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Original article

Stability Study of *Punaranavadi Guggulu*- an Ayurvedic Formulation for Pelvic Inflammatory Diseases with respect to Baseline Microbial Diagnostic Modalities

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ABSTRACT:
Background: Pelvic Inflammatory Diseases (PID) are caused by micro-organisms colonizing the endocervix and ascending to the endometrium and fallopian tubes. According to Ayurveda, *Shothahara* and *Shoolahara* drugs can be used. *Punarnavadi Guggulu* is having property of same. Aim: The present study was carried out to evaluate the stability study of powder of *Punarnavadi Guggulu* with respect to microbial contamination of sample prepared and store in different climatic conditions and temperature. Materials and methods: Sample of powder of *Punarnavadi Guggulu* was prepared and studied to check microbial contamination at regular time intervals. Results: Every time sample was subjected to the microbiological study from the date of the preparation to the date of completion of clinic study. No any contamination was found in microbiological study. Discussion: The stability test of powder of *Punarnavadi Guggulu* with respect to microbiological findings was negative at room temperature, warm and cold, dry and humid conditions, showed good shelf life. Conclusion: The present baseline microbial profile was supported that *Punarnavadi Guggulu* was suitable in different humidity and temperature after studied at regular interval of 1 month between October 2020 to January 2022.

Keywords: Ayurveda, microbiological study, *Punarnavadi Guggulu,* Stability test.

1. INTRODUCTION

Pelvic Inflammatory Disease (PID) is an upper genital tract infection, which may affect the uterus, fallopian tubes, ovaries and peritoneum. PID can begin as cervicitis, progress to endometritis, followed by involvement of the fallopian tubes as pyosalpinx and ultimately involve the ovary as a tubo-ovarian abscess [1]. In this condition, drug having *Shothahara*, *Aamapachana*, *Shoolahara* and *Vatanulomana* properties can be used. *Punarnavadi Guggulu*is having the same property [2].

Stability research provides proof of how the quality of a drug substance or formula changes over time, affected by a humidity, temperature, microbial contamination and storage condition.

The main purpose of this study to provide baseline microbial stability study and suitable for this use over time period. Ayurvedic Indian Formulary has also provided the time from the date of manufacture during which the formulations should be consumed for better results. According to ayurvedic manuscripts, '*Saviryata Avadhi'* term is mentioned in context of the time period during which the *Virya* (potency) of any drug remains unaffected due to environmental/microbial deterioration. According to ayurvedic pharmaceutical science, *Guggulu* preparations potent up to 5 years [3]. Thus, an effort has been made to evaluate the shelf life of *Punarnavadi Guggulu* with respect to microbial contamination of sample in different climatic conditions (temperature and humidity set ups). Thus a baseline microbial profile was studied at regularly in monthly intervals for total of 15 months (from 26/10/2020 to 05/01/2022). Aim of this article is to study the stability of *Punarnavadi Guggulu*at different climatic conditions (temperature and humidity set ups) to rule out any microbial contamination.

2. MATERIALS AND METHODS

Drug material

Punarnava, Devadaru, Haritaki, Guduchi and *Guggulu* were collected from the Pharmacy of Gujarat Ayurved University, Jamnagar. *Gomutra* was collected from local Gaushala of Jamnagar.Identification and authentication of all procured raw drugs were done from Pharmacognosy laboratory of ITRA, Jamnagar before manufacturing [Table 1].

Preparation of Drug

The drug was prepared in pharmacy of Gujarat Ayurved University, Jamnagar by adopting procedure given for *Guggulu* preparation.

Date of preparation: 01/08/2020

Method of preparation of *Punarnavadi Guggulu*

Impure *Guggulu* was added in filtered *Gomutra* overnight. The next day, it was boiled at a low temperature (100° C) without covering it. Continuous stirring was done. When it was converted into a soft mass, it was transferred to a small vessel to avoid the burning of *Guggulu* at the bottom of vessel. The Decoction was prepared from coarse powder of *Punarnava, Devadaru, Haritaki* and *Guduchi* with 8 parts of water. It was boiled until the water was reduced up to 4^{th} part. Then decoction was filtered.Purified *Guggulu* was added to filtrate decoction and was boiled till *Guggulu* was about to have *Asanna Pakva* phase.Then *Vati* of 500 mg was prepared and stored in airtight container under hygienic condition [4].

Methods

Microbial profile

Microbial contamination was assessed by two methods (Smear examination and Culture study) to check any mycological findings and bacteriological findings.

- **Smear Examination-**
  - A) Wet mount /10% K.O.H. Preparation
  - B) Gram's stain
- **Culture Study-**
  - A) Fungal culture
  - B) Aerobic culture

The details of the procedures followed are given below.

- **Smear Examination:**

o **Wet mount /10% K.O.H. Preparation:**

Objective: To rule out any mycological findings

Specimen: *Punarnavadi Guggulu*

Procedure for Wet Preparation

Take a clean grease free glass slide then put selected material and add distilled water (if needed). Cover with grease free cover glass, then Observe under the high power (40x) lens. Report as per findings (if found).

Procedure for 10% KOH Preparation

Take Potassium Hydroxides pelletsin distilled water to prepare 10% of the same in clean glass tube & mix well. Take a clean grease free glass slide and put a drop of specimen and add freshly prepared 10% KOH then cover with grease free cover glass. Allow it to rest for 15-20 minutes to remove extra debris other than fungal particles. Observe under high power (40x) lens and report as per findings (if found).

o **Gram's stain test:**

Gram staining is a differential staining technique that differentiates bacteria into two groups: gram positive and gram negative. The procedure is based on the ability of microorganisms to retain color of the stains used during the gram stain procedure. Gram negative bacteria are decolorized by any organic solvent (acetone or Gram's decolorizer) 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 color of the stains, based on the differences in the chemical and physical properties of the cell wall [5].

Aim: To rule out any bacteriological findings in the specimen.

Specimen: *Punarnavadi Guggulu*

Procedure for Gram' Stain

Take a 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 the material stick to the surface of slide & prevent autolytic changes). Then cover fixed prepared smear with **Gram's crystal violet** solution and allow remaining for mentioned time as per kit procedure. Washed off smear to remove excessive reagent with tap water than 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. Decolorize 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 color of primary dye (i.e. Gram's Crystal Violet) or as per kit procedure. Washed off smear to remove excess acetone with tap water than 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 than blot and allow to dry smear. Blot and allow to dry smear Examine under oil immersion lens and report as per findings (if found).

- **Culture Study:**

o **Fungal culture**

Sample 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.

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) by choosing appropriate selective solid media for inoculation purpose. Dry selective solid media in Hot Air Oven **before** specimen inoculation and allow it 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 37^0 c for 05 to 07 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 (if found).

- **Aerobic culture method:**

Sample of *Punarnavadi Guggulu* collected with sterile cotton swab for inoculation purpose on selected aerobic culture media (i.e. an artificial preparation)

Name of media : Mac Conkey 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.

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, then dry selective solid media in Hot Air Oven before specimen inoculation and allow it to cool dried 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 37^0c for 18-24 hours in incubator under aerobic or 10% CO_2 atmosphere. After that incubation period is examined the growth in form of colony and confirm growth by performing different related biochemical reactions and different related staining procedures (Figure1, 2 and 3). After that reports the isolates (if found).

Fig 1: Smear staining Procedure

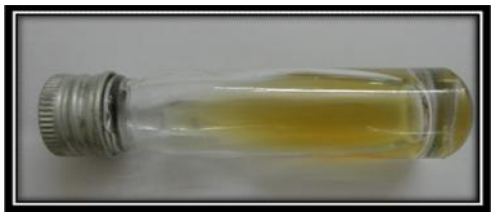

Fig 2: Sabouraud Dextrose Agar Base Media used for cultivation of any Fungal Contamination (SDA) bottle

Fig 3: MacConkey Agar (MA) preparation

3. RESULTS AND DISCUSSION

Each and every time before giving to the patients, sample of *Punarnavadi Guggulu* were subjected to the microbiological study from the date of the preperation to the date of last consumption of *Punarnavadi Guggulu*. Observation and results are shown in table no. 2.

Drug should be free from any type of microbial contamination for better safety and efficacy. Stability of drug is expressed in terms of its shelf-life. The intrinsic and extrinsic factors (FDA report 2001) affect stability of prepared drug. Intrinsic factors include moisture content, acidity, nutrient content, biological structure, redox potential, naturally occurring and added antimicrobials. Extrinsic factors include types of packaging, effect of time/temperature on microbial growth, storage/ holding conditions and processing steps (FDA report 2001). Microbial contamination should avoid increasing drug stability and storage time. *Punarnavadi Guggulu* was prepared and stored in tight container at room temperature. Sample was selected randomly for study of microbiological contamination. During this study, changes in temperature and humidity of environment was observed.

Optimum temperature for microbial growth is temperature at which microbes multiplies, this optimum temperature for psychrophilic bacteria (low temperate loving) is -20 to +10 °C while for mesophilic bacteria (moderate temperate loving) and thermophilic (high temperate loving) bacteria is 20-45 °C and 41-122 °C respectively. The region where the

drug was prepared and sample was stored was very proximal to sea coast, this area has longest sea shore and maximum number of sea ports, so relative humidity (RH) remains high in all the seasons of the year. Highest RH observed was 73 % in month of September while lowest relative humidity was 14% observed in month of November (as shown in Table 1). High RH may allow the growth of microbes [6], although air cannot be considered dry at RH more than 40%. Wet mount, fungal culture, gram stain and aerobic culture tests were used to rule out any fungal and bacterial contamination in the sample of monthly interval from 26/10/2020 to 05/01/2022. During this study period, any microbes were not isolated as a result of aerobic culture and any fungal pathogens were not isolated as a result of fungal culture (as shown in Table 1). Moisture content of drug play a key role in its long-term storage in sea coast area. Moisture contents also acts as an enzymatic activator which slowly decompose the drug resulting in its degradation as well as drug deterioration [7]. There were not found any microbial growth during above period. Hence, *Punarnavadi Guggulu* could be safe in relative humidity, temperature and also within shelf-life period as mentioned in Gazettes of India.

Table 1: Contents of *Punarnavadi Guggulu*

S.no.	Drug	Botanical name	Part used	Ratio
1	*Punarnava*	*Boerhaavia diffusa* Linn.	Whole plant	1.5 Part
2	*Devadaru*	*Cedrus deodara* Roxb. Loud.	Stem	1.5 Part
3	*Haritaki*	*Terminalia chebula* Retz.	Fruit	1.5 Part
4	*Guduchi*	*Tinospora cordifolia* Willd.	Stem	1.5 Part
5	*Gomutra*	---		1 liter
6	*Guggulu*	*Commiphora mukul* Engl.	Gum	6.5 Part

Table 2: Showing observations of sample of *Punarnavadi Guggulu* preserved in air tight container

Prepared batch	Date of investigations After prepa-ration of the sample	Temp. (°C)	Humi-dity	Observations of sample			
				Gram's Stain	Aerobic Culture	Wet mount/ 10%KOH Prepa-ration	Fungal culture
1	26/10/2020	35°	26%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated
2	02/12/2020	35°	29%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated
3	04/01/2021	32°	32%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated
4	03/02/2021	31°	30%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated
5	04/03/2021	37°	22%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated
6	06/04/2021	31°	42%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated
7	11/05/2021	40°	26%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated
8	02/06/2021	40°	30%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated
9	05/07/2021	37°	44%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated
10	18/08/2021	32°	55%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated
11	07/09/2021	32°	73%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated
12	06/10/2021	32°	59%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated
13	10/11/2021	34°	14%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated
14	06/12/2021	31°	29%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated
15	05/01/2022	27°	58%	Microorganism not seen	No organisms isolated	Fungal filament not seen	No fungal pathogen isolated

4. CONCLUSION

Microbiological safety is important factor to determine shelf-life of drugs. Hencemicrobiological study of the *Punarnavadi Guggulu* showed that the quality of *Guggulu* is in a standard condition. There were no any growth of bacterial and fungal microorganisms found, from the date of preparation i.e. 26th October 2020 till last consumption i.e. 05th January 2022 for total of 16 months, shows its good shelf life according to baseline microbiological study.

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