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DEVELOPMENT OF QUALITY CONTROL PARAMETERS FOR THE STANDARDIZATION OF *AEGLE MARMELOS* (ROXB.) SEED

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Keywords:

Pharmacognosy, Phytochemical analysis Physicochemical analysis, Adulteration, Macroscopic, Microscopic, Flavonoids

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ABSTRACT: The present investigation was undertaken to determine the requisite pharmacognostic standards for evaluating the plant material. The macroscopic, microscopic, powder study, physicochemical, qualitative phytochemical and florescence analysis of the seed were carried out. The microscopic study showed abundant large parenchymatous cells with aleurone grains, calcium oxalate crystals, lignified trichomes, endocarp and testa throughout the section. The powder microscopy showed the presence of lignified trichomes, rossete crystals of calcium oxalates, oil granules, *etc*. The physicochemical properties such as total ash, water soluble ash, acid insoluble ash and sulphated ash were 5.75%, 5.16%, 0.16% and 6.66% respectively. The aqueous soluble and ethyl acetate soluble extractive values were 34.07% and 16.66% respectively. The qualitative phytochemical analysis showed the presence of maximum amount of flavonoids followed by triterpenes, steroids and alkaloids respectively. The seed powder showed characteristic flourescence with various chemical reagents. The data generated in the present work could be used as reference for the standardization and quality control of *A. marmelos* seed. It will help in identifying and preventing intentional or unintentional adulteration of this plant material.

INTRODUCTION: *Aegle marmelos* (Roxb.) belongs to the family Rutaceae. This family consists of 162 genera and 1650 species distributed all over the world. In India, 23 genera with 70 species are reported. *Aegle marmelos* is used in indigestion, constipation diarrhea, dysentery, diarrhea, skin disorders leucorrhea, menstrual irregularities *etc*. The plant is commercially utilized as a bilvachurna.

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Various chemical constituents and components present are marmelosin, alloimperatorin, marmelide, tannic acid, marmin, umbelliferone, isoimperatorin, isopimpinellin, skimmin, marmesin, marmesinin, fatty acids, beta-sitosterol [1]. The plant is well known for various biological activities like anticancer [2], antioxidant [3], antimalarial [4], antibacterial [5] anti-cholestrol activity [6], anti-microbial [7]. This plant is prone to adulteration; the adulteration in bael fruit pulp is done with wood pulp (*Feronia limonia*) and mangosten (*Garcinia magostana*).

The use of herbal drugs as medicine is the ancient form of healthcare and it is used in all cultures throughout history. Ancient humans are well known their dependence on nature for a good

healthy life and since that time humankind depended on variety of plant resources for food, shelter, clothing and medicine to cure immensurable diseases. However, a common problem with herbal medicine or herbal drugs is adulteration. Adulteration, in a broad and legal sense, is the debasement of any article, which involves conditions such as inferiority, spoilage, deterioration, admixture, sophistication, and substitution. Adulterating the crude drugs by any of the said conditions is considered undesirable in the drug industry.

Adulteration is of many types and there are numerous examples of adulteration. Adulteration with substandard commercial varieties for *e.g*. *Nux-vomica* seeds (*strychnos nux-vomica*) are adulterated with *Strychnos nux-blanda* or *Strychnos potatorum* seeds; Adulteration with superficially similar but inferior drugs for *e.g.* Saffron (*Crocus sativus*) with dried flowers of *Carthamus tinctoria*; Adulteration with artificially manufactured substances *e.g*. Paraffin wax is tinged yellow and adulterated with yellow bees wax, while artificial invert sugar is mixed with honey; Replacement by exhausted drugs for *e.g*. volatile oil containing drugs like clove, coriander, fennel are adulterated by this method; Adulteration of powders *e.g.* red sandal (*Pterocarpus santalinus*) wood in capsicum powder and powdered bark of drugs adulterated with brick powder [8, 9].

There are many reasons of adulteration. Some examples are as follows: Confusion in vernacular names *e.g. Casuarina equisetifolia* for *Tamarix indica* and *Aerva lanata* for *Berginia ciliate*; Lack of knowledge about authentic source *e.g*. *Mesua ferrea* is adulterated with flowers of *Calophyllum inophyllum*; Similarity in morphology *e.g. Mucuna pruriens* is adulterated with *M. utilis* and *M. deeringiana*. To prevent all such incidences, standardization of medicinal plants is of utmost importance. The most easy, simplest and authentic way is to do pharmacognostic studies. Various parameters involved are shown in **Fig. 1**. Pharmacognostic study has been done in various other plants as reported by other researchers [10 - 13].

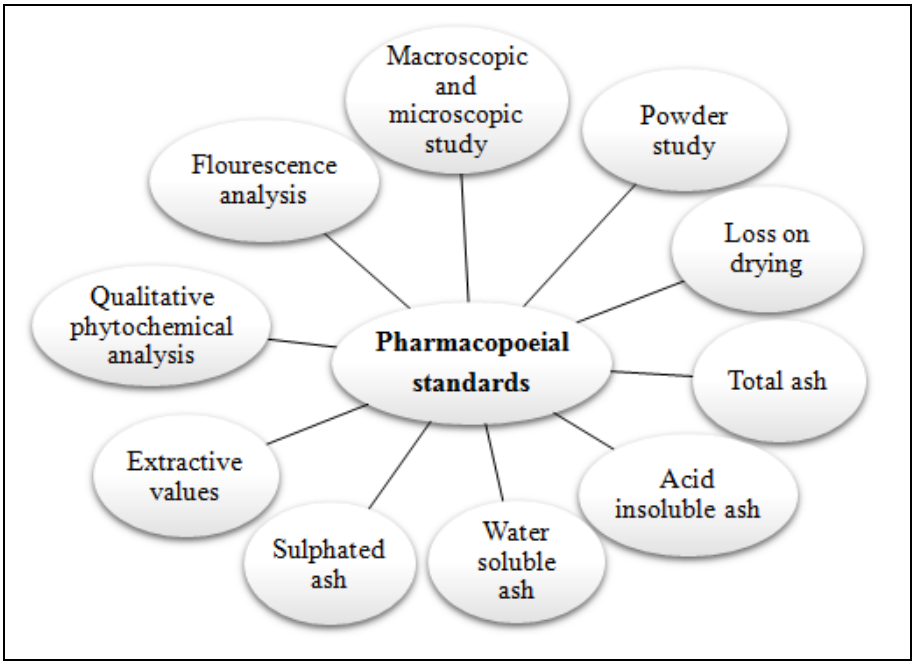


FIG. 1: STANDARDIZATION PARAMETERS

Considering the above, in the present work, *A. marmelos* seed was subjected to various pharmacognostic studies. It involved evaluation of organoleptic, macroscopic, microscopic and powder study; physicochemical, phytochemical and fluorescence analysis was also done.

MATERIAL AND METHODS:

Plant Collection and Extraction: The seeds of *Aegle marmelos* (Roxb). were collected in August, 2016 from Rajkot, Gujarat, India. The plant was compared with voucher specimen (Voucher specimen number PSN104) deposited at

Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India. The seeds were separated from fruit, washed thoroughly with tap water, shade dried and homogenized to fine powder and stored in closed container for further studies. For physicochemical studies, 10 g of dried powder of seeds was extracted in solvents of different polarities (petroleum ether, toluene, ethyl acetate, methanol and water) by cold percolation method. The solvent was evaporated to dryness and dried crude extracts were stored in air tight bottles at 4ºC. Macroscopic and microscopic characters were studied by standard methods. Photographs at different magnifications were taken by using digital camera.

Pharmacognostic Studies:

Macroscopic Studies: Macroscopic studies were carried out by using organoleptic evaluation method. The shape, size, colour, odour, taste, texture, margin, apex of seeds were observed [14].

Microscopic Studies: Microscopic studies were carried out by preparing thin sections of seed. The thin sections were further washed with water, stained with congo red, malachite green and mounted in glycerine for observation and confirm its lignifications (10x, 40x). The powder microscopic studies were also carried out and the specific diagnostic characteristic features were recorded [15].

Physicochemical Analysis: The physicochemical parameters like loss on drying, total ash, acid-insoluble ash, water-soluble ash, sulphated ash and extractive values were determined as per WHO guidelines in dry powder of *A. marmelos* seeds [16-17]. The procedure followed is as described earlier.

Determination of Loss on Drying: Two grams of crude powder of *A. marmelos* seeds were taken in a silica crucible and then dried in an oven at 105 ºC - 100 ºC till constant weight was obtained. The weight after drying was noted and loss on drying was calculated. The percentage was calculated on the basis of sample taken initially [16].

Determination of Total Ash: Two grams of dried powder of seed were taken in a silica crucible and was put in Muffle furnance at 500 ºC for 5 h. It was allowed to cool and then weighed. It was white in colour indicating the absence of carbon. Total ash value was calculated as mg/g of dried material [18].

Determination of Acid Insoluble Ash: 25 ml of hydrochloric acid (70 g/L) was added to the crucible containing total ash. It was covered with a watch-glass and heated gently for 5 min to boil. The watch-glass was rinsed with 5 ml of hot water and this liquid was added to the crucible. The insoluble matter was collected on an ashless filter paper and it was washed with hot water until the filter was neutral. The filter paper containing the insoluble matter was transferred to the original crucible; then it was put in Muffle furnance at 500ºC for 2 h. It was allowed to cool and then weighed. Acid insoluble ash was calculated as mg/g of air dried material [18].

Determination of Water Soluble Ash: To the crucible containing the total ash, 25 ml of water was added and boiled for 5 min. The insoluble matter was collected on an ash less filter paper. It was washed with hot water and heated in a crucible for 15 min. Weight of insoluble matter was subtracted from the weight of total ash. The content of water soluble ash was calculated as mg/g of air dried material [18].

Determination of Sulphated Ash: Two grams of crude powder of *A. marmelos* seeds were taken in a silica crucible and 2 ml 0.1N H_2SO_4 was added and incubated at room temp for 10 min, then it was put in Muffle furnance at 500 ºC for 5 h. It was allowed to cool and then weighed. The content of sulphated ash was calculated as mg/g of air dried material [17].

Qualitative Phytochemical Analysis: Qualitative analysis for the detection of phytoconstituents in the powdered seeds was carried out following the procedure of Harborne [19]. The details of the procedure followed is as described earlier [20]. Alkaloids were detected by using three reagents *viz.* Dragondroff's reagent, Mayer's reagent and Wagner's reagent separately. The tests were scored positive on the basis of orange precipitate, creamish precipitates and brown precipitate respectively. Flavonoids were detected by alkaline reagent test. The crude powder of seeds was treated with a few drops of diluted sodium hydroxide. Formation of intense yellow colour which turned colourless on addition of few drops of diluted HCl indicated the presence of flavonoids.

Tannins were detected by $FeCl_3$ test. The crude powder of seeds was treated with alcoholic ferric chloride ($FeCl_3$) reagent. Appearance of blue colour indicated the presence of tannins. Cardiac glycosides were detected by Keller-kiliani test. The crude powder of seeds was treated with 5% $FeCl_3$ and glacial acetic reagent to which few drops of concentrated H_2SO_4 was added. Appearance of greenish blue colour within few minutes indicated the presence of cardiac glycosides. Steroids were detected by Liebennann-Burchard test. The chloroformic solution of the crude powder of seeds was treated with acetic anhydride and a few drops of concentrated H_2SO_4.

Appearance of blue green ring indicated the presence of steroids. Anthocyanins were detected by the appearance of blue colour on treatment of crude powder of seeds with NaOH. Saponins were determined by Frothing test; the formation of stable froth upon vigorous shaking of crude powder of seeds with distilled water indicated a positive test. Triterpenes were detected by the addition of concentrated H_2SO_4 to the chloroform extract of crude powder of seeds. Appearance of reddish brown ring indicated the presence of triterpenes.

Fluorescence Analysis: Fluorescence study of crude powder of seeds was performed as per Chase and Pratt [21]. A small quantity of the dry powder of seeds was placed on grease free clean microscopic slide and 1 - 2 drops of freshly prepared reagent solution were added, mixed by gentle tilting of the slide and waited for a few minutes. Then the slide was placed inside the UV chamber and observed in visible light, short (254 nm) and long (365 nm) ultra violet radiations. The colours observed by application of different reagents in different radiations were recorded.

RESULTS:

Organoleptic and Macroscopic Characteristics of *A. marmelos*: Organoleptic and macroscopic characteristics of *A. marmelos* seed is given in **Table 1** and **Fig. 2**.

Seed: The macroscopic study showed that the shape of the seed was sub globular, with 0.5 - 1 cm long and 0.5 - 1 cm wide and the helium was raised type. The seed was creamiest white in colour with aromatic odour and taste. The seed was extremely hard and silky hair radially arranged on the surface. Many dense appressed unicellular lignified covering trichomes were also present on the upper surface of seed **Fig. 2**.

TABLE 1: ORGANOLEPTIC FEATURES OF *AEGLE MARMELOS* (ROXB.) SEED

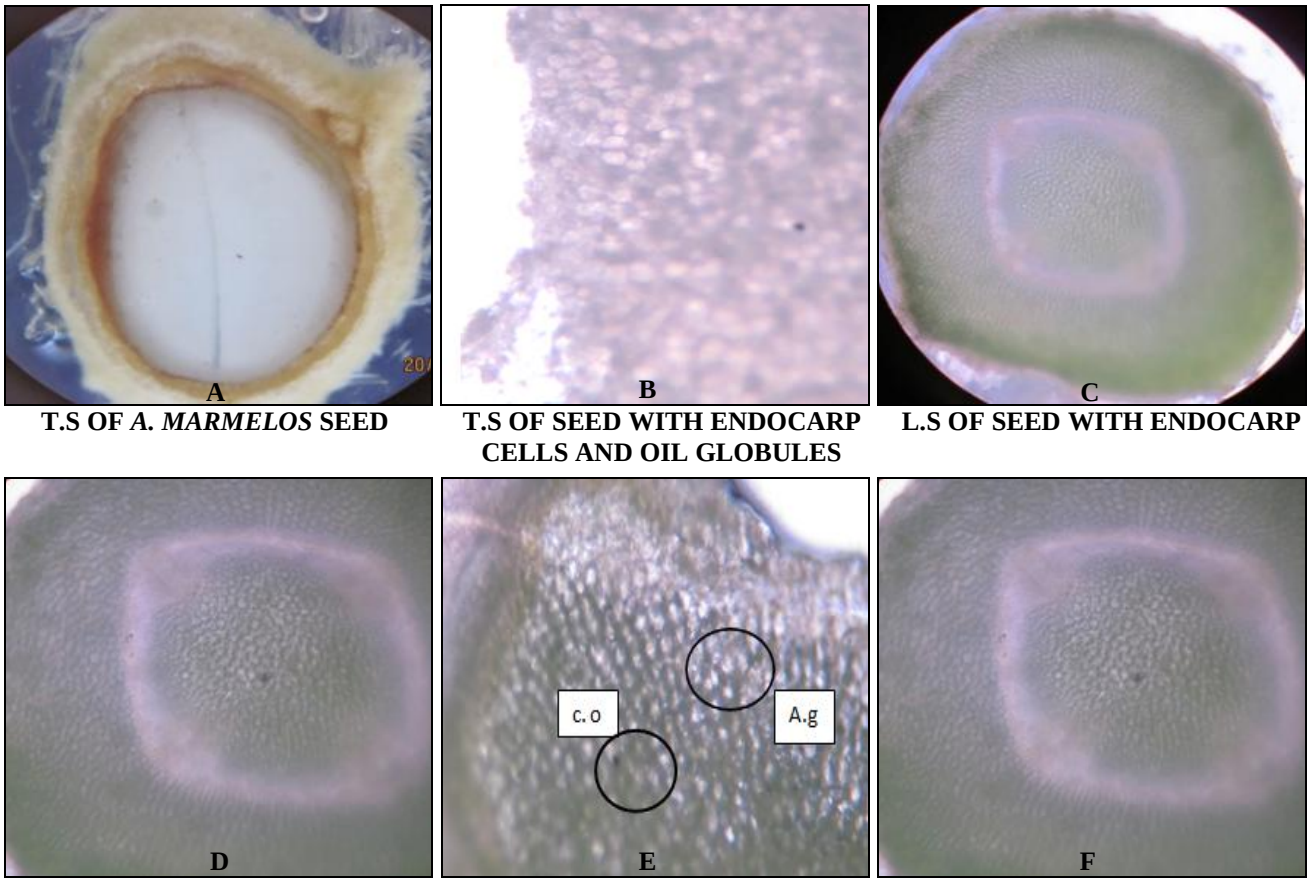
Characters	Observation
Part	Seed
Hilum	raised type
Size	0.5 - 1 cm long, 0.5 - 1 cm wide
Shape	Sub- globular
Colour	creamish white
Odour	Aromatic
Taste	Strongly aromatic
Appearance	Thick smooth
Placentation	Axile placentation in fruit
Apex	Stylopod
Base	Symmetrical
Pedicel	Short
Texture	Slite rought
Seed type	Orthospermous seed

A- *Aegle marmelos* (Roxb) fruit **B- *A. marmelos* seed** **C- Seed**

FIG. 2: MACROSCOPIC CHARACTERISTICS OF *A. MARMELOS*

Microscopic Characteristics:

Seed: The transverse section of *A. marmelos* seed is shown in **Fig. 3**. The seed was divided into two parts testa and endocarp **Fig. 3a**. The endocarpic cells consisted of oil globules **Fig. 3b**. The longitudinal section of seed endocarp contained thick walled cellulosic parenchymatous cells **Fig. 3c** and plasmodesma was in between the walls of endocarpic cells **Fig. 3d**. The endocarp consisted of aleurone grains and parenchymatous cells with rosette crystal of calcium oxalate **Fig. 3e**. The upper part of testa was single layered epidermis covered with lignified trichomes and oblique linear pits with large thick walled cells **Fig. 3f**.



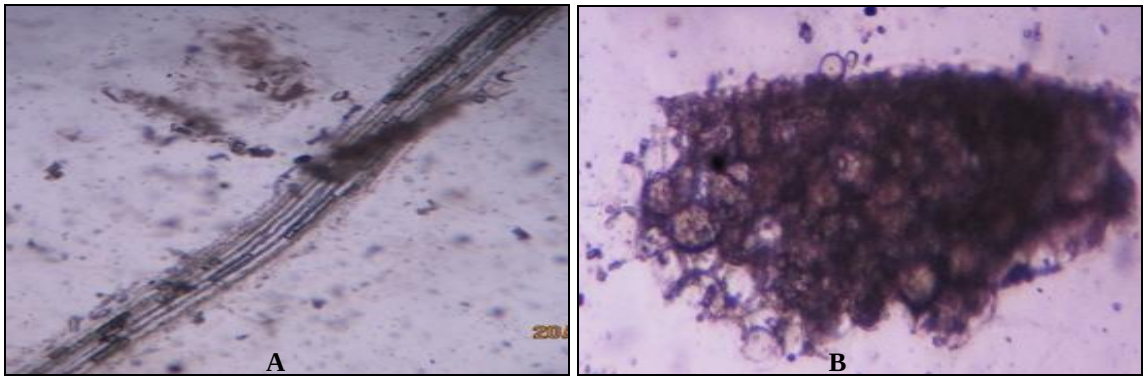
T.S OF *A. MARMELOS* SEED | **T.S OF SEED WITH ENDOCARP CELLS AND OIL GLOBULES** | **L.S OF SEED WITH ENDOCARP**

L. S. OF SEED PASSING THROUGH PLASMODESMA | **ALEURONE GRAIN AND MICRO ROSETTE CRYSTAL CALCIUM OXALATE** | **LIGNIFIED TRICHOMES**

FIG. 3: MICROSCOPIC CHARACTERISTICS OF *A. MARMELOS* SEED

Powder Microscopy of Seed: The crude powder of *A. marmelos* seed was whitish cream in colour, fine, aromatic odour with strongly aromatic taste. The powder microscopy characteristics are given in **Fig. 4**. The specific characteristics determined from the powder study under microscopic investigation showed different types of lignified trichomes, endosperm with aleurone grains and oil globules, rosette crystal of calcium oxalate, polygonal parenchymatous cells, *etc*.

Physicochemical Analysis: The physicochemical analysis of *A. marmelos* seeds is given in **Table 2**. The moisture content of dry powder of seeds was 8%. This seems to be lower than that necessary to support the growth of microbes such as bacteria, fungi or yeast. The ash value was determined in three forms such as total ash, water soluble ash, acid insoluble ash. The total ash in seed was 5.75%, while water soluble ash and acid insoluble ash was 5.16% and 0.16% respectively. The sulphated ash of seed was 6.66%. The extractive value of seed is shown in **Table 3**. The maximum soluble extractive value was found in ethyl acetate seed extract (16.66%) and minimum soluble extractive value was found in petroleum ether seed extract (11.67%). The water soluble extractive value of seed was 34.07%.



A LIGNIFIED TRICHOMES

B ENDOSPERM - ALEURONE GRAIN AND OIL GLOBULES

C ROSETTE CRYSTAL OF CALCIUM OXALATE

D ENDOSPERM POLYGONAL CELLS AND OIL GLOBULES

FIG. 4: MICROSCOPIC POWDER STUDY OF *A. MARMELOS* SEED

TABLE 2: PHYSIOCHEMICAL PARAMETER OF *AEGLE MARMELOS* SEED

S. no.	Parameters	% Value (w/w)
1	Loss on drying	92
2	Total ash	5.75
3	Water soluble ash	5.16
4	Acid insoluble ash	0.16
5	Sulphated ash	6.66
6	Petroleum ether soluble extractive value	11.67
7	Toluene soluble extractive value	14.05
8	Ethyl acetate soluble extractive value	16.66
9	Methanol soluble extractive value	11.78
10	Water soluble extractive value	34.07

Qualitative Phytochemical Analysis: The qualitative phytochemical screening of the crude powder of *A. marmelos* seed is given in **Table 3**. In the seed flavonoids were present in maximum amount followed by triterpenes and steroids. Other phytoconstituent were present in trace amount and anthocyanins and phlobatanins were absent.

Fluorescence Analysis: The fluorescence analysis of *A. marmelos* seeds crude powder treated with various chemical reagents showed characteristic fluorescence at 366 nm and 254 nm wavelength **Table 4**. Some constituents show fluorescence in the visible range in daylight. The UV light produce fluorescence in many natural products which do not visible fluoresces in daylight. Hence crude drugs are often evaluated qualitatively in this way and it is a significant parameter for pharmacognostic evaluation of crude drugs.

TABLE 3: QUALITATIVE PHYTOCHEMICAL ANALYSIS OF *AEGLE MARMELOS* SEED

S. no.	Phytochemicals	Observations
1	Alkaloids	
	Mayer's reagent	+
	Dragondroff's reagent	-
	Wagner's reagent	++
2	Flavonoids	++++
3	Tannins	+
4	Phlobatanins	-
5	Saponins	+
6	Steroids	++
7	Cardiac glycosides	+
8	Triterpenes	+++
9	Anthocyanins	-

Note: (++++) very more amount, (+++) more amount, (++) less amount (+) very less amount (-) absent

TABLE 4: FLUORESCENCE ANALYSIS OF *AEGLE MARMELOS* SEED

S. no.	Treatment	Visible light	Under UV light Short Wave length (254 nm)	Under UV light Long Wave length (365 nm)
1	1N NaOH (aq.)	Light yellow	Black	Dark brown
2	1N NaOH (alco)	Yellow	Black	Light green
3	Ammonia	Light yellow	Dark green	Light yellow
4	Picric acid	Brown	Black	Dark yellow
5	Petroleum ether	Light yellow	Black	Brown
6	50% HCl	Dark brown	Black	Light yellow
7	50% H_2SO_4	Brownish yellow	Yellowish black	Light black
8	Ethyl acetate	Light yellow	Light black	Light yellow
9	Ethyl alcohol	Dark green	Black	Greenish yellow
10	Methanol	Dark yellow	Black	Light yellow
11	50% KOH	Blackish yellow	Dark black	Dark green
12	50% HNO_3	Brown	Dark black	Light yellow
13	Acetic acid	Light yellow	Black	Brown
14	Iodine in water (1%)	Light yellow	Black	Yellowish white
15	$FeCl_3$	Black yellow	Dark black	Dark Black

DISCUSSION: Pharmacognosy is the scientific study of crude drugs from natural sources. The standardization is a very important step in establishing the correct identity, purity, safety and quality of crude drugs and it should be established before it can be successfully included in pharmacopoeia [22]. Recently, there has been an emphasis in standardization of medicinal plants of therapeutic potential. In spite of modern techniques, pharmacognostical evaluation is still more reliable for identification and evaluation of plants. World Health Organisation recommends that the macroscopic and microscopic evaluation is most important in establishing the identity and purity of the plants [22].

In the present study, pharmacognostic standardization of seeds of *A. marmelos* was done which included morphological, microscopic, physicochemical, phytochemical and fluorescence analysis. This evaluation provides the simplest, quickest and cheapest means to establish the identity and purity of drug and also acts as a reliable tool for detecting adulteration.

Adulteration of the original plant material is the main cause of dilapidation of original therapeutic effect of plants used in traditional systems of medicine [23, 24]. In physicochemical parameters, ash values like total ash, acid insoluble ash, water soluble ash, sulphated ash and extractive values were evaluated which serve as a reliable aid for detecting adulteration and identification of plant. Ash values give an idea about inorganic composition, other impurities present along with drug while extractive values are useful for the determination of exhausted and adulterated drugs. The chemical constituents of crude drug that are soluble in particular solvents can be known by extractive values [25, 26]. Moisture content of drug should be at minimal level so that bacteria yeast or fungi will not grow during storage [27 - 28].

The preliminary phytochemical analysis showed the presence of maximum amount of flavonoids followed by terpenoids, steroids, cardiac glycoside, saponins and tannins. Fluorescent analysis under day light and UV light by treatment with different chemical reagents showed different colours. This is attributed to the UV light which produces fluorescence in many natural products that do not visibly fluoresce in daylight. Thus fluorescecne is used for qualitative assessment of crude drug. Pharmacognostic studies are important in herbal technology as it ensures plant identity lays down standardization parameter which will help and prevent adulterations. Such studies will help in authentication of the plants and ensures reproducible quality of herbal products which will lead to safety and efficacy of natural products.

CONCLUSION: Standardization of a crude drug is very important for its proper and correct identification. The parameters evaluated in the present work will work as diagnostic parameters for identification of this plant. It will ensure authenticity and prevent the plant being adulterated and mishandling of the crude drug can be avoided. The therapeutic efficacy of the plant can be maintained.

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CONFLICT OF INTEREST: The authors declare that they have no competing interest.

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