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ORIGINAL ARTICLE **OPEN ACCESS**

Determination of chlorophyll content and foliar micro-morphological studies of Hibiscus species

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ABSTRACT
In World health organisation guidelines, quality control of medicinal plants is evaluated with different parameters using organoleptic, microscopical, physical, chemical and biological characterization. By studying these parameters, it becomes easier to evaluate the quality and quantity of authentic drugs concerning their adulterated/substituted form. To study the comparative study between different leaves varieties of Hibiscus using leaf constant parameters and evaluating chlorophyll contents using Arnon method. The foliar micro-morphological study was performed with different leaf varieties, using 80% sulphuric acid, chloral hydrate reagent, phloroglucinol reagent and concentrated hydrochloric acid on the slides. The chlorophyll a, chlorophyll b and total chlorophyll contents were studied on fresh leaf samples using Arnon method. The presence of anisocytic type of stomata in the red and yellow varieties were found to be more in the abaxial surface more than the adaxial. All the varieties were amphistomatic and containing cutin when examined with sulphuric acid. Among all the varieties, the stomatal number and stomatal density of red variety was found to be stomatal frequency /mm²14±0.32 (adaxial), 20.6±0.24 (abaxial); stomatal index (%) 22±0.31 (adaxial), 24.4±0.24 (abaxial) respectively. The percentage of chlorophyll a is found greater than chlorophyll b in the plant samples.
Keywords: *Leaf constants, stomatal index, chlorophyll, stomatal density, cutin, adaxial, abaxial*

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INTORDUCTION

Authentication of a plant species can be achieved through the micro-morphological evaluations. These parameters are supposed to benefit when the plants are deprived of their floral parts and are unable to distinguish with their different species as well as varieties which are to be used as adulterants/substituents. Micro-morphological and leaf architectural evaluation include stomatal density, stomatal index, vein islet, veinlet terminations, and crystal arrangements in foliar apparatus. These above-mentioned features are considered as the most valuable and important analytical tool for plant species. All these parameters have been used in the systematic study of flora concerning biology, phylogeny, palaeobotany, biostratigraphy and assessment of environmental parameters [1-8].

Hibiscus is a genus of flowering plants comes in the mallow family and it belongs to Malvaceae family, respectively. It is widely spread in warm to the temperate region as well as tropical to subtropical region throughout the world with the number of different varieties. The tea made from Hibiscus is known to be very famous. It has different vernacular names, *Asbissap* in West Africa, *Karkadé* in Egypt and Sudan, *Flor de Jamaica* in Mexico, G*udhal* in India, and *Gongura* in Brazil [9,10]. The plant was indigenous to tropical Asia and was cultivated in China, Japan and the Pacific islands. It is believed that the deep-red colour of the Hibiscus has an Asian origin. Therefore, the name "*rosa-sinensis*" meaning *rose* of China[11].

Chemically, it contained tannins, anthraquinones, quinines, phenols, flavonoids, alkaloids, terpenoids, saponins, cardiac glycosides, protein, free amino acids, carbohydrates, reducing sugars, mucilage, essential oils and steroids. The cyclopropanoids, methyl sterculate, methyl-2-hydroxy sterculate, 2-hydroxysterculate, malvalate and beta-sitosterol were also present as active ingredients. The major anthocyanin present in the flower was cyanidin-3-sophoroside [11-17].Traditionally, the red variety was utilised more as compared to other varieties in medicine. The roots and leaves were used as anodyne and emmenagogue. It helps in the regulation of menstruation cycle and even in the proper blood circulation. It

has abortifacient property and helps to stimulate the expulsion of the placenta after the birth of the child. The flowers were used for liver disorders, high blood pressure, as an antitussive, stomache and as an aphrodisiac. The young and fresh leaves and flowers were used in headache [18-20] Chlorophyll is a naturally occurring dye obtained from green leafy plants and algae. The pigment is used to synthesize carbohydrates from carbon dioxide through photosynthesizing using energy from the sun. Higher plants contain chlorophyll a and chlorophyll b present in the chloroplasts. Apart from its photosynthetic role, it has been used as a natural colourant in many foods and cosmetic products. The percentage of chlorophyll degrades during ageing of plant tissues that would result in a colour change [21].

The present study was performed to evaluate the foliar micro-morphological study and chlorophyll content present in four different varieties of Hibiscus leaf. While evaluating the leaf constants, leaf orientation, vein pattern, it becomes easier to distinguish the different varieties with their adulterants/substituents, both qualitatively and quantitatively.

MATERIAL AND METHODS

Plant collection and identification

Fresh leaves of different varieties of Hibiscus were collected from GLA University campus, Mathura, India and were authenticated with the help of Pharmacognosist, Mrs Sonia Singh. Fresh leave samples were chosen for qualitative and quantitative micro-morphological and leaf anatomical studies.

Foliar micro-morphological evaluation

The foliar micro-morphological examination of Hibiscus leaves includes leaf orientation, type of stomata and its morphology; stomatal frequencies, stomatal index, presence of trichomes were obtained manually using standard method [22].

Evaluation of venation pattern and stomatal determination

The fresh leaves were excised and primarily fixed in the solution of formalin, acetic acid and ethyl alcohol (1:1:3 v/v solution). The resultant fixed leaves were cleared with 70% ethanol to remove the chlorophyll pigment and then bleached with 5% NaOH for 24 hours, which was rinsed three times in distilled water. The sections were then allowed to stain with chloral hydrate solution and used for the further study [22,23].

The leaf sections were stained with chloral hydrate reagent; phloroglucinol reagent and concentrated hydrochloric acid (1:1) and then examined under phase contrast microscope for the identification of micro-morphological and leaf architectural parameters. The data for various parameters like stomatal frequency and stomatal index were calculated by the methods suggested by Salisbury and et al method [24]. Types of stomata have been studied by Prabhakarand et al method [25]. The evaluation of venation pattern and leaf orientation was studied as mentioned in the book, accordingly [26].

Leaf impression method

Leaf epidermal impressions were made directly onto the slides from cellulose acetate or cellulose nitrate or a clear polished of polyvinyl chloride. Solvents such as alcohol or acetone were applied directly onto a fresh leaf to give a more flexible film. Sometimes, leaves were simply coated with spray lacquer, allowed to dry. The clear transparent packing tape was applied to one of the surfaces and gently removed it from the adhered side after getting the impression of the leaf surface. The impression containing tape was then pasted on the slide and observed under the phase-contrast microscope. The precaution was to be taken as the tape should be gently adhered to the applied coat and gave the impression of the epidermal layer along with the stomata [27,28].

Determination of Chlorophyll using Arnon method

Plant material used

Fresh leaves of different varieties of Hibiscus were taken for chlorophyll determination.

Determination of Chlorophyll a, Chlorophyll b and Total chlorophyll

Chlorophyll a Chlorophyll b and Total chlorophyll content was carried out by weighing 2g of fresh plant leaf samples and homogenised with 10 ml of 80% acetone, using a laboratory blender. The resultant sample mixtures were then centrifuged at 10,000 rpm for 15min at 40°C. The supernatants were discarded and the obtained mixtures (0.5mL) were diluted with 4.5ml of the respective solvent. The solution mixtures were analysed for chlorophyll a, chlorophyll b and total chlorophyll in a spectrophotometer at different absorbance, respectively [21]. The equation used for the determination of chlorophyll a, chlorophyll b, and total chlorophyll is mentioned below.

$$\text{Chlorophyll a (mg/g fresh weight)} = (12.7\ A_{663} - 2.69\ A_{645}) \times X/1000 \times n$$

$$\text{Chlorophyll b (mg/g fresh weight)} = (22.9\ A_{645} - 4.68\ A_{663}) \times X/1000 \times n$$

$$\text{Total chlorophyll (mg/g fresh weight)} = (20.2\ A_{645} + 8.02\ A_{663}) \times X/1000 \times n$$

Statistical analysis

The results were calculated as mean value, standard deviation (SD) and standard error mean (SEM) was calculated in the excel sheet (n=5).

RESULTS AND DISCUSSION

Comparative study of leaf anatomy and leaf constants of different varieties of Hibiscus leaves under a phase-contrast microscope can reveal a vast number of important features, which were associated with their habitat. The shrubs of different varieties were having some common features such as the leaves of all varieties were alternate, ovate-lanceolate or lance liked shape, usually toothed or dentate lobed margin. The leaves exhibited different shades of green colour on its adaxial and abaxial surface. Even the size of leaves may vary concerning different varieties as shown in the Figures .1 of the leaf of all varieties of Hibiscus (as shown in Table 1 and 2).

The epidermal cells were compactly arranged and interrupted by stomatal apparatus. Almost all the cells were amoeboid in shape and visibly larger than the stomatal guard cells. The paradermal sections of the abaxial epidermis illustrate the presence of large, distinct and undulated regular anticlinal walls. In addition to these, glandular and non-glandular trichomes were also observed.

Analysis of Stomata, Trichome and epidermal cells

The leave patterns of all varieties of Hibiscus are amphistomatic. Types of stomata present in different varieties of Hibiscus leaves are detailed in Table.3 and shown in Figure.2-5 .In Figure.2-5, it has been found that anisocytic stomata were present on both the adaxial and abaxial surfaces of all varieties of Hibiscus. In white and pink varieties, it is present abundantly on the adaxial surface and found in less amount on the abaxial surface as compared to adaxial surface. The length and width of stomata were found to be more on the abaxial surface than the adaxial surface for all the varieties of Hibiscus, among which the length and width of red variety were more than others, as shown in Table.3. In yellow variety, the length and width of stomata were almost similar i_e 4.82±0.08 (adaxial) and 4.94±0.06 (abaxial) on both surfaces. Stomatal frequency per mm^2in red variety was 20.6±0.24 (abaxial) and the value was higher among all the varieties. From the above data, it has been observed that the stomatal frequency was more on the abaxial surface than the adaxial surface in case of red and yellow varieties of Hibiscus. Similarly, the mean stomatal index % was observed to be more on the abaxial epidermal surface and less in number on the adaxial surface. Among all the varieties, red variety contains 24.4±0.24 stomatal index% on the abaxial surface while in the white variety, the stomatal index% was found to be in the least ratio (20.6±0.4) as shown in Table.3. Presence of guard cells and subsidiary cells in all the varieties of Hibiscus leaves were the same. The guard cells are having some intercellular spaces with some slight variation in all the varieties were observed. Anisocytic type of stomata was found in all the varieties. It has been found that in red and yellow varieties, the abaxial epidermal cell wall contains a higher proportion of stomatal density than the adaxial epidermis. Both the epidermal surfaces of leaves contain stomata (an amphistomatic type of leaves). Stomata are abundantly found on both the surfaces of red and yellow. Variation in the stomatal frequency and index were observed in the varieties of Hibiscus leaves respectively, in Table.3.

In all the varieties of Hibiscus leaves, the epidermal cells present on the abaxial surface are in the shape of pentagonal to polygonal. Whereas in the adaxial surface it has some irregular shapes along with cuticle striations. In red variety, the anticlinal cell walls of abaxial surface are straight whereas, in white and pink varieties, these walls are in slightly undulate shape. And the arc-shaped abaxial cell walls are observed in yellow variety. The adaxial cell walls in all the varieties of Hibiscus leaves are in slightly undulating form. Covering and glandular trichomes were observed in all the varieties. Druses of calcium oxalate were found in the leaves. These calcium oxalate prisms were either arranged solitary or in groups and scattered in the veins as well as in the mesophyll regions.

Analysis of venation pattern

It has been observed that the section of all varieties of Hibiscus shows the presence of prominent primary and secondary veins arose from the major veins. The veins were straight and exhibited reticulate venation pattern in all the varieties. Vein islets were scattered irregularly, polygonal and rectangular.

Microscopical evaluation of Leaves of different varieties of Hibiscus

The present research work revealed the presences of single-layered of cuticle with epidermal cells, cortical parenchyma cells, thin strips of collenchyma cells found beneath vascular tissues, lignified xylem with phloem in all the varieties of Hibiscus leaves. Figure .6 showed the presence of anisocytic stomata with the cuticle layer (Yellow variety).

Determination of chlorophyll contents

The concentration of chlorophyll a, b and total in all plant leaf samples on 0, 10 and 20 days shown in Graph 1. The literature revealed that chlorophyll a is the predominant natural pigment and chlorophyll b

is considered as a complementary [21]. The graph resulted that Chlorophyll a shows a greater percentage as compared to chlorophyll b.

Table 1 Macro-morphological parameters of different varieties of Hibiscus.

Varieties	Leaf attachment	Leaf organisation	Leaf shape	Apex shape	Base shape	Margin type
Red	Alternate	Simple	Ovate	Acute	Attenuate	Serrate, Notched
White	Alternate	Simple	Elliptical	Acute	Attenuate	Dentate
Pink	Alternate	Simple	Elliptical	Acute	Attenuate	Dentate
Yellow	Alternate	Simple	Ovate	Acute	Attenuate	Serrate, Notched

Table 2Macro-morphological parameters of different varieties of Hibiscus.

Varieties	Petiole attachment	Lobation	Venation pattern	Leaf colour
Red	Petiolate	Unlobed	Reticulate	Dark green
White	Petiolate	Unlobed	Reticulate	Light green
Pink	Petiolate	Unlobed	Reticulate	Light green
Yellow	Petiolate	Unlobed	Reticulate	Dark green

Table 3 Foliar Micro-morphological study of different varieties of Hibiscus

Parameters	White variety	Pink variety	Yellow variety	Red variety
Length(cm)				
Adaxial	4.18±0.12	3.38±0.18	4.82±0.08	5.52±0.14
Abaxial	4.56±0.23	4.16±0.34	4.94±0.06	5.76±0.24
Width				
Adaxial	3.6±0.18	3.62±0.07	4.12±0.05	5.16±0.02
Abaxial	3.7±0.18	3.8±0.2	4.14±0.12	5.12±0.07
Stomatal frequency(no. of stomata/mm2)				
Adaxial	18.6±0.24	19.4±0.24	13.8±0.37	14±0.32
Abaxial	13±0.31	13.6±0.24	19.8±0.37	20.6±0.24
Stomatal index(%)				
Adaxial	21.2±0.37	23.4±0.4	21.2±0.5	22±0.31
Abaxial	20.6±0.4	20.8±0.6	24.2±0.50	24.4±0.24
Type of stomata	Anisocytic	Anisocytic	Anisocytic	Anisocytic
Number of vein islets/mm2	13±0.4	11±0.34	13±0.56	15±0.55
Number of veinlet terminations/mm2	20.2±0.17	19.2±0.7	22±0.09	23±0.17

n=0

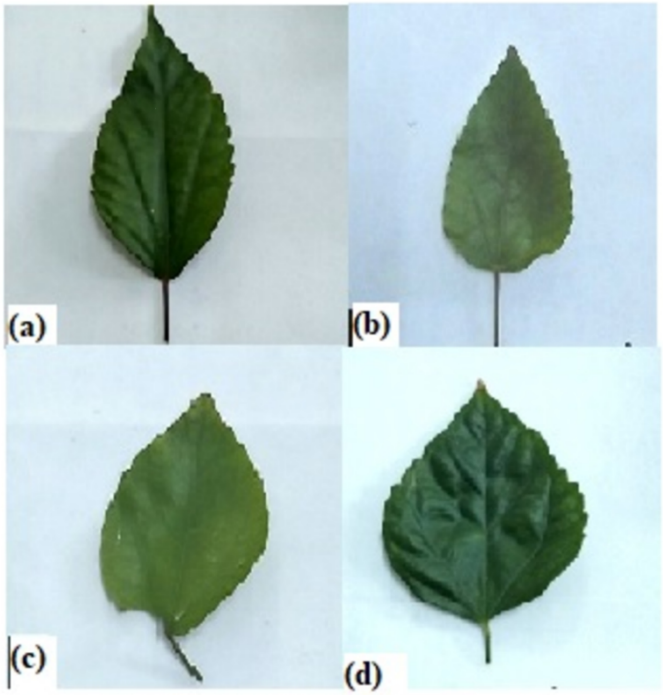
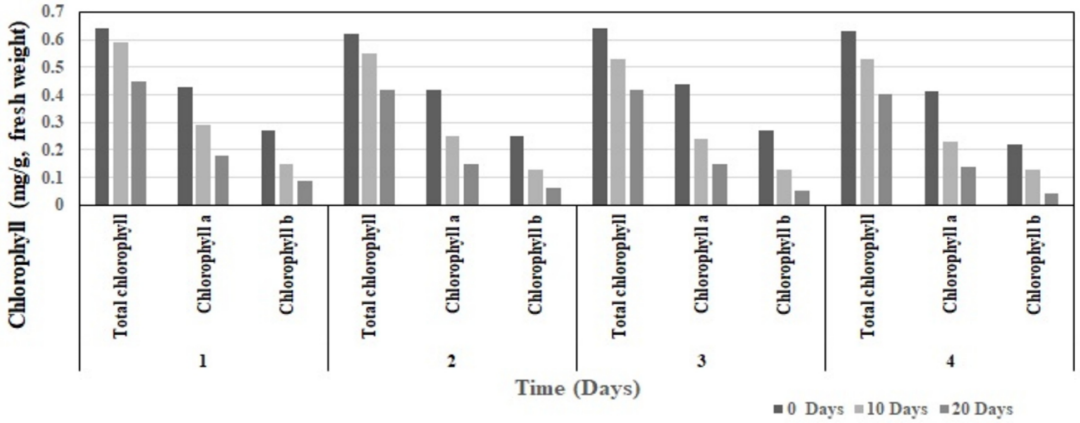


Figure 1 Leaves of different varieties of Hibiscus: (a) Red variety, (b) White variety,(c) Pink variety, (d) Yellow variety



Graph 1Chlorophyll contents present in Hibiscus leaf species. 1: White variety; 2: Pink variety; 3: Yellow variety: 4: Red variety.

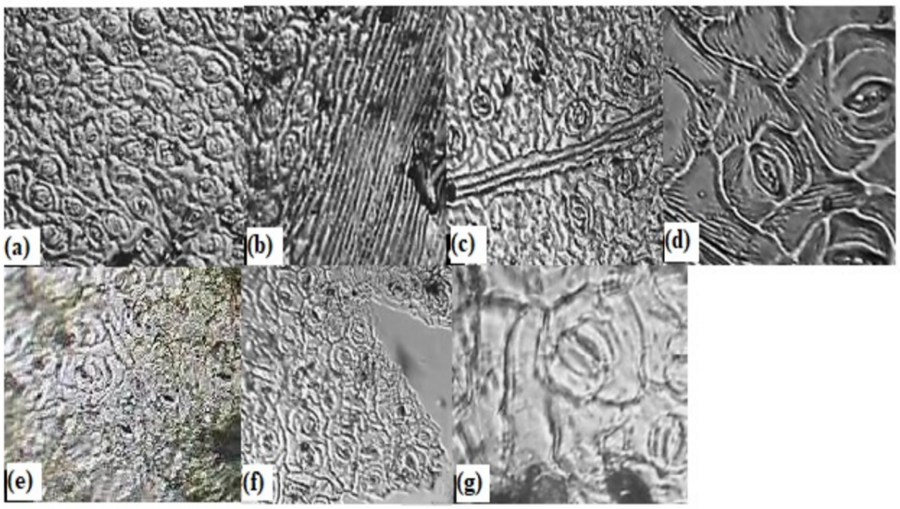


Figure 2 Foliar morphological evaluation of Red variety of Hibiscus, visualized under phase contrast microscope. Leaf impression sections: (a) Stomata on abaxial surface (10x), (b) epidermal layers (10x), (c) Stomata with epidermal layer on adaxial surface (10x), (d) Anisocytic stomata (40x); Leaf constants evaluation: (e) Stomata on the abaxial surface (10x), (f) stomata with epidermal cells on the adaxial surface (10x), (g) Anisocytic stomata (40x).

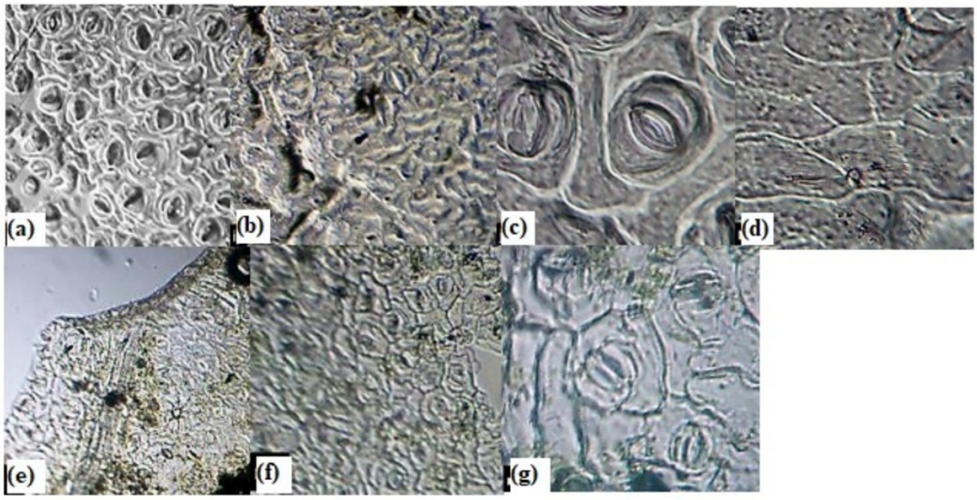


Figure 3 Foliar morphological evaluation of Yellow variety of Hibiscus, visualized under phase contrast microscope. Leaf impression sections: (a) Stomata on abaxial surface (10x),(b) Stomata with epidermal layer on adaxial surface (10x), (c) Anisocytic stomata (40x), (d) epidermal layers (10x); Leaf constants evaluation: (e) Stomata on the adaxial surface (10x), (f) stomata with epidermal cells on the abaxial surface (10x), (g) Anisocytic stomata (40x).

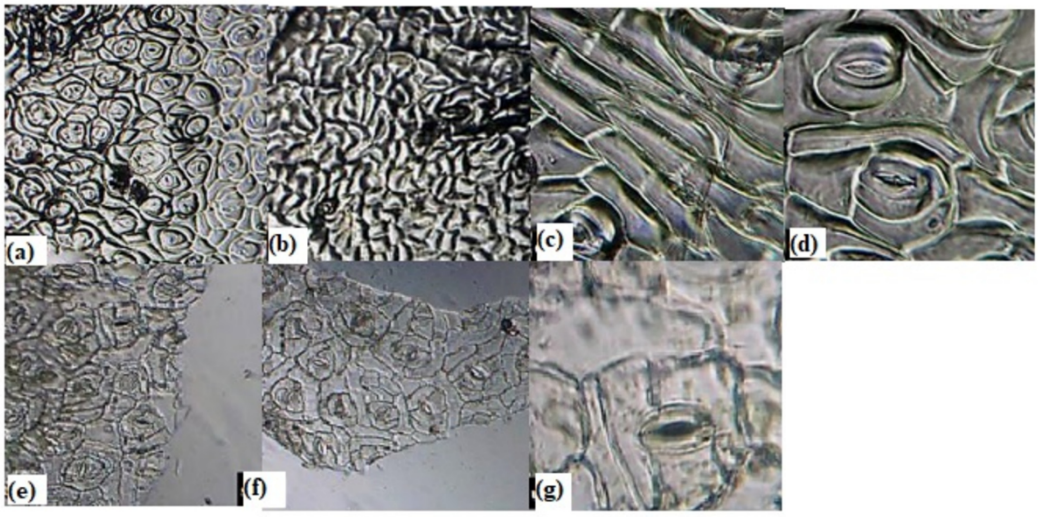


Figure 4 Foliar morphological evaluation of white variety of Hibiscus visualized under phase contrast microscope. Leaf impression sections: (a) Stomata on abaxial surface (10x), (b) Stomata with epidermal layer on adaxial surface (10x), (c) epidermal layers (10x), (d)Anisocytic stomata (40x); Leaf constants evaluation: (e) Stomata on the abaxial surface (10x), (f) stomata with epidermal cells on the adaxial surface (10x), (g) Anisocytic stomata (40x).

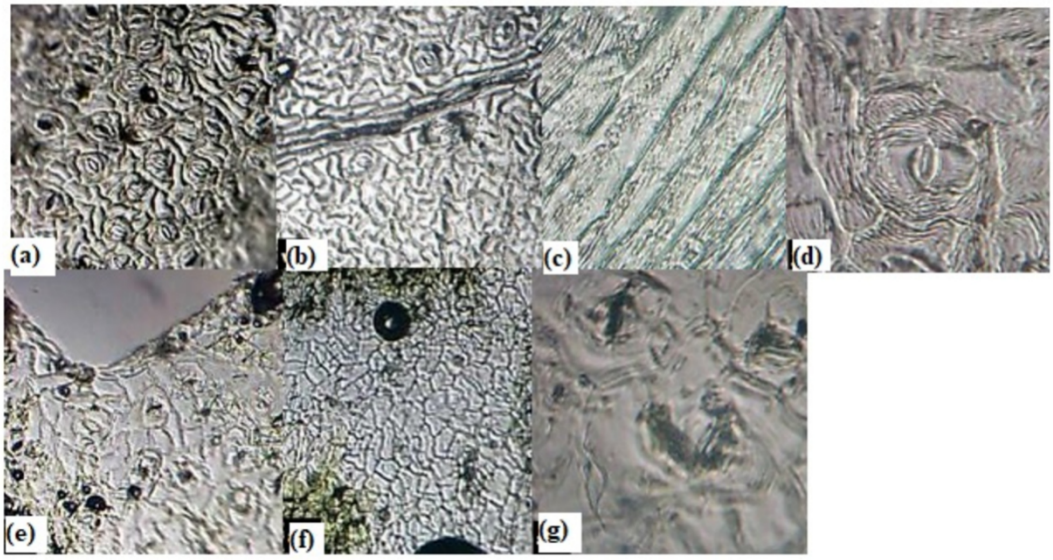


Figure 5 Foliar morphological evaluation of pink variety of Hibiscus, visualized under phase contrast microscope. Leaf impression sections: (a) Stomata on abaxial surface (10x), (b) Stomata with epidermal layer on adaxial surface (10x), (c) epidermal layers (10x), (d)Anisocytic stomata (40x); Leaf constants evaluation: (e) Stomata on the abaxial surface (10x), (f) stomata with epidermal cells on the adaxial surface (10x), (g) Anisocytic stomata (40x).

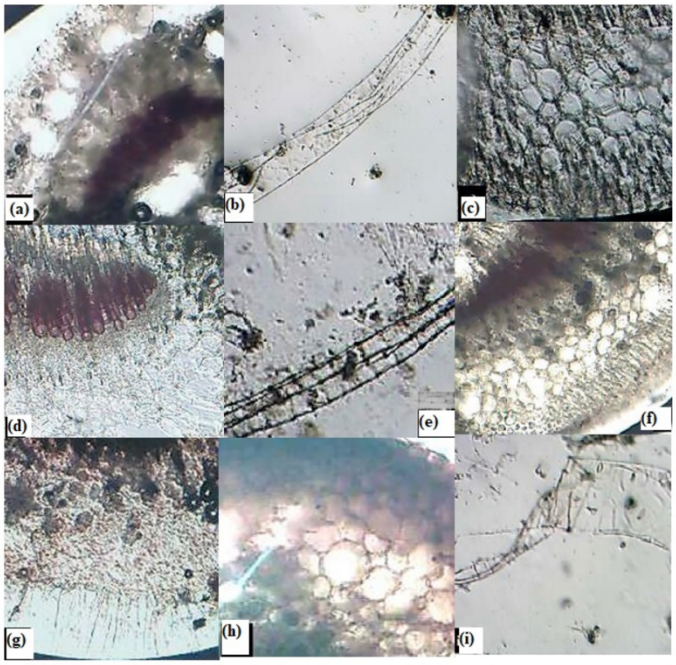


Figure 6 Transverse section of different varieties of Hibiscus under Phase contrast microscope (10x). Pink variety :(a) Lignified tissues with cortical parenchymal cells (b) cuticle layer; Red variety: (c) thin strips of collenchyma cells, (d) arc shaped vascular bundles containing lignified tissues (e) presence of cuticle layer, stained with 80% sulphuric acid; White variety: (f) xylem and phloem with collenchyma cells (g) cuticle with epidermal cells; Yellow variety: (h) collenchyma cells and (i) cuticle with stomata.

CONCLUSION

The present experimental work revealed the importance of foliar studies in the present scenario. Such studies are having some beneficial aspects in the systematic and diagnostic studies of this plant in future. The determination of the micro-morphological evaluation could serve as an important factor which helps in collecting the information of adaptability and physiological changes of the plant with the environmental influence. The present study may give a new direction or new way to analyse the effect as well as the response of micro-morphological parameters under various climatic conditions. Such research will provide an idea for further addition of electron microscopy for clear and enlarged observation of floral parts of the leaf section.

CONFLICT OF INTEREST

The authors confirm that the present research experiment has no conflict of interest.

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