African Journal of Plant Science Vol. 7(1), pp. 35-42, January 2013
Available online at http://www.academicjournals.org/AJPS
DOI: 10.5897/AJPS12.136
ISSN 1996-0824 ©2013 Academic Journals

Full Length Research Paper

Anatomical study of four species of *Heliotropium* L. (Boraginaceae) from Saudi Arabia

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Accepted 29 June, 2012

The leaf anatomy and distribution of types of foliar trichome of four *Heliotropium* L. species have been investigated. The aim of this study is to evaluate the systematic relevance of their diversity compared to the recent findings of systematic relationships within the genus. The results of leaf anatomy patterns, especially venation, vascular system, various types of foliar trichome and localization of crystals are surprisingly of high systematic value and proof.

Key words: *Heliotropium* L., leaf anatomy, Kranze-cells, C3 and C4 plants, venation, stomata, trichomes, crystals.

INTRODUCTION

The compositions of microparticles source of information taxonomy (Stace, 1969) are used in taxonomic studies that rely virtually on the qualities and characteristics of anatomy of species for distinguishing between species close to each other, and to confirm the deemed status of many plant species (Fritsch, 1903). The importance of anatomical studies in the definition and classification of herbarium samples finds a recipe for documenting the taxonomic relationship between the different taxonomic units (Bailey, 1951).

The leaf is the most important part of the plant and can be studied histologically to find out its anatomical characteristics, with good taxonomic evidence (Hickey and Wolf, 1975). The distribution and forms of trichomes of important qualities are used to differentiate between the genera and species of plant (Metcalfe and Chalk, 1950). These qualities can vary depending on the environmental conditions (density, shape and composition) where many of the species are fixed.

This study aims to detect the types of vascular bundles of the four species of the genus *Heliotropium* sp., and their cells arrangement. Most species throughout the world are characterized by a pattern of Kranz anatomy in which a layer of large barrel-shaped cells, which is dark green due to abundant chloroplasts, is filled with starch and has a high potential for carbon fixation and retention (Freitag and Stichler, 2002). Vascular bundles (Freitag and Stichler, 2002) are anatomical structure of leaves that establish C4 pathway. These plants spread in the tropics and subtropics regions; they grow in arid places particularly where the environmental conditions are too harsh for natural plants like C3 plants. Such places are land rocks, hypersaline soils and regions which suffer from lack of carbon and have high temperature. Scientists consider that the tendency of plants to have this kind of metabolism as a way of adapting to these environmental conditions is appropriate (Schulze et al., 1996). Diane et al. (2002a) conducted a study on leaves anatomy of *Heliotropium* spp.; they divided them into C3 and C4. This helped them to classify the plants into further subsidiaries.

MATERIALS AND METHODS

The study relied on three herbarium samples (*Heliotropium curassavicum, Heliotropium strigosum* and *Heliotropium subulatum*) and one fresh sample collected from Riyadh Region (*Heliotropium digynum*).

Study of stomata and hairs on the surface of the leaves

Dried leaves were placed in boiling water for few minutes to soften

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until they became unfolded and ready for epidermal scrapping. Fresh leaves were used directly for anatomical studies. Leaf samples were prepared according to the modified method of Cotton (1974). The fresh or dried leaves were placed in a tube filled with 88% lactic acid and kept in boiling water bath for about 50 to 60 min. Lactic acid softened the tissue of the leaves so that peeling off was possible.

To prepare the abaxial surface, the adaxial surface of the leaves was kept upward and then flooded with 88% cold lactic acid. The adaxial epidermis was cut across the leaves using a sharp scalpel blade and scrapped away together with the mesophyll cells until only the abaxial epidermis remained on the tile. The epidermis was placed outside the uppermost layer of the adaxial surface and mounted on a clean 88% lactic acid. The same procedure was followed to prepare the adaxial epidermis but the leaves were placed on the uppermost layer of the abaxial surface instead of adaxial surface uppermost layer. The photographs of these mounted materials were taken, using a camera (35 mm) mounted on light microscope (LM).

The study of the internal structures of the leaves

Johansen's method (Johansen, 1940) was used to prepare permanent slides. Formaldehyde based fixative containing 95% ethanol, 5 ml glacial acetic acid, 10 ml formaldehyde (37%) and 35 ml distilled water were used to kill and fix leaves as soon as they were collected. After 12 h in water, lamina strips from the middle part of the leaves consisting of the mid-vein, inter-coastal area and the margin were excised from the leaves, dehydrated in a graded ethanol series, infiltrated, embedded in paraffin wax and sectioned by microtome.

The transverse sections thus obtained were stained with safranin and light green combination. All the leaves collected were treated on the transverse section in this manner and were permanently mounted on balsam. Temporary section was fixed in formalin acetic acid alcohol (FAA) fixing solution, stained in aniline sulphate and mounted in glycerine. All slides were examined under light microscope.

Leaf venation patterns

Leaves from the terminal part of the branch were collected from 10 representative plants. Leaves were immersed in 80% ethanol for 48 to 72 h with several changes of solvent to remove chlorophyll pigments. Leaf samples were then washed and treated with 3 to 5% NaOH at 60°C for 24 to 36 h. The digested leaf tissues were carefully brushed apart to obtain the leaf skeletons. They were further hardened by treating with saturated chloral hydrate solution for several days, washed, dehydrated and preserved. The major leaves were dipped in hydrochloride or in a solution of ammonium hydroxide solution for a period of 4 to 5 min. After this, the leaves were passed through a series of different concentrations of ethanol (50, 70, 90 and 100%). They were transferred to a solution of absolute ethanol and hydrochloric acid with a status of 1:1 ratio for 2 min; and finally to the center of a hydrochloric acid for 2 min as well. Then the leaves were positioned on glass slides, with drops of Canada balsam added to them; they were covered with blankets and placed in a specialized heater of 40°C.

The leaves were examined and venation was drawn. Their patterns were studied with the help of a photographic enlarger. To study minor venation patterns, small bits were cut from the central part of the skeletons of the leaves (excluding mid rib and marginal parts), stained with safranin and mounted on euparol. Absolute veinlet number and absolute vein termination number were calculated using Gupta's (1961) method. The terminology of Hickey and Wolf (1975) was used for the description of leaf architecture.

RESULTS

Starting from the upper epidermis through the mesophyll and vascular bundle to the lower epidermis of the leaf, the following results were obtained.

Epidermis

Epidermis consists of epidermal cells standard, stomata, and growth of the epidermis (trichomes).

Epidermal cells standard

Epidermal cells differ in different plant species in arrangement. They are distinguished as living cells that relate to each other being placed sideways fully outside the species, except in places of stomata. Figure 1 shows photographical image of the four species of epidermal cells standard under investigation, which is a rounded polygonal upper and lower epidermis with straight cell wall.

Stomata

Stomata (Table 1, Figure 2) of the four species studied were hypostomatic. They could be classified into three different types:

a) Tetracytic: The stomata are surrounded with 4 subsidiary cells. All species are included in these types of stomata (Figure 2a, b, c and d).
b) Actinocytic: The stomata are surrounded with 5 or more subsidiary cells. And all species are included in these types of stomata (Figure 2a, b, c and d).
c) Anisocytic: In anisocytic type, the guard cells are surrounded by three unequal sized subsidiary cells, and the common wall which is at right angle to the longitudinal axis of stoma. This type can be seen in *H. subulatum* only (Figure 2d).

Growth of the epidermis (*trichomes*)

Growth of the epidermal cells (trichomes) of the four species under investigation was found (Table 1) to be of two types.

Non-glandular trichomes: They are of two forms:

1. Single-cell subset is unbranched-unicellular.
2. Single-cell non-branching papillary base overgrown with unbranched-unicellular trichomes with strongly

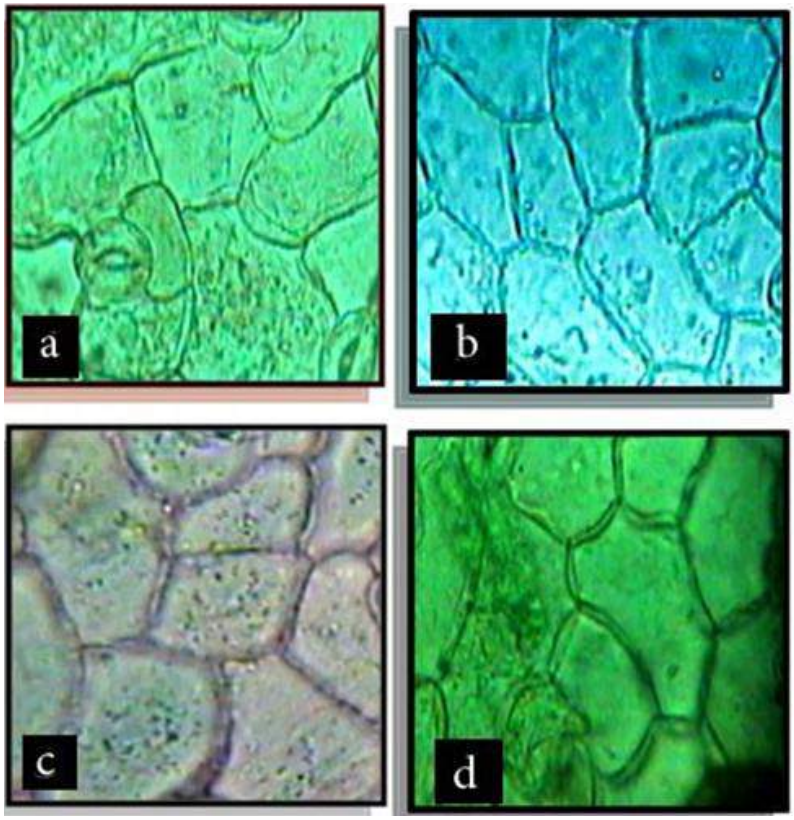

Figure 1. Photographical image of epidermis cells taken by light microscope (LM) polygonalroun: (a) *H. curassavicum*; (b) *H. digynum*; (c) *H. strigosum*; (d) *H. subulatum.*

Table 1. Characteristics of epidermal cells of the four *Heliotropium* species found in Saudi Arabia.

Species	Stomata			Trichome		
				Non-glandular		Stinging hair
	Tetracytic	Actinocytic	Anisocytic	Non-glandular unicellular	Papillose- hispid	
H. curassavicum	+	+	-	+	-	-
H. digynum	+	+	-	+	+	+
H. strigosum	+	+	-	+	+	+
H. subulatum	+	+	+	+	+	+

swollen base (papillose-hispid indumentums of uniform length).

Glandular trichomes: They are stinging hair. All species studied contained these types of trichomes mentioned in Table 1, except *H. curassavicum* which contained only unbranched-unicellular trichome as shown in Figure 3a.

Mesophyll tissue

Mesophyll tissue consists of two types of clorenchyma cells (palisade and spongy). The results in Table 2 and Figure 4 represent cells arrangement of palisade parenchyma and crystals found in the leaves of *Heliotropium* sp. The leaf anatomy can be distinguished into three categories:

1. Sub bifacial: Often discontinuous, one-layered palisade or palisade-like tissue on the abaxial side. The abaxial palisade cells are shorter than the adaxial ones. This type of leaf was found in *H. strigosum* and *H. subulatum* (Figure 4c and d).
2. Isobilateral, two-layered: An adaxial, two-layered palisade tissue and a two-layered palisade-like tissue of

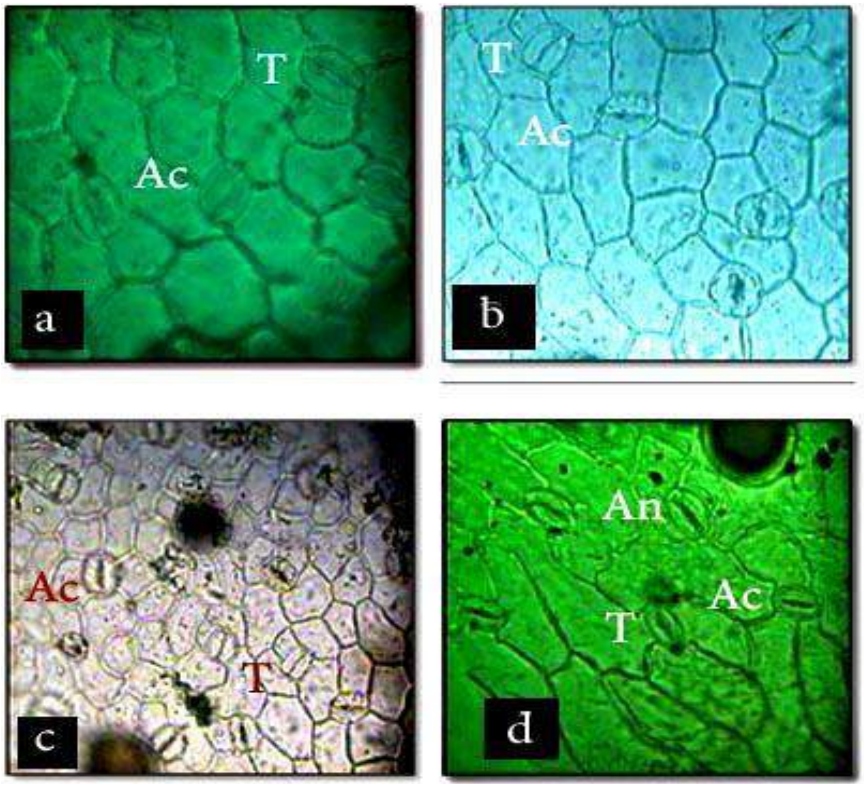

Figure 2. Photographs image by light microscope (LM) showing types of stomata: (a) *H. curassavicum*; (b) *H. digynum*; (c) *H. strigosum*; (d) *H. subulatum*.

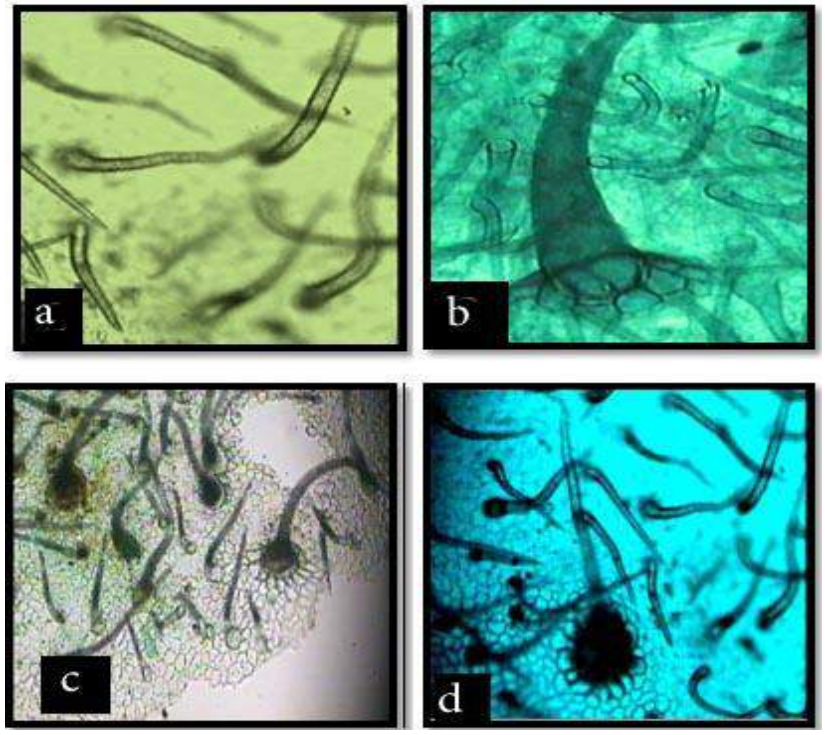

Figure 3. Photographs image by light microscope (LM) showing types of trichomes: (a) *H. curassavicum*; (b) *H. digynum*; (c) *H. strigosum*; (d) *H. subulatum.*

Table 2. The characteristics of the two types of cell arrangement of palisade parenchyma, succulent as well as crystals and Kranz-type in the four species *Heliotropium* found in Saudi Arabia.

Species	Types of cell arrangement palisade parenchyma			Crystal	Kranz-type
	Sub-bifacial	Isobilateral	Succulent	Druses	
H. curassavicum	-	-	+	-	-
H .digynum	-	+	-	+	-
H. strigosum	+	-	-	-	-
H. subulatum	+	-	-	+	+

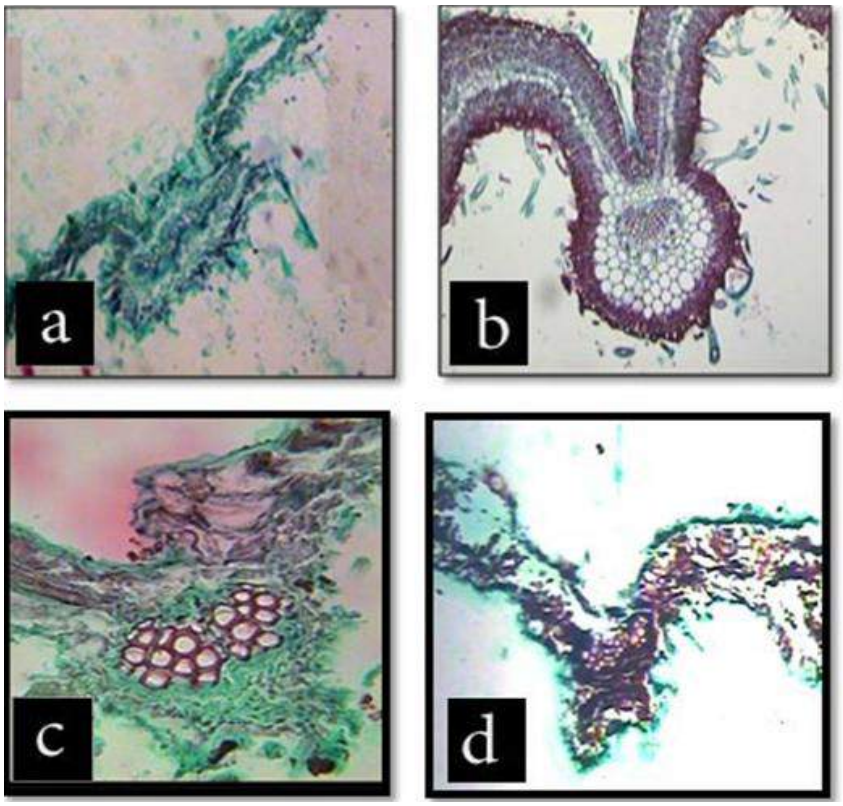

Figure 4. Photographical image by light microscope (LM) showing cross section of leaf anatomy: (a) *H. curassavicum*; (b) *H. digynum*; (c) *H. strigosum*; (d) *H. subulatum.*

shorter cells on the abaxial side, was present in *H. digynum* (Figure 4b).

3. Succulent: The succulent leaves of *H. curassavicum* are isobilateral and are not differentiated into palisade and spongy chlorenchyma (Figure 4a) since they are used as water-storage tissue and because this species is a halophytic one.

Idioblasts and crystals

Examination of the leaves of the four species resulted in finding calcium oxalate crystals deposited in mesophyll cells of *H. digynum* and *H. subulatu*m only (Table 2, Figure 5a and b).

Vascular system and Kranz-type

Leaf anatomy (Table 2) of all species investigated exhibited collateral bundles with adaxial xylem. Collenchymatic tissue was frequently associated to the main veins. Veins of higher order were surrounded by parenchymatous single layered bundle sheaths and their cells are mostly with and rarely without chloroplasts. Kranz-type of leaf anatomy was present in *H. subulatum* (Figure 6c and d) procumbens. The bundle sheath cells contained a large number of chloroplasts centripetally

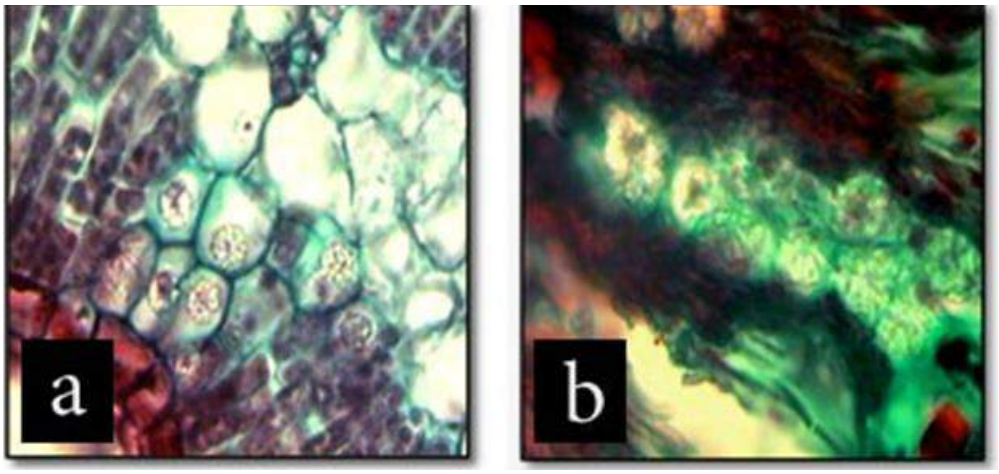

Figure 5. Photographs image by light microscope (LM) showing the types of druses crystals: (a) *H. digynum*; (b) *H. subulatum.*

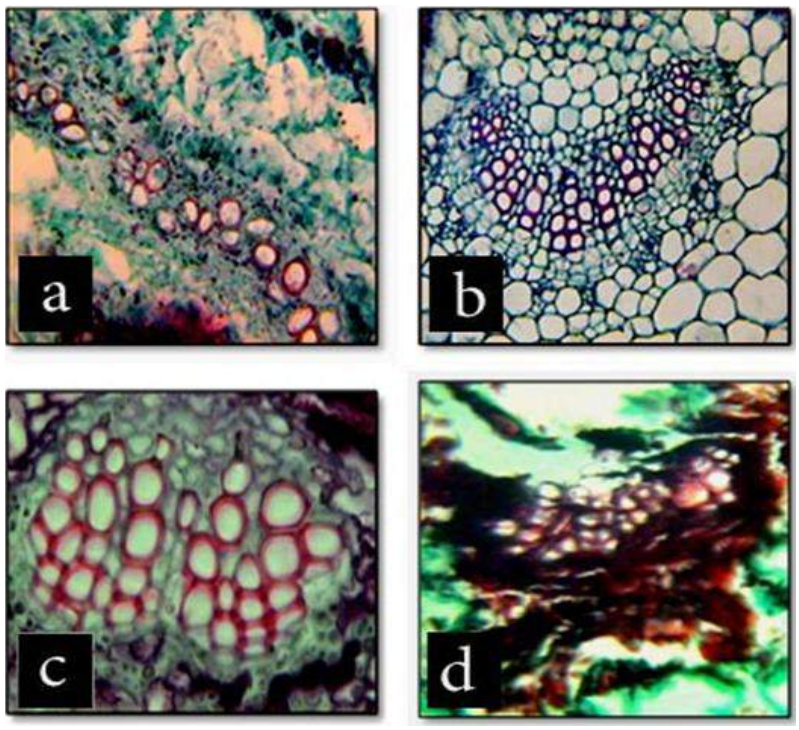

Figure 6. Photographs image by light microscope (LM) showing the types of vascular system: (a) *H. curassavicum*; (b) *H. digynum*; (c) *H. strigosum*; (d) *H. subulatum.*

appressed to the cell wall. There were no additional radially arranged mesophyll cells around the veins in *H. curassavicum*, *H. digynum* and *H. strigosum.*

Venations

In general, leaf venation was brochidodromous, differing in the prominence of secondary and tertiary veins on the abaxial surface, the orientation of the tertiary veins, or in the shape of the intercostal areas delimited by the secondary and tertiary veins.

Three types of venation were found (Table 3, Figure 7). According to the terminology of Hickey and Wolf (1975) and Ash et al. (1999), the descriptions of venations found in the four species are:

Table 3. Characteristics of venation types in the species of *Heliotropium* found in Saudi Arabia.

Species	Venation type			End of vein	
	Hyphodromous	Brochidodromous I	Brochidodromous II	Regular	Irregular
H .curassavicum	+	+	-	-	+
H. digynum	-	-	+	+	-
H. strigosum	-	+	-	-	+
H. subulatum	-	+	-	-	+

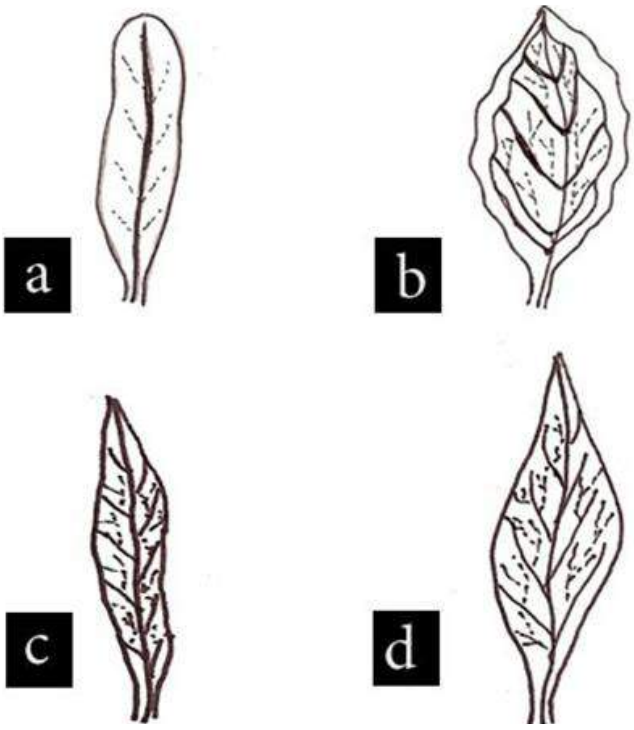

Figure 7. Drawing presentation of types of venations found in (a) *H. curassavicum*, (b) *H. digynum*, (c) *H. strigosum,* and *(*d) *H. subulatum.*

Hyphodromous

Only one prominent mid-vein was visible on the abaxial leaf surface. All higher order veins were weakly developed and immersed in the mesophyll. This condition was found in the succulent leaves of the halophytic *H. curassavicum*.

Brochidodromous I: Several fine and barely prominent secondary veins curve near the margin joined in a series of arches. The tertiary was very fine, not prominent, and randomly reticulate to weakly alternate percurrent, enclosing irregular four- to five-sided intercostal areas. This type of venation was found in *H. strigosum* and *H. subulatum*.

Brochidodromous II: Several prominent secondary veins curve near the margin joined in a series of arches. Near the margin, the secondary was often immersed in the mesophyll. Most or all tertiaries were not prominent. If elevated tertiaries were present, they were opposite to additional alternate percurrent right, and enclosing regular intercostal areas. This venation type was found in most *H. digynum*.

DISCUSSION AND CONCLUSION

General leaf morphology, size and shape of the leaves are of high variability within the genus *Heliotropium* in general. Therefore, these features are not appropriate for systematic studies on higher taxonomic level (Förther, 1998). These characters may be interpreted under ecological points of view. Semi-arid habitats promote small narrow- to linear-lanceolate leaves, sometimes with revolute leaf margins, and often a dense indumentum. This has evolved several times within most clades of *Heliotropium* L. On the other hand, humid tropical conditions or permanent water availability promote a broadleaved shape. The results of this study on leaf anatomy patterns, especially venation, vascular system, various foliar trichome types, and localization of crystals are of high systematic value.

Results obtained from studying the epidermal cells (upper and lower surface) in all the four species, indicate that the cells were rounded polygonal. These results are consistent with the results obtained in the study of Whang et al. (1998). It was also noted the presence of stomata on the lower and upper surfaces of the leaf on both hypoamphistoatic, as characterized by three different types of leaf stomata, and that leaf might contain one or combined types (Angel et al., 2003).

All the four species under study were characterized by indumentums, heavy relatively, but different in form and structure, as found in other studies (Rao and Kumar, 1995; Forther, 1998; Diane et al., 2002b). Mesophyll cells were arranged more or less radially around the bundle sheath. The vascular type *H. curassavicum* surrounded by several layers of cells, clorenchymic tissue interrelated, extended from the abaxial to adaxial surfaces of leaf epidermis but separated by the presence of vascular bundles. This type of vascular bundle was typical of succulent plants. These results are consistent with those of Diane et al. (2002a). Vascular bundles in *H. digynum* were surrounded by one layer of palisade

mesophyll to the upper epidermis; while at lower epidermis they were surrounded by four rows of parenchyma cells. Main vascular in *H. strigosum* showed the presence of layers of varying density of parenchyma tissue. Vascular system in *H. subulatum* was characterized by the arrangement of bundle sheaths. The sheath cells contained a large number of chloroplasts centripetally appressed against the cell wall. This arrangement indicated the Krantz-anatomy suggesting that this species is a C4-plant. These findings are consistent with other studies (Angel et al., 2003; Das and Raghavendra, 2003); but, one study found in the literature (Diane et al., 2002a) considered *H. subulatum* as a C3-plant and *H. strigosum,* a C4-plant. In the current study, results did not show the autopsy of the leaf but the presence of Krantz- cells around the vascular type mentioned. It is tempting to suggest that this metabolic pathway as a mutation for adaptation to environmental conditions might not be appropriate as it could be due to the different qualities of leaf histology based on the environment where the plant grows in.

Leaf size and shape may vary enormously within one group of plants, but leaf venation is usually constant (Sack and Michele, 2006). In general, results of this study on the leaves of *Heliotropium* showed a brochidodromous venation similar to other reports (Diane et al., 2002a). However, there were some differences in prominence of the secondary and tertiary veins, which might be immersed in the mesophyll, the orientation of the tertiaries, or in the shape of the intercostal areas. There was a brochidodromous venation with fine and barely developed veins of higher order and randomly reticulate to weakly alternate percurrent tertiaries enclosing irregularly four- to five-sided intercostals areas. Starting from this venation type, a reduction in hyphodromous venation characterized the leaves venation of the halophytic species (*H. curassavicum)* only. This result is consistent with another report of Diane et al. (2002a).

ACKNOWLEDGEMENT

This research project was supported by a grant from the Research Center of the Center for Female Scientific and Medical Colleges in King Saud University.

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