Antibacterial Activities of Spruce Bark (*Picea abies* L.) Extract and Its Components Against Human Pathogens

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Spruce is a used material in the wood industry and bark is regarded as a by-product. The aim of this study was to provide information about natural bioactive compounds from spruce (Picea abies L.) bark with potential therapeutic applications such as antibacterial activity against human pathogens. Spruce bark extract was obtained by the conventional aqueous extraction (EAM), and second with ultrasounds (USM). It was determined the total polyphenols by spectrophotometric methods and individual polyphenols by high-performance liquid chromatography (HPLC). For the determination of minimum inhibitory concentration was used microdilution technique. The following strains were tested: Staphylococcus aureus, Methicillin-resistant Staphylococcus aureus, Klebsiella pneumoniae, and Pseudomonas aeruginosa. The yield of extracted polyphenols in the case of spruce bark extract was of 33.8mg gallic acid/gfor EAM and 28.73 mg gallic acid/g for USM. The compounds identified in samples, by HPLC, were vanillic acid and taxifolin.It was found that both extracts have antibacterial capacity for all bacteria tested. The antibacterial effect of both extracts was stronger against Gram positive cocci compared with Gram negative bacilli.Antimicrobial spectra and activities of the fractions and pure isolated compounds from the spruce bark, can be suggested the use of spruce bark in pharmaceutical preparations and as well as a food preservative.

Keywords: antimicrobial, spruce bark, green biotechnology, biorefinery, polyphenols.

A major cause of infections and mortality are bacterial infections. Antibiotics were the preferred treatment for infections because of their strong results and cost-efficiency. Several studies have provided that the widespread use of antibiotics has led to the emergence of antibiotic resistant bacterial strains[1]. In this regard, the European Center for Disease Prevention and Control estimated that bacterial resistance causes the death of over 25,000 people every year [2].

Thus, attention has been focused on new products with antibacterial activity.The active principles of plants can represent a real alternative, with applications in different subject area: medicine, pharmacy, the food industry, cosmetics, agriculture etc.

Phenolic compounds, are part of the natural substances with complex biological activity with increased potential applicability. They are a heterogeneous group of organic compounds. Studying the effect of these compounds in living systems present a special scientific interest due to the numerous actions that they have: an antioxidative, immunostimulatory, antimutagenic, antibacterial, bioregulatory etc[3-6]. Furthermore, it was shown that these compounds have low toxicity to the living systems [1].

Referring to extraction of phenolic compound, it has been found that the main methods of extraction used was a classical aqueous extraction, microwave extraction and ultrasonique [7-9]. Most studies have focused on the characterization of plant extracts use techniques of high performance liquid chromatography (HPLC) coupled with MS [5,10]. Polyphenolic compounds present in plants, have a important role in defense against abiotic stress (UV) or biotic (predators, attacks pathogenic) [5]. In the studies performed, followed potential uses of such compounds as antioxidants in pharmaceutical applications as well as in cancer chemoprevention[11]. Polyphenolic extracts and essential oils were tested against pathogenic intestinal bacteria, highly resistant to drugs such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Proteus vulgaris*, *Bacillus cereus,* highlighting the growth inhibitory effect of bacterial colonies [12,13]. Aqueous and alcoholic extracts obtained from the leaves and wood (red heart) beech (*Fagus sylvatica* L.) were biologicalltested. It has been found that these extracts have a high antioxidant activity as fungicides and anti-cancer properties, showing inhibitory activity in vitro [7].

The literature data, gives extensive information on the influence of polyphenolic compounds (obtained from the various plant sources) in the form of global extracts, fractions or individual compounds in living systems [4,14-16].Lack of systematic studies on the correlation between structure and biological properties of the polyphenolic compounds. Consequently, the results achieved deserves to be developed further to establish correlations between the structure, functions and properties of polyphenols and to develop their practical applications, taking into account their multifunctionality. A number of modern techniques can be applied currently with results are certain, rapid and reproductible for toxicity evaluation of products with therapeutic application [17].

In this study we propose to provide information on separation and identification of natural bioactive compounds from spruce (*Picea abies* L.) bark with potential therapeutic applications such as antibacterial activity against human pathogens likemethicillin-sensitive*Staphyloccocus aureus (MSSA),*methicillin-resistant *Staphylococcus aureus (MRSA), Klebsiella pneumoniae* and *Pseudomonas aeruginosa.*

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Experimental part

Materials and methods

Spruce (*Picea abies*L.) bark was provided as waste from a wood processing company (Vatra Dornei, Romania). Prior extraction, the spruce 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 strainswere used: methicillin-sensitive *Staphylococcus aureus*(MSSA) ATCC 25923, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *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, Tirgu Mures).

Aqueous extraction

20 g of ground and dried spruce bark placed in an Erlenmeyer flask over which 125 mL distilled water were added. The mixture was kept for 45 min in a water bath at 85-90°C, shaking from time to time. Ultrasound assisted extraction was performed using 20 g of spruce bark, immersed in 125 mL distilled water in an Erlenmeyer flask, under ultrasounds action (using ultrasonic bath, Bandelin Sonorex), at 40 kHz frequency, 70°C and for 15 min.

Characterization of the extract

The aqueous extract from spruce bark (EAM and USM) was characterized in terms of total polyphenolic content using Folin Ciocalteau (FC) method. 1mL of plant extracts was mixed with 500µL of the FC reactiv, 2 mL of 10% sodium carbonate and 5 mL of water. The mixture was shaken thoroughly and was allowed to stand for 90 min. 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).

The HPLC analysis *of spruce bark extract*was performed with an UHPLC-DAD system, model Flexar FX-10(Perkin Elmer), with the following modules: binary pump, inline degaser, autosampler with Peltier system, column thermostat andDADdetector.The separation was achieved on Luna C18 (Phenomenex) column(150x4.6 mm, 3 µm) at 35 °C by delivering at a flow rate of 1 mL/min a mobile phaseconsisted of A-formic acid 0.1% V/V and B – acetonitrile; the elution gradient program was: 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 monitoring wavelengths were set at270 nm, 280 nm, 324 nm and 370 nm.For qualitative analysis, a methanol-water mixture of Sigma Aldrich reference substances (the concentration ofeach substance was 20 µg/mL) was analyzed after injecting a volume of 20 µL. Reference substances were: gallic acid (GAL), eleutheroside B (ELE B), catechine (CAT), epicatechine (EPICAT), vanillic acid (VANIL), sinapic acid (SINAP), taxifoline (TAXI) and quercetin (QUER).The quality of solvents and reagents assured the compatibility with HPLC analysis.

Microbiological activity

Minimum inhibitory concentration (MIC)

In order to determine the MIC of the obtained extract against tested bacterial strains, we used the microdilution method. From fresh (24 h) bacterial cultures, we prepared an inoculum of 0.5 McFarland density, this inoculum being afterwards diluted 1/1000. 100 µL of the tested extract were mixed in the first well of the microplate with 100µL of the diluted inoculum. From the first well,performed binary dilutions in Muller-Hinton broth medium were performed, up to the last well of the microplate. After incubating the plates in normal conditions (24 h at 37°C, O_2 atmosphere) we evaluated the MIC of the tested extracts in the first well with no bacterial growth. The MICs were calculated in mg/ml, by adjusting the obtained concentrations with the dilution factor.

Bacterial growth rate

In order to determinate if the EAM and USM solutions affects in any way the bacterial growth rate, we prepared 2 ml of stock solutions of the two extracts. The concentration of the stock solution was adjusted to correspond to the MIC that was assessed by the microplate method. On the same time, we prepared control solutions using sterile saline solutions instead of the studied extracts.

The frozen bacterial strains were revitalized on nutritientagar. From this fresh bacterial culture, we prepared an inoculum of 0.5 McFarland density from which we prepared working solution of1/1000 concentration in Muller Hinton broth. By mixing 100 µL of the diluted bacterial inoculum with the same amount of stock solution, we reproduced the conditions ftrom the microplate well in which theMIC was assessed.

In order to establish the total number of colony forming units/ml (CFU/mL) at time 0 (H0), we transferred 50 µL from 1/100 diluted working solution and from 1/100 diluted control on Mueller Hinton agar plates. After uniformly distributing the suspension on agar plates with an inoculating loop, the plates were incubated in normal conditions (24 h at 37°C, O_2 atmosphere).

Bacterial growth rate in the presence of the extracts was evaluated again after 3 (H2) respectively 6 h (H3). For this, the control solution and the working solution were incubated at 37°C for 3 h, when 50µL from 1/100 diluted working/control solution were seeded on agar plates. After another 3 h, the method was repeated, at this time 1/ 100,000 dilution being seeded.

The next day, after 24 h of incubation for each time point, the number of the colonies was counted using the colony counter IUL Flash & Grow. The number of CFU was adjusted at 1 mL, by adjusting with the inoculated volume and the dilutions, using the formula:

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

A = dilution adjustment in the MH media = 100 at H0 and H1/10000 at H2

B = volume of inoculation adjusting = 20

Statistical analysis

Using the calculated CFU/mL numbers from each time point, absolute growth curves were plotted. We used the next formula in order to assess the growth rates of the bacterial strains (*r*) for the tested sample and control, after 6 h of incubation:

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

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

Peaks of interest	GAL	ELE B	CAT	EPICAT	VANIL	SINAP	TAXI	QUER
270 nm	***	***	**	**	***	-	**	**
280 nm	**	**	***	***	**	-	***	-
324 nm	-	-	-	-	-	***	*	-
370 nm	-	-	-	-	-	*	-	***
Retention time (min)	*2.8*	*4.2*	*4.8*	*5.4*	*5.8*	*7.4*	*7.7*	*10.3*

***- High intensity, **- Moderate intensity, *- Low intensity

Table 1
SELECTIVITY AND SENSITIVITY OF THE ANALYSES AT DIFFERENT WAVELENGTHS

Results and discussions

Extract characterization

The spruce bark aqueous extract (EAM and USM) was characterized in terms of total content of polyphenols. The results summarized thatspruce bark extracts contain considerable quantities of bioactive aromatic compounds. The total contents of polyphenols for EAMwas 0.135mg GAE/mL (33.8mg GAE/g ±0.06) and for USM 0.114mg GAE/mL (28.73mg GAE/g ±0.08).

Identification of phenolic compounds using HPLC

It can be observed from table 1 that almost all components/peaks of interest are revealed at 270 nm and 280 nm, respectively. 324 nm seems to be the specific wavelength for SINAP and 370 nm for QUER.

The compounds identified in samples by HPLC were vanillic acid (4-hydroxy-3-methoxybenzoic acid) and taxifolin (dihydroquercetin) insmall amounts (fig. 2). The identification has been made by retention time's correspondence, multiwavelength analysis and addition standard method. Both VANIL and TAXI are in greater

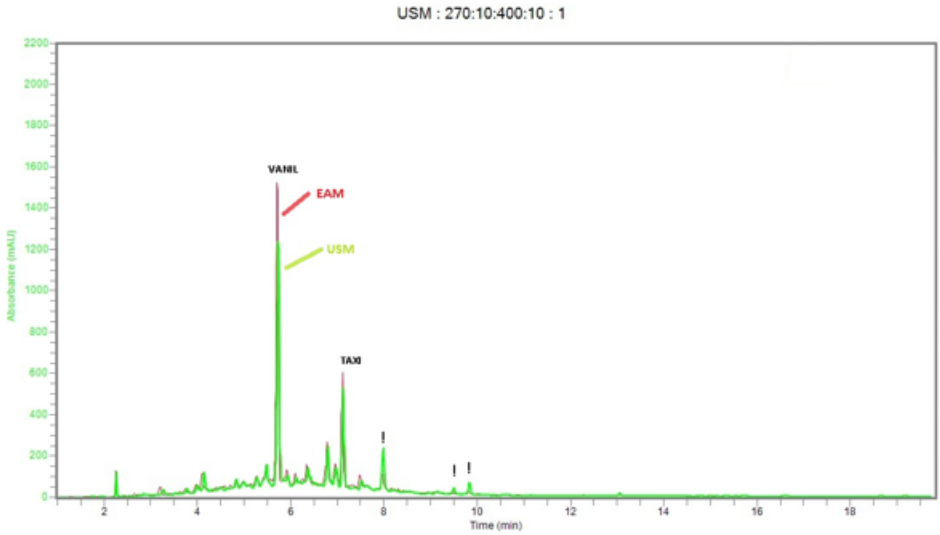

Fig. 1. Overlaid chromatograms of EAMand USM at 270 nm

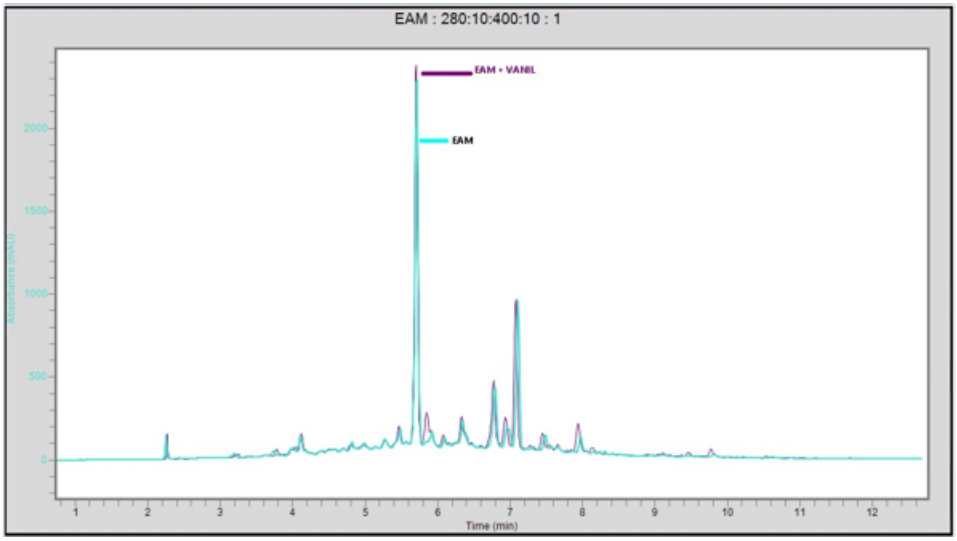

Fig.2. Overlaid chromatograms of EAM and EAM + VANIL

Bacteria	MICs – aqueous extract (EAM - mg/mL)	MICs – aqueous extract+US (USM - mg/mL)
(A) *Staphylococcus aureus*	3.75	7.5
(B) *Methicillin-resistant Staphylococcus aureus*(MRSA)	**0.23**	**0.23**
(C) *Klebsiella pneumoniae* ATCC 13883	7.5	7.5
(D) *Pseudomonas aeruginosa* ATCC 27853	15	15

Table 2
MINIMUM INHIBITORY CONCENTRATIONS (mg/mL) OF POLYPHENOLIC EXTRACTS AGAINST THE TESTED BACTERIA

quantity in EAM compared to USM. Unknown peaks at 8 min, 9.95 min, respectively are grater in USM. Additional peak appears in USM extract at 9.5 min. To prove the existence of VANIL in EAM, 1 mL extract EAM was marked with 20 µLstandard of VANIL. The chromatogram ilustrated below (fig. 3) shows that VANIL is certainly present in this extract (the peak which represents VANIL appears at the same retention time in both chromatograms).The presence of vanillic acid in EAM extracts in significant quantities were proved.

Microbiological activity

The minimum inhibitory concentrations (MICs) of polyphenolic extracts (EAM and USM) required for growth inhibition of the Gram-negative and Gram-positive bacteria are presented in table 2. It was found that both extracts have antibacterial capacity against *MSSA* and in specially for *MRSA,* with MICs of 0.23 mg/mL.The effect of polyphenolic extracts on Gram-negative bacteria was at a concentration of 7.5 mg/mL for *Klebsiella pneumoniae*, and 15 mg/mL for *Pseudomonas aeruginosa.*

As show in table 3, when the growth medium was enriched with both tested solutions (EAM and USM) the bacterial growth is significantly inhibited within six hours, compared with Control. At H1 time (after 3 h of incubation), spruce bark extract (EAM and USM) inhibited both MSSA and MRSA bacteria. After six of incubation both extracts presented bactericidal effect on *Staphylococcus aureus* (MSSA and MRSA) (fig. 3a,b and fig 4a.b). Aside the bactericidal effect the growth rate (r) was negtively affected by EAM and USM.

In the case of Gram-negative bacteria, after three hours of incubation, growth of all was significantly reduced (table

Tested solution	Bacteria strains	Experimental variants	CFU/ml			Growth rate (h^{-1})	Generation time (min)
			H0	H1	H2	r	g
EAM	*Staphylococcus aureus*	EAM	3.4×10^4	3.6×10^4	0	N/A	N/A
		Control	4.4×10^4	2.2×10^6	2.0×10^8	1.40	29.61
	Staphylococcus aureus, MRSA	EAM	6.0×10^4	7.0×10^4	0	N/A	N/A
		Control	7.6×10^4	5.4×10^5	1.4×10^8	1.26	33.11
	Klebsiella pneumoniae	EAM	4.2×10^4	1.8×10^5	3.8×10^7	1.13	36.68
		Control	7.0×10^4	3.0×10^6	4.0×10^8	1.44	28.87
	Pseudomonas aeruginosa	EAM	5.6×10^4	2.5×10^5	0	N/A	N/A
		Control	2.4×10^5	1.7×10^6	7.8×10^7	0.97	43.03
USM	*Staphylococcus aureus, MSSA*	USM	5.0×10^4	9.6×10^4	0	N/A	N/A
		Control	8.6×10^4	2.3×10^6	1.9×10^8	1.28	32.47
	Staphylococcus aureus, MRSA	USM	7.0×10^4	8.0×10^5	0	N/A	N/A
		Control	4.8×10^4	1.9×10^6	1.6×10^8	1.35	30.80
	Klebsiella pneumoniae	USM	2.8×10^4	2.8×10^4	0	N/A	N/A
		Control	9.8 x 104	3.2×10^6	4.3×10^8	1.40	29.71
	Pseudomonas aeruginosa	USM	4.0×10^4	4.0×10^5	2.0×10^5	0.27	155.01
		Control	2.8×10^4	1.1×10^5	1.7×10^7	1.06	39.07

Table 3
DATA PRESENTING THE GROWTH RATE OF TESTED BACTERIA IN PRESENCE OF EAM AND USM ($p < 0.0001$)

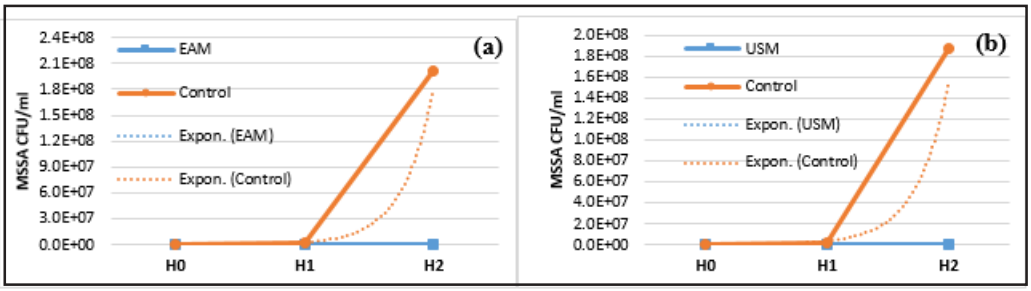

Fig. 3.Thegraphics representation of the growth rate for MRSA in the presence of EAM (a) and USM (b) comparing to the Control

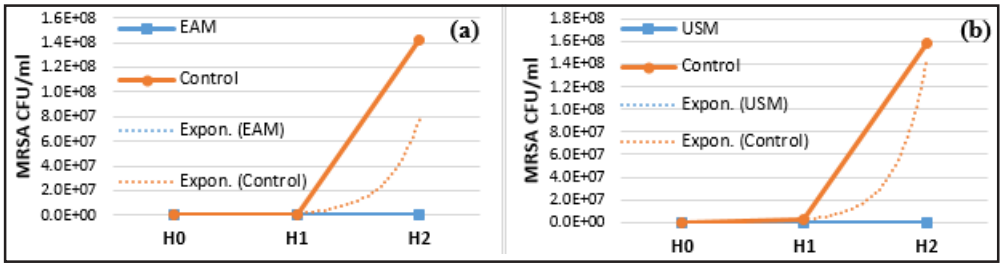

Fig. 4.Thegraphics representation of the growth rate for MSSA in the presence of EAM (a) and USM (b) comparing to the Control

3). At H2 time, USM presented bactericidal effect on *Klebsiella pneumoniae* (fig. 5b) and *Pseudomonas aeruginosa* (fig. 6b). EAM presented bactericidal effect only on *Pseudomonas aeruginosa* (fig. 6a)and further inhibited growth of *Klebsiella pneumoniae* (fig. 5a).

The reason for antimicrobial activity of EAM and USM extracts lies in phenolic nature. Phenolic compounds such as vanillic acid and taxifolin are known to affect the cell membranes of bacterial cells.The antibacterial activity of any compound from EAM/USM (VANIL or TAX) against Gram-positive and Gram-negative bacteria is partly due to their ability to reach the site of action. In accord withother research, the major processes underlying the antibacterial effects are: disruption of the bacterial cell membrane, generation of reactive oxygen species, penetration of the bacterial cell membrane and including interactions with DNA and proteins followed by induction of intracellular antibacterial effects [1].

Relatively little is known about the antibacterial activity of vanillic acid. Delaquis et al. [18] recently showed that the human pathogen *Listeria monocytogenes* is strongly inhibited by vanillic acid. Inhibition of a nonpathogenic *E. coli* strain has been reported [19]. In other study [20] a complete eradication of the inoculum was achieved after 7 days in juices supplemented with 10 mM vanillic acid. Populations fell at a faster rate at 15$^{R^{\circ}}$C, and the bacterium was not detected after 7 days in juices containing 10 mM vanillic acid.

The investigations of Yemis et al., [21] revealed that vanillic acid have direct bactericidal effects, inhibit the growth, and lessen the heat resistance of *Cronobacter* species in a microbiological medium. The thermal resistance of *Cronobacter* species was clearly diminished by the presence of vanillic acid.

In other research [22] was revealed that *Pinus pinaster* bark extract at the concentration 200 mg mL^{-1} showed bactericidal activity against two multidrug-resistant clinical isolates of *Acinetobacter baumannii* and antibacterial activity at lower concentrations, in vitro. This study reveald that *Pinus pinaster* bark extract contents vanillic acid, catechin, caffeic acid, ferulic acid and taxifolin. The conclusion of this study was that *Pinus pinaster* bark extract and its components could potentially find an application in treatment of multidrug-resistant *Acinetobacter baumannii*-infected patients. Thus, the presence of compounds such as vanillic acid and taxifolin, (found in EAM/USM) often reported as having antibacterial activity can be responsible for depletion of bacteria resistance mechanisms leading to increment in their susceptibility to these compounds.

It was found that, flavonoids have a strong ability to link with bacteria cell walls from complexes [23, 24] affecting the bacteria growth and survival. Thus, vegetal extracts with high content of phenolic compounds, like spruce bark aqueous extract, can be very useful when used in a

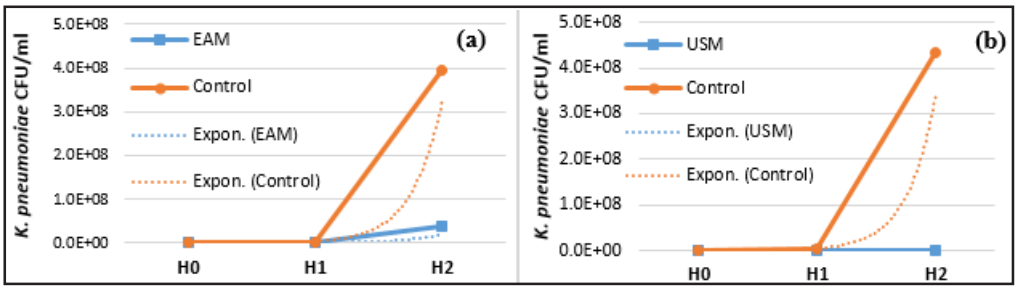

Fig. 5.The graphics representation of the growth rate for *Klebsiella pneumoniae*in the presence of EAM (a) and USM (b) comparing to the Control

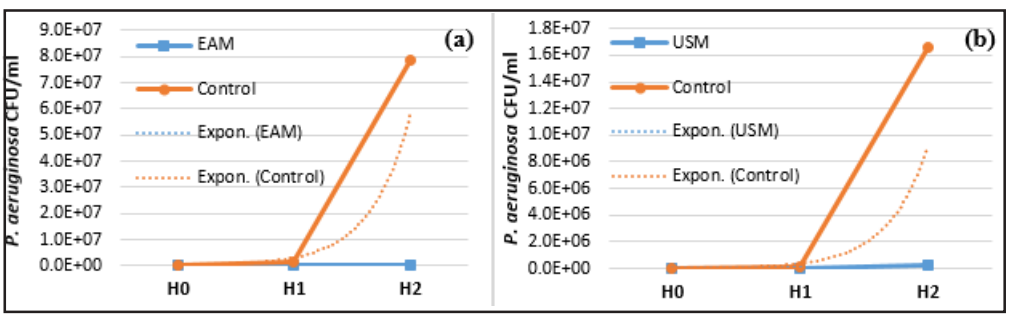

Fig. 6. The graphics representation of the growth rate for *Pseudomonas aeruginosa* in the presence of EAM (a) and USM (b) comparing to the Control

complementary therapy with commercial drugs due to their bacteriostatic effect.

Today, when bacteria are developing resistance to many types of antibiotics, it is very difficult to fight infectious diseases and cure patients. Natural extract are available alternative to antibiotics and appear to have high potential to solve the problem of the emergence of resistant bacteria. The current research of the antibacterial activity may contribute to the development of efficient antibacterial products.

Conclusions

The results of this study reveal that the spruce bark extract (obtaining by hot water with or without ultrasounds) contains a considerable amount of phenolic compounds. The compounds identified in samples, by HPLC, were vanilic acid, and taxifolin in both extract. Our results have shown that EAM/USM can be effective against both MRSA and MSSA and for Gram-negative bacterialike *Pseudomonas aeruginosa* and *Klebiella pneumoniae*, due to vanillic acid and taxifolin, which can act synergistically with each other against those bacteria. In the same time, antimicrobial spectra and activities of the EAM/USM fractions and pure isolated compounds from the spruce bark, can be suggestedthe use of this plant in pharmaceutical preparations and as well as a food preservative.By demonstrating antimicrobial capacity of polyphenolic extracts, we can follow a new direction of research, namely reducing the pharmacological resistance of microorganisms to antibiotics, by using polyphenolic extracts.Further research is needed to elucidate accurately the pathways and mechanisms used by these compounds against bacteria.

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