COMPARATIVE HISTO-ANATOMY AND CHEMICAL COMPOSITION OF TWO *AJUGA* SPECIES FROM THE ROMANIAN FLORA

ANCA HEMCINSCHI*, RAMONA GALEȘ**, URSULA STĂNESCU*, C. TOMA**

Abstract. The study deals with the anatomy and chemistry of two spontaneous *Ajuga* species (*A. genevensis* L. and *A. reptans* L.) of different populations from Romania. The purpose of this study is to analyze the inter- and intra-specific variation of some histo-anatomical and chemical characters in *Ajuga* sp. The chemical investigations use the TLC and UV-VIS spectrophotometry in order to quantify the composition in iridoids, flavonoids and polyphenolic acids.

Key words: *Ajuga*, anatomy, chemistry, variability, vegetative organs, flower, iridoids, polyphenols.

Introduction

In the flora of Romania [18] there are mentioned 8 species and 2 hybrids of *Ajuga*, out of which *A. reptans* L. is known as a medicinal plant for its bitter-astringent principles, a reason according to which it has recently been offered more attention, along with its close kin – *A. genevensis* L.

The anatomical structure of the plants from the *Lamiaceae* family was investigated by many authors as it is shown by the syntheses published by Metcalfe and Chalk (1972) on the anatomy of the dicotyledonous plants and by Napp-Zinn (1973, 1974) on the angiosperms in general; in these works the *Ajuga* genus is also mentioned when the main histological features of the stem and leaf are presented. Older literature [4], [5] refers to the structure of the aerial and underground organs, and most of the authors pay special attention to the secretory formations [7], [10], [11], [17]. Information about the hair structure may be found in some monographies or in the anatomic atlas relatively recently published in Romania [19].

Regarding the chemical composition of the essential oils of the *Lamiaceae* species, many data were found by us in the volumes published by Harley and Reynolds (1992) and Bruneton (1999), without special reference to the *Ajuga* taxons. The flourishing aerial parts of *Ajuga reptans* L. and *Ajuga genevensis* L. from the *Lamiaceae* family, common in orchards, hay fields and pastures, known under the name of bugle and blue bugleweed respectively are characterized by the content of tanins and iridoids, due to which, in folk medicine, both species are used, in the forms of infusions and tinctures, as anti-diarrhoeic, antileucorhoeic, vulnerar and hepatoprotecting remedies [1, 2].

Both species contain iridoids (harpagid, 8-O-acetilharpagid, ajugol), anthocyanosides, tanins, polyphenolcarboxilic acids (rozmarinic acid, caffeic acid), flavonoids [3], volatile

*“Gr. T. Popa” University of Iasi, Faculty of Pharmacy
**“Al. I. Cuza” University of Iasi, Faculty of Biology, ramona.gales@uaic.ro
oils, rezins, ozes, and also diterpens with a neo-clerodan skeleton, generically called ajugavensins [4, 5].

As the iridoidic compounds of the harpagoside/harpagide type are extremely interesting for therapeutics due to the anti-inflammatory and pain killing actions, the only used vegetal product in the present being the secondary tubers of *Harpagophytum procumbens* originating from South Africa (indicated in the treatment of the chronic forms of inflammatory rheumatism, and also in chronic gastro-intestinal inflammatory affections), the identification of a possible European source of such active principles would be an interesting challenge.

This paper analyzes two indigenous *Ajuga* species founded in the spontaneous flora of Romania, in order to identify the inter- and intra-specific variations of some hist-anatomical and chemical characters, observing the degree in which the iridoidic derivates, and also the flavons and polyphenylcarboxilic acids are to be found in the dry vegetal products in constant and sufficiently high quantities so as to be of interest for the phytomedicinal industry [6].

**Material and methods**

**Histo-anatomical investigations**

Plant material for the histo-anatomical investigations is represented by two *Ajuga* species (*A. reptans* and *A. genevensis*) from two different populations from Romania (Botosani County and Neamt County) collected in the anthesis stage of development.

The histo-anatomical researches were performed using light microscopy. Two methods were used to obtain the sections. The cross-sections through the vegetative organs (preserved in 70% ethylic alcohol) were made using a manual microtome, being coloured with iodine-green and ruthenium-red. The serial cross- and longitudinal sections through the flower (fixed in FEEA mixture) were performed using the standard paraffin-embedded protocol applied in plant histo-anatomical researches. Fixed samples were dehydrated by a passage through ethanol/water solutions and then embedded in paraffin at 65ºC for 24 hours. The embedded material was cut into 13 µm thick sections with a rotator microtome. The dried serial sections were deparaffinized, rehydrated in serial dilutions of ethanol (100%, 90%, and 70%), coloured with metilen-blue and ruthenium-red, and finally mounted in Canada balsam.

**Chemical investigations**

Plant material for the chemical researches is represented by the same two *Ajuga* species from 7 different populations: 4 (1 from Baisa, 1 from Guranda and 2 from Tg. Neamt) in case of *Ajuga reptans* L. and 7 (1 from Albesti, 1 from Baisa, 1 from Nemtisor and 4 from Mascateni) in case of *Ajuga genevensis* L. harvested in May, 2009.

The prelevated vegetal material was dried at environmental temperature, in a closed space, ventilated, and with no light, after which we achieved extracts in methanol 70% (DER=0.5:100g/ml) from this, knowing that both the iridoids and certain polyphenols have a raised hydrophilic degree.

The spectrophotometric determinations were performed due to the techniques known for flavonoids, polyphenolcarboxylic acids [7] and iridoids [8], while TLC was proceeded due to the classic procedure described by Wagner and Bladt [9].
Results and discussions

**Inter- and intra-specific anatomical variations in *Ajuga* species**

*The rhizome* structure (Plate I: Fig. 1-4) is relatively similar in the investigated *Ajuga* species. The stele has secondary structure; in the xylem ring the amiliferous parenchyma is dominant, the islands of conducting elements with slightly collenchymatous walls being in the neighbourhood of the cambium. The vessels diameter grows towards the edge of the secondary xylem ring, where the libriform fibres have thinner walls, and the cells of the medulary rays have a lignification tendency.

Some histological differences may be observed: 1. a greater number (25) of layers of cortical amiliferous parenchyma in *A. genevensis* (Fig. 1); 2. a visible Casparyan endodermis in *A. genevensis*; 3. cordons of sclerenchymatous elements in periphloemic position in *A. reptans*.

*The adventitious roots* (Plate I: Fig. 5, 6) formed on the rhizome have a polyarch stele (7-8 xylem bundles and as many phloem ones) (Fig. 2), a situation not very often found within the dicotyledonous plants. The cortex ends with a Casparyan endodermis. The passing from primary to the secondary structure (especially due to the cambium) takes place earlier in *A. genevensis*. In the secondary xylem structure there are a few libriform fibres and cellulosic parenchymatous cells.

The plant need for water and minerals is satisfied by both the great number of vessels, and, especially, by the great number of adventitious roots formed on the rhizome (and on the stolon, in *A. reptans*). These roots are branching, having stele of diarch or triarch type.

*The stolon of *A. reptans* presents some histo-anatomical peculiarities: epidermic cells full of anthocyans, stomata above the epidermic level, numerous glandular hairs, with tetracellular or octocellular secreting head, a continuous Casparyan endodermis, slightly developed collenchymas, secondary conducting tissue of annular type, the non-glandular hairs are missing (Fig. 3).

*The aerial stem* (Plate II: Fig. 7-9) is covered by a thin cuticle and the stomata are missing in the lower third of the organ.

Two major types of hairs are distinguished: 1. non-glandular hairs, which are long, multicellular, uniseriate, especially placed on the ribs (in *A. genevensis*) or in two opposite faces (in *A. reptans*) and 2. glandular hairs which are very short, multicellular, with bi-(Fig. 10), tetra- or octocellular secreting head gland (Fig. 11), located especially on the ribs in *A. reptans*.

The collenchymatous tissue is more developed in the ribs than in the rest of the stem, where it forms a 1-layered hypodermis. The cortex ends with a Casparyan endodermis, which is discontinuous, only in the lower third of the stem. In the external cortex there are numerous aeriferous lacunae.

The stele with a primary structure comprises numerous vascular bundles (big in the ribs and small in the rest of the stem), many of them being formed only of phloem (Fig. 4). In the lower third of the stem, the big vascular bundles present secondary structure, the vessels being separated by many libriform fibres with moderately thick but intensely lignified walls (Fig. 5).
The leaf limb (Plate II: Fig. 10, 11) is amphistomatic, with anomocytic and diacytic stomata and glandular hairs (Fig. 8). The mesophyll is slightly differentiated in palisade tissue (2-3 layers in *A. reptans* (Fig. 6) and 1-2 layers at *A. genevensis* (Fig. 7) with low cells) and lacunous tissue (4-5 layers in *A. reptans* and 2-3 layers in *A. genevensis*) with big aeriferous lacunae.

Comparing the structure of the individuals of different origin populations (in different climatic and soil conditions), we have observed some histological differences, noted below, only in the aerial stems.

Tabel 1. Variations of aerial stem histo-anatomical features

<table>
<thead>
<tr>
<th>Species</th>
<th>Variations of aerial stem histo-anatomical features</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neamt population</td>
</tr>
<tr>
<td><em>Ajuga reptans</em></td>
<td></td>
</tr>
<tr>
<td>Periphloemic mechanical elements</td>
<td>moderately thick walls</td>
</tr>
<tr>
<td>Secondary xylem ring</td>
<td>interrupted by cellulosic parenchymatous rays</td>
</tr>
<tr>
<td><em>Ajuga genevensis</em></td>
<td></td>
</tr>
<tr>
<td>Stomata</td>
<td>prominent above the epidermis</td>
</tr>
<tr>
<td>Periphloemic mechanical elements</td>
<td>visible</td>
</tr>
<tr>
<td>Mesophyll</td>
<td>homogenous of lacunous type with very big aeriferous cavities</td>
</tr>
</tbody>
</table>

The structure of flower (Plate III) is similar in both analyzed *Ajuga* species. On serial cross- and longitudinal sections through the flower some histological peculiarities may be observed. The epidermis of sepals and petals bears apart from papiliform cells and both categories of hairs: 1. very long non-glandular hairs (exclusively on the external face) and 2. short glandular hairs (on both faces), normally with a unicellular secretory gland (Fig. 9). The mesophyll is homogenous and consists of 3 layer of lacunous tissue. The transitory and tapet layers of stamen anther wall are early disorganized (Fig. 13). The anther exothecium consists of radially flattened cells and the endothecium has visibly radially elongated cells, with longitudinal thickenings in the form of lignified stripes. The ovary (Fig. 12) has a thick wall, with a homogenous mesophyll and inner epidermic cells visibly smaller than those of the outer epidermis. The anatropous ovule has only one integument, as in all the other *Lamiaceae* species.

Inter- and intra-specific chemical variations in *Ajuga* sp.
The TLC analysis for the absolute methanol extracts indicated the fact that in case of *Ajuga reptans* there are 3 major iridoid components while in case of *Ajuga genevensis* there are only two (Fig. 18, Pl. IV); likewise, from the quantitative point of view the iridoids seem to be better represented in bugle compared to blue bugleweed.

If, as we may notice, aucubin is absent from both species (as also shown by literature), in case of am Rf of approximately 0.6 we notice the existence of an obvious spot that is also to be found in the purified extract of *Harpagophytum procumbens* used by us as standard.

The spectrophotometric dosing performed on the extracts obtained with methanol 70% led us to the results in table 2.

Table 2. Bioaccumulation of some secondary metabolites in *Ajuga reptans* and *Ajuga genevensis* samples harvested in 2009.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Location</th>
<th>Bioaccumulation</th>
<th>Flavonoids (g% luteolin)</th>
<th>Polyphenolcarboxilic acids (g% chlorogenic acid)</th>
<th>Iridoids (g% aucubin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Ajuga reptans</em> L.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Tg.Neamt 1</td>
<td>0.3090</td>
<td>0.7685</td>
<td>1.5634</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Tg.Neamt 2</td>
<td>0.2449</td>
<td>0.5782</td>
<td>0.6190</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Baisa</td>
<td>0.2714</td>
<td>0.7231</td>
<td>0.8710</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Guranda</td>
<td>0.2834</td>
<td>0.6799</td>
<td>2.0090</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Ajuga genevensis</em> L.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Nemtisor</td>
<td>0.2574</td>
<td>0.5130</td>
<td>0.9419</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Baisa</td>
<td>0.3323</td>
<td>0.4270</td>
<td>0.8863</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Albesti</td>
<td>0.2946</td>
<td>0.4609</td>
<td>1.0274</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Mascateni1</td>
<td>0.3281</td>
<td>0.5422</td>
<td>0.9514</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Mascateni2</td>
<td>0.3090</td>
<td>0.7625</td>
<td>0.9828</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Mascateni3</td>
<td>0.2547</td>
<td>0.4343</td>
<td>1.0072</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Mascateni4</td>
<td>0.2614</td>
<td>0.4380</td>
<td>0.9136</td>
<td></td>
</tr>
</tbody>
</table>

Expressing the data graphically, we obtain the images in Fig. 19, Pl. IV.

Likewise, in case of the flavonoids, the intra- and interspecific variations exist, but are in close limits.

In case of the iridoids (Fig. 20, Pl. IV), the quantitative differences in the same species, *Ajuga reptans* L., are, as can be seen, extreme: the Tg. Neamt 1 population has an iridoid content of over 1.5g, while population 2, harvested in the same restricted perimeter, not greater than 200 m², contain by 2 and a half times less non-volatile monoterpenes; the Guranda population with over 2% proved to be the richest. For *Ajuga genevensis* L., the intraspecific differences are much smaller, fact also seen by comparing the 4 Mascateni populations (Fig. 21, Pl. V).
On the other hand, if we compare the flavonoid, polyphenolcarboxilic acid and iridoid contents determined in *Ajuga reptans* L. and *Ajuga genevensis* L. individuals prelevated from the same place (Baisa), where we assume the existence of the same pedoclimatic offer, the discriminating factor being, for sure, the genetic heredity, we get the image in Fig. 22, Pl. V.

As we see, the flavonoids are found in the Baisa populations of the two mentioned *Ajuga* species, in the smallest quantity, followed by the polyphenolcarboxilic acids, and the highest concentration is that of the iridoids.

If the flavonoids are quantitatively close for the two species, the content of the polyphenolcarboxilic acids is much raised at *Ajuga reptans* L. compared to *A. genevensis*, and the iridoids present almost equal values.

**Conclusions**

The results of our researches showed intra and inter-specific variations of certain anatomical and chemical characters in the investigated *Ajuga* species.

The histo-anatomical differences refer especially to the vegetative organs, being represented by the frequency and localization of the hairs (non-glandular and glandular), the development and lignification degrees of the mechanical tissues (periphloemic sclerenchymatous fibre cordons, libriform fibres from the secondary xylem), the ratio secondary phloem/secondary xylem, the number of xylem cells per surface unit, the endoderm and stele type in the root (polyarch in the adventitious roots formed on the rhizome and diarch or triarch in their branching), the thickness of the limb and the number of palisadic cell layers.

For the populations belonging of the investigated *Ajuga* species there is a certain intra- and inter-specific chemical variation, referring especially to the values registered for iridoids.

Nevertheless, to establish the clear interdependence regarding the quantitative variations of the active principles, one should achieve some multiannual studies (with a constant harvesting of the individuals in the same locations) with the simultaneous monitoring of both the climatic conditions of each year and the composition of the soil.

We consider that if future researches will establish that, especially for *Ajuga reptans* L., the total iridoidic is in its greatest part constituted of derivates of the harpagid type, it would be possible that the species furnish convenient raw material to obtain some preparations useful to the part of the population with LOHAS orientation (Life of Health and Sustainability).
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Explanation of plates

Plate I
Fig. 1-6. Cross-sections through the subterranean organs of *Ajuga* sp.: rhizome (1, 3. *A. reptans*; 2, 4. *A. genevensis*); adventitious root (5. *A. reptans*; 6. *A. genevensis*). scale bars = 100 µm

Plate II
Fig. 7-12. Cross-sections through the aerial vegetative organs of *Ajuga* sp.: stem upper third (7. *A. reptans*; 8. *A. genevensis*); stem lower third (9. *A. genevensis*); foliar limb (10. *A. reptans*; 11. *A. genevensis*); glandular hair from leaf (12. *A. genevensis*). scale bars = 100 µm

Plate III
Fig. 13-17. *Ajuga* sp. flower structure. 13. Cross-section through a sepal. 14. Cross-section through a petal. 15. Cross section through the ovary wall. 16. Longitudinal sections through the ovary. 17. Cross-section through the stamen anther. scale bars = 100 µm

Plate IV
Figure 18. TLC for iridoids in methanolic extyracts of *Ajuga reptans* and *Ajuga genevensis*, 2009
*Ajuga reptans*: 1=Tg. Neamt-1; 2=Tg. Neamt-2; 3=Baisa; 4=Guranda
*Ajuga genevensis*: 1=Nemtisor; 2=Baisa; 3=Albesti; 4=Mascateni1; 5=Mascateni2; 6=Mascateni3; 7=Mascateni4; **Standars**: Au=aucubosid; Hg= purified extract of *Harpagophytum procumbens*; β=β-sitosterol; O= oleanolic acid; U= ursolic acid

Figure 19. The flavonoidic content determined spectrophotometrically in *Ajuga reptans* and *Ajuga genevensis* L individuals prelevated in 2009.
*Ajuga reptans* L: 1-Tg Neamt 1; 2-Tg Neamt 2; 3- Baisa; 4- Guranda.
*Ajuga genevensis* L: 1-Nemtisor; 2-Baisa; 3- Albesti; 4- Mascateni 1; 5- Mascateni 2; 6- Mascateni 3; 7- Mascateni 4

Figure 20. The iridoid content spectrophotometrically determined in *Ajuga reptans* L and *Ajuga genevensis* L. individuals prelevated in 2009.
*Ajuga reptans* L: 1-Tg Neamt 1; 2-Tg Neamt 2; 3- Baisa; 4- Guranda.
*Ajuga genevensis* L: 1-Nemtisor; 2-Baisa; 3- Albesti; 4- Mascateni 1; 5- Mascateni 2; 6- Mascateni 3; 7- Mascateni 4

Plate V
Figure 21. The intraspecific variability of iridoids in populations of bugle and blue bugleweed, developed in a small perimeter.
*Ajuga reptans* L: 1-Tg Neamt 1; 2-Tg Neamt 2.
*Ajuga genevensis* L: 1- Mascateni 1; 2- Mascateni 2; 3- Mascateni 3; 4- Mascateni 4.

Figure 22. The interspecific variability of some pharmacologically active secondary metabolites in *Ajuga reptans* L and *Ajuga genevensis* L. determined in 2009.
ANCA HEMCINSCHI and colabs.

PLATE I

1

2

3

4

5

6
ANCA HEMCINSCHI and colabs.  

PLATE II

Images 6, 7, 8, 9, 10, 11, 12
Fig. 21.

Fig. 22.