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Morphological, anatomical and phytochemical characterization of *Neanotis montholonii* (Hook. F.) W.H. Lewis

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Abstract

Neanotis montholonii (Hook F.) W.H. Lewis belongs to family Rubiaceae. *Neanotis* genus has been represented by ca. 10 species in Maharashtra state. The morphological, anatomical and phytochemical characteristics are pivotal in diagnostics taxa at taxonomic level. In the present investigation an attempt has been made to identify alkaloids, saponins and tannin by using histochemical tests of root, stem and leaf. The organoleptic character and stomatal index (23/mm² area) were also recorded that aid in identification. Phytochemical study revealed the presence of active ingredients like alkaloids, flavonoids, glycosides, anthroquinones, amino acids, protein, saponins, steroids and tannins in all the parts those were subjected for analysis except starch that was absent in root. Among all four solvents viz. chloroform, ethanol, acetone and aqueous extract, ethanol showed positive tests in all plant parts. Histochemical and phytochemical information revealed in the present study can be used for developing standard parameters for the proper authentication of raw materials of pharmaceutical importance. The literature review reported that this study is the first of its kind in the *Neanotis montholonii* which is endemic to Maharashtra.

Keywords: Rubiaceae, *Neanotis montholonii*, histochemical, phytochemical

1. Introduction

Rubiaceae is the fourth largest family of Angiosperms containing highest number of taxa (Delprete *et al.* 2012) [5]. It comprises of 13,548 species belonging to 617 genera (Anonymous, 2016) [2]. This family is popularly known as the coffee, madder or bedstraw family. The plants are not only ornamental but also have applications in folk medicine formulations. Indeed, close to 60 species are used in over 70 medicinal preparations including for malaria hepatitis, eczema, oedema, cough, hypertension, diabetes and sexual dysfunction. Most of these plants exhibited presence of pharmacologically active ingredients having antimalarial, antimicrobial, antioxidant, anti-inflammatory, antihyperthermic and antidiabetic properties. Further, bioactive compounds such as indole, alkaloid, terpenoids, anthroquinones and tannins have also been reported (Simplice *et al.*, 2011) [25]. Genus *Neanotis* is represented by 33 species distributed in tropical and subtropical countries of Asia namely India, China and Malaysia. Ten species are reported from Maharashtra state, (Cook, 1967) [4]. *Neanotis montholonii* (Hook F.) W.H. Lewis (syn. *Anotis montholonii*) is commonly distributed in the open spaces within the state (Almeida, 1996) [1]. Moreover, presence of this species is perceived as a weed in cultivated crops like Groundnut, Maize, Jawar, Bajara, Rice, etc. It is an annual herb with sparsely hairy branches; terminal inflorescences; corymbose cymes; four stamens; two celled ovary; two to four seeded sessile capsule.

The macroscopic and microscopic illustrations of plant are crucial in identification and provide key peculiarities that aid systematic investigations. Nevertheless, such studies were not yet reported pertaining to *Neanotis montholonii*. Further, these studies are also important to ascertain evolutionary line of development as well as relationship between the closely related taxa. In the present report we made our efforts to document new information on morphology, anatomy and phytochemistry in this less investigated taxa. This information will also help in authenticating the herb during drug preparation.

2. Materials and methods

2.1 Authentication of the plant material: The plant material was collected in the month of September 2017 from Nashik (19°59'50.8344"N, 73°47'23.2908"E) district of Maharashtra. Authentication done by experts from Botanical Survey of India, Pune (Specimen voucher number was BSI/WRC/IDEN.CER./2016/800).

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2.2 Macroscopic studies: Macroscopic studies were carried out by using organoleptic evaluation method (Yadav *et al.* 2011)^[24]. Size, shape, odour, taste, colour, texture, base, apex, margin of leaves, petiole, stem, and root of *N. montholonii* were observed for recording key characters.

2.3 Microscopic studies: Microscopic studies (Wallis, 1950)^[23] were carried out by taking thin section of root, stem, leaf and petiole. Safranin and light green were used for staining and the sections were mounted in glycerin for microscopic investigations (Johanson 1940)^[10]. Thin sections were observed under binocular microscope (leica-DF450). Photographs at different magnifications were taken by using 12 megapixels Nikon digital camera.

2.4 Quantitative microscopy: Stomatal number and stomatal index pertaining to leaves were noted for quantitative microscopy.

a. Stomatal Number and Stomatal Index (SI): The upper and lower epidermis of leaf was peeled off to study the stomata. To remove adhering tissues and pigments it was immersed into hot alcohol, then stained with aqueous safranin and mounted in glycerine. Stomatal index was calculated using following formula (Trease & Evans, 1972)^[20].

$$SI \text{ (per mm}^2 \text{ area) \%} = \frac{S}{E+S} \times 100$$

Where **SI** - Stomatal index, **S** - no. of stomata per unit area, and **E** - no. of ordinary epidermal cells per unit area.

2.5 Histochemical Tests: The sections of root, stem, leaf and petiole were tested with the respective reagents. The detection and localization of alkaloids, saponins and tannin were carried out using methods given by Johanson, (1940) and Krishnamurthy (1988)^[10, 13].

1) Alkaloids: Detection of alkaloid was carried out using different reagents such as Wagner's reagent, Mayer's, reagent, Dragendorff's reagent and Hager's reagent (Fransworth, 1960)^[7].

2) saponin: Saponin test was carried out using method described by Trease and Evans, 2002^[21].

3) Tannins: Tannins were detected by Ferric chloride method, (Trease and Evans, 1972)^[20].

2.6 Phytochemical tests: Preliminary phytochemical test for the presence of Alkaloids by using Wagner's, Mayer's, Dragendorff's and Hager's tests), anthraquinone, amino acids, carbohydrates, flavonoids, flavanols, glycosides, phenols, protein, saponins, steroids, starch and tannins were carried out by standard methods of Horborne, (1973)^[8] and Trease & Evans, (2002)^[21].

3. Results and Discussions

3.1 Macroscopic:

a. Stem: Stem erect, four angled, cylindrical, diffusely branched, nodes swollen and covered with hairs.

b. Leaf: Leaves ovate-lanceolate, opposite, midrib prominent and pilose beneath, apex acute, base- rounded, exstipulate, and ciliate, size 4.9 cm x 2.9 cm, secondary venation very fine with reticulate venations, margin glabrous, pale green to dark green colour. Petiole 0.4 to 0.7 cm long, pale green.

c. The Inflorescence and Flower: Flowers onset was towards the August to September. Full blooming and beginning or fruit setting were took place at September to November. Flowers developed in the form of corymbose cyme inflorescence of about 10-15 cm long. Peduncle very short, 0.3-0.5 cm long, the flower numbers in the inflorescence was about 5-6 (Fig. 1B). Flowers are (Fig. 1C) purple in colour, pedicellate, actinomorphic, bisexual, tetramerous and pubescent on pedicels 0.1-0.2 cm long. Calyx green, 4 sepals, 0.6-1.0 cm long and 0.2-0.4 cm wide, gamosepalous, hairy, sepals end with bristle hairs, shape-ovate-lanceolate, apex acute. Corolla purple, petals - 4, 1.0-1.3 cm long and 0.5-0.7 cm wide, gamopetalous, tubular, alternate with sepals, shape ovate-lanceolate, apex acute, glabrous. Androecium consisted of four stamens, fertile, epipetalous, inserted, monothealous, basifixed, filament short or sub sessile. Gynoecium consisted of exserted, pilose, ovary bilocular with four ovules; style long, glabrous, terete, stigma bifid, basal placentation.

d. Fruit: capsule was compressed, 0.3-0.7 cm broad, dehiscing at the top, two lobed at apex, two cell fruit, calyx cluster long than the fruit, 2-4 seeds in fruits.

e. Seed: Seeds were ovate, almost round, compressed, 0.5 mm long and 0.4 mm wide, dark black, convex on the back, deeply excavated on faces.

Table 1: Macroscopic studies of *N. montholonii*

Sr. No.	Morphology	Observation		
		Root	Stem	Leaf
1	Colour	Brown	Pale green	Green
2	Odour	Characteristic	Characteristic	Characteristic
3	Taste	-	Bitter	Bitter
4	Size	5.2-6.7 cm long, 0.2-.03 wide	18-24 cm long, 0.3-0.4 cm wide	4.9-5.5 cm long, 2-3 cm wide
5	Shape	Tap root	Quadrangular with fistular	Lanceolate to ovate
6	Texture	-	-	Membranous
7	Fracture	Easy	Easy	Easy
8	Apex	-	-	Acute
9	Arrangement	-	-	Opposite
10	Appearance	Smooth	hairy	Smooth
11	Petiole	-	-	Short
12	Venation	-	-	Reticulate



Fig 1: Morphology of *N. montholonii*
(A-Habit, B-Inflorescence and C-Flower, D- Leaf, E- Root and F- Seeds)

3.2 Microscopic studies

a. Transverse section of root

T. S. of root showed distinct system composed of main tissue i.e. epiblema, cortex, endodermis as well as vascular system. The root epiblema was uniseriate, thin walled having irregular shape. Irregular parenchyma cells were without intercellular spaces. Moving inward, cell size increased while it reached maximum diameter at the middle of the cortex before decreasing again. The endodermis was uniseriate layer. The pericycle consisted of thin-walled parenchymatous cells with vascular tissues. The pith was wide. Bercu (2013) [3] demonstrated the anatomical study of the vegetative organs of *Gardenia jasminoides* Ellis (Rubiaceae) which corroborated well with inferences from present study of root anatomy. Pith was made up of small round oval cells and vessels are larger in size (Fig. 2a).

b. Transverse section of stem

T. S of stem was ribbed. Seldom unicellular and multicellular trichomes were observed on few epidermal cells. The epidermis layer consisted of uniseriate cells, irregular in shape with thin walled, irregular parenchyma cells having conspicuous intercellular spaces. The circular collenchyma cells were closely placed to the epidermis. The innermost layer of cortex was made up of 3-4 rows of hexagonal parenchymatous cells with thin walls. A distinct endodermis was also present. The vascular tissue was present in xylem, phloem as well as pith. The vascular bundles were relatively

different in size and number. There was large pith at the center consisted of hexagonal parenchymatous cells which tend to decrease in size towards the periphery. The small triangular intracellular spaces were visible. Hemcinschi *et al.* (2008) [9] investigated vegetative anatomy of two *Galium* species (Rubiaceae). They found the thick wall epidermis with unicellular trichomes, whose frequency decreased towards the upper and the lower level of the stem. In the ribs tangential collenchyma was present and the cortex ends with endodermis on the entire stem length, the outer cortical layer was collenchymatous (Fig. 2b).

c. Transverse section of leaf

Anatomy of leaf studied in midrib region and lamina as well. The midrib is well developed. The spongy tissue was composed of 4-5 layers of loosely arranged chlorenchymatous cells with intercellular spaces. Mechanical tissue was compact and surrounded the mid vein with vascular supply, thicken at the adaxial side. Well-developed, collateral vascular bundle was seen at the center of the midrib. Lower epidermal cells were elongated. The present study corroborated with that of Vieira *et al.* (2001) [22]. Lamina of the leaf exhibited dorsiventral composition and showed the upper epidermis with angular polygonal tubular cells covered with smooth cuticle. Mesophyll consisted of single layer of palisade parenchyma which consisted of radially elongated cells. Upper epidermis had unicellular and multicellular covering trichomes. Mussury *et al.* (2012) [16] studied the comparison of leaf

morphoanatomy of *Diodella radula* (Willd. & Hoffmanns. ex Roem. & Schult.) Delprete and *Diodella teres* (Walter) Small (Rubiaceae) and observed the presence of dorsiventral mesophyll cells, two or three layers of palisade parenchyma and three or four layers of spongy one, which was also the case in present investigation (Fig. 2c).

d. Petiole anatomy

The T. S. of petiole was of regular shape and consisted of the epidermal cells which were multiseriate with rectangular shaped. Single layer of circular collenchyma cells was located under the epidermis. The cortex consisted of orbicular

parenchymatous cells. Vascular bundles were of collateral type same as present in stem. The pith composed of polygonal parenchymatous cells with intracellular space. The vascular bundles were present in two ridge bundles. The pith was wide and formed of parenchymatous cells. Kocsis *et al.* (2003)^[12] described the petiole anatomy of some Rubiaceae genera and observed the outline of petiole is circular or oval as well as presence of unicellular hairs in the epidermis. The cell wall was made up of outer collenchymatous and inner parenchymatous cells. The main vascular bundle was collateral (Fig. 2d).

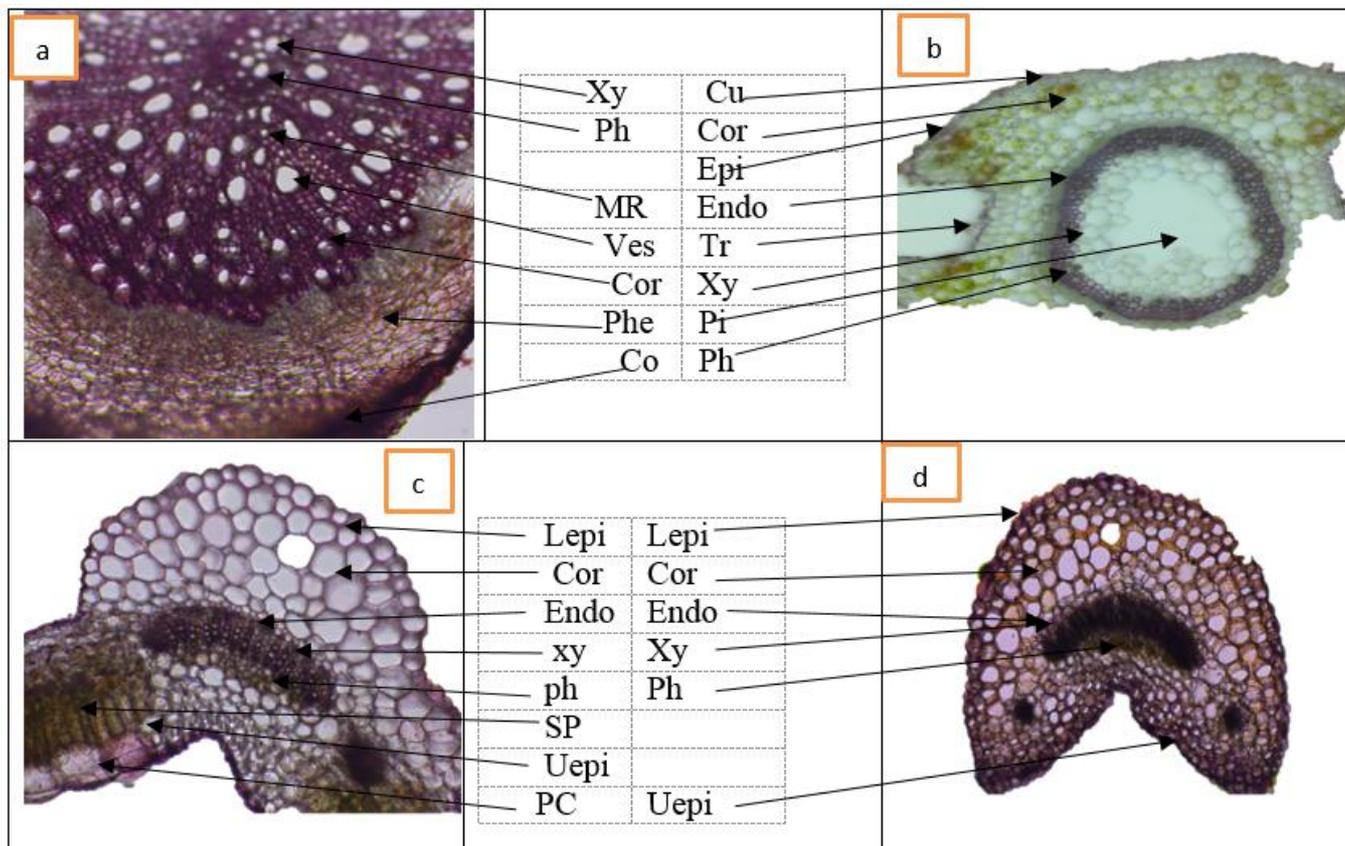


Fig. 2: Anatomy of different plant parts of *N. montholonii* (a- T.S. of Root, b- T.S. of Stem, c- T.S. of Leaf and d-T.S. of Petiole).

Tr: Trichome, **Cu:** Cuticle, **Epi:** Epidermis, **Co:** Cork, **Cor:** Cortex, **Phe:** Phellogen, **PC:** Palisade Cells, **SP:** Spongy Parenchyma, **Uepi:** Upper epidermis, **Lepi:** Lower epidermis; **Endo:** Endodermis, **Xy:** Xylem, **Ph:** Pholem, **Pi:** Pith, **MR:** Medullary rays, **Ves:** Vessels.

3.3 Quantitative microscopy

Stomata were discerned on upper and lower epidermis also. Paracytic type of stomata was recorded and stomatal index coincided with the observations of Mathew & Britto (2016)

^[14]. Stomatal index was recorded as 23 per mm² area. However, this differed with finding of Musmade *et al.* (2016)^[15], who reported hier Stomatal index (37.5/ per mm² area).

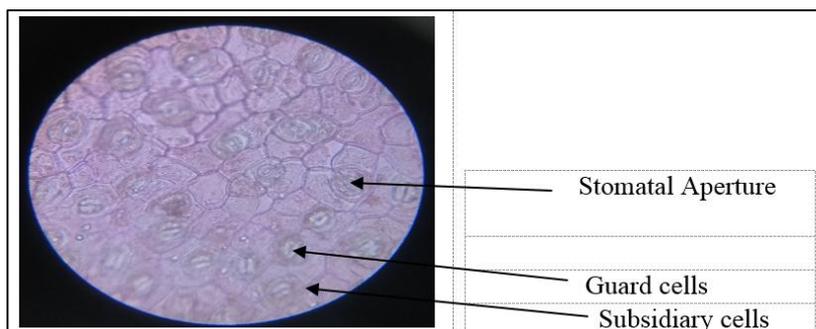


Fig 3: Quantitative microscopy of leaves of *N. montholonii*

3.4 Histochemical tests

N. montholonii is source of saponin, flavonoid, tannin, starch, protein and alkaloids so in the present study histochemical test of different plant parts was conducted. To analyze histochemistry of *N. montholonii* the free hand sections of root, stem, leaf and petiole were treated with different reagents.

Presence of alkaloids was detected by using different reagents such as Wagner's reagent that displayed reddish brown colour. Mayer's which displayed cream or pale yellow precipitate; that confirmed manifestation of alkaloids. Presence of alkaloids in Dragendroff's reagent was exhibited by formation of precipitation or development of turbidity.

Although Hager's reagent gave yellow precipitate indicating presence of alkaloids. All the above stated tests were positive for alkaloids in the parts investigated (Fig. 4, 5, 6, 7, a to d). For ratifying presence of saponins, another set of sections was treated with H_2SO_4 which indicated characteristic sequence of colour reactions beginning with instant occurrence of yellow, followed by red within 30 min and finally to yellowish green. This was detected in all the sections which confirmed that saponins were present in the plant parts (Fig. 4,5,6,7, e and f). For confirming presence of tannin, yet another set of sections was treated with dilute acidic ferric chloride solution which gave greenish colour.

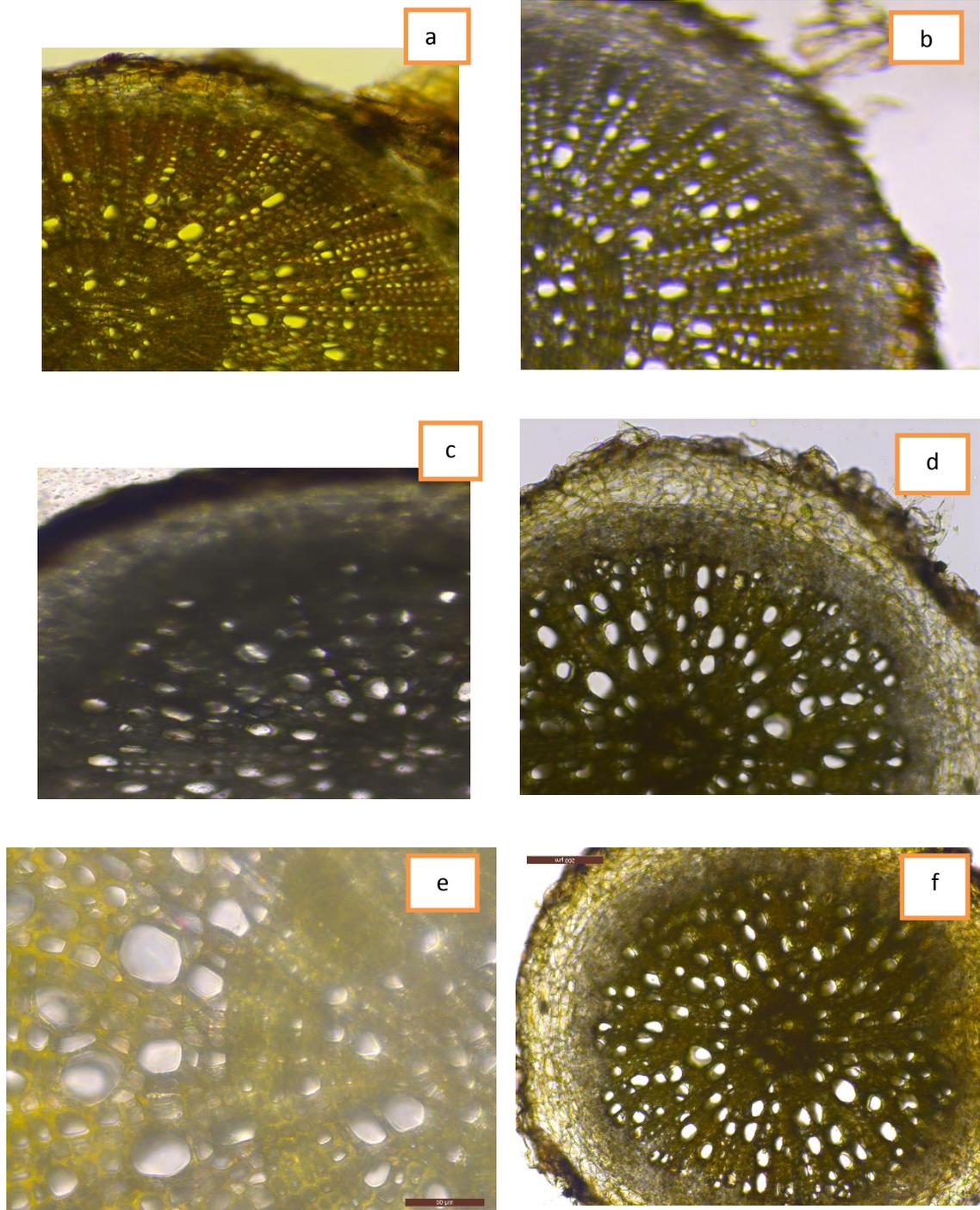


Fig 4: Histochemical tests of Root of *N. montholonii*
 a- Wagner's (reddish brown), b- Mayer's (pale yellow colour), c- Dragendroff's (ppt turbidity), d- Hager's (yellow ppt),
 e-Saponin (yellow colour), f-Tannin (Greenish).

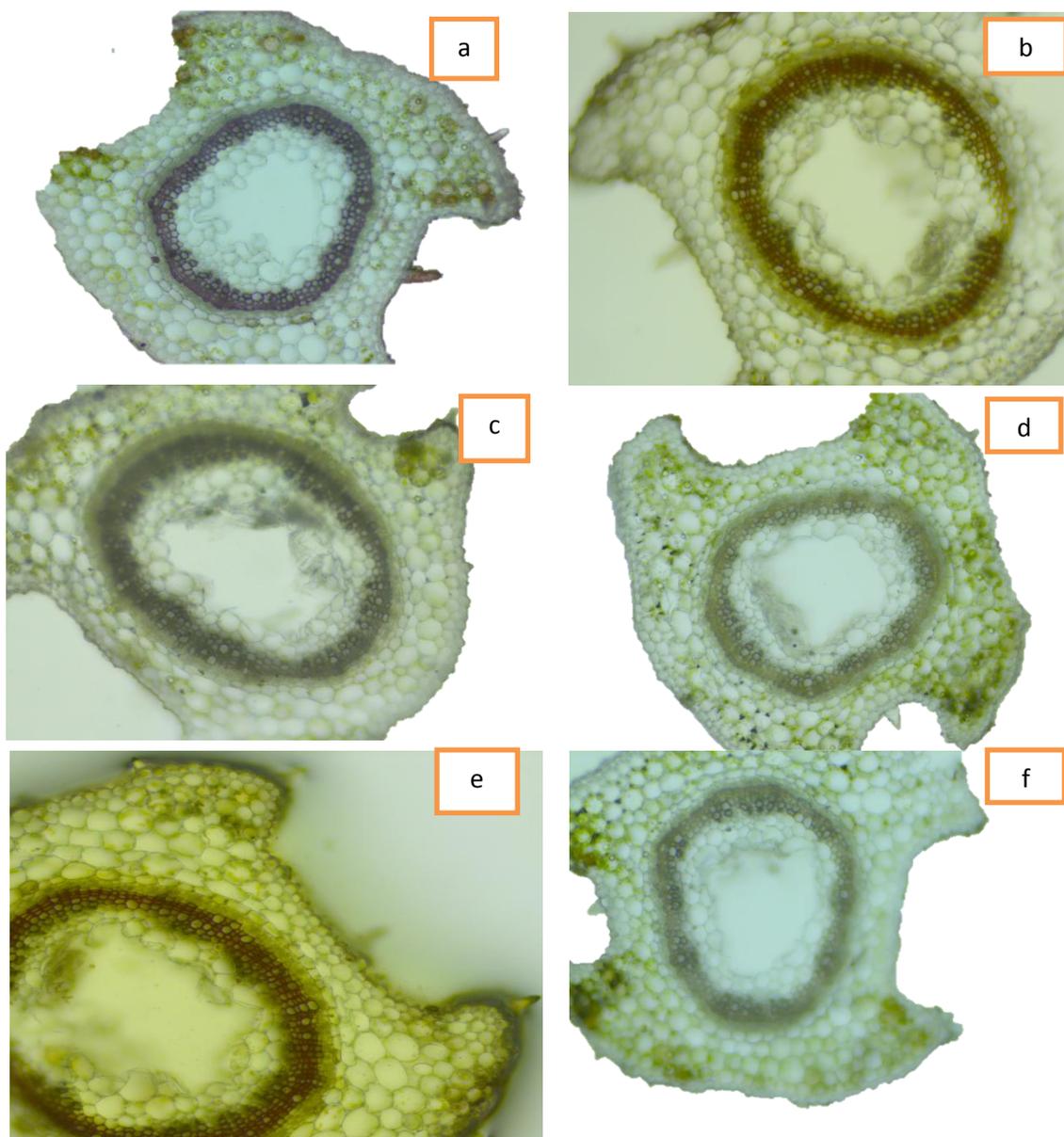
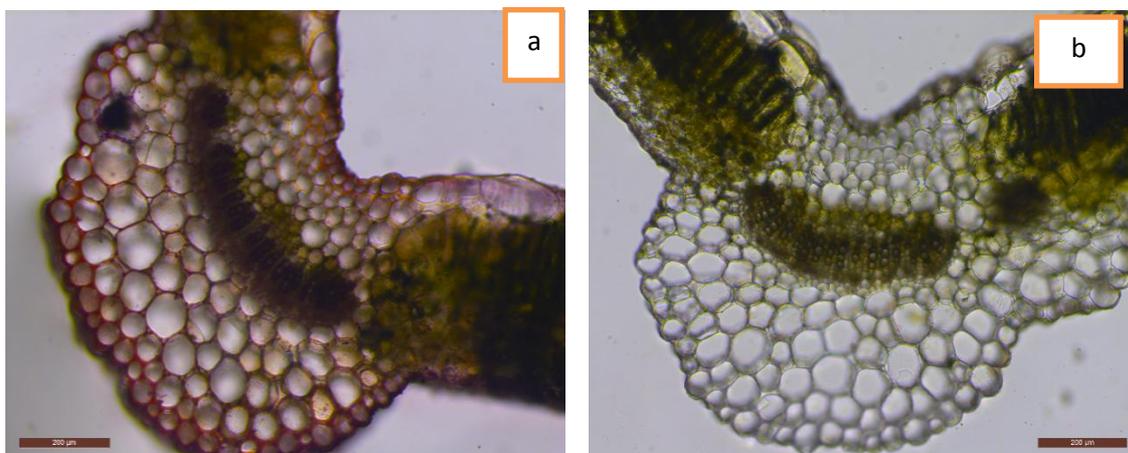


Fig 5: Histochemical tests of Stem of *N. montholonii*

a- Wagner's (redish brown), b- Mayer's (pale yellow colour), c- Dragendorff's (ppt turbidity), d- Hager's (yellow ppt), e-Saponin (yellow colour), f-Tannin (Greenish).



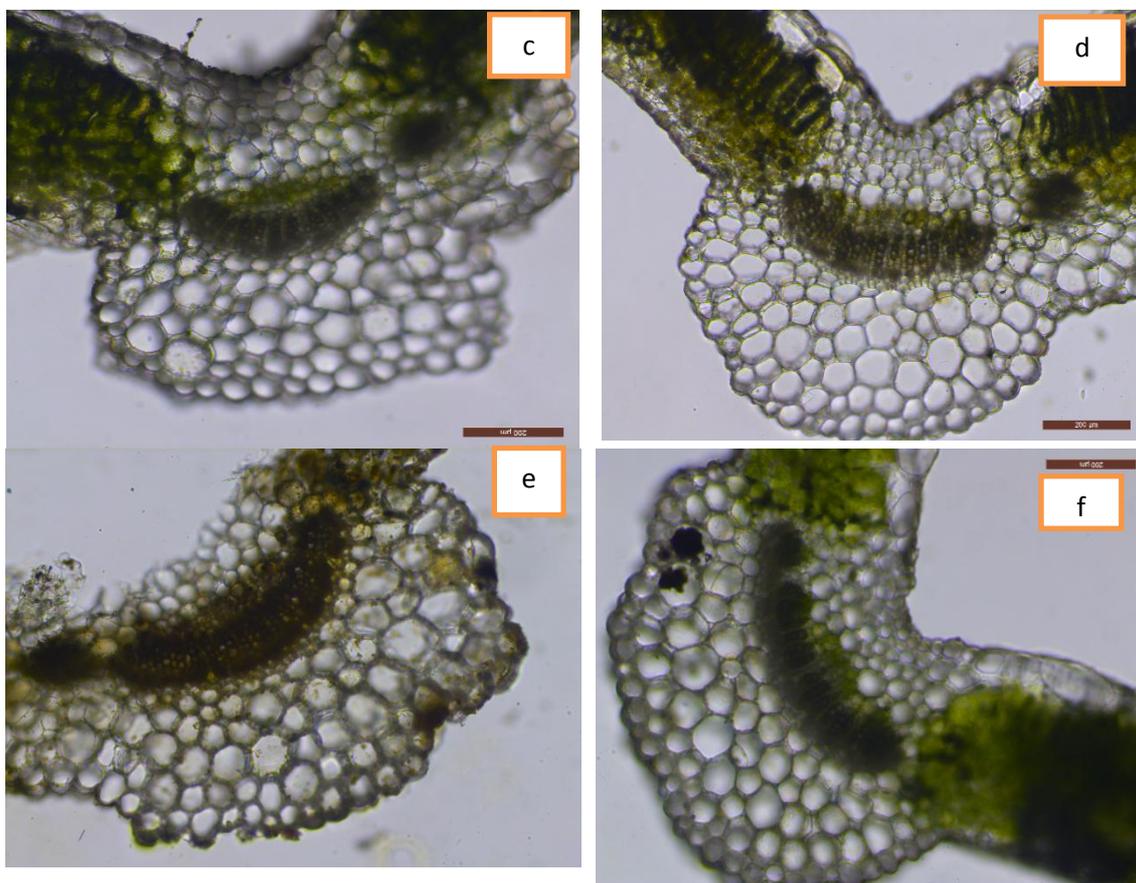
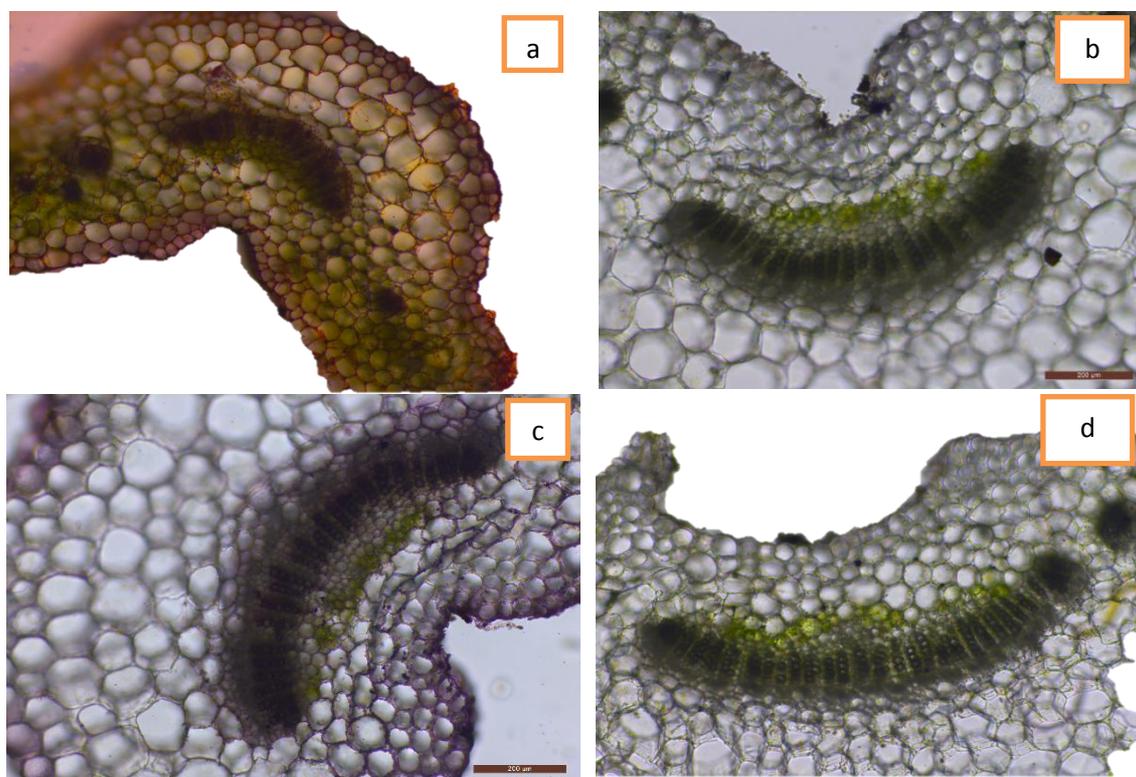


Fig 6: Histochemical tests of Leaves of *N. montholonii*
a- Wagner's (redish brown), b- Mayer's (pale yellow colour), c- Dragendroff's (ppt turbidity), d- Hager's (yellow ppt),
e-Saponin (yellow colour), f-Tannin (Greenish).



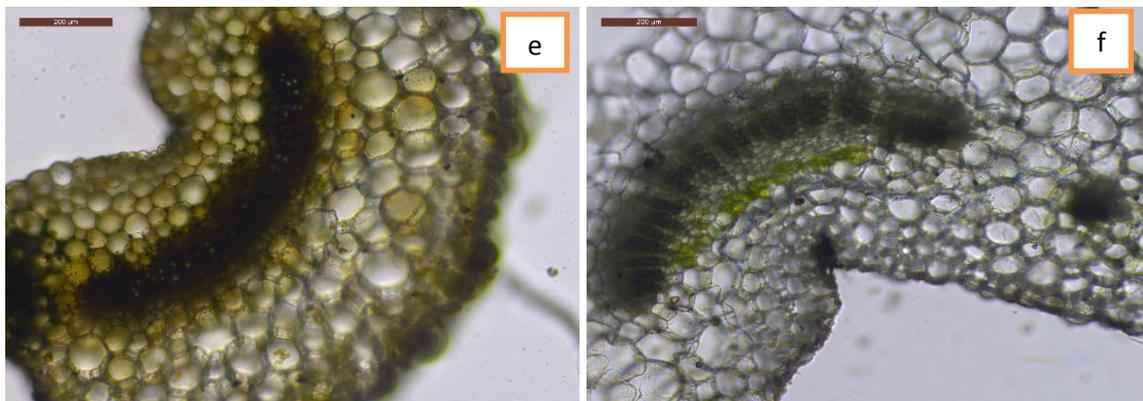


Fig 7: Histochemical tests of Petiole of *N. montholonii*

a- Wagner's (redish brown), b- Mayer's (pale yellow colour), c- Dragendorff's (ppt turbidity), d- Hager's (yellow ppt), e-Saponin (yellow colour), f-Tannin (Greenish).

3.5 Phytochemical analysis

Presence of crucial metabolites in crude drug was confirmed by pharmacological action. In the present investigation, the qualitative screening of alkaloids, flavonoids and glycosides were detected in alcoholic extracts of leaf, stem and root using different reagents. Alkaloids in all studied plant parts were detected using Wagner's, Mayer's, Dragendorff and Hager's reagents. It was found that they were found to be positive in ethanol and acetone whereas negative in chloroform and aqueous. Kannan *et al.* (2009) [11] observed the same results. More intensive colour was notice in leaf sample compare to root and stem indicating profound amount of alkaloid in the leaf tissue.

Carbohydrate was ubiquitous in all plant parts with positive reaction to Molisch's reagent. The positive test indicated occurrence of red cum violet ring. The presence of flavonoids and flavanols was confirmed by ammonia test. This test in ethanol and acetone imparted colour whereas it was negative in chloroform and aqueous extracts. Glycosides were also present in all parts those were subjected to Fehling test and Ferric chloride test that gave characteristic white precipitation. The test showed positive results in all solvents except for water extracts. Pathania *et al.* (2006) [17] recorded similar results in *Rubia cordifolia* L.

Water extract of root, stem and leaf was used to detect anthroquinones, amino acids, protein, saponins, steroids, starch, and tannins. Anthroquinones were detected in root, stem and leaf extracts, which gave pink to red colour in Borntrager's test. It was positive in chloroform and aqueous extracts but negative in ethanol and acetone. To confirm presence of amino acids and proteins in plant parts extracts when added to Million's and Ninhydrin reagent gave greenish black and blue colours respectively. Amino acids and proteins were present in all the solvents subjected to investigation. Saponins were present in all the plant parts and positive test pertaining to all the solvents was indicated by occurrence of foam.

Steroids were confirmed in all plant parts by Salkowski test indicated by occurrence of lower layer that turned to cherry red colour. Steroids were found to be positive in chloroform and ethanol whereas negative in acetone and aqueous extracts respectively. I₂KI reagent gave bluish black precipitate to the extract which confirmed presence of starch in leaf, stem; but was conspicuously absent in root extracts. Further, it was positive in all solvents except aqueous extract. Root, stem and leaf extract when added with acidic FeCl₃ and Trease and Evans test gave greenish and blue green colour respectively

that confirmed presence of tannins and phenols. It was positive in all solvents except aqueous extract.

These results were in agreement with Devi & Siril (2013) [6] who performed phytochemical screening of the powdered plant parts of *R. cordifolia*. Contrasting results were reported for alkaloids, steroids, tannin, flavonoids and phenols, in leaf, stem and root extracts.

Table 2: Phytochemical analysis of extracts of *N. montholonii*

Compounds	Name of Test	Extracts															
		Root			Stem			Leaf									
		1	2	3	4	1	2	3	4	1	2	3	4				
Alkaloids	Wagner's test	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-
	Mayer's test	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-
	Dragendorff's test	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-
	Hager's test	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-
Anthroquinone	Borntrager's test	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	-
Amino acids	Ninhydrin test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Carbohydrates	Molisch's test	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
Flavonoids	Ammonia test	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-
Flavanols	Ammonia test	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-
Glycosides	Fehling test	+	+	+	-	+	+	+	+	-	+	+	+	-	+	+	-
	Ferric chloride test	+	+	+	-	+	+	+	+	-	+	+	+	-	+	+	-
Phenols	Trease and Evans test	+	+	+	-	+	+	+	+	-	+	+	+	-	+	+	-
Protein	Million's test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Saponins	Foam test	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Steroids	Salkowski test	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-
Starch	Iodine test	-	-	-	-	+	+	+	+	-	+	+	+	-	+	+	-
Tannins	Ferric chloride test	+	+	+	-	+	+	+	+	-	+	+	+	-	+	+	-

1: Chloroform; 2: Ethanol; 3: Acetone; 4: Aqueous; +: Present; -: Absent.

4. Conclusions

The present study has enormous practical implications to authenticate genuine material that has been used in drug trade based on family Rubiaceae in general and *N. montholonii* in particular. The detailed investigation pertaining to macroscopic and microscopic features, along with biochemical investigations shall aid in confirming and further selecting appropriate plant based drug. The findings of the present investigation might define key features of *N. montholonii* as paracytic stomata were observed on both sides with stomatal index 23/mm² area; presence of alkaloids, saponin and tannin in root, stem and leaf; biochemical tests revealed presence of alkaloids, flavonoids and glycosides in alcoholic extracts and anthroquinones, amino acids, protein, saponins, steroids, starch, as well as tannins in water extract of root, stem and leaf. The present investigation thus highlighted urgent need to undertake detailed scientific

research to explore this common, endemic and least studied plant for its potential uses in herbal drug preparation.

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