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BIOCONTROL ACTIVITY OF AROMATIC AND MEDICINAL PLANTS AGAINST SOIL-BORNE PATHOGENS

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Globally, soil-borne phytopathogens can significantly impair horticultural and grain crops, causing substantial losses. Synthetic pesticides remain the primary choice in plant disease management due to their high efficiency and ease of application. However, strict regulations and growing environmental concerns have made the search for sustainable alternatives more urgent than ever. In addition to integrating botanicals into farming practices, incorporating aromatic and medicinal plants into crop systems can be an effective strategy for managing plant diseases by supplying nutrients and modifying soil microbial populations.

However, these techniques are not universally accepted and may negatively impact soil fertility if not carefully controlled. The present study aims to evaluate the biocontrol activity of tinctures prepared from aromatic and medicinal plants native to North Macedonia against certain soil-borne phytopathogens.

The antimicrobial potential of the tinctures was evaluated using the well diffusion method and micro-broth dilution method with 96-well microtiter plates, which allowed for the determination of the minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC). The tinctures were subjected to serial dilutions in descending concentrations, starting from 50 % and ending with a concentration of 0.39 %. Fifteen aromatic and medicinal plants and six commonly known phytopathogens were used in this experiment.

Generally, the tinctures were found to be active, with the MIC ranging from 0.39 % to 25 %, and the MFC ranging from 0.78 % to 50 %. However, of all the tested fungi, *Fusarium oxysporum* FNS-FCC 103 and *Aspergillus niger* FNS-FCC 33 were the most resistant microorganisms, while *Botrytis cinerea* FNS-FCC 23 was the most sensitive.

According to the findings of this study, the tested aromatic and medicinal plants exhibited relatively high antimicrobial activity against all the tested phytopathogenic fungi. The study suggests that tinctures from these plants could serve as a potential source of natural antifungal agents. Following this screening experiment, further research should be conducted to explore the antimicrobial activities in more detail.

Keywords: antimicrobial; tincture; 96-well microtiter plates; aromatic plant; soil-borne pathogen

INTRODUCTION

The world's population is growing daily, and the demand for food is rising as well. These factors have placed significant strain on soil health, leading to the degradation and exhaustion of agricultural fields, which in turn results in decreased yields and productivity [1–4]. Reducing crop loss due to fungal diseases is a critical goal in a world with a growing population and rising hunger. Fungi have a greater impact on crop illnesses and productivity losses than other plant pathogens.

Most phytopathogens naturally exist as soil fungi, contaminating important food crops at both the pre- and post-harvest stages. These soil-borne pathogen contaminations pose a global threat to food safety, affecting the quality and marketability of a variety of food crops [2]. Although effective synthetic fungicides against phytopathogens are available, their excessive use leads to the develop-

ment of resistance and long-term negative effects on human health [5, 6]. Today, the global trend is shifting towards reducing the use of synthetic fungicides, creating a strong and growing demand for safer and more ecological alternatives to combat plant diseases.

Biological control is an environmentally friendly method that utilizes various bioactive substances produced by other organisms, which can act as inhibitors or suppressors of phytopathogen development. Natural product-based fungicides offer the advantage of distinct mechanisms of action and low mammalian toxicity, along with the ability to break down quickly, reducing environmental risk [7, 8].

An environmentally friendly solution may be the use of plant extracts, which are composed of secondary metabolites from plant cells. Although the quality and quantity of these compounds depend on factors such as plant species, environmental growth conditions, pathogen incidence, harvesting season, and extraction method, their biopesticidal properties are generally broad and effective against a variety of plant pathogens. Furthermore, they are biodegradable and cause minimal harm to the environment. Therefore, plant extracts can serve as a natural alternative to synthetic fungicides for treating potatoes against post-harvest storage phytopathogens [6, 9]

In light of this, we report the *in vitro* antifungal activity of fifteen aromatic and medicinal plants against six commonly known phytopathogenic fungi in this study.

MATERIAL AND METHODS

Preparation of plants

To prepare the tinctures for the study, either the entire plant or specific plant components were used (Table 1). The collected plants or their components were washed with water to remove dirt and dust. After being thoroughly shaded and dried, they were ground into a fine powder and stored in airtight jars.

Table 1. Evaluated plants in the study

	Common name	**Latin binomial name**	**Family**	**Plant part**
1	St. John's-wort	*Hypericum perforatum*	Hypericaceae	flowers
2	Yarrow	*Achillea millefolium*	Asteraceae	leaves
3	European centaury	*Centaurium erythraea*	Gentianaceae	flowers
4	Blackberry	*Rubus fruticosus*	Rosaceae	leaves
5	Common horsetail	*Equisetum arvense*	Equisetaceae	aerial part
6	Summer savory	*Satureja hortensis*	Lamiaceae	leaves
7	Common nettle	*Urtica dioica*	Urticaceae	leaves
8	Breckland wild thyme	*Thymus serpyllum*	Lamiaceae	leaves
9	Common dandelion	*Taraxacum officinale*	Asteraceae	flowers
10	Horse chestnut	*Aesculus hippocastanum*	Sapindaceae	flowers
11	White man's foot	*Plantago major*	Plantaginaceae	leaves
12	Lemon balm	*Melissa officinalis*	Lamiaceae	leaves
13	Sage	*Salvia officinalis*	Lamiaceae	leaves
14	Peppermint	*Mentha piperita*	Lamiaceae	leaves
15	Clove	*Eugenia caryophyllata*	Myrtaceae	flower buds

Tinctures preparation

The most recent version of the European Pharmacopoeia was followed in preparing the tinctures. Each dried herb was combined with 35 % (V/V) ethanol in a 1:10 ratio (10 g of herbs per 100 ml ethanol) and left to stand for 5 days at room temperature in the dark with occasional shaking. Afterward, the mixture was filtered and then refrigerated at 4 °C.

Test microorganisms

The target fungi used in this study were plant pathogenic species*: Botrytis cinerea* FNS-FCC 23, *Fusarium oxysporum* FNS-FCC 103, *Plasmopara viticola* FNS-FCC 65, *Alternaria alternata* FNS-FCC 624, *Aspergillus niger* FNS-FCC 33, and *Aspergillus ochraceus* FNS-FCC 50 (Table 2). These fungi are part of the Culture Collection of the Department of Microbiology and Microbial Biotechnology, Faculty of Natural Sciences and Mathemat-

ics, Skopje, Macedonia. Fungal cultures of these phytopathogenic strains were maintained on Potato Dextrose Agar (PDA) at 4 °C.

The spore suspension of the fungal strains was prepared by washing spores from the surface of the pure cultures on the PDA medium into 0.85 % NaCl with Tween (0.02 %) and standardized to the 0.5 McFarland standard (equivalent to 10^6 CFU/ml) on the day of the experiment [10].

Table 2. Phytopathogens used in this study

	Fungus	Attack	Diseases
1	*Fusarium oxysporum* FNS-FCC 103	tomato, tobacco, legumes, cucurbits, sweet potatoes, banana, eggplant and pepper plants	fusarium wilt
2	*Botrytis cinerea* FNS-FCC 23	chickpeas, lettuce, broccoli, beans, grape, strawberry, and raspberry	gray mold
3	*Alternaria alternata* FNS-FCC 624	tomato, tobacco, apple, cherry, bean, strawberry	leaf spot
4	*Plasmopara viticola* FNS-FCC 65	grape	brown rot; downy mildew of grapevine; grey rot
5	*Aspergillus ochraceus* FNS-FCC 50	opportunistic storage mold on dried fruits, nuts and grains	production of ochratoxin A
6	*Aspergillus niger* FNS- FCC 33	fruits and vegetables such as grapes, apricots, onions, and peanuts	black mold

In vitro antagonistic activity assay

The antagonism assay was performed on PDA in Petri dishes using the well diffusion method. A layer of PDA medium, inoculated with the plant pathogen, was spread on 90 mm Petri dishes. After solidification, wells with a diameter of 8 mm were created. In each well, 20 µl of tincture was added. The activity against each phytopathogen was tested in triplicate. Antagonistic activity was assessed after 96 hours of incubation at 25 °C by measuring the radius of the inhibition zones (mm)—areas around the wells with no visible growth of the tested microorganism.

Microdilution method

The biological activities of the tinctures were assessed using the microdilution method [11]. The antimicrobial assay was performed using a sterile 96-well plate, and the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values were determined. The test plates were prepared by dispensing 50 µl of Potato Dextrose Broth into each well. A volume of 50 µl from the stock solution of the tested tincture was added to the first column of the plate, and two-fold serial dilutions of the extracts were performed. Then, 10 µl of the spore suspension of fungal strains was added to all wells. Each test plate included a growth control (without tested tinctures, only medium and test cultures) and a sterility control (without test cultures, only medium and tested tinctures). The MIC was defined as the lowest concentration of tinctures at which microorganisms showed no visible growth after seven days. All tests were performed in triplicate, and the MIC values were consistent.

The wells that demonstrated inhibitory activity (with no visible growth) were further tested for fungicidal activity. A sample from each well that tested positive for inhibitory activity was inoculated onto fresh sterile Potato Dextrose Agar plates and incubated for an additional 5 days at 25°C. The absence of colonies was regarded as positive for fungicidal activity, while the growth of colonies was considered negative. The MFC was defined as the lowest concentration of the tincture that resulted in microbial death. All tests were performed in triplicate, and the MFC values were consistent.

RESULTS AND DISCUSSION

Before technology advanced and synthetic pesticides were created and widely accepted for their effective management of numerous crop diseases, the use of plant products to manage pests was part of indigenous knowledge systems [12]. Consequently, the use of plant products for this purpose declined until scientists realized the harmful effects of synthetic pesticides on both the environment and human health. Many ethnic groups have historically used medicinal plant species to treat a variety of ailments in both domestic animals and humans [13, 14]. However, several species of

medicinal plants have shown promise in agriculture as fungicides to protect crops from infections [14–17].

Primarily due to the presence of secondary metabolites, aromatic and medicinal plants have sparked human interest for their potential therapeutic benefits. It is evident that nearly all microbes cannot infect plants due to their inherent defense mechanisms. Therefore, secondary metabolites such as alkaloids, phenols, flavonoids, terpenoids, essential oils, and others are responsible for the antimicrobial properties of plant extracts [18].

To ensure the validity of the extract's potential, the selection of assays used to assess medicinal plant extracts remains crucial. A variety of screening techniques or assays are employed to evaluate the antifungal efficacy of plant extracts. The most widely used methods are disk diffusion tests and microplate dilution assays, with the former being the most commonly used to assess the antifungal efficacy of plant extracts against phytopathogenic molds. The agar diffusion method has some drawbacks, such as difficulties in reproducing results across different laboratories and challenges in diffusing extracts with varying polarity, which make it unsuitable for assessing the antibacterial activity of plant extracts [19].

It is difficult, if not impossible, to compare the antimicrobial activities reported as zones of inhibition for different extracts tested in different laboratories, due to the dependence of the zone of inhibition measurement on several factors, including the size of the inoculum, the medium volume of the agar, and the concentration and volume of the test extracts [19]. Therefore, in this paper, we used two techniques simultaneously. The disk diffusion technique served as a screening method to provide preliminary insight into whether the plant tinctures possessed any biological properties, before proceeding with more detailed determinations of MIC and MFC.

This study provides valuable insights into the *in vitro* biocontrol activity of plant tinctures against certain phytopathogens. Initial screening of the antifungal activity of the 15 tinctures was conducted against six test fungal isolates (the findings are displayed in Table 3). The growth of the tested microorganisms was inhibited by the plant tinctures, as indicated by the results of the well diffusion assay. Distinct inhibition zones were observed in all tested plant tinctures against all fungi. Of all the tested fungi, *Fusarium oxysporum* FNS-FCC 103 and *Aspergillus niger* FNS-FCC 33 were found to be the most resistant microorganisms, with the smallest zones of inhibition, while *Botrytis cinerea* FNS-FCC 23 was the most sensitive. As for the biological activity of the tinctures, the most potent were those obtained from *H. perforatum*, *S. hortensis*, *U. dioica*, and *T. serpyllum*. According to Savary et al. (2006), *Aspergillus flavus*, *Alternaria alternata*, and *Botrytis cinerea* are among the common pathogens that cause diseases in many economically important crop species. Plant tinctures can be effective against phytopathogenic molds, fungi that cause diseases in plants. Research has shown that certain plant extracts possess antifungal properties that can inhibit the growth of these harmful molds. For example, a study on Canarian plant extracts found that some ethanolic extracts were effective against phytopathogenic fungi such as *Botrytis cinerea*, *Fusarium oxysporum*, and *Alternaria alternata* [21]. These extracts can serve as natural bio fungicides, offering an eco-friendly alternative to chemical fungicides.

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were used to assess the exact biological activity of the tinctures. Eight rows (A to H) and twelve columns (1 to 12) of 96-well microplates were used. Table 4 presents the data obtained. Our findings demonstrated the tinctures' effectiveness against all the tested molds. However, the degree of growth suppression varied depending on the type of microorganism involved. The tinctures were shown to be active in all cases, with MICs ranging from 0.78 % to 50 %. At an MIC of 0.39 %, the only mold that showed increased susceptibility was *Botrytis cinerea* FNS-FCC 23. With minimum inhibitory concentrations (MICs) of >50 %, *Fusarium oxysporum* FNS-FCC 103 and *Aspergillus niger* FNS-FCC 33 were the most resistant microorganisms. Additionally, a thorough analysis of the tinctures' activity was conducted to determine the lowest fungicidal concentrations. The molds showed a similar pattern in sensitivity as observed with the MICs. The most resistant molds, requiring the highest tincture concentrations for destruction, were *Fusarium oxysporum* FNS-FCC 103 and *Aspergillus niger* FNS-FCC 33, while the most sensitive was *Botrytis cinerea* FNS-FCC 23, with an MFC of 0.78 %.

Table 3. Average value of zone of inhibition (mm) of tested tinctures against different phytopatogenic microorganisms

	Fusarium oxysporum FNS- FCC 103	*Botrytis cinerea* FNS- FCC 23	*Alternaria alternata* FNS- FCC 624	Plasmopara viticola FNS- FCC 65	*Aspergillus ochraceus* FNS- FCC 50	*Aspergillus niger* FNS- FCC 33
Hypericum perforatum	13.45	20.51	18.58	17.55	16.44	13.88
Achillea millefolium	9.33	18.72	15.37	16.94	16.31	11.23
Centaurium erythraea	7.13	19.11	16.66	17.89	15.72	10.12
Rubus fruticosus	9.56	18.25	13.27	12.47	14.72	9.68
Equisetum arvense	10.03	18.30	12.68	11.37	13.62	11.12
Satureja hortensis	11.67	19.89	18.44	16.03	16.81	13.77
Urtica dioica	14.12	22.03	19.37	17.38	18.11	13.72
Thymus serpyllum	12.85	21.85	17.90	16.20	16.91	11.56
Taraxacum officinale	10.33	18.10	15.11	17.00	17.27	9.52
Aesculus hippocastanum	8.45	19.64	16.35	15.44	15.96	8.77
Plantago major	7.11	18.10	15.14	14.17	13.22	8.93
Melissa officinalis	6.73	17.13	16.38	13.36	12.48	8.47
Salvia officinalis	9.33	16.45	13.19	12.81	11.95	10.05
Mentha piperita	10.28	17.06	15.22	16.29	16.48	10.45
Eugenia caryophyllata	11.45	16.52	13.05	14.51	12.88	10.98

Data are presented as means of three repetitions

Table 4. Antimicrobial activity of tinctures against test microorganisms

		Fusarium oxysporum FNS- FCC 103	*Botrytis cinerea* FNS- FCC 23	*Alternaria alternata* FNS- FCC 624	*Plasmopara viticola* FNS- FCC 65	*Aspergillus ochraceus* FNS- FCC 50	*Aspergillus niger* FNS- FCC 33
Hypericum perforatum	MIC[1]	25	0.39	1.56	3.125	0.78	50
	MFC[2]	50	0.78	6.25	6.25	1.56	>50
Achillea millefolium	MIC[1]	>50	0.78	6.25	25	25	>50
	MFC[2]	>50	1.56	12.5	50	50	>50
Centaurium erythraea	MIC[1]	>50	1.56	6.25	25	6.25	>50
	MFC[2]	>50	3.125	12.5	50	12.5	>50
Rubus fruticosus	MIC[1]	>50	1.56	6.25	6.25	25	>50
	MFC[2]	>50	3.125	25	12.5	50	>50
Equisetum arvense	MIC[1]	>50	1.56	6.25	6.25	25	>50
	MFC[2]	>50	3.125	25	12.5	50	>50
Satureja hortensis	MIC[1]	50	0.39	3.125	0.78	3.125	50
	MFC[2]	>50	0.78	6.25	1.56	6.25	>50
Urtica dioica	MIC[1]	25	0.39	0.78	0.78	0.78	25
	MFC[2]	50	0.78	1.56	1.56	1.56	50
Thymus serpyllum	MIC[1]	50	0.78	3.125	3.125	0.78	25
	MFC[2]	>50	1.56	6.25	6.25	1.56	50
Taraxacum officinale	MIC[1]	>50	0.78	25	25	6.25	>50
	MFC[2]	>50	1.56	50	50	25	>50
Aesculus hippocastanum	MIC[1]	>50	1.56	6.25	6.25	6.25	>50
	MFC[2]	>50	3.125	25	12.5	12.5	>50
Plantago major	MIC[1]	>50	1.56	6.25	25	25	>50
	MFC[2]	>50	3.125	25	50	50	>50
Melissa officinalis	MIC[1]	>50	0.78	25	25	6.25	>50
	MFC[2]	>50	1.56	50	50	25	>50
Salvia officinalis	MIC[1]	>50	0.78	25	6.25	25	>50
	MFC[2]	>50	1.56	50	12.5	50	>50
Mentha piperita	MIC[1]	>50	0.78	6.25	6.25	6.25	>50
	MFC[2]	>50	1.56	12.5	25	25	>50
Eugenia caryophyllata	MIC[1]	>50	1.56	6.25	6.25	6.25	>50
	MFC[2]	>50	3.125	25	25	12.5	>50

[1]MIC= Minimum inhibitory concentration (% tincture, V/V); [2]MFC= Minimum fungicidal concentration (% tincture, V/V)
(Data are presented as means of three repetitions)

As for the tinctures obtained from different plants, all the tested tinctures demonstrated some biological activity against the tested molds. However, four plants stood out as the most potent in terms of inhibition and fungicidal action on the test microorganisms. These are tinctures obtained from *Hypericum perforatum*, *Satureja hortensis*, *Urtica dioica*, and *Thymus serpyllum*, which showed the largest zones of inhibition in the diffusion test (Table 3). In the microtitration test, these same tinctures were effective against all molds, even at the lowest concentrations. These findings align with those of Chebli et al. (2003), who showed that extracts of *Thymus* spp. completely inhibit *B. cinerea*. Additionally, Mehdi et al. (2012) demonstrated that thyme extracts exhibited the best growth inhibition against *B. cinerea*.

Plant products contain several constituent antibacterial substances, and their synergistic effects may help prevent the development of resistance when used against fungal diseases [24, 25]. It can be argued that products derived from medicinal plant species are eco-friendly, have low toxicity to humans, and are relatively safe [26]. Additionally, natural compounds, especially those derived from plants, tend to be unstable at high temperatures, decomposing quickly and not lingering in the environment as long as traditional synthetic fungicides [27].

Our research indicates that the tinctures exhibited strong *in vitro* activity and microbicidal effects, suggesting that they may be used as natural antifungal agents against phytopathogenic molds. Herb extracts contain several active compounds, in contrast to synthetic fungicides, which typically consist of a single active ingredient. These 'herbal cocktails' have the ability to work synergistically, offering unique benefits without negative side effects. Plant diseases pose significant challenges to the commercial agricultural sector and represent real financial risks to both conventional and organic farming systems. The presence of many pathogen types further complicates disease management. A variety of worms, bacteria, viruses, and fungi can affect a single crop. Organic vegetable growers face additional challenges because they are not permitted to use conventional synthetic fungicides and typically grow a variety of vegetable crops. They must continue to provide high-quality, disease-free food with a reasonable shelf life, as the global market remains highly competitive. Therefore, disease control is a crucial factor in the production of organic vegetables. The use of natural products for controlling fungal diseases in plants is considered a promising alternative to synthetic fungicides due to their reduced environmental impact.

From these findings, we can conclude that these plant tinctures exhibit antifungal activity and can be effective for mold management. This work paves the way for the development of a naturally bioactive substance with phytosanitary applications that is both environmentally safe and potentially profitable.

CONCLUSIONS

Our research showed that the tinctures obtained from fifteen different medicinal and aromatic plants contain compounds that are active against six investigated phytopathogenic molds. The tinctures from the following plants demonstrated the greatest potential for use in developing a protection regimen against phytopathogenic molds: *Hypericum perforatum*, *Satureja hortensis*, *Urtica dioica*, and *Thymus serpyllum*. These tinctures exhibited a broad spectrum of biological activity against phytopathogens under *in vitro* conditions and achieved the highest reduction in the growth of phytopathogens, with the lowest MIC and MFC values. An additional significant advantage of using these tinctures is the low cost of their production.

These preliminary results, obtained from *in vitro* experiments, can be further supported by more comprehensive *in vivo* studies, both under controlled greenhouse and outdoor conditions, to practically evaluate the use of these extracts in integrated pest management. These findings support the idea that plant-derived extracts are promising sources of biopesticides for such applications. However, further studies will be necessary to identify the bioactive components and explore their potential as biofungicides. Additionally, it is of great interest to deepen our understanding of the molecular mechanisms of action of plant extracts, not only against microorganisms but also in plant biology.

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СПОСОБНОСТ ЗА БИОКОНТРОЛА НА АРОМАТИЧНИ И ЛЕКОВИТИ РАСТЕНИЈА КОН ПАТОГЕНИ МИКРООРГАНИЗМИ ОД ПОЧВАТА

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На глобално ниво, фитопатогените кои се пренесуваат преку почвата може сериозно да го нарушат градинарството и житните култури, предизвикувајќи огромни загуби. Синтетичките пестициди и денес остануваат прв избор во системите за управување со растителни болести поради нивната голема ефикасност и леснотија на примена, но строгите закони и растечките грижи за животната средина ја зголемуваат потребата од одржливи алтернативи во земјоделието. Освен интегрирањето на ботаниката во земјоделските методи, различните системи за одгледување со ароматични и лековити растенија, исто така, може да бидат корисна стратегија за справување со растителните болести преку снабдување со хранливи материи и менување на микробната популација на почвата.

Сепак, овие техники не се универзално прифатени и може негативно да влијаат на плодноста на почвата доколку нивната примена не е темелно контролирана. Оваа студија има цел да ја утврди биоконтролната активност на тинктурите подготвени од ароматични и лековити растенија кои растат во Северна Македонија против некои фитопатогени кои се пренесуваат преку почвата.

Антимикробниот потенцијал на тинктурите беше евалуиран со помош на методот на дифузија и методот на разредување со употреба на микротитарни плочи со 96 бунари, што овозможи одредување на минималната инхибиторна концентрација (MIC) и минималната фунгицидна концентрација (MFC). Тинктурите беа подложени на сериски разредувања во опаѓачки концентрации, почнувајќи од концентрација од 50 % и завршувајќи со концентрација од 0,39 %. Во овој експеримент беа користени 15 ароматични и лековити растенија и шест општопознати фитопатогени.

Општо земено, се покажа дека тинктурите се активни, со MIC во опсег од 0,39 % до 25 %, и со MFC од 0,78 % до 50 %. Сепак, од сите тестирани габи, Fusarium oxysporum FNS-FCC 103 и Aspergillus niger FNS-FCC 33 се најдени како најотпорни микроорганизми, додека најчувствителен беше Botrytis cinerea.FNS-FCC 23.

Според наодите од овој труд, тестираните ароматични и лековити растенија покажаа релативно висока антимикробна активност против сите тестирани фитопатогени габи. Оваа студија сугерира дека тинктурите од овие растенија се потенцијален извор на природни антифунгални агенси. Сепак, по овој скрининг експеримент, треба да се изврши понатамошна работа за подетално да се опишат антимикробните активности.

Клучни зборови: антимикробно; тинктура; микротитарска плоча со 96 бунарчиња; ароматични растенија; почвени патогени