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COMPREHENSIVE ANALYTICAL PROFILING AND STANDARDIZATION OF *BILWADI AGAD* USING PHYSICOCHEMICAL, MICROBIAL, AFLATOXIN, AND HPTLC METHODS

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ABSTRACT

Bilwadi Agad is a classical polyherbal formulation described in classical *samhitas* for the management of various toxic and systemic conditions. Considering its wide therapeutic utility, scientific standardization is essential to ensure identity, purity, safety, and quality consistency. The present study aimed to develop a systematic analytical profile of *Bilwadi Agad* using modern evaluation techniques. **Materials and Methods:** Organoleptic characteristics were assessed through sensory parameters such as appearance, colour, taste, and odour. Physicochemical evaluation included determination of moisture content, ash values, extractive values, and pH following standard procedures. Powder microscopy was performed to observe characteristic diagnostic features. Safety evaluation comprised aflatoxin estimation and assessment of total microbial load. Chromatographic characterization was carried out using High-Performance Thin Layer Chromatography (HPTLC) to establish a distinctive fingerprint pattern. **Results**: Organoleptic evaluation confirmed acceptable sensory

characteristics. Microscopic examination revealed diagnostic features indicating authenticity of ingredients. Physicochemical parameters were found within permissible limits. Microbial load and aflatoxin levels complied with safety standards. HPTLC profiling exhibited distinct peaks and Rf values, indicating the presence of multiple phytoconstituents and a characteristic fingerprint profile. **Conclusion:** The analytical findings affirm the quality, safety, and consistency of *Bilwadi Agad*. The generated data may serve as reference standards for routine quality control and further standardization of the formulation.

KEYWORDS: *Bilwadi Agad*, Analytical Profiling, Standardization, Physicochemical Analysis, HPTLC, Aflatoxin, Microbial Safety.

INTRODUCTION

Bilwadi Agad is a classical polyherbal formulation described in classical texts. It is mentioned in *Ashtanga Hridayam* (*Uttarsthana* 36/84–85) and *Ashtanga Sangraha* (*Uttarsthana* 42/87–88). The formulation is indicated in the management of various poisonous conditions such as snake bite, spider poisoning, rat bite, scorpion sting, and artificial toxicity. It is also used in certain systemic disorders including indigestion and fever.[1,2] *Bilwadi Agad* is prepared using multiple herbal ingredients processed according to classical pharmaceutical procedures and is administered through different routes based on clinical requirements.

Classical polyherbal formulations have been used in traditional practice for centuries and continue to remain an important part of healthcare. However, their scientific validation and quality assurance are necessary to ensure safety, consistency, and wider acceptance. Establishing standard analytical parameters helps in confirming the identity, purity, and reproducibility of such formulations.[3,4]

Although the formulation has been used therapeutically for a long time, systematic analytical documentation is still required to establish reliable quality control standards. Therefore, the present study was undertaken to develop a comprehensive analytical profile of *Bilwadi Agad* using physicochemical, microbial, aflatoxin, and HPTLC evaluation methods.

MATERIALS AND METHODS

Test formulation- *Bilwadi Agad*

Bilwadi Agad was selected as the test formulation for the present study. The formulation comprises *Bilwa* (***Aegle marmelos***), *Surasa* (***Ocimum sanctum)***, *Karanja* (***Pongamia***

pinnata), *Nata* ***(Valeriana wallichi)***, *Suravaha* ***(Cedrus deodara)***, *Haridra* ***(Curcuma longum)***, *Daruharidra* ***(Berberis aristata)***, *Haritaki* (***Terminalia chebula***), *Vibhitaki* (***Terminalia bellirica***), *Amalaki* (***Emblica officinalis***), *Shunthi* (***Zingiber officinale***), *Maricha* (***Piper nigrum***), and *Pippali* (***Piper longum***).*Basta Mutra*(**Goat urine**)was used as the *Bhavana Dravya* during preparation, and three *Bhavanas* were administered.

Bilwa moola

Surasa pushpa

Karanja phala

Nata

Devdaru

Amalaki

Haritaki

Vibhitaki

Shunthi

Maricha

Pippali



Haridra ***Daruharidra*** ***Bast mutra***

Collection of Raw Drugs

The therapeutic efficacy of medicinal substances is largely influenced by their natural source, habitat, and appropriate season of collection. Therefore, all raw materials required for the preparation of *Bilwadi Agad* were procured during their respective optimal collection periods. The *Moola* of *Bilwa (**Aegle marmelos**)* and *Pushpa* of *Surasa (**Ocimum sanctum**)* were collected in June 2025 (*Grishma Ritu*) from the botanical garden of Rishikul Campus, Haridwar. The *Kanda Sara* of *Suravaha (**Cedrus deodara**)* was collected from New Tehri, Uttarakhand during august 2025. The fruits of *Karanja (Pongamia pinnata)* were collected from Dehradun, Uttarakhand, during August 2025. The remaining dried raw drugs, namely *Nata* **(*Valeriana wallichii*)**, *Haritaki* **(*Terminalia chebula*)**, *Vibhitaki* **(*Terminalia bellirica*)**, *Amalaki* **(*Emblica officinalis*)**, *Shunthi* **(*Zingiber officinale*),** *Maricha* **(*Piper nigrum*)**, *Pippali* **(*Piper longum*)**, *Haridra* **(*Curcuma longa*)**, and *Daruharidra* **(*Berberis aristata*)**, were collected from the Pannalal brijlal pharmaceutical crude drugs, Haridwar in the month of July 2025.

Authentication of Raw Drugs

All raw materials used in the formulation were authenticated by subject experts from the Postgraduate Department of Dravyaguna, Rishikul Campus, Uttarakhand Ayurved University under reference number **DG/RC/UAU-278,** dated **18/08/2025**.

Preparation of *Bilwadi Agad Churna*

Following authentication, all raw materials were thoroughly cleaned, shade-dried, and pulverized separately to obtain fine powders. As classical references do not specify exact proportions, all ingredients were taken in equal quantities. The powders were passed through sieve no. 80 to ensure uniform particle size and homogeneity. Subsequently, three Bhavana

were administered using Basta Mutra in accordance with classical guidelines to enhance uniformity and therapeutic efficacy. The final prepared *churna* was stored in clean, airtight containers for further analysis.

Place of study

The organoleptic and physicochemical evaluation of *Bilwadi Agad Churna* was conducted at Alarsin Pharmaceuticals, Mumbai **(Ref. No. AL/OT/25/L/08)** in December 2025. HPTLC fingerprint profiling and microbial safety analysis were performed at Patanjali Research Foundation (Drug Discovery & Development Division), Haridwar, under **COA No. PRF/2026/016** and **PRF0121** during December 2025. Aflatoxin analysis was carried out at Sanskar Ayush Medicare Private Limited **(Ref. No. AYF20251218001)** in December 2025. The study was undertaken after approval from the Institutional Ethics Committee, Uttarakhand Ayurved University **(IEC No. UAU/RC/IEC/2025/PG/269)**, in August 2025.

Analytical profile of *Bilwadi Agad*

Organoleptic Evaluation

The collected samples were visually inspected using the unaided eye and a magnifying lens. Organoleptic evaluation was carried out to assess colour, odour, taste, texture, and other observable physical characteristics in accordance with standard pharmacognostical procedures to ensure the quality and authenticity of the raw materials.

Physicochemical Analysis

Physicochemical standardization of the formulation was performed by assessing parameters such as moisture content, aqueous and alcoholic extractive values, total ash, acid-insoluble ash, water-soluble ash, and pH value, following the procedures prescribed in the Ayurvedic Pharmacopoeia of India (API). The pH of the formulation was determined using pH indicator paper by matching the developed colour with a standard pH chart, and the value was recorded as 3, indicating its acidic nature.

Powder Microscopy

The powdered drug was mounted in glycerine on a glass slide and examined under a light microscope for preliminary observation. Separate mounts were prepared using iodine solution to detect the presence of starch grains. For detailed observation of cellular structures, another portion of the powder was treated with potassium hydroxide (KOH) and gently heated for a few minutes to clear the tissues. After cooling, the material was stained with safranine and

mounted on a slide. The prepared slides were then observed under the microscope to study the diagnostic microscopic characters of the drug.

Safety Evaluation Aflatoxin Analysis

The estimation of aflatoxins in *Bilwadi Agad* (powder) was carried out in accordance with the standards prescribed in the Ayurvedic Pharmacopoeia of India (API) using validated analytical methods. The analysis was performed at a certified laboratory under controlled conditions to ensure accuracy and reliability of the results. The sample was evaluated for the presence of aflatoxin B_1 and total aflatoxins (B_1, B_2, G_1, and G_2) employing appropriate analytical techniques as per API guidelines. The results were expressed in parts per billion (ppb) and compared with the permissible limits specified in the pharmacopoeia.

Total Microbial Count

The microbial quality of *Bilwadi Agad* (powder) was evaluated in accordance with the procedures specified in the Ayurvedic Pharmacopoeia of India (API), Part I, Volume VIII (2011). The analysis was performed under aseptic conditions employing the serial dilution technique followed by the standard pour plate method. For the estimation of Total Aerobic Microbial Count (TAMC), appropriately diluted samples were inoculated into sterile Petri plates and mixed with molten nutrient agar medium. The plates were incubated at 37°C for 24–48 hours to allow the growth of aerobic microorganisms. For the determination of Total Yeast and Mould Count (TYMC), the sample was similarly inoculated onto Sabouraud Dextrose Agar (SDA) plates and incubated at 25–28°C for 3–5 days. Following incubation, the developed colonies were enumerated, and the microbial load was calculated and expressed as colony forming units per gram (CFU/g) of the sample.

HPTLC Fingerprinting

High Performance Thin Layer Chromatography (HPTLC) fingerprinting of *Bilwadi Agad* powder was carried out to establish its characteristic phytochemical profile using standard chromatographic techniques. The sample was applied on pre-coated silica gel 60 F254 HPTLC plates in the form of bands of 8 mm width, with an application volume of 6 µL, using an appropriate applicator. The chromatographic development was performed in a solvent system consisting of chloroform: methanol (9:1 v/v). The development was carried out up to a migration distance of 70 mm under controlled experimental conditions. After development, the plates were air-dried and visualized under ultraviolet light at 254 nm and 366 nm.[5] The chromatograms showed distinct bands at different Rf values, indicating the presence of

multiple phytoconstituents in the formulation. The obtained HPTLC fingerprint profile serves as a reliable tool for identification, quality control, and standardization of *Bilwadi Agad***.**

RESULT

Organoleptic Evaluation

Table 1: Organoleptic characters of *Bilwadi Agad.*

S. No.	Parameter	Result	Inference
1.	Appearance	Dried fine powder	Passes the test
2.	Colour	Dark brown	
3.	Taste	Characteristic	
4.	Odour	Characteristic	

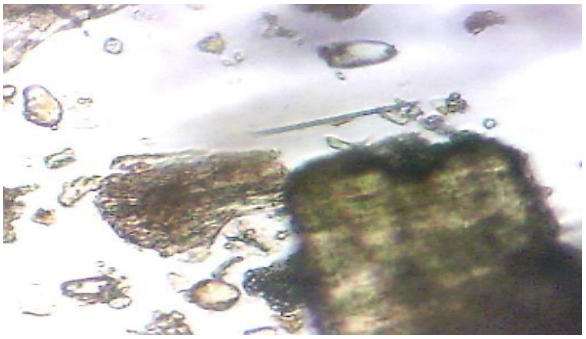
Physicochemical Analysis

Table 2: Physicochemical parameters of *Bilwadi Agad.*

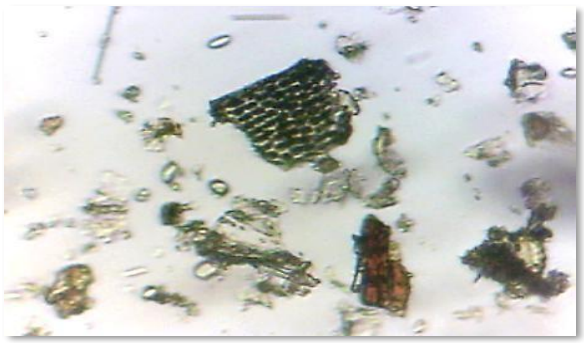
S. No.	Parameter	Result	Inference
1.	Moisture Content	0.8%	-
2.	Total Ash	18.68%	-
3.	Acid Insoluble Ash	4.05%	-
4.	Water Soluble Ash	9.12%	-
5.	Alcohol Soluble Extractive Value	19.25%	-
6.	Water Soluble Extractive Value	41.06%	-
7.	pH	3	Acidic

Powder Microscopy

Microscopic examination revealed diagnostic cellular structures including starch grains, sclereids, tracheids, and oleoresin, confirming authenticity of the formulation.



Starch Grain **Sclerides**



Tracheid **Oleoresin**

Aflatoxin Analysis

S.No.	Test Parameters	Test Method	Result	Reference value
1.	Aflatoxin B1	API/ IHS	ND	NMT- 2.0 ppb
2.	Aflatoxin B1+B2+G1+G2	API/ IHS	ND	NMT- 2.0 ppb

***API: Ayurvedic Pharmacopoeia of India, ND: Not detected, IHS: In House specification,**

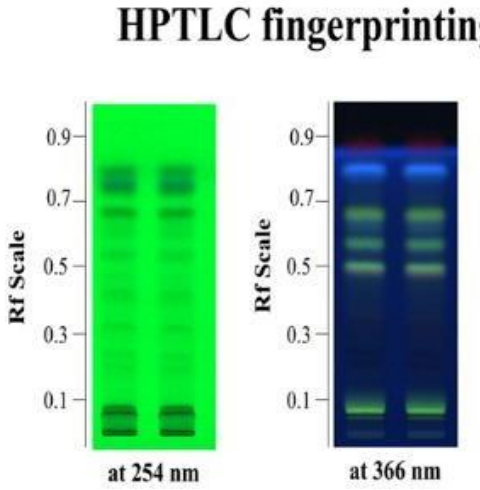
ppb: Parts per billions

Total Microbial Count

S.No.	Test Parameters	Test Method	Result	Unit
1.	Total Aerobic Microbial Count	API/ IHS	<10	CFU/g
2.	Total Yeast and Mould Count	API/ IHS	<10	CFU/g

***API: Ayurvedic Pharmacopoeia of India, CFU: Colony forming unit**

HPTLC Fingerprinting



254 nm			
Peak	**Start Position**	**End Position**	**Area %**
1	0.18 Rf	0.25 Rf	8.59%
2	0.25 Rf	0.30 Rf	6.32%
3	0.30 Rf	0.38 Rf	10.23%
4	0.41 Rf	0.49 Rf	16.38%
5	0.50 Rf	0.54 Rf	4.34%
6	0.54 Rf	0.61 Rf	17.30%
7	0.62 Rf	0.66 Rf	8.64%
8	0.68 Rf	0.74 Rf	28.19%

366 nm			
Peak	**Start Position**	**End Position**	**Area %**
1	0.42 Rf	0.50 Rf	19.44%
2	0.51 Rf	0.56 Rf	5.10%
3	0.56 Rf	0.63 Rf	22.60%
4	0.63 Rf	0.70 Rf	16.03%
5	0.70 Rf	0.75 Rf	36.82%

DISCUSSION

Standardization of herbo-mineral formulations is essential to ensure their quality, safety, and therapeutic efficacy. In the present study, *Bilwadi Agad* was evaluated using a comprehensive set of analytical parameters. The organoleptic characteristics, including dark brown colour,

characteristic odour, and taste, along with fine powdered appearance, were found to be consistent with classical descriptions, indicating proper formulation and absence of organoleptic abnormalities. Physicochemical evaluation revealed significant findings related to the formulation's stability and composition. The low moisture content **(0.8%)** suggests reduced susceptibility to microbial contamination and prolonged shelf life. The total ash value **(18.68%)** reflects the total inorganic content, while acid insoluble ash **(4.05%)** indicates the presence of siliceous matter, possibly from plant sources or processing. Water soluble ash (9.12%) denotes the presence of soluble inorganic salts. The extractive values showed higher water-soluble extractive **(41.06%)** compared to alcohol soluble extractive **(19.25%),** suggesting the predominance of polar constituents in the formulation. The acidic pH **(3)** may contribute to stability and could influence drug solubility and absorption. Microscopic analysis plays a vital role in authentication of herbal ingredients. The presence of diagnostic features such as starch grains, sclereids, tracheids, and oleoresin confirms the identity of crude drugs used and indicates absence of adulteration. These structural elements serve as important pharmacognostic markers.

Safety evaluation through aflatoxin analysis revealed that aflatoxin B1 and total aflatoxins were not detected, confirming that the formulation is free from harmful mycotoxins and complies with permissible safety limits. Similarly, microbial analysis showed that total aerobic microbial count and total yeast and mould count were below detectable limits **(<10 CFU/g)**, indicating good manufacturing practices and hygienic handling.

HPTLC analysis of *Bilwadi Agad* revealed multiple peaks at different Rf values, indicating the presence of diverse phytoconstituents. At 254 nm and 366 nm, distinct peaks with significant area percentages were observed, suggesting the presence of major and minor components. The peak area distribution indicates a complex phytochemical composition, which can be used as a reference fingerprint for standardization.

Collectively, these analytical findings validate the identity, purity, and safety of *Bilwadi Agad* and support its standardization using modern scientific parameters.

CONCLUSION

The present study establishes a comprehensive standardization profile of *Bilwadi Agad* based on organoleptic, physicochemical, microscopic, microbial, aflatoxin, and chromatographic evaluations. The formulation was found to be within acceptable limits for all tested parameters,

confirming its quality, purity, and safety.

The obtained data can be utilized as a reference for quality control and standardization of this formulation in future studies. Further pharmacological and clinical investigations are recommended to substantiate its therapeutic efficacy.

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