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Phytochemical Characterization and Evaluation of Anti-Inflammatory and Antibacterial Activities of Bilvadileha: An Ayurvedic Formulation

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KEYWORDS

Bilvadileha, HPTLC, phytochemical screening, anti-inflammatory activity, antibacterial activity, heavy metal analysis.

ABSTRACT:

Introduction: The necessity for scientific validation and standardization of traditional herbal formulations is highlighted by the growing demand for Ayurvedic pharmaceutical products worldwide. Irritable bowel syndrome (IBS) and other digestive conditions are frequently treated with *Bilvadileha*, a traditional semi-solid polyherbal Ayurvedic preparation. However, batch-to-batch consistency and quality variations may arise from conventional manufacturing methods, highlighting the significance of verified analytical and quality control parameters. Through thorough phytochemical analysis, physicochemical standardization and biological activity evaluation, the current study sought to analyse *Bilvadileha.*

Objectives: The present study aimed to evaluate the phytochemical characteristics, physicochemical parameters, and biological activities of *Bilvadileha* in order to establish preliminary quality control measures and scientifically validate its traditional therapeutic application.

Methods: The formulation was analysed using organoleptic evaluation, physicochemical parameters, qualitative phytochemical screening, high-performance thin layer chromatography (HPTLC) fingerprint profiling, heavy metal analysis and in vitro biological assays including anti-inflammatory and antibacterial activities.

Results: Organoleptic analysis revealed a brownish semi-solid formulation with a characteristic aromatic odour and a sweetish-bitter taste due to the presence of jaggery. Physicochemical parameters such as moisture content, ash values, extractive values and pH were found to be within acceptable limits and consistent with standards described in the Ayurvedic Pharmacopoeia of India (API). Phytochemical screening confirmed the presence of important bioactive constituents including alkaloids, flavonoids, glycosides, saponins, steroids, tannins and triterpenoids. HPTLC fingerprint profiling demonstrated multiple characteristic peaks indicating the presence of diverse phytoconstituents. The formulation exhibited notable anti-inflammatory activity through inhibition of protein denaturation and moderate antibacterial activity against selected bacterial strains.

Conclusions: The findings of the present investigation provide scientific support for the traditional use of Bilvadileha and establish preliminary quality control parameters for its standardization.

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1. Introduction

The Indian medical system known as Ayurveda is still among the oldest living traditions and has a strong philosophical and experimental foundation. It is a method of living that emphasizes customized treatment and a comprehensive approach to health. It is well known to be an all-encompassing medical system that included intellectual, emotional and physical wellness.[1] It is crucial to standardize herbal formulations to evaluate medicine quality. It is based on metrics from in vitro, in vivo, physical, chemical and phytochemical processes as well as the concentration of their active principles. In order to comply with regulatory standards and safety, herbal product must first undergo a quality assessment. The absence of distinctive pharmacological and analytical validation for herbal medications and their formulations is one of the key issues that Ayurvedic doctors deal with. Hence, the government of India's AYUSH department is now developing standard operating procedures for the manufacturing process of ayurvedic preparations. This can be accomplished if the herbal products are assessed and analyzed during and after production utilizing both Ayurvedic and contemporary approaches of standardization. [2]

Gastrointestinal diseases affect the gastrointestinal (GI) tract. IBS-irritable bowel syndrome is a umbrella term for a few illnesses that affect the GI tract and are a major cause of morbidity in the general population. Colitis, mucous colitis, spastic colon, and spastic bowel are just a few other names for IBS. The IBS is characterized by persistent stomach pain, discomfort, abdominal bloating and altered bowel patterns, and most commonly found in women over the age of 35 years.[3-4] IBS is a persistent, crippling functional GI condition that has a prevalence of 9-23% of population globally.[5] IBS is a new condition in the realm of clinical medicine because it is understood fairly in past century. Since Ayurveda is developed entirely on fundamental concepts of Morphometry and Physiology, no specific Ayurvedic ailment can be exactly correlated with IBS. But IBS and various other illnesses share a few clinical traits. *Pakvashayagata Vat, Grahani, Pravahika* and *Atisara* are some of them. [6] Modern medicine has identified several formulations for IBS; while these medicines may sometimes offer rapid relief, they also frequently cause unwanted side effects and have no discernible long-term treatment. This makes patients seek novel treatments that cause less inconvenience. The clinical efficacy of *Bilvadileha,* an intriguing herbal medicinal formulation that has been regularly recommended by Ayurvedic doctors without any adverse effects for millennia, is being examined for the condition of IBS.[3] The formulation contains 12 medicinal herbs which includes *"Bilva"* (aegle marmelos) main ingredient, *Musta (nutgrass*), *Jirak*a (cumin), Long *Pippali (pepper)*, *Sunthi (ginger)* , *Ela* (cardamom) , *Tvak* (cinnamon), *Marica* (black pepper), *Nagakesara* (*mesuaferrea*), Ghee (clarified butter), Guda (Jaggery) and Honey.[2] Pharmacopeia has been developed by various regulatory bodies responsible for overseeing and maintaining the Quality of Prescribed medicine, traditional or allopathic, being produced in the Market. [7-9]

2. Objectives

The study aims to validate the *Bilvadileha* protocol through comprehensive scientific evaluation, encompassing organoleptic qualities, physical parameters and chemical analysis. In addition, it seeks to characterize the phytochemical groups using HPTLC and assess the quality and effectiveness of *Bilvadileha* through ICP-OES, antimicrobial, and anti-inflammatory assays, while benchmarking the findings against established regulatory standards.

3. Method

Materials: One bottle of *Dabur Bilvadi Lehya* was procured from a reputed Ayurvedic pharmacy, Medical Practitioners' Co-operative Pharmacy and Stores Ltd., Mumbai, India, for the present study.

Organoleptic Evaluation: Characteristics like flavour, aroma, colour and consistency were noted. Utilizing 1g of *Bilvadileha* dispersed in a 2ml of distilled water. Few drops of the resulting mixture were dried out on a clean and dry slide and finally, it was examined under a binocular microscope equipped with a camera. [10]

Pharmaceutical Study: According to the procedure outlined in The Ayurvedic Pharmacopoeia of India, several physicochemical characteristics, including moisture content, ash values, extractive values, loss on drying, foreign matter, PH were recorded [11].

Extraction: 10g sample was mixed in 45ml distilled water and 5ml 0.5M HCL, and the solution was kept in

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water bath for 20 minutes. After cooling, 25ml ethyl acetate was added to separate the organic compounds. The ethyl acetate fraction was collected using a separating funnel. The collected layer of ethyl acetate was evaporated at 60ºC till it dried.

HPTLC Analysis: HPTLC analysis was performed using a CAMAG system comprising an Automatic TLC Sampler 4 (ATS 4), TLC Scanner IV, TLC Visualizer 2, CAMAG Derivatizer, and TLC Plate Heater 3 controlled by winCATS software. Chromatographic separations were carried out on silica gel 60 F254 aluminum-backed HPTLC plates (10 × 10 cm).

Approximately 300 mg of extract was dissolved in 3 mL methanol, sonicated briefly, and centrifuged at 15,000 × g for 10 min. The clear supernatant was used for HPTLC application.
Samples were applied as 8 mm bands using the ATS 4 automatic applicator fitted with a 100 µL Hamilton syringe. Four tracks were applied, with tracks 1 and 2 receiving 2 µL and tracks 3 and 4 receiving 4 µL of the sample solution.
Plates were developed in a twin-trough chamber pre-saturated with the mobile phase for 20 min to a migration distance of 70 mm. Plates were visualized using the CAMAG TLC Visualizer 2 under white light, UV 254 nm, and UV 366 nm before and after derivatization. Densitometric scanning was performed in absorbance mode at 254 nm and 366 nm, and at 540 nm after derivatization, using a scanning speed of 20 mm/s and data resolution of 100 µm/step.

HPTLC profiling was carried out to screen for major phytochemical classes including alkaloids, flavonoids, tannins, triterpenoids, saponins, and glycosides using class-specific solvent systems and derivatization reagents.

Heavy metals: The *Bilvadileha* material was digested using a microwave-assisted digestion system (MARS 6, CEM Corporation, USA) operated at 650 psi and 120 °C. Following digestion, the samples were filtered, and the final volume was adjusted to 25 mL with distilled water. The digested solutions were then subjected to Inductively Coupled Plasma–Optical Emission Spectroscopy (ICP-OES) for heavy metal analysis.

Antibacterial activity analysis using agar well diffusion technique: The agar well-diffusion technique described by Singh et al. [12] was employed to evaluate the in vitro antibacterial activity of aqueous *Bilvadileha* extract and its components against six bacterial species. Sterile nutrient agar media was prepared under aseptic conditions for *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi*, and *Proteus vulgaris*. Additionally, two blood agar plates were prepared for *Enterococcus faecalis*. To ensure uniform bacterial growth, secondary cultures were spread evenly on nutrient agar and blood agar plates using sterile spreaders, followed by incubation at 37 °C for 24 hours. Wells of 9 mm diameter were then created using a sterile cork borer.

Different concentrations of aqueous *Bilvadileha* extract (200, 400, 600, 800, and 1000 mg/mL) were introduced into the wells (100 µL each). Control wells were loaded with standard antibiotics, Tetracycline and Streptomycin (50 mg/mL). All plates were incubated at 37 °C for 24 hours, after which the zones of inhibition were measured to assess antibacterial activity.

Anti-inflammatory activity analysis:

Albumin denaturation method: The anti-inflammatory potential of *Bilvadileha* was evaluated using the albumin denaturation method, adapted from Mizushima et al. [13,14] with slight modifications. A series of concentrations of *Bilvadileha* formulations were prepared and mixed with an aqueous solution of bovine serum albumin (BSA, 1%). The mixtures were incubated at 37 °C for 20 minutes, followed by heating at 57 °C for 20 minutes. Tris buffer was used as the blank, while 1% BSA was used as the control. Aspirin (100 mg/mL) was included as the standard reference drug. After cooling, turbidity was measured spectrophotometrically at 660 nm.

The percentage inhibition of protein denaturation was calculated using the formula:

$$Percentage\ Inhibition = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} X100$$

Proteinase inhibitory action method: The assay was performed following the modified method of Parveen R. et al. [15,16]. The reaction mixture (2 mL) consisted of

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trypsin (0.06 mg), 20 mM Tris-HCl buffer (pH 7.4) and *Bilvadileha* extract at three different concentrations (1 mg/mL, 10 mg/mL, and 100 mg/mL). After incubation at 37 °C for 5 minutes, 1 mL of 0.8% (w/v) casein solution was added, and the mixture was further incubated for 20 minutes. The reaction was terminated by adding 2 mL of 70% perchloric acid. The mixture was centrifuged, and the absorbance of the resulting supernatant was measured at 210 nm, using buffer as the reference. Each trial was performed in triplicate. The percentage inhibition of proteinase activity was calculated using the formula:

$$Percentage\ Inhibition = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} X100$$

4. Results

Organoleptic Evaluation:

In the present study, *Bilvadileha* exhibited a brown colour, semi-solid paste-like consistency, sweetish taste, and a characteristic aromatic odour. The sweet taste is primarily attributed to the presence of jaggery used during preparation, while the aromatic odour arises from volatile phytoconstituents present in the herbal ingredients.

Physicochemical Evaluation:

Evaluation of Ayurvedic drug products using standardized analytical parameters helps in understanding their physicochemical characteristics and expands the criteria for quality assessment. The moisture content, indicating water holding capacity was found to be 43.39 ± 0.0164% (±0.04%). The total ash value, representing the inorganic content of the formulation, was 0.0145 ± 0.000926% (±6.38%), while the acid insoluble ash was 0.01567 ± 0.00274% (±17.50%). The water-soluble ash was almost negligible, recorded as 0.008 ± 0% (±0.00%). The water extractive value was 76 ± 1.645% (±2.16%), whereas the alcohol extractive value was 63.3 ± 5.483% (±8.66%). The pH of the formulation was 6, indicating a near-neutral nature. All parameters were expressed as % w/w ± SD at 90% confidence interval (n = 3).

HPTLC Fingerprinting:

HPTLC fingerprint analysis of the methanolic extract of *Bilvadileha* revealed multiple peaks corresponding to different phytochemical constituents. Three prominent bands were observed at Rf values 0.41, 0.54 and 0.63, indicating the presence of several phytoconstituents in the formulation as shown in figure 1. The Rf values obtained are comparable to those reported in previous studies on polyherbal Ayurvedic formulations [10]. The densitogram analysis further confirmed the presence of multiple compounds contributing to the complex polyherbal composition of *Bilvadileha*.

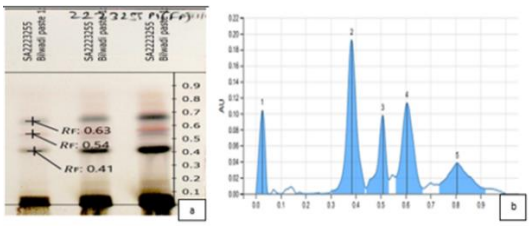

Fig 1: a) Fingerprint plate at white light b) Densitogram of methanolic extract at 540 nm, showing different peaks (bands) of *Bilvadileha*.

Table 1. Peak table with retention factor (RF) values, height (H) and area of methanolic extract of *Bilvadileha* (at 540 nm)

peak	Start		Max			End		Area	
	Rf	H	Rf	H	%	Rf	H	A	%
1.	0.011	0.0131	0.044	0.0418	5.87	0.066	0.0000	0.00213	2.57
2.	0.111	0.0000	0.139	0.0280	2.29	0.181	0.0000	0.00108	1.30
3.	0.269	0.0025	0.395	0.4533	37.07	0.461	0.0537	0.02985	36.02
4.	0.463	0.0663	0.542	0.2254	18.43	0.579	0.1296	0.01711	20.64
5.	0.579	0.1296	0.640	0.3054	24.97	0.710	0.0599	0.02398	28.93
6.	0.710	0.0599	0.735	0.0709	5.80	0.768	0.0555	0.00371	4.47
7.	0.769	0.0554	0.802	0.0682	5.58	0.910	0.0002	0.00503	6.07

Qualitative Phytochemical investigation:

Preliminary phytochemical screening of the methanolic extract of *Bilvadileha* revealed the presence of alkaloids, flavonoids, tannins, triterpenoids, saponins, glycosides and steroids as shown in figure 2. The presence of these bioactive compounds supports the therapeutic potential of *Bilvadileha* in digestive disorders. Steroids

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chromatogram developed (with N-butanol: methanol: water (3:1:1)) derivatized (with ASR- Anisaldehyde Sulphuric Acid Reagent) showing purple bands at visible light at Rf 0.81, no bands observed at UV 366nm. For triterpenoids the sample was developed using N-hexane: Ethyl acetate (1:1) solvent derivatized with ASR displaying fluorescent bands at Rf 0.13,0.19,0.37 under UV 366nm. The saponins and glycoside both were detected at white light range showing blue bands at Rf 0.94 and Rf 0.67,0.76 respectively when derivatized using ASR and solvent mixture Chloroform: acetic-acid: methanol: water (6.4:3.2:1.2:0.8) for saponins and Ethyl-acetate: Methanol: Water (100:13.5:10V/V/V) for glycoside. The plate for alkaloids developed using Toluene: Ethyl acetate: Methanol: Ammonia 25% (30:30:15:1) and dragandorffs dipping reagent showed significant yellow band at Rf 0.77 in the methanolic extracts of *Bilvadileha* all the solvent mixture were prepared for 10ml solution volume. The plates were scanned at 540 nm for densitograms to validate the presence of flavonoids, steroid, triterpenoids, saponin, glycoside and alkaloids from chromatograms after derivatization.

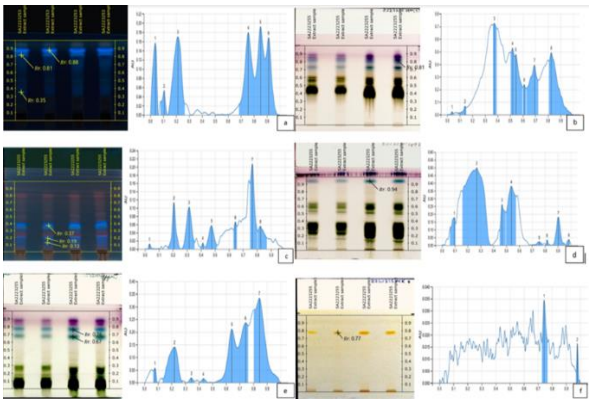

Fig2: HPTLC developed chromatograms of phytochemicals after derivatization and methanolic extract at 540 nm, showing different peaks (bands) of phytoconstituents in *Bilvadileha*. Concentration of sample 100 mg/ml. a) Flavonoids b) Steroids c) Triterpenoids d) Saponin e) Glycosides f) Alkaloids

Heavy Metal Analysis:

Heavy metal analysis was carried out using ICP-OES, a highly sensitive technique recommended for trace element analysis in herbal products. The concentration of lead was found to be 1.45 ppm, while cadmium was below the limit of quantification (BLQ). Mercury and arsenic levels were recorded as 2.95 ppm and 2.83 ppm, respectively. While lead and cadmium levels were within acceptable limits as per WHO and AYUSH guidelines (permissible limits: lead 10 ppm, cadmium 0.3 ppm), mercury was found to be slightly higher than recommended values (permissible limit: 1 ppm).

Antibacterial activity analysis using agar well diffusion technique:

The results (Figure 3) demonstrated that *Bilvadileha* exhibited moderate antibacterial activity, particularly against *Enterococcus faecalis, Pseudomonas aeruginosa, Salmonella typhi* and *Proteus vulgaris*. However, relatively lower inhibition was observed against *Escherichia coli* and *Staphylococcus aureus*. The differential sensitivity observed may be attributed to variations in cell wall structure and permeability between gram-positive and gram-negative bacteria.

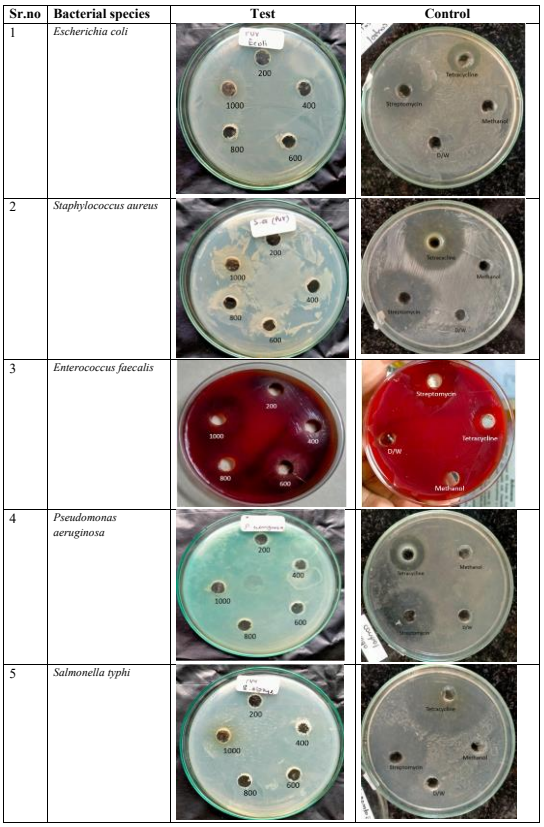

Fig: 3 Antibacterial activities of *Bilvadileha* aqueous extract against gram-positive and gram-negative bacterial species.

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Anti-inflammatory activity analysis: The formulation demonstrated significant inhibition of protein denaturation in a dose-dependent manner, with the highest inhibition of 92.90 % at 1 mg/ml concentration. The observed inhibition is comparable to that of standard anti-inflammatory drugs. Similarly, the proteinase inhibitory activity assay revealed a maximum inhibition of 54.30 % at 1 mg/ml concentration, further supporting the anti-inflammatory potential of the formulation. Proteases released during inflammatory responses can damage surrounding tissues, and inhibition of these enzymes is an important mechanism of anti-inflammatory action. The observed anti-inflammatory activity may be attributed to the presence of flavonoids, tannins, and triterpenoids, which are known to inhibit inflammatory mediators and oxidative stress pathways. [15,16]

Therapeutic Relevance to Gastrointestinal Disorders:

Bilvadileha is traditionally prescribed in Ayurveda for the management of digestive disorders, particularly conditions resembling irritable bowel syndrome (IBS).[3] IBS is a functional gastrointestinal disorder affecting 9–23 % of the global population, characterized by abdominal pain, altered bowel habits, and intestinal inflammation.[5] The combined anti-inflammatory and antibacterial properties observed in the present study may contribute to the therapeutic potential of *Bilvadileha* in managing gastrointestinal disturbances. The synergistic action of multiple herbal constituents may help regulate intestinal motility, reduce inflammation, and control microbial imbalance in the gut.[3,16] Thus, the findings of the present investigation provide scientific validation for the traditional use of *Bilvadileha* and establish preliminary quality control parameters for its standardization.

5. Discussion

This study aimed to scientifically evaluate and standardize the traditional Ayurvedic formulation *Bilvadileha*, commonly used in the management of gastrointestinal disorders. Organoleptic evaluation is considered an important preliminary step in the standardization of herbal formulations, as sensory characteristics often reflect formulation quality and authenticity.[19] The observed sweet taste is primarily attributed to the presence of jaggery used during preparation, while the aromatic odour may arise from volatile phytoconstituents present in the herbal ingredients. [24] These characteristics are consistent with the typical properties of Lehya preparations described in Ayurvedic pharmaceutics, where herbal decoctions are processed with sweetening agents to produce semisolid formulations with improved palatability and stability. [2,17] Organoleptic properties also serve as primary quality control indicators and can help detect adulteration or deterioration of herbal formulations. [19,25] Physicochemical parameters such as moisture content, ash values, extractive values, and pH provide essential information regarding the quality and composition of herbal formulations. Standardized analytical parameters are widely recommended for evaluating Ayurvedic drug products and establishing quality benchmarks (API guidelines). These parameters help determine the inorganic content, extraction efficiency, and overall physicochemical stability of the formulation.

HPTLC fingerprinting is widely used for qualitative evaluation and fingerprint profiling of herbal medicines and is recommended by WHO for herbal drug standardization. [20] The presence of multiple peaks in the chromatographic profile reflects the complex phytochemical composition of polyherbal formulations. Similar Rf patterns have been reported in earlier studies on Ayurvedic formulations. [10] Chromatographic fingerprinting provides a reliable tool for the identification, authentication, and quality control of herbal formulations by generating characteristic chemical profiles. [9,23] Preliminary phytochemical screening confirmed the presence of alkaloids, flavonoids, tannins, triterpenoids, saponins, glycosides, and steroids, which are known to exhibit various pharmacological activities including antioxidant, anti-inflammatory, antimicrobial, and gastroprotective effects. [15,18] Flavonoids are particularly known for their antioxidant and anti-inflammatory effects, while tannins possess astringent and antimicrobial properties that may help control intestinal infections. Alkaloids and triterpenoids have been reported to exhibit antimicrobial and anti-inflammatory activities, while saponins contribute to immunomodulatory effects. [18,21] The major ingredient of the formulation, *Bilva* (aegle marmelos), has been widely reported to contain

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flavonoids, tannins, and alkaloids that contribute to its antidiarrheal and antimicrobial properties. [3,16] In Ayurvedic medicine, Bilva is traditionally prescribed for digestive disorders such as Atisara and Grahani, as described in classical texts including the Charaka Samhita. [6]

The antibacterial activity observed in the present study may be attributed to the presence of polyphenolic compounds and flavonoids present in the constituent herbs. Previous studies have reported that herbal formulations rich in polyphenols exhibit antibacterial activity against both gram-positive and gram-negative bacteria. [12,18,22] The presence of tannins and alkaloids may contribute to antibacterial effects through mechanisms such as membrane disruption and enzyme inhibition. Inflammation plays a critical role in the pathogenesis of gastrointestinal disorders including irritable bowel syndrome (IBS). The inhibition of protein denaturation observed in this study indicates significant anti-inflammatory potential because protein denaturation is a known cause of inflammatory responses. [13,14] Similarly, inhibition of protease activity suggests the ability of the formulation to reduce tissue damage associated with inflammatory processes. The anti-inflammatory activity observed may be attributed to flavonoids, tannins, and triterpenoids, which are known to inhibit inflammatory mediators and oxidative stress pathways. [15,16] Heavy metal contamination remains an important safety concern in herbal medicines. Chronic exposure to heavy metals can lead to serious health hazards including organ toxicity and carcinogenic effects. In the present study, mercury levels were found to exceed recommended limits, highlighting potential safety concerns. Mercury contamination may arise from environmental sources such as soil contamination, water quality, or raw material sourcing. These findings emphasize the need for stringent quality control and monitoring during the manufacturing of herbal formulations to ensure compliance with regulatory guidelines. [11] *Bilvadileha* is traditionally used in Ayurveda for the management of digestive disorders resembling irritable bowel syndrome (IBS). [3] IBS is a functional gastrointestinal disorder affecting approximately 9–23 % of the global population and is characterized by abdominal pain, altered bowel habits, and intestinal inflammation. [5] The combined antibacterial and anti-inflammatory activities observed in this study may contribute to the therapeutic potential of *Bilvadileha* in managing gastrointestinal disturbances. The synergistic action of multiple herbal constituents may help regulate intestinal motility, reduce inflammation, and control microbial imbalance in the gut. [3,16]

Overall, the findings of the present investigation provide scientific support for the traditional use of *Bilvadileha* and establish preliminary quality control parameters for its standardization.

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