

Pharmacognostic evaluation of Bilva [*Aegle marmelos* (L.) Correa] root bark

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Aegle marmelos L., family Rutaceae, is a sacred plant mentioned in various Hindu scriptures. It has both medicinal as well as cultural importance. In the present investigation, macroscopy, microscopy, and powder microscopy studies, physicochemical analysis, detection of heavy metals, analysis of aflatoxins, screening of microbiological parameters and High Performance Thin Layer Chromatography (HPTLC) fingerprint profile of methanolic extract were performed. Analysis of 04 aflatoxins (A1, B1, A2, and B2) was performed and found that no aflatoxins were present, authenticated by comparing the R_f value and colour of the standards spot with sample on TLC plate. Qualitative microbiological analysis of pathogenic bacteria, i.e., *Staphylococcus aureus*, *Salmonella sp.*, *Pseudomonasaeruginosa* and *Escherichia coli* were done and found that no bacterial pathogens are present in the *Aegle marmelos* root bark extract. HPTLC chromatographic fingerprint of *Aegle marmelos* root bark was done by using mobile phase toluene: ethyl acetate: formic acid (7.5: 2.5: 0.4). TLC plate was derivatized by using derivatizing reagent 5% Methanolic - sulphuric acid reagent and plate was heated at 105°C till the bands are clearly visible. Major spots R_f values and colour were noted at 254 nm, 366 nm, after derivatization 366 nm and UV light. Therefore, established parameters may be used as a reference tool for proper recognition and confirmation of right plant material and monitoring of batch to batch consistency of finished herbal products using *Aegle marmelos* as an ingredient. This study may also helpful in the preparation of *Aegle marmelos* root bark monograph.

Keywords: *Aegle marmelos*, HPTLC fingerprints profile, Microscopy, Physicochemical

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Aegle marmelos (L.) is known as a Bilva in Sanskrit (Indian language), one of the most prominent plant used in traditional as well as an ayurvedic system of medicine in India. The species is tree native, found in Sri Lanka, Pakistan, Thailand, Malaysia, India, western Himalaya and Andaman Nicobar. Various parts of this plant like leaves, root bark, stem bark and fruits are widely used in preparation of ayurvedic drugs and as in folklore medicines¹.

In Ethnomedicine, it is used in the treatment of *Vatavyadhi* (Arthritis), *Sotha* (Swelling), *Sula* (pain), an important ingredient in many formulations². *Aegle marmelos* root bark is the very most ingredient of Dasamula (ten roots), which is widely used in recovering the loss of appetite^{3,4}. Its root is also used to treatment of hypoglycaemic and rheumatism⁵.

In the Ayurvedic system of medicine, the species is used as a brilliant remedy for the diarrheal disorder. *Aegle marmelos* fruit has important medicinal value used in treatments of many ailments, such as the unripe fruits are acrid, astringent, aids digestion and

stomach irritation. The half-ripe fruit is astringent, digestive and anti-diarrheal^{6,7}. The species is also used in the preparation of many ayurvedic formulations such as Chyavanprash, Amrtarista, Dantyadyarista, Agastya Haritaki Rasayana, Dasamularista, Dasamula Kwatha Curna and Bilvadi Leha^{8,9}

Aegle marmelos, a moderate-sized tree, 8-12 m long, 2.5 cm long spines. Leaves tri to five-foliolate and leaflets ovate. Flowers are short and in greenish colour. Fruit shape is globose and yellowish in colour with woody rind^{10,11}.

It contains a number of phytochemical compounds viz. coumarins, alkaloids, polysaccharides, fatty acids and essential oils. The species also contain tannins (tannin and riboflavin), organic acids and phenolics, fatty acid and ricinoleic acid^{12,13}.

This research work includes macroscopic, microscopic and powder microscopic study, physicochemical parameters, phytochemical screening, qualitative microbiological analysis, HPTLC fingerprints, and aflatoxin analysis. Due to the therapeutically importance, the industrial demand of the species day by day is increasing. Therefore, the

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study was designed to develop a standard protocol for standardization and identification of the right plant material of *Aegle marmelos*, root bark.

Materials and methods

Collection and processing of plant raw material

The 03 samples of fresh root bark of *Aegle marmelos*, were collected from three different localities viz. one from Arogyadham campus of Chitrakoot, Satna, Madhya Pradesh [Sample code PRS, (Phytochemical Reference Sample)]; in the month of March 2017; second from SMPU, RARIMD, Bangalore [Sample code BRS, (Botanical Reference Sample)] in the month of March 2017; and third sample was purchased from Karwi, Dist. Chitrakoot, Uttar Pradesh, [Sample code

MRS (Market Reference Sample)]; in the month of March 2017. All Samples were identified and authenticated by Dr RLS Sikarwar (Senior Scientist), Deendayal Research Institute Chitrakoot. The voucher specimen (AD/AS/125/2017) prepared as per standard procedure¹⁴ and kept in the herbarium section of Arogydham, Deendayal Research Institute, Chitrakoot, Satna (MP) for further reference.

Macroscopic and Microscopic study

Macroscopic or organoleptic characters like appearance, colour, odour and taste were evaluated. Fresh *Aegle marmelos* root bark section was cut by free hand sectioning and numerous sections examined microscopically and finally best section's photographs were taken by Digi-eye camera^{15,16}.

Powder microscopic study

For detailed powder microscopic study, dried *Aegle marmelos* root bark sample was grinded through electrical grinder to make a fine powder. About 2 g of powder gently warmed with chloral hydrate solution, washed with potable water or distilled water and a small quantity of powder put in microscopic glass slide, mounted in glycerine. A small portion of powder put in different glass slide and treated with iodine solution, sudan III solution separately and mounted in glycerin and observed under microscope at 40X x 10X magnification of the Trinocular Research Microscope¹⁷.

Physico-chemical parameters

Physico-chemical parameters like moisture content (loss on drying at 105°C), alcohol soluble extractive value, water soluble extractive value, hexane soluble extractive value, total ash value and acid insoluble ash value were done¹⁸.

Heavy metals tests

Heavy metals are toxic and generally occur through earth in plants. Mainly 04 types of heavy metals harmful to us are Pb, Cd, As and Hg. These heavy metals were detected through Atomic Absorption Spectrophotometer as per standard method¹⁹.

Preliminary phytochemical screening

Preliminary phytochemical analysis were performed in ethyl alcohol extract and water extract for the confirmation of present/ absent of various phyto-constituents in *Aegle marmelos* root bark²⁰.

High Performance Thin Layer Chromatography (HPTLC) study

For High performance thin layer chromatography (HPTLC) analysis, fine powdered 5 g of each samples (PRS, BRS & MRS) were macerated with 100 mL of ethyl alcohol 6 h, kept in resting period for 18 h then filtered and concentrated. These samples were used for spotting on pre-coated silica-gel aluminium plate 60 F₂₅₄ (5x10 cm with 0.2 mm layer thickness Merk Germany) using Camag Linomat -5 sample applicator and a 100 µL Hamilton syringe. 6 mm bands length of each samples, were spotted 15 mm distance from the bottom, 15 mm from left margin the plate and 10 mm part. The Plates were developed in mobile phase toluene: ethyl acetate: formic acid (7.5:2.5:0.4). The plate was developed in 10x10 cm twin through glass chamber equilibrated with 20 min saturated mobile phase at room temperature. The plate was dried with the help of heating plate for 5 min at 105°C. The plate was captured with Camera photo documentation system Camag Reprstar 3. Visualization of bands were made at 254 nm, 366 nm and ultra violet light with Wincat software, before and after derivatization (with 5% Methanolic - sulphuric acid reagent) and R_f values with colour were noted²¹.

Test for Aflatoxins

Aflatoxins are highly dangerous for human body. This test will help for detection of aflatoxins such as B₁, B₂, G₁ and G₂ in any plant origin materials. Three sample of *Aegle marmelos* root bark were checked for micotoxin, i.e., Aflatoxin with standard markers B₁, B₂, G₁ and G₂²².

Microbiological limit tests

Microbial limit tests useful for the estimation of viable aerobic micro-organisms present in the samples. Following tests were performed to determine the microbial load in 03 samples of *Aegle marmelos* root bark powder²³.

Enumeration of *Salmonella sp/g*
 Enumeration of *Staphylococcus aureus/g*
 Enumeration of *Escherichia coli*
 Enumeration of *Pseudomonasaeruginosa/g*
 Determination of Yeast & Mould
 Determination of total microbial count (TBC)

The microbiological tests were determined using specified agar media and enrichment media from Himedia, Pvt. Ltd. Mumbai.

Result

Macroscopic characters

Aegle marmelos root bark -curved, 4 to 6 cm long, 2 to 3 cm in width and 0.5 to 1 cm in thickness, surface, rough, covered with closely placed several lenticels arranged on longitudinal and transversely running lenticels which often gets burst out at places, forming vertical slits and furrows, exfoliated sometimes exposing the creamish or light yellow coloured inner surface, faint aromatic odour and taste astringent (Fig. 1 & Fig. 2).



Fig. 1 — Habit & Habitat



Fig. 2 — Pieces of dried root bark of *Aegle marmelos*

Microscopic characters

Diagrammatic *Aegle marmelos* root bark outline shows outer irregularly running lenticillate cork, narrow cortex embedded with sclereids, wider phloem with concentric bands of fibres (Fig. 3).

Detailed TS shows outer stratified cork consisting of alternate, narrow bands of 5 to 6 rows of compactly arranged thick walled, suberised cells alternating and vertical 2 to 4 rows of rectangular, wide lumened lignified cells, a very narrow band of cork cambium being located underneath this, followed by 9 to 12 rows of parenchymatous secondary cortex embedded with groups of thick walled transversely running sclereids, stone cells and oil cells containing yellowish coloured volatile oil, large sized solitary sclereids are also occasionally found to be present, phloem is a considerably broad region consisting of tangentially arranged concentric bands of lignified wide lumened sclereids, and groups of phloem fibres, sieve tubes and parenchyma which get obliterated at places especially towards the peripheral region forming bands of ceratenchyma, alternating with vertically running biseriate sinuous medullary rays, getting widened or funnel shaped towards the periphery. Secretory ducts schizogenous are embedded throughout the phloem region. Prismatic crystals of calcium oxalate and starch grains are present throughout the parenchymatous cells of section (Fig. 4).

Powder microscopic characters

Pale brown coloured gritty powder with faint aromatic odour, and bitter, pungent and astringent taste. Under microscope examined powder shows cork in surface view and in sectional view, sclereids of various sizes, shape and thickness, fragments of isolated and groups of thick walled fibres; isolated schizogenous oil glands scattered as such and embedded in the parenchymatous cells; prismatic

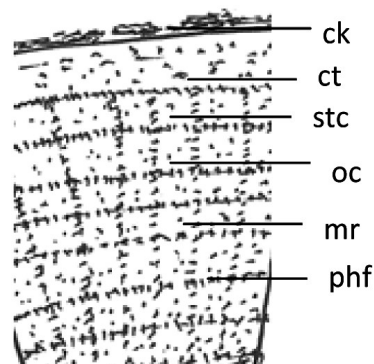


Fig. 3 — Diagrammatic TS of *Aegle marmelos* root bark

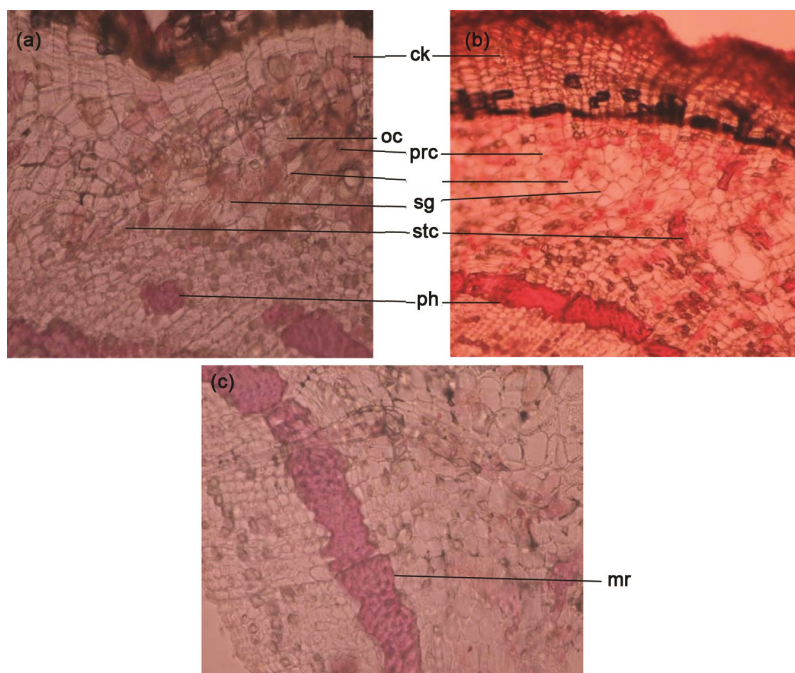


Fig. 4 — (A,B,C) Detailed TS of *Aegle marmelos* root bark Abbreviation- ck-cork, ct- cortex, mr-medullary rays, oc- oil cell, sc-sclereids, stc-stone cells, ph-phloem, phf-phloem fibres, sg- starch grains

crystals of calcium oxalate and simple starch grains are found (Fig. 5).

Physico-chemical analysis

The physico-chemical parameters of *Aegle marmelos* root bark were performed and results are given in (Table 1).

Preliminary phyto-chemical investigation

Quantitative phyto-chemicals analysis were performed in ethyl alcohol extract and water extract of *Aegle marmelos* root bark. Various phytochemicals like protein, tannin, flavonoids, saponin and alkaloids are present in the sample.

HPTLC finger print profile

HPTLC study of the ethanolic extract 03 spots of the *Aegle marmelos* root bark sample extract applied in pre-coated TLC plate. Applied 10 μ L of the test solution as 8 mm bands and develop the plate in a solvent system toluene: ethyl acetate: formic acid (7.5:2.5:0.4) to a distance of 8 cm. Dry the developed plate in room temperature and examined under at 254 nm and at 366 nm. Derivatized the plate using 5% Methanolic - sulphuric acid reagent and heating the plate at 105°C till the bands are clearly visible. Major spots Rf values with colour were recorded under, at 254 nm, at 366 nm, after derivatization 366 nm and UV light. Chromatogram profile and Rf values are given (Fig. 6 & Table 2).

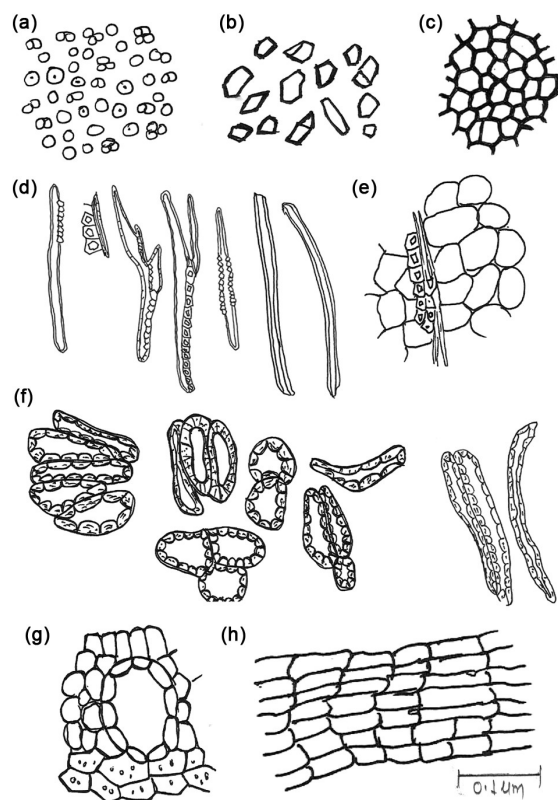


Fig. 5 — Powder microscopy of *Aegle marmelos* root bark (A) Simple starch grains (B) Prismatic crystals of calcium oxalate (C) Cork cell in surface view (D) Thick walled fibres and crystal fibres (E) Fragments of parenchyma with crystals and crystal fibres (F) Different shape and size Sclereids (G) Sclereids (H) Sclereids

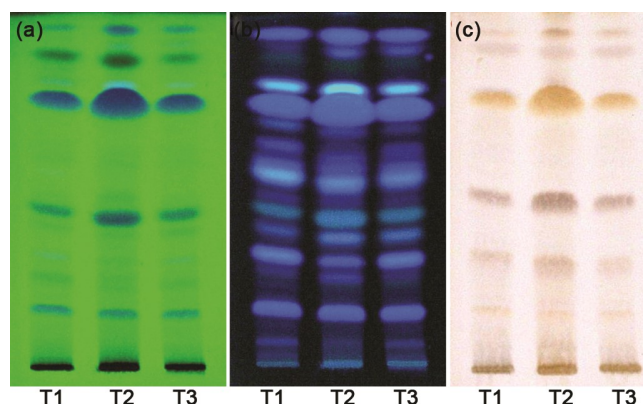


Fig. 6 — HPTLC fingerprints of *Aegle marmelos* root bark (A) 254nm before derivatization (B) 366nm before derivatization and (C) UV light after derivatization Where Tracks: T₁=Sample BRS T₂=Sample PRS; T₃= Sample MRS

Table 1 — Physico-chemical analysis of *Aegle marmelos* root bark

S. N.	Name of Parameters	Results		
		PRS	BRS	MRS
1	Foreign Matter	2%	2%	2.5%
2	LOD at 105 °C (%w/w)	4.34%	4.07%	2.73%
3	Alcohol soluble extractive value(% w/w)	18.35%	13.12%	14.14%
4	Hexane soluble extractive value(% w/w)	5.92%	4.19%	7.13%
5	Water soluble extractive value(% w/w)	22.30%	20.41%	25.43%
6	Total ash value (% w/w)	11.2%	13.00%	12.5%
7	Acid in soluble ash value (% w/w)	2.5%	3.0%	2.5%

Table 2 — R_f values of HPTLC fingerprints profile of *Aegle marmelos* root bark

R _f value	Before derivatization		After derivatization	
	254nm	366nm	366nm	UV light
R _{f1}	0.12 (blackish blue)	0.12(sky blue)	0.10 (sky blue)	0.12 (brown)
R _{f2}	0.48 (blackish blue)	0.20 (sky blue)	20. (sky blue)	0.24 (brown)
R _{f3}	0.70 (blackish blue)	0.48 (yellowish blue)	0.48 (yellow)	0.48 (brown)
R _{f4}	0.76 (blackish blue)	0.60 (sky blue)	0.66 (blue)	0.76 (brown)
R _{f5}	0.80 (blackish blue)	0.70 (sky blue)	0.80 (blue)	0.80 (brown)
R _{f6}	-	0.80 (sky blue)	-	-

Table 3 — Determination of heavy metals of *Aegle marmelos* root bark

S. N.	Name of Tests	Results			WHO/ API Limits
		PRS	BRS	MRS	
1	Lead (Pb)	0.7514 ppm	0.7514 ppm	0.6595 ppm	10 ppm
2	Cadmium (Cd)	0.0154 ppm	0.0167 ppm	0.0109 ppm	0.3 ppm
3	Arsenic (As)	12.3420 ppb	9.0981 ppb	11.5794ppb	03 ppm
4	Mercury (Hg)	1.5467 ppb	1.6712 ppb	1.8512ppb	01 ppm

Heavy metals tests

Heavy metal elements (Pb, Cd, As and Hg) test were performed and found under limits as per guideline WHO and results are given in (Table 3).

Microbiological limit tests

Microbiological profile of the *Aegle marmelos* root bark powder was found satisfactory under limits as per guideline WHO. Results are given in (Table 4).

Test for Aflatoxins

Aflatoxins (B₁; B₂; G₁ and G₂) study of the ethanolic extract was performed by three spots of the *Aegle marmelos* root bark sample and four standards of Aflatoxins (B₁; B₂; G₁ & G₂) applied in precoated TLC plate. Applied 10 μL of the test solution was applied as 8 mm bands and developed the plate in a solvent system *toluene:ethyl acetate:formic acid* (7.5: 2.5: 0.4) to a distance of 85 mm. Dry the developed plate in room temperature, examined under at 366 nm and major spots R_f values with colour were recorded (Fig. 7 & Table 5).

Discussion

Plants are one of the primary sources of dietary supplements that help in maintaining good health²⁴. Previous metabolite screening data reveals that bioactive compounds are mainly responsible for the potential of the plants²⁵⁻²⁷. Established macroscopic characters, microscopic characters and powder microscopic distinguished characters have been will be helpful to identification and authentication of *Aegle marmelos* root bark. Physicochemical tests will be used for checking the adulteration in the drug and

Table 4 — Microbiological limit tests of *Aegle marmelos* root bark

S.N.	Tests	PRS	BRS	Market	Permissible limits as per WHO/ API
1	<i>Staphylococcus aureus</i> /g	Absent	Absent	Absent	Absent
2	<i>Salmonella sp.</i> /g	Absent	Absent	Absent	Absent
3	<i>Pseudomonas aeruginosa</i> /	Absent	Absent	Absent	Absent
4	<i>E.coli</i>	Absent	Absent	Absent	Absent
5	Total microbial plate count(TPC)	12 cfu/g	14 cfu/g	17 cfu/g	10 ⁵ / cfu/g
6	Total Yeast and Mould	11 cfu/g	12 cfu/g	15 cfu/g	10 ³ / cfu/g

Table 5 — R_f values in test solution for Aflatoxin in *Aegle marmelos* root bark at 366 nm

R _f Values	Standard Aflatoxin				Results		
	B ₁	G ₁	B ₂	G ₂	PRS	BRS	MRS
R _{f1} *	-	-	-	0.40	Not Seen	Not Seen	Not Seen
R _{f2} *	-	0.44	-	-	Not Seen	Not Seen	Not Seen
R _{f3} *	-	-	0.50	-	Not Seen	Not Seen	Not Seen
R _{f4} *	0.54	-	-	-	Not Seen	Not Seen	Not Seen

Fluorescent colour

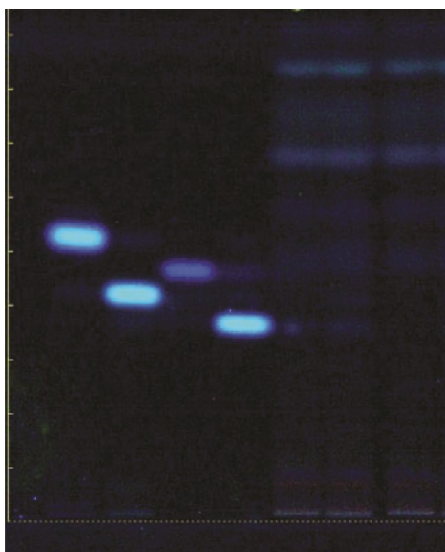


Fig. 7 — Aflatoxine analysis of *Aegle marmelos* root bark at 366 nm before derivatization Where Track 1=Aflatoxin standard marker B₁; Track 2=Aflatoxin standard marker G₁; Track 3=Aflatoxin standard marker B₂ and Track 4=Aflatoxin standard marker G₂, Track A= sample PRS; Track B= Sample BRS and Track C= Sample MRS

developed HPTLC finger print profile helps in identification of various phyto-constituents present in the crude drug. The Aflatoxins were absent in the *Aegle marmelos* root bark samples which indicates the safety of drug. Heavy metal elements are found under limits as per guideline WHO and microbial limits test of the *Aegle marmelos* root bark were found satisfactory. Total microbial plate count (TBC), Yeast & Moulds counts were reported less than the limit as per suggested by WHO and pathogenic bacteria i.e.,

Staphylococcus aureus, *Salmonella sp.*, *Pseudomonas aeruginosa* and *Escherichia coli* were found to be absent. All findings are indicating samples are genuine and free from any adulterations. These finding could be helpful in the development of new herbal formulation in future using *Aegle marmelos* root bark as an ingredient.

Conclusion

A simple, accurate and precious HPTLC finger print method was developed for the *Aegle marmelos* root bark, which can be recommended for the routine analysis of the herbal drugs. In view of the advantages of reliability and sensitivity for the HPTLC finger print of the species developed through HPTLC protocol, the study have wide application in identifying and assessing the quality of *Aegle marmelos* root bark containing as raw material in the herbal formulation. However, developed data may be used as a reference tool for proper confirmation of right plant material and monitoring of batch to batch consistency of finished herbal drugs using *Aegle marmelos* root bark as an ingredient.

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