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REVIEW ARTICLE / ARTÍCULO DE REVISIÓN

NATURAL COMPOUNDS TO REDUCE THE BACTERIAL LOAD IN THE ORAL CAVITY: A REVIEW ARTICLE

COMPUESTOS NATURALES PARA REDUCIR LA CARGA BACTERIANA DE LA CAVIDAD ORAL: UN ARTÍCULO DE REVISIÓN

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ABSTRACT

Currently, there is a wide range of pharmaceutical products that offer great benefits for the treatment of various oral diseases. Most of these products are of synthetic origin with antibacterial properties but there are the numerous side effects associated with their use. An alternative is the use of natural products from plants and insects in the reduction of the bacterial load of the oral cavity as chamomile (*Chamaemelum nobile* (L.) All.), cocoa (*Theobroma cacao* L.), aloe (*Aloe vera* L.), moringa (*Moringa oleifera* Lam.), oregano (*Origanum vulgare* L.), coconut (*Cocos nucifera* L.), garlic (*Allium sativum* L.), clove (*Syzgium aromaticum* L.), cardamom (*Elettaria cardamomum* L.), stevia (*Stevia rebaudiana* Bertoni), honey bee and propolis, addressed in this literature review. This review attempts to address the use of different natural compounds in reducing the bacterial load of the oral cavity. It can be concluded that there are several studies about the effects of natural products on the part of man in medicine, where there are a large number of works and publications related to natural substances with active ingredients in reducing the bacterial load of the oral cavity.

Keywords: Dentistry – Microorganism – Natural products – Phytopharmaceuticals

RESUMEN

Actualmente, existe una amplia gama de productos farmacéuticos que ofrecen grandes beneficios para el tratamiento de diversas enfermedades orales. La mayoría de estos productos son de origen sintético con propiedades antibacterianas, pero existen numerosos efectos secundarios asociados con su uso. Una alternativa es el uso de productos naturales de plantas e insectos en la reducción de la carga bacteriana de la cavidad oral como la manzanilla (*Chamaemelum nobile* (L.) All.), el cacao (*Theobroma cacao* L.), el aloe (*Aloe vera* L.), la moringa (*Moringa oleífera* Lam.), el orégano (*Origanum vulgare* L.), coco (*Cocos nucifera* L.), ajo (*Allium sativum* L.), clavo (*Syzgium aromaticum* L.), cardamomo (*Elettaria cardamomum* L.), stevia (*Stevia rebaudiana* Bertoni), miel de abeja y propóleos, abordados en esta revisión de la literatura. Esta revisión

intenta abordar el uso de diferentes compuestos naturales para reducir la carga bacteriana de la cavidad oral. Se puede concluir que existen varios estudios sobre los efectos de los productos naturales por parte del hombre en la medicina, donde hay una gran cantidad de trabajos y publicaciones relacionadas con sustancias naturales con ingredientes activos para reducir la carga bacteriana de la cavidad oral.

Palabras clave: Fitofarmacéuticos – Microorganismos – Odontología – Productos naturales

INTRODUCTION

The microbiota of the oral cavity is made up of thousands of microorganisms, including bacteria, fungi, and viruses, which find their ideal niche in it. The presence of pathogenic bacteria and their products of metabolism interfere with the homeostasis of the oral tissue, which leads to the development of diseases such as caries, periodontitis, and respiratory diseases (Mayta *et al.*, 2012; Falsetta *et al.*, 2014; Jakubovics, 2015).

Various investigations, such as epidemiological studies, laboratory tests, and animal tests, confirm the association between *Streptococcus mutans* Clarke 1924, dental caries, and poor hygiene of the oral cavity. *S. mutans* is a Gram-positive bacterium with coconut morphology, with optional anaerobic respiration, and presents lactic acid as the final product of its fermentation. The excessive production of this acid by this coconut in the presence of carbohydrates causes a drop in the pH (below 5.0) and the demineralization of dental tissues, giving rise and development to caries. This disease is multifactorial and global in scope and affects children and adults. In some parts of the world, it is endemic due to the minimal hygiene instruction of the population and the poor local conditions (non-fluoridated water, lack of adequate dental treatment, and a cariogenic diet) (Akthar *et al.*, 2014; Dalmasso *et al.*, 2015; Oda, 2015). *S. mutans* is also associated with cases of non-oral infection such as bacterial endocarditis. Therefore, it is crucial to decrease the number of bacteria in the saliva before invasive procedures, avoiding the appearance of bacteremia, and the consequent endocarditis (Lambert *et al.*, 2001; Shan *et al.*, 2011; Ricatto *et al.*, 2014; Kaur *et al.*, 2015).

Another important microorganism present in the oral cavity is the *Enterococcus faecalis* Orla-Jensen 1919 bacteria. It is an optional Gram-positive and anaerobic coco microorganism. This bacterium can colonize a wide range of habitats, such as the gastrointestinal tract and the vagina. In the oral cavity, it is free in saliva, and, when present in the root canals, it is associated with failure and the need for endodontic retreatment (Wang *et al.*, 2012; Aparicio *et al.*, 2019; Bernardino *et al.*, 2019). Improvements in biological material collection techniques and molecular identification methods helped reveal this microorganism in root canals (Sedgley *et al.*, 2006; Alarcón *et al.*, 2016; Escalante & Martínez, 2020; Moreno *et al.*, 2020). *E. faecalis* has some mechanisms that aid in survival and permanence within the tooth, where the decaying dental pulp creates an extremely inhospitable environment. These mechanisms include high nutritional adaptation, survival in an extremely alkaline environment, and mobility to penetrate deep into the dentinal tubules (Gursoy *et al.*, 2013; Bumb *et al.*, 2014; Zhang *et al.*, 2015; Reyes *et al.*, 2018).

The use of a toothbrush, paste, and dental floss combined with a correct oral hygiene technique are among the options found by humans for the mechanical elimination of pathogenic microorganisms. However, some patients cannot reach certain places or even lack the manual dexterity necessary to carry out effective oral hygiene, do not satisfactorily eliminate microorganisms, and, consequently, favor the evolution of pathologies (Almas & Al, 2004; Gunsolley, 2010; Al & Kasi, 2012). As an alternative to this problem, mouthwashes are recommended, since they can reach places that are difficult for the patient to access, in addition to destabilizing and helping to eliminate microorganisms (Santos, 2003; Xu *et al.*, 2011; Gonçalves, 2013).

All these factors result in the current market having a wide range of pharmaceutical products that offer great benefits for treating various oral diseases. Most of these products are of synthetic origin with healing properties. There is no report of products intended to decrease the bacterial load of the oral cavity, only pharmaceutical forms manufactured for the treatment of inflammation of the oral tissues (Shon *et al.*, 2004; Enrile de Rojas & Santos, 2005; Rasooli *et al.*, 2009).

The use of natural compounds as a treatment agent for different local and systemic disorders has been practiced

for thousands of years in various countries, mainly in eastern countries. According to the World Health Organization (WHO), 80% of the world population depends on traditional herbal medicine for their primary health needs (Borba & Macedo, 2006; García *et al.*, 2007).

The use of certain plants with natural medicinal properties has always been used to produce or use some drugs related to current therapeutics. In pharmaceutical laboratories, some plants undergo some processes such as extraction, purification, among others, and then they are used for some pharmaceutical functions (Dagli *et al.*, 2015; Jakubovics, 2015). In addition to plants, compounds generated by insects such as bees have also been used in the development of natural medicines.

The aim of this research is to review the use of compounds natural in reducing the bacterial load of the oral cavity.

Some natural compounds to reduce the bacterial load of the oral cavity are presented below:

Chamomile (*Chamaemelum nobile* (L.) All.)

Chamomile has been considered since past times as a natural and traditional medicinal plant. It can be anti-inflammatory and antioxidant, thus helping relaxation, disinflammation and also acts as a sedative, having reasonable control of bacteria and other microorganisms in regards to the oral cavity. It has been proven that it reduces signs of gingivitis, such as inflammation and bleeding at the gum level (López, 2015; Borja, 2017). Chamomile is titled as an ideal natural medicine due to its composition since this product does not produce stains; taste alterations, are non-toxic and can be used at all ages, both in children, the elderly, and pregnant women (López, 2015).

The antibacterial effect that chamomile has on the bacteria *S. mutans*, has been raised through studies for that it can be used as a mouthwash. Since it is easily accessible, low cost, and it can be found within everyone's reach, and it does not have harmful side effects for those who choose it possible occupy chamomile as a natural medicine (Schencke *et al.*, 2016; Fazio *et al.*, 2010).

Cocoa (*Theobroma cacao* L.)

One of the most predominant pathologies in the oral cavity due to *S. mutans* is caries and periodontal disease. Dentistry has been in charge of investigating among numerous natural agents to decrease the bacterial effects within the oral biofilm, among them cocoa complies with properties against acids and glucan synthesis, allowing its decrease and favoring the bacteriostatic qualities of the mentioned agent (Mariani *et al.*, 2010).

The properties of cocoa focus on the action that the extract of her seeds and powder can generate in dental plaque. Attributing the leading role to the polyphenols in cocoa that are responsible for the inhibitory activity on the glucosyltransferase enzyme, showing bacteriostatic behavior that reduces to some extent, the action of the *S. mutans* (Mariani *et al.*, 2010).

Aloe (*Aloe vera* L.)

Taking into account the anti-inflammatory, antimicrobial, and tissue healing effects of aloe vera, its application in dentistry is extensive. Periodontal disease and dental caries are multifactorial pathologies of high prevalence worldwide. They have an infectious component with tissue destruction; in this sense, the regenerative effect has been demonstrated in soft and hard tissues (Boonyagul *et al.*, 2013). On the other hand, scarring requires the absence of microorganisms. Therefore, the use of this plant could solve pathologies such as periodontal disease, loss of dentin tissue, bone tissue after tooth extraction, and other pathologies, in an economical and relatively safe way (Moreno *et al.*, 2011; Boonyagul *et al.*, 2013).

More than seven decades after the first publication of the application of *Aloe vera* in Dentistry, scientific work is scarce, as periodontal disease is the most studied. According to the results, the products or derivatives of this plant could be a pharmacological alternative for many oral diseases of an infectious, inflammatory, and tissue loss nature (Moreno *et al.*, 2011).

Moringa (*Moringa oleifera* Lam.)

Although research on herbal medicine applied in dentistry is scarce, moringa offers excellent potential for applications in diseases such as dental caries and periodontal disease, highly morbid pathologies, where their ubiquity makes it one of the problems of most important public health worldwide. In this sense, herbal formulations with antiseptic and antiplaque action play an essential role in the prevention of these diseases (Dinesh, 2016). Both dental caries and periodontal disease have their origin in the bacterial biofilm, an entity formed by the colonization and accumulation of microorganisms in the microflora, immersed in a matrix of glucans. *S. mutans* produces glucosyltransferase, an enzyme that synthesizes these glucans, essential for the adherence and

survival of biofilm microorganisms, since they form a barrier that prevents the diffusion of acids generated by these bacteria, in addition to creating poor environment oxygen (Dinesh *et al.*, 2016; Fazio *et al.*, 2019).

Oregano (*Origanum vulgare* L.)

Oregano is an aromatic plant. This plant has an essential oil that is carvacrol and thymol. This plant is well known commercially for being used as a species, seasoning, and medicinal properties. It also has antibacterial, antifungal, antiparasitic, antimicrobial, and antioxidant properties. The oil that comes from this plant has an antimicrobial effect against gram-positive and gram-negative bacteria. This antibacterial effect is because oregano has a high content of polyphenolic compounds (Albado *et al.*, 2001; Rasooli *et al.*, 2009).

Earlier it was mentioned that oregano contains compounds such as carvacrol and thymol; these compounds affect the permeability of the cell membrane. The effect on phospholipids causes changes in the composition of fatty acids (Pérez *et al.*, 2020).

Studies carried out to verify the effectiveness of oregano against dental caries show the following (Karadaĝlioĝlu *et al.*, 2019; Liu *et al.*, 2019):

"The findings of this study show that *O. vulgare* (Oregano) at low concentrations (1%, 5%, and 10%) do not have any antibacterial effect on the in vitro growth of *S. mutans*. However, at high concentrations (20%, 40%, 60%, 80%, and 100%), they have an antibacterial effect against the growth of *S. mutans*. So it was determined that the minimum inhibitory effect was achieved at 20% corresponding to 4 g of oregano in 200 ml of water.

According to the results of the study, the higher the dilution of oregano concentration, the greater the inhibition halos (Karadaĝlioĝlu *et al.*, 2019; Liu *et al.*, 2019).

Coconut (*Cocos nucifera* L.)

Coconut oil has antibacterial properties as a natural product. Also, it has antibiotic properties that come from a high concentration of lauric acid, which is characterized by the increased antibacterial and antiviral properties of the body. For this reason, when applied, coconut oil will be more effective than any other synthetic product, since it can minimize consequences, acting as an inhibitor of *S. mutans*, as the main bacteria involved in the generation of dental caries (Joy *et al.*, 2019).

An *in vitro* experimental research carried out (Lambert, 2001) in which coconut oil was applied to a culture of *S. mutans* strains, to identify the inhibitory effect by measuring inhibition halos, found the following:

"In a research study, homemade coconut oil was made, in which a diluent was used to obtain different concentrations of 50%, 75% and 100%, which is pure oil, whose average inhibition results were 12.96 mm for 100% concentration, 12.05 mm at 75% concentration and 11.17 mm at 50% concentration, which shows that *S. mutans* turns out to be "Sensitive or inhibited" when presenting halos of inhibition greater than 8 mm".

Streptococcus mutans was shown to be sensitive to coconut oil in concentrations of 50%, 75% and 100%, taking into account that there are no significant differences between the inhibitions produced by the three different concentrations (Joy *et al.*, 2019).

Garlic (*Allium sativum* L.)

In a study in India, he stated that an abscessed tooth is an infection with unsupportable pain that occurs when these bacteria enter the root of the tooth through a cavity or crack between them. These cavities occur when certain substances or bacteria, whether external or internal, enter between these cracks and make your enamel susceptible and weak. The symptoms are closely related to redness, burning, and swelling of the gums (Saha & Bandyopadhyay, 2019).

The mixture of the flavonoids that have sulfur in garlic heals a specific part of the swelling and relieves pain. It contains a substance called Allicin that its action is to function as a natural antibiotic that can make it possible to destroy some other bacteria. Garlic is a local anesthetic that gives pain relief by numbing the infected area and aiming to reduce pain temporarily (Saha & Bandyopadhyay, 2019).

Garlic, when used as a natural agent, can be used alone or mixed with other natural ingredients to make a treatment with improvement and effectiveness. There are several ways on how to apply garlic (topical use), place the garlic clove in direct contact with the infected area, rinse with garlic water, fill garlic powder, garlic paste and salt with hot water as an antiseptic property, garlic ointment as an antibiotic, garlic and cloves as a pain reliever and garlic paste and Curcuma (Saha & Bandyopadhyay, 2019).

Clove (*Syzgium aromaticum* L.)

According to Radünz *et al.* (2019) deduced that thanks to the small properties provided by cloves, a natural and effective remedy could be obtained to combat cavities and prevent the severe accumulation of tartar and to combat bad breath. To keep the teeth and the entire oral cavity healthy, we must maintain adequate hygiene and, for this, more effectively, natural agents such as rinsing cloves against dental caries. Cloves have excellent antiseptic properties (Radünz *et al*., 2019).

Most toothpaste and rinses contain ingredients that could be harmful to the oral cavity, such as alcohol, parasites, and sulfates. This natural agent serves to complete the cleaning of the mouth and to provide good breath after the correct brushing of teeth. With this, it can facilitate the elimination of microorganisms, either 50% of which cause cavities, bad breath, and inflammation of the oral cavity. However, this can be supplemented by its help with alcohol and natural ingredients such as cloves (Radünz *et al*., 2019).

Clove is an aromatic spice originating from Indonesia, and its high antiseptic power prevents cavities and any oral infection, it has analgesic properties for toothache. Its intense smell is a remedy for bad breath and is anti-inflammatory for the gums and prevents gingivitis (Radünz *et al*., 2019).

Cardamom (*Elettaria cardamomum* L.)

Cardamom is an aromatic condiment with highly valued medicinal agents since it helps digestion, it is a remedy for lack of salivation, a natural antiseptic, and a remedy for halitosis and bacteria that cause cavities. This plant is usually harvested in countries of India, it is a family of ginger, and therefore it grows and has excellent roots (Maheswari *et al*., 2020).

It has large flowers and green leaves, its seed has a bitter taste, but it is edible since it has its benefits as it is a natural medicine for bad breath since this plant is a source of cineole, this is a natural antiseptic that helps kill bacteria that cause cavities (Maheswari *et al*., 2020).

Stevia (*Stevia rebaudiana* Bertoni)

Stevia is used as pills in natural medicine, in the medicine of Asian countries, it is considered to be very good to fight allergies, increase defenses, control cholesterol and is also a natural treatment for hypertension, diabetes, obesity, caries and oral health. On the other hand, in the Mediterranean and American countries, its properties are less known, because people are unaware of its properties and its healing capacity, it is also known as the sweet herb of Paraguay. It is edible, and we find it as a sunflower family (Tiwari *et al*., 2018).

It has effective agents for treatments of oral conditions such as caries, the extracts it has been lethal for the bacteria that cause caries and dental plaque. These discoveries are important not only because these microorganisms do not grow because there is no sugar, but also because the activity is bactericidal, that is, it kills the bacteria that drive these diseases. This opens a handy field for stevia in other pharmacological applications. It can be used as the main compound in mouthwashes, toothpaste, among others, due to its high content of vitamin C (Tiwari *et al*., 2018).

Honey bee

Bee honey has essential medicinal properties so much so that since ancient times they have been considered for helping in treatments against infections caused by both bacteria and fungi. Manuel de la Rosa and José Prieto maintain that (De la Rosa & Prieto, 2010):

"Honey is a natural product, made by bees based on the nectar of flowers, bees enrich and transform this nectar with substances that they generate in their bodies, and deposit it and store it in the combs where they mature.t presents an acidic pH ranging from 3.2 to 4.5".

The antibacterial characteristics that it presents are mainly due to its osmolarity. Its content is related to the demand for water present, its low pH, among other specific components that each plant possesses, these will transfer its qualities and elements positive and essential to the nectar collected by bees (Romero, 2013).

The honey has been investigated against *S. mutans* infections, dental plaque and caries, gingivitis and halitosis. Honey was also useful in preventing side effects associated with treatment of cancers of the head and neck, namely, radiation induced mucositis, xerostomia and poor wound healing (Ramsay *et al*., 2019).

Propolis

Sánchez (2017), defines propolis as being: "distinguished by being a resinous substance, bitter at times with a pleasant and sweet aroma, propolis, performs an antibacterial action such as inactivating the membrane potential and inhibiting protein synthesis as for bacteria, it is concerned."

When talking about the bacterium *S. mutans* (the primary driver of caries), the focus is on damage to the plasma membrane. The antibacterial action that propolis develops consists of exclusive chemical composition in flavonoids such as apigenin, identified by having the inhibitory quality of glucosyltransferases in bactericidal response, avoiding the synthesis of glucans since these can affect the oral microbiota (Sánchez, 2017). This bacterium mentioned above has acidogenic properties which promote the production of extracellular and intracellular polysaccharides, under the effects of propolis (Mayta *et al*., 2012).

Antimicrobial-inhibitory effects of compounds natural on microorganisms in the oral cavity.

In Table 1, it is shown the antimicrobial inhibitory effects of natural compounds from plants and insects on microorganisms, being that different parts of the plants (fruit, endosperm, seed, bulb, shell, stem and leaves) are extracted with chemical or natural solvents, in the control of microorganisms, in research *in vivo* and *in vitro*.

Table 1. Antimicrobial-inhibitory effects of compounds natural on microorganisms in the oral cavity.

Plant/part or Compounds natural	Extract	Micro-organism control	Application	References
Cymbopogon citratus Spreng/ fruit	Ethanol	Enteriobacteriacae, *Staphylococcus aureus* Rosenbach, 1884	*in vitro*	(Ramsay *et al.*, 2019)
Allium sativum L./ Bulb	Ethanol	Enteriobacteriacae, *Candida* spp. Berkhout, 1923	*in vitro*	(Ramsay *et al.*, 2019)
Thymus vulgaris L./ leaves	Thymol, Linalol Carvacrol	*Listeria monocytogenes* Pirie, 1940, *Escherichia coli* Escherich, 1885, *S. typhimirium* Rosenbach, 1884 *S. aureus*	*in vitro*	(Radünz *et al.*, 2019)
Verbena officinalis L./ leaves	Borneol Geraniol	*S. aureus,* *E. coli,* *S. typhimirium,* *L. monocytogenes*	*in vitro*	(Rasooli *et al.*, 2009)
Libanothamnus neriifolius Ernst / leaves	Oil	*S. aureus,* *Enterococcus faecalis* Orla-Jensen 1919, *E. coli,* *Klebsiella pneumoniae* Trevisan 1885, *Pseudomonas aureginosa* Schroeter, 1872, *Candida albicans* Berkhout, 1923, *Candida krusei* Berkhout, 1923	*in vitro*	(Reyes *et al.*, 2018)
Theobroma cacao L./ shell.	Oil	*P. aeruginosas,* *Proteus* sp. Hauser 1885. *Enterobacter* sp., *E. coli,* *Pseudomonas putida* Trevisan 1889	*in vitro*	(Romero, 2013)

Continua Table 1

Continua Table 1

Aloe vera L./gel	Glycerol	*Helicobacter pylori* Marshall *et al.* 1985, *E. coli,* *E. faecalis,* *S. aureus,* *Streptococcus mutans* Clarke 1924	*in vitro*	(Saha & Bandyopadhy, 2019)
Cocos nucifera L./ endosperm	Oil	*Listeria ivanovii,* *L monocytogenes*	*in vitro*	(Sánchez, 2017)
Moringa oleífera Lam./ Seed	Water	*E. coli,* β-*Lactamasas*	*in vivo*	(Santos, 2003)
Stevia rebaudiana Bertoni/ leaves	Ethanol	*S. sanguinis* White & Niven, 1946 *Actinomyces viscosus* (Howell *et al.*, 1965) Georg *et al.*, 1969.	*in vitro*	(Sedgley *et al.*, 2006)
Salvadora persica L./ stem	Water	*S. mutans* *Lactobacillus* spp. Beijerinck 1901 *S. aureus*	*in vivo*	(Schencke *et al.*, 2016)
Juglans regia L./ stem	Methanol Ethanol chloroform Acetone Water	*S. mutans,* *Porphyromonas gingivalis* Coykendall *et al.,* 1980	*in vivo*	(Shan *et al.*, 2011)
Camellia sinensis L./ leaves	Polyphenol Ethanol	*S. mutans*	*in vitro*	(Shon *et al.*, 2004)
Morus alba L./ leaves	Ethanol	*S. mutans,* *Actinobacillus actinomycetemcomitans* Klinger, 1912, *P. gingivalis,* *Prevotella intermedia* (Holdeman & Moore 1970) Shah & Collins 1990, *Streptococccus mutans* Clarke 1924, *Streptococcus mitis,* *S. sanguinis,* *Actinomycete viscosus* (Howell *et al.*, 1965) Georg *et al.*, 1969, *Lactobacillus acidophilus* Johnson *et al.,* 1980 *Lactobacillus delbrueckii* subsp. *lactis* (Orla-Jensen 1919) Weiss et al. 1984	*in vitro*	(Tiwari *et al.*, 2018)
Mentha spicata L./ leaves	Oil	*S. mutans*	*in vivo, in vitro*	(Wang *et al.*, 2012)

Continua Table 1

Continua Table 1

Eucalyptus globulus LaBill./ stem	Oil	*P. gingivalis* *Actiobacillus actinomycetemcomitans* Klinger, 1912 *Fusabaterium nucleatum* Knorr, 1922 *S. mutans* *Streptococcus sobrinus*	*In vivo, In vitro*	(Xu *et al*., 2011)
Bee honey, Propolis	Water	*Bacillus cereus* Frankland & Frankland, 1887, *Bacillus subtilis* Ehrenberg, 1835, *E. coli,* *Salmonella enteritidis* (Gaertner 1888) Castellani & Chalmers 1919, *Salmonella typhimurium* Le Minor & Popoff, 1987, *S. aureus*	*In vitro*	(Zhang *et al*., 2015)

CONCLUSIONS

According to the literature, it can be concluded that there are several studies concerning the effects of natural products on the part of man in medicine, where there are a large number of works and publications related to natural substances with an active ingredient of biological interest with phytopharmaceuticals. However, in dentistry, there is also this trend, more than a few publications that allow a scientific endorsement for or use of these products, mainly the development of mouthwashes with a preventive and alternative means of reducing bacterial load in the oral cavity of the individual.

Conflict of interest

The authors declare that they do not have any conflict of interest regarding the study topic.

Ethical aspects

The authors declare that they have abided with ethical guidelines regarding the study.

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