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"STABILITY STUDY OF *MAHATRIPHALADI GHRITA* IN THE TREATMENT OF *SHUSHKAKSHIPAKA* (DRY EYE DISEASE) USING BASELINE MICROBIAL DIAGNOSTIC MODALITIES."

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ABSTARCT:

Prevalence of dry eye disease is estimated to be 5% to 35% worldwide i.e., 1 out of every 3 to 7 patients could have this condition. The prevalence of Dry eye disease is higher in India than the global prevalence, ranging from 18.4% to 54.3%. Artificial tear supplements and preservation of natural tears are the of management of Dry Eye Disease. Mainly this has a local effect only and, it can't break the root cause of pathology which often results in recurrence. *Mahatriphaladi Ghrita* is a choice of polyherbal compound selected for the treatment of *Shushkakshipaka* (Dry eye disease). To study 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 *Mahatriphaladi Ghrita* was carried out. It was stored in plastic bag during different climacteric conditions were studied at regular intervals for a period of 9 months to analysis presence bacteria or microbes by Smear and culture study respectively. At the end of study *Mahatriphaladi Ghrita* had no presence of microbes or bacteria throughout the study 9 months from the day sample preparation even in different climate and temperature. **Result:** In present study the stability test of *Mahatriphaladi Ghrita* with respect to microbiological findings was negative at room temperature, warm and cold, dry and humid conditions.

KEY WORDS: Microbial profile, Stability, *Mahatriphaladi Ghrita*

INTRODUCTION:

Mahatriphaladi Ghrita is an important formulation used by Ayurveda practitioners mainly in ayurveda ophthalmology (*Netra Chikitsa*). Most of the drugs in this formulation are having *Madhura rasa, Sheeta Virya, Snigdha, Rasayana* properties and are mainly *Vata-Pitta Shamaka.* [i,ii, iii] Here it was selected for the breaking of the pathology of *Shushkakshipaka*. The present study was chosen due to prevalence of dry eye disease is estimated to be 5% to 35% worldwide i.e., 1 out of every 3 to 7 patients could have this condition.[iv] And also the prevalence of Dry eye disease is higher in India than the global prevalence, ranging from 18.4% to 54.3%.[v] Integrating Ayurveda with modern medical treatments can offer a holistic approach to eye care, potentially reducing the need for high medication doses and minimizing adverse effects. As Furthermore, the use of herbal medicine has significantly increased in line with the global trend of people returning to natural therapies. This growing public reliance on botanicals (drugs and other products derived from plants) is driving efforts to evaluate the health claims of these agents and to establish standards for their quality and manufacturing.

There is significant drug wastage due to unused medications, which exacerbates the issue of affordability for many people since medications are already expensive. Also, there is a common belief that Ayurveda drugs do not have an expiry date, but this is a misconception because Ayurveda includes the concept of "*Saviryata Avadhi*," which denotes the shelf life of these drugs. The term "*Saviryata Avadhi*," comparable to shelf life, refers to the time period during which the "*Virya*" (therapeutic potency) of a drug remains unaffected.[vi] Texts such as Vangasena,[vii] Sharangadhara Samhita[viii], and Yogaratnakara[ix] have specifically mentioned shelf life for different Ayurvedic dosage forms. In conventional science, stability is defined as "the capability of a particular dosage form, in a specific container or closure system, to remain within its physical, chemical, microbiological, therapeutic, and toxicological specifications."[x] The World Health Organization (WHO) states that various environmental factors such as ambient temperature, humidity, and light, as well as product-related factors like the chemical and physical properties of the active ingredient and pharmaceutical excipient, the dosage form and its composition, the manufacturing process, the nature of the container closure system, and the properties of the packaging material all influence the stability of finished pharmaceutical products.[xi]

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.

Microbiological analysis is performed for the estimation of the no. of viable aerobic micro-organism presence and for detecting the presence of designated microbial species in pharmaceutical substance. 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.

AIM: To study the stability and to check microbial contamination in the finished product at different time interval with at different climatic conditions, temperature and humidity set ups.

Materials and Methods:

Collected sample of *Mahatriphaladi Ghrita* prepared (stored at room temperature) and finished product studied to check microbial contamination at regular intervals for a period of 9 months (upto drug used). Microbiological study has been carried out in Microbiology Laboratory, I.T.R.A., Jamnagar. Mainly 02 studies have been carried out to rule out that presence of any bacteria or fungi in the prepared drug as a final finished product.

The initial microbiological study was done on 27th 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.

Ingredients of *Mahatriphaladi Ghrita*: [xii] (Table 1)

Sr No.	Drugs	Botanical Name	Part use	Proportion
***Drava Dravya*:**				
1.	*Haritaki*	*Terminalia chebula* Retz.	Fruit	1 Part
	Amalaki	*Emblica officinalis* Gaertn.	Fruit	
	Bibhitaki	*Terminalia bellirica* Roxb.	Fruit	
2.	*Shatavari*	*Asparagus racemosus* Willd.	Root	1 Part
3.	*Bhringaraja*	*Eclipta alba* Hassk.	Whole	1 Part
4.	*Vasa*	*Adhatoda vasica* Medic.	Leaves	1 Part
5.	*Guduchi*	*Tinospora cordifolia* Willd.	Stem	1 Part
6.	*Amalaki*	*Emblica officinalis* Gaertn	Fruit	1 Part
7.	*Ajadugdha*	Goat milk	-	1 Part
Kalka Dravyas:				
1.	*Haritaki*	*Terminlia Chebula* Retz.	Fruit	1/8 Part
	Amalaki	*Emblica officinalis* Gaertn.	Fruit	
	Bibhitaki	*Terminalia bellirica* Roxb.	Fruit	
2.	*Pippali*	*Piper longum* Linn.	Fruit	
3.	*Nilotpala*	*Nymphaea nouchali* Linn.	Leaves	
4.	*Draksha*	*Vitis vinifera* Linn.	Fruit	
5.	*Kantakari*	*Solanum xanthocarpum* Sch.	Whole	
6.	*Kshira-Kakoli*	*Fritillaria*-Hook.F	Root	
7.	*Yashtimadhu*	*Glycyrrhiza glabra* Linn.	Root	
8.	*Gambhari*	*Gmelina arborea* Roxb.	Root	
9.	*Sharkara*	Sugar candy	-	
10.	*Go-Ghrita*	Cow ghee	-	1Part

Date of Drug Preparation: 05th August, 2023

Storage:

Finished product of *Mahatriphaladi Ghrita* was stored in air-tight food grade, plastic bag , stored in the open light area in the department at room temperature. Clean and dry stainless steel spoon was used to take medicine.

Microbial profile:

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

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: -

1. <u>PROCEDURE FOR 10% KOH PREPARATION:</u>

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 than cover 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) lens

↓

Report as per finding

2. GRAM'S STAIN TEST:

Procedure for Gram's Stain: -

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 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: -

1. 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.

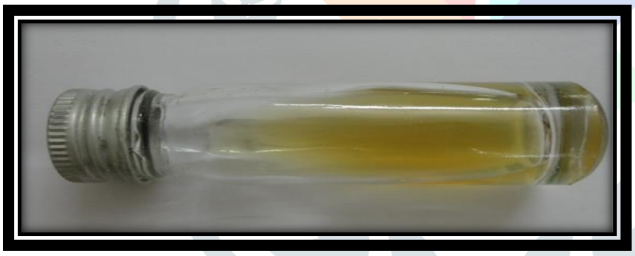


Figure3. Sabouraud Dextrose Agar Base (SDA) bottle

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

↓

Inoculate selected specimen 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]

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

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.

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

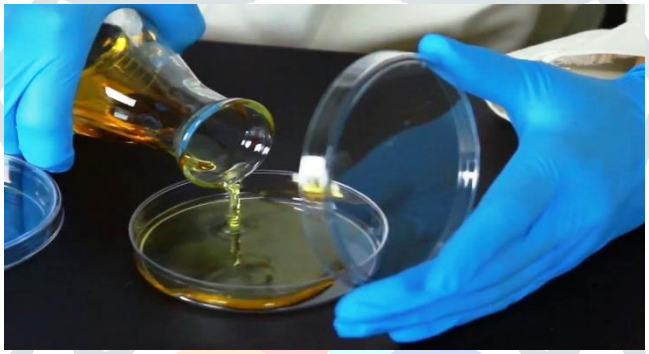


Figure 4: Procedure for Fungal culture

2. 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

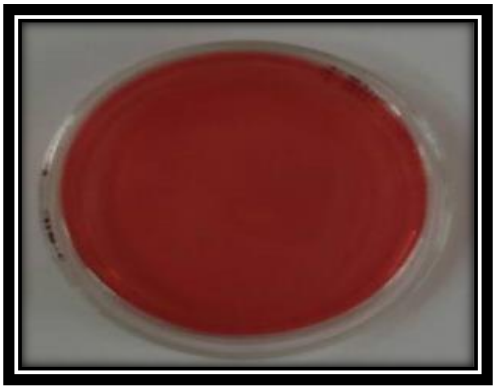


Figure 5: Mac Conkey Agar (MA)

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 specimen 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

↓

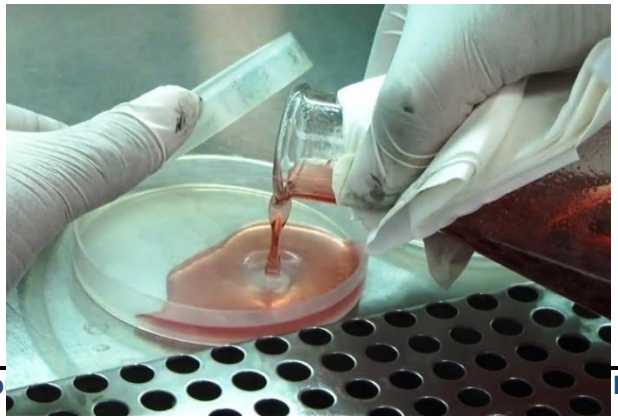
After selected incubation period examined growth by naked eye in form of colony and confirm growth by performing different related biochemical reactions and different related staining procedures. After that report isolates

↓

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.

↓

Inoculate selected specimen 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 cultured. One loopful of the specimen is transferred onto the surface of



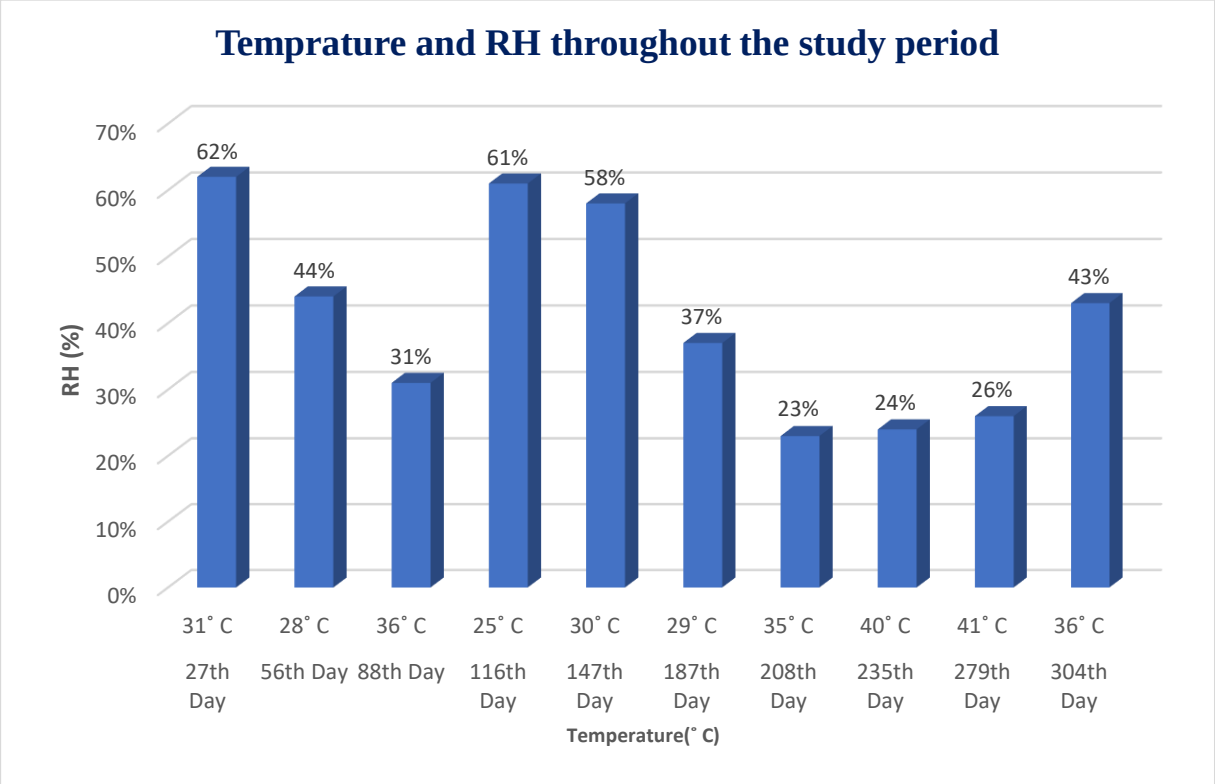
Every time sample (in which drug preserved) were subjected to the microbiological study from the date of the preperation to the date of last microbiological study.

Results are shown in table no 1.

Table 1: Showing observations of *Mahatriphaladi Ghrita* preserved at room temperature after preparation of *Ghrita* [xiii] (Drug Preparation Date: 05/08/2023)

Sr. No.	Date & Days of investigations After preparation of the sample	Temperature (˚ C)	Relative Humidity(RH) (%)	Observations of sample			
				Gram's Stain	Aerobic culture	Wet mount/ 10% KOH Preparation	Fungal culture
1.	31/08/23 27th Day	31˚ C	62%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
2.	29/09/23 56th Day	28˚ C	44%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
3.	31/10/23 88th Day	36˚ C	31%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
4.	28/11/2023 116th Day	25˚ C	61%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
5.	29/12/23 147th Day	30˚ C	58%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
6.	31/01/24 187th Day	29˚ C	37%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
7.	28/02/24 208th Day	35˚ C	23%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
8.	26/03/24 235th Day	40˚ C	24%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated

9.	**09/5/24** **279th Day**	**41˚ C**	**26%**	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
10.	**03/06/24** **304th Day**	**36˚ C**	**43%**	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated



DISCUSSION:

Mahatriphaladi Ghrita planned for the research work at ITRA to break the pathology of *Shushkakshipaka* (Dry eye disease), has shown very promising results in the current study. Therefore, a microbial study was conducted to assess the stability of *Mahatriphaladi Ghrita*, focusing on microbial contamination in samples prepared and preserved under various climatic and temperature conditions. Stability is usually expressed in term of shelf-life, which is the time period from when the product is produced until the time it is intended to be consumed or used. Microorganism needs water, humidity and temperature at suitable environmental conditions to develop in any media, surface or article temperature setups ranged from minimum temperature 25 ˚ C to maximum temperature 41 ˚ C, which proved as standard temperature for various typed of bacteria to overgrow. At the end of study, it was found that sample was not showed presence of any microbes which was preserved at room temperature at Jamnagar region. High Relative Humidity (RH) allows the growth of the microbes. *Mahatriphaladi Ghrita* which was prepared and stored at the Jamnagar region, proximal to sea coast, where RH remains high throughout a year. As shown in Table 2, highest humidity was 62 % on 31st August, 2023 while lowest was 23 % on 31st January, 2024. Although RH remained variable throughout the study period, microbial growth was not observed

during the study. Thus, a baseline microbial profile was studied at regular interval of 30 days average up to consumption of *Mahatriphaladi Ghrita*. This indicates that manufacturing and storage procedure adopted in this study were of high quality.

CONCLUSION: Microbiological study of the *Mahatriphaladi Ghrita* showed that the quality of *Ghrita* is in a standard condition. There were no growth found of microorganisms (bacterial or fungal),till 3rd june 2024 i.e. total 9 months (upto consumption of drug) from the date of preparation, shows its good shelf life.

FINANCIAL SUPPORT:
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CONFLICTS OF INTEREST:
There are no conflicts of interest.

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