Stability study of granular form of *Pippalikhanda* along with *Trivrittavaleha*, used in treatment of Amlapitta (Hyperacidity) - with respect to baseline microbial diagnostic modalities

Neha Keshari[1], Dr. Cholera MS[2], Prof. A. B. Thakar[3] , Dr. Rahul Gandhi[4] , Dr. N.N.Bhatt[5]

1. MD 3rd year scholar, Department of Panchakarma, IPGT & RA, GAU Jamnagar.
2. Head, Microbiology Laboratory, IPGT & RA, GAU Jamnagar.
3. Director and H.O.D. Department of Panchakarma, IPGT & RA, GAU Jamnagar.
4. Assistant Professor, Department of Panchakarma, IPGT & RA, GAU Jamnagar.
5. Panchakarma physician**,** Department of Panchakarma, IPGT & RA, GAU Jamnagar

Abstract-*Amlapitta* is the disease which is occure mainly due to unhealthy diet and faulty life style. There are several sign and symptoms appears in *Amlapitta* some of them are *Daha*, *Amlodgara*, *Shula*, *Avipaka*, *Chhardi* etc. In modern it is correlated with Hyperacidity. Amlapitta is found to be a disease of *mandagni*. In *Amlapitta* mainly *Rasavaha Raktavaha* and *Annavaha srotas* involved.

Aims: To carried out study for granular form of *Pippalikhanda* along with *Trivrittavaleha* in order to observe stability against microbial contamination.

Materials and Methods: Sample of granular form of *Pippalikhanda* and *Trivrittavaleha* was studied to check microbial contamination at regular time intervals.

Results: From the date of the preparation to the date of last microbiological study at every regular interval sample was subjected to the microbiological study. There was no any contaminations found in microbiological study.

Discussion: Thus this study was done to observe the stability of granular form of *Pippalikhanda* and *Trivrittavaleha* in order to check Microbial Contamination of the drug prepared and store in suitable climatic conditions and temperature. Thus a baseline Microbial profile was studied at regular interval of 1 month for total 10 month (i.e. time for consumption of prepared drug) and finally at the end of study it was found that there was not presence of any microbial contamination in the drug prepared.

Conclusion: In microbiological study of the granular form of *Pippalikhanda* and *Trivrittavaleha,* no growth for microorganisms had been found (bacterial or fungal), till 16th Dec 2018 i.e. approximately 10 month from the date of preparation, shows how stable it is along with its good shelf life. Hence in present study the stability test

of granular form of *Pippalikhanda* and *Trivrittavaleha* with respect to microbiological study was found to be negative at room temperature, dry and humid, warm and cold conditions.

Keywords: *Amlapitta*, Hyperacidity, granular form of *Pippalikhanda* and *Trivrittavaleha***,** microbial contamination.

Introduction- *Pippalikhanda*[i] *granules as shamana* drug and *Trivrittavaleha*[ii] as *Virechana drug* is mentioned in the management of *Amlapitta*. Most of the ingredients are having properties to control acidic condition and improve digestion. *Amlapitta* is one of the most common disease in the present era and highly prevalent due to hectic life style and faulty diet habit. In term of modern medicine it is known as Hyperacidity. Several enzymes and Hydrochloric acid releases from the wall of the stomach which causes gastric inflammation and finally indigestion that leads to Hyperacidity. The pathogenesis of *Amlapitta* involve three important factor i.e. *Agnimandya*, *Ama* and *Annavaha Srotodusti*. Along with this, vitiation of *Pitta* leading to qualitative and quantitative increase of *Pachaka Pitta* especially in its *Amla* and *Drava guna*, *Amlapitta* is the disease which occur due to having *Atiamlata* that leads to *Atidravata* which leads to *Agnimandya* and finally cause *Shuktpaka* of *Rasadhatu* that leads to *Amlapitta*. To overcome the problem of fungus formation in *Khanda kalpana*, *Pippalikhanda* has been prepared in granules form and *trivrittavaleha* remains as usual in *Avaleha* form. To overcome this problem *Pippalikhanda* is selected for *shamana* drug and *Trivrittavaleha* is selected as *Virechana* drug.

Amlapitta is *Amashaya and Grahani sthanajanya vyadhi Vyadhi* that causes distubence in *pittasthana* that leads to indigestion which causes *mandagni* so *Deepana* and *Pachana* administered before the *Virechana* to improve the condition of *agni,*which is followed by *Snehana* and *swedana* that leads to the detachment of doshas from periphery to *koshtha,* and finally *Virechana* causes *Samprapti vighatan of Amlapitta* by removing the *vitiated doshas.*

Most of the drugs in *trivrittavaleha* has *Madhura rasa, Laghu ruksha guna, Ushna virya* and *Katu vipaka. Madhura and Tikta rasa is responsible for the shamana of vitiated pitta. Tikta rasa* causes *Shoshana* of *vitiated Drava guna* of *Pitta.*

Pippalikhanda is selected as *shamana* drug for the treatment of *Amlapitta*. It containing both *ushna* and *sheeta virya* drug, *Sheeta virya* drugs decreases *ushna* and *tiksha Guna* of *vitiated pitta while Ushna virya drugs* decreases *drava guna of vitiated pitta.*

Pippalikhanda was prepared in pharmacy of Gujarat Ayurved University, Jamnagar and *Trivrittavaleha* was prepared in RS & BK department of Gujarat Ayurved University, Jamnagar. Any type of preservative was not added to both the drugs. Preparation of Pippalikhanda was done on 9 February 2018 and Preparation of *Trivrittavaleha* was done on 6 June 2018. Finished product was stored at room temperature in airtight plastic container. Thus in this study an attempt has been made to check stability of *granules* and *avaleha* with respect

to its Microbial profile at different climatic conditions and temperature setups at regular interval for a period of 10 months for *Pippalikhanda* and 7 months for *Trivrittavaleha* from date of preparation.

Aim

To evaluate and study the stability of finished product i.e. *Pippalikhanda* and *Trivrittavaleha* and to check the microbial contamination in the final product at different time interval and at different climatic conditions and temperature.

Material and Method

Sample of granular form of *Pippalikhanda* and *avaleha* form of *Trivrittavaleha* was prepared (stored at room temperature) and finished product studied to check microbial contamination at regular intervals for a period of 10 months for *Pippalikhanda* and 7 months for *trivrittavaleha* from date of preparation (upto drug used). The study for Microbiological contamination has been carried out in Microbiology Laboratory, I. P. G. T. & R. A., Jamnagar. There are mainly two studies have been carried out to rule out the presence of any kind of bacteria or fungi in the prepared final finished product. The initial microbiological study was done on 145 days for *Pippalikhanda Granules* and 28 days for *Trivrittavaleha* of drug preparation. Then at regular interval of time sample from same bag was subjected to the microbiological study until the drug finished from the same bag.

Drug material

All the raw drugs were collected from Pharmacy of Gujarat Ayurved University, Jamnagar.

Date of Drug Preparation: 09th February 2018 (*Pippalikhanda Granules*) - First Batch

: 04th February 2019 (*Pippalikhanda Granules*) - Second Batch

: 6th June 2018 (*Trivrittavaleha*) – Single Batch

Storage:

Finished product of granular form of *Pippalikhanda* along with *Trivrittavaleha* was stored in air-tight food grade, plastic containers, in the open light area in the department of *Panchakarma* at room temperature. Dry and clean spoon of stainless steel was used for taking medicine.

Microbial profile:

There are two methods to check the Microbial contamination and possibility of presence of 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 evaluate the presence of any mycological findings.

Specimen: Granular form of *Pippalikhanda* along with *Trivrittavaleha*

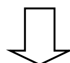
Procedure for Wet Preparation

Clean grease free glass slide was taken

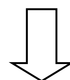
Selected material drawn on it

Added distilled water (if needed)

Covered with grease free cover glass

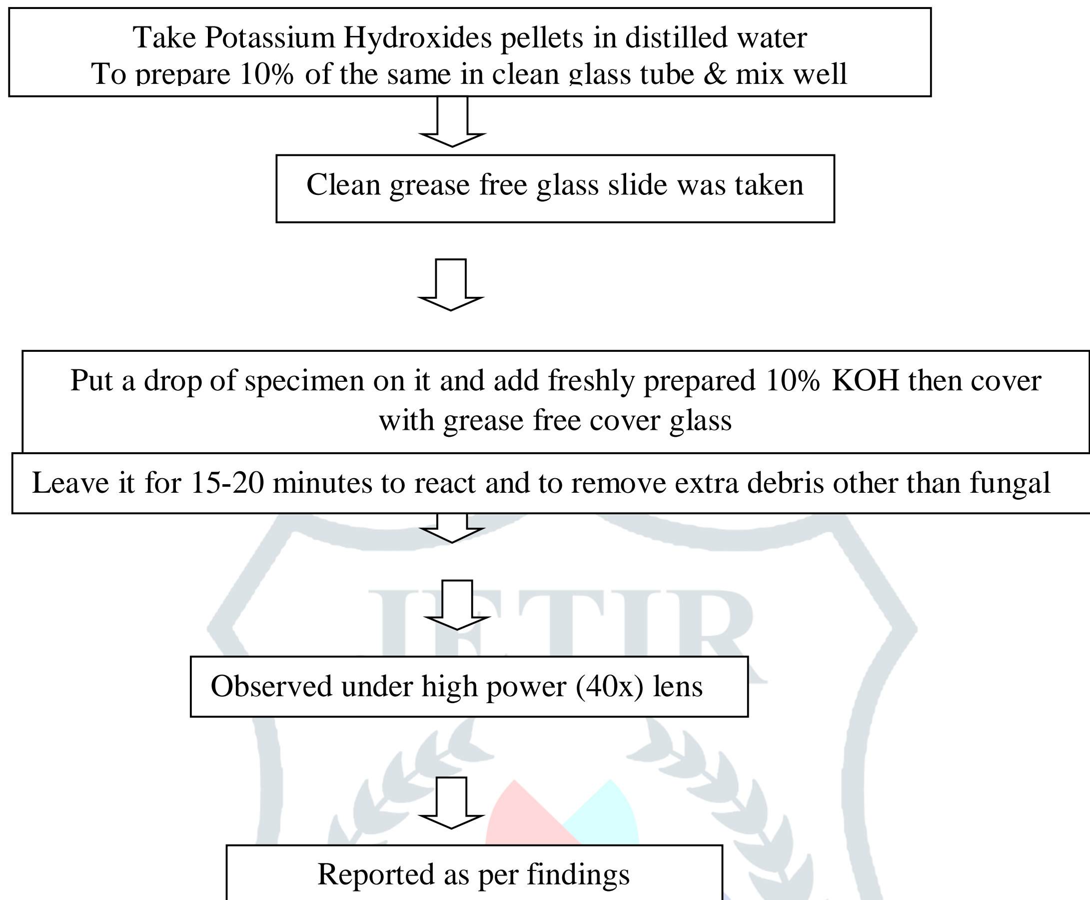


Observed under the high power (40x)



Reported as per findings

Procedure For 10% KOH Preparation



B. Gram's stain test:

Gram staining is a type of differential staining technique that causes differentiatation of bacteria into two groups: one is Gram positive and other is Gram negative. This procedure is fully depend upon the ability and capacity of microorganisms to capture and retain the colour of the stains used during the gram staining procedure.

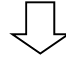
Gram negative bacteria shows decolorization by any organic solvent (acetone or Gram's decolorizer) while Gram positive bacteria do not show any decolorization and as the result primary dye retained by the cell and bacteria will remain as before i.e. purple. After decolorization step, there is a counter stain effect that is found on Gram negative bacteria and as a result 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)[iii].

Aim: To rule out any bacteriological findings.

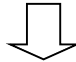
Specimen: *Pippalikhanda Granules* and *Trivrittavaleha*

1. Procedure For Gram's Stain

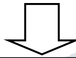
Take clean grease free glass slide to prepare dry equal thick preparation (i. e. smear)



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)



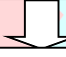
Cover fixed prepared smear with **Gram's crystal violet** solution and allow to remain for mentioned time as per kit procedure



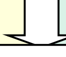
Washed off smear to remove excessive reagent with tap water

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



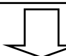
Decolourize smear with **Gram's decolourizer** by holding the slide at slope position and pour gram's decolourizer – acetone from its upper end upto removal of colour of primary dye (i.e. Gram's Crystal Violet) or as per kit procedure



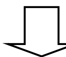
Washed off smear to remove excessive reagent with tap water

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

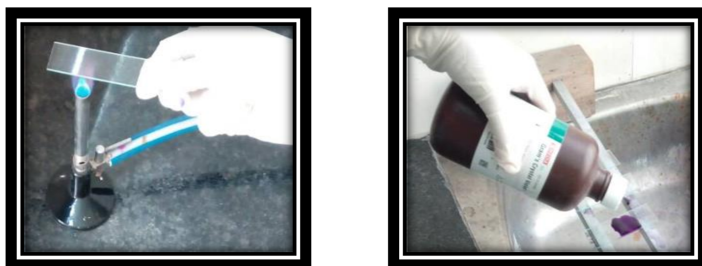


Figure 1, 2. Smear staining Procedure

1. Culture Study

A. Fungal culture method:

Necessary materials were collected with sterile cotton swab for the purpose of inoculation 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.

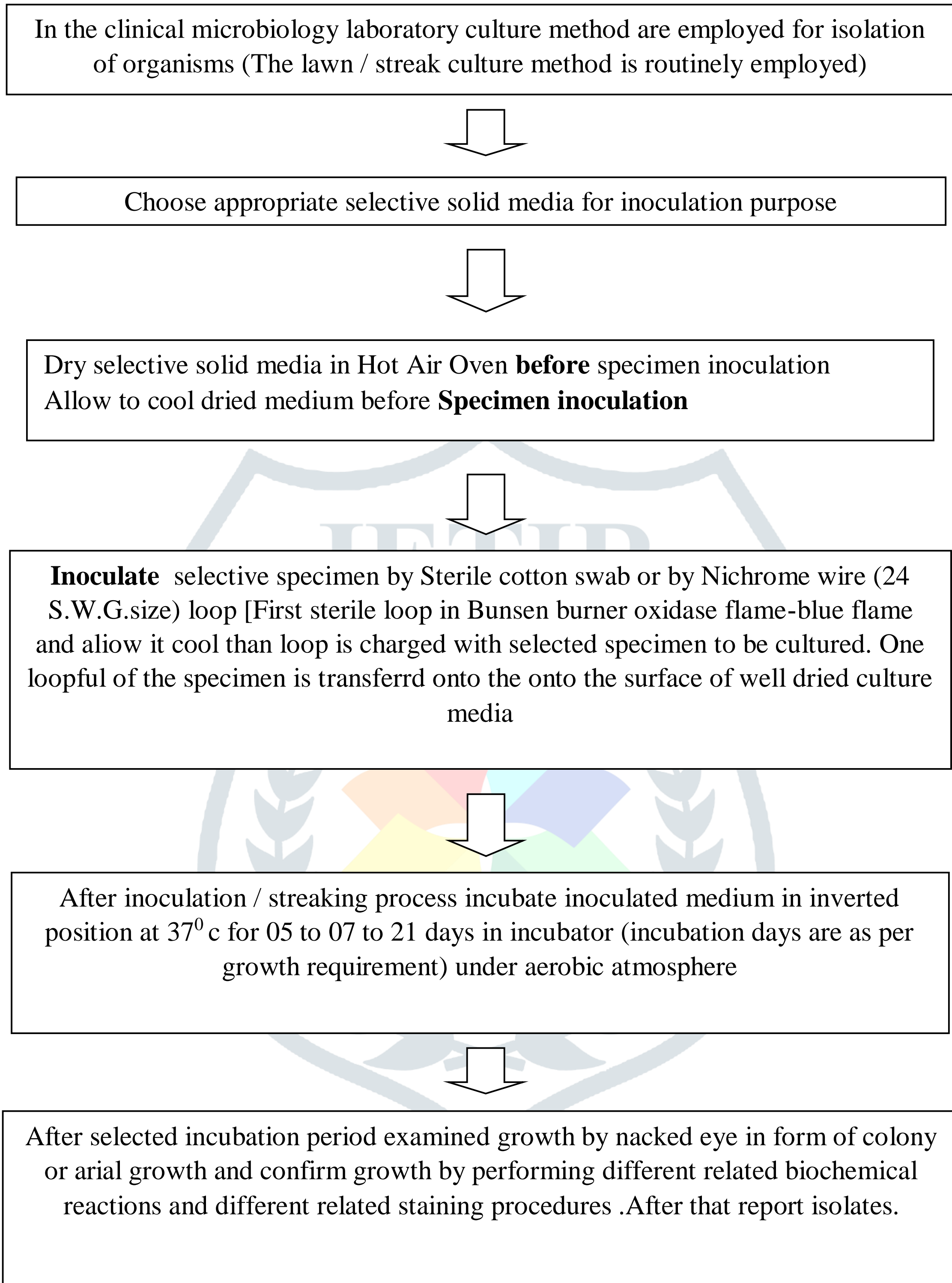
Required time duration : 05 to 07 days

Required temperature : 37 ºC

Use of media : For selective cultivation of pathogenic fungi.

Figure 3. Sabouraud Dextrose Agar Base (SDA) bottle

Procedure For Fungal Culture



B. Aerobic culture method:

Necessart materials collected with sterile cotton swab for the purpose of inoculation 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.

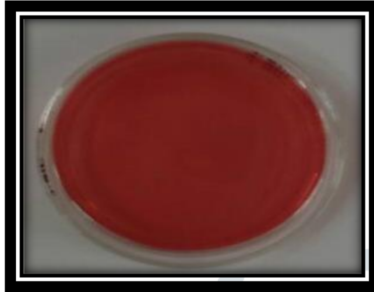
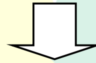


Figure 4. Mac Conkey Agar (MA)

Figure 5. Dark bloody red.

Procedure For Aerobic Culture

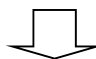
In the clinical microbiology laboratory culture method are employed for isolation of organism (The streak culture method is routinely employed)



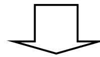
Choose appropriate selective solid media for **inoculation** purpose

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 sreaks 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



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 selected incubation period **examined growth** by nacked 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 01: Results of microbiological study of *Pippalikhanda Granules*- Batch 1

Sr. No.	Days of investigations After preparation of the sample at	Batch and container	Date of Sample given	Temp[iv]. (^{0}C)	humidity[v]	Observations / Findings			
						Gram's Stain	Aerobic culture	Wet mount/ 10% KOH Preparation	Fungal culture
1.	145 Days	Batch-1 Cont.-1	4th July 2018	32^0C	66%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
2.	145 Days	Batch-1 Cont.-2	4th July 2018	32^0C	66%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
3.	145days	Batch-1 Cont.-3	4th July 2018	32^0C	66%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
4.	173 Days	Batch-1 Cont.-4	1st august 2018	31^0C	57%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
5.	207 Days	Batch-1 Cont.-5	5th September 2018	28^0C	66%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
6.	241 Days	Batch-1 Cont.-6	9th October 2018	36^0C	31%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
7.	282 Days	Batch-1 Cont.-7	20th November 2018	31^0C	37%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
8.	305 Days	Batch-1 Cont.-8	13th December 2018	24^0C	31%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated

Table 02: Results of microbiological study of *Pippalikhanda Granules*- Batch 02

Sr. No.	Days of investigations After preparation of the sample at	Batch and container	Date of Sample given	Temp. (^{0}C)	Humidity	Observations / Findings			
						Gram's Stain	Aerobic culture	Wet mount/ 10% KOH Preparation	Fungal culture
1.	2 Days	Batch-2 Cont.-1	6th February 2019	25^{0}C	52%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
2.	52 Days	Batch-2 Cont.-2	26th March 2019	36^{0}C	14%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated

Results of Microbiological study of the Modified *Pippalikhanda Granules* showed that the quality of Granules is in a standard condition. There were no growth of micro-organisms (Bacterial or fungal)till it was in use for about 10 months in Batch-01, and upto its use in Batch-02 from the date of preparation, shows its good shelf life.

Table 02: Results microbiological study of *Trivrittavaleha:*

Sr. No.	Days of investigations After preparation of the sample at	Date of Sample given	Temp. (^{0} C)	humidity	Observations / Findings			
					Gram's Stain	Aerobic culture	Wet mount/ 10% KOH Preparation	Fungal culture
1.	28 Days	4th July 2018	32^{0}C	66%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
2.	42 Days	18th July 2018	26^{0}C	100	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
3.	56 Days	1st august 2018	31^{0}C	57%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
4.	91 Days	5th September 2018	28^{0}C	66%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
5.	126 Days	9th October 2018	36^{0}C	31%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
6.	168 Days	20th November 2018	31^{0}C	37%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated

7.	191 Days	13th December 2018	24^{0}C	31%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated

Results of Microbiological study of the *Trivrittavaleha* showed that the quality of *Avaleha* is in a standard condition. There were no growth of micro-organisms (Bacterial or fungal) till 31th December 2018 i.e. 07 months from the date of preparation, shows its good shelf life.

DISCUSSION-

It is very necessary that the drug should be free from any type of microbial contamination for the better life time, safety and efficacy. Self-life is a term which is use to show the stability of the drug. intrinsic and extrinsic factors are the term which is use to affect the stability of prepared drug. Moisture content, acidity, nutrient content, biological structure, redox potential, naturally occurring and added antimicrobials are included in intrinsic factors while types of packaging, effect of time/temperature on microbial growth, storage/holding conditions and processing steps (FDA report 2001) included under extrinsic factor. To increase the stability of drug and its storage time Microbial contamination should be avoided. *Pippalikhanda Granules* and *Trivrittavaleha* was prepared and stored at room temperature. There was random selection of Sample for study of microbiological contamination. There was Changes in humidity and temperature of environment that was observed during study period. The temperature at which microbes multiplies is known to be Optimum temperature for microbial growth. This optimum temperature is found to be -20 ^{0}C to +10 ^{0}C for psychrophilic bacteria (low temperate loving) while for mesophilic bacteria (moderate temperate loving) it is 20-45 ^{0}C and for thermophilic (high temperate loving) bacteria it is 41-122^{0}C respectively. The region was very proximal to sea coast where the drug was prepared and sample was stored, that area has longest sea shore and maximum number of sea ports, this is the reason why the relative humidity (RH) in that area remains high in all the seasons of the year. When relative humidity is very high then it may allow the growth of microbes[vi]

Any fungal and bacterial contamination in the sample was ruled out with the help of fungal culture, gram stain, wet mount and aerobic culture tests at monthly interval from 09th February 2018 to 13 December 2018 for *Pippalikhanda* in batch 01 and from 4 February 2019 to upto its use in batch 02 and 6th June 2018 to 13 December 2018 for *Trivrittavaleha*. During this study period it was found that as a result of aerobic culture no any microbes were isolated and as a result of fungal culture no any fungal pathogen were isolated (as shown in Table 1 & 2). There is an important role of Moisture content of drug for its long term storage, it is found to be the main causative factor in drug deterioration, it also act as an enzymatic activator which resulting in its degradation by slowly decomposition of the drug[vii].

In *Ayurvedic* classics both the drugs i.e. *Pippalikhanda* and *Virechana* by *Trivrittavaleha* play important role to remove the vitiated dosha responsible for *Amlapitta* and improve the condition of *Agni*. Both the drugs showed very result in the treatment of *Amlapitta*

CONCLUSION- The length of time for which a food item can be stored before it goes bad or became unsafe to eat is known as shelf-life. Shelf life is the length of time that a commodity may be stored without becoming unfit to use, consumption or sale[viii]. There are several factors which are responsible for determination of product's shelf-life, which ranges from organoleptic qualities to microbiological safety. Thus Microbiological study of the granular form of *Pippalikhanda* and *Trivrittavaleha* showed that the quality of granular form and *avaleha* form is in a standard condition. No any Bacterial and fungal growth was there in microorganism till 13 December 2018 i.e 10 months for *Pippalikhanda and* 07 months for *Trivrittavaleha* from the date of preparation it showed a very good shelf-life of the product.

Temperature ranges from 24^{0}C to 36 ^{0}C and Humidity ranges from 14% to 100% proved that at this at this temperature and humidity range the medicine remains safe to use.

References-

[i] *Bhaishajya Ratnavali, Amlapitta / 124-128*

[ii] *Charak Samhita Kalp 7/ 24-25*

[iii] Alfred E Brown (2001), Benson: Microbiological Application, 8th Edition, the McGraw – Hill Companies, P. 64.)

[iv] www.timeanddate.com

[v] www.timeanddate.com

[vi] Bruce J, Drysdale EM. Trans shell transmission. Microbiology of Avian egg. Chaman and Hall, London 1994, 63-91.

[vii] Sharma R, Amin H, Shukla VJ, Kartar D, Galib R, Prajapati PK Quality control evaluation of *Guduchi Satva* (solid aqueous extract of *Tinospora cordifolia* – Willd. Miers): An herbal formulation Int. J Green Pharm. 2013; 7(3):258-263.

[viii] Oxford English Dictionary, 2nd edition.