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Investigation into the Antimicrobial Activities of Tincture Prepared from Twenty Six Plants Growing in Macedonia

Natalija Atanasova-Pancevska[1*], Dzoko Kungulovski[2]

[1]Department of Microbiology and Microbial Biotechnology, Institute of Biology
[2]Faculty of Natural Sciences and Mathematics, "Ss. Cyril and Methodius" University, Skopje, Macedonia

Abstract

Antibiotic resistance has become a global concern. In recent years there has been increasing incidence of multiple resistance in human pathogenic microorganisms, largely due to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. This has forced scientists to search for new antimicrobial substances from various sources like medicinal plants.

The study was designed to determine the antimicrobial properties of a tincture prepared from 26 plants growing in Macedonia. The antimicrobial potential of the tincture was evaluated using the micro-broth dilution method using 96-well microtiter plates, which enabled determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) or minimal fungicidal concentration (MFC). The tincture was subjected to serial dilutions in descending concentrations starting from a concentration of 50% and finishing with a concentration of 0.39%. Thirteen reference bacterial strains and eight fungal strains were used.

Generally, the tincture was found to be active, with MIC ranging from 0.39 to 25%, and with MBC and MFC ranging from 0.78 to 50%. However, of the tested fungi, *Fusarium* sp. and *A. niger* ATCC 1052 were found to be the most resistant of the examined microorganisms, the most resistant appeared to be *P. aeruginosa* ATCC 9027 and *S. enteridis* FNS-BCC 98.

The present study suggests that the tincture from these plants is a potential source of natural antibacterial and antifungal agents. After this screening experiment, further work should be performed to describe the antimicrobial activities in more detail.

Keywords: antimicrobial, tincture, 96-well microtiter plates

Резюме

Резистентността към антибиотици се превърна в глобален проблем. През последните години се наблюдава нарастваща честота на множествена резистентнос на патогенните микроорганизми по човека. Този феномен до голяма степен се дължи на безразборното използване на търговски антимикробни средства при лечението на инфекциозни заболявания. Това принуждава учените да търсят нови антимикробни вещества от различни източници като напр. лечебните растения.

Настоящото проучване е предназначено да определи антимикробните свойства на тинктура, приготвена от 26 растения, отглеждани в Македония. Антимикробният потенциал на тинктурата се оценява по метода за разреждане на микроорганизмите, използвайки микротитърни плаки с 96 ямки, които позволяват определянето на минимална инхибираща концентрация (MIC) и минимална бактерицидна концентрация (MBC) или минимална фунгицидна концентрация (MFC). Тинктурата се подлага на серийни разреждания в низходящи концентрации, започвайки от концентрация 50% и завършваща с концентрация 0.39%. Използвани са тринадесет референтни бактериални щамове и осем гъбични щама.

Установено е, че тинктурата проявява антимикробна активност. Определената MIC варира от

** Corresponding author: Natalija Atanasova-Pancevska*
E-mail: natalijaap@gmail.com

0,39 до 25%, а MBC и MFC варират от 0,78 до 50%. Резултатите, обаче показват, че от изследваните микроорганизми най-резистентни са гъбите *Fusarium* sp. и *A. niger* ATCC 1052, а от бактериите - *P. aeruginosa* ATCC 9027 и *S. enteridis* FNS-BCC 98.

Настоящото проучване показва, че тинктурата от тези растения е потенциален източник на естествени антибактериални и противогъбични средства. След този скринингов експеримент трябва да се извърши по-нататъшна работа, за да се опишат антимикробните дейности по-подробно.

Introduction

The use of plants for treating diseases is as old as the human species. For centuries, the therapeutic properties of various medicinal plants have been used to treat human diseases. It has been estimated that between 60-90% of the populations of developing countries use traditional and botanical medicines almost exclusively and consider them to be a normal part of primary healthcare (WHO, 2002).

According to the WHO, traditional medicine includes health practices, approaches, knowledge and beliefs incorporating plants, animals and mineral based medicine, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses and maintain well-being. The therapeutic use of natural products is the early medical practice. To date, in the world there are more than 85,000 plant

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	milk thistle	*Marianum silybum*	Asteraceae	flowers
5	greater celandine	*Chelidonium majus*	Papaveraceae	leaves
6	white birch tree	*Betula alba*	Betulaceae	leaves
7	blackberry	*Rubus fruticosus*	Rosaceae	leaves
8	common horsetail	*Equisetum arvense*	Equisetaceae	aerial part
9	smooth rupturewort	*Herniaria glabra*	Caryophyllaceae	aerial part
10	summer savory	*Satureja hortensis*	Lamiaceae	leaves
11	common nettle	*Urtica dioica*	Urticaceae	leaves
12	great yellow gentian	*Gentiana lutea*	Gentianaceae	leaves
13	common agrimony	*Agrimonia eupatoria*	Rosaceae	aerial part
14	wall germander	*Teucrium chamaedrys*	Lamiaceae	aerial part
15	breckland wild thyme	*Thymus serpyllum*	Lamiaceae	leaves
16	mountain germander	*Teucrium montanum*	Lamiaceae	aerial part
17	common dandelion	*Taraxacum officinale*	Asteraceae	flowers
18	horse chestnut	*Aesculus hippocastanum*	Sapindaceae	flowers
19	white man's foot	*Plantago major*	Plantaginaceae	leaves
20	lemon balm	*Melissa officinalis*	Lamiaceae	leaves
21	sage	*Salvia officinalis*	Lamiaceae	leaves
22	dog rose	*Rosa canina*	Rosaceae	flowers
23	shepherd's purse	*Capsella bursa-pastoris*	Brassicaceae	leaves
24	common knotgrass	*Polygonum aviculare*	Polygonaceae	aerial part
25	peppermint	*Mentha piperita*	Lamiaceae	leaves
26	clove	*Eugenia caryophyllata*	Myrtaceae	flower buds

species that have been documented for medical use. This indicates that plant-derived natural products hold great promise for the discovery and development of new pharmaceuticals against diverse human ailments (Qurishi *et al*., 2010).

The expanding bacterial resistance to antibiotics has become a growing concern worldwide (Gardam, 2000). Increasing bacterial resistance is prompting a resurgence in research of the antimicrobial role of herbs against resistant strains (Hemaiswarya *et al*., 2008; Alviano and Alviano, 2009). A vast number of medicinal plants have been recognized as valuable resources of natural antimicrobial compounds (Mahady, 2005).

The screening of plant products for antimicrobial activity have shown that the higher plants represent a potential source of novel antibiotic prototypes (Afolayan, 2003).

The aim of this study was comparison of a spectrum of antimicrobial properties of a tincture prepared from 26 plants growing in Macedonia, to create a reserve for new sources of antimicrobial agents, some of which may be extracts from plants.

latest edition of the European Pharmacopoeia. Each of the dried herbs was mixed with ethanol 35% (V/V) in a 1 to 10 ratio (10 g herbal drug per 100 ml ethanol) and allowed to stand for 5 days in the dark at room temperature with occasional shaking; then they were filtered and combined. Then the tincture was stored under refrigeration at 4°C for further studies.

Test Microorganisms

Antimicrobial activities were tested against thirteen bacteria and eight molds (Table 2). The microorganisms were provided from the collection of the Microbiology Laboratory at the Faculty of Natural Sciences and Mathematics in Skopje.

Culture Media

Test bacteria were cultured in Mueller-Hinton broth and Mueller-Hinton agar (Merck, Darmstadt, Germany), and for the molds Sabouraud Dextrose Broth and Sabouraud Dextrose agar (Merck, Darmstadt, Germany) was used.

Table 2. Test microorganisms used in the study.

	Bacteria	Molds
1	*Bacillus subtilis* ATCC 6633	*Aspergillus ochraceus*
2	*Bacillus pumilus* NCTC 8241	*Alternaria alternata*
3	*Bacillus* sp.	*Fusarium* sp.
4	*Staphylococcus citrus*	*Botrytis cinerea*
5	*Staphylococcus albus*	*Penicillium* sp.
6	*Staphylococcus aureus*	*Plasmopara viticola*
7	*Micrococcus luteus*	*Aspergillus niger* ATCC 1052
8	*Listeria monocytogenes*	*Penicillium commune*
9	*Sarcina lutea*	
10	*Escherichia coli* ATCC 8739	
11	*Pseudomonas aeruginosa* ATCC 9027	
12	*Salmonella typhimurium*	
13	*Salmonella enteridis*	

Materials and Methods

Processing of medicinal plants

The whole plant or parts of plants (Table 1) were used to prepare extracts for the study. The plants collected were washed with water to remove the soil and dust particles. Then they were dried in a thoroughly shaded place, and blended to form a fine powder and stored in airtight containers.

Preparation of tinctures

The tinctures were prepared according to the

Standardization of Microbial Suspension

Microbial suspension was prepared by the direct colony method. The turbidity of the initial suspension was adjusted by comparison with 0.5 McFarland's standard (Andrews, 2005). The initial suspension contained about 10^8 colony forming units (CFU)/mL.

Microdilution Method

The antimicrobial activities of the tincture were assessed using the microdilution method with

Table 3. Antimicrobial activity of tincture against test Gram-positive and Gram-negative bacteria.

Bacteria	MIC[1]	MBC[2]
Gram-positive bacteria		
B. subtilis ATCC 6633	25	50
B. pumilus NCTC 8241	12.5	25
Bacillus sp.	12.5	25
S. citrus	0.39	0.78
S. albus	6.25	25
S. aureus ATCC	6.25	12.5
M. luteus	3.125	6.25
L. monocytogenes	12.5	25
S. lutea	0.39	6.25
Gram-negative		
E. coli ATCC 8739	6.25	12.5
P. aeruginosa ATCC 9027	25	50
S. typhimurium	12.5	25
S. enteridis	25	50

[1]MIC= Minimum inhibitory concentration (% tincture, V/V)
[2]MBC= Minimum bactericidal concentration (% tincture, V/V)

resazurin as an indicator of microbial growth (Sarker *et al.* 2007). The antimicrobial assay was performed by using a sterile 96-well plate, and the minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/ MFC) values were determined. The test plates were prepared by dispensing 50 µL of Mueller-Hinton broth/ Sabouraud dextrose Broth into each well. A volume of 50 µL from the stock solution of tested tincture was added into the first column of the plate and then two-fold serial dilutions of extracts were performed. Each test plate included growth control and sterility control. MIC was defined as the lowest concentration of tested tincture that prevented a resazurin color change from blue to pink. All tests were performed in triplicate and MIC values were constant.

The wells that demonstrated inhibitory activities were further tested for bactericidal/ fungicidal activity. A sample from each well that tested positive for inhibitory activity was inoculated on fresh sterile Mueller-Hinton agar/Sabouraud dextrose Agar plates and incubated additionally for 24 h at 37°C for bacteria, and 5 days at 25°C, for molds. Absence of colonies was regarded as positive for bactericidal/fungicidal activity, while growth of colonies was regarded as negative. MBC/ MFC was defined as the lowest concentration of the tincture that resulted in microbial death. All tests were performed in triplicate and MBC/MFC values were constant.

Results and Discussion

Medicinal plants have generated the interest of man for therapeutic values chiefly because of the presence of secondary metabolites. It is obvious that plants have their own builtin defense mechanism against infection by almost all microorganisms. The antimicrobial properties of plant extracts, therefore, are a result of the presence of secondary metabolites, such as alkaloids, phenols, flavanoids, terpenoids, essential oils etc (Cowan, 1999).

The present study offers some interesting aspects about the antimicrobial activity of ethanolic plant preparations *in vitro.*The tinctures' antimicrobial activity was evaluated by determining the minimum inhibitory concentration (MIC) and minimum bactericidal (MBC) and fungicidal concentration (MFC). We used 96-well microplates disposed in 12 columns (1 to 12) and eight lines (A to H). The tested tincture was assessed for its antibacterial properties against all test Gram-positive and Gram-negative bacteria. The resulting data are presented in Table 3.

Our results showed that the tincture was active against all the bacteria used. However, the power of bacterial growth inhibition depended on the bacterial species. In most cases the tincture was found to be active with MICs ranging from 6.25-12.5 %. Only *S. citrus* and *S. lutea* were more sensitive, with MICs of 0.39%. The most resistant bacteria were *B. subtilis* ATCC 6633, *P. aeruginosa* ATCC 9027 and *S. enteridis,* all with MICs of 25%.

Furthermore, the activity of the tincture was examined in detail determining the minimal bactericidal concentrations. Similar sensitivity was shown by the tested bacteria when we observed MBCs. The tincture exerted good to moderate effects against Gram-positive and Gram-negative bacteria. However, the presented results show that Gram-positive bacteria were more sensitive than Gram-negative bacteria. Cos *et al.*(2006), reported that Gram-negative bacteria are generally more resistant compared to the Gram-positive ones. Also, Shan *et al.* (2007), reported that Gram-positive bacteria were generally more sensitive to the tested extracts than Gram-negative. A possible explanation for these observations may lie in the significant differences in the outer layers of Gram-negative and Gram-positive bacteria. Gram-negative bacteria possess an outer membrane and a unique periplasmic space not found in Gram-positive bacteria (Duffy and Power, 2001).

The resistance of Gram-negative bacteria towards antibacterial substances is related to the hydrophilic surface of their outer membrane, which is rich in lipopolysaccharide molecules, presenting a barrier to the penetration of numerous antibiotic molecules. It is also associated with the enzymes in the periplasmic space, which are capable of breaking down the molecules introduced from outside (Nikaido, 1994; Gao *et al.,* 1999). Gram-positive bacteria do not possess this type of outer membrane and cell wall structure. Antibacterial substances can easily destroy the bacterial cell wall and cytoplasmic membrane and result in a leakage of the cytoplasm and its coagulation (Kalemba and Kunicka, 2003).

Plant diseases create challenging problems in commercial agriculture and pose real economic threats to both conventional and organic farming systems. Disease management is complicated by the presence of multiple types of pathogens. For any one crop the grower must deal with a variety of fungi, bacteria, viruses and nematodes. This situation is even more complicated for organic vegetable growers because they usually produce a wide array of vegetable crops and are prohibited from applying conventional synthetic fungicides. The world market continues to be extremely competitive and continues to require that growers supply high-quality diseasefree produce with an acceptable shelf life. Disease management is therefore a critical consideration in organic vegetable production. The use of natural products for the control of fungal diseases in plants is considered as an interesting alternative to synthetic fungicides due to their less negative impacts on the environment (Cao and Forrer, 2001).

The tincture was assessed also for its antifungal properties against eight test molds. The resulting data are presented in Table 4. According to these results, the most sensitive molds, with MIC of 0.39% and MFC of 0.78%, were *A. ochraceus, B. cinerea* and *P. commune.* However, *Fusarium* sp. and *A. niger* ATCC 1052 were the most resistant molds, with MFC more than 50% tincture. Despite these findings, from the results it can be concluded that this tincture showed antifungal activity and can be used for effective control of molds. This study has paved the way for the development of a bioactive natural product with phytosanitary applications, with the added benefits of being an environmentally safe and economically viable product.

Table 4. Antimicrobial activity of tincture against test molds.

Molds	MIC[1]	MFC[2]
A. ochraceus	0.39	0.78
A. alternata	25	50
Fusarium sp.	>50	>50
B. cinerea	0.39	0.78
Penicillium sp.	25	50
P. viticola	6.25	12.5
A. niger ATCC 1052	>50	>50
P. commune	0.39	0.78

[1]MIC= Minimum inhibitory concentration (% tincture, V/V)
[2]MFC= Minimum fungicidal concentration (% tincture, V/V)

According to our findings, the tincture showed a good microbicidal effect and *in vitro* activity, so it has the potential of a natural antimicrobial agent against both bacteria and molds.

Unlike modern drugs that invariably comprise a single active species, herb extracts and/or prescriptions contain multiple active constituents. Natural compounds contained in these “herbal cocktails” can act in a synergistic manner within the human body, and can provide unique therapeutic properties with minimal or no undesirable side-effects (Kaufman *et al*., 1999).

A screening of biological properties of medical plant tinctures demonstrated that herbal extractions are a potential reserve for the creation of new sources of antimicrobial agents, particularly for antibiotic-resistant clinical strains of microorganisms, which are formed under the constant influence of synthetic and semi-synthetic drugs.

Conclusions

The use of crude extracts of plant parts and phytochemicals of known antimicrobial properties can be of great significance in therapeutic treatments. In recent years, a number of studies have been conducted in various countries to prove such efficiency. Many plants have been used because of their antimicrobial traits, which are due to the secondary metabolites synthesized by the plants. These products are known by their active substances like phenolic compounds, which are part of essential oils, as well as of tanning agents.

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