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Anatomical and ultrastructural peculiarities in the laticifers of *Euphorbia antiquorum* L.: A potential source for the biofuel production

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Abstract

The present study was conducted to examine the anatomy, distribution and ultrastructure of laticifer system in phylloclades of *Euphorbia antiquorum* L. by light and transmission electron microscopy. The phylloclade shows several anatomical characteristics well adapted to the xerophytic environment. Transverse section of *E. antiquorum* phylloclade displays three distinct regions namely cortical tissue, vascular cylinder and the pith region. Non-articulated laticifers are present in three tissues, but their frequency varies with the tissue type. Highest laticifer frequency was observed in vascular cylinder (14%) followed by cortex (3.9%) and pith regions (3%). In contrast, laticifer index was greatest in the pith (9.7%) followed by vascular cylinder (6.9%) and in the cortex (4.9%). Laticifers were well recognized by the presence of nucleus and dense cytoplasm rich in ribosomes, mitochondria, plastids, endoplasmic reticulum, two types of vacuoles and osmophilic bodies when compared to the adjacent cells. Histo-chemical tests revealed the presence of phenolics, proteins, terpenoids, starch, alkaloids, indicating that this species may be useful as a potential feedstock for the production of biofuels in future from semiarid or arid environments. The discovery of laticifer system in *E. antiquorum* was not described earlier could also be of taxonomic value.

Keywords: Biofuel, *Euphorbia antiquorum,* latex, laticifer, phylloclade and ultrastructure

INTRODUCTION

Biofuel is becoming increasingly attractive fuel of choice due to high fossil fuel prices, concerns about national energy security, sovereignty, burden on foreign exchange, harmful impacts on the environment and global climate change. The major research focus has already been given on the production of biofuels from crops such as Sugarcane, Sugar beet, Corn (maize), Jatropha, Soybean, Canola, Switch grass, Miscanthus and Poplar, which are relatively less water use efficient and have competition for land and water. Therefore, cultivation of high energy yielding biomass feedstocks that require minimal inputs of water and nutrients, grow on marginal lands, not competing with food and feed crops could be a sustainable answer to the increasing demands for biofuels.

Plants grown in deficient water sources are succulent in nature and exhibit mostly the Crassulacean Acid Metabolism (CAM) pathway. Succulent plants are productive in semiarid regions because they assimilate carbon at night thereby reduce the diffusive gradient of water out of leaves and shown to improve water use efficiency [1, 2]. Several investigations were conducted for screening of laticiferous as well as resinous species as sources of high-energy, easily extractable compounds suitable for conversion to liquid biofuels [3, 4]. The family Euphorbiaceae is one of the principal flowering plants with 300 genera and 8000 species. *Euphorbia* is one of the largest genera with over 2000 species distributed all over the world and found mostly growing on dry lands in subtropics and temperate regions [5]. Serious concern has already been given to the option of extracting biocrude from latex bearing species of *Euphorbia*, such as *E. antisyphilitica* Zucc, *E. antiquorum* Linn, *E. lathyris* Linn, *E. tirucalli* Linn, *E. caducifolia* Haines, *E. neerifolia* Linn, *E. royleana* Boiss [6, 7], since this genera appears to be one of the most promising possibilities for obtaining petroleum substitutes and other useful chemical feedstock [8, 9].

Laticifers may be defined as a tube-like network of specialized cells contains latex including water-soluble components, rubber and other cytoplasmic organelles [10]. The principal components of latex are rubbers, resins, sugars, proteins, enzymes and alkaloids, terpenoids, triglycerides, waxes, tannins, lipid, phytosterols and other modified isoprenoid compounds [11, 12]. Based on the cell number, complexity and mode of origin and development, articulated and non-articulated laticifers have been recognized [13, 14]. Taxa of the genus *Euphorbia* reported to have non-articulated branched laticifers [15]. Ultrastructure, distribution and cytological analysis of laticifers in species of *Euphorbia pulcherrima* Willd, *Euphorbia kansui* Liou and *Euphorbia characias* Wulfenii have been reported [16-18]. However, there has been no report on the distribution, morphology and ultrastructure of laticifers in *E. antiquorum* L.

E. antiquorum is a huge, succulent shrub with CAM pathway naturally grows on rocky areas and ravine lands, distributed in various habitats majorly on arid and semiarid regions. In addition, these plants possess high adaptability to grow on very poor and marginal soils and show tolerance to different levels of moisture and temperature [19]. The phylloclades are quadrangular branched with

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divaricated stipules and covered with spines. The phylloclades are tough and fleshy and in particular their central portion contains large number of mucilage cells. The phylloclades display number of anatomical peculiarities and represent adaptations to the xerophytic environment. On injury to any part of the phylloclade, sticky, white, milky latex flows out in abundance. The ingredients of latex reported to act as defence against herbivores [20]. The latex reported to have novel phytosterols and triterpenes [21] and extensively used as folk medicine due to its purgative, rubefacient expectorant and antispasmodic properties [22], as well as possible biofuel [23]. The present study was conducted to examine the anatomy, distribution and ultrastructure of laticifer system in phylloclades of *E. antiquorum* by light and transmission electron microscopy. Laticifer index and frequency were calculated in cross sectional view of the phylloclade in different regions of the phylloclade. Histochemical tests were further performed to identify the principal components of latex using a variety of stains.

MATERIALS AND METHODS

Plant material

Young phylloclades of about 2 to 3 cm long and 1 cm diameter were collected from *E. antiquorum* (approximately 4cm to 5cm height) growing on Guvalchervu natural forest areas of YSR District of Andhra Pradesh, India.

Anatomy of phylloclades, morphology and distribution of laticifers

Phylloclades were cut into about 2 mm^2 size and fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 12 h at 4 ^{0}C. Samples were washed three times in buffer and post-fixed in 1% OsO_4 for 1 h at 4^{0}C. The samples were dehydrated in ascending grades of acetone, infiltered and embedded in araldite CY 212 (TAB, UK). Ultrathin sections of about 1 µm were made with an ultra-microtome, fixed on to clean slides and stained with an aqueous Toluidene blue O for the study of phylloclade anatomy, morphology and distribution of laticifers. The stained sections were observed at 40X and photographed by a Carl Zeiss Axioscope A1 fluorescent microscope (Carl Zeiss, Germany). Images were digitalized using a CCD camera (Prog Res C3 Jenoptik).

Laticifer index (LI) and Laticifer frequency (LF) were calculated based on the number of laticifer cells and undifferentiated cells under a microscopic field area (10 X). Per cent distribution of laticifers were calculated as [L/ (NL + L)] × 100; where L and NL are the number of laticifer cells and non-laticifer cells under a microscopic field, respectively [24]. Data are the means of thirty separate observations made from different cross sections from at least three different phylloclades (Table 1).

Table 1 Laticifer differentiation in *E. antiquorum* phylloclade*

Plant part	Region	Laticifer Index(LI)	Laticifer frequency(LF)
Phylloclade	Cortex	4.9 ± 0.9	3.9 ± 0.4
	Vascular system	6.9 ± 0.9	14.0 ± 1.9
	Pith	9.7 ± 2.5	3.0 ± 0.5

* Results are means ± SD of thirty separate observations

Ultrastructure of laticifers by Transmission Electron Microscopy (TEM)

After gross observation of the area and quality of the tissue fixation, ultrathin sections of grey-silver colour interference (70-80 nm) were cut and mounted on 300 mesh copper grids for transmission electron microscope observation. Sections were stained with alcoholic uranyl acetate and alkaline lead citrate, washed gently with distilled water and observed under a Morgagni 268D transmission electron microscope (Fei Company, The Netherlands) at an operating voltage of 80 kv. Images were digitalized using a CCD camera (Mega view III, Fei Company).

Histochemical analysis

The principal components of the latex were determined on fresh free hand transverse sections of the phylloclade by using a variety of stains: (1) Sudan IV for lipids [25]; (2) Nile blue for neutral and acidic lipids [26]; (3) Ruthenium red for pectins [27] and acidic polysaccharides [28]; (4) Wagner reagent and Dittmar reagent for alkaloids [29]; (5) Hellram reagent and Vanillin-HCl for flavonoids [30], in particular, flavanols [31] and condensed tannins [32]; (6) Ethanol-HCl (2:1), for leucoanthocyanins and anthocyanins [33]; (7) Cresyl blue and toluidene blue for phenols and (8) Eosin for proteins [27]. The stained sections were observed at 10X and photographed by a Carl Zeiss Axioscope A1 fluorescent microscope (Carl Zeiss, Germany). Images were digitalized using a CCD camera (Prog Res C3 Jenoptik).

RESULTS

Phylloclade anatomy

Transverse section of E. antiquorum phylloclade displays three distinct regions namely cortical tissue, vascular cylinder and the pith region. Epidermis is an outmost layer with single layer of cells, protected with a thick cuticle. The epidermal cells have conspicuous thick walls and possess conical shaped papillae. The sub-epidermal layer is made with quadrangular and rectangular cells. The dimensions of sub-epidermal cells are comparatively greater than those of typical epidermal cells. The cortical region is organized of two distinct regions namely an outer chlorophyllous palisade-like tissue and inner ground parenchyma. Chlorophyllous palisade tissue consists of few layers and occupied chief proportion of the cortex. Palisade cells are positioned perpendicularly to the surface with copious intercellular spaces (Fig.1a). The ground parenchyma is made by more than three cell layers directly over the vascular region. The cells of ground parenchyma appear isodiametric in shape with thin walls. Numerous large central cavities were found in ground parenchyma (Fig. 1b). The size of the primary laticifer cells can be easily characterized from the neighboring parenchymatous cells by their dark staining content (Fig. 1c). The vascular system is collateral, radial developing a ring in which the xylem is innermost and abundant and the phloem is outermost and formed by typical large cells. The vascular system contains large number of xylem elements, massive radial parenchyma and several large sieve cells (Fig. 1d). The pith region contains large rectangular, thin walled parenchyma cells with copious intra cellular spaces, with no central cavity present (Fig. 1e).

Laticifer distribution and morphology

Ultrathin microtome sections of E. antiquorum phylloclade were observed under light microscope to examine the distribution and morphology of laticifers. Laticifer frequency varies with the tissue type viz., in the cortical, vascular and pith regions of phylloclade (Table 1). However, non-articulated branched laticifers were observed in the transverse section of phylloclade. The primary laticifers are distributed in the pith, vascular region and in the ground cortical parenchyma. Primary laticifers are appeared as thick-walled rectangular cells surrounded by parenchyma cells. Their size is similar to the surrounding cells, but they are obvious due to dark staining (Fig. 1a and 1c). The secondary laticifers are restricted to palisade tissue of the cortical region. The cells looks like long tube branched without cross-walls and are rather irregularly interspersed among the elongated cells of the palisade tissues (Fig. 1a). The highest laticifer frequency was observed in the vascular cylinder (14%) followed by cortex (3.9%) and pith regions (3%). In contrast, maximum laticifer index was observed in the pith (9.7%) whereas it was 6.9% and 4.9% in vascular cylinder and cortex respectively (Table1).

Ultrastructure of laticifer

Transmission Electron Microscopic images of laticifer revealed dissimilar ultrastructure as compared to the neighboring cells. Laticifers were well distinguished by the presence of cell wall, nucleus, dense cytoplasm rich in ribosomes, small and big vacuoles, mitochondria, plastids, endoplasmic reticulum, and osmophilic bodies (Fig. 2a). The laticifer cell wall was thicker and darker than those of the neighboring parenchymatous cells. A thin layer of cytoplasm was restricted to the periphery of the laticifer cell. A round or sometimes elongated and lobed nucleus with the nucleoli and nuclear membrane was observed (Fig. 2a). Plastids were lens-shaped and possess well-organized grana and thylakoid membrane system with numerous translucent plastoglobuli in the homogenous stroma (Fig. 2b). Some greatly altered plastids with an exceptionally electron-dense stroma and a series of parallel membranes were also present in the laticifer. The mitochondria were either spherical or elongated in section with cristae and part of the envelope was unclear (Fig. 2c and 2d).

The globular osmophilic bodies are present in the peripheral cytoplasmic region of the cell (Fig. 2e). The endoplasmic reticulum is appeared either in long or short form; sometimes dilated and coalescence to form vacuoles. The resultant small vacuoles become enlarged and began to accumulate latex particles of various sizes and those vacuoles become electron transparent (Fig. 2f). Endoplasmic reticulum was distributed around mitochondria, plastids or globular osmophilic bodies. The dictyosomes were high in number and secreted numerous vesicles or small vacuoles during vacuolization. The laticifers could be easily distinguished from other cells by the manifestation of numerous small vacuoles or vesicles (Fig. 2a).

Histochemical tests

Examination of free hand transverse sections of the phylloclade with various stains revealed differences in cytochemical properties among cell types of E. antiquorum. All cells of the phylloclade including those of laticifers were non-specifically stained with Cresyl blue (Fig. 3a), Dittmar, Hellram and Toluidene blue O stains. However, laticifers stained more intensive than the adjacent cells. Furthermore, the intensity of staining with Eosin, Ruthenium red, Sudan IV, Vanillin-HCl and Ethanol-HCl stains varied among different cell types. The staining characteristics of different cell types observed through fresh hand sections of phylloclades are summarized in Table 2. It was found that phylloclade tissues of E. antiquorum were rich in fatty acids as confirmed by the blue staining of Nile blue (Fig. 3d).

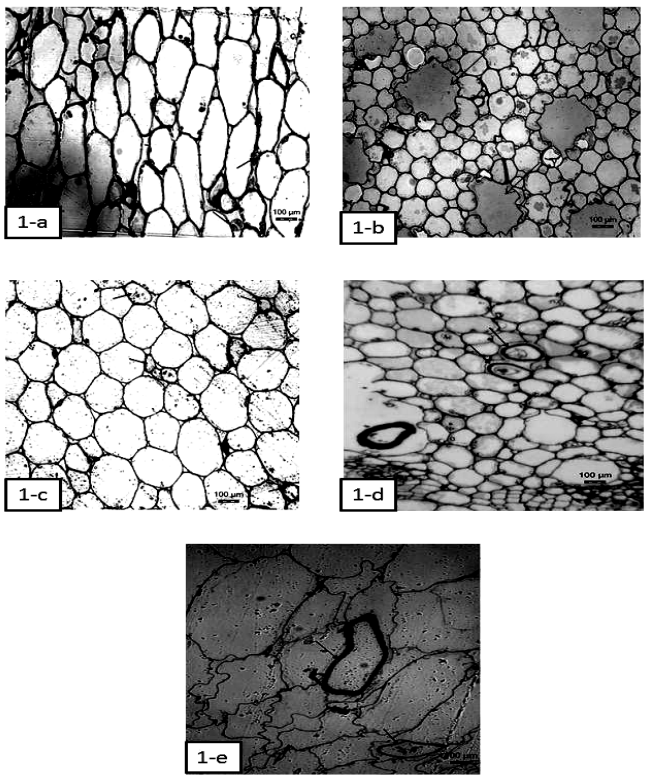

Fig. 1. Laticifers in transverse section of *E.antiquorum* phylloclade by ultra microtome (40X); **a** – Laticifers are interspersed between palisade tissue (arrows) stained blue with Toluidene blue O. Bars = 100µm; **b** – Large intercellular cavities are present in between the parenchymatous tissue of the cortex (arrows). Bars = 100µm; **c** – Laticifer cells are present in between the parenchymatous tissue of the cortex (arrows). Bars = 100µm; **d –** The sieve cells are present above the vascular region (arrows). Bars = 100µm; **e** – The thick walled tube like laticifer cells is present in between the irregularly shaped parenchymatous tissue of the pith region (arrows). Bars = 100µm.

The cytoplasm of the laticifer cells imparted strong staining reactions to Cresyl blue (Fig. 3a) as well as Toluidine blue O (Fig. 3e) specifying the presence of phenols. However visual observation of Cresyl blue stained cells of the phylloclade appears to be in blue-green and Toluidine blue O stained all cells in purple-blue and laticifer cells in dark blue colour. The results obtained with Toluidine blue O (Fig. 3e) designates that cells of phylloclade walls are rich in phenols. Sieve elements and laticifer cells stained light red with Eosin and dark brick red with Ruthenium red demonstrating the presence of protein and acidic polysaccharides, respectively. The cytoplasm of the laticifers displays dark brown when stained with Wagner reagent (Fig. 3f) reveals the presence of protein. Further

cells of phylloclade staining brown with Dittmar reagents confirm the sparse distribution of starch and alkaloids (Fig. 3b). The Vanillin tests (Hellram test (Fig. 3c) and Vanillin-HCl) colored the laticifer content an intense cherry-red revealing the presence of flavonoids. Further, the thin sections were treated with Ethanol-HCl colored cells in light pink confirm the presence of sparse amounts of Anthocyanidins. All cells of the phylloclade stained brown with Dittmar reagent suggesting the presence of Alkaloid. The intensity of the staining was also more for the laticifers as well as the sieve elements.

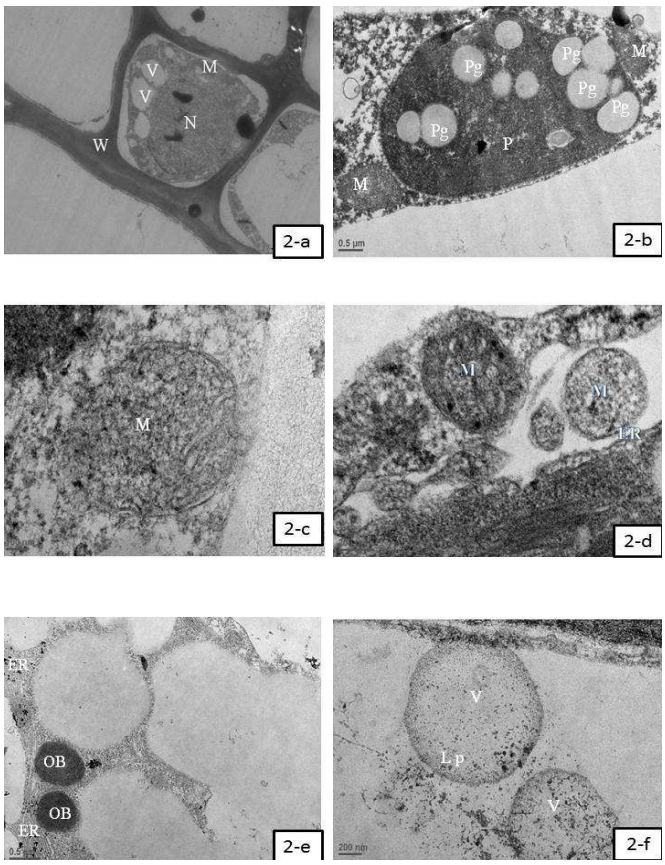

Fig. 2. TEM micrographs of laticifer cells; **a –** A cytoplasmic mass (**C**) with prominent nucleus (**N**), mitochondria (**M**), vacuole (**V**) are present and enclosed by a cell wall (**W**). Bars = 2 µm; **b –** A swollen lens shaped Plastid (**P**) showing stacks of grana with numerous plastoglobilin (**Pg**). Bars = 0.5µm.; **c –** Mitochondria (**M**) with numerous cristae are present and a part of the envelope is not clear. Bars = 100nm.; **d –** A round shaped Mitochondria (**M**), and many vesicles (**Vc**) originating from endoplasmic reticulum (**ER**) nearer to the plastid (**P**). Bars = 200nm;**e –** Numerous osmophilic bodies (**OB**) are scattered in the cytoplasm (**C**). Bars = 0.5µm; **f –** Latex particles (**LP**) of various shapes are accumulated in the electron transparent vacuoles **(V).** Bars = 200nm.

DISCUSSION

E. antiquorum phylloclade displayed the presence of thick cuticle and sub-epidermal layer which acts as an additional barrier to avoid water loss by transpiration. Such layers are formed as a result of the reduction and occasional loss of the leaf blade. It is considered to be an important xeromorphic character, has been proposed for E. tirucalli [34]. The cortical cells differentiated into outer chlorophyllous palisade tissue and inner ground parenchyma with isodiametric cells. The occurrence of chlorophyllous palisade tissue in phylloclade has also been correlated with leaf blade reduction and has also been reported to occur from Pteridophyta to Liliate [35]. More number of chloroplasts is concentrated in a relatively small area of the palisade tissue which considered being a specialized tissue. Collateral bundles are present in which the xylem strand oriented towards the center of the phylloclade, hugely as observed in the stem: this proves the fact that phylloclade is infact a modified assimilating stem. Similar anatomical observations have also been reported in E. tirucalli and E. nicaeensis [34, 36]. Laticifers revealed constant characters such as non-articulated, ramified, polygonal shape with thick walls in transverse section. Anastomoses between laticifers are believed to be involved in metabolite supply which is crucial for efficient latex production and facilitate latex flow through the development of a network structure. Similar observations were also made in other species of E. characias, E. nicaeensis, E. kansui and E. tirucalli [16, 18, 34 and 36].

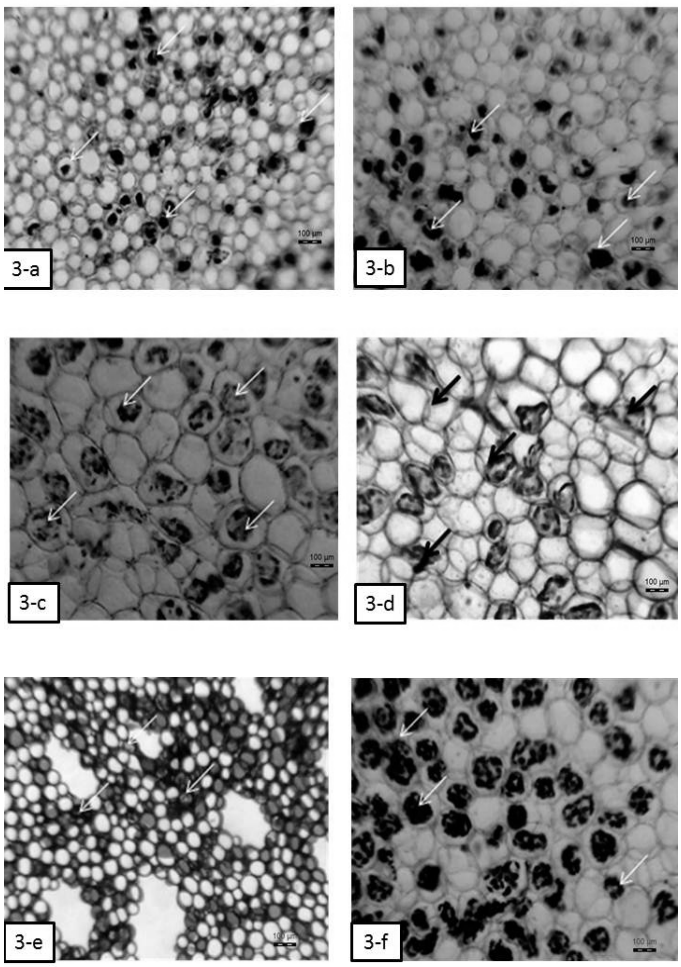

Fig. 3. Histochemical tests on hand sections for laticifers (10X)
a – Laticifer cells in cortical cells are stained dark blue with cresyl blue (arrows) indicates phenols. Bars = 100µm; **b –** Laticifers stained with Dittmar reagent shows dark brown (arrows) in cortical region indicates the presence of alkaloids and starch. Bars = 100µm; **c –** The cytoplasm of the laticifers stained light cherry red with Hellram reagent indicates the presence of flavonoids (arrows). Bars = 100µm; **d –** Laticifer cells stained blue with Nile blue indicates the presence of fatty acids (arrows). Bars = 100µm; **e –** Laticifer cells stained blue (arrows) more than the neighboring cells with Toluidine blue O indicates the presence of phenols**.** Bars = 100µm; **f** – The

laticifer contents stained dark brown (arrows) with Wagner reagent indicates the presence of protein. Bars = 100µm.

Table 2: Response of different phlloclade cells of *E. antiquorum* to histochemical tests

Reagent	Target compound	Observed color	Intensity of Staining[a] Phylloclade	Other cells with staining
Cresyl blue	Phenols	Blue	+++	All cells. Laticifer cells intensely stained
Eosin	Protein	Red	++	Sieve cells and cytoplasm of the laticifer cells
Hellram	Flavonoids	Light Cherry Red	+	Laticifers and Xylem elements
Vanillin HCl	Flavonoids	Light Cherry Red	++	Laticifers and sieve elements
Dittmar	Alkaloids Starch	Light brown Brown	+ ++	All cells; and intensity more for laticifers and sieve elements
Ethanol HCl	Anthocyanidins	Pink Color	+	Vascular elements and laticifers
Nile blue	Fatty acids Phospho lipids & Neutral lipids	Blue Brown Red brown	++ - -	Laticifers
Wagner	Protein	Dark Brown	++	cytoplasm
Sudan IV	Total lipids	Dark Orange	++	Laticifer and cuticle
Ruthenium red	Acid polysaccharides	Dark Brick Red	++	Sieve elements
Toluidene Blue	Phenols	Dark Blue	+++	All cells

[a]++++, very strong staining; +++, strong staining, + weak staining; – no staining

The present study constitutes the first report of ultrastructure of non-articulated laticifer in E. antiquorum. The observations of ultrastructure are in agreement to those reports on E. kansui [18] and Campotheca accuminata [37]. Laticifers were well recognized by the presence of nucleus and dense cytoplasm rich in ribosomes, mitochondria, plastids, endoplasmic reticulum, two types of vacuoles and osmophilic bodies when compared to the adjacent cell. Similar observations have been reported in E. pulcherrima [17], E. characias [38]. The plastids present in the laticifer cells did not contain starch grains [37]. Normally, the laticifers comprise amyloplasts which lack membranes and starch grains [39]. A distinctive feature of laticifers is the accumulations of electron dense masses as reported in E. kanusi, which is not found in other cell types [18]. These findings suggest that common model with non-articulated laticifers is the synthesis of most of the latex components in the cytoplasm and then transported to the vacuole, which is consistent with the results of a study on E. pulcherrima [17]. In the present study, it can be speculated that vacuoles play a key role in separating the latex droplets from the protoplasm and thus maintaining a normal metabolism. The latex particles are then transported and release into the central vacuole. Similar postulate is also available for E. kansui [18]. The lens shaped plastids play a key role in the synthesis of lipophilic substances [40] and in general the biosynthesis of terpenes has already been demonstrated [41].

The histochemical analysis of phylloclade tissues of E. antiquorum revealed the rich presence of fatty acids and phospholipids as confirmed by staining with Nile blue. The results obtained with Toluidine blue O indicates that cells of phylloclade walls are rich in acidic polysaccharides and phenols. These findings are reliable with the results published on E. pulcherrima [39]. The presence of phenols may possibly signify that the latex plays defensive role against fungal and bacterial invasion and studies indicate that there are very high concentrations of antifungal and antibacterial substances in latex [42]. Similarly, a large number of compounds from many different classes such as flavonoids, terpenes and lipids were recorded from the members of the family Euphorbiaceae [43]. This finding is in accordance with the report published on Campotheca acuminate Decne [37].

CONCLUSION

The present study contributes for the first time important information about phylloclade anatomy, distribution, ultrastructure of laticifer and latex composition of E. antiquorum. In addition, histochemical tests revealed the presence of lipophilic compounds in the phylloclades of E. antiquorum indicating that this species may be useful as a potential feedstock for the production of biofuels in future from semiarid or arid environments.

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