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"Baseline Microbial Diagnostic Modalities for Stability Study of *Triphaladi Churna* (*Anubhuta Yoga*) used in Treatment of *Amaja Abhishyanda* (Acute Infective Conjunctivitis)."

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Abstract: Conjunctivitis is a commonly encountered condition in ophthalmology clinics throughout the world which has high prevalence and recurrent rate in the general population of developing countries. If left untreated or undiagnosed, chronic conjunctivitis can cause permanent eye damage. Integrating Ayurveda alongside modern medical treatments can provide a holistic approach to eye care, potentially reducing the need for high doses of medications and minimizing their adverse effects. *Triphaladi Churna* (*Anubhuta Yoga*) is a choice of polyherbal compound selected to breakdown the pathology of *Amaja Abhishyanda* (Acute Infective Conjunctivitis). To check the stability and to check microbial contamination in the finished product at different time interval- at different climatic conditions, temperature and humidity set ups. **Materials and Methods:** In present study, stability with respect to its Microbial profile of *Triphaladi Churna* (*Anubhuta Yoga*) was carried out. It was stored in plastic bag during different climacteric conditions were studied at regular intervals for a period of eight months to analysis presence no microbial growth by Smear and culture study respectively. At the end of study *Triphaladi Churna* (*Anubhuta Yoga*) had no presence of microbes or bacteria throughout the study 8 months from the day sample preparation even in different climate and temperature. **Result**: In present study, the stability test of *Triphaladi Churna* (*Anubhuta Yoga*) with respect to microbiological findings was negative at room temperature, warm and cold, dry and humid conditions.

Key Words: Ayurveda, Microbial profile, *Triphaladi Churna.*

1. INTRODUCTION:

Triphaladi Churna (*Anubhuta Yoga*) is a modified polyherbal compound mentioned in classical text in the form of *Kwatha* (~decoction) which has *Tridosha Shamaka, Amapachana, Deepaniya* properties. *Triphaladi Churna* (*Anubhuta Yoga*) having ingredients *Amalaki, Haritaki, Bhibhitaki, Patola, Nima* and *Vasa Churna* in equal proportion is selected as a drug of choice in *Chikitsa* (~treatment) for *Amaja* (1) *Avastha* of *Netra Roga* which can be counted under the context of *Amaja Abhishyanda* (Acute Infective Conjunctivitis). The present study was chosen due to high prevalence and recurrent rate of Conjunctivitis in the general population of developing countries. (2) Integrating Ayurveda alongside modern medical treatments can provide a holistic approach to eye care, potentially reducing the need for high doses of medications and minimizing their adverse effects. (3) Moreover, the use of herbal medicine has increased remarkably in line with the global trend of people returning to natural therapies. The growing use of botanicals (drug and other products derived from plants) by public if forcing moves to assess the health claims of these agents and to develop standard of quality and manufacture.

Microbiological analysis is performed for the estimation of the number of viable aerobic micro-organism presence and for detecting the presence of designated microbial species in pharmaceutical substance. Stability, often gauged by shelf-life, denotes the duration from production to consumption, crucial for product integrity. Microbial proliferation

necessitates optimal environmental conditions: temperatures spanning 20°C to 40°C (68°F to 104°F), and relative humidity levels between 60% and 90%. These conditions foster microbial growth on various surfaces and articles. However, precise environmental control within this range is paramount for product preservation. Understanding these parameters aids in designing effective storage protocols to mitigate microbial contamination and extend shelf-life.

2. AIM:

To evaluate the stability and monitor microbial contamination in finished pharmaceutical products over varying time intervals and under diverse climatic conditions, temperature, and humidity settings, aiming to ensure adherence to standardization protocols for study drug production.

3. Materials and Methods:

Collected sample of prepared drug- *Triphaladi Churna* (*Anubhuta Yoga*) (stored at room temperature) studied to check microbial contamination with two different methods at regular intervals for a period of eight-months (upto drug used). Microbiological study has been carried out in Microbiology Laboratory, I.T.R.A., Jamnagar.

The initial microbiological study was done on 43rd day of preperation before starting patient enrollment. Then samples from same container were subjected to the microbilogical study regularly with random intervals during different seasons.

Drug material:

The raw drug materials were collected from the pharmacy department, I.T.R.A., Jamnagar. All the herbal dried ingredients of *Amalaki*, *Haritaki*, *Bhibhitaki*, *Patola*, *Nima* and *Vasa* was made into *Churna* (powder) form equal part of each drug separately. Then all *Churna* (powder) of ingredients was mixed thoroughly to prepare a homogeneous blend.

Ingredients of *Triphaladi Churna* (*Anubhuta Yoga*): - (Table 1).

Sr. no.	Name of Drug	Latin/English name	Part used	Proportion
1.	*Haritaki*	*Terminalia chebula* Retz.	Fruit	1 Part
2.	*Bibhitaki*	*Terminalia bellirica* Roxb.	Fruit	1 Part
3.	*Amalaki*	*Emblica officinalis* Gaertn.	Fruit	1 Part
4.	*Nimba*	*Azadirachta indica*	Leaves	1 Part
5.	*Vasa*	*Adhatoda vasica* Medic	Leaves	1 Part
6.	*Patola*	*Trichosanthes dioica Roxb.*	Leaves	1 Part

Date of Drug Preparation: 19th July, 2023

Storage:

Finished product of *Triphaladi Churna* (*Anubhuta Yoga*) was kept in air tight plastic container placed in the open light area in the department at room temperature. Clean and dry stainless steel spoon was used to pack the medicine into small plastic bag.

Microbial profile:

Microbial contamination was assessed by two methods to check any mycological findings and bacteriological findings. (4)

1. SMEAR EXAMINATION-

A) 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:

A. SMEAR EXAMINATION: -

➢ **PROCEDURE FOR 10% KOH PREPARATION:**

Take Potassium Hydroxides pellets (of HiMedia Lab. Pvt. Ltd.) in distilled water to prepare 10% of the same in clean glass tube & mix well

⇩

Take clean grease free glass slide

⇩

Put a-drop of specimen and add freshly prepared 10% KOH then cover it with grease free cover glass

⇩

Allow it to react for 15-20 minutes to remove extra debris other than fungal particles

⇩

Observe under high power (40x) microscopic lens

⇩

Report as per finding

➢ **PROCEDURE FOR GRAM'S STAIN TEST:**

Take clean grease free glass slide to prepare dry equal thick preparation (i.e. smear)

⇩

Fixed prepared smear by passing 3-4 times over the flame of Bunsen burner (the fixation kills vegetative form of microbes and render them permeable to stain, make material stick to the surface of slide & prevent autolytic changes)

⇩

Cover the fixed prepared smear with Gram's crystal violet solution and allow to remain for mentioned time as per kit procedure

⇩

Washed off smear to remove excessive reagent with tap water

⇩

Cover smear with Gram's Iodine solution and allow to remain for mentioned time as per kit procedure

⇩

Washed off smear to remove excessive reagent with tap water

Decolorize smear with Gram's decolorizer by holding the slide at slope position and pour gram's decolorizer - acetone from its upper end upto removal of colour of primary dye (i.e. Gram's Crystal violet) or as per kit procedure

Washed off smear to remove excess acetone with tap water

Cover smear with Safranin solution and allow to remain for mentioned time as per kit procedure

Washed off smear to remove excessive reagent with tap water

Blot and allow to dry smear

Examine under oil immersion lens and report as per findings

B. CULTURE STUDY: -

➢ **FUNGAL CULTURE METHOD:**

Respected materials collected with sterile cotton swab for inoculation purpose 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 to 07 days
Required temperature: 37 ºC
Use of media: For selective cultivation of pathogenic fungi.

Figure 1. Sabouraud Dextrose Agar Base (SDA) bottle

Procedure for Fungal Culture:

In the clinical microbiology laboratory culture method are employed for isolation of organisms (The lawn / streak culture method is routinely employed)

Choose appropriate selective solid media for inoculation purpose

Dry selective solid media in Hot Air Oven before specimen inoculation, allow to cool dried medium before specimen inoculation

↓

After inoculation / streaking process Incubate inoculated medium in inverted position at 37°C for 05 to 07 to 21-days in Incubator (incubation days are as per growth requirement) under aerobic atmosphere

↓

Inoculate selected *Triphaladi Churna* by Sterile cotton swab or by Nichrome wire (24 5.W.G. size) loop [First sterile loop in Bunsen burner oxidase flame blue flame and allow it to cool than loop is charged with selected specimen to be cultured. One loopful of the specimen is transferred onto the surface of well dried culture media]

↓

After selected incubation period examined growth by naked eye in form of colony or arial growth and confirm growth by performing different related biochemical reactions and different related staining procedures. After that report isolates.

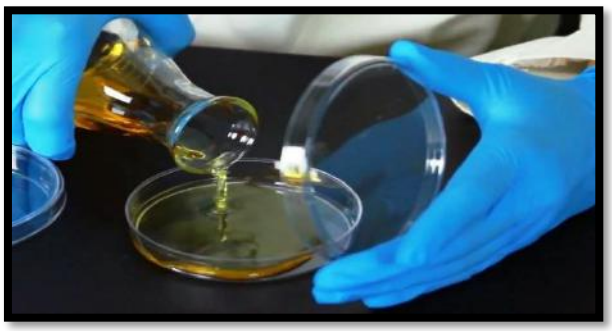


Figure 2: Procedure for Fungal culture

➢ **AEROBIC CULTURE METHOD:**

Respected materials collected with sterile cotton swab for inoculation purpose on selected fungal 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 to 48 hours
Required temperature: 37 ºC
Use of media: For selective cultivation of pathogenic bacteria.

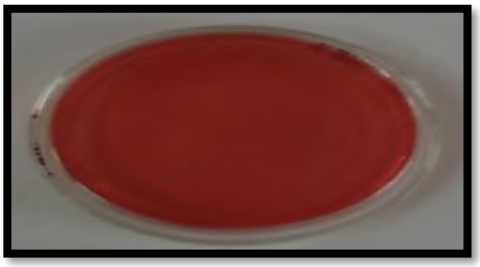


Figure 3: Mac Conkey Agar (MA)

Procedure for Aerobic Culture: -

In the clinical microbiology laboratory culture method are employed for isolation of organisms (The streak culture method is routinely employed)

⇩

Choose appropriate selective solid media for inoculation purpose

⇩

Dry selective solid media in Hot Air Oven before specimen inoculation, allow to cool dried medium before specimen inoculation

⇩

Inoculate selected *Triphaladi Churna* by four flame method (the loop should be flamed and cooled between the different sets of streaks i.e. four time) on surface of cool dried medium with Nichrome wire (24 S.W.G. size) loop [First sterile loop in Bunsen burner oxidase flame-blue flame and allow it to cool than loop is charged with selected specimen to be culture. One loopful of the specimen was transferred onto the surface of well dried plate]

⇩

After streaking process Incubate inoculated medium in inverted position at 37°C for 18-24 hours in Incubator under aerobic or 10% CO_2 atmosphere.

⇩

After selected incubation period examined growth by naked eye in form of colony and confirm growth by performing different related biochemical reactions and different related staining procedures. After that report isolates

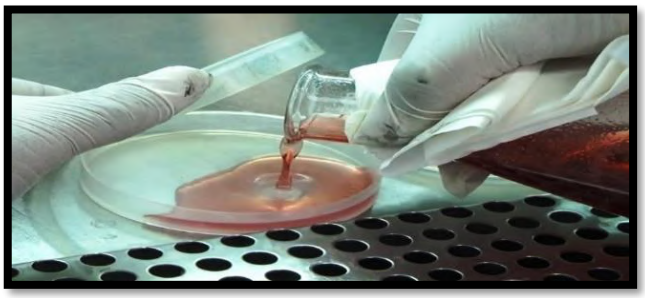


Figure 4: Procedure for Aerobic culture

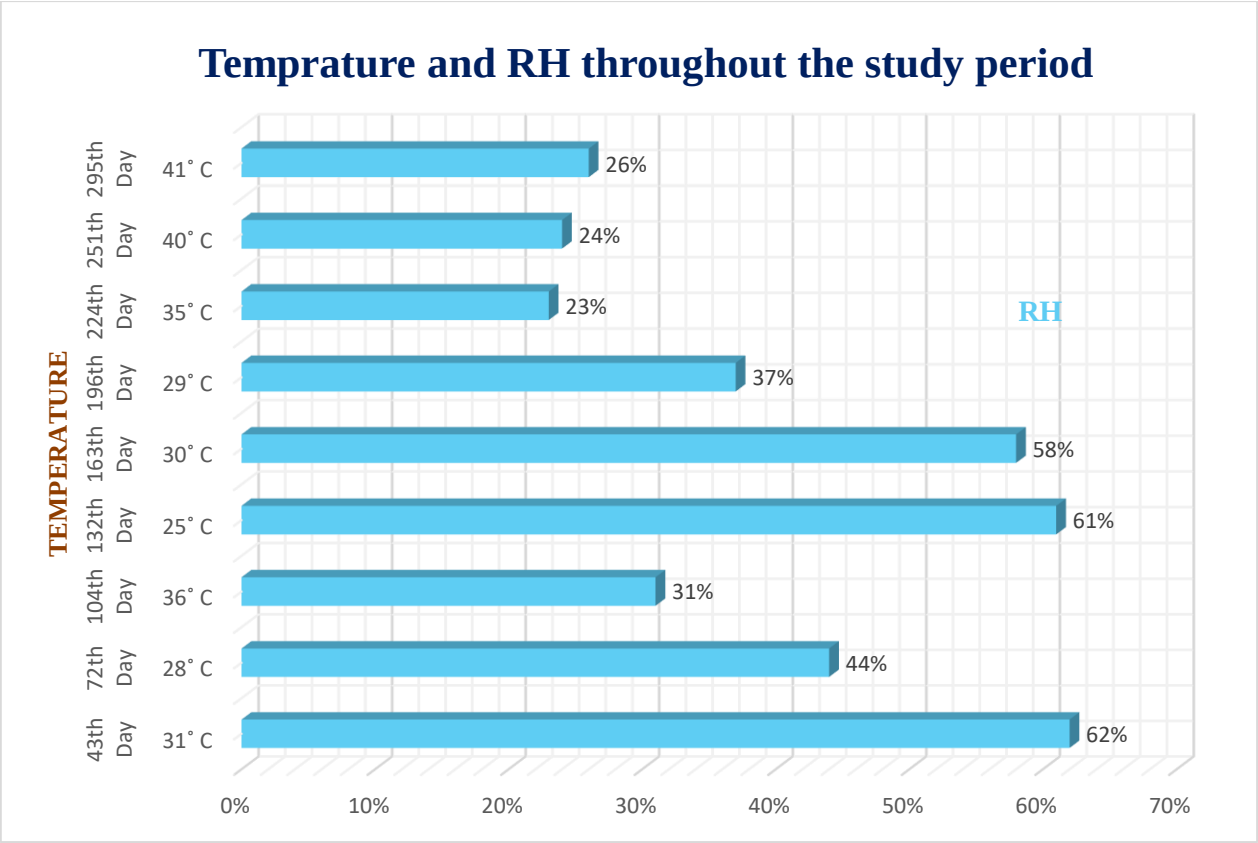
Every time sample (in which drug preserved) were subjected to the microbiological study from the date of the preperation to the date of last microbiological study.

4. RESULTS: Shown in table no 2. of *Triphaladi Churna* (*Anubhuta Yoga*).

- **Date of Drug Preparation: 19th July, 2023**

Table 2: Showing observations of sample preserved at room temperature after preparation of *Churna.* (5)

Sr. No.	Date & Days of investigations After preparation of the sample	Temperature (˚ C)	Humidity (%)	Observations of sample			
				Gram's Stain	Aerobic culture	Wet mount/ 10% KOH Preparation	Fungal culture
1.	31/08/23 43th Day	31˚ C	62%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
2.	29/09/23 72th Day	28˚ C	44%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
3.	31/10/23 104th Day	36˚ C	31%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
4.	28/11/2023 132th Day	25˚ C	61%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
5.	29/12/23 163th Day	30˚ C	58%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
6.	31/01/24 196th Day	29˚ C	37%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
7.	28/02/24 224th Day	35˚ C	23%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
8.	26/03/24 251th Day	40˚ C	24%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
9.	09/5/24 295th Day	41˚ C	26%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated



Graph 1: shows the timeline of temperature and RH through the study periods

5. DISCUSSION:

Ayurveda as an adjuvant therapy is widely used in *Amaja Abhishyanda* (Acute infective Conjunctivitis). *Triphaladi Churna* (*Anubhuta Yoga*) planned for the research work at ITRA to break the pathology of *Amaja Avastha* of *Amaja Abhishyanda* (Acute infective Conjunctivitis) as its prevalence rate and fatality is high in developing if left untreated or not addressed at its root cause.

Microbial study was carried out to observe microbial contamination and the stability study of *Triphaladi Churna* (*Anubhuta Yoga*) prepared and preserved in different temperature and various humidity conditions. Microbial growth depends on various factors, including temperature and humidity.

Triphaladi Churna (*Anubhuta Yoga*) prepared and stored temperatures ideal for bacterial growth at room temperature, minimum temperature 25 ˚ C to maximum temperature 41 ˚ C, astoundingly remains microbe-free. Situated in Jamnagar's coastal region, known for its high relative humidity throughout the year, defied expectations. Despite RH levels ranging from lowest range 23% on 31st January, 2023 to highest range 62% on 31st August, 2023 as shown in Table 2, no bacterial or fungal growth was observed upto drug used till study completed.

Thus, a baseline microbial profile was studied at regular interval of 30 days average upto consumption of *Triphaladi Churna* (*Anubhuta Yoga*). This indicates that manufacturing and storage procedure adopted in this study was up to the mark.

6. CONCLUSION:

The microbiological study of *Triphaladi Churna* (*Anubhuta Yoga*) confirms its preparation and storage under standard conditions, exhibiting no microbial growth, bacterial or fungal, for a remarkable eight-month period up to its intended use by May 19, 2024, highlighting its impressive shelf life. *Triphaladi Churna* (*Anubhuta Yoga*) emerges as a proven and effective remedy for treating *Amaja Abhishyanda* (Acute Infective Conjunctivitis), showcasing its potential to serve as a widely accessible solution for a broader population.

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CONFLICTS OF INTEREST:
There are no conflicts of interest.

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