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COMPARATIVE PHARMACOGNOSTIC CHARACTERIZATION OF AERIAL PART AND LEAF CALLUS OF *MOLLUGO PENTAPHYLLA* L. A POTENT ETHNO MEDICINAL HERB

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Keywords:

In-vivo and *in-vitro*, Leaf callus, Macroscopy, microscopy, *Mollugo pentaphylla* and pharmacognostic

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ABSTRACT: *Mollugo pentaphylla* L. (Molluginaceae), commonly known as carpetweed, has tremendous medicinal importance. Although the plant has been scientifically evaluated for its various biological activities but no work has not been carried out till date on comparative pharmacognostic characterization of the *in-vivo* plant and leaf callus. This study aims to investigate the comparative pharmacognostic and physicochemical standards for aerial parts and leaf callus of *M. pentaphylla*. The measures taken were macroscopic, organoleptic study, anatomy, powder microscopy, ash values, loss on drying, fluorescence analysis and extractive yield. This is the first study providing complete pharmacognostic profile of *M. pentaphylla* and hence will be useful for identification and authentication of the species for future studies.

INTRODUCTION: *Mollugo pentaphylla* L. (Molluginaceae) annual herb, commonly known as carpet weed, is distributed throughout tropical regions of India, China, Malaysia, Japan, Taiwan, Australia, Nepal [1, 2]. In India, it is distributed in Karnataka, Maharashtra, Bengal, Tamil Nadu and Kerala. It is fairly a common weed in cultivated land, road sides and waste land and is seasonal. In Indian folk medicine, *Mollugo pentaphylla* has been used as an emmenagogue, stomachic, aperients and antiseptic properties. An infusion of the plant is given to women to promote the menstrual discharge. It is used as blood purifier, improves digestion, stimulate the action of liver and cures burning sensation and skin diseases.

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Traditionally, the plant is also used as diuretic, anthelmintic, anti-inflammatory [3], digestive, constipating, spermicidal, antioxidant [4], antimalarial, antiviral property [5] and anticancer activity [6]. Many rural people are consume this plant as a green vegetable mainly leaf and stem portion. But the other part like stem and root of this plant is also has a great medicinal value. The urban people used this plant medicinally in the form of orally and externally for the treatment of skin allergic condition [5].

Despite the tremendous medicinal importance of *Mollugo pentaphylla*, there is insufficient information available on the pharmacognostic parameters for identification and standardization of the species. In this connection, whole aerial part (leaves, stem, flowers and fruits) with leaf callus of this plant were examined. The present work is an attempt to provide comprehensive report on the quality control and standardization parameters of *M. pentaphylla*. The pharmacognostic constant of plants, the diagnostic microscopic features and

standards reported could be useful for the compilation of a suitable monograph for their proper identification.

METHODS AND MATERIALS:

Plant Material: Healthy and disease free aerial parts of *M. pentaphylla* **Fig. 1A**, was collected from Karnatak University campus, Dharwad, state of Karnataka during the month of August. The collected specimens were identified and deposited in Karnatak University Herbarium (KUD) under voucher number KUD/BOT/AN/JM/002.

Processing of Plant Material and Preparation of Extract: The aerial part of plant was washed thoroughly and dried in room temperature. The dried plant samples were ground in to fine powder using electric grinder and 15-20 gram of aerial part powder thus obtained were extracted using Soxhlet extractor for 10-16 hrs at 30-60 ºC with the solvents of increasing order of polarity namely, petroleum ether, chloroform, acetone, ethanol, and water using Soxhlet apparatus for 24 hrs [7]. The extracts were used for the phytochemical analysis, powder was used for the further powder microscopy, macroscopy and phytochemical studies **Fig. 1D *and* 1F.**

Induction, Processing of Leaf Callus and Preparation of Leaf Callus Extract: Healthy leaves were selected and washed thoroughly under running tap water for 15 min with two drops of Tween 20 detergent solution for 10 min. Subsequently, they were thoroughly washed under running tap water until the traces of Tween 20 is removed and then rinsed with distilled water. The remaining steps of surface sterilization were carried out under aseptic conditions in the laminar airflow chamber. The leaf explants was then subjected to 70% ethanol treatment for 30 sec and again washed with distilled water at least three minutes. After washing with distilled water, surface sterilization was done with mercuric chloride (0.1%w/v $HgCl_2$) solution for 2 min and rinsed four to five times with sterilized distilled water to remove traces of mercuric chloride. Thus sterilized leaf explants were inoculated on to Murashige and Skoog (MS) medium for induction of callus. The medium was also supplemented with various plant growth regulators, which include auxin NAA (naphthalene acetic acid) and cytokinin BAP (6 benzylaninopurine) in different concentrations and combination (0.1-0.5 mg/L). The pH of the media was adjusted to 5.8 before autoclaving. All media were autoclaved at 121ºC for 15 min. The cultures were incubated in a growth chamber lab temperature of 25±2 ºC with relative humidity 55±5 and 16-h photoperiod.

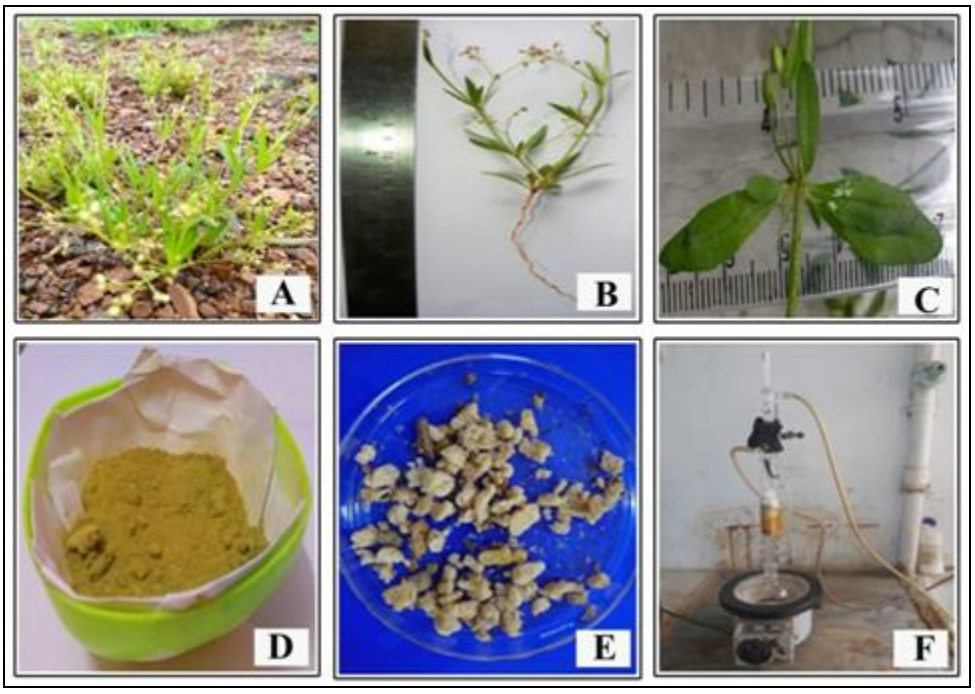

FIG. 1: MACROSCOPIC STUDY OF *MOLLUGO PENTAPHYLLA* L. AND LEAF CALLUS. A. Habit B. Size of the plant- 5 to 10 cm. C. Size of leaf-1 to 2 cm. D. Aerial part plant powder. E. Leaf callus. F. Soxhlet extraction apparatus.

Callus induction from leaf explants on MS medium with plant growth regulators observed regularly till required quantity of callus is available or grown **Fig. 1E** and **Table 2**. The experiment was set up in complete randomized design with 3 replicates, each replicate consisted of 12 ramets (explants). The leaf callus thus obtained was carefully taken out from the culture tubes and washed with slightly warm sterile distilled water to remove the agar traces and air-dried in the oven in the laboratory at lab temperature for 4 to 5 weeks. The dried callus were ground into a fine powder using the mechanical machine and the powder thus obtained was subjected extraction using petroleum ether, chloroform, acetone, ethanol and water solvent using Soxhlet extractor for 10-16 hrs at 30-60 ºC depending on the solvent used [8].

Statistical Analysis: From the day of inoculation and to initial callus formation, the morphology and color of the callus were recorded. At the end of the observation period, percentage of the explants forming callus as well as the degree of callus formation was measured. Data was analyzed using IBM SPSS (Version 20). Mean values obtained for above parameters were compared and grouped using Duncan Multiple Range Test (DMRT) at 5% level of probability. ANOVA was performed to study significance of the results.

Histological Investigation of Callus: Leaf callus were fixed in Carnoy's fluid for 24 hours. The fixed callus was later washed thoroughly with running tap water till the trace of fixative is removed. Then the callus was subjected to the process of dehydration, infiltration and embedding by following conventional methods. Later the embedded material was cut into paraffin blocks.

These blocks later were cut with the help of semi automatic Leica microtome at 8µ-10µ thickness. Then the paraffin ribbons containing sections were affixed on to clean micro slides with the help of gelatin adhesive and kept for drying and stored. Later these micro slides were processed and stained with saffranin to study the developmental stages of callus as well as embryo [9].

Pharmacognostic Studies:

Organoleptic Evaluation: Fresh and healthy plants of *M. pentaphylla* were assessed for their morphological characteristics. The colour, size, shape, odour, taste, margin, base, texture, apex, venation, arrangement, of leaves and stem of plant were observed. Macroscopic and microscopic characters were observed and recorded as described in quality control method [7, 9].

Anatomy: Transverse sections of fresh materials of different aerial parts of *M. pentaphylla* were cut with the help of sharp blades. Peels were obtained from fresh leaves by forceps. Different peels were stained with safranin and fast green, observed under microscope and photographed [7, 9].

Powder Microscopy: The plant powder analysis, undertaken with pinch of fine powder mixed with the Phloroglucinol: HCL (1:1) ratio on glass slide and observed under the compound microscope and photographed [9].

Moisture Content (Loss on Drying): The weight after drying was noted and loss on drying calculated. The percentage calculated using the formula:

Percentage loss on drying = Loss in weight in sample (Powder) / (Moisture content) Weight of the sample (Powder) × 100

Ash Value: The percentage of ash value, calculated with total sample taken by using the formula:

% Total ash value = Weight of total ash / Weight of plant powder taken × 100

Sulphated Ash Value: Silica crucible was heated to redness for 10 minutes and cooled in a desiccator and weighed. A known quantity of sample was accurately weighed into the crucible and ignited gently until the substance was thoroughly charred. Residue was cooled and moistened with 1.0 ml of sulphuric acid, heated gently until no white fumes evolved. Ignited at 800 ºC ± 25 ºC till all the black particles disappeared. The crucible was allowed to cool and few drops of sulphuric acid was added and heated. Later ignited, cooled and weighed. Repeated the operation until 2 successive weights [9].

Determination of Extractive Value:

Ether Soluble Extractive: The percentage of ether soluble extractive, calculated with reference to the air-dried drug.

% of extractive value = Weight of residue / Weight of plant powder taken × 100

Alcohol Soluble Extractive: The percentage of alcohol soluble extract, calculated with reference to the air-dried drug.

% of extractive value = Weight of residue / Weight of plant powder taken × 100

Fluorescence Analysis: Fluorescence character of powder drug done under visible light, UV (short 254) light, UV (long 365) and after treatment with different reagents [10].

RESULT:

Macroscopic Description: *M. pentaphylla* is an annual, diffuse, glabrous, erect slender up to 5-10 cm height, stems with many more quadrangular leafy dichotomously arranged branches. Leaves 3-4, linear-lanceolate, obtuse, sometimes apiculate, much narrowed at the base, petioles obscure. Flowers are white, numerous, terminal cymes, peduncles and pedicels filiform, bracts lanceolate, Calyx glabrous, sepals long, broadly elliptic-oblong, parallel-nerved. Stamens usually 3-4, styles 3-4, short, linear, Capsules sub-globose slightly longer than the sepals with thin walls.

Ovary is bicarpellary syncarpus and is inferior. Seeds numerous, roundish-reniform, compressed, covered with raised tubercular points, dark-brown. Branched taproot of2-3 cm. Rooting occurs at the nodal region and fruit is a capsule. The organoleptic evaluation of *M. pentaphylla* is arranged in a tabulated form **Table 2**. Leaf callus was green compact obtained in combination of plant growth regulator cytokinin and auxin 0.5 mg/L of BAP and 0.5 mg/L of NAA with weight of callus 787.01±82.2 **Fig. 1B** and **1C.**

TABLE 1: INDUCTION OF LEAF CALLUS OF *MOLLUGO PENTAPHYLLA* L.

Sl. no.	MS medium supplemented with combination and concentrations of hormones (mg/L)		Weight of leaf callus(mg)
	BAP	NAA	
1.	0.1	0.1	287.54 ± 6.82^{c}
2.	0.2	0.2	504.18 ± 2.65^{b}
3.	0.3	0.3	674.52 ± 47.08^{b}
4.	0.4	0.4	725.23 ± 32.17^{a}
5.	0.5	0.5	787.01 ± 82.2^{ab}

Each value represents the mean ± Standard error of three replicates, followed by superscript letters through columns that differ significantly at P<0.005 level when subjected DMRT followed by SPSS. BAP, benzylaminopurine; NAA, naphthaleneacetic acid.

TABLE 2: ORGANOLEPTIC FEATURES OF AERIAL PART OF *MOLLUGO PENTAPHYLLA* L.

Characters	Observation	Observation
Part	**Leaves**	**Stem**
Arrangement	Whorled	-
Size	2-3 cm long, 1cm wide	0.2 cm thickness, 20-30 cm height
Shape	Linear- lanceolate	Straight cylindrical
Colour	Green	Green
Odour	-	-
Taste	Bitter	Bitter
Appearance	Smooth	Herbaceous
Margin	Entire	-
Apex	Acute	-
Base	Attenuate	-
Petiole	Small	-
Texture	Smooth	-
Veination	Reticulate	-
Outer surface	-	Green colour smooth surface

Microscopy:

T. S of Leaves: The transverse section of leaf of *M. pentaphylla*, is showed **Fig. 2A** leaf lamina dorsiventral in nature. The epidermis is single layer consists of rectangular parenchymatous cells. Epidermal layer is followed by 3-5 layers. The cells are thick and lignified and these are present above and below the midrib. Four groups of vascular bundles are present in the midrib region. The groups of vascular bundles are surrounded by parencymatous cells and xylem vessels are surrounded by the xylem parenchyma cells.

T. S of Stem: The transverse section of stem of *M. pentaphylla*, is showed **Fig. 2B** the epidermis layered with thick walled narrow and elongated cells. The epidermis was surrounded by cuticle. It is cylindrical in shape, the cortex consists of 3-4 layers, vascular bundles were surrounded by 2-3 layers of parenchyma cells and collenchymatous cells with starch granules which are followed by endodermis. Xylem is very prominent and occupies major portion of the stem. Vessels are arranged in radial rows with circular or polygonal in shape. Phloem tissue surrounds the xylem with inner centrally placed pith cells.

Histology of Leaf Callus: Transverse section of leaf callus showed endarc condition **Fig. 2C**, somatic embryos **Fig. 2D**, parenchyma cells **Fig. 2E** and vascular tissues **Fig. 2F.**

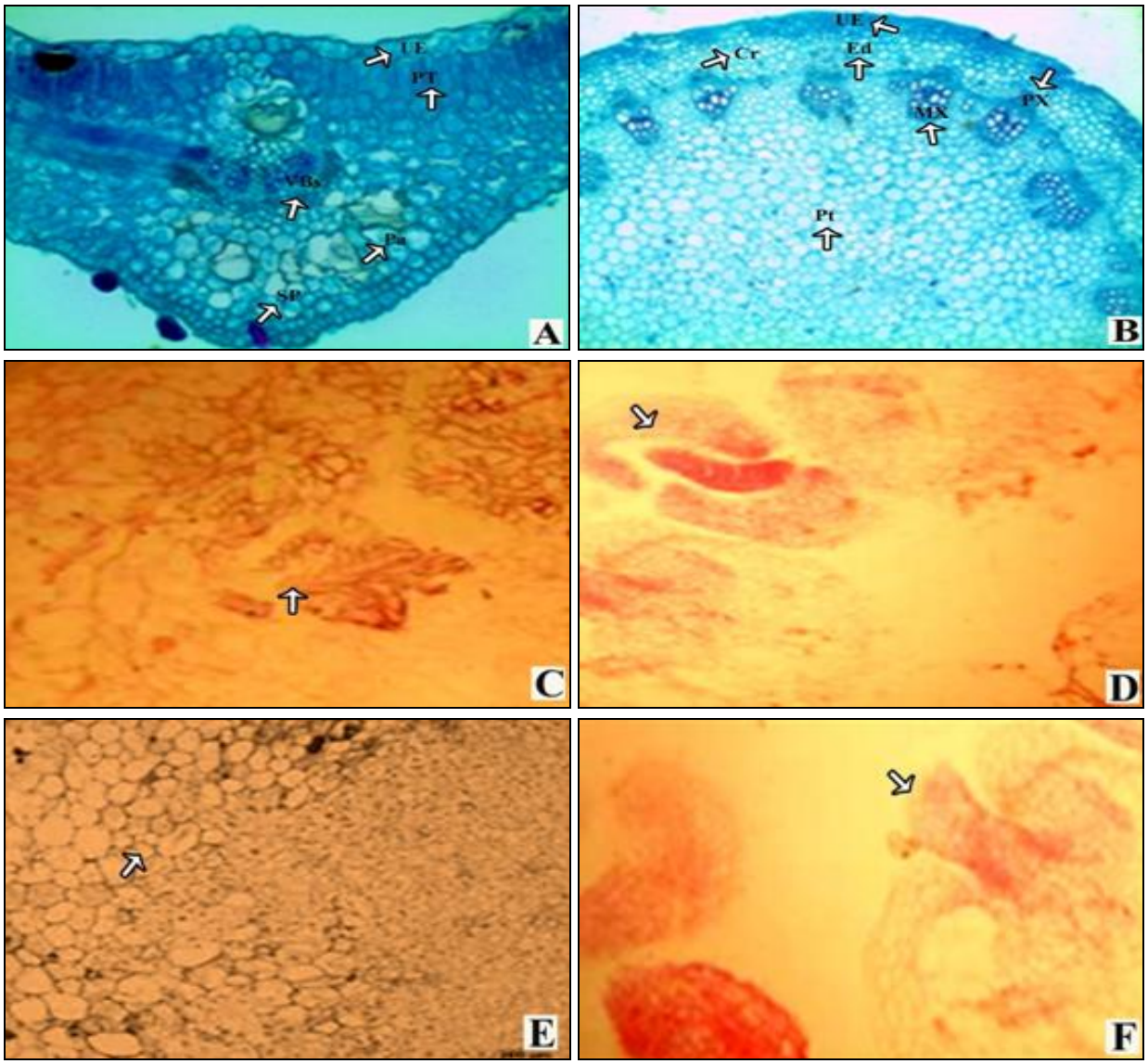

FIG. 2: MICROSCOPIC STUDY OF *IN-VIVO* AERIAL PART AND HISTOLOGY OF LEAF CALLUS OF *MOLLUGO PENTAPHYLLA* L. A. T.S of Leaf. B. T. S of stem. C. Section of leaf callus showing endarc condition. D. Section of leaf callus showing somatic embryos, E. Section of leaf callus showing parenchyma cells and F. Section of leaf callus showing vascular tissues.

Leaf Peel: Peels obtained from fresh leaves revealed a huge number of paracyticstomata with wavy epidermal cells. Stomata are present on both the surfaces, where upper surface has fewer in number of paracytic stomata than the lower surface **Fig. 3A** and **3B.**

Powder Microscopy: The powder microscopy characteristics determined from the powder study under microscopic investigation showed spiral vessel **Fig. 3C**, Bordered pitted vessel **Fig. 3D**, fibers **Fig. 3E**, pitted vessel **Fig. 3F**, Starch cells **Fig. 3G** and tannin cells **Fig. 3H.**

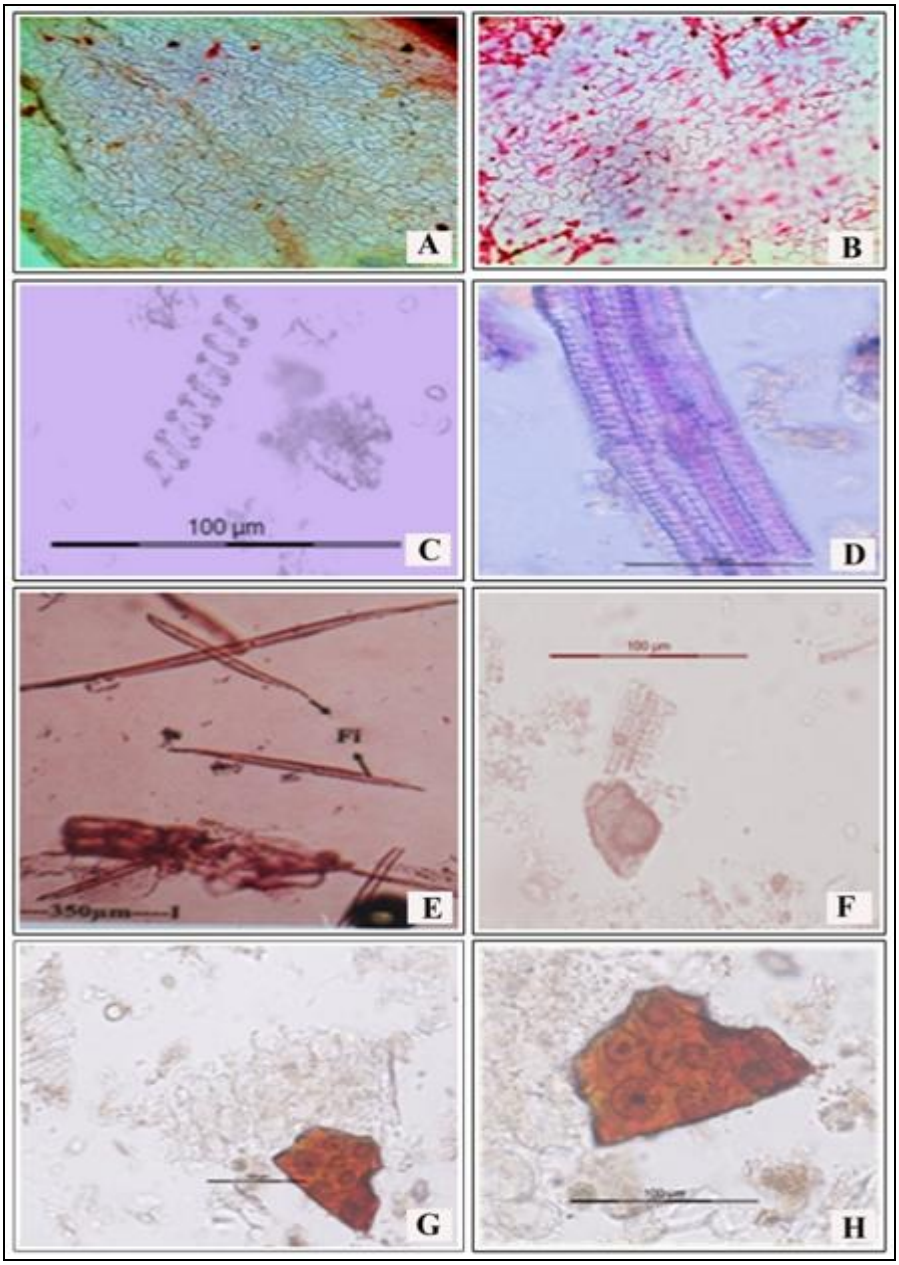

FIG. 3: STOMATA AND POWDER MICROSCOPIC STUDY OF *MOLLUGO PENTAPHYLLA* L. A. Upper epidermis stomata. B. Lower epidermis stomata. C. Spiral vessels. D. Bordered pitted vessel. E. Fibers. F. Pitted vessels. G. Starch cells. H. Tannin cells.

Physicochemical Analysis: The physicochemical analysis of *in-vivo* aerial part and leaf callus of *M.pentaphylla*. The results are recorded in a tabulated form **Table 3**. Total ash in *in-vivo* plant is 10.30±1.45% and in callus it is 5.40±1.40% and sulphated ash in plant is 13.11±3.72%, and in callus it is 4.06±2.03. Extractive values varied among the tested categories, ether soluble in wild plant is 12.32±3.59% and in callus it is 5.68±1.05%, in ethanol soluble plant is 13.50±0.73% and in callus it is 3.79±01.06% and in water wild plant is 13.00±2.61% and in callus it is 4.43±1.32%.

TABLE 3: PHYSIOCHEMICAL PARAMETERS OF *IN-VIVO* AERIAL PART AND LEAF CALLUS OF *MOLLUGO PENTAPHYLLA* L.

Sl. no.	Physicochemical evaluation	*In-vivo* plant	*In-vitro* leaf callus
1.	Moisture content	12.59±9.22 %	51.77±9.33%
2.	Ash value	10.30±1.45%	5.40±1.40%
	Water soluble ash value	5.43 %	3.80 %

	Acid insoluble ash value	6.21%	5. 09 %
3.	Sulphated ash value	13.11±3.72%	4.06±2.03%
4.		Extractive value	
a.	Ether soluble	12.32±3.59%	5.68±1.05%
b.	Ethanol	13.50±0.73%	3.79±1.06%
c.	Water	13.00±2.61%	4.43±1.32%

Fluorescence Analysis: Fluorescence analysis of *in-vivo* aerial part and leaf callus of *M. pentaphylla*, the powder is examined under fluorescent and UV light (365nm) to observe behavior crude drug and to produce different colour reactions. The results are arranged in tabulated forms **Table 4.**

TABLE 4: FLUORESCENCE ANALYSIS OF POWDERS OF *IN-VIVO* PLANT AND LEAF CALLUS OF *MOLLUGO PENTAPHYLLA* L.

Material with chemicals	*In vivo* plants			Leaf Callus		
	Light	UV light (Short)	UV Light (Long)	Day light	UV light (Short)	UV Light (Long)
Powder + Benzene	Light green	Dark green	Black	Dark green	Brown	Brown
Powder + Ethyl acetate	Greenish	Lemon yellow	Saffron	Green	Dark green	Black
Powder + 50% aqueous ethanol	Green	Dark green	Saffron	Yellow green	Green	Saffron
Powder + 1M HCl	Brown	Dark green	Brick red	Dark green	Brown	Brown
Powder + 1N NaOH aqueous	Dark Green	Transparent light green	Brick red	Light brown	Green	Brown
Powder + 1N NaOH alcoholic	Brown	Light green	Dark green	Greenish yellow	Green	Brown
Powder + Acetic acid	Light green	Dark green	Saffron	Green	Brown	Saffron
Powder + Nitric acid + Ammonia	Brown	Dark green	Brown	Light brown	Dark green	Saffron
Powder + Conc. Nitric acid	Yellow	Dark green	Black	Greenish yellow	Dark green	Brick red
Powder + 50% H_2SO_4	Green	Greenish	Brick red	Green	Dark green	Yellow

DISCUSSION: Plants are the rich source of secondary metabolites which gives them a wide variety of medicinal properties it's therefore, essential to identify these constituents from medicinal plants responsible for the treatment and cure of various ailments. In the past two decades, nearly two thirds of approved new drugs were obtained from natural plant products but the major drawback to the use of herbal medicine is the lack of standardization which in turn paves way for wrong identification, unintentional substitution of closely related species and adulteration of genuine herbs with low grade ones. Hence, in order to meet the growing demands of the market, WHO emphasized the need of taking certain suitable steps that ensure the quality of medicinal plants and their products by applying various parameters and standard procedures [11]. With this perspective pharmacognostic standardization of different parts of *M. pentaphylla* was carried out. Macroscopic and organoleptic description of a medicinal plant is the initial step towards establishing its identity and should be completed before any tests are undertaken [12, 13]. In the present study, macroscopic and organoleptic characters of whole plant part of *M. pentaphylla* were recorded with the aim of instantaneous identification of the plant in the natural habitat as well in its dried form. Microscopic evaluation of the plant material includes anatomy as well as powder microscopy, which in turn is indispensible for the identification of raw materials. The anatomical features are used as criteria for separating the species, genera and even families. The anatomy gives the idea of the internal study of the plant which forms the important parameters for the quality control and standardization of herbal drugs. In the present investigation, anatomical studies of leaf, stem of *M. pentaphylla* revealed some diagnostic characteristics like epidermal cells in leaves and hexagonal pith cells containing secretory functions in stem. Herbal characterization by powdered analysis is based on the cyto-morphological parameters of the plant material such as

collenchyma, parenchyma, sclereids, trichomes, vessels, secretory cells etc.; and cell inclusions namely, pollen grains, starch grains, calcium oxalate crystals etc. In the present investigation, various powdered materials (leaf, stem and callus) of *M. pentaphylla* were examined. The features observed were spiral vessel, bordered pitted vessel, fibers, pitted vessel, starch cells, tannin cells and parenchyma cells.

The power of plant hormones to promote development in tissue culture has been amazed and baffled plant scientists for many years. Growth regulators, as one kind of signal molecule, have been shown to play an important role during the callus formation [19]. Histological analysis of the tissues of leaf callus of *M. pentaphylla* indicated that the cells comprising certain regions of these tissues were small, compact typically contained a dense cytoplasm, indicating that such regions were embryogenic. In the present study histological of leaf callus revealed the presence of endarc condition, somatic embryos, parenchyma cells and vascular tissues.

Physico-chemical parameters prove to be important for the standardization and quality control of crude drugs. The results revealed that majority of the samples were free from any visible foreign matter. Loss on drying is a commonly used procedure for analyzing the moisture content in the powdered materials which in turn can be regarded as a quality control function. It was observed that the moisture content in *in-vivo* plant 12.59±9.22 % and *in-vitro* leaf callus 51.77±9.33% instudied test samples, clearly indicating that moisture content is within the limits in the test sample except in leaf callus. Thus it could avoid any kind of microbial contamination as the general requirement for moisture content in crude drug should not be more than 14% [14]. Ash content of the drug represents the inorganic salts, naturally occurring in the drug or intentionally added to it for the purpose of adulteration. For analyzing crude drugs different methods of ash values are considered namely total ash, acid insoluble ash, water soluble ash and sulphated ash value. The total ash value is the total amount of material that remains after incineration and includes both physiological ash as well as non physiologicalash. In acid insoluble ash, amount of silica and earthy material contaminants is measured. Water soluble ash values give the idea of water soluble portion of total ash [15]. In the present study water soluble ash content (5.43 %) was less as compared to acid insoluble ash content (6.21%) in the *in-vivo* plantand *in-vitro* water soluble ash content (3.80 %) and acid insoluble ash content (5.09%) thereby indicating very less silica and earthy material contaminants. Swelling index of the plant material is due to the presence of gums and mucilage, hemicellulose or pectin. In this investigation swelling index of leaves of *M. pentaphylla* is more.

Extractive value gives an idea about the active constituents present in the crude drug. This helps to identify the presence of several types of adulteration and exhausted materials. In the present study Soxhlet extraction methods were used. The results revealed that ethanolic solvent gives the highest yield in all studied samples as compared to other solvents. This in turn revealed that there is more number of polar compounds in the studied plant sample.

Fluorescence analysis is an important pharmacognostic parameter that depends on the nature of chromophores present in a test material. Some of the constituents in the plant samples show fluorescence in the visible range in daylight while as some other constituents are responsible for fluorescence character under ultraviolet light. With the aid of this technique quality of the crude drug can be evaluated which ultimately can check for its adulteration. This parameter can be used for standardizing the quality of the drug and hence, this property can be used as a fingerprint for identification of plant material [11]. In the present study, *in-vivo* plant and *in-vitro* leaf callus were analyzed for their florescence character both under visible light as well as UV light. The results depicted that *in-vivo* plant and *in-vitro* leaf callus showed characteristic florescence when treated with specific reagents and hence can be considered as a diagnostic color for the plant material.

CONCLUSION: The present study on *in-vivo* aerial part and leaf callus of *Mollugo pentaphylla* L. revealed the pharmacognostic features and phytochemicals indicated that different chemical compounds which are of pharmacologically bioactive. The present result used for development

of new potent drug, as they are already used as crude drug by rural people both in the form of green vegetable and traditional medicines.

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