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VARIOUS MICROBIAL DIAGNOSTIC MODALITIES TO ESTABLISH STABILITY OF MODIFIED *PIPPALYADI RASAYANA*

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Abstract*:*
Introduction: Ensuring drug safety is a central tenet across medical systems, including Ayurveda. Herbal formulations are susceptible to chemical, physical and microbiological alterations over time, which may compromise their safety profile. To address this concern, a stability study was undertaken on modified *Pippalyadi Rasayana*, a herbo-mineral preparation indicated for pediatric bronchial asthma. Aim: To evaluate the stability of modified *Pippalyadi Rasayana* by monitoring microbial contamination at defined intervals under varying climatic conditions, temperature ranges and humidity levels. Materials and Methods**:** The formulation was prepared at the pharmacy of ITRA, Jamnagar, Gujarat, India, in accordance with established standard operating procedures (SOPs). Samples was stored in airtight, food-grade plastic containers in a dark and dry place. Microbiological testing was performed monthly to ensure its stability at different temperatures and humidity, employing smear examination with wet mount /10% K.O.H. Preparation and gram stain, culture study by aerobic culture and fungal culture examination. Results**:** Throughout the study period, ambient temperature ranged between 22 °C and 31 °C, while relative humidity fluctuated between 38% and 80%. No evidence of microbial contamination was detected in any of the samples during the twelve- month observation period. Discussion: *Pippalyadi Rasayana*, classified as a *Leha* (semi-solid Ayurvedic preparation) used for bronchial asthma. The stability evaluation demonstrated that the formulation remained free from bacterial and fungal contamination under diverse climatic conditions. Conclusion: The twelve-month stability study (April 2024 – March 2025) confirmed that modified *Pippalyadi Rasayana* was microbiologically safe. The absence of contamination validates both the manufacturing and storage protocols, affirming the formulation's stability.

Key– words: Stability study, Microbial profile, *Pippalyadi Rasayana*, *Leha*

INTRODUCTION

The primary aim of any medical treatment is to ensure both safety and efficacy of the medicines. Disease management should never result in the onset of another illness and drug safety is therefore a fundamental right of consumers, guaranteeing access to pure and reliable medicines.[1] The ancient Indian system of medicine - Ayurveda, practiced since 1500 BC, has consistently emphasized patient safety and therapeutic benefit.[2] Drug safety is a core principle across all medical traditions, including Ayurveda. A common misconception is that Ayurvedic medicines do not expire; however, herbal formulations undergo chemical, physical and microbiological changes over time, which may compromise their safety. Classical texts describe the concept of *Saviryata Avadhi* (shelf life), stressing that medicines should not be consumed beyond this period to preserve efficacy and prevent harm.[3] The Ayurvedic Indian Formulary further specifies the duration from the date of manufacture within which formulations should be consumed to ensure optimal efficacy and patient safety.[4] Climate related factors such as – humidity, temperature, light; drug related factors like active and passive ingredients, handling, packaging material, container closure system, dosage form etc. can accelerate deterioration, sometimes leading to microbial contamination. This risk is particularly high in pediatric care, where contaminated drugs may cause infections or illness because of the lower immune state of children.[5] To safeguard patients, a scientific stability protocol is essential for Ayurvedic formulations. Such a protocol should define batch and sample, storage conditions, container systems, sampling plans and testing frequency.[6] In pharmaceutical science, stability refers to a product's

ability to remain within its physical and chemical changes over time, which may compromise its safety.[7] There are two types of stability study – accelerated stability study and long - term stability study. Long term stability study is ideal for establishing shelf life of the drug.

Among Ayurvedic dosage forms, *Leha* holds a unique place. Widely mentioned in classical texts, *Leha* is valued for both nutritional and medicinal purposes. Children are reluctant to consume medicines as *Choorna* (powder), *Vati* (tablet) and *Kwatha* (decoction).[8] The palatability and ease of preparation of *Lehana* make it especially suitable for children. *Kashyapa Samhita*, a foundational text in Ayurvedic pediatrics, dedicates an entire chapter—*Leha Adhyaya*.[9] The chapter contains indications, contraindications and various *Leha* preparations for pediatric-specific requirements. *Sahastrayoga* describes *Pippalyadi Rasayana*, a herbo-mineral preparation used as a *Vyadhi Pratyanika Rasayana* (disease-specific rejuvenation therapy) for *Tamaka Shwasa*. For pediatric use, the classical formulation of *Pippalyadi Rasayana* was modified to meet age-specific requirements. The preparation was carried out at the Pharmacy, ITRA, Jamnagar, under aseptic conditions and stored in airtight, food-grade plastic containers at room temperature in a dark, dry environment. Since no prior stability study of the modified *Pippalyadi Rasayana* existed, it was selected for evaluation under varying climatic conditions over twelve months. The drug, prepared on 14 March 2024, was periodically analyzed for stability. After confirming the absence of microbial contamination, it was administered to pediatric patients suffering from bronchial asthma. The shelf life of *Leha* formulations was not explicitly documented in classical Ayurvedic texts; therefore, the present study was undertaken to establish the shelf life of *Pippalyadi Rasayana*, a *Leha* preparation.

AIM

To evaluate the stability of modified *Pippalyadi Rasayana* by monitoring microbial contamination at defined intervals under varying climatic conditions, temperature ranges, and humidity levels.

MATERIAL AND METHODS

The drug-modified *Pippalyadi Rasayana* was tested for microbial contamination at the microbiology laboratory, ITRA, Jamnagar, Gujarat, India. The drug was prepared on date 14//03/2024. The drug was examined at random time interval for 12 times throughout the year. The first testing was done on 03/04/2024. Mainly, two cultures – aerobic culture and fungal culture were performed. The microbial safety of the drug was ensured before drug delivery to the patients.

Table No. 1: Ingredients of modified *Pippalyadi Rasayana*[10]

Sr. No.	Drug	Latin Name	Part used	Proportion
1	*Pippali*	*Piper longum* Linn.	Dry Fruit	½
2	*Vidanga*	*Embelia ribes* Burm.	Dry Seed	1/4
3	*Nagara*	*Zinziber officinalis* Rosc.	Rhizome	1/4
4	*Bharangi*	*Clerodendrum serratum* Linn.	Dry Bark	1/4
5	*Jeeraka*	*Cuminum ciminum* Linn.	Dry Fruit	1/4
6	*Maricha*	*Piper nigrum* Linn.	Dry Fruit	1/4
7	*Chitraka*	*Plumbago zeylanica* Linn.	Dry Root	1/8
8	*Ayo Raja*	Fe_2O_3	-	1/8
9	*Bhringaraja*	*Eclipta alba* Hassk.	Dry Whole Plant	1/8
10	*Twaka*	*Cinnamomnm zeylanicum* Breyn.	Dry Bark	1/24
11	*Ela*	*Elleteria cardamomum* Maton	Dry Fruit	1/24
12	*Patra*	*Cinnamomnm tamala* Linn.	Dry Leaves	1/24
13	*Amalaki*	*Emblica officinalis* Gaertn.	Dry Fruit	1/24
14	*Bibhitaki*	*Terminalia bellirica* Roxb.	Dry Fruit	1/24
15	*Haritaki*	*Terminalia chebula* Retz.	Dry Fruit	1/24
16	*Jati*	*Jasminum officinale* Linn.	Dry Leaves	1/8
17	*Takkolaphala*	*Illicium verum* Linn.	Dry Fruit	1/8
18	*Madhu*	-	-	4
19	*Sharkara*	-	-	1/2

Method of preparation

All the ingredients were procured from the pharmacy, ITRA, Jamnagar, except *Jati, Takkolaphala* and Honey. *Jati* and *Takkolaphala* werw procured from the local market of Jamnagar city and have been authenticated at the Pharmacognosy laboratory, ITRA, Jamnagar. Honey was procured from the forest department, Jamnagar. All the ingredients were taken as mentioned quantity. After removing physical impurities, all the ingredients except honey have been converted into powder form and sieved through a mesh with a size of 60 and mixed well. Then, after honey has been mixed into the powder and stirred well. The mixture has been kept in *Dhanya* (Barley) for fifteen days, as it is mentioned in the classical text.

Storage

The final product has been stored in a food grade air tight plastic container at room temperature. The containers were stored at dark and dry place at the Department of Kaumarbhritya, ITRA, Jamnagar.

Microbial Profile:

Microbial contamination was assessed by two methods to check any mycological findings and bacteriological findings.

1. Smear Examination-

A. Wet mount /10% K.O.H. Preparation

B. Gram's stain

2. **Culture Study-**

A. Fungal culture

B. Aerobic culture

The details of the procedures followed are given below.

1. **Smear Examination:**

1.A Wet mount /10% K.O.H. Preparation:

Procedure for Wet mount Preparation

A clean, grease-free glass slide was taken and a drop of the specimen was placed on it. Distilled water was added and the preparation was covered with a grease-free cover slip. The slide was then observed under the high-power objective (40X) of the microscope and the findings were reported.

Procedure For 10% KOH Preparation

For the preparation of 10% potassium hydroxide (KOH), KOH pellets were dissolved in distilled water to obtain the required concentration. To examine the sample, a clean, grease-free slide was taken and a drop of the specimen was placed on it. A drop of freshly prepared 10% KOH solution was added and the preparation was covered with a grease-free cover slip. The slide was allowed to stand for 15–20 minutes so that excess debris and keratin were cleared, leaving fungal elements intact. Finally, the slide was examined under the high-power objective (40X) of the microscope and the findings were recorded.

1.B Gram's stain test

Gram staining was performed as a differential staining technique that differentiated bacteria into two groups: gram-positive and gram-negative. The procedure was based on the ability of microorganisms to retain the color of the stains used during the process. Gram-negative bacteria were decolorized by an organic solvent such as acetone or Gram's decolorizer, whereas gram-positive bacteria were not decolorized because the primary dye was retained by their cell walls and they remained purple. After the decolorization step, a counterstain effect was observed on gram-negative bacteria, which appeared pink, while gram-positive bacteria continued to appear purple. Thus, the Gram stain procedure enabled bacteria to retain the color of the stains based on differences in the chemical and physical properties of their cell walls.

Procedure for Gram's stain[11]

A clean, grease-free slide was taken to prepare a smear of the sample. The prepared smear was fixed by passing it three to four times over the Bunsen burner; this fixation killed the vegetative forms of microbes, rendered them permeable to stain, ensured the material adhered to the slide surface and prevented autolytic changes. The fixed smear was then covered with Gram's crystal violet solution and allowed to remain for the time specified in the kit procedure. The smear was washed with tap water to remove excess reagent. It was subsequently covered with Safranin solution and left for the prescribed time, after which it was again washed with tap water to remove excess stain. The smear was decolorized using Gram's decolorizer (acetone) by holding the slide in a sloping position and pouring the reagent from the upper end until the primary dye (Gram's crystal violet) was removed. The smear was washed with tap water to eliminate residual acetone. Gram's iodine solution was then applied to the smear and allowed to remain for the required time before being washed off with tap water. The smear was blotted gently and allowed to dry. Finally, the slide was examined under the oil immersion lens and the findings were reported.

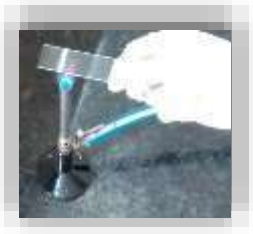

Figure 1 & 2: Smear staining Procedure

Figure 3: Stained smear ready for examination

2. Culture Study

2.A Fungal culture method:

Sabouraud Dextrose Agar Base (SDA), modified (Dextrose Agar Base, Emmons) from HIMEDIA Laboratories Pvt. Ltd. was collected using sterile cotton swabs for the purpose of inoculating selected fungal culture media. This medium is utilized for the selective growth of pathogenic fungi

Figure 4: Sabouraud Dextrose Agar Base (SDA) bottle

Procedure for Fungal Culture

In the clinical microbiology lab, the culture technique was used for organism isolation. Suitable selective solid media was selected for the purpose of inoculation. Selective solid media was dried in a Hot Air Oven prior to specimen inoculation, then permitted to cool before specimen inoculation. The sterile cotton swab was used to inoculate the selective specimen. Following the inoculation/streaking, place the inoculated medium in an inverted position in the incubator at 37°C for 5 to 7 to 21 days in an aerobic environment. Following the chosen incubation period, the growth was assessed visually as colonies or aerial growth and this was validated by conducting various relevant biochemical reactions and different staining techniques. Following that report's isolation.

2.B Aerobic culture method

MacConkey Agar (MA) and Columbia Blood agar (BA) from HIMEDIA Laboratories Pvt. Ltd. were obtained using sterile cotton swabs for inoculation on chosen aerobic culture media; the necessary incubation time was 24 to 48 hours. The media was utilized for the targeted growth of pathogenic bacteria

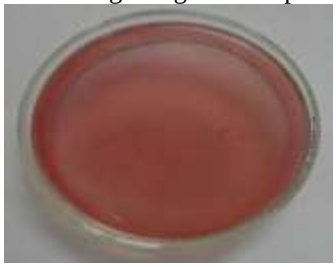

Figure 5: Aerobic culture media (MA)

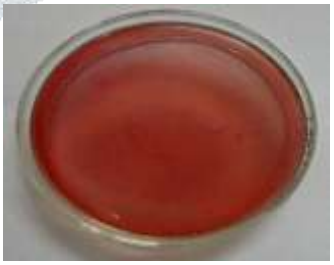

Figure 6: Aerobic culture media (BA)

Procedure for Aerobic Culture

The clinical microbiology lab utilized the culture method to isolate organisms. Suitable selective solid media was selected for the purpose of inoculation. Selective solid media was dried in a hot air oven prior to specimen inoculation and allowed to cool before specimen inoculation. The chosen specimen was inoculated using the four - flame technique (the loop was flamed and cooled between different sets of streaks, totaling four times) on the surface of cool, dried medium with a nichrome wire loop (24 S.W.G. size). Initially, the sterile loop was heated in a Bunsen burner's oxidizing flame—blue flame—and allowed to cool before being charged with the selected specimen for culturing. A loopful of the specimen was placed onto the surface of a thoroughly dried plate. Following the streaking procedure, incubate the inoculated medium upside down at 37°C for 18-24 hours in an incubator with either

aerobic or 10% CO2 conditions. Following the chosen incubation period, growth was assessed visually as colonies and verified through various relevant biochemical reactions and staining methods. After the isolation of that was reported.

OBSERVATION AND RESULTS

Table No. 2: Showing observations of modified *Pippalyadi Rasayana* preserved at room temperature

Sr. No.	Date of investigations	Days of study after drug preparation	Temp. (˚C)	Humidity	Observations of the sample			
					Gram's Stain	Aerobic culture	Wet mount/ 10% KOH Preparation	Fungal culture
1.	03/04/2024	19	29	53%	Microorganism not seen	No organism isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
2.	08/05/2024	53	31	63%	Microorganism not seen	No organism isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
3.	12/06/2024	88	31	70%	Microorganism not seen	No organism isolated	Fungal filaments not seen.	Aspergillus flavus Isolated
4.	10/07/2024	116	29	77%	Microorganism not seen	No organism isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
5.	07/08/2024	144	26	80%	Microorganism not seen	No organism isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
6.	18/09/2024	185	28	76%	Microorganism not seen	No organism isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
7.	14/10/2024	211	27	62%	Microorganism not seen	No organism isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
8.	06/11/2024	234	25	53%	Microorganism not seen	No organism isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
9.	04/12/2024	261	23	55%	Microorganism not seen	No organism isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
10.	01/01/2025	289	22	38%	Microorganism not seen	No organism isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
11.	05/02/2025	324	23	38%	Microorganism not seen	No organism isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
12.	04/03/2025	351	26.5	43%	Microorganism not seen	No organism isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated

DISCUSSION

Microbial safety plays a vital role in drug standardization. Appropriate procurement, preparation, storage and handling are crucial for preserving the microbial safety of the medication. *Pippalyadi Rasayana* is a *Leha* utilized for treating bronchial asthma in children. It underwent stability testing under various climatic conditions for twelve months. Tests including 10% KOH mount, fungal culture, Gram staining and aerobic culture, were conducted to assess fungal and bacterial contamination in the samples at regular time intervals. The study was carried out from the third April, 2024 (before drug delivery to the patients), until the fourth March, 2025, the date of completion of drug consumption. These procedures ensured continuous monitoring of microbial safety throughout the study period, and the findings were documented in Table No. 2. The initial test took place on April 3, 2024 – 19 days after the drug was prepared and the final testing occurred on March 4, 2025, after 351 days of preparation. The medication was evaluated for bacterial and fungal proliferation at the microbiology laboratory, ITRA, Jamnagar, Gujarat, India. Jamnagar is situated close to the coastal edge, leading to increased humidity. Fungal development is more likely in moist conditions. In the current study, the relative humidity (RH) varied between 38% and 80%. Elevated humidity (60-90%+ RH) encourages substantial bacterial and fungal proliferation, while moderate humidity (40-60% RH) is regarded as an optimal range for managing bacterial growth.[11] Temperature plays a crucial role in the stability of medications. Various kinds of bacteria thrive at distinct temperatures.

Psychrophiles thrive in low temperatures, specifically 0-15°C, while mesophiles develop in moderate temperatures ranging from 20-45°C. Pathogens that affect humans are typically mesophilic. Thermophiles thrive at elevated temperatures (50-80°C) and are located in hot springs. In the current study, the sample was examined at various temperatures between 22 and 31 ˚C. This temperature range is ideal for mesophiles; however, no bacterial growth was detected throughout the study duration. The minimum temperature recorded was in January 2025 at 22 ˚C, while the maximum occurred in May – June 2024 at 31 ˚C. The least humidity was noted in January - February 2025 at 38%. In August 2024, the rainy season was there in Jamnagar. The relative humidity reached 80%, marking the peak level of humidity throughout the study period. The rainy season generally promotes microbial growth due to increased moisture and humidity, which create favorable conditions for bacteria and fungus.[13] However, no microbial growth was observed during the study.

This finding revealed that the drug preparation, handling, and storage were maintained appropriately. The stability of the drug was assessed periodically and after confirming its microbial safety, the drug was prescribed to patients. Due to time constraints, the study was limited to twelve months, although a longer duration would provide stronger evidence regarding the actual stability of the drug. The study was conducted in Jamnagar city, and the results may vary depending on geographical location.

CONCLUSION

The trial drug, modified *Pippalyadi Rasayana*, was found to be microbiologically safe throughout the twelve-month study period. No contamination was observed, which indicated that the manufacturing process was carried out according to standard protocols and that the storage conditions were properly maintained. These findings confirmed the stability and safety of the drug during the study duration. This study can serve as a valuable reference for future stability studies and may provide a basis for extended evaluations of stability over longer time periods.

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