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Fomes fomentarius (L.) Fr. extracts as sources of an antioxidant, antimicrobial and antibiofilm agents

Original Article

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Abstract:

This paper evaluated antioxidant, antimicrobial and antibiofilm activities of ethanol, methanol, acetone and chloroform extracts of lignicolous fungal species *Fomes fomentarius* (L.) Fr. (Polypodiaceae). Antiradical activity was evaluated by using DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The antimicrobial screening was carried out via disc diffusion and microdilution methods in order to estimate minimum inhibitory (MIC) and minimum bactericidal concentration (MBC) of analyzed extracts against seven standard bacteria and one yeast: *Escherichia coli* NRRL B-3704, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 13315, *Acinetobacter baumanii* ATCC 19606, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *S. haemolyticus* ATCC 43252 and *Candida albicans* ATCC 10231. *In vitro* antibiofilm activities were investigated based on crystal violet binding assay. All the extracts showed higher antioxidant activity compared to BHT (butylated hydroxytoluene) which was used as a standard. Ethanol, methanol and acetone extracts of the tested macrofungus also showed higher antimicrobial effect against *P. aeruginosa* ATCC 27853 in comparison to the antibiotic penicillin (P10). The lowest MIC was recorded by ethanol extract against *S. haemolyticus* ATCC 43252 (0.625 µg/mL). The highest antibiofilm activity was also noticed against biofilm formed by *S. haemolyticus* ATCC 43252. The results indicated that the *F. fomentarius* tested represent potential source of natural bioactive compounds with respect to antimicrobial, antibiofilm and antioxidant activities.

Key words:

antimicrobial, antibiofilm, antioxidant activity, *Fomes fomentarius*

Apstract:

Fomes fomentarius (L.) Fr. ekstrakti kao izvori antioksidanasa, antimikrobnih i antibiotskih sredstava

Ovaj rad se bavi procenom antioksidativne, antimikrobne i antibiofilm aktivnosti etanolnog, metanolnog, acetonskog i hloroformnog ekstrakta lignikolne gljive Fomes fomentarius (L.) Fr. (Polyporaceae). Antiradikalna aktivnost je utvrđena korišćenjem DPPH (2,2-difenil-1-pikrilhidrazil) eseja. Antimikrobni skrining je izvršen putem disk difuzione i mikrodilucione metode kako bi se utvrdile minimalna inhibitorna (MIK) i minimalna baktericidna koncentracija (MBK) analiziranih ekstrakata u odnosu na sedam standardnih bakterija i jednu gljivu: Escherichia coli NRRL B-3704, Pseudomonas aeruginosa ATCC 27853, Proteus vulgarisATCC 13315, Acinetobacter baumaniiATCC 19606, Bacillus subtilis ATCC 6633, Staphylococcus aureus ATCC 25923, S. haemolyticus ATCC 43252 and Candida albicans ATCC 10231. In vitro antibiofilm aktivnost je ispitana korišćenjem kristal violet eseja. Svi ekstraktu pokazali su veću antioksidativnu aktivnost u poređenju sa BHT (butilovanim hidroksitoluenom) koji je korišćen kao standard. Takođe, etanolni, metanolni i acetonski ekstrakti testirane makrogljive su u poređenju sa antibiotikom penicilinom (P10) pokazali veći antimikrobni efekat na P. aeruginosa ATCC 27853. Najniža MIK vrednost je zabeležena zza etano0lni ekstrakt i to protiv S. haemolyticus ATCC 43252 (0.625 µg/mL). Najviša antibiofilm aktivnost uočena je kod biofilma formiranog od strane S. haemolyticus ATCC 43252. Rezultati su ukazalu da testirana vrsta F. fomentarius predstavlja potencijalni izvor prirodnih bioaktivnih jedinjenja u smislu antimikrobne, antibiofilm i antioksidativne aktivnosti.

Ključne reči:

antimikrobna, antibiofilm, antioksidativna aktivnost, *Fomes fomentarius*

Introduction

In recent years, the problem of antibiotic resistance, where bacterial and fungal pathogens developed numerous defense mechanisms against antimicrobial agents is increased, and scientific studies presented new and more powerful agents as an alternative to antibiotic therapy (Heleno et al., 2013). The interest in natural compounds has been a common point for most biotechnology companies to be used in the production of new antimicrobial drugs (Butler, 2004).

Microbial biofilms are communities of bacteria, embedded in a self-producing matrix, forming on living and nonliving solid surfaces (Vasudevan, 2014). They are considered as an important virulence factor that causes persistent chronic and recurrent infections; they are highly resistant to antibiotics and host immune defenses. Biofilm resistance is due to several reasons, like restricted diffusion of antibiotics into biofilm matrix, expression of multidrug efflux pumps, type IV secretion systems, decreased permeability, and the action of antibiotic-modifying enzymes (Alekshun and Levy, 2007). The increased biofilm resistance to conventional treatments enhances the need to develop new control strategies (Simões et al., 2007; Sánchez et al., 2016).

It is common to believe that it is difficult to prevent and treat biofilm infections. Current findings regarding the fact that biofilms have a highly heterogeneous structure necessitates the development of new treatment strategies unlike the treatment strategies used in the destruction of microorganisms in the planktonic phase (Clatworthy et al., 2007). Besides its antimicrobial effects, some natural product extracts are known to prevent biofilm formation. Due to the inadequate antimicrobial therapies in combating biofilms, researchers are directed towards the discovery and identification of new antimicrobial agents, especially natural (Karaca et al., 2017).

Antioxidants have the ability to protect the body against damage caused by oxidative stress. The interest in natural antioxidants is increasing day by day. For example, polyphenols, found in medicinal plants and foods, can help prevent oxidative damage (Silva et al., 2005). Mushrooms are rich sources of antioxidant compounds such as phenolic compounds (phenolic acids and flavonoids) and tocopherols (Cheung et al., 2003; Ferreira et al., 2009; Heleno et al., 2010; Sun et al., 2011; Dündar et al., 2016).

Fomes fomentarius (L.) Fr., Polyporaceae, tinder fungus is a woody, perennial fungus, large in size which develops as a parasite or saprophyte on the beech (*Fagus sylvatica* L.) and other deciduous species. It is a white root fungus causing heart rot of the wood. In recent years, the active compounds of *F. fomentarius* have been studied extensively and most of its biological activities have been put forward. It is found that *F. fomentarius* have significant effects such as antioxidant (Lee, 2005; Vazirian et al., 2014; Dündar et al., 2016; Bal et al., 2017), antimicrobial (Kolundžić et al., 2016), antifungal (Dresch et al., 2015), anti-inflammatory (Vazirian et al., 2014), cytotoxic (Kolundžić et al., 2016), antitumor (Chen et al., 2008), antiviral (Aoki et al., 1993), DNA protective (Bal et al., 2017), fibrinolytic activity (Sánchez-Santillán et al., 2015).

This extended study was to evaluate the antimicrobial, antibiofilm and antioxidant activities of ethanol, methanol, acetone and chlorofom extracts of *F. fomentarius*. Although there have been extensive researches to reveal antioxidant and antimicrobial activity of *F. fomentarius*, studies on antibiofilm activity have not been found in the literature. We also aimed to determine antibiofilm potential of the four extracts obtained from *F. fomentarius* against important pathogen microorganisms for the first time.

Materials and Methods

The macrofungal material

Fomes fomentarius was collected from Çanakkale, Turkey on decaying stump in January, 2018 and identified by Dr. Ersin KARABACAK.

Preparation of extracts

The sample of the dried macrofungus (15 g) was extracted with four solvents- ethanol, methanol, acetone and chloroform (150 ml) using the Soxhlet apparatus (ISOLAB). The extraction was continued until the final extract was colorless according to Khan et al. (1988). The solvents were removed under reduced pressure and dried using a rotary evaporator (Heidolp, Laborota 4001, Germany) at 55 °C. Dry extract was taken into glass bottles with dark lid and stored at +4 °C. For the experiments, dissolution in dimethyl sulfoxide (DMSO) (10%) and different concentrations of the prepared extract were sterilized by a membrane filter (0.2 µm) and prepared for screening.

Evaluation of antiradical activity
DPPH assay

The antioxidant activity of the extracts was measured by using commercial free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) following the procedure described by Brand-Williams et al. (1995), with slight modifications. The extracts were evaporated to dryness and re-suspended in DMSO. Butylated hydroxytoluene (BHT) solution was used as a positive control. The absorbance

values of the samples were measured on a UV-VIS spectrophotometer (PG Instruments T+80) at 517 nm against a blank. The experiment was repeated three times and the arithmetic mean of the readings was taken. The radical scavenging activity of each sample was calculated using the following equation and the results were expressed as % inhibition.

Inhibition (%) = $[(A_0 - A_1) / A_0] \times 100$

A_0: Extract or non-standard control absorbance,

A_1: Extract or standard absorbance

Evaluation of antimicrobial activity

Sterilized antibiotic discs 6 mm in diameter (Schleicher and Schull No. 2668, Dassel, Germany) were impregnated with 50 µL of each extract (10 mg/disc) at concentration of 200 mg/mL.

Gram negative bacteria - *Escherichia coli* NRRL B-3704, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 13315, *Acinetobacter baumanii* ATCC 19606, Gram positive bacteria - *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *S. haemolyticus* ATCC 43252 and yeast culture - *Candida albicans* ATCC 10231 were used as the test microorganisms.

All the bacteria were incubated at 35 ± 0.1 °C for 24 h by inoculation into Nutrient Broth (Difco Laboratories, MI, USA) and the yeast culture studied was incubated in Malt Extract Broth (Difco Laboratories, MI, USA) at 25 ± 0.1 °C for 48 h. An inoculum containing 10^6 bacterial cells or 10^8 yeast cells/mL was spread on Mueller Hinton Agar (MHA) (Oxoid Ltd., Hampshire, UK) plates (1 ml inoculum/plate). The discs injected with extracts were placed on the inoculated agar by pressing slightly. Petri dishes were placed at ±4 °C for 2 h and incubated for 24 h and 48 h for bacteria and yeast, respectively (Collins et al., 1989). At the end of the period, inhibition zones formed on the medium were evaluated in millimeters. Studies were performed in triplicate. Treatments with penicillin (P10), and nystatin (NYS30) served as positive controls and treatments with ethanol, methanol, acetone and chloroform without fungal materials served as negative controls.

For quantitative antimicrobial analyses, minimum inhibitory concentration (MIC) values of all samples were determined. MIC and MBC were investigated as recommended instruction of the Clinical and Laboratory Standards Institute (CLSI, 2006). Briefly, stock solution of each extract was diluted in the Muller Hinton Broth (MHB) in two-fold serial dilutions to obtain final concentrations range of 20-0.156 mg/ml at a total volume of 100 µl per well in 96-well microtiter plates. The lowest concentration of extracts inhibiting visible growth of each test microorganisms was taken as the MIC. The medium, 0.1% (w/v) streptomycin (ST), nystatin (NYS30) and 10% DMSO were used as the non-treated, positive and negative controls, respectively (Teanpasian et al., 2017).

Confirmation of MIC and establishment of the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC), 10 µL of the following dilutions (40 to 2.5 µg/ml) were inoculated into plates with MHA to evaluate microbial growth. After 24 h of incubations at 35 ± 0.1 °C for bacteria and 48 h at 25 ± 0.1 °C for the yeast culture, the plates with no apparent CFU of surviving microorganisms were determined. Each experiment was repeated three times (Pacheco-Ordaz et al., 2018).

Biofilm inhibition assay

Microplate biofilm method (Merrit et al., 2005) was used to evaluate the inhibition of biofilm formation by *F. fomentarius* extracts against tested microorganisms. Cultures were incubated in 5 ml Tryptic Soy Broth (TSB) medium containing 5% glucose. Cultures were diluted 1:100 in TSB and loaded into each well in 4 sterile microplate. Different concentrations of fungal extracts (MIC and sub-MIC concentrations: 50, 25, 12.5% of MIC) were prepared and transferred to each microplate well. After incubation at 37 ± 0.1 °C for 48 h planktonic bacteria were removed from the wells and wells were washed twice with distilled water. Crystal violet solution (0.1%, 200 µl) was added to each well and incubated for 20 minutes. The crystal violet bounded extracts were poured and washed until the crystal violet was finally removed. The microtiter plates were inverted and the remaining liquid was drained and dried in room heat. Finally, the adhered biofilm bounded crystal violet was eluted in ethanol (95%) and the absorbance was measured at 550 nm by using an automated Elisa reader (BioTek, UK). All experiments were repeated in triplicate. The calculation of the antibiofilm effect of the extract was made by the reduction formulation percentage.

$$\textbf{\% Inhibition} = (A_{control} - A_{sample} / A_{control}) \times 100$$

$A_{control}$: Absorbance of the control (containing 100 µL TBS instead of fungal extract) reaction,

A_{sample}: Absorbance of the tested compound

Results and discussion

Two polar (ethanol and methanol), one intermediate polar (acetone) and one nonpolar (chloroform) solvent were selected for comparison of biological activity of *F. fomentarius*. The choice of solvents depends on the type of macrofungi, part of the materials to be extracted, nature of the bioactive

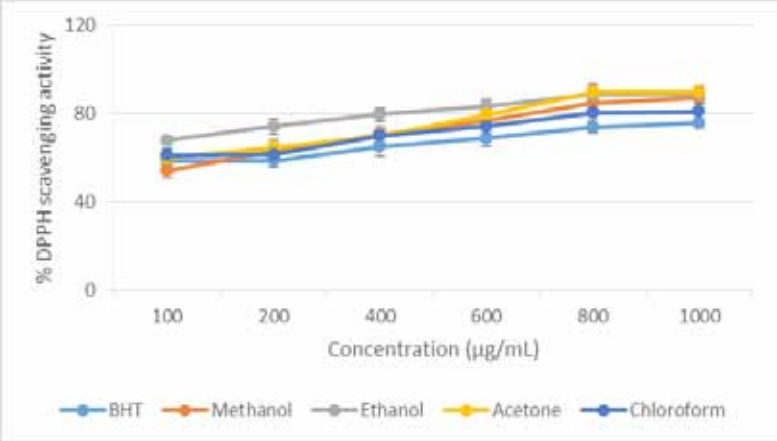

Fig. 1. DPPH free scavenging activity of *F. fomentarius* extracts

Studies have shown that the biological activities of the extracts can vary depending on the season, type of extract, dose and duration of application. Depending on the extraction method, the level of bioactive substances can be observed (Anlas et al., 2017). Mircea et al. (2015) and Kolundžić et al. (2016) reported that DPPH radical scavenging activity of *F. fomentarius* methanol extract results correlated with the polyphenol content. In addition, *F. fomentarius* methanol and ethanol extracts have been reported to have the strongest DPPH activity comparing to other solvents (Bojin et al., 2020). The present study indicated that ethanol extract showed slightly higher antioxidant activities than the other solvents.

compounds, the availability of solvent and the literature. Polar solvents such as ethanol, methanol and acetone were used in extraction of polar compounds, whereas nonpolar solvents such as chloroform were used in extraction of nonpolar compounds such as terpenoids, flavonoids, fats and oils (Abubakar and Hague, 2020). The findings showed that polar solvents, especially ethanol, are more successful in extraction of secondary metabolites with antioxidant, antimicrobial and antibiofilm activity.

DPPH assay is widely used in determination of the antioxidant activity of compounds and different fungal extracts. In this work, it was found that the inhibition percentage of ethanol extracts from *F. fomentarius* was slightly higher (89.95±0.21) than when acetone, methanol and chloroform solvents were applied (88.88±0.18, 87.34±0.11 and 80.93±0.07, respectively) (**Fig. 1**). BHT which was used as a standard showed lower antioxidant activity (75.81±0.03) compared with all other extracts, obtained by different solvents.

The antimicrobial activities of the macrofungal extracts against different test strains were assessed according to inhibition zones diameter and MIC and MBC or MFC values (**Tab. 1**). All extracts showed higher antimicrobial effect against *P. aeruginosa*, *P. vulgaris*, *S. haemolyticus*, *C. albicans* except chloroform extract with inhibition zone of 10-13 mm. Ethanol, methanol and acetone extracts of macrofungi also showed higher antimicrobial effect against *P. aeruginosa* in comparison to antibiotic P10. The lowest MIC value was recorded by *F. fomentarius* ethanol extract against *S. haemolyticus* (0.625 µg/ml). Also, ethanol extract showed higher MIC against *E.coli*, *B. subtilis*, *S. aureus* and *S. haemolyticus* bacteria when compared to antibiotic streptomycin probably due to the polar component that the ethanol extract contain.

There are many scientific reports about *F. fomentarius* antimicrobial activity (Zhao et al., 2013; Dündar et al., 2016; Kolundžić et al., 2016; Gedik et

Table 1. Antimicrobial activity of *F. fomentarius* extracts

Test microorganisms	*Disc Diffusion[a]				Control antibiotics		MIC (µg/mL)					Control antibiotics	MBC (MFC)				MBC (MFC)/ MIC			
	E1	E2	E3	E4	P10	NY 100	E1	E2	E3	E4	ST	NY 100	E1	E2	E3	E4	E1	E2	E3	E4
E.coli	7.0	7.0	10.0	8.0	16.0	Nt	2.5	2.5	10	20	4.0	Nt	40	2.5	40	40	16	1	4	2
P. aeruginosa	10.0	11.0	10.0	7.0	8.0	Nt	10	10	10	20	1.0	Nt	40	40	40	40	4	4	4	2
P. vulgaris	13.0	10.0	10.0	7.0	13.0	Nt	20	5	10	20	4.0	Nt	40	40	40	40	2	8	4	2
A. baumanii	7.0	11.3	8.6	9.0	12.0	Nt	5	5	10	20	2.0	Nt	40	5	40	40	4	1	4	2
B. subtilis	13.0	9.0	8.3	8.0	14.0	Nt	2.5	2.5	10	20	4.0	Nt	40	2.5	40	40	16	1	4	2
S. aureus	13.0	10.6	8.3	9.0	15.0	Nt	2.5	5	10	20	4.0	Nt	40	5	40	40	16	1	4	2
S. haemolyticus	11.6	11.6	10.3	8.0	14.0	Nt	0.6	5	10	20	5.0	Nt	40	10	40	40	64	2	4	2
C. albicans	10.0	11.0	10.0	7.0	Nt	16.0	5	5	5	20	NT	5.0	40	40	40	40	4	8	8	2

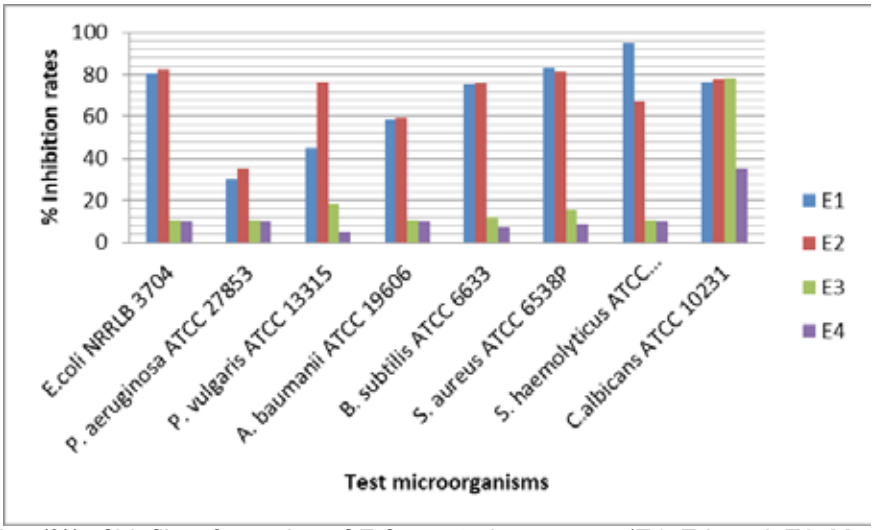

Fig. 2. Inhibition (%) of biofilms formation of *F. fomentarius* extracts (E1: Ethanol, E2: Methanol, E3: Acetone, E4: Chloroform)

al., 2019). Similar to the present study, Kolundžić et al. (2016) tested the antimicrobial activity of *F. fomentarius* extracts of different polarity, especially against Gram-negative and Gram-positive bacteria. They indicated that their *F. fomentarius* extracts (cyclohexane, dichloromethane, methanol and aqueous) displayed strong antimicrobial activity. Gedik et al. (2019) also showed that ethanol and aqueous extracts of *F. fomentarius* have higher antibacterial activity against *K. pneumoniae*, *A. baumanii*, *S. aureus*, vancomycin-resistant enterococci (VRE+), *E. coli* against standard antibiotics. In our study, a similar situation was obtained against *P. vulgaris*, *B. subtilis*, *S. aureus* bacteria.

The results of Zhao et al. (2013) demonstrated weak antimicrobial activity in isolated phenyl-ethanediols from the fruiting bodies of *F. fomentarius*. Moreover, methanolic extracts of *F. fomentarius* have been found to be effective against *M. luteus*, but no activity was detected against *S. aureus, E. coli, P. aeruginosa, B. subtilis, Enterococcus faecalis* (Kolundžić et al., 2016).

In the present study we obtained different results for antimicrobial activity of *F. fomentarius* against the tested microorganisms; that is possibly due to differences of fungal species habitat factors, ecological status, seasonal differences and variety of extraction methods. The results of potential inhibition of test microorganism's biofilm formation by four extracts of *F. fomentarius* were shown in **Fig. 2**. The MIC values of *F. fomentarius* extracts were used to determine antibiofilm rates. It can be seen that antibiofilm rates of four extracts ranged from 5.03 to 95.2%.

The results indicated that ethanol and methanol extracts of *F. fomentarius* could inhibit the biofilm formation of all the tested strains, significantly. The highest antibiofilm activity was noticed against biofilm formed by *S. haemolyticus*. Both acetone and chloroform extracts showed very low antibiofilm effects compared to ethanol and methanol, except for *C. albicans* (**Fig. 2**).

Although recent studies on the evaluation of medically important *Fomes* species in this direction are few in the literature (Bin et al., 2012; Solmaz et al., 2013; Alves et al., 2014; Petrović et al., 2014; Signoretto et al., 2014; Carvalho et al., 2016; Karaca et al., 2017), there is no study on the antibiofilm effects of the *F. fomentarius* on the related pathogen strains. Alves et al. (2014) tested the antibiofilm capacity of different macrofungal species against *E. coli, P. aeruginosa*, *A. baumannii* and *P. mirabilis* strains. They found that all macrofungi methanol extracts showed strong antibiofilm activity against pathogens. Petrović et al. (2014) also reported that hot water extract from *Agaricus blazei* could influence on biofilm formation, twitching and swimming activity, pyocyanin production which are part of anti-quorum sensing activity. Karaca et al. (2017) investigated antibiofilm potentials of methanolic and ethanolic extracts of the three medicinal macrofungi species and found that the highest antibiofilm activity was observed with *Ganoderma lucidum* methanolic extract. Therefore, the findings obtained from the present study are presenting the important contributions to the literature data on this subject.

Conclusions

In the present study, it was determined that *F. fomentarius* demonstrated high antioxidant,

antimicrobial and antibiofilm activities. In conclusion, it was determined that analyzed *F. fomentarius* extracts could be used after chemical characterization and toxicity testing as natural resources in the fields of medicine, pharmacology, nutrition and cosmetics due to their strong antioxidant and antimicrobial activities.

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