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: Bioccontrol, 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 (Dalaero 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 immunotoxic 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 (Bluna 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 water 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 Mirmostaafa (2003), with some modifications: Briefly, 50 µL of several dilutions of the extracts were plated 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*.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Microorganisms</th>
<th>Inhibition (mm)</th>
<th>SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thymus vulgaris</em></td>
<td><em>A. flavus</em></td>
<td>11.4</td>
<td>1.14018</td>
<td>0.00009*</td>
</tr>
<tr>
<td></td>
<td><em>A. ochraceus</em></td>
<td>16.6</td>
<td>1.14018</td>
<td></td>
</tr>
<tr>
<td><em>Rosmarinus officinalis</em></td>
<td><em>A. flavus</em></td>
<td>14.6</td>
<td>1.14018</td>
<td>0.03527*</td>
</tr>
<tr>
<td></td>
<td><em>A. ochraceus</em></td>
<td>15.2</td>
<td>0.81666</td>
<td></td>
</tr>
</tbody>
</table>

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*.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Microorganisms</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thymus vulgaris</em></td>
<td><em>A. flavus</em></td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td><em>A. ochraceus</em></td>
<td>0.25</td>
</tr>
<tr>
<td><em>Rosmarinus officinalis</em></td>
<td><em>A. flavus</em></td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td><em>A. ochraceus</em></td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.04</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Microorganisms</th>
<th>MFC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Thymus vulgaris</em></td>
<td><em>A. flavus</em></td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td><em>A. ochraceus</em></td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.098</td>
</tr>
</tbody>
</table>

**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 Sergiev-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 *Rosmarinus 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 Averbeek, 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 microbicidal 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 cytoplasmic 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.

**REFERENCES**


