STABILITY STUDY OF MODIFIED MUSTA-TRIPHALADI AVALHEA WITH RESPECT TO BASELINE MICROBIAL PROFILE USED IN THALASSEMA MAJOR

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ABSTRACT

Introduction: stability study of Modified Musta- Triphaladi Avaleha was done for 12 months (1 year) to know the stability of formulation in reference of its phyto-constituents and microbial growth therein. Modified Musta-Triphaladi Avaleha have been used in Thalassemia children as an adjuvant therapy prepared with various herbal product. Methods: Modified Musta-Triphaladi Avaleha was made in Pharmacy with standard classical method (Avaleha Kalpana) as per AFI part-1 (The Ayurvedic formulary of India, part-1 part- A) In present study, stability with respect to its Microbial profile of Modified Musta-Triphaladi Avaleha carried out. Avaleha was stored in 2 plastic containers during different climatic condition. Avaleha were studied at regular intervals for a period of 1 month to analysis Mycological findings and presence of Microorganisms by wet mount preparation and Gram stain test respectively. Results: At the end of study both Avaleha container has not present of microbes after 1 year of preparation, even in different climate and temperature. Conclusion: Since it is traditional Avaleha kalpana, the shelf life period was about one year (Sharangdhar Samhita) The presented study reflects that Microbiological findings of Modified Musta-Triphaladi Avaleha was negative at room temperature, warm and cold, dry and humid condition.
Keywords: Microbial profile, Modified Musta-Triphaladi Avaleha, Climate condition, Stability.

INTRODUCTION

Thalassemia is a monogenic disorder characterized by abnormal synthesis of hemoglobin due to defects in the globin chain. This causes early excessive destruction of red blood cells leading to hypochromic, microcytic anemia. In Ayurvedic authentic texts, it cannot be found a disease similar to Thalassemia. But the methodology of understanding the unknown disease has been mentioned in Charaka Samhita based on Aaptopadesha Pramana (Authoritative instruction). An effort has been made to correlate Thalassemia major with Beejadushtijanya Paud (Thalassemia Major). An ideal drug to treat Thalassemia should have Deepana (Appetizer), Aamapachana (Digestion of immature juice of food), Srotovishodhana (Opening of circulating micro channels), Tridoshahara, Rochana (Relish), Jwarahara (Antipiretic), Pittasaraaka (Pitta excretor), Lohashodhana (Iron purifier from body), Lohamarana (Iron Cheltor), Lekhana (Scrapping), Bhedana (Purgative), Raktashodhana (Blood Purifier), Raktaprasadana (Blood nutritive), Shonitasthanana (Haemostasis), Varnya (Glowing body complexion), Balya (Strengthen Drug), Brimhana (Anabolic), Rasayana (Enhance longevity & delays ageing) and Vayahasthana (Age Prolonger) properties. Modified Musta-Triphaladi Avaleha is such a combination of all properties. Avaleha is a Good palatable drug for child.

Table 1: Ingredients of Modified Musta-Triphaladi Avaleha

<table>
<thead>
<tr>
<th>No.</th>
<th>Drug Name</th>
<th>Latin Name</th>
<th>Part Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Musta</td>
<td>Cyprus rotundus Nust.</td>
<td>Dry Rhizome</td>
</tr>
<tr>
<td>2</td>
<td>Aamalaki</td>
<td>Emblica officinalis Gaertn.</td>
<td>Dry Fruit</td>
</tr>
<tr>
<td>3</td>
<td>Haritaki</td>
<td>Terminalia chebula Retz.</td>
<td>Dry Fruit</td>
</tr>
<tr>
<td>4</td>
<td>Vibhitaki</td>
<td>Terminalia bellerica Roxb.</td>
<td>Dry Fruit</td>
</tr>
<tr>
<td>5</td>
<td>Katuki</td>
<td>Picrorhiza kurroa Royle ex Benth.</td>
<td>Dry Root</td>
</tr>
<tr>
<td>6</td>
<td>Kakamachi</td>
<td>Solanum nigrum Linn.</td>
<td>Dry Whole plant</td>
</tr>
<tr>
<td>7</td>
<td>Kutaja</td>
<td>Holarrhena antidysenterica Wall.</td>
<td>Dry Bark</td>
</tr>
<tr>
<td>8</td>
<td>Haridra</td>
<td>Curcuma longa Linn.</td>
<td>Dry Rhizome</td>
</tr>
<tr>
<td>9</td>
<td>Vidanga</td>
<td>Embelia robusta Burm</td>
<td>Dry Fruit</td>
</tr>
<tr>
<td>10</td>
<td>Guduchi</td>
<td>Tinospora cordifolia Willd.</td>
<td>Dry Stem</td>
</tr>
<tr>
<td>11</td>
<td>Shweta Punarnava</td>
<td>Trianthema portulacastrum Linn.</td>
<td>Dry Root</td>
</tr>
<tr>
<td>12</td>
<td>Sharapunika</td>
<td>Tephrosia purpurea Linn.</td>
<td>Dry Root</td>
</tr>
<tr>
<td>13</td>
<td>Apamarga</td>
<td>Achyranthus aspera Linn.</td>
<td>Dry Whole plant</td>
</tr>
<tr>
<td>14</td>
<td>Kadali</td>
<td>Musa paradisiacaL Linn,</td>
<td>Dry Rhizome</td>
</tr>
<tr>
<td>15</td>
<td>Shatavari</td>
<td>Aspergus recemosus Willd.</td>
<td>Dry Root</td>
</tr>
<tr>
<td>16</td>
<td>Shigru</td>
<td>Moringa oleifera Lam.</td>
<td>Dry Root bark</td>
</tr>
<tr>
<td>17</td>
<td>Vasa</td>
<td>Adhatoda vasicca Nees</td>
<td>Dry Leaves</td>
</tr>
<tr>
<td>18</td>
<td>Daruharidra</td>
<td>Berberis aristata DC</td>
<td>Dry Root</td>
</tr>
<tr>
<td>19</td>
<td>Sariva</td>
<td>Hemidesmus indicus R.Br.</td>
<td>Dry Root</td>
</tr>
<tr>
<td>20</td>
<td>Manjishtha</td>
<td>Rubia cordifolia Linn.</td>
<td>Dry Root</td>
</tr>
<tr>
<td>21*</td>
<td>Agminantha</td>
<td>Clerodendrum Phlomidis.Linn.</td>
<td>Dry Root</td>
</tr>
<tr>
<td>22*</td>
<td>Rohitaka</td>
<td>Teconomella undulate seem.</td>
<td>Dry Root</td>
</tr>
<tr>
<td>23*</td>
<td>Agatsya</td>
<td>Seshbana grandifolia linn.</td>
<td>Leaves</td>
</tr>
<tr>
<td>24*</td>
<td>Kumari</td>
<td>Aloe barbadensis Mill.</td>
<td>Leaves</td>
</tr>
<tr>
<td>25*</td>
<td>Devadar</td>
<td>Cedrus deodara Roxb.</td>
<td>Dry Bark</td>
</tr>
</tbody>
</table>
The test drug was prepared in the Pharmacy, Gujarat Ayurved University, Jamnagar, by following Standard Operating Procedures (S.O.P.) of preparation of *Avaleha* as practiced in the pharmacy. *Avaleha* is palatable for Children. No any preservative was added to the test drug. Drug preparation was finished on 07.01.2017. Finished product was stored in airtight, sterilized food grade plastic containers at room temperature under hygienic condition. Modified *Musta Triphaladi Avaleha* administered in the Thalassemia major children. It was necessary to prepare the formulation in a better dosage form which is also free from microbial contamination, stability of a pharmaceutical product is the capability of a particular formulation in a specific container or closure system, to remain within its physical, chemical, microbiological therapeutic specifications. Thus in the present study on attempt was taken to check stability of *avaleha* with respect to its Microbial profile at different climacteric conditions and temperature setups at regular interval for a period of 1 year.

**Aim:** To study the microbial contamination in the finished product at different time interval at different climacteric conditions, temperature and humidity set ups.

**Materials and Methods:** Sample; of Modified *Musta Triphaladi Avaleha* were 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, of Institute. Mainly 02 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 on second day of preparation, Before issuing to the patients. Then samples from containers were subjected to the microbiological study regularly with random intervals during different seasons.

**Contents of Samples:**

The sample contents 1 gm of Modified *Musta Triphaladi Avaleha*, 25 ingredients added with Honey & Sarkara, also added Prakshepadravya used specific proportion of all contents it was followed by fix dosage form of *Avaleha* which will easier to take for children. The whole plant of *Sharapunkha* (*Tephrosiapi purpurea* Linn.) was purchased from the local market and rhizomes of *Kadali* (*Musa paradisiacal* Linn.) were collected from the behind of pharmacy, GAU, Jamnagar and *Agatsyapatra* collect from Kodinar, Gujarat. other remaining drugs were provided by raw drug store of Pharmacy. *Agatsyapatra* collected from Kodinar district, Gujarat.

**Preparation Time:** The drug was prepared in the Pharmacy, Gujarat Ayurved University, Jamnagar, by following Standard Operating Procedures (S.O.P.) of preparation of *Avaleha* as practiced in the pharmacy with the utmost caution to avoid any sort of contamination.

**Date of preparation:** 07 January 2017

**Storage:** Finished product of Modified *Musta Triphaladi Avaleha* was stored in air tight, sterilized food grade, plastic containers, smeared with ghee inside, stored in the open light area in the department at room temperature. Clean and dry stainless steel
spoon was used to take medicine. After emptying the container A, container B and C were used respectively in the same manner.

**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 (Chart No. 1&2):**
      Aim: wet mount test for to rule out mycological findings in Avaleha
      Specimen: As above mentioned

   **Chart 1:** Procedure for Wet mount /10% K.O.H. Preparation

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

   **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

2. **Culture Study**
   A) Fungal culture
   B) Aerobic culture

**B. Gram’s stain test (Chart No 3):** 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 (Alfred E Brown, 2001).**

**Aim:** Gram’s stain is to rule out any bacteriological findings from Avaleha.

**Specimen:** As Mentioned above

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**Figure 1 & 2:** Smear staining Procedure

**Chart 3:** 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**
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 upto 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. **Washed off smear to remove excessive reagent with tap water**
10. **Cover smear with Safranin solution and allow remaining for mentioned time as per kit procedure**
11. **Blot and allow to dry smear**
12. **Examine under oil immersion lens and report as per findings**
2. **Culture Study**

**A Fungal culture method as given in Chart No. 4:**
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 3:** Sabouraud Dextrose Agar Base (SDA) bottle

**Chart 4:** 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 onto the surface of well dried culture media]
- After inoculation / streaking process incubate inoculated medium in inverted position at 37°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.

**B. Aerobic culture method as given in Chart No. 5:**
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 4:* MacConkey Agar (MA)

**Chart No 5:** Procedure for Aerobic Culture

1. **Choose** appropriate selective solid media for *inoculation* purpose
2. **Dry** selective solid media in Hot Air Oven *before* specimen inoculation, Allow to *cool* dried medium *before* specimen *inoculation*
3. **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 loopful of the specimen is transferred onto the surface of well dried plate]
4. **After streaking process** *incubate* inoculated medium in inverted position at 37°C for 18-24 hours in incubator under aerobic or 10% CO₂ atmosphere
5. **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

**Observations And 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.
Observation are shown in Table 1.

Table 1: Showing Observation of sample preserved at room temperature.

<table>
<thead>
<tr>
<th>Days of investigations After preparation of the sample at</th>
<th>Date of Sample given</th>
<th>Temperature</th>
<th>Humidity</th>
<th>Observations of sample</th>
<th>Aerobic culture</th>
<th>Fungal culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>23 Days</td>
<td>22nd Feb 2017</td>
<td>32°C</td>
<td>32%</td>
<td>Microorganisms Not Seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
</tr>
<tr>
<td>53 Days</td>
<td>23rd March 2017</td>
<td>36°C</td>
<td>18%</td>
<td>Microorganisms Not Seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
</tr>
<tr>
<td>77 Days</td>
<td>27th April 2017</td>
<td>37°C</td>
<td>73%</td>
<td>Microorganisms Not Seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
</tr>
<tr>
<td>106 Days</td>
<td>22nd May 2017</td>
<td>38°C</td>
<td>34%</td>
<td>Microorganisms Not Seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
</tr>
<tr>
<td>137 Days</td>
<td>23rd June 2017</td>
<td>32°C</td>
<td>75%</td>
<td>Microorganisms Not Seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
</tr>
<tr>
<td>167 Days</td>
<td>24th July 2017</td>
<td>27°C</td>
<td>89%</td>
<td>Microorganisms Not Seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
</tr>
<tr>
<td>203 Days</td>
<td>29th August 2017</td>
<td>28°C</td>
<td>92%</td>
<td>Microorganisms Not Seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
</tr>
<tr>
<td>230 Days</td>
<td>25th Sep 2017</td>
<td>32°C</td>
<td>57%</td>
<td>Microorganisms Not Seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
</tr>
<tr>
<td>261 Days</td>
<td>26th Oct 2017</td>
<td>35°C</td>
<td>26%</td>
<td>Microorganisms Not Seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
</tr>
<tr>
<td>288 Days</td>
<td>23rd Nov 2017</td>
<td>23°C Light rain</td>
<td>28%</td>
<td>Microorganisms Not Seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
</tr>
<tr>
<td>316 Days</td>
<td>21st Dec 2017</td>
<td>28°C Partly sunny</td>
<td>20%</td>
<td>Microorganisms Not Seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
</tr>
<tr>
<td>377 Days</td>
<td>30th Jan 2018</td>
<td>32°C Suny</td>
<td>24%</td>
<td>Microorganisms Not Seen</td>
<td>No organisms isolated</td>
<td>Fungal filaments not seen.</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The unscientific methods of collection, storage, transportation and congenial climatic conditions to allow raw materials for herbal drugs prone to fungal & bacterial infestations. The raw materials collected using unscientific methods commonly exposed to many pathogenic contaminants and are often deteriorated by pathogenic microorganisms during handling and storage. Therefore, lack of regulation for herbal supplement presents potential health risk, largely their contamination chances with pathogenic. Present study was figured to observe the stability study of Modified Musta Triphaladi Avaleha with respect to Microbial Contamination of sample prepared and preserved in different climacteric and temperature conditions. Which has been used in Research work in IPGT & RA in 2017 shows a very good and promising result in Thalassemia. It is needed to keep at safe place will be helpful for safe usage for a long time. Hence primary 10% KOH/wet mount test was used; it detects fungal elements present but may not necessarily identify the species of the fungi. **Gram's method** of staining used to distinguish gram-positive bacteria and gram-negative bacteria. Aerobic & Fungal Culture was also performed in every sitting. It studied at regular interval of 1 month for 1 year. At the end of study, it was observed all three containers were not showed presence of any Microbes.

**CONCLUSION**

Stability is usually expressed in term of Serviceable life, which is the time period from when the product is produced until the time it is intended to be consumed or used. Hence Microbiological study of the Modified Musta-triphaladi Avaleha showed that the quality of Avalehas in a standard condition. There were no growth found of microorganisms (Bacterial or fungal) till 30th Jan 2018 i.e. 01 year from the date of preparation, shows its good shelf life. It can help in future for maintaining its optimum quality and safety.
and also provide guideline to the type of packaging and storage conditions.

REFERENCES


Source of Support: Nil
Conflict of Interest: None Declared