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PHARMACOGNOSTICAL AND PHYSICOCHEMICAL EVALUATION OF *AEGLE MARMELOS* (L.) CORREA. LEAF EXTRACT.

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ABSTRACT

Plants serve as a vast source for varied phyto-constituents exhibiting varied pharmacological properties. Identifying such potential plants is of significance in medicine. So it becomes necessary to study the pharmacognostic characteristic and physicochemical parameters of the plant before its use in the field of research and also in pharmaceutical formulation. Moreover, it also helps in distinguishing it from other allied species and adulterants. In this connection, the pharmacognostical characteristics of the leaf of *Aegle marmelos* (L.) Correa. were examined. The macro and microscopical characteristics like, vein islet numbers, palisade ratio, stomatal index (upper and lower surfaces of the leaf) etc were studied. Physicochemical parameters evaluated include ash values, extractive values and loss on drying. These findings will be helpful towards establishing pharmacognostic and physiocochemical standards on identification, purity, quality and classification of the plant, which is gaining relevance in plant drug research.

KEYWORDS: *Aegle marmelos (L.) Correa., Pharmacognostic standardization, physicochemical evaluations*.

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INTRODUCTION

In the indigenous system of medicine, the plants in crude form, either fresh or dried are utilized for their curative effects against a variety of mankind's ailments. However, due to some morphological similarities and lack of correct identification, the crude drugs are often adulterated or substituted in commerce, which obviously results in the loss of drug efficacy. Correct identification of a herbal drug is the foundation for the safe use of plant based natural health products. Without proper identification as a starting point, the safe use of quality products cannot be guaranteed[1]. Hence, adulteration in market samples has become one of the greatest drawbacks in promotion of herbal products[2]. Dried products sold in the market are generally difficult to identify, as many useful diagnostic characters are lost during drying. At the same time other numerous problems are confronted to taxonomists in the identification of traded herbal drugs. The existence of several common names for the same plant species in different areas may confuse end users for selection and utilization of genuine drug. Another problem is superficial resemblance of plant species within the same tribe or family[3]. Problem of adulteration in medicinal plants arose due to the potential use of different species for similar ailments[4].The quality control of herbal drugs and their bio-constituents is of paramount importance in justifying their acceptability in modern system of medicine. One of the major problems faced by user industry is non-availability of rigid quality control profiles for herbal raw materials and their formulations. With the advent of new analytical tools and sophisticated instrumental technology, it is possible to suggest a practicable quality assurance profile for a crude drug or its bioactive constituent[5].Over the years, nature and degree of evaluation of crude drugs has undergone a systematic change. Initially, the crude drugs were identified by comparison only with standard description available. Due to advancement in the chemical knowledge of crude drugs, at present, evaluation also includes method of estimating active constituent present in the crude drug, in addition to its morphological and microscopic analysis. With the advent of separation techniques and instrumentation analysis, it is possible to perform physical evaluation of a crude drug, which could be both of qualitative and quantitative nature. The biological behaviour of crude drug extracts constitutes pharmacological evaluation.The crude drugs can be identified on the basis of their morphological, histological, chemical, physical and biological studies.Each and every plant has its unique external and internal body structure (morphology) whether it is from the same or different genus. Various types of tissues / cells form the plants. These cell types can become markers for their identity. For confirmation of the same, the evaluation of the external and the internal morphology of the leaves of *Aegle marmelos* (L.) Correa..in fresh and powdered form was done using standard procedures from Indian Herbal Pharmacopoeia[6].Physicochemical analysis includes the study of various parameters such as determination of foreign organic matter, ash content, water & ethanol extractives, loss on drying and percentage moisture content. The evaluation of these parameters gives a clear idea about the specific characteristic of the medicinal plant under examination, besides it's macro-morphological or cyto-morphological characters.So also most of the crude drugs (plant material) are usually put in quarantine store and they remain there for long time. During storage proper ventilation, humidity controls, suitable temperature and light conditions should be ensured to maintain their original pharmacological action; however, it is observed that, crude plant materials, before being taken for processing, are not analyzed and can lead to changes in their original characteristics. To avoid this, the crude drugs should be tested for all the physicochemical parameters as per the United State Pharmacopoeia (USP)[7] and Indian Herbal Pharmacopoeia (IHP)[6].This paper reports the pharmacognostical standardization and physicochemical parameters which includes macroscopical, microscopical and quantitative microscopy studies. The study also includes determination of physico-chemical constants of leaves of *Aegle marmelos* (L.) Correa. Therefore the present work has been undertaken to establish the various pharmacognostical and physicochemical parameters, which could serve as a measure of authentication and quality control for commercial samples of the crude drug.

MATERIALS AND METHODS

Leaves of *Aegle marmelos* (L.) Correa. were collected from Mumbai and Talegaon – Dabhade (district - Maval, Pune). The plant samples *Aegle marmelos* (L.) Correa.(Acc. no.-08649,08652)was authenticated by the taxonomist Dr. Rajendra Shinde, Blatter Herbarium, Xavier's College-autonomous, Mumbai.

Pharmacognostical Study

The external and the internal morphological characteristics of the leaves of *Aegle marmelos* (L.) Correa. were studied in fresh form. For the microscopic study of the plant powder, the plant material was dried and processed as explained under collection.

Macroscopic studies of fresh plant leaves

External morphology of the said plant leaves were studied by naked eye and with the help of dissecting microscope.

Microscopic analysis of fresh plant leaves

The fresh leaves of *Aegle marmelos* (L.) Correa. were separated and washed thoroughly. Thin transverse sections of the leaves were taken and temporarily double stained with saffranin and hematoxylene by the standard procedure. The slides were observed under microscope with different magnifications, anatomical characteristics of the four leaves were noted down and photomicrographic records were made.

Quantitative Microscopy of fresh plant leaves
Determination of Stomatal Index

Leaf fragments of the plants under study (about 5 x 5 mm in size) were placed in test tube containing about 5ml chloral hydrate solution and heated in a boiling water bath for about 15 minutes or until the fragments became transparent. A fragment was transferred to a microscopic slide mounted, in chloral hydrate solution

and a small drop of glycerol, ethanol solution on one side of the cover glass to prevent the preparation from drying. Examination was done at 240x. A microscopical drawing apparatus was attached to the eyepiece A cross (x) was marked on the drawing paper for each epidermal cell and a circle (o) for each stomata. The Stomatal Index was calculated by using following formula[5]

$$\text{Stomatal Index (I)} = \frac{S * 100}{S + E}$$

Where, S: Number of stomata per unit area
E: Number of ordinary epidermal cells in the same unit area.
For each sample of leaf, minimum of ten determinations were made and the average index was calculated.

Determination of Palisade Ratio

Leaf fragments of the plants under study (about 5 x 5 mm in size) were placed in a test tube containing 5ml of chloral hydrate solution and heated in a boiling water bath for about 15 minutes or until the fragments became transparent. A fragment was transferred to a microscopic slide mounted; the upper epidermis in chloral hydrate solution and a small drop of glycerol solution was put on one side of the cover glass to prevent the preparation from drying. Examination was done at 240x. A microscopical drawing apparatus was attached to the eyepiece was marked on the drawing paper. Four adjacent epidermal cells on paper were traced; gently focused downward to bring the palisade into view and sufficient palisade cells were traced to cover the area of the outlines of the four epidermal cells. When the cell is intercepted, it was included in the count only when more than half of it was within the area of epidermal cells. The average number of palisade cells beneath one epidermal cell dividing the count by 4 was calculated[5].

Determination of Vein - Islet Number

Pieces of leaf lamina were taken with an area of not less than 4 square millimeters from the central portion of the lamina and excluding the midrib and the margin of the leaf. The pieces of lamina were cleared by heating in a test-tube containing chloral hydrate solution on a boiling water bath for 30 to 60 minutes or until clear and a mount on glycerol solution was prepared or, if desired, stained with saffranin solution and the mount prepared in Canada Balsam. The stage micrometer on the microscope stage was placed and examination was done at 240x by drawing a line representing 2 mm on sheet of paper by means of a microscopical drawing apparatus and construct a square on the line representing an area of 4 square millimeters. The paper was moved so that the square was seen in the center of the field of the eyepiece. The slide was placed with the cleared leaf piece on the microscope stage and drawn in the veins and veinlets included within the square, completing the outlines of those vein-islets, which overlap the two adjacent sides of the square. The number vein-islets within the square including those overlapping on two adjacent sides and excluding those intercepted by the other two sides were counted. The result obtained is the vein islets in 4 square millimeters. For each sample of leaf make not fewer than three determinations and the average number of vein islets per square millimeter was calculated[5].

Fluorescence analysis and Powder microscopy of the dry plant powders

For fluorescence analysis, the plant leaf powders were examined directly under UV light and day light by adding different reagents (Table No. 2). The powder microscopy was done by staining the dry plant powders with dilute aqueous saffranin for two minutes. At the end of two minutes, the stained material was washed to remove the excess stain and mounted in Dextrin plasticizer xylol (DPX) to make a permanent mount. The slides were studied under light microscope at different magnifications and photomicrographs were taken.

Physico-chemical parameters

Foreign Organic Matter

Medicinal plant materials should be entirely free from visible signs of contamination, i.e. moulds, insects, and other animal contamination, including animal excreta. It is seldom possible to obtain marketed plant materials that are entirely free from some form of innocuous foreign matter. However, no poisonous, dangerous or otherwise harmful foreign matter or residue should be allowed. Any soil, stones, sand, dust and other foreign organic matter must be removed before medicinal plant materials are cut or ground for testing. Macroscopic examination can conveniently be employed for the determination of foreign matter in whole or cut plant materials. In the present study, foreign organic matter of the plant leaf powders was determined as per WHO guidelines (1998)[8].

Calculation

$$\% \text{ Foreign organic matter} = \frac{(M_1 - M)}{M_2} \times 100$$

Where,
M = Weight of empty dish in gm.
M_1 = Weight of dish with foreign matter in gm.
M_2 = Weight of sample (whole plant material) in gm.

Extractable Matter

This method determines the amount of phyto-constituents extracted with solvents from a given amount of medicinal plant material. Here, according to Indian Herbal Pharmacopoeia (1998)[6], British Pharmacopoeia (2009)[9], British Herbal Pharmacopoeia (1990) and United States Pharmacopoeia, (1994)[7] as mentioned in (Mukherjee, 2002)[10], ethanol and water were used as solvents to determine the extractable matter.

Ash Content

The ash remaining after the 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 measures the total amount of material remaining after ignition including 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 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. In the present study, the total ash value**,** acid insoluble ash value, water-soluble ash values of the four plant leaf powders were determined as per WHO guidelines (1998)[8] as mentioned in (Mukherjee, 2002)[10] and Indian Pharmacopoeia, (1996)[11].

Loss on Drying

Loss on drying and the moisture content of the plant samples were determined according to Indian Herbal Pharmacopoeia (1998)[6], British Herbal Pharmacopoeia (1990) and United States Pharmacopoeia, (1994)[7]as mentioned in (Mukherjee, 2002)[10].

Moisture Content

Karl-Fischer titrimetric method

Moisture content of the *Aegle marmelos* (L.)Correa. was estimated using Digital Automatic Karl Fischer Titrator (microprocessor based). The powder of the leaves of *Aegle marmelos* (L.)Correa. was weighed and added to the titration vessel and the titration was allowed to complete. The readings were noted and the percentage moisture was calculated using following formula.

$$\text{Percentage of moisture} = \frac{\text{Titre factor x reading x 100}}{\text{Weight of sample (in mg)}}$$

RESULTS AND DISCUSSION

Pharmacognostical Study

The observations and the results of the pharmacognostic study of the leaves of *Aegle marmelos* (L.) Correa. are discussed as follows –

Macroscopic Analysis of the fresh plant leaves (Figure– 1)

The leaves of *Aegel marmelos* (L.) Correa. are alternate, pale or dark green, compound, trifoliate ; glabrous or grey-pubescent, smooth, alternate, membranous, odd pinnate of 3 leaflets (sometimes 5 leaflets present); lateral leaflets opposite and nearly sessile, ovate-lanceolate, 4.5 cm long and 2.2 cm wide ; terminal leaflet with long rachis, 4-6 cm long and 2-5 cm broad, ovate-lanceolate, entire or crenate, acute, reticulate, covered with glands, rachis 1.5 to 3 cm long, petiole not winged about 3.2 cm long; odour like lemon, taste sweetish, aromatic, slightly like lemon. New foliage is glossy and pinkish-maroon. Mature leaves emit a disagreeable odor when bruised.

Figure 1
Showing external morphology of the leaves of Aegle marmelos (L.) Correa.

Microscopic analysis of fresh plant Leaves

Transverse section (T.S.) of the leaf shows, single layered upper epidermis composed of polygonal barrel shaped cells with straight thick anticlinal walls covered with thick cuticle, cells of lower epidermis are smaller in size, stomata anomocytic, present on both the surfaces but abundant on lower surface; mesophyll differentiated into 2 or 3 layers of small palisade cells which are continuous on midrib and very compactly arranged spongy parenchyma containing chloroplast; large, circular secreatory canals surrounded by single layer of epithelial cells present in mesophyll. Midrib slightly pronounced towards lower surface; shows single layered epidermis covered with thick cuticle, cells of upper epidermis are bigger in comparison to lower surface; lower epidermis of midrib differentiated by 2 or 3 layers of collenchyma, remaining ground tissue is parenchymatous; meristele are arc shaped, vascular

bundle consists of radially arranged xylem and encircled by phloem, pericycle represented by patches of sclerenchymatous fibres. Rare trichomes, found mainly on the lower surface of mid-rib region, unicellular and 399 – 696 µm long. (Figure – 2 and 3)

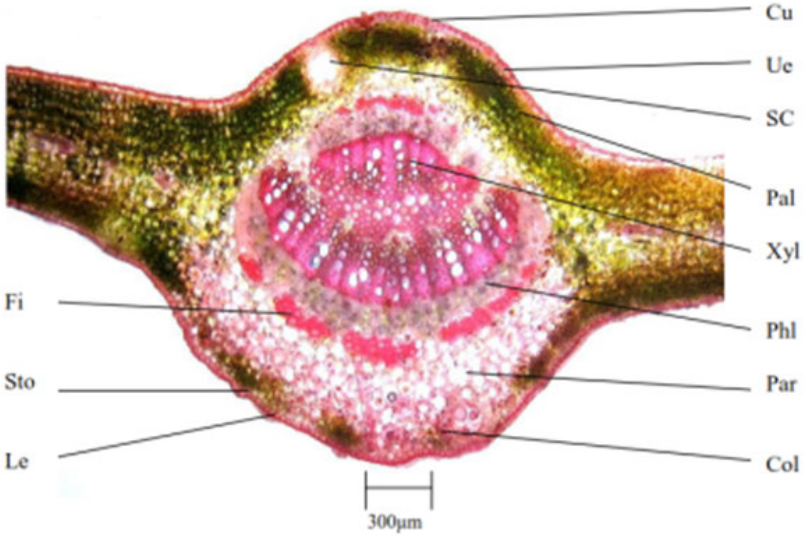

Figure 2

Transverse section of the midrib portion of the leaf of Aegle marmelos (L.) Correa. (Fi: Fibers; Sto: Stomata; Le: Lower epidermis; Col: Collenchyma; Par: Parenchyma; Phl: Phloem; Xyl: Xylem; Pal: Palisade cells; SC: Secretary Canal; Ue: Upper epidermis; Cu: Cuticle)

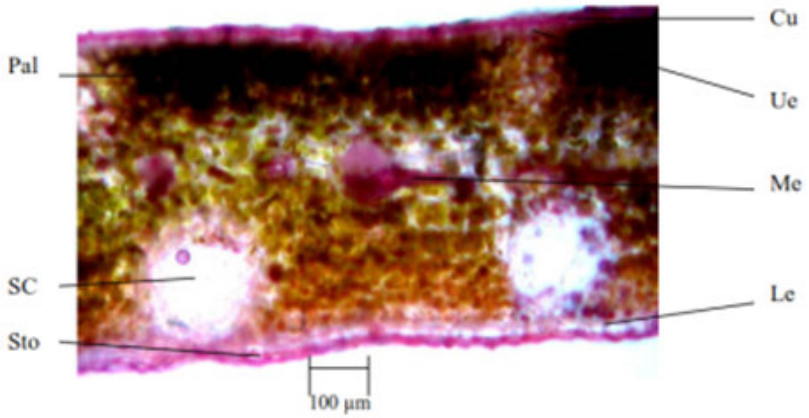

Figure 3

Transverse section of the lamina portion of the leaf of Aegle marmelos (L.) Correa. (Sto: Stomata; Le: Lower epidermis; Pal: Palisade cells; SC: Secretary Canal; Ue: Upper epidermis; Cu: Cuticle; Mes: Mesophyll)

Quantitative microscopy of the plants under study

The result of the quantitative microscopic analysis (determination of the leaf constants) of the fresh leaves of *Aegle marmelos* (L.) Correa. is listed in Table – 1.

Table1

Quantitative microscopy of the leaves of Aegle marmelos (L.) Correa.

Sr. No.	Plant Sample	Stomatal Index		Palliade ratio (under one epidermal cell)	Vein Islet number per square mm
		Upper epi.	Lower epi.		
1	*Aegle marmelos* (L.) Correa.	4 - 8	8.5 - 11.5	1.5 – 2.5	9 - 18

Fluorescence analysis of the plant leaf powders

The fluorescence characters of the leaf powder of *Aegle marmelos* (L.) Correa. Is tabulated in Table - 2. Many phyto-compounds fluoresce when suitably illuminated. The fluorescence colour is specific for each compound. A non-fluorescent compound may fluoresce if mixed with impurities that are fluorescent. The fluorescent method is adequately sensitive and enables the precise and accurate determination of the analyte over a satisfactory concentration range without several time consuming dilution steps prior to analysis of pharmaceutical samples[12].

Table 2
Showing the effect of different chemical reagents on the fluorescence behaviour of crude drug powder of Aegle marmelos (L.) Correa.

Sr. No.	Treatment	Day light	UV light (254 nm)	UV light (365 nm)
1	Distilled water	Light Yellow	Light Green	Fluorescent Dark Green
2	Alcohol	Yellow	Yellow	Fluorescent White
3	Acetic acid	Green	Green	Yellowish Green
4	1N Hydrochloric acid	Yellow Ochre(Yolk colour)	Light Green	Fluorescent light Green
5	2N Sulphuric acid	Green	Green	Fluorescent Dark Green
6	50% Nitric acid	Light Green	Light Green	Fluorescent Green
7	10% Sodium hydroxide	Yellowish Green	Green	Fluorescent Green

Powder microscopy of the plant leaf powders
The leaf powder is bright green in colour and shows following diagnostic features - Fragments of epidermal cells with anomocytic stomata; stone cells; unicellular trichomes; fragments of sclerenchymatous fiber patches and spiral thickening pitted vessels (Figure – 4).

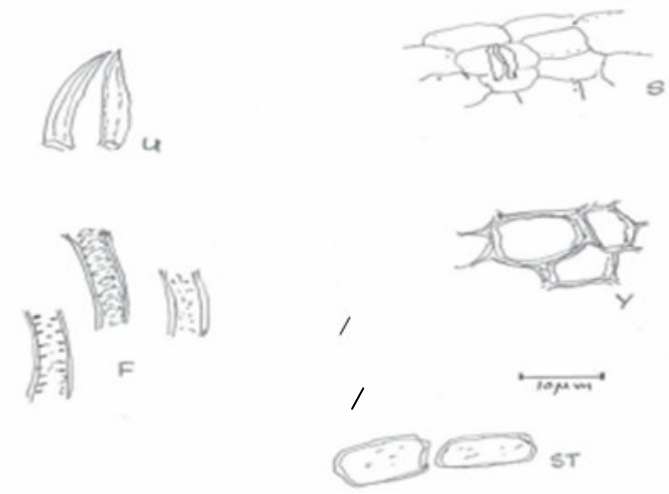

Figure 4
Powder microscopy of the leaf powder of Aegle marmelos (L.) Correa. (S: Epidermal cells with stomata; F: Sclerenchymatous fibers; ST: Stone cells; U: Unicellular trichomes; Y: Yellow coloured polygonal sclerenchymatous cells.)

The findings of the pharmacognostic study of the leaf powder of *Aegle marmelos* (L.) Correa. analysed in the present study were almost complying with the previous reports[13, 14]. Some difference in the analysis might be attributed to the conditions on which the plant is harvested along with environmental parameters[15, 16].

Physico-chemical parameters
The results of all the proximate parameters studied are presented in Table 3 and Figure 5. Study of these parameters is required for checking the quality of raw material of plant samples i.e., leaves of *Aegle marmelos* (L.) Correa. before going for processing. However, these quality control parameters also depend on cultivation, handling of the raw material and storage condition.

Table 3
Results of the Proximate Analysis of the leaves/leaf powders of Aegle marmelos (L.) Correa.

Sr. No.	Parameter	% Content in *Aegle marmelos* (L.) Correa.
1	Foreign organic matter	0.0346 ± 0.0105
2	Ethanol soluble extractive	6.79 ± 0.25
3	Water soluble extractive	27.61± 0.36
4	Total ash	7.05 ± 0.62
5	Acid-insoluble ash	0.63 ± 0.41
6	Water soluble ash	2.87 ± 0.56
7	Loss on drying	9.15 ± 2.12
8	Moisture content	8.839± 2.43

(*All values are expressed as mean ± SD for three determinations)

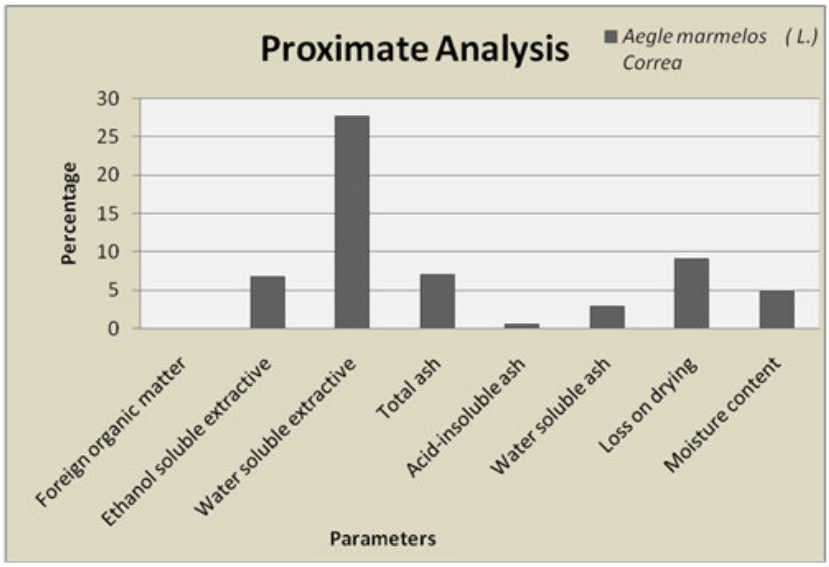

Figure 5
Bar diagram showing the results of the Proximate Analysis of the leave/leaf powder of Aegle marmelos (L.) Correa.

Physico-chemical parameters i.e. ash value of a drug gives an idea of the earthy matter or the inorganic composition and other impurities present along with the drug. Since the ash value is constant for a given drug, this value is also one of the diagnostic parameters of the drug. In certain drug, the percentage variation of ash from sample to sample is very small and any marked difference indicates the change in quality. Unwanted parts of drug, some time possess a character that will raise the ash value. Ashing involves oxidation of the components of the product. A high value is indicative of contamination, substitution, adulterations or carelessness in preparing the crude drug for marketing. In the present study, the total ash value, acid insoluble ash value, water-soluble ash values of the four plant leaf powders were determined as per WHO guide lines[8]. The ash value of the leaf powders of *Aegle marmelos* (L.) Correa. shown in table-3 revealed a high concentration of total ash. The total ash, water soluble ash and acid insoluble ash which are important parameters for detecting the presence of inorganic substances were found to be 7.05 $\pm$ 0.62 % w/w, 2.87$\pm$ 0.56 % w/w and 0.63$\pm$ 0.41 % w/w respectively in the leaf powder of *Aegle marmelos* (L.) Correa. The acid insoluble ash values were found to be very low indicating that the plant drugs were collected afresh[17].Extractive values are primarily useful for the determination of exhausted or adulterated drugs. The water and ethanol soluble extractive values which are indicator of total solvent soluble component are - 27.61$\pm$ 0.36 % and 6.79$\pm$ 0.25% for the leaf powder of *Aegle marmelos* (L.) Correa. respectively. The extractive values of water were found to be more than the ethanol extractive values for the plant leaf powders. The results of various types of ash and extractive values may provide a basis to identify the quality and purity of the drug[13].Loss on drying of the leaf powders of *Aegle marmelos* (L.) Correa. revealed an average of around 9% of moisture in the drugs.The results obtained in the present investigation are encouraging and can be used as reference data for the standardization of leaves of *Aegle marmelos* (L.) Correa. and the formulations containing the plant leaves as a main ingredient.The percentage of loss on drying, total ash values, water soluble ash, acid insoluble ash and resin content of the leaf powder of *Aegle marmelos* (L.) Correa. were determined by Siddique, *et al*., (2010)[14]. The results noticed by them were - loss on drying (0.7433%), total ash (6.3027%), water soluble ash (1.2796%), acid insoluble ash (2.5525%) and resin (0.2100%). Tiwari, *et al*., (2010), have also reported the values for some proximate analytical parameters studied by them using leaves/leaf powder of *Aegle marmelos* (L.) Correa[18]. Their study report indicated –moisture content - ranging between 64-66.5%, total ash - ranging between 6.7-6.95%,, water soluble ash - ranging between 2.35-2.45% and acid insoluble ash - ranging between 0.50-0.53%.The results of our study were found to be in almost in accordance with the study reports[14, 15].In our study, the total ash, water soluble ash and acid insoluble ash values were found to be – (7.05 $\pm$ 0.62 % w/w), (2.87$\pm$ 0.56 % w/w) and (0.63$\pm$ 0.41 % w/w) respectively in the leaf powder of *Aegle marmelos* (L.) Correa.

CONCLUSION

The microscopic evaluation is an indispensable tool for identification of medicinal herbs and is one of the essential parameters in modern monograph. The leaf of the plant under study *viz. Aegle marmelos* (L.) Correa. is used extensively in the traditional system of medicine for the treatment of number of ailments like peptic ulcer, diabetes mellitus, jaundice, constipation accompanied by edema.catarrh, fever, etc.Although it is a very common plant having less possibilities of adulteration but to get highest efficacy of an herbal drug or its finished product cent per cent genuine plant material should be the source material. In this regard the important microscopic features of the leaves of *Aegle marmelos* (L.) Correa. have been documented in this study; such as, T.S. of the fresh leaves, the study of the powder characters of the leaf powders and the quantitative microscopic studies.Fluorescence analysis

of the leaf powder of *Aegle marmelos* (L.) Correa. showed the presence of fluorescence compounds which would serve as valuable information, for the scientist engaged in research, on the medicinal properties of these plant.All the above said characters of the leaf powders reflect the diagnostic features of the *Aegle marmelos* (L.) Correa. plant parts in fresh and/or powdered form and can be used to check adulteration. Also, the evaluation of the various proximate parameters for the leaves of *Aegle marmelos* (L.) Correa. has given a clear idea about the specific characteristics of these crude drugs under examination, in their powder form. While these diagnostic features would enable the analyst to know the nature and characteristics of these plant drugs, further evaluation of different parameters is necessary to indicate their acceptability by criteria other than the proximate analysis. The data obtained in the present study is expected to serve as valuable tool for identification, authentication and detection of adulterants, standardization and quality control of the drugs. The developed technique was also thought to be useful for the standardization of formulations containing leaf powder of *Aegle marmelos* (L.) Correa. Hence, it can be concluded that the present study on the pharmacognostic and physicochemical characters can serve as a vital source of information and provide suitable standards to determine the quality of the leaf powders of *Aegle marmelos* (L.) Correa for future investigations.

CONFLICTS OF INTEREST

Conflict of interest declared none.

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