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The antimicrobial effect of various formulations obtained from *Fomes fomentarius* against hospital isolates

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Abstract: The purpose of this study was to investigate the antimicrobial activity of formulations of *F. fomentarius*. The antimicrobial efficacy levels of extracts of *F. fomentarius* prepared from ethanol, water and of various formulations (emulsion, ointment, paste, cream and gel) were determined. For this purpose, the disk diffusion method was used to test the extracts for antimicrobial activity against hospital isolates including *Klebsiella pneumoniae, Acinetobacter baumannii, Staphylococcus aureus*, vancomycin-resistant enterococci (VRE+), *Escherichia coli, Candida krusei, C. albicans, C. tropicalis, C. guilliermondii* and *C. glabrata*. Some standard antibiotics were used for comparison. The ointment formulation (No. 4) showed a good antimicrobial effect on bacteria compared to the antibiotics (9.6 - 16.1 mm.). The extracts were found to be more effective than the antibiotics against *Candida* species. Formulation No. 4 was considered to be more effective because of the protective nature of its cetyl alcohol and sodium lauryl sulfate (SLS) content. *F. fomentarius* ethanol extract, *F. fomentarius* water extract and gel formulation showed similar activity; however, other formulations (emulsion, paste, cream) were generally more effective due to the stabilizing effects of the polymers, oil and alcohols present in their preparations. The findings of this study establish the possibility of discovering new clinically effective antibiotic drugs and could be useful in understanding the relationship between traditional remedies and modern medicines.

Key words: *Fomes fomentarius*; β-glukan; antimicrobial activity; sodium lauryl ether sulphate; cethyl alchol

Fomes fomentarius'dan elde edilen çeşitli formülasyonların hastane izolatlarına karşı antimikrobiyal etkisi

Öz: Bu çalışmanın amacı, *F. fomentarius* formülasyonlarının antimikrobiyal aktivitesini araştırmaktır. Etanol ve sudan hazırlanan *F. fomentarius* özütlerinin ve çeşitli formülasyonların (emülsiyon, merhem, pat, krem ve jel) antimikrobiyal etkinlik seviyeleri belirlendi. Bu amaçla ekstreleri, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*,

vankomisine dirençli enterokok, (VRE +), *Escherichia coli, Candida krusei; C. albicans, C. tropicalis, C. guilliermondii* ve *C. glabrata* içeren hastane izolatlarına karşı antimikrobiyal aktivite açısından test etmek için disk difüzyon yöntemi kullanılmıştır. Karşılaştırma için bazı standart antibiyotikler kullanılmıştır. Merhem formülasyonu (No. 4), antibiyotiklere kıyasla bakteriler üzerinde iyi bir antimikrobiyal etki göstermiştir (9.6 - 16.1 mm.). Ekstrelerin *Candida* türlerine karşı antibiyotiklerden daha etkili olduğu bulunmuştur. 4 Nolu formülasyonun, setil alkolünün ve sodyum lauril sülfat (SLS) içeriğinin koruyucu doğası nedeniyle daha etkili olduğu düşünülmüştür. *F. fomentarius* etanol ekstresi, *F. fomentarius* su ekstresi ve jel formülasyonu benzer aktivite göstermiştir; bununla birlikte, diğer formülasyonlar (emülsiyon, pat, krem), preparasyonlarında bulunan polimerlerin, yağın ve alkollerin stabilize edici etkileri nedeniyle genellikle daha etkili olmuştur. Bu çalışmanın bulguları, klinik olarak etkili yeni antibiyotik ilaçları keşfetme olasılığını ortaya koymaktadır ve geleneksel ilaçlar ile modern ilaçlar arasındaki ilişkiyi anlamada faydalı olabilir.

Anahtar kelimeler: *Fomes fomentarius*; β-glukan; antimikrobiyal aktivite; sodyum lauril eter sülfat; setil alkol

Introduction

Fungi have long been used as traditional medicine worldwide (Bal et al., 2017); Among them, polypore fungi, especially *Fomes fomentarius* (L.) Fr., Summa Veg. Scand., Sectio Post. (Stockholm): 321 (1849) have been widely applied as alternative remedies in recent years. The tinder fungus, in the *Polyporaceae* family, is a woody, perennial fungus, large in size, which develops as a parasite or sapropbe on beech (*Fagus sylvatica* L.) and other deciduous species (Vetrovsky et al., 2011). It is a white root fungus which causes heart rot in the wood. Shape likes a horse's hoof, it is 5–50 cm in length, 3–25 cm in width, 2–25 cm in height and without a stem (Breitenbach et al., 1986). As this fungus develops, it appears as gray-colored concentric zones of varying thickness. These zones are formed as remnants from past years are covered with new parts that grow every year and are stacked on top of each other. In traditional medicine, *F. fomentarius* has been used to relieve pain and to treat rheumatism, painful menstruation (dysmenorrhea), hemorrhoids and bladder disorders. Furthermore, esophageal, gastric and uterine cancers are treated with the fungus. Moreover, compounds with potential antitumor, immuno-modulatory and anti-inflammatory activity have been identified in *F. fomentarius*, which have also shown potential in the treatment of diabetes (Grienke et al., 2014).

The most important compounds of *F. fomentarius* are polysaccharides,, which exhibit an anti-proliferative effect and power to promote the secretion of TNF-alpha, IFN-gamma and IL-2 (Wei et al., 2011, Gao et al., 2009), while the second class includes polyphenolic compounds. Bal et al., investigated anti-oxidative activities of *Trametes gibbosa* (Pers.) Fr., *Fomes fomentarius* (L.) Fr., *Fuscoporia torulosa* (Pers.) T. Wagner and M. Fisch., *Daedalea quercina* (L.) Pers., *Inonotus hispidus (*Bull.) P. Karst. and *Trichaptum biforme* (Fr.) According to this study, researchers concluded that cinnamic, caffeic and benzoic acid content determined in *I. hispidus*, *F. fomentarius* and *F. torulosa* mushrooms (Bal et al., 2017). Thus, antioxidant, antimicrobial and cytotoxic activities are among the different biological activities possessed by the fungi (Heleno et al., 2015). For this reason, the antimicrobial activities of *F. fomentarius* extracts and a variety of simple topical formulations were tested in this study, with focus on the antimicrobial activity against hospital infections like *Candida* (Berkhout), *Escherichia coli* (Migula), *Staphylococccus aureus* (F.J.Rosenbach), *Klebsiella pneumoniae* (Schroeter), vancomycin-resistant enterococci and *Acinetobacter baumannii* (Bouvet and Grimont).The objective of the study was to determine the antimicrobial effect of various formulations of *F. fomentarius*. Additionally, the enhanced antimicrobial efficacy was explored by using different pharmaceutical excipiants in the formulations with the goal of designing simple, producible, cheap and effective topical formulations to be used for treatment.

Materials and Methods

Reagents and chemicals

Two emulsifiers, borax and sodium lauryl sulfate (SLS), were chosen for the cream and ointment formulations, with cold cream (United States Pharmacopeia 21) used as the cream base material. Other materials used in the study, including cetaceum, cera alba, liquid paraffin, dimethicone, cetyl alcohol, gelatin, glycerine, carboxy methyl cellulose (CMC) and castor oil, were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Double-distilled water was used throughout the study. Mueller Hinton agar (Sigma-Aldrich) was chosen for the antimicrobial activity test. Ethanol (Merck, Darmstadt, Germany) was used to

obtain the fungus extract and dimethyl sulfoxide (DMSO) was used for impregnation in the prepared formulations.

Mushroom material

The aerial parts (fruit bodies) of *F. fomentarius* in the Figure 1 were collected by Hakan Allı on 25 March 2014. on *Liquidambar orientalis* Mill., Gard. Dict. ed. 8: n.º 2 (1768). in Köycegiz in the Toparlar region of Mugla, Turkey. A sample of the fungus was authenticated and a voucher specimen (No: 5379) was deposited in the herbarium of the Mugla University Faculty of Sciences. The fungus parts were ground to a powder using a porcelain mortar and pestle.

Figure 1. *F. fomentarius* on the body of a tree (Photo by Hakan Alli, Mugla City, Turkey, March 25, 2014).

Preparation of the extracts

Seven different formulations were prepared. The first and second formulations were fungus extracts. In formulation No. 1, 15 g of the dry powdered fungus material was mixed with 150 mL of 99% ethanol/water and processed for 12 h in a Soxhlet extractor. The extract was filtered using Whatman Grade 1 Qualitative Filtration Paper (Sigma-Aldrich) and the filtrate solvent was evaporated under vacuum using a rotary evaporator at 55°C. The extract was then kept in a sterile black glass bottle at + 4°C. Approximately 2 g of the extract in the form of a sticky, black substance was dissolved in 0.1 mL of DMSO (5 mg/g) before testing.

The aqueous extract for formulation No. 2 was obtained via extraction of 5 g fungus powder with 100 mL distilled water at 80°C for 30 min using a water bath. The extract was filtered through Grade 1 Whatman paper and the filtrate was then used instead of water to prepare formulation Nos. 3, 4, 5, 6 and 7.

Preparation of formulations

The different compositions included: emulsion (No. 3), ointment (No. 4), paste (No. 5), cream (No. 6) and gel (No. 7) formulations without permeation enhancers or preservatives.

Emulsion formulation: The CMC powder (0.8 g) was added to the filtrate (20 g), and castor oil (8.0 g) was then mixed with it for the emulsion formulation.

Ointment formulation: All the aqueous phase materials (*F. fomentarius* water extract 5 % 3 g, SLS 0.2 g) and the oil phase ingredients (dimethicone 8 g, cetyl alcohol 3 g) were placed in two separate porcelain containers and heated to above 75°C. The water phase was then added to the oil phase using continuous agitation.

Paste formulation: Gelatin powder (3 g) was added to the filtrate (7 g) under continuous stirring, and was then dissolved at above 70°C. Glycerine (8 g) was added and mixed with hydrated gelatin under continuous stirring at 37°C until the paste was formed.

Oil-in-water cream formulation: All the aqueous phase materials (*F. fomentarius* water extract 5 % 40 mL, borax 1 g) and the oil phase ingredients (cetaceum 15 g, cera alba 14 g, liquid paraffin 66 g) were placed in two separate porcelain containers and heated at above 75°C. The oil phase was then added to the water phase under continuous agitation. The semisolid emulsion (O/W) was then cooled to approximately 40°C.

Gel formulation: Carbomer 940 powder (1 %) was added to the fitrate. Triethanolamine was then added and mixed with it enough to gel.

Bacterial cultures

All test microorganisms, including *Klebsiella pneumoniae, Acinetobacter baumannii, Staphylococcus aureus,* vancomycin-resistant enterococci (VRE)+, *Escherichia coli, Candida albicans* (C.P. Robin) *C. tropicalis* (Castell.) *C. krusei* (Kudryavtsev), , *C. guilliermondii* (Kurtzman and Suzuki) and *C. glabrata* (H.W. Anderson) were obtained from the Duzce University Research Hospital (Duzce, Turkey). The microorganisims were stored in a refrigerator at +4°C prior to the study.

Screening antimicrobial activity

The disk diffusion method for antimicrobial susceptibility testing was carried out according to the Clinical and Laboratory Standards Institute (CLSI) technique to assess the antimicrobial activities of the fungal extracts and formulations (Clinical and Laboratory Standards Institute, 2006). All cultures (adjusted to 0.5 McFarland standard) were transferred equally to Mueller Hinton agar plates using a sterile swab and 50 µL of each formulation were impregnated onto sterile disks (6mm.) in order to determine the antimicrobial activity spectra. Standard commericial antibiotic disks (erythromycin, gentamicin, ampicillin, amphotericin B, fluconazole and ketoconazole) were used as a control group. In addition, the antimicrobial activity of all chemicals used in preparing the formulations was checked. In this context, each formulation was used as a control for the same formulation without extractions. The discs soaked in each of the formulations were placed and slightly pressed onto the inoculated agar and then incubated at 35°C for 24 h for bacteria, and at 25°C for 72 h for yeast. At the end of the incubation period, the inhibition zones were measured in mm and evaluated.

Results

The disk diffusion method for antimicrobial susceptibility testing was carried out according to the Clinical and Laboratory Standards Institute (CLSI) technique to assess the antimicrobial activities of the fungal extracts and formulations (Clinical and Laboratory Standards Institute, 2006). The antimicrobial activities of the various formulations of *F. fomentarious* are shown in the Table 1. The control studies included only the formulation carriers (no extracts) and displayed no antimicrobial activity. The emulsion and cream formulations of *F. fomentarius* exhibited good results against bacteria and fungi, respectively, while the other formulations showed moderate antimicrobial effects against the test organisms. In particular, the emulsion formulation showed the highest effect on *K. pneumoniae*, with an inhibition zone of 15.2 mm from the test bacteria as compared to the commercial antibiotics gentamicin and ampicillin. The water extract did not demonstrate inhibition against bacteria or fungi. Suprisingly, the ointment formulation was observed to have the greatest effect of all the formulations against all test bacteria. The disk diffusion zones varied from 14 mm to 16.1 mm. Cetyl alcohol is used as a preservative in the medical and cosmetics sectors in addition to its other properties as a lubricant, softener and transporter. Sodium lauryl sulfate (SLS) is an emulsifier that denatures protein and breaks down cell membrane structures. Formulation No. 4 (ointment) was seen to be more effective because of the protective nature of the cetyl alcohol and SLS in its content. Except for VRE+ , with an inhibition zone of 14.8 mm, and *C. krusei*, with an inhibition zone of 7.0 mm, the paste formulation displayed no other effect against the test microorganisms. When the antimicrobial effect of the cream formulation was examined, among the yeasts, a high effect (19.2 mm) on *C. glabrata* was observed. Moreover, this was found to be the highest value when compared with the antimicrobial effects of the other formulations.

Table 1.Antibacterial and antifungal activity of various extracts from *F. fomentarius.*

Test Microorganisms	Inhibition zones (mm)* 1	2	3	4	5	6	7	E	GN	AM	AMB	FLU	KTC
Klebsiella pneumoniae	8 ±0.36	-	15.2 ±0.52	16.1 ±0.75	-	7 ±0.26	10.3 ±0.79	NT	-	7	NT	NT	NT
Acinetobacter baumannii	11 ±0.75	-	8 ±0.5	15.2 ±0.95	-	-	-	NT	9	10	NT	NT	NT
Staphylococcus aureus	8 ±0.5	-	10.3 ±0.17	14.6 ±0.87	-	10 ±0.3	10.5 ±0.43	14	NT	11	NT	NT	NT
VRE+	-	-	12.5 ±0.65	14 ±0.8	14.8 ±0.85	-	-	10	12	9	NT	NT	NT
Escherichia coli	7 ±0.26	-	13.2 ±0.79	14.6 ±1.0	-	8.2 ±0.34	7 ±0	11	NT	-	NT	NT	NT
Candida krusei	9 ±0.3	-	9.5 ±0.52	11 ±0.51	7 ±0.51	10.5 ±0.98	11 ±0.26	NT	NT	NT	9	-	-
C. albicans	8 ±0.17	-	8 ±0.51	13.8 ±0.55	-	-	7 ±0.45	NT	NT	NT	9	8	9
C. tropicalis	10 ±0.52	-	10 ±0	12.4 ±0.52	-	-	9 ±0.17	NT	NT	NT	-	-	-
C. guilliermondii	7±0	-	14 ±0.72	9.6 ±0.26	-	13.2 ±0.36	10.2 ±0.4	NT	NT	NT	8	-	-
C. glabrata	11 ±0.36	-	13.8 ±0	16 ±0	-	19.2 ±0.26	10 ±0.62	NT	NT	NT	-	-	15

*1: Formulation of *F. fomentarius* ethanol extract; 2: Formulation of *F. fomentarius* water extract; 3: Emulsion Formulation (castor oil 8 g, carboxy methyl cellulose 0.8 g, *F. fomentarius* water extract 5% 20 g; 4: Ointment Formulation (dimethicone 8 g, cetyl alcohol 3 g, sodium lauryl sulfate 0.2 g , *F. fomentarius* water extract 5% 3 g; 5: Paste Formulation (gelatin 3 g, glycerine 8 g, *F. fomentarius* water extract 5% 7 g; 6: Cream Formulation (cetaceum 15 g, cera alba 14 g, liquid paraffin 66 g, borax 1 g, *F. fomentarius* water extract 5% 40 mL; 7: Gel Formulation (*F. fomentarius* water extract 5 % 50 mL, Carbomer 940 1 g, triethanolamine q.s.; VRE+: Vancomycin resistant Enterococci, E: Erythromycin 15 µg; GN: Gentamicin 30 µg; AM: Ampicillin 10 µg; AMB: Amphotericin B 100 µg; FLU: Fluconazole 25 µg; KTC: Ketoconazole 10 µg; NT: Not tried. The extracts were performed under sterile conditions in duplicate and repeated three times.

Discussion

Similar to the present study, Kolundžić et al. tested the antimicrobial activity of *F. fomentarius* extracts of different polarity, especially against Gram-negative and Gram-positive bacteria (*Staphylococcus aureus, S. epidermidis* (Winslow and Winslow)*, Micrococcus luteus* (Schroeter))*, Bacillus subtilis* (Ehrenberg)*, Enterococcus faecalis* (Andrewes and Horder)*, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Schröter) They indicated that their *F. fomentarius* extracts (C-cyclohexane, D-dichloromethane, M-methanol and A-aqueous) displayed strong antimicrobial activity (Kolundžić et al., 2016). Recently, other scientists have demonstrated the potential therapeutic effects of *F. fomentarius* resulting from compounds that exhibit strong antiviral activity against the human immunodeficiency virus HIV-1 and antimicrobial properties against *Candida albicans* and *Helicobacter pylori* (Marshall et al. 1985) C.S.Goodwin, J.A.Armstrong, T.Chilvers, M.Peters, M.D.Collins, L.Sly, W.McConnell, W.E.S.Harper, Int. J. Syst. Bacteriol. 39: 397-405 (1989) (Senyuk et al., 2011). Moreover, it was seen to inhibit the growth of several other pathogenic bacteria, including *Pseudomonas aeruginosa, Serratia marcescens* Bizio, Biblioteca Italiana o sia Giornale di Letteratura, Scienze e Arti 30(8): 275-295 (1823)*, Staphylococcus aureus, Bacillus subtilis* (Ehrenberg 1835) F.Cohn, Untersuchungen über Bakterien. Beitrage zur Biologie der Pflanzen, 1(2), 127-224 (1872)*,* and *Mycobacterium smegmatis* (Trevisan 1889) K.B.Lehmann, R.Neumann, Lehmann's Medizin, Handatlanter X. Atlas und Grundriss der Bakteriologie und Lehrbuch der speziellen bakteriologischen Diagnostik. 2 Aulf., JF. Lehmann, München, 1-497 (1899) (Senyuk et al., 2011). Formulation Nos. 1, 2 and 7 exhibited similar activity; however, the other formulations were generally more effective due to the stabilizing effects of their polymers, oils and alcohols.

The results of Zhao et al. (2013) demonstrated weak antimicrobial activity in isolated phenyl-ethanediols from the fruting bodies of *F. fomentarius* (Zhao et al, 2013). The presence of polysaccharides in the polar extracts was considered to be very important because a previous study of Senyuk et al. (2011) had shown that a water-soluble melanin-glucan complex (containing 80 % melanins and 20 % β-glucans) completely inhibited the growth of *C. albicans* (Senyuk et al., 2011). Methanol is rich in total polyphenol content and as a result, the *F. fomentarius* extracts were generally found to be more effective than the standard antibiotics for all *Candida*

species (C. krusei, C. albicans, C. tropicalis, C. guilliermondii and *C. glabrata*).

Several classes of metabolites were identified: Primary metabolites (i.e., proteins), polysaccharides (-glucans), polysaccharide–protein complexes, and secondary metabolites such as triterpene glycosides (1444001-94-8, tuberoside), esters and lactones (fungisterollinoleate, betulin 28-O-acetate), alcohols (7-ergostenol, -sitosterol), aldehydes and ketones (protocatechualdehyde), (22E)-ergosta-7,22-dien-3-one, organic acids, benzofurans (paulownin), coumarins (daphnetin), and volatile components (Grienke et al., 2014). The most important compounds with clinically beneficial activity are -glucans. In vitro studies have suggested that large molecular weight or particular -glucans can directly activate leukocytes, stimulating their phagocytic, cytotoxic and antimicrobial activities, including the production of reactive oxygen and nitrogen intermediates (Akramiene et al., 2007).

Numerous studies and clinical trials have been conducted with soluble yeast β-glucans and whole glucan particulates, ranging from the impact of β-glucans on post-surgical nosocomial infections to the role of yeast β-glucans in treating anthrax infections. Post-surgical infections are a serious challenge following major surgery, with post-surgical infection rate estimates of 25–27%. Alpha–Beta Technologies conducted a series of human clinical trials in the 1990s to evaluate the impact β-glucan therapy had on controlling infections in high-risk surgical patients. In the initial trial, 34 patients were randomly (double-blind, placebo-controlled) assigned to treatment or placebo groups. The patients who received PGG-glucan had significantly fewer infectious complications than the placebo group (1.4 infections per infected patient for the PGG-glucan group versus 3.4 infections per infected patient for the placebo group). Additional data from the clinical trial revealed decreased use of intravenous antibiotics and shorter stays in the intensive care unit (ICU) for patients receiving PGG-glucan versus patients receiving the placebo.

Studies conducted with humans and animal models further support the efficacy of β-glucan in combating various infectious diseases. One human study demonstrated that the oral consumption of whole glucan particles increased the ability of immune cells to consume a bacterial challenge (phagocytosis). The total number of phagocytic cells and the efficiency of phagocytosis in the healthy human study participants increased when a commercial particulate yeast β-glucan was consumed, showing the potential for yeast β-glucan to increase the reaction rate of the immune system to infectious challenges. The study concluded that the oral consumption of whole glucan particles was demonstrated to be a good enhancer of natural immunity (Vetvicka et al., 2002, Onderdonk et al., 1992).

The β-glucan from oats has been shown to have antimicrobial effects against *Escherichia coli* and *Bacillus subtilis*. On comparing cationic and native β-glucans, the latter was seen to inhibit the growth of these bacteria by approximately 35 %, while the cationic one led to 80% inhibition in both microorganisms, indicating that β-glucan amination promotes antimicrobial effects. In this same study, cationic β-glucan was found to be more effective against *E. coli* (Gram-negative) than against *B. subtilis* (Gram-positive), which can be explained by the interaction of the polycations with the negatively charged bacterial surface, which altered membrane permeability and thereby inhibited growth (Shin et al., 2005).

The ointment formulation (No. 4) was seen to be more effective because of the protective nature of cetyl alcohol and SLS. Cetyl alcohol is also used as a preservative in the medical and cosmetics sectors in addition to having other properties as a lubricant, softener, transporter and emulsifier. Sodium lauryl sulfate (SLS) is an emulsifier which denatures protein and breaks down the cell membrane structure (Committee for Human Medicinal Products, 2018). The presence of an anionic surfactant such as SLS was considered to enhance the activity of the glucan in the extract and thus increase its antimicrobial activity. High concentrations of SLS in glucan-synthesizing mixtures have been shown to inhibit the production of glucans, whereas low concentrations of SLS increase the production of glucans (Tadamichi et al., 1981). It was believed that the low concentration of SLS in formulation No. 4 led to the increased glucan concentration which enhanced its antimicrobial efficacy.

In our study, it was observed that the SLS and cetyl alcohol enhanced the antimicrobial effect significantly by increasing the beta glucan activity in the structure of the fungus. As a result, the natural extracts obtained can be formulated with substances such as SLS and cetyl alcohol to heighten their activity. Today many natural products are being used to facilitate the treatment of infections. Antibiotic resistance has become a significant public health problem and consequently, scientists have accelerated the search for new antimicrobial molecules. In particular, the search for antibiotics of natural origin is progressing rapidly, which points to the need for further studies exploring the utilization of the therapeutic agents from *Fomes fomentarius.*

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