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Antifungal Activity of Extracts of *Rosmarinus officinalis* and *Thymus vulgaris* against *Aspergillus flavus* and *A. ochraceus*

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Abstract: The antifungal activity of ethanolic extracts of *Rosmarinus officinalis* and *Thymus vulgaris* were tested against strains of *Aspergillus flavus* and *A. ochraceus*, since these two species are common contaminants of cereals and grains and are able to produce and accumulate mycotoxins. The methodology used is based on measuring the inhibition halos produced by discs impregnated with the extracts and establishing their Minimum Inhibitory Concentration (MIC) as well as the Minimum Fungicide Concentration (MFC). The results obtained suggest that the assayed extracts affect the proper development of *A. flavus* and *A. ochraceus*; leading to a lower MIC (1200 ppm) and MFC (2400 ppm) for *T. vulgaris* extract against *A. ochraceus* than against *A. flavus*. The results show, that the extracts of *Rosmarinus officinalis* and *Thymus vulgaris* used at low concentrations could have significant potential for the biological control of fungi in foodstuffs.

Key words: Biocontrol, *Aspergillus* sp., natural products, *Thymus vulgaris*, *Rosmarinus officinalis*

INTRODUCTION

Some foodstuffs are most susceptible to fungal infection such as vegetable products, including fruits, green vegetables and cereal grains. During their growth, harvesting and storage, the microbiology of cereals is highly dominated by fungi, being *Penicillium*, *Aspergillus* and *Fusarium* the most commonly isolated genera. These genera produce most of the damage to cereals by their presence itself, but due to their capacity to produce and accumulate mycotoxins (Del Castillo, 2007), it represents a threat to the wholesomeness of the food and thus constitutes an important health risk for consumers (Desjardins *et al*., 2000; Thompson and Henke, 2000; Bennett and Klich, 2003).

Currently, about 300 fungal toxins have been identified, being aflatoxins, fumonisins, ochratoxins, T-2 toxin and Deoxynivalenol (DON) the most frequently found (Dalcero *et al*., 1997; Dutta and Das, 2001; Bennett and Klich, 2003). Aflatoxins have an immediate toxic effect, as well as immunosuppressive, mutagenic, teratogenic and carcinogenic properties. It has also been demonstrated that ochratoxin A is nephrotoxic, carcinogenic, teratogenic and inmunotoxic for both animals and humans (Del Castillo, 2007).

The decontamination of mycotoxins in food may be carried out by means of physical, chemical or biological methods. These methods should be efficient, economic and should not significantly modify the nutritional value of the food. In addition, treatment by these methods should not leave residuals that could adversely affect animal or human health (Miller, 1995; Murphy *et al*., 2006). The use of the physical and chemical methods available for the detoxification of mycotoxin contaminated agricultural products is restricted due to health security issues and the possible decrease in the nutritional quality of the food. These methods are also of limited efficiency and involve expensive procedures, which has led to the search for alternative strategies such as biological control (Kabak *et al*., 2006).

The increase in the demand for natural and increasingly safe food products has led to the use of methods that biologically monitor microorganisms that contaminate foodstuffs. Among these is the addition of natural products with antimicrobial properties that guarantee the quality of the food (Kabak *et al*., 2006; Rasooli and Mirmostafa, 2003).

It has been shown that some vegetable extracts have an inhibitory activity on the growth of various moulds (Lelono *et al*., 2009; Moslem and El-Kholie, 2009) that are commonly found in foodstuffs (Bluma *et al*., 2008; Cakir *et al*., 2005; Velluti *et al*., 2004).

Some aromatic plants with known antiseptic and antimicrobial properties and mainly used as spices in food

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preparations, have been studied for use in the control of mycotoxins (Bakkali and Averbeck, 2008; Bluma *et al*., 2008; Kumar *et al*., 2008). The objective of this study is to assess the antifungal effect of extracts of *Rosmarinus officinalis* and *Thymus vulgaris* against *Aspergillus flavus* and *A. ochraceus*.

MATERIALS AND METHODS

This study was carried out at the end of 2008 and beginning of 2009 in the Department of Health and Animal Anatomy at the Universitat Autònoma de Barcelona (Spain).

Ethanolic extraction: Fresh *Rosmarinus officinalis* and *Thymus vulgaris* were acquired in supermarkets of Barcelona, Spain. The ethanolic extraction was performed according to the technique proposed by Bluma *et al*. (2008) the dried plants were ground until a fine powder was obtained and the material obtained was subsequently weighed. The dry weight of the thyme was 3 g and that of the rosemary was 6 g. The extraction was performed with an 80% ethanol solution in a 1:3 for each plant for 48 h at 25°C; after this time it was filtered with Watman No. 1 filter paper and the supernatant was left to dry at 37°C. The dry weight of each extract was determined and resuspended in sterile water a proportion of 1:3. The entire procedure was performed under sterile conditions.

Antifungal activity of the extracts: The antifungal activity of the extracts was determined according to a modification of the methodology described by Evandro *et al*. (2005): sterile discs of filter paper 6 mm in diameter were impregnated with the extracts obtained and they were placed on Sabouraud Dextrose Agar (SDA) Petri dishes, which had previously been surface-plated with 100 μL of a conidia suspension of *Aspergillus flavus* (FVB51) belonging to the laboratory's fungi collection and *Aspergillus ochraceus* (ATCC 2948) in a concentration of 10^6 conidia mL^{-1}. The dishes were incubated for 72 h at 28°C. Controls were produced using Petri dishes with SDA inoculated with *A. flavus* and *A. ochraceus*. The results were observed after 72 h of incubation and the inhibition halos formed were determined by measuring their total diameter. The test was carried out five times to assure repeatability.

On the other hand, this test was performed with a control of the ethanolic extraction system used to assure that the potentially inhibitory effects of the extracts was because of the extracts themselves and not from the ethanolic solution.

Determination of minimum inhibitory concentration (MIC): The MIC was determined using the methodology described by Rasooli *et al*. (2008) and Rasooli and Mirmostafa (2003), with some modifications; Briefly, 50 μL of several dilutions of the extracts were placed in the wells of an ELISA microplate which contained 100 μL of yeast-sucrose extract culture medium with 50 μL of conidia suspension with an approximate concentration of 10^6 conidia mL^{-1}. The microplate was incubated at 28°C for 48 h. The minimum inhibitory concentration was the highest level of dilution, which presented no fungal growth. Controls without inoculating extracts were also performed. In order to provide evidence of the presence of viable microorganisms, after incubation, 60 μL of iodonitrotetrazolium chloride (Sigma, Ref: I-8377) diluted in sterile water was added to each well. The microplate was therefore inoculated at room temperature for an hour and the color changes were checked every 30 min. A pink color in the wells meant the viability of the microorganisms.

Determination of Minimum Fungicide Concentration (MFC): The Minimum Fungicide Concentration (MFC) was also determined, with aliquots of the tubes that showed no fungal growth seeded in Petri dishes with Sabouraud Dextrose Agar, to thereby determine whether the inhibition of growth was reversible or permanent; the MFC was in the SDA dish with the highest dilution level, in which no fungal growth occurred.

RESULTS

Table 1 shows the diameters of the inhibition halos produced by the ethanolic extracts of *Thymus vulgaris* and *Rosmarinus officinalis* when in contact with *Aspergillus ochraceus* and *A. flavus*. The results obtained show that both extracts influence the growth of both *A. flavus* and *A. ochraceus*.

The Minimum Inhibitory Concentration (MIC) and the Minimum Fungicide Concentration (MFC) of the ethanolic extracts of *Thymus vulgaris* and *Rosmarinus officinalis* are shown in Table 2 and 3, respectively.

In Table 2, it can be observed that for both fungi, MIC is higher when using *Rosmarinus officinalis*, the extract of *T. vulgaris* has a lower MIC for *A. ochraceus*, with a dilution of 0.008, corresponding to a concentration of 1,200 ppm.

In Table 3, results for *Thymus vulgaris* CMF are showed, indicating that this concentration is lower against *A. ochraceus*.

Table 1: Inhibition halos produced by ethanolic extracts of *Thymus vulgaris* and *Rosmarinus officinalis* when in contact with *Aspergillus ochraceus* and *Aspergillus flavus*

Extracts	Microorganisms	Inhibition (mm)	SD	p-value
Thymus vulgaris	*A. flavus*	11.4	1.14018	0.00009*
	A. ochraceus	16.6	1.14018	
Rosmarinus	*A. flavus*	14.6	1.14018	0.03527*
officinalis	*A. ochraceus*	16.2	0.83666	

SD: Standard deviation. *p<0.05 significant

Table 2: Minimum Inhibitory Concentration (MIC) of the ethanolic extracts of *Thymus vulgaris* and *Rosmarinus officinalis* on *Aspergillus flavus* and *Aspergillus ochraceus*

		Minimum Inhibitory Concentration (MIC)						
Extracts	Microorganisms	0.40	0.25	0.20	0.125	0.10	0.08	0.004
Thymus	*A. flavus*	+	+	+	+	-	-	-
vulgaris	*A. ochraceus*	+	+	+	+	+	+	-
Rosmarinus	*A. flavus*	-	-	-	-	-	-	-
officinalis	*A. ochraceus*	-	-	-	-	-	-	-

+: No growth, -: Growth

Table 3: Minimum Fungicide Concentration (MFC) of extracts of *Thymus vulgaris* on *Aspergillus flavus* and *A. ochraceus*

		Minimum Fungicide Concentration (MFC)				
Extract	Microorganisms	0.40	0.25	0.20	0.125	0.008
Thymus	*A. flavus*	+	+	-	-	-
vulgaris	*A. ochraceus*	+	+	+	-	-

+: No growth, -: Growth

Statistical analysis: The results obtained were statistically analyzed by means of Student t-test. The p-value was <0.05 in both cases, indicating that the differences were statistically significant.

DISCUSSION

The inhibition halos (diameters) obtained in this assay were similar to those obtained by Rasooli *et al.* (2008), who determined the activity of *R. officinalis* against *A. parasiticus*. Likewise, Kumar *et al.* (2008), also observed strong inhibitory effects of oily extracts of *T. vulgaris* on *A. flavus*.

The Minimum Inhibitory Concentration and the Minimum Fungicide concentration of the ethanolic extracts coincide with those provided by Bozin *et al.* (2006) and Sergvic-Klaric *et al.* (2007) and suggested that the tested extracts, applied in low concentrations could have significant potential for the biological control of fungi in foodstuffs.

The active components of these two species have medicinal properties with tonic, stimulating and antimicrobial effects. In addition, since they are aromatic herbs, they are also nutritious and they are used as flavoring in the preparation of food (Sitte *et al.*, 2004). The main components of *Thymus vulgaris* are thymol, carvacrol and borneol and those of *Rosmarunus officinalis* are pinene, limonene and piperitone, all of which are phenolic compounds that confer antimicrobial properties to the natural extracts or essential oils obtained from these plants (Velluti *et al.*, 2004; Bakkali and Averbeck, 2008; Kumar *et al.*, 2008; Rasooli *et al.*, 2008).

The biological activity of the natural extracts is probably due to the synergy among their different components, since each component shows a lower activity separately compared to when tested together. The mechanism of action is not well known, although their microbiostatic or microbiocide activity is associated with an overload of the plasma membranes of the microorganisms, producing a loss of cellular symmetry and integrity thus preventing cell multiplication (Krishnamurthy and Shashikala, 2006; Evandro *et al.*, 2005). In general, they cause cytoplasmatic granulation, rupture of the cytoplasmatic membrane and the inactivation and/or inhibition of the synthesis of intra and extracellular enzymes, which impedes mycelial germination (Evandro *et al.*, 2005). The results obtained here demonstrate that the extracts of *Rosmarinus officinalis* and *Thymus vulgaris* possess antifungal properties against *Aspergillus flavus* and *A. ochraceus* and could be used as an alternative for the decontamination of food contaminated by fungi.

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