



Stability Study of *Shatavari-Shatapushpa Choorna* for the Assessment of Baseline Microbial Profile Used in Non-OBESE PCOS

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ABSTRACT:

PCOS is a heterogeneous, multisystem endocrinopathy in women. *Shatavari-Shatapushpa Choorna* is one of the herbal formulations mentioned which was used in clinical trial to treat non-obese PCOS. Objective: In the present study, stability with respect to its microbial profile in different climate condition of *Shatavari-Shatapushpa Choorna* carried out. Methods: *Shatavari-Shatapushpa Choorna* was stored in plastic bag of 500mg each with numbers given to each bag in cool, dark and dry place during different climatic condition. The drug was studied at different intervals for a period of one year from July 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 24% with 41°C temperature and at maximum humidity of 24% with 41°C temperature. Contamination in form of the bacteria and fungus at minimum humidity of 25% with 42°C temperature and at maximum humidity of 96% with 29°C temperature found in prepared drug.

Key words: Microbial profile, *Shatavari-Shatapushpa Choorna*, Stability study, Climate condition.

INTRODUCTION

PCOS is a global health issue/ burning problem for women's health in current scenario due to changes in life style, dietary habits and mental stress from menarche to menopause. It is Complex syndrome showing the clinical features of hyperinsulinemia and hyperandrogenism. Clinically it is characterized by menstrual abnormalities in the form of oligomenorrhea, amenorrhea, hypomenorrhea, hirsutism, acne etc.ⁱ Globally, prevalence estimates of PCOS are highly variable, ranging from 2.2% to high as 26%.ⁱⁱ PCOS is strongly associate with obesity but a small proportion of patients present with normal body mass index or low BMI (≤ 25 kg/m²) These cases of PCOS are termed as non-obese PCOS. So, for the clinical study, *Shatavari-Shatapushpa Choorna* was selected and for the stability of the finished drug the microbial profile was checked. *Shatavari-Shatapushpa Choorna* 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 16/06/2022. Then, the prepared drug was packed in plastic bag of 500 mg each and given numbers to them. These bags are kept in cool, dark and dry place in the department. This finished drug was given to the patients of non-obese PCOS. 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 approximately one year.

AIM:

To study the microbial contamination in *Shatavari-Shatapushpa Choorna* at different time interval at different conditions of weather i.e., temperature, humidity etc.

MATERIALS AND METHODS:

Sample of *Shatavari-Shatapushpa Choorna* was prepared (stored at room temperature) and studied to check microbial contamination at regular intervals for a period of one year. 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 0.25 gm of *Shatavari-Shatapushpa Choorna* which includes only two ingredients i.e., *Asparagus racemosus* Willd. and *Anethum sowa* Kurz.

Shatavari Choorna was procured from ITRA Pharmacy and *Shatapushpa Seed* was purchased from Local market of Jamnagar. Seed was lightly roasted and grinded. Then, powder was passed through sieve no 80. After that Mixture of *Shatavari Choorna* and *Shatapushpa Choorna* was packed in air tight container.

Preparation Time:

Drug was prepared under SOP with the utmost care to avoid any sort of contamination.

Date of preparation: 16 June 2022

Storage:

Finished product, *Shatavari-Shatapushpa Choorna* was stored in plastic bags of 500 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

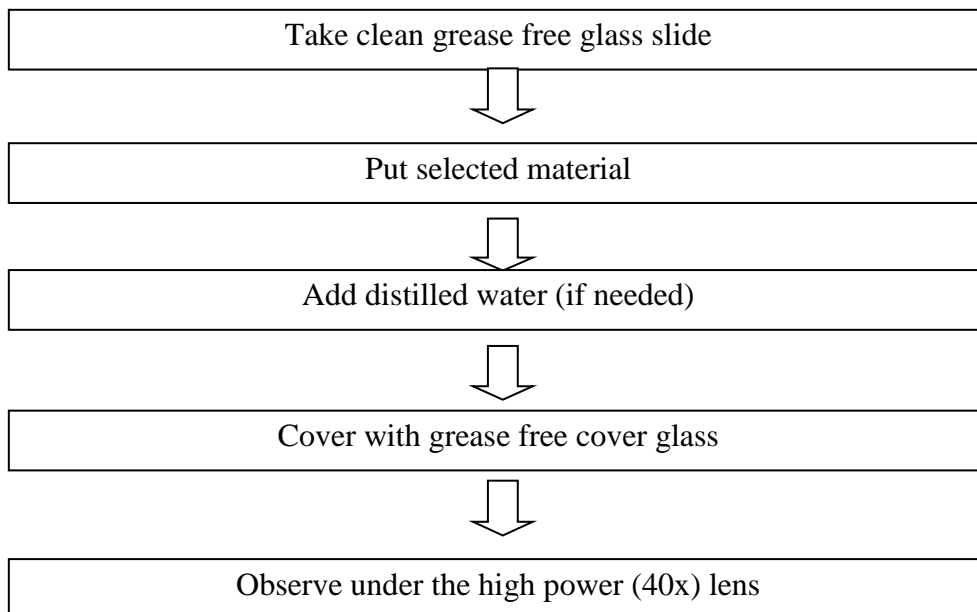
- A) Fungal culture
- B) Aerobic culture

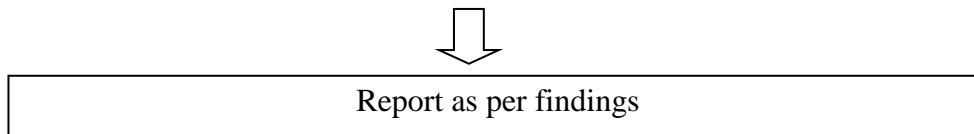
The details of the procedures of each specimen is as follow-

1. Smear Examination:**A. Wet mount /10% K.O.H. Preparation:ⁱⁱⁱ**

Aim: To rule out any mycological findings.

Specimen: *Shatavari-Shatapushpa Choorna*

Procedure for Wet Preparation:

**Procedure For 10% KOH Preparation:**

Take Potassium Hydroxides pellets in distilled water to prepare 10% of the same in clean glass tube & mix well

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graph TD; A[ ] --> B[Take clean grease free glass slide];
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Take clean grease free glass slide

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graph TD; A[ ] --> B[Put a drop of specimen and add freshly prepared 10% KOH, then cover with grease free cover glass];
```

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

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graph TD; A[ ] --> B[Allow it to react for 15-20 minutes to remove extra debris other than fungal particles];
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Allow it to react for 15-20 minutes to remove extra debris other than fungal particles

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graph TD; A[ ] --> B[Observe under high power (40x) lens];
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Observe under high power (40x) lens

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graph TD; A[ ] --> B[Report as per findings];
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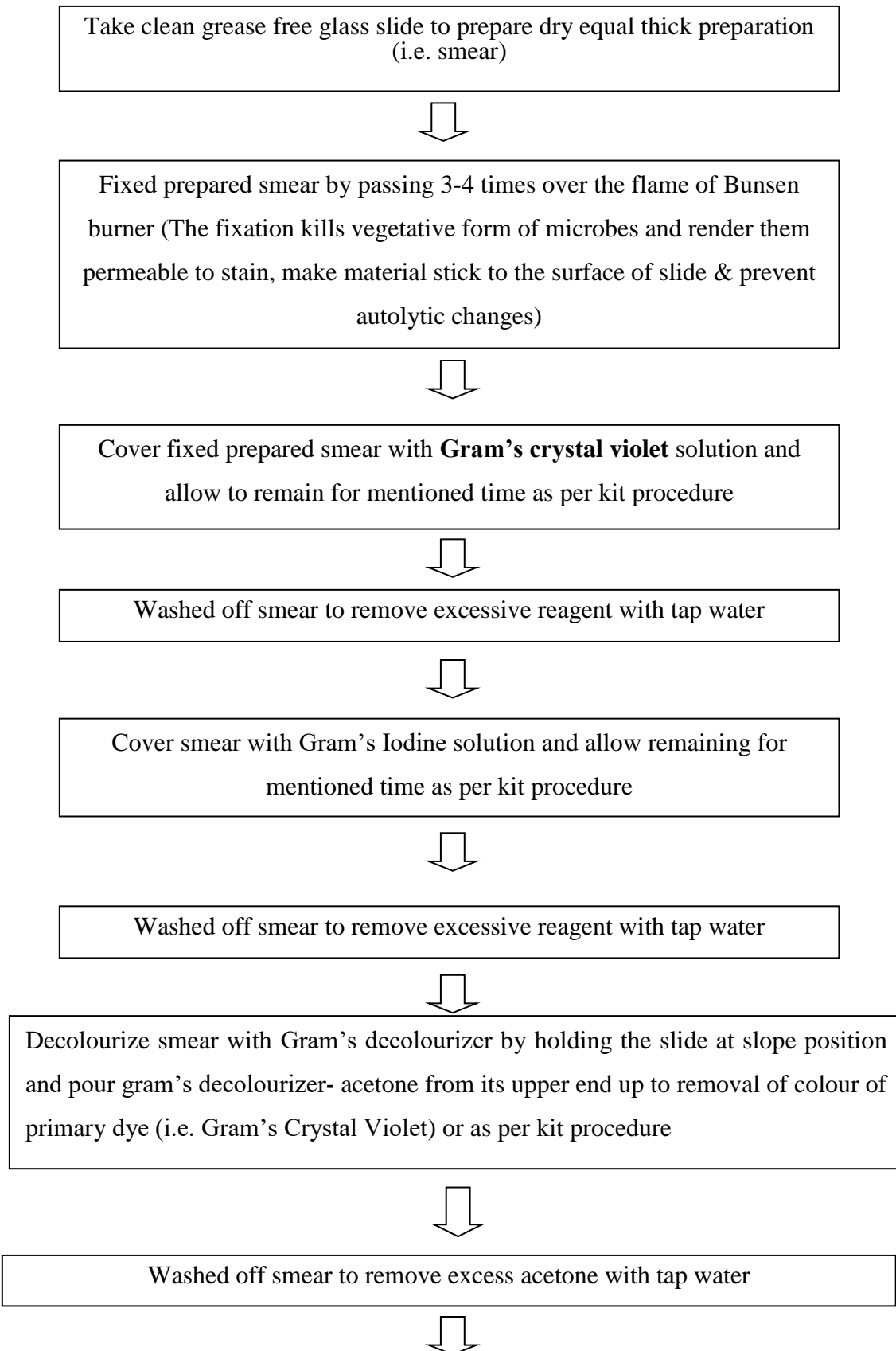
Report as per findings

B. Gram's stain test:^{iv}

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)^y

Aim: To rule out any bacteriological findings.

Specimen: *Shatavari-Shatapushpa Choorna*

Procedure for Gram's Stain

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:^{vi}

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 °C

Use of media: For selective cultivation of pathogenic fungi.



Figure 4 -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



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



After inoculation / streaking process incubate inoculated medium in inverted position at 37⁰ C for 5 to 7 to 21 days in incubator (incubation days are as per growth requirement) under aerobic atmosphere



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

B. Aerobic culture method:^{vii}

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 °C

Use of media : for selective cultivation of pathogenic bacteria.

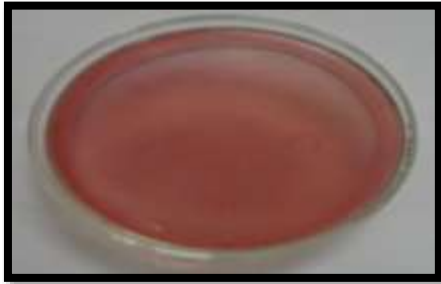


Figure 5: Aerobic culture media (MA)

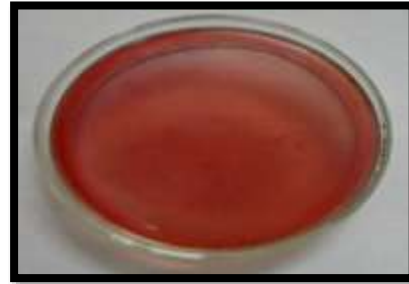


Figure 6: Aerobic culture media (BA)

Procedure for Aerobic Culture

In the clinical microbiology laboratory culture method are employed for isolation of organism (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 specimen to be cultured. One loop full of the specimen is transferred onto the surface of well dried plate)



After streaking process incubate inoculated medium in inverted position at 37⁰c for 18-24 hours in incubator under aerobic or 10% CO₂ 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

Observation and result:

Table 1: Showing observations of sample preserved at room temperature of *Shatavari-Shatapushpa Choorna*

Sr no	Days of investigation after preparation of the sample at	Bag No. & Date of Sample given	Temperature and humidity	Gram's Stain	Aerobic culture	Wet mount/ 10% KOH Preparation	Fungal culture
1.	42 nd day	Bag No. 1 29/07/2022	34° C, 90%	Many gram neg ative rods were seen	Escherichia coli	Fungal filaments not seen.	No fungal pathogen isolated
2.	47 th day	Bag No. 4,5,6,7,8 03/08/2022	33° C, 90%	Many gram negative rods were seen	Escherichia coli	Fungal filaments not seen.	No fungal pathogen isolated
3.	60 th day	Bag No. 9 16/08/2022	29°C, 96%	Many gram-negative rods were seen	Escherichia coli	Fungal filaments not seen.	No fungal pathogen isolated
4.	73 rd day	Bag No. 9 29/08/2022	33°C,91%	Microorganism s not seen	Organisms not seen	Fungal filaments not seen.	No fungal pathogen isolated
5.	163 rd day	Bag No. 10 28/11/2022	33°C,34%	Microorganism s not seen	Organisms not seen	Fungal filaments not seen.	No fungal pathogen isolated
6.	193 rd day	Bag No. 11 28/12/2022	30°C,66%	Microorganism s not seen	Organisms not seen	Fungal filaments not seen.	No fungal pathogen isolated
7.	226 th day	Bag No. 13 31/01/2023	28°C,71%	Microorganism s not seen	Organisms not seen	Fungal filaments not seen.	No fungal pathogen isolated

8.	302 nd day	Bag No. 13 17/04/2023	42°C,25%	Many gram-negative rods were seen	Escherichia coli	Fungal filaments not seen.	No fungal pathogen isolated
9.	309 th day	Bag No. 14 24/04/2023	41°C,24%	Microorganisms not seen	Organisms not seen	Fungal filaments not seen.	No fungal pathogen isolated
10.	338 th day	Bag No. 15 23/05/2023	41°C,78%	Many gram-negative rods were seen	Escherichia coli	Fungal filaments not seen.	No fungal pathogen isolated
11.	375 th day	Bag No. 16 30/06/2023	31°C,99%	Microorganisms not seen	Organisms not seen	Fungal filaments not seen.	No fungal pathogen isolated

DISCUSSION:

Ayurveda, a science of life, gives promising results in many diseases like non-obese PCOS in which no promising results are established. In this research study, *Shatavari-Shatapushpa Choorna* has been chosen to give the patients enrolled in the clinical study for the management of non-obese PCOS. For the safety purpose, it is needed to be proved safe on microbiological profile. Hence the present study was carried out to observe the stability study of *Shatavari-Shatapushpa Choorna* with respect to microbial contamination of sample prepared and preserved at different climacteric and temperature conditions. The area where the medicine was manufactured, and the sample was kept as close to the seaside; also boasts the most extended seashore and the most seaports. Therefore, relative humidity (RH) is consistently high throughout the year, regardless of the season. The highest RH^{viii} recorded was 99% in June 2023, while the lowest RH was recorded in April 2023 at 24%. High RH & specific temperature provided facilitate medium for cultivation of microorganisms.^{ix} For long term storage, moisture content of drug plays a key role in seacoast area. Moisture contents also acts as an enzymatic activator which slowly decompose the drug resulting in its degradations well as drug deterioration.^x Thus, a baseline Microbial profile was studied at regular interval of 1 month after preparation of *Shatavari-Shatapushpa Choorna* approximately for about one year (i.e., total time duration for consumption of prepared drug from drug preparation time i.e. 16th June 2022) At the end of study, it was observed that microbial contamination in the prepared drug at some temperature and humidity of the atmosphere as per shown in the table of observation.

CONCLUSION:

At different time interval, prepared drug named *Shatavari-Shatapushpa Choorna* checked at Microbiology laboratory, ITRA to rule out microbial contamination in prepared form (powder) of final product. 24% minimum humidity with 41°C temp and 99% maximum humidity with 31°C temp and common temperature range varies from 28°C to 42°C was found during total study period from July 2022 to June 2023. There was microbes cultivation found in prepared drug after 42nd, 47th and 60th day of drug preparation. E coli bacteria isolated in vitro at 37°C & accuweather shows 34°C temp & 90% humidity, 33°C temp. & 90% humidity & 29°C temp. & 96% humidity respectively. After dry heat sterilization at 75°C for 7 days in hot air oven. *Shatavari-Shatapushpa Choorna* tested at 73rd days, 163rd days, 193rd days & 226th days after drug preparation date and *Shatavari-Shatapushpa Choorna* found free from microbes. At 302nd day & 338th day after drug preparation accuweather shows at 25% Rh with 42°C and 78% Rh with 41°C, *Shatavari-Shatapushpa Choorna* again found contamination with E. coli bacteria and again reprocessed remaining product by means of dry heat sterilization at 75°C for 7 days in hot air oven. *Shatavari-Shatapushpa Choorna* tested at 309th days, 375th days after drug preparation date and *Shatavari-Shatapushpa Choorna* found free from microbes. Stability of prepared drug found at minimum humidity of 24% with 41°C temperature and at maximum humidity of 99% with 31°C temperature.

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