Standardising Bael Leaf Across Regions: Leaf Constants, Markers and HPTLC

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***Abstract*—Background: Bael (Aegle marmelos (L.) Correa, Rutaceae) leaves are used in Indian medicine for diabetes, dysentery, fever and inflammation and are collected commercially across the country. Most published quality data describe one source at a time, so values from different papers cannot be compared directly.**

Objective: To review what is known about the pharmacology, chemistry, safety and standardisation of bael leaf, and to ask how far leaf constants and chromatographic markers can be expected to vary between regions.

Findings: Leaf extracts and the alkaloid aegeline show antidiabetic, hepatoprotective, antimicrobial and antioxidant activity in animal and in vitro work, but dosing is not standardised and one toxicity study used the intraperitoneal route. Validated UHPLC, HPLC and HPTLC methods exist for aegeline, coumarins and phenolics, and leaf essential oils from different origins have different dominant components. We found no indexed abstract that reports quantitative leaf constants for bael leaf. Studies of other Indian plants show that marker content varies with provenance, altitude, season and genotype, and that common-garden designs are needed to separate environment from genetics.

Conclusion: A comparative study of leaf from four regions, collected in one month from several trees per site, with leaf constants, physicochemical values and a validated two-marker HPTLC method, would fill the main gap. The design proposed here is conceptual.

***Index Terms*—Aegle marmelos; bael leaf; leaf constants; HPTLC; marmelosin; aegeline; regional variation; standardisation**

I. INTRODUCTION

Aegle marmelos (L.) Correa, the bael tree, belongs to the family Rutaceae. It is sacred to Hindus and has long been used in Ayurveda and by ethnic communities of the Indian subcontinent; leaves, bark, stem, fruit and seeds are all used as medicines, and the fruit is the part traditionally used for chronic diarrhoea, dysentery and peptic ulcers [1]. The leaf is the part that attracts most laboratory interest. Aegeline, an alkaloidal amide isolated from the leaves, lowered blood glucose in diabetic rats and plasma triglycerides in dyslipidaemia hamsters [2], and leaf extracts have been tested for antidiabetic, hepatoprotective, antimicrobial and antiproliferative effects (Section 3).

A raw drug that is collected in many places raises an obvious question: is a leaf from Lucknow the same drug as a leaf from Bengaluru or Odisha? For bael the genetic background is wide. Eighty wild genotypes and two cultivars from the north-western plains of India showed wide variation in fruit traits and fell into three subpopulations by SSR markers [3], and SRAP markers showed that 70% of the molecular variation lay between populations in 24 genotypes from two agro-ecological zones [4]. Leaf chemistry is not the same as fruit morphology, but these studies make a uniform leaf composition across India unlikely, and the leaf literature should be read with that in mind.

This narrative review summarises the leaf pharmacology and safety evidence, the chemistry and analytical methods, the pharmacogenetic tools used to standardise leaves, and what other medicinal plants

teach about regional variation. It is built from abstracts and publisher pages of peer-reviewed papers; it contains no new experimental data, and the proposal in Section 7 is a design, not a result.

II. PLANT PROFILE AND TRADITIONAL USE

A. marmelos is a medium-sized tree with trifoliate leaves and a large, hard-shelled fruit (Fig. 1A). The leaves are used in diabetes, dysentery, fever and inflammatory conditions. A recent review lists antioxidant, antidiabetic, antimicrobial, hepatoprotective, cardioprotective and anticancer effects as the six activities with the most support; its abstract names no compounds and no doses [1]. In one comparison, methanolic leaf extract was the most active of leaf, bark and fruit in DPPH scavenging and the richest in total phenolics (Fig. 1B) [5], which is one reason the leaf is preferred in laboratory work.

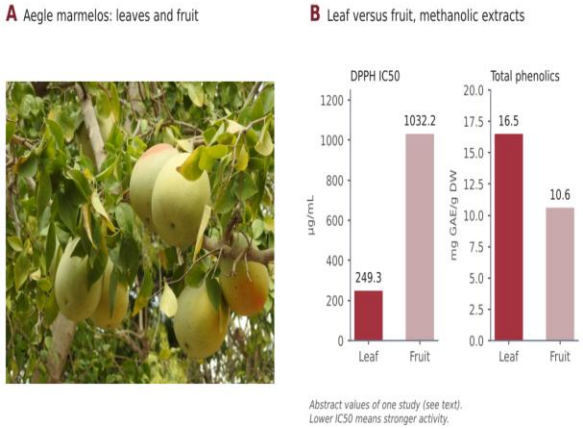


FIGURE 1 (A) Aegle marmelos, leaves and fruit (photograph supplied with the project synopsis). (B) DPPH IC50 and total phenolic content of methanolic leaf and fruit extracts, plotted from the abstract values of Gupta et al. [5]. One study; the bark value is not shown.

III. LEAF PHARMACOLOGY AND SAFETY

3.1 Antidiabetic and hepatoprotective activity

Aegeline isolated from the leaves lowered blood glucose in diabetic rats by 12.9% at 5 h and 16.9% at 24 h at 100 mg/kg, and lowered plasma triglycerides by 55% in dyslipidaemia hamsters at 50 mg/kg; computational modelling in the same paper suggested a possible beta-3 adrenergic agonist action, which was a hypothesis and not an experimental finding [2]. In C2C12 myotubes, aegeline increased glucose uptake through Akt and Rac1 signalling, with a separate PI3K-Rac1-PAK1-cofilin pathway for the cytoskeletal changes [6]. An ethanolic leaf extract (150 mg/kg for 30 days) restored antioxidant defences in streptozotocin-diabetic rats and protected the beta cells of the pancreas [7].

In carbon tetrachloride liver injury in rats, a leaf extract at 50 mg/kg acted comparably to silymarin, and 25 mg/kg of extract became significantly protective only when combined with piperine (20 mg/kg); IL-10 was not reduced by any treatment, so the anti-inflammatory mechanism remains open [8]. A methanolic leaf extract protected against CCl4 hepatotoxicity in mice and its LC-MS/MS profile is discussed below [9]. All of these are animal studies; none of the abstracts reports a standardised extract with a defined marker content, which limits the comparison between papers.

3.2 Antimicrobial and cytotoxic activity

Serial petroleum ether, chloroform and methanol extracts of the leaves inhibited seven bacteria and three fungi, with zones of 10-22 mm and MICs of 1.25-10 mg/mL [10]. Leaf extracts of 18 varieties were screened, and the variety Pant Aparna had the highest alkaloids, flavonoids and phenols [11]. The essential oil of Bangladeshi leaves, with ledene oxide-(II) as its main component (about 18.2%), had inhibition zones of about 9.3-12.3 mm; the MIC units in that abstract are inconsistent [12]. Ethanolic leaf extract inhibited colon and breast cancer cell lines at 100 µg/mL, and one fractionated sample inhibited a leukaemia line with an IC50 of 12.5 µg/mL; HPTLC of that fraction identified the furanocoumarin imperatorin (marmelosin) as a major constituent [13]. Antimicrobial and cytotoxic effects in vitro are only a starting point for drug quality, but the variety effect in particular shows that the plant itself is a source of variation.

3.3 Safety

Four leaf extracts were tested in rats by the intraperitoneal route; the acute LD50 values of three of them were 1445-1660 mg/kg, and there was no gross or microscopic change in organs at 50 mg/kg for 14 days; the authors concluded that the margin of safety was broad [14]. The route is not the oral route of herbal use, so the study does not settle oral safety.

The more serious signal comes from outside the plant itself. A cluster of acute hepatitis in Hawaii in 2013 was linked to the supplement OxyELITE Pro: of 76 reports, 44 met the case definition and 36 of those 44 had used the product in the preceding two months; two patients needed liver transplantation and one died, and the mechanism was not identified [15]. The abstract does not name aegeline. Others have re-analysed the clinical data and concluded that the cluster was better explained by a range of liver diseases and that causation by the supplement was not shown [16]. A review of aegeline notes that it occurs as a racemate, that most studies used plant-isolated material, and that individual enantiomers and their safety profiles need study [17]. Taken together, aegeline is a pharmacologically active constituent whose safety at supplement doses is not resolved, and a quality study that measures it should say so.

IV. LEAF CHEMISTRY AND CHROMATOGRAPHIC METHODS

Table 1 collects the chemistry reported for the plant, with the plant part marked, because composition differs among parts. A UHPLC-PDA method quantified aegeline and six coumarins (umbelliferone, scopoletin, marmesinin, 8-hydroxypsoralen, angelicin and marmelosin) in leaf, fruit, root and bark, with linearity over 0.5-250 µg/mL, LOD 0.1 µg/mL and LOQ 0.5 µg/mL, and a separate chiral method resolved the aegeline enantiomers [18]. An HPLC method for the alkaloids and coumarins of the leaf, with a C18 column and an acetonitrile-water gradient, gave recoveries of 99.27-99.98% and was described as the first extensive quantitative HPLC report on markers in bael leaves; the wavelength and recovery range quoted in the abstract differ slightly from those in the body of the paper [19].

TABLE 1 Constituents and analytical methods reported for Aegle marmelos, by plant part. Details are from the abstracts of the cited papers; "not stated" means the abstract does not give the item.

Constituent(s)	Part	Method and finding	Ref.
Aegeline	Leaf	Isolation; antihyperglycaemic and antidyslipidaemic in animals	[2]
Aegeline and six coumarins	Leaf, fruit, root, bark	UHPLC-PDA-MS; linearity 0.5-250 µg/mL; LOD 0.1, LOQ 0.5 µg/mL; chiral HPLC-ToF-MS for aegeline	[18]
Alkaloids and coumarins (five compounds)	Leaf	HPLC, C18, acetonitrile-water gradient; recovery 99.27-99.98%; detection limit 5 ng	[19]
Six alkaloids, rutin, kaempferol-3-O-rutinoside	Leaf	LC-MS/MS of methanolic extract; qualitative, no validation data in the abstract	[9]
Rutin, quercetin, gallic acid	Leaf	HPTLC; toluene:ethyl acetate:formic acid:methanol 3:4:0.8:0.7; 254 nm; RSD < 2%; LOD 4.2-5.27 ng/spot	[20]
Seven phenolic acids	Leaf, bark, fruit	RP-HPLC; leaf total phenolics 16.5 ± 0.3 mg GAE/g DW	[5]
Marmelosin, umbelliferone, scopoletin	Fruit	RP-HPLC, methanol:water 55:45 with 0.1% acetic acid; marmelosin recovery 98.8-102.9%	[21]
Umbelliferone, psoralen	Fruit pulp	HPTLC validated per ICH guidelines; conditions not stated	[22]
Marmelosin, marmesin, aegeline	Fruit pulp	Enriched fraction 48.61% coumarins; marmelosin 31.2%, marmesin 8.9%, aegeline 0.72%	[23]

Constituent(s)	Part	Method and finding	Ref.
Marmelosin	Root, small branches	HPLC; 0.0565% in root and 0.0145% in small branches	[24]

For HPTLC, a validated method for rutin, quercetin and gallic acid in the leaf of A. marmelos and Psidium guajava used silica gel 60 F254, toluene:ethyl acetate:formic acid:methanol (3:4:0.8:0.7), detection at 254 nm, Rf values of 0.08 (rutin), 0.76 (quercetin) and 0.63 (gallic acid) and inter-analyst RSD below 2% [20]. HPTLC with ICH validation has also been used for umbelliferone and psoralen in the fruit pulp [22]. For marmelosin, an isocratic RP-HPLC method (methanol:water 55:45 with 0.1% acetic acid, 12 min run) was developed on fruit, with recoveries of 98.8-102.9% for marmelosin; the abstract and tables of that paper are not fully consistent about the linearity data, so we quote only the recoveries [21]. We did not find an indexed HPTLC method for marmelosin and aegeline together in the leaf, which is the method the project would need.

Leaf essential oils differ. Leaf oil analysed by GC and GC-MS contained 35 compounds (99.6% of the oil), 93.6% of it monoterpenes, with limonene (26.8%), β-phellandrene (16.2%) and α-pinene (6.6%) among the main constituents [25]; an oil from the Western Ghats gave 31 compounds, dominated by p-mentha-1,4(8)-diene (33.2%) with limonene at 13.1% (the plant part is not stated in the abstract) [26]; and Bangladeshi leaf oil was led by ledene oxide-(II) [12]. A GC-MS study of the oil of leaves screened for heavy metals, pesticides and microbial contamination identified 62 compounds [27]. The three oils were obtained and analysed under different conditions, so they do not prove a regional effect, but they show that the volatile profile is not fixed.

V. PHARMACOGNOSTIC STANDARDISATION OF THE LEAF

Standardisation of a leaf drug normally combines macroscopy and microscopy, quantitative leaf constants (stomatal number, stomatal index, vein-islet number, veinlet termination number and palisade ratio), ash and extractive values, fluorescence behaviour and preliminary phytochemistry. Table 2 shows how these are reported for other medicinal leaves. Dillenia indica leaf was characterised by anomocytic stomata, unicellular trichomes, xylem fibres and calcium oxalate crystals, with the leaf constants and WHO-type parameters listed in the abstract [28]. Four Spermacoce species could be separated by epidermal and trichome features, leaf constants, physicochemical and extractive values [29]. Cnicus wallichi leaf was standardised on the same set of constants [30], and Myrica esculenta leaf by anatomy and HPTLC [31]. Two Aster species were separated by macroscopic and microscopic features and by physicochemical and HPTLC data [32].

TABLE 2 Pharmacogenetic standardisation parameters reported for medicinal leaves. The abstracts list the parameters; numerical values are given in the body of the papers and are not reproduced here.

Leaf	Parameters reported in the abstract	Ref.
Aegle marmelos	Soxhlet extracts (hexane, petroleum ether, chloroform, ethanol); antiproliferative activity; HPTLC identified imperatorin; leaf constants not in the abstract	[13]
Dillenia indica	Anomocytic stomata, trichomes, fibres, crystals; stomatal number and index, vein-islet and veinlet termination numbers; ash, extractive values, swelling index	[28]
Spermacoce spp. (four)	Midrib sections, epidermal characters, leaf constants, extractive values; phenolics and flavonoids	[29]
Cnicus wallichi	Morphology, anatomy, powder microscopy; vein-islet and termination numbers, stomatal number and index, palisade ratio; ash and extractive values	[30]
Myrica esculenta	Anatomy, powder microscopy, leaf constants, fluorescence, HPTLC fingerprint	[31]
Aster flaccidus, A. diplostephioides	Macroscopy, microscopy, WHO-based physicochemical limits, HPTLC of methanol extracts	[32]

For bael itself, the literature we could verify at abstract level is thin. A pharmacogenetic standardisation of the

leaf was reported together with antiproliferative activity, but its abstract does not give the constants [13]. We therefore quote no stomatal, vein-islet or palisade values for bael leaf. The monthly collection study in the next section also reports leaf morphology and microscopic features that varied with season, without values in its abstract [33].

5.1 What changes stomatal counts

The choice of constants matters because some of them respond to the environment. A review of stomatal density and morphology notes that stomatal size and density adjust to the environment over longer periods [34]. In a perennial grass, moderate water deficit raised stomatal density and severe deficit lowered it [35]. In poplar, the stomatal density and index of new leaves fell as the CO2 around the lower mature leaves rose, and were generally higher at low vapour pressure deficit [36]. In Salix, higher rainfall increased leaf area, which diluted lower-face stomatal density, while stomata per leaf were site-specific and independent of rainfall [37]. The lesson for a regional study of bael is practical: leaves of matched size and developmental stage must be compared, stomatal number should be read together with stomatal index and epidermal cell counts, and the constants should be treated as hypotheses about regional effects, not as fixed identity marks.

VI. REGIONAL, SEASONAL AND GENETIC VARIATION

The closest bael-specific evidence is seasonal. Leaves collected monthly over a year from different locations varied in morphology and microscopic features across seasons; ethanol and water extract yields and aegeline concentration were higher in winter, but the abstract gives no values and does not state the analytical method [33]. In the other direction, one study of 18 accessions found variety to be a source of variation in alkaloids, flavonoids and phenols [11], and the genetic studies cited above show variation between populations [3,4].

Plants that have been studied more systematically show what to expect (Table 3). HPLC fingerprints of 24 Bacopa monnieri accessions from Odisha and West Bengal, treated with HCA, PCA and discriminant analysis, fell into two groups that matched geographic origin [38]. In 43 neem provenances, kernel azadirachtin A ranged from 556.9 to 3030.8 mg/kg and differed significantly among provenances; high and low trees occurred in every provenance, and the authors pointed to individual genetic differences and not to rainfall, humidity or temperature [39]. Sixty accessions of Centella asiatica grown under identical conditions still showed wide variation in madecassoside (up to 5.67 ± 0.08%) and asiaticoside, three accessions had near-zero levels of both, and no correlation with the elevation of the collection site was found, so the differences were attributed to genetics [40]. In Paeonia emodi leaves from different altitudes, phenolics, flavonoids and tannins rose with altitude ($p < 0.01$) [41]. Bacopa accessions grown at one site differed by accession and season [42].

TABLE 3 Studies of variation in marker content or related traits with origin, altitude, season or genotype (abstract-level information).

Plant and material	Design and method	Reported finding	Ref.
Aegle marmelos leaf	Monthly collection over a year, several locations	Morphology, microscopy and yields varied with season; aegeline higher in winter	[33]
Aegle marmelos genotypes	80 wild genotypes and two cultivars; 27 SSR markers (17 polymorphic)	Three subpopulations; wide variation in fruit traits	[3]
Aegle marmelos populations	24 genotypes, two zones; SRAP, 13 traits	70% of molecular variation between populations; pulp phenols 11.65-24.38 mg GAE/g	[4]
Bacopa monnieri (24 accessions)	HPLC fingerprint, 12 common peaks; HCA, PCA, discriminant analysis	Two groups matching geographic origin	[38]
Bacopa monnieri (14 accessions)	Common site, one year	Accession and season effects significant; summer harvest suggested	[42]

Plant and material	Design and method	Reported finding	Ref.
Neem kernels (43 provenances)	RP-HPLC of azadirachtins; ANOVA	Azadirachtin A 556.9-3030.8 mg/kg; provenance effect significant	[39]
Centella asiatica (60 accessions)	Common garden; HPTLC-densitometry	Madecassoside up to 5.67 ± 0.08%; no link with elevation	[40]
Paeonia emodi leaf	Samples from different altitudes	Phenolics, flavonoids, tannins rose with altitude ($p < 0.01$)	[41]

Two conclusions follow. Provenance can matter, but so can the individual tree, so a regional contrast supported by one tree per site would be unconvincing. And when plants are grown in a common garden, genetic differences remain, so a field-collection design measures the combined effect of environment and genotype; that combined effect is what a buyer of raw leaf actually receives, but it must not be described as an environmental effect alone.

VII. A PROPOSED REGIONAL COMPARISON

Figure 2 outlines a design for a comparison of bael leaf from four regions of India: the north (Lucknow, Uttar Pradesh), west (Ahmedabad, Gujarat), south (Bengaluru rural, Karnataka) and east (Khordha, Odisha). It is a proposal for a project, not a protocol that has been run. The elements follow from the evidence above. Collection is restricted to a single calendar month, so that season does not confound region [33]; at least three trees are sampled per site, so that tree-to-tree differences can be separated from site differences [39,40]; and a herbarium specimen is retained for every collection, because identity rests on authentication and not on the label. Leaves of matched size and age are used for the stomatal constants [36,37].

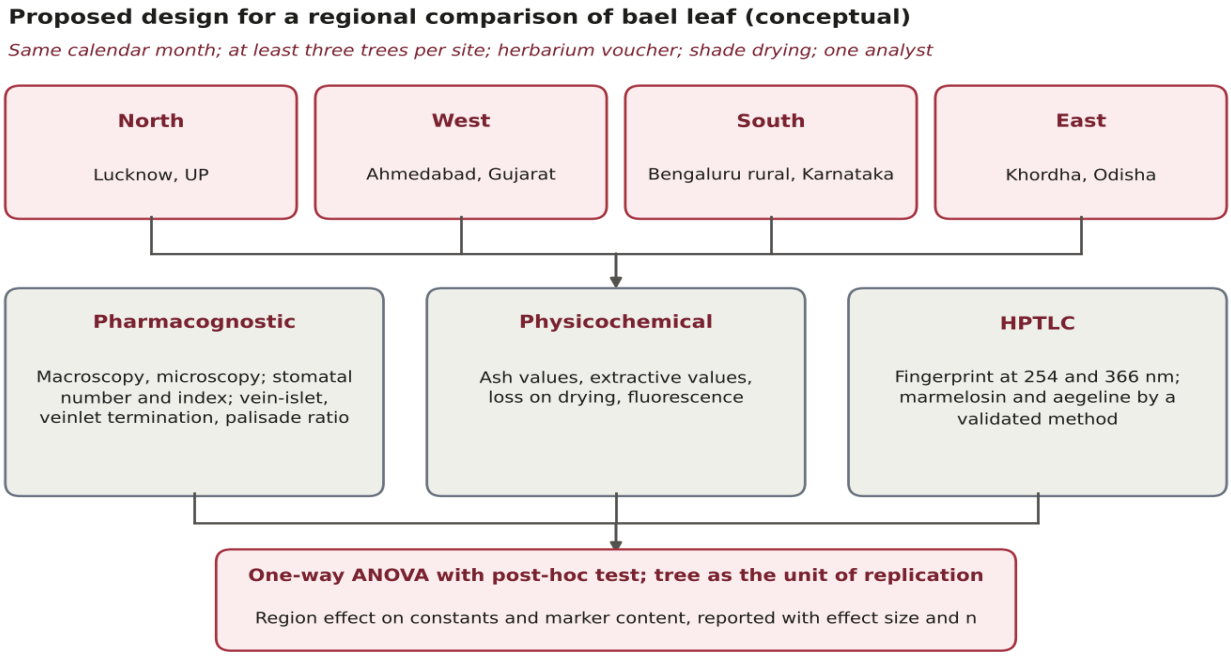


FIGURE 2 Conceptual design for a regional comparison of Aegle marmelos leaf. The scheme is a proposal built from the literature reviewed here and does not show experimental data.

The analytical streams run on the same material by one analyst: the pharmacognostic constants, the physicochemical values, and an HPTLC fingerprint with a validated method for two markers, marmelosin and aegeline. The method should be validated to the standard shown by earlier work: linearity, precision, accuracy, LOD and LOQ, specificity and robustness, with chromatographic conditions reported in full

[18,20]. Because aegeline occurs as a racemate, an achiral HPTLC measure reports total aegeline only [17]. The statistical analysis is a one-way ANOVA with a post-hoc test, with the tree as the unit of replication; the number of trees, not the number of plate applications, defines the sample size. The result is expressed as the size of the regional effect with its confidence interval. If the leaf constants and markers do not differ between regions, that is itself useful, since it would support a single monograph limit.

VIII. QUALITY CONTROL IN CONTEXT

Bael leaf is also a supply-chain problem. A paper on herbal quality assurance argues that composition and quality depend on the value chain linking producers to markets and that metabolomic and HPTLC tools can assess chemical variability along it [43]. A review of quality control practices covers standardised processes, contaminant detection, authentication and regulatory compliance [44]; neither abstract names WHO guidelines on collection practice. A review of microscopic authentication, covering 28 studies of 508 commercial products from 13 countries, found about 59% of the products authentic and 41% adulterated, and concluded that microscopy is rapid and cost-efficient but performs poorly on highly processed samples [45]. Powdered leaf is a processed form, so microscopy alone is unlikely to be enough and a chromatographic fingerprint will be needed beside it. Contamination screening belongs to the same picture: bael leaves screened for heavy metals, pesticide residues and microbial contamination in one GC-MS study passed all three [27].

IX. GAPS AND CONCLUSION

Five gaps limit what can be said about bael leaf today. First, we found no indexed abstract that gives quantitative leaf constants for A. marmelos. Second, a validated method for marmelosin and aegeline together in the leaf is not available in the abstracts we read; the marmelosin methods used fruit [21] or root and branches [24]. Third, the marker data that exist are single-source, so regional contrasts rest on inference from other plants and from the three essential-oil reports. Fourth, the seasonal study has no values in its abstract [33]. Fifth, the safety of aegeline at supplement doses is contested and unresolved [15–17].

Bael leaf is pharmacologically interesting and widely collected, but its standardisation data are incomplete and are drawn from separate sources. A regional comparison collected in one month, with several trees per site, matched leaves for the constants, a validated two-marker HPTLC method and a tree-level statistical design, would give the first comparable dataset. Whether the regional differences are large enough to need region-specific limits is the question that such data would answer.

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