ORIGINAL PAPER

ANTIFUNGAL ACTIVITY EVALUATION OF WHITE AND RED GRAPE POMACE EXTRACTS AGAINST SOME PHYTOPATHOGENS

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Abstract. *The main objective of this research is to assess the antifungal activity of grape pomace extracts of four local varieties (i.e., two white, Fetească Albă and Tămâioasă Românească, and two red, Fetească Neagră and Negru de Drăgășani), knowing their valuable chemical profile. The antifungal activity of the extracts was evaluated using two fungi strains (Aspergillus niger and Alternaria alternata). Despite the classical extraction method, using absolute ethyl alcohol (99–100%): water=1:1 as a solvent, the obtained grape pomace extracts presented high antifungal effectiveness. Thus, Tămâioasă Românească (TR), Fetească Neagră (FN), Fetească Albă (FA), and Negru de Drăgășani (ND) pomace extracts were shown to have superior antimicrobial activity against Aspergillus niger due to their higher concentration of polyphenols. On the other hand, FN and ND extracts did not exhibit antifungal activity against Alternaria sp., although the growth inhibition efficiency was lower compared to the growth inhibition of Aspergillus sp. In conclusion, this study emphasized a strong correlation between the antifungal activity of analyzed grape pomace extracts and their excellent antioxidant potential.*

Keywords: *pomace grape; antifungal inhibition; Aspergillus niger; Alternaria alternata; Kirby-Bauer method.*

1. INTRODUCTION

Grape berries host a complex microbial community consisting of various species of yeasts, bacteria, and molds, all of which play a crucial role in the quality of grapes and the production of wine. Within this community, some environmental bacteria and molds grow on the grape berries. Molds may exist on grapes as saprophytes, opportunistic pathogens, or

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parasites. For instance, *Botrytis cinerea*, *Rhizopus sp*., *Penicillium sp*., *Aspergillus sp*., and *Alternaria alternata* are linked to grape rot [1].

Postharvest fungal spoilage of table grapes is a major problem affecting the quality of these fruits during storage. The storage life is limited by several pathogens, with *Aspergillus niger*, *Botrytis cinerea*, *Rhizopus stolonifer*, *Penicillium spp*. and *Mucor sp.* being the prevalent postharvest pathogens [2]. Sulfur dioxide (SO_2) generators are commonly used to control fungal diseases in cold-stored table grapes. However, excessive use of SO_2 can have negative consequences, including early browning of the rachis, cracking, or detachment of grape berries from the bunches. These effects may pose potential health risks to consumers [3]. Concerns among consumers regarding chemical residues and environmental health issues due to the use of synthetic substances (i.e., preservatives) have led to the exploration of alternative methods for maintaining the quality of table grapes after harvest. These alternatives must be without any toxicity to humans, environment, and plants [4-7]. In addition, the industry requires alternative methods that are not only effective against pathogen invasion, but also economically feasible [8].

A variety of plant-based products have been recognized for their antifungal activity and have recently attracted the attention of the food industry for their effectiveness in controlling post-harvest fungal spoilage of fruits [2, 9, 10]. In addition, the development of effective biocontrol strategies could contribute to reducing the dependence on synthesized fungicides, promoting sustainable practices to improve the quality and safety of table grapes stored at low temperatures [11]. Phenolic compounds play a crucial role in the plant defense system against pathogenic fungi. These bioactive compounds are secondary metabolites synthesized by plants under stress conditions (i.e., infections, UV radiation, etc.), presenting different chemical structures, from simple (phenolic acids) to complex molecules (flavanols, flavonols, anthocyanins, stilbenes) [4, 12-14]. In grapes, phenolic compounds are mainly found in the skin and seeds [4, 6, 7].

The primary objective of this study was to evaluate the antifungal activity of extracts obtained from four varieties of grape pomace: two white varieties, Fetească Albă and Tămâioasă Românească, and two red varieties, Fetească Neagră and Negru de Drăgășani. This evaluation was conducted in strong correlation with their phytochemical profiles, including total polyphenol content, total flavonoid content, and antioxidant activity, data already published by authors [4, 6, 14]. Based on the determined correlations (antifungal activity/phenolic compounds), the hypothesis that the grape pomace extracts can be used for viticulture as an alternative to synthetic fungicides was emphasized. However, there is concern regarding the use of these extracts because, in high concentration, alcoholic or aqueous extracts can be considered to be a risk for biodiversity and human health. The research aimed to *(i)* isolating some fungal species from natural environments; *(ii)* testing the antimicrobial activity of the extracts, and optimizing the nature of the extractant/extract concentration; *(iii)* obtaining the highest possible antifungal activity and, as far as possible, with a broad spectrum of action.

2. MATERIALS AND METHODS

2.1. MATERIALS AND REAGENTS

Grape samples from four native grape varieties (i.e., white - Fetească Albă and Tămâioasă Românească, coded FA and TR, respectively -, and red - Fetească Neagră, and Negru de Drăgășani, coded FN and ND, respectively -) were collected manually, early morning in the harvested period (i.e., September 2023), taking into consideration several maturity parameters of grapes including sugar content, titratable acidity, and pH; these data were obtained from specialists of Dealu Mare vineyards (i.e., Domeniile Săhăteni, for white grapes, and Viile Metamorphosis, for red grapes). Representative samples were used for antifungal activity determination using the Kirby-Bauer disc diffusion method. The grape pomace extracts were obtained by usual fermentation/maceration, according to the condition recommended by producers from two aforementioned vineyards.

2.2. SAMPLE PREPARATION

The collected grapes were washed, separated from bunches, grain by grain, pressed, and then placed in a fermentation vessel for seven days. Several parameters, such as pH, acidity, and refractive index, were determined during the seven days of the fermentation process. Finally, the grape pomace (skin and seeds) was lyophilized and kept in the refrigerator (4°C) for the subsequent analyses. Before the determination of the antifungal activity of pomace extracts, all samples were sterilized by irradiation in UV. The grape pomace extracts were prepared according the procedure described in the previous study of the authors [4].

2.3. MICROORGANISMS ISOLATION FROM NATURAL ENVIRONMENTS

In order to determine the antifungal activity of grape extracts, fungal species (Fig. 1) were isolated from natural environments (fruits, cereals, air). They were subcultured on specific culture media (MMA - malt wort agar and PDA - potato dextrose agar) so that the strains of interest were obtained in pure cultures.

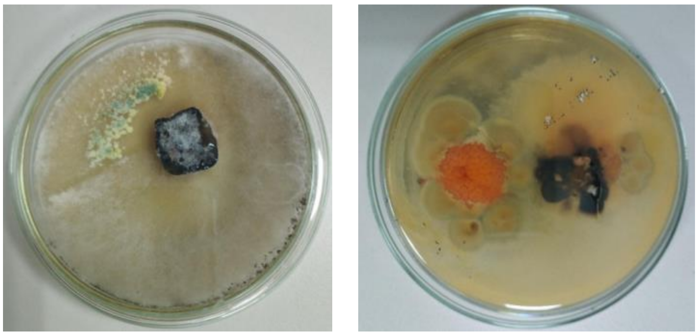


Figure 1. Heterogeneous microbiota stage. Selection of fungal strains of interest in the study.

2.4. KIRBY-BAUER DISC DIFFUSION METHOD

The Kirby-Bauer diffusimetric method [15] is the most widely used technique, being extremely simple, economical, and versatile in determining the antimicrobial/antifungal activity of extracts in relation to a target microorganism. The principle of this method consists of diffusing the extract from impregnated discs placed on the surface of the chosen solid culture medium, seeded with the tested fungal species. As the extract diffuses into the medium, a concentration gradient is created. Sensitive microorganisms cannot grow in areas where the extract concentration exceeds the minimum inhibitory concentration (MIC), resulting in clear zones of inhibition around the discs. The diameter of these zones is measured and compared with standard values to classify the microorganism as sensitive, intermediate, or resistant to each tested sample according to the standardized method recommended by the National Committee for Clinical Laboratory Standardization (NCCLS).

3. RESULTS AND DISCUSSION

3.1. RESULTS

3.1.1. Antifungal activity of grape pomace extracts against *Aspergillus niger*

Aspergillus niger is a common microorganism responsible for food spoilage and is, also, a frequent contaminant of grapes (Figs. 2 and 3).

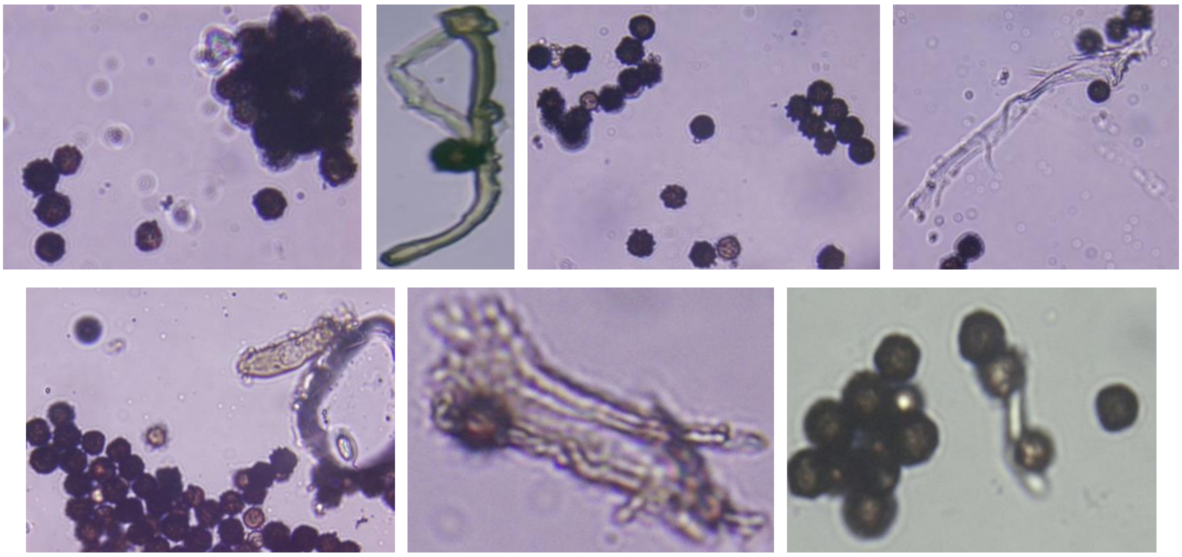


Figure 2. Microscopic aspect (10 x 100) of the *Aspergillus niger* species tested for the antifungal activity of grape pomace extracts.

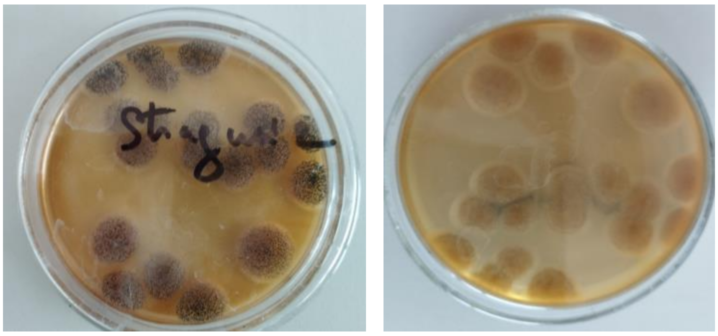


Figure 3. Macroscopic aspect (front/back colonies developed on PDA medium) of the *Aspergillus niger* species tested for the antifungal activity of grape pomace extracts.

All extracts obtained from grape pomace showed antifungal activity, the level of efficiency being different within the sample group (Fig. 3). The values of the diameter of the zone of inhibition of the growth of *Aspergillus* sp. varied within limits between 5.00 ± 0.00 mm (TR-1) and 9.66 ± 1.15 mm (ND-2) (Fig. 4 and Table 1).

Table 1. Antimicrobial activity of grape pomace extracts against *Aspergillus niger*

Sample code	Diameter of the inhibition zone [mm]	Minimum inhibitory concentration (MIC) [μg/mL]
TR-1	5.00 ± 0.00	2500
TR-2	5.33 ± 0.58	2500
FN-1	6.33 ± 0.58	2000
FN-2	7.66 ± 0.58	1000
FA-1	6.00 ± 0.00	2000
FA-2	6.33 ± 0.58	2000
ND-1	6.33 ± 0.58	1500
ND-2	9.66 ± 1.15	500

The ND-2 extract (Table 1) is noteworthy, for which the two determined parameters (including minimum inhibitory concentration) support the potential for use in various applications, including the control of *Aspergillus* sp. in viticulture.

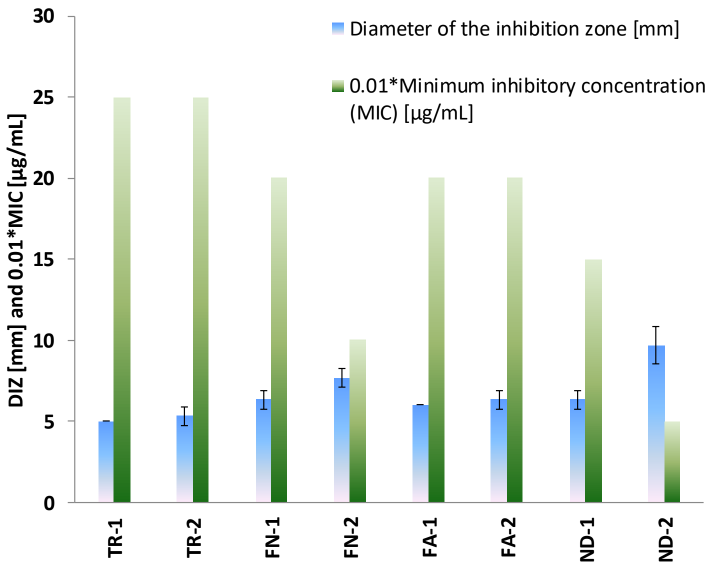


Figure 4. Antimicrobial activity of grape pomace extracts against *Aspergillus niger*

The TR-2, FN-2, FA-2, and ND-2 extracts were shown to have superior antimicrobial activity against *Aspergillus niger* compared to TR-1, FN-1, FA-1, and ND-1, respectively, an aspect due, probably, to their higher concentration of polyphenols [4, 6, 14]. The differences between the diameters of the growth inhibition zones for samples TR-1/TR-2 and FA-1/FA-2, respectively, did not reach the magnitude of those determined for the pairs of samples FN-1/FN-2 and ND-1/ND-2, respectively. Thus, the extracts with the indicator ’’-2’’ showed increased efficiency in terms of antifungal action compared to the extracts with the indicator ’’-1’’, as follows: 5.55% (FA), 6.66% (TR), 21% (FN), and 52.6% (ND), respectively.

3.1.2. Antifungal activity of grape pomace extracts against *Alternaria* sp.

Pathogenic species, such as the genus *Alternaria* (Fig. 5), are present in many agronomically important plants, including grapevines. The fungus *Alternaria alternata* (Fr. Keissler) has often been isolated from grapevine leaves and grapes during both the pre- and postharvest periods [16]. *A. alternata* causes spots on grapevine leaves, leading to the formation of necrotic lesions that quickly progress through the leaf blade. This results in premature leaf drop, which reduces agricultural production by impairing photosynthesis and directly affecting grape quality. Additionally, *A. alternata* is responsible for black rot of grapes during both fruit development in the vineyard and postharvest storage.

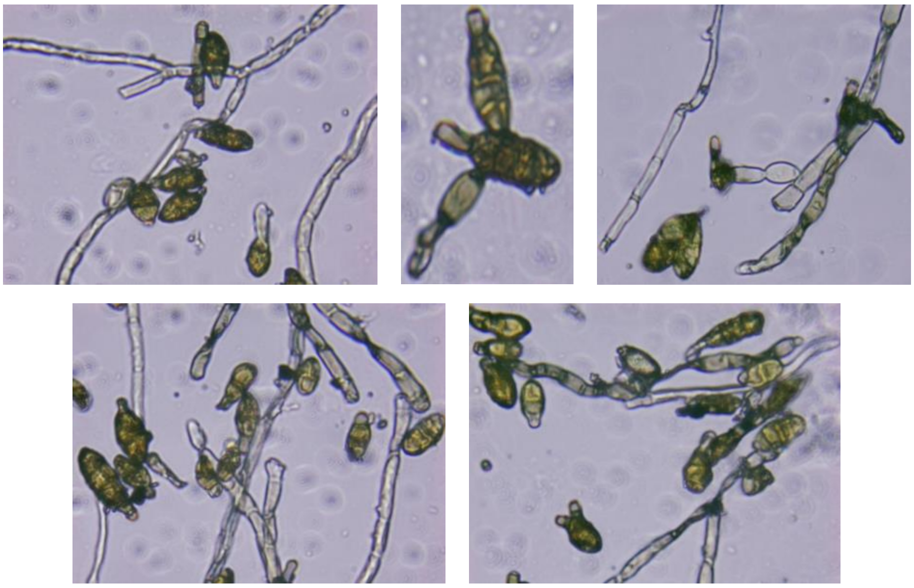


Figure 5. Microscopic aspects (10 x 100) of the *Alternaria* species tested for the antifungal activity of grape pomace extracts.

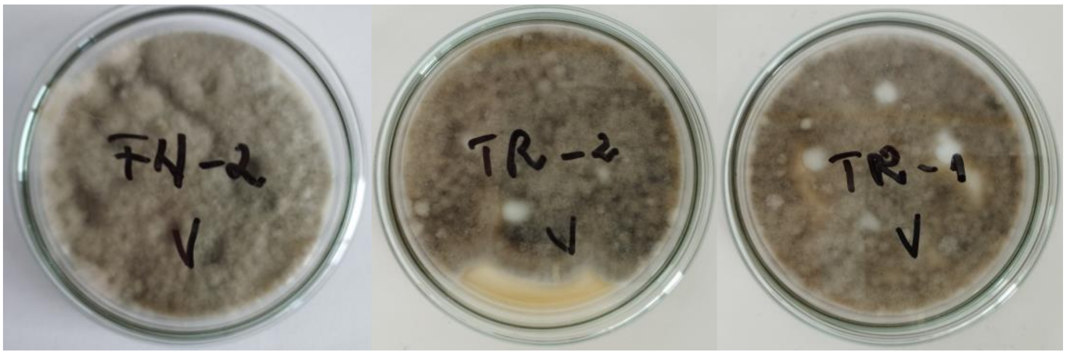


Figure 6. Highlighting antifungal activity by disk diffusimetric technique (PDA culture medium).

Within the group of samples analyzed, at the tested concentrations of grape pomace extracts, samples FN-2 and ND-1 did not exhibit antimicrobial activity against *Alternaria sp*. (Fig. 7 and Table 2).

Table 2. Antimicrobial activity of extracts obtained from grape pomace against *Alternaria* sp.

Sample code	Diameter of the inhibition zone [mm]	Minimum inhibitory concentration (MIC) [μg/mL]
TR-1	5.66 ± 0.58	2000
TR-2	6.00 ± 0.00	1500
FN-1	6.33 ± 0.58	2000
FN-2	0.00 ± 0.00	0
FA-1	6.00 ± 0.00	1500
FA-2	5.33 ± 0.58	> 2000
ND-1	0.00 ± 0.00	0
ND-2	7.33 ± 1.15	1000

It is also noted in this case that the antifungal activity of the ND-2 extract was lower, although the growth inhibition efficiency was lower compared to the growth inhibition of *Aspergillus sp*. Also, by comparatively analyzing the data in both Figs. 4 and 7, it is observed that the order of its inhibition capacity is FN-1 > FN-2 and FA-1 > FA-2. In the case of sample FN-1, equal values of the diameter of the growth inhibition zone of the two tested fungal species were determined.

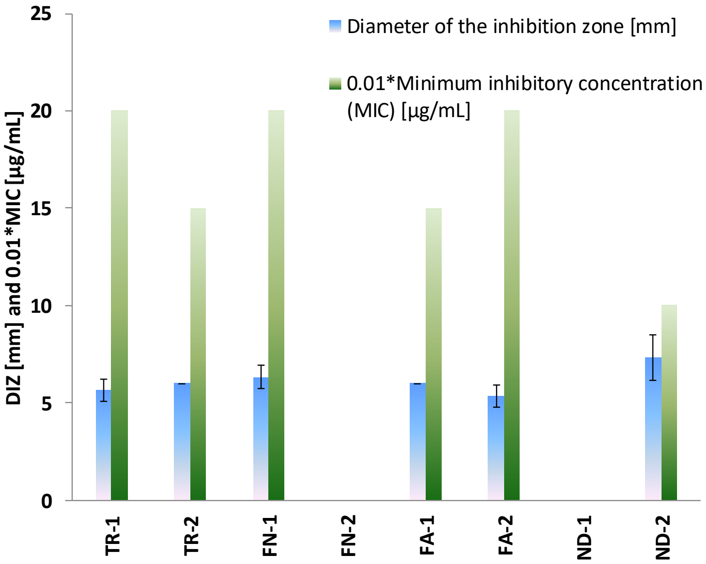
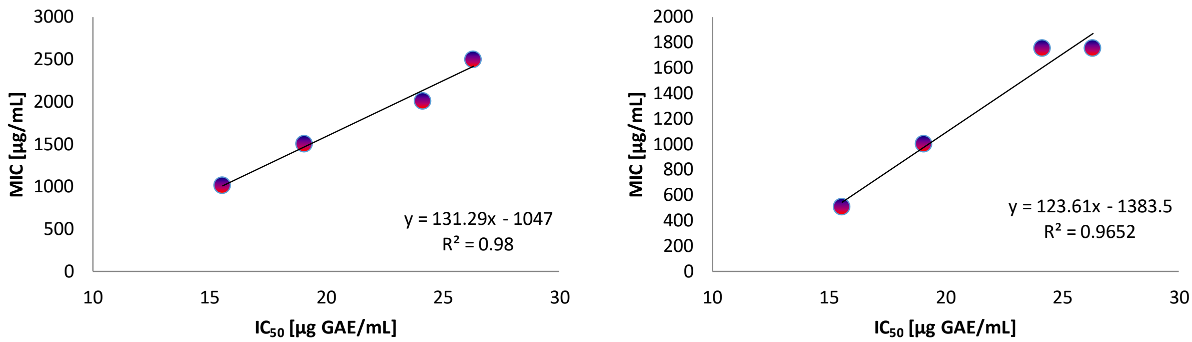


Figure 7. Antimicrobial activity of grape pomace extracts against *Alternaria* sp.

The variations of minimum inhibitory concentration with the antioxidant activity expressed as half maximal inhibitory concentration (IC_{50}) are shown in Fig. 8 for both tested fungal species. The values of IC_{50} (expressed as µg GAE/mL) were reported by Radulescu et al. (2024) in the previous study [4].



a) **b)**

Figure 8. Minimum inhibitory concentration *vs.* antioxidant activity: a) *Aspergillus niger; b) Alternaria* sp.

3.2. DISCUSSIONS

Various fungal species are associated with postharvest spoilage of fruits. Among them, *Aspergillus carbonarius* and *Aspergillus niger* are very important opportunistic pathogens, causing grape bunch or berry rot and raisin mold. Also, *Aspergillus niger* and *Aspergillus carbonarius* are the main fungi responsible for the contamination of grapes with ochratoxin A, a mycotoxin that causes negative effects on human and animal health [17]. Ochratoxins are a group of extremely dangerous secondary metabolites, classified as possible human carcinogens (group 2B) by the International Agency for Research on Cancer [2]. Therefore, it is essential to control the survival and growth of *Aspergillus sp*.

Essential oils from plants and some of the active components of the oils, which act as natural antifungals, are believed to inactivate *Aspergillus niger* [8]. Two components of *Melaleuca alternifolia* (tea tree) essential oil, namely α-terpineol and terpen-4-ol, had the strongest inhibitory action on *A. niger*, with the most severe damage being to the cytomembrane permeability, morphology, and metabolic pathways of the fungus. In addition, studies confirmed that α-terpineol and terpen-4-ol had the highest efficacy in inhibiting postharvest black rot caused by *A. niger* in grapes, at levels even higher than the essential oil [8]. In order to prevent the development of *Aspergillus carbonarius*, the potential preservative effect of essential oils obtained from eight plant species was investigated [18]. Essential oils obtained from oregano and thyme significantly limited the growth of *A. carbonarius* on postharvest grapes, which was correlated with high concentrations of carvacrol or thymol.

Tanapichatsakul et al. (2020) studied another *Aspergillus* species, namely *A. aculeatus*, which causes grape bunch rot disease, an aggressive disease that occurs in postharvest grapes and requires control [19]. The authors determined that *Cuminum cyminum* essential oil can be an alternative biological fungicide to control the disease caused by *A. aculeatus*. Treatment with *C. cyminum* essential oil, whose major volatile compounds identified were cuminaldehyde (33.94%) and α-terpinen-7-al (32.20%), was shown to delay grape ripening [19].

Dos Santos et al. (2012) demonstrated the potential of the combination of chitosan and essential oil of *Origanum vulgare* L. at sub-inhibitory concentrations to control post-harvest pathogenic fungi in fruits, in particular, *Rhizopus stolonifer* and *Aspergillus niger* in grapes [20]. Moreover, the authors found that the applied treatment preserved the quality of the grapes, measured by their physical and physicochemical attributes, while some of the sensory attributes improved during the storage period evaluated. Structural changes in the mycelium of the two fungi were determined as a result of the application of chitosan in combination with the essential oil. The minimum inhibitory concentration values, against both *R. stolonifer* and *A. niger*, were 10 mg/mL for chitosan and 10 μL/mL for the essential oil, respectively [20].

The antifungal activity of perylaldehyde (PAE), a monoterpene compound with antioxidant, antimicrobial and antidepressant activity, the main component of the essential oil of Perilla frutescens, was evaluated against *Aspergillus niger*. PAE showed notable antifungal activity against *A. niger*, with a minimum inhibitory concentration (MIC) and a minimum fungicidal concentration (MFC) of 0.25 and 1 μL/mL, respectively. Mycelial biomass accumulation was also inhibited by PAE in a dose-dependent manner [2]. According to the authors, PAE appears to ‘‘target’’ ergosterol biosynthesis as a way to inhibit the growth of *A. niger*, leading to significant damage to the cell membrane of this pathogen [2].

Biocontrol involves the use of organisms (such as bacteria, fungi, yeasts) or biologically derived compounds to manage plant diseases or pests. Yeasts have gained attention as potential biocontrol agents due to their simple nutritional requirements, ability to colonize dry surfaces for long periods, and rapid growth on inexpensive substrates [3]. Torres-Palazzolo et. al. (2024) studied the potential of indigenous yeasts and bioactive compounds from natural sources (chitosan and hydroalcoholic extracts obtained from vine ropes and bunches, respectively) to act as biocontrol agents against *Alternaria alternata* present in cold-stored table grapes [11]. For both extracts, concentrations of 5% and 25 % were most effective, while undiluted extracts were ineffective against mold growth. The extract from grapevine rope was found to be more effective against *Alternaria* sp. compared to that obtained from grape bunches [11].

Chitosan was also effective as a biocontrol agent in postharvest *in vivo* tests against *A. alternata*, resulting in similar fungal incidence rates to those obtained with SO_2 (traditional control) in the prolonged cold storage of grapes. Equally beneficial results were obtained by applying different doses of chitosan, such that the option of using the lowest doses would reduce chitosan residue in grapes to be marketed. The authors suggest that chitosan would work better as a curative treatment of grapes that come with a certain concentration of Alternaria alternata from the vineyard [21].

Gray mold, caused by *Botrytis cinerea*, is one of the most destructive post-harvest diseases in grape production. Recently, a new biocontrol agent for B. cinerea was investigated, which included *Brevibacillus brevis* and *Bacillus velezensis* [22]. The mixed microbial combination effectively suppressed the growth of *B. cinerea in vitro* and reduced the percentage of grape and vine leaf decay in vivo. Controlling *B. cinerea* infection using synthetic fungicides can lead to increased resistance among pathogen populations. An alternative to synthetic products is the development of natural fungicides derived from bioactive plant compounds. A new water-soluble bioproduct with antifungal properties has been created by extracting polymeric proanthocyanidins from the seeds of the "Fetească neagră" grape variety [23]. This bioproduct, which has a total polyphenol content of 0.625 mg GAE mg^{-1}, demonstrated a moderate effect on the radial growth of *B. cinerea mycelium*. Furthermore, at concentrations approximately ten times lower, it exhibited effective antifungal activity in inhibiting spore germination [23]

Bhosale et al. (2024) highlighted the significant role of endophytic bacteria isolated from grape leaves as antifungal tools, including against *Alternaria* sp. [24]. Endophytic bacteria can indirectly promote plant growth due to their ability to combat plant pathogens. The antimicrobial activity was also supported by the ability of endophytic bacteria to secrete hydrolytic enzymes (mainly cellulase and chitinase, but also amylase, protease, and lipase), which played a role in the destruction of fungal cell walls [24].

Thirty-one yeast species in total, isolated from grape must (*A. pullulans, K. mangrovensis, P. fermentans, P. kluyveri, W. anomalus,* etc.) were shown to exhibit antagonistic activity against pathogens (including *Aspergillus niger* and *Alternaria alternata*), giving these yeasts the potential to be used as biological control agents with antifungal activity [1]. The study largely confirmed that various mechanisms, including degradation of the pathogen cell wall, inhibition of cell wall polysaccharide biosynthesis, and inhibition of spore germination through the production of volatile organic compounds, can be expressed by yeasts interacting with filamentous fungi [1].

The possibility of increasing the aggregate value of the huge amount of residues generated by the wine industry stimulates studies using pomace to obtain functional ingredients. Currently, consumers generally prefer natural and safe products, both in the food and cosmetic fields. For these reasons, supercritical fluid extraction is of great importance due to the purity of the obtained extracts [25]. Extracts from Merlot and Syrah grape pomace, obtained using supercritical CO_2, had, as main components, gallic acid, benzoic acid, vanillic acid, and epicatechin. These extracts were shown to have antimicrobial activity against four strains of bacteria and three strains of fungi [25].

Black grape skin possesses a substantial amount of polyphenolic antimicrobial compounds, which can be used to control the growth of pathogenic microorganisms [26]. As an antimicrobial agent, polyphenols can penetrate the semipermeable cell membrane, where they react with cytoplasmic or cellular proteins. The antimicrobial potential of polyphenols is higher in the extract obtained from grape skin since phenolic acids are present in an undissociated form. Therefore, these highly negatively charged antimicrobial polyphenolic compounds can be used to prevent the growth of antibiotic-resistant pathogenic bacteria and toxin-producing molds. From an extraction point of view, different solvents can be used since the solubility of polyphenols depends on the aqueous and non-aqueous medium [26, 27].

Grape skin was subjected to polyphenol extraction using different solvents, namely water, ethanol, acetone, and methanol. Extracts with concentrations of 260, 540 and 1080 mg

TAE/mL (TAE = tannic acid equivalent) were tested against the growth of the fungi *P. chrysogenum*, *P. expansum*, *A. niger and A. versicolor*. The trend of inhibition of fungal growth was as follows: methanolic extracts > acetone extracts > ethanolic extracts > aqueous extracts. The minimum percentage of growth inhibition, of only 15%, was determined against *Aspergillus niger*, at a concentration of 1080 TAE/mL, both in the case of aqueous extracts and in the case of methanolic extracts obtained from grape skin [26].

Sagdic et al. (2011) determined the phenolic compounds correlated with the antioxidant and antifungal properties of ethanolic extracts obtained from five commercial grape varieties (three red and two white) [28]. The antifungal activity of the extracts was evaluated both in vitro (agar well diffusion test) and in situ (growth assay in apple and orange juices), using pure cultures of *Zygosaccharomyces rouxii* and *Zygosaccharomyces bailii*. Although the antifungal effects of the grape pomace extracts could be attributed to their phenolic content; stilbenes, present in small amounts, were found to be more active antifungal agents than phenolic acids and flavonoids [28].

Extracts obtained from leaves of different varieties of *Vitis vinifera*, in whose phenolic composition was identified isoferulic acid (30.98%), quercetin (14.62%), rutin (28.73%), catechin (24.92%), demonstrated antimicrobial activity against *A. niger*, the maximum inhibition being 81.81% [29].

The growth of *Aspergillus niger* was inhibited both by extracts obtained from grape skin, with a high content of polyphenols (1080 tannic acid equivalents/mL), and by those obtained from grape seeds (growth inhibition zone of 15.00 ± 0.81 mm at a concentration of 1000 μg/mL) [30].

Ethanolic extracts obtained from *Cinnamomum zeylanicum*, *Amomum subulatum*, *Trigonella foenum graecum* and *Mentha piperita*, respectively, tested by the disk diffusion method at a concentration of 100μg/disk, showed antifungal activity against *A. niger*, with the diameter of the growth inhibition zone varying between 6.0 ± 0.1 mm and 10.0 ± 0.1 mm [31]. Rutin, kaempferol, and quercetin were identified as the polyphenols in the plant composition that exerted antifungal action. The authors emphasize the correlation between the antifungal activity of the extracts and their excellent antioxidant potential. This could be due to the fact that plant flavonoids have been reported to inhibit biofilm formation by stimulating disruption of the cell membrane integrity, which reduces the size of fungal cells and causes leakage of intracellular components. Phenols are also oxidized to their respective quinones in response to infection, which can subsequently inactivate fungal enzymes. Polyphenols have ortho-para groups (which tend to donate electrons) that may contribute to their antioxidant and antifungal properties [31].

From our knowleadge, related to studies already mentioned, it has not been reported data in terms of correlations between antioxidant and antifungic activities of grape pomace extracts. In this respect, these investigations highlighted that the minimum inhibitory concentration of the grape pomace extracts is direct proportional with the antioxidant activity (as IC_{50}), with good linearity demonstrated by the R^2 values: 0.9800 in the case of *Aspergillus niger* and 0.9652 for *Alternaria* sp.

4. CONCLUSIONS

The obtained grape pomace extracts demonstrated a variable activity of inhibiting the growth of the two tested fungal species. The ND-2 sample showed good efficiency against both *Aspergillus sp*. and *Alternaria sp*., molds recognized for affecting the life cycle of the vine, for post-harvest degradation of grapes, and the production of mycotoxins, respectively.

The high concentration of bioactive compounds and the antioxidant capacity of the ND-2 extract may be responsible for the antifungal activity exhibited by it. The results of the study can thus be used to develop new applications of extracts obtained from grapes, contributing to sustainable wine production, the protection of human health, and, last but not least, the environment.

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