grape skins generally contain condensed tannins that mainly comprise prodelphinidins, as is indicated here by the % P value and the high mDP values (thus low amounts of catechin, epicatechin and their dimeric forms) (Souquet *et al.,* 1996).

2. Anti-downy mildew evaluation of tannin extracts

2.1. Evaluation of the inhibitory capacities of tannin extracts on sporulation

First, the five tannin extracts from different plant sources (nut gall, chestnut wood, grape skin and grape seeds) were studied for their capacity to inhibit the sporulation of downy mildew in grapevine foliar disc assays. To our knowledge, this is the first time that antimicrobial activities of diverse

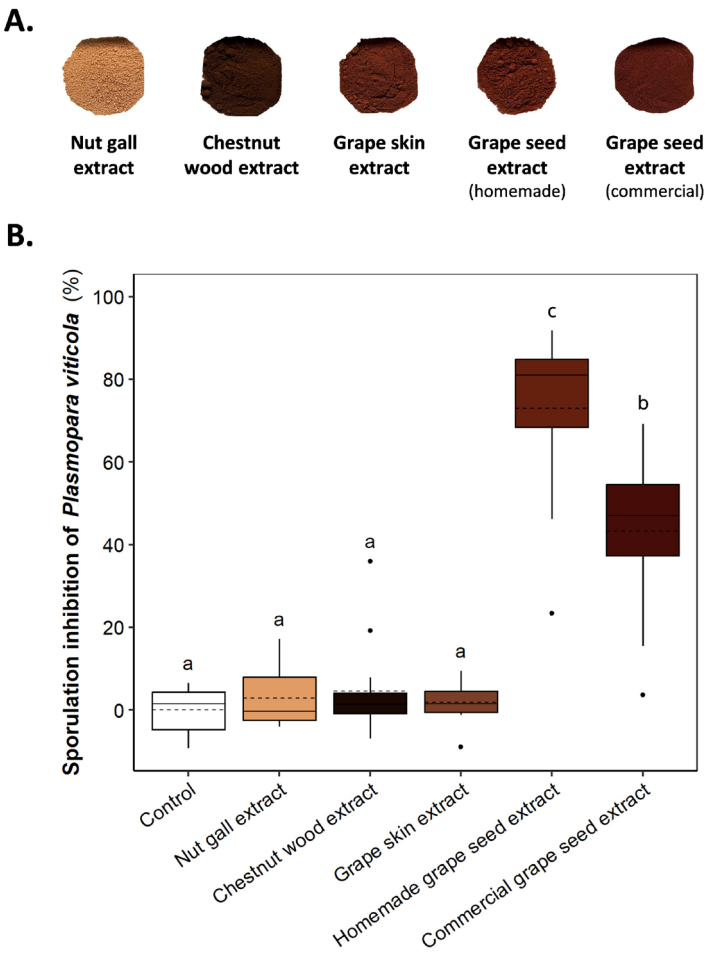


FIGURE 1. Effect of five tannin extracts (A) on the sporulation of *Plasmopara viticola* (B).

Tannin extracts in EtOH 0.2 % were tested at 400 mg/L. "Control" corresponds to treatment with EtOH 0.2 %. The mean of sporulation inhibition is represented by a dotted line and the median of sporulation inhibition by a plain line in each box. Letters represent the significant differences between tannin extracts ($p < 0.05$). Experiments were carried out four times each, with three biological replicates per extract in each experiment.

tannin extracts have been reported with regard to *P. viticola.* Figure 1B showed the inhibition levels of the different extracts (400 mg/L in EtOH 0.2 %). The tannin extracts of nut gall, chestnut wood and grape skin did not inhibit the oomycete sporulation at the concentration tested. Only the grape seed extracts showed inhibition: the homemade grape seed extract inhibited approximatively 73 % of oomycete sporulation relative to the "control", while the commercial grape seed extract inhibited 43 %. These results show that not all tannin extracts inhibit downy mildew sporulation. The fact that not all of the three condensed tannin extracts (grape skin and seed extracts) were able to trigger inhibition and that the two active extracts were from grape seeds indicates that the monomeric composition of the tannins determined their antimicrobial activity. Indeed, the seed tannins are procyanidin tannins comprising flavanol monomers that all exhibit a catechol B-ring, whereas the skin tannins comprise prodelphinidins composed of pyrogallol B-rings (de Hoyos-Martínez *et al.,* 2019; Mattivi *et al.,* 2009). Given that the homemade extract was the most active of the two grape seed extracts (homemade and commercial), and had the highest mDP, we hypothesise that the most active tannins in the inhibition of the sporulation of downy mildew are the oligomers/polymers of procyanidins with higher molecular weight.

The inhibitory capacity that tannins play in plant defense may originate from their capacity to complex and precipitate proteins (Pizzi, 2019). A study conducted by Vignault *et al*. (2020) evaluated the percentage of inhibition/precipitation of laccases from *Botrytis cinerea,* which are enzymes strongly associated with virulence in fungal *Botrytis* spp., in the presence of different oenological tannins: nut gall, oak wood, grape skin and grape seeds extracts; the grape seed tannins were found to be the most inhibiting, followed by nut gall (gallotannins), grape skin and oak wood (ellagitannins) tannins. Therefore, since genes encoding for laccase have been reported to be present in *P. viticola* (Luis *et al.,* 2013), a similar reaction to laccase of this oomycete may occur with tannins of the grape seed extracts, impeding its development.

We carried out further study on the two grape seed extracts, as they were the only extracts to display sporulation inhibition against downy mildew, performing additional experiments to determine their IC_{50} on sporulation inhibition, zoospore motility and zoospore release.

The homemade grape seed extract was tested at concentrations ranging from 0 to 550 mg/L (Figure 2A). At 300 mg/L, it inhibited 24 % of downy mildew sporulation. We observed a dose-effect response in the inhibition of *P. viticola* sporulation and we determined an IC_{50} of 353 mg/L ($R^2 = 0.96$). The IC_{100} (*i.e*., the concentration that totally inhibits the sporulation of the oomycete) was nearly achieved at 550 mg/L, with an inhibition of 99 %. We also noted that at 400 mg/L an inhibition of 70 % was obtained, which is consistent with the previous results presented in Figure 1B (73 % of inhibition).

Assays were also performed on the commercial grape seed extract (Figure 2B), in which the same concentration range was extended to 700 mg/L in order to better compare it with the homemade grape seed extract. At 300 mg/L, the commercial extract inhibited only 7 % of downy mildew sporulation, which was not significant relative to the control condition (EtOH 0.2 %). Significant inhibition was first triggered at 350 mg/L with 22 % of inhibition. Similar to the homemade extract, a dose-response effect was observed and an IC_{50} of 438 mg/L ($R^2 = 0.98$) was determined. At 700 mg/L, the sporulation of *P. viticola* was nearly completely inhibited with a value of 98 %.

The determined IC_{50} of the two grape seed extracts confirmed that the homemade grape seed extract was more active on the

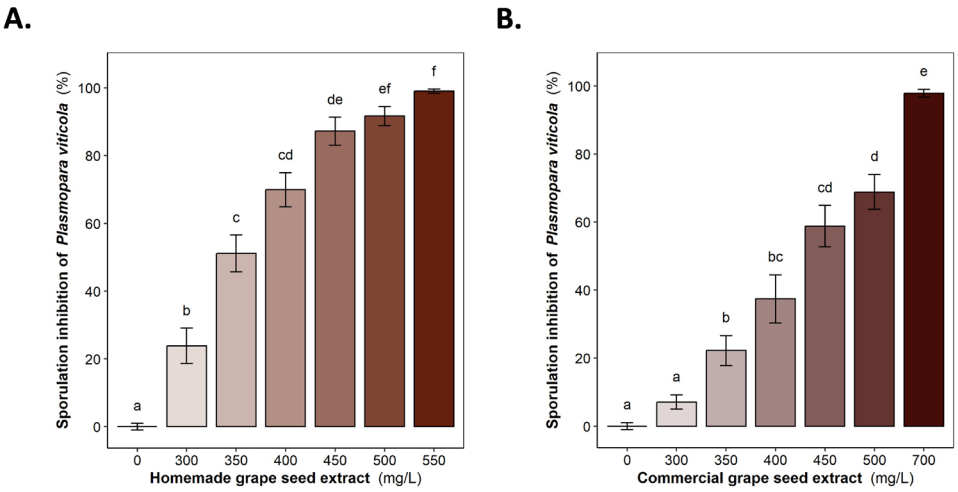


FIGURE 2. Sporulation of *Plasmopara viticola* in the presence of homemade grape seed extract (A) and commercial grape seed extract (B) at different concentrations.

Control condition (EtOH 0.2 %) corresponds to "0" (*i.e.*, 0 % of downy mildew sporulation inhibition). Values are expressed as a percentage of mean sporulation inhibition (relative to control conditions) ± standard error (SE). Lowercase letters represent significant differences between each concentration of extract tested ($p < 0.05$). Experiments were carried out seven times on the commercial grape seed extract and five times on the homemade grape seed extract, with three biological replicates per concentration in each experiment.

inhibition of downy mildew sporulation than the commercial one, with an IC_{50} 1.24-fold lower.

While, to our knowledge, this is the first time that the IC_{50} of tannin extracts has been reported for *P. viticola* sporulation inhibition, a number of studies have reported the IC_{50} of other plant extracts enriched in other polyphenols. For example, a grapevine trunk and root extract enriched in stilbenes (grapevine phytoalexins) displayed an IC_{50} of 70 mg/L with regard to *P. viticola* sporulation (Taillis *et al.,* 2022), and Gabaston *et al*. (2017) found IC_{50}s of 60 mg/L, 120 mg/L and 210 mg/L for stilbene-rich wood, root and cane extracts, respectively. Thus, while our tannin-rich grape seed extracts were less antimicrobial than the abovementioned extracts rich in stilbenes, their activity was not at all negligible. In addition, Dai *et al*. (1994) reported that the leaves of a downy mildew resistant grapevine (*V. rotundifolia* var. Carlos) show greater quantities of catechic tannins than those of a susceptible cultivar (*V. vinifera* var. Grenache). As regards other tannin extracts (bay laurel, black oak and white oak extracts) and their activity on oomycetes, Stong *et al*. (2013) evidenced that extract with the highest content of condensed tannins, the foliar extract of black oak, was the most toxic towards *Phytophthora ramorum*. This corroborates the results obtained in our analyses of the two grape seed extracts, which were also rich in condensed tannins and showed antimicrobial activity towards a plant oomycete, *P. viticola*.

Thus, the sporulation inhibition activity of grape seed tannin extracts towards *P. viticola* may interfere in the cycle of this pathogen by reducing secondary infections.

2.2. Evaluation of the inhibitory capacities of tannin extracts on zoospore motility

The homemade grape seed extract was assessed for its effect on zoospore motility (Figure 3A). Zoospores are motile structures that are released from sporangia and infect grapevine tissues (Hardham, 2007). The IC_{50} and the IC_{100} of both grape seed extracts for the inhibition of zoospore motility were determined 5 min after applying the extract, as the rate of inhibition of zoospore motility has been reported to depend on the reaction time of the extract tested (Taillis *et al.,* 2022). At 25 mg/L, there was a decrease in zoospore motility of approximatively 10 %, after which the rate of inhibition was proportional to the concentration (dose-response effect). An IC_{50} of 76 mg/L ($R^2 = 0.96$) was determined. At 150 mg/L, maximal inhibition (IC_{100}) was reached. We noticed a strong dose effect between 75 and 100 mg/L with 41 and 81 % inhibition, respectively. This may be due to the high sensitivity of zoospores as they lack a cell wall (Gleason & Lilje, 2009); thus, above 75 mg/L of tannin extract, their viability may be compromised. Moreover, their higher sensitivity is also clearly indicated by the 4.6-fold lower IC_{50} values for zoospore motility than those of sporulation inhibition.

The commercial grape seed extract was assessed using the same concentration range as the homemade one, with the added concentration of 175 mg/L. We observed the same trend as for the homemade grape seed extract, with a dose-effect response (Figure 3B). However, as observed in the *P. viticola* sporulation assays, the commercial grape seed extract was less antimicrobial than the homemade one: only 24 % inhibition was observed at 75 mg/L compared to 41 % for the homemade extract (significantly different at this concentration). The commercial extract had a 1.24-fold higher IC_{50} than the homemade one at 94 mg/L ($R^2 = 0.94$), and its IC_{100} was also higher than that of the homemade extract (175 versus 150 mg/L, respectively).

The motility of zoospores of *Aphanomyces cochlioides* (an oomycete plant pathogen) has been found to be remarkably inhibited by extracts from the stem bark of *Lannea coromandelica,* and this inhibition was followed by lysis of

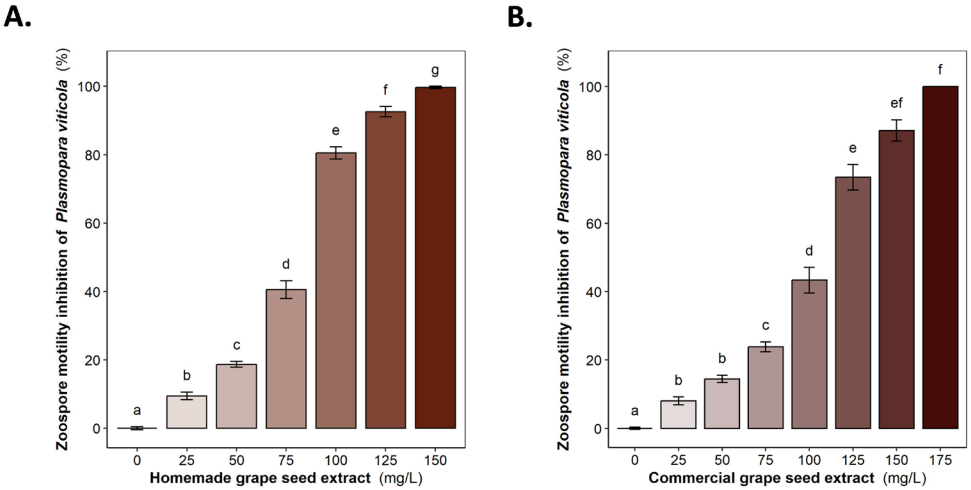


FIGURE 3. Inhibition of *Plasmopara viticola* zoospore motility in the presence of homemade grape seed extract (A) and commercial grape seed extract (B) at different concentrations.

Control condition corresponds to "0" (EtOH 0.2 %). Values are expressed as a percentage of mean motility inhibition (relative to control) ± standard error (SE). Lowercase letters represent significant differences between each concentration of extract tested ($p < 0.05$). Each experiment was carried out three times on both grape seed extracts, with three biological replicates per experiment.

the zoospores (Islam *et al.,* 2002). The authors chemically characterised their plant extract to find that it was rich in tannins, which were suggested to be responsible for the zoosporicidal activity. Moreover, they noticed that these extracts attacked the structures of the flagellum (used for swimming), consequently rapidly halting the zoospores, followed by the fragmentation of the inner cellular materials of the spores within 30 min. Such a phenomenon may have occurred in the present study during the exposure of *P. viticola* zoospores to the grape seed tannins. Another study showed that crude foliar extracts of grapevine with different levels of downy mildew susceptibility affected *P. viticola* zoospore motility differently: the extract from a resistant grapevine (*Vitis rotundifolia* var. Carlos) showed an IC_{50} of 117.6 mg/L and as much as 1000 mg/L for an extract of susceptible grapevine (*V. vinifera* var. Grenache) (Dai *et al.,* 1994). The authors stated that the leaves of the resistant grapevine contained higher quantities of catechic tannins than those of the susceptible cultivar. This therefore supports our hypothesis that the condensed tannins present in our grape seed extracts have a role in the inhibition of *P. viticola* zoospore motility.

2.3. Evaluation of the inhibitory capacities of tannin extracts on zoospore release

The two grape seed extracts were tested for their capacity to inhibit the release of zoospores from sporangia.

First of all, we noticed that the percentage of zoospore release in the control condition (EtOH 0.2 %) was 55 % after 2 h of sporangia incubation, which is consistent with the literature (Riemann *et al.,* 2002). In the control (0 mg/L), the inhibition of zoospore release was set to 0 %, and the inhibition rates of the different extract concentrations were calculated relative to this control.

As regards the homemade grape seed extract, 24 % inhibition of zoospore release was observed at 5 mg/L, (Figure 4A), increasing to 86 % at 80 mg/L. A dose-response effect was once again observed, and an IC_{50} of 17 mg/L ($R^2 = 0.96$) was determined.

The commercial grape seed extract was tested using the same concentration range as the homemade extract. Compared to the homemade extract, we noted similar inhibition of zoospore release of 27 % at 5 mg/L, (Figure 4B), and exactly the same inhibition of 86 % at the highest concentration of

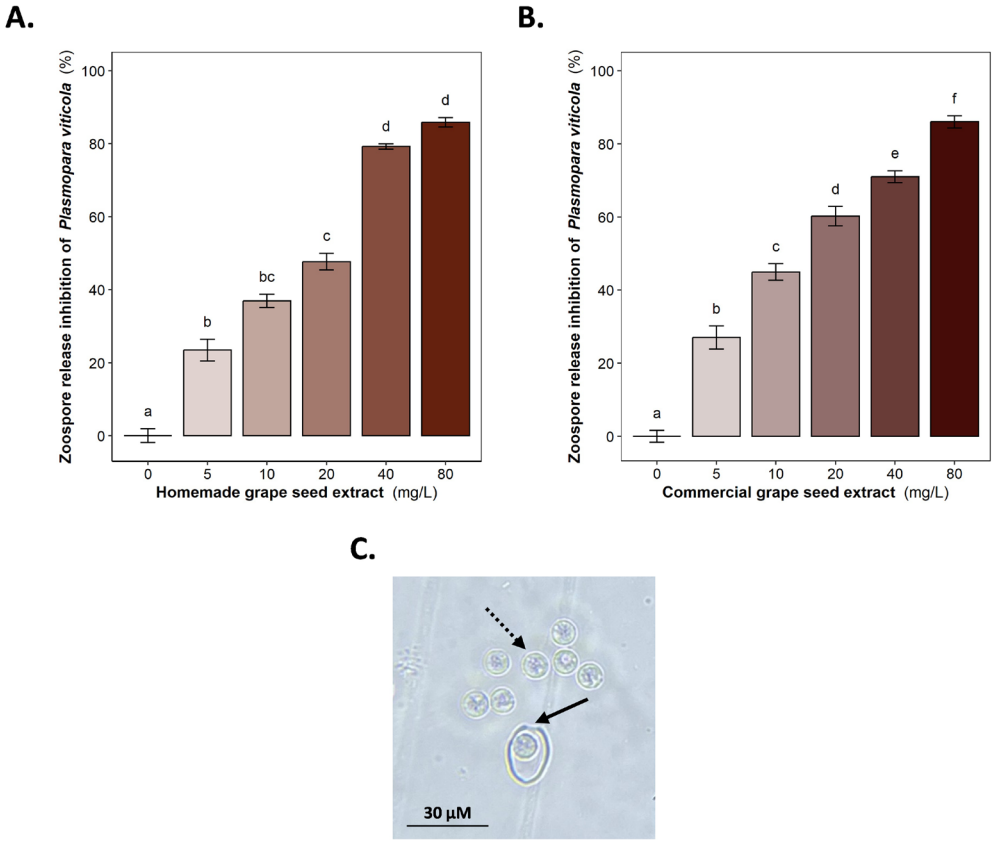


FIGURE 4. Inhibition of *Plasmopara viticola* zoospore release in the presence of homemade grape seed extract (A) and commercial grape seed extract (B) at different concentrations.

Control condition "0" corresponds to EtOH 0.2 %. Values are expressed as a percentage of mean zoospore release inhibition (relative to control condition) ± standard error (SE). Lowercase letters represent significant differences between each concentration for an extract tested ($p < 0.05$). Experiments were carried out three times each on both grape seed extracts, with three biological replicates in each experiment. (C) Picture of zoospores being released from sporangium of downy mildew (optical microscopy, x400) in the control conditions. The broken-line arrow is pointing to a zoospore and the unbroken-line arrow the open papilla of the sporangium.

80 mg/L. The IC_{50} of the commercial extract was 14 mg/L (R^2 = 0.99), which is only slightly lower than that of the homemade extract (not significant). The IC_{50} of the two grape seed extracts for zoospore release inhibition were thus found to be lower than the ones determined for zoospore motility and sporulation inhibition.

In order to release zoospores, sporangia need to be hydrated. The influx of water through the sporangial cell wall causes hydrostatic pressure within the sporangium that leads to the rupture of an apical papilla and the consequent discharge of zoospores (Hardham, 2007; Koledenkova *et al.,* 2022; Lamour & Kamoun, 2009). Figure 4C clearly shows the open papilla of a sporangium (indicated by the unbroken-line arrow) in the control condition (EtOH 0.2 %). Meanwhile, it is also known that tannins can complex with proteins and other macromolecules, such as cellulose, a polysaccharide (Hanlin *et al.,* 2010; Le Bourvellec *et al.,* 2005), and that the cell wall of *P. viticola* is mainly composed of cellulose and glucans (Koledenkova *et al.,* 2022). Therefore, we can hypothesise that tannins interact/bind with essential macromolecules of *P. viticola* sporangial cell walls, increasing cell wall rigidity and/or preventing their hydration, rendering the mechanical break down of the cell wall more difficult, and thus preventing the release of zoospores. To our knowledge, this study is the first to evaluate the inhibitory effects of grape seed tannin extracts on *P. viticola* zoospore release. A number of studies have reported the effects of plant extracts on zoospore release. Dai *et al*. (1994), already mentioned, found a crude foliar extract of a downy mildew resistant grapevine (enriched in catechic tannins) to inhibit zoospore release (IC_{50} of 164.2 mg/L), and Krzyzaniak *et al*. (2018) reported a plant extract to fully inhibit sporangia release from *P. viticola* at 0.5 g/L. In addition, Besrukow *et al*. (2023) reported that three grape cane extracts (from *V. vinifera* L. cv. Pinot noir, *V. vinifera* L. cv. Accent, and a company (Vineatrol® extract)) rich in stilbenoids at 5 g/L inhibited 42.7, 57 and 75.3 % of zoospore release, respectively. Thus, the grape seed tannin extracts in the present study that exhibited antimicrobial activity in the mg/L range (with an IC_{50} of around 15 mg/L) were far more active than the abovementioned plant extracts, which were in the g/L range. This is highly likely due to different modes of action and, above all, the capacity of tannins to interact/bind with macromolecules.

2.4. Fractionation of the homemade grape seed extract and inhibition of downy mildew sporulation

Based on our results, especially those obtained on sporulation and zoospore motility, we hypothesised that the oligomers or polymers with the higher molecular weight were the most active of the condensed tannins of grape seeds (procyanidins) against *P. viticola*. We therefore generated different fractions of the homemade grape seed extract each based on the polymerisation degree of the oligomers and polymers. Fraction 1 was mainly composed of monomers (catechin and epicatechin) and small oligomers (42 % such as dimers), and had an mDP of 1.61. Fraction 2 was composed of oligomers (99 %) and had an mDP of 3.51. Fraction 3 contained polymers (96 %) and had an mDP of 8.67. A more detailed characterisation of these fractions is available in Table S1.

We studied the antimicrobial activity of the three different fractions of the homemade grape seed extract to evaluate their capacity to inhibit *P. viticola* sporulation. All the

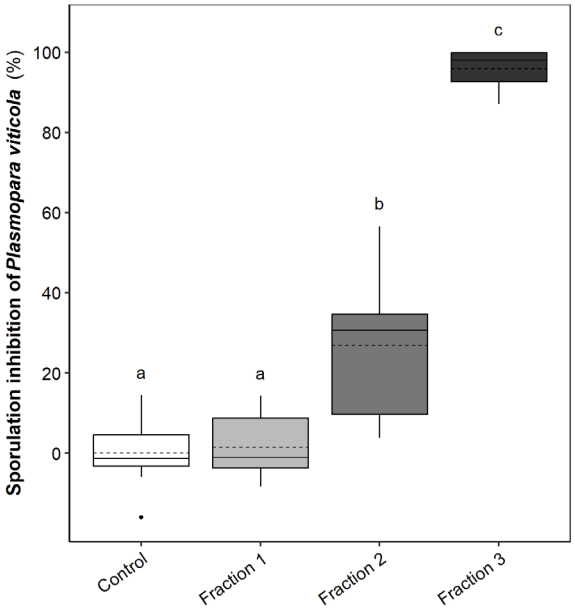


FIGURE 5. Sporulation of downy mildew in contact with different polymeric fractions of the homemade grape seed extract.

The three fractions were tested at the final concentration of 400 mg/L. The control corresponds to the treatment with EtOH 0.2 %. In each box, the mean is represented by a dotted line and the median by an unbroken line. Lowercase letters represent significant differences between each extract tested ($p < 0.05$). Experiments were carried out four times each, with three biological replicates per extract in each experiment.

fractions, dissolved in EtOH 0.2 %, were tested at a final concentration of 400 mg/L. The results are shown in Figure 5. Fraction 1 had no effect on downy mildew sporulation. Fraction 2 had an intermediate effect with an inhibition of 27 % relative to the control, and Fraction 3 showed almost total capacity for inhibition (96 %). Fractions 2 and 3, which almost exclusively contained procyanidins, were more active than Fraction 1, which comprises only 42 % procyanidins, in accordance to the results presented by Dai *et al*. (1994). Moreover, Fraction 1 had a low mDP value of 1.61, while Fractions 2 and 3 had values of 3.51 and 8.67, respectively, highlighting that the polymers of procyanidins with the highest molecular weight were the most antimicrobial toward *P. viticola*, thus confirming our hypothesis. These results align with those obtained in the previous experiments, showing better antimicrobial activity of the homemade grape seed extract (higher oligomers and polymers, mDP = 4.9) than that of the commercial extract (lower oligomers and polymers, mDP = 3.1).

To our knowledge, this is the first report on the influence of the polymerisation degree of procyanidins on anti-mildew activity. Meanwhile, when studied in the context of human health, it has been shown that, in most cases, the biological activities of procyanidins correlate with their degree of polymerisation, but with no clear conclusions being drawn (Yang *et al.,* 2021).

CONCLUSION

In this study, we first chemically analysed five tannin extracts from four botanical sources: nut gall, chestnut wood, grape skin and grape seed, which all showed a high content of polyphenols. The chestnut wood and nut gall extracts were characterised by the presence of hydrolysable tannins (*e.g*., ellagitannins for chestnut wood). The grape extracts (seeds and skin) showed a high content of condensed tannins, being higher in the two grape seed extracts (commercial and homemade). The skin extract contained prodelphinidins and procyanidins, whereas seed extracts only contained procyanidins.

Here, we report for the first time the antimicrobial activities of different tannin extracts on downy mildew, among which, only the two grape seed extracts inhibited the sporulation of *P. viticola* at 400 mg/L. The homemade extract was found to be the most active, with an IC_{50} for the inhibition of sporulation, zoospore motility and sporangia release of 353, 76 and 17 mg/L, respectively. These multi-target antimicrobial activities, acting at distinct critical stages of the downy mildew life cycle, show high potential for combating this highly concerning disease. Furthermore, the fractionation of the homemade grape seed extract based on the polymerisation degree of procyanidins revealed that the procyanidins with the highest molecular weight inhibited sporulation the most. Thus, grape seed extracts rich in procyanidin polymers and derived from by-products to be valorised could be used in the future as biopesticides in vineyard to help control downy mildew.

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