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Stability study of Katupila paste with respect to baseline microbial profile

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Abstract
Stability studies ensuring the maintenance of product quality, safety and efficacy throughout the shelf life are considered as pre-requisite for the acceptance and approval of any pharmaceutical product. The paste of *Katupila* leaves has been described by the medico-ethno botanist as a wonderful drug for the treatment of wound. Microbial contamination should be taken care of to increase shelf life of any formulation. Pastes of *Katupila* leaves powder in two media i.e. water and sesame oil were prepared and studied to check microbial contamination at different climatic conditions at different time interval. To analyze mycological findings and presence of microorganisms in paste, wet mount test and Gram stain test where applied respectively. At the end of the study, sample of *Katupila* paste in any media did not show any kind of microbial contamination with the changing in climatic conditions.

Keywords: Katupila paste, baseline microbial profile

1. Introduction
Delivery of drugs direct to the skin is an effective and targeted therapy because it avoids first pass effects, gastrointestinal irritation and metabolic degradation associated with oral administration [1]. Due to first pass effect only 25-45% of orally administered dose reaches the blood circulation [2]. In order to bypass these advantages topical formulation has been selected as topical application. The application of medicinal substances to the skin or various body orifices is a concept as old as humanity. Medications are applied to the skin or inserted into body orifices in liquid, semi solid, solid form. Pastes, ointments, creams and gels are semi solid dosage form intended for topical application [3]. USP defines pastes as semisolid dosage forms that contain one or more drug substances intended for topical application. Pastes adhere reasonably well to the skin and are poorly occlusive. Because of their physical properties, paste may be removed from the skin by use of mineral oil or a vegetable oil [4]. The base may be anhydrous or water soluble. Their stiffness makes them useful as protective coatings [5].
Securinega leucopyrus, (Willd) Muell. belonging to family Euphorbiaceae and known as *Katupila* (in Sri Lanka), *Thumri* (Sanskrit name), *Humari* (in Hindi), *Shinavi* (in Gujarat) and also called as "Spinous fluggea" in English [6]. It has been described by the medico-ethno botanist as a wonderful drug for the treatment of wound. *Katupila* possesses *kashaya* and *Tiktarasas*; *Lagu*, *Ruksha*, *Tikshna gunas*; *Ushna veerya* and *Katu vipaka*. Leaves of this plant act as an antiseptic and its paste is used in folklore to extract any extraneous materials from body tissues without surgery [7]. Sesame oil has *Vrana Shodhan* (wound cleaning) and *Vrana dahanashaka* (relief in burning pain) properties will be used along with the paste [8]. Many clinical trials confirm the potential effects of pastes made up form leaves powder of *S. leucopyrus* in diabetic wound when used in form of paste with sesame oil [9, 10]. Stability of a product like paste is the capability of a particular formulation in a specific container or closure system, to remain within its physical, chemical, microbiological, therapeutic and toxicological specifications at a defined storage condition [11].
Microbial communities are extremely complex in structure and function, can be affected by climate and other global changes in many ways. Thus the present study was designed to study the stability of *Katupila* paste with respect to microbial contamination.

1.1 Collection of raw Material
The fresh leaves of *S. leucopyrus* were collected from the surrounding area of Jamnagar in month of August 2016 (22º21'37.656"N70º11'1.056"E) and authentication of drug (Specimen no. IPGTRA/PHM/6275/17-18) was done in Pharmacognosy laboratory, IPGT & RA, Gujarat Ayurved University, Jamnagar.

Morphological characters were compared to the characters mentioned in various floras [12]. The collected leaves were washed, shade dried, pulverized and sieved through 120 mesh and preserved in an airtight vessel for further studies.

1.2 Preparation of the *Katupila* paste

Paste of drug was prepared in two media i.e. water and sesame oil. For making paste in water, 1 g of leaves powder was socked in 3 ml of distilled water and for paste in sesame oil, 1g of leaves powder was soaked in 1 ml of sesame oil and were kept overnight before use.

2. Materials and Methods

Pastes of *Katupila* leaves powder were prepared and studied to check microbial contamination at different climatic conditions at different time interval. The study was conducted at microbiology laboratory, Institute for Post Graduate Teaching and Research in Ayurveda, Jamnagar, Gujarat, India.

Wet mount, Fungal Culture, Gram stain and Aerobic Culture tests were used to rule out away fungal and bacterial contamination in the test drug or standard drug. The paste samples of *Katupila* leaves powder were selected for the same study at different climatic and temperature conditions on weekly interval for 5 months.

2.1 Microbial profile

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

2.2 Smear Examination

A) Wet mount /10% K.O.H. Preparation
B) Gram's stain

2.3 Culture Study

A) Fungal culture
B) Aerobic culture

2.4 Smear Examination

A. Wet mount /10% K.O.H. Preparation

Aim: To rule out any mycological findings.

Specimen: Paste of *Katupila* in water and in sesame oil

Procedure of wet preparation: Selected material was poured on clean grease free glass slide and covered with grease free cover glass. Sample was observed under the high power (40x) lens and report as per finding.

2.4.1 Procedure for 10% KOH preparation

10 % of potassium hydroxides solution was prepared in distilled water.

A drop of specimen was poured on clean grease free glass slide and freshly prepared 10% KOH was added and covered with grease free cover glass than allowed to react for 15-20 minutes to remove extra debris other than fungal particles. Sample was observed under high power (40x) lens for report as per findings.

B. Gram's stain

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 decolourizer) while Gram positive bacteria are not decolorized as primary dye retained by the cell and bacteria will remain as purple. After decolourization step, a counter stain effect found on Gram negative bacteria and 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 [13].

Aim: To rule out any bacteriological findings.

Specimen: Paste of *Katupila* in water and in sesame oil

2.5 Procedure for Gram's Stain

Clean grease free glass slide was taken to prepare dry equal thick preparation (i.e.smear). Prepared smear was fixed by passing 3-4 times over the flame of Bunsen burner (The fixation kills vegetative form of microbes and renders them permeable to stain, make material stick to the surface of slide & prevent autolytic changes). Fixed prepared smear was with Gram's crystal violet solution and allowed to remain for mentioned time as per kit procedure. After that smear was washed off to remove excessive reagent with tap water and covered with Gram's Iodine solution than allowed to remain for mentioned time as per kit procedure.

Again smear was washed off to remove excessive reagent with tap water and 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. Again smear was washed off to remove excess acetone with tap water and covered with Safranin solution and allowed remain for mentioned time as per kit procedure. Smear was again washed off to remove excessive reagent with tap water than blot and allowed to dry smear and examined under oil immersion lens for report as per findings.

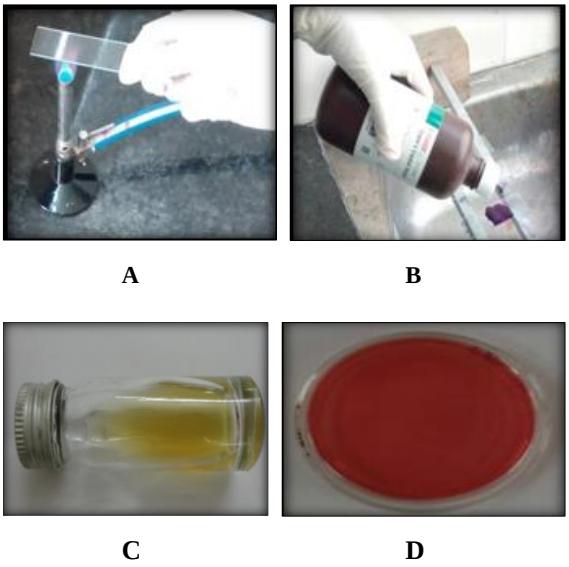

Fig 1: A & B) Smear staining Procedure; C) Sabouraud Dextrose Agar Base (SDA) bottle; D) MacConkey Agar (MA)

2.6 Culture Study

A. 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: 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.

2.6.1 Procedure for fungal culture
In microbiology laboratory culture method was employed for isolation of organisms (The lawn / streak culture method is routinely employed). For culture appropriate selective solid media was chose for inoculation purpose than selective solid media is dried in hot air oven before specimen inoculation and allowed to cool.
Selective specimen was inoculated 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 surface of well dried culture media]. After inoculation / streaking process inoculated medium was incubated in inverted position at 37^0 c for 05 to 07 days in incubator (incubation days are as per growth requirement) under aerobic atmosphere. After selected incubation period growth was examined by necked eye in form of colony or arial growth. Growth was confirmed by performing different related biochemical reactions staining procedure and after that report was prepared.

B. Aerobic culture method
Respected materials 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.

2.7 Procedure for aerobic culture
Appropriate selective solid media was chose for inoculation purpose. Selective solid media was dried in hot air oven before specimen inoculation and allowed to cool. Selected specimen was inoculated 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 inoculated medium was incubated in inverted position at 37^0c for 18-24 hours in incubator under aerobic or 10% CO_2 atmosphere. After selected incubation period growth was examined by necked eye in form of colony. Growth was confirmed by performing different related biochemical reactions staining procedure and after that report was prepared.

3. Observations and Results
Both pastes of *Katupila* which were made freshly on weekly interval and stored at room temperature did not show presence of any mycological and bacteriological contamination on wet mount test, fungal culture, gram stain and aerobic culture test at the end of study period. Results regarding the temperature, humidity and other observations like microorganisms, fungal filaments, and fungal pathogens at different time interval are shown in table below.

Table 1: High and low weather summery of temperature during study period.

Time period	Temperature (16 January 2018 to 05 June 2018)		
	Temp. (°C)		Date & time
January	High	34	31 Jan; 14:30
	Low	14	18 Jan; 05:45
February	High	36	27 Feb; 14:30
	Low	16	4 Feb; 08:30
March	High	41	30 Mar; 17:30
	Low	19	12 Mar; 08:30
April	High	42	28 Apr; 17:30
	Low	24	4 Apr; 05:30
May	High	43	17 May; 14:30
	Low	25	12 May; 05:30
June	High	42	05 Jun; 17:30
	Low	26	06 June; 05:30

Table 2: High and low weather summery of relative humidity during study period.

Time period	Humidity (16 January 2018 to 05 June 2018)		
	Humidity (%)		Date & time
January	High	100	18 Jan; 08:30
	Low	14	19 Jan; 14.30
February	High	100	2 Feb; 05:30
	Low	12	8 Feb; 14:30
March	High	98	12 Mar; 05:30
	Low	08	30 Mar; 17:30
April	High	91	27 Apr; 05:30
	Low	06	23 Apr; 11:30
May	High	87	10 May; 05:30
	Low	05	23 May; 14:30
June	High	91	06 June; 05:30
	Low	07	01 Jun; 17:30

Table 3: Observation of samples of *Katupila* pastes made in water and sesame oil and preserved at room temperature

Time (16.01.18 to 05.06.18)	Observation of samples			
	Gram Stain (Microorganisms)	Aerobic culture (Organisms isolated)	Wet mount (Fungal filament)	Fungal culture (Pathogen)
16.01	-	-	-	-
23.01	-	-	-	-
30.01	-	-	-	-
06.02	-	-	-	-
20.02	-	-	-	-
27.02	-	-	-	-
07.03	-	-	-	-
14.03	-	-	-	-
20.03	-	-	-	-
27.03	-	-	-	-
03.04	-	-	-	-
10.04	-	-	-	-
17.04	-	-	-	-
25.04	-	-	-	-
01.05	-	-	-	-
09.05	-	-	-	-
15.05	-	-	-	-
22.05	-	-	-	-
30.05	-	-	-	-
05.06	-	-	-	-

4. Discussion
Drug should always be free of microbial contamination for better efficacy and longer storage. Stability is usually expressed in terms of shelf life. The factors which may be considered when determining whether a prepared product

requires time/temperature control during storage, distribution, sale and handling may be categorized under intrinsic, extrinsic and other factors (FDA report, 2001). Intrinsic factors include moisture content, pH and acidity, nutrient content, biological structure, redox potential, naturally occurring and added antimicrobials. Extrinsic factors include types of packaging/atmospheres, effect of time/temperature conditions on microbial growth, storage/holding conditions and processing steps (FDA report, 2001). Microbial growth should be avoided to increase its stability period and drug can be stored normally.

Katupila paste was prepared and stored at room temperature. Sample was selected randomly for study of microbial contamination. Changes in temperature and humidity were may be at maximum during whole duration. In this study highest and lowest temperature observed was 43°C in the month of May and 14°C in January respectively. As shown in table-1 Average 30.167 °C temperature during whole study period. Optimum temperature for bacterial growth is temperature at which bacteria multiplies. This optimum temperature for psychrophilic bacteria (cold loving bacteria) is 15-20°C while for mesophilic (middle living) and thermophilic (heat loving) bacteria it is 30-37°C and 50-60°C respectively. Optimum temperature for psychrophilic and mesophilic bacteria falls in range of temperature observed during period of study. The region where the drug was prepared and sample was stored is very proximal to sea coast, this part of state has longest sea shore and most number of sea ports. So relative humidity (RH) remains high in almost all the seasons of year. Highest RH observed was 100% in month of January and February while lowest humidity was 05% noted in month of May. As shown in table-2 high RH can allow the growth of microbes [14]. Relative humidity remained constantly high during study duration, although air cannot be considered dry at RH more than 40%. Wet mount, Fungal culture, Gram stain and Aerobic culture tests were used to study fungal and bacterial contamination in the samples on weekly interval from 16 January 2018 to 05 June 2018. During this study period no any organisms were isolated as a result of aerobic culture**.** No fungal pathogen isolated as a result of fungal culture as shown in table-3. Sample which was stored in condition open to all climatic changes did not showed any kind of microbial contamination. These results may contribute to some properties of formulation. Moisture content of formulation plays most important role. Naturally it is desired that the moisture content should be minimum, which will help, in long storage of the product. Moisture content is the main causative factor in product deterioration. Moisture in a product is sufficient to activate different enzymes, which slowly decompose the product resulting in its degradation [15]. *Katupila* is having anti-microbial activity, this property of plant may played role in inhibition of microbial contamination [16].

5. Conclusion

Stability of a pharmaceutical product depends largely on many factors. Randomly selected samples of *Katupila* pastes made in water and in sesame oil showed negative finding for bacterial as well as mycological contamination. The product is free from microbial contamination revealing its safety aspects. More studies should be carried out on *Katupila* pastes made with different formulation to determine a common standard for herbal pastes.

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