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"Microbial Evaluation of *Dhatriadi* Eyedrops for *Amaja Abhishyanda* (Acute Infective Conjunctivitis): A Stability Study"

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ABSTARCT:

Conjunctivitis is a commonly encountered condition in ophthalmology clinics throughout the world which has high prevalence and recurrent rate in the general population of developing countries. If left untreated or undiagnosed, chronic conjunctivitis can cause permanent eye damage. Integrating Ayurveda alongside modern medical treatments can provide a holistic approach to eye care, potentially reducing the need for high doses of medications and minimizing their adverse effects. *Dhatriadi* Eyedrops is a choice of polyherbal compound selected to breakdown local ocular pathology of *Amaja Abhishyanda* (Acute Infective Conjunctivitis). To check the stability and to check microbial contamination in the finished product at different time interval- at different climatic conditions, temperature and humidity set ups. **Materials and Methods:** In present study, stability with respect to its Microbial profile of *Dhatriadi* Eyedrops was carried out. It was stored in plastic bag during different climacteric conditions were studied at regular intervals for a period of eight months to analysis presence no microbial growth by Smear and culture study respectively. At the end of study *Dhatriadi* Eyedrops had no presence of microbes or bacteria throughout the study eight months from the day sample preparation even in different climate and temperature. **Result**: In present study, the stability test of *Dhatriadi* Eyedrops with respect to microbiological findings was negative at room temperature, warm and cold, dry and humid conditions.

KEY WORDS: Ayurveda**,** Microbial profile, Stability, *Triphaladi Churna* (*Anubhuta Yoga*)

INTRODUCTION:

Aschyotana is a foremost procedure among *Kriyakalpa* [i] in the treatment of *Amaja Netra Abhishyanda* (Acute Infective Conjunctivitis). *Aschyotana* consumes less time and easy to do but it is difficult to store and maintain the hygiene of *Dravya*. On this basis, scientifically the *Ark Kalpana* was used to convert this drug into Eyedrop formulation. The pharmacology of eye drops is influenced by drug administration route, solubility and bioavailability, absorption surface (conjunctiva, cornea, etc.), vascularity of tissues, physical drug state, patient compliance, and excretion dynamics.[ii] Aqueous solutions are standard for more effect in less time.

Dhatriadi Eyedrops was selected for present study on *Amaja Netra Abhishyanda* (Acute Infective Conjunctivitis) which have main ingredients *Dhatriphala*[iii] having *Tridosha Shamaka*, *Vrushya* and *Rasayana* property. Another and *Saindhava* (*~Sodii chloridum*)[iv] and *Madhu* (*~honey*) [v] act as *Netra Amayharam* and alleviates *Kapha Dosha* in *Netra*.

Microbiological analysis is performed for the estimation of the number of viable aerobic micro-organism presence and for detecting the presence of designated microbial species in pharmaceutical substance. Shelf-life, a vital metric for product stability, marks the duration between production and consumption. Microbial growth thrives within specific environmental parameters: temperatures ranging from 20°C to 40°C (68°F to 104°F) and relative humidity levels of 60% to 90%. These conditions offer an ideal breeding ground for bacteria and fungi on surfaces and articles. Maintaining control within this range is critical for preserving product quality. Understanding these factors facilitates the implementation of effective storage strategies to curb microbial contamination and prolong shelf-life. Precise temperature and humidity regulation uphold product integrity, ensuring consumer confidence and safety.

AIM: Comprehensively to assess the stability of *Dhatriadi* Eyedrops (finished product) and monitor microbial contamination under varying time intervals, diverse climatic conditions, and fluctuations in temperature and humidity.

OBJECTIVE: -To ensure adherence to rigorous standardization protocols in the production of study drugs, thereby enhancing overall quality control and efficacy.

Materials and Methods:

Collected sample of *Dhatriadi* Eyedrops (sterile packaging and stored at room temperature) studied to check microbial contamination at regular intervals for a period of eight months (upto drug used). Microbiological study has been carried out in Microbiology Laboratory, I.T.R.A., Jamnagar. Studies have been carried out to rule out that presence of any bacteria or fungi in the prepared drug as a final finished product. Initially microbiological study was done on 43th day of preperation before starting patient enrollment and done till the cpmplition of thr study regularly with random intervals during different seasons.

Drug material:

The raw drug materials -fresh *Amalaki Phala* was collected from the Jamnagar local market. They were washed properly with clean water and then *Amalaki Phala Kalka* (made seedless) was prepared. Within *Kalka,* eight parts of distillate water were mixed in sterile round bottomed flask and subjected for Distillation. At the end of proper distillation process, *Arka* was collected and lastly one sixteen parts of each- *Madhu* and *Saindhav* was added to the solution.

Table 1: Ingredients *Dhatriadi* Eyedrop- [vi]

Sr. no.	Name of Drug	Latin/English name	Part used	Proportion
1.	*Amalaki*	*Emblica Officinalis* Gaertn.	Fruit	1 Part
2.	*Saindhava Lavana*	*Rock salt*	-------	1/16 Part
3.	*Madhu*	*Honey*	------	1/16 Part

Date of Drug Preparation: 24th August, 2023

Storage:

Arka was transferred to a sterile (autoclave) glass bottle. Packing in plastic air tight bottles was done in aseptic area. Store in a cool, dry place away from direct sunlight and heat.

PACKAGING: -

Packing of eye drops was done at Indiana Ophthalmics, Surendra Nagar in appropriate place under aseptic conditions. The procedure in brief is as given below-

Filtration by 2.0 microns glass filter was followed by 0.2 microns nylon filter. Filtered solution was filled in sterile plastic bottles under Laminar Air Flow in aseptic conditions. Plugging and capping was done in aseptic area. Thus, prepared distillate was passed into different microbial filter papers and pressure vessels ultimately obtained product packed into 10ml sterile bottle.

Microbial profile:

Microbial contamination was assessed by two methods to check any mycological findings and bacteriological findings.[vii]

1. SMEAR EXAMINATION-

A) 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:

A. SMEAR EXAMINATION: -

1. PROCEDURE FOR 10% KOH PREPARATION:

Take Potassium Hydroxides pellets (of HiMedia Lab. Pvt. Ltd.) 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 *Dhatriadi* Eyedrops 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 finding

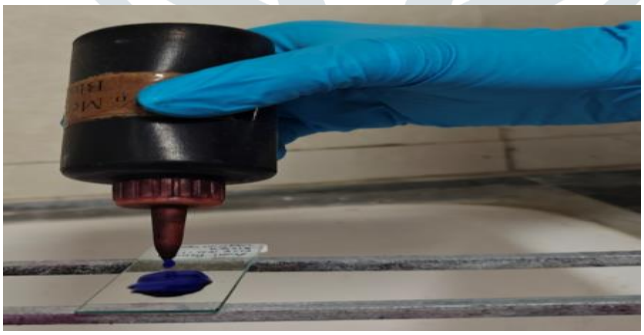


Figure 1. Smear staining Procedure

2. GRAM'S STAIN TEST:

Procedure for Gram's Stain: -

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

↓

Fixed prepared smear (*Dhatriadi* Eyedrops) 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 the smear with Gram's Iodine solution and allow to remain for mentioned time as per kit procedure

↓

Washed off smear to remove excessive reagent with tap water

↓

Decolorize smear with Gram's decolorizer by holding the slide at slope position and

Washed off smear to remove excess acetone with tap water

↓

Cover smear with Safranin solution and allow to remain 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

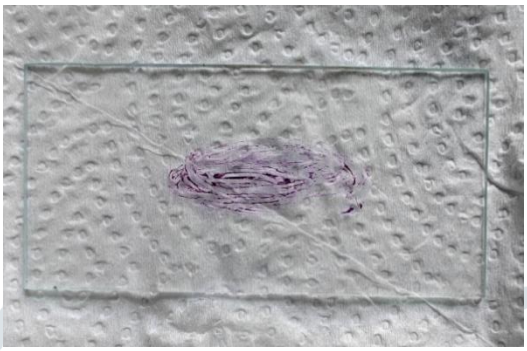


Figure 2. Stained Smear Procedure

B. CULTURE STUDY: -

1. <u>FUNGAL CULTURE METHOD:</u>

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 ºC

Use of media: For selective cultivation of pathogenic fungi.

Procedure For Fungal Culture:

In the clinical microbiology laboratory, culture method was employed for isolation of organisms (The lawn / streak culture method is routinely employed)

↓

Sabouraud Dextrose Agar Base (SDA) selected as solid media for inoculation purpose

↓

Dry selective solid media in Hot Air Oven before specimen inoculation, allowed to cool dried medium before specimen inoculation

↓

Inoculate *Dhatriadi* Eyedrops by Sterile cotton swab or by Nichrome wire (24 5.W.G. size) loop [First sterile loop in Bunsen burner oxidase flame blue flame and allow it to cool than loop was charged with *Dhatriadi* Eyedrops and cultured. One loopful of the *Dhatriadi* Eyedrops was transferred onto the surface of well dried culture media]

↓

After inoculation / streaking process, Incubate inoculated medium kept 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 the growth by naked eye in form of colony or arial growth and confirm growth by performing different related biochemical reactions and different related staining procedures. After that report isolates.

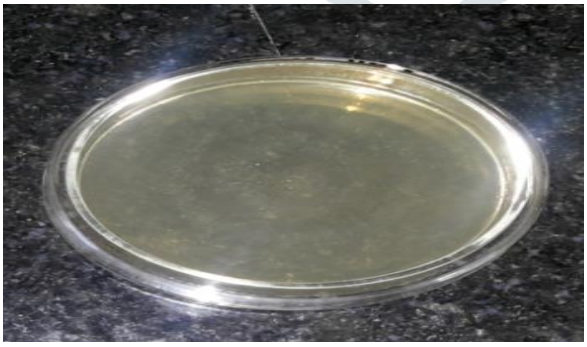


Figure 3 Fungal culture media preparation with Sabouraud Dextrose Agar Base (SDA)

2. AEROBIC 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)

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 organisms (The streak culture method is routinely employed)

↓

MacConkey Agar (MA) was selected as 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 *Dhatriadi* Eyedrop 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 was charged with *Dhatriadi* Eyedrops to be culture.

↓

After streaking process Incubate inoculated medium in inverted position at 37°C for 18-24 hours in Incubator under aerobic or 10% CO2 atmosphere

↓

After selected incubation period, examined the 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

Figure 3 Aerobic culture media preparation with MacConkey Agar (MA)

Figure 4 MacConkey Agar (MA)

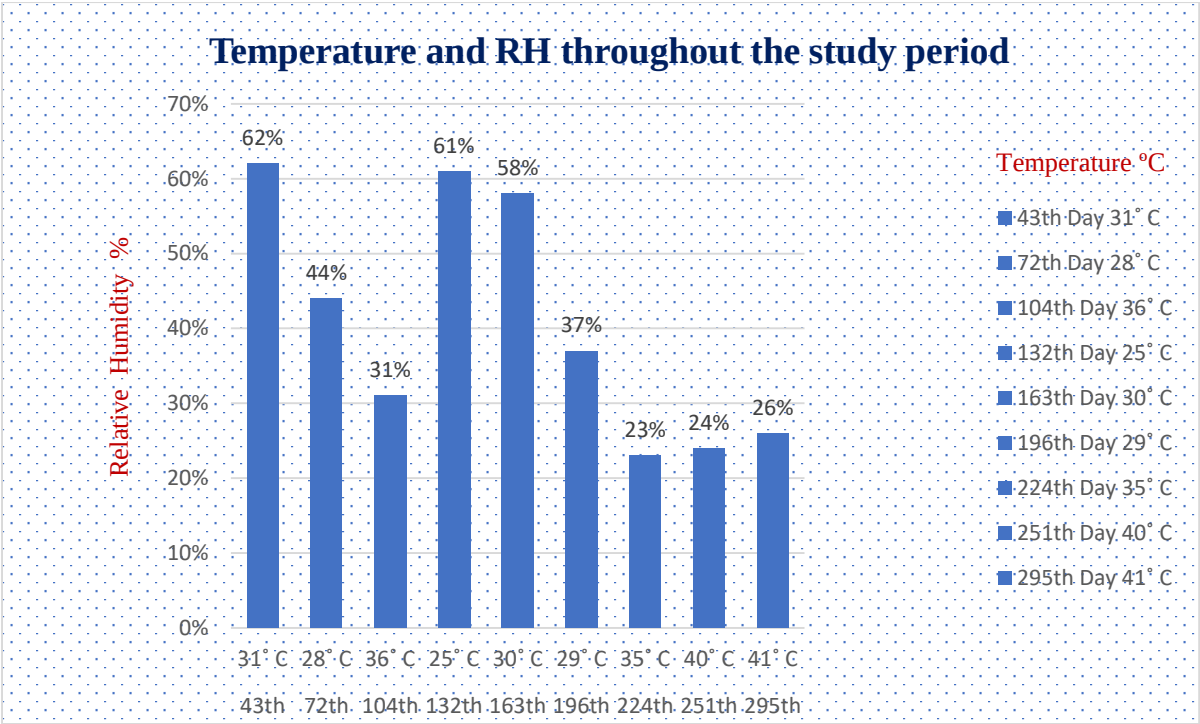
Every time sample (in which drug preserved) were subjected to the microbiological study from the date of the preperation to the date of last microbiological study.

RESULTS: Shown in table no 2. of *Dhatriadi* Eyedrop.

- **Date of Drug Preparation: 24th August, 2023**

Table 2: Showing observations of *Dhatriadi* Eyedrop preserved at room temperature after the preparation of Eyedrop.[viii]

Sr. No.	Date & Days of investigations After preparation of the sample	Temperature (˚ C)	Humidity (%)	Observations of sample			
				Gram's Stain	Aerobic culture	Wet mount/ 10% KOH Preparation	Fungal culture
1.	31/08/23 43th Day	31˚ C	62%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
2.	29/09/23 72th Day	28˚ C	44%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
3.	31/10/23 104th Day	36˚ C	31%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
4.	28/11/2023 132th Day	25˚ C	61%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
5.	29/12/23 163th Day	30˚ C	58%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
6.	31/01/24 196th Day	29˚ C	37%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
7.	28/02/24 224th Day	35˚ C	23%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
8.	26/03/24 251th Day	40˚ C	24%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated
9.	09/5/24 295th Day	41˚ C	26%	Microorganisms Not Seen	No organisms isolated	Fungal filaments not seen.	No Fungal Pathogen Isolated



Ayurveda as an adjuvant therapy is widely used in *Amaja Abhishyanda* (Acute infective Conjunctivitis). *Aschyotana* is a foremost procedure among *Kriyakalpa* for the treatment of *Amaja Abhishyanda* (Acute infective Conjunctivitis). *Aschyotana* consumes less time and easy to do but it is difficult to store and maintain the hygiene of *Dravya*. On this basis, scientifically the *Ark Kalpana* was used to convert this drug into Eyedrop formulation. *Dhatriadi Eyedrop* planned for the research work at ITRA to break the pathology of *Amaja Avastha* of *Amaja Abhishyanda* (Acute infective Conjunctivitis) as its prevalence rate and fatality is high in developing if left untreated or not addressed at its root cause.

The pharmacology of eye drops is influenced by drug administration route, solubility and bioavailability, absorption surface (conjunctiva, cornea, etc.), vascularity of tissues, physical drug state, patient compliance, and excretion dynamics. Eye drop effectiveness relies on drug solubility and local conditions at absorption sites like the conjunctiva, favoring water and lipid-soluble drugs for mucous membrane absorption. Aqueous solutions are standard due to their effective mixing in tears. Water-soluble drugs penetrate the stroma, requiring amphipathic properties for full corneal layer access, while high drug concentrations aid passage to the anterior chamber through mass action.[ixx] *Dhatriadi* Eyedrop meets all pharmacological requirements to enhance efficacy and provide rapid therapeutic effects.

A microbial study investigated contamination and stability of Dhatriadi Eyedrop across varying temperatures and humidity levels. *Dhatriadi* Eyedrop prepared and stored in temperatures ideal for bacterial growth at room temperature, minimum temperature 25 ˚ C to maximum temperature 41 ˚ C, astoundingly remains microbe-free. Situated in Jamnagar's coastal region, known for its high relative humidity throughout the year, defied expectations. Despite RH levels ranging from lowest range 23% on 31st January, 2023 to highest range 62%

on 31st August, 2023 as shown in Table 2, no bacterial or fungal growth was observed upto drug used till study completed.

Thus, a baseline microbial profile was studied at regular interval of 30 days average upto consumption of *Dhatriadi* Eyedrop. This indicates that no microbial growth was observed throughout the study, affirming the efficacy of the manufacturing and storage procedures.

COCLUSION: -

The microbiological assessment of *Dhatriadi* Eyedrop concludes its robust shelf life, remaining free from microbial growth, both bacterial and fungal, for a significant eight-month duration until its scheduled utilization by May 19, 2024, affirming its consistent quality and adherence to standard storage practices.

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CONFLICTS OF INTEREST: There are no conflicts of interest.

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