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Antibacterial potential of methanolic extract of *Syzygiumaromaticum* (clove) against different bacterial strains

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Abstract

The flower buds of *Syzygium aromaticum* (Cloves) are a well-known spice, prescriped for treatment from some microbial diseases since ancient civilizations and in traditional medicine today. In the currenrt investigation, the methanolic extract of cloves was tested against 4 Gram-positive bacteria , namely *Bacillus cereus* ATCC10876, *Staphylococcus saprophyticus* ATCC 43867, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus epidermidis* ATCC 12228. In addition to 5 strains of Gram-negative bacteria , namely *Proteus vulgaris* ATCC 6380, *Klebsiella pneumoniae* ATCC 27736, *Salmonella typhimurium* ATCC 14028, *Shigella flexneri* ATCC 12022 and *Escherichia coli* ATCC 25922, using two diffusion methods (Cup-plate and disc diffusion methods), besides MIC and MBC testings. The extract showed noticable dose-dependant antibacterial activity against all tested bacterial strains with varied degrees, and there was no significant differences between the results of the cup-plate diffusion assay and the disc-diffusion assay. Moreover, MIC values were between 3.9 to 125 mg/ml, and MBC values ranged between 7.8 to 125 mg/ml, which was higher than the MIC's, the MBC/MIC ratio indicating that the extract has a bactericidal attitude, which makes it suitable source for the formulation of new antibacterial drugs.

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Introduction

Plants are an enormous renewed source for diverse biochemical molecules that have various physiological and pharmaceutical advantages on animal body, with approximately between 250,000 to 500,000 known plant species on Earth, while human and animals utilize only around 10 % (Borris, 1996;Cowan, 1999; Abdallah, 2011). The World Health Organization (WHO) has estimated that between 65 to 80% of the population in the developing countries use plants in healthcare, and the global markets of these natural products are significantly growing, with a sum of $83 billion US Dollars in 2011, where 25% of these products are used in modern pharmaceutical industries (Palhares *et al.*, 2015). Accordingly, plants are the main source of remedies as well as food and miscellaneous purposes.

Over the past decades, the production and synthesis of huge quantities of antibiotics has led to pandemic health crisis, known as antibiotics-resistance phenomenon, where many Gram-positive and Gram-negative bacteria has induced mutation against almost all current antibiotics, putting human communities under the threat of increasing prevalence of hard-to-treat multidrug-resistant bacterial infections (Harold and Neu, 1992, Zaman*et al.*, 2017). Unfortunately, the antibiotics arsenal is very limited and cannot competing the amazing ability of bacteria to make mutations in short period of time, as an example of our incompetence, the alarming observation reported by scientists that there are no new antibiotics discovered since the mid-to-late 20th century (Richardson, 2017).

Consequently, there is an increasing need for development and innovation of new antibacterial drugs. Numerous plant products (seeds, leaves, fruits, roots…) have reported potent antibacterial activity against different pathogens. For example, but not limited to, plants like *Nerium oleander*, *Artemisia herba-alba*, *Withania somnifera*, *Ficus sycomorus*, *Allium sativum* and *Eucalyptus camaldulensis*, which showed significant inhibitory effects against clinically isolated bacteria (Amenu, 2014). This antibacterial potential is attributed to secondary metabolites secreted by medicinal plants known as phytochemical compounds, such as alkaloids, flavonoids, tannins, terpenes, quinones and resins, these molecules are unlike antibiotics, have varied mechanisms of inhibiting the bacteria (Compean and Ynalvez, 2014).

Spices like cinnamon, thyme, mint, oregano, and clove are well known since ancient civilizations and used in medicine, cosmetics, food seasoning and as preservatives. Clove, the aromatic flower bud of a tree Syzygium aromaticum L. from the family Mirtaceae, is a precious spice native of Indonesia and also cultivated in some other tropical or sub-tropical countries such as India, Sri Lanka, Malaysia, Tanzania and Madagascar (Cortés-Rojas *et al.,* 2014). In literature, some studies reported that the clove has represented noticeable antioxidant, antibacterial, antifungal, anti-inflammatory, anticarcinogenic and insecticidal capacities. It was also found to be rich in some important phytochemical molecules such as phenolic compounds, sesquiterpenes, monoterpenes and hydrocarbons (Mittal *et al.*, 2014).

As a continued search for antibacterial agents from natural products, this current study came to evaluate the antibacterial capacity of cloves (*Syzygium aromaticum* L.) against different Gram-positive and Gram-negative bacterial strains.

Materials and methods

Plant material

Dried flower buds of *Syzygium aromaticusm* (*S. aromaticum*), known as clove (Figure 1), were purchased from an herbal market at Al-Rass town, Qassim region, Saudi Arabia. The merchant showed evidence that its source of origin is Indonesia. Cloves were ground to a fine powder using mechanical blender and kept in an airtight dark bottle in refrigerator until used for the extraction processing.

Extraction

20 grams of the dried powder of cloves was soaked in 200 ml of 80% Methanol (Merck, Germany) for up to

5 days in dark tighten bottle with frequent shaking. Then, filtered using Whatman No.1 filter papers, the filtrate was allowed to evaporate until dryness using an incubator at 40 °C for up to 3 days. and the dried methanolic crude extract was kept in sterile dark bottle under refrigerated conditions until use.

Test organisms

The studied microorganisms were American Type Culture Collection (ATCC) strains, 4 of Gram-positive bacteria , namely *Bacillus cereus* ATCC 10876, *Staphylococcus saprophyticus* ATCC 43867, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus epidermidis* ATCC 12228. In addition to 5 strains of Gram-negative bacteria , namely *Proteus vulgaris* ATCC 6380, *Klebsiella pneumoniae* ATCC 27736, *Salmonella typhimurium* ATCC 14028, *Shigella flexneri* ATCC 12022 and *Escherichia coli* ATCC 25922.

Determination of antibacterial activity

The antibacterial activity of cloves (*S. aromaticum*) was determined using two difussion tests the cup-plate agar diffusion method (Babu and Uma, 2010) and disc diffusion method (Abdallah and Elsharkawy, 2019), with minor modifications. For the cup-plate agar diffusion method, 20 ml nutrient agar was put into sterile Capped Bottle (size 50 mL) and autoclaved, and then poured on a sterile disposable Petri-dish and left to solidify. Thereafter, 200 µL of standardized bacterial stock suspension (10^8 -10^9 CFU/mL) was poured over the Petri-dish containing nutrient agar and distributed above the nutrient agar using sterile cottown swab. Subsequently, 5 cups, 6mm in diameter, were cut using a sterile Cork Borer and the agar discs were removed. Cups were filled with 0.5 mL of re-constituted methanol extract (two replicates of two concentrations 500 and 250 mg/mL) and Chloramphenicol (2.5 mg/mL) using microliter-pipette and allowed to diffuse at room temperature for two hours. 80% methanol showed no antibacterial effect on the pre-experimental phase. The plates were then incubated using an Incubator adjusted at 37° C for 18 hours. After incubation period, the diameter of the inhibition zones around the cups were measured in millimeters and the mean values were calculated. For the cup-plate agar diffusion method, similar procedure was followed for the preparation of the nutrient agar plate, then sterile discs (6 mm in diameter) was saturated with approximately 20 µL of the re-constituted extract (500 and 250 mg/mL) and the chloramphenicol (2.5 mg/mL) was put over the media and inhibition zones were taken after overnight incubation.

MIC and MBC

Tube dilution method was used to evaluate the Minimum inhibitory concentration (MIC) (Akinyemi *et al.,* 2006), briefly, two-folds dilutions of the methanolic extract of *S. aromaticum* were made (3.9, 7.8, 15.62, 31.25, 62.5, 125 and 250 mg/mL) in previously autoclaved nutrient broth tubes to get an equal volumes of broth and the serially diluted extract. Then, 100 µL of the previously adjusted inoculums of the tested bacterial strains were loaded to each tube. A positive control (containing antibiotic instead of extract) and a negative control (containing 80% methanol instead of extract) was also prepared for test quality. MIC was deemed as the lowest concentration of the extract which showing no visible growth (no turbidity) when compared with the control tubes. For the minimum bactericidal concentration (MBC) (Doughari, 2006), 50µL of the MIC test dilutions were pipetted, transferred to sterile nutrient agar plates, incubated overnight at 37° C and inspected for visible growth. The MIC inoculum which showed no visible growth on agar medium was considered as MBC.

Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of two replicates. Paired Sample T-test was used to compare between the data of cup-plate and disc difussion tests. The program used in tabulation, graphing and statistical analysis was SPSS software, version 11.

Results

A total of 9 different bacterial strains representing the Gram-positives and the gram-negatives were

investigated in the current study against the methanol extract of cloves (*S. aromaticum*). Two different methods were used to evaluated the antibacterial potential of this extract, the cup-plate and the disc diffusion methods. The study showed that, the extract was effective against all tested bacterial strains with different degrees. As represented in (Table 1) and (Figure 2), the cup-plate diffusion method, revealed noticable antibacterial activity of S. aromaticum against all tested bacteria strains and the potency was dose dependant, At the dose 500 mg/ml, the highest susceptible bacterium was *Enterococcus faecalis* (15.5±0.7 mm zone of inhibition), followed by *Staphylococcus epidermidis* (14.5 ±0.7 mm), *Salmonella typhimurium* (14.5 ±0.7 mm), *Escherichia coli* (14.5 ±0.7 mm), *Shigella flexneri* (13.5 ±0.7 mm), *Staphylococcus saprophyticus* (13.5 ±1.4 mm), *Bacillus cereus* (11.5 ±0.7 mm), *Proteus vulgaris* (11.0 ±1.4 mm) and *Klebsiella pneumoniae* (11.0 ±1.4 mm), respectively. Similar results-to some degree- were recorded with the disc-diffusion method (Table 2) and (Figure 3), where the highest susceptible bacteria (At 500 mg/ml) were *Enterococcus faecalis*, *Staphylococcus saprophyticus* and *Shigella flexneri* (14.5±0.7mm zone of inhibition).

Table 1. Antibacterial activity of the methanol extract of cloves using cup-plate diffusion method.

Test	Gram-positive bacteria				Gram-negative bacteria				
	Bc	Ss	Ef	Se	Pv	Kp	St	Sf	Ec
Extract (500 mg/mL)	11.5 ±0.7	13.0 ±1.4	15.5 ±0.7	14.5 ±0.7	11.0 ±1.4	11.0 ±1.4	14.5 ±0.7	13.5 ±0.7	14.5 ±0.7
Extract (250 mg/mL)	9.0 ±1.4	8.5 ±0.7	11.5 ±0.7	12.5 ±0.7	7.0 ±0.0	7.5 ±0.7	10.0 ±0.0	8.5 ±0.7	11.0 ±1.4
Chloramphenicol (2.5 mg/mL)	30	32	33	27	32	30	35	31	30

*Diameter of blank paper disc= 6 mm, Bc= *Bacillus cereus* ATCC 10876, Ss= *Staphylococcus saprophyticus* ATCC 43867, Ef= *Enterococcus faecalis* ATCC 29212, Se= *Staphylococcus epidermidis* ATCC 12228, Pv= *Proteus vulgaris* ATCC 6380, St= *Salmonella typhimurium* ATCC 14028, Kp= *Klebsiella pneumoniae* ATCC 27736, Sf= *Shigella flexneri* ATCC 12022, Ec= *Escherichia coli* ATCC 25922.

Table 2. Antibacterial activity of the methanol extract of cloves using disc diffusion method.

Test	Gram-positive bacteria				Gram-negative bacteria				
	Bc	Ss	Ef	Se	Pv	Kp	St	Sf	Ec
Extract (500 mg/mL)	9.5 ± 0.7	14.5 ±0.7	14.5 ±0.7	13.5 ±0.7	10.0 ±0.0	11.0 ±0.0	13.5 ±0.7	14.5 ±0.7	14.0 ±0.0
Extract (250 mg/ mL)	8.5 ±0.7	11.5 ±2.1	11.0 ±1.4	12.5 ±0.7	9.0 ±0.0	8.5 ±0.7	10.0 ±0.0	9.5 ±0.7	7.5 ±0.7
Chloramphenicol (2.5 mg/ mL)	34	35	30	25	33	32	35	33	30

*Diameter of blank paper disc= 6 mm, Bc= *Bacillus cereus* ATCC 10876, Ss= *Staphylococcus saprophyticus* ATCC 43867, Ef= *Enterococcus faecalis* ATCC 29212, Se= *Staphylococcus epidermidis* ATCC 12228, Pv= *Proteus vulgaris* ATCC 6380, St= *Salmonella typhimurium* ATCC 14028, Kp= *Klebsiella pneumoniae* ATCC 27736, Sf= *Shigella flexneri* ATCC 12022, Ec= *Escherichia coli* ATCC 25922.

Followed by *Escherichia coli* (14.0±0.0 mm), *Staphylococcus epidermidis* (13.5±0.7 mm), *Salmonella typhimurium* (13.5±0.7 mm), *Klebsiella pneumoniae* (11.0±0.0 mm), *Proteus vulgaris* (10.0±0.0 mm) and *Bacillus cereus* (9.5±0.7 mm), respectively. Interestingly, our data shows there is no statistically significant difference between the two antibacterial diffusion tests used to evaluate the extract's activity ($P>0.05$). Figure 4 shows representative photo of the two tests employed to

evaluate theantibacterial activity of *S. aromaticum*. As shown in (Table 3), the minimum inhibitory concentrations (MIC)of the methanol extract of cloves were varing between 3.9 and 125mg/mL, whereas the minimum bactericidal concentrations were ranging between 7.8 to 125 mg/mL, and the MBC/MIC ratios were from 2 to 1.

Table 3. MIC and MBC values of cloves methanol extract against tested microorganisms.

Bacterial strains	MIC (mg/ mL)	MBC (mg/ml)	MBC/MIC ratio
Bacillus cereus	7.8	15.62	2
Staphylococcus saprophyticus	3.9	7.8	2
Enterococcus faecalis	7.8	15.62	2
Staphylococcus epidermidis	31.25	62.5	2
Proteus vulgaris	31.25	62.5	2
Klebsiella pneumoniae	7.8	15.62	2
Salmonella typhimurium	3.9	7.8	2
Shigella flexneri	125	125	1
Escherichia coli	125	125	1

Discussion

According to the results, methanol extract of *S. aromaticum* exhibited remarkable dose-dependent antibacterial activity against all tested bacterial strains (Gram-positives and gram-negatives).

Fig. 1. Dried flower buds of the clove (*S. aromaticusm*).

The current findings are in harmony with previous studies; Methanol extract of *S. aromaticum* showed good antibacterial activity against food-borne pathogens (*Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*) and methanol was the best solvent and showed good results with that plant product (Pandey and Singh, 2011).

Also, the methanol extract of clove revealed high antibacterial effects against some oral pathogens such as *Serratia* sp. and *Staphylococcus* sp. (Okmen *et al*, 2018), and methanol extract was the better extract for cloves, which showed varied remarkable activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Vibrio cholera*(Hemalatha*et al*., 2016).

Moreover, the essential oils recorded noticeable antibacterial activity against some fish pathogens and it is claimed that eugenol which was found in high percentage in the clove is the potential chemical compound of antibacterial characteristics (Pathirana *et al*., 2019).

Accordingly, it can be considered that Dried flower buds of clove (*S aromaticusm*) have a broad-spectrum antibacterial activity and it is a good candidate for antibacterial drugs industry.

This claim is supported by the results of MIC and MBC experiments conducted in this study which recordedinfluential effects of clove extract at low concentrations, as the lowest concentration that

inhibits the growth of tested bacterial strains (bacteriostatic) were ranging between 3.9 and 125mg/ml, whereas thelowest concentration that kills these bacteria (Bactericidal) were ranging between 7.8 and 125mg/ml, and the MBC/MIC ratio which was between 1 to 2 indicates that the extract have a bactericidal nature.

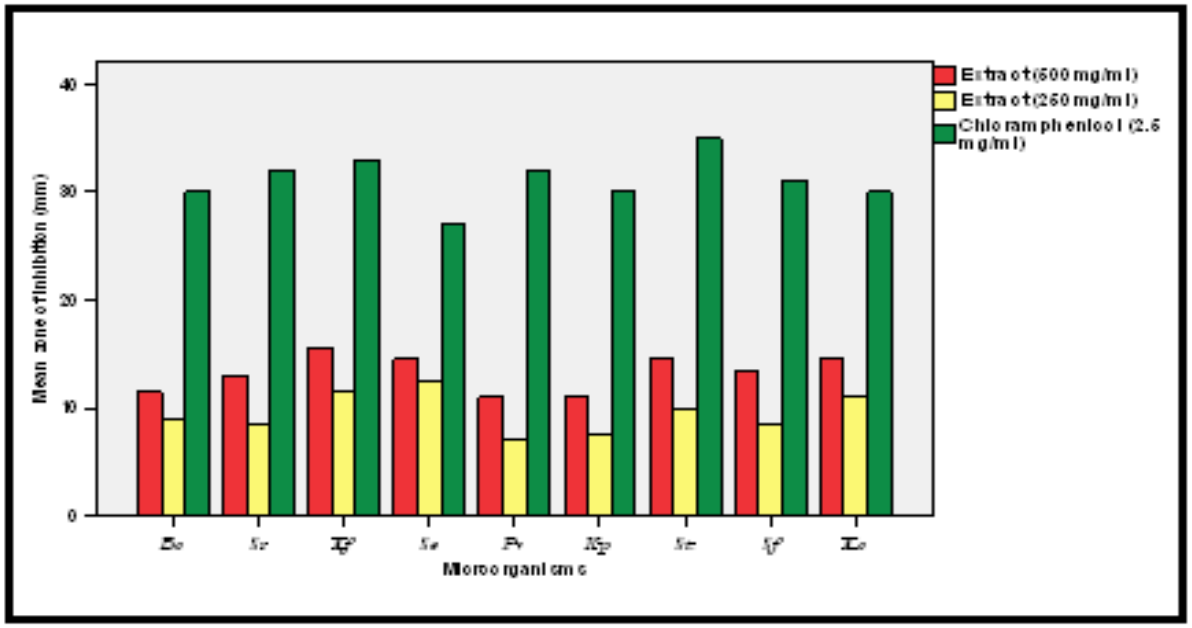

Fig. 2. Antibacterial activity of cloves methanol extract compared with chloramphenicol using cup-plate diffusion assay.

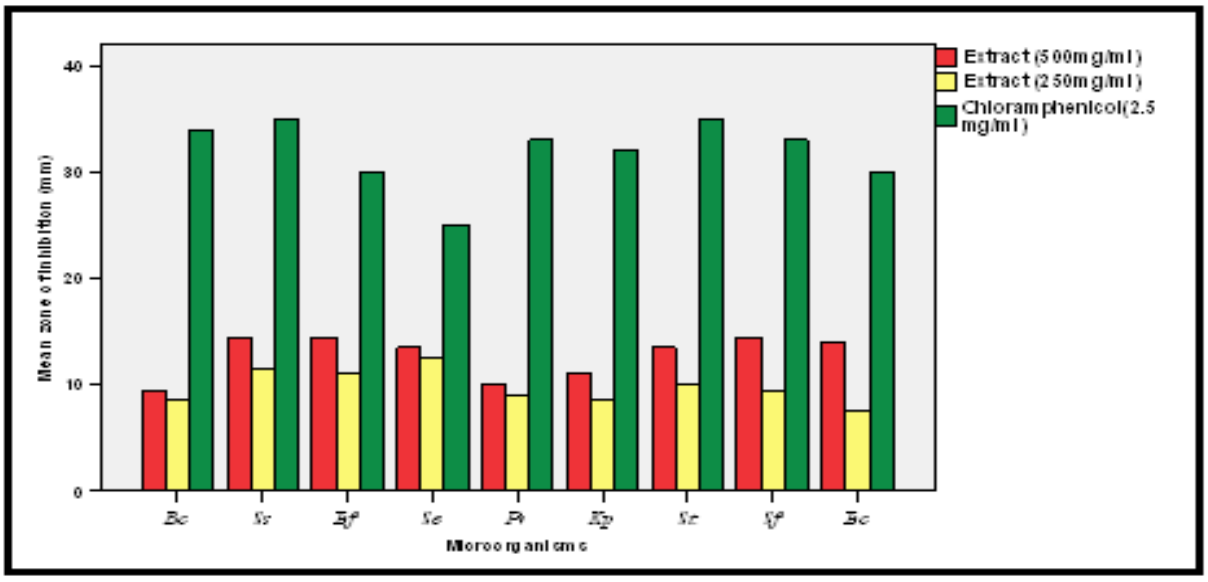

Fig. 3. Antibacterial activity of cloves methanol extract compared with chloramphenicol using disc diffusion assay.

Plant extracts with bactericidal attitude are promising sources for antibacterial drugs (Abdallah, 2016). On the other side, the current study noticed that, although there were some variations between the disc- diffusion assay and the cup-plate diffusion assay, but the "Paired Sample T-test" recorded no statistical significant between them, which means that both tests are appropriate for antibacterial evaluation of natural products, however the inhibition zones are clearer with the cup-plate diffusion assay (Figure 4).

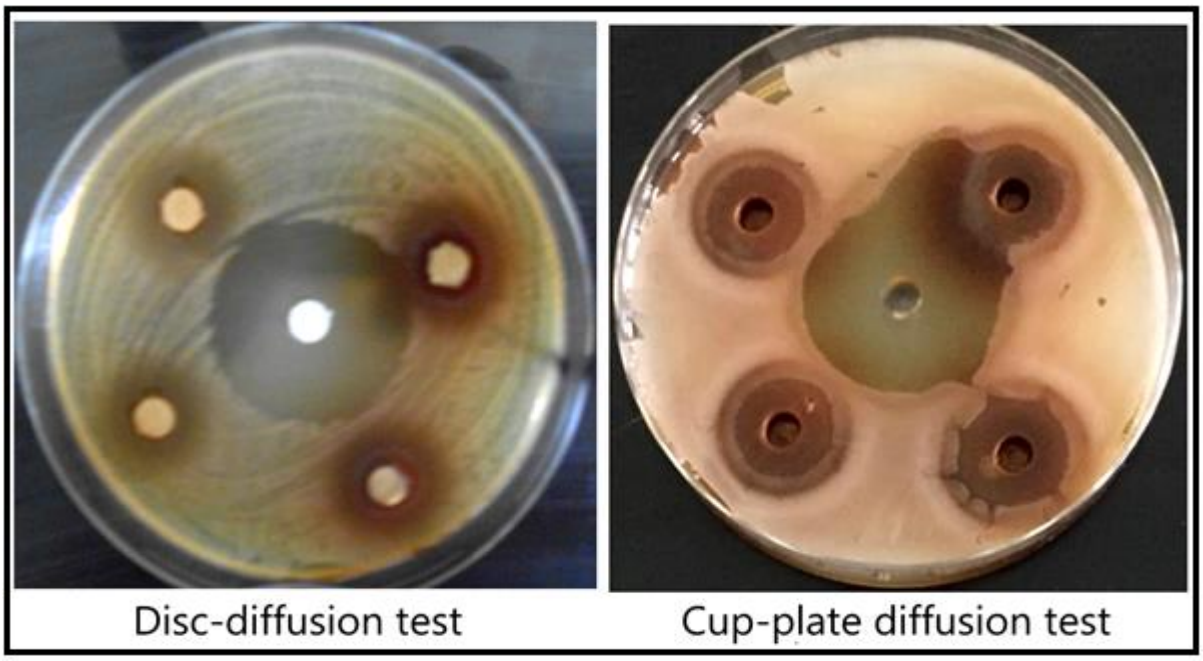

Fig. 4. Representative photos showing different methods of detection of antibacterial potential of cloves.

Conclusion

Natural products of medicinal plants continue to provide new phytochemical molecules with potent antibacterial activity. According to the findings of the current study, the methanol extract of the dried flower buds of *S. aromaticusm* (clove) has good antibacterial activity with bactericidal attitude. Accordingly, cloves are good potential source for new broad-spectrum antibacterial agents which can be formulated as effective antibiotics. Although, further studies are required, the active ingredients should be isolated, identified and characterized and additional *in vitro*, *in vivo* and pharmacological studies should be conducted.

Conflict of interest

No conflict of Interest.

Funding

Nil

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