Exploring the powerful phytoarsenal of white grape marc against bacteria and parasites causing significant diseases

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Abstract:

Natural extracts containing high polyphenolic concentration possess antibacterial, antiparasitic and fungicidal activities. The present research characterises white grape marc, a winemaking by-product describing their physicochemical features and antimicrobial capacities. Main components of 2 different extracts generated were phenolic acids, flavan-3-ols and their gallates, and flavonols and their glycosides. As a result of this complex composition white grape marc extracts showed pronounced bioactivities with potential uses in agricultural, pharmaceutical and cosmetic industries, among others. Specifically, polyphenol compounds were extracted by using hydro-organic solvent mixtures from the by-product of Albariño white wines (Galicia, NW Spain) production. In the present work the "*in vitro*" antimicrobial activity of the bioactive extracts was evaluated using two different hydro-organic mixtures (HO_L & HO_P). The microorganisms tested included Gram positive and negative bacteria, two Apicomplexan parasite species and one Oomycota parasite. Microbial species investigated are causing agents of several human and animal diseases, such as foodborne illnesses (*Bacillus cereus*, *Eschericha coli*, *Salmonella enterica*), skin infections (*Staphylococcus aureus*), mastitis (*Streptococcus uberis*), parasite infections as Malaria (*Plasmodium falciparum*) or Toxoplasmosis (*Toxoplasma gondii*), and plant infections as "chestnut ink" in chestnuts or "root rot" in avocado, both diseases caused by *Phytophthora cinnamomi*. Both extracts verified activity against all the tested species demonstrating their potentiality to be used for the development of biocides to control a wide range of pathogenic agents; at the same time that they contribute to winemaking industry residues valorisation.

Key words: grape marc, natural extract, polyphenols, antiparasitic, antibacterian, winemaking by-products valorisation.

Introduction:

Grapes are one of the largest fruit crops in the world. According to the Food and Agricultural Organization (FAO) of the United Nations, >67 million tons of grapes are produced annually worldwide, and during the production of wines, there is a big amount of the grapes that end up as byproducts. Among the mentioned byproducts, grape marc (peel, seeds, and stems after wine production) constitutes a very non-expensive material with numerous interesting activities due to its composition. That composition, in general, depends on the variety of the grape, type of soil, climate and wine making techniques[1].

Grape marc also contains several bioactive compounds which are different to those found in grapes and wine, opening a new path for exploring its potential anti-pathogenic activity. Activity of wine byproducts against pathogenic bacteria, virus, fungi toxins and parasites has been proved. This fact, together with the interest risen in the last decades, about finding natural bioactive compounds against different diseases due to antibiotic resistance among other issues, makes grape marc a good candidate to find new effective treatments and therapeutic strategies. Its composition is very rich and complex. Some of the compounds included are anti-oxidant phenolic compounds, which have been described as potential agents against several pathogenic diseases. The high phenolic content of grape marc is due to the fact that some of these bioactive compounds are poorly extracted into the wine during the vinification process [2,1].

An antioxidant agent is a molecule that delays, prevents or clears oxidative damage in a target cell[3]. They can act in biological systems by different mechanisms, including electron donation (acting as reducing agents), metallic ion chelation (deleting potential free radicals), or by regulation of genic expression[3]. This group of substances act at low concentrations and significantly inhibit or retard the oxidative process while they are oxidized. Some examples of antioxidants are ascorbic acid, uric acid and some polyphenols as resveratrol[4]. Their employment as additives is broadly distributed in industry field to delay, prevent or eliminate damage caused by oxidation. Antioxidant dietary fibre as well as extractable and non-extractable polyphenol content in bagasse obtained during winemaking usually ranges from 50 to 75 %, 1 to 9 % and 15 to 30% by dry matter, respectively. Due to this complex composition, grape marc extracts have a great potential to display extensive uses in agricultural, pharmaceutical and cosmetic industries among others[5,6,7,8]. The grape marc contains the following antioxidative polyphenolic categories: phenolic acids, flavonoids, lignans, and stilbenes[9].

The white grape marc is rich in polyphenols. The health promoting and disease preventing benefits of different types of grape polyphenols are well documented [14,15]. Phenolic acids, flavan-3-ols and their gallates, and flavonols and their glycosides, are the main phenolic constituents of white grape marc [16].

The present work describes the polyphenolic profile and antimicrobial capacities of bioactive extracts obtained from the white winemaking byproducts to give them a new use and valorisation. Bioactive polyphenols were extracted with hydro-organic solvent mixtures from the byproduct of the production of high quality Albariño white wines (Galicia, NW Spain). We then evaluated the "in vitro" antimicrobial activity of two extracts from Albariño grape marc using two different hydro-organic mixtures (HO_L & HO_P). Extracts were used against relevant microorganisms, including Gram positive and negative bacteria, two Apicomplexan parasite species and one Oomycota parasite.

Natural extracts as the ones studied in the present work, have raised interest in the last years as an alternative to general antibiotics and anti-parasitic treatments[17]. Extensive use of drugs has generated an increase in antimicrobial resistances being a worrying issue in the present. The use of natural extracts with antimicrobial capacity can be a good alternative that could avoid the generation of resistances[17,18]. On this way, the microbial species studied in the present work are involved in several diseases in humans and animals, such as foodborne illnesses (*Bacillus cereus, Eschericha coli, Salmonella enterica* subsp. *enterica*), skin infections (*Staphylococcus aureus*), and mastitis (*Streptococcus uberis*).

Besides, human and animal parasite infections as Malaria (*Plasmodium falciparum*) or Toxoplasmosis (*Toxoplasma gondii*) have been considered. *Plasmodium* genre´s species cause complex diseases and constitutes a serious health problem around the world. The World Health Organization estimated a number of 219 million people infected by *Plasmodium* distributed in 87 countries, almost 500,000 deaths and a considerably large population at risk of infection by this parasite in 2017[19]. *Toxoplasma gondii* is a protozoan parasite belonging to the phyla Apicomplexa. It is the causing agent of toxoplasmosis, a zoonotic

disease of worldwide distribution, which generates a significant problem on public health and on global economy. It is considered a high risk zoonotic agent by the European Food and Safety Agency (EFSA) [20] with an estimated one-third of the world's population infected[21].

Last, to evaluate the potential of the Albariño extracts in plant infections, we chose *Phytophthora cinnamomi* an oomycete species that causes known plant diseases as "chestnut ink" in chestnuts or "root rot" in avocado. The oomycetes form a phylogenetically distinct group of eukaryotic microorganisms that includes some of the most notorious pathogens of plants. Among these, members of the genus *Phytophthora* cause enormous economic losses on crop species as well as environmental damage in natural ecosystems. *P. cinnamomi* is the most widely distributed *Phytophthora* species, with nearly 1000 host species[22,23]. Although oomycetes have a filamentous growth habit they are not related to fungi and possess distinct mechanisms for pathogenicity. Consequently, fungicides rarely control them and the few anti-oomycete products are often overcome by resistant pathogen variants.

Due to climate change and migration[25], among other factors, a large proportion of the population is at risk of infection with parasites and other infectious agents, being thus, the study of infectious diseases an emerging field[26]. Trying to find new treatments to these diseases becomes a relevant topic of study nowadays mainly due to the lack of effectiveness or appearance of side effects with the existing ones, emergence of drug-resistance or just nonexistence of treatment.

Material and methods:

***Extracts production and polyphenolic evaluation*:** The extraction procedure is a green and straightforward process with few steps, under gentle conditions and using non-contaminating materials, while preventing the obtained eluates from containing suspended solids. Raw material is white grape marc from *Vitis vinifera* var. Albariño. Extract and process are patent-protected (Lores, 2014: ES 2 443 547; WO 2014/013122 A1) and can be obtained on lab, pilot or industrial scales.

***Anti-bacterial assays*:** a cellular suspension of the microorganisms to test was incubated in different extract concentrations at 20, 10, 5, 2.5,1.25, 0.625 and 0% at 37 C° for different times depending on the species. Time was adjusted to 3h for *E. coli* ATCC 8739 and *S. enterica* subsp. *enterica* CECT 554, to 2h for *B. cereus* CECT193 and to 1,5h for *S. uberis* CECT 994 and *S. aureus* CECT 59. The assay was performed in a sterile 96 multiwell plate, with 200 µl of final volume and incubated for 1,5-3h at 37ºC. The incubation time can be adjusted according to the survival of the positive control (optimal survival 50-200 colony forming units (CFU) from a 10^{-6} dilution). Some strains are more sensitive, and their survival is affected under the test conditions. To estimate the survival after incubation, samples of 20 µl with different concentrations from a serial dilution were grown on agar plates. After 16h of incubation at 37ºC, the CFU were counted and used to calculate IC_{50} values, with Quest Graph™ IC50 Calculator[27].

***Anti-parasitic assays*:** growth inhibition of three species was evaluated; one plant parasite, *P. cinnamomi* CECT 20919 and two human and animal parasites, *P. falciparum* 3D7 and *T. gondii* RH Type 1.

P. cinnamomi

The inhibition's percentage in the growth of *P. cinnamomi* was determined in both extracts, at the concentrations of 0% 4% and 10%. *P. cinnamomi* was grown on plates of 90mm in diameter, with 50mL of potato-dextrose-agar medium (PDA) per plate with the different extract's concentrations at 10, 4 and 0% for 10 days at 22°C in the dark. The antifungal capacity has been determined according to the growth inhibition respect to the untreated cultures (0% concentration). Quest Graph™ IC50 Calculator[27] application was used to calculate $IC_{50s.}$

Toxoplasma gondii

Human foreskin fibroblasts (HFF) were cultured in black optical bottom 96-well culture plates until confluence was reached. At this point, freshly egressed red-fluorescent *T. gondii* tachyzoites were washed and resuspended in culture medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with Penicillin-Streptomycin antibiotics and a 10% of Fetal Bovine Serum (FBS) without phenol red (Gibco BRL Life Technologies, Rockville, Md.))[1]. Each well of the HFF culture plate was then infected with approximately 500 parasites. Parasites were treated with the different extracts at concentrations of 1, 0.5,

0.25, 0.125, 0.065 and 0% (v/v drug/medium) in triplicate and incubated for 7 days at 37°C under 5% CO_2 and 100% humidity conditions. Fluorescence was read daily in a PHERAstar FS plate reader, and data from each drug concentration replicates averaged. Both, excitation (540 nm) and emission (590 nm) were read using the bottom optics option in the reader. Each experiment included triplicate controls for the uninfected host cells cultured in medium containing the extracts, as well as triplicate controls of *T. gondii*-infected host cells cultured in medium containing only the solvents used to resuspend the extracts[2]. Quest Graph™ IC50 Calculator[27] application was used to calculate IC_{50s} from day 2 to 7.

Plasmodium falciparum

P. falciparum was cultured in RPMI 1640 containing 11 mM glucose, 0.5% (w/v) Albumax II, 200 μM hypoxanthine and 20 μg/ml gentamycin (PAA) in human red blood cells (RBC) at 5% (w/v) haematocrit, as previously described (2, 3). Parasite cultures were maintained at 37ºC under an oxygen reduced atmosphere containing 1% (v/v) O_2, 3% (v/v) CO_2 and 96% (v/v) N_2[28]. To analyse IC_{50} concentrations of the extracts, wild type parasites were cultured in black optical bottom 96 well plates at 0.3% parasitemia and 2.5% haematocrit for 72h at 37ºC in culturing chambers with reduced oxygen. Infected RBC were exposed to 1:2 dilutions of the extracts in triplicates from a concentration of 2% to 0.004% (v/v) including no drug controls. Similar triplicate conditions were also set up for solvent-only controls in each plate. After incubation time, plates were frozen overnight at -20ºC. The plates were then thawed at RT for 3-4 hours and each well added with an equal volume of a 1x solution of SYBR green[29]. After incubation for 1h in the dark, fluorescent signal was acquired in a PHERA star FS plate reader using a 485-520 filter. Average values for each triplicate were then used to calculate IC_{50} concentrations using the software Quest Graph™ IC50 Calculator[27].

Results:

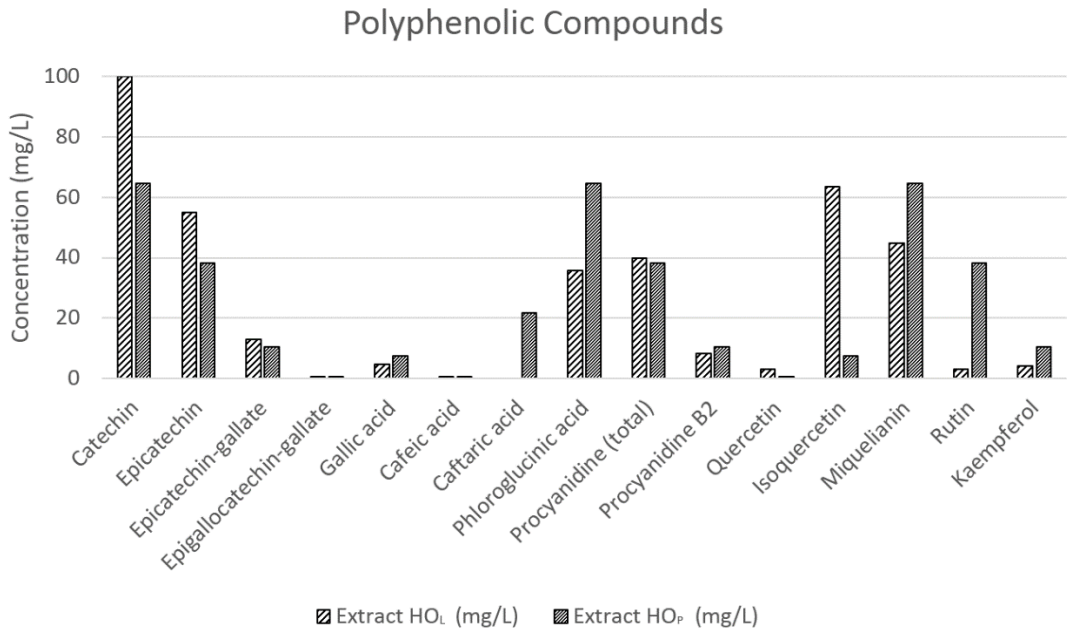
Polyphenol rich extract composition:

White grape marc extracts obtained showed a high content in polyphenols as it can be observed in Table 1. Nevertheless, there was a relevant difference in the composition between the 2 extracts. HO_L showed higher levels of catechin, epicatechin and isoquercetin than HO_P, while HO_P presented a considerably higher concentration of phologlucinic acid, miquelianin and rutin; also it is more concentrated in kaempferol and it has caftaric acid which is not present in HO_L. Polyphenolic compounds abundance and differences between the extracts can be observed in Figure 1. Stability studies of both extracts showed that their bioactivities are kept at room temperature for at least one year. In addition, the extracts maintain their polyphenolic profile and show thermal stability up to a temperature of 120 ºC, which opens up many possibilities for obtaining formulations containing them

Table 1. Main Polyphenols in white grape marc extracts. Concentration expressed in mg polyphenol/L extract (Testing method: LC-MS/MS Analysis).

Polyphenolic Compound	Extract HO_L (mg/L)	Extract HO_P (mg/L)	Polyphenolic Compound	Extract HO_L (mg/L)	Extract HO_P (mg/L)
Catechin	100.1	64.6	Procyanidine (total)	40.0	38.3
Epicatechin	54.9	38.3	Procyanidine B2	8.2	10.4
Epicatechin-gallate	13.0	10.4	Quercetin	2.9	0.15
Epigallocatechin-gallate	0.31	0.15	Isoquercetin	63.5	7.3
Gallic acid	4.7	7.3	Miquelianin	44.7	64.6
Cafeic acid	0.007	0.02	Rutin	3.0	38.3
Caftaric acid	--	21.6	Kaempferol	4.0	10.4
Phloroglucinic acid	35.7	64.6			

Figure 1. Representation of Main Polyphenols in white grape marc extracts. Concentration expressed in mg polyphenol/L extract (Testing method: LC-MS/MS Analysis).



Antibacterial activity:

Both types of bacteria, Gram negative and positive, were analysed. The extracts were active against both types. As indicated in table 2, for most of the bacterial species analysed, the IC_{50} is lower than 1,25% with exception of *S. uberis* which showed a slightly higher value in HO_P. In general, the HO_L showed a higher activity being its concentration lowest to reduce the growth in 50%.

Table 2. Inhibitory concentration 50% (IC_{50}) for anti-bacterial assays. Concentration expressed in % (v/v).

Species	IC_{50} (Extract HO_L)	IC_{50} (Extract HO_P)
Staphylococcus aureus	0,809	<<0,625
Bacillus cereus	<<0,625	<<0,625
Eschericha coli	0,718	0,795
Streptococcus uberis	<<0,625	1,349
Salmonella enterica subsp. enterica	0,752	1,025

Average values for triplicates were used to calculate IC_{50} concentrations using Quest Graph™ IC50 Calculator

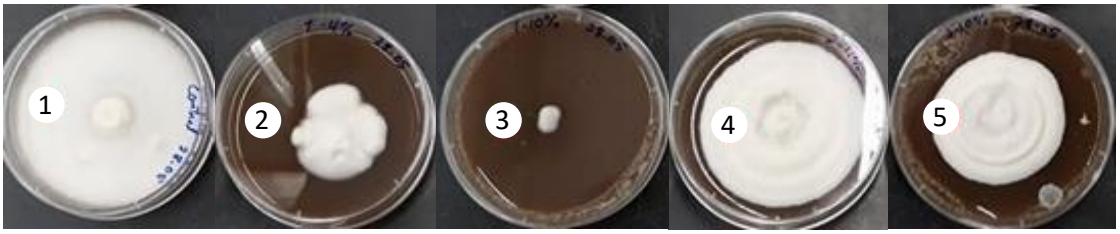
Anti-parasitic activity:

Extracts were also tested against the plant parasite *P. cinnamomi* and the human and animal parasite *T. gondii* and *P. falciparum*.

On *P. cinnamomi* assay HO_L showed a significative higher activity than HO_P extract as it can be clearly observed in pictures 2 and 3 of Figure 2. The concentration needed of HO_L to reach the same activity is 6 times lower than the quantity necessary for HO_P.

Figure 2. Inhibitory concentration 50% (IC_{50}) for *P. cinnamomi*. Illustrative results of the experiment, 1- Control (0%), 2- Extract HO_L 4%, 3- Extract HO_L 10%, 4- Extract HO_P 4%, 5- Extract HO_P 10%. Concentration expressed in % (v/v).

Control	IC_{50} (Extract HO_L)	IC_{50} (Extract HO_P)
	3,17	20,23



Average values for triplicates were used to calculate IC_{50} concentrations using Quest Graph™ IC50 Calculator

The anti-parasitic activity was assessed by growth inhibition of *T. gondii* and *P. falciparum* trophozoites. Both grape marc extracts were able to reduce parasite load as monitored by the IC_{50} values obtained by fluorimetry, in the case of both Apicomplexan parasite species. Neither extract nor vehicle treatments were toxic to fibroblast cell cultures as it was observed in the controls included. Although both extracts showed activity, HO_L showed a more efficient rate of anti-parasitic activity as the IC_{50} values obtained were lower than the ones for HO_P

Regarding *T. gondii* and *P. falciparum* the opposite effect could be observed, being in this case more active HO_P extract as observed in Table 3. In *P. falciparum* assay the concentrations necessary to inhibit the growth were lower in both cases, compared to *T. gondii* assay.

Table 3. Inhibitory concentration 50% (IC_{50}) for *T. gondii* and *P. falciparum*. Concentration expressed in % (v/v).

Species	IC_{50} (Extract HO_L)	IC_{50} (Extract HO_P)
Toxoplasma gondii	1,23	0,57
Plasmodium falciparum	1,07	0,26

Average values for triplicates were used to calculate IC_{50} concentrations using Quest Graph™ IC50 Calculator

Discussion and conclusions:

Grape related industry has a big economic value worldwide and there are several grape derived products like wine, which can be presented in many different formats depending on the grape type, elaboration process, etc. Due to the huge development of wine industry, a big amount of wine by-products is produced yearly. Finding a valuable use of these by-products will contribute to waste reduction adding a new source of economical profitability to the wine industry. At the same time, a more sustainable production process could report more benefits, due to the current trend in general society of developing environmentally-friendly products; finding a use for a residue will influence positively the public opinion about wine industry which could also be returned in an increase of consumption. As said, grape marc is one of the most abundant by-products of wine industry being in winemaking countries such as Spain around 1200tonnes per year [30]. Grape marc can also be considered a low-cost source of polyphenols, which could have interesting applications in many different industries[31,32,33] along with its upgrading to become a high valuable by-product[34].

Polyphenol Rich Extracts

In this work, the chemical composition and the anti-pathogenic effect of white grape marc extracts were evaluated. The polyphenolic composition of the HO_L and HO_P extracts was determined by LC-MS/MS, and

the major components identified are listed in Table 1. Grape marc extracts compositions vary depending on the method and solvent used for the extraction[35]. Both extracts resulted to be very rich in polyphenols and some differences were highlighted. One of the major differences is the content in phologlucinic acid, miquelianin, rutin and kaempferol, which could have potential implications in their capacity as anti-microbiological agents.

Activity against major infectious diseases

Several studies demonstrate the activity of the different polyphenols purified as anti-microbiological[36,37] and anti-parasitic[38,39,40] agents. Recently, their potential as synergic agents and their interaction with drugs when used as anti-microbiological[41] and anti-parasitic[42,43] agents, as well as the increase of the activity of such polyphenols when they are used in a combined way, has been a topic of interest[36,42,43]. All this together seems to indicate that natural extracts similar to the one studied in the present work, and obtained with the aim to conserve the synergistic activity of their specific polyphenolic content, are in fact, an efficient and economically viable approximation for the treatment of bacterial and parasitic derived infectious diseases.

Bacteria

Grape marc and grape seed extracts-based films have been reported to show activity against bacteria[44,45,46,47]. Seed and skin extracts from grape winery byproducts showed anti-bacterial and antifungal activity highlighting their potential to be used as anti-microbiological agents[48] in previous studies. Grape marc was found to be even richer in bioactive compounds than skin extracts and its potential to treat food to prevent deterioration has already been emphasised[49,50,51]. Table 1 shows the composition of the polyphenols present in both extracts. Many polyphenols are shared in both extracts, but their total composition and concentration possess significant differences that may be related to their dissimilar behaviour in the antimicrobial tests performed.

Parasites

The effect of the 2 different extracts from grape marc collected in Galicia, Spain, on Apicomplexan and Oomycota parasites, *T. gondii*, *P. falciparum* and *P. cinnamomi* has been also investigated in this study.

Parasite resistance to current treatments has generated the necessity to find new drugs against different infectious diseases. Natural products appear as a promising source to find potential solutions and have been used in traditional medicine extensively[52,53,54,55,56]. Among the natural products that have shown activities against different bacteria, fungi, and parasites, products obtained from grapes have been described to be involved in plant defence against pathogens as *P. cinnamomi*. Some of these products are phenolic compounds also active against human or animal pathogens, like *Toxoplasma gondii* [57], a parasite belonging to the phylum Apicomplexa causing the global foodborne disease toxoplasmosis. Apicomplexan diseases comprehend worldwide distribution infectious diseases such as toxoplasmosis or malaria.

As it can be observed in Figure 2 and Table 3, anti-parasitic effect of the target objectives studied in the present work is clear. Specifically, activity against oomycete *P. cinnamomi*, is observable for both extracts, being higher for HO_L. In fact, otherextracts from natural origin and similar to the ones described in this study, have shown activity against this species[58]. Differences in anti-parasitic capacity against *P. cinnamomi* between both extracts can be due to the different polyphenol concentrations they possess. Cathechin and epicathechin concentrations are higher in OH_L. Catechin demonstrated their antifungal capacities against Oomycota[59]. Likewise, high levels of epicathechins increase plant resistance to *P. cinammomi* infections[60].

On the contrary, the HO_P extract was the most active against *T. gondii* and *P. falciparum* and the anti-parasitic activity of the extract increased in a concentration and time-dependent manner. Since both of the extracts reduced the parasite load, we can suggest at this point that their activity is related to their polyphenol content. Other natural polyphenols have shown activity against *T. gondii* in previous studies[61,13].

There are significative differences between both extracts in the concentrations of some particular polyphenols, like rutin and kaempferol, being higher in the HO_P, which is the more efficient one. For this

reason we could infer a potential action of these specific polyphenols which have already been described as anti-malarial agent in different studies[62,63,64,42].

In other cases, natural polyphenols were used to complement activities of other antiparasitic treatments as it is the case for resveratrol combined to sulfamethoxazole-trimethoprim against *T. gondii*[65]. Anti-Plasmodium activity has also been tested several times by using polyphenols[66,67,68]. Our results demonstrate, for the first time, that both extracts of white grape marc reduce *T. gondii* infection in HFFs and that *P. falciparum* growth is also severely affected. Grape marc hydroalcoholic immunomodulatory and anti-immflamatory extracts were described to stimulate humoral immune responses in vaccine design for different parasitosis[69]; this topic could be of potential interest for further research with white grape marc extracts in the future.

To summarize, this is the first time that the same wine natural extract has been shown to be effective against both bacteria and parasites that attack humans, animals and plants; and this fact is confirmed for the two formulations obtained from white grape marc. Both extracts showed high antimicrobial potential, being effective for several kinds of bacteria and parasites belonging to different clades, all of them of economical and global health importance. The extracts assessed present a marked anti-pathogenical activity.

Accordingly, these results open up promising ways to valorise white grape marc, a by-product of wineries activity, due to the potential application of the target extracts as preservatives in cosmetic and food industry, sanitizing agents and phytosanitary products. In addition, it allows us to dream of a plant phenolics-based therapy against very concerning human diseases. Both extracts were obtained with solvents that are generally regarded as safe (GRAS).

In this way, anti-pathogenic activity and chemical composition assessed by LC-MS/MS, indicate the high potential of the polyphenols from grape marc to act as possible anti pathogenicals with large scope of action, which could lead this work to further studies concerning the development of therapeutic products of natural origin, targeted to the treatment of relevant infectious diseases.

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