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ANTIBACTERIAL ACTIVITIES OF BEECH BARK (*Fagus sylvatica* L.) POLYPHENOLIC EXTRACT

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Abstract

The study provides information about separation and identification of natural bioactive compounds from beech (*Fagus sylvatica* L.) bark with potential therapeutic applications such as antibacterial activity against human pathogens. Beech is a common material used in the wood industry, but its bark is separated from the wood and is considered a by-product. In this study, natural compounds with biological activity were obtained from beech bark by hot water extraction. The high-performance liquid chromatography (HPLC) was used to analyze the phenolic compounds in the beech bark extracts. Spectrophotometric methods were employed for the determination of total phenolic content. Microdilution technique was used for testing the antimicrobial activity of the extract. The following strains were tested: *Staphylococcus aureus, Methicillin-resistant Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa*. The yield of extracted polyphenols was of 22.952 mg gallic acid/g dry bark. The compounds identified by HPLC were vanilic acid, catechin, taxifolin and syringin. The extracts were active against *Staphylococcus aureus* and *Methicillin-resistant Staphylococcus aureus*. The effect of polyphenolic extract on Gram-negative bacteria was absent at a concentration of 30 mg/mL beach bark extract. Altogether, the use of pure water for extraction of polyphenols from beech bark proved to be an effective eco-friendly method. This method sustains the concept of "green" chemistry by involving the use of renewable plant resources and also by using water as solvent.

Key words: antibacterial, beech bark, green biotechnology, biorefinery, polyphenols

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1. Introduction

Today, with the evolution of technology triggering a great number of changes, humankind has adopted an attitude of returning to nature. The active principles of plants can represent a real alternative, with applications in different subject areas, tied to the specialty of the biologist (medicine, pharmacy, the food industry, cosmetics, and agriculture). Currently great interest is given to the separation and characterization of the natural compounds. Also their application and introduction in different research fields is intended using the principles of green chemistry and biorefinery in the context of sustainable development (Barbosa-Pereira et al., 2014; Fierascu et al., 2017; Huang et al., 2010; Pereira et al., 2016; Shahina et al., 2006). Plants are an inexhaustible sources of bioactive compounds with antioxidant

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properties. In the literature of specialty, there are several studies dealing with separation and augmentation of the polyphenolic compounds from waste resulting from different manufacturing processes (Balasundram et al., 2006; Ignat et al., 2013; Moure et al., 2001; Tanase et al., 2014). Studies have revealed the importance of waste from the timber industry. The rhytidome, the nodes of spruce (Ignat et al., 2013; Tanase et al., 2014) and the wood (red heart) of the common beech (*Fagus sylvatica* L.) contain important quantities of chlorogenic acid, catechin, quercetin, kaempferol (Hofmann et al., 2008; Hoppe et al., 2016; Omar et al., 2000; Vek et al., 2013a; 2013b).

The phenolic compounds present in plants have an important role in the protection against abiotic stress (UV rays) and biotic factors with negative influence (predators, pathogen attacks) (Ignat et al., 2013). The emergence of infectious diseases caused by drug resistant bacteria has become one of the most serious problems in medicine. Although the use of antibiotics has reduced the incidence of these diseases, nowadays, there are a number of bacterial strains resistant to antibiotics (Lazau et al., 2013).

Phenolic compounds (polyphenolic acids, tannins, flavonoids, anthocyanins), synthesized by plants as a response to microbial infections, have a high capacity for action against a wide range of microorganisms. Several studies have been reported about the use of polyphenolic plant extracts in medicine (Carraturo et al., 2014; Friedman et al., 2006; Haghi, 2008; Huang et al., 2010; Morteza-Semnani et al., 2011; Omar et al., 2000; Pereira et al., 2016; Szabo et al., 2010).

The majority of phenolic compounds are found in the leaf and stem of the plants. In stem, polyphenols are especially concentrated in the rhytidome. Thus, the extracts obtained from the rhytidome have a high antibacterial and antifungal activity (Omar et al., 2000). For example, it was found that the ethanolic extract obtained from the *Picea abies* L. rhytidome presents antibacterial activity against Gram-positive and Gram-negative bacteria (Ignat et al., 2013). The aqueous and alcoholic extract obtained from the bark of the common beech (*Fagus sylvatica* L.) were also biologically tested. It was found that these beech bark extracts present a high antioxidant activity (Hofmann et al., 2015b).

The aims of this study were: (1) to provide information on separation and identification of natural bioactive compounds with potential therapeutic applications from beech (*Fagus sylvatica* L.) bark, a renewable plant resources, using water as solvent; (2) to test the antibacterial activity of the beech bark extract on human pathogens like methicillin-sensitive *Staphyloccocus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa.*

2. Material and methods

2.1. Materials

Beech (*Fagus sylvatica* L.) bark was provided as waste by a wood processing company (Vatra Dornei, Romania). Prior to extraction, the beech bark was air-dried at room temperature (10.5 % humidity) and milled in a GRINDOMIX GM 2000 mill to a mean particle size diameter of 0.5 mm. The biomass was directly used without any pre-treatments.

To determine the antibacterial activity, the following bacterial strains were used: methicillin-sensitive *Staphylococcus aureus* (MSSA) ATCC 25923, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, and *Pseudomonas aeruginosa* ATCC 27853. The bacterial strains were selected from the collection of Laboratory of Microbiology, Virology and Parasitology (Faculty of Medicine - University of Medicine and Pharmacy, Tîrgu Mureş).

2.2. Aqueous extraction

Extractions were performed using 20 g of ground and dried beech bark placed in an Erlenmeyer flask over which 125 mL distilled water was added. The mixture was introduced and kept for 45 min in a water bath at 85 – 90°C, shaking from time to time. Collected extract were filtered and the extracted material was subjected to a second extraction with fresh distilled water. This operation was repeated 2 or 3 times until full beech bark exhaustion (colorless extract). All extracts were cumulated in a 500 mL volumetric flask and marked up to volume with distilled water.

2.3. Characterization of the extract

The aqueous extract from beech bark (EAF) was characterized in terms of total dry matter and organic matter content, and total polyphenolic content using Folin Ciocalteau (FC) method. Dry matter content in the extract was determined by evaporation of 25 mL extract on water bath and drying at 105°C till constant mass, using a porcelain crucible. After that, the crucible was placed into a muffle furnace at 600°C for 6 hours to establish dry and organic matter content. Spectrophotometer readings at 420, 520, 620 nm, of the extract were cumulated to determine the color intensity. Folin Ciocalteu (FC) method was used to determine the total polyphenolic content (TPC). The principle of this colorimetrically method is based on the reducing properties of phenolic compounds in contact with FC reagent, which present a dark blue coloration with a maximum absorbance at 765 nm. About 1mL of plant extract was mixed with 500 µL of the FC reagent, 2 mL of 10 % sodium carbonate and 5 mL of water.

The mixture was shaken thoroughly and was left to stand for 90 minutes. Then the absorbance at 765 nm was determined against a blank which contain all reagents without the samples or the gallic acid in the same conditions. The total phenolic content was expressed as the number of equivalents of gallic acid (GAE).

For *qualitative analysis of beech bark extract by HPLC with multiple wavelength detection* an UHPLC system, Flexar FX – 10 (Perkin Elmer) was used, consisting of binary pump, inline degaser, autosampler, column thermostat and PDA detector. All solvents were HPLC grade and the used reagents presented the highest available purity. The chromatographic conditions were: column - Luna C18 (Phenomenex), 150x4.6 mm, 3 µm; mobile phase A – formic acid 0.1 % V/V and B – acetonitrile; the elution gradient program: 0 – 0.1 min: 90 % A, 10 % B; 0.1 – 20.1 min: 90 % to 20 %A; 20.1 – 25.1 min: 20 %A; 25.1 – 26.1 min: 20 %A to 90 %A; 26.1 – 30.1 min: 90 %A. The mobile phase was delivered at a flow rate of 1 mL/min and the column was maintained at 35 °C. The monitoring wavelengths were 270 nm, 280 nm, 324 nm and 370 nm. A methanol-water mixture of Sigma Aldrich reference substances containing gallic acid monohydrate (GAL) (99%), eleutheroside B (ELE B) (98%), catechin (CAT) (99%), epicatechin (EPICAT) (90%), vanillic acid (VANIL) (97%), sinapic acid (SINAP) (99%), taxifolin (TAXI) (85%) and quercetin (QUER) (95%), with the concentration about 20 µg/mL for each substance, was analyzed after injecting a volume of 20 µL. Autosampler temperature was set at 20°C.

2.4. Microbiological activity

2.4.1. Minimum inhibitory concentration (MIC)

The microplate method was used to test the antimicrobial activity of the obtained extract, based on CLSI (2015). In order to establish the minimum inhibitory concentration (MIC) of the tested substance, 96 wells microplates were used. The bacterial inoculum was prepared by mixing 10 µL of bacterial suspension (0.5 McFarland) with 9990 µL of Muller-Hinton broth medium. In the first well of the microplate, 100 µL from the bacterial inoculum were mixed with 100 µl of the tested extract. From this well, binary dilutions were performed in Muller-Hinton broth medium. Control wells were also prepared: two for negative controls (culture medium and culture medium plus beech bark extract) and growth controls for each of the five bacteria (culture medium plus bacterial inoculum). The microplates were incubated at 37°C for 24 hours, in normal atmosphere. The minimum inhibitory concentration of the substance (MIC) was assessed in the first well (dilution), where no visible bacterial growth was detected by the unaided eye. For calculation of the MIC in mg of dry matter/mL, the concentration was adjusted mathematically by the dilution factor.

2.4.2. EAF growth rate effect

The growth rates were assessed for MSSA and MRSA by Spread Plate Procedure (Sanders, 2012). From fresh bacterial culture, a 0.5 McFarland suspension in sterile saline was prepared, and 10 µl of suspension was transferred into 9990 µL liquid culture medium (Muller Hinton broth). In Eppendorf tubes, 2 mL working solution of EAF was prepared, with a concentration corresponding to the well where the MIC was noted in the microplate assay. In the control solution the tested extract was substituted with sterile saline solution. From both control and sample, a series of dilutions in sterile saline were prepared.

From the dilution 1/100, 50 µL was seeded on Mueller Hinton agar plates (previously maintained at 37 °C). The seeded media were incubated at 37°C for 18 – 24 hours. This first phase was used to assess the total number of colony forming units/mL (CFU/mL) at time 0 (H0). The working solution and the control were incubated at 37°C for 3 hours and 6 hours, and in each of these time points, a new series of serial dilutions and seeding were performed. This allowed the assessment of CFU/mL after 3 hours and 6 hours of incubation (H1, H2). At the 3-hour time point, the 1/100 dilution was used for seeding, while at the 6-hour time point, the 1/100.000 dilution was used.

After 18 – 24 hours of incubation at 37°C, from the plates corresponding to each dilution and each time, the colonies were counted automatically using the "IUL Flash & Grow" colony counter. Mathematical adjustments were performed to compensate the dilution and the inoculation volume, using the formula:

$$\frac{CFU}{mL} = number\ of\ colonies\ x\ dilution\ factor\ x\ A\ x\ B \quad (1)$$

A = dilution adjustment in the MH media = 100 at H0 and H1/10000 at H2;
B = volume of inoculation adjusting = 20 (mL).

Absolute growth curves were plotted using the CFU/mL numbers from each time point. The specific growth rates *r* for the tested sample and control were assessed using the CFU/mL values obtained after 6 hours of incubation, by the formula:

$$r = \frac{LN(CFU/mL\ for\ H2 - CFU/mL\ for\ H0)}{no.hours\ for\ H2} \quad (2)$$

2.5. Statistical analysis

The statistical significance was assessed by GraphPad InStat 3 software, at a significance threshold value of $p < 0.05$.

3. Results and discussion

3.1. Extract characterization

The beech bark aqueous extract (EAF) was characterized in terms of dry matter and organic

matter, and total content of polyphenols. The results are summarized in Table 1. Beech bark extract contain considerable quantities of bioactive aromatic compounds. The total polyphenolic content for EAF was 0.918 mg GAE/mL.

3.2. Identification of phenolic compounds using HPLC

The chromatograms of a mixture of standards at different wavelengths are shown in Fig. 1. It can be observed that almost all components/peaks of interest are revealed at 270 nm and 280 nm, respectively. On the other hand, 324 nm seems to be the specific wavelength for SINAP and 370 nm for QUER. Based on the studies of Dübeler et al. (1997) and Hofmann (2015a) the major phenolic compounds from beech bark have been tentatively identified, including: (+)-catechin, (−)-epicatechin, quercetin-O-hexoside, taxifolin-O-hexosides, taxifolin-O-pentosides, B-type and C-type procyanidins, syringic acid and coumaric acid-di-O-glycosides, coniferyl alcohol and sinapyl alcohol-glycosides, (+) and (-) glucodistylin (Dübeler et al., 1997; Hofmann et al., 2015a). Comparing to this results the compounds identified in our extract by HPLC were catechin, vanillic acid, taxifolin and eleutheroside B (syringin) in small amounts.

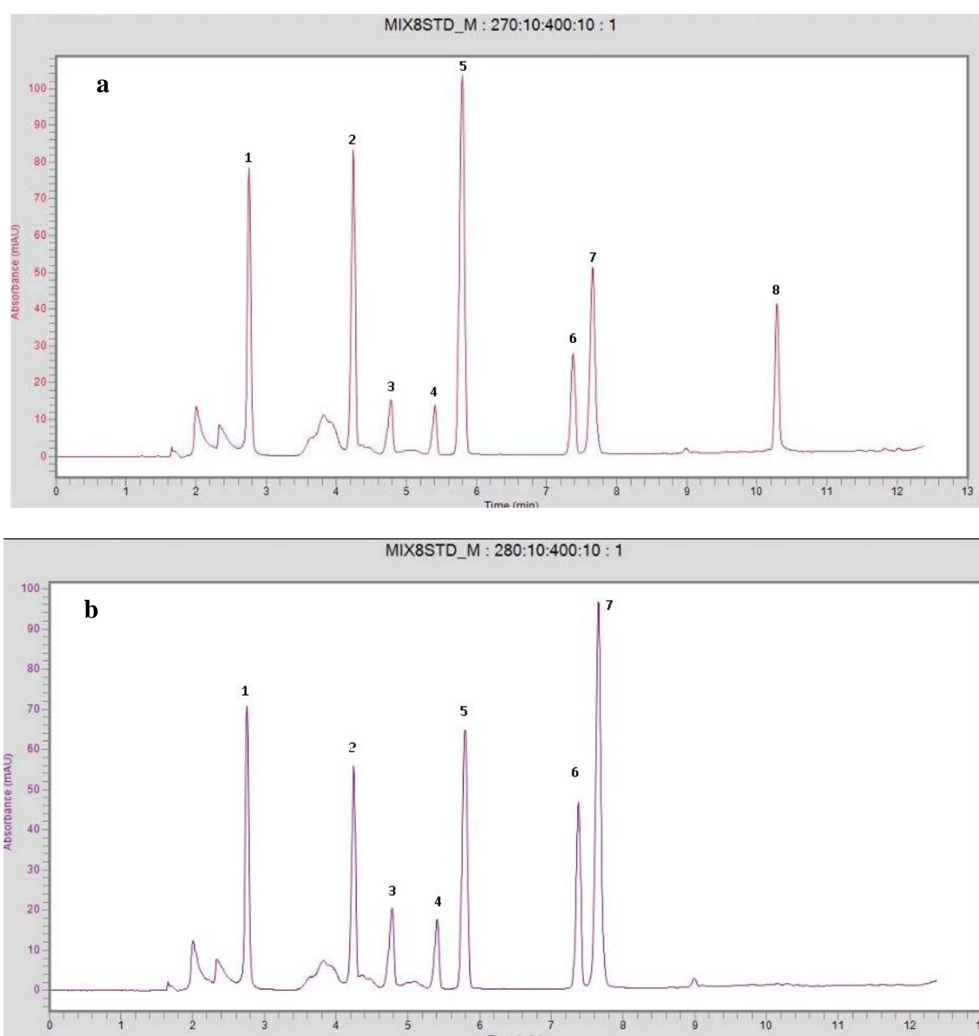
Vanillic acid being for the first time reported in the beech bark aqueous extract. The identification has been made by retention time's correspondence, multiwavelength analysis and addition standard method.

3.3. Microbiological activity

The minimum inhibitory concentrations (MICs) of polyphenolic extract required for growth inhibition of the Gram-negative and Gram-positive bacteria are presented in Table 2 and in a supportive image from Fig. 2. It was found, that EAF have antibacterial capacity against MSSA and MRSA. The effect of polyphenolic extract on Gram-negative bacteria was absent at a concentration of 30 mg/mL.

Table 1. Characteristics of beech bark aqueous polyphenolic extract

Dry matter content, g/L extract	*Organic matter content, g/L extract*	*Color intensity*	*pH (at 25°C)*	*TPC, mg GAE/g* **dry bark**
0.58 ± 0.09	0.41 ± 0.04	0.27 ± 0.03	4.5 ± 0.15	22.95 ± 0.07



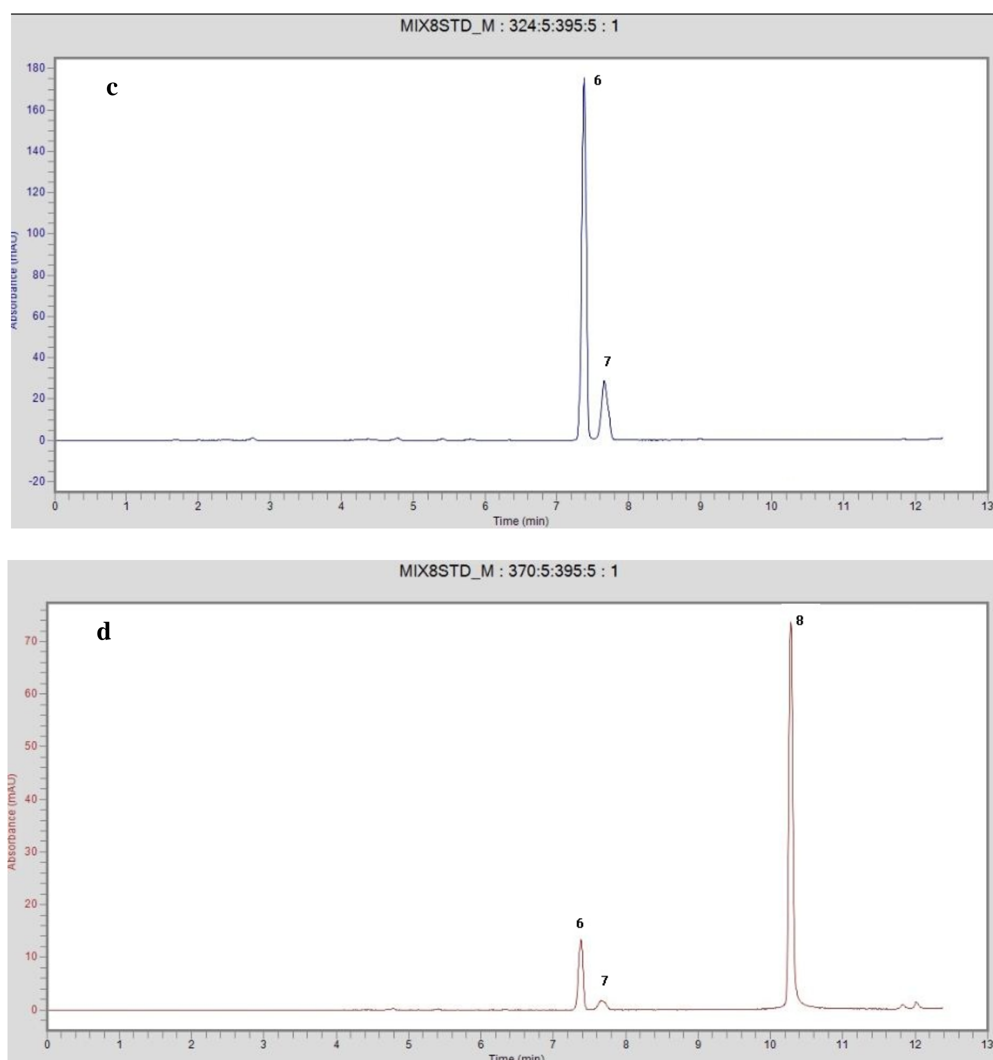


Fig. 1. Chromatograms of a mixture of eight standards, at different wavelengths: a) 270 nm; b) 280 nm; c) 324 nm; d) 370 nm. Order of elution: 1- gallic acid GAL, t_R=2.76 min; 2 – eleutheroside B ELE B, t_R=4.24 min; 3 – catechine CAT, t_R=4.78 min, 4 – epicatechine EPICAT t_R=5.41 min; 5 - vanillic acid VANIL, t_R=5.79 min, 6 - sinapic acid SINAP, t_R=7.38 min, 7 – taxifoline TAXI, t_R=7.65 min; 8 – quercetin QUER, t_R=10.28 min

Table 2. Minimum inhibitory concentrations (mg/mL) of EAF against the tested bacteria

Bacteria	*MICs – aqueous extract, mg/mL*
(A) *Staphylococcus aureus*	15
(B) *Methicillin-resistant Staphylococcus aureus*(MRSA)	**30**
(C) *Escherichia coli* ATCC 25922	-
(D) *Klebsiella pneumoniae* ATCC 13883	-
(E) *Pseudomonas aeruginosa* ATCC 27853	-

Thus, compared with MSSA and MRSA, which are gram-positive bacteria that only have the plasma membrane as their cell wall but do not have the bacterial outer membrane, it took longer for EAF compounds to penetrate into and interact with *E. coli*, *K. pneumoniae*, *P. aeruginosa*; thus, it may take a longer time for the gram-negative bacteria to be killed.

As shown in Fig. 3 and Table 3, when the growth medium was enriched with EAF, the bacterial growth was significantly inhibited within 6 hours compared to the control. After three hours of incubation (H1), EAF significantly inhibited the bacterial growth for both MSSA and MRSA ($p < 0.0001$). After six hours of incubation (H2) EAF presented bactericidal effect on MRSA and further inhibited MSSA growth (Figs. 3 and 4). Beside the bactericidal effect, the growth rate r was also negatively affected by EAF. Fig. 5 presents a supportive image of the inoculated plates after 24 hours of incubation (MSSA and MRSA), corresponding to the tree time-points (initial time, 3 hours and 6 hours). In a study by Friedman et al. (2006) it was shown the antimicrobial activities of catechin from tea extract. Most phenolic compounds

presented a higher activity than antibiotics used in medicine, such as tetracycline or vancomycin, at comparable concentrations. The conclusion of this study was that the bactericidal activities of the teas are due to the presence of catechins. The antimicrobial activity of EAF consists in its phenolic nature. The antibacterial activity of any compound from EAF (CAT, VANIL, TAX or ELEB) against Gram-positive bacteria is partly due to their ability to reach the site of action. In bacteria, various enzymes, especially components of energy-converting systems such as electron transport chains and ATPases, are embedded in the plasma membrane (Shahina et al., 2006). The extract of the beech bark have good efficacy of inhibiting bacterial growth and can be considered a potential pharmaceutical or food preservative.

Chemical constituents like catechin, vanillic acid, taxifolin, and syringin (found in EAF) have an antibacterial activity which leads to the increase of susceptibility of bacterial cells to these compounds.

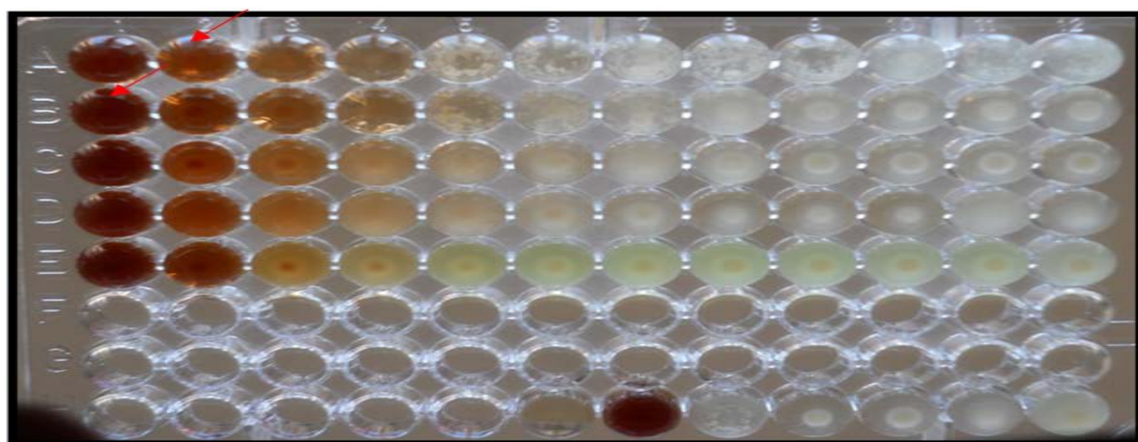


Fig. 2. Aspect of microplate for determined of MIC of beech bark polyphenolic extract obtained by hot water. Rows A-E: MSSA, MRSA, *E. coli, K. pneumoniae*, and *P. aeruginosa* respectively. Columns 1-12: Descending concentrations (binary dilutions) of beech bark extract, starting with 30 mg/mL (column 1) down to 0.015 mg/mL (column 12)

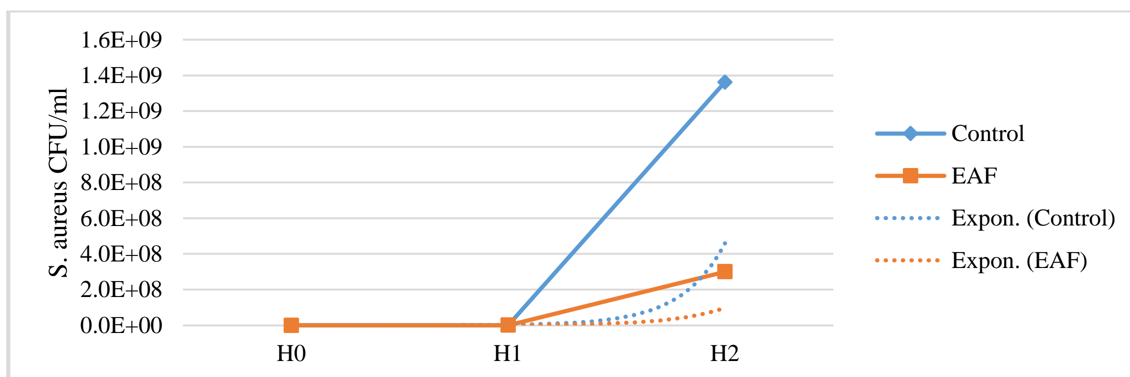


Fig. 3. The graphic representation of the growth rate for MSSA in the presence of EAF and Control (initial time – H0, 3 hours – H1 and 6 hours – H2)

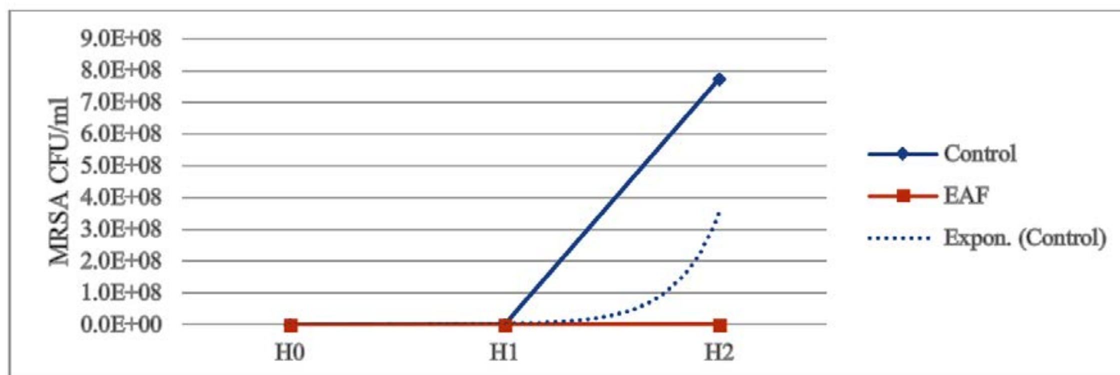


Fig. 4. The graphic representation of the growth rate for MRSA in the presence of EAF and Control (initial time – H0, 3 hours – H1 and 6 hours – H2)

Table 3. Data presenting the growth rate of *S. aureus* and *MRSA* in presence of EAF

		CFU/mL			*Specific Growth rate, h^{-1}*	*Generation time, min*
		H0	H1	H2	*r*	*g*
MSSA	EAF	6.2×10^4	1.5×10^5	3×10^8	1.41	29.40
	Control	7×10^4	3.72×10^5	1.36×10^9	1.65	25.26
	$p < 0.0001$					
MRSA	EAF	3.8×10^4	2.6×10^4	0	N/A	N/A
	Control	4×10^4	5.3×10^5	7.74×10^8	1.65	25.28
	$p < 0.0001$					

Taxifolin extracted from *Hypericum japonicum* Thunb., inhibits the growth of S. aureus (MRSA) by delaying the protein synthesis and adversely affecting the synthesis of enzymatic systems and nucleic acids (Grosso et al., 2007). These plant compunds also increase the membranes permeability to drugs, leading to a bacteriostatic effect. It was found that aromatic compounds have a good ability to link with bacteria cell walls and can exert bacteriostatic effect (Mahboubi and Haghi, 2008; Morteza-Semnani et al., 2011). Thus, plant extracts with high content of phenolic compounds (e.g. beech bark aqueous extract) can be efficient when used in complementary with commercial drugs due to their bactericidal effect.

It was found that recently work has been done on the antioxidant properties of hot water beech bark extracts (Hofmann et al., 2015b, 2017). According to Hofmann et al. (2017) the most efficient antioxidants in beech bark were found to be the (+) catechin, procyanidin B dimer 2, (−) epicatechin and coniferin isomer 2. The authors conclude that under high temperature pressurized environments water can be an extraction solvent as effective as solutions containing alcohols. The positive results related to antioxidant activity could be closely connected to the antibacterial properties of the EAF on *S. aureus* including methicillin-resistant strains.

Our results have shown that EAF can be effective against both MRSA and MSSA due to high content of phenolic compounds, which can act synergistically with each other against those bacteria. Further research is needed to elucidate accurately their pathways and mechanisms of action against bacteria.

4. Conclusions

The results of this study reveal that the beech bark extract contains a considerable amount of phenolic compounds. Pure water can be an efficient solvent, supporting the concept of green extraction. The compounds identified by HPLC were vanilic acid, catechin, taxifolin and syringin. The antimicrobial tests demonstrated antimicrobial activity against *Staphylococcus aureus* including methicillin-resistant strains.

The results of this study have led to a new research direction orientated on reducing the pharmacological resistance of microorganisms to antibiotics, by using polyphenolic plant extracts.

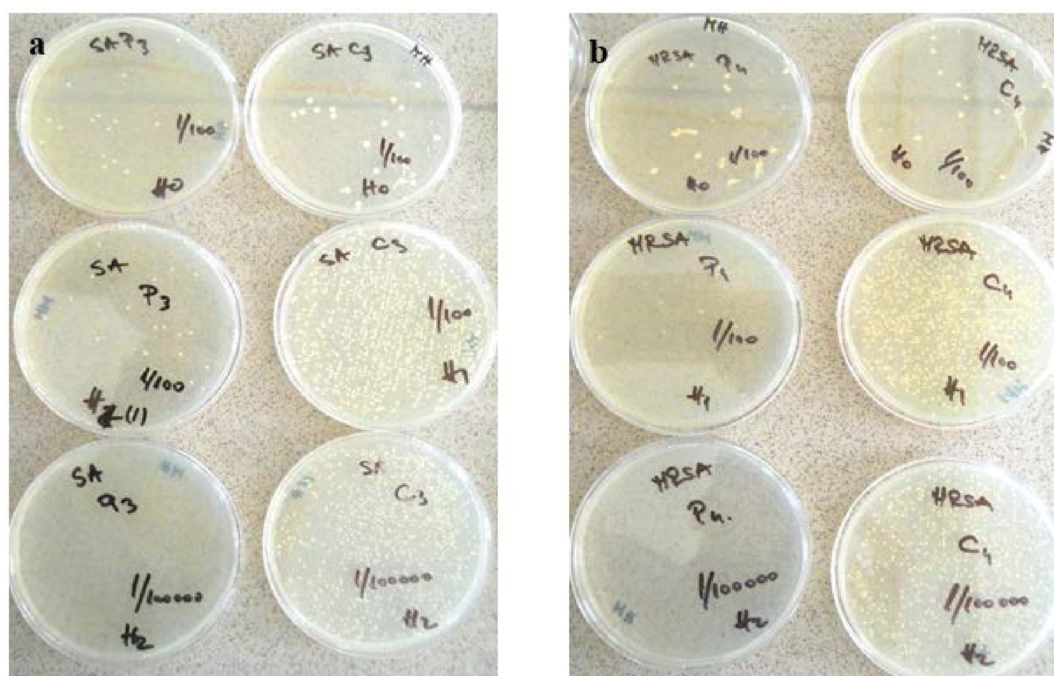


Fig. 5. Supportive image of the inoculated plates after 24 hours of incubation, corresponding to the tree time-points (initial time – H0, 3 hours – H1 and 6 hours – H2) for MSSA (a) MRSA (b) and Control

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