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Pharmacognostical study and establishment of quality parameters of *Hibiscus radiatus* cav. leaves as per WHO guidelines

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Abstract
Hibiscus radiatus Cav. (Malvaceae) Leaves have long been used as a traditional remedy by the tribe of India to treat various ailments. Pharmacognostical evaluation of *Hibiscus radiatus* leaf has been carried out to determine its macro and microscopic characters, and also some of its quantitative characters as per WHO guidelines. The Pharmacognostical evaluation of *Hibiscus radiatus* leaves revealed the presence of characteristic microscopic features of the crude drug like small epidermal cells, parenchyma greatly in shape, xylem vessels is numerous, Phloem is small in size etc. The Physicochemical analysis showed significant values for moisture content, water soluble extractive, alcohol soluble extractive and total ash which are within the World Health Organization (WHO) standards for crude drug from medicinal plants. All the parameters evaluated in the study will aid to identify the authenticity of *Hibiscus radiatus* Cav. leaf even from the crushed or powdered form.

Keywords: WHO, extraction, organoleptic, microscopy, physico-chemical

Introduction
Medicinal plants are playing most active role in traditional medicines for the treatment of various diseases [1-7]. As there is no evidence of documentation and absence of stringent quality control measures of most of the medicinal plants, there is a need for the record of all the research work carried out on traditional medicines in the form of documentation. With this drawback, it becomes extremely important to make surety about the standardization of the plant and parts of plant to be used as a medicine. For the process of standardization, we can use different techniques and methodology to achieve our goal in the stepwise manner e.g. Pharmacognostical and phytochemical studies. These steps and processes are helpful in identification and standardization of the plant material. Correct characterization and quality assurance of starting material is an essential step to ensure reproducible quality of herbal medicine which will help us to justify its safety and efficacy [8-11]. For this purpose we have performed pharmacognostical studies of *Hibiscus radiatus* leaves.
Hibiscus radiatus Cav. also known as monarch rosemallow, is an ideal crop for developing countries as it is relatively easy to grow, can be grown as part of multi-cropping systems and can be used as food and fibre. The genus Hibiscus (Malvaceae) includes more than 300 species of annual or perennial herbs, shrubs or trees [12].
The plant is about 3m tall and has a deep penetrating taproot. It has a smooth or nearly smooth, cylindrical, typically dark green to red stems. Leaves are alternate, 7 to 11cm long, green with reddish veins and long and short petioles. Leaves of young seedlings and upper leaves of older plants as simple; lower leaves are deeply 3 to 5 or even 7 – lobed and the margins are toothed [13].

Materials and Methods
Collection of plant material
The plant *Hibiscus radiatus* Cav. belonging to the family *Malvaceae* were collected from local area of Basna, district - Mahasamund, Chhattisgarh, India and was identified and authentified by M. Ahmedullah scientist 'E' Botanical Survey of India (BSI), Deccan Regional Centre, Hyderabad 500048, Telangana (Establishment under the Ministry of Environment & Forests, Government of India). The plant voucher No. is BSI/DRC/2015-16/Tech./664/07 dated 30-10-2015.

Fig 1: *Hibiscus radiatus* Cav. flower and leaves

Table 1

Scientific Classification	
Botanical Name	***Hibiscus radiatus* Cav.**
Kingdom	Plantae
Subkingdom	Tracheobionta
Super division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Dilleniidae
Order	Malvales
Family	*Malvaceae*
Genus	Hibiscus
Species	radiatus

Instruments and Chemicals

Compound microscope, stage micrometer, camera lucida, drawing sheets, glass slides, cover slips, watch glass and other common glassware were the basic apparatus and instruments used for the study. Microphotographs were taken using a Leica (Dissecting Microscope Lighting System-DMLS) microscope attached with Leitz (Magnification Power System- MPS) 32 camera. Solvents viz., 95% Methanol (MeOH), Petroleum ether (PE), chloroform and reagents such as phloroglucinol, glycerin, hydrochloric acid, chloral hydrate, and sodium hydroxide were procured from Merck Specialities Pvt. Ltd., Balanagar, Hyderabad, Telangana.

Macroscopic and Organoleptic Characterization

The macroscopic and organoleptic measurement were performed for important characters of fresh leaves like phyllotaxy, size, shape, colour, venation, presence or absence of petiole, apex, margin, base, lamina, texture, surface, odour and taste [14, 15].

Microscopic Characterization - Anatomical studies of the leaf

Free hand transverse sections of leaf lamina and midrib were prepared, stained with safranin, mounted on glass slides using glycerine and observed under light microscope by reported methods [16, 17]. Photomicrographs of the microscopical sections were taken with the help of Digital Microscope (MOTIC) provided with MOTIC IMAGE PLUS 2.0 software.

Determination of Stomatal Number and Stomatal Index

A small piece of leaf was cleaned by boiling with sodium hypochlorite solution. The upper and lower epidermis was peeled separately. The peeled epidermis was placed on slide and mounted with glycerine water. Average number of stomata per mm2 of the epidermis of the leaf (Stomatal number) is calculated from the microphotographs taken using camera attached microscope [18, 19]. Values for upper and lower epidermis were determined separately using the equation:

Stomatal index (SI) = S × 100 /E+S.

Where, S= the number of stomata per unit area and E = the number of epidermal cells in the same unit area of leaf.

Determination of Vein-Islet Number and Vein-Let Termination Number

Vein islet is the minute area of photosynthetic tissue encircled by the ultimate division of the conducting strands. Vein termination number is the number of veinlet terminations per mm^2 of leaf surface. A piece of the leaf was cleared by boiling in chloral hydrate solution and camera lucida and drawings board were arranged and 1 mm. line was drawn with help of stage mm. A square was constructed on this line in the centre of the field. The slide was placed on the stage. The veins included within the square were traced off, completing the outline of those islets which overlap two adjustment side of the square. The average number of vein islet from the four adjoining square, to get the value for one square mm was calculated [20]. The number of veinlet termination present within the square was counted and the average number of veinlet termination number from the four adjoining square to get the value for 1 square mm was found known as vein termination number.

Determination of Palisade Ratio

A piece of the leaf was boiled in chloral hydrate and was placed under microscope. Camera lucida and drawing board were arranged and the outline of four cells of the epidermis was traced using 4 mm objective. Then, palisade layer was focused down and sufficient cells for covering the tracing of the epidermal cells were traced off. The outline of those palisade cells which were intersected by the epidermal walls was completed. The palisade cells under the four epidermal cells (including cells which are more than half and excluding cells which are less than half within the area of epidermal cells) were counted. The determination for five groups of four epidermal cells from different part of the leaf was repeated. The average number of cells beneath epidermal cells was calculated known as palisade ratio [21].

Extraction

The authenticated plant materials were subjected for soxhlet extraction in which the solvent vapour generated by gently heating the reservoir condenses and is allowed to drip back onto the porous sample cup. The liquid condensate that drips onto the sample performs the extraction which then passes through the container and back into the reservoir. The cycle is repeated continuously and can be sustained as long as needed. The dried materials were coarsely powdered; the powder was packed in filter paper and loaded into the thimble of soxhlet extractor. The solvent was used for extraction was poured into flask. The soxhlet extraction was carried out for 72 hours. Later the extracted solvent was evaporated under reduced pressure to get waxy extract. The extractive value of the extraction was obtained by using the relation,

$$\% \text{ of extraction} = \frac{\text{Weight of dried extract}}{\text{Weight of fresh material}} \text{X100}$$

Determination of Extractive Values

5 g of air dried coarse powder of extracts macerated with100 ml. of solvent (petroleum ether, chloroform, alcohol and water) in a glass-stoppered conical flask with frequent shaking for 6 hours and then allowed to stand for 18hours. There after it was filtered rapidly taking care against loss of

solvent. About 25ml. of the filtrate was evaporated in a flat-bottomed dish to dryness using water bath and then dried at 105^0C for 6 hours, cooled in a desiccator for 30 minutes and weighed immediately [22].

Physicochemical Analysis

The fresh leaves were separated from the collected plants, thoroughly washed with fresh water, shade dried and powdered. The physicochemical parameters like ash value, acid-insoluble ash value, water soluble ash value, moisture content, foaming index and swelling index of *Hibiscus radiatus* leaves were determined according to the quality control methods for medicinal plant materials [23, 24].

Determination of Ash

The ash remaining following ignition of medicinal plant materials is determined by three different methods which measure total ash, acid-insoluble ash and water-soluble ash. The total ash method is designed to measure the total amount of material remaining after ignition. This includes both "physiological ash", which is derived from the plant tissue itself, and "non-physiological" ash, which is the residue of the extraneous matter (e.g. sand and soil) adhering to the plant surface. Acid-insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth. Water-soluble ash is the difference in weight between the total ash and the residue after treatment of the total ash with water [25].

Total Ash value

The method for determination of total ash is designed to measure the total amount of material remaining after ignition. This includes both “physiological ash”, which is derived from the plant tissue itself, and “non-physiological ash”, which is residue of extraneous matter (e.g. sand and soil) adhering to the plant surface. When vegetable drugs are incinerated, they leave an inorganic ash in some plants called the total ash.

Four grams of the ground air-dried sample was weighed into previously ignited, dried and tarred silica crucible. The material was spread evenly as a thin layer. Kept on a gas burner under a low flame and ignited slowly to obtain a carbonized residue. It was then placed in the muffle furnace and the temperature of the muffle was adjusted to 450-500 0C and heated for 3 hours, cooled in a desiccator and weighed [25].

Determination of Acid Insoluble Ash

Acid insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth. Total ash treated with dilute hydrochloric acid reacts with minerals to form soluble salts and the residue which consists mainly of silica is the acid insoluble ash.

To the Silica crucible containing the total ash obtained 25 ml of hydrochloric acid was added, covered with a watch glass and boiled gently for 5 minutes on a hot plate. The watch glass was rinsed with 5 ml of hot water and these washings added to the crucible and filtered. The insoluble matter was collected on an ashless filter paper by filtration. The filter paper was rinsed repeatedly with hot water until the filtrate was neutral /free from acid. The filter paper containing the insoluble matter was transferred to the original crucible, dried on a hot plate and ignited to a constant weight in the muffle furnace at 450-500 ºC. The silica crucible was removed from the muffle furnace and allowed to cool in a desiccator for 30 minutes, and then weighed without delay [25].

Determination of Water-Soluble Ash

To the crucible containing the total ash, add 25 ml of water and boil for 5 minutes. Collect the insoluble matter in a sintered-glass crucible or on an ashless filter-paper. Wash with hot water and ignite in a crucible for 15 minutes at a temperature not exceeding 450 °C. Subtract the weight of this residue in mg from the weight of total ash. Calculate the content of water-soluble ash in mg per g of air-dried material [25].

Determination of Moisture Content or Loss on Drying

About 5 g. of each raw material were accurately weighed. The air dried material was taken in a previously dried and tarred flat weighting bottle in IR moisture balance and the temperature was adjusted to 105 ^{0}C and heating was done for 5 minutes. The procedure was repeated for three times for different samples and the loss in weight of the formulation was calculated with respect to the original weight [25].

$$\text{The formula used for calculating LOD is} = \frac{W_1}{W_2} x\,100$$

W_1 - weight of raw material after heating

W_2 - Original weight of the raw material

Determination of Foaming Index

Many medicinal plant materials contain saponins that can cause persistent foam when an aqueous decoction is shaken. The foaming ability of an aqueous decoction of plant materials and their extracts is measured in terms of a foaming index. Finely divided (sieve No. 1250) plant powder 1g was kept into a 500 ml flask containing 100 mL of boiling water for 30 min. Then cooled and filtered into a 100ml volumetric flask and sufficient water was added to make up the volume. The prepared decoction was transferred into 10 stoppered test tubes each 1 mL, 2 ml up to 10 ml. The volume of the liquid in each tube was adjusted to 10 mL with water. The tubes were duly stoppered and shaken in a lengthwise motion for 15 sec (two shakes per second) and allowed to stand for 15 min. The foam height in each tube was measured [25].

Determination of Swelling Index

Many medicinal plant materials are of specific therapeutic or pharmaceutical utility because of their swelling properties, especially gums and those containing an appreciable amount of mucilage, pectin or hemicellulose. Leaf powder (1g) was taken in a measuring cylinder (25 mL) and suspended in 25 mL distilled water for 1h by thorough mixing every 10 min. After 3 h, volume in mL occupied by the plant material including any sticky mucilage was measured. The experiment was repeated three times for accuracy and the swelling index was calculated [25].

Preliminary Phytochemical Investigations

The qualitative chemical tests carried out for the identification of the different nature phytoconstituents present in the powered crude drugs by standard procedures. They are usually tested for the presence of alkaloids, flavonoids, phenols, tannins, cardiac glycosides, triterpenes, steroids and saponins [26, 27].

Florescence Analysis

A small quantity of dry leaf powder is placed on oil free clean

microscopic slide and 1-2 drops of freshly prepared reagent solution is added, mixed by gentle tilting the slide and wait for few minutes. Then the slide is kept inside the UV chamber and observe the colour in visible light, short (254 nm) and long (365 nm) ultra violet radiations. The color observed by application of different reagents in different radiations is recorded [28, 29].

Some constituents exhibit fluorescence in the visible range in daylight. The ultra violet light produces fluorescence in many natural products which do not visibly fluoresce in daylight. If substance themselves are not fluorescent, they may usually convert into fluorescent derivatives or decomposition products by applying different reagents. Hence crude drugs are often assessed qualitatively in this way and it is an important factor for pharmacognostic evaluation of crude drugs [30].

Results and Discussion

Macroscopic and Organoleptic Characterization

Macroscopic and organoleptic characters of the fresh leaves of *Hibiscus radiatus* were noted and the results were presented in the Table 1.

Table 1: Macroscopic and organoleptic characters of *Hibiscus radiatus* leaf

S. No	Macroscopic parameters	Observation
1	Colour	Green
2	Odour	Odourless
3	Taste	Mucilaginous
4	Phyllotaxy	Alternate or sub opposite
5	Shape	ovate to sub orbicular
6	Venation	Palmate
7	Base	Hastate
8	Apex	Acuminate
9	Margin	Incised

Microscopic Characterization

Transverse section of the leaf shows two distinguished regions, midrib region and laminar region.

Midrib: T.S. of midrib of leaf showed chained, numerous and small epidermal cells. The mesophyll layer is irregular and comprised of 5 - 7 layers. Cells of parenchyma varied greatly in shape and size and were sometimes, elongated or lobed. The xylem vessels were numerous, very big (size) and circular in shape. Phloem vessels were small (size), numerous and circular in shape. Calcium oxalate crystals were dark stained and numerous in mesophyll parenchyma.

Lamina: T.S. of lamina showed cuticle and thick walled cells in lower and upper epidermis. Epidermal cells were large in size, elongated and compact. Palisade parenchyma showed 3 or 4 layers of large, compact and dark cells. Dark stained crystals were present in mesophyll layer. The spongy mesophyll was wider comprising of 7-8 layers of lobed tightly interconnected cells. Trichomes were absent on both upper and lower surfaces. Vascular bundles had compact parallel rows of xylem vessels and fibres. (Fig. 2, 3)

Quantitative Leaf Microscopy

A quantitative leaf characteristic which includes Stomatal no., Stomatal index, vein-islet number, vein-let termination number and Palisade Ratio were observed and the results were shown in the Table - 2.

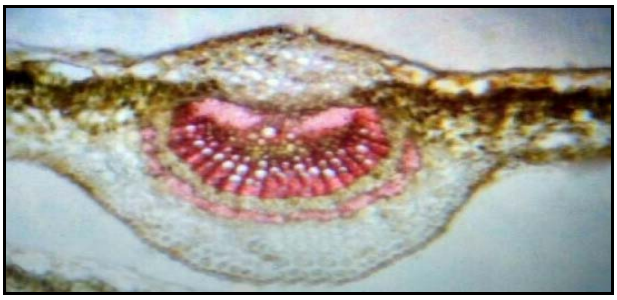

Fig 2: Transverse Section of Leaf Midrib.

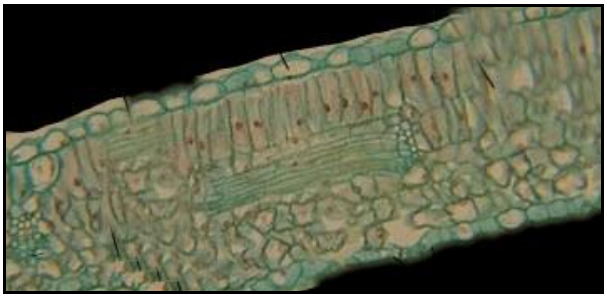

Fig 3: Transverse Section of Lamina.

Table 2: Quantitative Leaf Microscopy of *Hibiscus radiates*

S.no.	Parameter	Range
1	Stomatal number	8
2	Stomatal index	12.26
3	Vein islet number	12
4	Vein let termination number	13
5	Palisade Ratio	08

Physicochemical Parameters

The results of the various physicochemical constants of raw material lie within the limit which is given in Table 3; this signifies that the purity and quality of raw material was good enough. Insufficient drying may lead to spoilage by moulds and bacteria and makes possible the enzymatic destruction of active principles. Not only the ultimate dryness of the drug is important, equally important is the rate at which the moisture is removed and the condition under which it is removed thus the determination of moisture content also provide the method of preparation of drug and it is observed that the moisture content of the drug was found to be 4.3 ± 2.25% w/w which signify that the drug is properly dried and properly stored.

The total ash is particularly important in the evaluation of purity of drugs, i.e. the presence or absence of foreign matter such as metallic salts or silica. An analytical result for total ash was found to be 6.5% w/w. The amount of acid-insoluble matter present was 6.63% w/w. The water soluble ash was found to be 2.41% w/w; this parameter is used to detect the presence of material exhausted by water. As the ash values of the crude drugs lies within the fair limit which signify its quality and purity and gives idea about the total inorganic content. The water soluble extractive values indicated the presence of sugar, acids and inorganic compounds.

The water soluble extractive value found to be 11.72 ± 0.35% w/w and the alcohol soluble extractive was found to be 6.03 ± 0.23% w/w which signify that the large amount of constituents of leaves was soluble in water and alcohol.

Table 3: Physicochemical parameters of *Hibiscus radiatus* leaves

S. no.	Parameters	Values
1	Total Ash value	6.5% w/w
2	Acid insoluble ash	6.63% w/w
3	Water soluble ash	2.41% w/w
4	Moisture content (loss on drying)	4.3 ± 2.25% w/w
5	Foaming index (10ml.conc.)	1ml.
6	Swelling index	5.21 ml.
7	Alcohol-soluble extractive	6.03 ± 0.23% w/w
8	Water-soluble extractive	11.72 ± 0.35% w/w

Values are expressed as mean ± SD of six values

Phytochemical Screening

The qualitative chemical tests were carried out for the identification of the different nature of phytoconstituents present in the crude drugs of *Hibiscus radiatus* by standard procedures. They are usually tested for the presence of alkaloids, flavonoids, phenols, tannins, cardiac glycosides, triterpenes, steroids and saponins**.** The results were shown in Table no.4.

Table 4: Phytochemical screening of different extracts of *Hibiscus radiatus* leaves.

Tested Group	Ether Extract	Chloroform Extract	Methanol Extract	Aqueous Extract
Alkaloids	---	---	+++	+++
Glycosides	---	---	+++	---
Phenolic compound	---	---	+++	+++
Steroids & Sterols	+++	---	---	---
Saponins	---	---	---	+++
Flavones & Flavonoids	---	---	+++	+++
Proteins, Amino acids	+++	---	---	---
Carbohydrates	---	---	---	---
Tannins	---	---	---	---

Note: (+++) Present (---) Absent

Fluorescence Analysis

The fluorescence analysis of the powder drug was done and results are given in Table No. 5. The powder was treated with various reagents and the mixture was observed under UV light (254nm and 366nm). Fluorescence study is an essential parameter for first line standardization of crude drug. In fluorescence the fluorescent light is always of greater wavelength than the exciting light. Light rich in short wavelengths is very active in producing fluorescence and for this reason ultraviolet light produces fluorescence in many substances which do not visibly fluoresce in daylight.

Table 5: Observations of *Hibiscus radiatus.* leaves powder under visible light and UV (254nm and 366nm)) light.

S. No.	Treatment	Visible light	Observation (Colour developed)	
			UV-254nm	UV-366nm
1	Sample as such	Light Green	Dark Green	Black
2	Powder + 1N aq. NaoH	Brown	Green	Black
3	Powder + 1N alc. NaOH	Dark Brown	Green	Black
4	Powder + 1 N HCl.	Green	Black	Black
5	Powder + 50% HNO_3	Brown	Greenish Black	Black
6	Powder + 50% H_2SO_4	Dark Green	Black	Black
7	Powder + Methanol	Reddish brown	Green	Black
8	Powder + NH_3	Brown	Light Green	Greenish Black
9	Powder + I_2	Greenish Brown	Dark Green	Black
10	Powder + $FeCl_3$	Brown	Green	Black

Conclusion

In the present study as per establishment of pharmacognostical standard and quantitative parameter, a great bulk of information on identity, purity and quality of plant material is gained while evaluating the macroscopy, microscopy, powder and physicochemical characters. Thus, all studied standardization parameter like pharmacognostical study; phytochemical screening and physicochemical parameter provide the knowledge in the identification and authentication of leaf of *Hibiscus radiatus.*

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