Canli et al., Afr J Tradit Complement Altern Med. (2016) 13(4):42-46
doi: 10.21010/ajtcam.v13i4.7

IN VITRO ANTIMICROBIAL ACTIVITY SCREENING OF XYLARIA HYPOXYLON

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Abstract

Background: Fungi have a potential of using both as nutritive and medicinal foodstuff. Because of containing several therapeutic agents, they are reported to be used for hundreds of years to treat several diseases caused by bacteria, fungi, viruses and parasities. The aim of this study is to determine the in vitro antimicrobial activity of Xylaria hypoxylon, which were collected from Yomra, Trabzon, Turkey.

Materials and Methods: X. hypoxylon samples were air dried and extracted by using ethanol. Antimicrobial activity of X. hypoxylon ethanol extracts were investigated against 21 bacterial and 2 fungal strains, namely, Bacillus subtilis DSMZ 1971, Bacillus subtilis ATCC 6633, Candida albicans ATCC 10231, Candida albicans DSMZ 1386, Enterobacter aerogenes ATCC 13048, Enterococcus durans, Enterococcus faecalis ATCC 29212, Enterococcus faecium, Escherichia coli ATCC 25922, Escherichia coli CFA1, Klebsiella pneumoniae, Listeria monocytogenes ATCC 7644, Pseudomonas aeruginosa DSMZ 50071, Pseudomonas fluorescense P1, Salmonella enteritidis ATCC 13075, Salmonella infantis, Salmonella kentucky, Salmonella typhimurium SL 1344, Staphylococcus aureus ATCC 25923, Staphylococcus carnosus MC1.B, Staphylococcus epidermidis DSMZ 20044 and Streptococcus agalactiae DSMZ 6784 by using the disk diffusion method.

Results: It is observed that ethanol extracts of X. hypoxylon has antimicrobial activity against several Gram positive and Gram negative microorganisms tested. As a result of the study, an antimicrobial activity of X. hypoxylon found against most of strains used in the study.

Conclusion: The results of our study clearly puts forward that X. hypoxylon could have a possible medicinal use.

Keywords: Xylaria hypoxylon, fungi, antimicrobial activity, antimicrobial screening, ethanol extract.

Introduction

From the beginning of the history humankind natural products have provided a variety of medicines (Webster et al., 2008). It has been known for many years that natural products have potential of containing therapeutic agents as a source for analgesics, anti-inflammatories, anti-neoplastic drugs, medicines for asthma, anti-arrhythmic agents, anti-hypertensives and anti-infective agents against several infectious diseases (Clardy and Walsh, 2004; Webster et al., 2008; Altuner et al., 2010a,b). Natural products play a central role in the health care of about 80% of the world’s population (Baker et al., 1995).

Microbial evolution and antibiotic resistance as a result of the increase in use of anti-infective drugs worldwide has been defined as the major threat for the public health in the 21st century by World Health Organization (Syed et al., 2010). This dramatic increase is being associated with important morbidity and mortality in immune compromised patients (Kontoyiannis et al., 2003; Kauffman, 2006).

A tremendous progress has been made in human medicine in the last decades, but bacterial, fungal and viral diseases are still threatening the public health in the developing countries (Cos et al., 2006). The major problem in these countries is the extensive drug resistance which has a large impact on human health (Okeke et al., 2005), thus there is a continuous need to discover new antimicrobial compounds having diverse chemical structures and novel mechanisms of action (Rojas et al., 2003; Ertürk et al., 2006).

In order to prevent spreading of infection by antibiotic resistant microbes, scientists have been conducting intensive researches to determine new alternative antimicrobial agents (Paudel et al., 2008; Örkan et al., 2015). Huge number of studies presented that natural products have been used for hundreds of years to treat several diseases caused by bacteria, fungi, viruses and parasites (Jones, 1996; Onbaşılı et al., 2011; Onbaşılı et al., 2013). Recent researches showed that natural products have a potential of providing opportunities for new drug leads. As far as the current literature is concerned, it is obvious that only a very small amount of the available diversity among living organisms have yet been explored for such purposes (Cos et al., 2006, Altuner, 2011).

After Fleming has discovered penicillin accidentally from Penicillium fungi, scientific attention has widely been attracted for the potential role of fungi as antimicrobial agents, which has lead to the discovery and development of new antimicrobial agents (Bala et al., 2011).

Fungi have a potential of using both as nutritive and medicinal food (Bonatti et al., 2004; Agrahar-Murugkar and Subbulakshmi, 2005; Cheung and Cheung, 2005; Imtiaj and Lee, 2007).
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Some species such as *Agaricus bisporus* (J. E. Lange) Imbach (Agaricaceae) are frequently used as a food source, while some are very toxic such as *Amanitas*. In addition, some fungi have been used as medicines to prevent diseases such as hypertension, hypercholesterolemia and cancer (Bobek et al., 1995; Bobek and Galbavy, 1999).

Several compounds have been isolated and identified by researchers originating from fungi until now, which show medicinal properties, such as immune modulatory, liver protective, antifibrotic, anti-inflammatory, antiadipetic, antiviral and antimicrobial activities (Düger et al., 1999; Gunde-Cimerman, 1999; Ooi, 2000; Wasser and Weis, 1999a,b; Düger et al., 2005b). *Xylaria hypoxylon* (L.) Grev. belongs to family Xylariaceae Tul. & C. Tul. (Ascomycota). It is a very common species, can be collected during the year on dead wood of deciduous trees (Breitenbach and Kränzlín, 1984; Breitenbach and Kränzlín, 1986; Hansen and Knudsen, 2000).

In this study the antimicrobial activity of *X. hypoxylon* is investigated against *Bacillus subtilis* DSMZ 1791, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Candida albicans* DSMZ 1386, *Enterobacter aerogenes* ATCC 13048, *Enterococcus durans*, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium*, *Escherichia coli* ATCC 25922, *Escherichia coli* CFAI, *Klebsiella pneumoniae*, *Listeria innocua*, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* DSMZ 50071, *Pseudomonas fluorescens* P1, *Salmonella enteritidis* ATCC 13075, *Salmonella infantis*, *Salmonella kentucky*, *Salmonella typhimurium* SL 1344, *Staphylococcus aureus* ATCC 25923, *Staphylococcus carnosus* MC1.B, *Staphylococcus epidermidis* DSMZ 20044 and *Streptococcus agalactiae* DSMZ 6784 by using the disk diffusion method.

**Materials and Methods**

**Macrofungi samples**

*X. hypoxylon* samples used in this study were collected from Yomra, Trabzon, which is located in the Black Sea region of Turkey. Voucher specimens were deposited for further reference in Herbarium of Ankara University (ANK) Faculty of Science, Department of Biology, Ankara, Turkey.

**Extraction procedure**

All *X. hypoxylon* samples were dried out after collection and the samples were grounded by a mortar and a pestle. In order to extract active substances ethanol (Merck, Germany) was chosen as an extraction solvent. Grounded samples were shaken in ethanol at 100 rpm for 3 days at room temperature. All the extracts were filtered through Whatman No. 1 filter paper into evaporation flasks. The filtrate was evaporated by a rotary evaporator (Heidolph Hei-Vap Value HL/HB-G1) at 30°C. After evaporation the residues were collected and used to prepare 9 mg.mL⁻¹ extracts.

**Microorganisms**

A wide range of Gram positive and Gram negative bacteria and yeasts were selected to test the antimicrobial effect of *X. hypoxylon*. These strains are *Bacillus subtilis* DSMZ 1791, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Candida albicans* DSMZ 1386, *Enterobacter aerogenes* ATCC 13048, *Enterococcus aerogenes* ATCC 29212, *Enterococcus faecium*, *Escherichia coli* CFAI, *Klebsiella pneumoniae*, *Listeria innocua*, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* DSMZ 50071, *Pseudomonas fluorescens* P1, *Salmonella enteritidis* ATCC 13075, *Salmonella infantis*, *Salmonella kentucky*, *Salmonella typhimurium* SL 1344, *Staphylococcus aureus* ATCC 25923, *Staphylococcus carnosus* MC1.B, *Staphylococcus epidermidis* DSMZ 20044 and *Streptococcus agalactiae* DSMZ 6784.

The strains were chosen from standard strains as much as possible. Other strains which are not standard were all isolated from food and identified in Ankara University, Faculty of Science, Department of Biology.

**Preparation of inocula**

All bacterial strains were incubated at 37 °C for 24 hours (Canlı et al., 2015). But since the requirements for *C. albicans* is different, *C. albicans* was inoculated at 27 °C for 48 hours. Inocula were prepared by transferring morphologically similar colonies of each organism into 0.9% sterile saline solution until the visible turbidity was equal to 0.5 McFarland standard having approximately 10⁸ cfu.mL⁻¹ for bacteria and 10⁵ cfu.mL⁻¹ for *C. albicans* (Hammer et al., 1999; Altuner et al., 2012a,b; Canlı et al., 2014).

**Disk diffusion method**

Disk diffusion test was performed as described previously by Andrews (Andrews, 2003). The culture medium was poured into 120 mm sterile Petri dish to give a mean depth of 4.0 mm ± 0.5 mm (Altuner and Çetin, 2009; Altuner and Akata, 2010). 60 µL, 100 µL and 150 µL aliquots of each extract was applied on sterile paper disks of 6 mm diameter end up with 550 µg.mL⁻¹ and 1375 µg.mL⁻¹ sample on each disk (Mahasneh and El-Oqlah, 1999; Silici and Koc, 2006). To get rid of any residual solvent which might interfere with the results, disks were left to dry overnight at 30°C in sterile conditions (Silici and Koc, 2006; Altuner and Çetin, 2010, Altuner and Canlı, 2012). The surface of the plates was inoculated using previously prepared inocula containing saline suspension of microorganisms. Inoculated plates were then left to dry for 5 minutes at room temperature before applying the disks (Altuner et al., 2014). Disks were firmly applied to the surface of the plate which had an even contact with the agar. Plates were incubated and inhibition zone diameters were expressed in millimetres (Altuner et al., 2011a,b).
Empty sterile disks and extraction solvent (ethanol) loaded on sterile disks which were dried at sterile conditions to remove solvent as done in the study were used as negative controls.

Statistics

All extracts were tested in triplicate and MACANOVA (version 5.05) was used for statistical analysis of the data. P values of <0.05 were considered statistically significant.

Results and Discussion

In this test, extracts were loaded on empty sterile disks and these disks were then applied on a culture medium inoculated with microorganisms. If the extracts were active against these microorganisms, they have caused an inhibition zone. The diameters of the inhibition zones recorded in millimetres are given in Table 1. No activity was observed for the negative controls; extraction solvent and empty sterile disks.

|  | Disk diffusion test results (Inhibition zones in mm) |
|---|---|---|---|
|  | 30µL | 60µL | 150µL |
| B. subtilis ATCC 6633 | - | 7 | 11 |
| B. subtilis DSMZ 1386 | 10 | 11 | 13 |
| C. albicans ATCC 10231 | 7 | 8 | 9 |
| C. albicans DSMZ 1386 | - | - | - |
| E. aerogenes ATCC 13048 | 7 | 7 | 7 |
| E. durans | 8 | 8 | 9 |
| E. faecalis ATCC 29212 | - | - | - |
| E. faecium | 9 | 10 | 12 |
| E. coli ATCC 25922 | 7 | 7 | 10 |
| E. coli CFAI | - | - | - |
| K. pneumonia | 7 | 8 | 8 |
| L. innocula | 7 | 7 | 9 |
| L. monocytogenes ATCC 7644 | 10 | 12 | 13 |
| P. aeruginosa DSMZ 50071 | - | - | - |
| P. fluorescens P1 | - | 7 | 9 |
| S. enteritidis ATCC 13075 | - | - | - |
| S. infantis | - | - | - |
| S. Kentucky | 7 | 7 | 7 |
| S. typhimurium SL 1344 | - | - | 8 |
| S. aureus ATCC 25923 | 16 | 16 | 16 |
| S. carnosus MC1.B | 7 | 7 | 9 |
| S. epidermidis DSMZ 20044 | 7 | 8 | 11 |
| S. agalactiae DSMZ 6784 | - | - | 8 |

"-": No activity observed.

Results given in Table 1 show that 30 µL (550 µg.µL⁻¹) of X. hypoxylon samples caused an inhibition zone of 16 mm against S. aureus ATCC 25923, 10 mm against B. subtilis DSMZ 1386 and L. monocytogenes ATCC 7644, 9 mm against E. faecium, 8 mm against E. durans, 7 mm against C. albicans ATCC 10231, E. aerogenes ATCC 13048, E. coli ATCC 25922, K. pneumoniae, L. innocula, S. kentucky and S. epidermidis DSMZ 20044. 60 µL (917 µg.µL⁻¹) of X. hypoxylon samples caused an inhibition zone of 16 mm against 16 mm against S. aureus ATCC 25923, 12 mm against L. monocytogenes ATCC 7644, 11 mm against B. subtilis DSMZ 1386, 10 mm against E. faecium, 8 mm against C. albicans ATCC 10231, E. durans, K. pneumoniae and S. epidermidis DSMZ 20044, 7 mm against B. subtilis ATCC 6633, E. coli ATCC 25922, L. innocula, P. fluorescens P1 and S. kentucky, where 150 µL (1375 µg.µL⁻¹) of X. hypoxylon samples caused an inhibition zone of 16 mm against S. aureus ATCC 25923, 13 mm against B. subtilis DSMZ 1386 and L. monocytogenes ATCC 7644, 12 mm against E. faecium, 11 mm against B. subtilis ATCC 6633 and S. epidermidis DSMZ 20044, 10 mm against E. coli ATCC 25922, 9 mm against C. albicans ATCC 10231, E. durans, L. innocula, P. fluorescens P1 and S. carnosus MC1.B, 8 mm against K. pneumoniae, S. typhimurium SL 1344 and S. agalactiae DSMZ 6784, 7 mm against E. aerogenes ATCC 13048 and S. kentucky.

On the other hand, no inhibition zone was observed against C. albicans DSMZ 1386, E. coli CFAI, P. aeruginosa DSMZ 50071, S. enteritidis ATCC 13075 and S. infantis.

It is a fact that Gram-negative bacteria are more resistant to a large number of chemotherapeutic agents than Gram-positive bacteria (Nikaido, 1998). It was previously reported that antibiotics of natural origin showed >90% lacked activity against E. coli strains, although they were active against Gram-positive strains (Vaara, 1993). Thus, any antimicrobial activity against E. coli can be very important.

As far as the current literature considered there are no results reported regarding the antibacterial activity of any Fungi samples or any plant samples against E. coli CFAI until now. However, there are several studies reported by using other E. coli strains. For example, Dulger et al. (2005a) showed that the methanolic extract of H. cupressiforme (30mg.ml⁻¹) presented 12.2 mm of inhibition zone against E. coli ATCC 11230. When the results reported by Dulger et al. (2005a) were compared with our results, which were maximum 10 mm inhibition zone against E. coli ATCC 25922, it can be concluded that ethanolic extract of X. hypoxylon
presented lower antimicrobial activity. But since the E. coli strain used in this study is different than the study conducted by Dulger et al. (2005a), this difference is not comparable.

It was also pointed out previously that Gram-negative bacteria are the dominant killers among bacterial infections in the Intensive Care Units (ICU) (Villegas and Quinn, 2004). Klebsiella is one of the Gram-negative microorganisms that cause death in ICUs (Villegas and Quinn, 2004), so having antibacterial activity against K. pneumoniae may be very important.

Quereshi et al. (2010) identified that 40 µg mL⁻¹ of Ganodema lucidum caused 11.30 mm of inhibition zone against K. pneumoniae. In our study we observed 8 mm of inhibition zone with 917 µg mL⁻¹ of X. hypoxylon extract. By comparing these two studies it can be concluded that ethanolic extracts of X. hypoxylon has lower antimicrobial activity against K. pneumoniae when compared to G. lucidum, but on the other hand 8 mm of inhibition zone can be accepted as a moderate activity when compared to other studies.

The pathogenicity of B. subtilis is described as low or absent (De Boer and Diderichsen, 1991). B. subtilis is only known to cause disease in immunocompromised patients (Galieni and Bigazzi, 1998). Several researchers study antimicrobial activity of some plant extracts on B. subtilis strains. For example, Khalid et al. (2011) compared four different methanolic plant extracts, namely Zingiber officinale, Swertia chirata, Polygonum bistorta and Pistacia integerrim. In this study maximum 30 mg of extracts were loaded on sterile antibiotic disks and inhibition zones were found to be 17 mm for Z. officinale, 12 mm for P. integerrim, 12 mm for S. chirata and 11 mm for P. bistorta. In our study we observed 13 mm zone for 1.375 mg of X. hypoxylon extract which is about 22 times lower than the amount used for study conducted by Khalid et al. (2011). Comparing these results clearly puts forward how X. hypoxylon is active against B. subtilis when compared to some higher plants.

It was reported that although serovar Typhimurium has a less alarming public image than serovar Salmonella it is a bigger health problem and it is thought by researchers to be underreported (McClelland et al., 2001). There are probably hundreds of millions of cases every year in the world in which serovar Typhimurium kill twice as many people as serovar Typhi, which were mostly infants and the elderly patients (McClelland et al., 2001). According to results, 150 µL of X. hypoxylon extract showed low antibacterial activity against S. typhimurium SL 1344. Since the inhibition zone is quite low, increasing the active substance loaded on the empty sterile antibiotic disks may also increase the activity.

### Conclusion

As a result, it can be concluded that there is clear antimicrobial activity of X. hypoxylon sample against most of the strains tested. The results of our study clearly puts forward that X. hypoxylon could have a possible medicinal uses especially against B. subtilis ATCC 6633, B. subtilis DSMZ 1386, E. faecium, L. monocytogenes ATCC 7644 and S. aureus ATCC 25923. But further researches are needed to be conducted in order to analyse the active substances in details.

### Conflict of Interests: The authors declare that there is no conflict of interests regarding the publication of this paper.

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