ORIGINAL ARTICLE

ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACTS FROM *FAGUS SYLVATICA* L. AND *JUGLANS REGIA* L. LEAVES

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Abstract

The use of medicinal plants is well known and modern science investigates the scientific bases for the effects of numerous plant extracts against a great variety of diseases. Therefore, the aim of this study was to explore the antibacterial activity of the leaves from two Romanian indigenous species: European beech (*Fagus sylvatica* L.) and walnut (*Juglans regia* L.). The leaves were dried, then grinded and polyphenols were extracted using ethanol 70%. Afterwards, polyphenols and flavonoids were assayed and identified accurately by HPTLC. Antimicrobial activity of the extracts was tested against several pathogenic bacteria using the agar diffusion method and minimum inhibitory concentration determination. Our results showed a better activity against Gram positive bacteria, and the most susceptible bacterial strain was *Staphylococcus epidermidis*.

Rezumat

Este bine cunoscută utilizarea plantelor medicinale, metodele moderne de investigație fiind folosite pentru determinarea bazelor științifice ale efectelor a numeroase extracte din plante. Astfel, scopul acestui studiu a fost determinarea acțiunii antibacteriene a frunzelor din două specii indigene din România: fagul european (*Fagus sylvatica* L.) și nucul (*Juglans regia* L.). Frunzele au fost uscate și măcinate, iar polifenolii au fost extrași în etanol 70%. Ulterior, au fost identificații principalii polifenoli și flavonoide prin HPTLC și, de asemenea, s-au determinat concentrațiile acestora. Activitatea antimicrobiană a extractelor s-a testat împotriva unor specii bacteriene patogene, folosind metoda difuzimetrică și determinarea concentrației minime inhibitorii. Rezultatele noastre au arătat o mai bună activitate împotriva tulpinilor Gram pozitive, iar tulpina cea mai susceptibilă a fost *Staphylococcus epidermidis*.

Keywords: *Fagus sylvatica*, *Juglans regia*, antibacterial activity, polyphenols, Gram-positive, *Staphylococcus epidermidis*

Introduction

In the last decades the over-use of antibiotics led to an alarming increase in resistant microorganisms, a phenomenon that has become increasingly worrisome as it is more difficult and more expensive every day to treat infections. It was assessed that just urinary tract infections led to costs such as 1.6 billion/year [19]. In this context, the focus on natural products increased gradually and nowadays a large number of new treatments are based on natural extracts [5]. From these natural compounds, a very important class are the polyphenols that proved to have antioxidant, anti-inflammatory, antibacterial, antidiabetic, neuro-protective, antifungal, sedative, antihaemolytic, hypo-glycaemic and antiviral properties [3, 4, 8, 12, 23, 24].
Previous studies stated that *Fagus sylvatica* L. (*Fagaceae*) leaves contain manganese, molybdenum, copper, zinc, iron, cobalt ions and sulphur compounds, catechins, cis-coniferin and cis-syringin, saponins, ginsenoside derivatives and C, K vitamins or α-tocopherols [10, 11, 14, 22]. There are also references to the antibacterial effect of these vegetal products against *Helicobacter pylori* [7].
Regarding *Juglans regia* L. (*Juglandaceae*) there are extensive studies on fruits, bark or green husks, and less on leaves; however it was revealed that they contain caffeic acid derivatives, coumaric acid, quercetin and some of its derivatives [13]. The leaves were used in folk medicine for the treatment of inflammations, ulcer, as antiseptic and astringent [2].
The aim of the present study was to evaluate the antibacterial effect of polyphenolic extracts from *Fagus sylvatica* L. and *Juglans regia* L. leaves against some pathogenic bacterial strains.

Materials and Methods

Plant material
Fagus sylvatica folium raw material were harvested from Romanian Carpathian Mountains, Sinaia region and *Juglans regia folium* was acquired from Fabiol

S.A. (Romania). The taxonomic identification was performed by the botanist's team of the National Institute of Chemical-Pharmaceutical R&D (INCDCF), Bucharest, Romania. Voucher specimens have been deposited in INCDCF Plant Material Storing Room as follows: European beech leaves (FSAM20-24), walnut leaves (JRAM-06). Studies were carried out after the leaves were shade dried and minced as medium-size plant powder.

Obtaining and characterisation of the extracts

The polyphenolic extracts were obtained as follows: 100 g powder of plant material [16, 17] were twice (heat-assisted, 1 hour, continue stirring) extracted with 1000 mL of 70% (v/v) ethanol prepared with distilled water. The resulting pairs of extracts were filtered through filter paper resulting in 1700 mL beech leaves ethanolic extract further used for analytical studies. For microbiological studies, in order to avoid false positive results, the ethanolic extracts were concentrated at residue, which was dissolved into 20% propylene glycol reagent in a manner to assure 5 mg total phenols (gallic acid equivalents - GAE) *per* 1 mL sample.

Extracts characterisation

Total phenolic content was estimated by the Folin-Ciocâlteu assay, as previously described [9] and total flavones content was estimated using the method described by Fernandes *et al* [6].

HPTLC method was used for the identification of the phenolic compounds. Briefly, volumes measuring from 0.5 to 3 µL test vegetal product, as well as reference samples (mixtures of 3-5 Fluka and Sigma-Aldrich phenolics) were loaded as 8 mm band length in the 10 × 10 cm silica gel 60F HPTLC plate (Merck, Darmstadt, Germany) using Linomat 5 CAMAG instrument (Muttentz, Switzerland). The loaded plate was then kept in TLC twin developing chamber at 18 - 19°C with the mobile phase (ethyl acetate - acetic acid - formic acid - water/100:12:12:26) up to 90 mm. The developed plate was dried and then immersed into the identification reagents (Natural Product followed by PEG4000). The dried plate was next disposed in the photo-documentation chamber, and the images were captured at UV 366 nm. Spots' assignment has been done using reference compounds data and plant product literature data as well [20, 25].

Microbiological studies

Bacteria strains and inoculum preparation

In this study, the following bacterial strains were used: two Gram positive strains - *Staphylococcus aureus* ATCC 6538 and *Staphylococcus epidermidis* ATCC 12228 - and two Gram negative strains - *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027.

All bacterial strains used in this study were grown on casein soya agar medium (Merck, Germany) (CaSoA). The strains ware activated by culturing the cells on CaSoA and incubated for 18 - 24 hours at 35°C.

Antibiotics used as reference

As reference, we used ciprofloxacin for tests against *Escherichia coli* and *Pseudomonas aeruginosa* and oxacillin against *Staphylococcus epidermidis* and *Staphylococcus aureus.* The standards were purchased from Sigma-Aldrich, USA. For both antibiotics there were several tests performed in order to determine the most suitable concentration for the diffusion method.

Antimicrobial assay using the diffusion method

The tests were performed in sterile Petri dishes, each containing 15 - 20 mL of culture medium previously inoculated with 10^4 - 10^5 CFU/mL. On each dish, 4 stainless steel cylinders of 8 mm diameter were placed on the solidified surface of the medium. Afterwards, in each cylinder there were added 0.2 mL test sample or control (20% propylene glycol). The Petri dishes were incubated 24 hours at 35°C. After the incubation period, the inhibition zones were measured and the results were expressed as mean of three independent measurements. Antimicrobial activity was evaluated on the basis of the diameter of the growth inhibition zone as follows: < 10 mm - no antimicrobial activity; 10 - 15 mm - weak antimicrobial activity; 16 - 20 mm - moderate antimicrobial activity; 20 mm > - high antimicrobial activity [21].

MIC determination

The microdilution method was employed, as follows: in 96-well plates, casein soya broth medium (CaSoB) was added, previously inoculated with 10^4 - 10^5 CFU/mL of one of the test bacteria. For each plant extract, serial dilutions were obtained, from 2500 µg GAE/mL to 156.25 µg GAE/mL and the plates were incubated for 24 hours at 35°C and then the optical density was read at 600 nm.

Results and Discussion

The extracts used in this study were analysed and the phenolic composition was determined using the HPTLC method we described earlier. The results showed that the european beech leaves contain a series of derivatives of apigenin and quercitin, caffeic acid, chlorogenic and neochlorogenic acids [18], while walnut leaves mainly contain quercetin derivatives such as hyperoside, isoquercitrin, avicularin and quercetrin, caffeic acid derivatives (chlorogenic and neochlorogenic acids), but also apigenin derivatives: isovitexin and juglanin [16].

Regarding the microbiological assays, we used both the diffusion method and MIC determination because the first one has a major restriction, namely the possibility that an extract may contain large molecules that can migrate with difficulty in the agar [1]. The results for the agar diffusion assay are shown in Table I.

Table I

Results of the agar diffusion assay

Sample	Bacterial strain	Inhibition zone (mm)
Fagus sylvatica extract (5 mg GAE/mL)	*Staphylococcus aureus* ATCC 6538	17 ± 0.10
	Staphylococcus epidermidis ATCC 12228	29.33 ± 0.57
	Escherichia coli ATCC 8739	12 ± 0.10
	Pseudomonas aeruginosa ATCC 9027	12.33 ± 1.15
Juglans regia extract (5 mg GAE/mL)	*Staphylococcus aureus* ATCC 6538	< 8
	Staphylococcus epidermidis ATCC 12228	16 ± 0.16
	Escherichia coli ATCC 8739	< 8
	Pseudomonas aeruginosa ATCC 9027	11 ± 0.10
Oxacillin (2 µg/mL)	*Staphylococcus aureus* ATCC 6538	25.33 ± 0.57
	Staphylococcus epidermidis ATCC 12228	16.66 ± 0.57
Ciprofloxacin (4 µg/mL)	*Escherichia coli* ATCC 8739	23.5 ± 0.5
	Pseudomonas aeruginosa ATCC 9027	18 ± 0.10

The European beech leaves have previously been tested against *E. coli* and *S. aureus* [18] and in the present study we assayed the antibacterial activity against two new strains. As shown in Table I, *Fagus sylvatica* extract is more potent against Gram positive bacteria, showing certain activity against *S. epidermidis*. In the same previous study [18], it was determined that the extract from *Fagus sylvatica* leaves was among the four extracts that showed antibacterial activities against both bacterial strains tested (*Aronia melanocarpa*, *Lythrum salicaria*, *Fagus sylvatica* and *Epilobium hirsutum*).

As a comparison, it can be noted that the European beech leaves showed a better antibacterial activity than the walnut leaves, which presented antibacterial activity against only two of the tested strains (*S. epidermidis* and *P. aeruginosa*).

The second part of the study, the MIC determination led to some interesting results, which are shown in Table II.

Table II

MIC determination for the extracts studied

Sample	Bacterial strain	MIC (µg GAE/mL)
Fagus sylvatica extract	*Staphylococcus aureus* ATCC 6538	156.25
	Staphylococcus epidermidis ATCC 12228	156.25
	Escherichia coli ATCC 8739	625
	Pseudomonas aeruginosa ATCC 9027	2500
Juglans regia extract	*Staphylococcus aureus* ATCC 6538	312.5
	Staphylococcus epidermidis ATCC 12228	156.25
	Escherichia coli ATCC 8739	1250
	Pseudomonas aeruginosa ATCC 9027	312.5

As a general observation, it seems that Gram positive bacteria are more susceptible than Gram negative ones, the values of MIC being lower for *S. aureus* and *S. epidermidis*, for both extracts. Furthermore, it is important to mention an apparent discrepancy for the *Fagus sylvatica* leaves: while MIC is the same for both *Staphylococcus* strains (156.25 µg GAE/mL), the inhibition zone in the first test differed considerably (17 mm for *S. aureus* and 29 mm for *S. epidermidis*). However, this could also be due to the fact that 156.25 µg GAE/mL was the lowest concentration tested.

For the *Juglans regia* leaves, it is worth mentioning that the MIC value against *S. aureus* is quite low (312.5 µg GAE/mL), while in the agar diffusion assay it showed no activity. This could possibly be due to the inability of some compounds from the extract to diffuse in the agar medium. However, these results are consistent with the MIC reported for an aqueous extract from walnut leaves against clinical isolates of *S. aureus* [13].

Conclusions

In this study, two plants were used as a source of polyphenolic compounds: leaves from European beech and walnut. The extracts obtained proved to contain different phenolic and flavonoidic compounds such as caffeic, gallic, chlorogenic or neochlorogenic acids, hyperoside, apigenin or myricetin derivatives, juglanin or quercitrin. The microbiological studies revealed a tendency towards better results against Gram positive bacteria rather that Gram negative ones. Also, the tests showed that the European beech leaves have a higher antibacterial activity than the walnut leaves.

In conclusion, leaves from indigenous plants as *Fagus sylvatica* or *Juglans regia* could be considered as potential natural sources for the treatment of some bacterial infections, further studies being needed in order to validate our results.

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