Antifungal activity of essential oils from *Cinnamomum cassia*, *Myristica fragrans* and *Syzygium aromaticum* against *Rhodotorula mucilaginosa*

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*Rhodotorula* genus comprises yeasts from Sporidiobolaceae family. Considered as non-pathogenic until the last two decades, different species of *Rhodotorula* are emerging as human pathogens. *Rhodotorula mucilaginosa* is the most prevalent species commonly involved in infections which range from simpler clinical conditions such as skin manifestations to more severe cases as meningitis and endocarditis. The major facilitating agents for the emergence of these infections are invasive procedures such as catheter implants. The primary drugs of choice used to treat these infections are amphotericin B and fluconazole. However, some strains of this yeast show different degrees of resistance to these substances, thus justifying the search for new therapeutic agents. Considering this, the present study aims the investigation of the antifungal activity of the essential oils of *Cinnamomum cassia* (cinnamon), *Syzygium aromaticum* (clove) and *Myristica fragrans* (nutmeg) against clinical isolates of *R. mucilaginosa*. The essential oils were obtained by hydrodistillation and characterized by GC-MS. The investigation of the antifungal activity was performed by the agar disc-diffusion test followed by the Minimum Inhibitory Concentration (MIC) determination. All the essential oils are characterized by the presence of phenylpropanoids, with eugenol (77.6 to 94.4%) as the main compound of clove and *E*-cinnamaldehyde (90.4 to 100%) of cinnamon. Nutmeg oil is characterized by the presence of the phenylpropanoids myristicin (1.8 to 12.8%) and elemicin (4.3 to 11.1%), besides the monoterpenes sabinene (28.2 to 44.4%) and terpinen-4-ol (16.0 to 19.5%). In the investigation of antifungal activity, all the oils showed potential action against clinical isolates of *R. mucilaginosa*, with MICs ranging from 8 to 500 µg/mL. The results demonstrate that these oils are promising candidates in the search for new anti-*Rhodotorula* agents, enabling the treatment of aforementioned infections.

**Keywords**: *Rhodotorula mucilaginosa*, essential oils, antifungal.

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**Introduction**

*Rhodotorula* is a yeast fungus commonly found in nature. It can be found in different types of samples, such as soil, water, milk, fruit juices, and in the air. Although it has been considered a non-pathogenic microorganism until the last two decades, different species of *Rhodotorula* are emerging as human pathogens. Invasive procedures such as catheter implantation, especially in immunosuppressed patients, are a major factor in the emergence of secondary infections, such as fungemias, endocarditis, meningitis, peritonitis, among others (1).

*Rhodotorula* spp. appear to be more frequent in tropical regions (2). The first case of fatal endocarditis attributed to species of this genus was published in the 1960s in a 47-year-old rheumatic woman with heart disease (3). Since then, other reports of infection in humans have been described in the scientific literature, mainly in the last two decades (4). *Rhodotorula mucilaginosa* is the most commonly involved in human infections, followed by *R. glutinis* and *R. minuta* (2). The first choice of treatment involves the administration of amphotericin B and fluconazole. A study conducted by Franconieri et al. (5), including eleven case studies of peritonitis caused by *R. mucilaginosa*, found that amphotericin B and fluconazole had the strongest anti-*Rhodotorula mucilaginosa* activity. The tests performed to check its presence allow the visualization of the growth of its colonies after 24 to 48 hours of incubation, commonly showing smooth and mucous, reddish-orange appearance in Sabouraud dextrose agar culture medium with chloramphenicol. The maximum growth temperature varies from 25 to 37 °C (6,7).

*Cinnamomum cassia* (L.) J. Presl (cinnamon-of-china) is an aromatic plant, belonging to the Lauraceae family widely used in cooking and fragrance production (8). It originates in China and is widely cultivated in Indonesia, India, Malaysia, Laos, Taiwan, Thailand, and Vietnam (9). In folk medicine, it is used to treat inflammatory processes, fever, and hypertension. The essential oil has exhibited antifungal and antibacterial activity, in addition anti-inflammatory, antioxidant, anti-tyrosinase actions, among others (10–15). Regarding *E*-cinnamaldehyde, the main component of essential oil, the anti-inflammatory and antioxidant (16), antifungal (17) and antibacterial (18) activities have been proven.

*Syzygium aromaticum* L. Merr. & L.M. Perry (clove) is a large tree (12 to 15 meters tall), belonging to the Myrtaceae family and widely cultivated in Tanzania, Brazil, Sri Lanka, Indonesia, and Madagascar (19). It has...
been popularly used due to its flavoring properties, in food preservation, oral hygiene products, and also in gastrointestinal disorders. Several studies show its importance as a bactericidal and fungicidal, antioxidant, and anti-inflammatory agent, among others (20–23). The antimicrobial activity observed may be related to eugenol, the main constituent of the essential oil of the floral bud of *S. aromaticum*, a compound for which antimicrobial activities have already been proven (24–26), in addition to anti-inflammatory and antioxidant (16).

*Myristica fragrans* Houtt. (nutmeg) is a tree in the Myristicaceae family that can reach up to 12 meters in height as an adult (27). The largest producer is Indonesia, followed by Granada, Sri Lanka and Trinidad and Tobago (28). This species has been used in cooking as a food spice, as a flavoring and also in treatments for diseases such as stomach pain, rheumatism and vomiting during pregnancy (29). For the nutmeg essential oil, antimicrobial, antiseptic, antiparasitic, anti-inflammatory and antioxidant activities have been demonstrated (29–32). To date, there are no reports in the specialized literature describing the action of this species against *Rhodotorula mucilaginosa*. Thus, this work aims to extract the essential oils from *Cinnamomum cassia*, *Syzygium aromaticum* and *Myristica fragrans*, perform their chemical characterization through GC-MS and evaluate their antifungal activity against *R. mucilaginosa*, looking for new antifungal prospects for the market.

### Materials and Methods

#### Samples

Samples of *Cinnamomum cassia* (barks), *Syzygium aromaticum* (floral bud), and *Myristica fragrans* (fruits) were commercially acquired in the state of Rio Grande do Sul. The oils were obtained by hydrodistillation in a Clevenger type apparatus, according to the methodology recommended by the Brazilian Pharmacopoeia 6th ed. (2019).

The microorganisms used in this study are clinical isolates of *R. mucilaginosa* resistant to classic antifungals (approval by the Ethics Committee No. 4.069.576, CEP-UFRGS), provided by the Biomicolab Laboratory (Mycology Laboratory of the Federal University of Rio Grande do Sul). Five clinical isolates of *R. mucilaginosa* were selected. The stock cultures were kept in freezing soybean trypticase broth (TSB; Himedia®) plus 10% glycerol at -18 °C. To reactivate the strains, 10 µL of the stock culture were inoculated into tubes containing Sabouraud dextrose agar with chloramphenicol (Kasvi®) and incubated at 30 °C for 48-h. As positive controls, fluconazole (Polydrug Laboratory Private Limited/India), ketoconazole (Aairt Drugs Limited/India), amphotericin B (North China Pharmaceutical/China) and itraconazole (Cecon/Brazil) were used.

### Essential Oils Chemical Analysis

For chromatographic analysis, the oils obtained were diluted to 2% in ethyl ether (v/v) (Tedia®). The analysis was performed using a gas chromatograph coupled to a mass detector (GC-MS), model Shimadzu QP5000, equipped with a capillary column of fused silica Durabond-DB-5 (John Wiley & Sons Scientific, US, 30 m x 0.25 mm x 0.25 µm) for separating the constituents. The source temperature, quadrupole and injector were set at 230 °C, 150 °C and 220 °C, respectively. Detector temperature was set at 250 °C. The column temperature was programmed with a heating ramp from 60 °C to 300 °C with an increase of 3 °C / min, using ultrapure helium as the carrier gas at 80 kPa and flow of 1 mL/min. The mass detector was operating at 70 eV.

The components were identified by comparing their relative retention index, calculated by relative linear interpolation for the retention time of a series of *n*-alkanes (C<sub>9</sub> to C<sub>32</sub>), and their mass spectrum, with data obtained in the literature (33), as well as, by comparison with mass spectrums of the acquisition spectrothea (NIST 62 and 12 - National Institute of Standards and Technology, Kyoto, JP) and standards from our research group repository. Relative amounts of each compound were calculated from the peak areas by normalization.

### Antifungal Activity

Initially, antifungal activity against *R. mucilaginosa* was investigated by the paper disc diffusion technique (Kirby Bauer method). The tests were performed on sterile Petry plates containing Sabouraud agar. In summary, a suspension in yeast Sabouraud agar (90%) was spread on the plate agar. Paper discs were impregnated with 15-µL of the essential oil and placed on the surface of the previously inoculated medium. The plates were incubated in an oven at 37 °C and the inhibition halos were read after 48-h. The measurement scale was as follows: the zone of inhibition >15 mm was considered to be strongly inhibitory; the zone of inhibition 10-15 mm moderately inhibitory and <10 mm non-inhibitory (34). The test was performed in duplicate. The essential oils that exhibited strong inhibition were subjected to the test to determine the Minimum Inhibitory Concentration (MIC). As for antifungals, it was performed with the itraconazole disc to verify the antifungal action of this compound on the yeast *R. mucilaginosa*.

The determination of the Minimum Inhibitory Concentration (MIC) against *R. mucilaginosa* was carried out by the broth microdilution method, as recommended by the Clinical and Laboratory Standards Institute - CLSI (35). The oils were dissolved in dimethyl sulfoxide - DMSO (Nuclear®). The concentration range used for essential oils was 0.488 to 1000 µg/mL (36). The *R. mucilaginosa* culture (103 CFU/mL) in RPMI 1640 (Sigma Aldrich®) was added to the microplate wells to detect the action of the oil. The microplates were incubated at 30 °C and inspected after 24 and 48 h for visual verification of growth (35,37,38). The experiment
was carried out in duplicate. MIC was considered to be the lowest concentration of oil capable of completely inhibiting microbial growth visible to the naked eye. For results evaluation, the following cut-off points were used for the antifungals tested according to CLSI: fluconazole: sensitive (S) ≤ 8 µg/mL; dose-dependent sensitive (SDD) 16-32 µg/mL; resistant (R) > 64 µg/mL; itraconazole: S ≤ 0.12 µg/mL, S-DD 0.25-0.5 µg/mL, R > 1 µg/mL; amphotericin B: R ≥ 1 µg/mL. (37). For ketoconazole, the following cut-off point S ≤ 1 µg/mL, S-DD 2-4 µg/mL, R > 4 µg/mL was used.

Results and Discussion

The data regarding the samples are shown in Table 1. Three samples of cloves (identified by OSA1 to OSA3), five samples of cinnamon (OCC1 to OCC5) and two of nutmeg (OMF1 and OMF2) were considered. The percentage yield was calculated by the relationship between the volume of oil obtained by the dry mass of the plant. Considering the levels of essential oil obtained, there are some peculiarities, as in the case of Syzygium aromaticum, with a high variation in the oil content obtained from the three commercial samples (average of 9.8 ± 4.03%). This result may be related to the supplier, as in the case of Cinnamomum cassia, these authors also observed. According to Desai (39) obtained 11.35% yield for the essential oil extracted from the referred plant, corroborating the average value obtained in the present study.

Table 1. Percentage yield of essential oils obtained by hydrodistillation from Syzygium aromaticum (floral bud), Cinnamomum cassia (barks) and Myristica fragrans (fruits).

| Species | Supplier | Acquisition date | Expiration date | Yield [%] |
|---------|----------|-----------------|----------------|-----------|
| S. aromaticum | | | | |
| OSA1 | A | 18/09/2018 | 19/03/2020 | 14.1 |
| OSA2 | A | 02/10/2018 | 11/03/2020 | 9.3 |
| OSA3 | B | 03/10/2018 | Not informed | 6.1 |
| C. cassia | | | | |
| OCC1 | A | 12/09/2018 | 20/07/2020 | 0.5 |
| OCC2 | C | 14/09/2018 | 01/08/2020 | 0.2 |
| OCC3 | A | 26/09/2018 | 11/06/2020 | 0.3 |
| OCC4 | A | 27/09/2018 | 11/06/2020 | 0.2 |
| OCC5 | A | 28/09/2018 | 20/07/2020 | 0.5 |
| M. fragrans | | | | |
| OMF1 | A | 24/09/2018 | 12/06/2020 | 1.7 |
| OMF2 | D | 25/09/2018 | 06/07/2019 | 2.6 |

For the samples of Cinnamomum cassia, it is observed that the variation was not so great, presenting an average yield of 0.3 ± 0.14%. This value is close to that found in the literature. Tao et al. (40) obtained an average yield of 1.48% in the essential oil extracted from Cinnamomum cassia. Considering the suppliers there is no difference among them. Finally, for Myristica fragrans, the two samples showed a difference in their essential oil yield value with an average of 2.1 ± 0.64%, which may be related also to the supplier. This yield value is slightly below that reported in the literature for that plant (41). Regarding the expiration date, all samples were within the validity period (the ones that had this information).

The chemical characterization of essential oils obtained by hydrodistillation was performed by GC-MS. The results are described in Table 2. All the species selected for this study are characterized by the presence of phenylpropanoids. The analysis of the Syzygium aromaticum samples highlighted the majority of eugenol (77.6 to 94.4%) followed by its eugenyl acetate derivative (4.8 to 20.7%). The samples of essential oil of Cinnamomum cassia presented E-cinnamaldehyde as the main constituent, sometimes accounting for the totality of the oil (90.4 to 100%). Finally, the essential oil of Myristica fragrans was characterized by the presence of myristicin (1.8 to 12.8%) and elemicin (4.3 to 11.1%). In the oil of this species, monoterpens were also present in large quantities, represented mainly by sabinene (28.2 to 44.4%) and terpinen-4-ol (16.0 to 19.5%).

Souza et al. (42) studied the chemical composition of 16 essential oils, including those extracted from Syzygium aromaticum, Cinnamomum cassia and Myristica fragrans. The results associated with the composition of S. aromaticum oil reported in that study showed that it was mainly composed of eugenol (80.67%) and eugenyl acetate (11.92%). These values corroborate the analysis carried out in the present study and reported in Table 2, where an average concentration of 84% eugenol and 15% eugenyl acetate was found in clove oils. Still, according to these same authors, the essential oil of Cinnamomum cassia is composed mainly of E-cinnamaldehyde (84.52%), also in agreement with the results found in this study (Table 2).
The investigation of antifungal activity was performed to verify the susceptibility of R. mucilaginosa yeast to Syzygium aromaticum (OSA), Cinnamomum cassia (OCC) and Myristica fragrans (OMF) oils. Pooling was performed to determine the samples used in the test. Initially, a screening was carried out to select the samples that demonstrated action on yeast. For this purpose, the Kirby Bauer method was used which consists of impregnating paper discs with the oil samples to be tested. Itraconazole was used to check the susceptibility of the microorganism. The results obtained in this stage are shown in Figure 1, and Table 3 describes the diameters of the inhibition halos for each essential oil and for itraconazole. It can be seen that R. mucilaginosa was resistant to itraconazole, but sensitive to the three oils used. Thus, the three oils were sent to the next step, which consisted of determining the minimum inhibitory concentration (MIC). At this time, the antifungal agents itraconazole (ITR), amphotericin B (AMP), ketoconazole (KET), fluconazole (FLU) were also used to check yeast susceptibility. The MIC for each sample was determined in duplicate and on two different days. The concentration ranges tested were: 0.488 - 1000 µg/mL for essential oil samples; 0.0312 - 16 µg/mL of ANF; 0.03 - 16 µg/mL CET; 0.125 - 64 µg/mL of FLU and 0.0312 - 16 µg/mL of ITR. MICs values were defined as the lowest concentration of oil and amphotericin B samples in which R. mucilaginosa did not show visible growth. For itraconazole (ITR), ketoconazole (CET), fluconazole (FLU) it was defined as being 50% of the positive control. This value represents the drug concentration that inhibits the growth of 50% of the isolates. In general, all essential oils tested showed promising activity, with MICs ranging from 8 to 500 µg/mL (Table 3). Considering the classic antifungals, it was observed that all clinical isolates were resistant. It is interesting to note

Table 2. Chemical characterization of essential oils obtained from different samples of Syzygium aromaticum, Cinnamomum cassia and Myristica fragrans by GC-MS.

| RRI  | Constituents          | Syzygium aromaticum | Cinnamomum cassia | Myristica fragrans |
|------|-----------------------|---------------------|-------------------|-------------------|
|      | OSA1 | OSA2 | OSA3 | OCC1 | OCC2 | OCC3 | OCC4 | OCC5 | OMF1 | OMF2 |
| 1281 | -    | -    | -    | -    | -    | -    | -    | -    | -    |      |
| 1365 | 77.6 | 80.0 | 94.4 | -    | -    | -    | -    | -    | -    |      |
| 1406 | 1.6  | 0.5  | 0.9  | -    | -    | -    | -    | -    | -    | 1.9   |
| 1447 | -    | -    | -    | 2.0  | 0.2  | -    | -    | -    | -    | trace |
| 1487 | -    | -    | -    | trace| trace| Trace| -    | -    | -    | 12.8  |
| 1519 | -    | -    | -    | -    | -    | -    | -    | -    | -    | 4.3   |
| 1528 | 20.7 | 19.6 | 4.8  | -    | -    | -    | -    | -    | -    |      |
| 1538 | -    | -    | -    | -    | -    | -    | -    | -    | -    |      |
| 1556 | -    | -    | -    | -    | -    | -    | -    | -    | -    |      |

Compounds listed in order of elution in DB5 column; RRI: relative retention index. OSA: essential oil of Syzygium aromaticum. OCC: essential oil of Cinnamomum cassia. OMF: essential oil of Myristica fragrans.

Table 3. Inhibition halos size (values in mm) of the Syzygium aromaticum, Cinnamomum cassia and Myristica fragrans essential oils and Itraconazole against five clinical isolates of the yeast R. mucilaginosa.

| Clinical isolate | OSA | OCC | OMF | ITR |
|------------------|-----|-----|-----|-----|
| #1               | 28  | 40  | 15  | R   |
| #2               | 25  | 40  | 18  | R   |
| #3               | 25  | 40  | 10  | R   |
| #4               | 30  | 40  | 15  | R   |
| #5               | 35  | 40  | 20  | R   |

OSA: essential oil of Syzygium aromaticum, OCC: essential oil of Cinnamomum cassia, OMF: essential oil of Myristica fragrans. ITR: itraconazole. R: resistant.

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the difference in response according to the clinical isolate, which can be related to variations in intrinsic or acquired resistance, possibly induced by the environment. Among the essential oil samples tested, the one that showed the best result was Cinnamomum cassia, for which all clinical isolates tested were susceptible, with a MIC value of 8 µg/mL being observed. The oil of Myristica fragrans showed the best result for isolate 4 (MIC 8 µg/mL). The oil of Syzygium aromaticum showed action against all isolates, with MIC ranging from 62 to 125 µg/mL.

Figure 1. A) Results associated with clinical isolate 1 from essential oil of Syzygium aromaticum L. Merr. (OSA), Cinnamomum cassia (L.) J. Presl (OCC), Myristica fragrans Houtt (OMF) and itraconazole (ITR), from left to right, respectively; B) results associated with clinical isolate 2 from OSA, OCC, OMF and ITR; C) results associated with clinical isolate 3 from OSA, OCC, OMF and ITR; D) results associated with clinical isolate 4 from OSA, OCC, OMF and ITR; E) results associated with clinical isolate 5 from OSA, OCC, OMF and ITR.

The essential oil extracted from Cinnamomum cassia has been the subject of scientific studies due to its biological properties. In a study conducted by Cavalcanti and Almeida (10), the oil of this species demonstrated antifungal activity against strains of Candida albicans isolated from HIV positive patients and standard strain (ATCC 76845), with a MIC value of 64 µg/mL being observed for the most part of the strains analyzed. MA et al. (9), have already proved the antifungal property of C. cassia oil against pathogenic fungi present in Panax
Some scientific studies have reported the antifungal activity of the essential oil obtained from Syzygium aromaticum (46–50). Rajkowska et al. (50), for example, evaluated the antifungal action of Syzygium aromaticum against nine species of Candida, with MIC values varying between 0.03 and 8%. In the research conducted by Pinto et al. (48), antifungal activity of S. aromaticum oil was confirmed against strains of some species of Candida, Aspergillus and dermatophytes. As reported, these results were related to the high concentration of eugenol present in the essential oil. In another study, the action of eugenol alone against Candida albicans and in combination with amphotericin B was demonstrated, showing synergistic potential, enabling the use of lower concentrations of this substance in the treatment of C. albicans (51). Such findings are in agreement with the results presented in this work, demonstrating the potential antifungal effect of the oil against several species of fungi.

The antifungal action of Myristica fragrans essential oils has also been demonstrated against strains of Aspergillus niger, Fusarium axysporum, Penicillium glabrum, Rhizopus oryzae and Mucor recemosus (28) as well as against Candida albicans (52). These results corroborate the present study, where antifungal activity against Rhodotorula mucilaginosa was also proven (Table 4).

Table 4. Minimum Inhibitory Concentration (MIC) (values in µg/mL) of the Syzygium aromaticum, Cinnamomum cassia and Myristica fragrans essential oils against five clinical isolates of the yeast R. mucilaginosa.

| Clinical isolate | OSC | OCF | OMF | OFP | OMP | OKT | OIU | ORB |
|------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| OSA              | 250 | 62  | 500 | 16  | 16  | 64  | 16  | 16  |
| OCC              | 62  | 125 | 500 | 64  | 16  | 64  | 16  | 16  |
| CIN              | 125 | NT  | 500 | 64  | 16  | 64  | 16  | 16  |
| MYR              | 62  | 31  | 8   | 64  | 16  | 64  | 16  | 16  |
| SYZ              | 125 | 8   | 31  | 16  | 64  | 16  | 16  | 16  |

OSA: essential oil of Syzygium aromaticum, OCC: essential oil of Cinnamomum cassia, OMF: essential oil of Myristica fragrans. AMP: amphotericin B, KET: ketoconazole, FLU: fluconazole; ITR: itraconazole. NT: not tested.

Some studies have shown that essential oils that contain cinnamaldehyde, thymol, carvacrol or eugenol have high antimicrobial activity (42,53). Thus, the presence of E-cinnamaldehyde and eugenol identified in the chemical composition of the Cinnamomum cassia and Syzygium aromaticum essential oils, respectively, may be associated with the potential antifungal activity against R. mucilaginosa observed in the present study, aiming at the prospect of new therapeutic agents.

It has already been found that the same essential oil can present different antifungal activity and mechanism of action when it is tested against different pathogens (54,55). Such a result may be associated with the complexity and diversity of antifungal mechanisms (9). According to studies in the field, the mechanism of antifungal action of the Cinnamomum cassia essential oil may be associated with the CHO group present in the molecule of the cinnamaldehyde. Thus, considering the fact that CHO group is hydrophilic, the cinnamaldehyde can easily absorb the hydrophilic constituents of the surface of the fungi, facilitating its penetration into the cell wall and, consequently, causing the death of the cell (9). For the essential oil of Syzygium aromaticum, the mechanism of antifungal action may be related to eugenol, its main constituent, and its ability to disturb the integrity and permeability of the fungal cell membrane, which may cause irreversible damage to the cell. In addition, such a constituent can destroy yeast proteins and inhibit their synthesis (56). The presence of phenylpropene terpinen-4-ol and phenylpropanoid elemicin among the main constituents of Myristica fragrans essential oil may be related to the antifungal action verified for this substance in the present study. According to results reported in the literature, the mechanism of antifungal action of phenylpropenes is potentially associated with their lipophilicity, which allows them to act on the permeability of the cell membrane, in addition to inhibiting specific cellular processes or enzymes (57).

Conclusions

This study reports for the first time the activity of the essential oil extracted from Syzygium aromaticum, Cinnamomum cassia and Myristica fragrans against clinical isolates of R. mucilaginosa, demonstrating the originality of the study. The observed results allowed to verify the potential effect of the tested oils against this yeast, presenting MIC of up to 8 µg/mL. Chemically, the essential oils of these species are characterized by the presence of phenylpropanoids such as eugenol, E-cinnamaldehyde, myristicin and elemicin, and phenylpropanes as terpinen-4-ol, compounds with recognized antifungal action and that are probably responsible for the observed action. Thus, the results presented in this article are promising and justify the continuation of the work in this area. As a proposal for future work is the analysis of the genotoxicity of oils extracted from these plants.

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Conflict of interest

The authors declare no conflicts of interest.

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