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Pharmacognostical and Phytochemical Investigation of *Cynodon dactylon* L. Pers. leaves.

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Abstract
Pharmacognostical parameters for *Cynodon dactylon* were studied with the aim of drawing the pharmacopoeial standards for this species. Macroscopical and microscopical characters, physicochemical contents, extractive values of the dry leaves powder and its reaction with chemical reagents were studied. The determination of these characters will aid in future for pharmacological analysis of this species.

Key Words
Cynodon dactylon, Leaf microscopy, extracts, phytochemical screening.

Introduction
Cynodon dactylon (Family: Poaceae) is an important ingredient in various Ayurvedic preparations. It is reported[1-3] to have important properties like anabolic, antiseptic, astringent, cyanogenetic, demulcent, depurative, laxative, diuretic and emollient. A decoction of the root is used as a diuretic in the treatment of dropsy and secondary syphilis. An infusion of the root is used to stop bleeding from piles. The juice of the plant is astringent and is applied externally to fresh cuts and wounds. Internally it is used in the treatment of chronic diarrhea and dysentry. It is also useful in the treatment of catarrhal ophthalmia. The cytomorphological report of the leaves of *Cynodon dactylon* has been found inadequate, therefore the detailed pharmacognostical study of the leaves of *Cynodon dactylon* was decided to undertake to bring out the salient diagnostic features which would help in crude drug identification as well as standardization of the quality and purity of the drug in crude form. The generated data can also be used for the development of monograph on this plant. The present investigations are based on macroscopical, microscopical, physicochemical and phytochemical studies of *Cynodon dactylon*.

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Material and Methods
Collection and Authentication of the Plant Material
The fresh leaves of *Cynodon dactylon* was collected from the college campus of IFTM, Moradabad (U.P., India) in the month of October 2008. The plant material was identified and authenticated by Prof V.K. Lal, Director College of Engineering and Technology, Sitapur. The voucher specimen was also preserved for future reference. The collected leaves were shade dried for 15 days and size reduced by laboratory grinder into coarse powder. It was stored in a well closed container free from environmental climatic changes until usage.

Morphological investigation
The healthy plants and their normal parts were selected for the morphological investigation. The morphological characters like shape, size, colour and odor of the leaves were observed.

Microscopical Investigation
Preparation of specimen
The healthy leaves were selected carefully for the microscopical characters. The specimen leaves of *Cynodon dactylon* were fixed in FAA (formalin;5ml + acetic acid;5ml + 70% ethyl alcohol;90ml). After 24 h of fixing, the specimens were dehydrated with a graded series of TBA (*tert*-butyl alcohol) as per the schedule[4]. Infiltrations of the specimens were carried out by gradual addition of paraffin wax (melting point 58-60 ^{o}C) until the TBA solution attained super saturation. The specimens were cast into paraffin blocks.

Sectioning of specimen

The paraffin embedded specimens were sectioned with the help of rotary microtome. The thicknesses of the sections were 10-12 µm. De-waxing of the section was done by customary procedure[5]. The sections were stained with toluidine blue as per the methods[6]. Since toluidine blue is a polychromatic stain, the staining results were remarkably good and some cytochemical reactions were also observed. The dye rendered a pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage and blue to the protein bodies. Whenever necessary, sections were also stained with safranin, fast-green and N/10 iodine to identify the presence of lignified cells and starch grains. To study the histology of stomata, venation pattern, trichome distribution, and paradermal section (parallel to the surface of leaf) were taken. The clearing of leaf was done with a 5% NaOH or epidermal peeling by partial maceration, employing Jeffrey's maceration fluid[4]. The mounting of macerated/cleared materials was performed with glycerine. Powdered material of different parts was cleared with NaOH solution and mounted in a glycerine medium after staining. Different cell components were studied and measured.

Photomicrographs

Microscopic description of tissues can be described with micrographs whenever necessary. Photographs of different magnifications were taken with a Nikon labphoto-2 microscopic unit. For normal observation a bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since, these structures have birefringent properties under polarized light; they appear bright against dark background. Magnifications of the figures are indicated by the scale-bars.

Physicochemical Evaluation

Physical constants of crude drugs like loss on drying, total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive and water soluble extractive value were determined by using official methods[1-7].

Preparation of extracts

The dried and coarsely powdered leaves (100 g) were extracted successively with petroleum ether (40-60 ºC), chloroform (59.5-60ºC), and ethanol (90%) in a Soxhlet extractor by continuous hot percolation[8]. Finally the marc was macerated with chloroform water. Each time before extracting with the next solvent of higher polarity the powdered drug (marc) was dried in a hot air oven below 50 ºC for10 min. Each extract was concentrated by distilling off the solvent, which was recovered subsequently. The concentrated extracts were evaporated to dryness and the extracts obtained with each solvent were weighed. All the extracts were concentrated in vacuum and preserved. The colour, consistency and percentage yield of the extracts were also noted.

Preliminary phytochemical investigation

The extracts obtained during the extraction process were subjected to preliminary phytochemical screening to determine the presence of various phytoconstituents like alkaloids, carbohydrates and glycosides, phytosterols, fixed oils and fats, saponins, phenolic compounds, triterpenes, flavonoids, proteins and amino acids, gums and mucilage, tannins and volatile oils by reported methods[8-11].

Thin layer chromatographic examination

After concentration and drying of each extract in a vacuum dessicator, identification of phytoconstituents was carried out by thin layer chromatography using different detecting reagents[12]. The test extract was dissolved by using the appropriate solvent in a concentration of 1 mg/ml and subjected for spotting. Silica gel was used as a stationary phase and two solvent systems were used as a mobile phase; Toluene-Propanol (70:30) and Toluene-Propanol (75:25). Spots were detected by using KOH (for glycoside), Dragendroff's reagent (for alkaloids), UV light (for flavonoids) and vanillin-sulphuric acids (for saponin and volatile oils) as detecting reagents. The R_f values were also noted.

Fluorescence analysis[13]

A small quantity of dried and finely powdered leaves and extracts was placed on a grease free clean microscopic slide and added 1-2 drops of the freshly prepared reagent solution, mixed by gentle tilting the slide and waited for 1-2 min. Then the slide was placed inside the UV viewer chamber and viewed in day light, short (254 nm) and long (365 nm) ultraviolet radiations. The colours observed by application of different reagents in different radiations were recorded.

The Fluorescence nature of the different extracts like petroleum ether, chloroform, ethyl acetate, ethanol and aqueous were observed in U.V.254 nm, U.V 366 nm and day light.

Results and Discussion

The following results were obtained under different headings.

Morphological investigation

Morphological examination of *Cynodon dactylon* reveals the following interpretations: Leaves 2-10 cm x 1.25-3 mm, narrowly linear or lanceolate, acute, glaucous-green and soft. Spikes were 2-6 in no. radiating from slender ascending peduncle, green or purplish, grain- 1.05 mm long. Root was fibrous, cylindrical, upto 4 mm thick, minute hair like roots arising from the main root; cream coloured. Stem was slender, prostrate, upto 1 mm thick, jointed, leafy, very smooth, and yellowish-green in colour.

Microscopic investigation

Microscopic examination revealed that the leaf was bilateral, mesomorphic and stomatiferous. The surface of the leaf was even, smooth on the ad axial side and roughly echinate on the lower side.

Structure of the leaf-blade along the distal (terminal) part

The leaf is broadly un-shaped in outline (Fig. 1.1). It has fairly prominent mid vein; the lateral veins are more or less equal in prominence. The lamina is smooth on the abaxial side and ridged on the abaxial side. The region where a lateral vascular bundle is situated, the surface is raised; in between the vascular bundles, the surface is furrowed. The midrib part is 100-120µm thick; the middle part of the lamina is 80-90µm thick; the marginal part is 40µm thick. The adaxial epidermal layer lying over the vascular bundles has small circular cells with prominent eclinate projections (Fig 1.2). The epidermal cells in between the vascular bundles are modified into motor cells or bulliform cells. There is shallow furrow in the region of the bulliform cells, where the epidermal cells gradually increase in size; the middle cell of the epidermis is the largest beneath the largest cell, a vertical row of three small cells. Thus, the bulliform apparatus consists of a vertical band of dilated cells with an adaxial furrow (Fig. 1.3). The largest bullifofm cell is 30x50µm in size. The abaxial epidermis is uniform having squarish or circular cells and very thick, smooth cuticle.

Midrib vascular- bundle is collateral comprising of two metaxylem elements and large mass phloem in between and beneath the xylem elements (Fig. 1.3). The vascular bundle is surrounded by a single layer of sclerenchyma cells. The cells at the lower portion are smaller and thick walled; those at the upper portion are wider and comparatively thin walled.

Median lateral vascular bundles (Fig. 2.1)

The vascular bundles in the apical median (distal middle) part are smaller than the midrib bundle. The adaxial epidermis has circular cells with papillate walls. Abaxial epidermis is smooth with circular cells and thick cuticle. The leaf blade has shallow furrows where the epidermal cells and a vertical row of mesophyll cells form bulliform-apparatus. The vascular bundles are collateral small, less distinct nests of xylem and phloem. The vascular tissues are enclosed all around by five or six dilated chloroplast bearing bundle sheath cells, without any gap in the sheath (Fig.2.1).

Distal Marginal Portion

The epidermal layers on the both adaxial and abaxial sides are smooth; the cells are small squarish and thickly cuticularised (Fig. 2.2). There is a distinct marginal vascular bundle which is circular having a single layer of chlorophyllous bundle sheath cells; no sclerenchyma layer or sclerenchyma pads are evident.

Middle portion of the Lamina

The middle part of the lamima is nearly 3mm wide. The adaxial middle portion is slightly concave and margins are slightly curved upward. The adaxial surface is indulate with shallow ridges and furrows due to the presence of the projecting vascular bundles (Fig. 3.1 & 3.2). The abaxial side is smooth and more or less even.

Midrib region

The midrib region is 130µm thick. It has a collateral vascular bundle with two wide metaxylem elements, a wide mass of phloem, sclerenchymatous inner sheath and inverted urn-shaped, chlorenchymatous bundle sheath with dilated cells; the lower open end of the bundle sheath has thick band of sclerenchyma cells; no sclerenchyma elements are seen on the adaxial end (Fig. 3.3).

Middle region- Marginal part

The marginal portion is 50µm thick. It is bluntly conical and have heavily thick walled epidermal layer of cells (Fig. 4.1). The vascular bundle is smaller than the midrib and median bundles. The

bundle sheath is open on the lower end (no bundle sheath cells). The sclerenchyma pads and sclerenchymatous inner layer are lacking. The vascular bundles are less prominent.

Middle region- Mid part

The mid portion is 110µm thick. It has echinate adaxial epidermis and smooth thick walled abaxial epidermis (Fig. 4.2). The vascular bundle has liracket-shaped, chlorophyllous, dilated outer bundle sheath and thin layer of seriate sclerenchymatous inner bundle sheath. The adaxial portion of the bundle sheath has nonchlorophyllous, hyaline, thick walled cells. Thick pads of sclerenchyma cells occur both at the adaxial and abaxial ends of the vascular bundle.

Proximal part (lower part) of the Leaf blade

The lower part of the leaf blade has wide shallow concavity on the adaxial with more or less flat lateral wings. It is nearly 3mm wide. It has prominent echinate adaxial epidermis and smooth or echinate cells at certain place (Fig. 5.1 & 5.2). The midrib bundle is the big and the lateral bundles are gradually reduced in size as they reach the margins.

Midrib Vascular bundle

The vascular bundle of the midrib is circular with two metaxylem elements and a thick mass of phloem. The structure is similar to the vascular bundle of the middle part of the lamina. There is an inner thin layer of sclerenchymatous bundle sheath which has wide cells at the upper end; outer to the sclerotic layer, there is a bracket shaped layer of chlorophyllous dilated bundle sheath cells; the cells at the upper end of the bundle sheath, there is a group of two or three hyalive cells. Sclerenchyma pads are seen both on the upper and lower ends of the vascular strand (Fig 5.3).

Proximal part: Marginal portion (Fig. 6.1)

The marginal portion is blunty conical and slightly directed above. It is 50µm thick. The epidermal layers are smooth, the cells are small and the walls are much thicker. There is a small circular vascular bundle with reduced xylem and phloem strands. There is no inner sclerenchyma bundle sheath. The outer chlorenchymatous bundle sheath is present; it consists of two or three larger lateral cells and smaller cells at upper and lower ends. Outer to the bundle sheath occurs a rosette of radially oblong chlorophyllous palisade tissue.

Proximal middle portion

The middle portion of the lamina is 110µm thick. It has echinate adaxial epidermal layer and similar echinate epidermis on the abaxial side as well. The bulliform apparatus is wider towards the adaxial side and conical along the lower side. The vascular bundle has well developed collateral xylem and phloem tissues (as in the midrib region); the bundle has inner sclerenchyma bundle sheath whose cells are wider at the upper end; the outer chlorenchymatous bundle sheath has large dilated cells. The dilated outer sheath is incomplete on the upper and lower regions where there are small hyalive cells. A thin layer of radially aligned palisade cells occur on the side of the vascular bundle. A horizontal thick pad of sclerenchyma cells occur at the lower and upper ends of the bundle.

Physicochemical evaluation

Physical evaluation revealed that loss on drying was 7.09%, total ash- 6.35%, acid insoluble ash- 3.83%, water soluble ash- 5.76%, alcohol soluble extractive- 3.86%, water soluble extractive- 5.36% w/w in leaves of *Cynodon dactylon.* The results are presented in Table 1.

Average extractive values

During successive solvent extraction of leaves of *Cynodon dactylon*, the percentage yield was determined in petroleum ether extract (0.85%), chloroform extract (1.25%), ethanol extract (2.85%) and in water extracts (3.65%). The colour of each extract was also noted during the extraction process (Table 2).

TLC profile of hydro-alcoholic extracts

The preliminary phytochemical studies with the help of thin layer chromatography (TLC) revealed the prominent presence of protein and amino acids in the extracts (Rf value is 0.25, 0.47, 0.58 and 0.23, 0.44, 0.55, respectively in two solvent systems). The results are shown in Table 3.

Fluorescence analysis

The fluorescence nature of the leaf powder of *Cynodon dactylon* with different reagents was studied in day light, U.V. 254 nm and U.V. 366 nm. The Fluorescence nature of the different extracts like petroleum ether, chloroform, ethyl acetate, ethanol and aqueous was also observed in day light, U.V. 254 nm and U.V. 366 nm (Table 4 & 5).

Preliminary Phytochemical investigation

The preliminary phytochemical screening with the various chemical tests (qualitative) revealed the

presence of different chemical constituents in ethanolic and aqueous extracts. The results were shown in Table 6.

This study on micro-morphological features of *Cynodon dactylon,* revealed a set of anatomical parameters which may enable to those who handle this plant to maintain its quality control. Morphological as well as microscopical studies of plants are the primary steps to establishing their botanical standards before going to other studies. As per WHO norms, botanical standards are the proposed as a protocol for the diagnosis of the herbal drug. The pharmacognostical parameters are helpful for the future identification and authentification of the plant in the herbal industry. The physical parameters, such as loss on drying, ash values and extractive values will be helpful to identify the authenticity of the drug even from the crushed or powdered plant materials. It will serve as a standard data for the quality control of the preparations containing this plant in future. The information obtained from the ash values and extractive values are useful during the time of collection and also during extraction process. Using these standards, the plant can be differentiated from other related species. The leaf constants can be included as microscopical standards in the Indian Herbal Pharmacopoeia. The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, saponins, sterols etc. Thus the preliminary screening tests may be useful in the detection of bioactive principles and subsequently may lead to the drug discovery and development. Further, these tests facilitate their quantitative estimation and qualitative separation of pharmacologically active chemical compounds. Phytochemical study was also useful to isolate the pharmacologically active principles present in the drug. More phytochemical research work is required for isolation, purification and characterization of biologically compounds.

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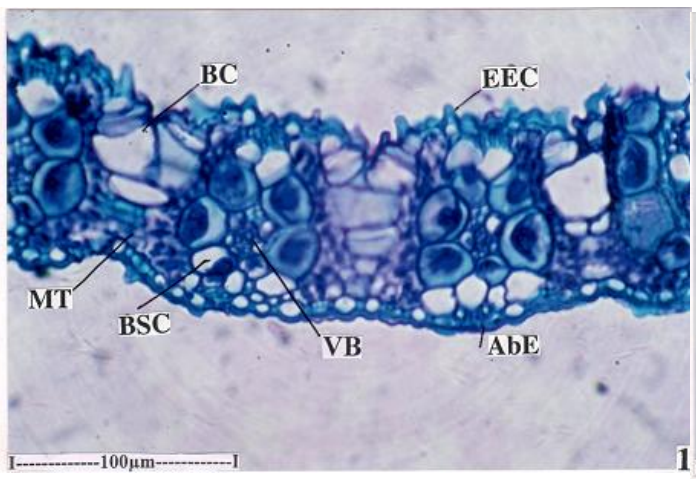

Fig. No. 2.1: T.S of Lamina

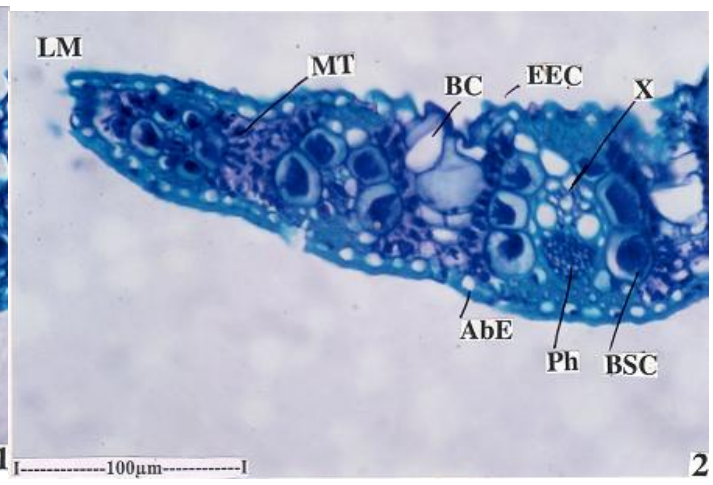

Fig. No. 2.2: T.S of Leaf Margin

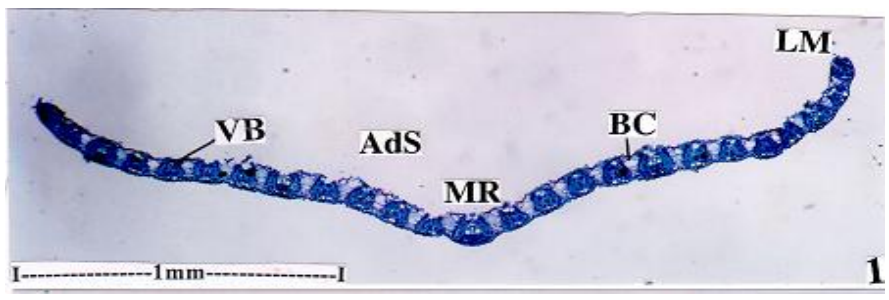

Fig. No. 3.1: T.S of Leaf through midrib with lamina (Middle portion)

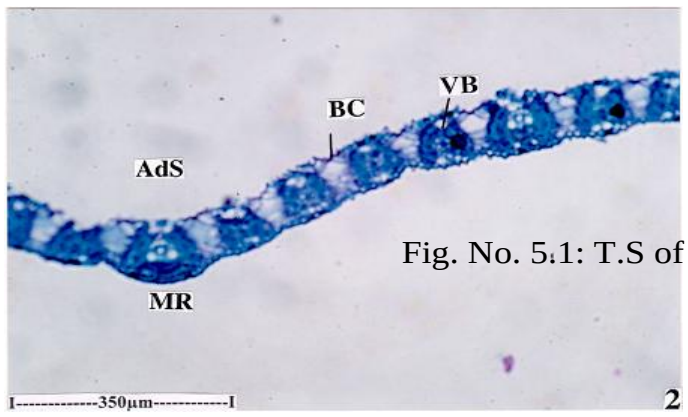

Fig. No. 3.2: A sector of midrib with lamina magnified

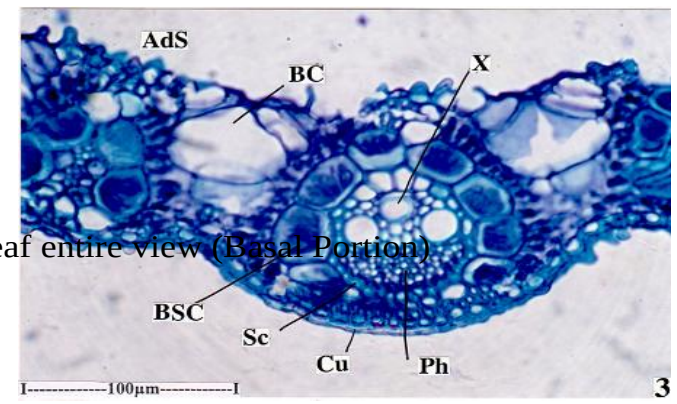

Fig. No. 3.3: Midrib vascular bundle enlarged

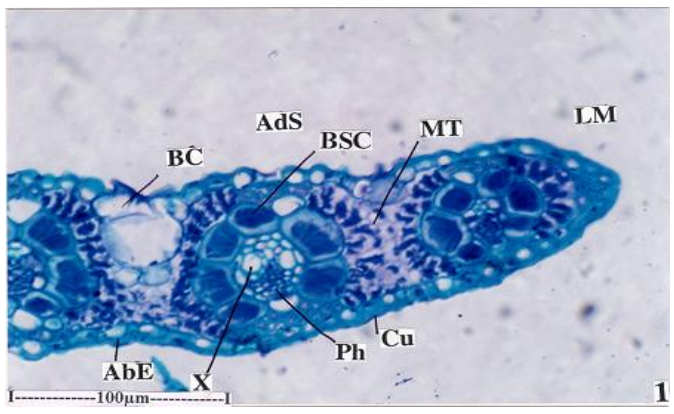

Fig. No. 4.1: T.S of the Leaf Margin

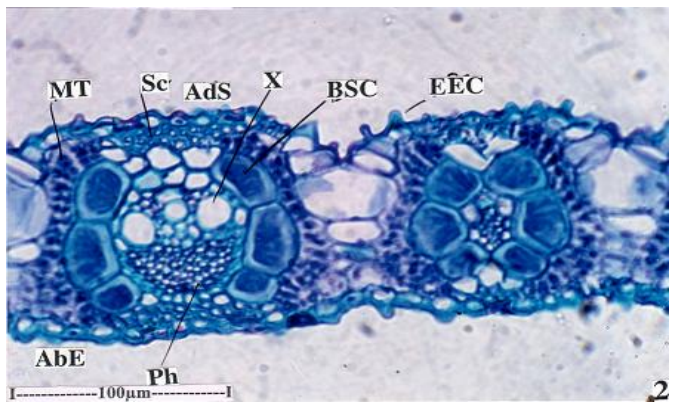

Fig. No. 4.2: T.S of Lamina through lateral vein

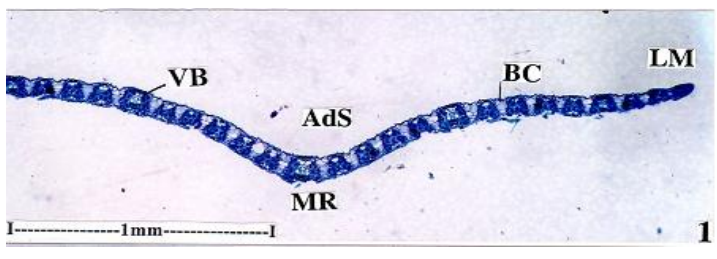

Fig. No. 5.1: T.S of the Leaf entire view (Basal Portion)

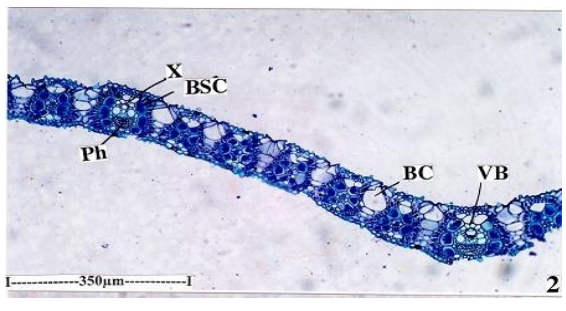

Fig. No. 5.2: A portion of Midrib with Lamina enlarged

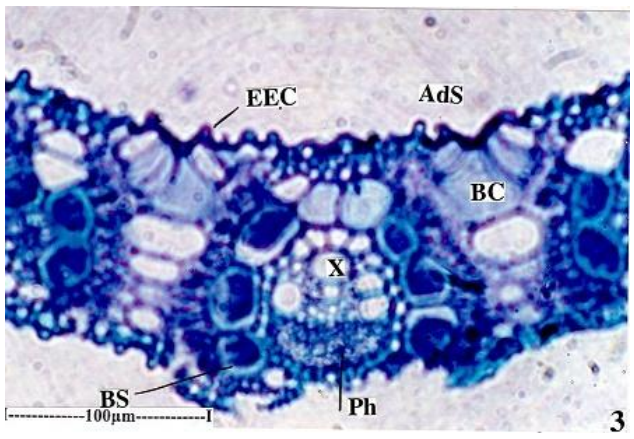

Fig No.5.3- Midrib vascular bundle enlarged

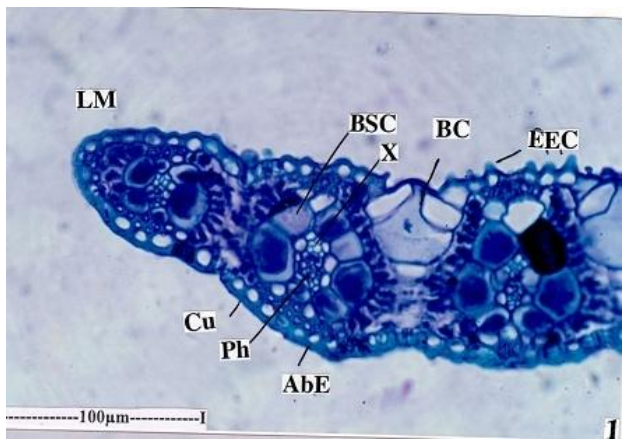

Fig. No. 6.1: T.S of the Leaf Margin

Table 1: Physical parameters of leaves of *Cynodon dactylon* (L.) Pers.

S. No.	Parameters	Observed value (%w/w)
1.	**Loss on Drying**	7.09%
2.	**Ash values**	
	Total ash	6.35%
	Acid insoluble ash	3.83%
	Water soluble ash	5.76%
3.	**Extractive values**	
	Alcohol soluble extractive	3.86%
	Water soluble extractive	5.36%

Table 2: Percentage yield of various extracts of leaves of *Cynodon dactylon* (L.) Pers.

S. No.	Extract	Colour	% Yield (w/w)
1.	Petroleum ether extract	Yellowish green	0.85
2.	Chloroform extract	Deep green	1.25
3.	Ethanol extract	Blackish green	2.85
4.	Aqueous extract	Dark green	3.65

Table 3: TLC of hydro-alcoholic extract of leaves of *Cynodon dactylon* (L.) Pers.

S. No.	Solvent system	Rf value	No. of spots
1.	Toluene : Propanol (70 : 30)	Rf_1=0.25 Rf_2=0.47 Rf_3=0.58	3
2.	Toluene : Propanol (75 : 25)	Rf_1=0.23 Rf_2=0.44 Rf_3=0.55	3

Table 4: Fluorescence analysis of leaf powder (treated) of *Cynodon dactylon* (L.) Pers.

S. No.	Treatment (Powder + reagent)	Day light	U.V. Light (254 nm)	U.V. Light (366 nm)
1.	Powder + Wagner reagent	Brown	Green	Deep black
2.	Powder + Mayer's reagent	Brown	Blackish green	Black
3.	Powder + Dragendroff's reagent	Yellowish brown	Light green	Deep black
4.	Powder + Iodine solution	Dark green	Green	Black
5.	Powder + Millon's reagent	Yellowish brown	Light green	Blackish green
6.	Powder + Ninhydrin solution	Light green	Light green	Blackish green
7.	Powder + 5% Lead acetate solution	Light green	Green	Black
8.	Powder + Tannic acid	Yellowish green	Greenish	Blackish green
9.	Powder + 10% Picric acid	Yellowish green	Green	Deep black
10.	Powder + 5% $FeCl_3$ solution	Yellowish brown	Yellowish green	Blackish green
11.	Powder + dil NH_3 solution	Dark green	Greenish	Blackish
12.	Powder + dil HCl	Greenish brown	Greenish	Blackish green
13.	Powder + Conc. H_2SO_4	Black	Blackish green	Deep black
14.	Powder + Conc. HCl	Dark green	Green	Blackish
15.	Powder + 5% NaOH solution	Yellowish green	Green	Blackish green
16.	Powder + Conc. HNO_3	Reddish yellow	Dark green	Blackish
17.	Powder + dil H_2SO_4 solution	Light green	Green	Blackish

Table 5: Fluorescence analysis of extracts of leaves of *Cynodon dactylon* (L.) Pers.

S. No.	Extract	Day light	U.V. Light (254 nm)	U.V. Light (366 nm)
1.	Petroleum ether	Deep green	Greenish brown	Blackish
2.	Chloroform	Green	Greenish black	Black
3.	Methanol	Yellowish green	Greenish brown	Blackish
4.	Aqueous	Green	Greenish black	Deep black

Table 6: Preliminary phytochemical screening of extracts of *Cynodon dactylon* (L.) Pers.

S. No.	Test	Aqueous extract	Alcoholic extract
1.	Alkaloids	-	-
2.	Glycosides (cardiac)	-	-
3.	Glycosides (anthraquinone)	-	-
4.	Organic acids	++	-
5.	Carbohydrates	+	-
6.	Proteins & Amino acids	++	++
7.	Saponins	-	-
8.	Gums & Mucilage	-	-
9	Terpenoids	-	+
10.	Steroids	-	-
11.	Flavonoids	+	-
12.	Tannins/ Phenolic compound	+	-
13.	Reducing sugars	-	-

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