



# **Antifungal Efficacy of Triple Antibiotic Paste, Double Antibiotic Paste with Fungicide and Calcium Hydroxide with Chitosan as a Vehicle against *Candida albicans*: An *In vitro* Study**

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## **Authors' contributions**

This work was carried out in collaboration among all authors. Author SSN designed the study, performed the statistical analysis, conducted the protocol. Author VR and KB analysed the study. Author CG managed the literature searches. Authors GH drafted the final manuscript. Author KKS conducted the protocol and performed the statistical analysis. All authors read and approved the final manuscript.

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## **ABSTRACT**

**Introduction:** The purpose of current research is to assess the in vitro antimicrobial efficacy of different medicaments with two different vehicles against *Candida albicans* (*C. albicans*).

**Materials and Methodology:** An agar well diffusion assay was used to determine the experimental medicaments' efficacy against *C. albicans*. Medicaments were divided into six groups, which includes Triple antibiotic powder (TAP) with saline or chitosan, Double antibiotic powder with fungicide (DAPF) with saline or Chitosan, and calcium hydroxide with saline or Chitosan. The diameters of growth inhibition zones were recorded and compared for each group for three days,

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i.e., 1,4,7 days. The differences between groups were analyzed by Kruskal-Wallis and Mann-Whitney U tests.

**Results:** The largest inhibition zones were observed for the double antibiotic paste + fungicide with chitosan and the smallest for Ca(OH)<sub>2</sub> with saline. Over a week, zones of inhibition were consistent only with group V – double antibiotic paste with fungicide and chitosan as the vehicle.

**Conclusion:** DAPF with chitosan is more efficient against *Candida albicans*. Since the endodontic infections are polymicrobial in origin, the combined local drug delivery of antimicrobial agents such as ciprofloxacin, metronidazole, doxycycline along with antifungal drugs such as fluconazole with an efficient drug carrier (chitosan) is recommended to combat the microbial load.

**Keywords:** Antimicrobial efficacy; calcium hydroxide; *Candida albicans*; chitosan; double antibiotic paste; triple antibiotic paste; intracanal medicaments; root canal medicaments.

## 1. INTRODUCTION

Root canal system serves as a harbour of microorganisms which provides an inherent habitat with low oxygen concentration for the growth of numerous bacteria and fungi. Endodontic infections are polymicrobial in origin with obligatory anaerobes being the dominant species [1]. Although facultative anaerobic bacillus such as *E.faecalis* is the most common organism associated with periradicular pathogenesis, fungi are also predominant organisms isolated from 3-18% of infected root canals.

The most potent and frequently isolated fungus from endodontic infections is *Candida albicans* while others being *Candida tropicalis*, *Candida kefyr*, *Candida parapsilosis*, *Candida glabrata* responsible for endodontic failures [2].

The foremost objective of endodontic treatment is rendering the root canals free of bacterial overload and reduce the risk of reinfection. However, because of the complex nature of the root canal system and the presence of many inaccessible areas chemomechanical preparation alone cannot eliminate all the microorganisms, so the use of interappointment medicament is recommended [3].

Calcium hydroxide [Ca(OH)<sub>2</sub>] has been widely used as an intracanal medicament due to its antimicrobial properties. However it has limited action against *C.albicans*. The high pH level of Calcium hydroxide alters the lipopolysaccharide structured cell wall of gram-negative bacteria, which inactivates membranes' transport and ultimately leads to cell death [4].

Recent studies revealed that Triple antibiotic paste, a combination of metronidazole, ciprofloxacin, and minocycline, has been used as an intracanal medicament successfully for

disinfecting the root canal system during regenerative procedures. Since the infected root canal biofilm is inaccessible to the local immune system, the drug concentration that reaches the canal space post administering systemic antibiotics is minimal and the action of the latter is uncertain. Therefore, the local application of intracanal medicaments within the root canal system may be a more effective mode of delivering the blend of drugs [5].

Chitosan has a wide range of applications in the field of medicine and dentistry mostly known for its antimicrobial; antifungal properties, pharmaceutical drug delivery and as vaccine adjuvant. It is used as a drug carrier where it has the added advantage of slow and controlled release of intracanal medicament and increases the medicament's constancy. The second most abundant natural polysaccharide is chitin, the basic component of crustacean exoskeletons. The partial deacetylation of which produces chitosan. It is composed of β (1→4) linked N-acetyl glucosamine units [6].

In continuation to our previous study on *E.faecalis* [7], this study aimed to evaluate the antifungal efficacy of Triple antibiotic paste, Double antibiotic paste with fungicide and Calcium hydroxide along with two different vehicles, saline and Chitosan as root canal medicaments used in endodontic therapy against *Candida albicans*.

## 2. MATERIALS AND METHODS

Ethical approval is obtained from the Institution's Ethics Committee. The present in vitro study was done at the Department of Conservative and Endodontics in collaboration with the Department of Microbiology at Anil Neerukonda Institute of Dental Sciences, Visakhapatnam, Andhra Pradesh, India.

The materials tested were:

- Triple antibiotic paste (ciprofloxacin, metronidazole and doxycycline).
- Double antibiotic paste with fungicide (ciprofloxacin, metronidazole, fluconazole).
- Calcium hydroxide
- Saline
- Chitosan

The methodology used in this study is done by an agar diffusion method.

### 2.1 Sample Preparation - Agar Diffusion Method

Standard resistant strains of *Candida albicans* are obtained (American Type Culture Collection [ATCC] 1827) for this study and were cultured on Sabouraud's Dextrose agar medium. Sterile glass petri dishes were used to prepare the agar plates and kept overnight at 37°C for sterility. After ensuring sterility, the strains were inoculated within the sterile saline, and the turbidity was compared using McFarland's turbidity standard tube No 0.5. These inoculations were used to make the organism's lawn culture using sterile cotton swabs on Sabouraud's Dextrose agar. Rendering to Kirby Bauer's punch well method, the holes were punched in the cultivated agar plates (4mm in-depth, 6mm in diameter). A total of 60 wells in 60 Sabouraud's Dextrose agar plates for *E. faecalis* were prepared (1 plate = 1 well). The medicaments tested were in powdered form. A sterile spatula was used to place the medicament prepared into each well. The plates were then incubated at 37°C under appropriate atmospheric conditions (80% N<sub>2</sub>, 10% CO<sub>2</sub>, 10% H<sub>2</sub>) for 24 hours under anaerobic conditions in a CO<sub>2</sub> incubator. The diameters of the zones of bacterial growth inhibition around the wells containing the test medicaments were then recorded after the period of incubation. The inhibitory zone was determined in millimeters by measuring the shortest distance between the

outer margin of the well and initial microbial growth. The readings were recorded on the 1<sup>st</sup> day, 4<sup>th</sup> day and 7<sup>th</sup> day.

### 2.2 Statistical Analysis

The results were analyzed using SPSS 20.0 software and were expressed by the mean ± standard deviation. The data were analyzed with Mann-Whitney U tests to check the microbial inhibition zones of differences between the groups. The p-value was considered significant at ( $P < 0.05$ )

## 3. RESULTS

Tables 1, 2, 3 show the mean counts and standard deviation of different diameters of microbial inhibition zones between 6 different groups. All the intra canal medicaments tested reduced the fungal load significantly irrespective of the vehicle used. The zones of inhibition are represented in Figs. 1, 2. The experimental medicaments with chitosan as a vehicle showed significantly better results among which Group V- double antibiotic paste with fungicide + chitosan exhibited better antifungal efficacy against *C.albicans*. Group III - Ca(OH)<sub>2</sub> + saline showed the least antibacterial efficacy. Over a week, zones of inhibition were consistent only with group V – double antibiotic paste with fungicide and chitosan as the vehicle.

## 4. DISCUSSION

The golden rule of successful endodontic therapy is the broad elimination of infection and three-dimensional obturation of the root canal system to attain a hermetic seal, thus preventing recurrent infections. Chemo mechanical preparation alone could not eliminate bacteria because of the complexity of the root canal system and limitation of access by instruments and irrigants, so the use of different intracanal medicaments was proposed to eliminate the bacteria [8].

The details of medicaments tested against *C.albicans* were as follows:

Groups	Description
I	Triple antibiotic paste (TAP) + Saline
II	Double antibiotic paste with fungicide (DAFP) + Saline
III	Calcium hydroxide (Ca(OH) <sub>2</sub> ) + Saline
IV	Triple antibiotic paste + Chitosan
V	Double antibiotic paste with fungicide + Chitosan
VI	Calcium hydroxide + Chitosan

**Table 1. Antifungal activity against C.albicans (TAP with saline and chitosan)**

Day	Candida Albicans					P Value
	TPA with SALINE		TPA with CHITOSAN		Difference Mean±SD	
	Mean	SD	Mean	SD		
1	35.60	1.71	60.00	9.14	24.40±7.43	0.000 S
4	32.60	2.27	42.60	12.50	10.00±10.23	0.023 S
7	24.20	4.87	40.80	12.14	16.60±7.27	0.001 S

Statistically significant if  $P < 0.05$

**Table 2. Antifungal activity against C.albicans (DAPF with saline and chitosan)**

Day	Candida Albicans					P Value
	DAPF with SALINE		DAPF with CHITOSAN		Difference Mean±SD	
	Mean	SD	Mean	SD		
1	39.80	8.78	62.20	6.84	22.40±1.94	0.000 S
4	39.60	2.46	56.00	4.57	16.40±2.11	0.000 S
7	30.80	8.26	55.20	3.79	24.40±4.47	0.000 S

*C.albicans* in comparison to its counterparts, appear to possess superior environmental adaptability, to evade the host defenses in adverse habitats such as the endodontic ecosystems despite efficient biomechanical preparation, irrigation due to its superior virulence attributes such as tenacious adhesion to host surfaces, and subsequent biofilm formation, production of hydrolytic enzymes, phospholipases, haemolysins and proteinases, and phenotypic switching [9].

The choice of culture media in the present study was Sabouraud's Dextrose agar, as this media is most commonly used for *C.albicans* incubation. In our study to determine the antimicrobial efficacy of the test medicaments agar diffusion test was used which is an accepted and standardized method making it reproducible, simple to perform and relatively inexpensive [10].

Calcium hydroxide is insufficient for the elimination of some symptoms, and thus, antibiotic pastes are employed as potential alternative [11, 12] due to their good antimicrobial and biocompatible properties [13-15]. One of the most widely used antibiotic pastes is TAP, which consists of equal portions of metronidazole, ciprofloxacin, and minocycline. However, TAP always causes tooth discoloration [16]. So, the current study evaluated the effect of adding an antifungal drug to DAP.

The ideal or optimum vehicle for the delivery of antibiotics in root canals should have the ability

to facilitate better diffusion of medicament through dentinal tubules and anatomical aberrations like fins, isthmuses, and blocked canals. In this study, Chitosan and saline were used as vehicles for the antimicrobials tested [17].

Chitosan is a  $\beta$ -1, 4-linked polymer of glucosamine (2-amino-2-deoxy- $\beta$ -D-glucose) natural polysaccharide comprising copolymers glucosamine and N-acetyl glucosamine. Due to its biodegradable and nontoxic properties, this helped prepare nanoparticles for various applications. It is insoluble in acidic conditions, and free amino groups on its polymeric chain protonate and donate to its positive charges. Its cationically charged amino group might combine with N-acetyl muramic acid, sialic acid, and neuramic acid, an anionic component on the cell surface, suppressing fungal growth by compromising the exchanges with medium, chelating transition metal ions, and inhibiting enzymes [18]. Therefore, incorporating Chitosan to TAP, DAPF,  $\text{Ca}(\text{OH})_2$  in a trial against *C.albicans* serves as a potential additive targeting their viability. Also, this explains the sustained antifungal effect of DAPF with chitosan as a vehicle.

TAP composed of ciprofloxacin, metronidazole, and minocycline, had an appropriate effect against *E.faecalis*, but not against *C.albicans* unlike DAPF. This result can be attributed to the fluconazole incorporated alongside antibiotic drugs.

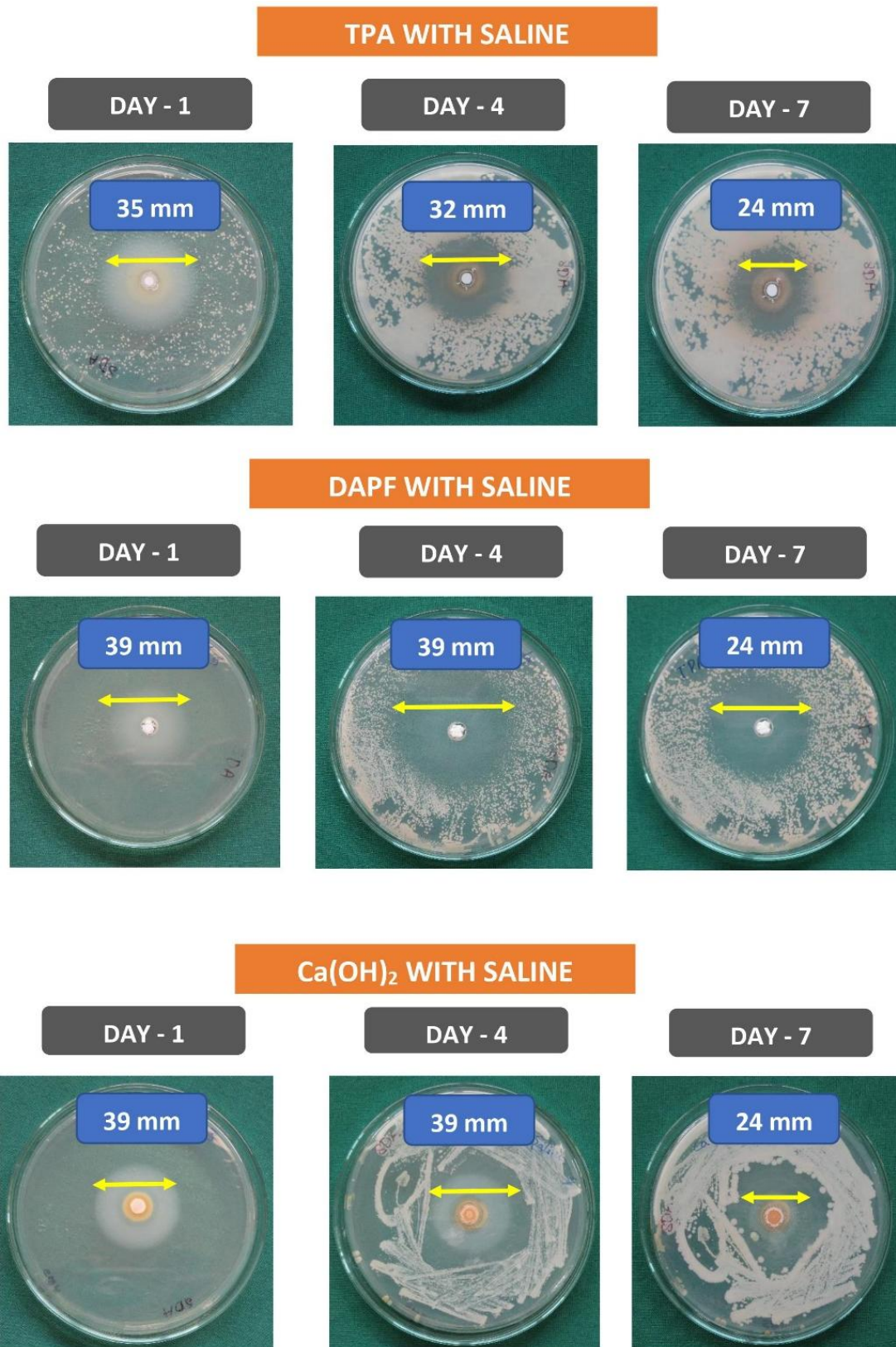


Fig. 1. Clinical representation of all mean zones of diameter in "mm" of various intracanal medicaments on the first, fourth & seventh day by saline treatment



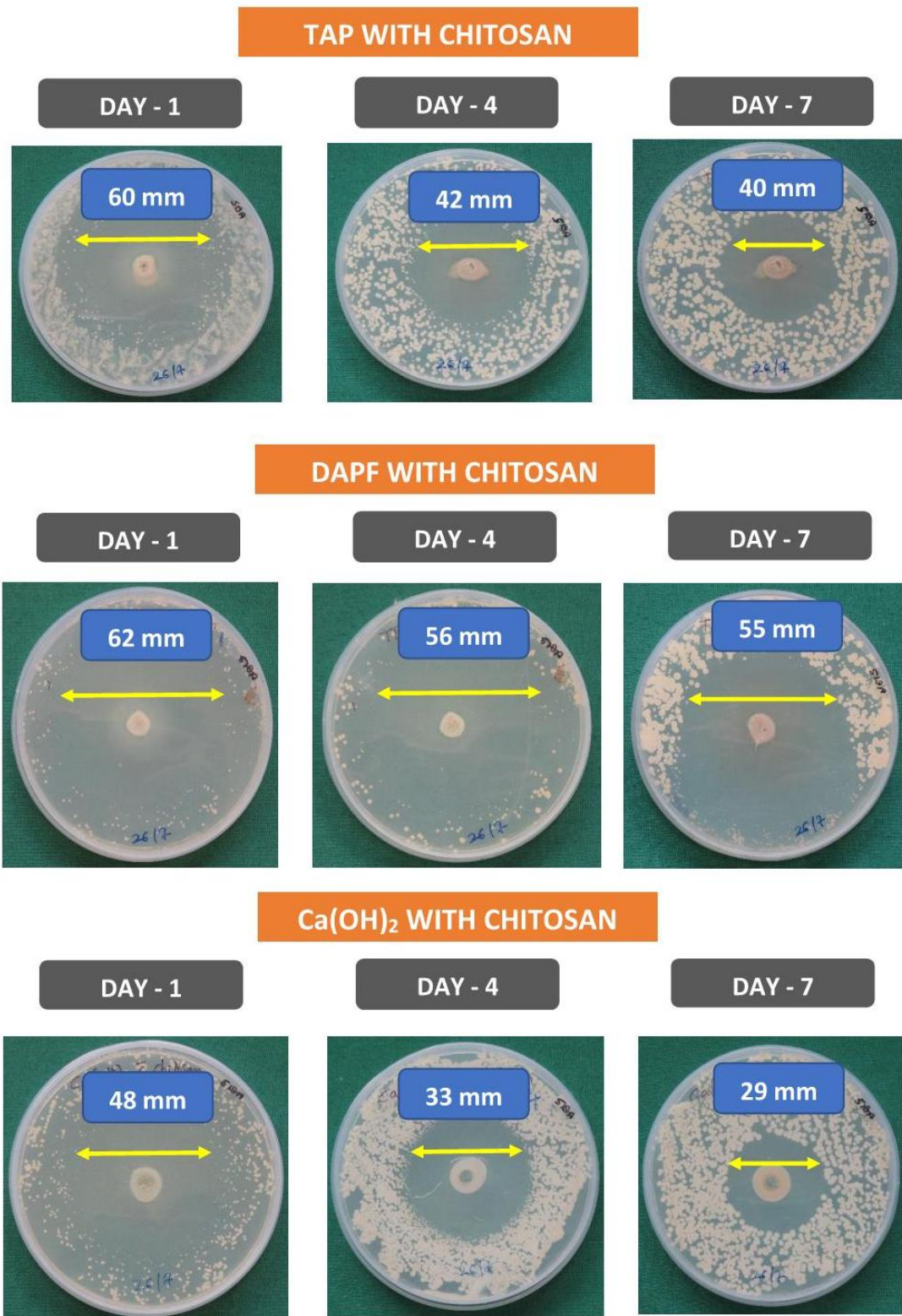


Fig. 2. Clinical representation of all mean zones of diameter in "mm" of various intracanal medicaments on the first, fourth & seventh day by chitosan treatment

**Table 3. Antifungal activity against C.albicans (CH with saline and chitosan)**

Day	Candida Albicans				Difference	P Value
	Ca(OH)2 with SALINE		Ca(OH)2 with CHITOSAN			
	Mean	SD	Mean	SD		
1	31.40	8.18	48.00	5.62	16.60±2.56	0.000 S
4	22.40	9.35	33.00	9.48	10.60±0.13	0.021 S
7	18.00	12.15	29.20	8.57	11.20±3.58	0.028 S

In extension to our previous study against *E.faecalis* [7], it is concluded that TAP with chitosan as a vehicle proved to have better efficacy against *E.faecalis* but DAPF with chitosan is more efficient against *Candida albicans*.

## 5. CONCLUSION

Since the endodontic infections are polymicrobial in origin, the combined local drug delivery of antimicrobial agents such as ciprofloxacin, metronidazole, doxycycline along with antifungal drug such as fluconazole with an efficient drug carrier (chitosan) is recommended to combat the microbial load.

## DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

Before carrying out the present in vitro study, institutional ethics committee approval was obtained from the college.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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