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A novel contribution to quality standards of *Trifolium pretense* L., A dietary supplement

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Trifolium pratense L. is an important medicinal plant distributed in Europe, United States, India and Australia. Flowering tops are used as dietary supplement for the relief of postmenopausal symptoms because of its isoflavone content. The present work was designed to study quality control parameters such as morphological, microscopical parameters, physicochemical along with fluorescence analysis, preliminary phytochemical analysis and HPTLC analysis of root, stem, leaf and flower of *T. pratense*. Morphological and microscopical evaluation of leaves, stem and flowers indicated the presence of long tricellular trichomes and prisms of calcium oxalate. Microscopy of root showed the presence of starch grains and abundant thin straight walled fibres. Preliminary phytochemical analysis showed the presence of alkaloid, flavonoids, tannins, phenolics and steroids as major phytochemicals of the plant while saponins were found to be present in flower. HPTLC fingerprinting was carried out using formononetin and biochanin A as a marker compound for an extract of root, stem, leaf and flower. All the extracts were found to contain both the isoflavone in different concentration. The results of the present study would serve as a source of valuable information for standardization and quality control of root, stem, leaf and flower of *T. pratense*.

Keywords: Ash value, Dietary supplement, Fluorescence analysis, HPTLC, Red clover, *Trifolium pratense* L.

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Introduction

Trifolium pratense L. (Fabaceae) is an erect biennial or perennial important villous forage herb. It is commonly known as "Red clover". The plant is indigenous to Central and South Western Asia, Europe, and Northern Africa and also widely distributed in the temperate zones such as North America, China, Australasia and northern India[1-3]. The available data showed that the plant was used in the treatment of human ailments first time in 1098, the flowers were first time recommended for the treatment of eye problems[4]. Traditionally, it is used to treat psoriasis, eczema, gout, pertussis, chronic fever, cancer, tonic, sedative and hard swelling[5,6]. Flowers are used in the form of herbal tea to treat the wound, insect bite, blood purification, cough and bronchitis[7]. The flowering tops of *T. pratense* are used throughout the world as a dietary supplement by women as a source of phytoestrogen. It is available in the form of herbal tea or liquid or capsule of isoflavone-rich extract[8]. Antispasmodic, anti-inflammatory, anti-neoplastic, antifungal, sedative and lipid-lowering activities of flowering tops and isoflavones are also reported[4,5,9]. The red clover extracts have also been studied clinically for the treatment of postmenopausal syndrome, the effect on bone density and as lipid-loweringagent[10,11]. Phytochemical reports showed that isoflavones are the major constituents of *T. pratense*. The highest amount of isoflavones is present in leaves than flowers and stem[12]. Flowers contain flavonoids and proanthocyanidin[13]. Pterocarpan, an isoflavonoidis present mainly in root and very less amount in leaf[14]. Flavonoids such as quercetin, myricetin and luteolin are mainly present in leaf and flowers[15]. Phenolic acids including protocatechuic acid, p-coumaric acid, salicylic acid, caffeic acid and gentisic acid have been reported in mainly leaves and flowers[16]. Clovamides, polyphenolic amides and essential oils are also reported in aerial parts of the plant[17,18]. Isoflavones and trifolirhizin (pterocarpan glycoside) are the only two compounds reported in the root of *T. Pratense*[19].

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Standardization of the medicinal plant is a crucial step before testing the plant for its medicinal value[20].WHO guideline includes morphological, microscopical, physico-chemical, phyto-chemical and chromatographic evaluation of crude drug to establish the quality standards of the crude drug. Fluorescence analysis is other important parameters for the standardization of crude drugs which provide valuable information about the fluorescence property of phytochemicals present in the crude drug. *T. pratense* has received tremendous importance throughout the world because of its isoflavone content. The flowering tops of *T. pratense* have been listed in United States Pharmacopoeia and British Herbal Pharmacopoeia as a dietary supplement. Recently, the flowering tops and aerial part of the *T. pratense* has been also introduced as a monograph in the therapeutic compendium of American Herbal Pharmacopoeia.

The quality control parameters like morphological and microscopical characteristic of flower, surface characteristic of the leaf and common microscopical characteristic of the stem have been reported in American Herbal Pharmacopoeia. The physico-chemical parameters like foreign organic matter, total ash, acid insoluble ash, loss on drying and water-soluble extractive value of flowering tops have been reported in USP[21]. HPTLC fingerprinting of hydroalcoholic extract of red clover blossoms and aerial part has been reported particularly for the identification of isoflavone[8].

But some important parameters which are very much required for identification, authentication and quality control such as detailed microscopy of stem including cell size measurement, leaf including detail cellular structure of leaf in transverse section, leaf constants have not been reported. The physicochemical parameters such as total ash value, acid insoluble ash, water soluble ash, sulphated ash, extractive values using different solvents like water, methanol, ethanol, ethyl acetate, chloroform, benzene and petroleum ether and fluorescence analysis for leaves, stem and flower separately have not been reported elsewhere. HPTLC fingerprinting for the individual part of *T. pratense* i.e. stem, leaf and flower separately have not been reported in the literature.

There are no scientific reports of quality control parameters for the root of this important plant available in the literature. Thus, the aim of the present work was to provide detail morphological, microscopical and physico-chemical, fluorescence analysis and phytochemical profile and TLC analysis of all parts including root, stem, leaf and flowers of *T. pratense* which will provide important additional information to develop quality standards for this plant. Also, the quantification of formononetin and biochanin A, the marker compound of *T. pratense* was also done in ethanolic extract of root, stem, leaf and flower of *T. pratense*.

Material and Methods

Plant material

The entire plant with flowers was procured in the month of August from Srinagar, Jammu and Kashmir, India. The flowering time of the plant is from May to September. All parts of the herb were identified and authenticated by the scientists of Botanical Survey of India, Pune, Government of India. A voucher specimen of all parts is deposited in the department for future reference. (Voucher Number –H 02) Roots, stems, leaves and flowers were separated and washed with water before drying. All parts were dried at 40°C in a tray drier. The dried material was powdered and the powder of root, stem, leaf and flower was used for the determination of extractive values, ash values, and phytochemical investigation and HPTLC analysis.

Morphology and microscopy

Morphology and microscopy of *T. pratense* root, stem, leaf and flower were studied using fresh as well as dried samples. To study microscopy, transverse sections of root, stem, leaf and flower were taken with the blade. The sections were cleared with clearing agent chloral hydrate and then washed with water, and later stained with different reagents such as a mixture of phloroglucinol and conc. HCl, 1% safranine, 1% toluidine blue and a mixture of toluidine blue and safranine. The photomicrographs were taken using CMOS Camera, Lawrence and Mayo, London and MOTIC photomicroscope provided with MOTIC IMAGES PLUS 2.0 software, Canada. The anatomical terminologies were used as per the book Esau's plant anatomy[22]. Histochemical reactions of transverse sections of all parts with different reagents were performed to check the presence of lignin, starch, lipophilic substances, mucilage and aleurone grain and phenolic compound respectively[23].

Powder drug analysis

Preliminary examination and microscopy of powdered root, stem, leaf and flower were studied as per the method described by Kulkarni *et al*[24].

Micrometry

The measurement of cell size and cell content and the leaf constants were determined by using Motic images plus 2.0 software.

Physico-chemical parameters

Ash value

The percentage of total ash, water-soluble ash, acid-insoluble ash and sulphated ash value were determined as per the procedure is given in Indian Pharmacopoeia[25].

Extractive value

Different solvents ranging from non-polar to polar solvents were used to find out the extractive value of root, stem, leaves and flower powder.

Fluorescence analysis

Fluorescence analysis of powdered root, stems, leaf and flower were carried out as per methods described by Chase and Kokoski. Ultraviolet cabinet (DESAGA, Germany)was used as a source of ultraviolet light. All tests were performed in a dark room. The distance between the light source and the powdered slide was approximately six inches. A black sheet was used as a slide holder. A small amount of powdered drug placed on a slide and was treated with aqueous NaOH, methanolic NaOH, 1N HCl, 50% HNO_3, 50% H_2SO_4 and, was observed under ultraviolet light while still wet[26,27]. The colour of fluorescence was recorded.

Preliminary phytochemical analysis

For the preliminary phytochemical analysis, 5 g of powdered root, stem, leaf and flower were extracted with petroleum ether (40-60 °C), benzene, ethyl acetate, chloroform, ethanol, methanol and water separately by cold maceration technique. The extracts were concentrated under vacuum, dried and weighed. Each extract was tested for the presence of different constituents as per the methods are given by Harborne and Kulkarni[28,29].

HPTLC fingerprinting and quantification

Exactly 50 mg of each extract was dissolved in 10 mL of ethanol in a volumetric flask. Prepared 10 mL ethanol extracts were used for HPTLC fingerprinting analysis using formononetin and biochanin A as a reference standard.

Exactly 10 µL ethanolic extract of root, stem, leaf and flowers were applied on pre-coated silica gel 60 F254 TLC plate with the thickness of 0.2 mm. (Merck, Darmstadt, Germany) using a DESAGA AS30applicator. The plate was developed in glass twin trough chamber (size of 15 X15 cm; silica gel 60 F_{254}TLC plates) using chloroform: methanol (9:1 v/v). After the development of the plate visualization of the plate was done using CabUVIS (DESAGA, Germany) visualizing chamber and scanned using DESAGA Densitometer CD60 scanner at 254 nm and 366 nm. Colour of spots, densitogram and R_f values were recorded using DESAGA ProQuant Windows software[23].

For the identification and quantification of marker compounds, 10 µLethanolic extracts of root, stem, leaf and flowers and 10 µL of marker compounds i.e. formononetin and biochanin A (200 µg/mL) were applied on the chromatographic plate and chromatogram was developed in chloroform: methanol (9:1) solvent system. The calibration curve was prepared using the concentration range of 25–500 µg/mL for formononetin and biochanin A. The plate was scanned after development at 254 nm and densitogram for each spot was recorded using DESAGA ProQuant Windows software.

Results

Morphology of *T. pratense* L.

Root- The roots are thin and tough. The roots are cylindrical with one side tapering and hairy rootlets. The diameters are 0.5 to 1.5 cm and the length is 10 to 20 cm. The colour of the root is a dark buff to brown. The surface is rough with 2 to 4 mm thick bark. The odour is characteristic and the taste is characteristics (Plate 1).

Stem- The stems are erect, cylindrical or angular. It is stout, straight and grows from a rhizome. The diameter of the stem is 1 mm to 4 mm. The stem is densely hairy on upper side while sparsely hairy towards the lower side. The stem is herbaceous and dark green in colour (Plate 1). Taste is acrid.

Leaves- The trifoliate compound leaves are arranged palmately. The leaves are petiolate with long petiole. Adnate type of stipule is present in the form of the wing at the base of the leaf which is attached with petiole. Stipule is buff green in colour with dark green veins on it. The stipule is oval, membranous with acuminate apex. The leaflet present at the base is oval in shape while the leaflets present on the upper side are obovate and very rarely elliptic with size 1.7 to 4.5 × 1-2.5 cm. The apex of the leaflet is obtuse.

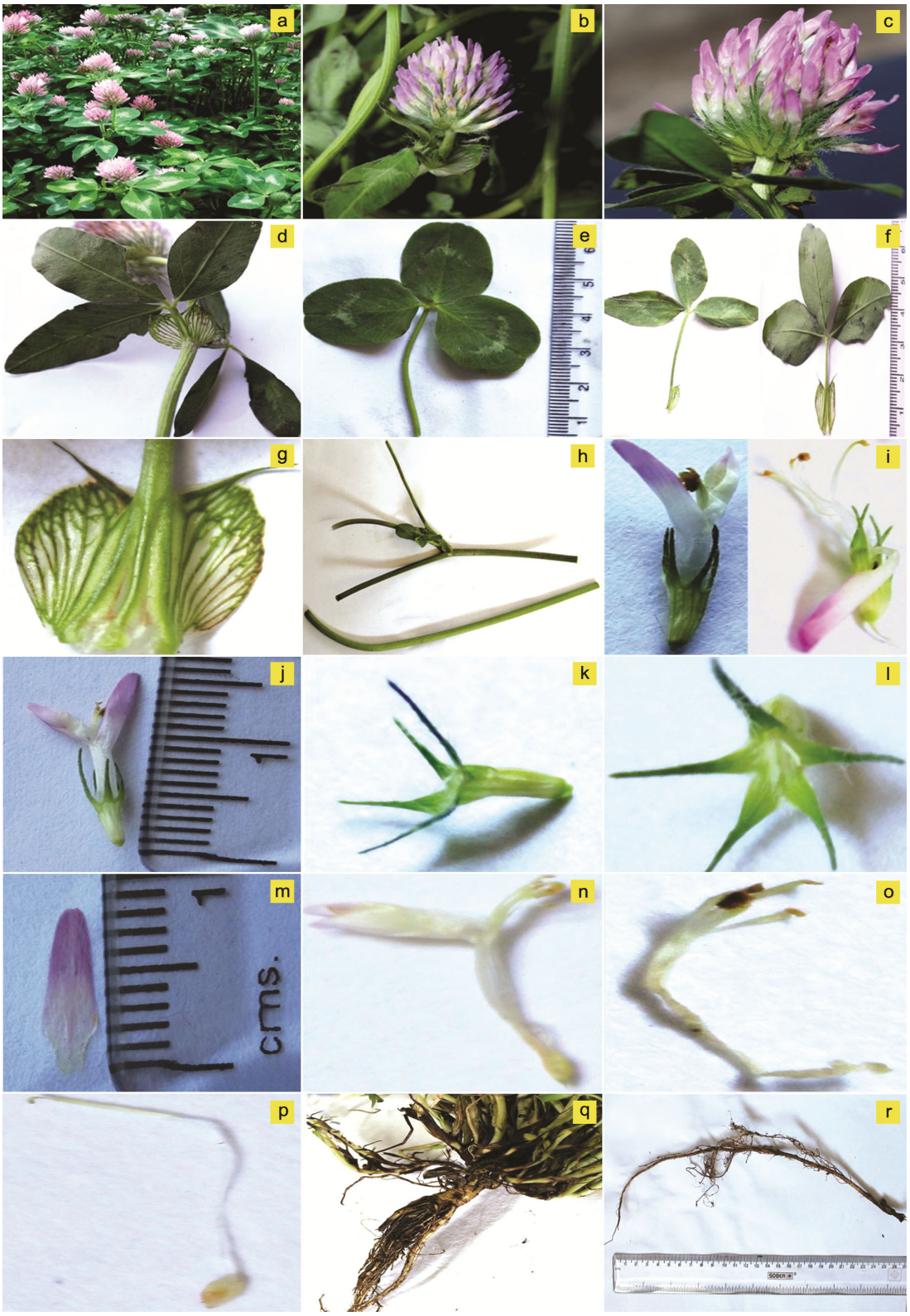

Plate 1 — Morphology of *T. pratense* L. in natural habitat at a) Flowering stage; b) Flower and cylindrical stem; c) Flowers; d) Trifoliate leaf with stipule; e) Basal leaf; f) Upper leaf; g) Wing shaped stipule; h) Stem; i-j) Flower; k-l) Calyx; m) Petal; n-p) Stamens and pistil; and q-r) Root.

The margin is ciliate and base of the leaflet is cuneate. The leaves having characteristics arrow shaped white spot towards the base on the upper side of each leaflet. Lateral veins are straight and thin (Plate 1).

Flower- Flowers are present in a terminal inflorescence with head, globose to ovoid, sessile to rarely pedunculate, subtended with an involucre of stipules. The calyx is tubular-campanulate, pubescent, subulate, teeth unequal, throat open, with a ring of hairs. Corolla is narrowly bell-shaped and hairy. The petals are purple to pink in colour, spatula te, the apex is rounded or retuse. The ovary is elliptic and anther is dorsified (Plate 1).

Microscopy of *T. pratense* L.

Root- The transverse section of root showed mainly three regions - periderm, secondary phloem and secondary xylem.

Periderm is composed of three layers i.e. phellem (cork), phellogen and phelloderm. Cork layer consists of 2 to 4 layers of thin-walled, tangentially elongated brown coloured parenchymatous cells which are arranged radially. Phellogen is indistinct but seen as a narrow layer of thin-walled parenchymatous cells with intercellular spaces. Phelloderm is made up of 10 to 12 layers of irregularly oblong to circular, thin-walled parenchymatous cells. Some of the parenchymatous cells show the presence of small globular starch grains.

Secondary phloem is composed of phloem parenchyma, sieve tubes, phloem fibre and medullary rays. Phloem parenchyma is made up of thin-walled, oval or circular parenchymatous cells some of which are filled with starch grain (Table 1). Phloem fibres are lignified, circular and present abundantly throughout the phloem region, either single or in the group. Initial few layers of phloem contain scattered fibres either single or in the group while inner layers of phloem region contained compactly arranged phloem fibres mostly in groups of four of five fibres together embedded in phloem parenchyma. Sieve elements are indistinct. It is traversed by conspicuous medullary rays and relatively covers a wider zone of the root. Medullary rays are 1-8 cells wide in the phloem region and divided the phloem parenchyma radially.

Vascular cambium is present in the form of the cambium ring. Cambium ring is made up of 10 to 12 layers of small, polygonal compressed parenchymatous cells which differentiate secondary xylem region from the secondary phloem region in the transverse section.

Table 1 — Histochemical colour reactions of transverse sections

Plant part	Reagent	Constituent	Colour	Histological zone
Root	Phloroglucinol + conc. HCl	Lignin	Pink	Vascular bundle, phloem and xylem fibre
	Weak iodine solution	Starch	Blue	Cortex parenchymatous cells
	Sudan red III solution	Oil globules	-	-
	Aqueous ferric chloride	Tannins	Bluish-black	Cork cells
	Dragendorff's reagent	Alkaloids	Light orange	Cortex, phloem
	Libermann-Burchard reagent	Steroids	Greenish	Cortex, phloem
	Millon's reagent	Protein	-	-
Stem	Phloroglucinol + Conc. HCl	Lignin	Pink	Vascular bundle
	Weak iodine solution	Starch	-	-
	Sudan red III solution	Oil globules	-	-
	Aqueous ferric chloride	Tannins	Bluish black	Cortex cells
	Dragendorff's reagent	Alkaloids	Light orange	Cortex, ground tissue
	Libermann-Burchard reagent	Steroids	Greenish	Cortex, ground tissue
	Millon's reagent	Protein	-	-
Leaf	Phloroglucinol + conc. HCl	Lignin	Pink	Vascular bundle
	Weak iodine solution	Starch	-	-
	Sudan red III solution	Oil globules	-	-
	Aqueous ferric chloride	Tannins	Bluish-black	Lamina
	Dragendorff's reagent	Alkaloids	Light orange	Lamina
	Libermann-Burchard reagent	Steroids	Greenish	Lamina
	Millon's reagent	Protein	-	-

Secondary xylem forms the major part of the root. Medullary rays traverse at regular intervals through the region. It consists of parenchyma, vessels and fibres. Xylem parenchyma is made up of small polygonal parenchymatous cells. Xylem fibres are small and lignified embedded in xylem parenchyma. Medullary rays are 1-6 cells wide in xylem region. Some of the medullary rays show the presence of starch grains. Pith is absent which is a characteristic of dicot root (Table 2, Plate 2).

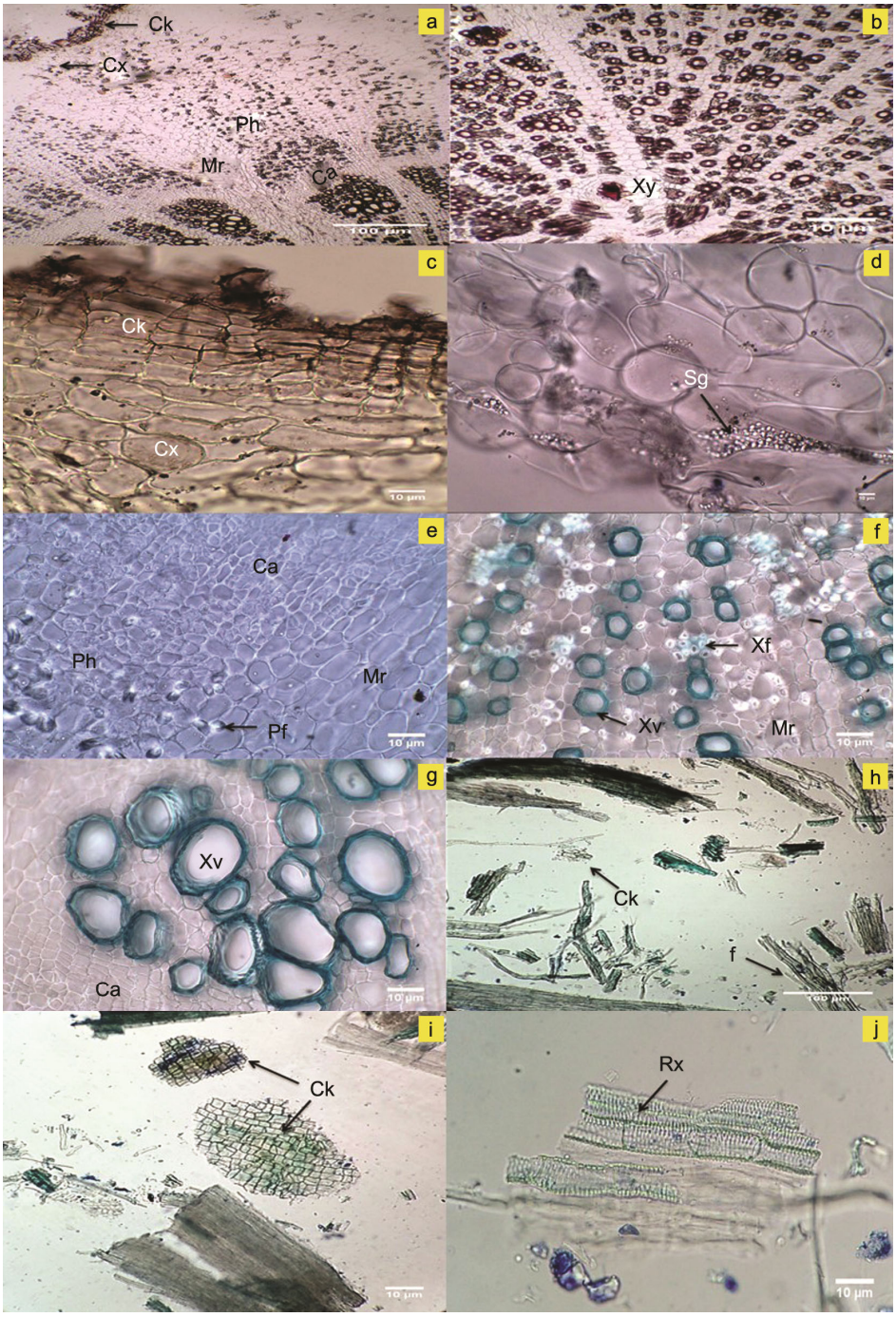

Plate 2 — Photomicrographs of transverse section and powder characteristic of root of *T. pratense* L. a) General view of transverse section of root; b) general view of xylem region; c) cork and cortex; d) starch grain in cortex parenchyma; e) phloem fibre and cambium; f) detail of xylem region; g) Xylem vessels; h) general view of powder characteristics; i) cork in surface view; and j) reticulated xylem vessels. Abbreviations: Ck, cork; Cx, cortex; Pf, phloem fibre; Ca, cambium; Xy, xylem; Xf, xylem fibre; Xv, xylem vessels; Rx, reticulated xylem; f, fibres; Mr, medullary rays; Sg, starch grain.

Stem- The transverse section of stem showed the presence of epidermis, cortex, vascular bundles and ground tissue (pith).

The epidermis consists of a single layer of cells covered with a cuticle. The epidermal cells are smaller in size and oval or oblong in shape. Some of the epidermal cells are converted into long tricellular covering trichomes with rounded basal cells, small middle cell and 800-900 µm long terminal cells with the narrow lumen and blunt apex. The surface of the stem shows ridges and below each ridge, a well-developed vascular bundle is observed.

Cortex occupies about 1/3rd of the diameter of the stem. It is made of oval or circular shaped parenchymatous cells. The cortex cells are thick walled with intercellular spaces. Cortex region is divided into three zones i.e. hypodermis, middle cortex and endodermis. The hypodermis consists of 1-2 layers of tightly arranged oblong parenchymatous cells. The cortical layer just below the hypodermis is made up of oval or rounded thin-walled parenchymatous cells with conspicuous intercellular space. The third zone consists of single-layered parenchymatous cells known as endodermis. Few cells of the endodermis surrounding the vascular bundle contain prisms of calcium oxalate crystals.

Vascular bundles are collateral, conjoint and open with endark xylem. Pericycle is well developed and present in the form of sclerenchymatous lignified fibres making a cap-like structure on phloem in each vascular bundle. In a young plant, this region shows the presence of collenchymatous cells and at the maturity of the plant, it is converted into lignified fibres (Table 1). Phloem is prominent with small parenchymatous cells, cambium is indistinct. The xylem vessels are well developed with spiral thickening (Table 2).

Ground Tissue (Pith) consists of large, parenchymatous cells with intercellular spaces (Plate 3).

Leaf- It is a dorsiventral compound leaf. The transverse section of the leaflet is mainly divided into two parts i.e. midrib and lamina.

Midrib- The section passing through the midrib showed boat shaped upper side and the protuberance on the lower side. The epidermis on both sides is made up of characteristics papillose epidermal cells. Collenchyma is absent on both sides of the leaf. Midrib shows the presence of 9-10 layers of polygonal compactly arranged cortical parenchymatous cells above lower epidermis while below upper epidermis it is made up of 5-6 layers of small polygonal cortical parenchymatous cells. In the centre of the midrib oval shaped vascular bundle is present. The vascular bundle is collateral and conjoint shows characteristics collenchymatous cap on both sides. Three to four layers of phloem cells are present on the ventral surface of the vascular bundle. Xylem vessels are lignified and show spiral thickening (Table 1). Protoxylem is present towards upper epidermis while metaxylem is present towards the lower epidermis.

Table 2 — Measurements of cells

Plant part	Type of cell	Size in µ (n=3)
Root	Cork	38.5 × 14.8 – 41.8 × 11.1
	Phellogen & Phelloderm	79.2 × 14.2- 34.9 × 11.2
	Cortex parenchyma	49.2 × 14.3 - 28.6 × 41.9
	Phloem fiber	12.8 × 12.3 - 10.1 × 10.1
	Xylem vessels	88.1 × 42.2 - 12.4 × 21.5
	Medullary rays	57.6 × 29.0 - 33.6 × 32.6
Stem	Epidermis	15.3 × 12.1 - 11.0 × 8.5
	Cortex	44.6 × 26.5 - 18.3 × 17.3
	Xylem	40.4 × 35.1 -7.3 × 9.3
	Phloem	13.4 × 7.9 - 6.7 × 4.6
	Cambium	11.0 × 8.7 - 4.6 × 4.5
	Pith	111.4 × 78.9 - 64.9 × 63.3
Leaf	Upper epidermis	49.6 × 25.6 – 20.0 × 32.3
	parenchyma	17.6 × 15.4 – 46.6 × 46.9
	Xylem vessels	15.5 × 16.8 – 6.7 × 7.6
	Phloem	16.8 × 12.2 – 4.5 × 4.8
	Trichomes	895.1- 151.1
	Lower epidermis	31.8 × 36.8 –13.8 × 13.9

Lamina- In the transverse section, the lamina showed the presence of mainly three parts i.e. upper epidermis, mesophyll and lower epidermis.

Epidermis- The upper epidermis as well as lower epidermis composed of papillose epidermal cells. Epidermal cells of the lower epidermis are wavy with anticlinal cell wall while epidermal cells of the upper epidermis are straight walled parenchymatous cells. Both the layers showed sward shaped tricellular trichomes made up of bulbous basal cell, small polygonal middle cell and very long outermost cell (700 to 800 µm) with the narrow lumen and an acute apex. Stomata and trichomes are present on both the epidermis.

Mesophyll- It is differentiated into palisade and spongy parenchyma. Palisade is made up of 1 to 3 layered, compact with radially elongated cells. Spongy parenchyma consists of 4-6 layered polygonal cells.

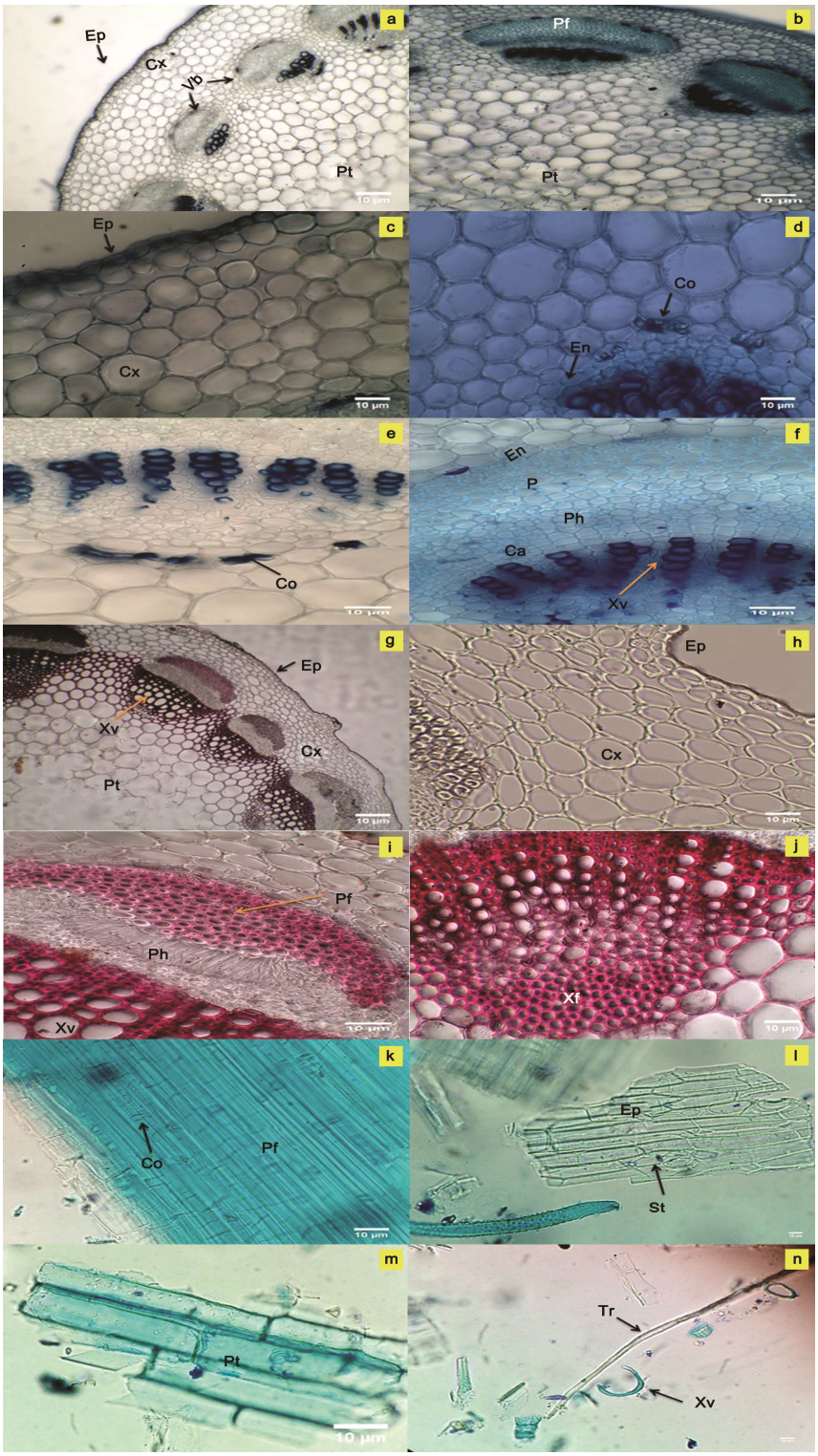

Plate 3 — Photomicrographs of transverse section and powder characteristic of stem of *T. pratense* L. a-b) General view of the transverse section young stem; c) epidermis and cortex; d) prisms of calcium oxalate in endodermal cells; e-f) Vascular bundle of young stem; g) general view transverse section of mature stem; h) epidermis and cortex of mature stem; i-j) Vascular bundle of mature stem; k) prisms of calcium oxalate on pericyclic fibre; l) epidermis with stomata; m) pith parenchyma; and n) trichomes and fragments of spiral xylem vessels. Abbreviations: Ep, epidermis; Cx, cortex; Vb, vascular bundle; Pf, pericyclic fibre; Ph, phloem; ca, cambium; Xv, xylem vessels; St, stomata; Pt, pith; Tr, trichome; Co, calcium oxalate; En,endodermis; P, pericycle; Xf, xylem fibre.

Surface view shows anisocytic and anomocytic stomata, covering trichomes and epidermal cells. Lower epidermis shows the presence of anomocytic stomata while upper epidermis shows the presence of more number of anisocytic stomata and very few anomocytic stomata on the surface. Stomata and trichomes are present on both the epidermis but more in number on lower epidermis compare to upper epidermis. Upper epidermal cells are straight walled polygonal parenchymatous cells while lower epidermis shows wavy walled polygonal parenchymatous cells. The surface preparation shows the presence of crystal sheath where calcium oxalate prisms are deposited in each parenchymatous cell surrounding the fibrovascular tissue (Plate 4 & 5, Table 2 & 3).

Powder drug analysis of root of *T. pratense* L.

Macroscopy- The powder is brownish buff in colour; texture is fibrous, acrid in taste with characteristics odour. Mucilage is found to be absent because the mucilaginous mass was not formed after the addition of a small quantity of water in powder. No greasy stain was found after pressing a small quantity

Table 3 — Leaf constants for *Trifolium pratense* L. leaf.

Leaf Constants	Value
Stomatal Number	Upper epidermis – 40 Lower epidermis – 70
Stomatal Index	Upper epidermis – 19.05 Lower epidermis – 25.92
Vein-islet Number	10 – 12
Vein-termination Number	07 – 10

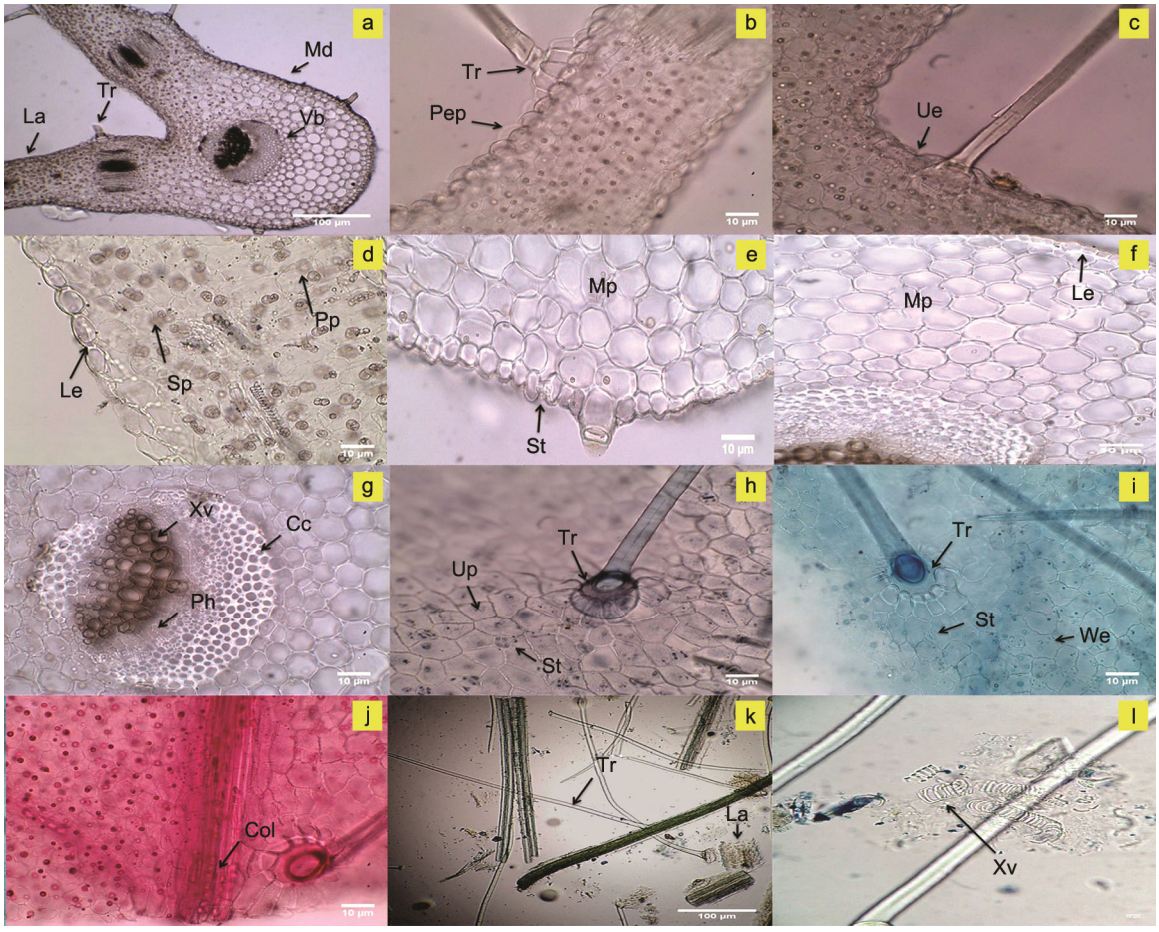

Plate 4 — Photomicrographs of transverse section and powder characteristic of leaf of *T. pratense* L. a) General view of midrib; b) general view of lamina; c-e) detail of upper and lower epidermis; f-g) detail of midrib; h) surface view of upper epidermis; i-j) Surface view of lower epidermis; k) detail of powder characteristics;andl) spiral xylem vessels. Abbreviations: La, lamina; Md, midrib; Pep, papillose epidermis; Ue, upper epidermis; Le, lower epidermis; Sp, spongy parenchyma; Pp, palisade parenchyma; Pf, pericyclic fibre; Ph, Phloem; Xv, xylem vessels; St, stomata; pt, pith; Tr, trichomes; Col, calcium oxalate layer; Mp, midrib parenchyma; Cc, collenchymatous cap; We, wavy epidermis.

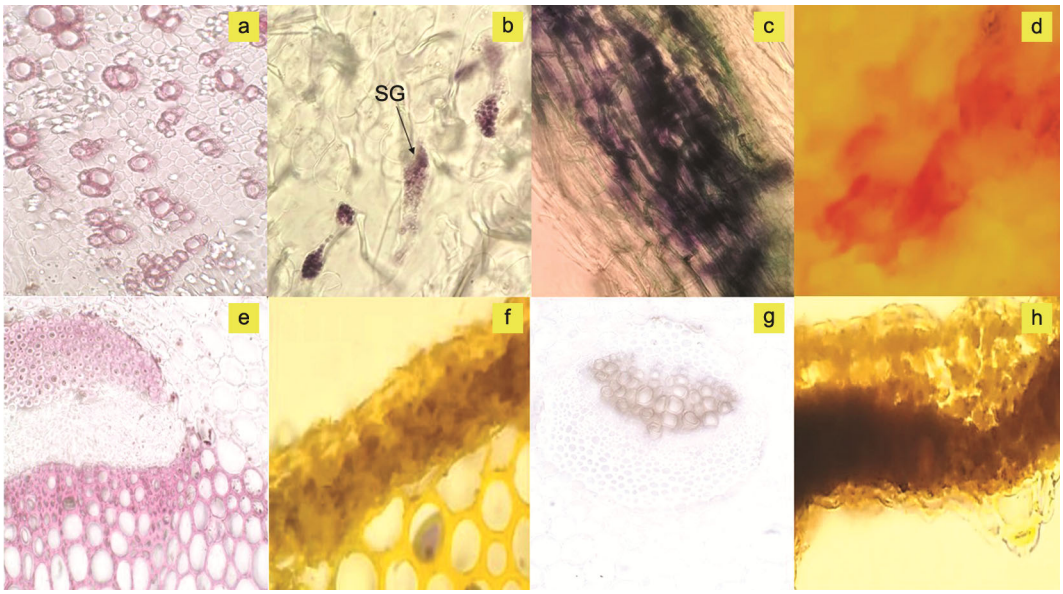

Plate 5 — Histochemical colour reactions of transverse sections. a) Root xylem after treatment with Phlorglucinol and Concentrated Hydrochloric acid;b) Cortex region of root after treatment with dilute iodine solution; c) Cork region of root after treatment with ferric chloride; d) Cortex region of root after treatment with Dragendorff's reagent; e) Vascular bundle of stem after treatment with Phlorglucinol and Concentrated Hydrochloric acid; f) Cork region of stem after treatment with $FeCl_3$; g) leaf xylem after treatment with Phlorglucinol and Concentrated Hydrochloric acid; and h) lamina region of leaf after treatment with $FeCl_3$.

of powder between filter paper which indicated the absence of fatty oils. No persistent foam was formed after shaking powder with water indicates the absence of saponins. Powder behaviour with different chemical reagents is shown in Table 4.

Microscopy-The powder showed the presence of polygonal light brown coloured cork cells. An abundant amount of lignified, thin, tubular fibres either single or in groups are present. Parenchymatous cells filled with circular starch grain are observed. Fragments of reticulated xylem vessels are found in group or isolated. The powder showed an absence of calcium oxalate crystals (Plate 1).

Powder drug analysis of stem of *T. pratense* L.

Macroscopy- The powder is greenish brown in colour, the texture is rough, taste is acrid and the odour is characteristics. After the addition of water in a small amount of powder mucilaginous mass was not formed indicates the absence of mucilage. Saponins were found to be absent because no foam was formed after shaking powder with water. The greasy stain was not observed after pressing powder with filter papers indicates the absence of fatty oils. Powder behaviour with different chemical reagents is shown in Table 4.

Microscopy-The powder showed the presence of fibres with prismatic calcium oxalate crystals. The fibres are long, tubular, lignified and present in groups. Unicellular, straight walled, long trichomes are present. Polygonal epidermal cells with stomata are also observed. Oblong parenchymatous cells of pith in longitudinal view are present. Fragments of spiral lignified xylem vessels are scattered through the slide. Pith cells in transverse view are also found (Plate 2).

Powder drug analysis of leaf of *T. pratense* L.

Macroscopy- The powder is green in colour, the texture is smooth, taste is acrid and the odour is characteristics. After the addition of water in a small amount of water mucilaginous mass was not formed indicated absence of mucilage. Saponins were found to be absent because no foam was formed after shaking powder with water. The greasy stain was not observed after pressing powder with filter papers indicates the absence of fatty oils. Powder behaviour with different chemical reagents is shown in Table 4.

Microscopy- Unicellular, sward shaped, blunt and very long trichomes with the bulbous base are present. Lignified fibrovascular tissue with prisms of calcium oxalate is present. Spiral xylem vessels are present. Anisocytic and anomocytic stomata with epidermal cells are observed. Fragments of lamina in the transverse view are observed (Plate 3).

Powder drug analysis of flower of *T. pratense* L.

Macroscopy- The powder is purplish brown in colour, the texture is smooth, taste is acrid and the

Table 4 — Behavior of root, stem, leaf and flower powder with different chemical reagents.

Plant part	Reagent	Colour/Precipitates	Constituents
Root	Conc. Sulphuric acid	Reddish	Steroid present
	Aqueous Ferric chloride solution	Blackish	Tannins present
	Iodine solution	Bluish-black	Starch present
	Picric acid solution	Yellowish	Alkaloid present
	Aqueous Mercuric chloride solution	Brownish	Alkaloid present
	Magnesium-Conc. hydrochloric acid	No change	Flavonoids absent
	Aqueous Silver nitrate solution	No precipitate	Protein absent
	Ammonia solution	No change	Anthraquinone glycoside absent
	Aqueous Potassium hydroxide solution	No change	Anthraquinone glycoside absent
Stem	Conc. Sulphuric acid	Reddish	Steroid present
	Aqueous Ferric chloride solution	Greenish black	Tannins present
	Iodine solution	No blue colour	Starch absent
	Picric acid solution	Yellowish	Alkaloid present
	Aqueous Mercuric chloride solution	Brownish	Alkaloid present
	Magnesium-Conc. hydrochloric acid	Reddish brown	Flavonoids present
	Aqueous Silver nitrate solution	No precipitate	Protein absent
	Ammonia solution	No change	Anthraquinone glycoside absent
	Aqueous Potassium hydroxide solution	No change	Anthraquinone glycoside absent
Leaf	Conc. Sulphuric acid	Reddish	Steroid present
	Aqueous Ferric chloride solution	Greenish black	Tannins present
	Iodine solution	No blue colour	Starch absent
	Picric acid solution	Yellowish	Alkaloid present
	Aqueous Mercuric chloride solution	Brownish	Alkaloid present
	Magnesium-Conc. hydrochloric acid	Reddish brown	Flavonoids present
	Aqueous Silver nitrate solution	No precipitate	Protein absent
	Ammonia solution	No change	Anthraquinone glycoside absent
	Aqueous Potassium hydroxide solution	No change	Anthraquinone glycoside absent
Flower	Conc. Sulphuric acid	Reddish	Steroid present
	Aqueous Ferric chloride solution	Greenish black	Tannins present
	Iodine solution	No blue colour	Starch absent
	Picric acid solution	Yellowish	Alkaloid present
	Aqueous Mercuric chloride solution	Brownish	Alkaloid present
	Magnesium-Conc. hydrochloric acid	Reddish brown	Flavonoids present
	Aqueous Silver nitrate solution	No precipitate	Protein absent
	Ammonia solution	No change	Anthraquinone glycoside absent
	Aqueous Potassium hydroxide solution	No change	Anthraquinone glycoside absent

odour is characteristics. After the addition of water in a small amount of water mucilaginous mass was not formed indicates the absence of mucilage. Saponins were found to be present because the persistent foam was formed after shaking powder with water. The greasy stain was not observed after pressing powder with filter papers indicates the absence of fatty oils. Powder behaviour with different chemical reagents is shown in Table 4.

Microscopy- Wavy epidermal cells of petals are present with spiral lignified xylem vessels. Broken fragments of style and stigma are present. The calyx with pointed, unicellular, long trichomes is present. Glandular trichomes are also present on the calyx. Polygonal parenchymatous cells filled with prismatic crystals of calcium oxalate are abundantly found in flower. Circular, yellowish brown coloured pollen grains are present. Dark brown anther lobes with pollen grains are present (Plate 6).

Physico-chemical parameters

The results of physico-chemical parameters are listed in Table 5-7.

Fluorescence analysis

Each powder shows characteristic fluorescence with different chemical reagents. The result of

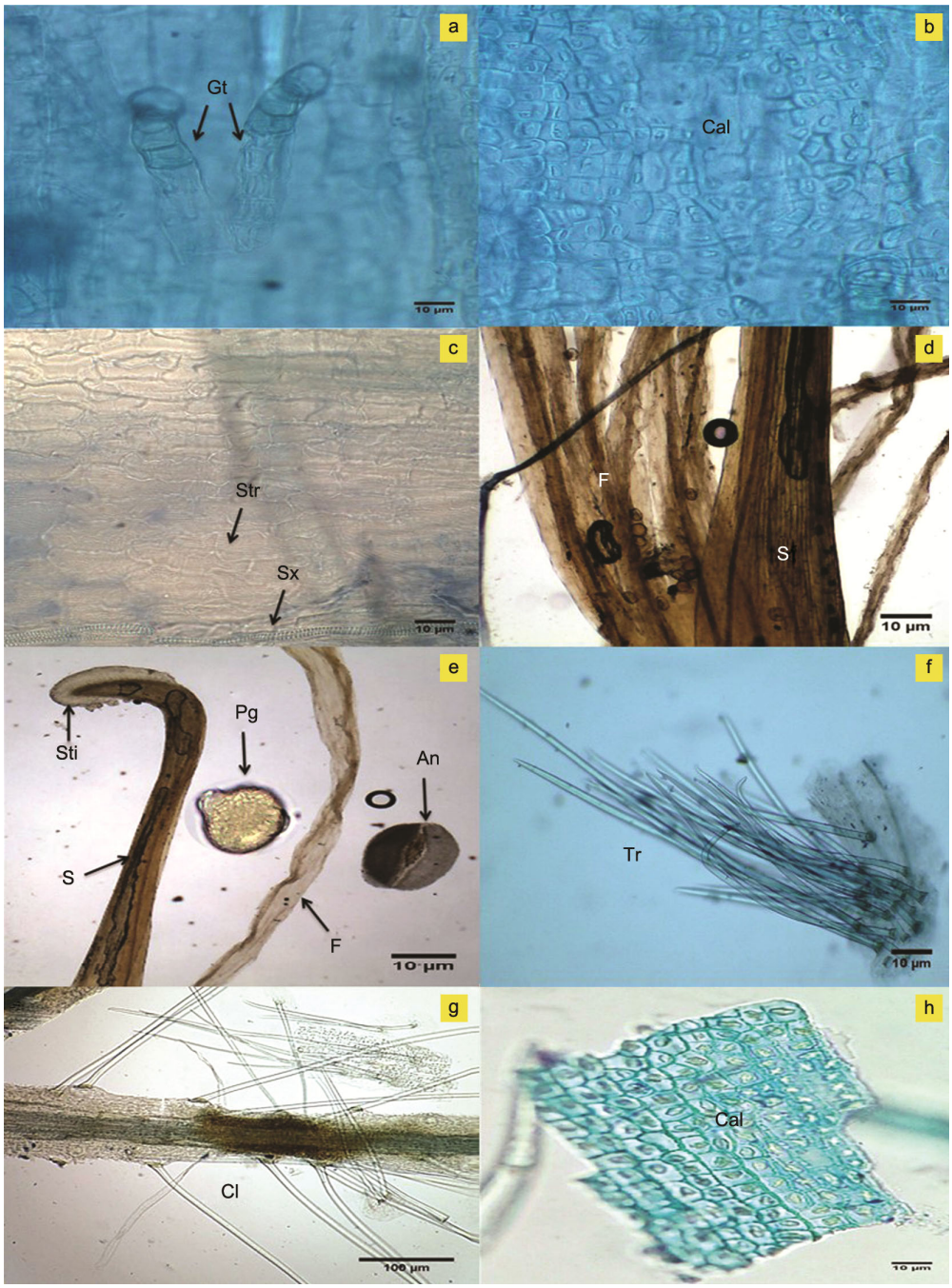

Plate 6 — Photomicrographs of powder characteristic of flower of *T. pratense* L. a-b) Surface view of calyx; c) epidermal cells of petal in surface view; d) stamen and pistil, e) detail of stamen and pistil; f) Bunch of trichomes; g) calyx lobe with trichomes; and h) crystal layer of calcium oxalate in parenchymatous cells of calyx. Abbreviations: Gt, glandular trichomes; Cal, calcium oxalate crystal layer; Tr, trichomes; Pg, pollen grain; An, anther; F, filament; S, style; Cl, calyx lobe; Sx, spiral xylem vessels; Str, striation; Sti, stigma.

fluorescence study of the powdered root, stem, leaf and flower with different chemical reagents were tabulated in Table 8.

Preliminary phytochemical screening

The results of the preliminary phytochemical screening of root, stem, leaf and flower extracts were tabulated in Table 9. The major phytochemicals present in the plants are steroids, carbohydrates, alkaloids, tannins and phenolics.

HPTLC Fingerprinting and quantification

The HPTLC fingerprint of ethanol extract of root, stem, leaf and flower was carried out using chloroform:

Table 5 — Ash value of root, stems, leaf and flower

Type of ash values	% W/W (Mean [a] ± SEM)			
	Root	Stem	Leaf	Flower
Total ash	8.35 ± 0.06	8.9 ± 0.10	14.18 ± 0.14	8.32 ± 0.20
Acid insoluble ash	2.75 ± 0.18	0.96 ± 0.09	3.47 ± 0.18	0.6 ± 0.18
water soluble ash	1.33 ± 0.16	3.83 ± 0.07	3.23 ± 0.04	5.17 ± 0.41
Sulphated ash	10.86 ± 0.11	10.98 ± 0.04	17.85 ± 0.48	11.32 ± 0.18

[a]Mean value of three readings.

Table 6 — Extractive values with different solvents of root, stems, leaf and flower

Type of Solvent	% Extractability (Mean [a] ± SEM)			
	Root	Stem	Leaf	Flower
Petroleum ether (60-80)	0.087 ± 0.007	0.24 ± 0.023	0.36 ± 0.012	0.42 ± 0.012
Benzene	0.32 ± 0.050	0.27 ± 0.067	1.013 ± 0.087	0.527 ± 0.064
Chloroform	0.553 ± 0.041	0.8 ± 0.012	0.98 ± 0.012	0.927 ± 0.053
Ethyl acetate	0.627 ± 0.114	0.62 ± 0.194	0.727 ± 0.029	0.673 ± 0.052
Ethanol	1.093 ± 0.137	1.033 ± 0.064	1.133 ± 0.186	1.293 ± 0.074
Methanol	3.34 ± 0.180	2.84 ± 0.081	2 ± 0.060	3.187 ± 0.064
Water	1.427 ± 0.255	3.827 ± 0.159	5.267 ± 0.350	5.847 ± 0.057

[a]Mean value of three readings.

Table 7 — Consistency, colour and fluorescence analysis of root, stems, leaf and flower extracts.

Plant parts	Extract	Consistency	Color in		
Root			Daylight	Short UV	Long UV
	Petroleum ether (60-80)	Sticky mass	"C" Green	Greenish black	Purplish pink
	Benzene	Solid mass	Brownish black	Greenish black	Brownish pink
	Chloroform	Solid mass	Brownish black	Black	Light pink
	Ethyl acetate	Semisolid sticky mass	Brownish black	Greenish brown	Purplish pink
	Ethanol	Semisolid mass	Brown	Greenish brown	Greenish black
	Methanol	Semisolid mass	Brownish orange	Greenish brown	Dark green
	Water	Solid mass	Orange brown	Brown	Brown
Stem	Petroleum ether (60-80)	Sticky mass	Green	Dark green	Orange brown
	Benzene	Semisolid sticky mass	Dark green	Dark green	Dark green
	Chloroform	Semisolid mass	Dark green	Dark green	Light brown
	Ethyl acetate	Semisolid sticky mass	Dark green	Dark green	Dark green
	Ethanol	Semisolid mass	Dark green	Dark green	Blackish brown
	Methanol	Semisolid mass	Dark green	Black	Blackish brown
	Water	Semisolid sticky mass	Brownish orange	Black	Blackish brown
Leaf	Petroleum ether (60-80)	Sticky mass	Greenish black	Greenish black	Reddish brown
	Benzene	Sticky mass	Greenish black	Black	Brown
	Chloroform	Sticky mass	Greenish black	Black	Reddish brown
	Ethyl acetate	Semisolid mass	Greenish black	Black	Reddish brown
	Ethanol	Semisolid sticky mass	Greenish black	Black	Blackish brown
	Methanol	Semisolid sticky mass	Greenish black	Black	Blackish brown
	Water	Solid mass	Orange brown	Brown	Blackish brown
Flower	Petroleum ether (60-80)	Sticky mass	Green	Reddish brown	Bright reddish brown
	Benzene	Sticky mass	Dark green	Black	Reddish brown
	Chloroform	Semisolid mass	Dark green	Black	Dark reddish brown
	Ethyl acetate	Semisolid mass	Dark green	Black	Brownish black
	Ethanol	Solid mass	Dark green	Black	Brown
	Methanol	Solid mass	Dark green	Black	Brown
	Water	Semisolid sticky mass	Orange brown	Colorless	Brownish yellow

Table 8—Fluorescence analysis of powdered root, stems, leaf and flower of *T. pratense* L.

Plant part	Sample	Colour in day light	Colour in short UV	Colour in Long UV
Root	Powder	Brownish buff	No fluorescence	No fluorescence
	Powder + Sodium Hydroxide in methanol	Light green	Greenish blue	Whitish blue
	Powder + Sodium hydroxide in water	Dark greenish black	No fluorescence	Greenish black
	Powder + 1N hydrochloric acid	Light green	No fluorescence	Orange brown
	Powder + 50 % nitric acid	Light green	No fluorescence	Orange brown
	Powder + 50 % sulphuric acid	Dark greenish black	No fluorescence	No fluorescence
Stem	Powder	Greenish brown	No fluorescence	No fluorescence
	Powder + Sodium Hydroxide in methanol	Bright yellow	Greenish yellow	Bright yellowish green
	Powder + Sodium hydroxide in water	Dull yellow	No fluorescence	Dull yellowish green
	Powder + 1N hydrochloric acid	Bright yellow	No fluorescence	Greenish yellow
	Powder + 50 % nitric acid	Bright yellow	No fluorescence	Greenish yellow
	Powder + 50 % sulphuric acid	Greenish yellow	No fluorescence	Dull green
Leaf	Powder	Green	No fluorescence	No fluorescence
	Powder + Sodium Hydroxide in methanol	Greenish yellow	Greenish	Yellowish green
	Powder + Sodium hydroxide in water	Dark Greenish yellow	No fluorescence	Light brown
	Powder + 1N hydrochloric acid	Light Greenish yellow	No fluorescence	Light brown
	Powder + 50 % nitric acid	Light Greenish yellow	No fluorescence	No fluorescence
	Powder + 50 % sulphuric acid	Brownish green	No fluorescence	Light brown
Flower	Powder	Light purplish brown	No fluorescence	No fluorescence
	Powder + Sodium Hydroxide in methanol	Brown	Light brown	Very light brown
	Powder + Sodium hydroxide in water	Greenish brown	No fluorescence	Greyish brown
	Powder + 1N hydrochloric acid	Creamish brown	No fluorescence	Greyish brown
	Powder + 50 % nitric acid	Creamish brown	No fluorescence	Greyish brown
	Powder + 50 % sulphuric acid	brown	No fluorescence	Greyish black

Table 9 — Qualitative phytochemical analysis of various extracts of powdered root, stems, leaf and flower of *T. pratense* L.

Plant Part	Type of Constituents	Petroleum ether	Benzene	Chloroform	Ethyl acetate	Ethanol	Methanol	Water
Root	Steroid	+	+	+	+	-	-	-
	Carbohydrate							+
	Alkaloids	–	–	–	–	+	+	-
	Glycosides							–
	Reducing sugar							+
	Flavonoids					–	–	–
	Tannins and Phenolic					+	+	+
	Protein							–
	Amino acid							–
	Acidic Compound							–
	Aleurone grain							+
	saponins							--
Stem	Steroid	+	+	+	+	+	+	-
	Carbohydrate							+
	Alkaloids	–	–	–	–	+	+	–
	Glycosides							–
	Reducing sugar							+
	Flavonoids					–	–	–
	Tannins and Phenolic					+	+	+
	Protein							–
	Amino acid							–
	Acidic Compound							–
	Aleurone grain							+
	Saponins							--

(*Contd*.)

Table 9 — Qualitative phytochemical analysis of various extracts of powdered root, stems, leaf and flower of *T. pratense* L. (*Contd.*)

Plant Part	Type of Constituents	Petroleum ether	Benzene	Chloroform	Ethyl acetate	Ethanol	Methanol	Water
Leaf	Steroid	+	+	+	+	+	+	-
	Carbohydrate							+
	Alkaloids	–	–	–	–	+	+	–
	Glycosides							–
	Reducing sugar							+
	Flavonoids					+	+	–
	Tannins and Phenolic					+	+	+
	Protein							–
	Amino acid							–
	Acidic Compound							–
	Aleurone grain							–
	saponins							–
Flower	Steroid	+	+	+	+	+	+	-
	Carbohydrate							+
	Alkaloids	–	–	–	–	+	+	–
	Glycosides							–
	Reducing sugar							+
	Flavonoids					+	+	–
	Tannins and Phenolic					+	+	+
	Protein							–
	Amino acid							–
	Acidic Compound							–
	Aleurone grain							+
	Saponins							+

Table 10 — Quantification of Formononetin and Biochanin A in ethanolic extract of root, stem, leaf and flower of *T. pratense* L.

Stationary Phase	Precoated Silica Gel 60 GF254
Mobile Phase	Chloroform: Methanol (9:1)
Calibration range of formononetin	0.25-5 µg/spot
Calibration range of Biochanin A	0.25-5 µg/spot
Detection	At 254 nm
Regression equation	Formononetin:Y = 5.3137x + 253.45
	Biochanin A : Y= 11.714x + 320.98
R^2 value	Formononetin: R^2 = 0.9895
	Biochanin A: R^2 = 0.9867
$R_f$value	Formononetin: 0.73
	Biochanin A: 0.80

methanol (9:1 v/v) as the mobile phase (Table 10). At the wavelength 254 nm root extract showed9 spots with R_f 0.05, 0.11, 0.27, 0.36, 0.43, 0.54, 0.64, 0.73 and 0.78. The ethanolic stem extract showed presence of 7 spots with R_f0.05, 0.11, 0.38, 0.40, 0.50, 0.71 and 0.77. The ethanolic leaf extract showed presence of 9 spots with R_f 0.05, 0.11, 0.27, 0.35, 0.41, 0.49, 0.60, 0.69, 0.76. The ethanolic flower extract showed presence of 8 spots with R_f0.10, 0.18, 0.24, 0.33, 0.47, 0.68, 0.75 and 0.94 (Table 11, Fig. 1)

The ethanolic extract of root, stem, leaf and flowers showed the presence of both the marker compounds i.e formononetin and biochanin A in different concentration at the R_f value 0.73 and 0.80 respectively (Fig. 2-6). The percentage of formononetin in the ethanolic extract of root, stem, leaf and flower was found to be 1.04, 0.35, and 0.06% w/w respectively. The percentage of biochanin A in the ethanolic extract of root, stem, leaf and flower was found to be 0.02, 0.29, 0.38, 0.12% w/w, respectively.

Discussion

It has been estimated that out of 250,000 plants 20,000 plants are used as a medicinal plant for treatment of various ailments worldwide[30] any plant material used for the medicinal purpose must be of

Table 11 — Description of TLC Fingerprint of ethanolic extract of root, stems, leaf and flower of *T. pratense* L.

Extract no/spot no.	Visible light	UV light 254	UV light 366	R_f
Ethanolic extract of root				
1	--	Dark green	Blue	0.05
2	--	Dark green	Blue	0.11
3	--	Dark green	Green	0.27
4	--	Dark green	Blue	0.36
5	--	Dark green	Green	0.43
6	--	Dark green	Bright Blue	0.54
7	--	Dark green	Bright blue fluorescence	0.64
8	--	Dark green	Green	0.73
9	--	Dark green	Blue	0.78
Ethanolic extract of stem				
1	--	Dark green	--	0.05
2	--	Dark green	Green	0.11
3	--	Dark green	Green	0.38
4	--	Dark green	Green	0.40
5	--	Dark green	Red	0.50
6	--	Dark green	Blue fluorescence	0.71
7	--	Dark green	Green	0.77
Ethanolic extract of leaf				
1	--	Dark green		0.05
2	--	Dark green	Green	0.11
3	--	Dark green	Red	0.27
4	--	Dark green	Red	0.35
5	--	Dark green	Green	0.41
6	--	Dark green	Red	0.49
7	--	Dark green	Blue fluorescence	0.60
8	--	Dark green	Blue	0.69
9	--	Dark green	Green	0.76
Ethanolic extract of flower				
1	--	Dark green	--	0.10
2	--	Dark green	--	0.18
3	--	Dark green	Green	0.24
4	--	Dark green	--	0.33
5	--	Dark green	Red	0.47
6	--	Dark green	Blue fluorescence	0.68
7	--	Dark green	Blue	0.75
8	--	Dark green	Green	0.94

standard quality and thus standardization of medicinal plants by means of botanical and chemical analysis is very important. Morphological and microscopical study of plant part either in dried or in the fresh form provides valuable information about the authenticity of the plant species[20]. Microscopic study of the crude drug either in transverse sections, longitudinal section or surface view or as isolated tissue in powder study also provides additional information about the adulteration or other substituents. The morphological characters of the leaves and flowers reported in the current study are similar to the previous reports[8]. The current study explained morphology of stem in detail while the morphology of the root is reported for the first time. The microscopy of the stem shows the presence of vascular bundle below each ridge with pericyclic fibre cap above the phloem and some of the parenchymatous cell surrounding vascular bundle showed the presence of prisms of calcium oxalate. The fibre cap is observed only in the stem of the mature plant and found to be undeveloped in the stem of the young plant. This is an important identifying characteristic of the stem of *T. pratense*. The results obtained after microscopy of leaf and flowers are comparable with the previousreports[8]. However, the leaf constants and cell size measurement for all the

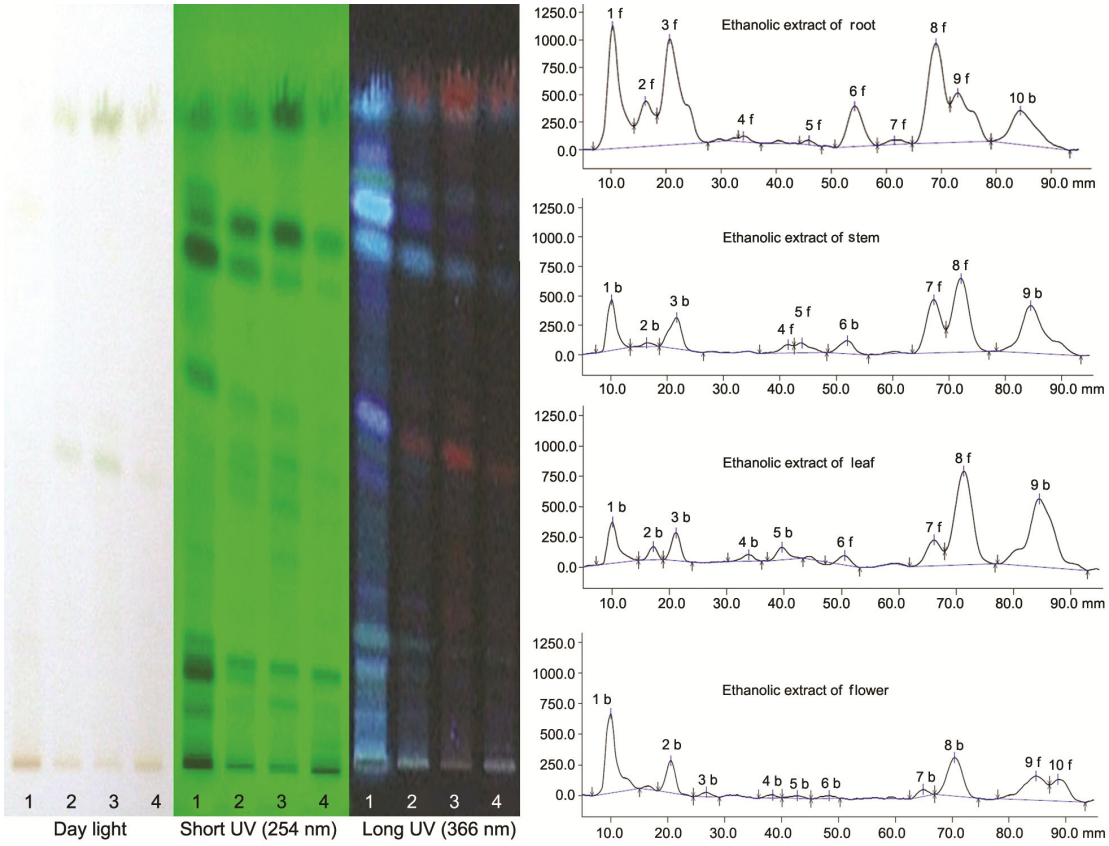

Fig. 1 — HPTLC fingerprint of ethanolic extract of root, stem, leaf and flower of *T. pratense* L. daylight, 254 and 366 nm.

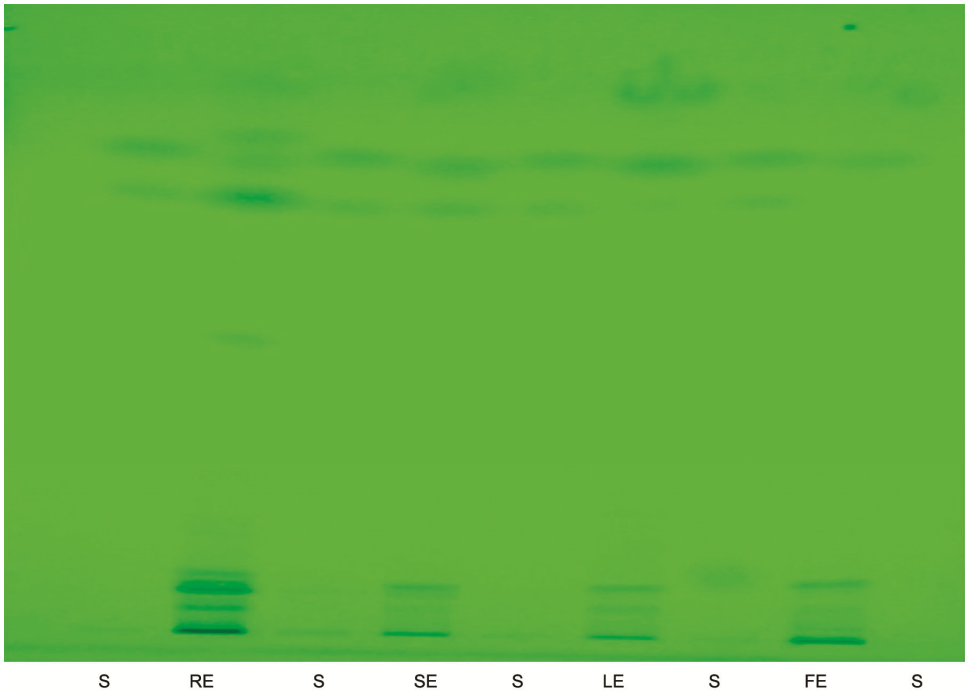

Fig. 2 — Densitogram of Formononetin and biochanin A compound at 254 nm, where S was formononetin and biochanin A, RE-root ethanolic extract, SE-stem ethanolic extract, LE-leaf ethanolic extract and FE-flower ethanolic extract, respectively.(Formononetin R_f = 0.73, Biochanin A R_f = 0.80)

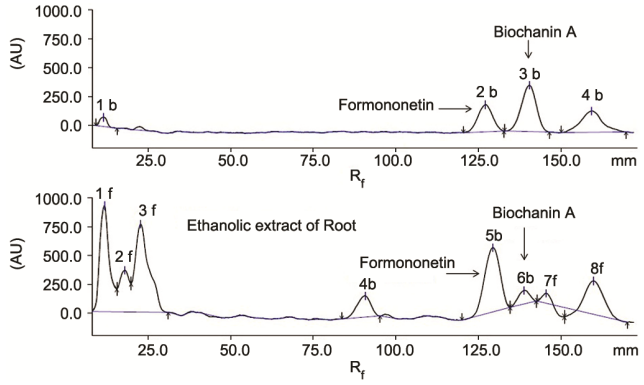

Fig. 3 — Chromatograms of standards (formononetin and biochanin A) and ethanolic root extract of *T. pratense* L.

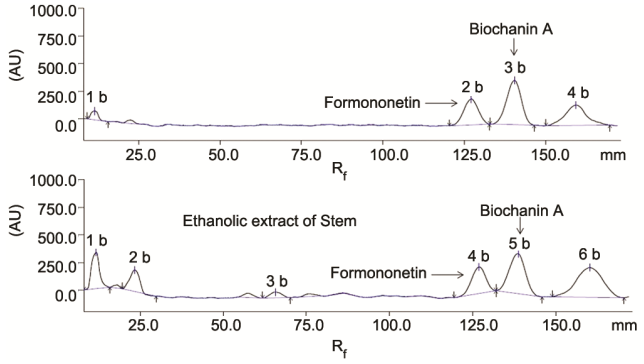

Fig. 4 — Chromatograms of standards (formononetin and biochanin A) and ethanolic stem extract of *T. pratense* L.

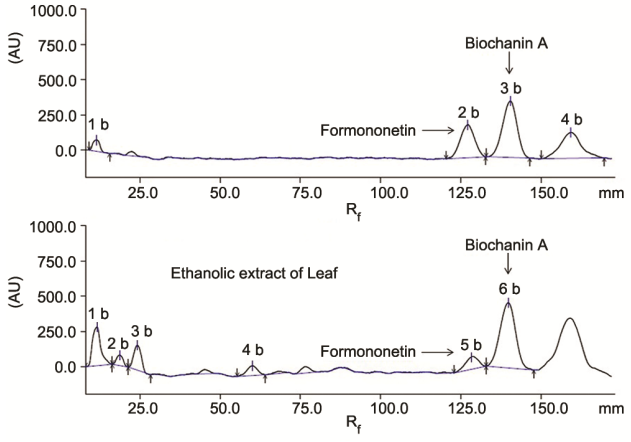

Fig. 5 — Chromatograms of standards (formononetin and biochanin A) and ethanolic leaf extract of *T. pratense* L.

type of cells have been reported for the first time in the current study which will provide additional information for the development of the quality standard of *T. pratense* plant. Only the root part of *T. pratense* shows the presence of globular starch grain in phelloderm region which is an important

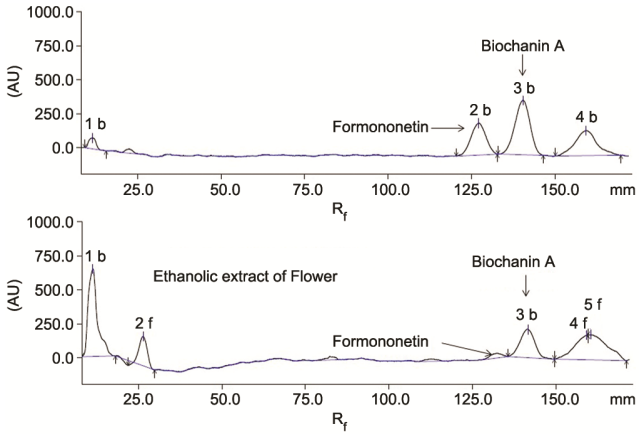

Fig. 6 — Chromatograms of standards (formononetin and biochanin A) and ethanolic flower extract of *T. pratense* L.

identifying character for roots of *T. pratense*. The major portion of the root is covered by phloem and xylem tissue. Secondary xylem and phloem are differentiated completely because of the well-developed vascular cambium. This study is the first endeavour to establish microscopy of the root of *T. pratense*.

Study of the physico-chemical property of medicinal plant with standard pharmacopoeial tests such as total ash value, acid insoluble ash value, water soluble ash, sulphated ash and extractive values provide valuable information about the quality of the crude drug.

The ash is generally made up of inorganic components and provides valuable information about the quality of the crude drug as each part of the plant has a specific ash value. The water-soluble extractive value and total ash of flowering top are available in the literature. However, total ash, acid insoluble ash, water soluble ash, sulphated ash and extractive value with different solvents have not been available in the literature for stem, leaves and root. The study results showed that the total ash value, acid insoluble ash and sulphated ash value are higher in leaf powder while water-soluble ash value is higher in flower powder. Thus, the results obtained in the present study will serve as an important tool regarding the chemical quality of root, stem, leaf and flower of *T. pratense* plant.

Extractive values of the crude drug with different solvents provide preliminary information about the quality of the crude drug. The study results revealed that the percentage extractability of flower powder with water, petroleum ether and ethanol is higher

compared to other parts. While percentage extractability of root power in methanol is greater compared to other powder. Most of the extracts are semisolid inconsistency and sticky in nature. Water extract of the flower is a solid mass inconsistency and hygroscopic in nature.

The results obtained after fluorescence analysis of the powdered crude drug is an additional important tool to provide unique information about the chemical property of the *T. pratense.* Ethyl acetate and petroleum ether extract of root showed unique purplish pink fluorescence in long UV light. Treatments of powdered root stem leaf and flower with sodium hydroxide in methanol give characteristic colour in daylight, short UV and long UV light.

Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, carbohydrates, saponins, steroids, tannins and phenolic as a major constituent of the plant. Alkaloid, steroid, tannin and phenolic compounds are distributed uniformly in root, stem, leaf and flower. However, saponins are present only in flower.

It is quite challenging to find precise marker compounds for herbal medicinal plants because herbal plants contain an abundant amount of chemical constituent and it varies with plant parts also. In such a situation, chromatographic fingerprinting of a specific part of the plant can provide valuable information about the chemical profile of the plant and their part. HPTLC fingerprinting of herbal medicine serves as a tool for authentication and identification of herbal medicine and their parts even though the concentration of the particular compound is not the same for different samples of crude drug[31].

HPTLC fingerprinting of ethanolic extract of root, stem, leaf and flower showed that some of the compounds are uniformly distributed throughout the plant with different concentration. The intensity of the chromatographic band showed that root contains more percentage of constituent followed by stem, leaf and flower for the majority of the compound with the same concentration of different extracts. In the ethanolic root extract, four compounds showed intense fluorescence at the R_f value of 0.78, 0.73, 0.64 and 0.54 at 366 nm. However, the compound separated at the R_f value 0.64 showed unique intense bright blue fluorescence at 366 nm which was not observed in the extracts of stem, leaf and flower. Some of the compounds separated in stem, leaf and flower showed the red colour band at 366 nm, while root extract showed the absence of a red colour band at 366 nm. Since *Trifolium* presence is known for its isoflavone all the four extracts were quantified using formononetin and biochanin A as a marker compound and chloroform: methanol as a solvent system for the separation of compounds. Formononetin was found in highest concentration in root followed by stem, leaf and flower. Ethanolic extract of the flower showed band for formononetin but concentration was not sufficient to quantify the compound. As per the result obtained in the present study biochanin A was found in a higher percentage of leaf followed by stem, flower and root.

HPTLC fingerprinting of root extract showed the presence of an abundant number of constituents. It also shows that along with aerial part of the plant, roots of *T. pratense* can also be considered as a source of isoflavone and other constituents for the medicinal purpose.

Conclusion

T. pratense is a dietary supplement, specifically used by women as a phytoestrogen. It has high medicinal value and many therapeutic actions. Thus, the current study on the development of quality control standards for different parts of the *T. pratense* will provide valuable information for its identification, authentication and quality control.

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