Stability study of *Amalakibhavita nisha* used in the management of type 2 diabetes (*Madhumeha*) - with respect to baseline microbial diagnostic modalities

Vasundhara Sharma, Sachin Kumar Sharma, MS Cholera, DH Pandya and Anup Thakar

**Abstract**

**Background:** Diabetes mellitus is a clinical syndrome characterised by hyperglycaemia caused by absolute or relative deficiency of insulin. In *Ayurveda* disease type 2 diabetes can be correlated with *Madhumeha*. *Madhumeha* (Type 2 Diabetes) is caused by the involvement of all Doshas and ten Dushyas. *Nisha Amalaki* is one of the *Ayurvedic* formulation widely used in the management of *Madhumeha* (Diabetes Mellitus). In the present study 7 *Bhavana* (trituration) of *Amlaki* (*Emblica officinalis*) Swaras (juice) have been given to *Haridra Choorna* (*Curcuma longa*).

**Aim:** To carried out study of *Amalakibhavita Nisha* with respect to its stability against microbial contamination

**Materials and Methods:** Sample of *Amalakibhavita Nisha* was prepared and studied at regular time intervals to check microbial contamination. *Vati* was stored in plastic container during different climatic conditions and temperature with respect to Microbial Contamination of prepared sample. Metabological findings and presence of bacteriological findings by Wet mount preparation and Gram stain test respectively. Sample was subjected to the microbiological study from the date of the preparation (17 January 2017) to the date of last microbiological study (30 April 2018). No any contaminations were found in microbiological study.

**Discussion:** This study was carried out to observe the stability study of *Amalakibhavita Nisha* store in different climatic conditions and temperature with respect to Microbial Contamination of prepared sample. Thus a baseline Microbial profile was studied at regular interval of 1 month for total 15 month (i.e. time for consumption of prepared drug). At the end of study sample was not showed presence of any Microbes.

**Conclusion:** At the end of study *Vati* container has not present of microbes even in different climate and temperature, after 15 months of preparation sample. Hence in present study the stability test of *Amalakibhavita Nisha* with respect to microbiological findings was negative at room temperature, warm and cold, dry and humid conditions.

**Keywords:** *Amalakibhavita Nisha*, climate conditions, microbial profile, stability, type 2 diabetes

1. **Introduction**

Diabetes mellitus is a clinical syndrome characterised by hyperglycaemia caused by absolute or relative deficiency of insulin. Hyperglycaemia, or raised blood sugar, is a common effect of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, especially the nerves and blood vessels. In *Ayurveda* disease diabetes mellitus can be correlated with *Prameha/Madhumeha*. It is *Tridosha* jain origin with involvement of ten Dushyas. *Nisha Amalaki* is very effective formulation mentioned in *Ashtanga Hridaya*. *Amalaki* (*Emblica officinalis*), family Euphorbiaceae, is sweet, sour, astringent, pungent and bitter in taste. The other ingredient is *Haridra* (*Curcuma longa*), family Zingiberaceae, it is *tridoshasamak*. *Haridra* due to bitter taste pacifies *Pitta* and because of hot potency pacifies *Vata* and *Kaphadosha*. Till date no scientific work has been reported on effect of *Bhavana* on *Haridra choorna* by *Amlaki* swaras.

According to the World Health Organization (WHO) report, India today heads the world with over 32 million diabetic patients and this number is projected to increase to 79.4 million by the year 2030. Recent surveys indicate that diabetes now affects a staggering 10-16% of urban population and 5-8% of rural population in India.
Ayurvedic management of Madhumeha (Type 2 Diabetes) aims not only to achieve a good glycaemic control but also to treat the root cause of disease and its prevention. For the first time the research work carried out for its authentication and microbial profile. The drug was prepared in pharmacy of Gujarat Ayurved University, Jamnagar by adopting standard operative procedure for Vati formation. No any preservative was added to the test drug. Drug preparation was finished on 17 January 2017. Finished product was stored in airtight plastic containers at room temperature. Thus in the present study on attempt was taken to check stability of Vati with respect to its Microbial profile at different climatic conditions and temperature setups at regular interval for a period of 13 months.

Aim
To study the stability and to check microbial contamination of Amalakibhavit Nisha at different time interval, at different climatic conditions, temperature and humidity set ups.

Materials and Methods
Sample of Amalakibhavit Nisha was prepared (stored at room temperature) and finished product studied to check microbial contamination at regular intervals of 1 month for a period of 13 months (upto drug used). Microbiological study has been carried out in Microbiology Laboratory, IPGT & RA, 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 86th day of preparation. Then samples from same container were subjected to the microbiological study regularly with intervals of 1 month during different seasons.

Drug material
All the raw drugs were obtained from Pharmacy of Gujarat Ayurved University, Jamnagar. The ingredients and the part used are given in table 1.

Table 1: Ingredients of Amalakibhavit Nisha (A.H.Utt.40/48)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Latin name</th>
<th>Part to be used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisha (Haridra)</td>
<td>Curcuma longa Linn</td>
<td>Rhizome</td>
</tr>
<tr>
<td>Amalaki</td>
<td>Embelica officinalis Gaertn.</td>
<td>Fruit juice</td>
</tr>
</tbody>
</table>
*Seven Bhavna of Amlaki swaras have been given to Nisha (Haridra) Choorna. After that Vati were prepared.

Date of drug preparation: 17 January 2017
Storage
Finished product of Amalakibhavit Nisha was stored in air-tight food grade, plastic containers, 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) 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 followed are given below

1. Smear Examination
   A. Wet mount /10% K.O.H. preparation
   Aim: To rule out any mycological findings.
   Specimen: Amalakibhavit Nisha

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)
- Report as per findings
Procedure for 10% KOH preparation

1. Take Potassium Hydroxide pellets in distilled water
2. To prepare 10% of the same in clean glass tube & mix well
3. Take clean grease free glass slide
4. Put a drop of specimen and add freshly prepared 10% KOH, then cover with grease free cover glass
5. Allow it to react for 15-20 minutes to remove extra debris other than fungal
6. Observe under high power (40x) lens
7. Report as per findings

Procedure for gram’s stain

1. Take clean grease free glass slide to prepare dry equal thick preparation (i.e. smear)
2. 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)
3. Cover fixed prepared smear with Gram’s crystal violet solution and allow to remain for mentioned time as per kit procedure
4. Washed off smear to remove excessive reagent with tap water
5. Cover smear with Gram’s Iodine solution and allow remaining for mentioned time as per kit procedure

B. 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 colour 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 (Alfred E Brown, 2001) [8].

Aim: To rule out any bacteriological findings.

Specimen: Amalakibhavit Nisha
**Procedure for gram’s stain**

1. Take clean grease free glass slide to prepare dry equal thickness preparation (i.e. smear).

2. Fix prepared smear by passing 3-4 times over the flame of Bunsen burner (to fix the vegetative form of microorganisms and render them permeable to stain, making material stick to the slide & prevent autolytic changes).

3. Cover fixed prepared smear with Gram’s crystal violet solution and allow to remain for mentioned time as per kit procedure.

4. Washed off smear to remove excessive reagent with tap water.

5. Cover smear with Gram’s Iodine solution and allow remaining for mentioned time as per kit procedure.

6. Washed off smear to remove excessive reagent with tap water.

7. 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.

8. Washed off smear to remove excess acetone with tap water.

9. Cover smear with Safranin solution and allow remaining for mentioned time as per kit procedure.

10. washed off smear to remove excessive reagent with tap water.

11. Blot and allow to dry smear.

12. Examine under oil immersion lens and report as per findings.

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Fig 1-2: Smear staining Procedure
Culture Study

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: MacConkey Agar (MA) and Columbia Blood agar (BA)

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 loopful of the specimen is transferred onto the surface of well dried culture media)

After inoculation / streaking process incubate inoculated medium in inverted position at 37ºC 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.

Fig 3: Stained smear ready for examination

Fig 4: Sabouraud Dextrose Agar Base (SDA)

Fig 5: Aerobic culture media (MA)

Fig 6: Aerobic culture media (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

Observations & Results
Every time sample (in which drug preserved) were subjected to the microbiological study from the date of the preparation to the date of last microbiological study. Results are shown in table 2.

Table 2: Showing observations of sample preserved at room temperature

<table>
<thead>
<tr>
<th>S. N.</th>
<th>Months of investigations</th>
<th>Temperature</th>
<th>Humidity</th>
<th>Gram’s Stain</th>
<th>Aerobic culture</th>
<th>Wet mount/ 10% KOH Preparation</th>
<th>Fungal culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3rd month (13/04/2017)</td>
<td>41°C</td>
<td>30%</td>
<td>Microorga-nisms not seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
<td>No fungal pathogen isolated</td>
</tr>
<tr>
<td>2</td>
<td>4th month (17/05/2017)</td>
<td>43°C</td>
<td>35%</td>
<td>Microorga-nisms not seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
<td>No fungal pathogen isolated</td>
</tr>
<tr>
<td>3</td>
<td>5th month (13/06/2017)</td>
<td>41°C</td>
<td>38%</td>
<td>Microorga-nisms not seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
<td>No fungal pathogen isolated</td>
</tr>
<tr>
<td>4</td>
<td>6th month (18/07/2017)</td>
<td>32°C</td>
<td>74%</td>
<td>Microorga-nisms not seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
<td>No fungal pathogen isolated</td>
</tr>
<tr>
<td>5</td>
<td>7th month (23/08/2017)</td>
<td>30°C</td>
<td>80%</td>
<td>Microorga-nisms not seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
<td>No fungal pathogen isolated</td>
</tr>
<tr>
<td>6</td>
<td>8th month (21/09/2017)</td>
<td>33°C</td>
<td>69%</td>
<td>Microorga-nisms not seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
<td>No fungal pathogen isolated</td>
</tr>
<tr>
<td>7</td>
<td>9th month (12/10/2017)</td>
<td>33°C</td>
<td>61%</td>
<td>Microorga-nisms not seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
<td>No fungal pathogen isolated</td>
</tr>
<tr>
<td>8</td>
<td>10th month (16/11/2017)</td>
<td>34°C</td>
<td>30%</td>
<td>Microorga-nisms not seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
<td>No fungal pathogen isolated</td>
</tr>
<tr>
<td>9</td>
<td>11th month (20/12/2017)</td>
<td>29°C</td>
<td>24%</td>
<td>Microorga-nisms not seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
<td>No fungal pathogen isolated</td>
</tr>
<tr>
<td>10</td>
<td>12th month (16/01/2018)</td>
<td>34°C</td>
<td>36%</td>
<td>Microorga-nisms not seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
<td>No fungal pathogen isolated</td>
</tr>
<tr>
<td>11</td>
<td>13th month (15/02/2018)</td>
<td>32°C</td>
<td>24%</td>
<td>Microorga-nisms not seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
<td>No fungal pathogen isolated</td>
</tr>
<tr>
<td>12</td>
<td>14th month (26/03/2018)</td>
<td>42°C</td>
<td>28%</td>
<td>Microorga-nisms not seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
<td>No fungal pathogen isolated</td>
</tr>
<tr>
<td>13</td>
<td>15th month (30/04/2018)</td>
<td>42°C</td>
<td>32%</td>
<td>Microorga-nisms not seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
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Ayurveda is widely used in management of Madhumeha (Type 2 Diabetes). In the present study Amalakibhavita Nisha has been used in the management of Madhumeha. In present study, it has shown very good and encouraging results in management of Madhumeha (Type 2 Diabetes). Hence the present Study was carried out to observe the stability study of Amalakibhavita Nisha with respect to Microbial Contamination in different climatic and temperature conditions. Thus a baseline Microbial profile was studied at regular interval of 1 month for total 13 months for Amalakibhavita Nisha. At the end of study it was found that sample has not shown presence of any Microbes. 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. Microorganisms needs water, humidity and temperature at suitable environmental conditions to develop in any media, surface or article. The factors affecting stability of prepared drug are categorized under intrinsic and extrinsic factor (FDA report 2001). 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). 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 80% in month of August while lowest relative humidity was 24% observed in month of February (as shown in Table 2). High RH may allow the growth of microbes, RH remain variable during whole study period. 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 13th April 2017 to 30th April 2018. During this study period no any microbes were isolated as a result of aerobic culture and no any fungal pathogen were isolated as a result of fungal culture (As shown in Table 2). Moisture contents main causative factor in drug deterioration, it also act as an enzymatic activator which slowly decompose the drug resulting in its degradation. 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. Microorganisms needs water, humidity and temperature at suitable environmental conditions to develop in any media, surface or article. The factors affecting stability of prepared drug are categorized under intrinsic and extrinsic factor (FDA report 2001). 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Conclusion

Shelf life is the time period from when the product is produced until the time it is planned to be consumed or used. Several factors are used to determine a product’s shelf-life, ranging from organoleptic qualities to microbiological safety. Hence Microbiological study of the Amalakibhavita Nisha showed the quality of Vati in standard condition. There were no growth of microorganisms (bacterial or fungal) found, till 30th April 2018 i.e. 15th month from the date of preparation of Amalakibhavita Nisha, which shows its good shelf life.

References