Microscopic evaluation, ethnobotanical and phytochemical profiling of a traditional drug *Viola odorata* L. from Pakistan

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Ethnobotany Research and Applications 25:4 (2023) - http://dx.doi.org/10.32859/era.25.4.1-24
Manuscript received: 22/11/2022 – Revised manuscript received: 15/01/2023 - Published: 15/01/2023

Research

Abstract

Background: The present study encompassed a highly traded medicinal plant *Viola odorata* L. (Violaceae) for detailed Light and Scanning Electron Microscopy, ethnobotany and phytochemical evaluation. Phytochemical evaluation included ascorbic acid, nutritional, and phytochemical profile, and essential and fixed oil study. Even though each feature has its own limited taxonomic value but collectively these characteristics may be systematically important especially for the discrimination and identification of complex and problematic taxa.

Methods: The aim was to study microscopy, histology and phytochemical composition of *Viola odorata* on the basis of ethnobotanical information cited in the literature. Methods: The microscopy, and phytochemical composition of *V. odorata* was studied using standard methods.

Results: Anatomy of the plant parts depicted dicot histology. Stomatal study under LM and SEM, revealed the presence of diacytic and anisocytic type of stomata. Stomata were numerous on the lower epidermis of the leaf. SEM of the powder drug showed the presence of trichomes, calcium-oxalate crystals, pitted vessels, fibers, trichomes, pollen grains, parenchyma cells, pith cells and root hair, but some unknown tissues were also seen. Ascorbic acid, nutritional, and phytochemical profile was investigated according to the standard methods. Different parts of the plant contained various chemical constituents such as alkaloids, mucilage, anthraquninon, saponins, tannins, fats and oil, protein and starch. Quantification of phytochemicals revealed mucilage and tannins to be the highest as compared to saponins and alkaloids. Leaves had 0.00143 % essential oil and 0.396 % fixed oil. Ascorbic acid, nutritional, and phytochemical profile, and oil study revealed vitamin C, proximate and phytochemical composition of *V. odorata*. Conclusion: Overall, this study can be helpful for plant taxonomists to further analyze the species for phytochemical isolation. This will improve the regulatory process and reduce the risk of a quality breach.

Keywords: Ethnobotany, Histology, Morphology, Phytochemical evaluation, SEM, *Viola odorata* L.

Background

There are about 250,000 medicinal plant species with therapeutic value that include a variety of bioactive compounds (tannins, alkaloids, flavonoids, terpenoids, saponins, phenols, and others) in variable concentrations, as well as minerals, vitamins, amino acids, and other nutrients. These plants are inexpensive, effective, and safe, and they have a variety of physiological effects on humans (Olafadehan *et al.* 2020). Herbal markets sell medicinal plants in their raw or powdered form, extracts, and fragments. Differences in variations and nomenclature systems in different topographical regions of the world are the primary grounds for adulteration in medicinal plants that will set the quality control requirements. The lack of standards for medicinal plants, as well as people's untrustworthy transactions, are the most pressing issues confronting the herbal sector as it seeks to develop new treatments. Herbal plant botanical descriptions are particularly valuable for traditional practices as well as academic and pharmaceutical research to find new products (Ahmed *et al.* 2019).

Anatomy and histology can be used to differentiate between taxonomic traits of closely related taxa and to authenticate and validate adulterants in crude herbal medicines. Because these things are meant for use in the herbal market, research institutes, and other enterprises, it can also play a vital role in the quality assurance of natural products (Fatima *et al.* 2018). SEM is now considered to be more advantageous than standard photomicroscopes for a better understanding of plant samples. Furthermore, SEM's high depth of field allows numerous microscopic structures to be in focus at the same time. Due to its great resolution, even minute details that are tightly spaced may be identified. Because electromagnets have replaced traditional lenses, the magnification of any object has grown by a factor of ten (Dastagir *et al.* 2021).

The will power of chemical components in plant material is the subject of phytochemical analysis. The majority of herbal extracts are derived from raw herbs, and the quantity of active ingredients might vary, which determines the therapeutic action of herbs depending on the source. Single active ingredients make some therapeutic herbs popular. Considering the whole situation, just a few therapeutic herbs have been standardized, and market research obviously favors those (Uza & Dastagir 2020).

V. odorata L. is a perennial herb. Leaves are heart shaped, broad with net veined. Flowers zygomorphic, bilaterally symmetrical. Seeds with straight embryo and fleshy endosperm. It flowers from January to June (Orhan *et al.* 2015). The plant is well known for its pharmaceutical significance in Ayurvedic and Unani medicinal system (Shahin 2021). Mehraban (2022) reported that *V. odorata* commendably controls ubiquitous manifestations of COVID-19 including cough, fever, myalgia, diarrhea and headache. Yazdi (2020) studied *its* anti-inflammatory and anti-asthmatic effects.

Keeping in mind its therapeutic potential as cited in the literature, it was aimed to study its micromorphology, and phytochemical analysis for its proper identification and authentication using standard methods.

Material and Methods

Review of Ethnobotanical Uses

The information regarding ethnobotanical uses of the plant were gathered from Google Scholar, Web of Science, Springer Link, Wiley, Baidu Scholar, and Technology Journal Database.

Collection and Preservation

The fresh specimens of *V. odorata* were collected in December 2019 from medicinal garden, Pakistan Forest Institute (PFI), Peshawar. Each specimen was cleaned, washed, separated and was dried in air for 15 days. These specimens were then ground with the help of a pestle and mortar and were preserved in an airtight bottle to combat climatic conditions and moisture.

Organoleptic Evaluation

The organoleptic evaluation of *V. odorata* included root, stem, petiole, leaf, and flower description both in fresh and dry forms following (Trease & Evans 2009) methods (Fig. 1).

Histological Differentiation

The hand-sectioning of fresh root, stem, petiole and leaf, was done with the help of sharp razor. The thin sections were stained with safranin (Puruis 1996) and dehydrated by different grades of alcohols i.e., 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 % and 90 %. After this, the dehydrated sections were put into a drop of light green, and then again dehydrated in absolute alcohol for 2-3 minutes. The sections were finally mounted in Canada balsam to make them permanent (Trease & Evans 2009). (Fig. 2).

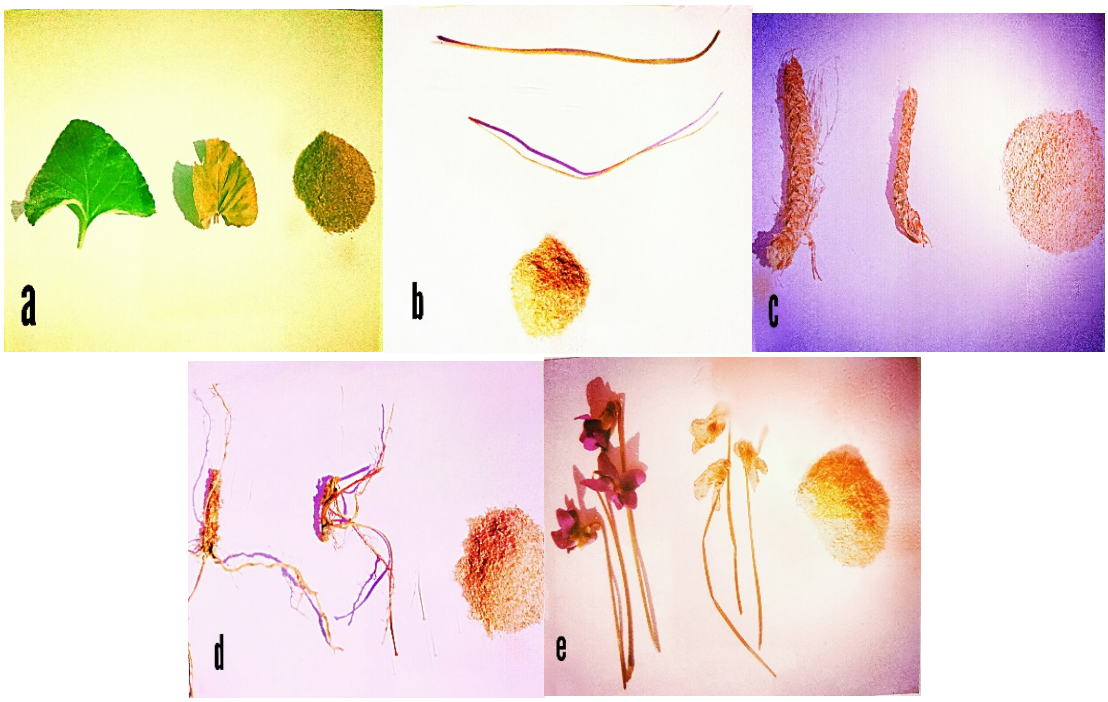

Figure 1. Organoleptic evaluation of *V. odorata* showing: a. Fresh, dry and powder form of leaf; b. Fresh, dry and powder form of petiole; c. Fresh, dry and powder form of stem; d. Fresh, dry and powder form of root; e. Fresh, dry and powder form of flower.

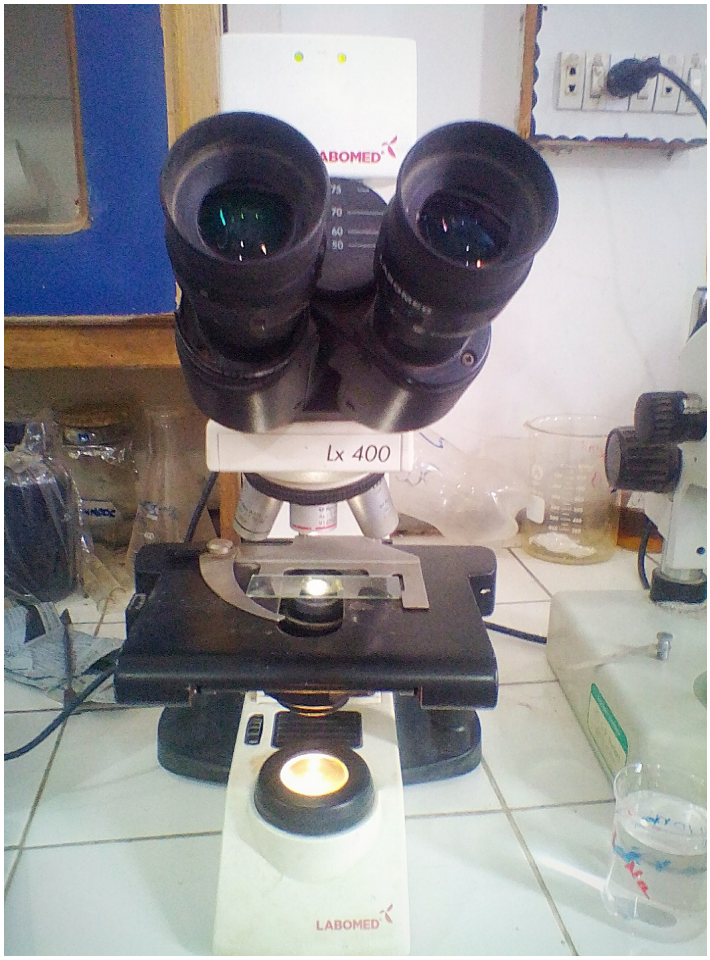

Figure 2 (i). Light microscopic study of *V. odorata.*

Stomatal Study (LM & SEM)

For LM, the fresh leaves were immersed in water to prevent desiccation, water restored turgidity of leaves and facilitated the procurement of epidermis. The abaxial and adaxial surfaces were peeled with the help of a razor and placed on a glass slide, mounted in fluid Canada balsam, and then examined under the microscope (Chaudhary & Imran 1997). The presence and absence of stomata on each epidermis, type of stomata, stomata distribution, position of stomata with respect to epidermal cells, number of stomata, number of epidermal cells, stomatal index, size of stomatal pore, size of guard cells, size of whole stomata, percentage of open and closed stomata were studied using light microscope (Labomed Lx 400) Model [Figure 2(i)]. Micrometery was done with the help of micrometer (Wallis 1985). The SEM of the stomata was done at Centralized Resource Laboratory, Department of Physics, University of Peshawar, using (SEM), Model (JSM-5910), Company (JEOL) made in Japan. Dried leaves were transferred to a metallic stub using double sided cellotape and coated with gold by using (JEE-420) Model, Vacuum Evaporator (JEOL). Finally, the examination was carried out on JEOL microscope (JSM-5910), and images were taken. Micrometry was done using micrometer (Wallis 1985; Uza & Dastagir 2021).

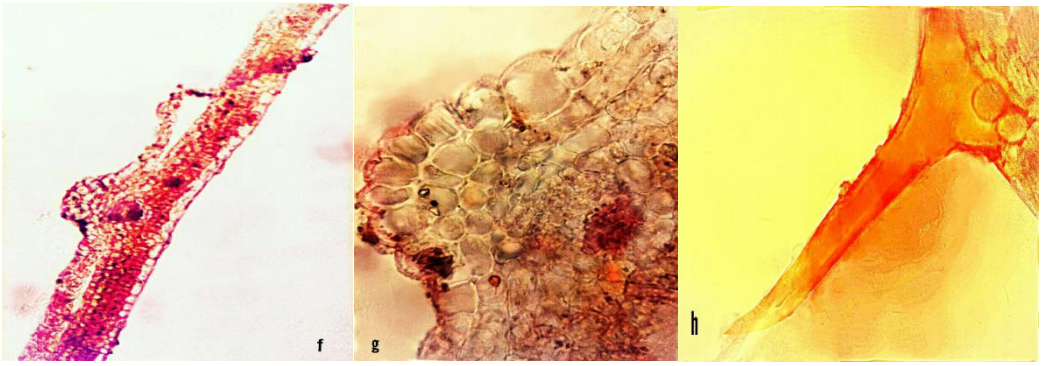

Figure 2 (ii). f: T. S of *V. odorata* leaf; g: Showing vascular bundle and lower epidermis; h: Lamina with an emerging trichome.

Powder Drug Study

The powder drug study included two aspects:

a. Organoleptic Evaluation

The macroscopic studies included color, odor, size and taste of powdered plant parts (Trease & Evans 2009).

b. SEM

The SEM of powder drug of *V. odorata* was done at Centralized Resource Laboratory, Department of Physics, University of Peshawar, using (SEM), Model (JSM-5910), Company (JEOL) made in Japan, following the method of Wallis (1985) and Uza & Dastagir (2021). Micrometry was done with the help of micrometer.

Estimation of Ascorbic Acid (Vitamin-C) & Nutritional Analysis

Estimation of ascorbic acid, and proximate analyses were carried out following A. O. A. C. (1984).

Phytochemical Tests & Oil Extraction

Qualitative and qualitative phytochemical tests were done at Department of Botany, University of Peshawar, Pakistan, using protocol of Trease & Evans (2009). The extraction of essential oil and fixed oil was done at PCSIR Laboratory Peshawar, according to the method of Cocking & Middleton (1935) and Peack & Tracy (1963).

Results and Discussion

Ethnobotanical Uses of *V. odorata*

Ethnobotanical uses of *V. odorata* are given in Table 1.

Organoleptic Evaluation

The organoleptic features of leaf, petiole, stem, root and flower are given in (Table 2 & Figure 1).

Other workers reported similar features of *V. odorata* such as Grieve (1979), Qaiser & Omer (1985) and Kathi (1991). The organoleptic characteristics of a plant can have an impact on the final quality of dried goods. Color, e.g., is a critical factor in determining the intrinsic quality of commercial products. Plant domestication relies heavily on organoleptic features (Khan *et al.* 2020). According to Carrillo-Galván *et al.* (2020), the organoleptic qualities of plants are a key aspect in determining whether or not they are medicinal, and they are also used to describe the alleviation illness.

Table 1. Ethnobotanical profile of *V. odorata*

Part used	**Phytochemical constituents**	**Folk medicinal uses**	**References**
Rhizomes	Glucosides	As an expectorant, in the treatment of cancer, inflammation of eyes.	Rao *et al.* (2015), Paniagua-Zambrana *et al.* (2020)
Roots	Salicylic acid, violin	As an expectorant, for treatment of cough, cold, bronchitis, headaches, migraine and visominia.	Rao *et al.* (2015), Paniagua-Zambrana *et al.* (2020)
Leaves	Salicylic acid, violin, stigmasterol	Leaves are used both internally and externally in the treatment of cancer, to allay the pain in throat and tongue cancer; have antidyslipidemic effect, blood pressure-lowering effect.	Siddiqi *et al.* (2012), Parsley *et al.* (2018), Narayani *et al.* (2017), Aslam *et al.* (2020)
Stem	Salicylic acid, violin	antibacterial, hepatoprotective, antipyretic, anti-inflammatory.	Gautam & Kumar (2012)
Flowers	Salicylic acid, violin, (4.0 %) anthocyanins, (1.1 %) flavonoids, (0.4 %) rutoside, (18.0 %) mucilage, (8.5 %) ash.	Flowers are emollient, demulcent, used in sleeplessness, inflammation of eyes, for lungs and to eliminate the hoarseness of the chest. Syrup (Sharbat-e-banafsha) from the petals is a remedy for infantile disorders and for respiratory problems, constipation and in jaundice. The flowers are edible and used in salad, jelly, candies and for decoration.	Jackson & Bergeron (2005), Mahboubi & Kashani (2018), Jalali *et al.* (2020), Sher *et al.* (2020).
Seeds	---	Seeds are given in urinary complaints.	Jamal *et al.* (2017), Mahboubi & Kashani (2018)
Essential oil	---	It is used as diuretic, expectorant, for heart, in gout, spleen disorder, headache, dizziness and AIDS	Jasim *et al.* (2018)
Whole Plant	alkaloid, glycoside, saponins, methyl salicylate, mucilage, vitamin C, violanthin, flavonoids, glycosides, stigmasterol, violaquercetin, saponins, alkaloids, vitamins, phenols, glucosides, violin, violanthin and violanin, vanillic acid, benzofuranone, glucopyranosides, shikimic acid, cycloviolacin, dimethyldodecane, dimethylheptane, glucopyranoside, violacin and peptide	The whole plant is antibacterial, hepatoprotective, antipyretic, anti-inflammatory, diaphoretic, diuretic, emollient, expectorant, and laxative. It is taken internally for the treatment of mouth and throat infection, jaundice, bronchitis, respiratory catarrh, coughs, asthma, breast cancer, lungs cancer, gastrointestinal, respiratory and vascular disorder.	Ebrahimzadeh *et al.* (2010), Monadi & Rezaie (2013), Mittal *et al.* (2015), Asheesh *et al.* (2017), Fazeenah & Quamri (2020), Janbaz *et al.* (2015).

Table 2a. Organoleptic features of leaf of *V. odorata*

Type	Duration	Insertion	Phyllotaxis	Presence of petiole	Presence of stipule	Venation	Margin	Apex	Surface	Fracture	Texture	Shape	Taste	Color	Odor	Size
Fresh Form																
Simple	Perennial	Radical	Alternate	Petiolate	Stipulate	Reticulate & 5-7 veins	Serrate	Obtuse	Hairy	Short	Herbaceous	Cordate	Acrid	Upper surface dark green & lower surface light green	Aromatic	Ave. Length = 5.5.cm Ave. Width= 5.7 cm
Dry Form																
Simple	Perennial	Radical	Alternate	Petiolate	Stipulate	Reticulate & 5-7 veins	Serrate	Obtuse	Hairy	Short & uneven	Herbaceous	Cordate but curved at margin	Better	Upper surface dull green & lower surface whitish green	Indistinct	Ave. length =5.3 cm Ave. Width= 5.4 cm

Table 2b. Organoleptic features of petiole of *V. odorata*

Color	Size	Texture	Fracture	Shape	Odor	Taste
Fresh Form						
Dull green	Ave. Length= 18.0cm & Ave. width =0.2cm	Herbaceous	Fibrous	Cylindrical	Indistinct	Bitter
Dry Form						
Whitish green	Ave. Length= 17.8cm & Ave. Width= 0.1cm	Herbaceous	Brittle, tough & uneven	Cylindrical & shrunken	Indistinct	Tasteless

Table 2c. Organoleptic features of stem of *V. odorata*

Color		**Size**		**Texture**		**Fracture**		**Shape**		**Odor**		**Taste**	
Fresh	**Dry**	**Fresh**	**Dry**	**Fresh**	**Dry**	**Fresh**	**Dry**	**Fresh**	**Dry**	**Fresh**	**Dry**	**Fresh**	**Dry**
Greenish brown	Same but slightly dull	Ave. length=2.5 cm & Ave. width=0.5 cm	Ave. length=2.3 cm & Ave. width=0.3 cm	Herbaceous	Same	Fibrous & flexible	Brittle, short & uneven	Hollow	Irregular	Distinct	Indistinct	Mucilaginous	Tasteless

Table 2d. Organoleptic features of root of *V. odorata*

Color		**Size**		**Texture**		**Fracture**		**Shape**		**Odor**		**Taste**	
Fresh	**Dry**	**Fresh**	**Dry**	**Fresh**	**Dry**	**Fresh**	**Dry**	**Fresh**	**Dry**	**Fresh**	**Dry**	**Fresh**	**Dry**
Yellowish brown	Dull brown	Ave. length=16 cm & Ave. width=0.4 cm	Ave. length=15.6 cm & Ave. width=0.3 cm	Herbaceous	Same	Short, complete & uneven	Weak & uneven	Cylindrical	Irregular	Distinct	Indistinct	Slightly bitter	Same

Table 2e. Organoleptic features of flower of *V. odorata*

Color		**Texture**		**Fracture**		**Odor**		**Taste**	
Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Purplish blue	Brown	Weak	Weak	Uneven	Uneven	Distinct	Indistinct	Sweet	Sweet

Histological Differentiation

Leaf

Cuticle: The epidermis was completely covered by a thin cuticle with (373.6 µm) thickness. **Epidermis:** A single layered epidermis was found on both surfaces of the leaf. **Upper epidermis** had rectangular and somewhat oval shaped cells while, the **lower epidermis** had papillose cells. The length of cells varied from (810.3-1071.2 µm) and width from (689.2-781.9 µm). **Stomata:** They were present mainly on the lower surface of the leaf. There were diacytic and anisocytic type of stomata. They were elongated and the guard cells were parallel to the subsidiary cells and some of the subsidiary cells were similar to the other epidermal cells. The average length of the whole stoma was (34.5 µm) and width was (23 µm). **Hypodermis:** It was composed of two layers of closely packed polygonal cells with an average length of (829-1138.6 µm), and width of (802.5-943.3 µm). **Parenchyma cells:** Below the hypodermis, parenchyma cells constitute a large portion of the midrib up to the phloem cells. Size of the cells increased successively towards the vascular bundle. The average length was (870.1-1015.2 µm) and width was (890.5-1051.3 µm). **Midrib:** It was prominent on the lower surface of the leaf and was demoted form parenchymatous cells by two layered endodermis. **Mesophyll tissue:** It was clearly differentiated into palisade and spongy parenchyma cells. **(a) Palisade parenchyma tissue:** It was single layered, and had closely packed, elongated cells. The length was (2713-2998 µm), and width was (2500-2935 µm). It had numerous plastids. **(b) Spongy parenchyma tissue:** Its cells were oval in shape and were present in vertical rows with large intercellular spaces. The diameter of each cell was (924.4-1156.3 µm). The length was (2700-2890 µm), and width was (2000-2150 µm). **Vascular bundle:** There was a crescent shaped closed vascular bundle. The phloem lied below the xylem. The xylem cells were (213.2-312.6 µm) in diameter while, phloem cells were (205-415 µm). The phloem cells were (213.2-289.1 µm) in length and (208.1-265.2 µm) in width. Similarly, xylem cells were (305-415 µm) in length and (178.1-256 µm) in width (Figure 2 ii).

Petiole

The transverse section of petiole was like midrib portion of leaf with wings at the two ends. **Cuticle:** A thick (274.56 µm) cuticle was present, all around the epidermal cells. **Epidermis:** It was two layered, made up of oval to elongated shaped cells. The length varied from (1082.4-1346.4 µm) and width from (937.2-1069.2 µm). **Hypodermis:** The length of hypodermal cells was (1069.2-1214.4 µm) and width was (937.2-1148.4 µm). **Cortex:** It had polygonal shaped cells without intercellular spaces. Its length varied from (2376-3300 µm) and width from (2310-3960 µm). **Vascular bundles:** There was a single median, crescent shaped vascular strand, with very much incurved ends, consisted of closed cylinder of phloem surrounded by closed cylinder of xylem cells. The length of phloem was (710-950 µm) and width was (680.3-806.4 µm) while that of xylem was (703-800 µm) and width was (516-723 µm). The diameter of xylem was from (680.2-1060 µm) and of phloem was (750-802 µm). **Pith:** The pentagonal pith cells had no intercellular spaces and were surrounded by extraxylary fibers. The cells had a diameter of (561-673.2 µm) (Figure 3).

Stem

Cuticle: Cuticle was 238 µm in thickness. **Epidermis:** It was single layered. The cells were rectangular in shape and were closely arranged. The length of the cells was (1632-1820.4 µm) whilewidth was (1361.1-1530.2 µm). **Cork cells:** Next to epidermis is broad zone of cork cells made up of 5-6 layers of cells. Cells were of barrel shaped, with length of (2361.3-2620.5 µm) and width of (2180.4- 2580 µm). **Cork cambium:** It was composed of narrow zone of elongated cells arranged in two layers. Cells were (1104.3-1410.1 µm) in length and (778-941.2 µm) in width. **Secondary cortex:** It consisted of oval and more or less spherical shaped cells, loaded with calcium oxalate crystals. The cells had average diameter of (298-410.4 µm). **Phloem:** There were narrow phloem patches separated by medullary rays most of which were one celled in width, but may be two celled, at the peripheral region. The length of the cells was (380.2-510.6 µm) and width was (318.2-385.6 µm). The cells were rectangular in shape. **Xylem:** It was composed of small spherical cells having length of (205-317 µm) and width of (150-190 µm). Xylem vessels were (801.2-992 µm) in diameter. Phloem and xylem were separated by narrow strip of cambium. **Pith:** It had oval shaped cells containing Ca-oxalate crystals. The diameter of each cell was (785-910.2 µm) (Figure 4).

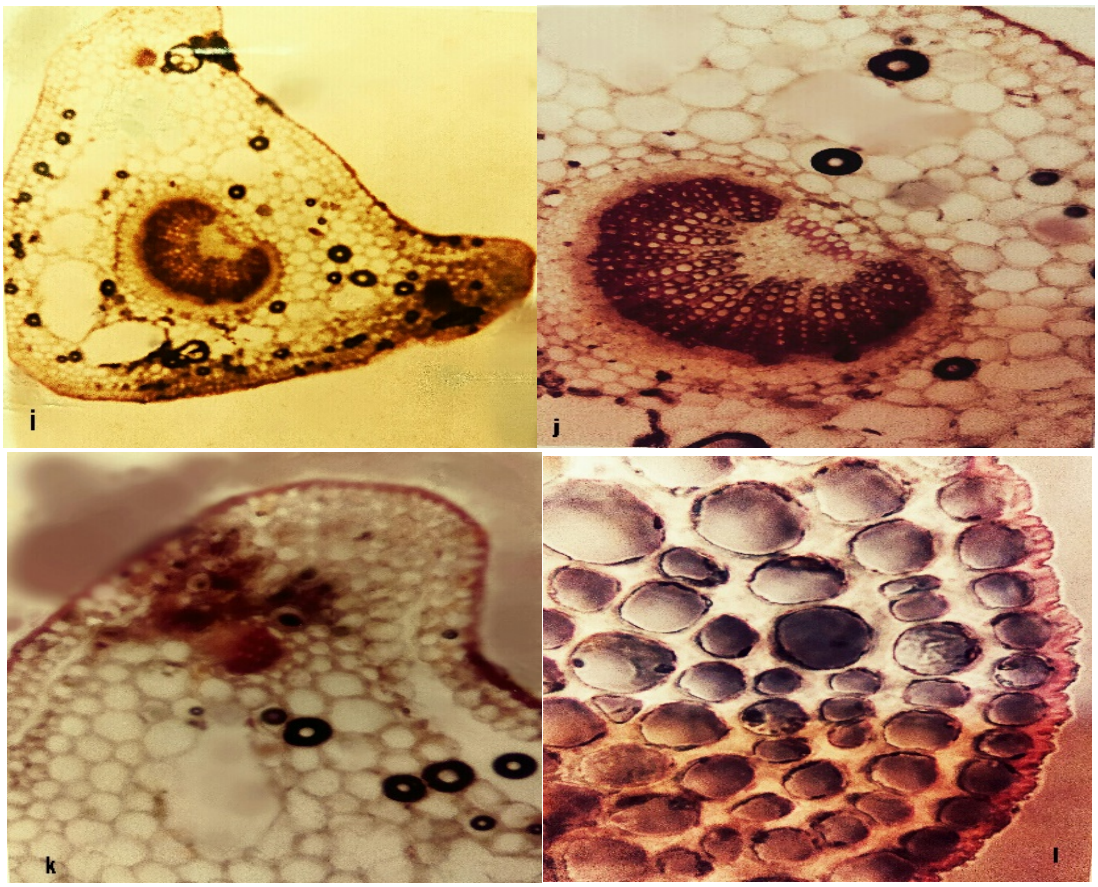

Figure 3. T. S of petiole showing: I-j: Vascular bundle; k: Wing of petiole, l: Cortex.

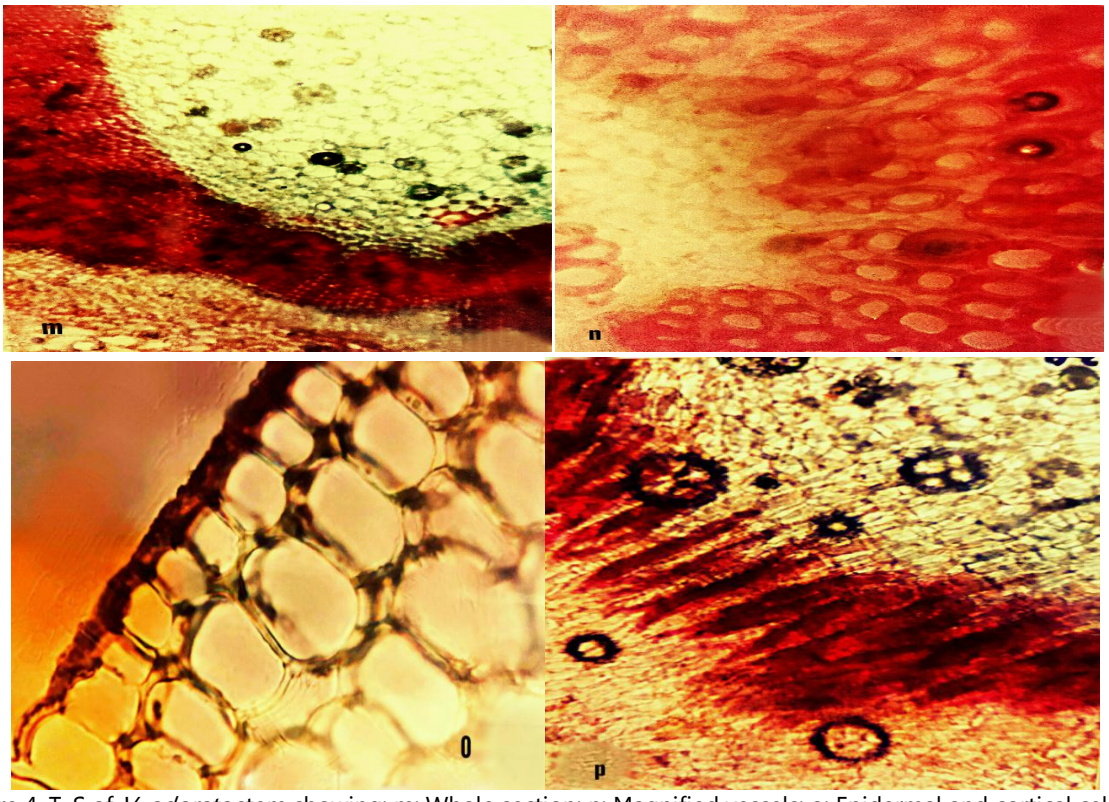

Figure 4. T. S of *V. odorata* stem showing: m: Whole section; n: Magnified vessels; o: Epidermal and cortical cells; p: Pith cells and vascular bundles.

The T. S. of root of *V. odorata* showed the following tissues.

Cuticle: The cuticle having 240.2 µm thickness was covering the entire surface. **Epiblema:** It was composed of small, rectangular cells. The length of the cell was (810-998.8 µm) and width was (718.3-810µm). Root hairs were present. **Cortex:** Cortex was composed of 10-12 layers of cells. The cells resembled to epidermal cells in shape more or less rectangular in shape. The length of cortical cells was (2210.3-2685 µm) while the width was (2180.1-2410.2 µm) (Figure 5p). **Ground tissue:** It was a broad zone of parenchymatous cells. In some of the cells, rosette aggregates of calcium oxalate were present. Numerous bundles were arranged in more or less distinct concentric rings. The xylem was (808.3-958.5 µm) in diameter while, phloem was (600-750 µm). The xylem was (415-620 µm) in length and (215-390 µm) in width while, phloem was (302.4-396.6 µm) in length and (308.0-405.2 µm) in width (Figure 5q). **Pith:** These cells were oval or oblong in shape and were about (382-518.2 µm) in diameter (Figure 5). Anwar *et al.* (1976) reported the anatomy of *Viola serpens* root. It showed the presence of cuticle followed by epidermis. Cortex had 4-7 layers of parenchymatous cells. Jurca *et al.* (2019) worked on histology of *Viola* species and reported similar results. Anatomy makes the first step to get knowledge about the diagnostic features, which are ascertained through the study of the tissue and their arrangement, cell wall and cell contents (Asheesh *et al.* 2017). It is one of the most basic and inexpensive approaches for determining the correct identity of source materials (Mownika *et al.* 2020). The anatomical features reveal potential taxonomic, ecological, and evolutionary consequences, and can be utilized as an additional tool for sub generic and species-level identification and categorization of herbal drugs (Uza & Dastagir 2021).

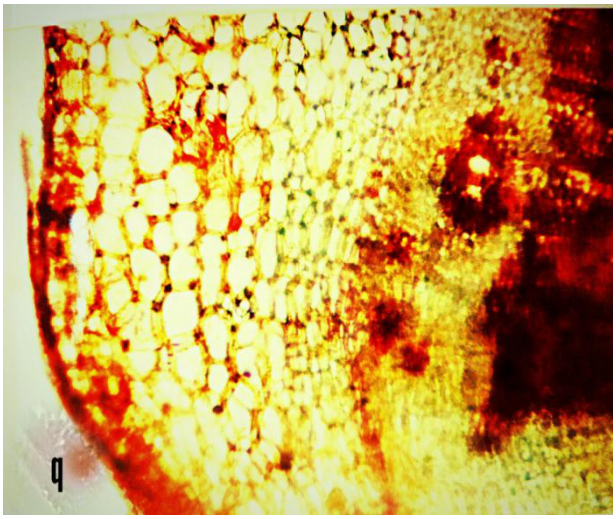

Figure 5. T. S of *V. odorata* root showing: Vascular bundles, medullary rays, cortex and epiblema.

Stomatal Study (LM & SEM)

Stomatal Study Under LM

The mean measurements taken for stomatal study under LM are given in the Table 3.

Upper epidermis:

The number of stomata on the upper epidermis of the leaf was 9.36 per mm^2. The stomatal index was 14.4. The average length and width of the stomatal pore and guard cells was 18.4 µm, 4.6 µm, 13.2 µm and 6.9 µm, respectively. The length of the whole stomata was 34.5 µm and width was 23.0 µm. The average of open stomata was reported to be 1 and of close it was 0.17. Open and closed stomata were 85.7 % and 14.3 %, respectively [Figure 6(i)r]. In the most dicotyledonous plant, the extensive stomata distribution observed at the lower foliar epidermal surface has been documented as a natural phenomenon (Zhigila *et al.* 2015). It is also suggested to be a way for angiosperms to adjust to changing environmental conditions and avoid losing too much water (Anifat *et al.* 2017).

Lower epidermis

The number of stomata and epidermal cells on the lower surface of leaf was 124 per mm^2 and 673.6 per mm^2. Stomatal index was 15.54. The average length and width of the stomatal pore was 20.7 µm and 5.75 µm, respectively. The average length and width of guard cells was 36.8 µm and 5.75 µm. The length of whole stomata was 23.0 µm while, width was 10 µm. The average of open and closed stomata was 12.8 and 2.7, respectively. Similarly, open and closed stomata percentage was 82.8 % and 17.2 % [Figure 6(i)s-t].

Table 3. Mean values of the stomatal study of *V. odorata* under LM

Leaf portion	**Type of stomata**	**Stomata W.R.T epidermal cells**	**Stomatal Distribution**	**No of half stomata**	**No of epidermal cells per mm² (mean) field area =0.125mm²**	**No of stomata per mm² (mean) field area = 0.125mm²**	**Epidermis**	**Stomatal index (mean)**	**Size (µ) Mean**						Open and close stomata		% of open and close stomata	
									Stomatal pore		**Guard cells**		**Whole stomata**					
									L	W	L	W	L	W	Mean open	Mean close	% open	% close
Upper	Diacytic	ASL	Scattered	Absent	Mean = $\frac{480}{6}$ = 80.0 $1mm^2 = \frac{80.0}{0.125}$ = 640	Mean = $\frac{7}{6}$ = 1.17 $1mm^2 = \frac{1.1}{0.12}$ = 9.36	Upper	14.4	18.4	4.6	32.2	6.9	34.5	23.0	1.0	0.17	85.7	14.3
Lower	Anisocytic & Diacytic	ASL	Scattered	Absent	Mean = $\frac{505}{6}$ = 84.2 $1mm^2 = \frac{84.2}{0.125}$ = 673.6	Mean = $\frac{93}{6}$ = 15.5 $1mm^2 = \frac{15}{0.1}$ = 124	Lower	15.54	20.7	5.75	36.8	5.75	23.0	10.0	12.8	2.7	82.8	17.2

Key: ASL = at the same level; W.R.T = with respect to; L= length, W= width

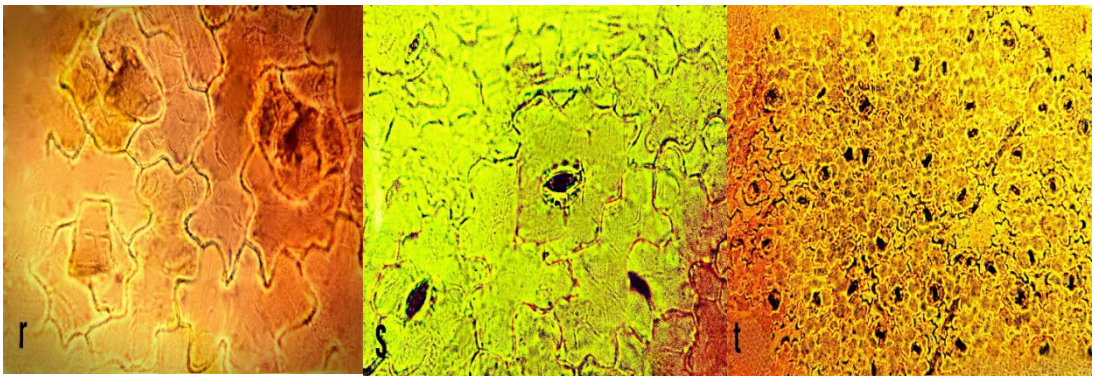

Figure 6(i). Stomatal study under LM showing: r: Upper surface of leaf; s-t: Lower surface of leaf.

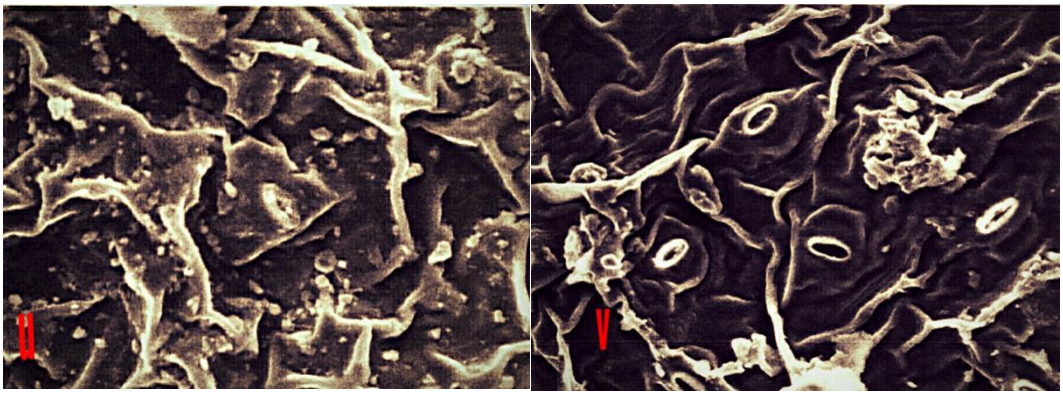

Figure 6(ii). Stomatal study under SEM showing: u: Stomata in upper epidermis; v: Stomata in lower epidermis.

Stomatal Study Under SEM

SEM gave high depth of resolution of both upper and lower epidermis of *V. odorata* leaves. It depicted clearer epidermii with epidermal cells, diacytic and anisocytic stomata and guard cells. The cells of the epidermii were with anticlinal walls. Diacytic and anisocytic type of stomata were scattered in great number on the lower epidermis. Most of the stomata were open, and few were closed. Upper epidermis contained only diacytic type of stomata, but these were few in number (Figure 6-ii). The stomatal features that were observed under LM and SEM were generally consistent with the earlier findings (Siddiqui *et al.* 1991; Ali *et al.* 2020; Uza & Dastagir 2021). The stomatal study helps in the correct identification of herbal drugs. During the past four decades, plant anatomists have emphasized the importance of epidermal studies and stomata in particular in solving some taxonomic riddles and phylogenetic problems (Khan *et al.* 2020). The size and shape of stomata, the guard cells, the subsidiary cells, and the types of trichomes are useful analytical features of foliar epidermal properties. Genetic variations or the diversity of their natural environments could explain the disparities in epidermal features between species (Fatima *et al.* 2018; Vaz *et al.* 2019).

Powder Drug Study (Macroscopy & SEM)

Macroscopic Study of Powder Drug

Leaf: The powder drug of leaf was green in color with aromatic odor and acrid taste (Figure 1a).
Petiole: The powder drug was dull green in color with indistinct odor and bitter taste (Figure 1b).
Stem : The powder drug was yellowish brown in color. Taste was mucilaginous and odor was indistinct (Figure 1c).
Root: The powder drug of root was yellowish brown in color having distinct odor and slightly bitter taste (Figure 1d).
Flower: The powder drug was yellowish brown in color having sweet fragrance and sweet taste (Figure 1e).

SEM

Leaf

SEM showed the fragments of epidermis with anisocytic stomata having (28 µm length & 10 µm width) and diacytic stomata with (28.2 µm length & 15 µm width), palisade parenchyma with (36.2 µm length & 12.5 µm width), unicellular and multicellular trichomes. The length of trichome was (29.4 µm) and width was (22.7 µm). An unknown tissue was also reported (Figure 7). The stomata and trichomes are structural features that are well preserved and

can be employed in taxonomic and phylogenetic approaches (Silva *et al.* 2018). Trichomes have the ability to synthesize, store, and secrete compounds. The metabolites including alkaloids, terpenoids, proteins, organic acids, and polyphenols are important in the pharmaceutical industry (Hussain *et al.* 2019).

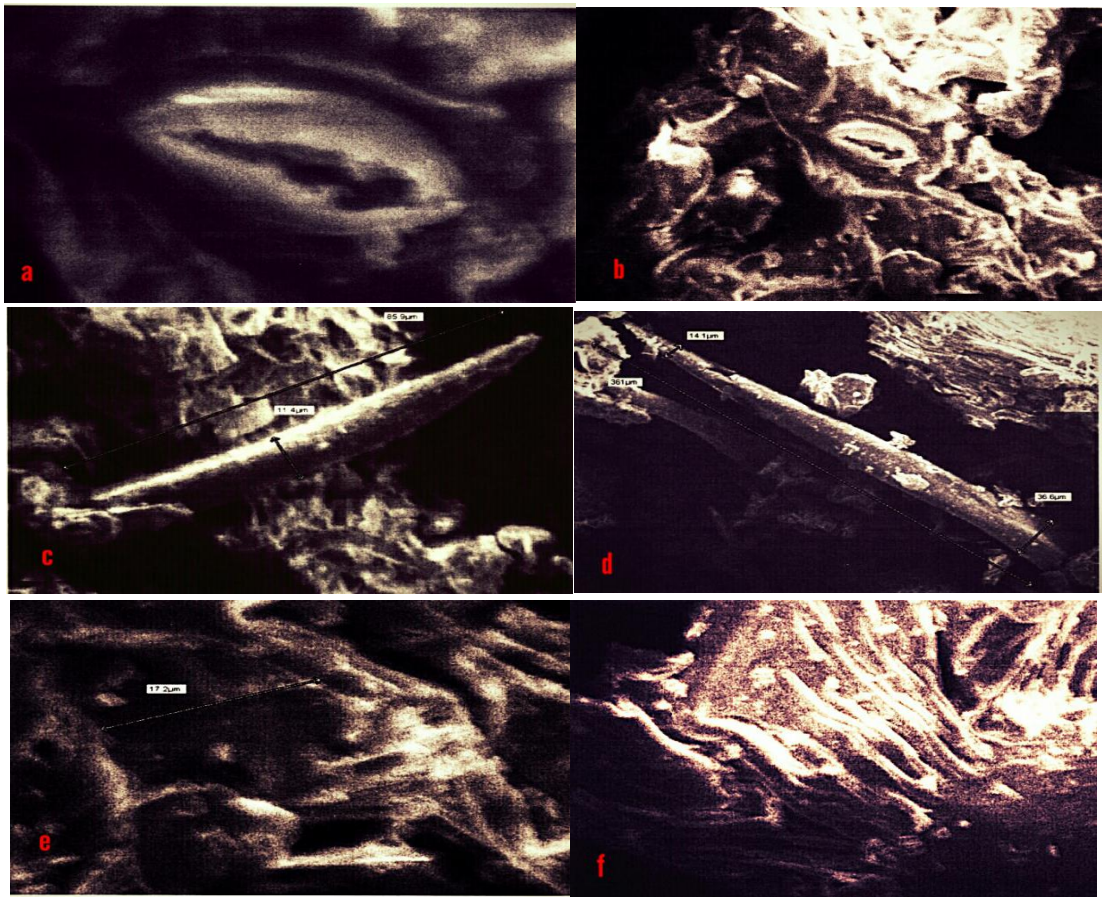

Figure 7(i). Powder drug SEM of *V. odorata* leaf showing: a: Magnified diacytic stoma; b: Anisocytic stomata; c-d: Trichome; e: Unknown tissue; f: Palisade parenchyma cells.

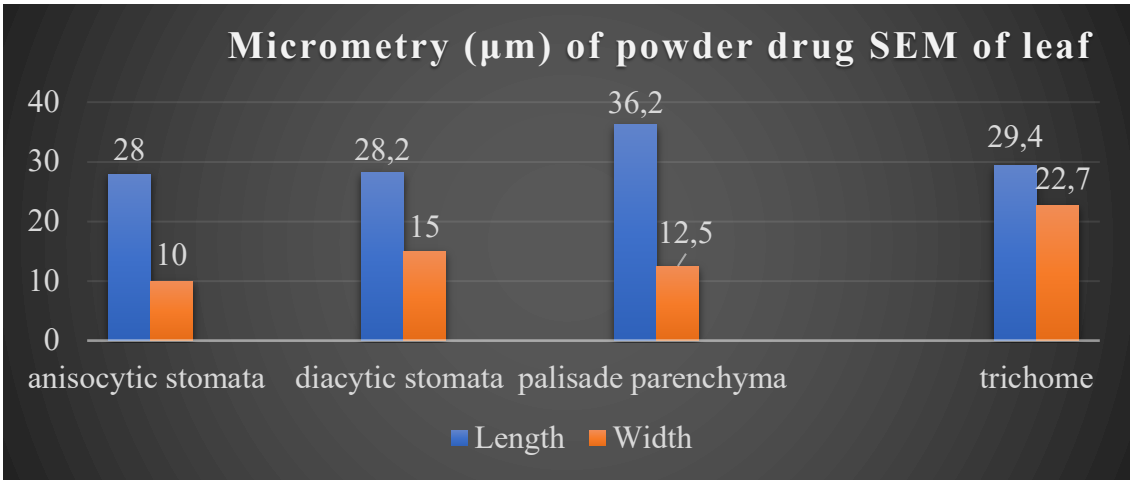

Figure 7(ii). Micrometry (µm) of powder drug SEM of leaf of *V. odorata*

Petiole

SEM study revealed the presence of fiber cells (18.7 µm length & 5.8 µm width), pitted vessels (34 µm length & 10 µm width), and conical trichomes (28 µm length & 21 µm width) (Figure 8). At the generic level, the presence or lack of trichomes is utilized to distinguish plants. Trichomes have long piqued the curiosity of scientists due to their unique properties (Xiao *et al.* 2017).

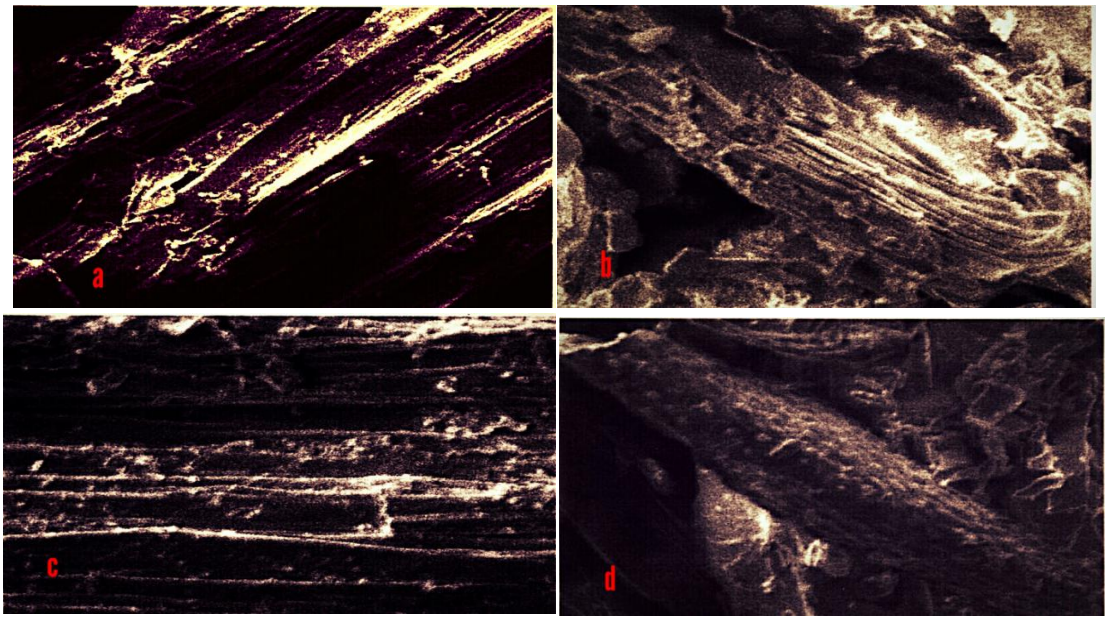

Figure 8(i). Powder drug SEM of *V. odorata* petiole showing: a-b: Fibers; c: Vessels; d: Trichome.

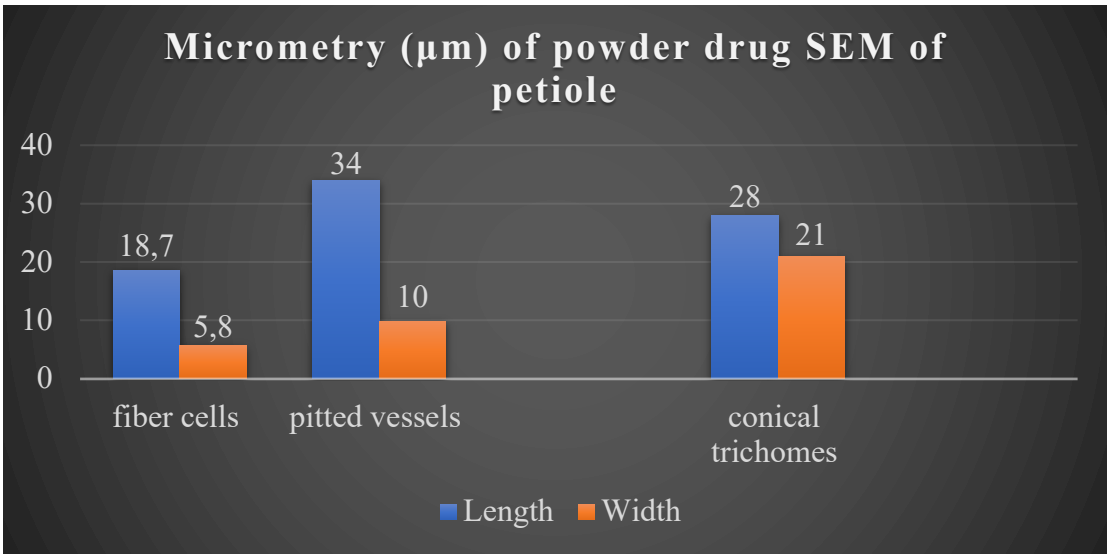

Figure 8(ii). Micrometry (µm) of powder drug SEM of petiole of *V. odorata*

Stem

The powder drug of stem showed pitted vessels (31 µm length & 26 µm width), fibers (20 µm length & 16 µm width), annular thickening (27 µm length & 12 µm width), fragments of vascular tissue and an unknown tissue (25 µm length & 10 µm width) (Figure 9).

Root

SEM of the macerated specimens of root showed the presence of epidermal cells with root hair (15 µm length & 5 µm width), pitted vessels (32.4 µm length & 16 µm width), cortical parenchyma cells (37 µm length & 19 µm width) and pith cells (31 µm length & 12 µm width) (Figure 10).

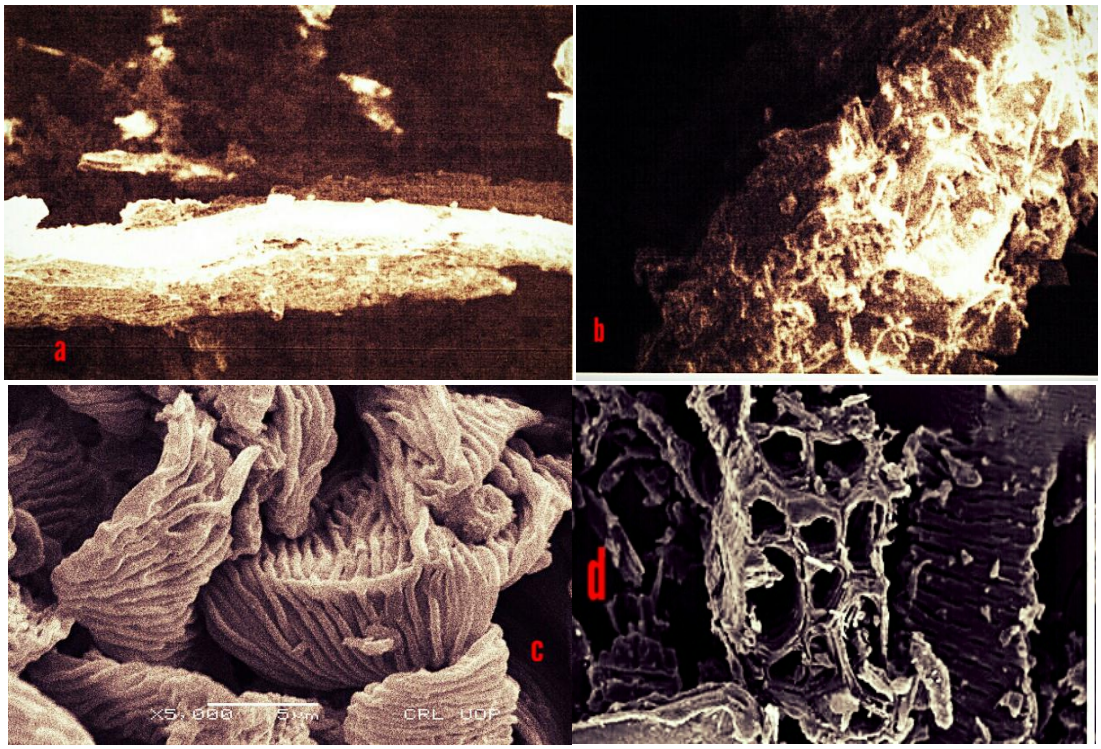

Figure 9(i). Powder drug SEM of *V. odorata* stem showing: a: Fibers & annular thickenings; b: Pitted vessels; c: Unknown tissues; d: Fragments of vascular tissue.

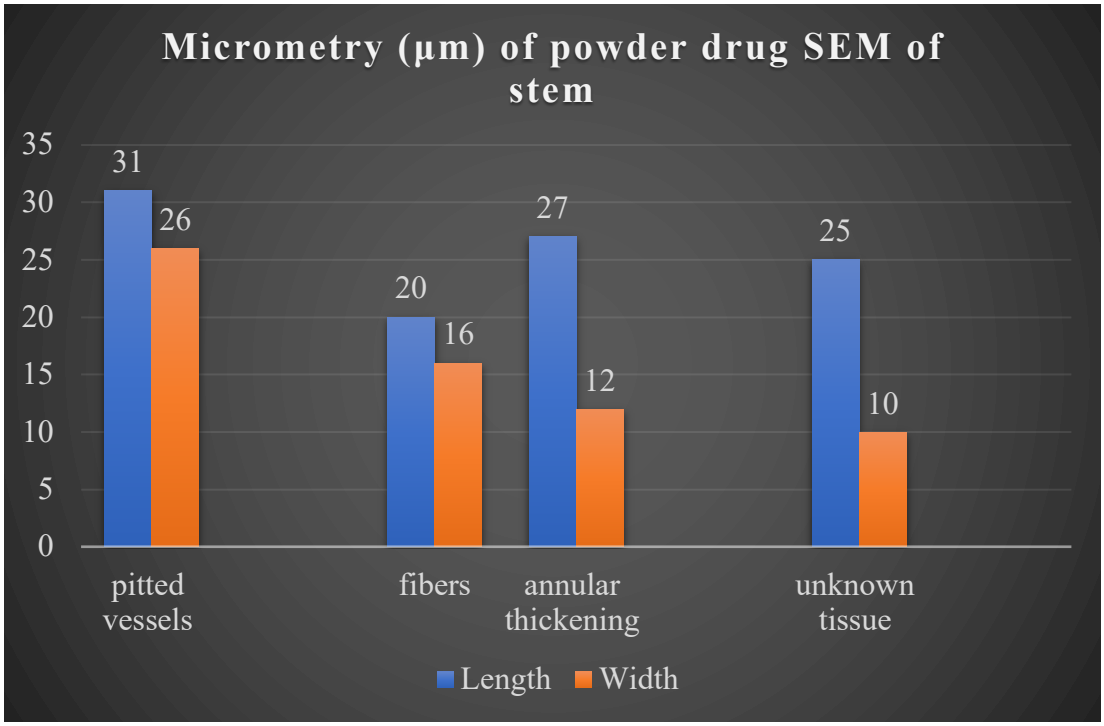

Figure 9(ii). Micrometry (µm) of powder drug SEM of stem of *V. odorata*

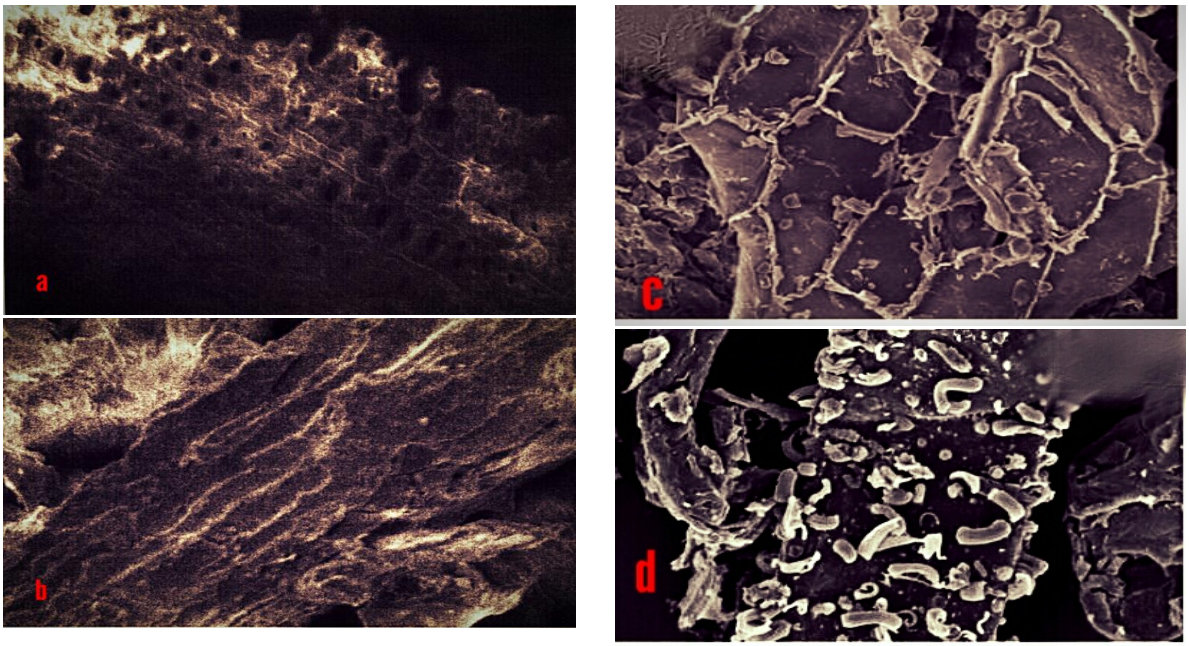

Figure 10(i). Powder drug SEM of *V. odorata* root showing: a: Pitted vessels; b: Cortical parenchyma cells; c: Pith cells; d: Root hair.

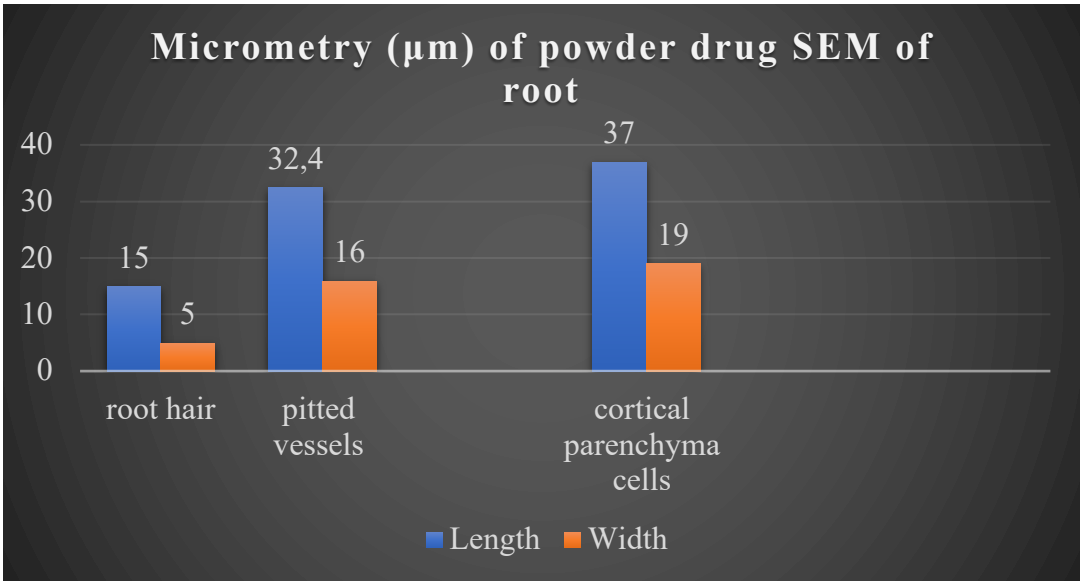

Figure 10(ii). Micrometry (µm) of powder drug SEM of root of *V. odorata*

Flower

SEM exhibited parenchyma cells with (6 µm length & 3.7 µm width), an unknown tissue with (10 µm length & 7 µm width), pollen grain with (12 µm length & 9.6 µm width), annular and spiral thickening with (2.5 µm length & 1 µm width) (Figure 11). Palynology has grown in popularity in the scientific community. Pollen grains are now intensively studied for taxonomic identification of blooming plants. Pollen morphology has been recorded by taxonomists and botanists in order to define flowering plants at the species and variety level (Huchelmann *et al.* 2017). Gavrilova & Nikitin (2012); Nansai (2020); Ahmed (2021) worked on SEM of various medicinal plants and their results are in line with the present findings. A SEM image can be compared with a sample viewed with a hand lens or a dissecting light microscope. But with its great depth of field and it's increased resolution, the SEM provides the user with a magnified image of specimens showing detail not visible with a light microscope (Dastagir *et al.* 2021). Microscopy of the powder drug has the advantage of requiring only a small quantity of material. This study provides useful references not only for forensic scientists and other engaged in the evaluation of powder drug but also to the analysts who one concerned with standardization and authentication of prepared drug material (Hameed *et al.* 2020).

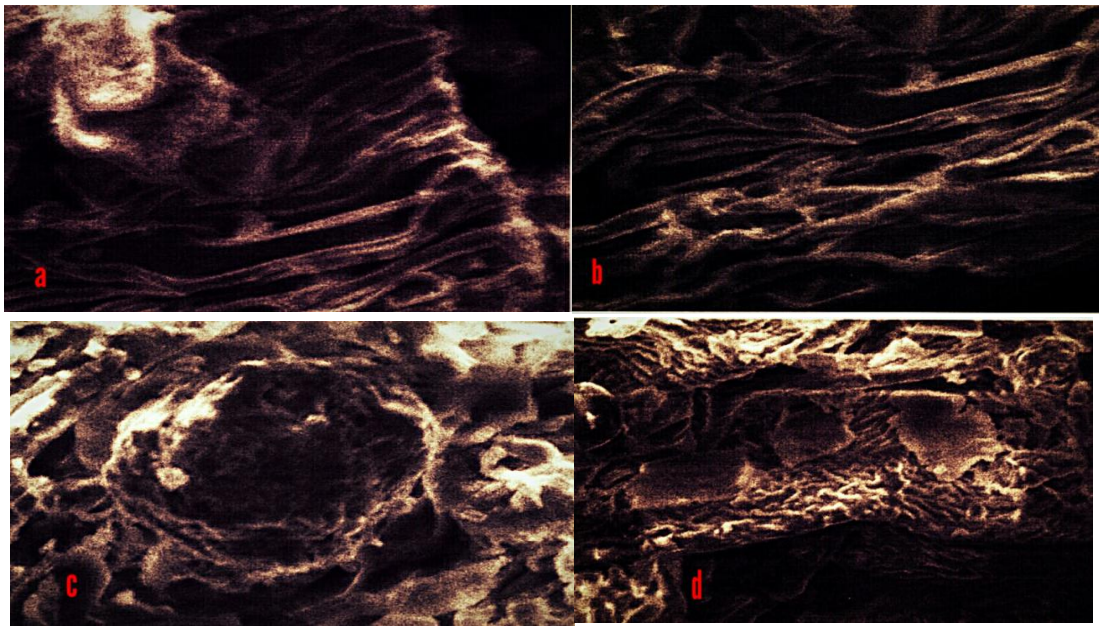

Figure 11(i). Powder drug SEM of *V. odorata* flower showing: a: Parenchyma tissue; b: An unknown tissue; c: Pollen grain; d: annular and spiral thickening.

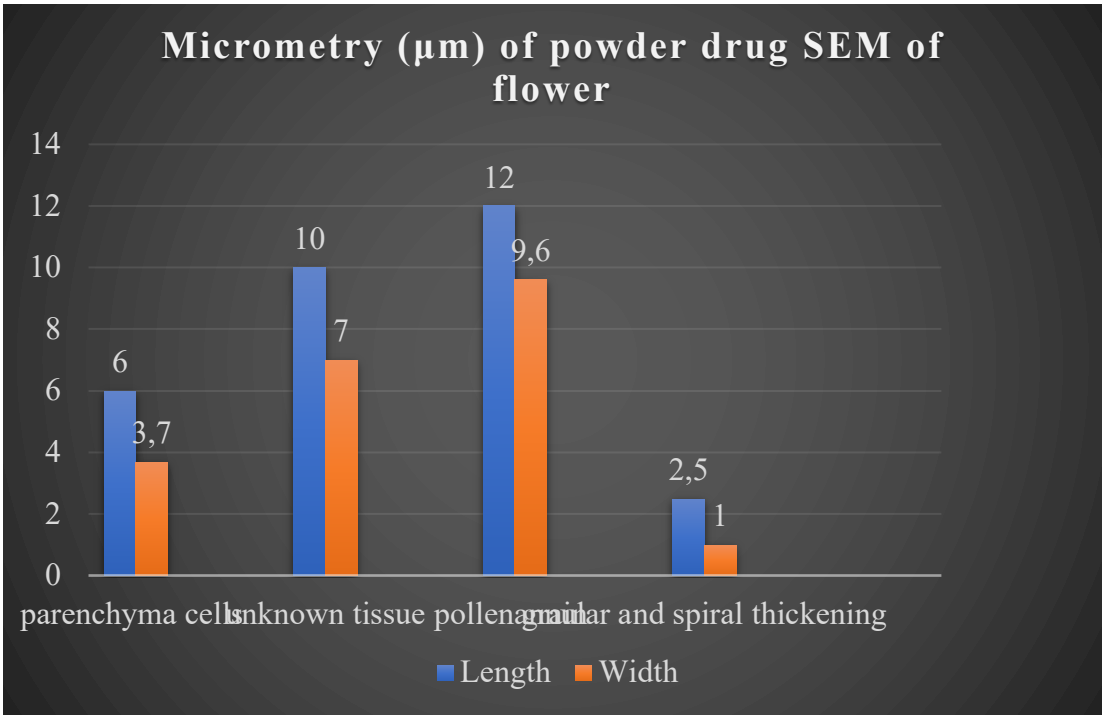

Figure 11(ii). Micrometry (µm) of powder drug S

Ascorbic Acid (Vitamin C)

Determination of ascorbic acid (vitamin C) in different parts of *V. odorata* was done. Largest quantity of ascorbic acid (20.83 mg) was found in 4ml solution of leaves, followed by 9.26 mg in 5ml solution of root and stem. 5ml solution of petiole had lowest content of ascorbic acid (7.4 mg) (Table 4). Diep *et al.* (2020), Abeysuriya *et al.* (2020) and Ahmed *et al.* (2020) quantified ascorbic acid from various plants and stressed that among the several forms of vitamin C, ascorbic acid (AA) is the most physiologically active, and it is reversibly oxidized into DHA, which is also involved in biological functions that help to sustain human health. Vitamins C is necessary for both immune defense (antioxidant) and calcium absorption. An adult's Vitamin C need is around 40-50 mg per day (NHWC 2002). Ascorbic acid protects proteins from oxidized lipid damage in the cell. Lipid peroxidation helps to prevent the onset and progression of chronic inflammatory and age-related illnesses such as atherosclerosis. It may aid to keep

metabolizing and transporter proteins in good working order, reducing the harmful effects of oxidized lipids (Mohamed *et al.* 2020). Mega-doses of ascorbic acid are used to treat the common cold, wound healing, and trauma. Its levels can have significant nutritional consequences due to a variety of inhibitory and boosting interactions with minerals and nutrients (Goel & Agarwal 2020). It is involved in cell division and osmotic regulation and also has strong antioxidant properties and aids in the prevention and scavenging of reactive oxygen species (Savych & Basaraba 2021). So, it is recommended that *V. odorata* is a rich source of Vitamin C, that indicates its potential nutritional and therapeutic value.

Nutritional Analysis

The different parts of *V. odorata* i.e., root, stem, leaf and petiole, on dry matter basis, were analyzed for, ash content, crude protein, crude fiber, fat, moisture contents, and carbohydrate and the results were given in (Table 5). Carbohydrates contents were highest among the nutrients. Petiole contained maximum contents (62.83 %) followed by stem (60.32 %), leaves (55.58 %) and root (51.72 %). Plants contain carbohydrates, protein, fats etc., essential for growth and development and are therefore considered as basic nutritional source for man and animals. These phytochemicals are therapeutically very important as they help to treat various disorders such as cancer, antimicrobial and anti-inflammatory diseases (Begum *et al.* 2018). They provide energy, as they are the body's main source of fuel, required for brain function, intestinal condition and waste elimination (Abiola *et al.* 2018). Fat and protein contents were minimum in all the plant parts (Table 5). High fats lead to heart problems. It was revealed that *Viola odorata* is safe for human consumption. Protein is a vital nutrient and main metabolite that is required for all physiological functions. The macromolecules amino acids are the building blocks of proteins, and they are required to treat a variety of physiological conditions (Kulal *et al.* 2020). Ash contents were highest in leaves (14.89 %) followed by petiole (13.45 %), stem (12.81 %) and root (10.22 %). The amount of minerals in a sample is determined by the ash content, which is vital in many biochemical activities as a co-enzyme and aids the physiological functioning of major metabolic processes in the body. Moisture contents were recorded under permissible limits recommended by World Health Organization (WHO). These were (7.35 %), (7.1 %), (7.06 %) and (6.62 %) in leaves, stem, root and petiole, respectively. Moisture is a measurement of how much water is contained in the sample. It's also employed as a metric for determining a sample's shelf life. Samples with high moisture contents are susceptible to microbial attacks (Dastagir *et al.* 2021). The highest gross energy was reported for leaves (173.52 K cal/100g) followed by petiole (160.40 K cal/100g), stem (90.90 K cal/100g) and root (87.08 K cal/100g) (Table 6). Gross energy or total caloric value of medicinal plants is generally used to interpret medicinal sample intake as consumption of food components (Uza & Dastagir 2021). Ghosh & Chatterjee (2020) and Alagbe (2020) supported the present study. It was revealed that a nutritional study is required to find new sources of bioactive chemicals with promising medical potential and to make the most use of available natural resources (Verma & Singh 2020). The above discussion claimed that *V. odorata* could be used as a nutrient supplement for both human and animals.

Phytochemical Tests

The saponins were present in all the parts including root, stem, petiole, leaf and flower of the plant. Starch and proteins were present only in leaf. Alkaloids were present in root and leaf, while absent in stem, petiole and flowers. Mucilage was present in root, stem, petiole and leaf, while absent in flowers. Anthraquinone was found only in stem and flowers, while absent in root, petiole and leaf. Tannins were present in stem, petiole and leaf, while absent in root and flowers. Fats and oils were present in stem, leaf and flowers and absent in root and petiole (Table 6a). Mittal *et al.* (2015) worked on phytochemistry of *V. odorata* and stressed that these phytochemicals are accountable for the pharmacological action of this plant. Saponins, alkaloids, mucilage and tannins were further quantified. Saponins were highest (58 %) in flower followed by leaves (57.2 %) and stem (52 %). Alkaloids were maximum in flower (48.5 %) followed by leaves (46 %). Similarly, mucilage and tannins were disclosed to be highest in leaves (65 %, 59 %) and stem (59 %, 53 %), respectively (Table 6b). Audu *et al.* (2018); Ezekiel *et al.* (2019); Alagbe (2020) worked on phytochemical screening of medicinal plants and reported similar results. Phytochemicals play an important part in the plant's defense mechanism against numerous harmful microorganisms (Verma & Singh 2020). They have antibacterial, antifungal, antiviral, anthelminthic, antioxidant, and other biological activities (Hyun *et al.* 2018). Antimicrobial, analgesic, antiplasmodic, and antimalarial properties have all been implicated in alkaloids. Saponins have antibacterial, antifungal, and antioxidant properties (Alagbe *et al.* 2020). Tannins are a diverse collection of plant secondary metabolites that are soluble in polar solutions and separate themselves from other polyphenolic chemicals by their capacity to precipitate proteins. In a wide variety of plant species, there are two forms of soluble tannins. The hydrolysable tannins (HTs) and condensed tannins are these (CTs). They have been shown to have antibacterial and antiviral properties. Phenols are powerful antioxidants that protect biomolecules like DNA from oxidative degradation (Alagbe 2020).

Essential & Fixed Oil Study

The essential oil was 0.00143 % in 349.55g of fresh leaves. The color, taste and odor of oil was yellowish, agreeable and aromatic, respectively (Table 7a). Anca *et al.* (2009), Akhbari *et al.* (2012) and Mohamed & Ghatas (2016) worked on essential oil of *Viola arvensis, V. odorata,* and *V. tricolor* and stated that it possessed antibacterial, insecticidal and phytotoxic properties. Like other characteristics, a medicinal plant is also important due to the presence of essential oils (Abd-ElGawad *et al.* 2020). Most of the essential oils give economic value to the medicinal plant as these oils can be used for different purposes as medicines, perfumes, etc. (Pavela *et al.* 2020).

The fixed oil was 0.396 % in 50g powder of leaf. The color, taste and odor of the oil was yellowish brown, bitter and pleasant, respectively. As both types of oils were found in very low quantity so, it was difficult to determine its refractive index and specific gravity (Table 7b). Since the ancient period, plant oils have been used as a form of therapy. Plant oils were extracted using a variety of processes, including cold pressing, hydrodistillation, steam distillation, hydrodiffusion, effleurage, solvent extraction, carbon dioxide extraction, and microwave assisted processing. The oils were valuable natural materials used in perfumes, cosmetics, aromatherapy, phytotherapy, spices and nutrition, and pesticides, among other businesses (Ammar *et al.* 2020). Gastroprotective, carminative, antiemetic, antibacterial, antifungal, antiviral, antiprotozoal, insect repellents, antioxidant, anticancer, antidiabetic, and antimutagenic properties are all found in fixed oils (Acimovic *et al.* 2020). For many years, herbal extracts and essential oils have been utilized in foods like floral beverages, functional foods, and traditional medicines, with little known "negative effects" on human health. Herbal medicines may be useful in lowering drug reliance and preventing antibiotic resistance (Hyun *et al.* 2018).

Conclusion and Recommendations

The current study contains novelty. Local populations have traditionally employed *V. odorata* to treat a variety of diseases. We confirm that this is the first comprehensive study from Pakistan, based on its systematics and therapeutic applications. Because there has previously been no such work on this medicinally robust plant, the current study was done to develop the parameters that could be beneficial in determining its authenticity. Anatomy of the plant parts showed dicot histology. SEM of the powder drug showed the presence of trichomes, calcium-oxalate crystals, pitted vessels, fibers, trichomes, pollen grains, parenchyma cells, pith cells and root hair, but some unknown tissues were also seen. Ascorbic acid, nutritional, and phytochemical profile revealed that the different parts of the plant contained Vitamin C, other nutrients and various secondary metabolites such as alkaloids, mucilage, anthraquinone, saponins, tannins, fats and oil, protein and starch. Quantification of phytochemicals revealed mucilage and tannins to be the highest as compared to saponins and alkaloids. Leaves had 0.00143 % essential oil and 0.396 % fixed oil. It is suggested that the plant has a great nutritional and therapeutic potential. To meet the future ever-increasing demands, cultivation of *V. odorata* should be initiated on priority basis in the country to meet the local and pharmaceutical demand. As it is a highly medicinally an important plant, so useful information should be given to the different pharmaceutical companies in order to make effective preparations for the treatment of different ailments. The researchers should study the effect of this plant on human beings as it has got a number of biological activities and medicinal properties.

Declarations

Ethical approval and consent to participate: Not applicable.
Availability of data: The data used in this work are available.
Consent to publication: Not applicable.
Conflict of interest: The authors declare that there is no conflict of interest.
Funding: No research grant is involved in this research.
Authors' contributions: GD, SB designed the study; SB conducted the fieldwork, NUU, IA, S conducted the main statistical analysis, NUU wrote the manuscript, RWB revised the data analysis and the manuscript; all authors read, corrected, and approved the manuscript.
Conflicts of Interest
The authors declare no conflicts of interest.

Acknowledgements

The authors are thankful to Centralized Resource Laboratory, Department of Physics, University of Peshawar, for SEM facilities and to the PCSIR Laboratory Peshawar, for the extraction of essential oil and fixed oil.

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