Variations in the Pharmacognostic Properties of *Aegle marmelos* found across Five Geographical Locations in Sri Lanka

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Abstract

Aegle marmelos (Bale tree), family-Rutaceae is a very important medicinal plant used for the Ayurveda and traditional medical systems. This study is focused on pharmacognostic characteristics of the leaves, stem barks and the root barks of Aegle marmelos (A. maemelos) found in five geographical locations in Sri Lanka. A solvent system containing, Toluene, Ethyl acetate, Formic acid and Methanol was used as mobile phase for the leaves. For stem bark and root bark extracts, Toluene: Ethyl acetate: Chloroform: Hexane: Methanol solvent system was used as the mobile phase. TLC outcomes were developed by High Performance Thin Layer Chromatography (HPTLC) technique. According to this study moisture values of the selected plant parts were in the range of 7% to 12% and ash values were in the range of 5% to 10%. pH values of the selected plant parts were varied form 5-8. Microscopic view of the root bark and stem bark were observed and tracheid and xylem vessels were detected. One common compound was detected in leaves, stem bark and root bark, and two common compounds were detected in stem bark and root bark from TLC and HPTLC profiles. It was detected that the concentrations of the similar compounds vary with plant part as well with the origin of the plant. These findings can be used for the identification and authentication of A. marmelos plant parts that are used for medicinal purposes and herbal product manufacture. A comparative clinical study is needed to explore the therapeutic efficacy of these parts.

Keywords: Bael , HPTLC, microscopic , pH, Moisture , Ash

I. INTRODUCTION

Man is a creation of nature. Therefore, if a human gets an illness, there should be a cure for it through nature itself. The medicines available in the areas where we live are *sāthmya* for us. Although Sri Lanka is located in a small land area, it is rich in biodiversity due to its coastline, plains, lowlands and mountains. Since Sri Lanka is also known as a country with high biodiversity in South Asia , even though it is the same plant in Sri Lanka, diversity (Ashton *et al.*, 2004).

Aegle marmelos is commonly known as Bilwa or Bael which belongs to the family Rutaceae. It is an important indigenous medicinal plant in Ayurveda. The English name for Bael is stone apple, as its rather large fruit is like pale yellow to golden orange when ripe (Subedi and Bashyal, 2022). Due to urbanization and commercialized economy, nowadays we usually find the stem bark of the Bael tree is sold instead of the root bark. Therefore, when preparing Ayurvedic drugs it is vital to verify the quality of the raw materials originated from the Bael plant.

If there are similar fundamental chemical compounds found in the root bark and stem bark of the tree, the Bael tree's stem bark can be used in place of the root bark. When preparing the standard quality of Ayurveda drugs, it is needs to have a good understanding of the variation of the chemical compositions of the tree during different seasonals and in different geographical areas. This will assure a speedy recovery of a patient by a quality drug. Therefore, this study has been focused to determine variations in the pharmacognostic properties of *Aegle marmelos* found across five geographical locations in Sri Lanka.

II. LITERATURE REVIEW

Bael can thrive well in high altitude as high as 1,200 m and withstand without any significant growth retardation at 50°C and −7°C. In the prolonged droughts, fruiting may cease, but the plant can survive with shallow soil moisture. Bael

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trees generally require well-drained soil (pH:5–8)(Pathirana, Madhujith and Eeshwara, 2020). It is a subtropical, deciduous tree that grows well in various soil-climatic conditions and can also survive in alkaline soil, and is not injured by temperatures even as low as -7ºC (Shashank and Poonia, 2018). *Aegle marmelos is a* strong tree that grows 6m to 10m of height and bears aromatic trifoliate leaves (Singh, 2008).It has two varieties; *"Vanya*" is thorny and small fruited and "*Grāmya*" is less thorny large fruited (Department of Ayurveda, 1979). Bael plant contains various phytochemicals like alkaloids, tannins, essential oils, gums, resins, coumarin, polysaccharide that makes it useful in many ailments. It has numerous crucial therapeutic applications like antifungal, analgesic, anti-inflammatory, antipyretic, hypoglycemic, anti-lipidemic, and immune-modulatory, anti-proliferative, wound healing, anti-fertility and insecticidal (Mali *et al.*, 2020). Bale plant also mentioned in the specific classification systems (*Ghana vargīkarana*) in main Ayurveda texts as *Śothahara, Arśoghna, Asthāpanoga Mahakaşāy (caraka samhitha),* Var*unādi, Ambhasthādi, Brihat pancamula gana (Susruta Samhita) and Guducyādi varga (*Bhavaprakāśa) (Agrawal and Kumar, 2015). Ayurvedic Pharmacological properties of Bale plant described in Ayurveda text (Shrma)Rasa (taste)-Kaşāya,Tikta; Guna (properties)-Laghu, Rukşa; Vīrya (power) -Uşna, Vipāka Metabolism)-Katu and Karma(action)-Grāhi.

The different parts of *Aegle marmelos* are used for various therapeutic purposes such as for treatment of asthma, anemia, fractures, healing of wounds, swollen joints, high blood pressure, jaundice, diarrhea, healthy mind and brain typhoid troubles during pregnancy (Virendra, Rashmi and Pandey, 2018). Various pharmacological actions said by traditional healers are antioxidant, antibacterial, antifungal, antidiarrheal, antidiabetic, anti-proliferative, cytoprotective, hepatoprotective, antifertility, analgesic, anti-arthritis, contractile, antihyperlipidemic, cardioprotective, radioprotective, anticancer, antiviral, anti-ulcer, immunomodulatory and wound healing properties. (Bhar, Mondal and Suresh, 2019).

Chemical compounds found in various parts of *A. Marmelos* having biological influences in the body (Venthodika *et al.*, 2021). Also, the Rf of other phytocompounds of methanol extracts which are near to marmelosin range may be other compounds under the class coumarins (Nirupama *et al.*, 2012).

The root and stem bark have more identical compounds and so they have same pharmacological activities and therefore stem bark could be substituted or used along with root in any of drug preparation where root is of important.

III. METHODOLOGY

A. Sample Collection and Preparation

Samples from the different parts of the Bael plants collected from the aforementioned geographical areas were introduced to the National Herbarium of Sri Lanka and obtained authenticated prior to the study. When collecting the plant specimens, foreign matter like sand, soil, polyethene, fungus or insect infestations were avoided and samples were cleaned, packed packages and labeled accordingly. Sample labeling was done as follows and labeling numbers are referred here on for the convenience.1 -Colombo, 2- Kegalle, 3-Matale, 4-Galle & 5 – Puttalam. All samples were cleaned well and dried under sunlight while preventing any contaminants. Then they were grounded into fine powder by a clean grinder machine (Premier Xpress 750.India), sifted and stored in clean labeled pouches separately. Sifting was done through sieve number 125µm containing an aperture size of 0.125 mm and having a wire diameter of 0.090 mm.

B. Determination of Moisture Content

Moisture testing was conducted based on ISO 1573. Initially the empty weight of the moisture dish was measured (Wo). Then 5g of powders specimen was put into the dish and weight was measured (W_1). Then it was heated for 2 hours in the electric oven at 103^0C temperature. Heated dish was cooled down to room temperature inside a desiccator. Final weight of the cooled dish was measured (W_2).

C. Determination of Ash

Ash value determination was conducted based on the WHO quality control methods for medicinal plants. Initially the cleaned, empty crucibles were marked and the weights were measured. Initial weights (Wo) of the crucibles were recorded separately and about 5 g of each sample were put in to the crucibles accordingly. Weight of the crucibles with the samples (W_1) were recorded separately and then the crucibles were heated in

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an open flame to reduce the moisture. Then the samples were introduced to the muffle furnace (KJ-$MC_{1000-27}$LWQ , Zhenzhou Kejia Furnace Co., Ltd, China) to be heated at 500°C.Samples were heated for 4 hours until constant weights were gained for each sample. Then the samples were cooled down to room temperature inside the desiccator. Then then the final weights of the crucibles with the samples (W_2) were measured and recorded.

D. Determination of pH

pH meter (PH400S,APERA, Columbus, Ohio, US) was calibrated with standard buffer solutions of pH 3.01 , pH 7.00 and pH 13.01.pH probe was cleaned before and after each test.10g of the powdered specimens were extracted to 100 ml of hot water at 50^0C temperature and allowed to cool down. Then the insoluble content was removed and the pH of the extract was tested.

1) Thin Layer Chromatography and High-Performance Thin Layer Chromatography

Ten grams of powdered samples were placed into Erlenmeyer flasks, and 100ml of methanol was added to each flask. The flasks were then placed on an orbital shaker and operated for 24 hours. After that, the solution was filtered using filter papers, and the filtrate was introduced to a rotary evaporator. The solvent was evaporated, and the remaining extract was taken and labeled according to the test tube it was placed in. Drops from the extract were then introduced to the starting line of a TLC plate using separate capillary tubes. The TLC plate was then immersed in the prepared mobile phase inside the TLC chamber, and the samples were allowed to run. Once the mobile phase reached the end line, the TLC plate was taken out and allowed to dry at room temperature.

2) Preparation of the Mobile Phase

Mobile phase was prepared with several mixtures of solvents (Toluene: Ethyl Acetate: Methanol -16: 4: 1, Chloroform: Methanol -9:1, Toluene: Ethyl Acetate -i3: 2, Toluene: Ethyl Acetate -3: 1, Toluene: Ethyl Acetate- 5: 2, Ethyl Acetate: Toluene: Chloroform: Hexane: Methanol- 2: 2: 1: 4: 1, Ethyl Acetate: Toluene: Formic Acid: Methanol - 3: 3: 0.8: 0.2) in order to obtain the best combination for a perfect mobile phase. TLC plates with highest visibility were introduced to the HPTLC machine and separations were analyzed.

IV. RESULTS AND DISCUSSION

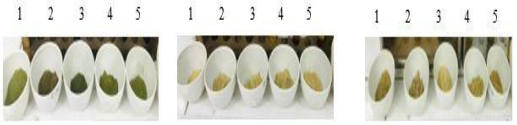

Figure 03: Powdered Samples of Bael Plant Parts
a: Leaf powder, b: Stem bark powder, c: Root bark powder

When analyzed the morphological characteristics of the dried and powdered leaves from the 05 regions shown in Figure 01, all samples exhibited a similar aromatic odor and their colors varied from dark green to light green. Since all samples were dried under similar conditions, this color variation should be due to the density of chlorophyll content and other compounds.

Dried stem bark powders were cream colour and the root bark powder was darker than the stem bark powders. Root bark powders were brownish cream in colour and both stem and root bark powders gave aromatic odour. Colour variations are mainly due to the presence of different types of chemical compounds and organelles in different concentrations.

The moisture content of Bael leaves, stem barks, and root barks were analyzed in (Figure 02) different districts, revealing distinct patterns. For Bael leaves, the moisture content ranged from 7.88% to 11.69%, with Colombo exhibiting the highest moisture content followed by Kegalle, Galle, Matale, and Puttalam. This variation is attributed to regional differences in humidity and rainfall, with areas having higher humidity areas having more moisture. In Bael stem barks, moisture content varied from 7.58% to 10.38%, with Kegalle having the highest moisture content, followed by Matale, Colombo, Galle, and Puttalam.

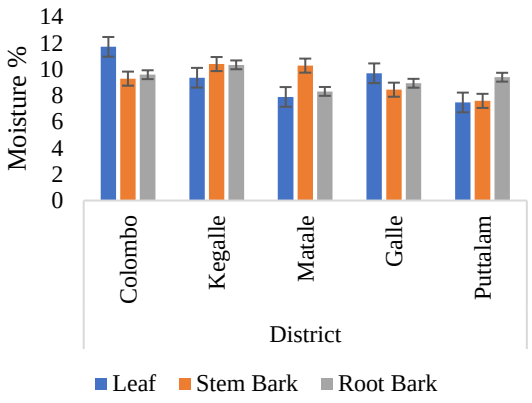

Figure 04: Moisture Variation in the Selected Districts

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The moisture content of Bael root barks ranged from 8.3% to 10.32%, with Kegalle showing the highest moisture content, followed by Colombo, Puttalam, Galle, and Matale. These variations are influenced by factors such as soil moisture and altitude, highlighting the importance of understanding regional differences for standardized production in industries utilizing Bael products.

Figure 03 displays the pH variations in Bael leaves, stem barks, and root barks collected from different districts. For Bael leaves, pH ranged from 5.92 to 7.3, with Colombo having the highest pH followed by Puttalam, Kegalle, Galle, and Matale. Acidic soils in certain regions, influenced by factors like minerals and rainfall, contributed to lower pH levels in leaves. Bael stem barks showed a pH range between 6.67 and 5.87, with Galle being the most acidic followed by Kegalle, Matale, Colombo, and Puttalam. Urban and industrial activities, along with microbial processes, affected stem bark acidity. Bael root barks exhibited a pH range of 7.42 to 6.29, with Colombo being the most basic followed by Galle, Puttalam, Matale, and Kegalle. Alkaline minerals and organic matter decomposition influenced higher pH levels in root barks.

These variations stem from a complex interplay of soil composition, environmental factors, microbial activity, and organic matter content in the respective regions. Understanding these pH differences is crucial for comprehending the quality and characteristics of Bael products from different districts.

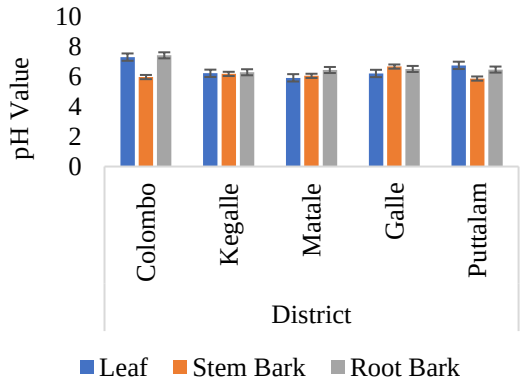

Figure 05: pH Variation in the Selected Districts among the Tested Bale Samples

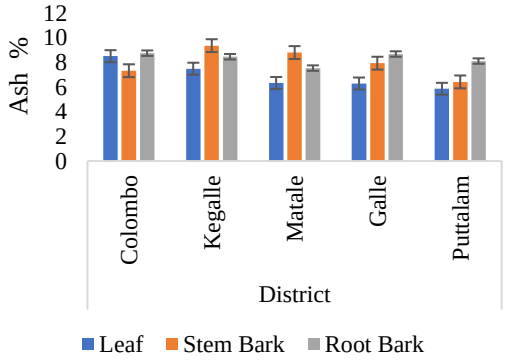

Figure 06: Ash Content Variation in the Selected Districts among the Tested Bael Samples

Figure 04 demonstrates the results of the ash content in Bael leaves, stem barks, and root barks of different regions. Ash content can be indicative of the mineral content in plant samples and is commonly used to assess the quality and nutritional value of plant material. The ash content in Bael leaves ranged from 8.51% to 5.86%, with the highest content in Colombo and the lowest in Puttalam. The descending order of ash content was Colombo > Kegalle > Matale > Galle > Puttalam. The mineral content in plants often reflects the composition of the soil they grow in. Different regions have varying soil compositions, which can influence the mineral uptake by plants.

Environmental factors such as rainfall, sunlight, and temperature can affect plant metabolism and nutrient absorption, leading to differences in ash content. Older or stressed plants might have different nutrient profiles compared to younger, healthier ones. Ash Content in Bael Stem Barks varied from 6.42% to 9.36% and the descending order of ash content was Kegalle > Matale > Galle > Colombo > Puttalam Similar to leaves, environmental factors play a significant role. Adequate water supply and nutrient availability in the soil can lead to higher ash content.

Ash Content in Bael rootbarks were in the range of 7.54% - 8.75% in the following descending order .Kegalle > Colombo > Puttalam > Galle > Matale. Plants absorb minerals not only from the surface but also from deeper layers of the soil. Variations in root depth across regions can lead to differences in mineral content. The pH of the soil can influence the availability of certain minerals. Plants in regions with more acidic or alkaline soils might have different ash content.

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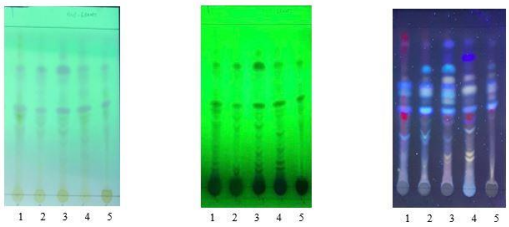
A) Analysis of TLC & HPTLC Observations

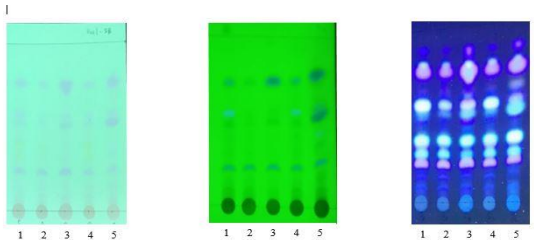


TLC Plate at 200 nm | TLC Plate at 254 nm | TLC Plate at 366 nm

Figure 07: TLC profiles of Leaf Extracts under Different Wave Lengths

(Left to right Spots :1-Colombo,2-Kegalle,3 -Matale,4-Galle and 5-Puttalam)

Figure 05 depicts the TLC profiles of leaf extracts under different wave lengths. The Solvent system used for the Bael leaves was different form the solvent system used for the stem and root barks due to the different compositions of the parts.

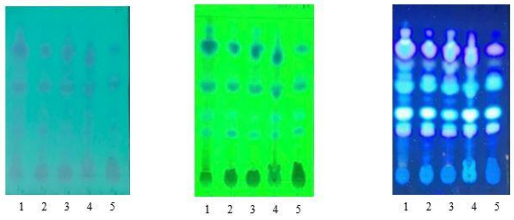


TLC Plate at 200 nm | TLC Plate at 254 nm | TLC Plate at 366 nm

Figure 08: TLC Profiles of Stem Bark Extracts under Different Wave Lengths

(Left to right Spots: 1-Colombo,2-Kegalle,3-Matale, 4-Galle and 5 -Puttalam)

Developed TLC profiles of stem bark extracts under different wave lengths described in Figure 6. Most clear visualization of the sperated spots of stem bark extracts were observed under 366nm wave length.



TLC Plate at 200 nm | TLC Plate at 254 nm | TLC Plate at 366 nm

Figure 09: TLC Plated of Root Barks under Different Wave Lengths

(Left to right Spots :1-Colombo, 2-Kegalle, 3-Matale, 4- Galle and 5- Puttalam)

Figure 07 described the TLC profiles of root bark Extracts at different wave lengths. The clearest visualization of the sperated spots of root bark extracts were observed under 366nm wave lenth.

The data provided on Table 03 indicate the presence of certain compounds in the samples, as represented by their Retention Factor (Rf) values and the corresponding areas under the peaks (which indicate the quantity of the compounds).

Table 01: Comparison of Rf Values and Peak Areas in HPTLC Readings on Rf Values and Peak Areas for Bael Leaves

Sample	Leaves of *Bale* plants from different geographical locations					
	Rf 01		Rf 02		Rf 03	
	Max Rf	Area %	Max Rf	Area %	Max Rf	Area %
1	0.4	23.85	0.11	3.9	0.23	0.51
2	0.4	11.12	0.12	2.69	-	-
3	0.39	3.78	-	-	-	-
4	0.39	4.02	0.11	3.29	0.23	0.43
5	0.42	1.87	0.12	6.69	0.24	0.49

(Sample Origin: 1-Colombo,2-Kegalle,3-Matale,4-Galle and 5-Puttalam)

Rf values indicate the presence of specific compounds in the samples. Each Rf value corresponds to a different compound or a group of compounds in the Bael leaves. The presence and quantity of compounds vary across regions. For instance, all 5 samples share some compounds (Rf around 0.4), but the quantities differ significantly. Additionally, certain compounds found in Colombo and Galle samples (Rf: 0.11, 0.23) are present in lower /higher quantities or absent in other regions. The differences in compound presence and quantity suggest that the chemical composition of Bael leaves varies based on the geographical location where the plants are grown. These differences could be due to soil composition, climate, or other environmental factors.

Best Solvent system for the TLC for Bael leaves is the mixture of Ethyl Acetate: Toluene: Formic Acid: Methanol in 3 :3 : 0.8 : 0.2 ratio and the Best solvent system for the TLC for Bael root bark

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and stem bark is Ethyl Acetate: Toluene: Chloroform: Hexane: Methanol mixture in 2: 2: 1: 4: 1 ratio. The best Wavelength to visualize the separations using the selected mobile phase is 366nm.

Table 04: TLC profiles of Root Bark and Stem Bark with Rf Values

Sample	Stem bark				Root bark			
	Rf 01		Rf 02		Rf 01		Rf 02	
	Max Rf	Area %	Max Rf	Area %	Max Rf	Area %	Max Rf	Area %
1	0.22	8.59	0.57	43.02	0.24	6.3	0.53	27.85
2	0.21	8.76	0.57	14.34	0.23	10.04	0.57	7.94
3	0.19	9.62	0.55	13.4	0.22	13.76	0.56	7.12
4	0.21	8.81	0.56	14.57	0.23	10.75	0.56	4.74
5	0.21	7.98	0.56	14.57	0.23	13.94	0.57	8.6

(Sample Origin:1-Colombo,2-Kegalle,3-Matale, 4-Galle and 5-Puttalam)

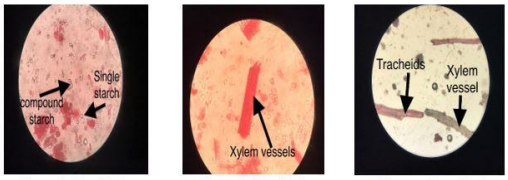
According to Table 04, root bark and the stem bark had 02 similar Rf values with higher peak areas. That implies, both stem bark and root bark have two similar chemical compounds in large concentrations. The highest area of common Rf 01 of the stem bark Sample was found in Colombo (8.59%) & Kegalle (8.76%) .Highest Area %: of common Rf 02 was also found in Colombo (43.02%), Kegalle (14.34%) samples. For Root Bark Samples the highest area was found in Puttalam (27.85%) and Colombo (6.3%) for common Rf 01 and for common Rf 02, highest area was found in Colombo (27.85%) and Puttalam (8.6%).

Colombo exhibits significant presence and high area percentages for both Common Rf 01 and Common Rf 02 in both stem bark and root bark samples. It stands out as having considerable quantities of the compounds represented by these Rf values. Kegalle shows notable presence for both Common Rf 01 and Common Rf 02 in stem bark samples, although the area percentages are comparatively lower than Colombo. In root bark samples, Kegalle's presence is moderate. These regions exhibit moderate presence and area percentages in both stem bark and root bark samples. They do not have the highest values but still have considerable quantities of the compounds represented by the given Rf values. Matale samples generally show lower presence and area percentages compared to the other regions. It has moderate presence in stem bark samples and relatively low presence in root bark samples.

Industries utilizing Bael bark for medicinal or herbal products should consider the regional differences in chemical composition for quality control and standardization purposes. It's crucial to ensure consistent quality and efficacy of the products derived from Bael bark across different regions. These variations highlight the importance of understanding regional differences in herbal products. The presence and concentration of specific compounds can influence the medicinal properties and overall quality of Bael leaves in different regions.

B. Microscopic View of the Different Parts of the Bael plant

Starch granules were not found in the root bark microscopic view but single and compound starch granules were found in Bael leaves. Tracheids and xylem vessels were found in the Bael root bark and Stem bark also showed xylem vessels in the microscopic view.



a b c

Figure 010: Microscopic view of a: Bael leaves, b: stem bark and c: root bark

VII. CONCLUSION

These results revealed that sustainable medicinal usage from the *Aegle marmelos* plant, there is a possibility to use stem bark of the plant as a substitute for root bark because both parts presented with many similar compounds. This replacement prevents the damage done to the root of the Bael plant by people when they search root barks for commercial purposes. This would be validated with clinical studies in therapeutic practice and their potential medicinal significance, additional analysis, such as mass spectrometry or chemical profiling.

Moreover, the results of this study would be used for further research to understand the specific compounds responsible for the medicinal properties of Bael leaves in each region. Such knowledge is crucial for both traditional medicine and the pharmaceutical industry to harness the full potential of this plant for medicinal purposes.

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