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In vitro antimicrobial screening of *Lycoperdon lividum* and determination of the ethanol extract composition by gas chromatography/mass spectrometry

Kerem Canli[1], Ergin Murat Altuner[2], Ilgaz Akata[3], Yavuz Turkmen[4] and Ugur Uzek[4]

[1]Department of Biology, Dokuz Eylul University, Turkey; [2]Department of Biology, Faculty of Science and Arts, Kastamonu University, Kuzeykent, TR 37100, Kastamonu, Turkey; [3]Department of Biology, Faculty of Science, Ankara University, Tandogan, TR 06100, Ankara, Turkey; [4]Ankara Police Forensic Laboratories, Gölbasi Campus of the General Directorate of Security, TR 06830, Ankara, Turkey.

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Abstract

The aim of this study was to investigate the antimicrobial activity of *Lycoperdon lividum* against 17 bacterial and 1 fungal strains and analyse the composition of ethanol extracts by GC/MS. The *in vitro* antimicrobial activity of *L. lividum* extracts having 15 mg/mL concentration was assessed against a wide range of strains by disk diffusion method. The ethanol extract of *L. lividum* had antimicrobial activity against several microorganism tested, but it was active especially against *S. carnosus*. The results obtained herein indicate that *L. lividum* contains several active metabolites.

Introduction

Although there is tremendous progress in human medicine; bacterial, viral and fungal issues are still threaten the public health especially in the developing countries (Cos et al., 2006). Relative unavailability of medicines in these countries and in addition to this the extensive antibiotic resistance has a large impact on human health (Okeke et al., 2005). Therefore, further researches about investigation of new antimicrobial substances should be conducted (Altuner et al, 2010).

Mushrooms have a potential of using both as nutritive and medicinal food stuff (Imtiaj and Lee, 2007; Bonatti et al., 2004; Cheung and Cheung, 2005; Agrahar-Murugkar and Subbulakshmi, 2005; Altuner et al., 2012). Mushrooms are not only sources of nutrients but also could be used to prevent diseases such as hypertension, hypercholesterolemia and cancer (Bobek and Galbavy, 1999).

Lycoperdon lividum Pers. grows on soil and dunes, solitary to gregarious, in forests, outside of forests, in meadows and cow pastures (Breitenbach and Kränzlin, 1986).

Researchers isolated and identified some compounds, originating from mushrooms; show other medicinal properties, such as immunemodulatory, liver protective, antifibrotic, antiinflammatory, antidiabetic, antiviral and antimicrobial activities (Wasser and Weis, 1999a-1999b; Gunde-Cimerman, 1999; Dülger et al., 1999; Ooi, 2000).

The aim of this study was to investigate the antimicrobial activity of *L. lividum* against 17 bacterial and 1 fungal strains and analyse the composition of ethanol extracts by GC/MS.

Materials and Methods

Fungi samples

L. lividum samples used in this study were collected from Trabzon, Yomra, Çamlıyurt village, in spruce forest, 40°44'N, 39°50'E, 1000 m, 22.09.2012, Akata 4702. Voucher specimens were deposited for further reference in Herbarium of Ankara University (ANK) Faculty of Science, Department of Biology, Ankara, Turkey.

Extraction procedure

L. lividum samples were air dried and samples were ground by a mortar and a pestle. In order to extract active substances, ground samples were shaken in ethanol (Merck, Germany) at 100 rpm for 3 days at room temperature. Then 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 and lyophilized. After lyophilization the residues were collected and used to prepare 15 $mg.mL^{-1}$ extracts.

Microorganisms

A wide range of bacteria (Gram positive and Gram negative) and yeast were selected to test the anti-microbial activity of *L. lividium*. The strains were chosen from standard strains as much as possible. Other strains which are not standard were all isolated from food and identified at the Department of Biology, Faculty of Science, Ankara University.

Bacillus subtilis ATCC 6633, *Candida albicans* ATCC 10231, *Enterobacter aerogenes* ATCC13048, *Enterococcus durans*, *E. faecalis* ATCC 29212, *E. faecium*, *Escherichia coli* ATCC 25922, *E. coli* CFAI, *Klebsiella pneumoniae*, *Listeria monocytogenes* ATCC 7644, *Salmonella enteritidis* ATCC 13075, *S. infantis*, *S. kentucky*, *S. typhimurium* SL 1344, *Staphylococcus aureus* ATCC 25923, *S. carnosus* MC1.B, *S. epidermidis* DSMZ 20044 and *Streptococcus agalactiae* DSMZ 6784 were used in the study.

Prepartion 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 incubated 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 for bacteria and 10^7 cfu/L for *C. albicans* (Canlı et al., 2015).

Disk diffusion method

Disk diffusion test was performed as described previously by Andrews (2003). The culture medium was poured into 120 mm sterile petri dish to give a mean depth of 4.0 ± 0.5 mm. 40, 60 and 100 µL aliquots of each extract was applied on sterile disks of 6 mm diameter end up with 550, 917 and 1375 µg sample on each disk. 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 (Altuner et al., 2010). The surface of the plates was inoculated using previously prepared inocula containing saline suspension of microorganisms. Inoculated plates were then left to dry at room temperature before applying the disks. Disks were firmly applied to the surface of the plate which had an even contact with the agar (Savaroglu et al., 2011). Plates were incubated and inhibition zone diameters were expressed in millimetres.

Gas chromatography-mass spectrophotometry method (GC-MS)

The GC-MS analysis of etanol extract of LC were quantitatively performed by GC-MS (Agilent 19091S-433, 30 x 0.25 x 0.25 HPS MS) based on the method defined previously by Hossain and Rahman (2011) with some modifications. Split less injection was performed with a purge time of 1.0 min. The carrier gas was helium at a flow rate of 1 mL/min. The column temperature was maintained at 60°C for 2 min, then programmed at 10°C/min to 300°C and waited for 10 min. The inlet temperature was 250°C, AUX was 290°C and the solvent delay was 4 min. The identification of the peaks was based on computer matching of the mass spectra with the National Institute of Standards and Technology (NIST Mass Spectrometry DATA CENTER, 2005) library and by direct comparison with published data (Canlı et al., 2014).

Controls

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

The statistical analysis was performed using a non-parametric method Kruskal-Wallis one-way analysis of variance. A value of $p<0.05$ was considered statistically significant.

Results

The diameter of the inhibition zones identified as the diameter of the zones in millimetres for the samples are given in Table I. No activity was observed for the negative controls; solvents and empty sterile disks.

Table I clearly shows that ethanol extracts of *L. lividum*

were presented antimicrobial activity against *E. aerogenes*, *E. coli* ATCC 25922, *K. pneumoniae*, *S. kentucky*, *S. carnosus, S. epidermidis* and *S. agalactiae*.

The GC-MS analysis of ethanol extract of *L. lividum* was identified and some of the chemical components found in the ethanol extract of *L. lividum* was isovaleric acid, pantoic lactone, pyrrolidinone, coumaran (2,3 dihydro benzofuran), 3-pyridine,1,2-proponediol, dihydroxy-toluene, niacinamide, 3-piperidino-1,2 propanediol, isobutyl p-hydroxybenzoate, dodecanoic asit, metacetamol (paracetamol), ethyl quinoline, tetradecanoic acid, pentadecanoic acid, ethyl-13-methyl-tetradecanoate, hexadecanoic acid, 9-hexadecenoic acid, ethyl hexadecanoate, propanoic acid 3-mercapto-dodecyl ester, heptadecanoic acid, linoleic acid, ethyl linoleate, oleic asit, stearic acid, 2-monopalmitin, monolinolein, 2-mono stearin, dihydroergosterol and ergesterol.

Table I

Disk diffusion test results (Inhibition zones in mm)

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

"-": No activity observed

Discussion

Results clearly show that *L. lividium*is active against several microorganisms but its antimicrobial activity is notable especially against *S. carnosus.* Among the microorganisms affected by *L. lividum* extracts are *E. coli*, *S. carnosus*, *S. Epidermidis, S. Agalactiae, E. aerogenes*, *S. kentucky* and *K. Pneumonia.*

It is well-known that Gram negative bacteria are in general more resistant to a large number of antibiotics and chemotherapeutic agents than Gram positive bacteria (Nikaido, 1998).

We have observed that *L. lividum* had a low antimicrobial activity against *E. aerogenes*, *E. coli*, *K. pneumoniae*, *S. kentucky*, *S. epidermidis* and *S. agalactiae*, when it is compared to *S. carnosus* for the concentrations used in the study.

On the other hand, the chemical composition of *L. livdium* was analysed and several compounds, namely isovaleric acid, pantoic lactone, pyrrolidinone, coumaran (2,3 dihydro benzofuran), 3-pyridine,1,2-proponediol, dihydroxytoluene, niacinamide, 3-piperidino-1,2 propanediol, isobutyl p-hydroxybenzoate, dode-canoic asit, metacetamol (paracetamol), ethyl quinoline, tetradecanoic acid, pentadecanoic acid, ethyl-13-methyl -tetradecanoate, hexadecanoic acid, 9-hexadecenoic acid, ethyl hexadecanoate, propanoic acid 3-mercapto-dodecyl ester, heptadecanoic acid, linoleic acid, ethyl linoleate, oleic asit, stearic acid, 2-monopalmitin, monolinolein, 2-mono stearin, dihydroergosterol and ergesterol, in which some of them have been proved to have antimicrobial activity before.

Unfortunately there have been no reports neither about the antimicrobial activity of *L. lividium*, nor the chemical composition of its ethanol extract in order to compare with our results, as far as the current literature is concerned. Results presented in this study are the very first data both about the antimicrobial activity and the chemical composition of the ethanol extract of *L. lividium*.

E. aerogenes was identified as one of the "*ICU* bugs" which could cause significant mortality and morbidity. In addition the infection management is complicated due to its resistance to multiple antibiotics (Hidron et al., 2008). Although a very low activity was observed against *E. aerogenes*, the results can be accepted as noteworthy. Probably increasing amount of extracts loaded on the empty sterile antibiotic disks may increase the activity.

There are several studies reported by using different*E. coli* strains. For example, Dulger et al. (2005) showed that the methanolic extract of *Hypnum cupressiforme* (30 mg/mL) presented 12.2 mm of inhibition zone against *E. coli*. When the results reported by Dulger et al. (2005) were compared with our results, which were maximum 7 mm inhibition zone, it can be concluded that *L. lividum* 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 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 ICUs (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 7 mm of inhibition zone against *L. lividum* extract. By comparing these two studies it can be concluded that ethanolic extracts of *L. lividum* has lower antimicrobial activity against *K. pneumoniae* when compared to *G. lucidum*.

It was previously uncommon but in the last decade an increase was observed in *S. kentucky* cases especially in Northeast Africa and Turkey. This strain displays high-level resistance to ciprofloxacin, one of the drugs used against *Salmonella* infections. In addition, secondarily acquired resistances to extended-spectrum cephalosporin and trimethoprim + sulfamethoxazole was also observed (Collard et al., 2007)

S. epidermidis is not usually pathogenic. But they often develop risk for infection for patients with a compromised immune system. These infections can be both nosocomial and community acquired, but they pose a greater threat to hospital patients. *S. epidermidis* is also a major concern for people with catheters because it is known to cause biofilms that grow on these devices (Queck and Otto, 2008; Salyers et al., 2002).

Several researchs conducted on the antibacterial activity of several higher plants against *S. epidermidis*. Mahida and Mohan (2007) tested 10 mg of methanolic extracts of 23 plant extracts, but the highest zone was found to be 20 mm for *Mangifera indica*. In another study *ethanol* extracts of 23 plants were tested against *S. epidermidis* and the highest inhibition zone diameter was given as 18 mm for *Stachys leptoclada* (Sarac and Ugur, 2007).

The antimicrobial nature of *L. lividum* extract can be explained by looking at its GC/MS analysis. The GC/MS analysis clearly puts forward the composition of *L. lividum* extract, which contains several compounds that have antimicrobial activities.

For example, tetradecanoic acid, which is also called myristic acid, is a saturated fatty acid having molecular formula $CH_3(CH_2)_{12}COOH$. It is previously presented that myristic acid is an antibacterial and antifungal agent (McGaw, 2002; Seidel and Taylor, 2004; Agoramoorthy et al., 2007). Skrivanova (2006) showed that myristic acid has antimicrobial activity against *Clostridium perfringens*. Batovska et al. (2009) found that myristic acid is active against *L. monocytogenes* with having 125 µg/mL MIC value. Narasimhan et al. (2006) tested the antimicrobial activiy of myristic acid derivatives and presented that myristic acid derivatives are more effective against Gram positive bacteria rather than Gram negative bacteria. It is also concluded that *S. aureus* is the most sensitive organism to myristic acid derivatives (Narasimhan et al., 2006). It is also reported that myristic acid has antifungal activity against *Alternaria solani* (Liu et al., 2008), *Aspergillus niger* (Altieri et al., 2007; Carballeira et al., 2005), *C. albicans* (Kabara et al., 1972), *Emericella nidulans* (Altieri et al., 2007), *Fusarium oxysporum* (Liu et al., 2008), *Penicillium glabrum* (Altieri et al., 2007) and *P. italicum* (Altieri et al., 2007).

Hexadecanoic acid, which is also known as palmitic acid, is the most common fatty acid found in animals, plants and microorganisms (Gunstone et al., 2007). It is mainly used to produce soaps and cosmetics. Hexadecanoic acid is also proposed as an antimicrobial agent in several researches (Glover et al., 1997; Risk et al, 1997; Bazes et al., 2009). Hexadecanoic acid also presented a significant antibacterial activity against *S. mutans* (Huang et al., 2011). In addition, it has antifungal activity against *Alernaria solani* (Liu et al., 2008), *A. niger* (Altieri et al., 2007), *A. terreus* (Altieri et al., 2007), *Cucumerinum lagenarium* (Liu et al., 2008), *Emericella nidulans* (Liu et al., 2008) and *F. oxysporum* (Liu et al., 2008).

Stearic acid is a saturated fatty acid having molecular formula $C_{18}H_{36}O_2$. Stearic acid is mainly used in the production of detergents and cosmetics, but an antimicrobial activity potential against diverse pathogenic microorganism is also proposed in several studies (Zheng et al., 2005; Nalina and Rahim; 2007; Chakraborty and Shah, 2011).

Antimicrobial activity of linoleic acid was also determined in several researches. Tsuchida and Morishida (1995) investigated its antimicrobial activity against some Gram positive and Gram negative strains. They found that linoleic acid has high antimicrobial activity mostly against Gram positive strains. It also presented antimicrobial activity against *B. larvae* (Feldlaufer et al., 1993). It is also identified that linoleic acid is active against *A. solani* (Liu et al., 2008), *C. albicans* (Kabara et al., 1972), *Crinipellis pernicosa* (Walters et al., 2004), *F. oxysporum* (Liu et al., 2008), *Pyrenophora avanae* (Walters et al., 2004), *Pythium ultimum* and *Rhizoctonia solani* (Walters et al., 2004).

It is also previously reported that linoleic acid has antibacterial activity (Knapp and Melly, 1986; Kabara et al., 1972; Sun et al., 2003; Zheng et al., 2005) and antifungal activity against *C. pernicosa*, *P. avanae*, *P. ultimum* and *R. solani* (Walters et al., 2004).

On the other hand, there are several studies that show pantoyl lactone has antimicrobial activity, especially against *S. equi* (Bashir, 2013) and some pyrrolidinone derivatives present antimicrobial properties (Daferner et al., 2002).

Conclusion

We observed antimicrobial activity against *E. aerogenes*, *E. coli*, *K. pneumoniae*, *S. kentucky*, *S. carnosus*, *S. epidermidis* and *S. agalactiae*. The ethanol extract of *L. lividum* contains several active metabolites and it is active against several microorganisms. Its antimicrobial activity is notable especially against *S. carnosus*.

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Conflict of Interest

Authors declare no conflict of interest

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Author Info
Kerem Canli (Principal contact)
e-mail: biyoloji@gmail.com