

RESEARCH PAPER

“DETECTION OF ADULTERATION AND STANDARDIZATION OF BILVADILEHA BY PHYSICOCHEMICAL AND TLC-BASED MARKER ANALYSIS”

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Abstract

In India, herbal medicine is commonly practiced through the traditional system of Ayurveda. Ayurvedic formulations such as Avaleha are widely used for the management of various disorders due to their therapeutic efficacy and natural origin. Bilvadileha is a classical Ayurvedic polyherbal formulation mainly indicated for gastrointestinal disorders such as diarrhea, dysentery, and digestive weakness. With the growing global acceptance of herbal medicines, standardization of Ayurvedic formulations has become essential to ensure their quality, safety, and efficacy. The World Health Organization has recommended suitable standardization parameters for the evaluation of crude drugs and finished herbal products. In the present study, Bilvadileha was thoroughly evaluated for its organoleptic and physicochemical characteristics to establish a routine standardization procedure for this Ayurvedic formulation. Parameters such as colour, odour, taste, and consistency were examined under organoleptic evaluation, while physicochemical studies included determination of pH, moisture content, total ash value, acid-insoluble ash, water-soluble extractive value, and alcohol-soluble extractive value. An adulteration study was additionally performed using microscopic examination and thin-layer chromatographic (TLC) profiling of *Aegle marmelos*-containing samples to assess the identity and consistency of the formulation. Modern analytical techniques such as TLC were employed for the identification and evaluation of major phytoconstituents present in the formulation. Furthermore, phytochemical screening was carried out for the detection of carbohydrates, alkaloids, tannins, glycosides, flavonoids, proteins, and saponins. The comparative TLC profiles and diagnostic microscopic characters provided supportive evidence for authentication and evaluation of possible adulteration. The obtained results may be useful in establishing quality control standards and ensuring the purity, safety, and consistency of Bilvadileha formulation.

Keywords: Bilvadileha; Ayurveda; Avaleha; standardization; adulteration; authentication; physicochemical evaluation; phytochemical screening; TLC fingerprinting.

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INTRODUCTION

Ayurveda is one of the traditional systems of medicine that has evolved over centuries through accumulated empirical knowledge, clinical experience, and systematic documentation. The system emphasizes the maintenance of health and prevention of disease through an integrated approach involving diet, lifestyle, medicinal plants, and pharmaceutical preparations [1]. Ayurvedic materiamedica comprises a wide range of medicinal plants and formulations, many of which continue to be used in traditional healthcare. With increasing interest in traditional and herbal medicines, scientific evaluation of Ayurvedic formulations has become important for establishing their identity, quality, consistency, safety, and reproducibility [2].

Ayurvedic pharmaceuticals, traditionally described under *Bhaishajya Kalpana*, encompasses various pharmaceutical dosage forms prepared from medicinal plants and other therapeutic substances, including *Swarasa*, *Kalka*, *Kwatha*, *Churna*, *Asava*, *Arishta*, *Taila*, *Ghrita*, and *Avaleha* [3]. These dosage forms involve specific pharmaceutical processes intended to facilitate extraction, preservation, administration, and utilization of the constituents of medicinal substances. Among them, *Avaleha Kalpana* represents an important category of semisolid oral preparations [4].

1.2 Avaleha and Bilvadileha: Formulation and

Traditional Significance

Avaleha or *Leha* preparations are semisolid Ayurvedic formulations generally prepared by concentrating herbal decoctions or extracts with prescribed sweetening and pharmaceutical ingredients, followed by incorporation of powdered medicinal substances [5]. The preparation involves several critical stages, including selection and authentication of raw materials, preparation of the herbal extract or decoction, concentration to the appropriate stage, incorporation of fine powders, and development of the desired consistency. Variations in raw-material quality, proportions, processing conditions, and storage may influence the physicochemical and phytochemical characteristics of the finished formulation [6].

Bilvadileha is a classical Ayurvedic semisolid polyherbal formulation belonging to the *Avaleha* category. It is traditionally prepared using *Bilva* (*Aegle marmelos*) as an important ingredient along with other medicinal substances prescribed according to the formulation [7]. Traditionally, Bilvadileha has been associated with the management of gastrointestinal disturbances and conditions involving impaired digestion and altered bowel function. The traditional properties attributed to its ingredients, including *Deepana*, *Pachana*, and *Grahi*, provide the Ayurvedic rationale for its use in such conditions [8].

As a polyherbal preparation, Bilvadileha contains constituents derived from multiple botanical sources. Consequently, the identity and quality of each ingredient, its proportion, pharmaceutical processing, and storage conditions can influence the characteristics of the final product. Maintenance of these factors is essential for achieving reproducible quality and consistency among different batches and sources [9].

1.3 *Aegle marmelos*: Botanical, Phytochemical and Analytical Significance

Aegle marmelos (L.) Corrêa, commonly known as Bilva or Bael, belongs to the family Rutaceae and is an important medicinal plant in Ayurveda. Different parts of the plant, including the roots, stem bark, leaves, fruits, and seeds, have been traditionally utilized for various purposes, particularly those associated with gastrointestinal health. Its importance in Bilvadileha makes the authentication and quality assessment of *A. marmelos* relevant to the standardization of the formulation [10].

Phytochemical investigations of *A. marmelos* have reported several classes of secondary metabolites, including coumarins, alkaloids, flavonoids, tannins, phenolic compounds, terpenoids, glycosides, and volatile constituents. Characteristic constituents such as marmelosin and umbelliferone have analytical relevance and may serve as chemical markers in appropriate analytical systems for the authentication and standardization of *A. marmelos*-containing preparations. The selection of a marker, however, should be based on its suitability for the specific plant material, formulation, analytical method, and study objective [11].

The phytochemical composition of *A. marmelos* may vary according to geographical origin, genetic characteristics, plant part, maturity, environmental conditions, harvesting season, processing, extraction conditions, and storage. Such variability can affect the concentration and profile of characteristic constituents. Therefore, botanical authentication together with appropriate physicochemical and chromatographic evaluation is important for maintaining the quality and reproducibility of Bilvadileha [12].

1.4 Quality Control and Standardization of Herbal Formulations

Herbal medicines differ from chemically defined synthetic drugs in that they contain complex mixtures of primary and secondary metabolites. Their chemical composition may be influenced by botanical identity, geographical origin, cultivation practices, soil and climatic conditions, harvesting period, post-harvest handling, extraction procedures, manufacturing processes, and storage. Such variability presents a significant challenge in establishing consistent quality of herbal formulations [13].

Standardization involves the establishment of defined and reproducible parameters for assessing the identity, purity, quality, strength, and consistency of a pharmaceutical preparation. In Ayurvedic formulations, standardization is particularly important because variations in raw materials and manufacturing practices can produce differences in the physicochemical and chemical characteristics of the finished product [14].

For Bilvadileha, quality assessment should include appropriate physical and physicochemical characteristics together with chemical profiling. Parameters such as colour, odour, taste, appearance, consistency, pH, moisture content or loss on drying, ash values, and extractive values can provide useful baseline information regarding the quality and general characteristics of the formulation. However, these parameters alone cannot establish the complete chemical identity of a complex polyherbal preparation. Therefore, their combination with chromatographic approaches can provide a more comprehensive quality profile [15].

1.5 Adulteration and Authentication Challenges in Polyherbal Formulations

The increasing production, commercialization, and distribution of herbal medicines have increased the importance of concerns related to adulteration, substitution, contamination, misidentification, and inadequate quality control. Herbal adulteration may involve the intentional or unintentional replacement, addition, or mixing of authentic medicinal material with inferior, exhausted, contaminated, or unrelated material. Intentional substitution may occur for economic reasons, whereas unintentional adulteration may result from incorrect botanical identification, collection of inappropriate plant parts, morphological similarity between species, poor procurement practices, or inadequate post-harvest handling [16].

Adulteration may be facilitated by limited availability of genuine plant material, increasing market demand, economic incentives, inadequate taxonomic identification, and insufficient quality-control systems. At the same time, natural variation in phytochemical composition may arise from differences in botanical source, geographical origin, environmental conditions, harvesting season, plant part, processing, extraction efficiency, and storage. Therefore, differences observed between herbal samples should not automatically be interpreted as evidence of adulteration; appropriate authenticated reference material and multiple complementary analytical parameters are necessary for a reliable assessment[17].

The problem is particularly challenging in polyherbal formulations because individual ingredients undergo processes such as drying, pulverization, extraction, heating, concentration, and mixing. These processes may obscure the original morphological and anatomical characteristics of the crude drugs, making identification of individual ingredients in the finished semisolid formulation difficult. Root and bark materials may present additional authentication challenges because dried materials from different botanical sources can exhibit similar external characteristics. In Bilvadileha, inappropriate substitution or use of inferior-quality *A. marmelos* material may alter the expected chemical profile of the formulation. Hence, analytical evaluation of the finished product is an important component of quality assurance[18].

1.6 Physicochemical and Chromatographic Approaches for Quality Assessment

Physicochemical evaluation provides measurable parameters that are useful for the routine quality assessment and comparison of herbal formulations. Organoleptic characteristics such as colour, odour, taste, appearance, texture, and consistency provide an initial assessment of the finished formulation. Although these parameters are simple, rapid, and economical, their subjective nature limits their ability to independently establish the quality and consistency of a complex polyherbal formulation[19].

Moisture content or loss on drying is an important quality parameter because excessive moisture may affect the stability and microbial quality of herbal preparations. Total ash provides information regarding the total inorganic residue, while acid-insoluble ash may provide an indication of insoluble extraneous matter. Extractive values provide an approximate measure of the constituents soluble in particular solvents and can be useful for assessing the consistency of herbal formulations. Collectively, these physicochemical parameters can provide baseline quality characteristics and facilitate comparison among different samples[20].

Thin-layer chromatography (TLC) provides an additional approach for the qualitative evaluation of herbal formulations. It enables the separation and visualization of multiple chemical constituents and produces a characteristic pattern of chromatographic zones under specified analytical conditions. The

number, position, colour, intensity, and distribution of the zones, along with their corresponding R_f values, can provide a useful fingerprint of the chemical constituents present in a formulation. Such TLC profiles can be compared among different Bilvadileha samples to identify variations in their chromatographic characteristics[21].

Marker-based TLC analysis further provides targeted information regarding selected characteristic phytoconstituents. The detection of a selected marker at an appropriate R_f value, together with the overall TLC fingerprint, can provide supportive evidence regarding the chemical consistency of the formulation. Thus, the combination of physicochemical evaluation and TLC-based analysis provides complementary information for assessing the quality and consistency of Bilvadileha and for identifying variations that may warrant further investigation for possible substitution or adulteration[22].

1.7 TLC Fingerprinting and Marker-Based Analysis

A chemical marker is a defined constituent that can be used as an indicator of the identity, quality, or consistency of a medicinal plant or formulation. Marker-based analysis can provide greater specificity than a general phytochemical screening approach when the selected constituent is appropriate for the botanical material and analytical objective.

TLC fingerprinting provides an overall qualitative representation of the chemical constituents present in an extract or formulation. The number, position, colour, intensity, and distribution of chromatographic zones, together with their corresponding R_f values, can generate a characteristic chemical profile. In a complex polyherbal formulation such as Bilvadileha, such fingerprinting can be particularly useful because the original morphological characteristics of individual botanical ingredients may be obscured during pharmaceutical processing[23].

For *A. marmelos*-containing preparations, characteristic constituents such as marmelosin and umbelliferone may have analytical relevance depending on the selected extraction system and chromatographic conditions. Detection of characteristic marker zones at appropriate R_f values can provide supportive evidence for the presence of the corresponding botanical material. However, marker detection should be interpreted together with the overall chromatographic fingerprint and other quality parameters rather than being considered as independent proof of authenticity[24].

Comparison of TLC profiles between an appropriately authenticated reference formulation and test samples may reveal differences in chromatographic zones or marker profiles. Such differences can provide analytical evidence of variation associated with raw-material quality, substitution, adulteration, extraction efficiency, processing, or storage and can therefore serve as a basis for further investigation[25].

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1.8 Research Gap and Rationale of the Present Study

Although considerable research has been conducted on the pharmacognosy, phytochemistry, and biological properties of *Aegle marmelos*, comprehensive analytical standardization of the finished Bilvadileha formulation remains comparatively limited. Existing investigations have provided information on the physicochemical characteristics and chromatographic profiles of selected Ayurvedic formulations and on the phytochemical characteristics of *A. marmelos*. However, an integrated approach combining physicochemical evaluation, chromatographic fingerprinting, marker-based analysis, and assessment of possible adulteration in Bilvadileha is warranted.

A further requirement is the establishment of formulation-specific analytical reference parameters for Bilvadileha. Such parameters may include characteristic physicochemical properties, TLC fingerprints, selected marker profiles, and acceptable analytical variation. Comparative assessment of appropriately authenticated and test or marketed samples can provide useful information regarding formulation consistency and deviations that may warrant investigation for substitution or adulteration.

The complex composition of Bilvadileha and the variability associated with its botanical ingredients therefore necessitate a systematic quality-control approach. Conventional organoleptic and physicochemical parameters can establish important baseline characteristics, while TLC fingerprinting and marker analysis can provide complementary chemical evidence. An integrated evaluation may consequently provide a more informative basis for authentication, quality assessment, and detection of possible adulteration than reliance on a single analytical parameter.

2. Materials and Methods

2.1 Collection and Procurement of herbal drugs

The primary crude drug, *Bilva Roots*, along with other herbal ingredients required for the preparation of *Bilvadileha*, was procured from a certified Ayurvedic raw material supplier, Welankar Ayurvedic Store, Sangli, India. The complete list of ingredients used in the formulation is provided in Table 1.1. The identity and quality of the procured materials were preliminarily verified based on standard organoleptic characteristics and supplier documentation, in accordance with Ayurvedic pharmacopoeial guidelines. For comparative analysis, two commercially available formulations of *Bilvadileha* were procured from local ayurvedic store. ie, MB1- Kerala ayurveda Vilwadi Lehyam and MB2- Arya Vaidya Sala Vilwadi Leham. Bilva plant and other ingredients were authenticated by Dr. S. M. Shendage, Dept. of Botany, Balwant College, Vita.

2.2 Preparation of Bilvadileha in laboratory

Take raw material of pharmacopoeial quality. Wash, dry, powder ingredient number 1 (KvāthaDravya) of the formulation composition and pass through sieve number 44 to obtain coarse powder. Clean, dry, powder the ingredients number 4 to 12 (Prakṣēpa Dravya) of the formulation composition and pass through sieve number 85 to obtain fine powder. Add specified amounts of water to the KvāthaDravya, heat, reduce to one fourth and filter through muslin cloth. Add jaggery to the Kvātha, boil to dissolve and filter through muslin cloth. Reduce the kvātha to thicker consistency by gentle boiling and stirring continuously during the process. Continue heating till the preparation attains the consistency of leha confirmed by the formation of a soft ball that doesn't disperse in water. Remove from heat source and allow to cool to room temperature. Add fine powders of Prakṣēpa Dravya, mix thoroughly to prepare a homogeneous mass. Pack it in tight closed containers to protect from light and moisture [26].

Table no 1.1: Composition of Bilvadileha formulation

Sr. No.	Ingredients	Latin names	Part used	Quantity by reference	Quantity used
1	Bilva API-mūla	<i>Aegle marmelos</i>	Root	1536 g	384 g
2	Jala API for decoction	Water	—	12.28 L	3.072 L
3	JīrṇaGuḍa (PurāṇaGuḍa) API	Old jaggery	—	768 g	192 g
4	Ghana (Musta API)	<i>Cyperusrotundus</i>	Rhizome	12 g	3 g
5	Dhānya (Dhānyaka API)	<i>Coriandrum sativum</i>	Fruit	12 g	3 g
6	Jiraka (Jiraka API)	<i>Cuminumcyminum</i>	Fruit	12 g	3 g
7	Truti (Sūkṣmailā API)	<i>Elettariacardamomum</i>	Seed	12 g	3 g
8	Tvak (Tvak API)	<i>Cinnamomumzeylanicum</i>	Stem bark	12 g	3 g
9	Keśara (Nāgakeśara API)	<i>Mesuaferrea</i>	Stamen	12 g	3 g

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10	Sunthī (Sunthī API)	<i>Zingiberofficinale</i>	Rhizome	12 g	3 g
11	Marica API	<i>Piper nigrum</i>	Fruit	12 g	3 g
12	Pippalī API	<i>Piper longum</i>	Fruit	12 g	3 g

Table no 1.2: Details of preparation of Kwath and Bilvadileha

Sr. No	Parameters	Result
1	Quantity of Bilva coarse powder taken	384 gm
2	Quantity of water added	3.072 L
3	Temperature maintained during heating	90–95°C
4	Kvātha used	1.0 L
5	Guda (Jaggery) added	192 gm
6	Prakṣepadavya added	3 g each

Synthesis of marker compound (7 hydroxy 4 methyl coumarin)

Dissolve resorcinol in ethyl acetoacetate. Cool concentrated sulfuric acid below 10°C and slowly add the resorcinol solution with stirring. Continue stirring for 30–40 minutes. Pour the mixture into ice to form the product (7-hydroxy-4-methylcoumarin). Filter, wash with cold water, and dry. Recrystallize using NaOH solution and precipitate with dilute HCl, then purify with methylated spirit.

3.0 ADULTERATION DETERMINATION STUDY:

3.1 Extraction

3.1.1 Extraction of crude herbal plant materials:

Plant materials of *Aegle marmelos* (L.) was cleaned and pulverized to make powder. Powdered material was sieved through 36 mesh sieve. Weighed quantity of 3gm of sieved powder (yielding 74 mg extracts of *Aegle marmelos* (L.) was taken in separate 50 ml volumetric flasks and 30 ml of ethanol 32.33 was added in it, ultrasonicated for 25 minutes and filtered. The filtrate was concentrated to 10 ml by heating at 40°C. This extract was used for further study.

3.1.2 Extraction of marketed herbal formulations (Samples):

3.1.2.1 Baidyanath Bilva Taila (BT):
A measured quantity of marketed Baidyanath Bilva Taila (1 ml) was transferred to a 50 ml volumetric flask, and 30 ml of ethanol was added. The sample was ultrasonicated for 25 minutes and subsequently filtered. The filtrate was concentrated to 10 ml by heating at 40°C. The resulting extract was used for further physicochemical and chromatographic analysis.

3.1.2.2 Punarvasu Bilvadi Churna (PBC):

A measured quantity of marketed Punarvasu Bilvadi Churna (3 g) was transferred to a 50 ml volumetric flask, and 30 ml of ethanol was added. The sample was ultrasonicated for 25 minutes and subsequently filtered. The filtrate was concentrated to 10 ml by heating at 40°C. The resulting extract was used for further physicochemical and chromatographic analysis.

3.1.3 Phytochemical Screening:

The alcoholic extracts of all the authenticated plants

and samples were subjected to qualitative chemical investigation. The following procedures were adopted to test the presence of various chemical constituents in the extracts. The obtained results are tabulated in table no 1.7.

3.1.4 Thin Layer Chromatography (TLC) of *Aegle marmelos* (L.) Corrêa

Extracts prepared above were subjected to chromatographic separation on silica gel 60 F₂₅₄ TLC plates using toluene:ethyl acetate (9:1 v/v) as the mobile phase. Marmelosin standard and the prepared extracts of the test formulations were applied on the TLC plate as spots of uniform diameter using a capillary tube. The plate was allowed to dry and was then developed in the previously saturated TLC chamber containing the mobile phase up to approximately three-fourths of the total height of the plate.

After development, the plate was removed from the chamber and air-dried. The developed chromatogram was observed under UV light at short wavelength (254 nm) and long wavelength (366 nm). The plate was subsequently exposed to an iodine chamber, and the spots obtained were recorded. The R_f values of the resolved spots were calculated by measuring the distance travelled by each spot and the distance travelled by the solvent front.

The TLC profiles of marmelosin standard, Bilvadileha, Baidyanath Bilva Taila and Punarvasu Bilvadi Churna extracts were compared with respect to the number, colour and R_f values of the resolved spots under visible light, iodine chamber, UV 254 nm and UV 366 nm. The observations were recorded in triplicate and tabulated accordingly. The characteristic spots corresponding to the marker compound were used for comparative identification of *Aegle marmelos* (L.) Corrêa in the investigated formulations.

4.0 Standardization of Different Marketed Brands and In-House Formulation Of Bilvadileha

4.1 Organoleptic Properties

The organoleptic characteristics of different marketed brands and the laboratory-prepared *Bilvadileha* were evaluated based on colour, odour, and taste. The findings of this evaluation are

presented in Table no 1.9.

4.2 Physicochemical Evaluation

Physicochemical Evaluation viz. pH, Phenolic content, Total solids, alcohol content, specific gravity etc. were determined as per the procedure given in Ayurvedic Pharmacopoeia of India. ^[10]

4.2.1 Determination of Alcohol-soluble extractive

The ethanol-soluble extractive value is determined by taking 5 g of the powdered sample and soaking it in 100 mL of 90% ethanol in a closed flask for 24

hours. During this time, the mixture is shaken frequently for the first 6 hours and then left undisturbed for the next 18 hours. After that, it is filtered carefully to avoid loss of ethanol. From the filtrate, 25 mL is taken and evaporated to dryness using a water bath. The remaining residue is then dried in a hot air oven at 105°C until a constant weight is obtained. It is cooled and weighed, and finally, the percentage of ethanol-soluble extractive value is calculated based on the weight of the residue compared to the original sample.

$$\text{Percentage of ethanol-soluble extractive} = \frac{(\text{Weight of residue})}{(\text{Weight of the drug})} \times 100$$

It is expressed as percent w/w to the air-dried drug

4.2.2. Determination of water soluble extractive

The water-soluble extractive value is determined by taking 5 g of the powdered sample and soaking it in 100 mL of water- chloroform in a closed flask for 24 hours. During this time, the mixture is shaken frequently for the first 6 hours and then left undisturbed for the next

18 hours. After that, it is filtered carefully to avoid loss of chloroform. From the filtrate, 25 mL is taken and evaporated to dryness using a water bath. The remaining residue is then dried in a hot air oven at 105°C until a constant weight is obtained. It is cooled and weighed, and finally, the percentage of water-soluble extractive value is calculated based on the weight of the residue compared to the original sample.

$$\text{Percentage of water-soluble extractive} = \frac{(\text{Weight of residue})}{(\text{Weight of the drug})} \times 100$$

4.2.3 Determination of Total Ash

Accurately weigh about 2–3 g of the powdered drug and place it in a previously weighed platinum or silica crucible. Incinerate it at a temperature not exceeding 450°C until it becomes completely free from carbon. Allow it to cool and then weigh it. If it is not possible to obtain carbon-free ash by this method, treat the charred residue with hot water, filter it through an ashless filter paper, and collect the insoluble matter. Incinerate the residue along with the filter paper. Combine the filtrate, evaporate it to dryness, and ignite again at a temperature not exceeding 450°C. Finally, calculate the percentage of ash with reference to the air-dried sample of the drug.

4.2.4 Determination of Acid-Insoluble Ash

Add 25 ml of dilute hydrochloric acid to the crucible containing the total ash. Filter the mixture through an ashless filter paper (Whatman No. 41) to collect the insoluble portion, and wash it with hot water until the filtrate becomes neutral. Transfer the filter paper along with the insoluble residue back into the original crucible. Dry it on a hot plate and then ignite it until a constant weight is obtained. Allow the crucible to cool in a desiccator for about 30 minutes and weigh it immediately. Calculate the percentage of acid-insoluble ash with respect to the air-dried drug sample.

4.2.5 Determination of Water-Soluble Ash

Boil the obtained ash with 25 ml of water for about 5 minutes. Filter the mixture to separate the insoluble

residue using a Gooch crucible or ashless filter paper. Wash the remaining residue with hot water, and then ignite it for 15 minutes at a temperature not exceeding 450°C. The weight of this insoluble residue is subtracted from the total ash weight. The resulting difference corresponds to the water-soluble ash. Finally, express the water-soluble ash as a percentage based on the air-dried sample.

4.2.6 Determination of Moisture Content (Loss on Drying)

The procedure is used to determine the moisture content (loss on drying) by measuring the amount of water or volatile matter present in the drug. About 10 g of the sample is accurately weighed (without prior drying) and placed in a clean, pre-weighed evaporating dish. If the drug is not powdered, it is cut into small pieces of about 3 mm thickness. Seeds and fruits smaller than 3 mm should be crushed carefully, avoiding high-speed grinding to prevent moisture loss. The sample should represent the original material properly.

The dish containing the sample is then dried at 105°C for 5 hours and weighed. Drying is continued with weighing at one-hour intervals until the difference between two successive weights is not more than 0.25%. A constant weight is achieved when two consecutive weighings, taken after drying and cooling in a desiccator for 30 minutes, do not differ by more than 0.01 g.

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4.2.7 Determination of pH

The pH value of a semisolid preparation can be determined potentiometrically it means of the glass electrode, a reference electrode and a pH meter either of the digital or analogue type. For Cali pH meter calibration, 3 types of buffer capsules were taken initially of pH 3, 7 and 9.2 and pH meter was calibrated. pH of both formulations after opening of bottles was recorded Same procedure was repeated in triplicate. [22]

4.3 Microbial limit test

4.3.1 Preliminary testing:

The procedures are invalid unless it can be proved that the test specimens to which they are applied do not hinder microorganism proliferation under the conditions that can be present.

4.3.2 Preparation of sample for preliminary testing:

1 gm of Bilvadileha of both test sample and standard sample i.e. in laboratory prepared Jirakadyarishta were taken to prepare fifty ml of fluid lactose medium. However, this medium proved insufficient for the growth of *Escherichia coli* and *Pseudomonas aeruginosa*. Thus, the relevant medium was prepared by taking 1 ml Jirakadyarishta of to prepare 100 ml of fluid lactose medium. Prior to doing the test, diluted specimen of substance being examined were inoculated with separate viable cultures of *E. coli*, *S. aureus*. This is done by adding 1 ml of the microorganisms' 24hour cultures to the initial dilution

of the test sample (in buffer solution pH 7.2, fluid lactose medium).

Initially, *E. coli* organisms fail to grow in the relevant medium. The procedure was modified by increasing the volume of diluents with the quantity of test material remaining the same, i.e. 1 ml of Jirakadyarishta of both standard and test to prepare hundred ml of fluid lactose medium. After this modification, growth of all the strains was observed.

4.3.3 Tests for specified microorganisms:

A. Test for *E. coli*:

1 gm of Bilvadileha was added to the MacConkey broth and incubated at 43-45°C for 18-24 hours. A subculture was created using the same medium and incubated for the same amount of time at the same temperature. The presence of *E. coli* is indicated by the growth of red, often non-mucoid colonies of gram-negative rods, occasionally surrounded by a crimson zone of precipitation. Therefore, the material will pass the test if no such colonies are grown.

B. Test for *S. aureus*:

The pre-treated material Bilvadileha was used and inoculated. It had to be incubated in Vogel- Johnson agar at 35-37°C for 24-48 hours. If there is no growth of the microorganism then the material passes the test. The presence of *Staphylococcus aureus* will be detected with the presence of blank colonies of gram-positive cocci often surrounded by a clear zone. [22]

4.4 Thin layer chromatography (TLC)

A) TLC Details –

Table no: 1.3: TLC details

Adsorbent	Silica Gel (Activated)
Plate size	10 – 20 cms
Thickness	0.4 mm
Activation temperature	110 c
Detecting system	UV cabinet

B) Selection of mobile phase

Selection of mobile phase was done by using preparative thin layer plates made by silica gel G on glass plate. As per literature survey different mobile phases were tried for proper identification of RF values different samples. From trial and error basis combination of mobile phase was selected.

Table no 1.4: Different mobile phases used for determination of Marker compound

Sr. no.	Mobile phase	Proportion
1	Ethyl acetate: acetone: water	8:1:1
2	n-Hexane: ethyl acetate	8:2
3	Toluene: ethyl acetate	8:2
4	Chloroform: ethyl acetate	8:2
5	Toluene:ethyl acetate: acetic acid	6:3:9

(C) Sample preparation

- 5 g of Avaleha was extracted with 75 ml of n-hexane under reflux on a water bath for 30 minutes.
- The extract was filtered and concentrated to 10 ml.
- Thin layer chromatography (TLC) was carried out using the concentrated extract.
- About 10 µl of the extract was applied on the TLC plate.

- After development, the plate was dried in air and examined under ultraviolet light at 366 nm.

4.5 Adulteration Study

Extraction of crud plant material -

Plant material of *aeglemarmalose* were cleaned and pulverized to make powder. Powder material was sieved through 36 mesh sieves. Weighed powder material taken in volumetric flask and 30ml ethanol was added it in. ultrasonicated for 25 min. and filtered. Filtrate was concentrated by heating. This

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extract was used for further study.

Extraction of Marketed herbal Formulation samples

Extraction of samples done by using ethanol as a solvent by maceration method.

- 1) Phytochemical Screening
- 2) TLC

Microscopic Study

Procedure

- Small quantity of Bilvādileha was taken and washed with distilled water to remove the semisolid base.
- The separated drug particles were mounted in glycerin water on a clean glass slide.
- Different mounts were prepared using suitable reagents such as phloroglucinol and hydrochloric acid for identification of lignified tissues.
- The prepared slides were observed under microscope under low and high power.
- Diagnostic characters of individual ingredients were identified and compared with standard microscopic features.

5.0 RESULT AND DISCUSSION

Bilvādileha was prepared according to the formulation and preparation procedure described in the Ayurvedic Pharmacopoeia of India (API). The

laboratory-prepared Bilvādileha was designated as the laboratory batch (LB). Two commercially available marketed Bilvādileha formulations were procured from different manufacturers and designated as marketed bilvādileha MB1 and marketed bilvādileha MB2, respectively. The laboratory-prepared and marketed formulations were subjected to comparative standardization based on organoleptic characteristics, physicochemical parameters, and chromatographic profiling. The obtained results were compared to evaluate the quality and consistency of the laboratory-prepared formulation with the marketed formulations. Figure 1- 4 illustrates the various stages involved in the preparation of laboratory-prepared Bilvādileha.

During preparation, the kvātha changed from light brown to dark brown on heating. After reduction to one-fourth, the filtrate became thick and viscous after addition of jaggery. Continuous heating and stirring produced semisolid leha consistency. Leha siddhi was confirmed by formation of a soft bolus that did not disperse in water. Fine powders of PrakṣepaDravya were mixed uniformly after cooling. Final Bilvādileha showed dark brown colour, semisolid consistency, characteristic odour, and sweet taste with slight bitterness. The formulation was packed in airtight containers and stored in a cool dry place.

Table no 1.5: Details of preparation of kwath

Sr.No	Parameters	Result
1	Quantity of Bilva coarse powder taken	1.500 kg
2	Quantity of water added	12 L
4	Temperature maintained during heating	90–95°C
5	Kvātha obtained after reduction	3 L
6	Weight of residue after filtration	1 kg
7	Total time taken for kvātha preparation	5 hr

Table no 1.6: Details of preparation of Bilvādileha

Sr.No	Parameters/Stage	Result
4	Initiation of leha consistency	After 2 hr heating
5	Leha siddhi obtained	Soft bolus stage
6	Quantity of Bilvādileha obtained	100 gm
7	Storage condition	Storage condition



Fig. 1: Weighing of herbal ingredients



Fig.2: Preparation of Kwath



Fig. 4: Leha Siddhi



Fig. 3: Laboratory Prepared Bilvadileha

4.3 Adulteration Study

1) Phytochemical Screening

All the two samples and plant extract were subjected to qualitative phytochemical investigation.

Table no. 1.7: shows result of phytochemical testing.

Phytoconstituents	Plant Extract	Marketed 1	Marketed 2
Alkaloid	+	+	+
Glycoside (anthraquinone & coumarin)	+	+	+
Cardiac & saponin	+	+	+
Flavonoid	+	+	+
Tannins and phenolic content	+	+	+
Proteins and amino acid	–	–	–
Steroids	+	+	+
Volatile oil	+	+	+

2) Chromatographic evaluation

A) TLC

TLC fingerprint profiles of standard plant extract, and two marketed formulations were compared. Table no.1.8 represents results of TLC fingerprint profiles which show comparison in R_f value with prepared plant extract and two marketed formulations

Adsorbent	Silica Gel (Activated)
Plate size	10 – 20 cms
Thickness	0.4 mm
Activation temperature	110 c
Detecting system	UV cabinet

Table no 1.8: R_F value of Different Extracts

Sr.no.	Sample	R _F value from TLC fingerprinting
1	Plant Extract	0.56
3	Marketed formulation1	0.56
4	Marketed formulation2	0.57

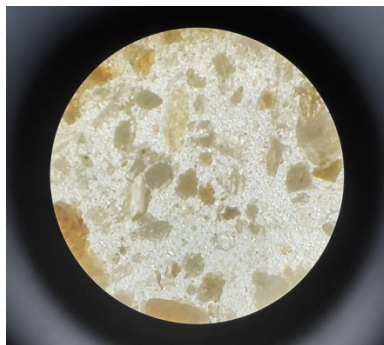
3) Microscopic Evaluation

Microscopic Characteristics Observed

- Multicellular and multiseriate trichomes observed (*Śvetajīraka*)
- Vittae fragments with elongated epithelial cells present
- Mesocarpic stone cells longer than broad observed

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- Parenchymatous cells containing rosette crystals of calcium oxalate seen (*Sūkṣmailā*)
- Bulbous perisperm cells packed with starch grains observed
- Epidermal and hypodermal cells crossing at right angles present
- Fibres with narrow lumen and oil cells identified (*Tvak*)
- Pollen grains and anther lobe fragments observed (*Nāgakeśara*)
- Oval to rod-shaped starch grains with eccentric hilum present (*Śuṇṭhī*)
- Scalariform vessels and fibre-sclereids observed (*Mustā*)
- Lignified stone cells present in groups (*Pippalī*)
- Hypodermal stone cells and parenchymatous cells observed (*Marica*)
- Sclerenchymatous cells with calcium oxalate crystals present (*Dhānya*)



4.4 Comparative Evaluation of Prepared Bilvadileha with Marketed Formulations

4.4.1 Organoleptic Evaluation

The organoleptic characteristics of the laboratory-prepared Bilvadileha (LB) and marketed formulations (MB1 and MB2) were evaluated comparatively. All three formulations exhibited a semi-solid consistency and aromatic odour. LB showed a dark-brown colour, whereas MB1 and MB2 were brown in colour. The taste of all formulations was predominantly sweet with slight astringency. The overall organoleptic profile of the laboratory-prepared formulation was comparable with that of the marketed formulations, indicating consistency in the characteristic sensory attributes of Bilvadileha.

4.4.2 Physicochemical Evaluation

The physicochemical characteristics of LB, MB1, and MB2 were comparatively evaluated to assess the quality and consistency of the prepared Bilvadileha. Preliminary phytochemical screening demonstrated the presence of alkaloids, glycosides, cardiac glycosides and saponins, flavonoids, tannins and phenolic compounds, proteins and amino acids, steroids, and volatile oils in all three formulations. The comparable phytochemical profile among the batches indicated the presence of major classes of phytoconstituents in both the laboratory-prepared and marketed formulations.

The loss on drying (LOD) was found to be 9.0% in LB, compared with 10.8% and 11.5% in MB1 and MB2, respectively. Thus, LB exhibited comparatively lower moisture loss than the marketed formulations. The total ash values were 1.9% for LB, 2.1% for MB1, and 2.2% for MB2. Similarly, the acid-insoluble ash values were 0.18%, 0.20%, and 0.21% for LB, MB1, and MB2,

respectively. The comparatively lower ash and acid-insoluble ash values observed in LB suggest a lower contribution of inorganic and acid-insoluble matter in the laboratory-prepared formulation.

The pH values of LB, MB1, and MB2 were approximately 7.51, 6.13, and 6.68, respectively. The variation in pH among the formulations may be attributed to differences in raw materials, processing conditions, and manufacturing practices. The water-soluble extractive value was highest in LB (70.0%), followed by MB2 (68.0%) and MB1 (67.0%). The higher extractive value of the laboratory-prepared formulation indicates a comparatively greater proportion of water-soluble constituents.

Overall, the laboratory-prepared Bilvadileha exhibited physicochemical characteristics comparable to the two marketed formulations, with relatively lower LOD, total ash, and acid-insoluble ash values and a higher water-soluble extractive value. These findings indicate satisfactory quality and consistency of the laboratory-prepared formulation and provide a physicochemical basis for its further comparative chromatographic standardization.

7) Microbial limit test

Preliminary testing:

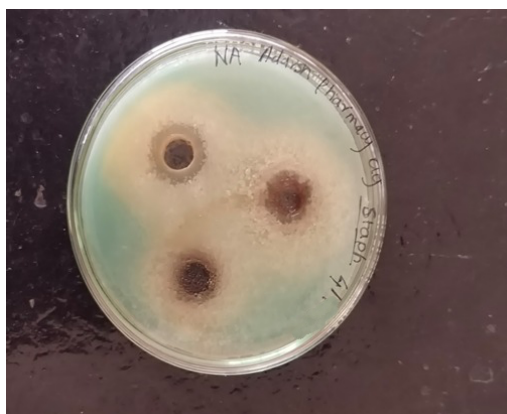
The procedures are invalid unless it can be proved that the test specimens to which they are applied do not hinder microorganism proliferation under the conditions that can be present.

Tests for specified microorganisms:

- A. Test for *E. coli*
- B. Test for *S. aureus*

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Sample	Zone of inhibition of streptococcus aureus	Zone of inhibition of E.coli
LB	18 mm	13 mm
MB 1	-	-
MB 2	12 mm	-



Zone of inhibition of E.coli



Fig. 5: Zone of inhibition of streptococcus aureus

Fig. 6:

4.3 Chromatographic evaluation

The TLC fingerprint profiles of the standard marker compound 7-hydroxy-4-methylcoumarin (Umbelliferon) and the laboratory-prepared Bilvadileha (LB) and two marketed formulations (MB1 and MB2) were comparatively evaluated. The standard marker compound was identified and confirmed by thin-layer chromatography. The

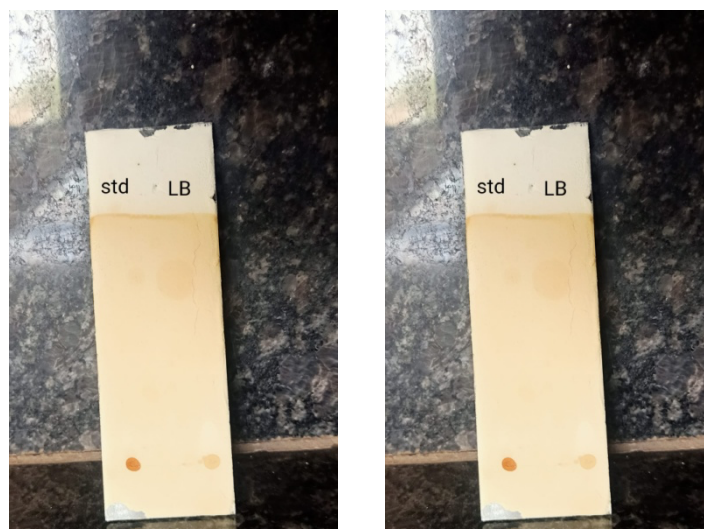
characteristic spot corresponding to 7-hydroxy-4-methylcoumarin was observed in the TLC profiles of LB, MB1, and MB2, with *R_f* values comparable to that of the standard marker. These findings confirmed the presence of the selected marker compound in the laboratory-prepared as well as marketed Bilvadileha formulations and demonstrated comparable TLC fingerprint profiles among the tested formulations.

Adsorbent	Silica Gel (Activated)
Plate size	10 – 20 cms
Thickness	0.4 mm
Activation temperature	110 c
Detecting system	UV cabinet

Table no 1.15: RF value of standard Marker compound and formulations

Sample	RF value from TLC fingerprinting
Umbelliferon	0.28, 0.51, 0.73
LB	0.31, 0.49, 0.76
MB 1	0.30, 0.49, 0.71
MB 2	0.27, 0.53, 0.75

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(A) TLC of standard Umbelliferon and laboratory prepared Bilvadileha (LB)
(B) marketed formulations MB 1, MB 2

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