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Foliar secretory structures in *Ekebergia capensis* (Meliaceae)

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Abstract

Ekebergia capensis is a medium-sized to large evergreen to deciduous tree ranging from southern Africa to Ethiopia. Two morphologically-distinct variants of *E. capensis*, southern and northern, may be recognized in southern Africa. Despite its wide distribution range there appear to be no published reports on the secretory structures occurring on the leaves. In very young leaves, colleters on the petiolules, adjacent portions of the rachis and the midrib of the adaxial leaflet surfaces, secrete fluid which at least partly covers these developing areas. This is the first record of colleters in Meliaceae. In addition, several extrafloral nectaries (EFNs) are found in variable positions on the abaxial side of the leaflets. No stomata are associated with the EFNs. The glandular tissue of active EFNs is surrounded by druse crystals of calcium oxalate and consists of secretory cells some of whose walls are separated by "strands" of amorphous lipophilic material, especially in a radial orientation. EFNs on developing leaves are inconspicuous but with time, frequently become more easily visible due to the accumulation of pinkish/reddish anthocyanins. Even on senescent leaves, shed in autumn, large droplets of nectar are frequently visible on the EFNs. The secretory tissue originates from protoderm and ground tissues. Slight differences in abundance, size, shape, position and structure exist between the EFNs of the southern and northern forms. Varying proportions of glucose, fructose and

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sucrose were detected in the rather viscous nectar with the most abundant sugar usually being fructose. Ants were only rarely observed feeding on the nectar. This finding is in conflict with the generally accepted idea that EFNs provide food for ants which in turn protect the plant from herbivores. More detailed studies of the chemistry of the nectar, which is relatively copious, may provide clues as to the function.

Keyword: Plant biology

1. Introduction

It is well established that nectar from extrafloral nectaries (EFNs), through its mutualistic association with mainly ants, provides plants with an indirect defence against herbivores (Rico-Gray and Oliveira, 2007; Walters, 2011; Yamawo et al., 2014). There is also mounting evidence that the ecological effects of this nectar source are much more profound as it is known to not only mediate multi-species interactions across trophic levels (Bezemer et al., 2014; Staab et al., 2016), but may even be herbivore-induced (Heil, 2015). Despite the considerable ecological importance of EFNs, their presence in many floras has not been carefully studied, and much remains to be learnt about their structure, biology and function (e.g., Koptur, 1994, 2005; Marazzi et al., 2013; Heil, 2015).

The Meliaceae is a family of about 51 genera and 700 species, comprising mainly tropical trees and shrubs (Mabberley, 2017). Members of this family usually have pinnately compound leaves which may persist or be deciduous. It is one of 109 angiosperm families with EFNs according to Weber et al. (2015) who record such nectaries in the following genera of the family: *Carapa* (two spp.), *Cedrela* (two spp.), monotypic *Cipadessa*, *Dysoxylum* (one sp.), *Guarea* (two spp.), *Pseudocedrela* (one sp.), *Swietenia* (three spp.) and *Trichilia* (five spp.). However, in an unpublished doctoral thesis (Clark, 1990), EFNs (referred to as "glandular bodies") have also been reported in *Ekebergia* (three spp.), *Heynea* (one sp.) and *Walsura* ('most species'—ten studied). For *Ekebergia* it is merely mentioned that these "glandular bodies" occur sporadically on the leaflets and are most probably EFNs. No significant information on their morphology, structure and nectar-secreting behaviour was supplied. Yamawo (2015) claimed a first report of EFNs in *Melia azedarach* L., but glands at the base of the petiole which seem to be the EFNs of Yamawo were earlier described by Jacobs (1961) in this taxon and in *Azadirachta excelsa* (Jack) Jacobs. It appears that Jacobs' reference to these genera has also been overlooked by other researchers of this family, e.g., Lersten and Rugenstein (1982) and Morellato and Oliveira (1994). Metcalfe and Chalk (1950) do not cite EFNs in the Meliaceae but record the presence of multicellular glandular hairs of various shapes and secretory cells usually located at the boundary between the palisade and

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spongy mesophyll. However, colleters are not mentioned, nor are they recorded for Meliaceae in the comprehensive review of these structures by Thomas (1991). Colleters are glandular structures which secrete a sticky substance, frequently composed of mucilage and terpenes, that may provide protection against excessive sunlight and loss of water to the apical meristem and expanding young leaves (Fahn, 1979; Evert, 2006; Oliveira et al., 2017) or act as a lubricant during floral development (Leitão and Cortelazzo, 2008).

Ekebergia capensis is a medium-sized to large tree growing in coastal and montane forest, occasionally savannah, in southern Africa and northwards to Sudan and Ethiopia. In southern Africa two morphologically distinct variants, northern (Fig. 1A) and southern (Fig. 1B), may be recognized (Van Wyk and Van Wyk, 2007: 19, 2013). The southern form has stout stems, relatively small leaves with a slightly winged rachis, leaflets with relatively short petiolules, and the petiolar bases of shed leaves are often transformed into persistent, woody phyllopodia. Plants of the northern form have more slender stems, larger leaves with a cylindrical rachis, leaflets with relatively long petiolules, and phyllopodia are usually absent. Both variants are evergreen in warmer areas but tend to shed their leaves in colder, drier parts, the latter quite noticeable when being cultivated outside their natural ranges.

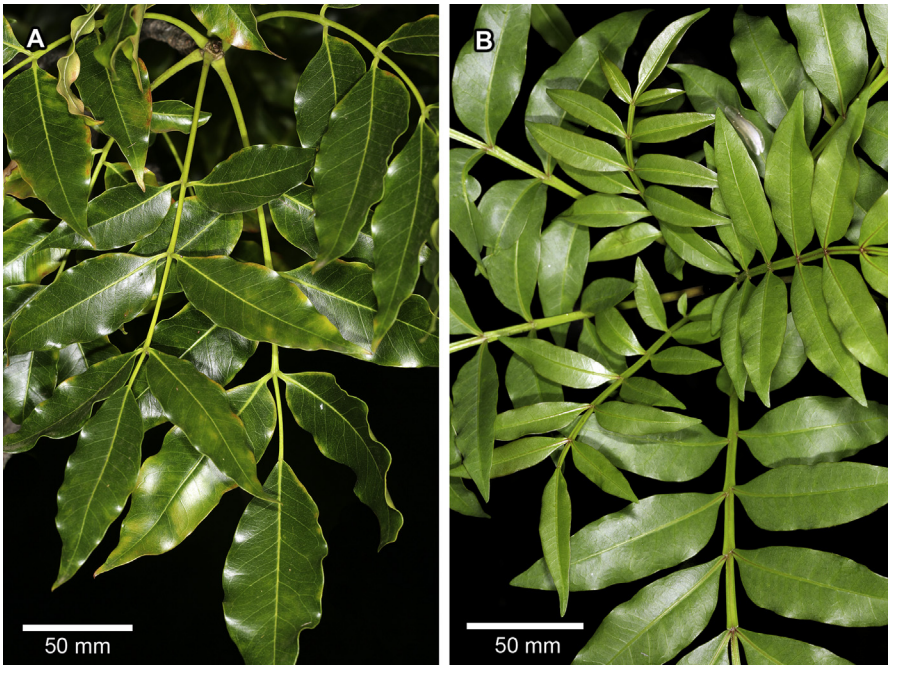

Fig. 1. Morphology of the pinnately compound leaves in the two forms of *Ekebergia capensis*. A: Northern form; leaves relatively large with a cylindrical rachis and leaflets with relatively long petiolules. B: Southern form; leaves relatively small, often with a slightly winged rachis, and leaflets with relatively short petiolules.

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Despite its widespread distribution and that it is widely cultivated, it seems that EFNs in *E. capensis* have not been described in the literature apart from a brief mentioning of their mere presence in the unpublished report of Clark (1990). Since EFNs have not previously been studied in any detail in this taxon, we provide information on their distribution, structure, initiation and the sugar composition of the nectar. We also report on observations relating to nectar consumers, and hypothesize on the possible functional significance of these glands. Northern and southern forms of *E. capensis* are recognized and both were included in the study. We also record, for the first time in Meliaceae, the presence of colleters in this species and their distribution and structure.

2. Materials and methods

2.1. Macroscopic and stereomicroscopic observations

Observations were made mainly on cultivated trees on the campuses of the University of Pretoria and University of Johannesburg periodically for a few years and also elsewhere, whenever possible, including the Pretoria National Botanical Gardens, Tshipise Forever Resort and the Kruger National Park. The appearance of the glands, evidence of secretion and the presence of any associated insects or other organisms were noted. Photographs were taken with a Canon PowerShot A1100 IS camera or an Olympus SZX 16 stereomicroscope using an Olympus ColorView Soft Imaging System. To determine the relative abundance of EFNs, 12 sun leaves from each of seven trees of both the southern and the northern variants growing on the campus of the University of Pretoria and in various suburbs of Pretoria, were used. The average number of laminar EFNs per leaf and the average number of EFNs per pinna were calculated.

2.2. Compound light microscopy (LM)

Leaf material for the anatomical study, representing both northern (four different trees) and southern (two different trees) forms of the species, was obtained from trees at the Universities of Johannesburg and Pretoria. Small portions of the leaflet, each containing a gland and a few millimetres of surrounding tissue, were cut and placed in formalin-acetic acid-alcohol (FAA) in the following proportions: 5 formalin: 5 acetic acid: 90 70% ethanol (Johansen, 1940), for at least 24 h. Leaflets in different stages of development were used, from where the lamina was first formed till maturity. The material was subsequently prepared for LM. The methods involved dehydration in a graded alcohol series, followed by infiltration and embedding in glycol methacrylate (Product 16800, Electron Microscopy Sciences, Pretoria) (Feder and O'Brien, 1968). Transverse sections, 3−5 μm thick, were stained and counterstained according to the periodic acid-Schiff (PAS)/toluidine blue method of Feder and O'Brien (1968)

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and mounted in Entellan (Product 7961, E. Merck, Darmstadt). Some sections were left unstained and examined under polarized light to facilitate study of the crystals. Histochemical tests with Sudan III (Johansen, 1940) were done on unstained sections to confirm the presence of lipids and cutin. Razor blades were used to cut transverse sections of some fresh laminar EFNs to study their natural appearance especially the pigmentation, as well as of EFN-bearing portions of petioles. Observations were made and photographs taken, with an Olympus CX41RF light microscope, the digital images with an Olympus ColorView Soft Imaging System (Stream Essentials 1.8).

2.3. Fluorescence microscopy

Confocal fluorescence microscopy with the stain auramine O (Sigma-Aldrich 861030, Johannesburg; 0.01% w/v in 0.05M Tris/HCl, pH 7.2) was used to study the cuticle/cutin. Sections of GMA-embedded material (see above) were placed in the stain for 15 min, rinsed with distilled water (Considine and Knox, 1979; Buda et al., 2009) and then dried before mounting in immersion oil and sealing with nail varnish. Confocal laser imaging was performed using a confocal laser scanning microscope (CLSM) (Model LSM 880, Zeiss, Germany). Auramine O was excited using a 460 nm argon laser, and emission was collected at 550 nm.

2.4. Scanning electron microscopy (SEM)

Fresh leaflet material with actively secreting EFNs was studied by SEM. Suitable portions of leaflets were critical-point dried, mounted on stubs using carbon tape, coated with gold and viewed in a SEM (Tescan, soft−VegaTS) at 8.0 kv. Colleter-bearing portions and whole immature leaflets were fixed in methanol after which they were transferred to dry absolute ethanol according to the method of Neinhuis and Edelmann (1996), as supported by Talbot and White (2013). Chemical drying with hexamethyldisilazane (Lee and Chow, 2012) was carried out before mounting the material on carbon tape, coating with carbon and viewing using a Zeiss Crossbeam 540 FE6 SEM at 3.0 kv. Please note that the two different SEMs and associated protocols used were necessitated by the availably of instruments at different times, rather than a particular intent.

2.5. Sugar analysis

Extrafloral nectar was obtained from 11 trees growing on the campus of the University of Pretoria and two in the Pretoria National Botanical Garden, Brummeria. Southern and northern variants were included and more than one sample was collected from some of the trees. To facilitate collection of the secretion from the EFNs for sugar analysis, the lower ends of leafy twigs were placed in a jar of water and the foliage enclosed in a plastic bag (to create a moisture-saturated environment). After several hours the

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accumulated exudate from the glands was absorbed onto small strips of Whatman's No. 1 filter paper and stored in a fridge. The samples were extracted with 50% acetonitrile (0.5 mL), sonicated, centrifuged and transferred to vials. The sugar composition (glucose, fructose and sucrose) was determined using the fast screening HILIC-MS method of Stander et al. (2013). The percentage of each of these sugars was calculated as were the glucose: fructose and glucose + fructose: sucrose ratios.

3. Results

3.1. Extrafloral nectaries (EFNs)

3.1.1. Macroscopic, stereomicroscopic and SEM observations

EFNs of both northern and southern forms of *Ekebergia capensis* occur on leaves, notably on the abaxial surfaces of leaflets (Fig. 2A–E). They were also observed on the petioles (Fig. 2A). Fewer were present on the rachis and petiolules. Varying numbers were seen on the abaxial surface of the leaflets with the southern form tending to have fewer and larger (maximum diameter *ca.* 500 μm vs. 200 μm) EFNs than the northern form. The average number of laminar EFNs per leaf and the average number of EFNs per pinna are recorded in Table 1.

On leaflets the EFNs are usually concentrated on or near the midrib or associated with smaller veins, especially distally (Fig. 2B–E). In the southern form, the EFNs were found mainly on or against the midrib (Fig. 2B) whereas those of the northern form tended to be close to the midrib (Fig. 2E). The EFNs in intercostal areas on the lamina surface are circular (Fig. 2C, D) whereas elsewhere, as on the midrib and larger side veins, they tend to be oval to elliptical (Fig. 2B).

The EFNs are usually green and inconspicuous in young leaves. However, with age they frequently become easily visible due to the accumulation of pinkish or reddish pigments, henceforth described as anthocyanins (Fig. 2A, C). The accumulation of anthocyanins is particularly evident in the northern form, the leaves of which also tend to have more reddish autumn colours (predominantly yellowish in the southern form).

We did not observe any secretion from the EFNs on young leaves but on mature and senescent leaves droplets of a clear, frequently viscous, secretion, were visible (Fig. 2D, E). These droplets were often relatively large compared to the size of the EFNs. Even on some senescent, though still fleshy, leaves from the previous season that had already been shed, droplets of secretion were seen (Fig. 2E).

SEM observations confirm the tendency for EFNs associated with veins to be oval or elliptical in outline and those associated with intercostal areas to be circular (Fig. 3A, B). The EFNs have a relatively smooth surface and lack stomata, even though the latter are abundant on the abaxial surfaces of the leaves (Fig. 3A, B).

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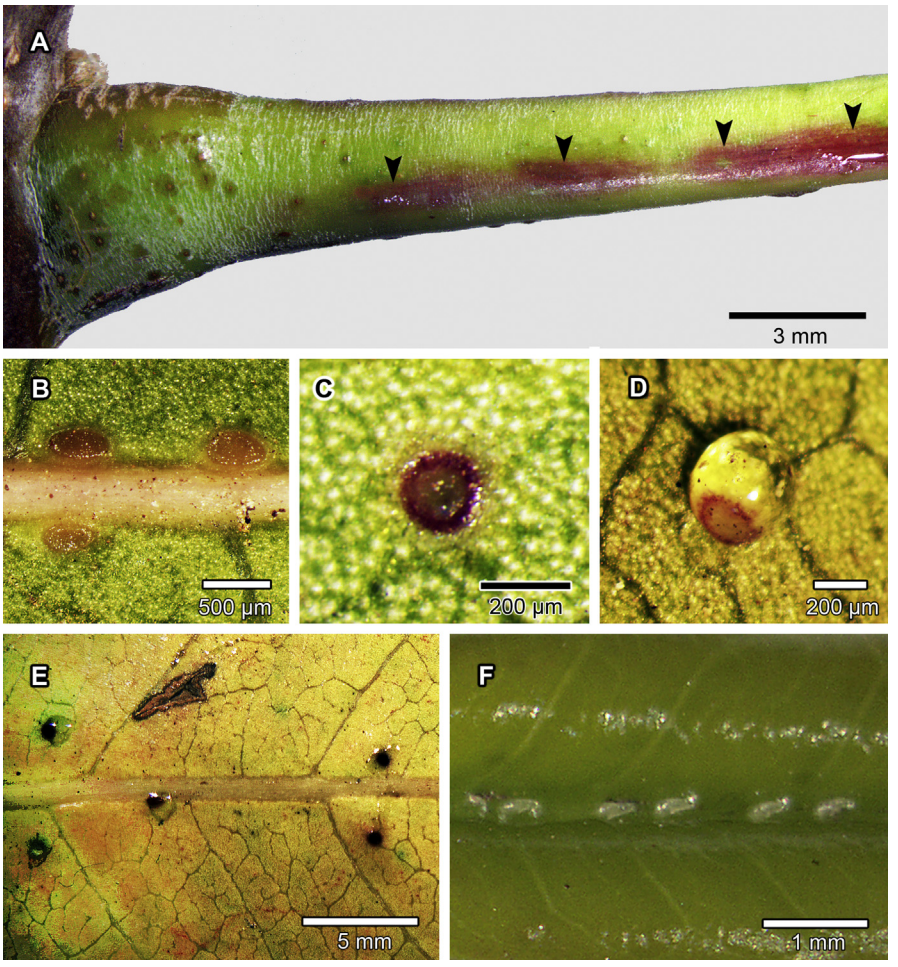

Fig. 2. Morphology of extrafloral nectaries (EFNs) and colleters in the northern (A, C–F) and southern (B) forms of *Ekebergia capensis*. A: Petiole with EFNs, the latter not to be confused with the lenticels on the thickened basal part. Non-secreting green EFNs are difficult to see but, when secreting, their presence is often revealed by a reddish colouration of the gland and its surrounding tissue. Position of glands is indicated by arrowheads, the far right-hand one with visible secretion. B: Three oval laminar EFNs without visible red anthocyanins and associated with the midrib. C: Round intercostal laminar EFN with slightly raised rim and presence of red anthocyanins. D: Actively secreting reddish intercostal laminar EFN completely covered by a relatively large drop of nectar. E: Five actively secreting dark red laminar EFNs on the abaxial surface of a senescent leaflet already displaying autumn colours. F: Adaxial surface of a very young leaflet showing a midrib with several translucent colleters.

3.1.2. Anatomy and histochemistry

The circular and oval EFNs have the same internal structure, but the northern and southern variants show slight differences (Fig. 4). These glands are generally more or less level with the leaflet surface but in the southern form the epidermis forms an indentation (groove) along the perimeter of the EFN (Fig. 4C). The epidermal cells associated with the indentation are more papillate and have a relatively thick cuticle. A distinct epidermis is seldom visible when

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Table 1. Relative abundance of EFNs on the leaves of southern and northern variants of *Ekebergia capensis*. For each tree twelve leaves were examined.

Tree	Average number of laminar EFNs per leaf	Average number of EFNs per pinna
Southern form		
1	72.1	7.8
2	19.0	2.0
3	25.4	2.8
4	29.8	2.6
5	30.3	2.6
6	10.9	1.3
7	39.8	4.5
	32.5 (average)	3.4 (average)
Northern form		
1	123.5	15.9
2	101.1	13.5
3	110.4	12.4
4	121.8	13.1
5	103.9	11.3
6	156.2	15.9
7	147.2	11.9
	123.4 (average)	13.4 (average)

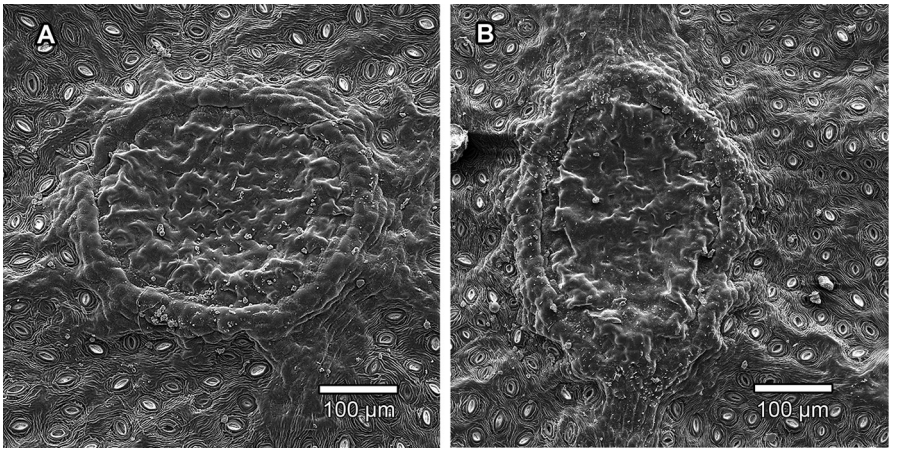

Fig. 3. SEM micrographs taken with a Tescan, soft−VegaTS SEM of laminar nectaries abaxially on leaflets of *Ekebergia capensis* (northern form). Stomata are absent from the secretory poles, but present on the surrounding laminar tissue. A: Circular intercostal gland. B: Oval gland on a vein.

mature (Fig. 4B, C). A thin cuticle is present but at the periphery of the EFN, it is thicker than elsewhere on the gland or lamina surface (Fig. 4B, C). Towards the centre of the gland it appears to be very thin or apparently absent (Figs. 4B, C and 5).

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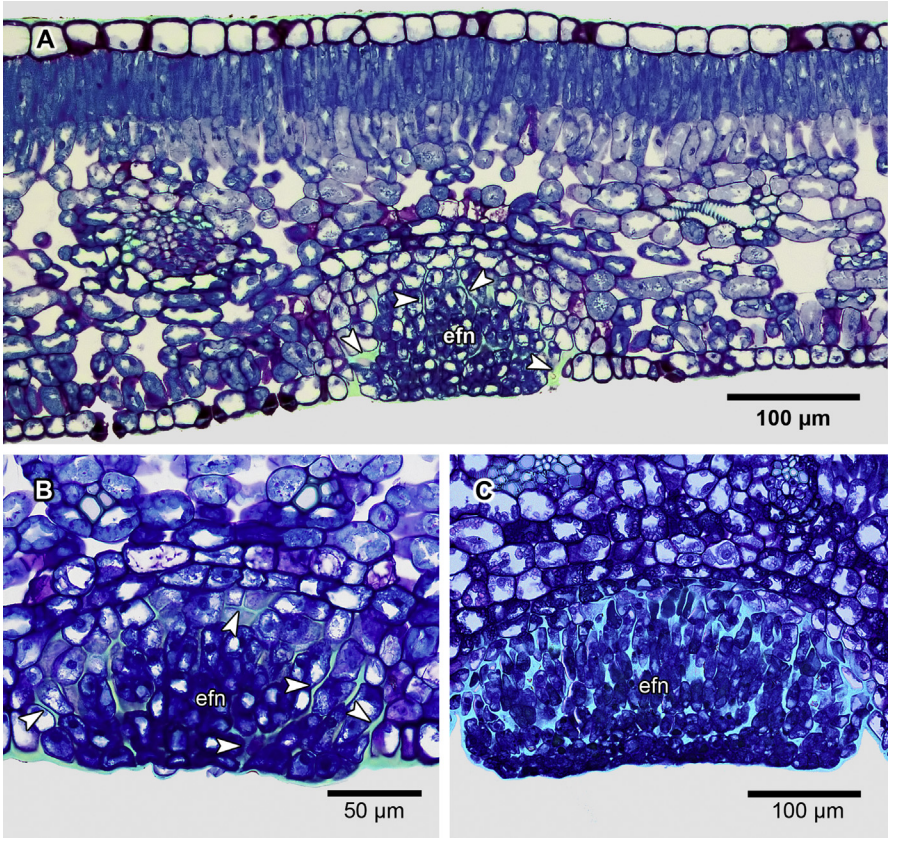

Fig. 4. Anatomy of laminar nectaries in the northern (A, B) and southern (C) forms of *Ekebergia capensis*. Leaflets in transverse section, with nectary (efn) on abaxial side. A: Tangential view of nectary. Note secretory tissue pervaded by "strands" of material (arrowheads) that stains the same colour as the cuticle. B: Details of a median longitudinal view of a nectary, showing "strands" of lipophilic material (arrowheads) in the secretory tissue. Cuticle relatively thin or absent on the secretory pole. C: Median longitudinal view of nectary showing secretory cells embedded in an extensive matrix of lipophilic material (staining pale blue-green).

Within the secretory tissue there tends to be a gradual increase in the intensity of the staining towards the surface of the EFN. The secretory tissue is composed of several layers of relatively small cells with dense cytoplasm and large nuclei typical of cells with a high metabolic activity. These cells are somewhat isodiametric in the northern form (Fig. 4A, B) but more elongated in the southern form (Fig. 4C). The walls of adjacent cells are often separated by "strands" of amorphous material that, with the PAS/toluidine blue reaction, stain similar to the cuticle (bright blue to greenish blue), especially in a radial orientation, and are henceforth referred to as "lipophilic material" (Fig. 4A–C). This gives the appearance of mainly radial "strands" traversing the gland; these deposits, which are continuous with the cuticle, form an intricate three dimensional matrix throughout much of the secretory tissue. Transverse sections treated with auramine O showed fluorescent enhancement of the cuticle and the radiating "strands" within the EFN, indicating the presence of cutin (Fig. 5A, B). In addition, staining with Sudan III yielded a similar result for the cuticle and

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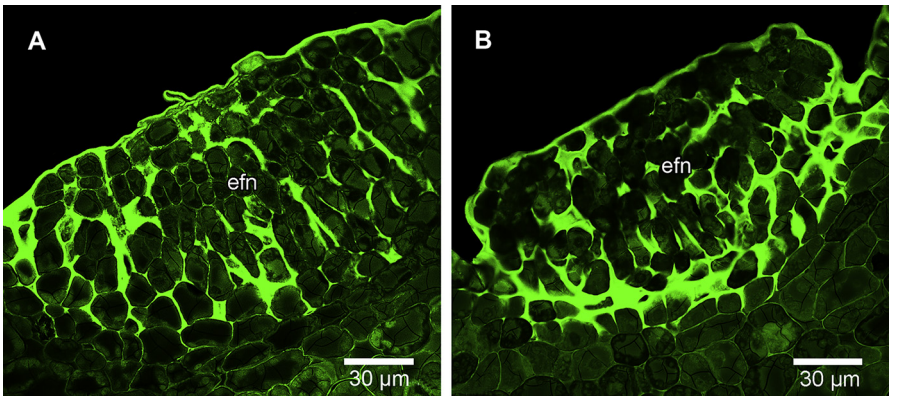

Fig. 5. Histochemical staining of laminar nectaries in the northern (A) and southern (B) forms of *Ekebergia capensis*. Transverse sections of portions of leaflets with GMA-embedded nectaries (efn) stained with auramine O as seen in median longitudinal view under CLSM. Note secretory tissue pervaded by "strands" of lipophilic material showing enhanced autofluorescence similar to that of the cuticle, thus supporting the presence of cutin. In the southern form (B) the intercellular matrix of lipophilic material is particularly abundant throughout the secretory tissue, especially towards the inner periphery of the nectary.

the "strands". In some EFNs of the southern form, this material was particularly abundant as an intercellular matrix throughout the secretory tissue, and in others around the periphery of the EFN (Figs. 4C and 5B).

The pinkish or reddish anthocyanins in many fresh EFNs tend to be concentrated around the perimeter of the gland although some EFNs were quite pinkish towards the centre as well (Fig. 6A−D). In unstained transverse sections of these EFNs, an increase in the concentration of anthocyanins in the vacuoles of the cells towards the surface was observed (Fig. 6C). Druse crystals of calcium oxalate are found as idioblasts in relatively large numbers surrounding the nectariferous tissue (Fig. 6E); elsewhere they are associated mainly with the phloem. Although the EFNs are usually close to vascular tissue, no direct cellular association was apparent. EFNs are similar on the petiole to those on the leaflets.

In *E. capensis* the secretory tissue originates from protoderm and ground meristem (Fig. 7A, B). Most of the initial divisions are anticlinal. During the early stages of development, calcium oxalate crystals are absent. The future secretory cells lack tanniniferous contents which are common in other cells of the mesophyll (tanniniferous idioblasts) (Fig. 7A, B).

3.1.3. Sugar analysis

Glucose, fructose and sucrose were detected in all samples of EFN secretions and occurred in varying proportions (Figs. 8 and 9). In the northern form the proportion of sucrose tended to be slightly lower than in the southern form. In general the most abundant sugar was fructose.

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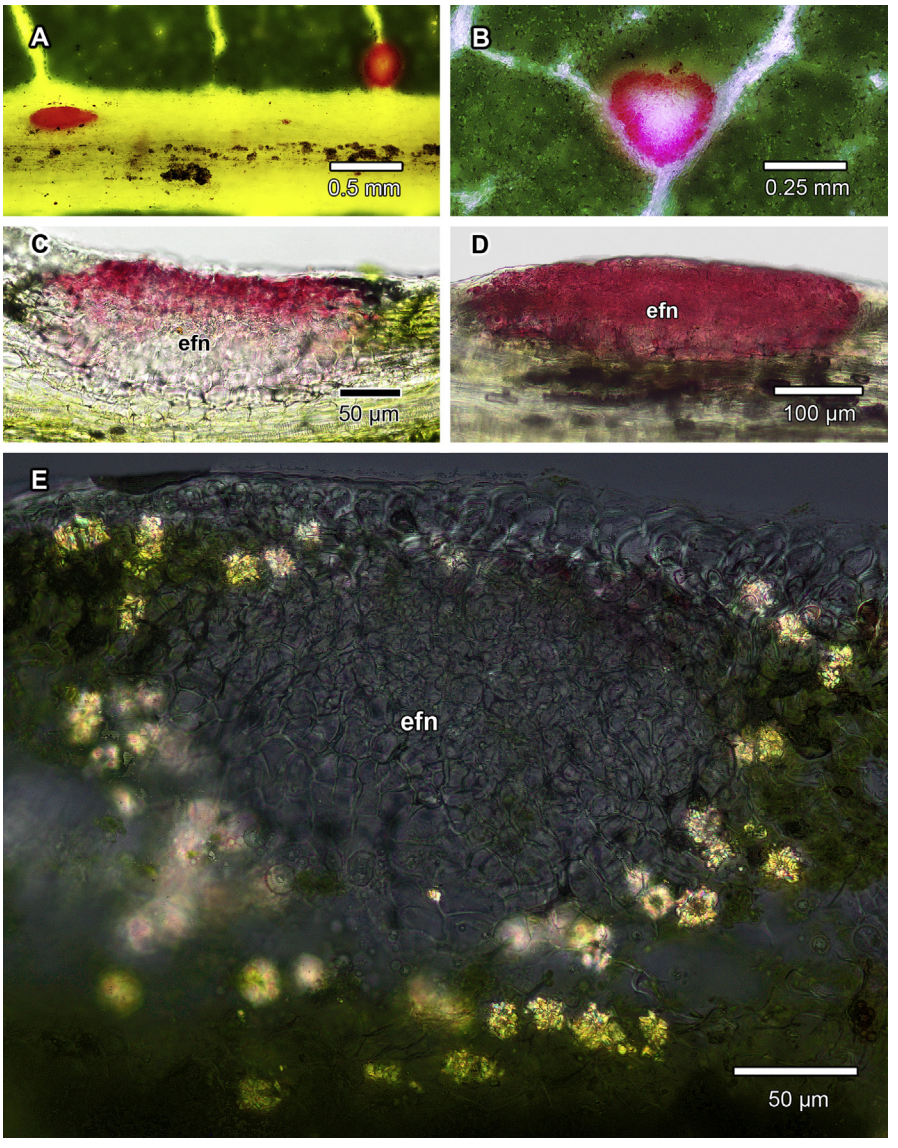

Fig. 6. Laminar nectaries of *Ekebergia capensis* (northern form) on abaxial surface of fresh leaflets as seen in intact leaflets under transmitted light (A, B), hand-cut transverse sections under transmitted light (C, D), and hand-cut transverse section under polarized light (E). A: Oval nectary on midrib and a nearby circular one, both with red anthocyanins. B: Intercostal nectary with red anthocyanins mainly confined to the periphery of the gland. C: Nectary (efn) with red anthocyanins concentrated in secretory cells towards the secretory pole. D: Nectary (efn) with red anthocyanins present throughout the secretory tissue. E: Druse crystals of calcium oxalate (glowing) as idioblasts in mesophyll and arranged in close proximity to the secretory tissue of the nectary (efn).

3.1.4. Visitors to EFNs

Very few visitors to the EFNs were recorded, even when they were actively secreting. Nymphs of psyllids, belonging to at least two species of *Pseudophacopteron* (Hemiptera, Psyllidae), were the most common relatively large organisms present on the leaves, but these are stationary and confined to dimples (pit-galls) on the

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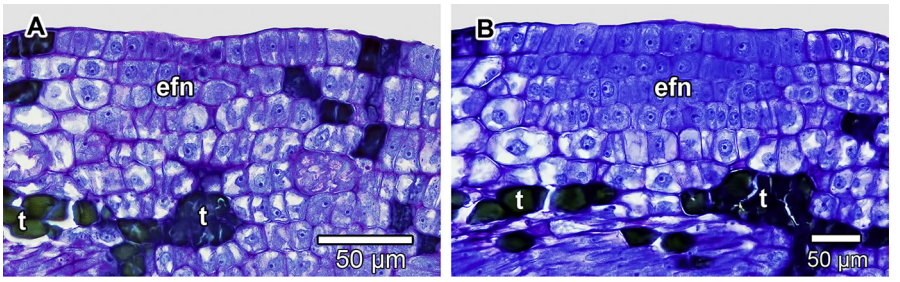

Fig. 7. Initial stages in the development of laminar nectaries in *Ekebergia capensis* (northern form). Leaflets in transverse section, with developing nectary (efn) on abaxial surface. A: Very young nectary developing from protoderm and ground meristem, usually in close proximity to procambium; initial cell divisions are mostly anticlinal. Tanniniferous cells (t) are usually absent from the future secretory tissue, but common elsewhere in the mesophyll. Associated druses in mesophyll surrounding the secretory tissue only appear once glands are functional. B: Developing nectary slightly older than the one depicted in A. Future secretory cells lack tanniniferous contents, but tanniniferous idioblasts (t) are common in the rest of the mesophyll.

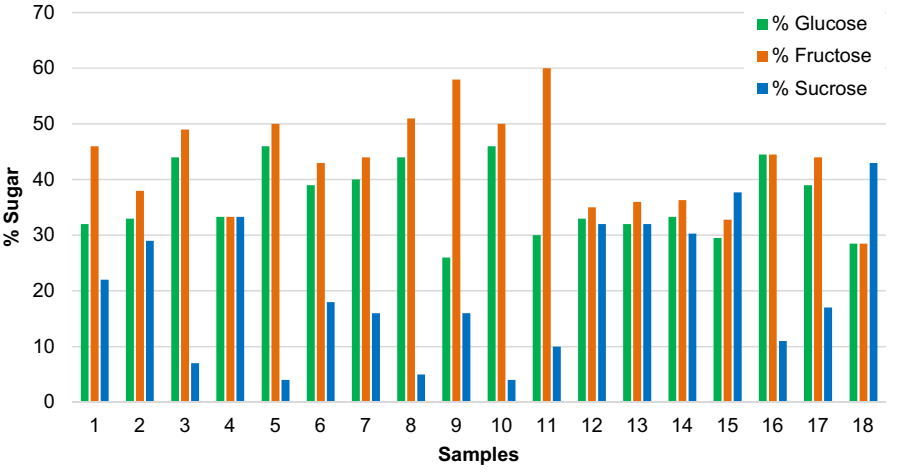

Fig. 8. Percentage sugars in the foliar nectary exudate of the two geographical forms of *Ekebergia capensis*. Variation is shown in 18 samples: 1−11 from the northern form; 12−18 from the southern form.

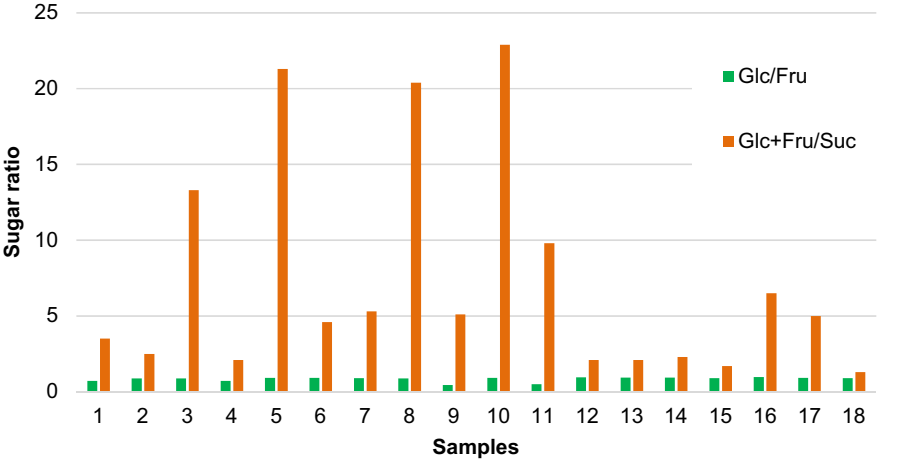

Fig. 9. Sugar ratios in the foliar nectary exudate of the two geographical forms of *Ekebergia capensis*. Variation is shown in 18 samples: 1−11 from the northern form; 12−18 from the southern form. Fru = fructose; Glc = glucose; Suc = sucrose.

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surface of the lamina and have no specific association with the EFNs. Each dimple manifests as a raised lump on the opposite surface of the leaflet. Nymphs are most numerous on the undersides of leaves and particularly common in trees of the southern form. Occasionally a small whitish mite was seen feeding on the nectar at an EFN. These were more commonly seen on the twigs, apparently hiding in cracks in the bark and in the leaf axils. Ants were very rarely observed even when they were abundant on the ground. During a visit to Jock Safari Lodge, Kruger National Park in September 2014, weevils were observed feeding at the EFNs of a large number of leaves of the *E. capensis* trees.

3.2. Colleters

3.2.1. Macroscopic, stereomicroscopic and SEM observations

On very young living leaves colleters were observed in the form of small (0.3−0.5 mm long), fleshy and translucent glands (Fig. 2F). They were present, sparsely, on the rachis, at the base of the petiolules, and in large numbers on mainly the adaxial surface of the still conduplicate developing leaflets particularly near the base and associated with the midrib (Figs. 2F and 10A). The secretion, which was sticky, accumulated around the colleters in relatively large amounts. Some remained as shiny spots or as a film visible on the surface of some leaves that had partially unfolded and expanded. The colleters persisted after the leaflets had unfolded and a sticky "string" of secretion was visible at the apex of some of the colleters. Under the SEM, some secretory material was usually visible apically as globose droplets and twisted "strands" (Fig. 10B). The possibility of these "strands" being the result of the chemical dehydration of the material cannot be ruled out. However, by the time the leaflets were approximately half the size of mature leaves many of the colleters had started turning brown and subsequently dried up and fell off. Thus they ceased to be functional and were no longer visible by the time active secretion by the EFNs became apparent.

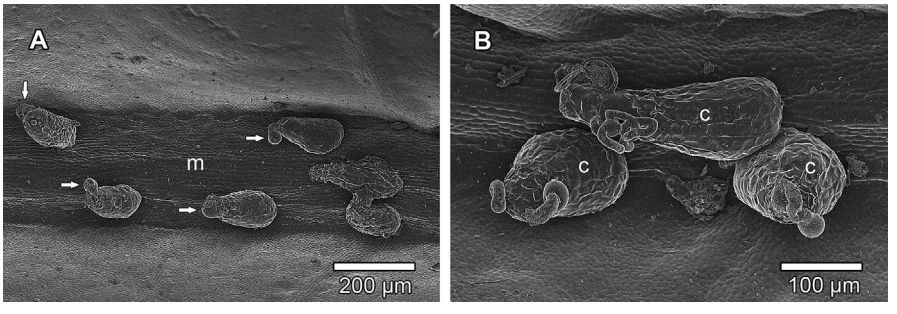

Fig. 10. SEM micrographs taken with a Zeiss Crossbeam 540 FE6 SEM of very young leaflets of the northern form of *Ekebergia capensis* showing colleters adaxially on the midrib (m). At this stage leaflets are still partly conduplicate and the lamina was gently pushed apart to better view the midrib. A: Six conical colleters, each with a small secretion droplet (some arrowed) at the apex. B: Three colleters (c) with secretory material visible apically as globose droplets and twisted "strands".

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The colleters are essentially sessile, conical in shape and under SEM the surface has a faint polygonal pattern imparted by the outline of the epidermal cells (Figs. 10 and 11). The exudate appears to be released mainly from the apex of the gland. In material viewed under the SEM some secretory material was usually visible apically on most glands as globose droplets and twisted "strands" (Fig. 10B).

3.2.2. Anatomy and histochemistry

The colleters are composed of a few layers of secretory cells usually with dense cytoplasm, surrounding a central axis of lightly-staining cells (Fig. 11A–B). The

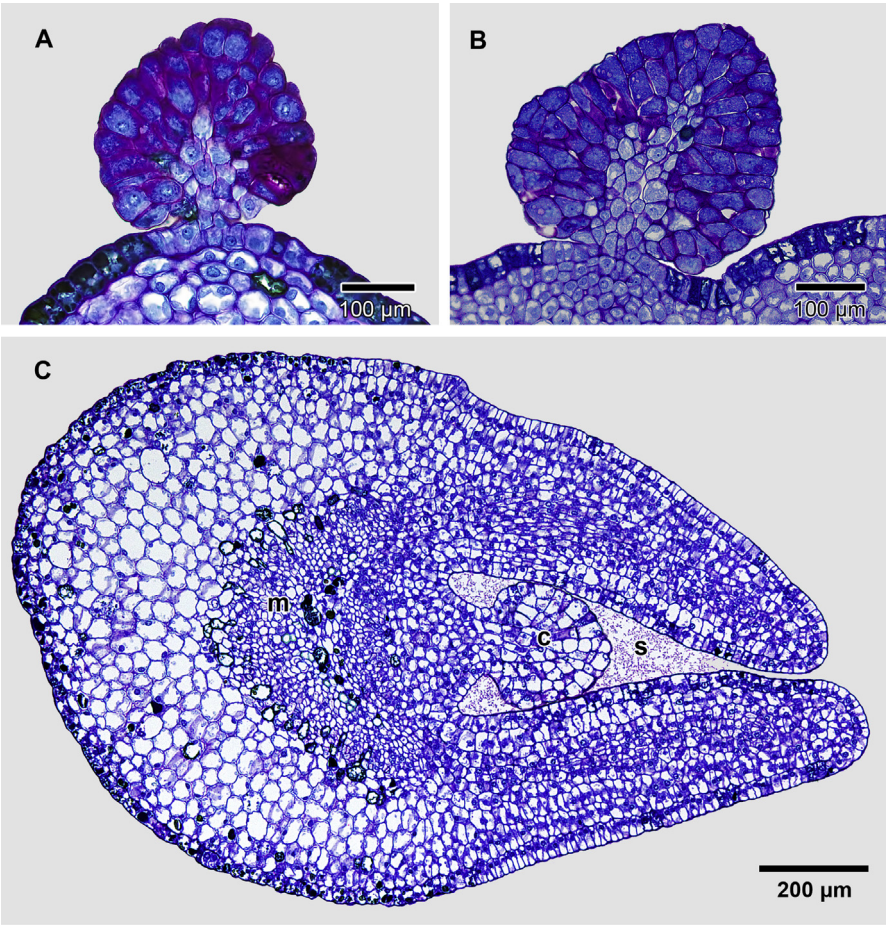

Fig. 11. Anatomy of colleters on developing leaflets of *Ekebergia capensis* (northern form). Transverse sections of very young leaflets. A: Parasagittal view of a relatively old colleter showing outer layers of secretory cells with dense cytoplasm, surrounding a central axis of lightly-staining cells. B: Mid-sagittal view of a relatively old colleter. Note its essentially sessile nature, and clear differentiation between a central axis of lightly-staining cells surrounded by a few layers of intensely-stained secretory cells with dense cytoplasm. C: Conduplicate leaflet showing a relatively young colleter (c) adaxially on the midrib (m), with the space surrounding the colleter filled with a granular secretion (s). Note lack of intensely-stained secretory tissue.

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peripheral secretory cells of older colleters (Fig. 11A, B) stain much darker with the PAS/toluidine blue reaction than those of relatively young actively secreting ones (Fig. 11C). Histochemical tests with Sudan III confirm the presence of a well-developed cuticle but no noticeable lipids. In transverse sections of still conduplicate leaflets, a granular material, interpreted as colleter-derived secretions, fills the space surrounding the colleters (Fig. 11C). With the PAS/toluidine blue reaction the granular material stains a pinkish colour.

Although the exudate is produced in relatively large quantities, the small size of the developing leaflets and their conduplicate vernation (which conceals the colleters at the time secretion is most active), made it difficult to unambiquously determine the chemical composition of the colleter exudate. It is nevertheless clear that at least that component of the exudate persisting apically on the colleters and visible under SEM does not dissolve in the methanol and ethanol that were used in the protocol for the chemical drying of the leaflets.

4. Discussion

4.1. Evolution, position and frequency of foliar EFNs in Meliaceae

Considering available phylogenetic hypotheses for Meliaceae (Stevens, 2001 onwards; Koenen et al., 2015; Muellner-Riehl et al., 2016), members with EFNs are known from both major monophyletic clades, namely that of subfamilies Cedreloideae and Melioideae (of which *Ekebergia* is a member). In the larger and more diverse Melioideae, EFNs are present, amongst others, in the two diverse clades representative of the tribes Trichilieae + Turraeae and the Guareeae + Aglaieae. A study about the repeated evolution and loss of EFNs in Meliaceae could be perceived, but the available sampling of genera specifically for this feature is still too limited to allow for meaningful results. It is nevertheless noteworthy that EFNs are also known in Simaroubaceae (Weber et al., 2015), which is sister to Meliaceae. This suggests that the propensity to develop EFNs may well be an ancestral feature for Meliaceae, having already been present in at least the most recent common ancestor of these two families.

In members of the Meliaceae, the position of the EFNs on the leaf varies. They are present on the petiole, rachis, petiolules, and both leaflet surfaces of *Swietenia* spp. (Lersten and Rugenstein, 1982). In *Ekebergia capensis* and *Cedrela fissilis* Vell. (Paiva et al., 2007) they are similarly widespread on the leaves except they are absent from the adaxial surface of the leaflets. This corresponds with most investigated taxa of the Meliaceae, where it appears that they are absent from the adaxial lamina surface of the leaflets or are present only in very small numbers [Zimmermann (1932) in *Carapa guianensis*, Lersten and Pohl (1985) in *Cipadessa baccifera* (Roth) Miq.,

https://doi.org/10.1016/j.heliyon.2018.e00541

Morellato and Oliveira (1994) in *Guarea macrophylla* Vahl. and Paiva et al. (2007) in *Cedrela fissilis*]. In *Cedrela fissilis* (Paiva et al., 2007), two morphotypes ("flattened or elevated, either circular or slightly elliptical") were distinguished. In the present study, we found circular and oval to elliptical morphotypes depending on the position on the leaf.

A noteworthy difference in the average number of laminar EFNs per leaf in the southern and northern forms of *Ekebergia capensis* was found (32 and 123 respectively). In other studies of members of the family, substantial differences between taxa have been reported, e.g., 25−35 per leaf in *Cipadessa baccifera* (Lersten and Pohl, 1985) and more than 300 per leaf in *Cedrela fissilis* (Paiva et al., 2007).

A study of herbarium specimens of other Meliaceae members at PRU revealed (unpublished observations) EFNs to be present in *Ekebergia pterophylla* (C.DC.) Hofmeyr and in *E. benguelense* Welw. ex C.DC., the only other species of *Ekebergia* in southern Africa as well as in species of *Entandophragma*, *Khaya*, *Nymania*, *Trichilia* and *Turraea*, but not in *Pseudobersama*. Clark (1990) described the occurrence of the EFNs in the three species of *Ekebergia* (*E. benguelensis*, *E. capensis* and *E. senegalensis* Fuss) he studied as seemingly sporadic. We found EFNs to be consistently present in the first two species and also *E. pterophylla*. We suspect that they are more widespread than is currently known and possibly occur in all members of *Ekebergia* but, largely because of their small size, they are easily overlooked especially in herbarium material. We confirmed the earlier reported presence of EFNs in *Nymania* (Dahlgren and Van Wyk, 1988), a monotypic genus of Meliaceae (previously Aitoniaceae) endemic to southern Africa.

4.2. Anthocyanins associated with EFNs

We have found no other records of reddish anthocyanins being present in EFNs of the family and this raises a question as to their function. Elsewhere anthocyanin-containing cavities were reported as being present throughout the EFNs of *Ricinus communis* L. (Euphorbiaceae), although they were often associated with the fringes of the vascular system which supplies the glands (Baker et al., 1978). In the same family anthocyanin-containing cells formed a round ring at the borders of the leaf-margin glands in *Sapium glandulosum* (L.) Morong (= *S. biglandulosum* Müll.Arg.) (Coutinho et al., 2010). It is generally assumed that anthocyanins may protect leaves in plants by functioning as antioxidants and sunscreens (Landi et al., 2015). Other suggested functions by these authors include serving as metal-chelating agents responsible for the delaying of foliar senescence. As structures potentially rich in sugars, one may also speculate that the reddish colour of the EFNs is an anti-herbivore defence mechanism, albeit at a very minute scale (Hughes and Lev-Yadun, 2015). However, Paiva (2012) reported a colour change in the *floral* nectaries of *Swietenia macrophylla* from pale yellow to intense red but stated that the nectary colour has a limited role as a pollinator attractant. Furthermore

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the apparent absence of secretory activity in very young leaves of *E. capensis* and the continued activity in old, including senescent, leaves add to the questions on the functionality, if any, of the pinkish colour. Paiva et al. (2007) reported secretory activity throughout the leaf's life in *Cedrela fissilis* and we also noted this in *Melia azedarach* (unpublished observations).

4.3. EFN structure

Although the occurrence of EFNs in the Meliaceae has been known for many years, anatomical studies have apparently only been carried out on three species of *Swietenia* (Lersten and Rugenstein, 1982), *Cipadessa baccifera* (Lersten and Pohl, 1985), *Guarea macrophylla* (Morellato and Oliveira, 1994) and *Cedrela fissilis* (Paiva et al., 2007). In all these studies, as with *Ekebergia capensis*, a lack of stomata or other structures on EFNs through which nectar could be released was apparent. To explain the continuous secretion observed in some glands including certain EFNs, Paiva (2016) proposed a cell-cycle model. This involves the protoplast contracting and expanding in successive cycles and thus mechanically causing the material surrounding the protoplast to cross the cell wall and cuticle. Although such a model largely presupposes that the cell wall is responsible for restricting secretion reflux, the present study suggests that the radial "strands" of lipophilic material in the nectariferous tissue may also play a role—see further on. Paiva (2017) mentions that in stomata-free nectaries, cuticular pores (hydrophilic pathways) and cuticle rupture or detachment provide the main means by which nectar can be exuded. Although the tests with Sudan III in the present study were carried out on chemically fixed material which may have affected the results, the cuticle stained the same as the "strands" of material traversing the EFN.

In LM sections of *Ekebergia capensis* there is generally an increase in the intensity of the staining towards the outside of the EFN but all the nectariferous cells within an EFN, which are more elongated in the southern form than in the northern form, appear similar in shape. However, Morellato and Oliveira (1994) noted in *Guarea macrophylla* a transition in form and size of the cells, which have dense cytoplasm, from the surface inwards; these cells being arranged in several strata. In *Swietenia* species, typically the outermost three or four layers are composed of somewhat palisade-like cells with dense cytoplasm subtended by a sheathing biseriate layer of almost clear rounded cells with thickened walls (Lersten and Rugenstein, 1982). Two distinct zones of differently staining cells are also seen in *Cedrela fissilis* (Paiva et al., 2007). The secretory tissue is similarly made up of elongated cells with dense cytoplasm but it appears more extensive. It is also surrounded by a sheath of lightly cytoplasmic cells having thick walls (impregnated with lignin and suberin). The nectariferous tissue in *Cipadessa baccifera* is composed of relatively small and somewhat irregularly shaped parenchyma cells (Lersten and Pohl, 1985). A sheath is absent.

https://doi.org/10.1016/j.heliyon.2018.e00541

In *Ekebergia capensis*, as in all other studied members of the Meliaceae, the EFNs are usually close to vascular tissue but no cellular connection has been reported. Lersten and Rugenstein (1982) observed five to eight layers of parenchyma cells between the phloem and the nectary sheath in leaflets flanking the midrib of *Swietenia* species, and Paiva et al. (2007) up to ten layers in the rachis of *Cedrela fissilis*. In various studies, involving different taxa, calcium oxalate crystals are reported within or in the region of EFNs e.g., Schnell et al. (1963), Elias (1983) and Tilney and Van Wyk (2004). Metcalfe and Chalk (1950) state that both solitary and clustered crystals of calcium oxalate are common in the tissues of all the organs of the Meliaceae but, in this family, they are mentioned only by Paiva et al. (2007) in connection with the EFNs. They report numerous calcium oxalate crystals (druses and prismatic) in the cortical parenchyma of the leaf rachis of *Cedrela fissilis* only in the EFN region; elsewhere they are associated with the phloem adjacent to the pericyclic fibres. In *Ekebergia capensis* we found them in relatively large numbers surrounding the secretory tissue of the EFNs and in the phloem. Paiva and Machado (2005) mention that the presence of such calcium oxalate crystals may be indicative of cells active in symplastic transport. It is, for example, known that calcium ions inhibit plasma membrane ATPase which is involved in the transport of sucrose in plants. By sequestering calcium, the crystals of calcium oxalate may remove calcium to facilitate the transport of sucrose (Nepi, 2007, and references therein). The EFNs in members of the family typically appear to have no direct vascular connection—although they are usually not far from the phloem—and calcium oxalate crystals may accumulate as a result of the transfer of material from the phloem to the EFNs.

The extensive intercellular pervasion of the nectariferous tissue of the EFNs in *E. capensis* by mainly radial "strands" of lipophilic material is quite pronounced and, to the best of our knowledge, has not previously been reported in EFNs (e.g., Zimmermann, 1932; Fahn, 1979; Nepi, 2007). However, the floral nectaries of *Catharanthus roseus* (L.) G.Don. (= *Vinca rosea* L.) are covered by a thick (*ca.* 5 μm) cuticle and nectar secretion is through stomata. Not only are the relatively large intercellular spaces between the epidermis and the parenchyma lined by a cuticle, but a cuticle also covered the parenchyma (secretory) cells adjacent to the epidermis (Rachmilevitz and Fahn, 1973). In some EFNs resembling the type in *E. capensis*, the nectariferous tissue is isolated from the surrounding mesophyll by a layer (sheath) of cells with walls that are lignified or composed of lipophilic material that is continuous with the epidermis (Zimmermann, 1932). These thickenings tend to affect mainly the radial walls and such EFNs are classified by Zimmermann as "Flachnektarien, *Beninncasa* type" (see e.g. his figures 3, 41−45) or "flat nectaries" of Elias (1983). Fahn (1979) speculated that these lignified or suberized walls may well be a mechanism ensuring that the sugar solution from the phloem reaches the secretory cells through the symplast, rather than through the apoplast. The state in *E. capensis*, however, differs from that described by Zimmermann (1932) in that the lipophilic material is deposited outside

https://doi.org/10.1016/j.heliyon.2018.e00541

the cell walls and pervades most of the nectariferous tissue. The function of these deposits remains a mystery but may in an as yet unexplained way facilitate the secretory output from the gland. This may account, at least in part, for the relatively large size of the secretion droplets commonly noticed.

The initiation of EFNs in *E. capensis* differs from that described by Paiva et al. (2007) in *Cedrela fissilis* where a few cells of the protoderm divide periclinally. In our study the initial cell divisions all appear anticlinal. Calcium oxalate crystals are not initially present in the surrounding mesophyll.

4.4. Nectar chemistry

Chemical analysis of the secretion of the EFNs confirmed that it is nectar. Variable proportions of glucose, fructose and sucrose were found with fructose generally being the most abundant. This variability corresponds with the findings of other studies which showed the sugar composition of EFN nectar to be more variable than that of floral nectars (e.g., Koptur, 1994, 2005; Nicolson et al., 2007). In a comparative study between floral and extrafloral nectars of *Inga* trees (Fabaceae: Mimosoideae), Koptur (1994) found that sucrose-hexose ratios in extrafloral nectars were generally much lower than those of floral nectars. This she explained is plausible since extrafloral nectar is more exposed thus leading to a faster breakdown of sucrose into its hexose components. Thus the composition of nectar could vary with age being the most sucrose-rich in newly produced nectar. This may, at least in part, be responsible for the variation recorded in *E. capensis* which also may not reflect a possible difference between southern and northern variants. Unfortunately no floral nectar analyses are available for *E. capensis*. Nicolson and Thornburg (2007) caution that the use of nectar sugar ratios can be misleading and should at least be supplemented by percentage sugar composition. Analysis of the sugar composition of the extrafloral nectar of *Melia azedarach* (Meliaceae) (unpublished results) yielded a similar composition to that of *E. capensis*.

Tilney and Van Wyk (2004) compared the sugar composition between floral and extrafloral nectar of *Terminalia phanerophlebia* Engl. & Diels (Combretaceae) and found a marked difference. A more or less balanced-sugar extrafloral nectar was found whereas the floral nectar was fructose-dominant. In another comparative study of floral and extrafloral nectar sugars (Sherbrooke and Scheerens, 1979), extrafloral nectars of *Erythrina flabelliformis* Kearney (Fabaceae) were fructose-glucose dominant and the floral nectar sucrose dominant. Further studies are obviously needed to gain a better understanding of the composition of extrafloral nectars and how they differ from floral nectars. In a study of EFNs on members of the Combretaceae (Tilney and Van Wyk, 2004), sugar crystals were visible on hot dry days on the surface of EFNs. Such crystals were never observed in *E. capensis.* The secretion in this latter taxon often appeared viscous which may reduce evaporation.

https://doi.org/10.1016/j.heliyon.2018.e00541

4.5. Nectar consumers

Psyllids are commonly associated with leaves of *E. capensis* and some years heavy infestations by the nymphs can cause considerable deformity of the leaves (Capener, 1973; Malenovsky and Burckhardt, 2009; unpublished observations). Recent findings have revealed that EFN secretion can be directly induced by herbivores (Heil, 2015, and references therein), hence our suspicion that in *E. capensis* there might well be a functional association between the psyllids and the EFNs. It is generally accepted that a mutualistic relationship exists between EFNs and aggressive arthopods, notably ants (Marazzi et al., 2013). Foraging ants are provided with food and in turn protect the plant against potentially destructive herbivores (Rico-Gray and Oliveira, 2007). However, we very rarely saw ants on the EFNs of *E. capensis* and when we did so, the visit appeared opportunistic. It is unclear as to why ants were not congregated around secreting EFNs. Ants feeding on nectar were also rarely seen on senescent leaves with large droplets of secretion that have fallen to the ground and therefore easily accessible to these insects. The small size of the EFNs is also very different from many other EFNs known to attract ants (e.g., Tilney and Van Wyk, 2004). Although by no means common, it has been claimed that some floral nectars contain ant-deterring compounds (Janzen, 1977; Junker et al., 2007). Although it seems counterintuitive to suspect such deterrents in the nectar of EFNs, the lack of interest shown by ants in the secreting EFNs of *E. capensis* (even if offered to them) suggests the likely presence of some form of deterrent.

The concentration of the EFNs on the distal portion of the leaflets (rather than proximally closer to the petiolule, as is often the case in other plants with foliar EFNs) suggests that the possible nectar consumers may be flying rather than crawling insects. In addition to ants, other predatory or parasitoid insects feeding on nectar, in particular wasps, have also been shown to have a protective function to plants with EFNs (e.g., Cuautle and Rico-Gray, 2003; Koptur, 2005). For example, relatively small EFNs in some species of *Passiflora* are not visited by ants, but preferentially by parasitoid wasps (Hymenoptera) that breed on larvae of *Heliconius*, a group of butterflies for which members of *Passiflora* serve as larval food plants (Bentley, 1983; Apple and Feener, 2001, and references therein). Although we have never seen parasitoid wasps feeding on nectar from the EFNs in *E. capensis*, the very small size of the EFNs may well make them accessible to these wasps which usually have small or short mouthparts, and are known to be attracted by nectar (Pemberton and Lee, 1996). As mentioned above, leaves of *E. capensis* are particularly prone to attack by nymphs of psyllids belonging to the genus *Pseudophacopteron*. This is especially noticeable in the southern form cultivated in inland regions such as Gauteng. It is well known that tiny parasitic chalcidoid wasps (Hymenoptera: Chalcidoidea) feed on and kill psyllids, many of which have received attention for their potential use as biocontrol agents of psyllid pests (e.g., Prinsloo, 1981;

https://doi.org/10.1016/j.heliyon.2018.e00541

Pemberton and Lee, 1996; Noyes, 2017). This makes the possible attraction of parasitoid wasps by the EFNs of *E. capensis* an attractive hypothesis to be investigated further.

Predaceous and fungivorous mites may also benefit from the presence of EFNs (Pemberton, 1993). Despite the lack of domatia, we have occasionally seen mites feeding on the nectar from EFNs while investigating leaves of *E. capensis* under the stereomicroscope. It has been claimed that in *E. capensis* the nymphs of the psyllid *Pseudophacopteron electum* Capener are heavily preyed on by a small mite, as well as the larvae of a lacewing (Neuroptera: Chrysopidae) (Capener, 1973). Mature and larval lacewings also feed on nectar and may be attracted by the EFN secretions (New, 1975). Further research is needed to determine the frequency of extrafloral nectar feeding by these beneficial mites and lacewings, and what its significance may be for both the predators and the plants.

4.6. Colleters in Meliaceae

The colleters in *E. capensis* are similar to the standard-type of Lersten (1974). Although multicellular glandular hairs are typical of the Meliaceae (Metcalfe and Chalk, 1950), to the best of our knowledge, colleters have not been reported previously in the family. It is, however, quite possible that some authors may have interpreted the colleters as "multicellular glandular hairs". We speculate that the exudate derived from the colleters and which collects between the folded young leaflet blades and at the petiolule bases could effectively protect the young developing leaves in some or other way. Pacini et al. (2003) found PAS-positive polysaccharides in the nectar of *Cucurbita pepo* L. that were not removed by fixation and dehydration suggesting that nectar contains unknown dissolved polysaccharides. The granular residue of the exudate of *Ekebergia capensis* colleters persisting in sections of embedded material, gave a positive result (pinkish colour) for polysaccharides to the PAS/toluidine blue reaction (Feder and O'Brien, 1968), and thus points to the likelihood of being composed of mucilage polysaccharides. The granular texture is most likely due to coagulation of the secretion following the various chemical treatments associated with fixation, embedding and staining. The observed early senescence, necrosis and eventual shedding of the colleters once the leaflets have expanded are in agreement with the pattern described for colleters in other plants (Thomas, 1991, and references therein).

5. Conclusions

Slight differences exist in the distribution, abundance and structure of EFNs between the northern and southern forms of *E. capensis*, thus calling for a re-assessment of the taxonomic status of these two forms. Structurally the EFNs approach the so-called

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"Flachnektarien, *Benincasa* type" of Zimmermann (1932), but they lack a sheath composed of thick-walled cells and have most of the nectariferous tissue pervaded by "strands" of amorphous lipophilic material outside the cell walls. The functional significance of this unusual presence of lipophilic material is unknown and requires further study. Another peculiarity of the EFNs is the accumulation of reddish anthocyanins in the nectariferous tissue. The nectar is quite viscous and contains glucose, sucrose and fructose, the latter usually being the most abundant sugar. Our observations and anatomical findings on the EFNs and their secretion in this species raised several questions as to the interpretation of the relationship between structure and function. The general absence of ants, or their rare visitation, on the EFNs appears to preclude the generally assumed strategy of attracting ants as a defence mechanism against herbivore attack. Even though the EFNs are smaller than the norm for EFNs in plants with known ant-associations, they have relatively large droplets of nectar, even in older, senescent leaves. Further studies on the composition of the nectar may well provide answers to some of these questions. The mechanisms whereby plants respond to herbivory are currently receiving much attention because of their potential in being exploited for pest management of various crops (e.g., War et al., 2012; Marazzi et al., 2013; Heil, 2015).

Colleters are described in *E. capensis* for the first time and their secretions are likely to play a role in the protection of young leaflets. This is also the first record of colleters in Meliaceae. It is now well established that foliar secretory structures, including EFNs, play a significant ecological role in both plant and animal communities (e.g., Wink, 1999; Marazzi et al., 2013). As judged from the questions emanating from our work on the foliar secretory structures of *E. capensis*, much remains to be learned of the structure, physiology and functional significance of these structures.

Declarations

Author contribution statement

Patricia M. Tilney, Abraham E. van Wyk: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Magda Nel: Performed the experiments; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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