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Stability Study of *Lodhradi Lepa* with respect to Baseline Microbial Different Diagnostic Modalities

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ABSTRACT:

Background: The market for herbal, herbo-mineral and traditional medicine has expanded rapidly during the past few decades. Lack of information regarding the stability and shelf life of traditional medicines is the primary obstacle to their widespread use. In order to observe the stability of *Lodhradi Lepa* against microbial contamination of sample prepared and stored in various climatic conditions and temperatures, the current investigation was conducted. **Aim:** To study the stability of *Lodhradi Lepa* and inspect microbial contamination in the finished product at different time intervals and at different climatic conditions (different temperature and humidity set ups). **Materials and Methods:** Samples of *Lodhradi Lepa* were studied to inspect microbial contamination at different climatic conditions. The study was conducted at Microbiology Laboratory, Institute of Teaching and Research in Ayurveda (ITRA), Jamnagar, Gujarat, India. **Observations & Results:** The initial microbiological study of *Lodhradi Lepa* was carried out on the thirtieth day if its preparation. Further studies were carried out at regular time intervals up to 215 days. **Conclusion:** In microbiological study of *Lodhradi Lepa*, growth of microorganisms either bacterial or fungal was not found till 215 days from the date of preparation, which shows its intact stability and good shelf life.

KEYWORDS: Stability, Microbial profile, *Lodhradi Lepa*, Climatic conditions

INTRODUCTION:

Stability is the capability of a drug or formulation in a particular container/closure system to remain within its physical, chemical, microbiological, toxicological, therapeutic specifications and is always expressed in terms of shelf life.[1] The shelf life of a product can be defined as the time

duration up to which it is expected to retain 90% of its active ingredients when stored in recommended condition. The purpose of stability testing is to provide evidence of how the quality of a pharmaceutical product or drug changes with time due to impact of a variety of environmental factors, namely temperature, humidity and light and product-related factors.[2] The duration of a pharmaceutical product's physical, chemical, microbiological, and pharmacokinetic features and characteristics over the course of its shelf life after manufacture can be referred to as the stability study of that product. Stability research aids in calculating how long the drug substance should be substituted for and how long it should be stored. It can be said that a stability study is a crucial component in determining a drug's quality.[3]

Stability study is necessary to determine and ensure the identity, potency and purity of ingredients as well as those of the formulated products is the most important step during the developmental stages.[4] 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 *Lodhradi Lepa* with respect to microbial contamination.

The term *"Saviryata Avadhi"* refers to the shelf life of recent era, is used in the Ayurvedic lexicon to describe the amount of time that a drug's *Virya*(potency) remains unaffected and above a certain threshold, after which it may lose some of its potency but not entirely if it is stored in the mentioned condition.[5] The word *Virya* is having different meanings as per Sanskrit-English dictionary, namely, heroism, valor, vigor, strength, virility, energy, firmness, courage, potency, efficacy, splendour, lustre, and dignity.[6] Usually, *Virya* is considered to be the most active principle of a drug among *Rasa, Guna, Vipaka,* and *Prabhava* responsible for overall effect of the same.

Churna form or powder preparations of medicine are widely used in Ayurveda pharmaceutical industry by the practitioners of Ayurveda for various ailments. According to Ayurvedic classical texts, preparations remain potent up to months to years depending on the dosage form. The *Saviryata Avadhi* of *Churna* (powder) form is described in Table no.1 below.

Table No. 1: *Saviryata Avadhi* of *Churna* (powder) as per Ayurvedic classics

Form of Preparation	*Sharangadhara Samhita*[7] (13^{th} century)	*Yoga Ratnakara*[8] (17^{th} century)	AFI (Ayurvedic Formulatory of India)[9]
Churna	2 months (60 days)	3 months (90 days)	2 years

The drug *Lodhradi Lepa* studied in present study was prepared at Pharmacy, Institute of Teaching and Research in Ayurveda (ITRA), Jamnagar under all possible hygienic conditions. No any preservative was added to the test drug. Drug preparation was finished on 21^{st} August 2023. Finished product was stored in airtight plastic bags at room temperature. In the present study, an attempt was made to check stability of *Lodhradi Lepa* with respect to its microbial profile at

different climatic conditions and temperature setups at regular intervals for a period of 215 days.

AIM:

To study the stability of *Lodhradi Lepa* by inspecting microbial contamination in the finished product at different time intervals and at different climatic conditions (different temperature and humidity set ups).

MATERIALS AND METHODS:

Samples of *Lodhradi Lepa* were studied to check microbial contamination at different climatic conditions. The study was conducted at Microbiology Laboratory, Institute of Teaching and Research in Ayurveda (ITRA), Jamnagar, Gujarat, India. Mainly two tests were performed to rule out the existence of any bacteria or fungi in the finished product sample of prepared drug. The initial microbiological study was done on thirtieth day of preparation, just before giving for its local application to the patients. Then samples from the airtight bags were subjected to the microbiological study regularly with random intervals during different seasons till seven months (upto drug used).

Drug materials

Lodhradi Lepa is a formulation of *Lodhra Churna*, *Dhanyaka Churna* and *Vacha Churna* in equal proportion.[10] All the three ingredients of formulation were procured from Pharmacy attached to Institute of Teaching and Research in Ayurveda (ITRA), Jamnagar. The formulation composition of *Lodhradi Lepa* is summarized at Table no. 2.

Table No. 2: Ingredients of *Lodhradi Lepa*

Sr. no.	Drug Name	Latin Name	Part Used	Proportion
1.	*Lodhra*	*Symplocos racemosa* Roxb.	*Twak* (Stem Bark)	1 part
2.	*Dhanyaka*	*Coriandrum sativam* Linn.	*Phala* (Fruit)	1 part
3.	*Vacha*	*Acorus calamus* Linn.	*Kanda* (Rhizome)	1 part

Preparation Time:

The whole process of formulation preparation of *Lodhradi Lepa* was carried out in the Pharmacy, Institute of Teaching and Research in Ayurveda (ITRA), Jamnagar, Gujarat, India. The process was completed by following Standard Operating Procedure (SOP) with the utmost care to avoid any sort of contamination.

Date of preparation of drug: 21st August, 2023.

Storage:

Stability study with respect to microbial and fungal contamination at regular time intervals, details of which are cited below. The finished product, *Lodhradi Lepa* was stored in airtight plastic-

bags at room temperature in a cool, dark and dry place. Samples of finished product were subjected to stability study with respect to microbial and fungal contamination at regular time intervals, details of which are cited below.

Microbial Profile:

Microbial contamination was assessed by two methods to check any mycological findings and bacteriological findings. Detail of Microbial profile is mentioned in Table no. 3.[11]

Table No. 3: Microbial profile

1. Smear Examination	A) 10% KOH Preparation
	B) Gram's stain Test
2. Culture Study	C) Fungal culture
	D) Aerobic culture

1. SMEAR EXAMINATION

A. 10% K.O.H. Preparation:

Aim: To rule out any mycological findings.

Specimen: ***Lodhradi Lepa- Churna***

Procedure For 10% KOH Preparation

Mix Potassium Hydroxides pellets in distilled water
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

↓

Note the findings

B. Gram's stain test:

Aim: To rule out any bacteriological findings.

Specimen: ***Lodhradi Lepa – Churna***

Procedure for Gram's Stain

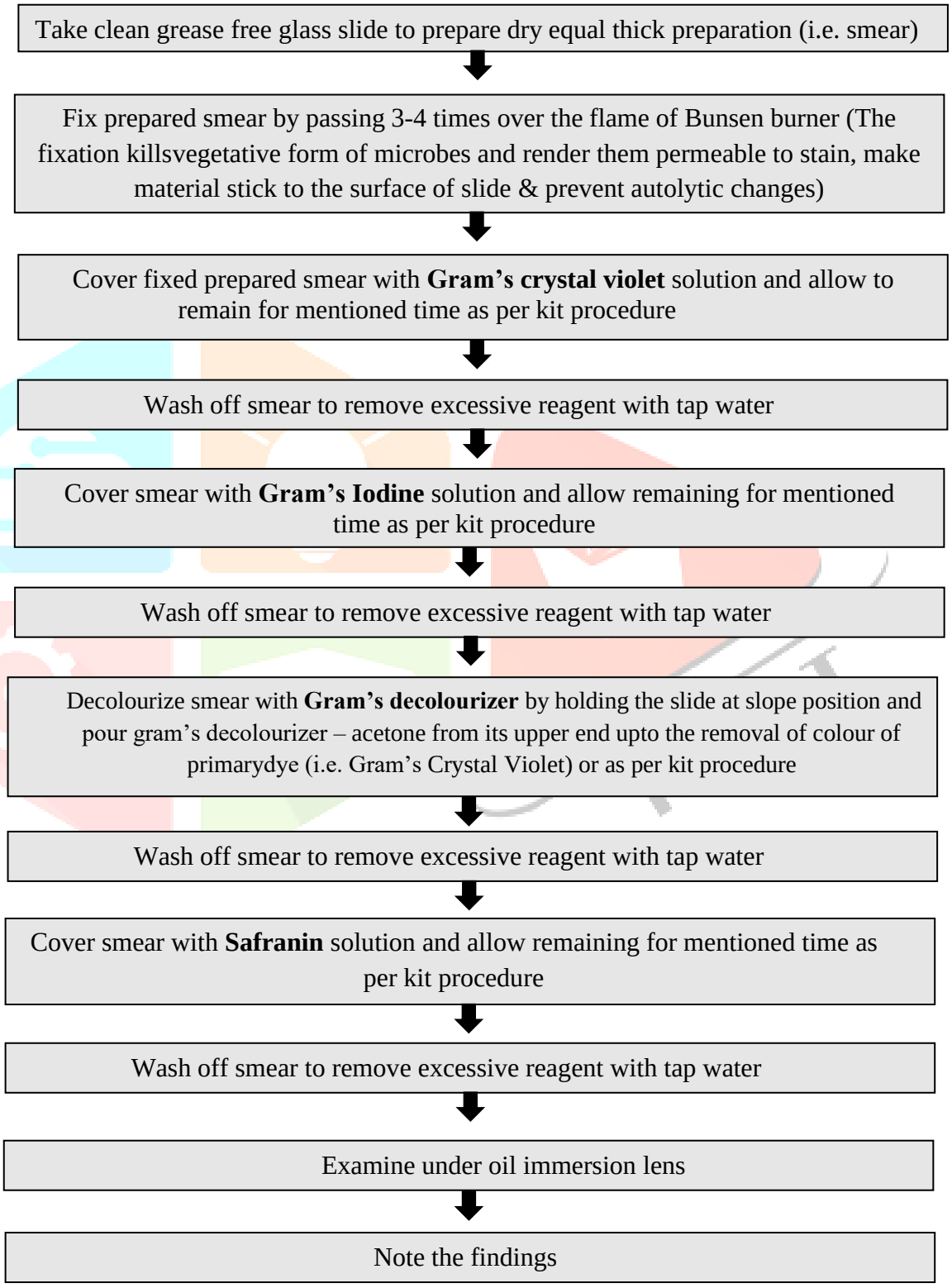


Fig. 1 & 2: Smear staining Procedure

2. CULTURE STUDY

C. Fungal culture

The materials collected with sterile cotton swab for inoculation purpose on selected fungal culture media (an artificial preparation). Details of a fungal culture media used in the study is described in Table no. 4.

Table No.4: Fungal Culture

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	37^0C
Use of media	For selective cultivation of pathogenic fungi.

Fig. 3: Sabouraud Dextrose Agar Base (SDA) bottles

Procedure For Fungal Culture

In the clinical microbiology laboratory culture method are employed for isolation oforganisms (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 to cool, then loop is charged with selected specimen to be cultured. One loop full 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 37^0 c for05 to 07 to 21 days in incubator (incubation days are as per growth requirement) under aerobic atmosphere

↓

After selected incubation period examined growth by necked eye in form of colony or arial growth and confirm growth by performing different related biochemical reactions and differentrelated staining procedures. After that report isolates.

↓

After that report isolates.

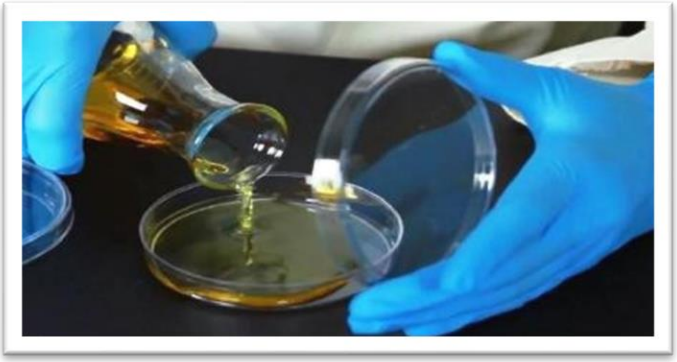


Fig. 4: Procedure for Fungal Culture

D. Aerobic Culture method

Respected materials collected with sterile cotton swab for inoculation purpose on selected aerobic culture media (an artificial preparation). Details of aerobic culture media used in the study is described in Table no. 5.

Table No.5: Aerobic Culture

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	37^0C
Use of media	For selective cultivation of pathogenic bacteria

Fig. 5: MacConkeyAgar (MA)

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 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 –blueflame 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 18-24hours 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 relatedstaining procedures.

After that report isolates

Fig. 6: Procedure for Aerobic culture

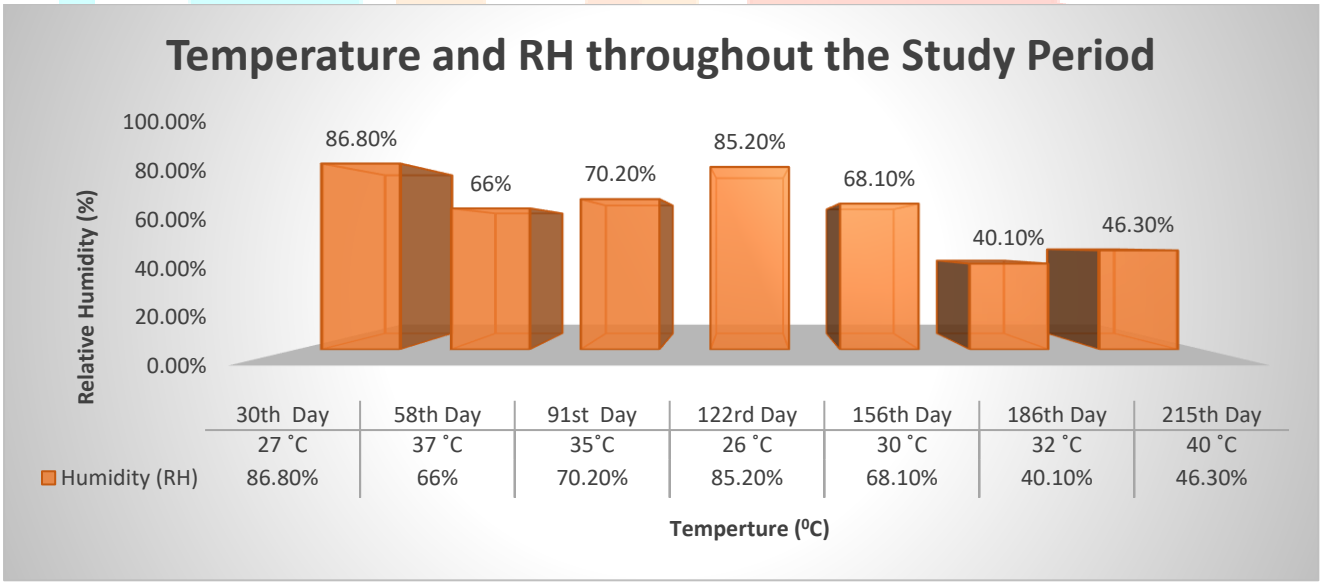
OBSERVATIONS AND RESULTS

Every time samples were subjected to the microbiological study from the date of the preparation to the date of last microbiological study. Results are shown in Table no 6.

Table No. 6: Obeservations and results of Stability tests

No.	Date of Sample given for test	Study conducted at (No. of days)	Avg. Temperature[12] (˚C)	Avg. Humidity[13] (%)	Observations/Findings			
					Gram's Stain	Aerobic culture	Wet mount/ 10% KOH Preparation	Fungal culture
1	19.09.23	30th Day	27 ˚C	86.6 %	Microorganisms not seen	No organisms isolated	Fungal filaments not seen	No fungal pathogen isolated
2	18.10 23	58th Day	37˚C	65.9 %	Microorganisms not seen	No organisms isolated	Fungal filaments not seen	No fungal pathogen isolated

3	20.11.23	91st Day	35 ˚C	70.2 %	Microorganisms not seen	No organisms isolated	Fungal filamens not seen	No fungal pathogen isolated
4	20.12.23	122rd Day	26˚C	82.5 %	Microorganisms not seen	No organisms isolated	Fungal filamens not seen	No fungal pathogen isolated
5	23.01.24	156th Day	30˚C	68.1 %	Microorganisms not seen	No organisms isolated	Fungl filamens not seen	No fungal pathogen isolated
6	22.02.24	186th Day	32˚C	40.1 %	Microorganisms not seen	No organisms isolated	Fungal filamens not seen	No fungal pathogen isolated
7	21.03.24	215th Day	40˚C	46.3 %	Microorganisms not seen	No organisms isolated	Fungal filamens not seen	No fungal pathogen isolated



Graph No.1: Timeline of Temperature and RH throughout the Study period

DISCUSSION:

The data generated during the stability test is an important requirement for regulatory approval of any drug or formulation.[14] The primary aim of pharmaceutical stability study of a drug is to give fair affirmation that the medications will stay at a proper norm of wellness/quality during the hour of which they are accessible to patients on the lookout and will be appropriate for their utilization before the patient purposes the eventual outcome unit.[15] The unscientific methods of collection,

storage, transportation and congenial climatic conditions allow raw materials for herbal formulations prone to fungal & bacterial infestations. The raw materials collected using unscientific methods are commonly exposed to many pathogenic contaminants and are often deteriorated by pathogenic microorganisms during handling and storage.[16]

Therefore, lack of regulation for herbal supplements presents potential health risk, largely due to their contamination chances with pathogenic micro- organisms. Other important aspect of drug safety is shelf-life of a drug which is defined as 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.

The city Jamnagar, the region where the drug was prepared and sample was stored is very proximal to sea coast where relative humidity (RH) is minimum 17.15% & maximum 96.4% [17] while temperature ranges from minimum 16 ^{0}C to maximum 43^{0}C.[18] High RH can trigger the growth of microbes[19], although RH remained variable throughout the study period, average air quality cannot be considered dry at RH exceeding 40%. Hence, 10% KOH, fungal culture, gram stain and aerobic culture tests were used to study fungal and bacterial contamination in the samples at monthly intervals from 19th September, 2023 to 21st March, 2024.

In present study microbiological stability study of *Lodhradi Lepa- Churna* was carried out. Samples were selected randomly for the study to rule out any microbiological contamination in entire batch of the finished product. Changes in temperature and humidity of environment were observed and noted during the study period. *Lodhradi Lepa* prepared and stored temperatures ideal for bacterial growth at room temperature, minimum temperature 26 ˚C to maximum temperature 40 ˚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 40.1% on 22nd February, 2024 to highest range 86.6 % on 19th September, 2023 as shown in Table 6, no bacterial or fungal growth was observed upto drug used till study completed.

During this study period, no any microbe was isolated as a result of aerobic culture and no any fungal pathogen was isolated as a result of fungal culture (Table 6). Thus, at the end of study, it was observed that finished drug samples studied at different time intervals and at different climatic conditions did not show any presence of microbes in them. The general concept of stability for Ayurvedic or modern medicine remains same but the parameters used to assess the stability may vary from product to product.

CONCLUSION:

Stability is usually expressed in terms 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. In present study microbiological study of *Lodhradi Lepa* showed that there wasn't any growth found of any bacterial

or fungal microorganisms till seven months from the date of its preparation which shows a good shelf life. The study also proved that quality of the drug was in a standard condition at different climatic conditions viz. relative humidity ranging from 40.1% to 86.6% and temperature ranging from 27 ˚C to 40 ˚C.

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CONFLICTS OF INTEREST:

There are no conflicts of interest.

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