



Antimicrobial and cytotoxic activity of *Alnus glutinosa* (L.) Gaertn., *A. incana* (L.) Moench, and *A. viridis* (Chaix) DC. extracts

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ABSTRACT

Introduction: The objective of the present study was to evaluate the antimicrobial and cytotoxic activities of water extracts of leaves and barks from *Alnus glutinosa* (L.) Gaertn., *A. incana* (L.) Moench, and *A. viridis* (Chaix) DC.

Methods: The antimicrobial activities of extracts were tested against gram-negative and gram-positive bacteria as well as yeast strains by the agar diffusion method. The cell viability was determined by the Trypan blue dye exclusion method.

Results: The largest diameters of inhibition zone (DIZ) were recorded with *Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* 168M. The highest percentage of cell viability was observed with water bark extracts of *A. glutinosa* (97.46%).

Conclusions: Potential antimicrobial properties of *A. glutinosa*, *A. incana*, and *A. viridis* demonstrated in this study, as well as their low levels of toxicity, make them an interesting subject for further studies.

Keywords: Antibacterial activity; antifungal activity; cytotoxic activity; *Alnus*

INTRODUCTION

Plants have been used as natural sources of medicinal agents from the beginning of human civilization. Interest for plants with potential medicinal usage has increased in recent years because of their effects, including antioxidant, antiviral, antibacterial, and anticancer activity. According to the

World Health Organization, nearly 50,000 people die each day throughout the world from infectious diseases (1). Recently, the effectiveness of many synthetic antibiotics has become a serious problem because of the emergence of resistance (2). Therefore, the plants are becoming more interesting as a potential source of new antimicrobial agents.

According to Mitchell and Wilkinson (3) and O'Rourke et al. (4), the *Alnus* genus (Betulaceae) include about 30 species of trees and shrubs found in the Northern Hemisphere (3,4). This genus is represented in Bosnia and Herzegovina by three species: *Alnus glutinosa* (L.) Gaertn. (black alder),

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A. incana (L.) Moench (gray alder), and *A. viridis* (Chaix) DC. (green alder).

The plants of this genus have been found active against hepatitis, HIV-1 viral replication, and cancer (5-7) due to the presence of plant secondary metabolites, including terpenoids (e.g. secodamarane-type triterpenoids), flavonoids, diarylheptanoids, phenols, steroids, and tannins (8,5). Traditionally, extracts of *Alnus* species are used for gastrointestinal, skin diseases, and bacterial infections of the mouth and throat (9-12). Extracts of some *Alnus* species have immunostimulatory properties and are used in anticancer therapy (13-15). There are no published data on antimicrobial or cytotoxic activity of three *Alnus* species that naturally grow in Bosnia and Herzegovina. Therefore the aim of our study was to examine possible antimicrobial and cytotoxic activity of the water extracts of three *Alnus* species.

METHODS

Plant material

The plant material (leaves and bark) of *A. glutinosa*, *A. incana*, and *A. viridis* was collected in June 2009 on the mountain Vranica in Central Bosnia. A voucher specimen is deposited in the Herbarium of the National Museum of Bosnia and Herzegovina. Leaves and bark of the three *Alnus* species were dried in the shade at room temperature in airy place. Twenty grams of the chopped, dried leaves and of bark were extracted with water by a Soxhlet apparatus. All extracts were kept at +4°C for up to 1 week until antimicrobial and cytotoxicity tests were carried out.

Antibacterial and antifungal assays

Antibacterial activity of the plant extracts was determined by the disc diffusion method (16). Three gram-positive bacteria (*Bacillus subtilis* 168M, *Staphylococcus aureus* ATCC 6538, *Micrococcus luteus* DSM 1790), three gram-negative bacteria (*Escherichia coli* K12 strain DH5alpha (BRL), *Pseudomonas aeruginosa* ATCC 27853, *Salmonella abony* NCTC 6017) and two yeast strains (*Candida albicans* ATCC 10231 and *Aspergillus brasiliensis* ATCC 16404) were used for the present study. The

bacterial strains were grown in Mueller Hinton Agar (Himedia, Mumbai, India) and the yeast strains were grown in Sabouraud Dextrose Agar (Himedia, Mumbai, India). Dried filter paper discs (5 mm in diameter) impregnated with 30 µl of the extracts and placed on the agar plates were uniformly seeded with the test organisms. The treated Petri dishes were left at room temperature for 10 minutes and then incubated at 37° ± 0.1°C for 24 hours for bacteria and 48 hours for fungi. At the end of the incubation period, inhibition zones of the extracts formed on the medium were measured and expressed in millimeters. Chloramphenicol (Caelo, Caesar & Loretz, Hilden, Germany) and Kanamycin (Duchefa, Haarlem, The Netherlands) in concentrations of 5 mg/ml, and 2 mg/ml of Nystatin (Caelo, Caesar & Loretz, Hilden, Germany), were used as positive reference standards to determinate the sensitivity of each of the tested microorganism strains. A negative control was sterile distilled water.

Cytotoxic assay

Cytotoxic activity of the water extracts of three *Alnus* species was analyzed by the Trypan Blue dye exclusion method adopted by Freshney (17). The human fibroblast cells were seeded at a density of 1x10⁵ cells/well. These cell lines were cultured in RPMI-1640 medium. The media were supplemented with fetal bovine serum, glutamine, penicillin, and streptomycin. The cells were plated onto 96-well plates and allowed to grow in CO₂ incubator for 24 hours (37°C, 5% CO₂). After 24 hours, the cells were treated with the plant extracts to final concentrations of 15 and 30 µl/ml. Culture medium was used instead of the plant extract for the negative control. At the end of 72 hours incubation, 20 µl of medium and equal volume of 0.4% trypan blue were added and cell viability was determined using hemocytometer. Cells were examined and counted in duplicates under a light microscope at 100x (Olympus, Japan). When observed under the microscope, non-viable cells are stained blue while viable cells remain unstained (18).

Statistical analysis

All experiments were carried out independently three times. Results are presented as mean ± standard deviation.

RESULTS

In our study, antimicrobial activity of water extracts obtained from the leaves and bark of *A. glutinosa*, *A. incana*, and *A. viridis* were tested against eight microorganisms (Table 1). The data indicated that the gram-positive *S. aureus* strain was the most sensitive to the water extracts of *A. viridis* (21.3 ± 1.15) and *A. glutinosa* (19.3 ± 1.15) leaves. All tested extracts (except *A. glutinosa* bark) showed inhibitory effect on the *E. coli* strain. It is important to mention the results on *P. aeruginosa* strain which is known to be very resistant (Table 1).

The water extract from *A. viridis* leaves and bark was the most active extract, and this result was comparable to the results obtained with the standards, Chloramphenicol (5 mg/ml) and Kanamycin (5 mg/ml). The tested extracts showed no inhibitory activity on the *C. albicans*, *A. brasiliensis*, and *S. abony* strains.

The results of the cytotoxicity test on the human fibroblast cells are shown in Table 2. The highest percentage of cell viability was observed using the water extracts of *A. glutinosa* bark (97.46%). The cell viability results demonstrate that two concentrations of plant extracts values were higher than 85%, which is similar to that of control. The Trypan blue test showed that the water extracts at concentrations of 15 and 30 $\mu\text{l/ml}$, obtained from leaves and bark of the three *Alnus* species, had no toxicity effects on the human fibroblast cells.

DISCUSSION

Numerous species of *Alnus* have been used as traditional herbal medicines since many components from this genus have showed significant bioactivities. Our previous results suggest that *A. glutinosa*, *A. incana*, and *A. viridis* have strong antimicrobial and antioxidant potential due to the presence of large quantities of phenols and flavonoids (12). In this study, the antimicrobial activity test showed that the leaf and bark extracts of *A. viridis* inhibited the growth of almost all the microorganisms, except *S. abony*, *C. albicans*, and *A. brasiliensis*. Stević et al. (11) showed that *A. incana* and *A. viridis* bark, cone, and leaf extracts inhibited *S. aureus* growth and had a synergistic effect against bacteria resistant to the known antibiotics. Middleton et al. (19) also reported antimicrobial activity of *A. glutinosa* against eight bacterial species and confirmed the usage of this species in traditional medicine. These results are consistent with previous reports on related plants regarding antibacterial activity (12). Earlier, it was noted that differences in the antibacterial activity are associated with secondary metabolites (tannin and phenol) (12,20).

According to our results, the leaf and bark extracts showed no toxicity within the concentrations tested on the human fibroblast cell cultures. This is the first study that evaluated the cytotoxic activities of *Alnus* species from Bosnia and Herzegovina, which is considered as a member of plant family reported

TABLE 1. Antibacterial and antifungal activity of three *Alnus* species

| Microorganism | Zone of inhibition (mm) | | | | | |
|---------------------------------|-------------------------|------------------------------|---------------------|------------------------------|------------------------------|----------------------------|
| | <i>Alnus glutinosa</i> | | <i>Alnus incana</i> | | <i>Alnus viridis</i> | |
| | Leaves | Bark | Leaves | Bark | Leaves | Bark |
| <i>Bacillus subtilis</i> | - | - | - | 17.6 \pm 0.57 ^b | - | 21 \pm 1.73 ^a |
| <i>Staphylococcus aureus</i> | 19.3 \pm 1.15 | - | - | - | 21.3 \pm 1.15 ^a | - |
| <i>Micrococcus luteus</i> | - | - | - | - | - | 15.3 \pm 5.03 |
| <i>Escherichia coli</i> | 15 \pm 6.24 | - | 13.6 \pm 2.3 | 10.6 \pm 0.57 ^b | 18 \pm 8 | 18 \pm 6.53 |
| <i>Pseudomonas aeruginosa</i> | - | 11.3 \pm 6.24 ^b | - | - | 19.6 \pm 6.35 | - |
| <i>Salmonella abony</i> | - | - | - | - | - | - |
| <i>Candida albicans</i> | - | - | - | - | - | - |
| <i>Aspergillus brasiliensis</i> | - | - | - | - | - | - |

- No inhibition zone, ^aExtract with stronger effect than Chloramphenicol, ^bExtract with lower effect than Kanamycin, Kanamycin (22 \pm 2.64), Chloramphenicol (18 \pm 0.57), and Nystatin (27 \pm 1.41) were used as positive controls for gram-positive bacteria, gram-negative bacteria, and yeast, Negative control was sterile distilled water

TABLE 2. Cytotoxic activity of three *Alnus* species

| Sample | Percentage of viability (%) | |
|--------------------------------|-----------------------------|----------|
| | 15 µl/ml | 30 µl/ml |
| <i>Alnus glutinosa</i> -leaves | 94.50 | 93.50 |
| <i>Alnus glutinosa</i> -bark | 97.46 | 91.23 |
| <i>Alnus incana</i> -leaves | 88.23 | 93.47 |
| <i>Alnus incana</i> -bark | 90 | 85.52 |
| <i>Alnus viridis</i> -leaves | 89.83 | 96.92 |
| <i>Alnus viridis</i> -bark | 89.28 | 93.30 |
| Control | 94.92 | |

*Culture medium was used instead of the plant extract for the negative control

to demonstrate interesting biological activities. Middleton et al. (19) evaluated *A. glutinosa* extracts, obtained with different solvents, for toxicity using the brine shrimp lethality assay. All three extracts of *A. glutinosa* showed low levels of toxicity toward brine shrimps (LD_{50} values were in the range of 1.29×10^{-1} to 8.30×10^{-1} mg/ml) (19).

The development of new studies using other methods for plant extraction is suggested as well as the use of extracts obtained in the presence of other solvents but always coupled with toxicity tests.

CONCLUSION

No data have been published on antimicrobial or cytotoxic activity for three examined *Alnus* species in Bosnia and Herzegovina. It is evident from this study that water extracts from leaves and bark of the three *Alnus* species exhibited moderate to good antibacterial activity against the tested organisms.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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