

Morphological Characterization of Homeopathic Medicinal Plants and Evaluation of their Biological Effect Against the *Microsporium canis*

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ABSTRACT

Objective: The aim of this study is to identify and characterize the homeopathic medicinal plants on the basis of their morphology and evaluation of their biological effect against the "*Microsporium canis*".

Materials and Methods: Taxonomic analyses (e.g., documentation of the biological origin and morphological characteristics) are essential for characterizing homeopathic drugs in a systematic manner to reach authentication, and thus maintaining homeopathic drug efficacy. The selected homeopathic medicinal plant drugs *Allium sativum* (Bulb), *Eucalyptus globulus* (Leaves), *Ficus religiosa* (Leaves), *Holarrhena antidysenterica* (Bark), *Ocimum sanctum* (whole plant) and *Terminalia chebula* (Fruit) were investigated morphologically including microscopical and quantitative micrometric analysis as per standard protocol. The mother tincture and potencies of homeopathic drugs were prepared as per standard HPI protocol and were evaluated for their biological activity against the *Microsporium canis* using agar disc diffusion assay as per guidelines of clinical and laboratory standard (M44-A) with slight modification. The diameters of zone (mm) of inhibition were measured, and the results were compared with the vehicle control followed by positive control Ketoconazole as reference standard fungicide. **Results:** Some peculiar characters in plants were observed like elongated or polyhedral cells followed by storage parenchymatous cells and poor vasculature in *Allium sativum*, secretory canal and curved eye shaped vascular bundle in *Eucalyptus globulus*, eye shaped vascular bundle in *Ficus religiosa*, hair pattern and quadrangular stem of *Ocimum sanctum*, rhomboidal prismatic crystals, laticifers and stone cells in *Holarrhena antidysenterica*. Mother tincture of *Terminalia chebula* was exhibited maximum zone of inhibition up to 13.6±1.1 mm followed by *Ocimum sanctum*, *Eucalyptus globulus*, *Allium sativum*, *Ficus religiosa* and *Holarrhena antidysenterica*. In case of potencies (3X, 6X and 12X), significant zone of inhibition was observed with many medicines especially *Allium sativum* at 6X (9.6±2.9 mm), *Ficus religiosa* at 3X (9.8±0.4) and 12X (9.2±1.1), *Ocimum sanctum* at 3X (9.8±1.3), 6X (11.6±1.7) and 12X (9.2±0.8), compared to vehicle control against *M canis*. These plants of medicinal importance were fully described macro- and micro-morphologically for easier and more accurate identification. **Conclusion:** The present study obtained results was that morpho-anatomical characters and biological activity not only provide characters for their correct taxonomic authentication, but also serve as standard data for the quality assessment of the pharmaceutical preparation of homeopathic drugs.

Keywords:

INTRODUCTION

Multiple approaches of taxonomical analysis (e.g., documentation of the biological origin and morphological characteristics) play important role for characterizing homeopathic drugs in a systematic manner to reach authentication and identification, for the aim to maintaining efficacy of any drug. It is commonly known that morphological data can be of dubious taxonomic reliability because of environmental interaction and the largely unknown mechanisms of genetic control of these traits^{1,2}. However, problems associated with the interpretation of morphological descriptions can be minimized by measuring traits in several environments or by limiting comparisons to those traits for which the

effects of environmental interaction are smallest. Besides, continued usage of morphological features to describe varieties indicates that these morphological markers retain popularity as descriptors.

Different plants and their parts have their own medicinal properties and are used extensively in indigenous, traditional and natural medicinal systems from ancient time. *Allium sativum* is used for the treatment of bronchitis, catarrhs, colic, constipation, cough, coxalgia³. Part of the plant having chemical defence system against insects is composed of volatiles and various allelochemicals, such as mono terpenes, which have deterrent activity⁴. *Eucalyptus globulus* Labill belonging to family Myrtaceae also known as Blue Gum is used for the treatment of a

Table 1: Effect of the homeopathic mother tincture (\emptyset) and potencies (3x, 6x and 12x) against fungal strain *Microsporium canis*. The values were expressed as mean \pm SD of diameter of zone inhibition (mm) and statistical data

S. No.	Name of plant	Family	Average zone of inhibition (mm)			
			Mother tincture	Potency (3X)	Potency (6X)	Potency (12X)
1	<i>Allium sativum</i>	Amaryllidaceae	7.0 \pm 1.7	6.2 \pm 0.4	9.6 \pm 2.9*	9.4 \pm 5.3
2	<i>Eucalyptus globulus</i>	Myrtaceae	7.8 \pm 2.4	8.0 \pm 1.0	8.0 \pm 0.7	8.0 \pm 0.7
3	<i>Ficus religiosa</i>	Moraceae	6.6 \pm 0.9	9.8 \pm 0.4*	8.6 \pm 1.1	9.2 \pm 1.1*
4	<i>Holarrhena antidysenterica</i>	Apocynaceae	6.6 \pm 1.3	6.8 \pm 1.3	8.0 \pm 2.0	6.0 \pm 0.0
5	<i>Ocimum sanctum</i>	Lamiaceae	7.8 \pm 1.1	9.8 \pm 1.3*	11.6 \pm 1.7*	9.2 \pm 0.8*
6	<i>Terminalia chebula</i>	Combretaceae	13.6 \pm 1.1*	7.2 \pm 1.6	7.6 \pm 1.5	7.4 \pm 1.1
7	Alcohol (90%)	7.0 \pm 1.6				
8	<i>Ketoconazole 10μg/ml</i>	19.4 \pm 2.6 mm*				

various diseases from thousands of years. Recently researchers from Serbia found evidence supporting the antimicrobial action of *Eucalyptus* (<https://www.medicalnewstoday.com/articles/266580.php>) Different parts of *Ficus religiosa* L., family Moraceae are extensively used in indigenous medicine, especially for their antibacterial action⁵ and anticonvulsive effect⁶, *Holarrhena antidysenterica* (Roth) Wall. ex A. DC., family Apocynaceae commonly known as Kutaja⁷ or Kurchi used as remedy for hematuria, bronchitis, spermatorrhoea, epilepsy, asthma, piles leprosy, eczema, diarrhoea, fever and jaundice^{8, 9}. Various parts of *Holarrhena antidysenterica* have been reported to possess antibacterial activity^{10, 11}. Barks of this plant also have many medicinal uses for colic, dyspepsia, chest affections diuretics and skin diseases¹². *Ocimum tenuiflorum* L. syn. *Ocimum sanctum* L., family Lamiaceae is also known as Tulsi or Holy basil, has been used the treatment of bronchitis, malaria, diarrhea, dysentery, skin disease, arthritis, eye diseases and insect bites etc. It has also been suggested to possess anti-fertility, anticancer, antidiabetic, antifungal, antimicrobial, cardioprotective, analgesic, antispasmodic and adaptogenic actions¹³. *Terminalia chebula* Retz. family Combretaceae is used in gastrointestinal affections, profuse salivations, flatulence with indigestion, and loss of appetite.

Microsporium canis belongs to the group of dermatophytes (a group of fungi known to cause skin infection, nail and hair infection in animal and human). This fungal species is widely distributed throughout the world and its most prevalence to cause *Tinea corporis* and *Tinea capitis* in many areas like North and South Africa, Central and Southern Europe and China^{14, 15}. In case of animal, it is the most common in cats, dogs and other animals, where cats are the most important reservoir hosts^{16, 17, 18}. It mainly affects elderly people, preadolescents, immune deficient human being and lead to cause hair loss, itching, skin scaling¹⁹⁻²¹ and decolourisation of nail. Because of the westernization and increasing popularity of keeping pets like dogs and cats, the chances of infection and disease caused by *M. canis* are rising day by day, both in animals as well as in human beings. There is number of problem occur in management of dermatophytosis such as limited resources of antifungal agents, their toxicity as well as their high cost. Although antifungal agents are used for the

treatment of dermatophytosis like terbinafine, clotrimazole, and ketoconazole²² but drug resistance, toxicity, and drug-drug interactions make them limited²³. Therefore there is need to develop a new drug that has to be safe and effective as antifungal remedy. Homeopathic system of medicine is very beneficial because of their efficacy, low cost and less side effect. There are reports available for supporting the homeopathic treatment in antifungal infection mainly dermatophytosis infection. Therefore In this study we have selected six homeopathic drugs[#] namely *Allium sativum* (All. sat.)²⁴ *Eucalyptus globulus* (Eucal. gl.)²⁵, *Ficus religiosa* (Ficus rl.)²⁶, *Holarrhena antidysenterica* (Hol. andy)²⁷, *Ocimum sanctum* (Oci. sant.)²⁸ and *Terminalia chebula* (Term. ch.)²⁹, were studied morphologically along with their antifungal effect against *M. canis*

MATERIAL AND METHODS

Plant collection and identification

The fresh and healthy plant parts of *Allium sativum* L. (Bulb), *Eucalyptus globulus* Labill. (Leaves), *Ficus religiosa* L. (Leaves), *Holarrhena antidysenterica* (Roth) Wall. ex A. DC. (Bark), *Ocimum sanctum* L. (whole plant) and *Terminalia chebula* Retz. (Fruit) were collected, identified/authenticated and supplied by the Centre for Medicinal Plants Research in Homoeopathy (CMPRH), Emerald, Tamil Nadu. For microscopical studies, supplied raw drugs were first kept in warm water followed by boiling for 30 minutes and then preserved in FAA (formaldehyde acetic acid) solution and for mother tincture and successive potencies preparation, dried raw drugs were used.

Chemicals and reagents

All the chemicals used for study were of analytical grade and procured from thermo Fischer scientific, Mumbai, India and E. Merk India Limited.

Microscopic evaluation

Transverse sections of leaves, bulb, bark and stem were studied under the light microscope. The preserved specimens in FAA solution were washed with distilled water and dehydrated by using Alcohol-TBA- Xylene series followed by embedding in paraffin wax for microtomy. The sections with thickness range of 30 to 40 micrometres were taken by using microtome (WESWOX MT-1090A). Sliced sections were heat fixed on the slide

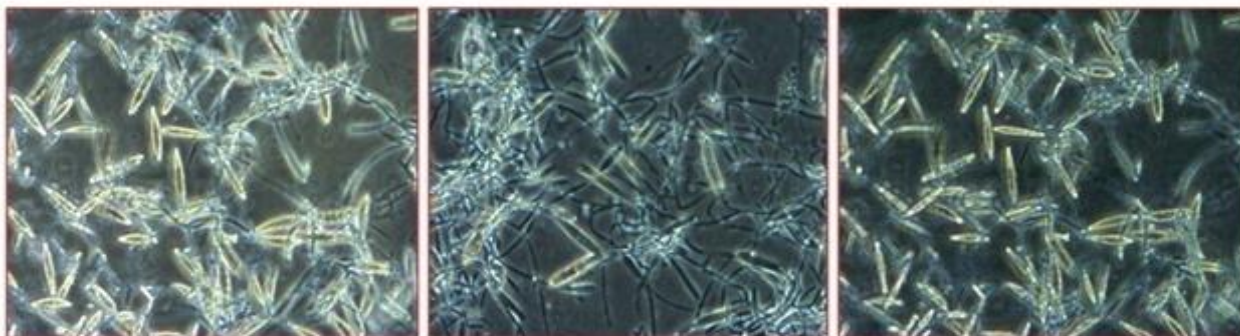


Figure 1: 10% KOH, Identification and characterization of fungal strain *M. canis.E*.

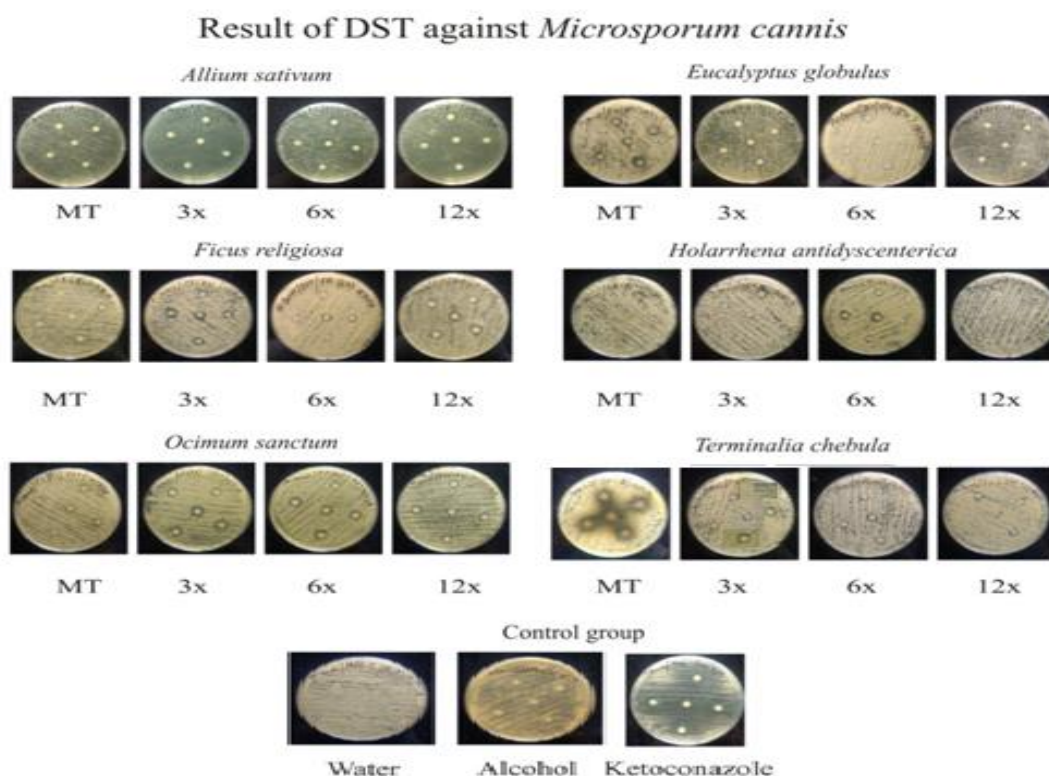


Figure 2: Effect of Homoeopathic medicines against fungal strain *Microsporium canis*.

using Gelatine and then double staining method was applied as per the method of Johnsons³⁰ Safranin and fast green of Fischer scientific were used as per standard procedures. Different diagnostic characters studied and noted down for identification of drug.

Leaf microscopy

For this study, leaves preserved in FAA solution were first washed with distilled water for about 30 minutes and then dipped in saturated chloral hydrate solution. The decolourized leaves were mounted in 50% glycerine solution and surface structures like trichomes, epidermal cells, type of stomata and its arrangement was observed using safranin and other reagents and also without any reagent. Different diagnostic characters were observed, recorded and photomicrography was done.

Stem, Bark, Bulb and Leaf Microscopy

The raw drugs were boiled and preserved in FAA solution was first washed with running water for 15-20 minutes

followed by dehydration method and embedding protocol for microtomy and staining. For dehydration, successive dehydration method was done keeping samples in different gradients of Alcohol-Butanol-Xylene series followed by wax-paraffin series for preparation of cubes embedded with samples. Microtomy was done by using WESWOX microtome and permanent slides were prepared as per standard protocol of Johnson. Stained and without stained sections of plant parts were observed under microscope. Different layers of cells and their diagnostic characters were recorded followed by photomicrography.

Quantitative microscopy

A quantitative microscopy is an important tool for the identification of raw drugs. In this method, leaves were decolourised and peelings were taken with help of forceps and stained with safranin. Peelings were mounted with 50% glycerine and different leaf characters were observed and parameters recorded.

Stomatal index determination

The percentage proportion of the ultimate divisions of the epidermis of leaves which can be converted into stomata is called as stomatal index. This can be calculated as following formula

$$\text{Stomatal index} = \frac{S \text{ (Stomatal cells)}}{S + E \text{ (Stomatal cells + Epidermal cells including Hairs)}} \times 100$$

Vein islets and vein termination determination

Vein-islet is the minute area of photosynthetic tissue encircled by ultimate divisions of conducting strands present in per square. Vein termination is the ultimate free termination of a vein let or branch of vein let in per square mm of leaf surface. A piece of leaf was de-pigmented by using saturated chloral hydrate solution followed by washing with water. The leaf now stained with safranin and mounted with glycerine for photomicrography. 1 mm square area was drawn and vein islets and vein termination number were counted³¹.

Palisade ratio determination

The average number of palisade cells present below the epidermal cells is called palisade ratio. A part of the leaf was first treated with saturated chloral hydrate solution, was mounted with 50% glycerine and focused under the light microscope in such a way so that both epidermal cells and palisade cells are visible simultaneously. The number of palisade cells present below each epidermal cell was counted (if cells are more than half then included otherwise excluded).

Preparation of mother tincture, potencies and standard:

Mother tinctures (ϕ) and their successive potencies of homeopathic drugs of *Allium sativum*, *Eucalyptus globulus*, *Ficus religiosa*, *Holarrhena antidysenterica*, *Ocimum sanctum* and *Terminalia chebula* were prepared and processed for different potencies (3X, 6X, 12X) according to the standard procedures mentioned in Homoeopathic Pharmacopoeia of India. The Ketoconazole (10 $\mu\text{g/ml}$) (Sigma-Aldrich, GmbH Germany) was used as standard antifungal drugs, alcohol and double distilled water used as vehicle control and negative control.

Test Microorganisms and Growth Media

The fungal culture of *Microsporium canis* (MTCC No.3270) were chosen based on their clinical and pharmacological importance and obtained as lyophilized freeze dried culture strain from Institute of Microbial Technology, Chandigarh, were used for evaluating antimicrobial activity. The freeze dried culture strain of *Microsporium canis* was aseptically open in Bio-safety cabinet and suspension was made as per protocol. 0.4 ml sterilized water taken in a micro centrifuge tube (MCT) and freeze dried culture were transferred in water, mixed well and let it stand for 20 minute before transferred it on solid media. Petri plates containing Sabouraud dextrose agar (SDA; Hi Media, Mumbai, India, Catalogue No. M063) medium incubated for 24-48 hrs at 35°C to give white round colonies against a yellowish background. Approximately 1 mm colonies were picked up and suspended in 5 ml of sterile SDA and kept as broth culture /stock culture. Microorganisms were repeatedly sub-cultured using

streaking method and maintained in order to obtain pure isolation on the SDA media for further drug sensitivity assay.

*Morphological Identification of Microsporium canis**Direct Microscopy by KOH stain*

Morphological features of *Microsporium canis* species were identified according to the method described. In brief, one drop of KOH stain was placed on centre of clean Grease free glass microscope slide and transferred a loop of culture growth from SDA media containing *Microsporium canis*, mixed gently with the stain and covered with a cover slip. The preparation was examined using the low power (10X, 20X) objective of the Inverted phase contrast microscope (RTC-7, Radical Microscope). High power (45X) resolution was used to confirm observations.

Preparation of disc for antifungal assay

For determining antifungal activity of different homeopathic mother tinctures as well as their different potencies, agar disc diffusion method was used. Whatman (No.-1 filter paper) was used to prepare discs approximately 6 mm in diameter, which were placed in a petri dish and sterilized in a hot air oven. Discs were soaked in selected homeopathic mother tinctures, as well as different homeopathic potencies of same drugs and let stand for 30 minute. Antifungal drug Ketoconazole was used as positive control in this study.

Preparation of Muller Hinton Agar, 2% Glucose with Methylene Blue for Antimicrobial Activity

Media containing relatively high concentration of Glucose (20%) was prepared by mixing Muller Hinton Agar, 2% Glucose with Methylene Blue (MHAGMB) (HIMEDIA, Mumbai, India, Catalogue No. M1825), distilled water and autoclaved at 121°C for 15 minutes. Twenty ml of molten (45°C) MHAGMB medium was aseptically transferred into each sterile Petri plates (100mm×15mm) and allowed to solidify in a biological safety cabinet.

*Antimicrobial Activity**Antifungal susceptibility tests*

Antifungal activities of mother tinctures and ultrahigh dilution potencies against pathogenic fungi *Microsporium canis* were investigated by the agar disk diffusion method. Each mother tincture and potencies were prepared in alcohol and stored at room temperature for the determination of zone of inhibition; All the homeopathic drugs were screened for their antifungal activities against *M. canis* MHAGMB (HIMEDIA, Mumbai, India, Catalogue No: M1825), The suspension of hyphal fragments of *M. Canis* were transferred to a sterile tube and adjusted by turbidimetry to obtain inocula of approximately 10⁶ cfu/mL. Plates were seeded with fungal strains and allowed to stay at 35°C for 24 hours. Control experiments were carried out under similar condition by using Ketoconazole for antifungal activity as standard drugs. The sensitivities of the microorganism species to the plant extracts were determined by measuring the sizes of inhibitory zones (including the diameter of disk) on the agar surface around the disks after 72 and 96 hours of incubation at 35°C, and values <6 mm were considered not active against microorganisms.

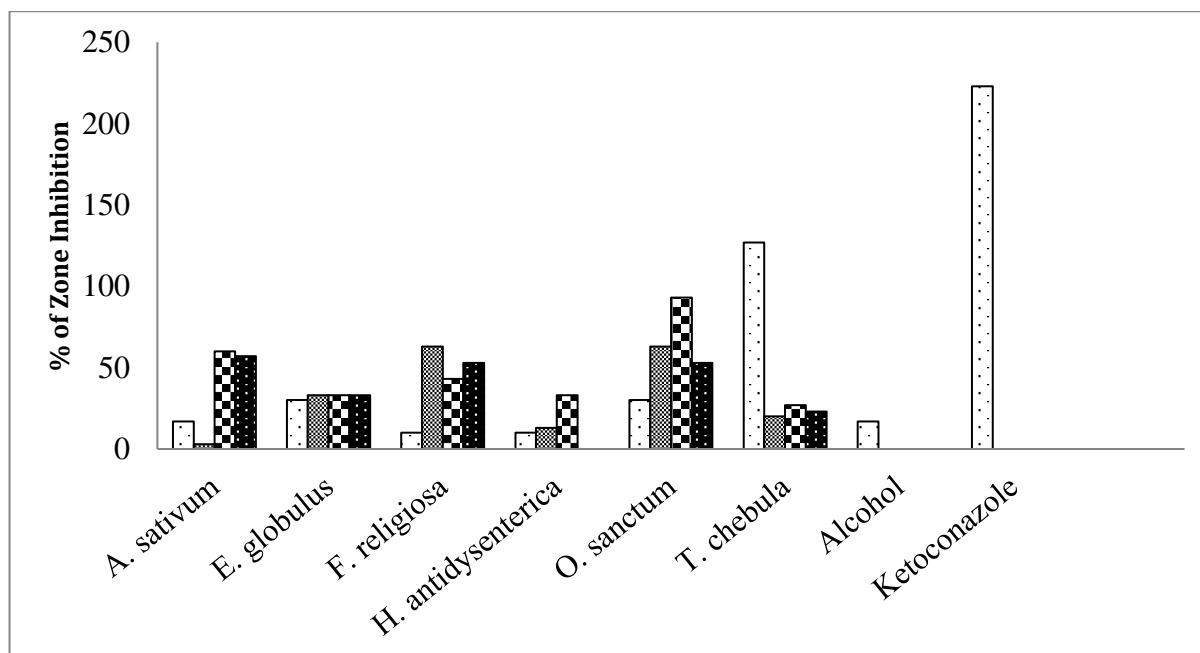


Figure 3: % Zone inhibition.

Statistical analysis

The values were expressed as mean diameter of zone inhibition (mm) and statistical data were analyzed by using one way analysis of variance (ANOVA) followed by Dunnett's post hoc test to monitor significance among groups using the Graph Pad prism version 7.0. $P < 0.05$ were considered as significant as compared to vehicle control.

RESULTS AND DISCUSSION

Morphological Characterization

Allium sativum: Outer epidermal cells were variable in shape, elongated or polyhedral, inner epidermis cells are longer than the outer epidermis cells. Epidermis followed by the storage parenchymatous region, some cells contained starch grains. Vascular bundles were radially arranged; poor vasculature, less developed xylem and phloem, central opening or hollow area present in the middle region of the section (Figure. 1 A & B).

Eucalyptus globulus: Transection of *E. globulus* leaf, lamina region showed distinct single layered epidermis with thick cuticle on both the side of the leaf, mesophyll has two types of parenchymatous cells viz. palisade and spongy parenchyma. Dorsi-ventral mesophyll with palisade layers on both adaxial and abaxial side of leaf, below the epidermis a 2 - 3 rows of palisade cells were present, spongy parenchyma were loosely arranged between the palisade layers, James and Bell, 2001 also reported on the presence of palisade layers on both side³². Secretary canals were arranged in the slightly upper region of the leaf that was arranged linearly up to the margin of leaf lamina. These canals have thin small, epithelial cells. Midrib region have a central, large stele surrounded by 2 - 3 layers of sclerenchyma cells (stone cells), Three vascular bundles were present viz. one large vascular bundle and two small vascular bundles, all three were forming curved C-shaped structure with opening on dorsal side of grooved

midrib. Up to 2 - 3 layered collenchymatous region was also present below epidermis on both sides; ground parenchyma cells were present with oil globules. Leaf amphistomatous with anomocytic stomata, stomatal index 18.68 in lower side and 9.21 in upper side, palisade ratio 4 - 6, vein islet 19.21 per mm square and vein termination 21.08 per mm square. (Figure. 1 C & D).

Ficus religiosa: Transection of *Ficus religiosa* leaf through lamina region showed distinct single layered epidermis with thin cuticle on both the side of the leaf, upper epidermal cells were larger than lower one. In midrib region epidermal cells are very smaller and gradually going toward margin region of lamina, these cells becomes larger. Mesophyll differentiated into small sized palisade cells and spongy parenchymatous cells, mesophyll with palisade layers on ventral side, loosely spongy parenchymatous cells were present below the palisade layers, and few prismatic crystals are also present in the mesophyll. Midrib region contains 'eye-shaped' or a ring form of closed vascular bundles having larger bundles on ventral and smaller on dorsal side, vascular bundles were surrounded by a discontinuous layer of several sclerenchyma cells. Vascular bundles consisted xylem which surrounded by a zone of phloem towards outer side. Epidermal cells are discontinued and interrupted with stomata and motor cells. The presence of these motor cells with the epidermal cells is also reported by the Shakir and Baji³³. According to Waman, the leaves of *Ficus* species are hypostomatic³⁴ but in this study leaves a amphistomatous having paracytic stomata with stomatal index 4.28 in upper side and 27.47 in lower side, palisade ratio 4 - 5, vein islet 12.43 per mm square and vein termination 10.15 per mm square. .

Ocimum sanctum

Transection of stem showed semi quadrangular to quadrangular outline with single layered, elliptical

isodiametric epidermal cells, there are two types of hairs present in the epidermis viz. glandular and non-glandular. Epidermis followed by one to two layered of collenchymatous hypodermis, cortical region have four to five layers of parenchymatous cells having intracellular spaces between the cells and sclerenchymatous fibres were present above the vascular bundles. Four large well developed vascular bundles at each corner of quadrangular stem were present, cambial ring formed due to activities of fascicular and intra fascicular cambium but remains active and functional normally only in fascicular region where as it was less active and showed abnormal division in intrafascicular region where it formed only uniseriately arranged thin walled parenchymatous cells towards inner side. Secondary phloem and secondary xylem developed where xylem vessels lignified and broad, vessels prominent with spiral and annular thickenings, tracheids showed simple pits; rays multiseriate; pith broad and parenchymatous cells, no cellular inclusion were seen in any region of stem.

Holarrhena antidysenterica

Transection showed outermost compressed zone of 3 - 10 layers of thin walled rectangular cells of cork; cork cambium 2 or 3 layered; secondary cortex parenchymatous, 10 - 20 layered, with rhomboidal prismatic crystals of calcium oxylate, sclereids and a few laticifers; stone cells with narrow lumen; phloem broad zone of sieve elements, companion cells and phloem parenchyma with rhomboidal crystals calcium oxalate; phloem fibres prominent, solitary with narrow lumen; rays 2 or 3 seriate, parenchymatous.

Antifungal activity

In the present study, Mother tincture and their potencies (3X, 6X and 12X) of homeopathic drugs *Allium sativum*, *Eucalyptus globulus*, *Ficus religiosa*, *Holarrhena antidysenterica*, *Ocimum sanctum* and *Terminalia chebula* were evaluated against the growth of human pathogenic fungi *M. canis*. Susceptibility of each drug was tested using agar disc diffusion method and a diameter zone of growth inhibition was measured in millimetre. Potassium hydroxide (KOH) preparation of the specimen reveals abundant macroconidia that were thick-walled and with many septa, Macroconidia were often hooked or curved at ends. Micro conidia were small and clavate (club-shaped) confirmed the identification of *M. canis*. Antifungal potential of Homeopathic drugs were assessed in terms of zone of inhibition of fungal growth. The results of the antifungal activities were presented in (Table, Figure). The percent zone of inhibition of each medicine was also calculated and depicted in Figure. The mother tincture of *Terminalia chebula* was found to be most potent against *M. canis*. It exhibited maximum zone of inhibition up to 13.6±1.1 mm followed by *Ocimum sanctum*, *Eucalyptus globulus*, *Allium sativum*, *Ficus religiosa* and *Holarrhena antidysenterica*. Significant zone of inhibition was observed with the mother tinctures of *Terminalia chebula* compared to vehicle control.

In case of potencies (3X, 6X and 12X), significant inhibition of zone was observed with many medicines especially *Allium sativum* at 6X (9.6±2.9 mm), *Ficus*

religiosa at 3X (9.8±0.4) and 12X (9.2±1.1), *Ocimum sanctum* at 3X (9.8±1.3), 6X (11.6±1.7) and 12X (9.2±0.8), compared to vehicle control. The anti-fungal activity of reference drug Ketoconazole was promising with significant zone of inhibition (19.4±2.6mm) compared to control group. The maximum percentage of zone inhibition (127%) was detected in mother tincture of *Terminalia chebula* as compared to other medicines used in the study. In case of potencies, percentage of zone inhibition was found in *Allium sativum* at 6X (60%) and 12X (57%), *Ficus religiosa* at 3X (63%) and 12X (53%), *Ocimum sanctum* at 3X (63%), 6X (93%) and 12X (53%) against *M. canis*.

As a result, the plant based homeopathic drugs which used in the present study showed a different level of antifungal activities with mother tincture and potencies.

CONCLUSION

These plants of medicinal importance were fully described microscopically for more accurate identification in addition to their respective antimicrobial activities. The conclusion of the obtained results was that morpho-anatomical characters and biological activity of the homeopathic plant based medicines not only provide correct taxonomic authentication, but also served as standard data for the quality assessment of the pharmaceutical preparation of as well as homeopathic herbal drugs. Anti-microbial evaluation suggested that these homeopathic drugs may be alternate therapy to control the growth of pathogenic fungi *M. Canis* however; clear conclusion could not be drawn due to lack of any fix pattern (decrease or increase) in the response achieved. Further investigations may require to analyzing the potential of homeopathic medicines as possible antimicrobial agent.

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