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Study of the Antimicrobial Properties of Oleoresins from Berries of Aguaribay (*Schinus molle* Linn) on Fungi and Food Spoilageyeasts

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Abstract: In this research the antimicrobial properties of the oleoresin extracted from berries of Aguaribay (*Schinus molle* L.) are tested on fungi commonly found in foods such as *Penicillium nalgiovence, P. citrinum, P. rugulosum, P. griseofolvum, P. brevicompactum, Aspergillus flavus, Alternariaalternata y Fusarium graminearum*. It was found that an oleoresin concentration of 0.125 mg mL^{-1} reduces the mycelial growth of these fungi, whereas in the cases of *Eurotium, Epicoccum, Trichoderma* and *Paecylomyces* effective oleoresin concentrations were 5, 10 and 15 mg mL^{-1}. In the case of Epicoccum, a concentration of 15 mg mL^{-1} produces 100% inhibition of mycelial growth. Studies of germination inhibition of conidia showed that the minimum lethal concentration (MLC) for *A. ochraceus* and *A. flavus* was 6 and 25 mg mL^{-1} for A. paraciticus. The MLC for *Saccharomyces piss*, *Rhodotorula* sp. and *Zygosaccharomyces* sp. was 11 and 16 mg mL^{-1} for *Zygosaccharomyces rouxii*. This oleoresin has no effect on *Saccharomyces cerevisiae* showing that it can be employedas a flavoring in baked goods.In summary, oleoresins from berries of Aguaribay can be used as antimicrobial to prevent food spoilage and are also obtained easily and with high yields.

INTRODUCTION

The concern among consumers of the potentially harmful properties to human health of most of the synthetic additives commonly used as food preservatives is increasing at present times. These chemical agents can cause several diseases, ranging from simple gastroenteritis to various types of cancer (Sacchetti *et al*., 2004 and references cited there in and Padin and Pollio (2009). One example of this kind of harmful preservatives is found in meat products preservation where nitrites and nitrates are employed. Nitrates can be metabolized to nitrites and these can react, under certain conditions with degradation products of amino acids forming nitrosamines which are

known carcinogens. Additionally, nitrites oxidize iron in hemoglobin from Fe(II)-(III) inhibiting it to transport oxygen. Another example is sulfur dioxide, one of the most effective and oldest preservatives, used in wines and frozen foodsas antimicrobial, antifungal and antioxidant. Sulfur dioxidedestroysvitamin E and thiamine (Vitamin B1), this last one essential for normal growth and development of the heart and the central nervous system.

For the reasons outlined, modern consumers are looking for high quality food and toiletry products which must be healthful as well as free of synthetic preservatives, so the interest in ingredients from natural sources has substantially grown. This type of ingredients can be found inherbs and spices which were used since, ancient times not only for flavoring foodsbut also as food preservatives, promoting conservation and thusextending their shelf life. Currently, the use of essential oils and oleoresins extracted from herbs and spices has a prominent place in the food industry, not just for flavoring and coloring foods and beverages but also for their antimicrobial, antifungal, antioxidant and radical-scavenging properties (Padin *et al*., 2005; Hayouni *et al*., 2008).

Fungi are one of the causative agents of food decay. Among these fungi are species of the genus *Aspergillus*, *Penicillium* and *Fusarium* (Nguefack *et al*., 2004). Another possible causative agent of food deterioration are yeasts, for example, species of the genus *Rhodotorula*, *Saccharomyces* and *Zygosaccharomyces* as reported by several authors (Pitt and Hoching, 1997; Soliman and Badeaa, 2002; Salo and Wirtanen, 2005). Some species of the above mentioned fungi can even produce mycotoxins such as aflatoxins, ochratoxin A and fumonisins which are known carcinogens. For example, aflatoxin B1 and B2 are produced by *A. flavus* (Nguefack *et al*., 2004) whereas *A. parasiticus* and *A. ochraceus* generate ochratoxin (Basilico and Basilico, 1999) and *A. niger* produces fumonisin and also ochratoxin. It is well documented thata flatoxins in particular are hepatocarcinogensin animals and in humans; Ochratoxin (OTA) in turn, is mutagenic and carcinogenic in animals and is also related to nephropathy in pigs and humans (Soliman and Badeaa, 2002). From other side, it has been shown that the essential oils of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), dictamus (*Origanum dictamus*) and marjoram (*Origanum marjoram*), to name a few are effective inhibitors of the radial growth and germination of conidia in *Penicillium digitatum* while essential oils of cinnamon (*Cinnamon umzeylanicum* L.), marigold (*Calendula officinalis* L.) and basil (*Ocimum basilicum* L.) inhibit the growth of *Aspergillus flavus*, *A. ochraceus*, *A. ochraceus* and *Fusarium moniliforme* as well (Daferera *et al*., 2002). These essential oils are obtained by hydrodistillation or by steam stripping processes, using any part of the plant such as leaves, stems, roots, flowers and fruits. Once obtained, the essential oils retain the characteristic aroma of the plant from which it is originated (Ribeiro, 2001. Oleoresins, in turn, are obtained by a solid-liquid extraction process, in which several organic solvents can be used such as ethanol, methanol or hexane. In this process, all aromatic constituents, flavorings and colorings are removed from the plant material. After the extraction process, the solvent is removed under reduced pressure leaving the extract or oleoresin. One of these oleoresins can be prepared from the fruits (berries) of an American tree of the Anacardiaceae family, Aguaribay (*Schinus molle* Linn). Aguaribay's berries are pink and have a mild spicy flavor similar to pepper, that's why they are known as "pink pepper". The antimicrobial activity of the essential oil of leaves and berries from Aguaribay has been extensively studied but this is not the case of the corresponding oleoresin. Just Padin *et al*. (2007) analyzed the antibacterial properties of Aguaribay´s oleoresin against bacteria commonly found in food and more recently the chemical composition of this oleoresin has also been determined (Caballero *et al*., 2014). To explore further the potential food preservative properties of the oleoresin extracted from berries of Aguaribay, we decided to testit against other microorganisms responsible for food spoilage such as fungi and yeasts. The results obtained are reported in the present paper.

MATERIALS AND METHODS

Preparation of oleoresins: Aguaribay berries were collected from trees located on public streets in the town of Quilmes, Buenos Aires, Argentina, during the months of December and January. Once collected the berries are stored refrigerated at -4°C if not used immediately for oleoresin preparation. For oleoresin preparation, ten grams of freshly collected berries were grounded and extracted with 100 mL of 96% ethanol at 40°C for 48 h (40 cycles min-1). The extract was filtered through filter paper (Whatman Nº4) and evaporated to dryness at 40°C under reduced pressure with a rotary evaporator (Heidolph Laborata 4000). Extraction yield (R%) is determined by the following equation:

$$R\% = \frac{\text{Oleoresin mass}}{\text{Berries mass}} \times 100$$

Microorganisms used: Fungi used were: *Penicillium nalgiovence*, *Penicillium citinum*, *Penicillium rugulosum*, *Penicillium brevicompactum*, *Penicillium griseofolvum*, *Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus paraciticus*, *Aspergillus clavatus*, *Aspergillus niger*,

Eurotium spp., *Epicoccum* spp., *Trichoderma* spp., y *Paecylomyces* spp. In the case of *Fusarium graminearum* and *Alternaria* reactivation of stored strains was performed in Malt extract broth. This must be done because pure fungi cultures used are deposited in a culture collection which is kept frozen at-20°C for conservation. Other species were reactivated in Malt Extract Agar (MEA) (all culture media were from Britania). Incubation for *F. graminearum* and *A. alternate* was performed with stirring for 7 days at 25°C. For the remaining species incubation was done for 7 days at 25°C. ACFC free incubator was used, 0°C-100°C range, ±2°C accuracy). The yeasts employed were: *Rhodotorula sp.*, *Saccharomyces cerevisiae*, *Saccharomyces piss*, *Zygosaccharomyces rouxii* and Zygosaccharomyces sp. All of them were reactivated in MEBG liquid medium, containing malt extract (20 g), peptone (1 g) and glucose (20 g) in 1 L of distilled water (Betts *et al*., 2000) and incubated with shaking for 24 h at 35°C before use. Both fungi and yeasts were donated by the Laboratory of Food Microbiology of the Universidad Nacional de Quilmes (Argentina).

Mycelial growth inhibition assay: The "poisoned-food" technique was use for fungal species *P. rugulosum*, *P. citrinum*, *P. brevicompactum*, *P.* and *P.* nalgiovence griseofolvum. Petri dishes were prepared as described previously (Dikshit *et al*., 1986 and references cited therein) inoculating with a spore suspension concentration 2.5×105 spores mL^{-1}, prepared in sterile distilled water. Then, 1 mL of each spore suspension was transferred to the center of a corresponding Petri dish (diameter 8.5 cm) containing malt extract agar. For *F. graminearum* and *A. alternata* the inoculation proposal of Liand coworkers was used (Li *et al*., 2005). This involves planting the mycelium in 3 mm diameterdiscs in the center of the plates. Oleoresin concentrations assayed were 0.125, 0.187 and 0.250 mg mL^{-1} with 16 mL final volume of each plate (Adam *et al*., 1998).

The strains belonging to the genus *Eurotium* sp., *Epicoccum* sp., *Trichoderma* sp. and *Paecylomyces* sp. were inoculated by puncture ansa needle (Pitt and Hoching, 1997). On plates containing MEA and sufficient quantities of oleoresin to obtain final concentrations in each plate of 5, 10 and 15 mg mL^{-1} with a 20 mL final volume in each plate (Sing *et al*., 2010). In all cases, the plates were incubated at 25°C in a CFC free incubator, 0-100°C range, ±2°C accuracy. Assays were performed in duplicate and the results were measured at day seven after sowing. My celial growth was measured and the percentage of growth inhibition was calculated with the following formula (Quiroga *et al*., 2002):

$$\text{Inhibition} = \frac{\left(\begin{array}{l}\text{Control mycelial growth-}\\ \text{mycelial growth extract}\end{array}\right)}{\text{Control mycelial growth}} \times 100$$

Effect on aspergillus sporesgermination (mcfungistatic and fungicide): This study was conducted on the fungi genus *Aspergillus* because they are important producers of mycotoxins such as aflatoxins (*A. flavus* and *A. paraciticus*) and ochratoxin (*A. ochraceus* and *A. niger*). These fungi were inoculated by seeding, by suspension or pour plate, 0.1 mL of a spore concentration 1×105 spores mL^{-1}, prepared with sterile distilled water, poured over malt extract agar prepared with different amounts of oleoresin of final concentrations ranging from 1-25 mg mL^{-1} (variation of 1 in 1). The final volume of each plate was 20 mL. Plates with ethanol were used as a control. The plates were incubated at 25°C for 7 days. All assays were performed in duplicate (Hitokoto *et al*., 1980). After the incubation time, the plates showing no growth were submitted to the technique described by Garber and Houston (1959) to determine MIC (minimum inhibitory concentration, fungistatic effect) and MLC (Minimal Lethal Concentration, fungicide effect).

Effect of ethanol in yeast: This effect was tested by a dilution technique of liquid medium (Quintas *et al*., 2000). The Yeasts used were: *Rhodotorula* spp, *Saccharomyces cerevisiae*, *Saccharomyces* piss, *Zygosaccharomyces rouxii y Zygosaccharomyces* spp. To an Erlenmeyer containing 20 mL of MEBG culture medium and 1 mL of a medium with activated yeast, ethanol was added in sufficient amount to obtain the following concentrations 1, 1.15, 1.25, 1.35 and 1.5% v/v. As a control, an Erlenmeyer flask containing only the culture medium was used for each type of yeast. The Erlenmeyer flasks were incubated at 35°C for 48 h. After that time 0.1 mL of each flask was taken and then plated on separate plates containing 20 mL of MEBG to determine survival.

Inhibitory power test in yeast: For this assayin liquid medium, the dilution technique proposed by Kalemba and Kunicka (2003) was used. The Yeasts used were *Rhodotorula* spp, *Saccharomyces* cerevisiae, *Saccharomyces* piss, *Zygosaccharomyces rouxii* y Zygosaccharomyces spp. Tubes containing MEBG were used and to different tubes enough oleoresin was added to concentrations ranging from 1-25 mg mL^{-1} (with variation of 1 in 1). The final volume of each tube was 5 mL. As a control culture medium, ethanol was added at a final concentration in the tube of 25 mg mL^{-1}. The tubes were incubated at 35°C for 48 h. Assays were performed in duplicate. For determination of the MLC, following incubation, 0.1 mL of the tubes were taken and plated on

half MEA. The plates were incubated at 35°C for 48 h. The value taken as the MLC corresponds to the no growth plate.

RESULTS AND DISCUSSION

Oleoresin preparation: The oleoresin obtained by the described procedure is greenish amber and has a spicy aroma consistent with reported literature data (Montes *et al*., 1961). Oleoresin extraction yield is about 23.5% starting from 10 g of ground berries. By comparison, Aguaribay essential oil preparation by hydrodistillation of leaves has a reported yield of only 0.9% starting from one kilo of processed leaves. The simplicity of the procedure of oleoresin preparation and the much better yield obtained are very important factors for the food industry.

Inhibition of mycelial growth: The results obtained in the study of mycelial growth inhibition are detailed in Table 1. It can be observed that 50% of the fungi studied (*P. citrinum*, *P. nalgiovence*, *F. graminearum* and *A. alternata*), after 7 days of incubation, show a higher percentage of growth inhibition with increasing oleoresin concentration. Gundidza (1993) study in antimicrobial capacity of essential oil of *Schinus molle* Linn found that it was effective in reducing the mycelial growth of *P. citrinum*, species that turned out to be the most sensitive to the use of the oleoresin compared to other species of *Penicillium* studied. In the case of *P. brevicompactum* and *P. rugulosum*, it is observed a decrease in inhibition of mycelial growth at 0.250 mg mL^{-1}, the highest oleoresin concentration tested and in the case of *P. griseofolvumno* inhibition is observed at 0.185 mg mL^{-1}. These results could be attributed to a growth stimulating effect sometimes observed at low inhibitor concentrations, a phenomenon which is called hormesis (Order, 2005). In fact, hormesis is the phenomenon that presents dose-response behavior stimulatory at low doses and inhibitory at high doses it is considered a natural phenomenon of all biological systems. The first report of hormesis dated to 1943 when Chester Southam and John Ehrlich were investigating the effect of the extract of red cedar on the metabolism of fungi (Davison *et al*., 2009). Previous works on inhibition of mycelial growth have been developed with oils from aromatic herbs extracted with dimethyl ether. It has been observed that a concentration of 1 mg mL^{-1} of lavender, rosemary and sage oil, produced an inhibition of mycelial growth of the genus *Penicillium* of 29.5, 24 and 9%, respectively while 0.25 mg mL^{-1} of oregano oil produces 100% inhibition in the growth of the same genus (Daferera *et al*., 2000). The inhibition values found in this work for *P. rugulosum* and *P. citrinum* with oleoresin concentration of 0.25 mg mL^{-1} of 13.35 and 16.19%, respectively are clearly lower than the value reported for oregano oil but compare quite well withthe rest of the oils assayed by Daferera *et al*. (2002) since, oleoresin concentration employed in this work is a quarter of those.

Table 1: Percentage of inhibition of mycelial growth

	Oleoresin Concentration (mg mL^{-1})		
Parameters	1	2	3
Fungi	0.125	0.185	0.250
P. rugulosum	9.54	14.12	13.35
P. citrinum	6.07	8.09	16.19
P. brevicompactum	0.96	8.21	4.83
P. griseofolvum	7.14	0	7.65
P. nalgiovence	0	1.27	7.65
A. alternata	2.8	12.93	15.51
F. graminearum	7.05	9.41	13.52

Table 2: Percentage of Inhibition of Mycelial Growth (Poisoned-food technique)

	Oleoresin Concentration (mg mL^{-1})		
Parameters	1	2	3
Fungi	5	10	15
Eurotium sp.	52.17	71.73	82.60
Epicocum sp.	44.60	78.64	100
Trichoderma sp.	27.21	41.77	85.88
Paecylomyces sp.	24.60	44.60	47.60

The results obtained in the study of mycelial growth inhibition by the poison-food technique are detailed in Table 2. It shows that at concentrations of 15 mg mL^{-1} 100% inhibition was obtained in the mycelial growth of *Epicoccum* sp. and values above 80% were registered for *Eurotium* sp. and Trichoderma sp. and 50% for *Paecylomyces* sp. In all cases, the percentage of inhibition of mycelial growth was higher as the oleoresin concentration increased. According to Vági *et al*. (2005), the ethanolic extract of marjoram presents an inhibition of 100% for *Trichoderma* sp. with a concentration of 500 mg mL^{-1}; in comparisonan inhibition of 85.8% is obtained with an oleoresin concentration of just 15 mg mL^{-1}.

Effect on spore germination of the genus Aspergillus (MC fungi static and fungicide): As mentioned above, inhibition of Aspergillus spore germination is very significant since, fungi of this genus are producers of mycotoxins. We report herein MIC and MLC values for *A. flavus* and *A. ochraceus* of 6 mg mL^{-1}. In the case of A. parasiticus, it is observed that at an oleoresin concentration of 17 mg mL^{-1} there is no growth. The fact that MIC and MLC are equal indicates that the effect of the oleoresin on these fungi is only fungicidal. In comparison, Briceño have found on *A. flavus* a MIC of

23.3 mg mL^{-1} of an ethanolic extract of lemon balm, concentration approximately 4 times higher than the one found in this study. Survival analysis, i.e., after applying the Garber-Houstontechnique, indicated that the plates of oleoresin concentrations from 17 mg mL^{-1} showed fungistatic effect (MIC) whereas the concentration of 25 mg mL^{-1} corresponded to a fungicidal effect (MLC). For the species A. niger no inhibition on the germination of spores was observed at any of the oleoresin c oncentrations assayed. In accordance, Davicino *et al*. (2007) reported that ethanolic extracts of leaves of *Schinus molle* L. and seeds of *Schinus terebenthifolius* were ineffective for the inhibition of *A. niger* spore germination. On the contrary, several authors reported positive results for the inhibition of *A. niger* spore germination: García *et al*. (2003) ethanol/hexane extract of houseleek (MIC 8 mg mL^{-1}), Carpinella *et al*. (2003) extract of the paradise tree (MIC 100 mg mL^{-1}, MLC of 200 mg mL^{-1}), Gulluce *et al*. (2003) essential oil of *Saturdeja hortensis* (MIC 31.25 mg mL^{-1}). In turn, Sokmen *et al*. (2004) informed inhibition of *A. niger* and *A. flavus* spore germination with extracts of *Origanum acutidens* (MIC 62.5 µg mL^{-1}).

Effect of ethanol on yeast: The study of yeast ethanol tolerance showed no growth inhibition at any of the concentrations tested. These findings ensure that ethanol does not interfere with the specific activity of the oleoresin, allowing the safe use of ethanol as dilution solvent. Medawar *et al*. (2003) have shown that an ethanol concentration of 91 mg mL^{-1} affects the growth of yeasts, contributing to the deterioration of wine. In turn, Quintas *et al*. (2000) report that *Z. bailli* and *S. cerevisiae* have a *similar* resistance to ethanol, maximum tolerance values of 180 and 175 mg mL^{-1}, respectively. The maximum ethanol concentration testedin this work (1.5 %v/v) corresponds to 11.82 mg mL^{-1}, a value significantly lower than those reported in the literature for tolerance trials.

Yeast growth inhibition: The results obtained are the following: for *S. piss*, *Rodotorula* spp. and *Zygosacharomyces* spp. MLC of 11 mg mL^{-1} and for *Zygosaccharomyces rouxii* MLC of 17 mg mL^{-1}. In the case of *Saccharomyces cerevisiae*, it was observed that the oleoresin is not effective at any of the concentrations tested. These findings are relevant because yeasts such as Z. *rouxii*, Z. *Bailiff*, *S. cereviciae* and *Rodotorula* spp. are reported food spoiling agents and grow in foods when they are not prepared following good manufacturing practices. Even more, there have been cases of gastroenteritis and allergic reactions where yeasts were suspected to be the causative agents (Salo and Wirtanen, 2005). The growth inhibition of *Z. rouxii* and Z. *bailli* has been previously reported by Ciani *et al*. (2003) with essential oils of Saturdeja montanaat a concentration of 0.12 mg mL^{-1} whereas Matan *et al*. (2006) found that a 1:1 mixture of essential oils of cinnamon and clove inhibits the growth of *Z. rouxii* employing the paper disc diffusion technique. In this case, the required concentration of oleoresin is much higher than the value reported by Ciani *et al*. (2002). Clearly, all these yeasts are sensitive to the use of natural antimicrobials. Accordingly, our results demonstrate that the oleoresin from Aguaribay berries has a lethal effect on most of the yeasts usually found on food. Also the negative result obtained for S. *cerevisiae* is significant, particularly for the production of baked goods where this oleoresin could be used as flavoring since it will not affect the yeast responsible for fermentation processes.

CONCLUSION

This research demonstrates that the oleoresin extracted from the berries of Aguaribay (*Schinus molle* Linn) shows antimicrobial activity inhibiting the growth of both fungi and yeasts responsible for food spoilage. Additionally, the process of obtaining the oleoresin is fast, inexpensive and presents a high yield, properties highly appreciated by the food industry.

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