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Evaluation Of The Inhibitory Activity Of Alcohol Extracts Of *Thymus Vulgaris* L. On The Fungi *Bipolaris Sorokiniana*, *Alternaria Alternata* And *Colletotrichum Gloesporioides*

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ABSTRACT

The identification of plant constituents and their biological applications is the aim of several scientific studies. To discover novel alternatives for biological control, alcoholic extracts of *Thymus vulgaris* L., obtained by cold extraction, were evaluated according to their inhibitory activities at different concentrations (5, 50 and 500 μg x mL) on the mycelial growth of *Bipolaris sorokiniana*, *Alternaria alternata* and *Colletotrichum gloesporioides* using the *in vitro* bioanalytical method. During the incubation period at 21 ± 2°C with a photoperiod of 12 hours of light and 12 hours of darkness, the growth of the colonies was obtained from the average of the orthogonal measurements of the colony diameter. The results were shown by the MGI (Mycelial Growth and/or Inhibition Index) and statistically analyzed ($p < 5$), the best results being observed with 500 μg x mL. The ethanol extract presented the highest activity at this concentration, with growth reductions of 47.76%, 15.21% and 34.01%, relative to the control, for *Bipolaris sorokiniana*, *Alternaria alternata* and *Colletotrichum gloesporioides*, respectively. The reductions observed were 46.87%, 9.57% and 23.53% using a methanol extract.

KEYWORDS

Biological activity;
Alternaria alternata;
Bipolaris sorokiniana;
Colletotrichum gloesporioides;
Thymus vulgaris L.

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INTRODUCTION

Aromatic medicinal plants have long been known for their curative activities. They are the benchmark of several scientific investigations for presenting very selective biological activities. The discovery of new biological activities is the aim of scientific explorations in the agronomic sector, where products obtained from medicinal plants can function in the control of pests, reducing the negative effects of pesticides and resulting in high quality products[1,2].

Among the aromatic species, *Thymus vulgaris* L, of the Lamiaceae family, is completely adapted to Brazil, being in the list of the 46 most cultivated aromatic plants of the state of Paraná. This plant, popularly known as thyme, has great scientific importance in phytopathology and entomology in the control of several fungi, bacteria and insects because of its active constituents, identified as thymol and carvacrol, and the high content of free phenols, tannins and flavonoids. Karaman et al.[3] evaluating the activity of the essential oil of *T. revolutus* in which the carvacrol was identified as a major constituent, observed a high anti-fungal and anti-bacterial activity. Essawi and Srour[4] studied the anti-bacterial activity of 15 medical plants on eight bacterial species. All of them presented activity, the organic and aqueous extracts of *T.vulgaris* and *T.origanium* being the highest.

From the agricultural point of view, fungi cause serious damage to agriculture. It is estimated that more than 30% of the agricultural production of the world is lost annually because of phytosanitary problems for which the fungi are the principal agent responsible for plant diseases[5]. Alves et al.[6] concluded that the greatest epidemiological agents affecting plants in the Lavras, MG region were the fungi, attacking 78.47% of the plants studied. Later, Pozza et al.[7] showed that the Deuteromycetes account for 82.5% of the incidence among the fungal classes. Fungal etiological agents were determined as follows: *Fusarium* sp. (12.1%), *Colletotrichum* sp. (11.5%), *Alternaria* sp. (7.6%), *Cercospora* sp. (6.4%) and *Oidium* sp. (5.8%). The symptoms of greatest occurrence were leaf lesions (48.9%), rot (16.3%) and wilt (9.5%).

One of the fungus species of huge economic importance is *Colletotrichum gloesporioides*, the principal infectious agent of post-harvest anthracnose. This disease is a common rot in mature fruits. It occurs with high frequency and severity, making the fruits inadequate for consumption. *Bipolaris sorokiniana* is the principal pathogen for wheat and other grasses, affecting seeds, leaves and roots. The diseases caused by this species, such as root rot and leaf lesions (helminthosporiosis), have caused considerable losses in production and the reduction of proteins in the grains[8,9]. Another important pathogen is *Alternaria alternata*, the most common species of Alternaria. It is responsible for diseases like black spot in several Solanaceae, vegetables of great importance as a food source. Because of the nutritional value of compounds from *T.vulgaris*, the potential it presents and the necessity of establishing selective control by environmentally friendly methods, the present work sought to evaluate the inhibitory activity of the alcohol extracts (ethanol and methanol) on the *C. gloesporioides*, *A.alternata* and *B.sorokiniana* pathogens as an alternative method of control.

EXPERIMENTAL

The plant material was collected from the medicinal plants garden of the Universidade Federal de Lavras (UFLA) in May, 2001, at 8:00 a.m., at a temperature of 21°C and a relative humidity (RH) of 60%. To obtain the extracts, 291 g of *T. vulgaris* leaves were dried to constant weight at 40°C in a forced air chamber and submitted to successive cold extractions with solvents of increasing polarity-hexane, chloroform, ethyl acetate, ethanol, methanol-by immersion in two liters of each solvent for a period of eight days per extraction. After the extraction with hexane, the material was filtered under vacuum, the filtered residue was dried to constant weight at 40°C in a forced air chamber and the residue was extracted with chloroform. This process was repeated with each solvent in the series. At the end of the series of extractions, this residue was discarded. The filtrate obtained from each extraction was concentrated on a Büchi R-114 rotatory evaporator under reduced pressure and dried to constant

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weight at 40°C in a forced air chamber.

The biological assays of the ethanol and methanol extracts began with standard fungus cultures obtained from the fungal collection of the phytopathology department of UFLA and identified as *Colletotrichum gloesporioides*, *Bipolaris sorokiniana* and *Alternaria alternata*. BDA (potato, dextrose and agar)[10] was used as the culture medium for the first two pathogens and BCA (potato, carrot and agar)[11] for the last. The *in vitro* bioanalytical method was utilized to evaluate the effects of the ethanol and methanol extracts in different concentrations on the mycelial growth and/or inhibition of fungal cultures.

In a laminar flow aseptic hood, samples of the methanol and ethanol extracts were diluted with the respective solvent and added to the previously sterilized media to obtain concentrations of 5, 50, and 500 μg/mL (treatments T_2, T_3, T_4, respectively). The T_1 treatment (control, 0 μg/mL) utilized only the culture media and was used for both extracts. Later, the media were poured into sterilized petri dishes. Inverted mycelial disks of 6 mm in diameter were placed in the center of the dishes. The petri dishes were sealed with plastic film and incubated for seven days in a culture chamber at 22 ± 2°C with a photoperiod of 12 hours of light and 12 hours of darkness[12]. The evaluations were performed 2, 4 and 6 days after initiating the experiment by daily measurements of the diameters of the mycelial growth. The averages were calculated from each two measurements. The mycelial growth index (MGI) was calculated by the modified formula of Nakagav and Maguire, adapted by Oliveira[13].

MGI = + + , for
MGI = mycelial growh index

C1, C2, Cn = Mycelial growth of the colonies in the first, second and last evaluations; N1, N2, Nn = number of days.

The experiment was set up in an entirely randomized design, with four replicates and a factorial scheme of 2 x 4 x 3 involving two plant extracts at four concentrations with three fungus species. The results were submitted to analysis of variance and regression in the SAEG system, version 5.0. The averages for each of the three fungus species were compared by the Tukey test at 5% probability. In the regression, linear and square effects of the $Y=a+bx+b_1x^2$ model and the square root of the model, i.e., $Y=a+bx^{0,5}+b_1x$., were determined. Amid each group of equations with the same number of estimated parameters, the equation that had a significant effect by the F test at 5% probability, and that had biological significance and a higher square sum, i.e., higher R^2, was chosen.

RESULTS AND DISCUSSION

The mycelial growth indices of the *B. Sorokiniana*, *A. Alternata* and *C.Gloesporioides* fungi were calculated and submitted to statistical analyses that revealed significant differences as well as significant interactions between the extracts, the treatments and the fungal species (TABLE 1). From the data in TABLE 1, it can be seen that the increase in the concentration of the ethanol extract caused a reduction in the MGI of *B.sorokiniana* and *C. gloesporioides*,

TABLE 1: Averages of the mycelial growth index (MGI) for three fungus species *(B.sorokiniana, A.alternata, C.Gloeosporioides)* submitted to four concentrations (0, 5, 50 and 500 mg x mL^{-1}) of two plant extracts

Extracts	Concentration (μg x mL^{-1})	MGI (cm)*		
		B. sorokiniana	*A. alternata*	*C. gloeosporioides*
Ethanol	0	4.48^a	3.55^a	3.91^a
	5	4.37^b	3.45^b	3.63^b
	50	4.14^c	3.40^b	3.29^c
	500	2.34^d	3.01^c	2.58^d
Methanol	0	4.48^a	3.55^a	3.91^a
	5	4.48^a	3.34^b	3.69^b
	50	4.42^a	3.35^b	3.51^c
	500	2.38^b	3.21^c	2.99^d

***Averages followed by the same letter in each column do not differ significantly at 5% of probability by the Tukey test**

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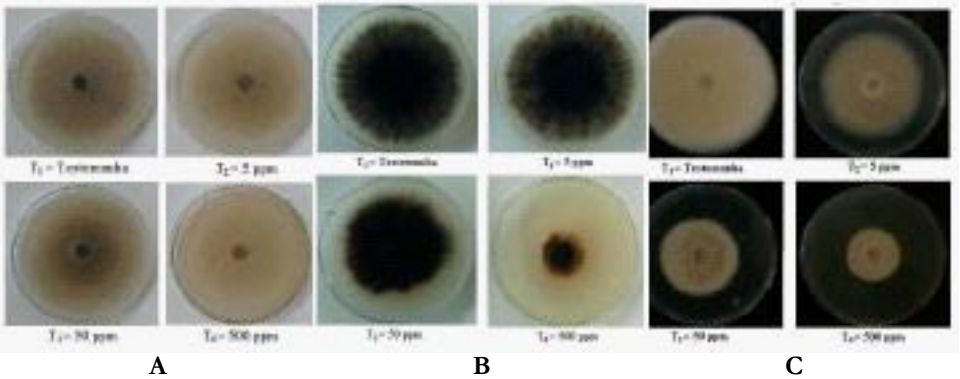
although no significant decrease was observed with 5 and 50 μg x mL^{-1}. No real reduction pattern with *A.alternata* was observed. The methanol extract only caused a significant decrease in the MGI of *B. sorokiniana* with 500 μg x mL^{1}. For *A.alternata*, a decrease similar to that observed with the ethanol extract occurred, with no significant variations in the MGI at the 5 and 50 μg x mL^{-1} concentrations. The highest inhibitory activity was observed at 500 μg x mL^{-1} for both extracts and for the three fungus species. The ethanol extract presented the highest activity at this concentration, with growth reductions of 47.76%, 15.21%, and 34.01% relative to the control for *B.sorokiniana, A.alternata* and *C. gloesporioides*, respectively. Aktug and Karapinar, in Reddy et al.[14], observed a high inhibitory activity at concentrations beyond 500 μg x mL^{-1} for the activity of the ethanol extract of *T.vulgaris* against the two bacterial species *S.aureus* and *V.parahaemolyticus*.

The respective reductions were 46.87%, 9.57% and 23.53% for the methanol extracts. There was no complete inhibition at the concentrations tested, suggesting that the extracts act in a fungistatic manner under these conditions. Economou et al.[15] observed a pronounced anti-oxidant activity for the methanolic extracts of the lamiaceae herbs, including *T.vulgaris*. Later, Chipault et al., cited by Matiucci[16], attributed the same activities to the ethereal and ethanol extracts that, according to Simões et al.[2], can be caused by the metabolite classes extracted by the solvents. One of these classes includes tannins, phenolics of confirmed fungicide activity that are present in several medicinal species[17].

The mycelial inhibition of the fungal cultures submitted to the different treatments was estimated from the regression equations for the data in TABLE 1. For the ethanol extracts, the equation obtained for *B.sorokiniana* was $Y = 4.46 - 0.02418\ C^{0.5} - 0.00317\ C$ ($R2 = 0.999$); for *C.gloesporioides*, $Y = 3.88 - 0.10049\ C^{0.5} + 0.00189\ C$ ($R^2 = 0.998$); for *A. alternata*, $Y = 3.53 - 0.01049\ C^{0.5} - 0.00023\ C$ ($R^2 = 1.00$). For the methanol extracts, the equation obtained for *B.sorokiniana* was $Y = 4.46 + 0.03316\ C^{0.5} - 0.00565\ C$ ($R^2 = 1.000$); for *C.gloesporioides*, $Y = 3.88 - 0.06225\ C^{0.5} + 0.00102\ C$ ($R^2 = 0.990$); for *A. alternata*, $Y = 3.50 - 0.03183\ C^{0.5} + 0.00087\ C$ ($R^2 = 0.77$). Equations with coefficients (R^2) over 99% were obtained for both extracts except for the methanol extract of *A.Alternata*. This result demonstrated a high correlation between the growth rate and the extract concentrations. The increase in the concentrations of the methanol and ethanol extracts caused reasonable reductions in the mycelial growth of the fungus colonies, and *B.Sorokiniana* presented the highest sensitivity to the increases in the concentration of the ethanol extract. The same was not observed for *A.alternata*, which, despite presenting a significant inhibitory activity, exhibited no increase in inhibitory activity in response to an increase in the extract concentration. The bioassays performed with the ethanol and methanol extracts on the fungi under study are displayed in figures 1 and 2.



A B C

Figure 1: Bioassays performed with the ethanol extract of *T.vulgaris*, on the mycelial growth of the fungi, *A.alternata* (A), *B.sorokiniana* (B) and *C.gloesporioides* (C) on the sixth day of evaluation

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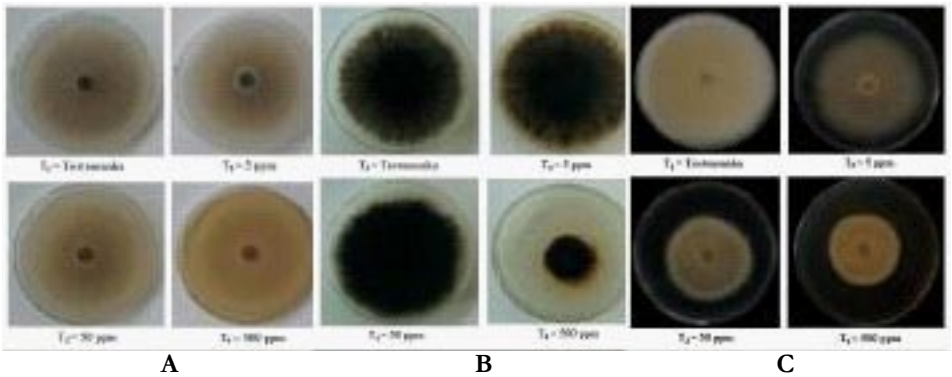

A B C

Figure 2: Bioassays performed with the methanol extract of *T. vulgaris*, on the mycelial growth of the fungi *A.alternata* (A), *B. sorokiniana* (B) and *C.gloesporioides* (C) on the sixth day of evaluation

CONCLUSIONS

The highest MGI values were obtained with alcohol extracts of *T.vulgaris* at the 500 μG x mL^{-1} concentration. The reductions in growth rate for the ethanol extracts relative to the control were 47.76%, 15.21% and 34.01% for *B.sorokiniana*, *A.alternata* and *C.gloesporioides*, respectively, while those for the methanol extract were 46.87%, 9.57% and 23.53%.

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