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Antibacterial and hemolytic activities of different extracts of *Amsonia orientalis* Decne (Apocynaceae)

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Abstract

In the current study, the antibacterial activities of the crude extracts (leaf and stem chloroform and methanol; leaf hexane, acetone, deionized water and ethanol; stem deionized water) of *Amsonia orientalis* Decne. (Blue Star) were investigated against 17 different strains of Gram-negative and Gram-positive bacteria. To evaluate the antibacterial and hemolitic activities of extracts standard antibiotic discs and human erythrocytes were used, respectively. The antibacterial activity was determed in the all extracts, except the hexane extracts of the leaves. It was observed that *Bacillus subtilis* ATCC 6633, *Legionella pneumophila* ATCC 33152 and *L. pneumophila* serogroup (SG) 2-14 are the most susceptible bacteria to the different extracts, while *Pseudomonas aeruginosa* ATCC 9327, *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 10541 and *Proteus mirabilis* are resistant bacteria. It was determined that chloroform (leaf and stem), hexane (leaf) and deionized water (stem) extracts did not show hemolytic activity, while ethanol (leaf) (≥5 mg/ml), methanol (stem and leaf) (≥10 mg/ml), acetone (leaf) (≥10 mg/ml) and deionized water (leaf) (≥20 mg/ml) extracts showed hemolytic activity.

Key words: *Amsonia orientalis*, Antibacterial activity, Hemolytic activity, Plant extract.
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Farklı *Amsonia orientalis* Decne (Apocynaceae) ekstraktlarının antibakteriyel ve hemolitik aktiviteleri

Özet

Çalışmamızda *Amsonia orientalis* Decne. (Mavi Yıldız)' nin ham ekstraktlarının (gövde ve yaprağın kloroform ve metanol; yaprağın hekzan, aseton, deiyonize su ve etanol; gövde dokusunun deiyonize su ekstraktları) 17 farklı Gram-negatif ve Gram-pozitif bakteriye karşı antibakteriyel aktiviteleri incelenmiştir. Ekstraktların antibakteriyel ve hemolitik etkileri sırasıyla standart antibiyotik diskler ve insan eritrositleri kullanılarak değerlendirilmiştir. Yaprakların hekzan ekstraktı ile yaprak+gövde dokusunun kloroform ekstraktı dışındaki tüm ekstraktlarda antibakteriyel aktivite saptanmıştır. *Pseudomonas aeruginosa* ATCC 9327, *Staphylococcus aureus* ATCC 6538, *Enterococcus faecalis* ATCC 10541 ve *Proteus mirabilis*' in denenen ekstraktlara karşı üreme inhibisyon zonu oluşturmadığı; *Bacillus subtilis* ATCC 6633, *Legionella pneumophila* ATCC 33152 ve *L. pneumophila* SG 2-14' ün ise inhibisyon zonu oluşturduğu

saptanmıştır. Yaprağın etanol (≥5 mg/ml), aseton (≥10 mg/ml) ve deiyonize su (≥20 mg/ml) ekstraktları ile yaprak+gövde dokusunun metanol (≥10 mg/ml) ekstraktı hemolitik aktivite gösterirken, yaprak+gövde dokusunun kloroform, yaprağın hekzan ve gövde dokusunun deiyonize su ekstraktlarının hemolitik aktivite göstermediği saptanmıştır.

Anahtar kelimeler: *Amsonia orientalis*, Antibakteriyel aktivite, Hemolitik aktivite, Bitki ekstraktı.

Introduction

When human beings suffer from diseases, the therapeutic properties of plants are studied in order to take advantage in these situations. Thanks to the development of technology, plant extracts which have medicinal value in the treatment or prevention of various diseases have been frequently used. Turkey is a rich country in terms of flora and medicinal plants; especially plant extracts and oils are commonly used for a variety of purposes. Since synthetic drugs have many side effects, the use of plants is encouraged as a source of alternative medicine. In recent years, it has been demonstrated in various studies that substances of biologically active plants have been used as natural antibiotics against resistant bacteria (Konar et al. 2000; Öztürk et al. 2000; Dushenkov and Raskin 2008). Numerous studies have been conducted on biologically active plants. *Amsonia orientalis* (syn. *Rhazya orientalis*) has been studied with respect to alkaloids (Evans et al. 1968; Dabine-Lengyel et al. 1986; Zongo et al. 2009) and flavonoid glycosides (Itoh et al. 2002). However, we observed an extremely limited number of studies on antibacterial activity (Akyalçın et al. 2006). Akyalçın et al. (2006) demonstrated that extracts of *A. orientalis* have a strong antibacterial activity against different microorganisms. Alkaloids as well as their pharmacological, toxicological and antioxidant effects of different species of genus *Amsonia* (*Amsonia stricta, Amsonia angustifolia, Amsonia tebernaemontana* and *Amsonia elliptica*) have been studied (Clauder et al. 1973; Zsadon et al. 1973; Zsadon et al. 1974; Ozaki 1989, 1990, 1994; Tanira et al. 1996; Iqbal et al. 2008).

A. orientalis is a rare plant and is endemic to Turkey and Greece (Tutin et al. 1968; Davis 1978). The observed plant was collected by Turhan Baytop from Balıkesir in 1988 and the specimen was cultivated in Istanbul University Alfred Heilbronn Botanical Garden. *A. orientalis* is categorized as critically endangered (CR) according to the International Union for Conservation of Nature and Natural Resources (IUCN 2001) and it will probably be extinct in near future in Turkey (Özen 2006).

We aim to investigate the antibacterial and hemolytic activities of deionized water, ethanol, methanol, acetone, chloroform and n-hexane extracts obtained from the different parts of *Amsonia orientalis* against 17 different strains of Gram-negative and Gram-positive bacterial groups.

Materials and methods

Plant material

In the current study, *A. orientalis* which was used as a biological material had been identified by Dr. Osman Erol from the Department of Botany (Fig. 1). Herbarium voucher specimens (40081 ISTF) are stored at the Herbarium, Faculty of Science, Istanbul University.

Figure 1. Habitus of *Amsonia orientalis* Decne.

Chloroform and methanol (leaf and stem); hexane, acetone, deionized water and ethanol (leaf); deionized water (stem) extracts were prepared from *A. orientalis*. All the solvents were obtained from Merck Company (Germany). Plant materials were collected freshly and divided into small pieces and were then extracted in different solutions using homogenizers. Crude extracts were kept in the refrigerator +4°C for 1 day, crept from the cheesecloth and was filtered by using Whatman no.1 filter paper after centrifugation. The filtrates were then evaporated under reduced pressure and dried using a rotary lyophilizator. All samples were tested for the antimicrobial activity 40 mg/ml dissolved with 0.8% NaCl. Stock solutions were sterilized by means of membrane filters (0.20 mm pore size) and were then diluted in various proportions (40, 20, 10 and 5 mg/ml).

Bacteria

The environmental and clinical isolates and reference strains belonging to different bacterial groups were selected for antibacterial assay: Gram-negative strains such as *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Salmonella choleraesuis* ATCC 14328, *Pseudomonas aeruginosa* ATCC 9327, *Legionella pneumophila* ATCC 33152, *Aeromonas hydrophila*, *Escherichia coli*, *Serratia marcescens, Proteus mirabilis, Legionella pneumophila* serogroup (SG) 1 and *Legionella pneumophila* SG 2-14; Gram-positive strains such as *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 10541, vegetative form of *Bacillus subtilis* ATCC 6633, *Staphylococcus epidermidis* and *Enterococcus faecalis*.

Antibiotics

Ten antibiotics were used to compare their antibacterial effects with those of plant extracts. The antibiotics used were Amikacin (30 µg), Ampicillin (10 µg), Chloramphenicol (10 µg), Erythromycin (15 µg), Gentamicin (10 µg), Kanamycin (30 µg), Nalidixic acid (30 µg), Penicillin G (10 units), Streptomycin (10 µg) and Vancomycin (30 µg). All of the antibiotics (each 6 mm diameter disc) were obtained from Oxoid (Hampshire, England). The inhibition zones, to be formed by antibiotic discs, were evaluated according to the CLSI (Clinical and Laboratory Standards Institute) criteria.

Preparation of bacterial inoculation

Gram-negative and Gram-positive strains were incubated at 37°C for 18-24 h on Mueller Hinton Agar (MHA) (Merck Company, Germany) and then the inoculation were adjusted in Mueller Hinton Broth (MHB) (Merck Company, Germany) to a concentration of 10^8 colony forming unit (CFU)/ml using McFarland standards, for antibiotic tests and antibacterial activity of plant extracts.

Legionella pneumophila bacteria were grown on buffered charcoal yeast extract agar (BCYE) to late log phase at 37°C in an atmosphere of 2.5% CO_2. After the cultures reached late log phase, cells were harvested and a suspension was turbidimetrically prepared in buffered yeast extract (BYE) broth to a concentration of 10^8 CFU/ml in terms of antibiotic tests and antibacterial activity of plant extracts (Kimiran Erdem et al. 2007). The total number of bacteria was confirmed by colony counts. All experiments were performed in triplicate.

Determination of antibacterial activity of plant extracts

In vitro antibacterial studies were carried out according to the agar disc diffusion method (Barry and Tornsberry 1981). MHA and BCYE Agar media were used to establish the antibacterial effects of plant extracts and antibiotics for Gram-negative and Gram-positive bacteria and *L. pneumophila*, respectively. In each plate, 100 µl of bacterial inoculum was placed on Mueller Hinton Agar (BCYE Agar for *Legionella*) by a Drigalski rod. The discs (6 mm in diameter) containing 20 µl of plant extracts

were inserted on the plates, while antibiotic discs were placed on agar media inoculated with the test bacteria. Negative controls were prepared using the same solvents employed to dissolve plant extracts. Then, bacterial cultures were incubated at 37°C for 18-24 h. At the end of incubation time, the antibacterial activity of both plant extracts and antibiotics was evaluated by measuring the diameter of the inhibition zone.

Measurement of hemolytic activity

Hemolytic activity was determined using the microdilution method (Kohlerova et al. 2004; Arslan Aydoğdu and Çotuk 2008). All hemolytic activity experiments were studied with human erythrocyte from healthy volunteers. Freshly collected blood from healthy volunteers with heparin was centrifuged (2000 rpm, 4°C and 15 minutes) and the erythrocytes obtained were washed three times in 0.85% saline. The washed erythrocytes were suspended to a final concentration of 2%. *A. orientalis* extracts were serially diluted in phosphate buffered saline (PBS) in round bottom microliter plates and 50 µl from 2% erythrocyte suspensions was added to each well. Plates were incubated at room temperature and the concentration required for complete lysis was determined visually after 1 h. In hemolytic effect assays, sterile PBS was used as a negative control.

Results

In the current study, the antibacterial and haemolytic activities of the crude extracts of *A. orientalis* were investigated. When examining the antibacterial effect of plant extracts, since other concentrations (20, 10 and 5 mg/ml) were not effective (data no shown), 40 mg/ml was used and antibacterial activity was evaluated using a concentration of 40 mg/ml of the extracts. The antibacterial activities of different extracts of *A. orientalis* plant were determined measuring the inhibition zone diameter observed (Table 1 and 3). In additional, the antibacterial activity of extracts was compared with the standard antibiotic discs (Table 2 and 4) in view of consisting inhibition zones.

Table 1. Antibacterial effect of the different extracts of *Amsonia orientalis* on Gram-negative bacteria.

	Stem and leaf (chloroform)	Stem and leaf (methanol)	Leaf (hexane)	Leaf (acetone)	Leaf (ethanol)	Leaf (deionized water)	Stem (deionized water)
	Zone of inhibition (mm)						
A. hydrophila	0	0	0	8	0	9	0
E. coli	7	8	0	8	0	7	0
E. coli ATCC 25922	8	8	8	9	0	8	7
K. pneumoniae ATCC 4352	0	6	0	6	7	7	0
S. marcescens	7	7	0	8	7	7	0
S. choleraesuis ATCC 43128	7	7	0	8	7	8	0
P. aeruginosa ATCC 93127	0	0	0	0	0	0	0
P. mirabilis	0	0	0	0	0	0	0
L. pneumophila ATCC 33152	10	11	0	14	0	0	0
L. pneumophila serogroup 1	0	0	0	0	15	9	8
L. pneumophila serogroup 2–14	22	12	0	18	15	9	0

Table 2. Antibacterial effects of antibiotics on Gram-negative bacteria.

	Zone of inhibition (mm)									
	E	GM	C	S	NA	PG	AK	K	VA	AP
A. hydrophila	14	21*	15-	16	0	27	25*	24	23	30*
E. coli	15	32*	26*	30*	27*	7	24*	24*	10	15-
E. coli ATCC 25922	15	22*	17-	26*	28*	8	29*	26*	8	23*
K. pneumoniae ATCC 4352	9	21*	17-	16*	24*	0	22*	20*	9	0
S. marcescens	0	18*	17-	21*	20*	0	16-	19*	0	0
S. choleraesuis ATCC 43128	10	19*	27*	10+	0	0	26*	21*	0	22*
P. aeruginosa ATCC 93127	12	20*	17	18	10	0	19*	20	0	10
P. mirabilis	0	21*	16	14	0	17	17*	21	27	17
L. pneumophila ATCC 33152	11	14	30	29	31	18	15	11	10	17
L. pneumophila serogroup 1	10	13	37	31	29	17	18	10	13	14
L. pneumophila serogroup 2–14	20	23	43	35	44	26	27	18	21	29

(E= Erythromycin, GM= Gentamicin, C= Chloramphenicol, S= Streptomycin, NA= Nalidixic acid, PG= Penicillin G, AK= Amikacin, K= Kanamycin, VA= Vancomycin, AP= Ampicillin)
*: susceptible
-: intermediate resistant
+: resistant

The results of antibacterial activity of the extracts against Gram-negative and Gram-positive bacteria show that they have antibacterial activity, except *P. aeruginosa* ATCC 93127, *P. mirabilis*, *S. aureus* ATCC 6538 and *E. faecalis* ATCC 10541. On the other hand, it was observed that the most sensitive bacteria against tested extracts was *L. pneumophila* serogroup 2-14 (in which Gram-negative bacteria), and *B. subtilis* ATCC 6633 (in Gram-positive bacteria).

Table 3. Antibacterial effect of the different extracts of *Amsonia orientalis* on Gram-positive bacteria.

	Stem and leaf (chloroform)	Stem and leaf (methanol)	Leaf (hexane)	Leaf (acetone)	Leaf (ethanol)	Leaf (deionized water)	Stem (deionized water)
	Zone of inhibition (mm)						
B. subtilis ATCC 6633	10	0	0	13	0	0	0
S. aureus ATCC 6538	0	0	0	0	0	0	0
S. epidermidis ATCC 12228	7	7	0	9	0	0	0
S. epidermidis	7	7	0	9	0	0	0
E. faecalis	0	8	0	9	0	0	0
E. faecalis ATCC 10541	0	0	0	0	0	0	0

Table 4. Antibacterial effects of antibiotics on Gram-positive bacteria.

	E	GM	C	S	NA	PG	AK	K	VA	AP
	Zone of inhibition (mm)									
B. subtilis ATCC 6633	17	32	25	27	27	31	31	29	24	36
S. aureus ATCC 6538	9	19*	15	20	24	8	19*	19*	10	9
S. epidermidis ATCC 12228	14	17	14	11	26	15	20	19	0	14
S. epidermidis	15	20	17	16	27	17	23	22	0	18
E. faecalis	0	19	17	16	26	16	21	20	0	20
E. faecalis ATCC 10541	0	23	16	15	27	11	22	19	0	15

(E= Erythromycin, GM= Gentamicin, C= Chloramphenicol, S= Streptomycin, NA= Nalidixic acid, PG= Penicillin G, AK= Amikacin, K= Kanamycin, VA= Vancomycin, AP= Ampicillin)
*: susceptible
-: intermediate resistant
+: resistant

When the hemolytic activities of the extracts were examined, it was observed that the chloroform (leaf and stem), hexane (leaf), distilled water (stem) extracts were not able to have hemolysis human erythrocytes. In these experiments, ethanol extracts of the leaves (≥5 mg/ml) were shown to have the strongest hemolytic activity, followed by methanol (leaf and stem) (≥10 mg/ml), acetone (leaf) (≥10 mg/ml) and distilled water (leaf) (≥20 mg/ml).

Discussion

In the present study, the results of discs diffusion methods indicated that the crude extracts of *A. orientalis* have antibacterial activity. It was shown that *Legionella pneumophila* SG 2–14 strain (zone of inhibition: 22 mm) was the most susceptible, while *Pseudomonas aeruginosa* ATCC 9327 and *Proteus mirabilis* strains were the most resistant strains. Among all extracts, hexane exhibited a good activity against *Legionella pneumophila* SG 2–14 strain. It was determined that, although *Legionella pneumophila* ATCC 33152 showed the highest susceptibility (zone of inhibition: 14 mm) against the acetone extracts of the leaves, *Legionella pneumophila* SG 1 strain (zone of inhibition: 15 mm) showed the highest susceptibility against the ethanol extracts of the leaves. *Bacillus subtilis* ATCC 6633, a Gram-positive rod, was observed to have the strongest activity against methanol extracts of the stem and leaf, and against acetone extracts of the leaves. When Gram-positive cocci were examined, *Staphylococcus epidermidis* ATCC 12228, *Staphylococcus epidermidis* and *Enterococcus feacalis* strains were observed to be sensible, while *Staphylococcus aureus* ATCC 6538 and *Enterococcus feacalis* ATCC 10541 strains were found to be resistant to all extracts.

Erythromycin and gentamicin is known to be effective antibiotics on bacteria of genus *Legionella* (Dedicoat and Venkatesan 1999). When the inhibition zones of these antibiotics compared with zones formed by the crude extracts, the acetone extract of leaf for *L. pneumophila* ATCC 33152 and the ethanol extract of leaf for *L. pneumophila* SG1 are observed to be important alternatives. At the same time, for *L. pneumophila* SG 2-14, the chloroform extract of stem+leaf is seen to be an alternative to erythromycin. On the other hand, for other studies microorganisms, crude extracts are less effective than the standard antibiotic discs. For this reason, we consider the active component(s) of extracts should be determined and investigated of their antimicrobial activities.

When the extracts were evaluated (according to the extracts), it was observed that among the extracts, the acetone extracts of the leaves exhibited the strongest activity

(71%), and the following were methanol (leaf and stem) (58%), chloroform (leaf and stem) and distilled water (leaf) (52%), ethanol (leaf) (41%), distilled water (stem) (24%) and hexane (leaf) (6%) extracts, in decreasing order. When the hemolytic activities of the extracts were compared, it was observed that the ethanol extracts of leaves has the strongest activity, followed by methanol (leaf and stem) and acetone (leaf), and distilled water (leaf).

The results concerning the evaluation of hemolytic and antibacterial activity have indicated that ethanol, acetone and distilled water extracts of the leaves, and the ethanol extracts of the leaf and stem may not be suitable to be used as antibacterial agents due to hemolytic activities.

When the obtained results were compared with those of standard antibiotics, it was determined that all bacteria examined were more susceptible to almost all extracts. Based on the results, it was concluded that the inhibition zones of some extracts were smaller than those of antibiotics. Moreover, the results have shown that some extracts had moderate bactericidal activity. Also in a similar study conducted by Akyalçın et al. (2006), it has been found that leaf extracts obtained from the same plant had strong antimicrobial activity against the tested microorganisms. It can be considered that this difference in the bacterial inhibition could be due to the differences in resistance of the tested bacteria, the solvent used for extraction and different chemical agents in the extract.

In addition, although hexane (leaf) and deionized water (stem) extracts are not able to show hemolytic property, they have weak antibacterial activity (6% and 24%, respectively). Accordingly, these extracts have been determined to have no pharmaceutical value. However, the chloroform extract of the leaf and stem has both hemolytic activity and antibacterial activity on bacteria; thus it can be concluded that these extracts are suitable for controlling pathogenic bacteria. In the other hand, it remains unknown which compounds are responsible of the antibacterial activity of *A. orientalis*. For this reason, in the further studies, these extracts will be fractionated / purified and will be determined as bioactive compounds.

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