Leaf age, position and anatomical influences on the distribution of the secondary metabolites, homonataloin and three isomers of aloesin in *Aloe hereroensis* (Aloaceae) leaves

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Twenty leaves belonging to five age groups from four different *Aloe hereroensis* plants were investigated by anatomical and TLC methods. These results indicated that most of the phenolic metabolites are located in the vascular bundle sheath. The homonataloin accumulates in the big inner bundle sheath parenchymatous cells and the three isomers of aloesin isomers are mainly located in the outer bundle sheath cells. Therefore, the different content of these secondary metabolites in the various leaf parts, and different leaf ages, of *Aloe hereroensis* are positively related to the density of the vascular bundles. Of all 20 leaves, the top thirds had similar density of vascular bundles, as well as the highest content of the secondary metabolites. However, between the top, middle and the base leaf thirds, both the density of vascular bundles and the content of secondary metabolites were significantly different. In the same leaf, the top third has the highest density of vascular bundles, the base the lowest and the middle an intermediate density. The more exposed the younger leaves and the leaf parts to consumption, the higher the content of the secondary metabolites. This distribution of phenolic metabolites in leaves seems to relate to defense strategies of a plant.

Introduction

*Aloe hereroensis* Engler (Aloaceae) is abundant in parts of South West Africa, the type of locality being Usakos. Southern Angola is the farthest north that this species has been found (Reynolds 1966, Van Wyk 1996). This plant species was introduced and grows well in the Introduction Garden at the Jacob Blaustein Institute for Desert Research at Sede Boker Israel (34°46'E, 30°52'N, 460m a.s.l.). Plants of this species are acaulescent or have short stems. The grayish-green leaves are about 30cm long and 6cm broad, 3 leaves arranged in a circumference (Figure 1). The shrub is usually densely rosulate, lanceolate-deltoid and sometimes is elongated double H-shaped with whitish spots. Margins are sinuate-dentate, with slight cartilaginous edges armed with red-brown to brownish, deltoid, pungent, spreading teeth, 3–4mm long, which are 8–10mm apart (Reynolds 1966).

It is known that the leaf exudate of the *Aloe* species contains a number of pharmaceutically active phenolic metabolites, including homonataloin, barbaloïn, nataloin, aloeresin-A, aloeresin-C, isoaloeresin-A, aloenin 5-hydroxyaloenin and others (Birch and Donavan 1955, Takayuki and Toshifumi 1983, Nakagomi et al. 1985, Reynolds 1985, Yamamoto et al. 1991, Speranza et al. 1985). These different phenolic metabolites were found to have different colours under UV light when located on TLC or paper chromatography. In general, barbaloïn is yellow and homonataloin is dark yellow or light purple, but aloeresin and aloenin are always strong blue (Haynes and Holdsworth, 1970, Franz and Grün, 1983). The accumulation of these natural products seem to relate to a defense strategy, which is significant in itself (Hess 1975).

It has been found that the barbaloïn or homonataloin content in different *Aloe* species is higher in younger leaves than in older leaves (Birch and Donavan 1955, Takayuki and Toshifumi 1983, Nakagomi et al. 1985, Groom and Reynolds 1987, Yamamoto et al. 1991). The terminal third of the leaves has the highest percentage of these secondary metabolites and the leaf base third, the lowest (Chauer-Volfson and Gutterman 1996, 1997, 1998).

Homonataloin occurs in about 15% of the *Aloe* species in the Kew collection that contains over 200 species from a total of over 300 described *Aloe* species, and is therefore a representative sample of the genus (Beaumont et al. 1984). In the Introduction Garden of the Jacob Blaustein Institute for Desert Research in the Negev Desert highlands of Israel, there is a collection of about 100 *Aloe* species that have
been introduced from South Africa over the last 20 years. 16% of these Aloe plant species contain homonataloin. Several anatomical studies of leaves of Aloe plants have been carried out (Cutler 1972, 1978, Beaumont et al. 1985, 1986, Smith and Van Wyk 1992). The general leaf structures are outlined and it is suggested that the phenolic metabolites mainly collect in the vascular bundles that are surrounded by the chlorenchyma of the leaves (Grindlay and Reynolds 1986, Reynolds and Dweck 1999). However, previous reports were mainly concerned with the comparison between the Aloe and their evolved allies and hybrids (Cutler 1972, Cutler et al. 1980, Smith and Van Wyk 1992).

The aims of this study were to determine the distribution of the contents of homonataloin and 3 isomers of aloeresin in the leaves and the relationship between the anatomical structure and the content of these metabolites. Leaves were tested according to their different ages and parts to determine the possible significance of the metabolite distribution in Aloe plants.

Materials and Methods

The Introduction Garden of the Jacob Blaustein Institute for Desert Research at Sede Boker, in the Negev Desert, contains a unique collection of species of Aloe planted in loess soil. These plants have flourished in desert conditions and are irrigated with only small amounts of water equivalent to about 200mm of rain per year, in addition to the average annual 100mm of rain in this area. Twenty leaves from four different A. hereroensis plants and five different developmental stages from top to base of a plant were harvested on 27 April 2000. The youngest leaf harvested was L3, then the fifth — L5, seventh — L7, thirteenth — L13 and the eldest was the twenty-first — L21 (Figure 1). Two parallel experiments were carried out.

Each leaf was cut into 3 parts: terminal (T), middle (M) and base (B). Each part was then cut into two sections: one 2–3cm thick slice for the anatomical experiments and the remainder which was weighed and then analysed for the content of homonataloin and three isomers of aloeresin.

Anatomical experiment

Each fresh leaf piece was cut into 15–20μm sized cross sections by hand. Some sections were not stained and were directly observed under a fluorescence microscope (UV light, at 365nm wavelength) in order to determine the location of the different phenolic metabolites according to their different colours under UV light. In other sections the secondary metabolites were dissolved with 2–5% NaOH solution and then observed under a light microscope. The leaf structure, in particular the details of a vascular bundle, were examined. The density of the vascular bundles in all leaf parts was measured and compared. The result of the density of the vascular bundles is expressed in average numbers (± se) of 1mm lengths. The structural diagrammatic figures of leaves were drawn according to the images seen through the microscope.

Chemical experiment

Exudate was collected from each leaf part, using a hand press, frozen immediately (-20°C) and then freeze-dried in vacuum (80°C, 10 millitorr, Freeze mobile, Virtis). The freeze-dried material (powder) was analysed. Samples (5–10mg) of powder were dissolved in 1ml methanol and were dripped onto the pre-coated thin-layer chromatographic sheets, silica-gel 60 on polyester (20 x 20). After drying the plates were placed in the TLC glass developing tank with solvent: ethyl acetate-methanol-water (100:16.5:13.5 v/v). Homonataloin (Rf 0.41–0.45) and three isomers of aloeresin, aloeresin-1 (Rf 0.10–0.15), aloeresin-2 (Rf 0.20–0.30), aloeresin-3 (Rf 0.32–0.35), were separated from the plant material. They were eluted with an exact volume of methanol (5–10ml) and measured absorbencies at the different λ max 342–344nm for homonataloin, 298–300nm for aloeresin -1, 300–302nm for aloeresin -2 and -3. The contents of homonataloin and isomers of aloeresin were determined using calibration curves (Diode-Array Spectrophotometer. Hewlett Packard 8452A) and were calculated as percentage of dry weight of the leaf part exudate.

The significance of the above anatomical and chemical results were calculated and tested by the One-way ANOVA at 95% followed by the Fisher PLSD test (Sokal and Rohlf 1981).

Results

Anatomical study

1) General leaf structure of A. hereroensis

In this species, there are four main parts of a leaf in cross section of this species. Each section consists of one layer of epidermis covered by a thicker cuticle, several undifferentiated chlorenchymatous cells, central mucilage tissues, and one ring of vascular bundles located at the boundary...
between the chlorenchyma and central tissue. Each bundle has one layer of outer bundle sheath cells (OBSC) and 6–8 large, well-developed, parenchymatous inner vascular bundle sheath cells (IBSC). The phloem and xylem were underdeveloped. Several yellowish bubbles were observed in the chlorenchymatous cells and in the OBSC, but not in the IBSC (Figures 2, 3). The bubbles and IBSC appeared as the same dark yellow to light purple colour under the UV light. The greenish, blue or bluish colour were also found in the OBSC and other parenchymatous cells.

2) Density of vascular bundles
There were significant differences in the density of vascular bundles in the different leaf parts of each leaf and the different leaf ages among the 20 leaves. The result indicated the means of vascular bundles (V.B) among three parts: Top part > Middle part > Base part. The base part was lowest (2.01 ± 0.03, V.B./mm), the top part had the highest density (2.59 ± 0.04, V.B./mm), the middle was 2.18 ± 0.04, V.B./mm, between the top and base parts. Among three parts, there were significant differences between base vs. middle (p = 0.0005), between base vs. top and middle vs. top (p = 0.0001) (Figures 2, 4a). The density of vascular bundles in the whole leaf in the five groups decreased from L3 > L5 > L7 > L13 > L21 (Figure 5a). However, there were no significant differences in the top parts among the 20 leaves. The density of vascular bundles in the middle and base parts differed significantly between the leaf parts in these leaves (Figure 6).

Chemical analysis
Twenty leaves of the five leaf Nos. L3, L5, L7, L13 and L21, from four plants, contained homonataloin and Aloe resin isomers-1, 2 and 3 (Figure 7).

1) Distribution of homonataloin and aloeresin (isomers-1, 2, 3) in different leaf parts
The average content of homonataloin and aloeresin (isomers-1, 2, 3) in the exudate was higher in the top third of the leaves than the middle and base parts. The differences between the percentage of the four metabolites in the top, middle and base parts of the leaves were significant (p-value < 0.01) (Figure 4b).

2) Homonataloin and aloeresin (isomers-1, 2, 3) content influenced by leaf age
The average content of homonataloin and aloeresin (isomers-1, 2, 3) in the whole youngest leaves (L3) was the highest, and in the oldest (L21) leaves was the lowest (Figure 5b). There were no significant differences among the top third parts of the 5 leaves. However, there were significant difference among some of the third middle parts and some of the third base parts when tested separately, except between the neighbouring leave: L3 vs. L5, L7 vs. L13, L13 vs. L21 (Figure 8). The correlation between the density of the vascular bundles and the content of four secondary metabolites is shown in Figure 9.

Discussion
There is a similar general leaf structure in Aloe hereroensis to the other Aloe species (Cutler 1972, 1978, Beaumont et al. 1985, 1986, Shen et al. 1999). The outstanding feature in Aloe species is the lack of differentiation in the chlorenchyma and well-developed inner bundle sheath cells that exist in the phloem pole of each vascular bundle (Figures 2, 3).

During this study, it was found that A. hereroensis leaves mainly contain four different phenolic metabolites: homonataloin and 3 isomers of aloeresin-1, 2 and 3. These are distributed in such a way that the tops of each leaf have a significantly higher content than lower parts. It was also found

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**Figure 2:** Schematic diagram of three leaf sections of an Aloe hereroensis leaf: top, middle and base, showing the different density of vascular bundles

**Figure 3:** Diagrammatic representation of cross section of one part of an Aloe hereroensis leaf: C: cuticle; Ch: chlorenchyma; E: epidermis; IBSC: inner bundle sheath cells; OBSC: outer bundle sheath cells; Mc: mucilage cells; Ph: phloem; Xy: xylem; Yb: yellowish bubbles. Scale bar: 50μm
that the uppermost younger leaves, which are near the apex of the plant, have a significantly higher content of these metabolites than lower older leaves (Figure 1). These results are similar to the distribution of barbaloin, aloeresin and aloenin in A. arborescens (Chausser-Volfson and Gutterman 1996, Gutterman and Chausser-Volfson 2000a) and in A. pubescens and A. juncunda (Groom and Reynolds 1987).

Some reports stated that the secondary phenolic metabolites of Aloe plants originate mainly from the vascular bundles of the leaves (Grindlay and Reynolds 1986, Reynolds and Dweck 1999), and possibly accumulate in the inner bundle sheath cells (IBSC), e.g. 'aloin cells' (Cutler 1972, Cutler et al. 1980, Beaumont et al. 1986). Our results also revealed that the accumulation of these phenolic secondary metabolites are mainly related to the vascular bundles. From our preliminary tests it seems that these different phenolic metabolites accumulate in different parenchymatous cell. They indicated different colours according to IBSC and OBSC, under the fluorescence microscope.

Therefore, it is clear that the major factor that affects the content of these phenolic metabolites appears to be directly related to the density of vascular bundles (Figure 9). In this study, we found three interesting results about the density of vascular bundles of A. hereroensis leaves. These are: the density of vascular bundles in the top parts of all leaves is highest, lowest in the base parts and the middle is between top and base; the density is higher in younger leaves than in old leaves; in all upper thirds of 20 leaves, the density of the bundles was similar. According to the results, we assume that the density of vascular bundles is the most important structural factor to determine the relative content of the metabolites in leaves. The explanation of these anatomical results, and the different distribution of the secondary phenolic metabolite content among different leaves and leaf parts in the same leaf, are logical. In the upper third, there is less leaf volume and a lower ratio of the central parenchymatous tissue to the whole cross section of a leaf compared with the other two parts (Figure 2). Therefore, the highest density of vascular bundles has, correspondingly the highest secondary metabolite content. Among the five groups of leaves, the leaves (L3), as in the upper third of a leaf, have the highest vascular bundle density, so this group reasonably has the highest metabolite content.

Between the neighbouring groups of leaves, there were no significant differences in the density of vascular bundles because of their close developmental ages. Hence, there is also no difference in content. An interesting question is: Why does the upper third of all the leaves have the highest content of these secondary metabolites and vascular bundle density but no difference and no age effect? Could this be a defense strategy of this plant species? Some studies have revealed that the phenolic metabolites in plants are known to

Figure 4: a. Comparison of the density of vascular bundles in three different leaf parts of Aloe hereroensis leaves. Each column is an average of Top, Middle and Base parts of 20 leaves. b. Comparison of the content of homonataloin, aloeresin-1, aloeresin-2 and aloeresin-3 in three different parts of Aloe hereroensis leaves. Each column is an average of Top, Middle and Base parts of 20 leaves.
be toxic or to inhibit digestion of proteins and polysaccharides. Herbivores tend to avoid feeding on plants that contain high amounts of such substances (Nahrstedt 1989, Klocke and Kubo 1991, Rosenthal and Berebaum 1991). *A. hereroensis* is stemless or only has a very short stem. All of the 25 or so leaves are arranged in a rosette located parallel to the soil surface and the top of each leaf has its own exposure space (Figure 1). Therefore, it is clear that the higher content and the distribution of the phenolic metabolites are more efficient against herbivores, if these metabolites are repellent to them. The youngest leaves and the top part of each leaf have the higher density of vascular bundles which guarantees the higher accumulation of the special defense metabolites. Such a strategy may prevent some small herbivores from eating the plant.

Is the ecological significance to plant survival that a ‘cocktail’ of four different phenolic metabolites is found in larger quantities in the exposed leaf tops and younger leaves as a more efficient defense strategy to prevent adaptation in the herbivores? A similar phenomenon was also found in *A. arborescens* (Gutterman and Chauser-Vollson 2000b). Such questions require further study.

In addition to defense against herbivores, the phenolic metabolites in *Aloe* plants are also involved in the protection of the plants from U.V. irradiation damage (Bennett and Wallsgrove 1994, Lee et al. 1997, Reynolds and Dweck 1999, Strickland et al. 1994). Therefore, the higher the concentration of these metabolites in the younger leaves or top parts of a leaf, which are more exposed to the sun, the higher the efficiency of protection against U.V. radiation. There is still an uncertainty waiting to be clarified regard-
Figure 7: The absorption spectrum of four phenolic metabolites in Aloe hereroensis: aloeresin-1 (A), aloeresin-2 (B), aloeresin-3 (C) and homonataloin (D) at the wave length range from 190 to 820 nm in MeOH (mg/ml).
Figure 8: Comparison of the content of homonataloin, aloesin-1, -2, and -3 in three different parts of Aloe hereroensis leaves Nos. L3, L5, L7, L13, L21 from four plants. Each column is an average of one part of four leaves which were analysed separately.

Figure 9: The correlation between the content of four metabolites and the density of vascular bundles in Aloe hereroensis leaves.

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