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**TITLE**

Identification and characterization of anti-caries natural compounds  
and their use in experimental slow release tablets as advancement of  
oral health strategies.

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A handwritten signature in black ink, appearing to be the name of the tutor, Prof. Aniello Ingenito.

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## **1. Introduction**

Dental caries has been identified as one of the most prevalent chronic condition and it is a major problem for children all over the world [Millo et al., 2017]. Despite the use of preventive systems (improved oral hygiene, usage of fluoride-containing toothpaste, fluoride content in drinking water, sealing), international data on childhood caries epidemiology confirm that dental caries remains a 'significant and consequential disease of childhood', being increasingly localized in a subgroup of high-risk children, both in developing and developed countries. In 2010, untreated caries in permanent teeth was the most prevalent health condition worldwide, affecting 2.4 billion people and untreated caries in deciduous teeth was the 10th most prevalent medical condition, affecting 9% of the global population [Lambert et al., 2017], impacting quality of life through pain, infection, diet, and loss of sleep. Caries can also lead to time lost from school for children and time off work for parents [Anop et al., 2015]. In addition, oral diseases affect psychologically, resulting in difficulty to socialize. In recent years, these conditions have been associated with a negative impact on children's quality of life: cross-sectional studies demonstrated that dental caries have been associated with a negative impact on the quality of life of children from different age groups [Martins et al., 2017]. Recent epidemiological surveys indicate a reduction in the prevalence of caries in Italy, which is in line with the trend observed in industrialized countries in the last decades. Despite this reduction, a polarized distribution of the disease has

recently been observed [Ferrazzano et al., 2016]. In some countries, this positive trend could deter action to further improve oral health or sustain achievements. It might also lead to the belief that caries is no longer a problem, at least in the developed countries, resulting in the limited resources available for caries prevention being diverted to other areas [Ferrazzano et al., 2016]. However, it must be stressed that caries as a disease has not been eradicated, but only controlled to a certain degree. The burden of oral disease and needs of populations are in transition and oral health systems and scientific knowledge are changing rapidly. The etiology of tooth decay is multifactorial and it is induced by three main factors: host, environment and bacteria [Fusao et al., 2007]. Today it is known that caries is characterized by an early acquisition and overgrowth of several species of cariogenic bacteria, such as *Streptococcus mutans*, *Streptococcus sanguinis*, and *Lactobacillus casei* [Millo et al., 2017]. Many studies have revealed that *S. mutans* represents about the 20-40 % of the cultivable flora in biofilms removed from carious lesion and gives its name to a group of seven closely related species collectively referred to as the mutans streptococci [Jane et al., 2016]. It is one of the main factors for triggering of dental caries because causes demineralization of inorganic tooth structure by metabolizing sucrose to lactic acid. It can also colonize tooth surfaces and initiate plaque formation through its ability to synthesize and bind extracellular polysaccharides (glucan) using the enzyme glucosyltransferase [Forssten et al., 2010]. Usually, the appearance of *S. mutans* in the tooth cavities is followed by caries after 6-24 months [Mayooran et al., 2000]. *S. mutans* and the other microorganisms involved in the pathogenesis of dental caries have been

considered very difficult to control, because they have developed tolerance and resistance to many antimicrobial agents routinely used in the clinical practice. Several antibiotics and antimicrobial agents have been used to eliminate cariogenic bacteria from the oral flora. However, their clinical use is limited due to undesirable side effects, including microorganism susceptibility, vomiting, diarrhea, and tooth staining. The most commonly used preventive and therapeutic mouth rinses in children is chlorhexidine. Chlorhexidine mouth rinse is considered the “gold standard” due to its bacteriostatic and bactericidal properties at low and high concentrations, respectively. It has been studied for nearly 40 years primarily for its ability to reduce gingivitis [Thomas et al., 2015]. Classified as an antimicrobial agent, it has been proven to inhibit the formation and development of dental plaque biofilm. However, it can cause a change in taste and produce yellow or brown pigments on tooth surfaces. Therefore, the use of chlorhexidine for caries prevention is controversial, especially in children [Sadat et al., 2015]. Due to indiscriminate use of antimicrobials, more and more pathogens are becoming resistant and posing a serious threat in rendering successful treatment of the diseases. So, the resistance of microorganisms against the antibiotics commonly used to treat oral infections, the increasing number of oral pathologies and the lack of medications without side effects stressed the importance of further research to develop alternative antibacterial agents from natural sources with focus on safety for humans and efficacy in the treatment and prevention of dental caries. Since the past, the bioactive principles of plant origin have been used for treatment of many diseases and microbial infections. Medicinal plants have been a great source of

novel drug compounds since ages. Plant derived products have made large contributions to the well being of human health [Giriraju et al., 2013]. In the last decades, the use of plants with preventive and therapeutic effects contributing to health care has increased. Scientists investigated many plant products in order to find their effectiveness in the prevention of dental plaque formation [Rajini Kanth et al., 2016]. Numerous medicinal plant extracts have been shown to inhibit the formation of dental biofilm by reducing the adhesion of microbial pathogens to the tooth surface or reducing the number of bacteria implicated in the caries pathogenesis. Natural phytochemicals would offer an effective alternative to antibiotics and drugs; hence, represent a promising approach in prevention and therapeutic strategies for prevention of dental caries and other oral infections [E.A. Palombo, 2011]. However, only few natural products have found therapeutic applications. The reasons of such limited use are due to different factors as: effectiveness, stability, smell, taste, and, not last, cost [Ferrazzano et al., 2013]. The challenge that we face is how best to deliver these new anti-caries entities at true therapeutic levels, over time, to favorably tip the caries balance. There are three major problems associated with drug therapy within the oral cavity: rapid elimination of drugs due to the flushing action of saliva or the ingestion of food, the non-uniform distribution of drugs within saliva on release from a solid or semisolid delivery system and patient compliance in terms of taste [Mizrahi et al., 2008]. Medicated mucoadhesive tablets could be an effective way for establishing sufficient concentrations of antibacterial agents in the oral environment to reduce the growth of plaque. Over the past few decades, mucosal drug delivery has

received a great deal of attention: mucoadhesive dosage forms may be designed to enable prolonged retention at the site of application, providing a controlled rate of drug release for improved therapeutic outcome. Application of dosage forms to mucosal surfaces may be of benefit to drug molecules not amenable to the oral route, such as those that undergo acid degradation or extensive first-pass metabolism [Rahamatullah et al., 2011].

## **2. Aims of the research.**

The aim of this research program was to elaborate a new methodology against dental caries, that is based on:

1. identification, characterization and validation of natural active compounds that have anti-caries activity and reduce cariogenic microflora pathogenicity.
2. The determination of the effectiveness of a novel route of anticaries bio-active molecules administration by the usage of mucoadhesive buccal drug delivery system that can satisfy the patient compliance.



### **3. Natural anti-caries active compounds**

The natural active compounds identified and used in the preparation of mucoadhesive tablets were Casein phosphopeptides (CPPs), *Stevia rebaudiana* Bertoni and the hydro-alcoholic extract of, polyphenol rich, pomegranate (*Punica granatum* L.) peel.

#### **3.1. Casein phosphopeptides**

Casein phosphopeptides (CPPs) are phosphorylated casein-derived peptides produced by proteolytic digestion of  $\alpha_1$ -,  $\alpha_2$ -, and  $\beta$ -casein during the natural digestive process in vivo, by action of proteolytic enzymes in vitro, and by proteolytic starter cultures during manufacturing of dairy products as fermented milk, yogurt and cheese [Bouhallab and Bouglé, 2004; Cai et al., 2003; Ramalingam et al., 2005; Walker et al., 2006]. CPPs, containing the sequence Ser(P)-Ser(P)-Ser(P)-Glu-Glu, stabilize nanoclusters of amorphous calcium phosphate (ACP) in metastable solution. These multiple phosphoserine residues of the CPPs bind to forming nanoclusters of ACP in supersaturated solutions, preventing growth to the critical size required for phase transformations. CPPs-ACP localize ACP in dental plaque, which buffers the free calcium and phosphate ion activities, helping to maintain a state of supersaturation with respect to tooth enamel, depressing

demineralization and enhancing remineralization [Cross et al., 2004; Cross et al., 2005]. In particular, CPPs stabilize calcium and phosphate ions under neutral and alkaline conditions forming metastable solutions that are supersaturated with respect to the basic calcium phosphate phases. Under these conditions, the CPPs bind their equivalent weights of calcium and phosphate. The preventive action of CPPs, in vivo, takes place when there are demineralising agents (acid pH), for example during a carious or erosive process. That situation can enhance the release of calcium from the CPP-ACP complex, thus increasing the Ca cation concentration and promoting a supersaturation condition: that will prevent demineralization and enhance the remineralization of early enamel caries [Reynolds et al., 2003]. On the basis of the generally accepted molecular formula for ACP  $[Ca_3(PO_4)_2 \cdot nH_2O]$ , ACP also may be considered a tricalcium phosphate. There is no conclusive evidence that ACP is an integral mineral component in hard tissues. It likely plays a special role as a precursor to bioapatite and as a transient phase in biomineralization [Azarpazhooh and Limeback, 2008].

Furthermore, Guggenheim et al. found that CPP-ACP taken with a cariogenic diet in rats significantly reduced the numbers of streptococcus sobrinus by interfering with bacterial adherence and therefore colonization [Guggenheim et al., 1995]. In addition, a commercial paste containing CPP-ACP has shown to remineralize initial enamel lesions [Kumar et al., 2008]. The application of a CPPs toothpaste and sodium fluoride (Colgate Neutrafluor 9000 ppm) (NaF) can provide significant additional prevention of enamel demineralization when resin-modified glass ionomer cement (RMGIC) is used for bonding molar tubes for orthodontic patient as

preventive actions [Sudjalim et al., 2007]. An in vitro study to evaluate the remineralization of incipient enamel lesions by the topical application of Casein Phosphopeptide-Amorphous Calcium Phosphate (CPPACP) using laser fluorescence and scanning electron microscope showed high scores of remineralization [Pai et al., 2008].

Other recent in vitro and in vivo experiments have demonstrated that both synthetic casein phosphopeptide-amorphous calcium phosphate (CPPs-ACP) nanocomplexes contained in mouthrinses and sugar-free chewing gum, and natural CPPs contained in dairy products (such yogurt) are anticariogenic [Ferrazzano et al., 2008; Iijima et al., 2004; Manton et al., 2008; Morgan et al., 2008; Shen et al., 2001].

In summary, CPP-ACP complexes have a multiple action mechanism: on one hand, providing an oversaturation of calcium and phosphate ions in the dental biofilm and saliva, conferring the potential to be biological delivery vehicles for calcium and phosphate; on the other hand, inhibiting adhesion of cariogenic bacteria to the hydroxyapatite making it possible to modulate the activity of plaque bacteria and determines colonization by less cariogenic bacteria.

Recent studies tested if adding casein phosphopeptide-stabilized amorphous calcium phosphate to the Powerade sport drink could be possible prevent erosive enamel lesions: enamel samples were analyzed at scanning electron microscope (SEM) after erosive immersion test with and without the protective biomolecules to evaluate the resulted surface profiles: it was assessed that CPP-ACP included in

sport drinks significantly reduced the beverage's erosion effect on dental enamel without affecting the product's taste [Ramalingam et al., 2005]

### **3.2. *Stevia rebaudiana* Bertoni**

*Stevia* Cav. is a genus of herbaceous and shrubby plants distributed exclusively in the American Continent, from the Southern United States to Central and South America.

In Central and South America, numerous *Stevia* species, such as *S. salicifolia* Cav. and *S. lucida* Lag., have long been known for their ethnopharmacological uses, ranging from anti-helminthic to anti-rheumatic and anti-inflammatory applications. Certain species are also used as an emetic (*S. rhombifolia* HBK), for the treatment of cardiac conditions (*S. cardiatica* Perkins) or as anti-diarrheal (*S. balansae* Hieron, *S. trifida*), whereas diuretic properties have been attributed to *S. eupatoria* (Spreng.) Willd. and *S. pilosa* Lag. [Soejarto et al., 1983]. Apparently, *S. rebaudiana* (Bertoni) Bertoni, which originated from Northeastern Paraguay, is a unique species containing the glycosides stevioside and rebaudioside A, responsible for the sweet taste of the leaves [Lemus-Mondaca et al., 2012]. It is a perennial shrub, spontaneously growing in the subtropical, mesothermal and humid habitats of South America ([Figure 1](#)) [Kinghorn et al., 2003].

*S. rebaudiana*, often referred to as *the sweet herb of Paraguay*, has been widely used in many countries, including China, Japan, Korea, Brazil, and Paraguay, either

as a substitute for sucrose in foods and beverages or as a household sweetening agent [Soejarto et al, 2002]. The plant is rich in carbohydrates (62% dry weight, dw), protein (11% dw), crude fibre (16% dw), minerals (K, Ca, Na, Mg, Cu, Mn, Fe, Zn), and essential amino acids [Aminha et., 2014].



**Figure 1.** Countries of South America where *S. rebaudiana* grows spontaneously.



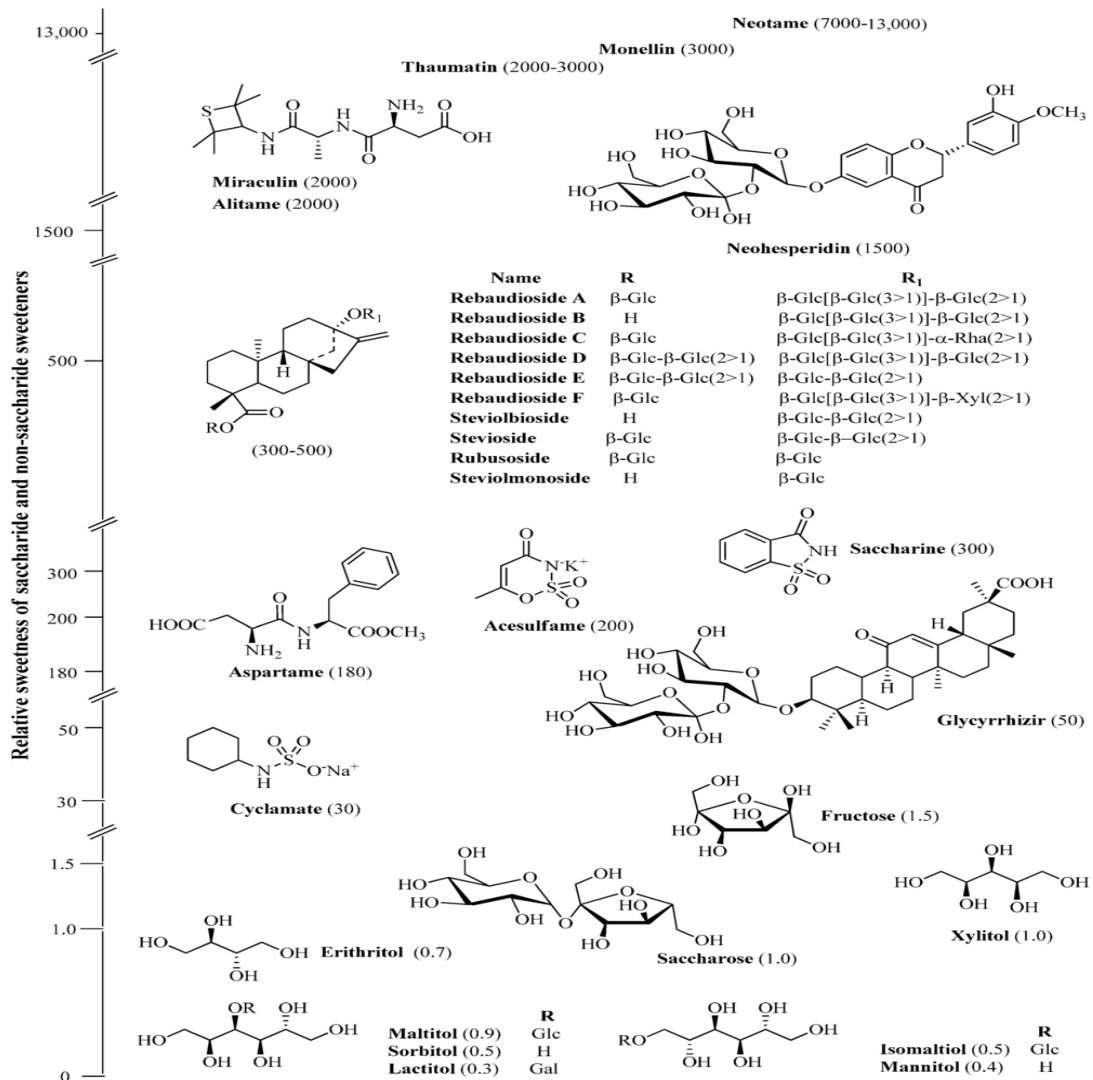
**Figure 2.** Regions of the world where it is possible to cultivate *S. rebaudiana*.

### **2.3. *S. rebaudiana* Chemical Constituents and Extraction Procedures**

The extracted active ingredient of *S. rebaudiana* is a white crystalline substance, and it has been used for centuries to sweeten food and beverages by the indigenous people of South America.

The compounds responsible for the natural sweetness of *S. rebaudiana* leaves include diverse diterpenoid glycosides derived from a steviol skeleton. These steviol glycosides also exhibit low calorific value, which is interesting for promising therapeutic applications, particularly for the treatment of disturbances in sugar metabolism.

The three major constituents of the leaf extract of *S. rebaudiana* were stevioside, rebaudioside A, and rebaudioside C (from 3% to 17%, by weight) [Kolb et al., 2001; Morlock et al., 2014]. Other compounds present at lower concentration are: steviolbioside, rebaudiosides B, D, E, F, and steviolmonoside [Chaturvedula et al., 2011, Ohta et al., 2010].



**Figure 3.** Sweetness of the most common artificial and natural sweeteners.

Stevioside, the main sweet component in the leaves of *S. rebaudiana* (Bertoni) Bertoni tastes approximately 300 times sweeter than sucrose. The structures of the sweet components of *S. rebaudiana*, which occur primarily in the leaves, are provided in Figure 3.

Isolated steviosides can be purified using various methods including column chromatography, TLC and HPLC methods. Finally the isolated compounds were analyzed and characterized using analytical methods such as UV, FTIR, MS, and NMR analyses.

#### ***Medicinal and Alimentary Uses of S. rebaudiana Glycosides***

There are three types of *S. rebaudiana*-based products: the regular products, which consist mainly of a stevioside; the Reva A products, which consist mainly of rebaudioside A; and the sugar metastasis product. In the regular products, the content ratio of stevioside to rebaudioside ranges from 7:3 to 8:2, while in Reva A, this ratio is approximately 1:3. Since rebaudioside has a very sweet taste, the quality of sweetness for Reva A products is higher than regular ones [Matsukubo et al., 2006]. Steviosides offer several advantages over other non-caloric sucrose substitutes: they are heat-stable, resistant to acid hydrolysis and non-fermentable [Giongo et al., 2014].

Further studies have suggested that in addition to sweetness, steviosides and their related compounds, including rebaudioside A and isosteviol (a metabolic



component of stevioside), may also offer therapeutic benefits. These benefits include: anti-hyperglycaemic, anti-hypertensive, anti-oxidant [Kelmer et al.,1985], anti-tumor [Jayaraman et al., 2008; Mizushima et al.,2005], anti- diarrheal, diuretic, gastro- [Shiozaki et al., 2006] and renal-protective [Melis et al., 1995], anti-viral [Takahashi et al., 2001], and immunomodulatory [Sehar et al., 2008., Boonkaewwan et al., 2006] actions.

Fengyang *et al.* [Fengyang et al., 2012] examined the anti-inflammatory properties of stevioside and discovered that stevioside exerts its anti-inflammatory effect by inhibiting the activation of NF- $\kappa$ B and mitogen-activated protein kinase signaling and the release of pro-inflammatory cytokines.

The effects of stevioside and its metabolite, steviol, on human colon carcinoma cell lines were studied from Boonkaewwan *et al.* [Boonkaewwan et al. 2008] in 2008. Their results demonstrated two biological effects of steviol in the colon: the stimulation of Cl(-) secretion and the attenuation of TNF-alpha stimulated IL-8 production.

The anti-hyperglycaemic and blood pressure-reducing effects of *S. rebaudiana* were investigated in 2003 by Jeppesen *et al.* [Jeppesen et al., 2003] in a long-term study of type 2 diabetic Goto-Kakizaki (GK) rats. According to their results, stevioside may determine an increasing of insulin secretion, inducing genes involved in glycolysis. It can also: improve the nutrient-sensing mechanisms, rise cytosolic long-chain fatty acyl-coenzyme A (CoA), and control down-regulation of phosphodiesterase 1 (PDE1). They concluded that stevioside demonstrates a dual positive effect: both antihyperglycemic and blood pressure-lowering actions.

As mentioned above, the steviol glycoside is currently used in several countries as a sweetener, and it has been extensively tested to demonstrate that its use is safe for humans. In 2002, *S. rebaudiana* ranked second in the sales of herbal supplements in the USA.

According to the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2004), the consumption of *S. rebaudiana* has been generally regarded as safe [Tandel et al., 2011].

Aqueous extracts of *S. rebaudiana* leaves have been approved since 2008 by the JECFA as sugar substitutes in many foods and beverages in the Western and Far East Asian countries. However, JECFA has requested additional information to change the temporary accepted daily intake (ADI) of 0–2 mg·kg<sup>-1</sup>·day<sup>-1</sup> for steviol glycoside. The European Union approved stevia additives in 2011 [Beck et al., 2011].

### ***Caries Prevention Activity of S. rebaudiana Extracts and Steviol Glycosides***

Presently, *S. rebaudiana* is the only species of the genus with recognized antibiotic properties. The antimicrobial effects of *S. rebaudiana* have been ascribed to the presence of stevioside and related compounds, but their role in caries prevention and dental health promotion is not fully understood. In 2010, Mohire and Yadav [Mohire et al., 2010] conducted a four week clinical study in patients with orodental problems to develop a chitosan-based polyherbal toothpaste (including *S. rebaudiana* extract). They also evaluated its plaque-reducing ability and efficacy in the reduction of dental pathogens using chlorhexidine gluconate (0.2% w/v) mouthwash as the positive control.

The study involved 18 subjects divided into three groups. The groups were treated as follows: Group-I, placebo, toothpaste without chitosan and herbal ingredients; Group-II, positive control, CHX (0.2% w/v) mouthwash; and Group-III, test (Polyherbal), toothpaste with chitosan, eugenol, and *Pterocarpus marsupium* (PM), *S. rebaudiana*, and *Glycyrrhiza glabra* aqueous extracts. Authors determined the total microbial count in order to obtain the reduction, in percentage, of oral bacterial count during the treatment period.

At the end of the study, the herbal extracts were shown to possess satisfactory antimicrobial activity against most of the dental pathogens. The chitosan-containing polyherbal toothpaste significantly reduced the plaque index from 70% to 47% and the bacterial count from 85% to 29%.

The authors concluded that chitosan-based polyherbal toothpaste represented a promising novel oral hygiene product compared with the currently available oral hygiene products. Nevertheless, in this study, the role of *S. rebaudiana* in reducing antimicrobial count is not clear: this effect, in fact, could be the result of synergic action of all active principles involved in the toothpaste.

In 2013, Giacaman *et al.* [Giacaman *et al.*, 2013] investigated the cariogenic and enamel demineralization potential of several sweeteners in an artificial caries model.

Bovine enamel slabs were utilized as the culture medium for *S. mutans* UA159 biofilm that were exposed to different sweeteners in powder or tablet form, as *S. rebaudiana* extracts, sucralose, saccharin, aspartame, and fructose, three times a day for five minutes. The caries-positive and caries-negative controls were 10%

sucrose and 0.9% NaCl, respectively. After five days, the biomass, bacterial counts, and intra- and extracellular polysaccharides of the biofilm were assessed. Surface microhardness was measured before and after the experiment to evaluate enamel demineralization, which was expressed as percentage of surface hardness loss (%SHL). The results of this study suggest less cariogenic effects and enamel demineralization for all tested sweeteners except sucrose. Compared to sucrose, *S. rebaudiana* extracts, sucralose and saccharin reduced the number of viable cells ( $p < 0.05$ ), and all sugar alternative sweeteners reduced extracellular polysaccharide formation. Nevertheless the primary limitation of this study is that the artificial substrate does not allow a biofilm formation rate comparable with a real clinical situation.

In 2012, Gamboa and Chaves [Gamboa et al., 2012] evaluated the antibacterial activity of *S. rebaudiana* leaf extracts against cariogenic bacteria. They prepared extracts from dried leaves in hexane, methanol, ethanol, ethyl acetate, and chloroform, and they evaluated, using well diffusion method, the antibacterial capability of the five extracts for 16 bacterial strains of the genera *Streptococcus* ( $n = 12$ ) and *Lactobacillus* ( $n = 4$ ). Lactobacilli were the most sensitive, with an inhibition zone between 12.3 and 17.33 mm. Moreover, Blauth de Slavutzky [De Slavutzky et al., 2010] conducted an *in vivo* study to evaluate the accumulation of dental plaque after rinsing with a solution of 10% sucrose four times daily for five days and compared it to rinsing with the same frequency using a 10% solution of *S. rebaudiana* extract, which was prepared with 100 g of *stevia* boiled for 2 h in 3 L of distilled water. Consequently, it was

demonstrated that *S. rebaudiana*, after rinsing, reduced dental plaque between 57%–82% than sucrose solution, when measured by Silness-Löe index and 10%–40% less when measured by O’Leary index of plaque.

In 2014, Brambilla *et al.* evaluated the effect of *S. rebaudiana* extracts on *in vitro* *S. mutans* biofilm formation and the *in vivo* pH of plaque. Three separate 10% solutions of stevioside, rebaudioside A and sucrose were prepared. The microbiological count *in vivo* was measured using a MTT assay. Twenty volunteers rinsed with each solution for one minute and then the plaque pH was analyzed seven times after the rinses. Higher *in vitro* *S. mutans* biofilm formation was observed with the sucrose solution ( $p < 0.01$ ). After 5, 10, 15, and 30 min, the *in vivo* sucrose rinse produced a statistically significantly lower pH value compared to the *S. rebaudiana* extracts ( $F = 99.45, p < 0.01$ ). Therefore, *S. rebaudiana* extracts can also be considered non-acidogenic [Brambilla *et al.*, 2014 16].

In 1992, Das *et al.* [Das *et al.*,1992] tested stevioside and rebaudioside A for cariogenicity in albino Sprague-Dawley rats. The authors divided sixty rat pups colonized with *S. sobrinus* into four groups and fed them their basal diets with added stevioside, rebaudioside A or sucrose as follows: group 1, 30% sucrose; group 2, 0.5% stevioside; group 3, 0.5% rebaudioside A; and group 4, no additional chemicals. Significant differences resulted in sulcal caries scores and *S. sobrinus* counts between group 1 and the other three groups. In fact, there was no significant difference between the stevioside, rebaudioside A and no-addition groups. Thus, neither stevioside nor rebaudioside A were cariogenic under the conditions of the study, whose primary limitation is the use of a not human sample.

Zanela *et al.* [ Zanela et al.,2002] investigated the effect of daily mouth-rinse use on dental plaque accumulation and on salivary *S. mutans* in 200 children in 2002. The solutions used were: a placebo solution composed of mentholated deionized water (group I); 0.12% chlorhexidine gluconate associated to 0.05% sodium fluoride (group II); 0.2% chlorhexidine digluconate (group III); and 0.5% stevioside mixed with 0.05% sodium fluoride at pH 3.4 (group IV). To verify the accumulation of plaque, it was assessed the Löe index method at the beginning and end of the experiment. Moreover, the analysis of cariogenic streptococci was accomplished on three saliva samples collected at three different times: before the first mouth-rinse, 24 h after the first mouth-rinse and one week after the last mouth-rinse. The mouth-rinsing routine was performed daily for 4 weeks.

The solution used by group III was the least accepted by children. Furthermore, as solution II was utilized by group II, it caused mild dental pigmentation. There were no statistically significant differences in the levels of *S. mutans*, most likely due to the low initial levels observed in each of the four groups (Table 1).

**Table 1-** Caries prevention activity of *S. rebaudiana* extracts and steviol glycosides.

Authors	Year	Source	Type of Study	Results
Mohire <i>et al.</i>	2010	<i>S. rebaudiana</i> aqueous extract (SR)	<i>In vivo</i>	Reduction of plaque index by 70.47%
Giacaman <i>et al.</i>	2013	<i>S. rebaudiana</i> aqueous extracts (SR)	<i>In vitro</i>	Reduction of extracellular polysaccharide formation
Gamboa <i>et al.</i>	2012	<i>S. rebaudiana</i> methanol and ethanol extracts (SR)	<i>In vitro</i>	Inhibition of growth of <i>Lactobacilli</i>
Blauth de Slavutzky	2010	<i>S. rebaudiana</i> aqueous extracts (SR)	<i>In vivo</i>	Reduction of plaque index
Brambilla <i>et al.</i>	2014	<i>S. rebaudiana</i> aqueous extracts (SR)	<i>In vitro</i> and <i>in vivo</i>	<i>S. rebaudiana</i> extracts are non-acidogenic
Zanela <i>et al.</i>	2002	Solution containing 0.5% Stevioside and Rebaudioside A	<i>In vivo</i>	Dental plaque reduction was not evident using stevioside mouthrinses
Das <i>et al.</i>	1992	Stevioside extracts	<i>In vitro</i>	Stevioside and rebaudioside A are not cariogenic.

### **3.3. Polyphenols.**

Polyphenols constitute one of the most common and widespread groups of substances in plants. Simple phenols consist of a single substituted phenolic ring; flavones and their derivatives -flavanoids and flavanols- are phenolic structures containing one carbonyl group [Cowan, 1999].

Vegetables are the main source of the polyphenols daily intake in human diet, but other strong contributors are tea, coffee, cereals and fruit, due to their high consumption.

The biological properties of polyphenols include antioxidant [Balz and Jane, 2003; Luczaj and Skrzydlewska, 2005], anticancer [Krishnan and Maru, 2004; Yamane et al., 1996; Zhang et al., 2002;] and anti-inflammatory [Sang et al., 2004] effects.

In the last years, polyphenols from some edible plants have attracted attention as potential sources of agents capable of controlling the growth of oral bacteria [Taguri et al., 2004].

Polyphenols could be able to influence the process of caries formation at crucial different stages. In fact, they have been shown to inhibit the adherence of mutans streptococci to saliva-coated hydroxyapatite [Smullen et al., 2007]. Polyphenols are able to interact with microbial membrane proteins, enzymes, and lipids, thereby altering cell permeability and permitting the loss of protons, ions, and macromolecules [Ikigai et al., 1993]. It has been, in fact, demonstrated that when *S. mutans* was pretreated with Sunphenon (a mixture, containing polyphenols), its cellular attachment to a saliva-treated hydroxyapatite surface was significantly



reduced, showing that the phenomenon was a consequence of a specific interaction with the bacteria [Otake et al., 1991].

In addition, several works have demonstrated that polyphenols inhibit in vitro the glucosyltransferases activity of *S. mutans* (GTases) [Hattori et al.,1990; Kashket et al., 1985;Ooshima et al.,1993; Sakanaka et al., 1989].

Experiments also demonstrate the inhibition of salivary amylase activity by polyphenols. The effect on salivary amylase may contribute significantly to reduce the cariogenicity of starch-containing foods [Kashket and Paolino; 1988].

### **3.3.1. Pomegranate.**

Pomegranate (*Punica granatum* L.) is a common fruit of a tree belonging to the family Punicaceae. It is native to the region from northern India to Iran and it has been cultivated and naturalized over the entire Mediterranean region since ancient times. The ripe fruit is about five inches wide with a deep red, leathery skin, grenade shaped with a pointed calyx. The fruit contains many seeds separated by white membranous pericarp. Each seed is surrounded by tart and red juice [Divyashree et al., 2014].

Pharmacological properties of pomegranate have a long history, but, in the recent decades, the interest in evaluating therapeutic effects of pomegranate has increased noticeably. Studies show that pomegranate juice has potent antioxidant activity (capability to scavenge free radicals) due to its high polyphenols content, including ellagitannins (hydrolysable tannins) and anthocyanins (condensed tannins). There is a range of phytochemical compounds in pomegranate that have

showed antimicrobial activity, but most of the researchers have found that ellagic acid and larger hydrolyzable tannins, such as punicalagin, have the most important activities. In many cases, the mixture of the pomegranate constituents offers the most advantage [Howell et al., 2013]. This fruit has also been used in traditional medicine for the treatment of dysentery, diarrhea and respiratory pathologies [Ismail et al., 2012; Dey et al., 2015]. Many studies indicate that pomegranate extracts may be employed as natural alternative for the treatment of a wide range of bacterial and viral infections due to their antimicrobial activity. Recent study indicate that both pomegranate aril and peel extracts have an effective antimicrobial activity, as evidenced by the inhibitory effect on the bacterial growth of two important human pathogens, including *Staphylococcus aureus* and *Escherichia coli*, often involved in foodborne illness [Pagliarulo et al., 2017]. In addition, experimental data strongly support the antibacterial activity of pomegranate extracts against oral pathogen such as *S. mutans*.

#### **4. Experimental Studies Section.**

The intent of this research program was to determine a new way in caries prevention. Literary evidences and previous studies conducted in the Department of Neuroscience, Reproductive and Oral Sciences, Section of Paediatric Dentistry, University of Naples, Federico II, Naples [Ferrazzano et al.,2011;Ferrazzano et al., 2012] had already demonstrated the regular efficacy of natural compounds such as CPPs in preventing dental caries.

Therefore, in this present research activity, we decided to perform experimental microbiological studies order to evaluate:

- 1.** The antibacterial activity of pomegranate extracts against cariogenic bacteria.
- 2.** The in vitro evaluation of cytotoxic effects of experimental mucoadhesive tablets, containing Stevia, CPPs and pomegranate extract, object of the research program.
- 3.** The in vivo compliance of mucoadhesive tablets as model drugs for sustained local action.

#### **4.1. In Vitro Antibacterial Activity of Pomegranate Juice and Peel Extracts on Cariogenic Bacteria.**

##### **Materials and Methods**

###### *Preparation of Extracts for Microbiological Assay.*

Fresh fruits of pomegranate (*P. granatum* L.) were collected from trees located in the countryside of Avellino (Southern Italy) during fruit season. The fruits were handpicked, washed, and peeled, and the arils, without seeds, were hand-crushed and then squeezed in order to obtain the juice. The peel was air dried a few days and then pulverized. The samples were stored at  $-20^{\circ}\text{C}$  for further analysis. The juice was defrosted at room temperature. Solution water/ethanol 25 ml 50% (v/v) was added to 5 g of juice. The same procedure was carried out for the peel powder. Each sample was mixed for 30 minutes, and then the extracts were filtered. The analysis of phenolic compounds of the pomegranate (juice and peel) was performed by reverse phase HPLC (RP) coupled offline mass spectrometry (MS) MALDI-TOF .

For microbiological assays, the ethanolic extracts of juice and peel were dried in Savant in order to calculate the percentage yield of total polyphenols. Each extract was reduced in volume in a rotavapor, transferred into a plastic tube, and finally lyophilized. The hydroalcoholic extracts of pomegranate peel and juice were used.

## Microorganisms and Growth Conditions.

The antimicrobial activity of the pomegranate extracts was evaluated against the strain *Streptococcus mutans* Clarke ATCC 25175 (LGC Standards, UK) isolated from carious dentine and *Rothia dentocariosa* clinical isolate Rd1, obtained from samples of dental plaque provided from the Pediatric Dentistry Department of “Federico II” University, Naples, Italy. Permission to take dental plaque samples was acquired according to the local planning authorities. Furthermore, approval for this study was granted by the ethics committee of the “Federico II” University, Naples, Italy (Protocol number 101/14).

The identification of clinical isolates was performed, from UOC of Clinical Microbiology, AOU “Federico II” of Naples, Italy, by mass spectrometry using the Matrix Assisted Laser Desorption/Ionization (MALDI) mass spectrometer (Bruker Daltonics, MALDI Biotyper, Fremont, CA, USA), a high-throughput proteomic technique for identification of a variety of bacterial and fungal species [Neville et al., 2011; Sogawa et al., 2011], and biochemical phenotyping method in an BD Phoenix Automated Microbiology System (Becton Dickinson, BD Franklin Lakes, NJ, USA), according to the manufacturer’s instruction. Bacteria were cultured aerobically in broth and agar media at 37°C. The media used were Brain Heart Infusion (BHI) (Oxoid, S.p.a., Rodano, Milano, Italy), Columbia CAN with 5% Sheep Blood with Colistin and Nalidixic Acid (Oxoid, S.p.a., Rodano, Milano, Italy), and Mueller-Hinton (Simad s.a.s., Naples, Italy). Microbial strains were maintained at 4°C on agar media. The isolates were stored frozen at –80°C in BHI broth supplemented with 10% glycerol (v/v) (Carlo Erba, Reagents, Milan, Italy) until use

and the working cultures were activated in the respective broth at 37°C for 15–18 h.

#### In Vitro Antibacterial Activity Assays.

The susceptibility of *S. mutans* ATCC 25175 and *R. dentocariosa* Rd1 to different concentrations of *Punica granatum* L. fruit extracts was determined by dilution tube method with  $1 \times 10^5$  CFU/ml as standard inoculums. The extracts were added in the series of tubes achieving final concentrations of 0, 5, 10, 15, 20, 30, 40, 60, 100, and 140  $\mu\text{g}/\mu\text{l}$ , and tubes were incubated at 37°C for 24 h. As positive control the bacterial strains were tested with ranging concentrations of Ampicillin (Sigma-Aldrich, Milano, Italy) and with extraction buffer as negative control. After incubation, the optical density at 600 nm was determined; subsequently an aliquot of each sample was spread into BHI-agar plates in duplicate and then incubated for 24–48 h for the evaluation of viable counts. Minimum inhibitory concentration (MIC) was assigned to lowest concentration of pomegranate extract, which prevents bacterial growth. The minimum bactericidal concentration (MBC) was defined as the minimum extract concentration that killed 99% of bacteria in the initial inoculums. To verify the effect of pomegranate juice and peel hydroalcoholic extracts on the fitness of *S. mutans* ATCC 25175 and *R. dentocariosa* Rd1, assays of bacterial growth and survival were performed in presence of increasing concentrations of the extracts. To evaluate the fitness of each strain, during the observation period (96 h), serial dilutions were spread on BHI-agar and incubated at 37°C for 24–48 h to evaluate viable counts. All experiments were performed in triplicate, with three independent cultures; the results obtained were analyzed and

graphically reported by using “GraphPad Prism6” software. Results are presented as mean  $\pm$  SD. The statistical significance was determined by the two-way ANOVA test with a Bonferroni correction ( $P$  value  $\leq$  0.05).

### **3. Results**

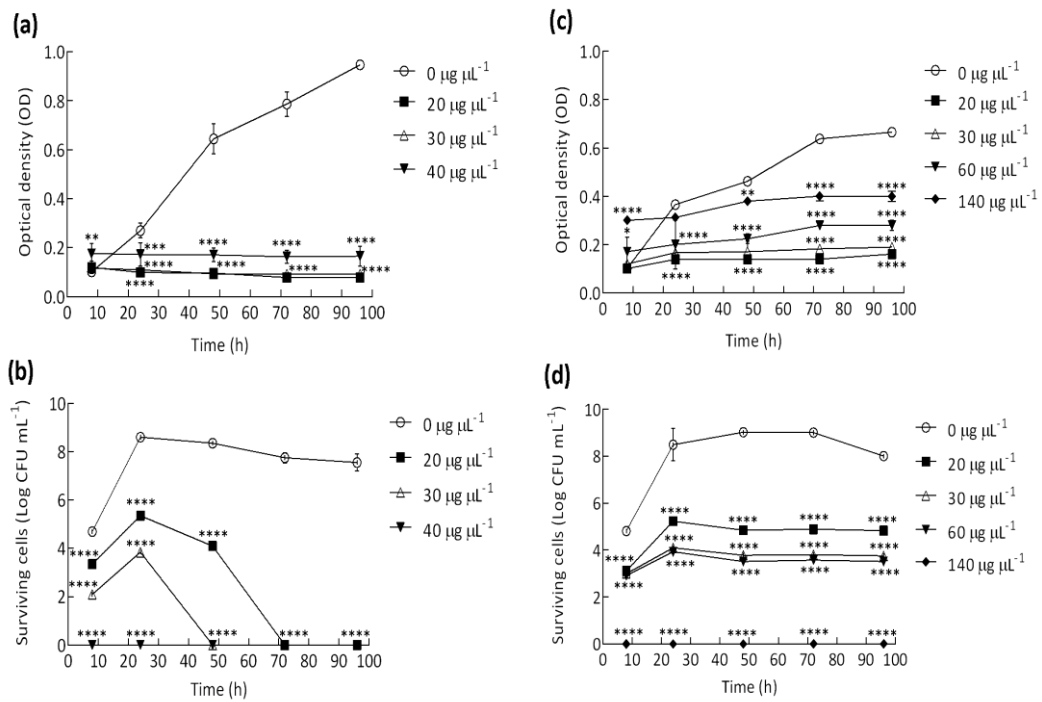
#### *3.1. In Vitro Antibacterial Activity of Pomegranate Extracts.*

The antimicrobial activity of pomegranate extracts against *S. mutans* ATCC 25175 cariogenic strain and *R. dentocariosa* Rd1 clinical isolate was evaluated by dilution tube method, according to the CLSI (Clinical and Laboratory Standards Institute) guidelines. Growth of *S. mutans* ATCC 25175 strain and *R. dentocariosa* Rd1 clinical isolate was inhibited with a concentration of pomegranate juice extract equal to 25  $\mu\text{g}/\mu\text{l}$  and 20  $\mu\text{g}/\mu\text{l}$ , respectively. Pomegranate juice extracts showed a MBC value of 40  $\mu\text{g}/\mu\text{l}$  against *S. mutans* ATCC 25175 and a MBC value of 140  $\mu\text{g}/\mu\text{l}$  against *R. dentocariosa* Rd1. The pomegranate peel extracts exhibited a MIC value of 10  $\mu\text{g}/\mu\text{l}$  and a MBC value of 15  $\mu\text{g}/\mu\text{l}$  against both microorganisms tested. Both the bacteria tested in this study are sensitive to ampicillin.

#### *Effects of Pomegranate Extracts on Bacterial Fitness.*

To verify the effect of pomegranate juice and peel hydroalcoholic extracts on the fitness of *S. mutans* ATCC 25175 cariogenic strain and *R. dentocariosa* Rd1 clinical isolate, the growth and survival were evaluated for 96 h, with increasing concentrations of hydroalcoholic extracts. The pomegranate juice extracts exhibited inhibitory effect on growth and survival of both strains (Figure 4). The growth evaluation was biased by the turbidity of the extracts, as clearly showed by growth

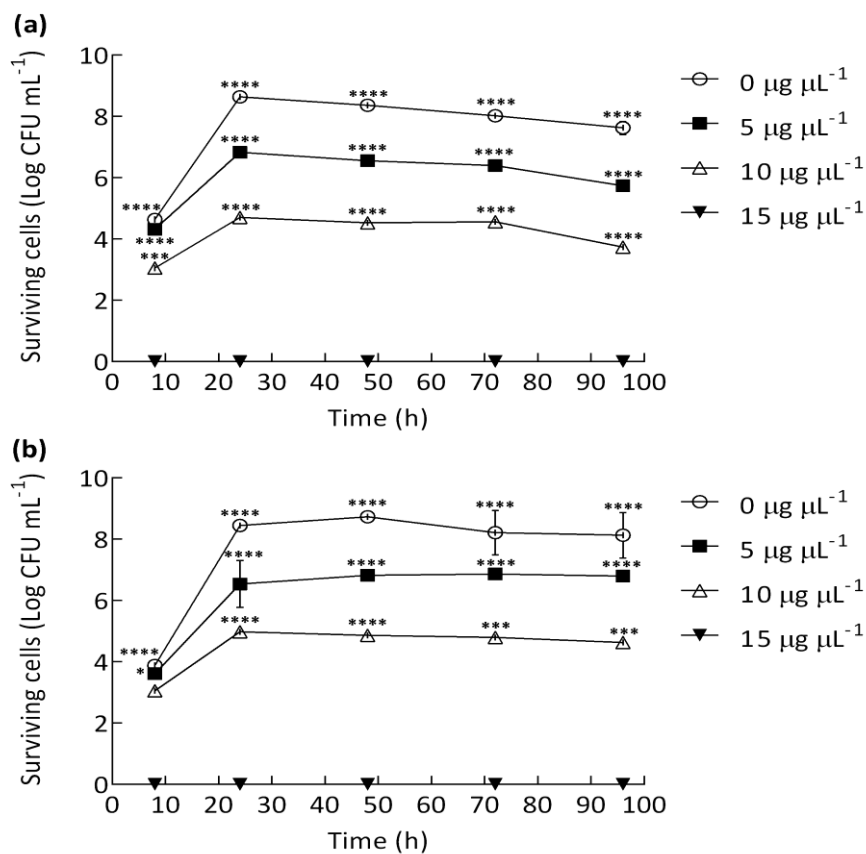
curves (Figures 4(a) and 1(c)). However, the evaluation of viable counts had highlighted a strong bactericidal activity of pomegranate juice hydroalcoholic extract with a concentration of 40  $\mu\text{g}/\mu\text{l}$  for *S. mutans* ATCC 25175 and a moderate bactericidal effect against *R. dentocariosa* Rd1 with a concentration of 140  $\mu\text{g}/\mu\text{l}$  (Figures 4(b) and 4(d)).



**Figure 4:** Effect of pomegranate juice extracts on (a) growth of *S. mutans* at different concentration (0, 20, 30, and 40  $\mu\text{g}/\mu\text{l}$ ); (b) survival of *S. mutans* at different concentration (0, 20, 30, and 40  $\mu\text{g}/\mu\text{l}$ ); (c) growth of *R. dentocariosa* at different concentration (0, 20, 30, 60, and 140  $\mu\text{g}/\mu\text{l}$ ); (d) survival of *R. dentocariosa* at different concentration (0, 20, 30, 60, and 140  $\mu\text{g}/\mu\text{l}$ ).



Interestingly, the pomegranate hydroalcoholic peel extract exhibited a strong inhibitory activity against both tested cariogenic strains (Figure 5). The hydroalcoholic peel extracts interfered with the bacterial growth, survival, and fitness in a dose dependent manner and with time-lasting effects, as previously described for other clinical isolates [Pagliarulo et al., 2016]. In addition the bactericidal activity is detectable at a very low concentration equal to 15  $\mu\text{g}/\mu\text{l}$  for both strains. The peel extracts in ethanol were cloudy so it was impossible to test it in the bacterial growth assay.



**Figure 5:** Effect of pomegranate peel extracts on survival of *S. mutans* (a) and *R. dentocariosa* (b) at different concentration (0, 5, 10, and 15  $\mu\text{g}/\mu\text{l}$ ).

## **Conclusions**

In vitro microbiological assays demonstrated that pomegranate (*Punica granatum* L.) hydro-alcoholic peel and juice extracts are able to counteract cariogenic bacteria of dental plaque. In fact, the extracts showed inhibitory effect on the growth and survival of *S. mutans* ATCC 25175 and *R. dentocariosa* Rd1 isolate, considered among the most important etiological agents of tooth decay. The strongly bactericidal power of the pomegranate fruit extracts against oral cariogenic bacteria suggests further deep investigation.

### **Development of new experimental tablets.**

As above mentioned, the drug therapy within the oral cavity is not completely effective in maintaining therapeutic concentrations at the site of action. Various disadvantages result in the short retention time of the drugs, such as the rapid loss of drug from the site of absorption by means of salivary action and mechanical stress, the inadequate distribution of drugs within the areas of oral cavity, the patient discomfort due to unpleasant taste sensations and the barrier effect of oral mucosa [Perioli et al., 2008].

A solution to these problems could be the design of mucoadhesive sustained release products capable of retaining the device in the oral cavity so it keeps the drug concentration within the therapeutic range, in order to require less frequent administrations. Therefore, mucoadhesive systems may represent valid alternatives in light of their easiness to use because they can be applied and removed directly by patients. In collaboration with the Nutrition Science Institute of Avellino, our scientific group performed new slow-release tablets, designed for being settled on the inner-mouth mucosal surface, in close contact with gingival tissues. In this way, the tablets can release their active load gradually, during the daily activities of the mouth. Tablets are designed to be applied to different regions of oral cavity, such as cheeks, lips, gums, and palate and can allow drinking, eating, and speaking without any major discomfort. The tablets main content consists of natural active anti-

carries compounds such as CPP-ACP, *stevia rebaudiana* Bertoni and pomegranate peel extract and a mixture of excipients was also used in the design .

#### **Manufacturing of medicated tablets.**

A powder or granule mixture containing all the ingredients was prepared, using a lab mixer (HulaMixer Sample Mixer); a physical blend magnesium stearate was homogeneously mixed with the blend in order to optimize the compression process. To get the final product the powder mix was compressed by a single punch machine (Matrix 2.2 A, Ataena Srl, Ancona, Italia) at room temperature. The tablets obtained were blistered and stored at temperatures below 20 ° C.

#### **4.3. In vitro cytotoxicity of slow-release tablets containing CPPs, Stevia and pomegranate extract, on human gingival fibroblasts.**

Slow-release tablets are designed for being settled on the inner-mouth mucosal surface, in close contact with gingival tissues. In this way, the tablet releases its active load gradually, during the daily activities of the mouth. As the prolonged contact of the tablets with the mucosa could possibly interfere with the normal turnover of the mucosal cells or determine detectable alterations, it was decided first to directly test in vitro the effects of our tablets laying down on a monolayer of human gingival fibroblast (HGF-1, ~80% of confluence) for a prolonged time (up to one week). In addition, the effect of the active content extracted from tablets with a solvent at increasing concentrations on the same cell strain was tested, as above.

#### **Materials and methods**

Slow-release tablets containing Pomegranate Peel extract, stevia and Cpps were kept in sealed blisters in the refrigerator at +4°C until used. One percent (1%) methylene blue solution for staining was prepared freshly by dissolving the dry powder (Sigma Aldrich) in ethanol/PBS (1:1). Human Gingival Fibroblasts (HGF-1) cell line was from ATCC (Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), containing 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, and 1% penicillin/streptomycin and maintained in 5% CO<sub>2</sub> at 37°C with

96% relative humidity (pH 7.4). Cell culture medium and serum were both purchased from Gibco (Invitrogen).

### *Treatments*

Cells - About  $3.5 \times 10^5$  cells were seeded in 60-mm tissue culture dishes. The experiments described below were initiated when the attached cells reached 80-90% of confluence.

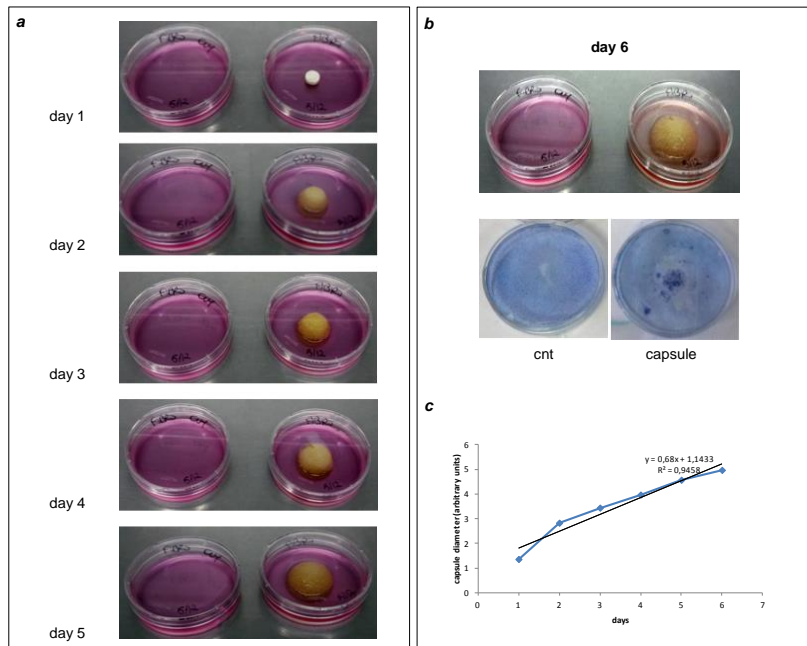
Intact tablets - Slow-release tablets were gently set down in the middle of the tissue culture dishes where remained immersed for 6 days. Every day, after cells washing with warm PBS (twice), the culture medium was carefully replaced. The sixth day, the tablets leftovers were cautiously removed and cells were stained for 2 hours with methylene blue in ethanol/PBS.

Dissolved tablets - In this case, fixed volumes (2 mL) of phosphate-saline buffer isotonic solvent (PBS) were slowly poured on a tablet with stirring. As the tablet matrices were insoluble, this material was separated by the clear supernatant by extended centrifugation and filtration. This clear solution (stock solution) was used immediately after its preparation. Aliquots of this solution (1 tablet/2 mL PBS) were added to cells (from 0 to 200  $\mu$ L). After 24 hours, cells were washed twice with warm PBS and finally stained for 2 hours with methylene blue in ethanol/PBS.

The medium around each tablet was gently aspirated and prudently replaced. This operation was repeated every day for one week. Finally, after the removal of tablets from the plates, the cells were stained with methylene blue. This procedure was to evaluate potential effects of tablets on cell survival and growth

## **Results**

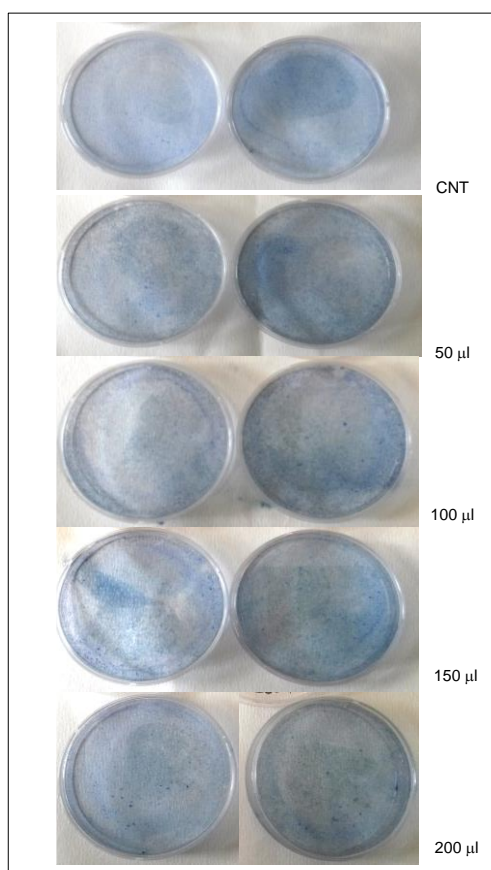
Slow-release tablet itself and its content do not interfere with physiologic cell growth in vitro. During the in vitro experiment, using the intact tablet, an increase of the tablet size was observed; this increase, due to its extended soaking, is certainly accompanied by a continuous release of its active content into the medium. Except for a factitious spot in the middle of the plate (where the tablet had been setting for a week), it appears that the cells continue to grow normally (Figure 6). Moreover, as result of the second test, performed using dissolved tablets, staining with methylene blue solution demonstrates that cell physiological growth was not affected in all tested conditions (Figure 7).



**Figure 6.** Monolayer of HGF-1 during and after treatment with slow-release tablet.

*Panel a: On the left: control cells; on the right: cells growing in the presence of the tablet. While releasing progressively its active content, the tablet appears to increase its size, as it got soaked. Pictures were taken daily. Panel b: The last day of treatment before (upper picture) and after tablet removal and cells staining (lower picture). Panel c: Approximate tablet diameter with soaking time.*





**Figure 7.** *Methylene Blue staining of HGF-1 monolayer after 24 hours of incubation with specified volumes of tablet extract. No significant alterations of cells growth are evident as compared to control (two similar, separate experiments were carried out simultaneously).*

### **Conclusions**

In this trial, toxicity studies were conducted to determine the possible toxic effects of Slow-release tablets containing Pomegranate Peel extract, stevia and Cpp on

human gingival fibroblast (HGF-1, ~80% of confluence) for a prolonged time (up to one week). In addition we tested the effect of the active content extracted from tablets with a solvent at increasing concentrations to provide credible information for the future application of the tablets. Our findings revealed that the Slow-release tablets experimented and its content do not interfere with physiologic cell growth in vitro. These results provide important information for the further use of slow release-tablets in the prevention of dental caries .

#### **4.3. In vivo evaluation of controlled release mucoadhesive tablets containing containing stevia rebaudiana Bertoni, CPPs and pomegranate peel extract.**

This trial describes the preliminary clinical evaluation of mucoadhesive slow release formulations containing *stevia rebaudiana* Bertoni, CPPs and pomegranate peel extract. Each formulation was characterized in terms of adhesiveness, tolerability, and patient's compliance.

#### **Materials and Methods**

The study was conducted in accordance with the Declaration of Helsinki (World Medical Association, 2001). Ethical approval was granted by the "Federico II" University of Naples, Italy ( Protocol number: 101/14).

The trial was carried out in May 2017 among a sample of 40, 12 to 14 years-old, children. Only Patients in good general health with caries-free, completely erupted first and second permanent molars were included in the study. Parents were informed about the study by a verbal and written explanation of the protocol and the aim and then they were invited to give their written consent to the study.

The study lasted seven days; volunteers for each day were instructed to press against gums, above upper second molar, the slow release tablets without moistening them before application. Residence tablet time, possible irritation, loss of fragments, bad taste, dry mouth or excessive salivation have been evaluated using self-report questionnaires.

The persistence of the adherence of the tablets was checked at the application (t0), after 6 hours (t1) and 12 hours (t2) by two examiners.

Two standardized dentists performed a comprehensive dental examination for all 40 patients under artificial light (portable 60w lamps) using a plane buccal mirror and a dental explorer.

To evaluate the parameters of adhesiveness and irritation, the examiners were calibrated at the Department of Neuroscience, Reproductive and Oral Sciences, Section of Paediatric Dentistry, University of Naples, Federico II, Naples, Italy. A subsample of fifty subjects was observed independently by the two examiners as a tool for standardizing examination procedures: agreement was assessed by means of kappa statistic ( $k = 0.935$ ) (CI 95% 0.777-0.975) for DMFT score.

At the end of the treatments the data were processed with the Statistical Package for Social Sciences (version 10.0, SPSS Inc., Chicago, Illinois, USA). A regression binary logistic analysis was made. Statistical significance level was established at  $p < 0.05$ .

## **Results**

Forty-one patients were consecutively enrolled in the study. Among them, four were dropped from the study because of discontinuation for the final assessment. Thirty-seven patients completed the study. Of the 37 patients, 16 (43.2%) were females and 21 (56.8%) were males with a mean age of  $15.05 \pm 14.7$  years (range: 14-16 years).

T0

At the first application tablets adhered in 93.8% of the cases, while 6.26% patients were not able to attach the tablets against the gum (**Table 2**). In 2.9% of the cases the first application of the tablet resulted uncomfortable for the patients, while in 97.1% patients did not feel any disturb. In 2.1% of the cases patients referred sensation of burning. In 81.5% of the cases the tablets did not have any taste.

Clinical examination.

Examiners referred that at T0 93.8% of the tablets were steadily adherent to the gum. At the first application no sign of inflammation of soft tissues was reported.

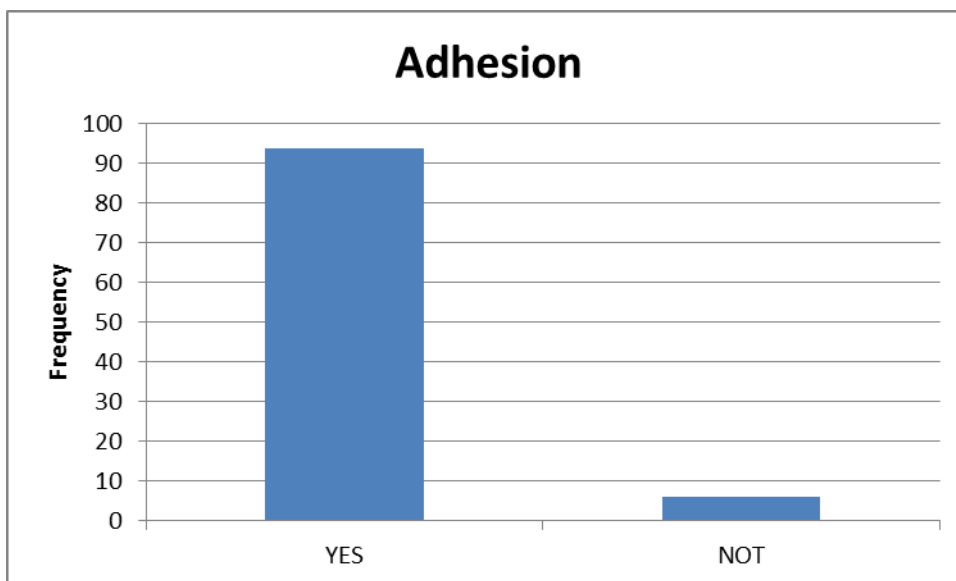


Table 2: adhesion at T0.

T1

6 hours after the application, tablets resulted lost in just 2.9% of the cases, while stayed attached in the remaining 97.1%. At T1 in 30.1% of the cases the tablet resulted uncomfortable for the patients, in 76.1 % of the cases they reported a slight discomfort, in 15.5% the discomfort was moderate and just in 8.4% of the cases patients referred a high annoyance. In 8.1% of the cases patients referred sensation of burning and only in 7.2 % of the cases sensation of dryness .In 97.5% of the cases the tablets did not have any taste.

Clinical examination

At T1 45.8% of the tablets were still intact, on the contrary the remaining 54.2% was partially solved. Patients tissue resulted slightly inflamed only in 28.8% of the cases, while in 168 cases (64.9%) no sign of inflammation or irritation was found. In 89.9% of the cases tablets were steadily adherent to the gum yet.

T2 .

12 hours after the application, tablets resulted lost in just 5,5% of the cases, while stayed totally attached in 94,5%. At T2 in 36.6% of the cases the tablet resulted uncomfortable for the patients, in 100% of the cases they reported a slight

discomfort, in 0% the discomfort was moderate or high .No one of the patients reported sensation of dryness or burning .In 80.8% of the cases the tablets did not have any taste.

#### Clinical examination

At T2 86.1% of the tablets were partially solved, on the contrary the remaining 13.9% was yet intact. Patients tissue resulted slightly inflamed only in 26.3% of the cases, while in 168 cases (64.9%) no sign of inflammation or irritation was found. In 89.9% of the cases tablets were steadily adherents to the gum yet.

#### Statistical analysis.

The differences in adhesiveness between T0 and T1, T0 and T2, T1 and T2 were not statistically significant, respectively (Fig.8).

	Sig.	OR	95,0%C.I.	
			Lower	Upper
T 0 - T1	.08	2.22	0.89	5.49
T1 - T2	.15	.50	.19	11.29
T0 - T2	.75	1.12	.53	2.40

Table3: Statistical analysis within the test gr statistical analysis concerning the degree of adhesion.

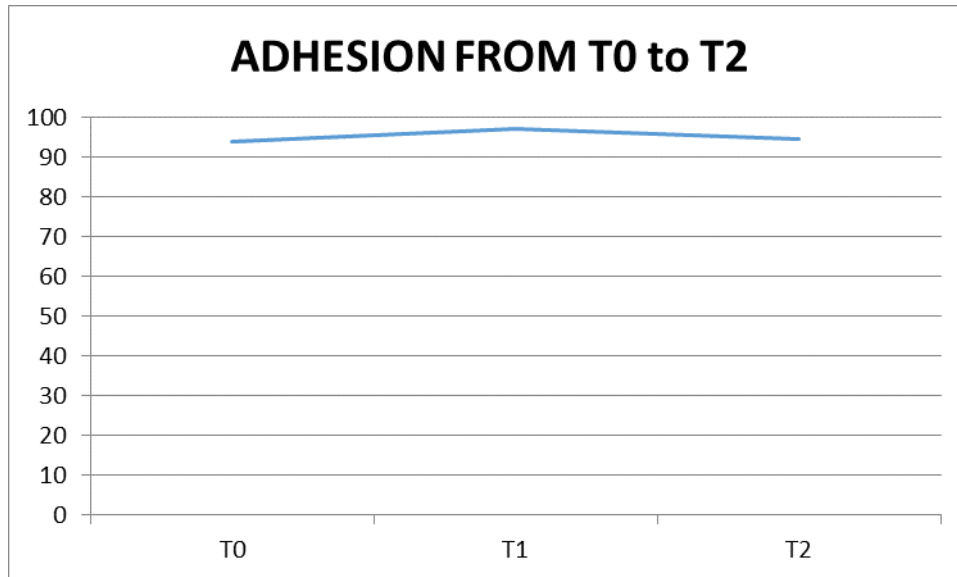


Figure 8.

### Conclusion

The tablets investigated in this study were acceptable to many patients and since there is a potential role for aslow-release tablets in preventing oral disease in this group its clinical effectiveness needs to be evaluated.



## 5. GENERAL CONCLUSIONS.

Dental caries is still the main health problem worldwide both in more developed and in lower income countries [Bourgeois et al., 2014] and bacteria have been suggested to cause the strongest effect on the prevalence or incidence of dental caries. The final score of this research was the creation of a new preventive methodology against dental caries by using new mucoadhesive formulations that could offer many advantages in comparison to traditional treatments and could be proposed as a new therapeutic tool against dental disease. The studies carried out in recent decades in our departments allow us to define that CPP-ACP could contrast dental caries and erosion; furthermore results of experimental protocols have supported the antibacterial role of polyphenols, such as pomegranate extract, and their potential use in the control of bacteria responsible of caries. In vitro microbiological assays demonstrated, indeed, that pomegranate (*Punica granatum* L.) hydro-alcoholic peel and juice extracts are able to counteract cariogenic bacteria of dental plaque. The in vivo trial suggests that the usage of mucoadhesive buccal drug delivery system could be a novel route of anticaries bio-active molecules administration by offering prolonged contact at the site of administration and satisfying the compliance of the patients. More studies, particularly in vivo and in situ, are necessary to clarify the synergic effects of the different active principles insert in the tables and their clinical applications in contrasting dental caries.

## 6. Bibliography.

1. Azarpazhooh, A.; Limeback, H. Clinical efficacy of casein derivatives: a systematic review of the literature. *J Am Dent Assoc* 2008;139(7):915-24.
2. Beck, A.; Cabaret, J.; Halberg, N.; Ivanova-Peneva, S.; Jespersen, L.M. Joint FAO/WHO Expert Committee on Food Additives 2008. Commission Regulation (EU) No 1131/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council with regard to steviol glycosides. *Off. J. Eur. Union* 2011, 54, 207
3. Boonkaewwan, C.; Toskulkao, C.; Vongsakul, M.J. Antiinflammatory and immunomodulatory activities of stevioside and its metabolite steviol on THP-1 cells. *J. Agric. Food Chem.* 2006, 54, 785–789.
4. Bouhallab, S.; Bouglé, D. Biopeptides of milk: caseinophosphopeptides and mineral bioavailability. *Reprod Nutr Dev* 2004;44(5):493-8.

5. Bourgeois, D.M.; Llodra, J.C. Global burden of dental condition among children in nine countries participating in an international oral health promotion programme, 2012–2013. *Int. Dent. J.* 2014, 64, 27–34.
6. Chatsudthipong, V.; Muanprasat, C. Stevioside and related compounds: Therapeutic benefits beyond sweetness. *Pharmacol. Ther.* 2009, 121, 41–54. Kinghorn, A.D. *Stevia: The Genus Stevia*; CRC Press: Boca Raton, FL, USA, 2003; p. 224.
7. Chaturvedula, V.S.P.; Prakash, I. A new diterpene glycoside from *Stevia rebaudiana*. *Molecules* 2011, 16, 2937–2943.
8. Committee on Herbal Medicinal Products (HMPC). Community herbal monograph on *Plantago lanceolata* L., folium EMA/HMPC/437858/2010, 22 November 2011.
9. Cross, K.J.; Huq, N.L.; Stanton, D.P.; Reynolds, E.C. NMR studies of a novel calcium, phosphate and fluoride delivery vehicle- $\alpha$ (S1)-casein(59-79) by stabilized amorphous calcium fluoride phosphate nanocomplexes. *Biomaterials* 2004;25(20):5061-9.
10. Cross, K.J.; Huq, NL, Palamara J.E., Perich, J.W.; Reynolds, E.C. Physicochemical characterization of casein phosphopeptide-amorphous calcium phosphate nanocomplexes. *J Biol Chem* 2005;280:15362-15369.
11. Das, S.; Das, A.K.; Murphy, R.A.; Punwani, I.C.; Nasution, M.P.; Kinghorn, A.D. Evaluation of the cariogenic potential of the intense natural sweeteners stevioside and rebaudioside A. *Caries Res.* 1992, 26, 363–366.
12. De Slavutzky, S.M.B. *Stevia* and sucrose effect on plaque formation. *J. Verbr. Lebensm.* 2010, 5, 213–216.
13. Divyashree P., Kunnaiah R. *Punica granatum*: A review on its potential role in treating periodontal disease. *Journal of Indian Society of Periodontology.* 2014;18(4):428–432.
14. E. A. Palombo, “Traditional medicinal plant extracts and natural products with activity against oral bacteria: Potential application in the prevention and treatment of oral diseases,” *Evidence-Based Complementary and Alternative Medicine* 2011.
15. Fengyang, L.; Yunhe, F.; Bo, L.; Zhicheng, L.; Depeng, L.; Dejie, L.; Wen, Z.; Yongguo, C.; Naisheng, Z.; Xichen, Z.; Zhengtao, Y. Stevioside suppressed inflammatory cytokine secretion by downregulation of NF- $\kappa$ B and MAPK signalling pathways in LPS-stimulated RAW264.7 cells. *Inflammation* 2012, 35, 1669–1675.
16. Ferrazzano, G. F.; Cantile, T.; Ingenito, A.; Chianese, L.; Quarto, M. New strategies in dental caries prevention: experimental study on casein phosphopeptides. *Eur J Paediatr Dent* 2007;8(4):183-7.

17. Ferrazzano, G.F.; Cantile, T.; Quarto, M.; Ingenito, A.; Chianese, L.; Addeo, F. Protective effect of yogurt extract on dental enamel demineralization in vitro. *Aust Dent J* 2008;53(4):314-9.
18. Ferrazzano, G.F.; Cantile, T.; Roberto, L.; Ingenito, A.; Catania, M.R.; Roscetto, E.; Palumbo, G.; Zarrelli, A.; Pollio, A. Determination of the in vitro and in vivo antimicrobial activity on salivary Streptococci and Lactobacilli and chemical characterisation of the phenolic content of a *Plantago lanceolata* infusion. *Biomed. Res. Int.* 2015, 2015.
19. Ferrazzano, G.F.; Roberto, L.; Catania, M.R.; Chiaviello, A. "Screening and scoring of antimicrobial and biological activities of italian vulnerary plants against major oral pathogenic bacteria," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 316280, 10 pages, 2013.
20. Ferrazzano, G.F.; Roberto, L.; Catania, M.R.; Chiaviello, A.; De Natale, A.; Roscetto, E.; Pinto, G.; Pollio, A.; Ingenito, A.; Palumbo G. Screening and Scoring of Antimicrobial and Biological Activities of Italian Vulnerary Plants against Major Oral Pathogenic Bacteria. *Evid Based Complement Alternat Med* 2013;2013:316280.
21. Ferrazzano, G.F.; Sangianantoni, G.; Cantile, T.; Ingenito, A. Relationship Between Social and Behavioural Factors and Caries Experience in Schoolchildren in Italy. *Oral Health Prev Dent.* 2016;14(1):55-61.
22. Forssten, S.D.; Björklund, M.; Ouwehand, A.C. Streptococcus mutans, Caries and Simulation Models. *Nutrients.* 2010 Mar; 2(3): 290–298.
23. Gamboa, F.; Chaves, M. Antimicrobial potential of extracts from *Stevia rebaudiana* leaves against bacteria of importance in dental caries. *Acta Odontol. Latinoam.* 2012, 25, 171–175.
24. Giacaman, R.A.; Campos, P.; Muñoz-Sandoval, C.; Castro, R.J. Cariogenic potential of commercial sweeteners in an experimental biofilm caries model on enamel. *Arch. Oral. Biol.* 2013, 58, 1116–1122.
25. Giongo, F.C.; Mua, B.; Parolo, C.C.; Carlén, A.; Maltz, M. Effects of lactose-containing stevioside sweeteners on dental biofilm acidogenicity. *Braz. Oral Res.* 2014, 28, S1806–S8324.
26. Giriraju; Yunus, Y. "Assessment of antimicrobial potential of 10% ginger extract against Streptococcus mutans, Candida albicans, and Enterococcus faecalis: An invitro study," *Ind Jour of Dent Research* 2103 vol. 24, pp. 397–400.
27. Guggenheim, B.; Neeser, J.R.; Golliard, M.; Schüpbach, P. Salivary pellicle modified by milk components mediated caries prevention. 1995.41st ORCA Congress Abstracts, pag 182. 75
28. Iijima, Y.; Cai, F.; Shen, P.; Walker, G.; Reynolds, C.; Reynolds EC. Acid resistance of enamel subsurface lesions remineralized by a sugar-free

- chewing gum containing casein phosphopeptide-amorphous calcium phosphate. *Caries Res* 2004; 38(6):551-6.
29. Javad, S.; Naz, S.; Ilyas, S.; Tariq, A.; Aslam, F. Optimization of the microwave assisted extraction and its comparison with different conventional extraction methods for isolation of stevioside from *Stevia rebaudiana*. *Asian J. Chem.* 2014, 26, 8043–8048.
  30. Jayaraman, S.; Manoharan, M.S.; Illanchezian, S. In-vitro Antimicrobial and antitumor activities of *Stevia rebaudiana* (Asteraceae) leaf extracts. *Trop. J. Pharm. Res.* 2008, 7, 1143–1149.
  31. Jeppesen, P.B.; Gregersen, S.; Rolfsen, S.E.; Jepsen, M.; Colombo, M.; Agger, A.; Xiao, J.; Kruhoffer, M.; Orntoff, T.; Hermansen, K. Antihyperglycemic and blood pressure-reducing effects of stevioside in the diabetic Goto-Kakizaki rat. *Metabolism* 2003, 52, 372–378.
  32. Kelmer, B.A.; Alvarez, M.; Brancht, A. Effects of *Stevia rebaudiana* natural products on rat liver mitochondria. *Biochem. Pharmacol.* 1985, 34, 873–882.
  33. Kolb, N.; Herrera, J.L.; Ferreyra, D.J.; Uliana, R.F. Analysis of sweet diterpene glycosides from *Stevia rebaudiana*: Improved HPLC method. *J. Agr. Food Chem.* 2001, 49, 4538–4541.
  34. Krishnan, R.; Maru, G.B. Inhibitory effect(s) of polymeric black tea polyphenol fractions on the formation of [(3)H]-B(a)P-derived DNA adducts. *J Agric Food Chem* 2004;52(13):4261-9.
  35. Kumar, V.L.; Itthagaran A, King NM. The effect of casein phosphopeptide-amorphous calcium phosphate on remineralization of artificial caries-like lesions: an in vitro study. *Aust Dent J* 2008; 53(1):34-40. 77
  36. Lemus-Mondaca, R.; Vega-Gálvez, A.; Zura-Bravo, L.; Ah-Hen, K. *Stevia rebaudiana* Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. *Food Chem.* 2012, 132, 1121–1132.
  37. Manton, D.J.; Walker, G.D.; Cai, F.; Cochrane, N. J.; Shen, P.; Reynolds, E. C. Remineralization of enamel subsurface lesions in situ by the use of three commercially available sugar-free gums. *Int J Paediatr Dent* 2008;18(4):284-90.
  38. Martins, M.T.; Sardenberg, F.; Bendo, C.B; Abreu,M.H.; Vale M.P., Paiva,S.; Pordeus, I.A. Dental caries remains as the main oral condition with the greatest impact on children’s quality of life. *PLoS One.* 2017; 12(10): e0185365.
  39. Matsukubo, T.; Takazoe, I. Sucrose substitutes and their role in caries prevention. *Int. Dent. J.* 2006, 56, 119–130.
  40. Mayoaran, B.; Robin, S.; John, R.T. Dental caries is a preventable infectious disease. *Aust. Dent. J.* 2000; 45 : 235–245.

41. Melis, M.S. Chronic administration of aqueous extract of *Stevia rebaudiana* in rats: Renal effects. *J. Ethnopharmacol.* 1995, 47, 129–134.
42. Millo, G.; Juntavee, A.; Ratanathongkam, A.; Nualkaew, N.; Peerapattana, J.; Chatchiwattana, S.; Antibacterial Inhibitory Effects of *Punica Granatum* Gel on Cariogenic Bacteria: An in vitro Study. *Int J Clin Pediatr Dent.* 2017 Apr-Jun; 10(2): 152–157.
43. Mizrahi, B.; Domb, A.J. Mucoadhesive polymers for delivery of drugs to the oral cavity. *Recent Pat Drug Deliv Formul.* 2008;2(2):108-19.
44. Mizushima, Y.; Akihisa, T.; Ukiya, M.; Hamasaki, Y.; Nakai, C.M.; Kuriyama, I.; Tkeuchi, T.; Sugawara, F.; Yoshida, H. Structural analysis of isosteviol and
45. related compounds as DNA polymerase and DNA topoisomerase inhibitors. *Life Sci.* 2005, 77, 2127–2140.
46. Mohire, N.C.; Yadav, A.V. Chitosan-based polyherbal toothpaste: As novel oral hygiene product. *Indian J. Dent. Res.* 2010, 21, 380–384. Morgan, M.V.; Adams, G.G.; Bailey, D.L, Tsao, C.E.; Fischman, S.L.; Reynolds, E.C. The anticariogenic effect of sugar-free gum containing CPP-ACP nanocomplexes on approximal caries determined using digital bitewing radiography. *Caries Res* 2008;42(3):171-84.
47. Morlock, G.E.; Meyer, S.; Zimmermann, B.F.; Roussel, J.M. High-performance thin-layer chromatography analysis of steviol glycosides in *Stevia* formulations and sugar-free food products, and benchmarking with (ultra) high-performance liquid chromatography. *J. Chromatogr.* 2014, 1350, 102–111.
48. Neville, M.C.; Keller, R.P; Casey, C.; Allen, J.C. Calcium partitioning in human and bovine milk. *J Dairy Sci* 1994;77(7):1964-75.
49. Nishikawara, F.; Yoshiaki, N.; Susumu, I; Senda, A.; Hanada, N. Evaluation of Cariogenic Bacteria. *Eur J Dent.* 2007 Jan; 1(1): 31–39.
50. Ohta, M.; Sasa, S.; Inoue, A.; Tamai, T.; Fujita, I.; Morita, K.; Matsuura, F. Characterization of novel steviol glycosides from leaves of *Stevia rebaudiana*. *J. Appl. Glycosci.* 2010, 57, 199–209.
51. Ohta, M.; Sasa, S.; Inoue, A.; Tamai, T.; Fujita, I.; Morita, K.; Matsuura, F. Characterization of novel steviol glycosides from leaves of *Stevia rebaudiana*. *J. Appl. Glycosci.* 2010, 57, 199–209. [Google Scholar] [CrossRef]
52. Pai, D.; Bhat, S.S; Taranath, A; Sargod, S; Pai, V.M. Use of laser fluorescence and scanning electron microscope to evaluate remineralization of incipient enamel lesions remineralized by topical application of casein phospho peptide amorphous calcium phosphate (CPP-ACP) containing cream. *J Clin Pediatr Dent* 2008;32(3):201-6.
53. Pol, J.; Varadova, O.E.; Karasek, P.; Roth, M.; Benesova, K.; Kotlarikova, P.; Caslavsky, J. Comparison of two different solvents employed for pressurized

- fluid extraction of stevioside from *Stevia rebaudiana*: Methanol versus water. *Anal. Bioanal. Chem.* 2007, 388, 1847–1857. *AAPS PharmSciTech.* 2008 Mar; 9(1): 274–281.
54. Perioli, L.; Ambrogi, V.; Giovagnoli, S.; Blasi, P.; Mancini, A.; Maurizio Ricci, M. and Rossi, C. Influence of Compression Force on The Behavior of Mucoadhesive Buccal Tablets. *AAPS PharmSciTech.* 2008 Mar; 9(1): 274–281.
  55. Rahamatullah, S.; Thakur, R.R.S.; Martin, J.G. A David Woolfson, A.D.; Ryan, F. Donnelly. Mucoadhesive drug delivery systems *J Pharm Bioallied Sci.* 2011 Jan-Mar; 3(1): 89–100.
  56. Rajini Kanth, M.; Ravi Prakash, A.; Sreenath, G.; Vikram Simha Reddy, V.; S Huldah, S. Efficacy of Specific Plant Products on Microorganisms Causing Dental Caries. *J Clin Diagn Res.* 2016 Dec; 10(12): ZM01–ZM03.
  57. Ramalingam, L., Messer, L.B.; Reynolds, E.C. Adding casein phosphopeptide-amorphous calcium phosphate (cpp-acp) to sports drinks to eliminate in vitro erosion. *Pediatr Dent* 2005; 27(1):61-7. 81
  58. Ramesh, K.; Singh, V.; Megeji, N.W. Cultivation of *Stevia* [*Stevia rebaudiana* (Bert.) Bertoni]: A comprehensive review. *Adv. Agron.* 2006, 89, 137–177.
  59. Rao, A.B.; Reddy, G.R.; Ernala, P.; Sridhar, S.; Ravikumar, Y.V.L. An improvised process of isolation, purification of steviosides from *Stevia rebaudiana* Bertoni leaves and its biological activity. *Int. J. Food Sci. Technol.* 2012, 47, 2554–2560.
  60. Reynolds, E.C.; Cai, F.; Shen, P.; Walker, G.D. Retention in plaque and remineralization of enamel lesions by various forms of calcium in a mouthrinse or sugar-free chewing gum. *J Dent Res* 2003; 82(3):206-11.
  61. Sadat Sajadi, F.; Moradi, M.; A. Pardakhty, A.; Yazdizadeh, R.; Madani, F. “Effect of fluoride, chlorhexidine and fluoride-chlorhexidine mouthwashes on Salivary *Streptococcus mutans* count and the prevalence of oral side effects,” *Journal of Dental Research, Dental Clinics, Dental Prospects* 2015; vol. 9, no. 1, pp. 49–52.
  62. Sehar, I.; Kaul, A.; Bani, S.; Pal, H.C.; Saxena, A.K. Immune up regulatory response of a non-caloric natural sweetener, stevioside. *Chem. Biol. Interact.* 2008, 173, 115–121.
  63. Shen, P; Cai, F.; Nowicki, A.; Vincent, J.; Reynolds E.C. Remineralization of enamel subsurface lesions by sugar-free chewing gum containing casein phosphopeptide-amorphous calcium phosphate. *J Dent Res* 2001; 80:2066-2070.
  64. Shiozaki, K.; Fujii, A.; Nakano, T.; Yamaguchi, T.; Dato, M. Inhibitory effects of hot water extract of the *Stevia* stem on the contractile response of the

- smooth muscle of the guinea pig ileum. *Biosci. Biotechnol. Biochem.* 2006, 70, 489–494.
65. Singh, S.D.; Rao, G.P. Stevia: The herbal sugar of 21st Century. *Sugar Tech* 2005, 7, 17–24.
  66. Soejarto, D.; Compadre, C.M.; Kinghorn, A.D. *Ethnobotanical Notes on Stevia*; Botanical Museum Leaflets Harvard University: Cambridge, MA, USA, 1983; pp. 1–25. [
  67. Soejarto, D.D. Botany of Stevia and Stevia rebaudiana. In *Stevia. The genus Stevia*, 1st ed.; Kinghorn, A.D., Ed.; Taylor & Francis Inc.: New York, NY, USA, 2002; pp. 18–39.
  68. Soejarto, D.D. Ethnobotany of Stevia and Stevia rebaudiana. In *Stevia. The Genus Stevia*; Kinghorn, A.D., Ed.; CRC Press: London, UK; New York, NY, USA, 2001; pp. 40–67.
  69. Soejarto, D.D.; Compadre, C.M.; Medon, P.J.; Kamath, S.K.; Kinghorn, A.D. Potential sweetening agents of plant origin. II. Field search for sweet-tasting
  70. Stevia species. *Econ. Bot.* 1983, 37, 71–79.
  71. Sudjalim TR, Woods MG, Manton DJ, Reynolds EC. Prevention of demineralization around orthodontic brackets in vitro. *Am J Orthod Dentofacial Orthop* 2007;131(6):705.e1-9.
  72. Takahashi, K.; Matsuda, N.; Ohashi, K.; Taniguchi, K.; Nakagomi, O.; Abe, Y.; Mori, S.; Okutani, N.K.; Shigeta, S. Analysis of anti-rotavirus activity of extract from Stevia rebaudiana. *Antivir. Res.* 2001, 49, 15–24.
  73. Tandel, K.R. Sugar substitutes: Health controversy over perceived benefits. *J. Pharmacol. Pharmacother.* 2011, 2, 236–243.
  74. Thomas, A.; Thakur,S.; Sanjana Mhambrey, S. Comparison of the antimicrobial efficacy of chlorhexidine, sodium fluoride, fluoride with essential oils, alum, green tea, and garlic with lime mouth rinses on cariogenic microbes. *J Int Soc Prev Community Dent.* 2015 Jul-Aug; 5(4): 302–308.
  75. Walker ,G.; Cai, F.; Shen, P.; Reynolds ,C.; Ward, B.; Fone, C.; Hond,S.; Koganej, M; Oda, M.; Reynolds E. Increased remineralization of tooth enamel by milk containing added casein phosphopeptide-amorphous calcium phosphate. *J Dairy Res* 2006;73:74-78.
  76. Yulia Anopa, Alex D. McMahon, David I. Conway, Graham E. Ball, Emma McIntosh, Lorna M. D. Macpherson. Improving Child Oral Health: Cost Analysis of a National Nursery Toothbrushing Programme. *PLoS One.* 2015; 10(8): e0136211.
  77. Zanela, N.L.; Bijella, M.F.; Rosa, O.P. The influence of mouthrinses with antimicrobial solutions on the inhibition of dental plaque and on the levels



- of mutans streptococci in children. *Pesqui. Odontol. Bras.* 2002, 16, 101–106.
78. Zhang, G.; Miura, Y.; Yagasaki, K. Effects of dietary powdered green tea and theanine on tumor growth and endogenous hyperlipidemia in hepatoma-bearing rats. *Biosci Biotechnol Biochem* 2002;66(44):711-6.