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Evaluation of *Spirulina platensis* and Propolis as Natural Promising Preservatives for Controlling of *Candida albicans* in Minced Meat

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Abstract | The goal of this study was to determine how well *Spirulina platensis* and propolis ethanolic extracts work as natural antifungal agents against *Candida albicans*, both *in vivo* and *in vitro*. The broth microdilution assay was used for testing how well *Spirulina platensis* and propolis extracts inhibit fungal growth. The minimum inhibitory concentrations (MIC) of *Spirulina platensis* (SPP) and propolis extracts (PEE) against *Candida albicans* were determined to be 0.5 mg/mL and 6 mg/mL, respectively. The initial work confirmed that *Spirulina platensis* and propolis ethanolic extracts at such concentrations significantly (p <0.05) inhibited 100% of the fungal growth. Meat samples were inoculation with *Candida albicans* into at an infected dose of 10^5 cfu/g. The initial group (control 1) did not get any inoculation or treatment. The second group (control 2) received an inoculation without treatment. The third and fourth groups were thoroughly mixed with 0.5 mg/ml of SPP and 6 mg/ml of PEE, respectively. All samples were refrigerated at 4°C for duration of 12 days (n = 4 for each group at each examination day). The obtained results confirmed that the tested concentrations of PEE significantly inhibited *Candida albicans* counts in treated minced beef samples, propolis extract has shown superior efficacy in reducing the *Candida albicans* counts (completely inhibited on day 6 of examination), improving organoleptic qualities, particularly in terms of color, as well as prolonging the shelf-life of examined samples to the end of the experimental trial on the day 12. *Spirulina platensis* and propolis are suggested as natural antifungal agents for the preservation of minced meat.

Keywords: Spirulina platensis, Propolis, Antifungal, Candida albicans

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INTRODUCTION

Recently, there has been a growing emphasis on improving food safety, leading to a rise in the use of natural preservatives that include antibacterial properties. Concurrently, there is an increasing inclination towards natural foods and ingredients, which are commonly perceived as being safer, healthier, and less susceptible to risks compared to those containing artificial additives. This is especially true for meat products, which are particularly susceptible

to the growth of microorganisms that can cause spoilage and foodborne illnesses in humans (Abd El-Malek, 2017).

Yeast is a ubiquitous organism found throughout nature. Yeasts contribute positively to the fermentation process of numerous substances, such as wine and beer. However, they are also responsible for causing food spoilage. In addition, specific yeast species, such as *Candida spp.,* can infiltrate the human body through the consumption of food and beverages, leading to a range of disorders (Riesute *et al*., 2021).

Among the yeast species representing a health issue is Candida species which are frequently occurring fungal pathogens that exploit opportunities in human hosts. *Candida albicans* (*C. albicans*) is the most widespread pathogen responsible for both mucosal and systemic fungal infections (Poulain, 2015). The pathogenicity of the *C. albicans* significantly contributes to host cell invasion and damage (Höfs *et al*., 2016).

Spirulina is a type of blue-green microalgae that has a spiral cellular structure. It belongs to the Arthrospira and Spirulina genera. Spirulina is classified as a "superfood" due to its composition, which consists of 55-70% protein and amino acids such as leucine, valine, isoleucine, tryptophan, phenylalanine, threonine, methionine, lysine, and gamma-linolenic acid (36% total PUFAs). It also contains glycolipids, sulfolipids, carbohydrates like mannose, glucose, galactose, xylose, and rhamnose, carotenoid (4000 mg/kg), 1.5–2% vitamins A, B, and E, minerals such as iron, calcium, manganese, zinc, magnesium, selenium, and potassium, as well as pigments like chlorophyll, carotenoids, xanthophylls, and the blue pigment known as phycocyanin (Pyne *et al*., 2017). *Spirulina platensis* is one of the algae that the FDA has categorized as generally regarded as safe (GRAS). The FDA classified Spirulina platensis as generally regarded as safe (GRAS) in 2017. However, the antifungal activities of Spirulina using meat products as substrates had received less attention.

Propolis is a mixture of pollen, waxes, essential oils, balsam, resins, and other substances, including enzymes and compounds related to bee metabolism. Propolis is composed of around 300 distinct chemical compounds, including flavonoids, phenolic acids, cinnamic acid, caffeic acid, terpenes, and esters. The variances in propolis originate due to differences in the plant source as well as the time and location of its collection. Ethanol is the predominant and widely employed solvent for extracting propolis. Propolis has antifungal properties because it can effectively combat many types of fungi by acting as both a fungicidal and fungistatic agent. Propolis possesses antioxidant, antibacterial, and antifungal properties, which make it an attractive option for use as a natural preservative in creative culinary uses (Shehu *et al*., 2016).

The antibacterial activities of propolis are attributed to its high amounts of phenolics, namely derivatives such as pinobanksin and pinocembrin. The antibacterial properties of propolis have been linked to a bioactive compound called caffeic acid phenyl ester (Gavanji *et al*., 2014). However, the use of propolis in the elimination of yeast contamination in meat and meat products is less investigated.

In sight of the previous information, this study was conducted to examine the impact of *Spirulina platensis* and propolis ethanolic extracts against the growth of *C. albicans* in minced meat and to improve the quality of the meat and extend its shelf life.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine at Benha University approved this research (approved number BUFVTM 03-12-23).

Sampling

Precisely, a total of 2.4 kg of beef flesh (with each piece weighing 200g) was bought from a butcher shop. The meat was promptly minced and transferred to the laboratory in pre-cooled insulated containers with ice packs, ensuring sterile conditions. Samples were grouped and inoculated with *C. albicans* without delay. The *Spirulina platensis* and propolis were acquired from the National Research Center in Dokki, Cairo, Egypt.

Preparation of Extracts

Spirulina platensis ethanolic extract (SEE): A quantity of 25g of algae powder was combined with 250mL at 1:10 ratio of 96% ethanol and placed in a Soxhlet device at a temperature of 60°C for duration of 24 hours in order to extract the active chemicals from the algae. The obtained extract was passed through a filter paper of Whatman No.1 and subsequently subjected to evaporation using a rotary vacuum evaporator (EYELA Auto jack NAJ-100, Tokyo, Japan) at a temperature of 45°C. Ultimately, the extract underwent a process of dehydration in a vacuum-drying oven at a temperature of 55°C for duration of 48 hours, as described by Saranya *et al*. (2014).

Propolis ethanolic extract (PEE): The 30g of raw propolis sample was crushed and dissolved in 100mL at 1:10 ratio of ethanol: water (80:20 v/v) while being stirred continuously at a temperature of 50°C for duration of 30 minutes. The suspension was passed through a Whatman no. 1 filter paper and then subjected to centrifugation at a speed of 600×g for duration of 20 minutes. The supernatant was concentrated using a rotary evaporator at 40°C under decreased pressure for three hours. The propolis powder was

prepared through freeze-drying (Jonaidi Jafari *et al*., 2018).

In vitro Study

Minimum inhibitory concentration (MIC): The antifungal properties of extracts from *Spirulina platensis* and propolis were assessed using the broth microdilution assay in a 96-well microdilution plate, using 5 concentrations of the two tested antimicrobials starting from the 0 concentration as a negative control and using 64 µg/mL of fluconazole as a positive control for antifungals against *C. albicans*. The used concentrations of SEE were 0, 0.25, 0.5, 1, 2 mg/mL and for PEE were 0, 1.5, 3, 6, 12 mg/mL. The M27-A3 technique established by the Clinical Laboratory Standards Institute (CLSI, 2008) was followed.

Preparation of *Candida albicans*: *Candida albicans* ATCC10231 (were sub-cultured once into Sabouraud's dextrose agar (SAD) and incubated for 24h at 37°C. Five colonies were transferred to sterile distilled water to make inocula (5 mL). After fungal growth, the cells were collected by centrifuging the conical tubes at 1,200 x g for 5 min at room temperature. Discard the supernatant and add 10 mL of PBS. Resuspend the cells and centrifuge again at 1,200 x g for 5 min at room temperature. Repeat the PBS wash and centrifugation twice more. After the third wash, resuspend the cells in 5 mL (according to the pellet size) of 2X RPMI-1640 medium. To ensure homogeneity, the suspensions were mixed for 15 seconds then adjust cell density with a spectrophotometer by adding sufficient sterile saline or sterile water to match the turbidity of a 0.5 McFarland standard.

Preparation of Spirulina platensis extract: The ethanolic extract of *Spirulina platensis* was dissolved in dimethyl sulfoxide (DMSO) and then weakened in peptone water using a two-fold serial dilution method. The dilution concentrations ranged from 2 mg/mL to 0.0675 mg/mL. El-Baz *et al*. (2013) reported that the non-toxic dose of the ethanolic extract of *Spirulina platensis* varied between 1.6 and 2 mg/ml.

Preparation of propolis extract: The ethanolic extract of propolis was dissolved in dimethyl sulfoxide (DMSO), and then diluted in peptone water using a serial two-fold dilution method. The concentrations ranged from 12 mg/mL to 0.1875 mg/mL.

About 100 µL of the inoculum was put into each well of the 96-well sterile culture plates. The inoculum contained varied quantities of *Spirulina platensis* and propolis extracts. Using 100 µL of *Candida albicans*, a negative control was established with 100 µL per well of DMSO, while a positive control was set up with 100 µL per well of *Candida albicans*. The plates were thereafter placed in an incubator set at a temperature of 37 °C for duration of 48 hours. Regarding the proliferation of *Candida albicans*. The entire technique was replicated three times. We found the minimum inhibitory concentration (MIC), which is the lowest concentration that stopped all fungal growth. This was done by adding propolis and *Spirulina platensis* to Sabouraud's Dextrose Agar (SDA) as antifungal agents and leaving it to grow at 37 °C for 48 hours.

In vivo Study

Preparation of inoculums: The preparation of inoculums involved cultivating a strain of *Candida albicans* ATCC10231 to obtain a stock culture with a population of approximately 10^9, as reported by Krifors *et al*. (2023). This stock culture was then artificially introduced into all meat samples that were being examined, except for one (referred to as the blank sample), resulting in a final concentration of 10^5 as specified by the APHA (1985). Following inoculation, the first group (not inoculated) received no treatment (control 1), while the second group (control 2) also received no treatment. The third and fourth groups were thoroughly mixed with SPP at a concentration of 0.5 mg/ml and PEE at a concentration of 6 mg/ml (both dissolved in DMSO), respectively. Treatment groups were preserved at 4°C for 12 days and examined on days 0, 3, 6, 9, and 12 (n = 4 for each examination point).

Preparation of samples: Each sample, weighing twenty-five grams, was thoroughly mixed with 225mL of 1% peptone water using a stomacher for duration of 1 minute. Subsequently, serial dilutions were made. Multiple replicates of one milliliter of selected dilutions were placed on petri dishes, and Sabauroud's dextrose agar was poured onto the dishes. The plates were then incubated at a temperature of 30 °C for a period of 3–5 days. By following the steps outlined by Cruickshank *et al*. (1975), we were able to count the number of *Candida albicans* colonies per gram after 0, 3, 6, 9, and 12 days of storage at 4 °C. The experiment was performed in triplicate.

Sensory evaluation (Horwitz, 2020): Personnel at the Animal Health Research Institute, specifically the Benha branch, assessed the samples. Every participant was required to evaluate the degrees of odor, color, consistency (firmness or succulence), and visual aspect on days 0, 3, 6, 9, and 12 of refrigeration. The representative samples were assigned random numbers, and the judges were kept ignorant of the experimental method. Samples of the various treatments were presented in small porcelain dishes that were covered. Each judge was given their own separate area where distractions, noises, and odors were minimized. They were then asked to score each sample based on its overall acceptance, taking into account factors such as color, odor, texture, and appearance. Overall acceptability is assessed

using a nine-point descriptive scale. The scale ranges from 9 (excellent) to 8 (very good), 7 (very good), 6 (good), 5 (medium), and S (spoiled).

Statistical Analysis

The collected data were subjected to analysis of variance (one-way ANOVA) using SPSS software, following the method described by Sabine and Brian (2014). A one-way analysis of variance (ANOVA) was conducted using Dunnett's multiple comparisons test to compare treatment groups with the control. Individual variances were estimated for each comparison, and the significance level was set at $p < 0.05$. The mean values were reported with their corresponding standard deviations (SD).

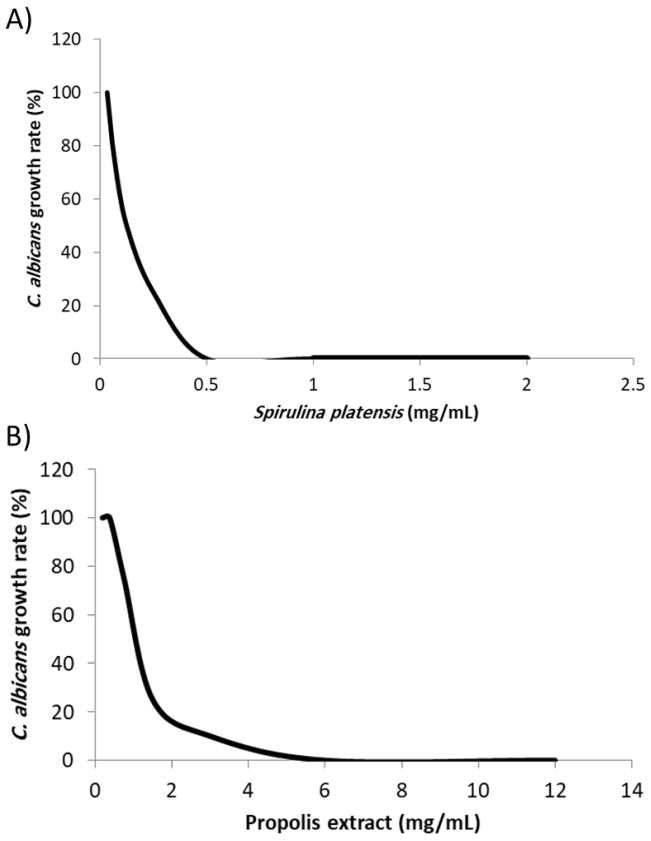

Figure 1: Minimum inhibitory concentrations of **A)** *Spirulina platensis* and **B)** propolis extracts on *Candida albicans*

RESULTS AND DISCUSSION

In vitro Study

The antifungal properties of *Spirulina platensis* and propolis extracts were demonstrated by the reduction in the growth rate of the tested yeast. Higher concentrations of the extracts improved their effectiveness, as shown in Figure 1. The findings demonstrated a noteworthy suppressive impact at various concentrations of SEE (0.25, 0.5, 1, 2 mg/mL) and PEE (1.5, 3, 6, 12 mg/mL). It was found that 0.5 mg/mL of *Spirulina platensis* and 6 mg/mL of propolis extracts were the lowest concentrations needed to stop the growth of Candida. This concentration was the minimum amount required to completely prevent the fungus from growing.

In vivo Study

Table 1 shows how *Spirulina platensis* and propolis extracts affect the number of *Candida albicans* colonies (cfu/g) in minced meat. The results revealed that the untreated samples (control 1 group) had a microbial load of 6.85 ± 0.02, which increased to 7.74 ± 0.02 on the 3rd and 6th days, and then decreased to 6.86 ± 0.02 on the 9th day and 5.63 ± 0.02 on the 12th day, respectively. *Spirulina platensis* caused a decrease in yeast counts to 5.86 ± 0.01, 4.25 ± 0.01, 2.78 ± 0.01, and 1.49 ± 0.01 on the zero, 3rd, 6th, and 9th days of storage, respectively. On the 12th day, yeast counts were not detectable. The application of propolis resulted in a reduction in yeast count in the samples, with counts of 6.03 ± 0.01, 3.58 ± 0.01, and 1.02 ± 0.01 on the zero, 3rd, and 6th days, respectively. Furthermore, on the 9th and 12th days, *Candida albicans* was not detected. The untreated sample remained free of any measurable quantities of yeast during the zero, 3rd, and 6th days of storage. However, the samples were already degraded by the sixth day.

Table 1: *Candida albicans* count (Log_{10}) in non-treated (control) and treated samples with spirulina and propolis during storage period at 4°C (Mean ± SE).

Storage Day	0	3	6	9	12
Control 1	ND	ND	ND	ND	ND
Control 2	6.85^{b} ± 0.02	7.74^{a} ± 0.02	7.74^{a} ± 0.02	6.86^{b} ± 0.02	5.63^{c} ± 0.02
Spirulina platensis	5.86^{a} ± 0.01	4.25^{b} ± 0.01	2.78^{c} ± 0.01	1.49^{d} ± 0.01	-
Propolis	6.03^{a} ± 0.01	3.58^{b}± 0.01	1.02^{c} ± 0.01	-	-

Means with different letters within the same row differed significantly at ($P < 0.05$), ND: Not detected

Sensory Evaluation and Overall Acceptance

The overall acceptance of minced beef samples held at 4°C revealed that both the control 1 and control 2 samples were fully ruined by the 6th day of cold storage. The inclusion of *Spirulina platensis* preserved the product's overall sensory acceptability until the ninth day. Additionally, adding *Spirulina platensis* to the meat gave it a green hue. On the other hand, the addition of propolis maintained the product's acceptability until the 12th day of cold storage, as shown in Table 2.

To assess the impact of *Spirulina platensis* and propolis on *Candida albicans* in minced beef, we initially conducted *in vitro* experiments to determine the appropriate dosage to

add to the meat. To our knowledge, this study is the first to examine the antifungal properties of spirulina and propolis against *Candida albicans* in beef mince. We tested *Spirulina platensis* and propolis against the testing strain of *Candida albicans* and found that they were both effective antifungals, with MIC values of 0.5 mg/ml for *Spirulina platensis* and 6 mg/ml for propolis.

Table 2: Sensory evaluation of the untreated and treated samples of minced meat during cold storage at 4°C.

Samples	0 day	3rd day	6th day	9th day	12th day
Control 1	9	6	5	S	S
Control 2	9	6	5	S	S
Spirulina platensis	8	7	6.5	5	S
Propolis	9	8.5	7	6	5

9: Excellent; **8:** Very very good; **7:** Very good; **6:** Good; **5:** Medium; **S:** spoiled.

The alga's action may be attributed to the intracellular and extracellular metabolites possessing antifungal characteristics (Al-Ghanayem, 2017). Overall, research has shown that the primary antifungal chemicals found in spirulina are predominantly polyphenols and polysaccharides. These compounds are effective in inhibiting the growth of microorganisms and can also directly destroy the cellular structures of fungi (Bajpai, 2016).

Likely, Marangoni *et al.* (2017) conducted a study to assess the effectiveness of a water extract from *Spirulina platensis* in inhibiting the growth of *Candida albicans* in a laboratory setting. Furthermore, it was discovered that the extract derived from microalgae also has fungicidal properties. According to Marangoni *et al.* (2016), the researchers found that the extract from the microalga has a strong antibacterial activity against *Candida albicans*. They observed that the MIC of the extract was 0.25 mg/ml, which was consistent with previous findings. In a study conducted by Usharani *et al.* in 2015, the antimicrobial activity of solvent extracts from *Spirulina platensis* against pathogenic fungi was examined. The researchers found that each solvent extract had a different MIC. The lowest MIC value was 2 mg/ml for the methanol extract, while the ethanol extract had a MIC of 8 mg/ml against *Candida albicans*. The minor variations observed in different studies are likely due to the extraction process used and the chemical composition of the extract (Vonshak, 1997).

Several authors have conducted studies on the antifungal properties of propolis against *C. albicans*, yielding varying findings depending on the origin of the propolis. Sowmya *et al.* (2021) reported that the MIC value of Indian propolis is 0.03 mg/mL. In contrast, Yusoff *et al.* (2016) recorded a MIC value of 500 mg/mL for propolis from New Zealand and South Australia. In a study conducted by Joya *et al.* in 2016, the researchers investigated the antifungal properties of ethanol extracts of propolis from different regions (Germany, Italy, Spain, and Venezuela) against *C. albicans*. The study found that all propolis extracts from the four regions exhibited significant fungistatic and fungicidal effects. However, there were variations in the MIC among the different propolis extracts.

The observed variations in chemical composition across different regions of the world are believed to be responsible for these results. It is hypothesized that the differences in minimum inhibitory concentration (MIC) are a result of the interaction between distinct propolis-specific compounds (de Souza *et al.*, 2013; Zhang *et al.*, 2015). The phenolics and flavonoids in propolis contribute to its antifungal activity. These compounds alter the permeability of the cytoplasmic membrane, resulting in cellular leakage and ultimately cell death.

During the 12-day storage period at 4°C, all the treated meat samples in the experimental group exhibited a notable decrease in Candida count.

The statistical analysis revealed a significant difference (p <0.05) between the control samples (non-treated) and each of the treated samples on the zero, 3rd, 6th, and 9th days of storage, respectively. Compared to Spirulina, propolis has shown greater efficacy in reducing the count. Spirulina possesses powerful antioxidants and free-radical scavengers that can hinder the growth of certain types of bacteria and yeast, including Candida albicans (Marangoni *et al.*, 2017). The polysaccharides derived from Spirulina possess qualities that inhibit tumor growth, prevent oxidation, slow down the aging process, and combat viruses (Choi *et al.*, 2019). Microalgae biomass or its separated components can be directly included as techno-functional additives in various processed meat products. The study conducted by de Medeiros *et al.* (2020) examined several meat products, such as sausages (Marti-Quijal *et al.*, 2019), burgers (Marti-Quijal *et al.*, 2019), pork liver pâté, turkey patties (Zamuz *et al.*, 2019), beef patties (Zugcic *et al.*, 2018), and chicken roti (Parniakov *et al.*, 2018).

Propolis exhibits antimicrobial properties that can be attributed to either its direct antimicrobial effects or its indirect effects on the immune system, which result in the elimination of a greater number of microorganisms. Research has shown that propolis has the ability to promote the production of antibodies, activate macrophages, and increase their ability to fight against microorganisms (Gavanji *et al.*, 2014). Researchers studied propolis extracts mixed in ethanol and found that they were antibacterial (Abdullah *et al.*, 2019), antifungal, antioxidant, and anticancer (Abubakar *et al.*, 2014; Abdullah *et al.*, 2019).

Pobiega *et al.* (2019) stated that propolis is a desirable ingredient for use in food production due to its chemical composition, namely its high concentration of bioactive compounds. Propolis is commonly used as a natural preservative in food to maintain food quality and prevent microbial growth during storage (Viera *et al.*, 2016). Foodborne saprophytic microbiota and foodborne pathogens can be diminished or eradicated by directly administering propolis powder and its extracts to food. The study observed that the inclusion of *Spirulina platensis* in minced meat resulted in a green color, which may not be deemed acceptable to consumers. In contrast, the blank, control, and propolis-treated samples exhibited a desirable bright red color. These findings align with the findings of Awadalla *et al.* (2020), who observed that the inclusion of Spirulina platensis had a negative impact on the color of uncooked meatball samples.

The sensory attributes of the treated samples were enhanced; resulting in a longer shelf life compared to the untreated samples during the 0, 3, 6, 9, and 12 days of refrigerated storage. According to Ismet Ozturk's 2014 study, samples treated with propolis exhibited increased firmness and brightness compared to the other samples. Propolis was found to effectively reduce the presence of yeasts and molds in sausages without causing significant changes to their color and scent attributes.

Among the practical applications of the current study is the potential use of propolis in controlling fungal growth of minced meat. While the limitations of the present study appear in using one meat product and in future using different types of meat products is highly recommended. The change in the color of meat after adding spirulina makes it difficult to use it in the meat industry and therefore finding methods to stabilize the red color of the meat while using spirulina is regarded as one of the future approaches in our research.

CONCLUSIONS AND RECOMMENDATIONS

From the results it could be concluded that 0.5 mg/ml of ethanolic extracts of *Spirulina platensis* and 6 mg/ml of propolis effectively inhibit the growth of *Candida albicans* in fresh minced beef that has been stored at 4°C. Compared to Spirulina, propolis has shown superior efficacy in reducing the count. Additionally, propolis exhibited superior sensory qualities, particularly in terms of color, and prolonged the meat preservation period for an extended duration. In samples treated with propolis, the meat began to deteriorate on the 12th day, but in samples treated with *Spirulina platensis*, it ruined on the 10th day. Propolis is suggested as a natural antifungal agent for the preservation of minced meat. Finding alternatives to stabilize the red color of the meat while using spirulina could be a challenge to get the highest benefit of this alga and will be one of our future work.

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AUTHOR'S CONTRIBUTION

All authors contributed equally to the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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