# *In vitro* antimicrobial effects of crude miswak extracts on oral pathogens

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في المختبر أظهرت الدراسات الوبائية والسريرية تأثيراً مفيداً لمضغ أعواد المسواك على صحة الفم .
كان الهدف من هذه الدراسة هو التقييم في شروط اختبار موحدة ، فيما إذا كانت خلاصة المسواك المخضرة من جذور الـــ سلفادورا بيرسيكا وباستعمال محاليل متنوعة فيما إذا كان لها تأثير مثبط على نمو بعض الجراثيم المختارة والتي لها علاقة بالالتهابات التي تصيب الانسان . ماء مقطر ومعقم ، ٩٦% ايثانول ، ٢% حمض الصفصاف وحلات الكحـــول . وتم اختبار قدرتها المثبطة على الجراثيم التالية المكورات العقدية المتحولة ، العصيات اللبنية الحامضة، الفطر الشعاعي العصوي ، العصوانية اللثوية ،المبـــيضات البـــيض والمزروعة في أوعية الاختبار واستعمل تركيز مختلف من خلاصة المسواك بدرجة حرارة ٣٧ درجة مئوية ولمدة ٧٢ ساعة وحدد أقل تركيز للمادة له قدرة على توقـــيف نموالجراثيم . أظهرت النتائج أن خلاصة المسواك في الكحول كانت الأكثر قدرة على تثبيط الجراثيم والأكثر تأثيراً على سلالة الموتانز . والأقل تأثيراً على الأسيدوفيلوس . والقيم المختلفة لخلاصة النباتات تراوحت من ١٠٠ ملغم إلى ٣٠٠ملغم . واعتماداً على هذه النتائج تبين أن خلاصة المسواك أظهرت قدرة منخفضة على قتل الجراثيم إذا ما قورنت مع ٠,٢% من محلول الكلوروهيكسيدين.

*In vitro*, epidemiological and clinical studies have demonstrated beneficial effects of chewing sticks on oral hygiene. The aim of this study was to assess under standardized test conditions whether miswak crude extracts prepared from *S. persica* roots and twigs using different solvents inhibited *in vitro* growth of some selected oral microbes involved in infections in humans. Sterile distilled water, 96% ethanol, 2% acetic acid and ethyl acetate were used as solvents. Reference strains of *Streptococcus mutans*, *Lactobacillus acidophilus*, *Actinobacillus actinomycetemcomitans*, *Actinomyces naeslundii*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Candida albicans* were tested for susceptibility to the antimicrobial effects of crude extracts using the broth microdilution method of Cai *et al*. Microbial growth was estimated spectrophotometrically at 650 nm in 96-well microtiter plates. Different concentrations of the *S. persica* extracts were incubated at 37$^{O}$C with each test strain for up to 72 hrs. The minimum inhibitory concentration (MIC) of the extracts against the individual test organisms was determined as the lowest concentration of the extract that limited turbidity to < 0.05 absorbance at $OD_{650nm}$. Results showed that the root-ethanolic extract was the most potent. The most susceptible strain was *S. mutans* whereas *L. acidophilus* was the least susceptible. MIC values for the various plant extracts ranged from 100mg/ml to 300 mg/ml. Based on these results, it was concluded that miswak extracts exhibited low antimicrobial activity against the test microorganisms when compared with 0.2% aqueous chlorhexidine.

## Introduction

The World Health Organisation has recommended and encouraged the use of chewing sticks.[1] Recently, chewing sticks have been comprehensively reviewed[2,3] and examination of their effectiveness as an oral hygiene aid has been encouraged.[4] The Arabic word miswak is used to describe a chewing stick used for cleaning teeth, tongue and gum. Miswak includes all types of sticks used as oral cleansing aids. In Sudan, miswak is prepared from stems, roots or twigs of *Salvadora persica L.* (order: Celastrales, family: Salvadoraceae). This shrub is commonly known as the arak tree[5] and has a wide geographical distribution over the Middle East and most of the African countries.[6] In Sudan, it is distributed in the arid areas of the flood plains along valleys and seasonal water courses known as "khors" in North and East Sudan, Red Sea Hills (Sinkat), Kassala (Gedaref and Dinder), White Nile (Dueim, Getaina), Khartoum and Kordofan and its main usage is currently as miswak for oral cleansing.[2,3] The promotion of good oral health by miswak is mainly attributed to mechanical cleansing, but may also be due in part to built-in antiseptics.[7]

"Siwak purifies the mouth and pleases Allah" Prophet Muhammad (PBUH) said.[8] Islam teaches the importance of cleanliness of the body as well as the mind and therefore introduced basic oral hygiene by incorporating it as a religious practice.[9] The religious and spiritual impact of miswak probably is the principal reason why it is extensively used in Islamic countries.

Different parts of the plant have shown various chemical components when analyzed by different methods.[10-14] Several *in vitro* studies have indicated that *S. persica* contains substances that possess dental plaque-inhibiting and anti-microbial properties against oral microbes.[15-21] The aim of this study was to compare the antimicrobial effects of *S. persica* root and twig extracts using different solvents, standardized test

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conditions and a panel of target microorganisms known to be involved in oral and systemic infections in humans.

## Materials and Methods

### *S. persica* authenticity, collection and grinding

Roots and twigs of *S. persica* were collected from Khor Adeit, Sinkat (north-eastern Sudan) in July 1999. Two plant taxonomists from the Department of Botany, University of Khartoum and School of Life Sciences, University of ElNeelen, Sudan, recommended this location since the plant is the dominant species in the area.[6] Prior to collection, a visit was made to the Botanical Garden of Khartoum to study the cultivated plant and the wild types in the area of collection. An agriculturist and some inhabitants of the area helped to identify these. After collection, the plant material was air-dried in a shaded area and then air-shipped to the Laboratory of Oral Microbiology, University of Bergen, Bergen, Norway, where the antimicrobial examinations were performed. Each plant sample was cut into small pieces using plant scissors and then finely ground using a grinder*. The powders were kept separately in sterile, dry screw-capped bottles which were stored in a dry and cool place, until extraction.

### Extraction and reconstitution

Sterile distilled water, 96% ethanol, ethyl acetate and 2% acetic acid were used for crude extractions. The extracts were prepared by mixing 50 g plant powder and 250 ml solvent in sterile, dry screw-capped bottles.[18] The bottles were maintained at room temperature in a shaker+ at 400 rotations per min. The solvents were changed every 24 hrs for 9 days, and the supernatants were separately collected in sterile screw-capped bottles and kept at 4-60$^0$C. The volume of each extract was reduced by evaporation under pressure at 35-38$^0$C and the remaining solvent was removed by air-drying for 2-4 days at room temperature. The yield of each extract was calculated as a percentage of the original weight. The dried extracts were then kept in a dry place at 4$^0$C (in a cold room) until its use for antimicrobial testing. Before testing, each crude extract was freshly reconstituted in 0.5% Tween 80® to prepare two stock solutions with concentrations of 300 mg/ml and 200 mg/ml respectively. They were then centrifuged at 15800 xg for 20 min using a cooled centrifuge** at 10$^0$C. The supernatants were sterilised by filtration through a 0.2 mm membrane filter‡. Each stock solution was used to prepare two 2-fold serial dilutions. The pH of the reconstituted extracts were determined using a pH-meter++.

### Cultivation of microbes

The following type-strains were used for the antimicrobial testing of the crude extracts: *Actinobacillus actinomycetemcomitans* ATCC (American Type Culture Collection) 43717, *Actinomyces naeslundii* 40110/87, *Candida albicans* ATCC 90028, *Lactobacillus acidophilus* CCUG (Culture collection University of Gothenburg, Sweden) 5917, *Porphyromonas gingivalis* W50 Black, *Prevotella intermedia* VPI (Virginia Polytechnical Institute) 4197 and *Streptococcus mutans* CCUG 11877. Fresh cultures were prepared from the strains stock cultures. All the bacterial strains were grown on blood agar (1.2% agar with 5% sheep blood) for anaerobes (LAB M fastidious anaerobes agar) and aerobes (Columbia agar Difico 0790-17), while the yeast was cultivated on Sabouraud dextrose agar (Oxoid CM 41). *P. gingivalis*, *P. intermedia*, *L. acidophilus* and *A. naeslundii* were incubated anaerobically while *S. mutans* and *A. actinomycetemcomitans* were incubated microaerophilically for 48 hrs using the Anoxomat System‡‡ and *C. albicans* was incubated aerobically at 37$^0$C for 24 hrs.

### Antimicrobial susceptibility testing

The microdilution method of Cai *et al.*[22] was applied using 96-well microtiter trays***. The growth medium employed was tryptic soy broth (3%)-yeast extract (0.5%) media (TSB-YEM) (Difico 0370-17-3) supplemented with cysteine hydrochloride (0.05%), menadione (0.02µg/ml), hemin (5µg/ml) and 0.02% potassium nitrate (supplemented TSB-YEM). Innocula were prepared for all test microbes to obtain about 3 x 10$^5$ colony forming units of test microbes in the growth medium. Suspension in sterile saline of each

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microbial test strain was adjusted to McFarland standard 1 (≈$3x10^8$ bacterial cells). A volume ratio of strain suspension in saline to medium (1µl:1ml) was prepared according to the amount needed per analysis. In different wells, 100µl of each innoculated or uninnoculated (background wells) TSB-YEM was added to 100µl of each of the crude extract concentrations or controls. Serial dilutions of chlorhexidine in sterile distilled water (final concentration 0.005%-0.0001562%) and tea tree oil in 0.5% Tween 80® (final concentration 0.5%-0.0625%) served as positive controls. Negative controls were sterile saline, medium without additives and 0.5% Tween 80®. Each test was carried out in triplicate. Innoculation times were determined from growth curves obtained individually for each microorganism in pilot experiments. According to this, the trays were incubated at $37^0$C for up to 72 hrs; C. *albicans* aerobically, *S. mutans* and *A. actinomycetemcomitans* were incubated microaerophillically, and the remaining strains were incubated anaerobically. Growth was estimated spectrophotometerically as turbidity by measuring the light absorption of the microbial mass as determined by the optical density (OD) readings at 650 nm using a microtiter plate reader**. Growth was checked at 0 hrs, 24 hrs and 48 hrs, and at 72 hrs for the slow growing strains *P. gingivalis* and *P. intermedia.*

Values calculated from the differences in $OD_{650nm}$ readings between inoculated TSB-YEM (the medium plus crude extract or control and bacteria) and uninoculated TSB-YEM (the medium plus crude extract or control alone) wells were used to assess the susceptibility of each strain to the different crude extracts. The antimicrobial activities were presented graphically as the mean of the triplicate $OD_{650nm}$ differences between wells with growth and background wells, y-axis; the x-axis showing the medium alone or with the different additives. The growth inhibition was assessed by subtracting $OD_{650nm}$ values of incubated medium supplemented with *S. persica* extracts or controls from those of incubated medium alone. The minimal inhibitory concentration (MIC) of each extract for each strain tested was defined as the lowest concentration of the extract that limited the turbidity to <0.05 absorbance at 650 nm.[22] These values were calculated according to the volume (100µl) of *S. persica* extract (mg/ml) or control (v/v %) added to each well.

Spot samples taken from microtiter plate wells with visible growth were cultivated on blood agar for the bacteria and on Sabouraud dextrose agar for *C. albicans* to check for microbial contamination. Gram-stained smears of the samples were prepared and examined microscopically using oil immersion at 1000 x magnification.

**Molecular Device®/E max/ for Macintosh® / version 2.3, Molecular Devices Corporation, Sunnyvale, CA, USA

## Results

### Antimicrobial susceptibility testing

The extraction yields ranged from 38.8 % for the root-acetic acid extract to 3.3% for the stem-ethyl acetate extract. The pH range was from 5.9 for the stem-water extract to 3.9 for the root-ethanolic extract. The pH of chlorhexidine standard solutions was 6.6.

The crude extracts either inhibited or enhanced the growth depending on which test strain was used (Table 1). There was no growth for *P. intermedia* in the final medium. *S. mutans* was the most susceptible strain to all extracts while *L. acidophilus* was resistant to all extracts except for the root-ethanolic extract. Compared with the other solvents, the ethanolic extracts showed the strongest antimicrobial activity. Within the ethanolic extracts the root extract was more potent than the twig extract. The stem-water extract was found to have the least effect. Saline and Tween 80® (serving as negative controls) showed negligible reduction in the $OD_{650nm}$ readings. As examples of graphically presented effects of crude extracts on the test bacteria after 48 hrs incubation, Figs. 1 and 2 show different effects of root and stem extracts, respectively, on *S. mutans* and Figs. 3 and 4 on *L. acidophilus.*

The MIC values of the various crude extracts and the positive controls varied with the test microbes. The MIC values ranged from 100 mg/ml to 300 mg/ml for the different crude extracts (Table 2). MIC values were not determined where no inhibition of growth was observed.

## Discussion

According to the findings of this study, the *S. persica* crude extracts inhibited, reduced or enhanced the growth of the test microorganisms. Most of the extracts exerted their antimicrobial activity only at the highest concentrations used while chlorhexidine and tea tree oil showed

**Table 1**. Antimicrobial activity of different S. persica extracts.

| Stem extracts | | | | | |
|---|---|---|---|---|---|
| **Microorganisms** | **Incubation periods** | **Ethanol** (96%) | **Water** | **Ethyl acetate** | **Acetic acid** (2%) |
| *C. albicans* | 24 | Reduction | Reduction | Reduction | Inhibition |
| | 48 | Reduction | Reduction only at the highest concentration otherwise no effect | Enhancement | Inhibition |
| | 72 | Not carried out | Not carried out | Not carried out | Not carried out |
| *S. mutans* | 24 | Enhancement | Enhancement | Inhibition | Inhibition |
| | 48 | Inhibition | Reduction | Inhibition | Inhibition |
| | 72 | Not carried out | Not carried out | Not carried out | Not carried out |
| *A. actinomycetem-comitans* | 24 | Inhibition | Enhancement | Inhibition | Inhibition |
| | 48 | Inhibition | Enhancement | Inhibition | Inhibition |
| | 72 | Not carried out | Not carried out | Not carried out | Not carried out |
| *L. acidophilus* | 24 | Enhancement | Enhancement | Enhancement | Enhancement |
| | 48 | Enhancement | Enhancement | Enhancement | Enhancement |
| | 72 | Not carried out | Not carried out | Not carried out | Not carried out |
| *A. naeslundi* | 24 | Inhibition | Enhancement | Inhibition | Inhibition |
| | 48 | Inhibition | Reduction | Inhibition | Inhibition |
| | 72 | Not carried out | Not carried out | Not carried out | Not carried out |
| *P. gingivalis* | 24 | Not carried out | Not carried out | Not carried out | Not carried out |
| | 48 | Inhibition | Inhibition | Not carried out | Reduction |
| | 72 | Inhibition | Inhibition | Not carried out | Reduction |
| **Root extracts** | | | | | |
| **Microorganisms** | **Incubation periods** | **Ethanol** (96%) | **Water** | **Ethyl acetate** | **Acetic acid** (2%) |
| *C. albicans* | 24 | Inhibition | Enhancement | Enhancement | Enhancement |
| | 48 | Inhibition | Reduction in the highest concentration otherwise no effect | Enhancement | Enhancement |
| | 72 | Not carried out | Not carried out | Not carried out | Not carried out |
| *S. mutans* | 24 | Inhibition | No growth was indicated | Inhibition | Inhibition |
| | 48 | Inhibition | Inhibition | Inhibition | Inhibition |
| | 72 | Not carried out | Not carried out | Not carried out | Not carried out |
| *A. actinomycetem-comitans* | 24 | Inhibition | Inhibition | Inhibition | Inhibition |
| | 48 | Inhibition | Inhibition | Inhibition | Inhibition |
| | 72 | Not carried out | Not carried out | Not carried out | Not carried out |
| *L. acidophilus* | 24 | Inhibition | Enhancement | Enhancement | Enhancement |
| | 48 | Inhibition | Enhancement | Enhancement | Enhancement |
| | 72 | Not carried out | Not carried out | Not carried out | Not carried out |
| *A. naeslundii* | 24 | Inhibition | Inhibition | Inhibition | Inhibition |
| | 48 | Inhibition | Inhibition | Inhibition | Inhibition |
| | 72 | Not carried out | Not carried out | Not carried out | Not carried out |
| *P. gingivalis* | 24 | Not carried out | Not carried out | Not carried out | Not carried out |
| | 48 | Inhibition | Inhibition | No growth was indicated | Enhancement |
| | 72 | Inhibition | Inhibition | No growth was | Enhancement |

**Table 2**. Minimal inhibitory concentration* of different *S. persica* crude extracts against various test microorganisms as determined by the Cai (1996) method.

| Strains | Extracts | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ethanol (96%) | | Water | | Acetic acid (2%) | | Ethyl acetate | |
| | Root | Stem | Root | Stem | Root | Stem | Root | Stem |
| *C. albicans* | | 150 | | | | | | |
| *A. naeslundii* | | 100 | 300 | | 100 | 100 | | 300 |
| *L. acidophilus* | 200 | | | | | | | |
| *S. mutans* | | 50 | 150 | | 300 | 200 | 300 | 200 |
| *A. actinomycete-mcomitans* | | 50 | | | | | 100 | |
| *P. gingivalis* | | | | 150 | | | | |
| *P. intermedia* | | | | | | | | |

Key of the table: = no growth on the final medium, = no minimal inhibitory concentration (MIC) was established,
*The MIC of each extract for each strain tested was defined as the lowest concentration of the extract that limited the turbidity to

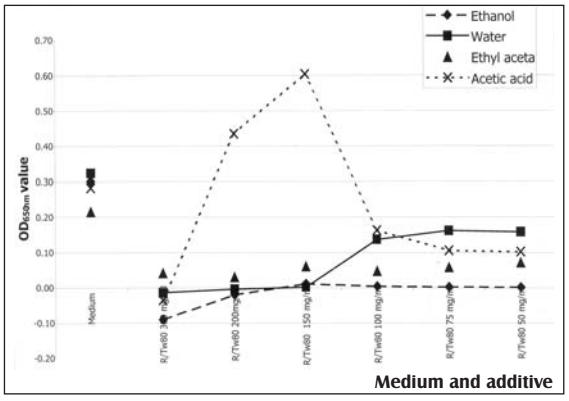


**Fig. 1**. Effect of *S. persica* root extracts on the growth of *S. mutans* after 48 hrs microaerophilic incubation at 37$^0$C.*

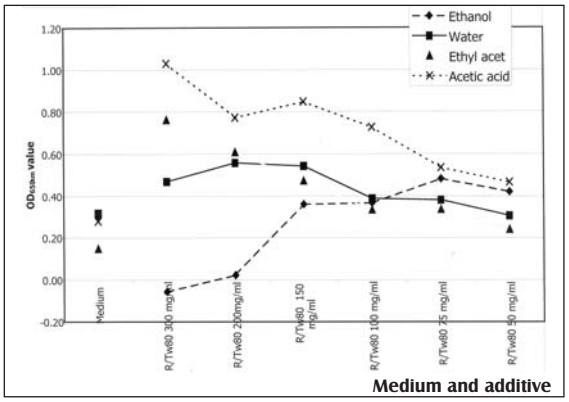


**Fig. 3**. Effects of *S. persica* root extracts on the growth of *L. acidophilus* after 48 hrs aerobical incubation at 37$^0$C.*

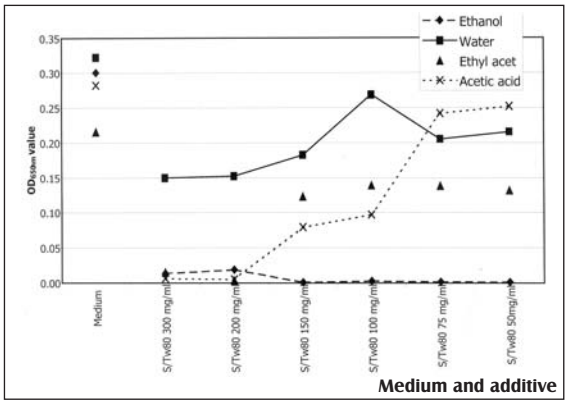


**Fig. 2**. Effect of *S. persica* stem extracts on the growth of *S. mutans* after 48 hrs. microaerophilic incubation at 37$^0$C. *

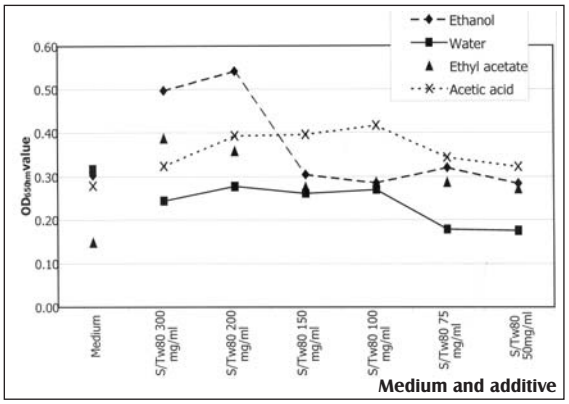


**Fig. 4.** Effects of *S. persica* stem extracts on the growth of *L. acidophilus* after 48 hrs. aerobic incubation at 37$^0$C.*

*The curves indicate experiments performed on four different days. Medium=medium (TSF-YEM) alone; R/Tw80 and S/Tw80=root (R) extract and (S) extract, respectively, reconstituted in 0.5% Tween 80®; CDX=0.2% chlorhexidine; and TTOil/Tw80=tea tree oil diluted in 0.5% Tween80®.

inhibition and growth reduction, respectively, at much lower concentrations. *S. mutans* was the most susceptible strain to all the extracts and *L. acidophilus* was the most resistant. Regarding inhibition, the most potent extract was the root-ethanolic extract and the weakest one was the stem-water extract. Their effects on growth were most likely due to the release of chemicals from the crude extracts into the medium when they were mixed. The different reactions of each strain to the various extracts indicated that each solvent extracted different chemical components of *S. persica* (miswak). The strength of the antimicrobial activity may also depend on the pH of the extracts since the lowest pH was shown by the root ethanolic extract while the stem water extract demonstrated the highest pH. This assumption is in agreement with a recent study by Almas in 1999.[15] However, according to our findings, it appears that miswak has a relatively low antimicrobial activity against the selected oral pathogens when compared with 0.2% aqueous chlorhexidine.

Our results didn't support some previous findings[16-20] that miswak extracts possess considerable antimicrobial activity. By using different antimicrobial assay in this study and evaluating the effects under standardized test conditions, we have inferred that miswak extracts manifested low antimicrobial activity. A case in point was the study conducted by Abo Al-Samh *et al.*[19] who used sodium hypochorite and compared it with miswak and discovered that the latter showed inhibition effects at much higher concentrations than NaOCl.

The MIC chosen for the present study is similar to that used by Cai *et al.*[22] because similar medium and wavelength were used. OD $_{650nm}$ value of 0.05 indicated no growth. Other studies have defined their MIC values according to the media used and the microbial strain tested.[23-24]

Many studies have been done to test the *in vitro* antimicrobial activity of *S. persica* miswak.[15-21] The present study is the first one reporting the *in vitro* antimicrobial activity of miswak twigs and roots collected from Sudan using four different solvents and standardised test conditions. The findings that *S. mutans* was the most susceptible strain and that the ethanolic extract was the most potent extract, agree with previous reports.[16-20] Al-Bagieh *et al.*[21] and Abo Al-Samh *et al.*[19] used OD readings at 600 nm and 420 nm, respectively, at macroscale. Other studies utilised the agar diffusion method which was also tried unsuccessfully in our laboratory.[25] A problem, however, arises when comparing our results with those of the studies which used agar diffusion tests. The latter did not quantify the microbial inhibition neither did they report MIC values. Only one of the studies used a standard antimicrobial agent for comparison.[19] Consequently, the effective antimicrobial activity reported by the authors who used the agar diffusion test needs verification using standardised testing and other methods. Our study was such an attempt.

## Conclusion

Based on our results, we concluded that:

1. Crude miswak extracts showed low to moderate antimicrobial activity when compared with standard antimicrobial agents like chlorhexidine and tea tree oil.
2. The different response of each microbial strain to the various crude extracts indicated that each solvent extracted different chemical components of *S. persica* and that these components either inhibited or enhanced the growth.
3. The strength of the observed antimicrobial activities may be due to the pH of the crude extracts.
4. More studies performed by independent researchers using other techniques and standardised test settings are needed to verify the previously published antimicrobial results of S. persica extracts obtained with the agar diffusion test.

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