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ORIGINAL RESEARCH ARTICLE- EXPERIMENTAL **OPEN ACCESS**

DIFFERENT DIAGNOSTIC MODES USED TO CHECK STABILITY STUDY OF G*ANDHAKADI* Y*OGA* (USED IN THALASSEMIA MAJOR AS ADJUVANT TREATMENT) FOR THE ASSESSMENT OF BASELINE MICROBIAL PROFILE

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Abstract:

Thalassemia major is a malignant type of genetic disorder and iron overload is the main complication of the disease which results due to frequent blood transfusions. *Gandhakadi Yoga* is one of the Herbo-mineral formulation mentioned which was used in clinical trial to treat Thalassemia major. **Objective:** In the present study, stability with respect to its microbial profile in different climate condition of *Gandhakadi Yoga Vati* was carried out. **Methods**: *Gandhakadi Yoga Vati* was stored in airtight plastic bag of 50 gm each with numbers given to each bag in room temperature, dark and dry place during different climatic condition. The drug was studied at different intervals for a period of 13 months from June 2022 to June 2023 for the assessment of mycological findings and presence of microorganisms by Wet mount preparation and Gram stain test respectively. **Results** and **Conclusions**: At the end of study, no contamination found in prepared drug at minimum humidity of 38% with 22°C temperature and at maximum humidity of 91% with 29°C temperature.

Keywords: Microbial profile, *Gandhakadi Yoga*, 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. *Vati* (tablet) preparations are widely and largely used in pharmacies as well as by practitioners of Ayurveda for various ailments. According to Ayurveda classics, *Vati* preparations remain potent up to 1 year [3,4] after which they start losing their efficacy, whereas the shelf-life of *Vati* has been mentioned as 3 years in Gazette of India.[5] *Gandhakadi Yoga Vati* (GYV) is a Herbo-mineral formulation used as an adjuvant in the management of Thalassemia Major. (Table.1) It is a modified form of the drug suggested for *Loha Sevanajanya Vikara Prashamana* (iron overloading) in Ayurveda Prakasha.[6] This is being used as iron chelator to decrease the iron overload. *Gandhakadi Yoga Vati* was evaluated earlier for its chelation effect in Iron Sorbitol induced iron overload in albino rats by Pramod Yadav *et al* (2011).[7] The pre-clinical studies of *Gandhakadi Yoga* had shown promising results as iron chelator. [8, 9] Till today microbial stability data on *Gandhakadi Yoga Vati* is not available in scientific domain. So, GY was selected and for the stability of the finished drug the microbial profile was checked. GY 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 03/06/2022. Then, the prepared drug was packed in plastic bag of 50 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 Thalassemia major as adjuvant 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 13 months.

AIM & OBJECTIVE:

To study the microbial contamination in *Gandhakadi Yoga* at different time interval at

different conditions of weather i.e., temperature, humidity etc.

MATERIALS AND METHODS:

Sample of *Gandhakadi Yoga* was prepared (stored at room temperature) and studied to check microbial contamination at regular intervals for a period of 13 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 approximately 0.5 gm (1-2 *Vati*) of *Gandhakadi Yoga* which includes only two ingredients i.e., *Shuddha Gandhaka* (~purified Sulphur) and *Vidanga* (*Embelia ribes* Burm. f.) was procured from ITRA Pharmacy. Fresh leaves of *Agastya* (*Sesbenia grandiflora* Linn.) for *Bhavna* (~lavigation) were purchased from local farmer and authenticated for quality and purity by the experts of Pharmacognosy laboratory, ITRA, Jamnagar. *Gandhakadi Yoga* were prepared at the department of *Rasashastra* and *Bhaishajya Kalpna* and pharmacy ITRA, Jamnagar; by following Standard Operating Procedures (SOP) of *Vati* preparation.

Table.1: Ingredients of *Gandhakadi Yoga Vati*.

Sr. No.	Drug name	English / Scientific name	Part used	Quantity
1.	*Shuddha Gandhaka*	Sulphur	As whole	1 part
2.	*Vidanga*	*Embelia ribes* Burm. f.	Dry Fruit	1 part
3.	*Agastya*	*Sesbenia grandiflora* Linn.	Green leaf (for *Bhavna*)	Quantity sufficient

Preparation Time:

Drug was prepared under SOP with the utmost care to avoid any sort of contamination. Date of preparation: 03 June 2022.

Storage:

Finished product, *Gandhakadi Yoga* was stored in plastic bags (HDPE-High-Density Polyethylene) of 50 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

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

Gram's stain

Culture Study

1. Fungal culture
2. Aerobic culture

The details of the procedures of each specimen are as follow-

1. Smear Examination:

Wet mount /10% K.O.H. Preparation:[10]

Aim: To rule out any mycological findings.

Specimen: *Gandhakadi Yoga*

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

B. 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

Gram's stain test: [11, 12]

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) [13]

Aim: To rule out any bacteriological findings.

Specimen: *Gandhakadi Yoga* (*Vati*)

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

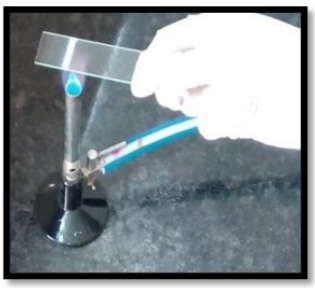



Figure 1 &2: Smear staining Procedure

Figure 3: Stained smear ready for examination

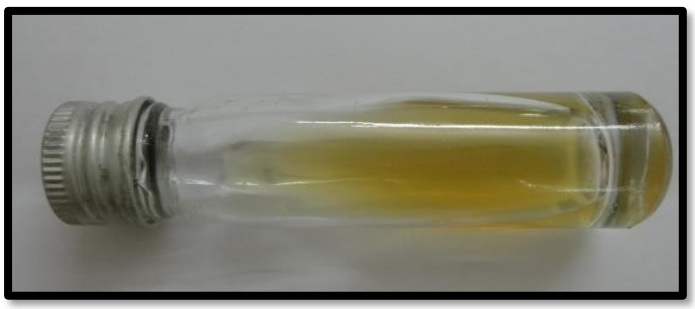


Figure 4 -Sabouraud Dextrose Agar Base (SDA)

1. Culture Study

A. Fungal culture method:[14]

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

: 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.

Procedure for Fungal Culture

A. Aerobic culture method: [15]

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

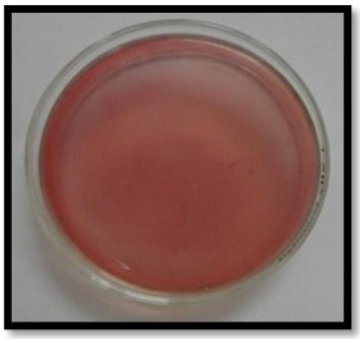
Respected materials collected with sterile cotton swab for inoculation purpose on selected aerobic culture media (i.e., an artificial preparation)

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.



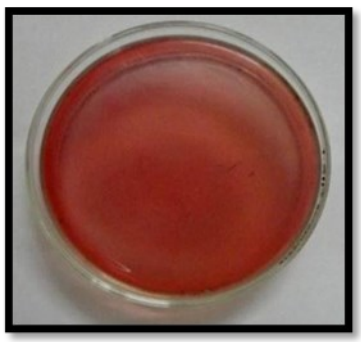


Figure 5: Aerobic culture media (MA) Figure 6: Aerobic culture media (BA)

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 *Gandhakadi Yoga*

Sr no	Days of investigation after	Bag No. & Date of	Temperature and humidity	Gram's Stain	Aerobic culture	Wet mount/ 10% KOH Preparation	Fungal culture
1.	3 Days	Bag No. 1 06/06/22	30° C, 55%	Micro-organism Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
2.	31 Days	Bag No. 9 04/07/22	29° C, 83%	Micro-organism Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
3.	62 Days	Bag No.	29°C, 91%	Micro-organism Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
4.	96 Days	Bag No. 41 07/09/22	28°C, 74 %	Microorganism Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated

5.	129 Days	Bag No. 50 10/10/22	27°C, 88%	Microorganism Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
6.	159 Days	Bag No. 58 09/11/22	20°C, 56%	Microorganism Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
7.	187 Days	Bag No. 60 07/12/22	18°C, 59%	Microorganism Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
8.	220 Days	Bag No. 60	16°C, 64 %	Microorganism Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
9.	249 Days	Bag No. 61 07/02/23	17°C, 72%	Microorganism Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
10.	283 days	Bag No. 68	22°C, 38%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
11.	313 Days	Bag No. 75 12/04/23	26°C, 45%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
12.	339 Days	Bag No. 77 08/05/23	28°C, 52%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
13.	3 Days	Bag No. 80 21/06/23	28°C, 69%	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. *Gandhakadi Yoga* 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- 370C and for Thermophilic bacteria (heat loving) is 50-60^{0}C. [16] In this study, as mentioned in table 2, temperature setups ranged from minimum 16 ^{0}C to maximum 30^{0}C, which proved as standard temperature for various types of bacteria to overgrow, but there were no any microbes isolated till 13 months of drug preservation after preparation. High Relative Humidity (RH) allows the growth of microbes. [17] 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 91% in the month of August 2022, while lowest humidity was 38% in month of March 2023. 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 *Gandhakadi Yoga* for 13 months (i.e., total time duration for consumption of prepared drug from drug preparation time i.e.,6th 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., 3rd June 2022 till last consumption i.e., 21st June 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 *Gandhakadi Yoga* for 13 months (i.e., total time duration for consumption of prepared drug from drug preparation time i.e.,6th 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., 3rd June 2022 till last consumption i.e., 21st June 2023 as per shown in the table of observation.

Limitations: Due to monitory restrictions and stipulated time frame, this study was carried out till 13 months only, which has given only a minimum idea of the stability period of GY. A

long-term stability study or an accelerated stability study is required to get the actual idea about shelf-life of the GY. These findings can be generalized for GY, 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 *Gandhakadi Yoga* can remain stable in various climatic conditions for a minimum of 13 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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