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Different diagnostic modes used to check stability study of *Guduchi-Mustaadi Kwath* for the assessment of baseline microbial profile.

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Abstract:

Background: In last few decades, market of herbal and traditional medicines has grown up in leaps and bounds, but majority of traditional medicines are lacking in its data of *Saviryatavadhi* (shelf-life period). Hence the present study was carried out to evaluate the stability of *Guduchi-Mustaadi Kwath* with respect to microbial contamination and fungal growth. **Material and method:** *Guduchi-Mustaadi Kwath Yavakuta* was prepared in the pharmacy ITRA on date 2nd June 2022 and stored in food grade air-tight plastic containers, in a well-ventilated, well lighted space at room temperature. A baseline microbial profile was studied at regular interval of 1 month for total eight months. Every time, the sample was subjected to microbiological studies like smear examination with wet mount - 10% K.O.H. preparation and Gram's stain; fungal culture; and aerobic culture at Microbiology Laboratory, ITRA, Jamnagar. **Results**: In this study, no any growth of micro-organisms (bacterial or fungal) was found till 28th January 2023 i.e., for eight months from the date of preparation. **Conclusion**: Hence this study shows that *Guduchi-Mustaadi Kwath* can remain stable in various climatic conditions for a minimum of eight months and remain free from any microbial contamination, which indicate that the manufacturing and storage practices adopted in this study was up to the mark of Good Manufacturing Practice (GMP) and could be used as quality standards by future researchers and drug manufacturers.

Key words: Microbial profile, *Guduchi-Mustaadi Kwath*, Stability study, Climate condition.

Introduction

Stability of a pharmaceutical product refers to the capability of a particular formulation in a specific container or closure system, to remain within its physical, chemical, microbiological, therapeutic and toxicological specifications at a defined storage condition. Stability research provides proof of how the quality of a drug substance

or product changes over time, affected by a number of environmental factors such as temperature, humidity, and light, as well as determining the substitution duration for the drug substance or product and prescribed storage. Hence, one can say that, stability study proves to be a necessary tool for assessment of the quality of any product. [1] The main purpose of pharmaceutical stability testing is to provide fair assurance that, the drugs will remain at an appropriate standard of fitness / quality during the time of which it is available to patients in the market and will be suitable for their consumption. [2] Ayurvedic Formulary of India (AFI) has also provided the time from the date of manufacture till the time they should be consumed; for better results. In the Ayurvedic literatures, the word "*Saviryata Avadhi*" (~shelf life) is stated in the sense of the time interval in which any drug's *Virya* (~potency) remains unaffected due to environmental or microbial degradation. *Kwatha* (decoction) preparations are widely and largely used in pharmacies as well as by practitioners of Ayurveda for various ailments. According to Ayurveda classics, *Kwatha* preparations use instant after preparation. [3] *Guduchi-Mustaadi Kwath* (GMK) is an Herbal formulation used as in the management of *Sthaulya* (~obesity) [4]. (Table.1) Till today microbial stability data on *Guduchi-Mustaadi Kwath* is not available in scientific domain. So, GMK was selected and for the stability of the finished drug the microbial profile was checked. GMK was made, in Pharmacy, ITRA, Jamnagar, under standard operating procedure and with proper precautions to avoid any contamination. The preparation of the drug was finished on 02/06/2022. Then, the prepared drug was packed in plastic bag of 150 gm each and given numbers to them. These bags are kept in room temperature, dark and dry place in the department. This finished drug was given to the patients of *Sthaulya* as Ayurvedic treatment. This formulation was first checked and assured with nil microbial contamination prior to give it to the patients. For that, this study has been planned to check stability of finished drug to its microbial profile at different climacteric conditions and temperature with regular interval of the time. The stability study was performed for eight months.

Aim & objective:

To study the microbial contamination in *Guduchi-Mustaadi Kwath* at different time interval at different conditions of weather i.e., temperature, humidity etc.

Materials and methods:

Sample of *Guduchi-Mustaadi Kwath* was prepared (stored at room temperature) and studied to check microbial contamination at regular intervals for a period of eight months. Microbiological study has been carried out in Microbiology Laboratory, ITRA, Jamnagar, Gujarat. Mainly two studies have been carried out to rule out that presence of any bacteria or fungi in the test drug. The initial microbiological study was done before giving it to the patients. Then samples from plastic bags were collected from plastic bags for the microbiological study regularly with random intervals during different seasons with different climates and temperatures.

Contents of samples:

The sample contents swab of *Guduchi-Mustaadi Kwath* which includes ingredients i.e., *Guduchi* (~*Tinospora*

cordifolia Linn.), *Musta* (~*Cyperus rotundus* Linn.), *Haritaki* (~*Terminalia chebula* Retz.), *Bibhitaki* (~*Terminalia belerica* Roxb.), *Amalaki* (~*Embelica officinalis* Gaertn.) was procured from ITRA Pharmacy. *Guduchi-Mustaadi Kwath Yavakuta* were prepared at pharmacy ITRA, Jamnagar; by following Standard Operating Procedures (SOP) of *Yavakuta* preparation.

Table.1: Ingredients of *Guduchi-Mustaadi Kwath*

Sr. No.	Name of the Ingredient	Botanical name/ English Name	Parts used	Proportion
1.	*Guduchi*	*Tinospora cordifolia* Linn.	Stem	1 Part
2.	*Musta*	*Cyperus rotundus* Linn.	Rhizome	1 Part
3.	*Triphala* • *Haritaki* • *Bibhitaki* • *Amalaki*	 *Terminalia chebula* Retz. *Terminalia belerica* Roxb. *Embelica officinalis* Gaertn.	 Fruit Fruit Fruit	1 Part

Preparation Time:

Drug was prepared under SOP with the utmost care to avoid any sort of contamination. Date of preparation: 02 June 2022.

Storage:

Finished product, *Guduchi-Mustaadi Kwath* was stored in plastic bags (HDPE-High-Density Polyethylene) of 150 gm each at room temperature in a dark and dry place. So, the bag no. was assigned for testing. Samples were subjected to stability study for microbial and fungal contamination at different intervals of time with proper precautions for avoiding contamination. Details of which are cited below.

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 of each specimen are as follow-

1. Smear Examination:
Wet mount /10% K.O.H. Preparation:[5]

Aim: To rule out any mycological findings.

Specimen: *Guduchi-Mustaadi Kwath*

A. Procedure for Wet Preparation:

Flow chart.1 Procedure for Wet Preparation

Take clean grease free glass slide
↓
Put selected material
↓
Add distilled water (if needed)
↓
Cover with grease free cover glass
↓
Observe under the high power (40x) lens
↓
Report as per findings

Procedure For 10% KOH Preparation:

Flow chart.2 Procedure For 10% KOH Preparation:

Take Potassium Hydroxides pellets 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 findings

B. Gram's stain test: [6, 7]

Gram staining is a differential staining technique that differentiates bacteria into two groups that is gram-positive and gram-negative. The procedure is based on the ability of microorganisms to retain colour of the stains used during the gram stain procedure. Gram- negative bacteria are decolorized by any organic solvent (acetone or Gram's decolourizer) while Gram-positive bacteria are not decolorized as primary dye retained by the cell and bacteria will remain as purple. After decolorization step, a counter stain effect found on Gram negative bacteria and bacteria will remain pink. The Gram stain procedure enables bacteria to retain colour of the stains, based on the differences in the chemical and physical properties of the cell wall (Alfred E Brown, 2001) [8]

Aim: To rule out any bacteriological findings.

Specimen: *Guduchi-Mustaadi Kwath* (decoction)

Procedure for Gram's Stain

Flow chart.3: 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 remaining for mentioned time as per kit procedure

↓

Washed off smear to remove excessive reagent with tap water

↓

Decolourize smear with Gram's decolourizer by holding the slide at slope position and pour gram's decolourizer- acetone from its upper end up to 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 remaining 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

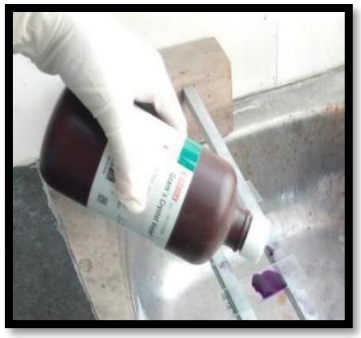


Figure 1 &2: Smear staining Procedure

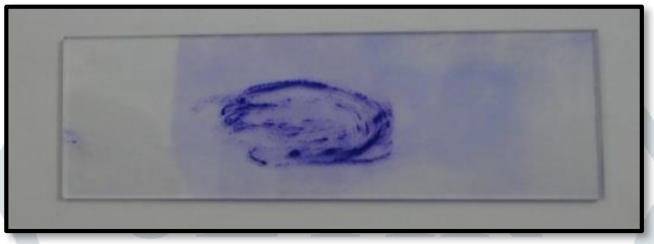


Figure 3: Stained smear ready for examination

1. Culture Study

A. Fungal culture method:[9]

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.

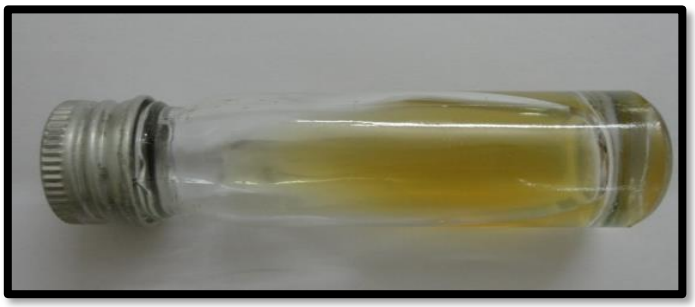


Figure 4 -Sabouraud Dextrose Agar Base (SDA)

Procedure for Fungal Culture

Flow chart.4: Fungal culture Method:

Take a suitable selective solid media

↓

Dry this media in hot air oven

↓

Infuse the specimen on this media by sterile cotton swab

or by Nichrome wire (24 S.W.G. size) loop

↓

In inverted position incubate the infused media at 37^0c

Under aerobic atmosphere for 5-7-21 days (as per requirement)

↓

Colony or Arial growth can be seen by naked eye after incubation period

↓

Verify the growth by related biochemical reactions & staining procedures

↓

Make a report

B. Aerobic culture method: [10]

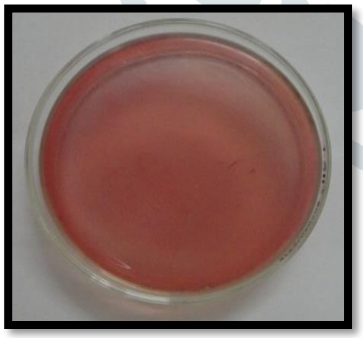
Respected materials collected with sterile cotton swab for inoculation purpose on selected aerobic culture media (i.e., an artificial preparation)

Name of media : MacConkey Agar (MA) and Columbia Blood agar (BA)

Company : HIMEDIA Laboratories Pvt. Ltd. Required time duration : 24 to 48 hours

Required temperature : 37 ºC

Use of media : for selective cultivation of pathogenic bacteria.



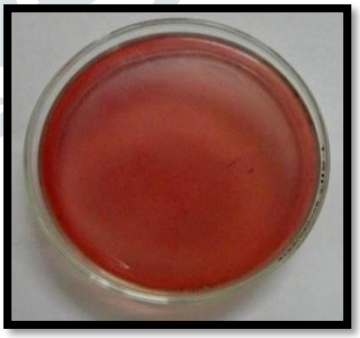


Figure 5: Aerobic culture media (MA) Figure 6: Aerobic culture media (BA)

Procedure for Aerobic Culture

Flow chart.5: Aerobic Culture Procedure:

The streak culture method is mostly employed for isolation of organisms.

↓

Take a suitable selective solid media

↓

Dry this media in hot air oven & let it be cool

↓

Infuse the sample on this media by applying four flame methods

By Nichrome wire (24 S.W.G. size) loop (streaking process)

↓

In inverted position incubate the infused media at 37^0c

Under aerobic or 10% CO_2atmosphere for 18-24 hours

↓

The growth can be seen by naked eye after incubation period

↓

Verify the growth by related biochemical reactions & staining procedures

↓

Make a report

Observation and result:

Table 2: Showing observations of sample preserved at room temperature of *Guduchi-Mustaadi Kwath*

Sr. No.	Study conducted at (No. of days)	Date of Sample given for test	Avg. Temperature ^{0}C (^{0}F) & humidity	Observations/Findings			
				Gram's Stain	Aerobic culture	Wet mount/ 10% KOH Preparation	Fungal culture
1	26th Day	27th June 2022	26.1 ^{0}C (79 ^{0}F) 48%	Microorganisms not seen	No organisms isolated	Fungal filaments not seen.	No fungal pathogen isolated
2	55th Day	26th July 2022	28.8 ^{0}C (83.8 ^{0}F) 56%	Microorganisms not seen	No organisms isolated	Fungal filaments not seen.	No fungal pathogen isolated
3	87th Day	27th August 2022	30.3 ^{0}C (86.6 ^{0}F) 63%	Microorganisms not seen	No organisms isolated	Fungal filaments not seen.	No fungal pathogen isolated
4	118th Day	27th September 2022	30.4 ^{0}C (86.8 ^{0}F) 69%	Microorganisms not seen	No organisms isolated	Fungal filaments not seen.	No fungal pathogen isolated
5	149th Day	28th October 2022	28.5 ^{0}C (83.4 ^{0}F) 78%	Microorganisms not seen	No organisms isolated	Fungal filaments not seen.	No fungal pathogen isolated
6	177th Day	25th	27.5 ^{0}C	Microorganisms	No	Fungal	No fungal

		November 2022	(81.5 ^{0}F) 81%	not seen	organisms isolated	filaments not seen.	pathogen isolated
7	210th Day	28th December 2022	27.7 ^{0}C (81.9 ^{0}F) 76%	Microorganisms not seen	No organisms isolated	Fungal filaments not seen.	No fungal pathogen isolated
8	241th Day	28th January 2023	28.7 ^{0}C (83.7 ^{0}F) 57%	Microorganisms not seen	No organisms isolated	Fungal filaments not seen.	No fungal pathogen isolated

Discussion:

Microbial contamination should be avoided to maintain the drug stability. There are many factors like temperature, humidity, moisture etc. which can have an impact on microbial growth in a drug. These factors should be taken care by adopting GMP (Good Manufacturing Practice), for better stability. *Guduchi-Mustaadi Kwath* was prepared and stored at room temperature. Optimum temperature for microbial growth is the temperature at which microbes multiplies, this optimum temperature for Psychrophilic bacteria (cold loving bacteria) is 15-20^{0}C while for Mesophilic bacteria (middle loving) is 30- 37^{0}C and for Thermophilic bacteria (heat loving) is 50-60^{0}C. [11] In this study, as mentioned in table 2, temperature setups ranged from minimum 26.1 ^{0}C to maximum 30.4^{0}C, which proved as standard temperature for various types of bacteria to overgrow, but there were no any microbes isolated till eight months of drug preservation after preparation. High Relative Humidity (RH) allows the growth of microbes. [12] The Jamnagar region, where this drug was prepared and stored in present study is proximal to the sea coast, where the RH remains high in all the seasons of the year. As shown in Table 2, highest RH observed was 81% in the month of November 2022, while lowest humidity was 48% in month of June 2022. Although RH remained variable throughout the study period, microbial growth was not observed during this study. Thus, a baseline Microbial profile was studied at regular interval of 1 month after preparation of *Guduchi-Mustaadi Kwath* for eight months (i.e., total time duration for consumption of prepared drug from drug preparation time i.e., 2nd June 2022) At the end of study, it was observed that no any growth of bacterial and fungal microorganisms found, from the date of preparation i.e., 2nd June 2022 till last consumption i.e., 28th January 2023 as per shown in the table of observation. This indicates that manufacturing and storage procedures adopted in this study was up to the mark.

Thus, a baseline Microbial profile was studied at regular interval of 1 month after preparation of *Guduchi-Mustaadi Kwath* for eight months (i.e., total time duration for consumption of prepared drug from drug preparation time i.e.,2nd June 2022) At the end of study, it was observed that no any growth of bacterial and fungal microorganisms found, from the date of preparation i.e., 2nd June 2022 till last consumption i.e., 28th January 2023 as per shown in the table of observation.

Limitations: Due to monitory restrictions and stipulated time frame, this study was carried out till eight months only, which has given only a minimum idea of the stability period of GMK. A long-term stability study or an

accelerated stability study is required to get the actual idea about shelf-life of the GMK. These findings can be generalized for GMK, which is prepared from the ingredients collected from same geographical regions; because changes in the concentration of phytochemicals of herbs are very common with a change in the geographical region.

Conclusion:

Hence this study shows that *Guduchi-Mustaadi Kwath* can remain stable in various climatic conditions for a minimum of eight months and remain free from any microbial contamination, which indicate that the manufacturing and storage practices adopted in this study was up to the mark of Good Manufacturing Practice (GMP) and could be used as quality standards by future researchers and drug manufacturers.

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Conflicts of interest

There are no conflicts of interest.

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