In vitro Antimicrobial Activity Screening of *Bovista nigrescens* Pers.

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Abstract

The aim of this study is to determine the antimicrobial activity of *Bovista nigrescens* ethanolic extracts against 17 bacterial and 1 fungal strains. *B. nigrescens* samples which were collected from Yomra, Çamlıyurt, Trabzon, TURKEY were air dried and extracted with ethanol. Extracts were investigated for *in vitro* antimicrobial activity against a wide range of strains such as *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Enterobacter aerogenes* ATCC13048, *Enterococcus durans*, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium*, *Escherichia coli* ATCC 25922, *Escherichia coli* CFAI, *Klebsiella pneumoniae*, *Listeria monocytogenes* ATCC 7644, *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 disc diffusion method. It is observed that ethanolic extract of *B. nigrescens* has antimicrobial activity against several gram positive and gram negative microorganims tested. As a result of the study, it could be concluded that ethanolic extract of *B. nigrescens* is active against several microorganisms but its antimicrobial activity is notable especially against *B. subtilis*, *K. pneumoniae* and *S. carnosus*.

Keywords: *Bovista nigrescens*, antimicrobial activity, antimicrobial screening, ethanolic extract.

Bovista nigrescens Pers.'in *in vitro* Antimikrobiyal Etki Taraması

Özet

Bu çalışmanın amacı, *Bovista nigrescens*'e ait etanol ekstraktlarının 17 bakteri, 1 mantar suşuna karşı gösterdiği antimikrobiyal aktivitenin incelenmesidir. Yomra, Çamlıyurt, Trabzon, Türkiye'den toplanan *B. nigrescens* örnekleri kurutulduktan sonra etanol ile ekstraksiyona tabi tutulmuştur. Bu ekstraktların; *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Enterobacter aerogenes* ATCC13048, *Enterococcus durans*, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium*, *Escherichia coli* ATCC 25922, *Escherichia coli* CFAI, *Klebsiella pneumoniae*, *Listeria monocytogenes* ATCC 7644, *Salmonella enteritidis* ATCC 13075, *Salmonella infantis*, *Salmonella kentucky*, *Salmonella typhimurium* SL 1344, *Staphylococcus aureus* ATCC 25923, *Staphylococcus carnosus* MC1.B, *Staphylococcus epidermidis* DSMZ 20044 ve *Streptococcus agalactiae* DSMZ 6784 gibi geniş yelpazedeki suşlara karşı *in vitro* antimikrobiyal aktiviteleri disk difüzyon yöntemi kullanılarak incelenmiştir. *B. nigrescens*'in etanol ekstraktlarının bazı gram negatif ve gram pozitif mikroorganizmaya etki ettiği gözlenmiştir. Bu çalışmanın sonucu olarak *B. nigrescens*'in etanol ekstraktarının bazı mikroorganizmalar üzerine aktivite gösterdiği, ancak *B. subtilis*, *K. pneumoniae* ve *S. carnosus* üzerine gösterdiği etkinin dikkat çekici olduğu sonucuna varılmıştır.

Anahtar Kelimeler: *Bovista nigrescens*, antimikrobiyal aktivite, antimikrobiyal tarama, etanol ekstraktı.

Introduction

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 (WHO, 2007; Syed et al., 2010).

Although a tremendous progress has been made in human medicine in the last decades; bacterial, fungal and viral diseases are still threatening the public health in the developing countries (Cos et al., 2006). In these countries the major problem is not only the relative unavailability of medicines but also, the extensive drug resistance has also a large impact on human health (Okeke et al., 2005).

When public health security is taken into account, determining new alternatives against spreading of infection by antibiotic resistant microbes in future is very important for preventing the evolution of 'super' pathogens (Paudel et al., 2008). For this

reason, scientists have been conducting intensive researches in order to determine new antimicrobial agents.

It has been known for many years that natural products have potential of containing therapeutic agents especially for infectious diseases (Clardy and Walsh, 2004; Altuner et al., 2010). Conducted huge number of studies supports that natural products have been used for hundreds of years to treat several diseases caused by bacteria, fungi, viruses and parasites (Jones, 1996). 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).

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). According to the recent studies fungi are not only sources of nutrients but also could be used to prevent diseases such as hypertension, hypercholesterolemia and cancer (Bobek et al., 1995; Bobek and Galbavy, 1999).

Several compounds were isolated and identified by researchers originating from fungi until now, which show medicinal properties, such as immune modulatory, liver protective, antifibrotic, antiinflammatory, antidiabetic, antiviral and antimicrobial activities (Dülger et al., 1999; Gunde-Cimerman, 1999; Ooi, 2000; Wasser and Weis, 1999a; Wasser and Weis, 1999b).

Bovista nigrescens belongs to the family *Agaricaceae* Chevall. (Agaricales, Basidiomycota). They grow on soil among grasses, often in open areas, also in hardwood and mixed forests in montane to alpine elevations. They can be found between summer to autumn and widely distributed in Europe and Asia (Breitenbach and Kränzlin, 1986; Calonge, 1998).

In this study the antimicrobial activity of *B. nigrescens* is investigated against *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Enterobacter aerogenes* ATCC13048, *Enterococcus durans*, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium*, *Escherichia coli* ATCC 25922, *Escherichia coli* CFAI, *Klebsiella pneumoniae*, *Listeria monocytogenes* ATCC 7644, *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 disc diffusion method.

Materials and Methods

Macrofungi samples

Bovista nigrescens Pers samples used in this study were collected from Yomra, Çamlıyurt, 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 *B. nigrescens* 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 8 $mg.mL^{-1}$ extracts.

Microorganisms

A wide range of gram positive (*Bacillus subtilis* ATCC 6633, *Enterococcus durans*, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium*, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 25923, *Staphylococcus carnosus* MC1.B, *Staphylococcus epidermidis* DSMZ 20044 and *Streptococcus agalactiae* DSMZ 6784), gram negative bacteria (*Enterobacter aerogenes* ATCC13048, *Escherichia coli* ATCC 25922, *Escherichia coli* CFAI, *Klebsiella*

pneumoniae, *Salmonella enteritidis* ATCC 13075, *Salmonella infantis*, *Salmonella kentucky* and *Salmonella typhimurium* SL 1344) and yeast (*C. albicans* ATCC 10231) were selected to test the antimicrobial effect of *B. nigrescens*. 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. 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 10^8 cfu.mL^{-1} for bacteria and 10^7cfu.mL^{-1} for *C. albicans* (Hammer et al., 1999).

Disc diffusion method

Disc 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). 40 µL, 60 µL and 100 µL aliquots of each extract was applied on sterile paper discs of 6 mm diameter end up with 400 µg.µL^{-1}, 600 µg.µL^{-1} and 1000 µg.µL^{-1} sample on each disc (Mahasneh and El-Oqlah, 1999; Silici and Koc, 2006). To get rid of any residual solvent which might interfere with the results, discs were left to dry overnight at 30°C in sterile conditions (Silici and Koc, 2006; Altuner and Çetin, 2010). 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 discs. Discs 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.

Controls

Empty sterile discs and extraction solvent (ethanol) loaded on sterile discs 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

The main aim of this study was to identify the antimicrobial activity of ethanol extracts of *B. nigrescens* samples. To do this, disc diffusion test was performed in the study. In this test, extracts were loaded on empty sterile discs and these discs 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 discs.

Results given in Table 1 clearly show that 40 µL (400 µg.µL^{-1}) of *B. nigrescens* samples caused an inhibition zone of 18 mm against *B. subtilis* ATCC 6633, 11 mm against *S. carnosus* MC1.B, 9 mm against *K. pneumoniae* and 7 mm against *E. aerogenes* ATCC13048 and *E. coli* CFAI. 60 µL (600 µg.µL^{-1}) of *B. nigrescens* samples caused an inhibition zone of 19 mm against *B. subtilis* ATCC 6633, 12 mm against *S. carnosus* MC1.B, 11 mm against *K. pneumoniae*, 8 mm against *E. coli* CFAI and 7 mm against *E. aerogenes* ATCC13048, where 100 µL (1000 µg.µL^{-1}) of *B. nigrescens* samples caused an inhibition zone of 19 mm against *B. subtilis* ATCC 6633, 13 mm against *S. carnosus* MC1.B, 8 mm against *E. coli* CFAI, *K. pneumoniae* and *S. epidermidis* DSMZ 20044 and 7 mm against *E. aerogenes* ATCC13048 and *S. typhimurium* SL 1344.

Table 1. Disc diffusion test results (Inhibition zones in mm)

	40µL	60µL	100µL
B. subtilis ATCC 6633	18	19	19
C. albicans ATCC 10231	-	-	-
E. aerogenes ATCC13048	7	7	7
E. durans	-	-	-
E. faecalis ATCC 29212	-	-	-
E. faecium	-	-	-
E. coli ATCC 25922	-	-	-
E. coli CFAI	7	8	8
K. pneumoniae	9	11	8
L. monocytogenes ATCC 7644	-	-	-
S. enteritidis ATCC 13075	-	-	-
S. infantis	-	-	-
S. kentucky	-	-	-
S. typhimurium SL 1344	-	-	7
S. aureus ATCC 25923	-	-	-
S. carnosus MC1.B	11	12	13
S. epidermidis DSMZ 20044	-	-	8
S. agalactiae DSMZ 6784	-	-	-

"-": No activity observed.

On the other hand, no inhibition zone was observed against *C. albicans* ATCC 10231, *E. durans*, *E. faecalis* ATCC 29212, *E. faecium*, *E. coli* ATCC 25922, *L. monocytogenes* ATCC 7644, *S. enteritidis* ATCC 13075, *S. infantis*, *S. kentucky*, *S. aureus* ATCC 25923 and *S. agalactiae* DSMZ 6784.

Among these microorganisms *B. subtilis* ATCC 6633, *S. carnosus* MC1.B and *S. epidermidis* DSMZ 20044 are gram positive where *E. aerogenes* ATCC13048, *E. coli* CFAI, *K. pneumoniae* and *S. typhimurium* SL 1344 are gram negative strains.

It is a well known fact that gram-negative bacteria are in general more resistant to a large number of antibiotics and chemotherapeutic agents than gram-positive bacteria (Nikaido, 1998). It was previously reported that antibiotics of natural origin showed >90% lacked activity against *Escherichia coli*, although they were active against gram-positive bacteria (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 antimicrobial activity of either any Bryophyte samples or other plant samples against *E. coli* CFAI until now. Because of this, it is not possible to compare our results with previous studies. But there are several studies reported by using other *E. coli* strains. For example, Dulger et al. (2005) 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. (2005) were compared with our results, which were maximum 8 mm inhibition zone, it can be concluded that *B. nigrescens* presented very low antimicrobial activity. But since the *E. coli* strain used in this study is different than the study conducted by Dulger et al. (2005), this difference is not surprising.

In addition it was also pointed out previously that gram-negative bacteria are the dominant killers among bacterial pathogens in the Intensive Care Units (*ICU*) (Villegas and Quinn, 2004). *Klebsiella* is one of these gram negative microorganisms that cause death in *ICU*s (Villegas and Quinn, 2004).

From this point of view, having antibacterial activity against *K. pneumoniae* may very important.

Quereshi et al. (2010) identified that *Ganoderma lucidum* (40 µg.mL^{-1}) caused 11.30 mm of inhibition zone against *K. pneumoniae*. In our study we observed 11

mm of inhibition zone against 600 $\mu g.\mu L^{-1}$ of *B. nigrescens* extract. By comparing these two studies it can be concluded that ethanolic extracts of *B. nigrescens* has lower antimicrobial activity against *K. pneumoniae* when compared to *G. lucidum*, but on the other hand 11 mm of inhibition zone can be accepted as a moderate activity when compared to other studies.

E. aerogenes was previously identified as one of the "*ICU* bugs" which could cause significant morbidity and mortality. In addition the infection management is complicated due to its resistance to multiple antibiotics (Fraser et al., 2007).

Dulger et al. (2005) tested *Phellinus torulosus*, a macrofungus, against wide range of microorganisms. Dulger et al. (2005) presented an inhibition zone of 10.4 mm for acetone extract and 10.8 mm for chloroform extract. When these results were compared with our results it can be concluded that *B. nigrescens* extracts showed very low activity against *E. aerogenes*. Probably increasing amount of extracts loaded on the empty sterile antibiotic discs may increase the activity.

It was reported that although serovar Typhimurium of *Salmonella* has a less alarming public image than serovar Typhi it is a bigger health problem and it is thought by researchers to be at least 30-fold 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 people (McClelland et al., 2001). According to results, 100 µL of *B. nigrescens* extract showed very 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 discs may also increase the activity.

Conclusion

As a result, it can be concluded that there is clear antimicrobial activity of samples against *B. subtilis* ATCC 6633, *E. aerogenes* ATCC13048, *E. coli* CFAI, *K. pneumoniae*, *S. typhimurium* SL 1344, *S. carnosus* MC1.B and *S. epidermidis* DSMZ 20044. Although Burk (1983) stated that *B. nigrescens* don't have any medicinal usage in terms of ethnopharmacology, but used as insulation material (Burk, 1983), the results of our study clearly puts forward that *B. nigrescens* could have a possible medicinal uses against *B. subtilis* ATCC 6633, *K. pneumoniae* and *S. carnosus* MC1.B.

But further researches are needed to be conducted in order to analyse the active substances in details.

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