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Morphological and histochemical studies on trichomes on different parts of *Boerhavia diffusa* L.

Gulshan Chaudhary & Prem Kumar Dantu*

Department of Botany, Dayalbagh Educational Institute (Deemed University), Dayalbagh, Agra 282110, Uttar Pradesh, India

e-mail: *deigulshan.chaudhary0@gmail.com, **premdantu@gmail.com

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ABSTRACT

Boerhavia diffusa (Nyctaginaceae) rich in several secondary metabolites is recommended for various ailments in the Ayurvedic system of medicine. Present study reveals that the medicinal properties of this plant are largely due to presence of digitiform and capitate trichomes on both vegetative and reproductive parts. These trichomes secret alkaloids, phenols, polysaccharides, proteins, saturated and unsaturated lipids. Present study deals with the morphological (LM and SEM) and histochemical studies on the trichomes in *B. diffusa*. The capitate trichomes were recorded on the surface of ovaries and apocarps only. Trichomes present on the surfaces of younger parts provide biotic and abiotic protection.

Keywords: Capitate, digitiform, trichomes, light microscopy, scanning electron microscopy, histochemical tests

INTRODUCTION

The genus *Boerhavia* (Nyctaginaceae) has 40 species, which are widely distributed in Australia, China, Egypt, Pakistan, Sudan, Sri Lanka, South Africa, USA, India and several Middle East countries. *B. diffusa*, commonly known as punarnava in Sanskrit is an important medicinal herb indigenous to India and is found throughout the warmer parts of the country up to an altitude of 2000 m in the Himalayan region. It grows well on wastelands and in fields after the rainy season. The plant grows in open sun and endures severe abiotic stresses as UV exposure, high temperature, water and nutrient deficiencies. In West Bengal, it is cultivated for its leaves used for medicinal purpose (Anonymous 1988).

B. diffusa has a long history of therapeutic uses in the indigenous tribal communities and in the Ayurvedic system of medicine in India. The root possesses anticonvulsant (Adesina 1979), antiviral (Verma & Awasthi 1979), antifibrinolytic (Jain & Khanna 1989), antibacterial (Olukoya *et al*. 1993), and hepatoprotective (Rawat *et al*. 1997) properties. The flowers and seeds are used as contraceptive (Chopra *et al*.1956). The plant as a whole is used for oedema and ascites (Anonymous 1988). Chemical analyses of the aerial parts and roots have revealed the presence of the alkaloid known as punarnavine (Agarwal & Dutt 1936), hypoxanthine-9-Larabinofuranoside (Ahmad & Hossain 1968), ursolic acid (Misra & Tiwari 1971), boeravinone A-F (Kodata *et al*. 1989, Lami *et al*. 1992), and punarnavoside (Jain & Khanna 1989). The medicinal value of this plant has been extensively reviewed recently by Chaudhary & Dantu (2011).

Trichomes in recent times have attracted the attention because of their secretory products, which

range from essential oils to being insect repellents and for biotechnological intervention for modifying their products and understanding molecular basis of their function (Wagner *et al*. 2004, Marin *et al*. 2008, Schilmiller *et al*. 2008). Trichomes because of their morphological and mechanical features and metabolites they produce influence several aspects of plant physiology and ecology (Wagner *et al*. 2004). The genus *Boerhavia* is richly covered with trichomes (Fadeyi *et al*. 1989, Struwig *et al*. 2011). The present paper is based on a detailed morphological, histological and histochemical study on the trichomes present on both vegetative and reproductive parts of *B. diffusa*.

MATERIAL & METHODS

Present study was conducted on ten marked plants of *Boerhavia diffusa* growing in the Herbal Garden, Department of Botany, Dayalbagh Educational Institute, Dayalbagh, Agra. A voucher specimen of the plant has been deposited in the Herbarium of the Department. The distribution of trichomes on dorsal and ventral surface of the third fully expanded leaf, young stem, bract, perianth, ovary and young and mature apocarp was determined under light microscope. Trichome density was determined in 25 mm^2 leaf, 1 mm^2 from stem and unfertilized entire ovary, 1.5 mm^2 from newly fertilized whole ovary and 6 mm^2 whole apocarps with the help of an ocular measuring grid.

Light Microscopy — For morphological studies, small pieces of stem, leaf, bracts, ovary, and apocarp were cut and fixed in formalin-acetic-alcohol (FAA) for 24 h. These were dehydrated, cleared, infiltrated and embedded in paraffin wax and sections were cut at 7-8 µm and stained in Toludene Blue O (0.1 % in 1 M phosphate buffer, pH 7.2) using the customary procedures described by Johansen (1940), Jensen (1962), and O'Brine *et al*. (1964). Length of stalk and diameter of head of trichomes was measured with the help of an ocular and stage micrometer. Data on 50 measurements per different types of trichomes were collected and analyzed. All observations for light microscopy were carried out using a Nikon Eclipse 200 epifluorescence microscope.

Scanning Electron Microscopy (SEM) — The pieces of stem, leaf, bracts, ovary, and apocarp were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.4 for 24 h at 4°C (De Mason *et al*. 1989). After washing in the same buffer, the material was dehydrated in a graded ethanol series, critical point dried, and coated with a thin layer of gold. The processed material was observed and photographed in a LEO 435 VP scanning electron microscope at an acceleration voltage of 15-30 kV at All India Institute of Medical Sciences, New Delhi.

Histochemistry — For histochemical localization of various metabolites in the trichomes fresh hand sections of leaf, stem, ovary, and apocarp were cut. NADI reagent was used for the localization of terpenoids (David & Carde 1964 as quoted by Machado *et al*, (2006); Periodic Acid-Schiff (PAS) reagent and Ruthenium Red for polysaccharids and pectin, respectively (Jensen 1962); Fehlings test for reducing sugars (Purvis *et al*. 1964); Sudan Black B for total lipids (Lison 1960 as quoted by Machado *et al*. 2006); Nile Blue A for neutral and acidic lipids (Cain 1947, Jensen 1962); Dragendorff reagent for alkaloids (Svendsen & Verpoorte 1983); ferric trichloride for phenolic compounds (Johansen 1940); mercuric bromophenol blue for total proteins (Mazia *et al*. 1953) and silver nitrate test was used for localizing calcium oxalate (Yusue 1969). Standard control procedures were carried out simultaneously for all the tests.

RESULTS & DISCUSSION

Distribution and morphology of trichomes — The trichomes on different plant parts were not evenly distributed and their number was higher on reproductive parts as compared to those on vegetative parts. The density of trichomes was much higher in the upper region of the stem and on young leaves. The trichomes had three distinct parts: (i) 2-4 basal cells embedded in the epidermis, (ii) variable number of uniseriate stalk cells and (iii) the single celled head at the top, distinct in shape, structure and secretions, but was also rarely branched. On the basis of the shape of the head, trichomes are of three types: (i) digitiform, (ii) capitates, or (iii) branched types.

Since *B. diffusa* grows in comparatively harsh abiotic conditions, the younger parts require more protection from desiccation, UV irradiation and insect attack and as the parts mature the number of trichomes reduce as also observed by Struwig *et al*. (2011). Larger numbers of trichomes on ovaries indicated that these require greater protection as compared to the apocarp. The ovary and apocarp had both capitate and digitiform trichomes while leaves and stem had only the digitiform trichomes.

Trichomes on vegetative parts — Only the digitiform trichomes were present on the vegetative parts. The distribution, density and size of trichomes varied on the stem and leaves.

Stem — Approximately 270±15 trichomes were present in 1mm^2 area of stem. On the basis of the length, the uniseriate stalks were of four types: short (26-28 µm), medium (40-50 µm), moderately long (72-75 µm) or long (94-96 µm). The short stalks consisted of three15-16 µm broad cells. The medium and moderately long stalks consisted of four cells (Fig. 1A-C), while the long stalk consisted of 94-96 µm long, 27-28 µm broad 4-6 cells. The trichome heads were at right angles to the stalk with the head cells always bending towards the apex (Fig. 1B & 1C). The head cell of all the trichomes on the stem was digitiform and 57-59 µm long. In short trichomes, the head was 10-11 µm broad, while in all other trichomes, the head was much broader (21-22 µm).

Leaves — The number of trichomes were slightly higher on the ventral surface (120 trichomes/sampling area) as compared to dorsal surface (110 trichomes/ sampling area). On both dorsal (Figs. 1D-F) and ventral surface (Figs. 1G-J) of the leaf only digitiform trichomes were present. The trichomes on the dorsal surface consisted of 28-32 µm long and 13-16 µm broad stalks with 2-4 cells. The head of digitiform trichome was 42-45 µm long and 19-23 µm broad. Variation in The size and type of trichomes head was much variable on the ventral surface. The stalk was 30-34 µm to 41-45 µm long and consisted of 3-4 cells. The digitiform head was 41-43 µm long and 22-25 µm broad (Fig.1 G & H). A limited number of trichomes consisted of two to three heads (Fig.1 I). Trichomes on both dorsal and ventral surface were bending towards leaf apex (Fig. 1 F & H). Presence of some substances on the surface of the trichome head were observed under SEM (Fig. 1J).

Trichomes on reproductive parts — The distribution, density and size of trichomes on different parts of the flower and apocarps were quite variable.

Bracts — On the bracts only the digitiform trichomes were present. Their head cell was oriented towards the apex (Figs. 1K & L). The stalk consisted of 64-65 µm long and 14-15 µm broad 3-5 cells. The head cell was much longer (78-79 µm) than the stalk but less broad (7-8 µm; Fig.1L). The surface of the head cell was rough as observed under SEM (Fig. 1K).

Parianth — Trichomes were present only on the outer surface of the perianth and were absent from the inner surface. There were two types of trichomes on perianth: (i) single digitiform trichomes (Fig.1M) and (ii) branched head trichomes (Fig 1N). The digitiform trichomes were 55-56 µm long and 14-15 µm broad with 3-4 uniseriate cells (Fig. 1M). The branched trichomes consisted of 55 µm long stalk with the head divided into two (Fig. 1N). One of the head was longer (176 µm) than the other (142 µm), while both were equally broad (10-12 µm).

Ovary — The ovaries consisted of higher number of trichomes/unit area. However, number decreased with the formation and maturation of the apocarp. The number of trichomes was higher on unfertilized ovary as there were approximately 490-500 trichomes/sampling unit area. Both digitiform type (Fig.1O) and capitate types of trichomes were present on the ovary. On the basis of stalk length, the digitiform trichomes were either short (80 µm long and 44 µm broad with 3-4 cells) or long (110 µm long and 51µm broad with 5-6 cells). The head cell of the short stalked trichome was small (43-44 µm long and 13-14 µm broad) as compared to that of long stalked trichome (67-68 µm long and 22-23 µm broad). The surface of the head cell in both short and long stalked trichomes was smooth.

Apocarp — Soon after fertilization, the ovary swelled and the trichome density also increased (645-650 in the sampling area of 1.5 mm^2). The mature apocarp of 6 mm^2 size consisted of 714 trichomes/sampling area. The trichomes were of two distinct types: (i) normal digitiform type and (ii) capitate type (Fig.1P). The

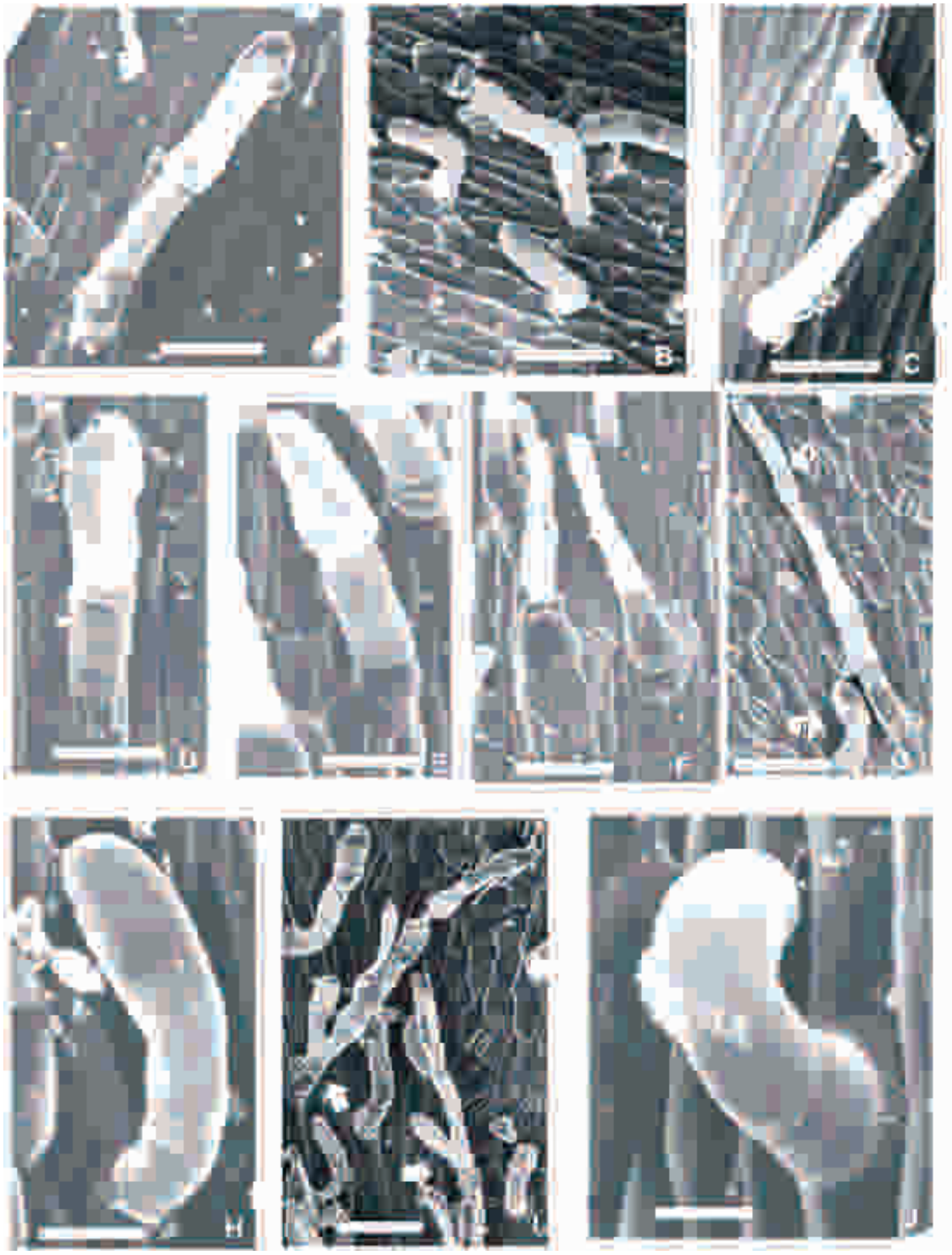

Fig. 1- SEM micrographs showing morphology of trichomes on the vegetative and floral parts of *Boerhavia diffusa.* **A-C.** Moderately long digitiform trichomes, **B & C.** Trichome with medium length stalk on stem; **D-F.** Three different types of digitiform trichomes on the dorsal surface of leaf, **G-J:** Different types of trichomes on the ventral surface of the leaf; **G:** A long (arrow marked i) and a short trichome (arrow marked ii); **H & J** Digitiform trichomes, Note the presence of dipositions on the trichome; **I:** Branched digitiform trihome (arrow marked)

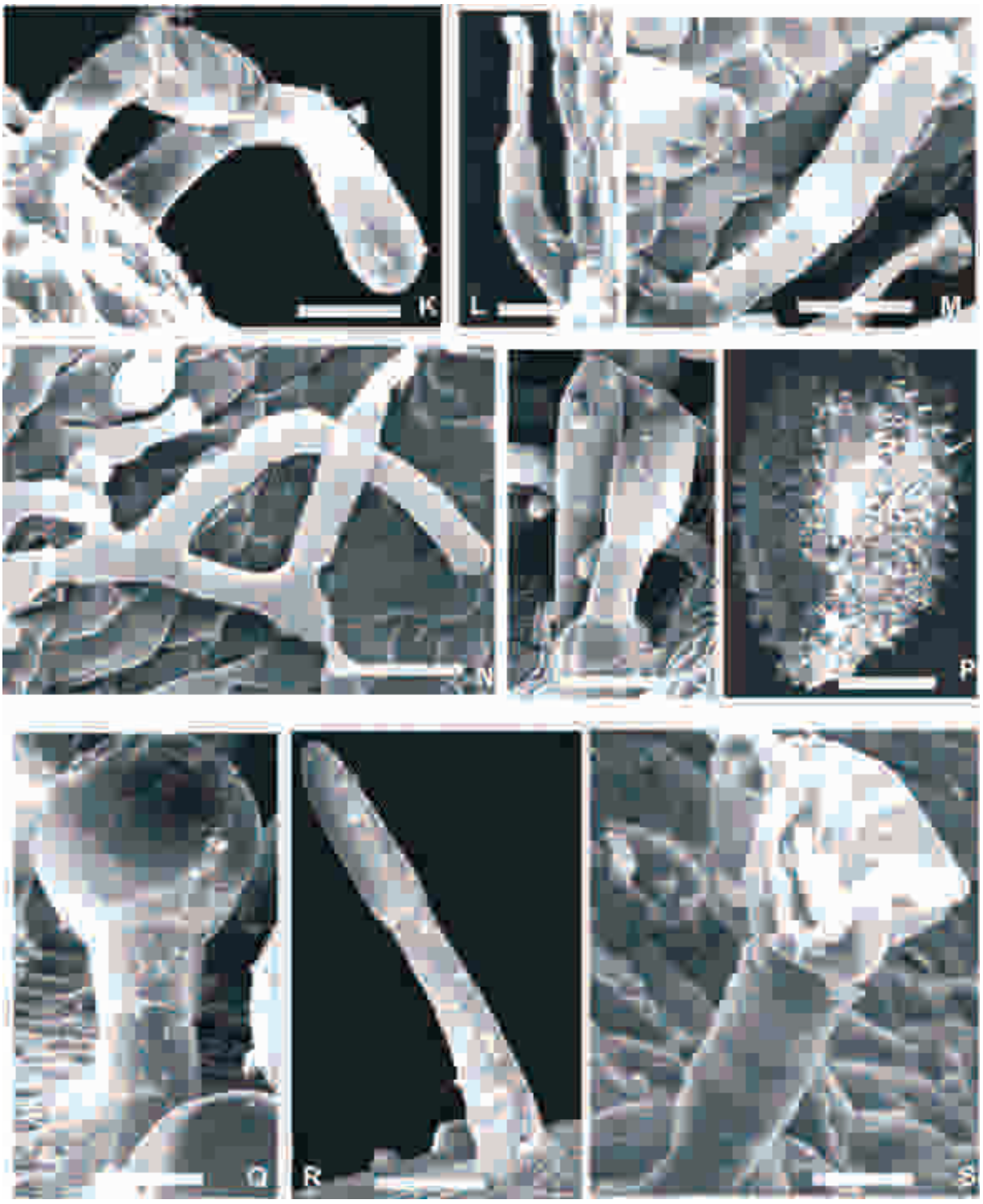

Fig. 1- K. Digitiform trichome on the bracts; **L.** Two types of digitiform trichomes on the bracts; **M & N.** Trichomes on the parianth; **M.** Normal digitiform trichome; **N.** Branched trichome on parianth; **O.** Digitiform trichome on the ovary; **P:** Capitate and digitiform trichomes; **Q.** Capitate trichome on pericarp; **R.** Digitiform trichome; **S.** Ruptured head of trichome. (bar size: Fig. A= 41 µm, B = 45 µm, C = 45 µm, D= 25 µm, E = 33 µm, F= 61 µm, G = 76 µm, H= 20 µm, I = 60 µm, J = 8 µm, K = 22 µm, L= 50 µm, M = 25 µm, N = 30 µm, O = 50, P = 750 µm, Q = 38 µm, R = 53 µm, S = 25 µm).

capitate trichome consisted of 2-4 celled stalk (86-87 µm long and 32-33 µm broad). The head of the capitate type was rough and spherical with a diameter of 76 µm (Fig. 1Q). The digitiform trichomes were much longer (122-123 µm) than broader (26-27 µm) with 5-6 cells arranged uniseriately. The head cell of the digitiform trichome was 92-93 µm long and 28 µm broad (Fig. 1R). The capitate trichomes showed seasonal variation and were totally absent during the monsoon season, but reappeared soon afterwards. The apocarp consisted of both digitate (Fig. 2 A) and capitate trichomes (Fig. B-D). The two basal cells of the stalk were embedded in the epidermis. The head cells were covered by a distinct cuticle and consisted of dense cytoplasm, prominent nucleus and a vacuole (Fig. 2 A-D). LM and SEM observations showed that the bursting of the head cell resulted in the discharge of substances (Fig. 1S).

The present SEM observations on *Boerhavia diffusa* clearly revealed that the heads of digitiform and capitate trichomes were single celled with a distinct nucleus. In a number of plants such as *Cucurbita pepo* (Kolb & Muller 2003), *Lippia scaberrima* (Combrinck *et al*. 2007), *Zeyheria montana* (Machado *et al*. 2006), *Salvia blepharophylla* (Bisio *et al*. 1999) trichome heads are multicellular. The secretion from head cell in glandular trichomes was caused by the breakdown of the cuticle as was also observed in *Salvia blepharophylla* (Bisio *et al*. 1999), *Thymus lykae* (Marin *et al*. 2008), *Cordia verbenacea* (Ventrella & Marinho 2008), *Teucrium scorodonia* (Antunes & Sevinate-Pinto 1991) and *Leonotis leonurus* (Ascensao *et al*. 1995) or through pores in the cuticle of the head cell as was shown in *Salvia officinalis* (Corsi & Bottega 1999) or even through diffusion from the cuticle as was in the case of *Salvia aurea* (Serrato-Valenti *et al*. 1997). Although trichomes with broken cuticle of the head cell were found in *B. diffusa* during present study, but no pores for secretions were found. Probably the secretions were released from the head of *B. diffusa* trichomes by the rupture of the cuticle caused by mechanical damage. The other likely explanation as proposed by Ascensao *et al*. (1995) may be that natural conditions e.g. high temperature and low air humidity caused the cuticle to burst releasing the essential oil. Other factors, such as contact with predators can also be involved in the rupture mechanism (Werker *et al*. 1993). Depositions on the surface of the heads of trichomes in *B. diffusa* indicated outward diffusion which could also be a mode for the cellular secretions to exude.

Histochemical studies — The trichomes on stem, leaf and apocarp tested positive for phenols by ferric chloride test (Fig. 3A). Phenolic compounds were also present in the head and the stalk cells of digitiform and capitate trichomes. Pectin was detected only in the cuticle on all the three parts of trichomes as tested by Ruthenium Red (Fig. 3B). Lipids as tested by Sudan Black B and Nile Blue A were also present (Figs. 3C & 3D). Polysaccharides tested positive with PAS in all the three parts of the trichomes (Fig. 3E). Protein tested positive only in the capitate trichomes on the ovary and apocarps with mercuric bromophenol blue (Figs. 3F). Alkaloids were detected in the capitate trichomes of the apocarps only, but were absent in the trichomes of stem and leaf as tested by Dragendorff reagent (Fig. 3G). Terpenes tested positive by NADI reagent in the trichome heads of the apocarps (Fig. 3H) and in the stalk of the trichomes on the stem and leaf. Reducing sugars and calcium oxalate were absent in the trichomes on all the three parts.

Histochemical tests in the present study revealed that the material secreted from capitate and digitiform trichomes of *B. diffusa* are lipids existing in the space enclosed by the cuticular membrane. Alkaloids and phenolic substances also occur in the secretary head of trichomes on apocarps. As reported in *Lundia cordata* (Lopes *et al*. 2002) and *Stevia rebaudiana* (Monteiro *et al*. 2001) in *Boerhavia diffusa* as well the detachment of the secretory sheath from trichome head cells may be occurring along a zone possibly containing pectins as revealed by the staining of the outer cuticle by Ruthenium Red.

Struwig *et al*. (2011) observed the accumulation of calcium and certain sticky substances in the trichomes of *Boerhavia*. The secretions which accumulate in the heads of the capitate and digitiform trichomes on the apocarps of *B. diffusa* stain positively for lipophilic substances, with lipids prevailing in fully secreting

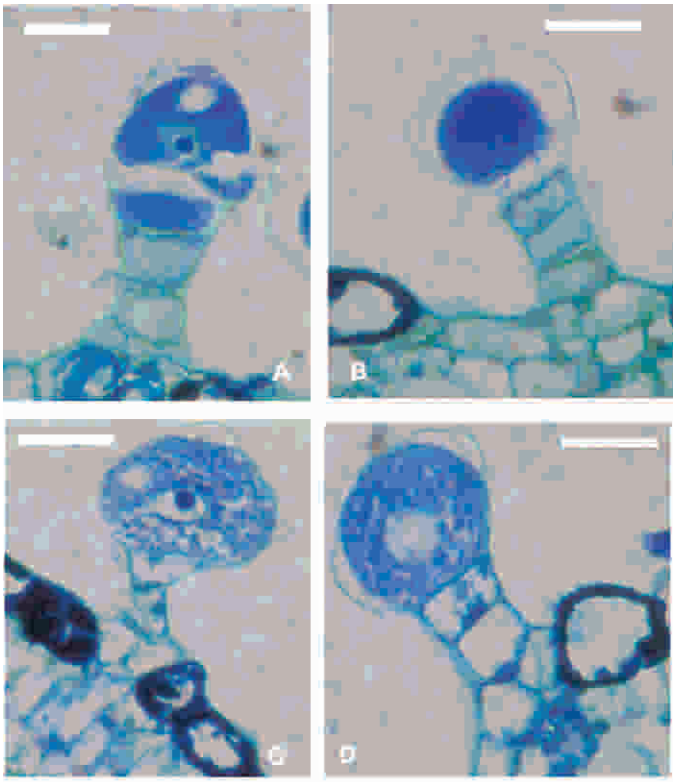

Fig. 2- Histology of trichomes on apocarp; **A:** Digitiform trichome; **B-D.** Capitate trichomes with dense cytoplasm a nucleus in C and a vacuole D. (Bar A & B= 30 µm, C= 60 µm, D =20 µm, E= 45 µm, F = 35 µm, G=40 µm).

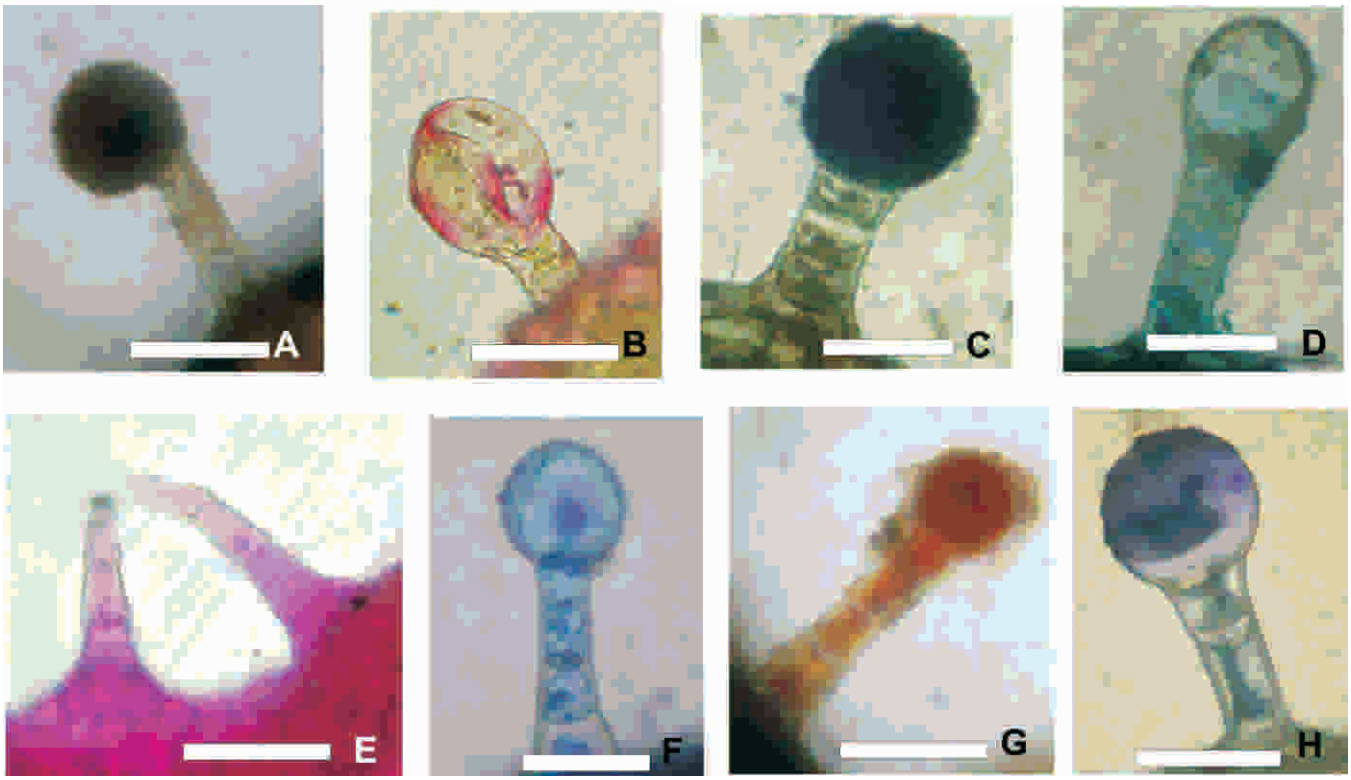

Fig. 3- A. Capitate trichome on apocarp showing the presence of phenolics, **B.** Pink colour indicates presence of pectin in the outer wall of the trichome head, **C.** Black colour shows the presence of saturated lipids, **D.** Blue colour shows the presence of unsaturated lipids, **E.** Polysaccharides in digitiform trichomes, **F.** Proteins **G.** Alkaloids, **H.** Terpenoid. (bar: A, B, F=80 µm, C=60 µm, D=40 µm, E=180 µm, G=90 µm, H=70 µm).

trichomes (Kolb & Muller 2004, Machado *et al*. 2006). The ferric trichloride test gave a positive reading for the presence of phenols in *B. diffusa* is based on the formation of intensively coloured precipitate from the reaction of iron with ortho-dihydroxyphenols (Johansen 1940). Alkaloids are not a common occurrence in the trichomes. However, the capitate trichomes of *B. diffusa* tested positively to Dragendorff reagent indicating the presence of alkaloids. Alkaloids have been reported in *Sigesbeckia jorullensis* (Heinrich *et al*. 2002) and *Salvia officinalis* (Corsi & Bottega 1999). The trichomes probably only store the alkaloids produced elsewhere and might not be the site of their synthesis (Schilmiller *et al*. 2008).

The capitate trichomes only on the apocarp of *B. diffusa* showed the presence of proteins. Presence of proteins in the trichomes in a limited number of plants such as, *Salvia aurea*, *Rosmarinus officinalis*, *Leonotis leonorus*, *S. blepharophylla* and *S. officinalis* was shown (Corsi & Bottega 1999, Bissio *et al*. 1999, Ascensao *et al*. 1995, Serrato-Valenti *et al*. 1997). Since trichomes are the site of synthesis of a number of metabolites it is obvious to expect a battery of enzymes within the trichomes and besides, some of these proteins could be playing a role in defence (Schilmiller *et al*. 2008). Terpenes were of common occurrence in the trichomes of several plants. In *B. diffusa* both the capitate and digitiform trichomes of the apocarps indicated the presence of terpenes in their head, while it was present only in the stalk of trichomes on the stem and leaf. Such variation in the presence of terpenes in trichomes was observed in *Salvia officinalis* (Corsi & Bottega 1999). Pectin was not detected from cellular contents of the trichome cells but was observed in the cuticle of entire trichomes of both the types in *B. diffusa* presently studied. However, in a number of plants pectins and other polysaccharides form a part of the cellular secretions of the trichomes (Serrato-Valenti *et al*. 1997, Ascensao *et al*. 1999, Bisio *et al*. 1999, Combrinck *et al*. 2007, Ventrella & Marinho 2008).

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