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Antimicrobial and antioxidant activity of the vegetative and reproductive organs of *Robinia pseudoacacia*

IOANA C. MARINAS[1], ELIZA OPREA[2]*, ELISABETA-IRINA GEANA[3], CARMEN CHIFIRIUC[1] and VERONICA LAZAR[1]

[1]*University of Bucharest, Faculty of Biology, Microbiology Department, 1–3 Portocalilor Way, Bucharest, Romania,* [2]*University of Bucharest, Faculty of Chemistry, 4–12 Regina Elisabeta, 030018 Bucharest, Romania and* [3]*National R & D Institute for Cryogenics and Isotopic Technologies – ICIT Rm. Valcea, 4 Uzinei Street, PO Raureni, 240050 Rm. Valcea, Romania*

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Abstract: This study was aimed at investigating the antimicrobial and antioxidant activity of ethanol extracts obtained from the leaves, seeds and sheaths of *Robinia pseudoacacia*. Total phenolic content (*TPC*, Folin–Ciocalteu method), antioxidant activity (trolox equivalent antioxidant capacity (*TEAC*) assay) and antimicrobial activity (agar disk diffusion method and broth dilution method) of the vegetative and reproductive organs of *R. pseudoacacia* were determined. The highest content of polyphenols (expressed as gallic acid equivalents, GAE) was found in the extract of *R. pseudoacacia* leaves (266.7 μg GAE mL^{-1} extract) followed by the extract of the seeds (232.2 μg GAE mL^{-1} extract). HPLC analysis showed the presence of catechin (0.925 μg mL^{-1}), rutin (0.831 μg mL^{-1}), resveratrol (0.664 μg mL^{-1}) and quercetin (0.456 μg mL^{-1}) in the leaf extract, and catechin (0.127 μg mL^{-1}), epicatechin (0.239 μg mL^{-1}) and rutin (0.231 μg mL^{-1}) in the seed extract. The results showed that the studied extracts exhibited a selective antimicrobial effect directed against Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*) bacterial strains. The combination leaf extract/antibiotic had the highest synergistic effect when compared to combinations with seed and sheath extracts. The same extract with penicillin G, kanamycin and rifampin had highest synergetic effect against methicillin-resistant *S. aureus* strain (MRSA), a strain that has gained great interest of microbiologists within the past decades. The chemical characterization of ethanol extracts from the vegetative and reproductive organs of *R. pseudoacacia*, the synergistic effects of certain antibiotics and acacia extracts and the potential to increase the antimicrobial activity of some commercial antibiotics against MRSA were investigated for the first time.

* Corresponding author. E-mail: eliza.oprea@g.unibuc.ro
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INTRODUCTION

Robinia pseudoacacia (black locust) is an invasive species transformer that is gaining increasing abundance throughout Europe. It is included on the list of the most dangerous invasive species due to its ability to spread quickly and its high growth rate, forming mono-dominant forests. Few species are tolerant to the allelopatic substances (robetrin, myricetin and quercetin) found in the ethanol extracts from the leaves of acacia.[1,2] Acacia plants (species) are still spreading and invading spontaneous vegetation types which are important and valuable. This species was brought from North America and planted for ornamental and melliferous purposes due to its quality in stabilizing eroded field slopes and sands.[1]

According to the literature, *R. pseudoacacia* contains polyphenolic compounds, such as tannins[3] and taxon specific monoterpenes (robinlin).[4] In addition, some natural compounds with antibacterial activity have been identified in the leaves.[5,6] Some phytocompounds of this species play a vital role in protecting against pathogen or other biotic attacks, being responsible for the natural durability of the solid wood of *R. pseudoacacia*.[7] It was reported that acacia bark has exceptional resistance to biodegradation, this property being assigned to its concentration of dihydrobinetin and robinetin.[8] Essentially the flavonoids with allelopatic properties were detected in high concentrations in willow bark including the chalcones: robtein (2′,3,4,4′,5-pentahydroxychalcone), butein (2′,3,4,4′-tetrahydroxychalcone) and 2′,4,4′-trihydroxychalcone; the flavanones: L-robtin ((2*S*)-3′,4′,5′,7-tetrahydroxyflavanone), ((2S)-3′,4′,7-trihydroxyflavanone) and liquiritigenin ((2S)-4′,7-dihydroxyflavanone); flavanonols: D-dihydrorobinetin ((2*R*,3*R*)-3,3′,4′,5′,7-pentahydroxyflavanone), which is the major component of the bark, and D-fustin ((2*R*,3*R*)-3,3′,4′,7-tetrahydroxyflavanone); flavonols: robinetin (3,3′,4′,5′,7-pentahydroxyflavone) and fisetin (3,3′,4′,7-tetrahydroxyflavanone), flavan-3-ol: L-robinetinidol (3,3′,4′,5′,7-pentahydroxy-2,3-*trans*--flavan), flavan-3,4-diols: leucorobinetinidin (3,3′,4,4′,5′,7-tetrahydroxyflavanhexol) and D-3,3′,4,4′,7-pentahydroxy-2,3-*trans*-3,4-*cis*-flavan. In addition, heartwood contains *β*-resorcylic acid and methyl *β*-resorcylate.[9–11] Traditionally, *R. pseudoacacia* flowers are used in medicine as antispasmodic agents, for soothing the feelings of heartburn, to reduce gastric hyperacidity, as a mild sedative and a cholagogue.[12] In addition, the content of polyphenols present in the flowers create a strong antioxidant potential.[13]

The antimicrobial effect of polyphenols could be caused or facilitated by a significant electrical charge, the redox potential or through the free radical scavenging activity of antioxidants.[14,15] This hypothesis demonstrated that the anti-

microbial effects of the derived polyphenols cause structural or functional damage to the bacterial cell membrane.[14] Microbial cells are negatively affected by derived substances from plants *via* various mechanisms of action that attack the phospholipid bilayer of the cell membrane and disrupt enzyme systems.[16] According to the literature,[17,18] a number of polyphenols (catechin, epicatechin, rutin and quercetin) that have been identified in some vegetal extracts show antimicrobial activity.

There are not data on HPLC analysis or the total phenol content of ethanolic extracts of *R. pseudoacacia*. Data on the chemical composition of acacia extracts are scarce. An aqueous extract was used to isolate five compounds from acacia leaves: luteolin 7-*O*-*β*-D-glucuronopyranosyl-(1→2)[*α*-L-rhamnopyranosyl-(1→6)]-*β*-D-glucopyranoside, apigenin 7-*O*-*β*-D-glucuronopyranosyl-(1→2)[*α*-L--rhamnopyranosyl-(1→6)]-*β*-D-glucopyranoside, acacetin 7-*O*-*β*-D-glucuronopyranosyl-(1→2)[*α*-L-rhamnopyranosyl-(1→6)]-*β*-D-glucopyranoside, acacetin 7-O-*β*-D-glucopyranosyl-(1→2)[*α*-L-rhamnopyranosyl-(1→6)]-*β*-D-glucopyranoside and acacetin 7-O-*β*-D-xylopyranosyl-(1→2)[*α*-L-rhamnopyranosyl-(1→6)]--*β*-D-glucopyranoside. These compounds were characterised by spectroscopic and chemical methods.[19]

In 2000, secundiflorol, mucronulatol, isomucronulatol, and isovestitol, identified by spectral analyses, were reported for the first time from this species in an ethanolic extract of the whole plant of *R. pseudoacacia*.[20]

Some literature data concerning antimicrobial activity were found. A low molecular weight cationic peptide was isolated from *R. pseudoacacia* seed and its *in vitro* antibacterial activity was investigated. The peptide inhibited the growth of the tested strains and *S. aureus* was found to be the most sensitive strain compared with others strains (*Corynebacterium michiganense*, *Bacillus subtilis*, *Erwinia carotovora*, *Pseudomonas syringae*, *Xanthomonas campestris* and *Escherichia coli*).[21]

Poor data on the antioxidant activity of *R. pseudoacacia* extracts exists. The antioxidant activity of lyophilized extracts of acacia leaves was evaluated by the oxygen radical absorbance capacity (ORAC) assay. The extract had a lower antioxidant capacity (1940 μmol trolox equivalent g^{-1}) compared with the other tested plants *Rhus typhina* (4651), *Acer rubrum* (3805) and *Rosa multiflora* (2533).[22]

Therefore, the aim of this study was to obtain and determine the antimicrobial and antioxidant activity of plant extracts from different vegetal and reproduction organs of the species *R. pseudoacacia* and to demonstrate potential synergistic effects with antibiotics commonly used in the clinic. The chemical characterization of the ethanol extracts from the vegetative and reproductive organs of *R. pseudoacacia*, synergistic effects of certain antibiotics and acacia extracts were realized for the first time. The potential to increase antimicrobial

activity of some commercial antibiotics against MRSA is very important because these strains produce nosocomial infections, which has become an area of great interest for microbiologists in the past decades.

EXPERIMENTAL

Plant material. The plants were harvested during the physiological maturity period (July–September) from a hilly area (Calimanesti – Valcea). The vegetative and reproductive organs taken into consideration for the *R. pseudoacacia* species were the leaves, seeds and sheaths. The plants were manually sorted and dried at room temperature. The species was identified by the Department of Botany and Microbiology from the Faculty of Biology, University of Bucharest. A voucher specimen was deposited in the Herbarium of the Botanical Gardens "Dimitrie Brandza" of the University of Bucharest (No. 400636).

Alcoholic extracts. 4g of dried plant material was extracted with 50 mL 70% ethanol. The extraction was performed without heating using an ultrasonic bath (Elma Sonic 80H), with frequencies ranging from 20 kHz to 2000 kHz, which increases the permeability of the cell walls causing cell lysis, thereby enabling the extraction of the biologically active compounds. The extract thus obtained was filtered. The plant material residual was extracted for three times and brought to 300 mL with the same solvent. The extracts were stored in tightly closed containers at 4 °C and away from sunlight.

Determination of total phenols (TPC). The total phenols content (TPC) was determined by the Folin–Ciocalteu method[23] by mixing 0.5 mL of sample or standard (gallic acid) with 5 mL of Folin–Ciocalteu reagent and 4 mL of 1 M sodium carbonate solution. The absorbance was measured at wavelength of 746 nm. The calibration curve was constructed using standard solutions of gallic acid in the concentration range from 5 to 150 mg L^{-1}. The equation of the standard curve was $y = 0.0061x + 0.0057$ ($R^2 = 0.9988$). The TPC was expressed as mg of gallic acid equivalents (GAE) in one gram of the plant and in 1 mL extract.

HPLC analysis. All standards (gallic acid, (+)-catechin, (−)-epicatechin, syringic acid, vanillin, *p*-coumaric acid, resveratrol, rutin and quercetin) were purchased from Sigma–Aldrich (Steinheim, Germany). Stock solutions of all the standards were prepared in methanol. Working standards were made by diluting the stock solutions with a mixture of methanol and water (50:50, *V*/*V*). Both stock and working standards were stored at 4 °C until further use. Formic acid, acetonitrile and methanol (LC grade) were obtained from Merck. Double distilled and demineralised water from a Milli-Q Millipore system (Bedford, MA, USA) was used for the preparation of the aqueous solutions. The phenolic compounds were evaluated by reversed phase-high performance liquid chromatography (RP-HPLC) with direct injection. Chromatographic analysis was performed with a Thermo Finnigan Surveyor Plus instrument equipped with a Surveyor Photodiode Array Detector (PDA), a Surveyor autosampler, a Surveyor LC Pump (Quaternary gradient) and a Chrome Quest Chromatography workstation. The separation was performed at 30 °C with Accuacore PFP (2.6 μm, 100 mm×2.1 mm) column. The flow rate was 0.4 mL min^{-1} and an injection volume 1 μL. Gradient elution of two solvents was used: solvent A consisted of water with 0.1 % formic acid and solvent B: acetonitrile with 0.1 % formic acid. The gradient programme used is given Table I. Detection was made at 280 nm.

The *R. pseudoacacia* extracts were injected into HPLC system after filtering through a 0.45 μm pore size membrane filter. The amount of phenolic compounds in the extracts were calculated as mg L^{-1} extract using external calibration curves, which were obtained for each phenolic standard. The linearity of the method was between 0 – 50 mg/L, for each compound.

Each determination was realized in triplicate and the mean is reported. Blank solution and control samples were analyzed in order to monitor performance related to variable factors or random error.

TABLE I. Solvent gradient conditions with linear gradients

Time, min	Solvent A content, %	Solvent B content, %
Initial	98	2
30	70	30
35	25	75
40	98	2
50	98	2

TEAC assay (trolox equivalent antioxidant capacity). The method is based on the ability of antioxidants to quench the long-lived $ABTS^{\bullet+}$, a blue–green chromophore with a characteristic absorption at 734 nm. The addition of antioxidants to the preformed radical cation reduces it to ABTS, causing a decolourization. A stable stock solution of $ABTS^{\bullet+}$ was produced by reacting an aqueous solution of ABTS with potassium persulphate. Then, the mixture was left standing in the dark at room temperature for 12–16 h before use. An $ABTS^{\bullet+}$ working solution was obtained by dilution of this solution with ethanol to an absorbance of around of 0.70, according to Pellegrini *et al.*[24]. The results for the test compounds were expressed relative to trolox, in mmol of trolox per mL extract.

Evaluation of the antimicrobial activity of the studied phytochemical mixtures. The antimicrobial activity was tested using reference and clinically isolated microbial strains, belonging to Gram-positive, (*Staphylococcus aureus*, *Bacillus subtilis* and *Enterococcus faecalis*), Gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*) bacteria and yeasts (*Candida famata*, *C. utilis* and *C. albicans*). These strains are part of the collection of the Laboratory of Microbiology, Faculty of Biology, University of Bucharest, Romania.

Qualitative screening of the antimicrobial activity. In order to test the degree of antimicrobial activity, microbial suspensions were adjusted to 1.5×10^8 CFU mL^{-1} 0.5 McFarland standard from 18–24 h cultures grown on solid medium. The antimicrobial activity was determined by the disc diffusion method. Stock solutions that were used to test the quality of antimicrobial activity were the alcoholic plant extracts and also solvent control, ethanol 70 %. The obtaining of a mixed inhibition area at the spot level of the culture area was interpreted as a positive result.

*Quantitative assessment of the minimal inhibitory concentration (*MIC*).* Quantitative analysis was performed by the binary serial microdilution method in liquid medium (broth for bacteria and Sabouraud for yeasts) in 96-well plates. The concentration range of the working stock solutions for alcoholic extracts was from 0.78 to 400 μL mL^{-1}. Simultaneously, serial dilutions were made with 70 % ethanol under the same working conditions in order to obtain a negative control. Each well was inoculated with 10 μL microbial suspension adjusted to 1.5×10^8 CFU mL^{-1} 0.5 McFarland standard from 18–24 h grown cultures. The *MIC* values were established both macroscopically, as the last concentration at which no microbial growth was observed, and spectrophotometrically. The absorbance of the microbial cultures was measured at 620 nm using an Apollo LB 911 spectrophotometer. An amount of 5 μL of the *R. pseudoacacia* leaves extracts of different concentrations from 200 to 6.25 μL mL^{-1} were spotted on solid medium to identify the minimal microbicidal concentration after 18–24 h.

Influence of plant extracts on the microbial adherence capacity to an inert substrate. Following the quantitative analysis protocol of the antimicrobial effect on adherence, the adherence of a biofilm biomass was assessed after fixation with cold methanol (5 min) and crystal violet staining (0.1 % concentration for 15 min) using the microtitre method. The optical density of the biological material resuspended in 33 % CH_3COOH (under stirring with Optic Ivymen System at 150 rpm for 15 min) was determined by reading the absorbance at 490 nm. The inert substrate was 96-well plates.

Sensitivity of bacterial strains to the alcoholic extracts and antibiotics commonly used in clinic. The antibiotic discs alone or with 10 μL of the stock solution of alcohol extracts were placed on the previously seeded solid media, as for a classic antibiogram*, using as controls simple antibiotic and antibiotic impregnated with 70% ethanol. Standard antibiotic discs were chosen according to the Clinical and Laboratory Standards Institute (CLSI)** and literature data.[25,26]

RESULTS AND DISCUSSION

The results concerning the total phenols and total antioxidant capacity are presented in Table II.

TABLE II. Content of total phenols (*TPC*) and the *TEAC* value of the *R. pseudoacacia* extracts

No.	Parameter	*R. pseudoacacia* leaf extract	*R. pseudoacacia* sheath extract	*R. pseudoacacia* seed extract
1	*TP* / μg GAE mL^{-1} extract	266.7	56.7	232.2
2	*TEAC* / mmol trolox mL^{-1} extract	902.17	172.91	625.23

The highest content of polyphenols was found in the leaf extract of *R. pseudoacacia* (266.7 μg mL^{-1} plant extract), compounds known for their strong antioxidant activity. HPLC chromatograms for the standard solution and for *R. pseudoacacia* leaf extract are presented in Figs. 1 and 2, respectively. By HPLC, four polyphenolic compounds were identified in the leaf extract, which represent 1.078 % of the TPCs quantified by the Folin–Ciocalteu method, and three compounds in the seed extract, which represent 0.257 % of the quantified TPCs. (These percentages were calculated by dividing the amount of phenols as determined by HPLC analysis at the total quantity of phenols obtained by Folin–Ciocalteu method). The identified polyphenolic compounds were catechin, rutin, resveratrol and quercetin in the leaf extract, and catechin, epicatechin and rutin in the seed extract, Table III). The major components are catechin for leaves extract and epicatechin for seeds extract. None of these compounds were identified in the sheath extract. Besides identified phenols in *R. pseudoacacia* leaf extract, HPLC analysis shows chromatographic separation of other compounds with a

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** CLSI. Performance Standards for Antimicrobial Susceptibility Testing, M100-S24, Clinical and Laboratory Standards Institute, Wayne, PA, 2014.

significant peak area whose identification was not possible due to the lack of the standards. In our future studies, we will increase the number of identified and quantified phenolic compounds in plant extracts.

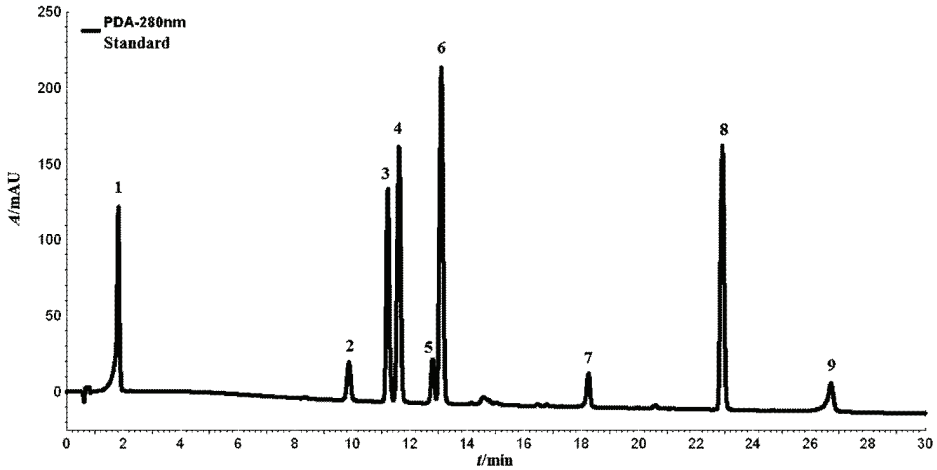

Fig. 1. HPLC chromatogram of standard solution. Peaks identification: 1 – gallic acid; 2 – (+)-catechin; 3 – syringic acid; 4– vanillin; 5 – (–)-epicatechin; 6 – *p*-coumaric acid; 7 – rutin; 8 – resveratrol; 9 – quercetin. Separation conditions: Accuacore PFP (2.6 μm, 100 mm×2.1 mm) column, temperature: 30 °C, flow rate: 0.4 mL min^{-1}, injection volume: 1 μL, gradient elution: solvent A consisted of water with 0.1 % formic acid and solvent B: acetonitrile with 0.1 % formic acid.

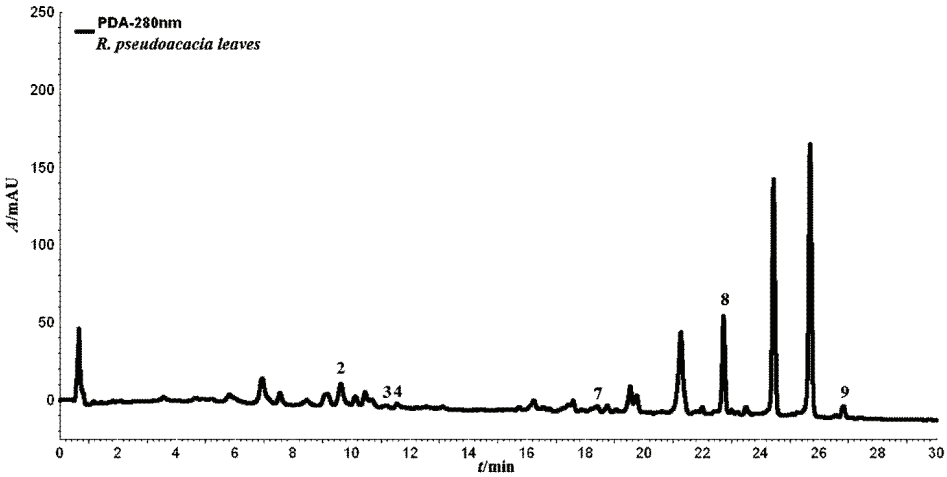

Fig. 2. HPLC chromatogram of *R. pseudoacacia* leaf extract. Peaks identification: 2 – (+)-catechin; 3 – syringic acid; 4 – vanillin; 7 – rutin; 8 – resveratrol; 9 – quercetin. Separation conditions: as in the legend to Fig. 1.

TABLE III. The concentration of polyphenols derivatives (μg mL^{-1} extract) in the hydro-alcoholic extracts of the vegetative and reproduction organs of *R. pseudoacacia*; bdl – below the instrumental detection limit

R. pseudo-acacia sample	Derivative								
	Gallic acid	Cate-chin	Syringic acid	Vanil-lin	Epica-techin	*p*-Couma-ric acid	Rutin	Resve-ratrol	Quer-cetin
Leaves	bdl	0.925	bdl	bdl	bdl	bdl	0.831	0.664	0.456
Sheaths	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
Seeds	bdl	0.127	bdl	bdl	0.239	bdl	0.231	bdl	bdl

The results of the qualitative assessment of the antimicrobial activity of the alcoholic extracts are given in Table IV. According to the qualitative assay, the alcoholic extracts showed different diameters of the inhibition zones for *K. pneumoniae* (14–16 mm for the leaf extract), *S. aureus* (8–12 mm for the leaf extract), *P. aeruginosa* (7–10 mm for the sheath extract) and *Candida* sp. (8–14 mm for all extracts) and lower than 6 mm for the solvent control. In addition, the 70 % ethanol solvent control showed some activity against *K. pneumoniae* strains, *A. baumannii* and *Candida* sp. These results are in accordance with literature data, *i.e.*, a 90 % ethanolic extracts of *R. pseudoacacia* leaves and barks were shown to be active against *E. coli*, *P. aeruginosa*, *Proteus vulgaris*, *Salmonella choleraesuis* and *C. albicans* strains.[27]

TABLE IV. Qualitative and quantitative assessment of the antimicrobial activity of alcoholic extracts obtained from different parts of the species *R. pseudoacacia*; –: 5 mm (disc diameter); +: 6–10 mm; ++: 11–20 mm

Microbial strain	Leaves		Sheaths		Seeds		Solvent control (ethanol 70 %)	
	Qualit. (5 μL)	*MIC* μL mL^{-1}	Qualit. (5 μL)	*MIC* μL mL^{-1}	Qualit. (5 μL)	*MIC* μL mL^{-1}	Qualit. (5 μL)	*MIC* μL mL^{-1}
S. aureus ATCC 6538	++	50	–	–	–	–	–	200
S. aureus MRSA 1263	+	200	–	–	–	–	–	400
B. subtilis 6683[a]	+	100	+	100	+	100	–	200
B. subtilis 12488[a]	+	200	+	200	+	400	–	400
E. coli ATCC 8739	–	–	–	–	–	–	–	200
E. coli $O_{126}B_{16}$	+	200	–	–	–	–	–	400
K. pneumoniae 134202[a]	++	50	–	–	–	–	+	200
K. pneumoniae 11[a]	++	50	–	–	+	–	+	200

TABLE IV. Continued

Microbial strain	Leaves		Sheaths		Seeds		Solvent control (ethanol 70 %)	
	Qualit. (5 μL)	*MIC* μL mL^{-1}	Qualit. (5 μL)	*MIC* μL mL^{-1}	Qualit. (5 μL)	*MIC* μL mL^{-1}	Qualit. (5 μL)	*MIC* μL mL^{-1}
P. aeruginosa ATCC 27853	++	50	+	100	+	50	–	200
P. aeruginosa 13202[a]	+	200	+	200	+	100	–	400
E. fecalis ATCC 29212	+	100	+	100	–	–	–	200
A. baumannii 77 sc	+	200	++	50	++	25	+	200
C. utilis	++	50	++	100	++	100	+	200
C. famata	++	50	+	200	+	200	–	200
C. albicans 945[a]	++	50	+	100	+	100	+	200
C. albicans 393[a]	+	200	+	100	+	100	+	200

[a]The clinical microbial strains were introduced in our laboratory collection from hospital units where they received these registration codes

Strains that were shown to be susceptible to the studied extracts in the qualitative screening were used for *MIC* determination of the extracts. Comparisons between *MIC* values of the extracts from vegetative and reproductive organs and also the differences between them and the solvent used are given in Table IV. Quantitative analysis indicated a moderate antibacterial activity of the alcoholic extracts of *R. pseudoacacia* (the *MIC* values ranged between 50–400 μL mL^{-1}) but only the leaf extract, which had the most active compounds, showed antibacterial activity on the MRSA strain. In case of the alcoholic extracts obtained from *R. pseudoacacia* leaves, a precipitate was observed which prevented both macroscopic and spectrophotometric determination of the *MIC* value. Thus, for the *MIC* determination, the microbial culture grown in the presence of the *R. pseudoacacia* leaf extract concentrations of 200 to 6.25 μL mL^{-1} was spotted on solid medium.

Microorganisms involved in invasive infections usually produce extracellular capsular polysaccharides known as capsules, slime or glycocalyx. The slime term is usually used to refer to closely related bacterial cell exopolysaccharides, which increase the viscosity of the liquid culture medium seeded with strains possessing the virulence factor. The production of slime is involved in the adhesion and colonization of medical devices.[28] Regarding the influence on the ability of adhesion, there was a significant decrease in absorbance measured at the wavelength of 490 nm. The influence of plant extracts on the microbial adherence to an inert substrate resulted in inhibition of microbial biofilm development. These results suggest the ability of the alcoholic extracts to interfere with

the expression of microbial adhesions involved in the initial step of colonization, essential for the initiation of an infectious process; thus, the synthesis of adhesins is therefore an important virulence factor for pathogenic microorganisms. Adhesion is a major ecologic advantage for the pathogenic bacteria in terms of provision of nutrients, protection from antibodies and lysozyme, *etc*. Microbial growth after adhesion occurs at a rate much higher than that of non-adherent cells.[29] The ability of adhesion to an inert substrate was inhibited by extract concentrations ranging from 12.5 to 100 μL mL^{-1} (Table V). The main virulence factor involved in the microbial pathogenesis lies in their ability to adhere to various cellular and inert surfaces, by microbial adhesins. Numerous studies showed that the inhibition of a single adhesin may often be sufficient to produce non-virulent pathogenic microorganisms. This led to the exploration of the interference with the activity of adhesins, as a strategy for the treatment of bacterial infections.[30] The development of a biofilm is closely related to the pathogenicity of the microorganisms and their sensitivity to antibiotics, and is also influenced by other factors, such as enzymatic factors, hydrophobicity *etc*.[31]

TABLE V. Quantification of the inhibitory effect of alcoholic extracts from various parts of *R. pseudoacacia* species on the adherence capacity (μL mL^{-1}) of the studied strains to the inert substratum

Microbial strains	*R. pseudoacacia* leaves	*R. pseudoacacia* sheaths	*R. pseudoacacia* seeds	Solvent control (ethanol 70%)
S. aureus (S.a.) ATCC 6538	12.5	–	–	100
MRSA 1263	100	–	–	200
B. subtilis (B.s.) 6683	50	50	50	100
B. subtilis (B.s.) 12488	50	100	100	200
E. coli (E.c.) $O_{126}B_{16}$	100	–	–	200
K. pneumoniae (KPN) 134202	25	–	–	100
K. pneumoniae (KPN) 11	25	–	–	100
P. aeruginosa (P.a.) ATCC 27853	25	50	25	100
P. aeruginosa (P.a.) 13202	50	100	50	200
E. faecalis ATCC 29212	25	50	–	100
A. baumannii 77	100	25	12.5	100
C. utilis	25	50	50	100
C. famata	25	100	100	100
C. albicans 945	12.5	50	50	100
C. albicans 393	50	50	50	100

The alcoholic extract of the *R. pseudoacacia* leaves potentiated the antimicrobial activity of currently used antibiotics against the nine tested bacterial strains: *E. coli*, *K. pneumoniae*, *B. subtilis*, *S. aureus* and *P. aeruginosa*. The most significant potentiation effects were observed in case of *E. coli* $O_{126}B_{16}$ for trimethoprim–sulfamethoxazole (SXT), ticarcillin–clavulanic acid (TIM) and piperacillin–tazobactam (TZP); *K. pneumoniae* 134202 for cefotaxime (CTX), aztreonam (ATM) and cephalexin (LEX); *P. aeruginosa* 13202 for ciprofloxacin (CIP), piperacillin (PIP) and colistin (CST); *P. aeruginosa* ATCC for CIP, PIP, CST, ceftriaxone (CRO) and ofloxacin (OFX); *S. aureus* ATCC for CIP, erythromycin (ERY) and penicillin (PEN); MRSA for rifampin (RIF), kanamycin (KAN), ERY and PEN); *B. subtilis* 6683 for KAN and PEN); *B. subtilis* 12488 for cefaclor (CEC) and vancomycin (VAN). On the contrary, in the case of the *K. pneumoniae* 11 strain, the plant extracts decreased the activity of the used antibiotics. Noteworthy, for kanamycin, a very strong potentiation effect was observed in case of the MRSA strain (Table VI).

TABLE VI. Quantification of the differences between the diameters (mm) of the inhibition zones of microbial growth caused by *R. pseudoacacia* leaf extract and an antibiotic; solvent: ethanol 70 %; –: resistant strain; negative values: the combination solvent:antibiotic was more active than the combination extract:antibiotic; positive values: the combination extract:antibiotic was more active than the combination solvent:antibiotic

Antibiotic	MRSA	S.a. ATCC	B.s. 6683	B.s. ATCC	E.c. $O_{126}B_{16}$	KPN ATCC	KPN 11	P a. ATCC	P.a. 132404
Amoxicillin (AM)						–	–		
Aztreonam (ATM)						3	–1		
Cefaclor (CEC)			0	4					
Cefotaxime (CTX)					–2	2	14		
Ceftriaxone (CRO)								8	8
Cephalexin (LEX)						4	0		
Chloramphenicol (CHL)			0	2					
Ciprofloxacin (CIP)	0	2			–2	–2	18	17	12
Colistin (CST)CL								5	4
Erythromycin (ERY)	4	10							
Kanamycin (KAN)	22	0	2	0					
Ofloxacin (OFX)						2	–2	1	0
Oxacillin (OXA)	9	0						–	–
Penicillin G (PEN)	18	8	3	0					
Piperacillin (PIP)					–4			7	8
Piperacillin–tazobactam (TZP)					2				
Rifampin (RIF)	13	2							
Ticarcillin–clavulanic acid (TIM)					9			–	0
Trimethoprim–sulfamethoxazole (SXT)					15				
Vancomycin (VAN)			–2	1					

Among the tested antibiotics, the penicillin activity (acting on the synthesis of bacterial cell wall) and kanamycin activity (active on bacterial protein synthesis) were significantly improved on the MRSA strain by the active substances found in the alcoholic extract of *R. pseudoacacia* leaves. Oxacillin for *S. aureus* is often associated with resistance mechanisms that determine the deactivation of other classes of antibiotics (aminoglycosides, fluoroquinolones, macrolides, lincosamides, ketolides, phosphomycin and rifampicin).[32,33]

Alcoholic extracts of *R. pseudoacacia* sheaths and seeds had a lower synergistic effect than the leaf extract. An improvement observed in the antimicrobial effect of seeds extracts was obtained for *B. subtilis* strains in combination with PEN, CHL, CEC, KAN, VAN and teicoplanin (TEC); *P. aeruginosa* 13202 for PIP, CRO, norfloxacin (NOR), ATM and TIM, and *K. pneumoniae* 11 for TIM and ATM (Tables VII and VIII).

TABLE VII. Quantification of the differences between the diameters (mm) of the inhibition zones of microbial growth caused by antibiotic: *R. pseudoacacia* seed extract plus the antibiotic; solvent: ethanol 70 %; –: resistant strain; negative values: the combination solvent:antibiotic was more active than the combination extract:antibiotic; positive values: the combination extract:antibiotic was more active than the combination solvent:antibiotic

R. pseudoacacia seeds and:	Microbe				
	B.s. 6683	B.s. ATCC	KPN 11	P.a. ATCC	P.a. 132404
Amoxicillin (AMX)			–		
Aztreonam (ATM)			5		
Cefaclor (CEC)	8	10			
Cefotaxime (CTX)			–1		
Ceftriaxone (CRO)				1	1
Cephalexin (LEX)			–5		
Chloramphenicol (CHL)	2	0			
Colistin (CST)				–3	–4
Kanamycin (KAN)	–2	–7			
Nalidixic acid (NAL)			–2		
Norfloxacin (NOR)				0	0
Ofloxacin (OFX)			–8	–	–
Oxacillin (OXA)					
Penicillin G (PEN)	0	–7			
Piperacillin (PIP)				–1	3
Teicoplanin (TEC)	–1	–4			
Ticarcillin–clavulanic acid (TIM)				9	2
Vancomycin (VAN)	–2	–3			

The alcoholic extract of *R. pseudoacacia* sheaths proved to be less effective on the synergistic effect of the antibiotics tested, the differences between the diameters of the inhibition zone for the extract:antibiotic and solvent:antibiotic

ranging between –6 and 9 mm and no change from resistant to susceptible category being noticed (Table VIII).

TABLE VIII. Quantification of the differences between the diameters (mm) of the inhibition zones of microbial growth caused by antibiotic: *R. pseudoacacia* sheaths extract and antibiotic; solvent: ethanol 70 %; –: resistant strain; negative values: the combination solvent:antibiotic was more active than the combination extract:antibiotic; positive values: the combination extract:antibiotic was more active than the combination solvent:antibiotic

R. pseudoacacia sheath extract and:	Microbe			
	B.s. 6683	*B.s.* ATCC	*P.a.* ATCC	*P.a.* 132404
Cefaclor (CEC)	4	6		
Ceftriaxone (CRO)			1	2
Chloramphenicol (CHL)	–2	0		
Colistin (CST)			–3	–4
Kanamycin (KAN)	5	–3		
Norfloxacin (NOR)			–2	–1
Oxacillin (OXA)			–	9
Penicillin G (PEN)	1	–5		
Piperacillin (PIP)			1	1
Teicoplanin (TEC)	–4	–6		
Ticarcillin–clavulanic acid (TIM)			9	1
Vancomycin (VAN)	–5	–3		

According to recent data,[34] antibiotics can produce genetic changes in different ways, including an increase in free radicals within the cell. These results demonstrate that polyphenolic compounds could have a double effect of increasing the antimicrobial activity and neutralizing the free radicals formed by the use of antibiotics.

CONCLUSIONS

Ethanol extracts from the vegetative and reproductive organs of *R. pseudoacacia* were chemically characterised and the synergistic effects of certain antibiotics and the acacia extracts were investigated for the first time.

The phytochemical extract of *R. pseudoacacia* leaves (0.27 mg GAE mL^{-1} extract) proved to be the richest in polyphenols, known for their strong antioxidant capacity and possible antimicrobial activity. Through HPLC, four compounds from the leaf extract, which represented 1.078 % of the TPC, and three compounds from the seed extract, which represented 0.257 % of the TPC, were identified for the first time.

The alcoholic extracts of *R. pseudoacacia* showed antimicrobial activity towards the tested strains belonging to the Gram-positive (*S. aureus*, *B. subtilis* and *E. faecalis*) and Gram-negative (*P. aeruginosa*, *E. coli*, *K. pneumoniae* and *A. baumannii*) bacterial and the yeasts (*Candida* sp.) strains. The minimum inhibitory concentrations of stock solutions from the studied phytochemical alcoholic

mixtures ranged from 25–400 μL mL^{-1}. The *R. pseudoacacia* active principles extracted from the leaves were proved to be the most active in terms of antimicrobial activity. The tested plant extracts also inhibited adherence of the microbial cells and their ability to form biofilms on an inert substrate. However, further studies are required in order to establish their efficiency on pre-formed biofilms. The antimicrobial activities of antibiotics commonly used in the clinic were enhanced in the presence of the studied vegetative plant extracts. Among the tested antibiotics, the activities of penicillin, kanamycin and rifampin on an MRSA strain were significantly improved by the active substances from the alcoholic extract obtained from the leaves of *R. pseudoacacia* and furthermore conversion from resistant to susceptible was obtained. These results are of special interest nowadays because MRSA has become resistant to many antimicrobial agents, and currently represents a large problem in clinical infections.

This study has proved that the tested vegetative extracts could be used as therapeutic agents complementing antibiotherapy; such an approach enables potentiation of the activity of antibiotics with different mechanisms of action, highlighting the necessity for molecular studies in order to establish the influence of vegetal extracts upon gene expression on certain genes involved in microbial virulence and resistance. The results also demonstrated that polyphenolic compounds can have a dual effect, *i.e.*, increasing the antimicrobial activity and neutralizing the free radicals formed by the employed antibiotics.

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ИЗВОД

АНТИМИКРОБНА И АНТИОКСИДАТИВНА АКТИВНОСТ ВЕГЕТАТИВНИХ И РЕПРОДУКТИВНИХ ОРГАНА БИЉКЕ *Robinia pseudoacacia*

IOANA C. MARINAS[1], ELIZA OPREA[2], ELISABETA-IRINA GEANA[3], CARMEN CHIFIRIUC[1] и VERONICA LAZAR[1]

[1]*University of Bucharest, Faculty of Biology, Microbiology Department, Bucharest, Romania,* [2]*University of Bucharest, Faculty of Chemistry, Bucharest, Romania и* [3]*National R & D Institute for Cryogenics and Isotopic Technologies - ICIT Rm. Valcea, Romania*

Испитивана је антимикробна и антиоксидативна активност етанолних екстраката добијених из лишћа, семена и изданака биљке *Robinia pseudoacacia*. Одређени су укупни феноли (TPC, Folin–Ciocalteu метод), антиоксидативна активност (TEAC тест) и антимикробна активност (метод дифузије на агару) вегетативних и репродуктивних органа *R. pseudoacacia*. Највећи садржај полифенола (изражен као еквивалент галне киселине, GAE) нађен је у екстракту лишћа (266,7 μg GAE mL^{-1} екстракта), а затим у екстракту семена (232,2 μg GAE mL^{-1} екстракта). HPLC анализа је показала присуство катехина (0,925 μg mL^{-1}), рутина (0,831 μg mL^{-1}), резвератрола (0,664 μg mL^{-1}) и кверцетина (0,456 μg mL^{-1}) у екстракту лишћа, односно катехина (0,127 μg mL^{-1}), епикатехина (0,239 μg mL^{-1}) и рутина (0,231 μg mL^{-1}) у екстракту семена. Екстракти су показали

селективни антимикробни ефекат спрам Грам-позитивних (*Staphylococcus aureus* и *Bacillus subtilis*) и Грам-негативних (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae* и *Escherichia coli*) бактерија. Најјачи синергистички антибактеријски ефекат имао је екстракт лишћа у комбинацији са антибиотиком. Екстракт лишћа, у комбинацији са пеницилином, канамицином и рифампицином испољио је највећи синергистички ефекат спрам метицилин-резистентног соја *S. aureus* (MRSA). У овом раду је, по први пут, извршена хемијска карактеризација етанолних екстраката вегетативних и репродуктивних органа *R. pseudoacacia* и испитан синергистички ефекат неких антибиотика и екстраката, показујући да екстракти могу повећати антимикробну активност комерцијалних антибиотика спрам MRSA.

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