

Confocal Laser Microscopic Evaluation of the Efficiency of Strawberry Extract as root canal irrigation with Different Activation Techniques on E- Faecalis Biofilm Eradication. (An In-Vitro Study)

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Aim: This study aims to conduct a comparative evaluation of the impact of sodium hypochlorite and strawberry extract as irrigation solutions through the utilization of cone focal laser scanning microscopy.

Materials & Methods: preparation and sterilization of single-rooted premolars (n = 21). A mature biofilm of *Enterococcus faecalis* was cultivated. The roots were separated into three groups based on the activation technique employed, namely ultrasonic, sonic, and positive control. The study involved the division of each group into two subgroups (n = 7) based on the type of irrigant utilized: strawberry extract and 2.5% sodium hypochlorite. The specimens were prepared by cutting and subsequently analyzed by confocal laser scanning microscopy. The fluorescent images were subjected to analysis using Zen imaging software. The data analysis was conducted utilizing the one-way analysis of variance (ANOVA) method, followed by Tukey's honestly significant difference (HSD) test for pairwise comparisons. The level of statistical significance was established at a significance level of 5%.

Results: The Ultrasonic and Sonic stimulation groups exhibited a greater proportion of deceased cells in comparison to the positive control group for both the NaOCl and Strawberry Extract groups. In the NaOCl group, the ultrasonic activation demonstrated the highest proportion of deceased cells, followed by the sonic activation and the positive control without activation, with percentages of deceased cells amounting to 57.229%± 3.998, 47.086%± 4.558, and 29.457%± 3.713, respectively.

Conclusion: it was concluded that Strawberry extract has been shown to exhibit more bacterial reduction with Ultrasonic activation than with Sonic or non-activation approaches, making it a viable irrigation alternative for endodontic therapy.

Keywords: Sodium hypochlorite, Strawberry extract, *Enterococcus faecalis* and Confocal laser scanning microscopy

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Introduction

The primary objective of endodontic treatment is to eradicate bacteria and mitigate the risk of subsequent reinfection of subsequent reinfection. While mechanical debridement does really lead to a large reduction in bacterial count, it is important to note that achieving complete cleanliness of the entire root canal system remains unattainable (1). *Enterococcus faecalis* is among the most resilient bacteria species found in endodontic infections, as it has the ability to endure and thrive in highly challenging environmental circumstances. Multiple studies have demonstrated that the incidence of *E. faecalis* in teeth that have undergone endodontic treatment might be as high as 90% of all cases. According to reports, there is evidence suggesting resistance of the target to commonly employed intracanal drugs, including calcium hydroxide, clindamycin, tetracycline, and erythromycin (2).

Various irrigation techniques and multiple activation systems, alongside mechanical preparations, have been employed in the field of endodontic therapy. The conventional endodontic needle (CEN) represents a long-standing approach in the field. Nevertheless, the current limitations of CEN in accessing canal extensions that are difficult to reach necessitate the exploration and advancement of alternate irrigation delivery devices and procedures. (3)

Sodium hypochlorite (NaOCl) possesses numerous features that align with the predicted characteristics of root canal irrigation solutions. As a result, it has emerged as the most optimal and extensively utilized agent among the various cleaning irrigation agents that have been produced in recent times. (4) Sodium hypochlorite (NaOCl) exhibits versatility in its applicability across many concentrations, with a prevalent utilization in root canal therapies ranging from 0.5% to 6%. (5) In

addition to its notable attributes such as strong antimicrobial efficacy, effective tissue dissolution, wide availability, and relatively affordable cost, sodium hypochlorite (NaOCl) also presents certain drawbacks including its inability to solely eliminate the smear layer, generation of unpleasant odor, potential development of emphysema, likelihood of allergic reactions, and toxic impact on adjacent tissues. In recent studies, it has been observed that there exists a negative influence on the elasticity and bending resistance of dentin. (6)

The strawberry fruit is widely consumed in Europe and is recognized for its significant contribution as a source of bioactive chemicals that exhibit antioxidant properties (7). The fruit in question is a notable source of folate, possesses a high concentration of vitamin C, and harbors a diverse array of phytochemicals. These constituents have the potential to significantly impact the nutritional attributes of this fruit. These compounds possess significant biological features such as antioxidant, anti-inflammatory, anti-cancer, and anti-neurodegenerative effects (8).

MATERIALS AND METHODS

The current study, which included twenty single-rooted permanent mandibular premolars removed for periodontal reasons, was authorized by the research ethics committee of October 6 University. The teeth were subjected to examination with a dental operating microscope (Zumax, Suzhou New District, China) set at a magnification level of $\times 8$, and radiographs were captured. Dental caries, fissures, fractures, resorption, or calcification, as well as teeth with multiple root canals, were disqualified from the study. An ultrasonic scaler (Satelec, Cedex, France) was used to mechanically clean the teeth in order to remove any calculus or soft tissues. Following that, the procedure of decoronation was executed by utilizing a

diamond wheel stone attached to a handpiece operating at a high speed. The objective was to focus on the cementodentinal junction, while also guaranteeing the availability of sufficient water for the purposes of cooling and lubrication. The length of the roots was adjusted to 16 millimeters. The roots were immersed in a solution comprising 5.25% sodium hypochlorite (NaOCl) for a period of 30 minutes. Subsequently, they were placed in a saline solution provided by El Nasr Pharmaceutical Chemicals Co., located in Cairo, Egypt.

Table 1: Sample Classification

GROUPS	NEGATIVE CONTROL (WITHOUT IRRIGATION)	GROUP A (ULTRASONIC ACTIVATION)	GROUP B (SONIC ACTIVATION)	GROUP C (POSITIVE CONTROL WITHOUT ACTIVATION)	TOTAL
SUBGROUP 1 (STRAWBERRY EXTRACT)		7	7	7	21
SUBGROUP 2 (2.5% NaOCl)		7	7	7	21
TOTAL	7	14	14	14	49

The chemo-mechanical preparation of all root canals was performed using WaveOne Gold (Dentsply Tulsa Dental, Tulsa, OK, USA), Additionally, a 2.5% sodium hypochlorite solution was utilized for thorough irrigation. The roots underwent autoclaving for a period of 30 minutes at a temperature of 121°C, with two repetitions. The *E. faecalis* strain 29212, sourced from the American Type Culture Collection (ATCC) and acquired from Nemro Co. in Cairo, Egypt, was introduced into a 7 mL volume of brain-heart infusion (BHI) medium. The culture was then subjected to incubation at a temperature of 37°C for a period of 24 hours. Following that, suspensions were prepared. The bacterial cells were resuspended in a solution of saline and then carefully adjusted to the #1 McFarland turbidity standard using sterile loops.

The inoculum was mixed with an equal volume (5 milliliters) of sterilized brain heart infusion (BHI) media. The pre-established root canals were inoculated with *E. faecalis* using sterilized micropipettes for

a period of 60 hours. After a 72-hour period of incubation, a re-inoculation was performed using a pure culture that had been generated and adjusted to satisfy the #1 McFarland turbidity standard. To promote the production of biofilm, the roots were subjected to a two-week incubation period under controlled conditions of high humidity and a temperature of 37°C.

The classification of roots was based on the activation method, which resulted in three distinct groups (A, B, and C). These groups were assigned based on the use of different methods, namely ultrasonic, sonic, and positive control. Each group was separated into two subgroups (1 and 2) based on the type of irrigant used: strawberry extract and 2.5% NaOCl (Egyptian Company for home detergents, Cairo, Egypt), respectively. A total of seven root samples were employed as a negative control in the absence of irrigation.

The irrigation of each root canal was conducted by administering 5 cc of the appropriate irrigant, utilizing a 23-gauge plastic syringe for the final flush. Group A did not get any form of stimulation. In Group B, the activation of the irrigant was performed by utilizing the IRRS ultrasonic tip (manufactured by VDW GmbH, located at Bayerwaldstr, Munich, Germany) for a duration of 2 minutes. Irrigant activation in Group C involved the utilization of the Eddy sonic tip (VDW GmbH, Bayerwaldstr, Munich, Germany) for a duration of 2 minutes.

The samples were further sectioned using a 0.3-mm IsoMet saw (IsoMet 4000 Precision Saw, Secunderabad, Telangana, India) while maintaining a consistent cooling process with sterile distilled water. Impression compounds were utilized for the purpose of securing the roots onto an IsoMet platform. Two pieces of 1 mm in thickness were extracted from each root, specifically from the apical and middle thirds.

The root portions were rinsed with 100 μ l of sterile distilled water for a duration of 1 minute and subsequently dried in a uniform manner. The root portions that had been cleansed were placed at the base of Eppendorf tubes, manufactured by Eppendorf, a company based in Hamburg, Germany. A specimen was stained by adding 100 μ l of 0.01% acridine orange (AO; Shanghai Yueteng Biotechnology Co., Ltd., Shanghai, China) (green fluorescence) and 10 μ L propidium iodide (PI; Shanghai Yueteng Biotechnology Co., Ltd., Shanghai, China) (red fluorescence). This staining process was conducted in a dark room and the specimen was allowed to remain stained for 15 minutes after being centrifuged for 10 seconds. The specimens were extracted from the tube and subsequently rinsed twice with aseptic 100 μ l of distilled water. The specimens were carefully put onto glass coverslips and subsequently coated with immersion oil prior to the imaging process. Confocal illumination was achieved with an argon laser microscope, with a laser wavelength of 500 nm for AO and 460 nm for PI. The light emitted by the labeled cells was observed using a Confocal Laser Scanning Microscope (Carl Zeiss, ZEISS, Jena, Germany). The dye exhibits a red fluorescence upon binding to RNA and a green fluorescence upon binding to DNA. Acridine orange (AO) can induce green fluorescence in viable cells. The fluorescent dye known as PI can permeate the membranes of non-viable cells, resulting in the emission of red fluorescence. The excitation and emission wavelengths for DNA staining using AO were measured at 500 nm and 526 nm, respectively. In contrast, the excitation and emission wavelengths for RNA staining using AO were found to be 460 nm and 650 nm, respectively. The utilization of sequential frame scan mode was implemented to mitigate the occurrence of crosstalk.

The specimens were analyzed with a $\times 40$ magnification oil immersion objective, which had a numeric aperture of 1.4. Additionally, a confocal pinhole of 88 Mm was employed for channel one, while channel two utilized a confocal pinhole of 164 Mm.

The fluorescent images were subjected to analysis using Zen imaging software, specifically Zen 2012 blue edition. Each specimen was subjected to deep scans, which were taken at a depth of 5-10 μ m into the dentin structure. The scans were performed in a format of 1024 \times 1024 pixels, with a step size of 2 μ m, resulting in a total of 20-40 sections. The calculation was performed to determine the percentage of deceased cells within the biofilm.

A one-way analysis of variance (ANOVA) was employed to examine the differences among groups using different activation strategies. This was followed by Tukey's honestly significant difference (HSD) test for pairwise comparisons, with a significance level (α) set at 0.05. The statistical analysis was conducted using the Statistical Package for the Social Sciences (IBM SPSS Statistics for Windows, Version 25.0). Armonk, New York, United States of America (USA).

RESULTS

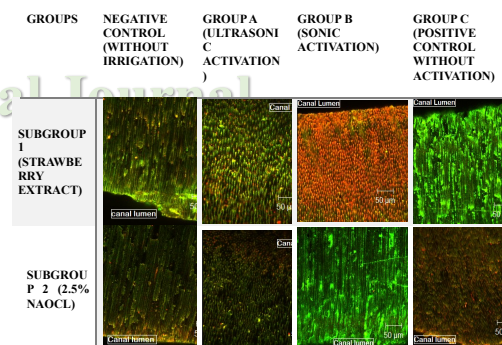


Figure 1. Representative confocal laser microscope images for different groups (where green colour represent live bacteria and red colour represent dead bacteria)

The comparison between the various test groups and the negative control group

revealed that all the test groups exhibited a statistically significant increase in the percentage of deceased cells for both NaOCl and Strawberry Extract.

The results of the Bonferroni t-test comparing the NaOCl group to the control group indicated a statistically significant difference (F value = 329.199, $P < 0.001$).

A comparable trend was observed in the Strawberry Extract group, wherein the Bonferroni t-test findings indicated a statistically significant distinction between the control and experimental groups (F value = 604.913, P -value < 0.001). (Figure 2)

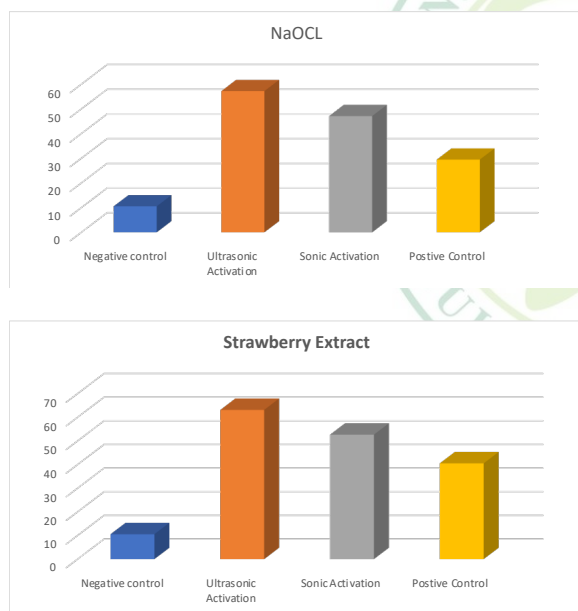


Figure 2. A Bar chart showing the percent of the dead cells for the different groups in the current research

The Ultrasonic and Sonic stimulation groups exhibited a greater proportion of deceased cells in comparison to the positive control group for both the NaOCl and Strawberry Extract groups. In the NaOCl group, the highest proportion of deceased cells was observed with ultrasonic activation, followed by sonic activation and the positive control without activation, with percentages of deceased cells equal to $57.229\% \pm 3.998$, $47.086\% \pm 4.558$, and $29.457\% \pm 3.713$, respectively. The Tukey pairwise comparison

test revealed a statistically significant distinction among the groups (F value = 82.051, P -value < 0.001).

In the Strawberry Extract group, the ultrasonic activation exhibited the largest percentage of dead cells, followed by the sonic activation and the positive control without activation, with percentages of deceased cells amounting to $63.100\% \pm 4.175$, $52.629\% \pm 2.378$, and $40.514\% \pm 3.186$, respectively. The Tukey pairwise comparison test yielded statistically significant results, indicating a substantial difference between the groups (F value = 80.710, $P < 0.001$).

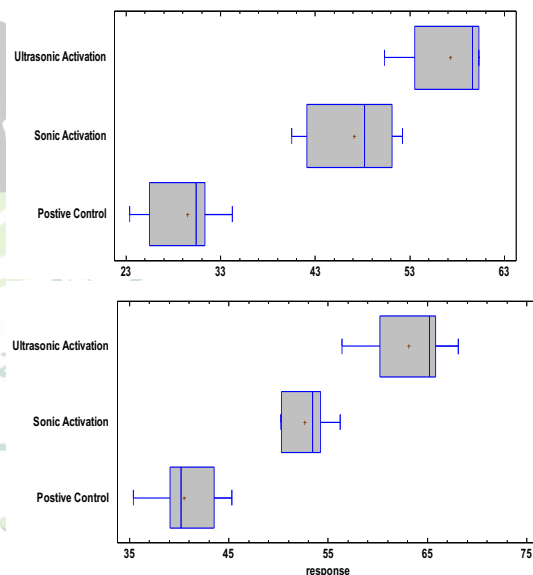


Figure 3. Box and Whisker plot for the percentage of dead cells for different activation techniques for different research groups. **Figure 3A** Box and Whisker plot for the percent of dead cells for different activation techniques for NaOCl group. **Figure 3B** Box and Whisker plot for the percent of dead cells for different activation techniques for Strawberry Extract group

Table 2: Mean and Standard deviation for the percentage dead cells for different tested irrigation methods and activation techniques

GROUPS	GROUP A (ULTRASONIC ACTIVATION)	GROUP B (SONIC ACTIVATION)	GROUP C (POSITIVE CONTROL WITHOUT ACTIVATION)	P- VALUE
SUBGROUP 1 (STRAWBERRY EXTRACT)	63.1%± 4.2 ^{aA}	52.6%± 2.4 ^{Ab}	40.5%± 3.2 ^{Ac}	<0.001*
SUBGROUP 2 (2.5% NAOCL)	57.3 %± 3.9 ^{aB}	47.1%± 4.6 ^{bB}	29.4%± 3.7 ^{Bc}	<0.001*
P-VALUE	0.038*	0.0145*	<0.001*	

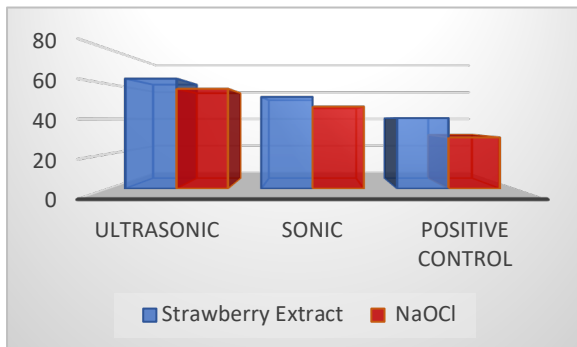


Figure 4. Bar chart showing mean values of percentage of dead cells the percentage dead cells for different tested irrigation methods and activation techniques.

Discussion

Dental caries is a highly widespread oral illness that is widely distributed on a global scale. The current research in addressing cariogenic bacteria centers around the eradication or mitigation of these bacteria, as well as the disruption of virulence factors, such as acid production. Numerous investigations have been undertaken to explore the potential of plant extracts as antimicrobial agents against cariogenic bacteria, specifically *S. mutans* and *S. sobrinus*. According to a study conducted by Vieira et al. (2012) (9), the adherence of *S. mutans* to an experimental pellicle treated with *Psidium* sp. was significantly reduced when compared to a pellicle that was not treated. (10)

Enterococcus faecalis has the ability to endure and withstand highly demanding conditions. Within the confines of root canals, these bacteria are somewhat protected

from the immune response of the host organism. The microorganism exhibits various virulence factors, including lytic enzymes, pheromones, aggregation material, cytolysin, and lipoteichoic acid, alongside its capability to build biofilms. (11)

The capacity to inhibit lymphocytes, hence resulting in endodontic failure, is indeed present. This study employed mature *Enterococcus faecalis* biofilm as a representative model of the clinical context, as opposed to planktonic bacteria which are characterized by their simplicity and susceptibility to eradication (12). Primary endodontic infections are characterized by the comparatively infrequent presence of enterococci. The prevalence of these microorganisms in secondary endodontic infections ranges from 29% to 77%. (13) In the field of antimicrobial research, *in vivo* studies have been widely recognized as the most effective approach. However, challenges related to standardization and ethical concerns have led researchers to explore alternate models such as removed teeth, *in situ*, animal, and *ex vivo* models, which offer more convenience (14). Human single-canal teeth that had been extracted were utilized in order to closely replicate the clinical scenario, while removing any anatomical variations and elements of complexity (15).

The efficacy of irrigation is contingent upon both the mechanical cleansing mechanism and the chemical capacity of irrigants to disintegrate tissue. In addition, the irrigation process facilitates the elimination of organic and dentinal debris as well as germs from the root canal. (16) The efficacy of syringe irrigation in terms of flushing action is very limited, as it is influenced by various factors including the root canal anatomy, needle implantation depth, and needle diameter. Research findings have indicated that irrigants have a

limited capacity to advance beyond 1 mm from the needle's tip. (17) The sole efficacious method for cleansing webs and fins involves the application of agitation to the irrigation solution. Ultrasonic and sonic stimulation serve as valuable supplementary methods in effectively cleansing challenging anatomical aspects. Previous studies have provided evidence that the utilization of an irrigant in combination with ultrasonic vibration, resulting in a continuous movement of the irrigant, is closely linked to the efficacy of root canal space cleansing. (18)

The remarkable efficacy of sodium hypochlorite (NaOCl) can be attributed to its approved effectiveness in eradicating intracanal microorganisms. Hypochlorous acid, the active component of NaOCl, acts as a potent oxidizing agent that exerts its antimicrobial activity by the irreversible oxidation of hydro-sulfuric groups present in bacterial enzymes. The inhibition of crucial enzymes leads to the disruption of metabolic processes within bacterial cells, ultimately leading to their demise. (19) Chlorine has the ability to bind to various components within bacterial cytoplasm, resulting in the formation of N-chloro composites that exhibit significant toxicity, ultimately leading to the eradication of the microorganisms. (20)

The application of ultrasonic activation has been observed to elevate the temperature of the irrigant, perhaps leading to enhanced antibacterial efficacy. Sodium hypochlorite (NaOCl) has the capability to eliminate biofilms within the root canal. However, a study conducted by Rosen et al. demonstrated that the use of sodium hypochlorite (NaOCl) resulted in the presence of live bacteria within biofilms. This finding suggests that the persistence of disease and endodontic failure may be attributed to the survival of bacteria in biofilms when NaOCl is used. (21)

The present study aimed to assess the impact of strawberry (*Fragaria x ananassa*) extracts on the in vitro biofilm development of *E. faecalis*. The strawberry extract exhibited a notable concentration of vitamin C (0.58 mg vitamin C per gram of fresh weight), polyphenols (2.52 mg Gallic Acid Equivalent per gram of fresh weight), and flavonoids (namely pro-anthocyanidin and anthocyanin, 0.66 mg Catechin Equivalent per gram of fresh weight). (22)

Several other research have been conducted on flavonoids, which have revealed that pro-anthocyanidin has inhibitory effects on *Staphylococcus aureus* biofilms, catechins inhibit *Streptococcus mutans*, and ellagic acid inhibits *Candida albicans*. have indicated that strawberry juice possesses antibacterial properties against *Streptococcus mutans*, hence exhibiting the potential to impede the production of biofilms. (23)

In recent times, confocal laser scanning microscopy has evolved as a highly effective technique for examining the structural characteristics of biofilms. This method enables non-destructive analysis of these complex ecosystems, facilitating the research of their hydrated spatial organization at the cellular level. (24)

Confocal laser scanning microscopy (CLSM) enables the acquisition of a sequence of optical slices with a thickness as low as 0.3 μ m, preserving the integrity and undisturbed nature of biological samples. The use of Confocal Laser Scanning Microscopy (CLSM) in conjunction with vital staining procedures is a prevalent approach for assessing the viability profile, architectural characteristics, and spatial distribution within microbial biofilms. (25) Propidium iodide and acridine orange are fluorescent dyes that possess distinct features, making them valuable tools for investigating the antibacterial activities and impacts of irrigation solutions on biofilm

through the utilization of confocal laser scanning microscopy (CLSM). (26)

Root canal irrigants have a substantial impact on the eradication of germs, disintegration of tissue, and elimination of debris and smear layer. The literature highlights several significant challenges associated with the utilization of irrigant solutions. One prominent issue is the limited capacity of these solutions to effectively reach the apical third and intricate anatomical structures. This limitation is particularly evident when employing conventional syringe irrigation, which is relatively weak and reliant on factors such as root canal anatomy, needle placement depth, and needle diameter. (27)

The utilization of ultrasonic activation or sonic activation has been found to enhance the eradication of bacteria and the smear layer within the canal system, thereby resulting in increased success rates for endodontic therapy. (28)

Ultrasonically activated files generate streaming patterns in close proximity to the file, resulting in the continuous movement of irrigants. This movement induces shear stress, which has the potential to harm biological cells. Consequently, the shear stress disrupts the bacterial biofilm, leading to the dispersion of bacteria in planktonic form. As a result, these bacteria become more vulnerable to the effects of antimicrobial irrigants. Moreover, the generation of cavitation could potentially induce a transient debilitation of the cellular membrane, therefore enhancing the susceptibility of bacterial cells to antimicrobial irrigants. (29)

Nevertheless, our findings contradict the results reported by Huffaker et al. (30) and Townsend and Maki (31), as they found no significant distinction between active and non-activated irrigation methods. However, in both experiments, the colony-forming unit was utilized as a means of evaluating bacterial eradication. The colony-forming

unit (CFU) method exhibits limited sensitivity when it comes to detecting viable cells at low concentrations and is unable to identify bacteria in a viable but nonculturable stage. (32)

While the ultrasonic activation resulted in a lower number of viable bacterial cells, there was no statistically significant difference observed between the two activation strategies. No method achieved complete eradication of bacteria within the root canal, resulting in the absence of a sterile root canal system. (33)

The null hypothesis is rejected due to the considerable reduction of bacterial counts in mature *E. faecalis* biofilm caused by irrigant activation.

Conclusion

Based on the constraints of the present investigation, it can be inferred that the activation of irrigants is a crucial procedure in diminishing bacterial populations within highly infected root canals. A promising natural antibacterial substitute is strawberry extract.

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