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Anatomy, Morphology, Palynology and Antimicrobial Activity of *Amsonia orientalis* Decne. (*Apocynaceae*) Growing in Turkey

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Abstract: In this study, anatomy, morphology, palynology and antimicrobial activity of *Amsonia orientalis* Decne., which is distributed only three localities in Turkey and evaluated as critically endangered according to IUCN categories, have been investigated. Leaf and stem sections were investigated anatomicaly. The leaf has a thick cuticula layer. The inflorecense, the leaf and floral morphology were studied morphologicaly. The inflorescence is corymbose, leaves have an alternate order and almost without petiols. The pollen grains are trizonoporate and the ornamentation is psilate. Leaves are glabrous except for margin and midrib, pubescent when young. The extras obtained from the plant heve strong antimicrobial activity against the tested microorganism used in this study.

Key words: Anatomy, apocynaceae, microbial activity, morphology, palynology, Turkey

INTRODUCTION

Amsonia Walter is a genus belonging to *Apocynaceae*, a tropical family, which includes about 180 genera and 1500 species throughout the world (Wilkinson, 1996). The genus has ornamental and medicinal taxa with wide distribution on the world (Cronquist, 1981). This study is focused on one of these species, *Amsonia orientalis* Decne. (syn. *Rhazya orientalis*), which is distributed in Turkey and north-east of Greece (Tutin *et al*., 1968). This species has been recorded from only three localities in Turkey (Istanbul, Bursa and Balikesir), indicating that it is very rare and almost extinct in Turkey (Davis, 1978). Moreover, our recent field work has shown that the species is found only in Balikesir province and it occupies less than 10 km^2. Thus, this species must be evaluated as critically endangered (CR) taxon (Özen, 2002) according to IUCN categories (IUCN, 2001). *A. orientalis* has 13 different alkaloids, some of which have anticancer activity (Dabiné *et al*., 1986; Rahman *et al*., 1989; Rahman and Zaman, 1988; Sauerwein, 1991). Furthermore, another study by our time revealed that the species is also very rich in glycosides (about 17 different glycosides) (unpublished data). Besides, in Western Europe and America, the small but profuse blue flowers of *A. orientalis* are used as an ornamental plant in gardens (Davis, 1978; Brickell, 1996). The present study aims to provide insight into leaf and stem anatomy, morphology, palynology and antimicrobial activity of this rare and nearly extinct species in Turkey.

MATERIALS AND METHODS

The *Amsonia orientalis* Decne samples used in this study were collected during the generative and vegetative growing periods from Balikesir in northwest of Turkey. The research was based on the examination of the herbarium specimens and living materials. The vaucher specimens were deposited in herbarium of the Uludağ University (BULU, specimen No: 18138). Fifty morphological measurements were taken from stem, leave and flower of the living specimens. Euromex Holland stereo microscope was used for morphological drawings. The cross sections obtained from stem and leaves were fixed in 70% ethyl alcohol for preservations. The cross sections were made by hand from fresh material. Photograph of stem and leaves were obtained from Olympus BX 50 using the magnification of 10×20. The pollens used in this study were obtained from the living plant materials. At least 30 pollen grains were scrutinezed on Nichon Alphobot YS light microscope. The pollen slides are made according to the techniques of Erdtman (1960, 1969), Woodhouse (1935) and SEM micrograph. On the light microscope, pollen grains P, E, exine, intine of the

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taxon were measured until the gausse curve is reached. SEM JSM 5600 (30 KV), which was used throughout this study, belongs to University of Kirikkale, Department of Physics Electron Microscope Laboratory. The photographs of pollen samples, which revealed the detailed surface ornamentations on their general appearence, were developed by the dimension ranging 1500 and 2000. The terminology of Erdtman (1960, 1969), Faegri and Iverson (1975) was followed.

Photograph of pollen grains were obtained from Olympus BX 50 using the magnification of 10×100.

Extraction of plants for microbial activity: Leaves of *Amsonia orientalis* was ground to fine powder under steril conditions. Twenty gram of the plant was extracted with 150 mL methanol, chloroform, or acetone for 24 h by using a soxhlet apparatus (Khan *et al*., 1988).

All the extracts thus obtained were injected into empty sterilized antibiotic discs having a diameter of 6 mm (Schleicher and Schul No. 2668, Germany) in the amount of 20 μL. Discs injected with pure methanol, chloroform, or acetone served as negative control.

Microorganisms: The bacteria *Escherichia coli* ATCC 11230, *Enterobacter aerogenes* ATCC 13048, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus epidermidis* ATCC 12228, *Bacillus cereus* ATCC 7064, *Bacillus subtilis* ATCC 6633, *Proteus vulgaris* ATCC 6895, *Pseudomonas aeruginosa* ATCC 27853, *Yersinia enterocolitica* ATCC 9610 and *Salmonella typhimurium* CCM 5445, the yeast cultures *Kluyveromyces fragilis* ATCC 8608, *Rhodotorula rubra* DSM 70403, *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* ATCC 9763 were used in this study as the test microorganisms.

Preperation of microorganism culture: All the bacteria mentioned above incubated at 30±0.1 °C for 24 h by inoculation into Nutrient Broth (Difco) and the yeast cultures studied were incubated in Malt Extract Broth (Difco) for 48 h. Mueller Hinton Agar (Oxoid) sterilized in a flask and cooled to 45-50 °C was distributed to sterilized Petri dishes having a diameter of 9 cm, by using pipettes in the amount of 15 mL after injecting cultures of bacteria prepared as mentioned above and yeast for 24 h in the amount of 0.01 mL (10^5 bacteria and yeast cultures per mL) and providing the distribution of food medium in Petri dishes homogenously. Dishes injected with extracts were located on the solid agar medium by pressing slightly and incubated at 37±0.1 °C for 24 h for bacteria and 30±0.1 °C for 48 h for yeast cultures (Collins *et al*., 1989; Bradshaw, 1992). On each plate an appropriate reference antibiotic disc was appilied depending on the test microorganisms.

RESULTS

Growth form, habitat and ecology: *Amsonia orientalis* (Blue Star) has 30-60 cm. (Fig. 1a, b) length and is a branched perennial plant. It grows in places which are wet during the winter in Balikesir. It can be found along margins of lakes and streams, together with other species such as *Hordeum bulbosum* L., *H. murinum* L. subsp. *glaucum* (Stuedel) Tzvelev, *Salix alba* L. and *Hypecoum procumbens* L. (Özen, 2002).

Indumentum (Trichome): It is glabrous except for margin and corolla tubes pubescent when young, with numerous lateral veins. Hairs in this section are unicellular and the form of simple hair type, while length of hair is 0.1-0.4 mm in corolla throat, it is 0.1-3.0 mm in edge of leave and middle vein.

Leaf morphology: Leaves are alternate arranged and almost without petiole, narrowly ovate or lanceolate, simple and coriaceous. Leave edges are entire, base cuneate or rounded, apex acute or acuminate (Fig. 2). Leaf venation is parallel venation. Leaves are glabrous except for edge and main vein but they are pubescent when young and are 5-7×1.5-3.5 cm in base and upper stem. Petiole is in base and upper stem 0.1-0.5 cm, in middle part 0.2-0.4 cm.

Leaf anatomy: Leaves are bifacials. Epidermis is one layered and hypodermis is absent. Upper surface of epidermis is covered with thick lines and fluctuated cuticle. Pallisade parenchyma is two layers beneath epidermis and one or two layers of loosely arranged spongy parenchyma. Chloroplasts of pallisade parenchyma are larger than those of spongy parenchyma. Cuticle is thinner than upper epidermis. Leaf is hypostomatic. Stomata is scarcely distributed in upper epidermis and it is amaryllis type with 2-6 neighbour cells. Main vein has hadrocentric vascular bundle; xylem is in center surrounded by phloem. (Fig. 3 a and b, c)

Vascular system is covered with one layered bundle. Main vein is distinguished in subsurface of leaf. Collenchyma tissue is five layered between main vein and upper epidermis while two layered between main vein and lower epidermis. In sections bringing pallisade parenchyma ending with collenchyma tissue.

Epidermis is one layered and regular. Cortex parenchyma is under epidermis and multi layered. Cells in first and middle layers have chloroplast. Cells in the lattest one or two layers of cortex parenchyma consist of starch.

Fig. 1a: General appearence of amsonia orientalis

Fig. 1b: General appearence of amsonia

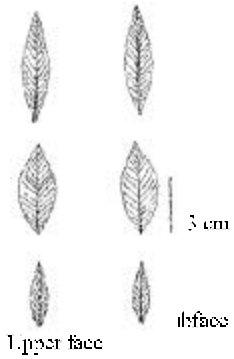

Fig. 2: Leaf morphology

Sclerenchyma forms a ring surrounding vascular system as clusters. Following this layer, there is phloem forming the mashed cells (Fig. 4) xylem forms a compherensive ring and consists of lignin. Parenchyma cells are located in centre.

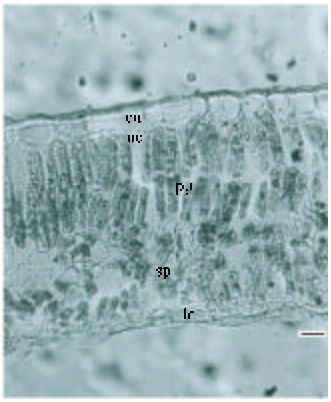

Fig. 3a: Leaf anotomy

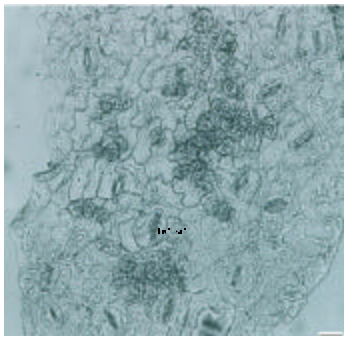

Fig. 3b: Amarllis type stomata

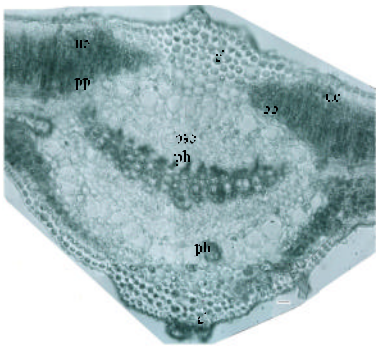

Fig. 3c: Leafe main vein

Inflorescence: Inflorescence is intense or loose, terminal. Corymb has multiflower and 4-13 cm in length. Bracts are 1.0-8.0 mm in length. Tips of bracts are acut. Edge of bracts is pubescent.

Floral morphology: Calyx is 3-6 mm in length. It is without tubes, consisting of five calyx lobes, gamosepalous. Corolla is little, light blue, hypocrateriform, 13-21 mm in length. Corolla tube is 10-12 mm in length with tidy throat. Lobes (Petal) is 4-5.5 mm in length. The aestivation of the corolla lobes is convolute. Anther is acute and surrounds

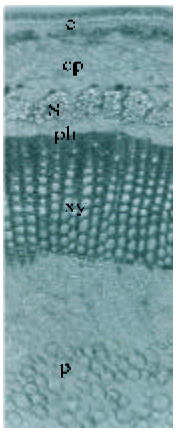

Fig. 4: Stem anatomy

Fig. 5a: General appearence of flower

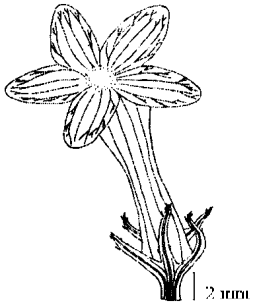

Fig. 5b: The lontitudinal appearence af *Amosonia orientalis* flower

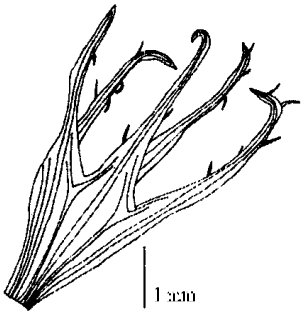

Fig. 5c: Calyx

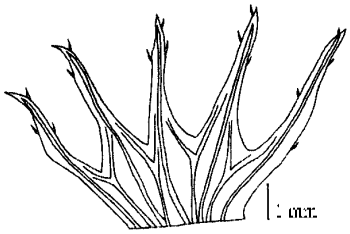

Fig. 5d: Caıyx

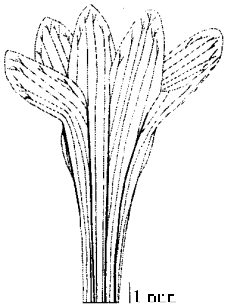

Fig. 5e: Corolla

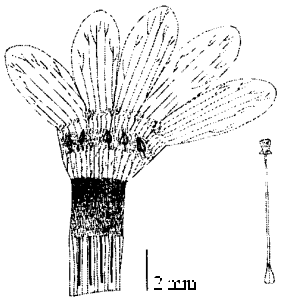

Fig. 5f: Corolla and pistil

stigma but not adjacent to it. Stigma is 1-2 mm in length. Style is 6-8 mm in length. Carpel is 1-2 mm in length, covered with a disc and has many ovules (Fig. 5a-f).

Fruit is a follicle, 2-7, 5 cm in length. The opening of the fruit is secund cleavage. Seeds are tuberculate, 6-11 mm in length.

Pollen morphology: The pollen grains of *Amsonia orientalis* are trizonaporate and in medium size. The P (Polar axis) value is 47.5-52.5 µm (W) and mean value is 50.36 µm. E (equatorial diameter) value is 56-60 µm (W) and mean value is 59.18 µm (W). Pollen shape is suboblatae and it is compressed in their polar areas. Because pollen grains were fixed only in polar area therefore P and E values couldn't be measured (Fig. 6a-d).

Exine is 2 µm (W, E). Ectexine and endexine are 1 µm (W,E). Mesoporium ranges from 37 to 42 µm (W) and from 31 to 42 µm (E) mean value is 40 µm (W) and 36.2 µm (E). Pore diameter is between 15-21 µm (W) and 10-14 µm (E),

Table 1: Antimicrobial activity of *Amsonia orientalis* Decne

Microorganisms	Zone of inhibition (mm)*						
	MeOH	Acetone	$CHCl_3$	SAM20	CTX30	VA30	NY
Escherichia coli ATCC 11230	14	15	16	12	10	22	-
Enterobacter aerogenes ATCC 13048	13	13	14	12	12	26	-
Staphylococcus aureus ATCC 6538P	14	11	12	16	12	13	-
Staphylococcus epidermidis ATCC 12228	18	19	22	16	14	15	-
Bacillus subtilis ATCC 6633	13	14	13	14	15	20	-
Bacillus cereus ATCC 7064	14	15	14	16	16	20	-
Proteus vulgaris ATCC 6895	15	16	17	16	18	20	-
Pseudomonas aeruginosa ATCC 27853	15	17	19	10	54	20	-
Salmonella thyphimurium CCM 5445	18	20	21	16	18	15	-
Yersinia enterocolitica ATCC 9610	12	14	14	18	22	20	-
Kluyveromyces fragilis ATCC 8608	14	12	14	-	-	-	14
Rhodotorula rubra DSM 70403	14	13	13	-	-	-	18
Candida albicans ATCC 10231	16	16	17	-	-	-	15
Saccharomyces cerevisiae ATCC 9763	15	14	16	-	-	-	12

*Values, including diameter of the filter paper disc (6.0 mm), are means of three replicates, SAM20 : Ampicillin (10 ug $disc^{-1}$), CTX30 : Cefotaxime (30 ug $disc^{-1}$), VA30 : Vancomycin (30 ug $disc^{-1}$), NY : Nystatin (8 ug $disc^{-1}$)

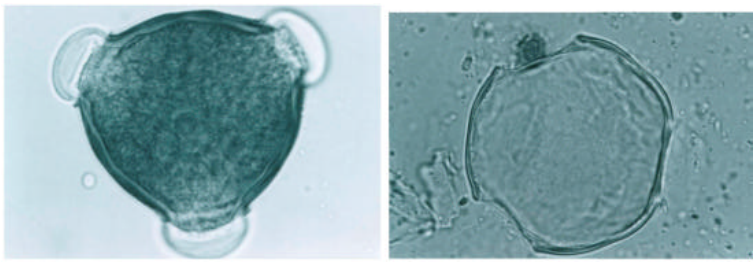

Fig. 6a: Pollen grain in LM (W), 6b: pollen grain in LM (E)

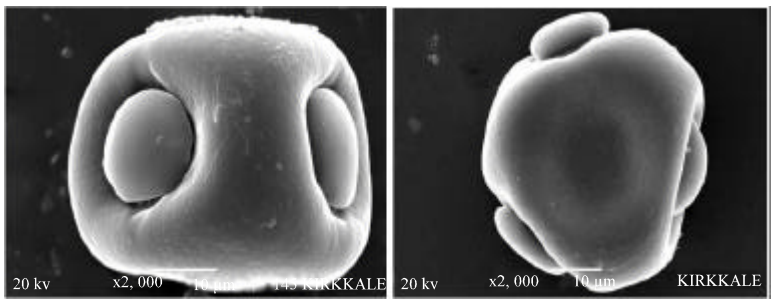

Fig. 6c: Pollen grain in equatorial view (SEM), 6d: poolen grain in polar region (SEM)

Table 2: Comparison of some characteristics between Flora of Turkey and present study of *Amsonia orientalis* Decne

	Flora of Turkey (Davis, 1978)	Present study
Calyx	2-3 mm	3-6 mm
Follicles	3,5-5 cm	2-7,5 cm
Seeds	6-8 mm	6-11 mm

mean value is 16.06 µm (W) and 11.66 µm (E). Pores have operculum, operculum diameter is 12.5-15 µm (W), mean value is 13.5 µm (W). Measurements couldn't be taken according to Erdtmans Method. AMB shape is triangular-rounded and its diameter between 55-60 µm (W) and 45-56 µm (E), mean value is 56.3 µm (W) and 44 µm (E). Intine is 0.5-1.00 µm and it is thickened differently from layer to layer. Ornamentation is psilate. The pollen surface in SEM micrograms have sparsely punctate to finely pe-forate and operculum surface is smooth.

Antimicrobial properties: Table 1 shows antimicrobial activities of the plant extracts. Besides, the inhibition zones formed by Standard antibiotic discs are indicated in the same table.

As can clearly be seen from Table 2, all extracts obtained from *Amsonia orientalis* show good growth inhibition against all bacteria used in this study. Extracts obtained from the plant inhibit *Salmonella typhimurium*, having an inhibition zone of 14-16 mm. When the result obtained were compared to those of Standard antibiotics,

it was determined that the extracts have higher antimicrobial effects against this microorganism. *Escherichia coli* and *Enterobacter aerogenes* are more susceptible to the extracts, as compared to Standard antibiotics except for VA. Similarly, *Pseudomonas aeruginosa* is seen to be more susceptible to all extracts, as compared to the the Standard SAM and VA. However, *Yersinia enterocolitica* and *Bacillus* species are more resistant. Although *Stapylococcus epidermidis* is more susceptible to the extracts, *Staphylococcus aureus* is resistant, as compared to standard antibiotics. It is explained that microorganisms variable sensivity to chemical substances relates to different resistance levels between the strains (Cetin *et al*., 1989).

The plant differ significantly in their activity against tested microorganisms. These differences may be attributed to fact that the cell wall in gram-positive bacteria of a single layer, whereas the yeast cell wall is quite complex (Yao and Moellering, 1995).

In general, the extracts obtained the plant have strong antimicrobial activity against the yeast cultures. Against *Kluyveromyces fragilis*, acetone and chloroform extracts are equavalent to the standard antibiotic nystatin. Notably, all extracts of the plant have higher antimicrobial effect than those of the Standard antibiotic against *Candida albicans* and *Saccharomyces cerevisiae*, but *Rhodotorula rubra* is resistant.

For the evaluation of plant that grow naturally in Turkey and are potentially useful resource, additional studies will be benefecial from medicinal and economic standpoints. The extracts of *Amsonia orientalis* can be used for protection against some bacteria and yeasts.

DISCUSSION

There is no anatomical, morphological, palynological and antimicrobial activity study on *Amsonia orientalis* in the literature. However some metric differences between Flora of Turkey (Davis, 1978) and present study were determined. These differences are shown in Table 2.

Because there is also no anatomical, morphological, palynological and antimicrobial activity study on *Amsonia* genus, *A. orientalis* can not be compared with the closely related taxa. Therefore, *A. orientalis* compared with *Nerium oleander*, which also belongs to family of Apocynaceae. In the *N. oleander* there is a thick cuticle layer in upper and lower epidermis of leaves. But it is not so thick in *A. orientalis*. Besides the upper epidermis (including hypodermis) is few layered in *N. oleander*, it is one layered in *A. orientalis*. Pallisade parenchyma of mesophyl is two layered in *N. oleander* while it is one layered in *A. orientalis*. The stomatas are sunken type in *N. oleander*, but it is in the same level with epidermis cells in *A. orientalis* (http://www.park.edu/bhoffman/courses/bi225/recaps/leavesii.htm, http://www.park.edu./bhoffman/courses/bi225/images/nerlf10lab.jpg). There is also some important differences between these two taxa from the point of view of stem, leaf, flower, fruit and seed size (Davis, 1978). Pollen grains are medium size and trizonoporate. Ornamentation is psilate. It has been stated that ornamentation in Plectania and Alstonia belond to Apocynaceae family is microfossulate to perforate and psilate (Kuijt and Van der Ham, 1997; Van der Ham *et al*., 2001)

Antimicrobial activity studies have shown that this plant is very suitable for ethnobotanical studies. As a result of future biochemical studies, it may be possible to use extracted the effective substances from *A. orientalis* in various diseases. As a summary, it was shown in this study that *A. orientalis* which has economical importance in terms of medical and ornamental characteristics were revealed.

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