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MICROBIOLOGICAL OBSERVATION CONCERNING THE STABILITY PROFILE OF *TRIPHALA CHURNA* WITH REFERENCE TO BASELINE STUDY

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ABSTRACT: Introduction: *Triphala Churna* a polyherbal Ayurvedic formulation – with significant therapeutic potential is used to treat a variety of oral ailments. To ensure product's quality and effectiveness, it is essential to evaluate its microbiological stability. **Materials and Methods:** *Triphala Churna* was prepared using standard procedures and stored in various containers for ten months under different real-world storage conditions, without specific control over temperature or humidity. Microbial contamination was assessed periodically through Gram staining for bacteria, wet mount preparation for fungal visualization, and culture methods for isolating and identifying potential microbes. **Discussion:** No bacterial or fungal growth was observed in any of the samples throughout the ten-month study period, even under conditions that could potentially support microbial growth. The preparation, storage, and inherent qualities of the individual ingredients in the formulation may have contributed to the observed findings. **Conclusion:** This preliminary study indicates good microbial stability of *Triphala Churna* under real-world storage conditions. However, further research using a wider range of microbial detection methods is needed for a more comprehensive evaluation.

INTRODUCTION: A significant obstacle Ayurvedic formulas face is the need to analyze their complex ingredients thoroughly. It is essential to evaluate these components to maintain the final product's quality, purity, and stability. Ensuring the microbiological stability of herbal preparations is crucial for their safety and effectiveness. This examination elucidates how various environmental factors, including temperature, humidity, and light, can impact the potency of the active ingredients over time.

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Additionally, it helps determine the product's retest duration and recommended storage conditions. Therefore, conducting a stability study is vital for assessing the overall quality of the product. Research on herbal products significantly impacts regulatory compliance, patient safety, and quality assurance. Contaminated herbal products pose a threat to health, and understanding microbial survival can aid in preventing adverse health effects.

Stability studies provide crucial insights into herbal medications' effectiveness, safety, and shelf life, ensuring they remain free of harmful microbes during storage and use. As herbal remedies are distributed worldwide, addressing microbial contamination becomes essential for protecting public health and considering the financial

implications for manufacturers and consumers due to the global sales of herbal remedies. Ayurveda texts prescribe several herbal and Herbo-mineral formulations to treat various ailments. *Triphala Churna* has been used in dentistry, especially to improve gingival health, since the time of *Charaka* and *Sushruta*. *Triphala Churna* has antimicrobial properties; it reduces plaque and helps anti-cavity medications effectively manage oral cavity diseases. *Triphala Churna* is a well-recognized and revered formulation in Ayurveda, containing fine powders of three herbal drugs *viz. Terminalia chebula* Retz., *Terminalia bellerica* Roxb., *and Emblica officinalis* Gaertn [1]. Several studies have evaluated the shelf-life and Microbial Stability of classic or novel Ayurveda formulations [1, 2]. However, the Microbial Stability of *Triphala Churna* is essential to prove the drug's safety before administration to the patients.

Factors affecting the shelf-life of *Churna* (Powder) are physical (temperature, moisture, *etc*.), chemical (hydrolysis, oxidation, *etc*.) and biological types. Exposure to microbes is an important factor affecting the shelf-life of *Churna* preparations [1]. Determining the product's microbial stability is crucial for determining the formulation's shelf life. We conducted this preliminary study to evaluate the microbiological contamination in the finished product at different times under varied climatic conditions, temperatures, and humidity levels. This study addresses the argument that the studies on stability and microbial contamination may only partially represent the real-world conditions in which herbal products are stored and used, as the product under investigation was stored as it would be in real life.

MATERIALS AND METHODS:

Preparation of Formulation: Each ingredient included to make *Triphala Churna* was purchased from a nearby market and verified by a pharmacognosy laboratory. Ingredients of *Triphala Churna* with their proportions are depicted in **Table 1**. The formulation was made using standard procedures for preparing *churna*. All of the ingredients of raw materials were ground into a fine powder and poured through an appropriate sieve in compliance with standard operating procedure, taking care to prevent contamination. The powders were equally combined, and the finished medication was created devoid of the addition of any preservatives. The finished product was stored in polyethene bags that were sealed tightly. The medication was manufactured and sealed in 2 kg numbered plastic bags. These bags were stored in a dry, dark, and cool atmosphere.

Storage Conditions: Samples of the finished product were kept for microbiological research at various stages in 10 distinct plastic containers. The samples were kept in regular storage without controls over temperature or relative humidity.

Parameters: For each month, maximum and minimum temperature, along with relative humidity, were recorded with the help of online tools. We assessed the final product's microbial contamination (mycological and bacteriological) using the following methods: 1. Smear examination (using a wet mount/10% KOH preparation and Gram stain); 2. Culture study (using fungal and aerobic cultures).

Frequency of Assessment: After the preparation of drug; on the 21st, 48th, 78th, 111th, 139th, 170th, 203rd, 225th, 259th, and 288th day, the sample was examined for contamination.

Smear Examination:
Wet Mount/10% KOH Preparation 1:
Specimen: *Triphala Churna* **Fig. 1.**

Aim: To rule out any mycological findings, the following procedure was adopted:

Procedure for Wet Preparation: Take a clean grease free glass slide and put selected material on the slide. Add distilled water if needed and cover with a grease free cover class. Then, observe under the high power (40x) lens.

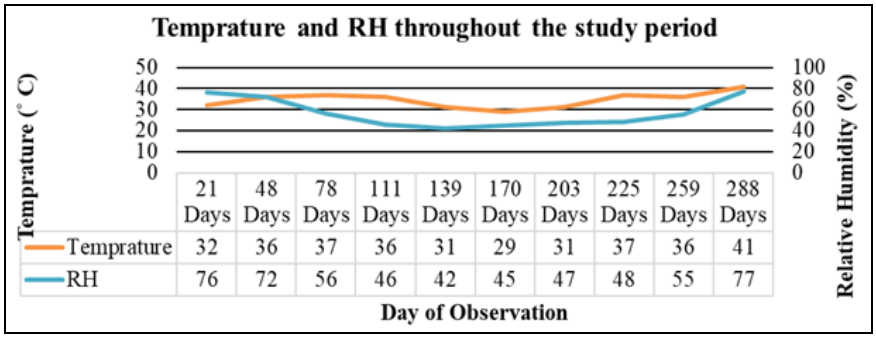
Procedure for 10% KOH Preparation: Potassium hydroxide (KOH) pellets were added to distilled water and mixed well to prepare a 10% solution. A sample drop was put on a clean, grease-free glass slide, and freshly prepared 10% KOH was added. The sample was covered with a grease-free cover glass and was allowed to react for 15–20 min to remove extra debris other than fungus particles. The cover glass was observed under a high-power (×40) lens.

Gram's Stain Test [1]: Gram staining is a differential staining technique that differentiates bacteria into two groups.

The Gram stain procedure enables bacteria to retain the colour of the stains based on the differences in the chemical and physical properties of the cell wall [1].

Aim: To rule out any bacteriological findings.

Specimen: *Triphala Churna* **Fig. 1.**



	21 Days	48 Days	78 Days	111 Days	139 Days	170 Days	203 Days	225 Days	259 Days	288 Days
Temprature	32	36	37	36	31	29	31	37	36	41
RH	76	72	56	46	42	45	47	48	55	77

FIG. 1: TEMPRATURE AND RELATIVE HUMIDITY OF STORAGE DURING STUDY PERIOD

Procedure for Gram's Stain: A clean, grease-free glass slide was taken, and a dry, equally thick smear was prepared. The smear was fixed by passing it over a Bunsen burner flame several times, which kills vegetative microbes, makes them permeable to stain, and prevents autolytic changes. The fixed smear was covered with Gram's crystal violet solution, and the kit procedure was followed. After washing off excess reagent with tap water, the smear was covered with Gram's iodine solution and allowed to remain for some time. Excess reagent was washed off. The smear was decolourised using Gram's decolouriser. We used Safranin solution to cover the smear; following the kit procedure, the excess reagent was washed off. The smear was allowed to dry. The slide was observed using an oil immersion lens **Fig. 2, 3**.

FIG. 2: SPECIMEN OF TRIPHALA CHURNA

FIG. 3: SMEAR STAINING PROCEDURE

Culture Study:

Aerobic Culture Method [1]: Respected materials were collected with sterile cotton swabs for inoculation purposes on selected aerobic culture media (i.e., an artificial preparation).

Name of Media: MacConkey Agar (MA) and Columbia blood agar (BA).

Company: HIMEDIA Laboratories Pvt. Ltd.

Required time Duration: 24–48 hours.

Required Temperature: 37°C

Use of Media: For selective cultivation of pathogenic bacteria.
Aim: To rule out any microorganism growth.

Specimen: *Triphala Churna* **Fig. 1.**

Procedure for Aerobic Culture: The streak culture method was employed in the isolation of an organism. Appropriate solid media, i.e., MA and Columbia BA, were selected for inoculation purposes. The selective solid media were dried in a hot air oven and allowed to cool before specimen inoculation. Samples were inoculated by sterile cotton swabs to the surface of well-dried culture media. After the streaking process, the inoculated medium was incubated in an inverted position at 37°C for 24–48 h in the incubator under the aerobic condition and 10% carbon dioxide atmospheric condition. After the incubation period, the growth was examined by the naked eye in the form of a colony and confirmed by performing different related biochemical reactions and different staining procedures **Fig. 4, 5.**

FIG. 4: STAINED SMEAR

FIG. 5: AEROBIC CULTURE MEDIA PREPARATION WITH MACCONKEY AGAR (MA)

Fungal Culture Method [1]: Respected materials were collected with a sterile cotton swab for inoculation purposes on selected fungal culture media (i.e., an artificial preparation).

Name of Media: Sabouraud dextrose agar base (SDA).

Modified (Dextrose Agar Base, Emmons)

Company: HIMEDIA Laboratories Pvt. Ltd.

Required Time Duration: 05–07 days.

Required Temperature: 37°C

Use of Media: For selective cultivation of pathogenic fungi.

Aim: To rule out any fungal pathogen.

Specimen: *Triphala Churna* **Fig. 1.**

Procedure for Fungal Culture: The streak culture method was employed for the isolation of organisms. Appropriate selective solid media, *i.e.*, SDA Base, modified (dextrose agar base, emmons) for inoculation purposes, was selected. The selective solid media were dried in hot airoven and allowed to cool before specimen inoculation. Samples were inoculated by sterile cotton swabs to the surface of well-dried culture media. After the streaking process, the inoculated medium was incubated in an inverted position at 37°Cfor 5–7 days. After the incubation period, the growth was examined by the naked eye in the form of a colony and confirmed by performing different related biochemical reactions and different staining procedures **Fig. 6, 7.**

FIG. 6: AEROBIC CULTURE MEDIA WITH MACCONKEY AGAR (MA)

FIG. 7: FUNGAL CULTURE MEDIA

RESULTS: Table 2 and **3** show the temperature and humidity observations for the entire period. Chart 1 displays the inverse relationship between temperature and relative humidity. Tests concerning microbial contamination, such as Gram's Stain, Aerobic culture, Wet mount/10% KOH Preparation, and Fungal culture test, were performed more than ten months after preparation and revealed no contamination. **Table 4** presents the results.

TABLE 1: CONTENTS OF TRIPHALA CHURNA

S. no.	Drug	Botanical Name	Part used	Proportion
1	*Haritaki*	*Terminalia chebula* Retz.	Fruitcarp	1Part
2	*Bhibhitaka*	*Terminalia bellerica* Roxb.	Fruitcarp	1Part
3	*Amalaki*	*Emblica officinalis* Gaertn.	Fruitcarp	1Part

TABLE 2: HIGH AND LOW WEATHER SUMMERY OF TEMPERATURE DURING STUDY PERIOD [13]

Months	Temperature			
	High	Date & time	Low	Date & time
August- 2023	35°C	30th August	25°C	11th August
September-2023	37°C	15th September	24°C	20th September
October-2023	39°C	24th October	22°C	19st October
November-2023	38°C	11st November	21°C	3rdNovember
December-2023	32°C	15th December	13°C	17th December
January-2024	32°C	25th January	11°C	20th January
February-2024	37°C	17th February	12°C	11th February
March-2024	41 °C	27th March	13°C	6th March
April-2024	44 °C	17th April	19 °C	7th April
May-2024	43 °C	7th May	23 °C	2nd May

TABLE 3: HIGH AND LOW WEATHER SUMMERY OF RELATIVE HUMIDITY DURING STUDY PERIOD [14]

Months	Relative Humidity				
	High	Date	Low	Date	Average
August- 2023	94.4 %	18th August	65.5%	2ndAugust	80.7%
September-2023	92.6%	24th September	52.9 %	29th September	77.9%
October-2023	95.5%	25th October	32.3%	29thOctober	65.5%
November-2023	95.5%	18thNovember	25.5%	7thNovember	59.8%
December-2023	91.0%	27th December	27.5%	28th December	54.8%
January-2024	96.4%	18th January	29.8%	1stJanuary	64.1%
February-2024	96.4%	2ndFebruary	29.5%	15th February	64.1%
March-2024	94.7%	9th March	17.1%	25th March	64.9%
April-2024	93.0%	14th April	22.4%	23rd April	70.1%
May-2024	90.3%	4th May	52.6 %	6th May	72.5%

TABLE 4: OBSERVATION OF SAMPLE *TRIPHALA CHURNA* [15]

Date of Sample given	Days of investigations After preparation of the sample	Bag No.	Temperature	Humidity	Observations of sample			
					Gram's Stain	Aerobic culture	Wet mount/ 10% KOH Preparation	Fungal culture
08/08/'23	21 Days	1	32˚ C	76%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
04/09/'23	48 Days	2	36˚ C	72%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
04/10/'23	78 Days	3	37˚ C	56%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
06/11/'23	111 Days	4	36˚ C	46%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
04/12/'23	139 Days	5	31˚ C	42%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
04/01/'2024	170 Days	6	29˚ C	45%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
06/02/'24	203 Days	7	31˚ C	47%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
28/02/'24	225 Days	8	37˚ C	48%	Microorganisms	No organisms	Fungal filaments	No Fungal Pathogen

02/04/'24	259 Days	9	36˚ C	55%	Not Seen Microorganisms No organisms isolated	Not Seen Fungal filaments not seen.	Isolated No Fungal Pathogen Isolated
01/05/'24	288 Days	10	41˚ C	77%	Not Seen Microorganisms No organisms isolated	Not Seen Fungal filaments not seen.	Isolated No Fungal Pathogen Isolated

DISCUSSION: This work represents an initial effort to assess *Triphala Churna* microbiological stability evaluation. Because microorganisms can compromise the quality of herbal treatments, there is a major risk to their safety and effectiveness. Therefore, evaluating the microbial load in herbal drugs is essential to protect public health [1].

Triphala Churna was prepared and stored at room temperature under standard conditions without specific temperature or humidity control. The study covered various seasons, reflecting significant climatic variations throughout the year. The ideal temperature for bacterial growth differs depending on the type of bacteria; the conventional classification categorises microorganisms into hyperthermophiles, thermophiles, mesophiles, and psychrophiles based on their preferred temperature ranges. These ranges are approximately as follows: above 60°C, above 45°C, in the 20–45°C range, and below 10°C, respectively. The temperatures observed during our study period aligned with the optimal conditions for psychrophilic and mesophilic bacteria [1].

The drug preparation and sample storage areas are located near the seaside; this state section boasts the longest coastline and the highest concentration of seaports. Relative humidity (RH) is hence high for practically the whole year. The recorded RH ranged from 17.1% to 96.4% at its highest point. **Table 3** High RH can allow the growth of microbes [1]. Except for November and December, when RH was lower than in other months, relative humidity remained consistently high throughout the study period. However, air cannot be deemed dry with RH greater than 40%.

Significant results during the ten-month analysis period observed the lack of microbiological contamination in the formulation. **Table 4** the rigorous preparation method, fine powdering, screening, and appropriate mixing guaranteed homogeneity and reduced the danger of contamination, among other aspects that may have contributed to the observed findings. This process may cause the preparation to become less wet, which would inhibit the growth of microorganisms. Antimicrobial characteristics are inherent in all constituents of the formulation. Each ingredient in the formulation has demonstrated significant antimicrobial activities, which may have helped to maintain microbiological stability.

Storage conditions simulating real-world conditions can be a unique aspect of the study. We accept that methods used to evaluate the microbiological stability may need to be more comprehensive to capture the full range of microbial interactions and potential contamination in the herbal products. However, our study has covered some of the most important organisms affecting the microbial stability of the herbal formulations.

The Drug and Cosmetic Act of India states that 25 °C and 60% relative humidity are ideal for storing medications. Based on observations, we may conclude that the product was unaffected by contaminants even if stored in routine conditions without any humidity or temperature control.

CONCLUSION: The microbiological stability study conducted on *Triphala Churna*, when stored under normal atmospheric conditions, indicates that the prepared pharmaceutical formulation remains uncontaminated for a duration of up to ten months. Since the study is preliminary in nature, more research is necessary to confirm the conclusions.

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