Anatomical Study and Phytochemical Screening of *Vitex negundo* L.

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***Abstract*—*Vitex negundo* L. (Lamiaceae), commonly known as Nirgundi, is an aromatic shrub used extensively in the Ayurvedic, Siddha and Unani systems of medicine. The present investigation was carried out to study the anatomy of the leaf, petiole, stem and root and to screen the leaf extract for some major groups of phytoconstituents. Free-hand transverse sections stained with safranin and fast green revealed a dorsiventral leaf with abundant glandular and non-glandular trichomes on the abaxial surface, anomocytic stomata and a nearly circular vascular cylinder in the midrib. The stem showed a wide zone of secondary xylem traversed by rays and a large parenchymatous pith. The petiole showed an arc-shaped vascular bundle with accessory bundles, and the root showed secondary xylem with prominent medullary rays and no pith. In the qualitative tests, Fehling's and Benedict's reagents indicated the presence of reducing sugars, while Mayer's, Dragendorff's and Millon's tests were negative and Wagner's test was doubtful. The anatomical characters recorded in this study may be useful in the identification and standardisation of the crude drug.**

***Index Terms*—*Vitex negundo*, Nirgundi, leaf anatomy, trichomes, phytochemical screening**

I. Introduction

Vitex negundo L., the five-leaved chaste tree, is a large aromatic shrub or small tree distributed throughout India, Sri Lanka, Malaysia, China and other parts of tropical Asia [1]. The genus was earlier placed in the family Verbenaceae but is now included in Lamiaceae [2]. In Sanskrit the plant is called Nirgundi [1], a name generally taken to mean “that which protects the body from diseases”.

II. Literature Survey

The leaves and roots are used in traditional medicine for the treatment of rheumatism, headache, cough, asthma, fever, wounds and skin diseases [3]. Experimental studies have shown anti-inflammatory, analgesic, antioxidant, antimicrobial, hepatoprotective and insecticidal properties [4], [5]. These activities have been attributed to iridoid glycosides such as agnuside and negundoside, flavonoids including vitexin and isovitexin, lignans, terpenoids, essential oil and alkaloids [1], [3]. Negundoside, an iridoid glycoside from the leaves, protected human liver cells against carbon tetrachloride-induced toxicity [6], and leaf extracts showed anti-inflammatory and analgesic activity in animal models [7].

Nirgundi leaf is sold as a crude drug in the herbal market and may be adulterated with leaves of related species such as *V. trifolia*. Microscopic characters such as the type of trichomes, stomata and vascular arrangement are therefore important for its correct identification [8].

Early pharmacognostic work on the species [9] described the microscopy of the leaf and root [10] and of the leaf [11]. Physicochemical constants, fluorescence analysis and TLC profiles of the leaf extract have also been reported for standardisation of the drug [12]. More recently, light and scanning electron microscopy showed dense, segmented, branched non-glandular hairs together with glandular hairs on the lower leaf

surface of *V. negundo* [13], and trichome characters have been used to separate species and varieties of *Vitex* [2]. In a comparative study of three medicinal *Vitex* species, the leaf of *V. negundo* showed anomocytic stomata, a dense cover of tomentose hairs and a closed midrib vascular bundle ensheathed by sclerenchyma [14]. Peltate and capitate glandular trichomes on the leaf follow distinct developmental patterns, and the peltate type forms large secretory cavities thought to release the aromatic compounds [15]. The official monograph of the leaf describes uniseriate non-glandular hairs, glandular scales with a four-celled head and a crescent- or U-shaped collateral bundle in the midrib [16]. Preliminary screening of root and stem fractions has revealed alkaloids, flavonoids, terpenoids, phenols and saponins [17].

III. Problem Definition

Nirgundi leaf is traded as a crude drug and is liable to adulteration or substitution with leaves of related species [8], [14]. Reliable microscopic and chemical markers are therefore needed for its identification, and these need to be documented for local populations, including those of Ratlam district. The present study was undertaken to (i) study the anatomy of the leaf, petiole, stem and root of *V. negundo* collected from the Tal region of Ratlam district, and (ii) carry out a preliminary qualitative screening of the leaf extract for alkaloids, proteins and reducing sugars.

IV. Methodology / Approach

Plant Material

Fresh leaves, petioles, young stems and roots of *V. negundo* were collected from the Tal region of Ratlam district, Madhya Pradesh. The samples were washed thoroughly under running tap water to remove soil particles and adhering debris.

Anatomical Study

Fresh plant material was fixed in FAA (formalin : acetic acid : 70% ethanol, 5 : 5 : 90) for 24 hours and later preserved in 70% ethanol. Thin free-hand transverse sections of the leaf passing through the midrib, the petiole, the young stem and the root were cut with a sharp razor blade, using potato pith as support wherever necessary. The sections were stained with 1% safranin, washed, counterstained with 0.5% fast green and mounted in 50% glycerine [18]. Epidermal peels were taken from both surfaces of the leaf for the study of stomata and trichomes. The slides were observed under a research microscope at 10×, 40× and 100× magnification and photographed. Trichomes and stomata were classified following the terminology of Payne [19] and Metcalfe and Chalk [20].

Preparation of Extract

The leaves were shade dried and ground to a coarse powder. About 50 g of the powder was extracted with 250 ml of ethanol and the extract was filtered through Whatman No. 1 filter paper. For the alkaloid tests, a part of the extract was warmed with 1–2% hydrochloric acid and filtered, and the acidic filtrate was used for testing [21].

Phytochemical Screening

The extract was tested for alkaloids, proteins and reducing sugars following standard procedures [21], [22], [23]. The tests performed are given in Table 1.

Table 1 Qualitative Tests Used for Phytochemical Screening

Test	Constituent	Procedure	Positive result
Mayer's	Alkaloids	2 ml acid filtrate + few drops Mayer's reagent (potassium mercuric iodide)	Cream or white precipitate
Wagner's	Alkaloids	2 ml acid filtrate + few drops Wagner's reagent (iodine in potassium iodide)	Reddish-brown precipitate

Test	Constituent	Procedure	Positive result
Dragendorff's	Alkaloids	2 ml acid filtrate + few drops Dragendorff's reagent (potassium bismuth iodide)	Orange-red precipitate
Millon's	Proteins	2 ml extract + few drops Millon's reagent, then warmed	White precipitate turning brick-red
Fehling's	Reducing sugars	1 ml Fehling's A + 1 ml Fehling's B + 2 ml extract, boiled in water bath	Brick-red precipitate
Benedict's	Reducing sugars	2 ml extract + 2 ml Benedict's reagent, boiled for 5 min	Green, yellow, orange or brick-red precipitate

V. Results and Discussion

Anatomical Study

Photomicrographs of the sections are presented in Figs. 1–3, and the diagnostic features are summarised in Table 2.

Leaf. The transverse section of the leaf showed a dorsiventral structure. The upper epidermis was single layered, made up of rectangular cells covered by a thin cuticle, and bore only a few trichomes. The lower epidermis was densely covered with uniseriate, multicellular non-glandular trichomes, which give the lower surface of the leaf its whitish grey colour. Glandular trichomes with a short stalk and a uni- to multicellular head were also present. The stomata were of the anomocytic type and were mostly confined to the lower surface (Fig. 2d). The mesophyll consisted of one or two layers of compactly arranged palisade cells and a loose spongy tissue with large intercellular spaces. The midrib was prominent on the lower side. Below each epidermis were a few layers of collenchyma, followed by ground parenchyma and a large, nearly circular vascular cylinder in which the xylem vessels were arranged in radial rows (Fig. 1).

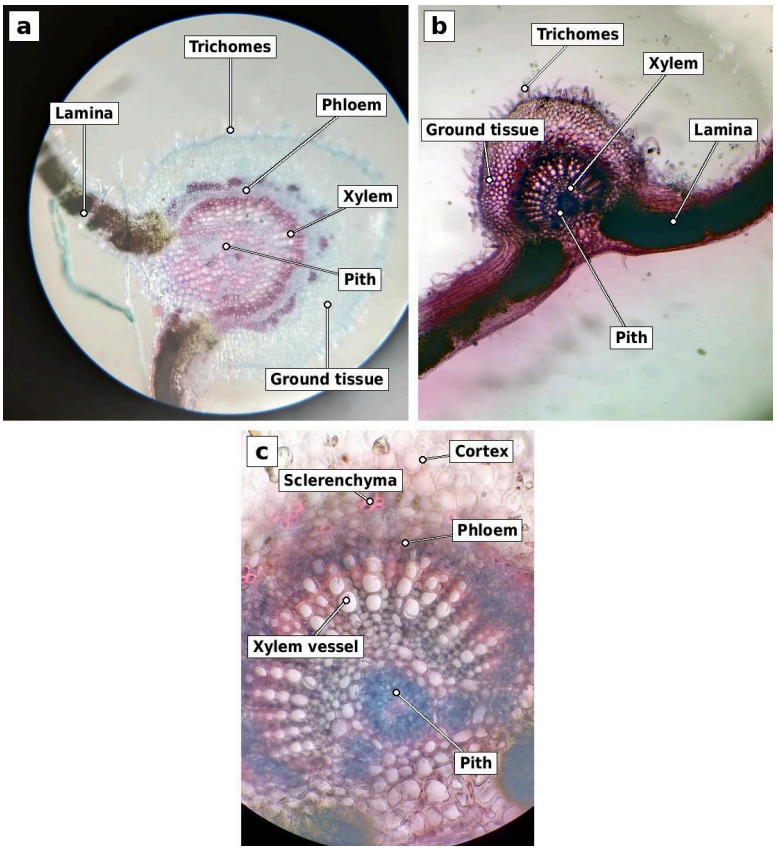


Fig. 1. Leaf anatomy of *V. negundo*. (a) T.S. of leaf through the midrib, low power, showing the bulging midrib, vascular cylinder and lamina. (b) T.S. through the midrib, higher power, showing trichomes along the epidermis, collenchyma and the vascular cylinder. (c) Midrib vascular tissue, enlarged, showing radially arranged xylem vessels.

Petiole. In outline the petiole is plano-convex to slightly grooved on the upper side. A single-layered epidermis with trichomes encloses a few layers of collenchyma and a parenchymatous ground tissue. The main vascular tissue forms a broad arc, with small accessory bundles towards the two upper wings (Fig. 3a).

Young stem. The young stem is quadrangular, a feature typical of Lamiaceae [24]. A single-layered epidermis bears trichomes. Thick patches of collenchyma fill the four corners, followed by a few layers of cortical parenchyma. Groups of pericyclic fibres cap the phloem. Secondary xylem forms a continuous ring of vessels, fibres and uniseriate rays, and a large parenchymatous pith fills the centre (Fig. 2a).

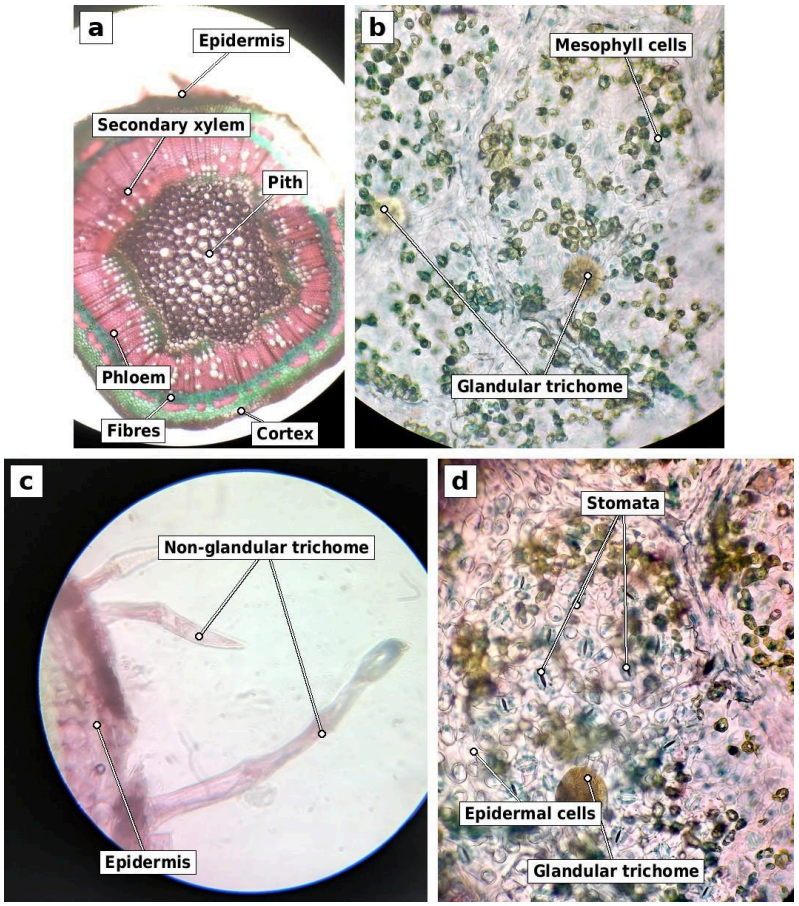


Fig. 2. Stem, trichomes and stomata. (a) T.S. of stem showing cortex, phloem, a broad ring of secondary xylem with rays, and a large central pith. (b) Leaf surface view with glandular (peltate) trichomes among chlorophyll-bearing cells. (c) Uniseriate, multicellular non-glandular trichomes. (d) Epidermal peel of the lower leaf surface showing anomocytic stomata and a peltate glandular trichome.

Root. The mature root shows a brown cork of several layers, a narrow secondary cortex and a cylinder of secondary phloem. Secondary xylem occupies most of the section, with solitary vessels scattered in radial rows and narrow, dark-staining medullary rays. The centre is occupied by primary xylem and no pith is present (Fig. 3b).

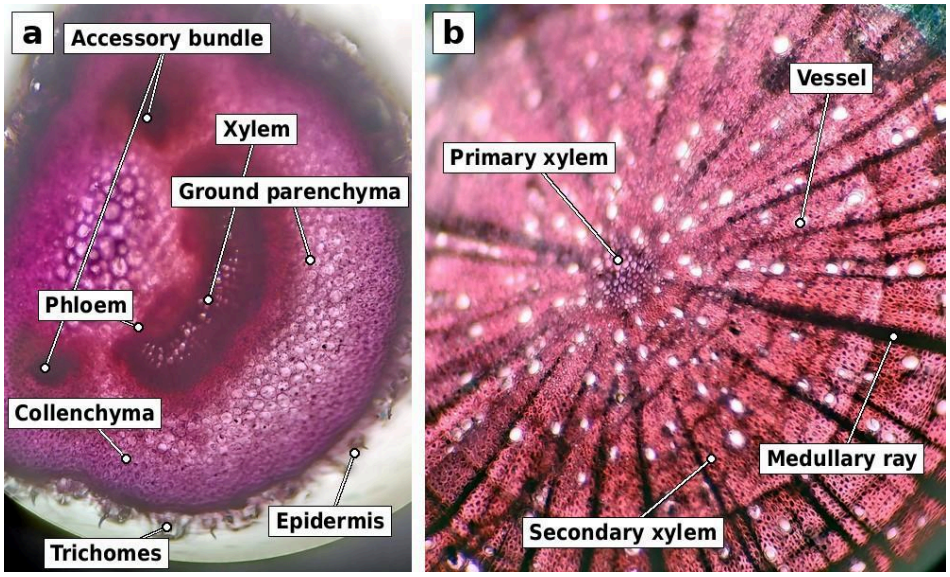


Fig. 3. Petiole and root. (a) T.S. of petiole showing trichomes on the epidermis, collenchyma, ground parenchyma, the arc-shaped vascular bundle with xylem and phloem, and accessory bundles. (b) T.S. of root showing central primary xylem, secondary xylem with vessels in radial rows, and medullary rays.

Table 2 Diagnostic Anatomical Features of *V. negundo*

Organ	Key diagnostic features
Leaf	Dorsiventral; dense non-glandular and glandular trichomes on lower surface; anomocytic stomata, mainly lower surface; nearly circular vascular cylinder in midrib
Petiole	Plano-convex outline; arc-shaped main bundle with accessory bundles
Young stem	Quadrangular outline; collenchyma at the angles; pericyclic fibres; large pith
Root	Cork layers; secondary xylem dominant; narrow rays; no pith

Phytochemical Screening

The results of the phytochemical tests are shown in Fig. 4 and summarised in Table 3.

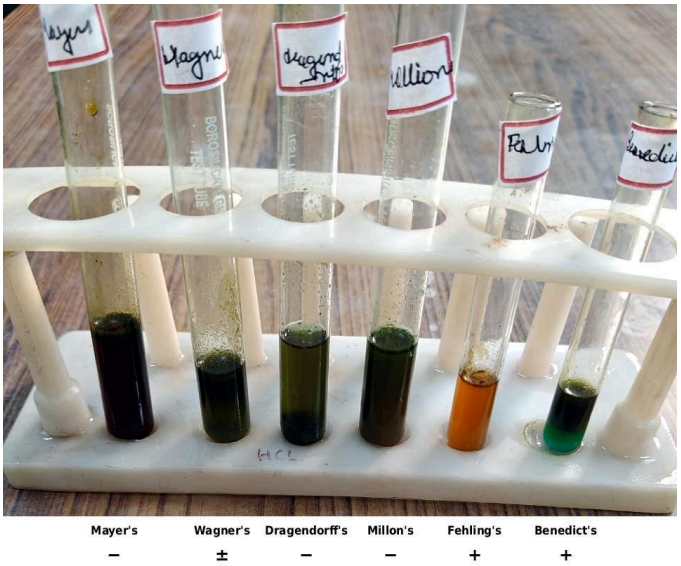


Fig. 4. Phytochemical tests on *V. negundo* leaf extract. Left to right: Mayer's, Wagner's, Dragendorff's, Millon's, Fehling's and Benedict's.

Table 3 Results of Phytochemical Screening of Leaf Extract

Test	Constituent	Observation	Result
Mayer's	Alkaloids	Deep reddish-brown solution; no cream precipitate	–
Wagner's	Alkaloids	Dark olive-green solution with fine particles on the tube wall; no distinct reddish-brown precipitate	± (doubtful)
Dragendorff's	Alkaloids	Olive-green solution with dark sediment; no orange-red precipitate	–
Millon's	Proteins	Dark olive-brown solution; no white or red precipitate	–
Fehling's	Reducing sugars	Blue reagent changed to clear orange-amber	+
Benedict's	Reducing sugars	Green colour with a dark upper layer	+ (low)
		+ present; ± doubtful; − absent	

Discussion

The dense covering of trichomes on the lower surface of the leaf was the most distinctive feature observed in the present study. Hadi et al. [13] also reported segmented, branched non-glandular hairs

together with glandular hairs in *V. negundo*, and noted that the hairs were so dense that the stomata could hardly be seen. The type and density of trichomes have been used to separate *V. negundo* from other species and varieties of *Vitex* [2], which supports their value in the identification of the drug. The presence of anomocytic stomata is in agreement with earlier reports on the genus [14], [24]. The crescent-shaped vascular tissue of the midrib and petiole also agrees with the official monograph of the leaf [16]. The secondary xylem of the stem and root, with vessels in radial rows and narrow rays, shows the usual pattern of woody dicotyledons [25]. The glandular trichomes are probably the site of secretion of the volatile oil responsible for the characteristic smell of the leaves, as peltate trichomes of this species develop large secretory cavities [15], [26].

Positive Fehling's and Benedict's tests indicated the presence of reducing sugars in the leaf extract. These may be present as free sugars or may have been released from glycosides such as agnuside and negundoside, which are known to occur in good amounts in the leaves [3].

Alkaloids were not clearly detected, although their presence in the leaves and stem of *V. negundo* has been reported earlier [17]. This may be because alkaloids are present in low concentration in the leaves, below the sensitivity of the precipitation tests [22]. The dark green colour of chlorophyll in the crude extract also made it difficult to see the cream, reddish brown or orange precipitates. A negative Millon's test was expected, as mature leaves contain very little free protein.

The tests used in the present study are qualitative and only indicate the presence or absence of a group of compounds. The alkaloid tests should be repeated on a defatted and decolourised acidic extract, and the results confirmed by TLC or HPTLC using suitable standards.

VI. Conclusion

The present study showed that *Vitex negundo* can be identified microscopically by its dorsiventral leaf, dense glandular and non-glandular trichomes on the lower surface, anomocytic stomata and a nearly circular vascular cylinder in the midrib, together with an arc-shaped vascular bundle in the petiole. Preliminary screening of the leaf extract showed the presence of reducing sugars, while alkaloids and proteins could not be detected clearly. These observations can serve as a reference for the authentication of Nirgundi leaf.

VII. Future Scope

The alkaloid tests should be repeated on a defatted and decolourised acidic extract, and the results confirmed by TLC or HPTLC with suitable standards [12]. Quantitative estimation of total phenolics, flavonoids and iridoid glycosides such as agnuside and negundoside [3] would add to the chemical profile of the drug. Comparative anatomical study of *V. negundo* and its common substitutes, such as *V. trifolia*, would help in detecting adulteration [2], [14]. Extracts of the Ratlam population may also be evaluated for antioxidant, antimicrobial and anti-inflammatory activity [4], [7].

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