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Antibacterial Activity of Oleoresin from Aguaribay (*Schinus molle* L.)

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Abstract: In this study the oleoresin extracted from Aguaribay (*Schinus molle* L.) berries using ethanol is tested for antibacterial activity. The screening of *Salmonella enteriditis*, *Staphylococcus aureus*, *E. coli*, *B. cereus* and *Bacillus subtilisby* using the paper disk agar diffusion method revealed that bacterial growth is inhibited at the analysed concentrations (2, 3 and 4 mg mL^{-1}).The minimal bactericidal concentration (MBC) was determined by the tube dilution test. The MBC value for *Listeria monocytogenes* was determined at 2 mg mL^{-1}, for *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* at 15 mg mL^{-1} and for *Escherichia coli* O 157:H7, *Salmonella enteriditis*, *Pseudomona aeruginosa* and *Klebsiella pneumoniae* at 14 mg mL^{-1}. These concentrations produced 100% growth inhibition. The storage effect was studied at 4 and 25°C for 30 days, the temperature effect at 40, 60 and 80°C and the inoculum density effect on *S. enteriditis* and *S. aureus* growth. A 15 mg mL^{-1} concentration and an inoculum density were used applying 0,1 0, 2 0, 3 and 0,4 mL of a culture over night. For both species, a 100% of growth inhibition was produced.

Key words: Spices, aguaribay, oleoresin, antibacterial effect

INTRODUCTION

The search of new compounds with biological activities resulted in an increase in the number of studies on the evaluation of antimicrobial activities of extracts and essential oils of medicinal and aromatic plants (Rodriguez *et al*., 1996). In recent years there has been an increasing interest in the use of essential oils and oleoresins as functional ingredients in foods, drinks, toiletries and cosmetics because they replace potentially harmful synthetic additives (Sachetti *et al*., 2005).

Since prehistoric times, spices and herbs have been added to food because they impart characteristic flavours and aromas. Gerhart (1975) defines the spices as part of some natural or dried plants (root, rhizome, bulb, bark, leaf, stem, flower, fruit, seed) and/or those undergoing mechanical elaboration. At present, the use of one of the constituents of the spices is widely known in food preparations (Benezet *et al*., 1975). The oleoresins, mixtures of volatile oils obtained by extraction with solvents, are responsible for the aroma and flavour of foods to which they are added. Studies on the biological activity of oleoresins of spices such as oregano (Adam *et al*., 1998), black pepper (Singh *et al*., 2004), rosemary (Deferera *et al*., 2000), curcuma and laurel (Ross *et al*., 1980), among others have been mentioned in literature for years.

The Aguaribay (Schinus molle L.) is an American tree of the Anacardiaceas family, Terebinthales order, widely spread in the centre and south of America (Montes *et al*., 1961). In Argentina, it occurs naturally from the Northern limit to Rio Negro. It is a rustic species resistant to cold and dry weather conditions. It was the Inca's holly tree and was originally named mulli by them. The name was then hispanicized to molle. At present, Aguaribay is also known as Gualeguay or Molle (Chirino *et al*., 2001). The fruit, called berry, is known as pink pepper. The grain is similar to that of the white pepper and it a has a very mild flavour and aroma, slightly pungent. The essential oil extracted from the leaves has demonstrated antimicrobial properties (Gundidza, 1993).

In this study, the antibacterial property of the oleoresin, ethanolic extract, extracted from the Aguaribay berries was analysed against different bacterial species responsible for the contamination of food products for human and animal consumption.

MATERIALS AND METHODS

Preparation of oleoresin: Aguaribay berries were collected from trees located on public streets of Florencio Varela locality, province of Buenos Aires, Argentina.

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For the preparation of the oleoresin, ten grams of berries ground using a laboratory mill (Moulinex 505 CE) were shaken in 100 mL ethanol 96 at 40°C for 49 h (40 cycles min^{-1}) (Quiroga *et al*., 2001). The insoluble material was filtered by filter paper (Whatman No. 4) and evaporated to dryness at 40°C under reduced pressure with a Rota Vapor (Heidolph Laborata 4000). The oleoresin obtained was resuspended with ethanol until 100 mg mL^{-1} concentration. It was kept in caramel-coloured bottles and refrigerated (4°C) until used.

Microorganisms: The following bacterial species were used for the different assays: *Salmonella enteriditis*, *Staphylococcus aureus*, *Escherichia coli*, *E. coli* O 157:H7, *B. cereus*, *Bacillus subtilis*, *P. aeruginosa*, *Listeria monocytogenes* and *Klebsiella pneumoniae*. These bacteria were obtained from Instituto Malbran (Argentina). They were kept in nutritive agar and refrigerated (4°C) until used.

Screening of antibacterial activity: For the antimicrobial assessment of essential oils, the agar diffusion method (paper disk or well) is used (Kalemba and Kunicka, 2003). It is a qualitative method which rapidly estimates the degree of microorganisms growth inhibition (Brock and Madigan, 199). This method was used for screening of antibacterial property of the oleoresin of Aguaribay berries. Petri dishes with nutritive agar (20 g of nutritive broth, 20 g of agar and 1 l of distilled water) were uniformly inoculated with the following bacterial species: *S. enteriditis*, *E. coli, B. cereus*, *B. subtilis* and *S. aureus*, from cultures over night. Sterile filter paper disks of 3 mm diameter (Whatman No. 4) were placed on the dishes. They were impregnated with different concentrations of ethanolic extract : 2, 3 and 4 mg mL^{-1}. The control was done with ethanol and all assays were done in duplicate The dishes were incubated at 35°C for 48 h.

Minimal inhibitory concentration: The Minimal Bactericidal Concentration (MBC) was determined using the dilution method (Sivropoulou *et al*., 1996; Dikshit *et al*., 1996; Kim *et al*., 1995). The following bacteria were used: *S. enteriditis*, *E. coli*, *E. coli* O157 H7, *B. cereus*, *S. aureus*, *P. Aeruginosa*, *L. monocutogenes* and *K. Pneumoniae*. For each bacterial species, a series of tubes were prepared with broth and different concentrations of ethanolic extract. The final volume of each tube was 5 mL and the concentrations assayed were: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20 and 25 mg mL^{-1}. Each tube was inoculated with 0.1 mL culture over night of the different bacterial species. The control tubes were filled with 0.1 ml of ethanol and incubated at 35° for 48 h. All assays were done in duplicate After 48 h, 0.1 mL of ethanol from each tube was poured in petri dishes containing nutritive agar to determine in which tube the bactericidal effect had occurred. The dishes were incubated at 35° for 24 h. In the case of *L. Monocytogenes*, the broth was replaced by medium for *Listeria,* in the form of broth for the tubes and in the form of agar for the dishes.

Stability of the oleoresin: The stability of the oleoresin was studied under different conditions of storage, temperature and inoculum density. Two species of those previously assayed were selected: *S. enteriditis* and *S. aureus*. The concentration of the ethanolic extract used for the tests was 15 mg mL^{-1} because this concentration produces 100% inhibition in the growth of both broths. The following tests were carried out:

Storage conditions: The oleoresin was stored at 4 and 25°C and the bactericidal effect was tested for 0 and 30 days.

Temperature: The oleoresin was heated up to 40, 60 and 80°C before used.

Influence of inoculum density: The tubes containing the same volume (5 mL) of broth and the adequate oleoresin to obtain a final concentration of 15 mg mL^{-1} were inoculated with 0.1, 0.2, 0.3 and 0.4 mL of culture over night. The same technique was used to carry out the different tests and to determine the MBC.

RESULTS AND DISCUSSION

The effectiveness of oleoresin is demonstrated by the size of the zone of microorganism growth inhibition around the disc or well and it is usually expressed as the diameter of this zone. Table 1 describes the halos of growth inhibition (in mm) produced by the different concentrations of oleoresin for the different studied bacteria. The mean value of the duplicates was reported. The ability of the oleoresin to inhibit growth of gram-positive and gram-negative bacteria was observed; this effect increases with increasing concentration of the ethanolic extract. Gram-positive bacteria are reported to be more susceptible than gram-negative to the essential oils of cinnamon, clove, cumin, garlic, oregano, black pepper, capsicum and thyme (Quattara *et al*., 1997). This effect was n ot observed in our work. Among the gram-positive bacteria, *B. cereus* was the most susceptible to the 4 mg mL^{-1} concentration, followed by *S. aureus* and *B. subtilis*. Regarding the gram-negative bacteria, *S. enteriditis* was more sensitive than *E. coli* considering the same concentration. This result agrees with that obtained by Nasar and Halkman[19] for the aqueous extract of sumac (*Rhus coriara* L.).The authors

sTable 1: Growth Inhibition halo (mm) produced by different concentrations of oleoresin

Concentration bacterium	2 mg mL^{-1}	3 mg mL^{-1}	4 mg mL^{-1}
S. aureus	6.5±0.7*	8.5±0.1	9.5±0.7
S. enteriditis	6.5±0.1	8.3±1.0	10.5±2.1
E. Coli	7.0±0.1	9.5±0.1	6.5±0.7
B. cereus	6.5±0.7	9.0±1.4	14.0±0.1
B. subtilis	8.5±2.1	9.0±1.4	7.5±2.1

*Standard deviation between the duplicates

determined that the gram-negative bacteria *S.enteriditis* proved to be more sensitive than *E.coli* to this extract. Inverse results were obtained for the gram-positive bacteria, because *B.subtilis* was more sensitive than *B.aureus* and *S. aureus*. It should be pointed out that the agar diffusion method presents preliminary results. Possibly because the volatile components can be evaporated during incubation while the poorly soluble components cannot diffuse in the agar. Yet, this method is the most commonly used to determine the antibacterial behaviour of the essential oils and oleoresin because it is easy to carry out and requires a small amount of sample. It is recommended for a screening to determine the biological activity of oleoresins.

Table 2 shows the results of MBC determination of the ethanolic extract of Aguaribay berries. The following MBC values could be determined: 14 mg mL^{-1} for *E.coli* 0157 H7, S.enteriditis and K. pneumoniae; 15 mg mL^{-1} for *P. aeruginosa*, *S. aureus* and *B. cereus*. *L. monocytogenes* was the most sensitive to the oleoresin with a value of 2 mg mL^{-1}. Dorantes *et al.* (2000) found that *L. monocytogenes* was the most sensitive to chilli compared with the rest of the bacteria analysed in his study. The MBC value for *P. aeruginosa*, 15 mg mL^{-1}, reported in the present study is an intermediate value compared with those found in literature. For instance, the MBC for the lemon extract and for the bay extract was 10 mg mL^{-1} and for the oregano extract 20 mg mL^{-1} (Hammer *et al.*, 1999). The determination of the value for the essential oil of Schinus molle, extracted by hydrodistillation, showed no significant differences with respect to the action on gram-positive or *S. molle* exhibit bactericidal effect on *S. aureus* at a MBC value of 15 mg mL^{-1}. Thus, Ross *et al.* (1980) determined that the essential oil of *Schinus molle* did not exhibit growth inhibitory effect against *E. coli* or *B. cereus* and it did display inhibitory effect against *P. aeruginosa* and *S. aureus*. Our results from the analysis of the oleoresin indicate bactericidal effect against the two former ones (*E. coli* and *B. cereus*, 14 and 15 mg mL^{-1}, respectively).

The results obtained from the stability tests of the Aguaribay oleoresin are shown in Table 3. The oleoresin maintains its inhibitory ability because it produces 100% growth inhibition of the bacteria studied after storage. Our results agree with those found by Dikshit *et al.* (1986). The ethanolic extract tolerates heat up to 80°C. It can be observed that during a period of 30 days the oleoresin retains its properties when kept either at refrigeration temperature (4°C) or at room temperature (25°C).

From our results we can conclude that the oleoresin extracted from Aguaribay berries exhibits an interesting potential to be used as a natural preservative in the food industry, due to its wide range of action on gram-positive and gram-negative bacteria and to its bactericidal effect on food spoilage microorganisms and/or those which cause food toxi-infections. It is important to highlight the bactericidal action of the oleoresin against *E. coli* 0157 H7. It would be interesting to assess its application on meat products, specially in those prepared with chopped meat. Also, the oleoresin stability should be

Table 2: Minimal bactericidal concentration (MBC)

Concentration Bacterium	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	20	25	C
S. aureus	ND	ND	ND	ND	-	ND	ND	ND	ND	-	-	-	-	-	+	+	+	-
B. cereus	ND	ND	ND	ND	-	-	-	-	-	-	-	-	-	-	+	+	+	-
L.monocytogenes	-	+	+	+	+	+	+	ND	ND	ND	+	ND	ND	ND	+	+	+	-
E. coli	ND	ND	ND	ND	-	ND	ND	ND	ND	-	-	-	-	-	+	+	+	-
E.coli O 157:H 7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	+	+	+	+	-
S. enteriditis	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-
P. aeruginosa	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	ND	ND	ND	+	+	+	-
K. pneumoniae	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	ND	-	+	+	+	+	-

C = control, (+) no growth was produced, (-) growth was produced, (ND) undetermined growth

Table 3: Stability of the oleoresin under different storage, temperature and inoculum density conditions

	Storage										
	4°C		25°C		Temperature (°C)			Inoculum density (mL)			
Bacterium	0 days	30 days	0 days	30 days	40	60	80	0.1	0.2	0.3	0.4
S. aureus	+	+	+	+	+	+	+	+	+	+	+
S. enteriditis	+	+	+	+	+	+	+	+	+	+	+

(+) = 100% of growth inhibition, The oleoresin concentration was 15 mg mL^{-1} in all the assays

considered under the following conditions: storage at 4°C and 25°C for 30 days, heating temperature at 40, 60 and 80°C and mainly the increase of the inoculum density, habitual conditions during the process of foodstuff production and its preservation.

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