Microscopic morphology of *Dichapetalum cymosum* (Hook.) Engl. as an aid in the identification of leaf fragments from the digestive tract of poisoned animals

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ABSTRACT

Dichapetalum cymosum (Hook.) Engl. (Poison leaf; gifblaar) is a major cause of acute livestock plant poisoning in southern Africa. Microscopic identification of leaf fragments found in the digestive tract of poisoned animals can assist in the diagnosis of poisoning when *D. cymosum* poisoning is suspected. The microscopic morphology of *D. cymosum* leaves are described using standard staining and microscopy methods for histopathology samples at many regional diagnostic laboratories. Morphological descriptions included structures in the epidermis and mesophyll that were discernible using H & E staining. The microscopic morphology of *D. cymosum* was used to differentiate between leaf fragments of *D. cymosum* and other species from the same habitat with macroscopic features that resemble those of *D. cymosum,* including *Euclea crispa, Combretum zeyheri, Burkea afrikana* and *Lannea discolor.*

Key words: *Burkea africana, Combretum zeyheri, Dichapetalum cymosum, Euclea crispa, Lannea discolor,* plant poisoning, monofluoroacetate, rumen content microscopy.

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INTRODUCTION

The economic losses and social impact of plant poisonings of livestock in southern Africa is considerable[5]. *Dichapetalum cymosum* (Hook.) Engl. (poison leaf; gifblaar), which occurs in southern Angola, north-eastern Namibia, Botswana, southern Zambia, western and southern Zimbabwe, southern Mozambique and northern and eastern South Africa, is highly toxic to livestock[2]. It causes around 8 % of annual cattle losses due to plant poisonings in South Africa[5].

The toxic principle of *D. cymosum* is monofluoroacetate[7]. Monofluoroacetate itself is not toxic, but it is converted in the animal to monofluorocitrate. Monofluorocitrate inhibits the enzyme aconitase, thereby the tricarboxylic acid cycle and cellular respiration[6]. It causes sudden death in animals, 4 to 24 hours after they have ingested lethal amounts of plant material[4].

Owing to the rapidity of death in animals that have died of *D. cymosum* poisoning, leaf fragments of the plant that caused the poisoning can often be found in the digestive tract of poisoned animals. The presence of *D. cymosum* leaf fragments in the digestive tract of an animal that has died due to suspected plant poisoning is therefore considered to be an important factor in the diagnosis of *D. cymosum* poisoning[4].

Microscopic examination of leaf fragments found in the digestive tract of poisoned animals can aid in the identification of *D. cymosum* leaf fragments recovered from rumen content. This study reports the characterisation of the microscopic morphology of *D. cymosum* leaves discernible by light microscopy using tissue sections prepared by standard histopathology procedures.

MATERIALS AND METHODS

Plant samples

Dichapetalum cymosum samples were collected from the northern slopes of the Magaliesberg in the vicinity of the Florauna suburb of Pretoria in the Gauteng Province of South Africa. Leaf samples were fixed in 10 % buffered formalin. Mature and immature leaf samples were obtained from 2 plants that differed morphologically regarding the density of trichomes (hairs) on the leaf surface. Leaves of plant species with reticulated leaf vein patterns, including *Burkea africana* Hook. f., *Combretum zeyheri* Sond., *Euclea crispa* (Thunb.) Guerke subsp. *crispa* and *Lannea discolor* (Sond.) Engl., were collected from the same habitat.

Tissue preparation

The method of plant tissue preparation followed standard procedures for the preparation and haematoxylin and eosin (H & E) staining of animal tissues for histological examination in the Pathology Section of the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria. The procedures used are widely known and commonly available at regional veterinary diagnostic laboratories.

Dichapetalum cymosum samples were also stained using McMannus' Periodic Acid Schiff's (PAS) method to compare it with H & E staining. PAS staining is commonly available at diagnostic laboratories for detecting fungi in histological tissue sections. It is an efficient stain for polysaccharides found in cell walls.

Microscopy

Microscopy was with a Leitz Laborlux D microscope fitted with ×10 eyepieces and ×10, ×25, ×40, and ×100 (oil) objectives. All diagnostic features could be discerned using ×10, ×25 and ×40 objectives.

RESULTS

Epidermis (outer cell layer)

The epidermis has 1 adaxial (facing the stem, usually the 'top' surface of the leaf and 1 abaxial (facing away from the stem) cell layer. The cuticle (waxy outer membrane) is well developed on both sides of the leaf.

Stomata (openings on leaf surface that allows for gas exchange) (Fig. 1) are rare or absent on the adaxial surface, but relatively common on the abaxial surface where they are randomly distributed.

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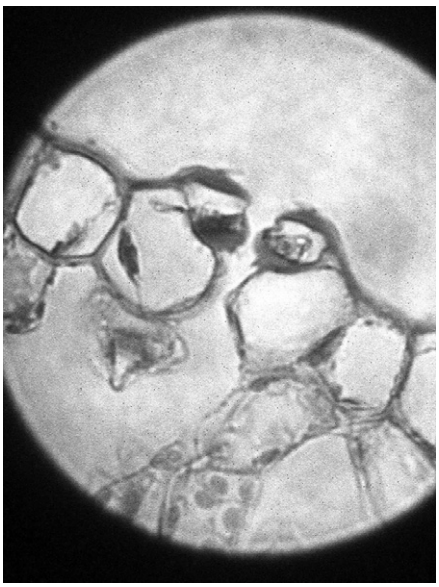

Fig. 1: **Stomatal structure in the abaxial epidermis of a *Dichapetalum cymosum* leaf.**

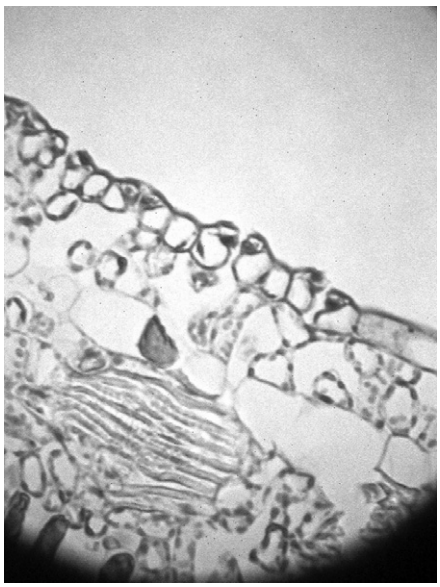

Fig. 2: **Abaxial side of a *Dichapetalum cymosum* leaf showing the epidermis with stomata, spongiform parenchyma and a secondary vein.**

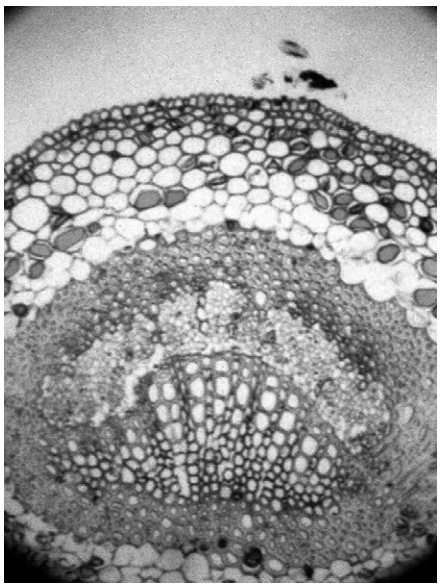

Fig. 4: **Large vein of a *Dichapetalum cymosum* leaf showing the bundle sheath, bundle sheath extensions, inner sheath, xylem and phloem.**

Stomata consist of 2 guard cells (cells lining the stomata openings) and 2 subsidiary cells (cells that connect guard cells with typical epidermal cells) and are paracytic, meaning that the longitudinal axes of the guard cells and the subsidiary cells are parallel to the stomatal apertures. Each guard cell is supported by a single subsidiary cell. Guard cells do not span the entire thickness of the epidermis. The guard cells are smaller than the surrounding epidermal cells, with diameters of one third to one half of subsidiary cells. The guard cells are raised above the surface of the epidermal layer. The cuticle extends onto the outside surface of the guard cells. It does not extend over the guard cells into the stomatal lumen. Guard cell wall protuberances are present on the outer guard cell walls. These protuberances are visible in dorsoventral sections as small, horn-like ridges in the centre of the outer cell walls and they appear to be part of the cuticle. In dorsoventral section, the guard cells and subsidiary cells usually appear rounded, compared to cube or brick-shaped surrounding epidermal cells.

Large, intercellular substomatal chambers consistently occur in mature leaves (Fig. 2), but are absent or small in immature leaves.

Trichomes (hairs) are unicellular and non-flattened. They vary in density from very dense to very sparse or absent.

Mesophyll (internal leaf tissues)

One hypodermal cell layer (layer/layers of cells beneath the epidermis that differ structurally from the tissues beneath them) is present under the adaxial epidermis (Fig. 3). No hypodermis occurs beneath the abaxial epidermis (Fig. 2).

Parenchyma cells that contain large vesicles with yellow to orange coloured content on H & E staining are scattered through all parenchymal cell types.

Palisade parenchyma (columnar cells with longitudinal axes perpendicular to the epidermis) occurs only on the adaxial side of the leaf (Fig 3). They occur in 1 to 3 layers with 2 layers being the most prevalent. The cell walls typically have a beaded appearance due to multiple areas of cell wall thickening.

Spongiform parenchyma (irregularly shaped cells) occurs only on the abaxial side of the leaf (Fig. 2). The cell walls typically have a beaded appearance similar to that of the palisade cells. Large intercellular spaces are present in the spongiform parenchyma of mature leaves, but may be small or absent in immature leaves.

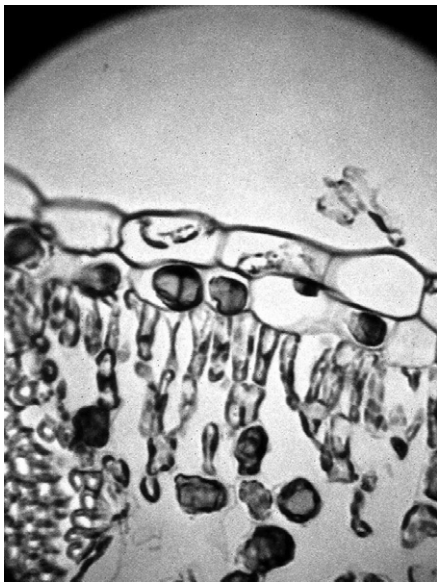

Fig. 3: **Adaxial side of a *Dichapetalum cymosum* leaf showing the epidermis and palisade parenchyma.**

Larger veins (Fig. 4) are surrounded by bundle sheaths, consisting of a single layer of tightly packed, thin-walled parenchyma cells, which are elongated in the direction of the veins. Multi-layered bundle sheath extensions, consisting of more or less elongated cells with unequally thickened walls, connect the veins with the epidermis and are present on both sides of the leaf where large veins are present. In these areas palisade and spongiform layers are absent.

Sclerenchymal cells, a supporting tissue consisting of multilayered, elongated cells with markedly thickened walls, form the inner sheath layer around larger veins (Fig. 4).

The xylem (Fig. 4) consists of variably shaped cells arranged in dorsoventral rows on the adaxial side of the vascular bundles of large veins. The phloem consists of variably shaped cells in a random pattern on the abaxial side of the xylem of large veins. The cells of the phloem stain more basophilic than the cells of the xylem and the bundle sheaths on H & E staining.

Venation characteristics

Venation is reticulate (Fig. 5). Side-veins branch in offset pairs and occasionally oppositely, from a central main vein, which runs from the leaf stem through the length of the leaf blade. Large side-veins anastomose with neighbouring large side-veins about three quarters of the way to the leaf margin to form a closed system. The central vein and large side

Table 1: **Anatomical features of leaves used to differentiate between *Dichapetalum cymosum, Burkea africana, Combretum zeyheri, Euclea crispa and Lannea discolor*.**

	Dichapetalum cymosum	*Burkea africana*	*Combretum zeyheri*	*Euclea crispa*	*Lannea discolor*
Venation	Reticulate	Reticulate	Reticulate	Reticulate	Reticulate
	Central vein with alternately to oppositely branching side-veins	Central vein with alternately to oppositely branching side-veins	Central vein with alternately branching side-veins	Central vein with alternately branching side-veins	Central vein with alternately branching side-veins
	Large side-veins anastomose about three quarters of the way to the leaf margin	Large side-veins anastomose close to the leaf margin	Large side-veins anastomose close to the leaf margin	Large side-veins anastomose about halfway between the central vein and the leaf margin	Large side-veins anastomose about three quarters of the way to the leaf margin
	Central vein and large side-veins form palpable ridges on the adaxial and abaxial leaf surfaces	Central vein and large side-veins form palpable ridges on the adaxial leaf surface while on the abaxial surface only the central vein is palpable and only towards the base of the leaf	Central vein and large side-veins form palpable ridges only on the abaxial leaf surface	Central vein and large side-veins form faintly palpable ridges only on the adaxial leaf surface while the central vein only forms a prominent ridge on the abaxial leaf surface	Only the central vein form palpable ridges only on the abaxial and adaxial leaf surfaces
	Areoles contain 1–3 terminal vein endings	Areoles usually contain terminal vein endings (up to 5)	Areoles are rich in terminal vein endings (up to 10)	Areoles contain 1–3 terminal vein endings	Terminal vein endings are absent from areoles or are low in number (1–2)
Epidermal cell layer	The epidermis consists of a single cell layer	The epidermis consists of a single cell layer	The epidermis consists of a single cell layer	The epidermis consists of a single cell layer	The epidermis consists of a single cell layer
	The cuticle is well developed on the adaxial and abaxial side	The cuticle is markedly thicker on the adaxial side	The cuticle is well developed on the adaxial and abaxial side	The cuticle is well developed on the adaxial and abaxial side	The cuticle is thicker on the adaxial side
Trichomes	Trichomes are unicellular, non-flattened hairs	No trichomes	Two trichome types: unicellular, uniseriate, non-flattened hairs and multicellular, sessile, flattened, peltate hairs	No trichomes	Multi-cellular, stellate trichomes
	Trichomes vary in density from very sparse to very dense on both leaf surfaces and are not always present		Trichomes common on both leaf surfaces		Trichomes only on the abaxial leaf surface
Stomata	Stomata mostly found in the abaxial epidermis	Stomata mostly found in the abaxial epidermis	Stomata mostly found in the abaxial epidermis	Stomata mostly found in the abaxial epidermis	Stomata mostly found in the abaxial epidermis
	Guard cells raised above leaf surface	Guard cells not raised above leaf surface	Guard cells not raised above leaf surface	Guard cells not raised above leaf surface	Guard cells raised above leaf surface
	Guard cell diameter is a third to half that of subsidiary cells	Guard cell diameter is about half that of subsidiary cells	Guard cell diameter is about a quarter that of subsidiary cells	Guard cell diameter is a third to half that of subsidiary cells	Guard cell diameter is a third to half that of subsidiary cells
	Cuticle does not extend into the stomatal lumen	Cuticle extends into the stomatal lumen	Cuticle extends into the stomatal lumen	Cuticle extends into the stomatal lumen	Cuticle extends into the stomatal lumen
	Cuticle forms a horn-like ridge on each guard cell	Cuticle forms 2 thin, curved ridges on each guard cell	Cuticle forms a horn-like ridge on each guard cell	Cuticle ridges on guard cells are low or absent	Cuticle forms a thin, curved ridge on each guard cell
Hypodermal cell layer	One hypodermal cell layer is present under the adaxial epidermis	No hypodermal cell layers	No hypodermal cell layers	No hypodermal cell layers	No hypodermal cell layers
Palisade parenchyma	Only on adaxial side of leaf	Only on adaxial side of leaf	Only on adaxial side of leaf	Only on adaxial side of leaf	On adaxial and abaxial sides
	1–3 cell layers	1–3 cell layers	1–3 cell layers	1–2 cell layers	1–3 cell layers on adaxial side; 1 layer on abaxial side
Spongiform parenchyma	Only on abaxial side of leaf	Only on abaxial side of leaf	Only on abaxial side of leaf	Only on abaxial side of leaf	Central
	Substomatal chambers present	Substomatal chambers present	Substomatal chambers present	Substomatal chambers present	Substomatal chambers present
Bundle sheath extensions	Present only on large veins	Absent only from very small veins	Absent only from very small veins	Absent only from very small veins	Absent only from very small veins

Table 2: **Summary of *Dichapetalum cymosum* morphological structures that are useful for the microscopic identification of leaf fragments.**

Structure(s)	Description
Venation	Reticulate Central vein with alternately to oppositely branching side-veins Large side-veins anastomose about three quarters of the way to the leaf margin Central vein and large side-veins form palpable ridges on the adaxial and abaxial leaf surfaces Areoles usually contain 1–3 terminal vein endings
Epidermal cell layer	The epidermis consists of a single cell layer The cuticle is well developed on the adaxial and abaxial side
Trichomes	Trichomes are unicellular, non-flattened hairs Trichomes vary in density from very sparse to very dense on both leaf surfaces and are not always present
Stomata	Guard cells raised above leaf surface Guard cell diameter is a third to half that of subsidiary cells Cuticle does not extend into the stomatal lumen Cuticle forms a horn-like ridge on each guard cell
Hypodermal cell layer	One hypodermal cell layer is present under the adaxial epidermis
Palisade parenchyma	Only on adaxial side of leaf 1–3 cell layers
Spongiform parenchyma	Only on abaxial side of leaf Substomatal chambers present
Bundle sheath extensions	Present only on large veins

veins form palpable ridges on both the adaxial and abaxial leaf surfaces. Smaller veins anastomose to form networks covering the whole leaf blade. Areoles contain 1–3 terminal vein endings that end blindly in the mesophyll. Except for areoles, the venation characteristics described above can be seen macroscopically on whole leaves and large leaf fragments.

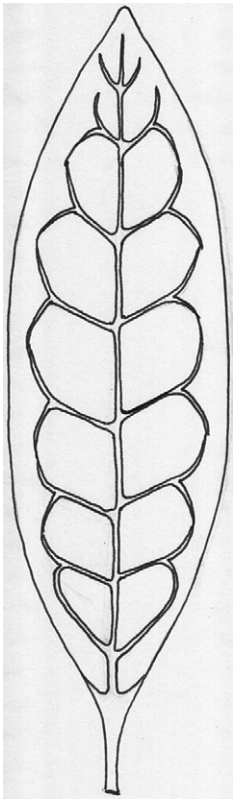

Fig. 5: **Schematic representation of the reticulate venation of *Dichapetalum cymosum* leaves.**

Comparison with other reticulately-veined plant species

Samples of other reticulately-veined plant species that share *D. cymosum* habitat in the Magaliesberg region of Gauteng, including *Burkea africana, Combretum zeyheri, Euclea crispa* and *Lannea discolor,* were compared with *D. cymosum.* Note that this does not represent a complete list of plants with leaf fragments that may resemble *D. cymosum* macroscopically, but only those that were found in association with *D. cymosum* at a single locality. All plants with similar growth forms to *D. cymosum,* such as *Parinari* capensis Harv., were not included if their leaf venation patterns were not reticulated and leaf fragments could easily be differentiated from that of *D. cymosum* macroscopically. The results are summarised in Table 1.

DISCUSSION

Post mortem examinations of animals that have died from *D. cymosum* poisoning usually do not reveal any lesions of diagnostic significance[4]. A high-performance liquid chromatographic technique has been developed for the estimation of monofluoroacetate concentrations in rumen contents[8], but the technique is not performed routinely and is not readily available. The presence of undigested *D. cymosum* leaf fragments in conjunction with circumstantial evidence is often the most practical method used to confirm a diagnosis of poisoning[4].

The macroscopic characteristics typically used to differentiate between *D. cymosum* and other species with similar growth forms, such as *Parinari capensis* (grysappel), *Pachystigma pygmaeum* (Schltr.) Robyns (gousiektebossie), *P. thamnus* Robyns (smooth gousiektebossie), *Pygmaeothamnus zeyheri* (Sond.) Robyns (goorappel), *P. chamaedendrum* (Kuntze) Robyns (small goorappel) and the shrublet-form of *Ochna pulchra* Hook. (lekkerbreek) include the leaf attachment pattern, the presence/absence of stipules, the relative colour of the adaxial and abaxial leaf surfaces, the leaf venation pattern and fruit and flower characteristics[4]. Identification of plant fragments in rumen content, however, requires a different approach because most of these characteristics can be lost after an animal chews and ingests leaves. Growth form is irrelevant when only leaf fragments are available for examination. The venation pattern is often the only recognisable macroscopic feature discernible in leaf fragments found in the gastrointestinal tract. The reticulate venation of *D. cymosum* leaves is not unique and is found in a number of other species that share the same habitat. Leaf fragment identification based on reticulate venation alone is therefore not a completely reliable proof that a poisoned animal had ingested *D. cymosum* leaves.

Microscopic examination of plant samples offers a means of identifying typical histological features[3,9]. It provides access to multiple identification features on relatively small leaf fragments. Histological sections of leaf fragments can be prepared using standardised, routine techniques that are available at many regional laboratories. H & E staining was found to be adequate for the visualisation of diagnostic features using light microscopy. In this study, PAS staining compared with H & E staining resulted in

more vivid staining of cell walls, and both methods resulted in satisfactory visualisation of diagnostic features. PAS staining may be useful if increased contrast is desired.

Direct comparison between unknown plant material and known standards, using the same instrumentation, magnification and settings, is a practical an effective approach to plant fragment identification[1]. It would be of practical value to those who might use this diagnostic approach to prepare reference slides containing stained and permanently mounted cross-sections of *D. cymosum* leaves. Reference slides should represent the morphological variability of the species in the area of interest, such as hairy/smooth leaves and mature/immature plants. Table 2 represents a summary of morphological characteristics of *D. cymosum* that are commonly discernible in leaf fragments and that are likely to be useful in the identification of leaf fragments. It would be imprudent to depend on a single characteristic for identification. Combining a number of characteristic histological features significantly reduces the risk of misidentification.

Use of microscopy to identify plant fragments can help to confirm the diagnosis of plant poisoning if the gastrointestinal content is examined shortly after ingestion of plant material. However, extended exposure to the digestive tract secretions and flora may degrade or destroy diagnostic morphological features. It should also be noted that the presence of potentially toxic plant fragments in the digestive tract does not prove the absorption of lethal doses of toxin, and should be interpreted with caution in the absence of supporting circumstantial and symptomatic evidence.

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