Università degli Studi di Napoli Federico II

Scuola di dottorato in medicina clinica e sperimentale

DIPARTIMENTO DI NEUROSCIENZE E SCIENZE RIPRODUTTIVE ED ODONTOSTOMATOLOGICHE

DOTTORATO DI RICERCA IN:
SCIENZE ODONTOSTOMATOLOGICHE
XXVI ciclo

Tesi

Anti-caries efficacy of biofunctional molecules of natural origin: in vitro and in vivo experimental study

Coordinatore: Prof.Sandro Rengo **Candidato: Dott.ssa LiaRoberto**

Tutor: Prof. Aniello Ingenito

Anni accademici 2011-2014

INDEX

1) INTRODUCTION

Oral diseases, especially dental caries, are the most common of the chronic diseases. They are important public health problems in industrialized countries and they are emerging public health problems in developing countries because of their prevalence, the cost of treatment and their impact on individuals and the society [Hamissi et al., 2008].

Oral diseases affect various aspects of children's quality of life, causing considerable pain, acute and chronic infections, eating and sleep difficulty, reduced body weight and loss of school days [Acs et al., 1992].

In addition, oral diseases affect not only physically but also psychologically, resulting in difficulty to socialize.

The prevalence of dental caries has declined in western countries in last decades [Campus et al., 2007]. Epidemiological studies have shown, particularly, that caries severity drastically decreased in the last thirty years; the values of the Italian DMFT (decayed, missing, filled teeth), for example, have decreased by almost 90% in 12 year-old children since the late 1970s: from 6.9 [Vogel et al., 1979] to 1.09 [Campus et al., 2006].

Significantly gains in oral health have been observed over the last few years in countries like Germany, England, USA, Scandinavia, Scotland, Norway and Australia [Cypriano et al., 2008].

Among Norvegian adolescents, between 1985 and 2000, the DMFT declined by 49%; 10.2 +/- 0.75 to 5.2 +/- 0.78 [Birkeland et al., 2002].

Oral health in Germany improved considerably between the years 1994 and 2000. The mean DMFT values for Germany decreased from 2.44 in 1994-1995 to 1.81 in 1997 and 1.24 in 2000.

Nevertheless, the results in different federal states showed a wide variation in caries prevalence [Pieper and Schulte, 2004].

A substantial decline in the prevalence of dental caries may be attributed to preventive systems,such as tooth-brushing, dietary control, topical and systemic use of fluorides and fissure sealants [Birkeland et al., 2002; Tinanoff et al. ,2002].

The most recent epidemiological data suggest that this decline has remained constant in the last years [Marthaler, 2004], and in some areas, children of low socioeconomic status and immigrants from outside Western Europe, still, generally have higher caries disease levels [Tinanoff et al., 2002].

The unequal distribution of caries needs the identification of individuals with the highest caries risk from tender age to adopt new preventive strategies suitable for these groups [Hamissi et al., 2008]. For these reasons, caries epidemiology will remain an indispensable part of dental public health [Birkeland et al., 2002; Ferrazzano et al.,2006; Marthaler, 2004].

A recent document has set out new oral health objectives for the year 2020. The new goals are: to increase the proportion of caries-free 6-year-olds, to reduce the DMFT particularly the D component (decayed) at age 12 with special attention to high-risk group within the population and to reduce the number of teeth extraction due to dental caries at age 18 [Hobdell et al., 2003].

It's well known that dental caries has a multi-factorial etiology [Kutsch and Young, 2011].

It is caused by three main factors: carbohydrate, bacteria and susceptible teeth (the host) [Lenander-Lumikari and Loimaranta, 2000].

Among the three above mentioned factors, bacteria are considered to play a leading role in the development and progression of caries [Marsh; 1994].

Streptococci mutans and *Lactobacilli casei* are known as cariogenic oral bacteria [Loesche, 1986; Seminario et al., 2005].

S. mutans synthesize adherent, water-insoluble glucans from sucrose by the action of glycosyltransferases (GTFs). The synthesis of glucans favours the firm adherence of the cariogenic

bacteria to tooth surface and contributes to the composition of the dental plaque polysaccharide matrix [Tanzer et al., 1974 and Koo et al., 2002].

The virulence key factor is the ability of bacterium to produce organic acids from carbohydrate (anaerobic glycolysis) [Loesche, 1986]. The accumulation of acids in dental plaque reduces the pH value of the bio-film to less than 5.5 and causes decalcification of tooth enamel and consequentelly dental decay [Freedman and Tanzer, 1974].

L. casei are acidogenic in presence of carbohydrates; they are acid tolerant [Crossner et al., 1989] and they are often isolated from established carious lesions [Loesche and Syed,1973; Staat et al., 1975].

Since these bacteria are the main factors in dental caries development, it is necessary to use antibacterial substances capable of reducing their pathogenicity [Palombo, 2014].

The research for foods and vegetables, naturally rich in bioactive compounds, already in use in traditional medicine, is considered as good alternatives to synthetic chemicals [Kim, 2005].

To date, numerous plant products have been analyzed for their effectiveness in the prevention of dental plaque formation. However, only a very small number of these natural products have been used for therapeutic applications. This limited use is due to various factors such as adequate effectiveness, stability, smell, taste and cost [Ferrazzano et al., 2009].

Vulnerary plants represent one of the largest legacies provided by folk medicine worldwide [Forrest, 1982]. The therapeutic activities of these plants are numerous and span widely. Some of them present astringent properties, other are endowed of anti-inflammatory power. Some plants, for example, may be immune-stimulant and/or possess recognized antimicrobial activity [Houghton et al., 2005]. For these reasons, vulnerary plants are an inexhaustible sources of research for such types of compounds, especially those aimed at fighting microbial infections. Indeed, Brantner and Grein demonstrated that about 60% of plant extracts used in traditional medicine exhibit antibacterial actions [Brantner and Grein., 1994].

In the scientific community there is a growing awareness that some dietary components may affect the overall health. Suitably, the role of food as an agent for improving health has been recognized, initiating the development of new classes of food, known as functional foods [Honkanen, 2009].

The concept of functional foods is to improve the general conditions of the body and to decrease the risk of illness and disease [Siró et al., 2008]. Bioactive compounds present as natural constituents in food have the potential to provide health benefits, beyond the basic nutritional value of the product [Biesalski et al., 2009].

Most of the bioactive molecules are derived either directly or indirectly from naturally occurring sources, especially, terrestrial food plants and marine species or from animal products [Shahidi, 2009; Venugopal, 2009].

Subsequently, these natural bioactive compounds are being incorporated in the form of ingredients in functional and novel foods, dietary supplements and even pharmaceuticals with the purpose of delivering specific oral health benefits.

In conclusion, there is a need for developing new natural agents which can act to prevent the carious lesion, or which can act to arrest the process of demineralization, when the dental caries has already been initiated.

2) SCIENTIFIC BACKGROUND

Polyphenols

In recent years, there has been an increased interest in polyphenolic compounds found in plant foods. This is probably due to the mounting evidence that several of these may have beneficial effects on humans.

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.].

Polyphenols occurr in all vegetative plant organs, and also in flowers and fruits.

Plants 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. Despite their wide distribution, the healthy effects of dietary polyphenols have come to the attention of nutritionists only in the last years. The main factor responsible for the delayed research on polyphenols is the variety and the complexity of their chemical structure.

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.

Experimental studies strongly support a role of polyphenols also in the prevention of cardiovascular disease, osteoporosis, diabetes mellitus and neurodegenerative disease [Scalbert et al., 2005].

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].

Following in vitro studies on plant extracts suggest an activity against several metabolic activities of mutans streptococci, resulting in a decrease in growth and virulence [Brighenti et al., 2008; Hirasawa et al., 2006; Otake et al., 1991; Matsumoto et al., 1999].

In particular, it has been proved that tea polyphenols can inhibit the adherence of Streptococcus mutans and Actinomyces viscosus to salivary acquired pellicle of the tooth surface [Xiao et al., 2000].

Smullen et al. have shown that the extracts from unfermented cocoa, green tea and red grape seeds have a bacteriostatic effect on *S. mutans*. and reduce its adherence to glass [Smullen et al., 2007].

Studies on the development of anti-plaque agents for prevention of dental caries have investigated the effect of some tea preparations and their individual components on the glucan synthesis catalyzed by glucosyltransferase (GTF) from mutans streptococci. Extracts of green tea and polyphenol mixtures showed appreciable inhibition in the synthesis of insoluble glucan [Hattori et al., 1990].

Experiments demonstrate also the inhibition of salivary amylase activity by extracts of a commercial tea. The effect on salivary amylase may contribute significantly to reduce the cariogenicity of starch-containing foods [Kashket et al.,1988]. Besides to these in vitro evidences, there is a correlation between subjects with a diet rich in tea respect to subjects with a different diet [Signoretto et al., 2006; Jones et al., 1999].

Infact, tea has been considered a healthful beverages since ancient times and, actually, for its health benefits, tea has been included, in particulary green tea, in the group of beverages with biofunctionally properties [Cabrera, 2006].

Stevia Rebaudiana

In the northern regions of South America, Stevia rebaudiana has been used by tribal people for centuries and since 1970s, extract and pure stevioside was utilized in Japan for sweetening and flavoring foods and beverage as a substitute for several synthetic sweeteners.

Stevia is being cultivated in continental China, Taiwan, Thailand, Korea, Brazil, and Malaysia and today it is also grown in Israel, Ukraine, UK, Philippines, Canada, Hawaii, California, and all over South America.

Stevia is a botanically derived from the *Stevia rebaudiana* Bertoni plant (from the Asteraceae family) and consists of glucoside compounds called steviol glycosides (SG).

Steviol glycosides (SG) are the secondary metabolites responsible for the sweetness of Stevia [Gardana et al., 2003].

They are synthesized by steviol glycosides biosynthesis pathway operating in the leaves.

Out of various SG, stevioside and rebaudioside A are the most abundant and most studied [Wölwer-Rieck, 2012].

An extract of these compounds may be up to 250 times sweeter than sucrose [Gardana et al., 2003].

Recent studies on absorption and metabolism of glycosides in rats and humans have shown that stevioside and rebaudoside are not metabolized by gastric juice in the stomach and they are not absorbed by the small intestine due to their high molecular weight [Koyama et al., 2003].

In the colon, glycosides are transformed into steviol by human microflora.

It is interesting to note that the human intestinal microflora is not able to metabolize steviol, the only metabolities found in feces.

A part of free-steviol are absorbed by the colon and trasported to the liver, where they are converted into steviol glucuronide and eliminated through the urine. The remaining part is escreted through feces [Genus, 2007].

In blood plasma, no stevioside, no free steviol metabolities were found [Genus, 2007].

In 1991, the FDA banned stevia, claiming it was an "unsafe food additive."

For this reason, nowdays stevioside and steviol have been subjected to extensive genetic testing [Yadav and Guleria, 2012; Williams and Burdock, 2009]. The majority of the findings show no evidence of genotoxic activity. Neither stevioside nor its aglycone steviol have been shown to react directly with DNA or demonstrate genotoxic damage in assays relevant to human risk.

Nowdays, it can be concluded that these substances do not pose any genetic damage following human consumption [Brusick, 2008].

Passing through the digestive process without chemically breaking down, stevia could replace sugar for those who need to control their blood sugar levels [Strauss, 1995].

Infact, stevia could become the ideal sweetener for anyone who suffers from diabetes, hypoglycemia, high blood pressure, obesity and chronic yeast infections.

The role of Stevia against diabetes is really importan but it has not been inequivocally demostrated [Shivanna et al., 2012].

Some authors assert that Stevia's utility is due to its antioxidant properties; this is supported by analysis of the phenols that may be extracted from the plant. Stevia has a large overall proportion of phenols, up to 91mg/g; it is proposed that these constituents extracted from the leaves are the major agents contributing to wards the antihyperglycemic activities exerted by the plant [Mohd-Radzman et al., 2013; Shivanna et al., 2013].

This is further supported by the fact that the leaves have a greater ability to scavenge free radicals and prevent lipidic peroxidation.

Products containing stevia also seem to interfere with bacterial metabolism [Giacaman et al., 2013]

Debnath has shown that leaf extracts in different solvent system has potential antimicrobial activity against medically important bacterial as E. coli, B. subtilis, S. mutans, S. aureus and fungal strains [Debnath, 2008].

Stevioside and rebaudioside A were tested for cariogenicity in albino Sprague-Dawley rats colonized with Streptococcus sobrinus. It was concluded that neither stevioside nor rebaudioside A are cariogenic under the conditions of this study [Das et al., 1992].

Experimental biofilm caries model were exposed to Stevia. Stevia determinned the reduction of the number of mutans streptococci with respect to sucrose [Giacaman et al., 2013].

Gamboa has evaluated the antibacterial activity of Stevia rebaudiana Bertoni leaf extracts against cariogenic bacteria by the well diffusion method. The zones of inhibition present at the MIC (Minimum Inhibitory Concentration) were from 9 mm to 17.3 mm [Gamboa and Chaves., 2012].

In conclusion, stevia is unique in its potential as a sweetening agent. It is natural, non-toxic, non-mutagenic, non-caloric and non cariogenic [Nikiforov and Eapen, 2008; Philippe et al., 2014; Williams and Burdock, 2009].

Even if these numerous properties of stevia are now widely recognized, many aspects relating to their in vivo metabolism and their relationship to the overall plant physiology, still need to be understood [Ceunen and Geuns, 2013]. For this reason further studies, in vivo and in vitro, are necessary.

3) EXPERIMENTAL STUDY

Aims

The intent of this research program was to determine a new way in caries prevention.

The score was the valuation of anti-caries effects of bio-active molecules of natural origin, in order to elaborate a new preventive methodology. The under discussion molecules were polyphenols.

For this aim, this research project has been divided in three parts.

Part 1: Screening and scoring of antimicrobial and biological activity of Italian vulnerary plants against major oral pathogenic bacteria.

Part 2: Antimicrobial properties of green tea extract against cariogenic microflora: an in vivo study.

Part 3: In vivo antimicrobial efficacy of Plantago Lanceolata extract mouth-rinse on salivary bacterial counts.

Part 1

Screening and scoring of antimicrobial and biological activity of Italian vulnerary plants against major oral pathogenic bacteria.

Abstract - This study aims to evaluate the activity of Italian vulnerary plants against the most important oral pathogenic bacteria**.** This estimate was accomplished through a fivefold process: a) a review of ethnobotanical and microbiological data concerning the Italian vulnerary plants; b) the development of a scoring system to rank the plants; c) the comparative assessment of microbiological properties; d) the assessment of potential cytotoxic effects on keratinicyte-like cells and gingival fibroblasts in colture by XTT cell viability assay; e) clinical evaluation of the most suitable plant extract as anti bacterial agent in a home-made mouthwash.

The study assays hexane (H), ethanol (E), and water (W) extracts from 72 plants.

The agar diffusion method was used to evaluate the activity against *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus casei*, *Actinomyces viscosus*. Twenty-two plants showed appreciable activity. The extracts showing the strongest antibacterial power were those from *Cotinus coggygria* Scop., *Equisetum hyemale* L*., Helichrysum litoreum* Guss, *Juniperus communis* L. and *Phyllitis scolopendrium* (L.) Newman subsp. *scolopendrium*. The potential citotoxic effect of these extracts was assessed. On the basis of these observations, a mouthrinse containing the ethanolic extract of *H. litoreum* has been tested *in vivo*, resulting in reduction of the salivary concentration of *S. Mutans*.

METHODS AND MATERIALS

Ranking procedure

Seventy-two plants, reported as vulnerary in at least three different ethno-botanical records, were ranked according to the following criteria:

a) - Indication of an established vulnerary use in traditional medicine of different Countries

- Extensively used: 3 points
- Used: 2 points
- Occasionally used: 1 point

b) - Specific use in the treatment of oral affections

- Extensively used: 3 points
- Used: 2 points
- Occasionally used: 1 point

c) - Available data on the antimicrobial activity of extracts or isolated principles from a selected plant

- Frequently reported: 3 points
- Some times reported: 2 points
- Rarely reported: 1 points

d) - Distribution

- Plant easily found, forming large population: 1 point
- Plant difficult to found or forming undersized populations: 0 points

Collection of plant samples

Plants were collected during spring and summer 2008 in the regional parks of Matese and Cilento, Campania, Italy, or in the Botanical Garden of University Federico II of Naples.

Shortly after collection, plants were oven-dried at 50 °C for 48 hours. Dried plants were finely grinded and the resulting powder-like materials stored at -20 °C. For each plant, a voucher sample was saved at the Department of Biological Sciences, University Federico II of Naples.

Preparation of plant extracts

Exactly 4 g of each powdered plant material were soaked in 40 ml of hexane, then in 40 ml of ethanol and finally in 40 ml of water. Each extraction was carried out in a ultrasonic bath for 30 minutes, followed by 24 hours continuous stirring (90 rpm) in a rotary shaker. Extracts were then filtered on paper (Whatman, n.1) and concentrated using a vacuum roto-evaporator at 38 °C. The dried material was finally stored at -20 °C. Powders were solubilized in aqueous DMSO (10%) before further use. To assess biological activity of on lymphocytic cells, the solutions were diluted 1:10 with water (final DMSO concentration was 1%).

Bacterial strains

The bacterial strains used for the screening were: *Actinomycesviscosus* (ATCC 19246) and *Lactobacillus casei* (ATCC 393), obtained from American type Culture Collection (ATCC; Rockville, MD, USA); *Streptococcus mutans* and *Streptococcus sobrinus* were from clinical specimens obtained at the Diagnostic Unit of Microbiology of the University of Naples "Federico II". Bacteria were grown on Trypticase Soy Agar II with 5% Sheep Blood (TSS; Becton Dickinson, USA) plates at 37°C in 5% CO_2 for 48h.

Antimicrobial tests

The initial screening of antibacterial activity was performed using the agar well diffusion method. Inocula were prepared from overnight cultures of each bacterial strain and adjusted to 0.5 McFarland standard of turbidity. Bacterial strains were evenly spread on the surface of TSS agar plates using sterile swabs and three wells of 8 mm diameter were punched into the agar medium.

The vacuum-dried extracts from water, ethanol and hexane, were re-dissolved in water containing 10% DMSO (Sigma Aldrich Milan, Italy); these stocks were serially diluted to give concentrations, referred to the dry powder, ranging from 200 to 12.5 mg/ml. The assay was initiated pouring 100µl of each of these solutions into separate wells (100 µl of 10% DMSO solution were used as negative control). A 0.3% Triclosan solution in DMSO was used as positive control. The plates were incubated at 37°C in 5% CO_2 atmosphere for 48h. The antibacterial activity of plant extracts was evaluated by measuring the diameter (expressed in mm) of inhibition zone observed around each well. All tests have been performed in triplicate and repeated twice.

The minimum inhibitory concentration (MIC) was measured by the standard microdilution method in 96-wells polystyrene plates using Brain-Heart Infusion (BHI) medium. The starting inoculum was 5 x 105 CFU ml^{-1} and the concentrations for the plant extracts ranged from 100 to 6.25 mg ml^{-1} (twofold dilution). The MIC was considered the lowest concentration of extract able to inhibit any visible bacterial growth. To determine the MBC (minimal bactericidal concentration) 50 µL of bacterial suspension from the wells containing extract concentrations equal or higher than the MIC, were inoculated in 5 mL of sterile BHI medium and incubated for 24 h at 37 °C in 5% CO_2 atmosphere. MBC was considered the lowest concentration that inhibited completely bacterial growth. Each extract was tested in triplicate; each experiment was performed twice.

Cell lines

Human gingival fibroblasts (HGF-1) and keratinocyte cell line HaCaT cell lines were both obtained from ATCC (Rockville,MD, USA). Both cell lines were cultured in Dulbecco's Modified Eagle Medium, containing 10% Foetal Calf Serum (FCS), 2 mM L-glutamine, and 50 µg/mL gentamicin (fibroblasts) or 100 µg/mL streptomycin + 100 units/mL penicillin (HaCaT). The media were changed every second day. Cell culture reagents were purchased from Life Technologies (San Giuliano Milanese, Italy).

Biologic assays on human normal (gingival fibroblasts) or immortalized (keratinocyte-like) cells

Cell viability was assayed by using the Cell Proliferation Kit II (XTT, Roche, MilanItaly). This assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active cells [Sun et al.,1997]; therefore, this conversion only occurs in viable cells.

The plant extracts, were provided to our laboratory in the relative extraction solvents having all a nominal concentration of 50 mg/ml. The extracts were dried under vacuum and re-dissolved in DMSO 10% in water. Samples were stored at -30°C until use. Before measurements, samples were brought to room temperature under agitation and added to culture media in a ratio 1 to 10. In details, 90 µl of suspensions of fibroblast or HaCaT cells (containing ~$1x10^4$ cellsin complete medium) were seeded into 96-well plates. Then, 10 µl of each extract (50 mg/ml in 10% DMSO) were added to each well so that the final extract concentration was 5 mg/ml, while the DMSO content was reduced to 1%. Cells were incubated in these conditions at 37°C for 24 hours in 5% CO_2 atmosphere. Triplicate samples were prepared for any individual condition.

As a positive control for cyto-toxicity we used Triclosan at low concentration (0.03% as compared with 3% used in toothpastes). This synthetic is a polychloro-phenoxyphenolan endowed with antibacterial and antifungal properties. For these reasons it is currently largely used in oral hygiene as additive of toothpastes to prevent gingivitis.

In vivo test: efficacy of *H. litoreum* ethanolic extract against *mutans streptococci*

The study enrolled 28 volunteers (12 males and 16 females) ranging in age from 12 to 18 years. The participants were recruited from young patients of the Departement of Paediatric Dentistry - University Hospital of Naples “Federico II”, Italy. The study plan was approved by the Local Committee for Medical and Health Research Ethics-University of Naples “Federico II”. Patients and their parents received verbal and written explanations about the study and written informed

agreementform tobe signed to partecipate. The study protocol was in accordance with the Helsinki Declaration of Human Rights.

Inclusion criteria were: good general health (ASA I-II) and agreement to strictly comply with the procedures indicated by the study protocol. Exclusion criteria were prior exposure (less than two weeks) to antibiotic treatment and/or prior use (<12 hours) of anti-bacterial mouthwashes. Similarly, individuals carrying fixed orthodontic appliances, were excluded from this study.

Participating volunteers were randomly distributed into two groups of 14 subjects: group A representing patients using *H. litoreum* mouthwash (a 1% ethanolic extract of *H. litoreum*, at a concentration of 12,5mg/ml), and group B representing those using placebo solution (20 ml of a 1% ethanol in water).

A first sample of saliva was collected from each patient before the treatment (t0) in order to establish the baseline levels of mutans streptococci. After the collection of first sample, all participants were instructed to mouth-rinse with 20 ml of *H. litoreum* extract (group A) or placebo solution (group B) for 1 minute. This procedure has to be repeated three times a day (after breakfast, after lunch and at the bed time), after normal oral hygiene procedures, for fourteen consecutive days. Saliva samples were collected at day 7 (t1) and 14 (t2) of treatment.

Mutans streptococci counts in saliva were determined by CRT bacteria test (IvoclarVivadent, Bologna, Italy), a method used in dental clinics for a semiquantitative evaluation of the main cariogenic bacteria in saliva [Dziedzic et al., 2013].

The saliva samples were collected in sterile containers and used to wet the blue Mitis-salivarius-agar with bacitracin for determination of mutans streptococci as indicated by the kit's manufacturer. Vials were incubated at 37 °C for 48 hours .

Statistical analysis

All data from *in vitro* tests (section 2.7) were expressed as mean ± SD. Significance was assessed by the Student's t test for unpaired data for comparisons between two means. Statistical significance was defined as *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$.

All data from *in vivo* samples (section 2.10) were processed with the Statistical Package forSocial 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

Plant selection and ranking

Data from Guarrera [Guarrera, 2006] and from the ethno-botanical database of Campania and South Italy [De Natale et al., 2009] were used in listing 312 vulnerary plants of Italian Flora. Since this number appears to be quite large and not easy to manage, only the species reported as vulnerary in at least three different ethno-botanical reports were selected and included in this study. On this basis, only 72 plants responded to this criterion. These plants were then ranked according to the scoring system outlined in the Method section. Table 1 resumes the score assigned to each plant associated with the estimated antibacterial activity. The antibacterial potential of the selected plants, assessed by using ethno-botanical data, ranked 20 species with a score ≥ 6 (group A), and the remaining 52 between 5 and 1 (group B).

Antimicrobial activity

Out of 72 plants tested only 20 (28%) exhibited variable degrees of inhibitory activity against one or more bacterial species (Table 1, last column). The most active plants belong all to the high rank group A, whereas only few extracts from group B were endowed of a measurable antibacterial activity. The only notable exception is represented by *Equisetum hyemale*, which at least at the highest concentration, was definitely effective towards three strains, *S. sobrinus*, *S.mutans* and *L.*

casei. Table 2 presents the results obtained in typical well-diffusion bioassays, compared to that shown by a Triclosan 0.3% solution.

The water extract from *Cotinus coggygria* engenders the major effects, being active against all the four bacteria at any concentration tested. The hexane extract of *Juniperus communis* inhibited the growth of all bacteria except that of *L. casei*. The ethanolic extract obtained from *Helichrysum litoreum* was effective against *S. mutans* and *A.viscosus,* while the ethanolic extract from *Phyllitis scolopendrium* subsp. *scolopendrium* was successful against *S. mutans* and *L. casei*. The ethanolic extracts of *Bellis perennis* and *Ceterach officinarium* Willd. s.l. showed a small inhibitory activity against *S. sobrinus*; when used at the highest concentrations, both extract induced a tiny reduction in the growth rate of *L.casei*. The ethanolic extracts of *Thymus vulgaris* L. s.l. exhibited a mild activity against *S. sobrinus* and *L. casei*.

A selective inhibitory activity towards *A. viscosus* was evidenced by the ethanolic extract of *Gentianalutea* L. s.l. The other plant extracts, but only at the highest concentration tested, were all endowed of a scarcely noticeable activity against two or even one bacterial strain.

The antimicrobial activity of more effective extracts was investigated also in terms of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The water and ethanolic extracts of *Cotynus coggyria* demonstrated a considerable activity against all the bacteria tested, while the extract from hexane appeared very selective against *S.sobrinus*. Similarly, the ethanolic extract of *Helychrisum litoreum* was very effective against *S. mutans* and *A. viscosus*. All the remaining extracts were all characterized by high MIC values (≥100 mg/ml).

Activity of plant extracts on human cells viability

Activity of the extracts was assessed observing the consequences of their action on the viability two eukaryotic cell lines, one normal (human gingival fibroblasts) and one immortalized (HaCaT cells). The assay was done by means of a specific test (XTT assay) that provides information on cell proliferation/impairment through the assessment of changes in mitochondrial specific enzymatic

activity of cells under observation. In general, no statistically significant changes were observed in cells treated with extracts from most plants included in this study (extracts from 72 plants). However, some interesting exceptions ensued, as among the extracts endowed of in vitro antibacteric activity, some were void and some were endowed of inhibitory properties on the growth of both fibroblasts and HaCaT cells. Specifically, the ethanolic extract from both *H. litoreum* and *E. hyemale* did not affect the viability of both cell lines, while the ethanolic extract from *P. scolopendrium* and the water extract from C. *coggigrya* were slightly but measurably inhibitory ($p<0,001$). The etanolic extract from C. *coggigrya* and the hexane extract from J. *Communis* appeared to be, in turn, frankly toxic to both cell lines ($p<0.0001$) The control of these experiments was provided by Triclosan, whose addition to cells at low concentration (0.03%, i.e. up to 100 times lower than that used in toothpastes) caused a profound reduction (up to >90%) in the cell viability as measured by XTT assay (Figure 1).

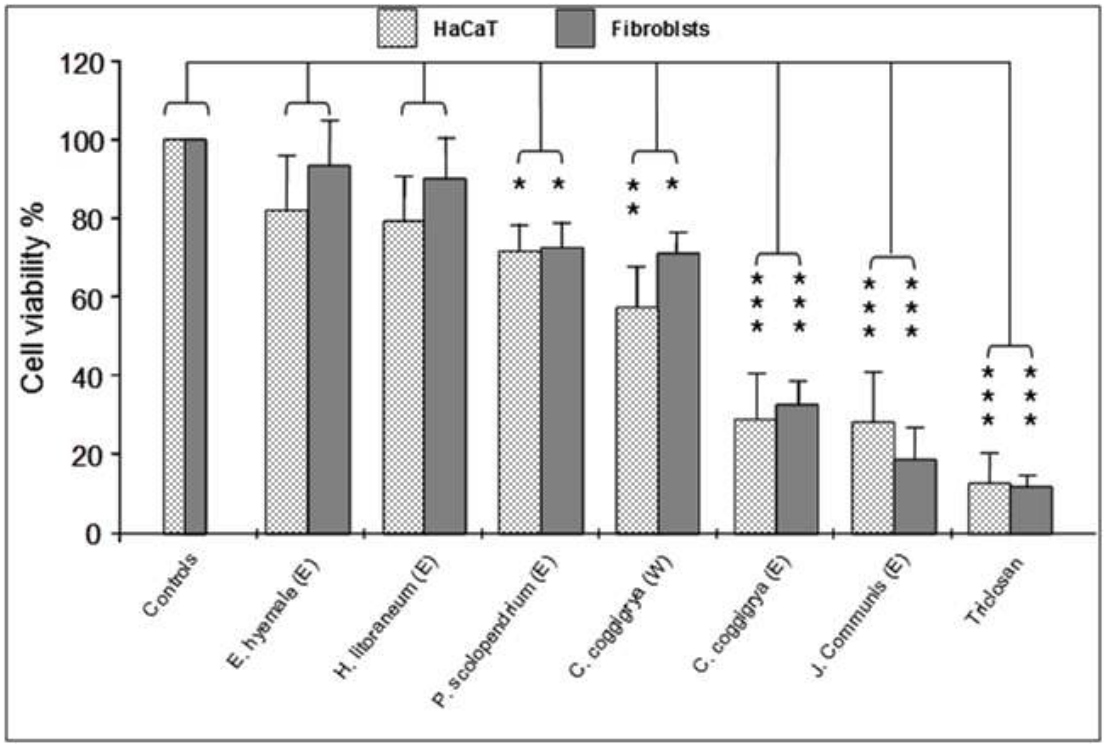

Figure 1: Effects of selected plant extracts (as indicated) on viability of keratonocyte-like cells and gingival fibroblasts as measured by XTT assay. Statistical significance is defined as *, p <0.01; **, p< 0.001; ***, p<0.0001.

Preliminary *in vivo* assessment on the efficacy of *H. litoreum* ethanolic extract against *Streptococcus* mutans

The CRT bacteria test results were expressed as a low (<10^5CFU) or a high (>10^5CFU) bacterial count. Variations in *S. Mutans* density of the CFU (CFU/ml) at t0, t1, t2 for the test group (**A**) are summarized in figure 2.

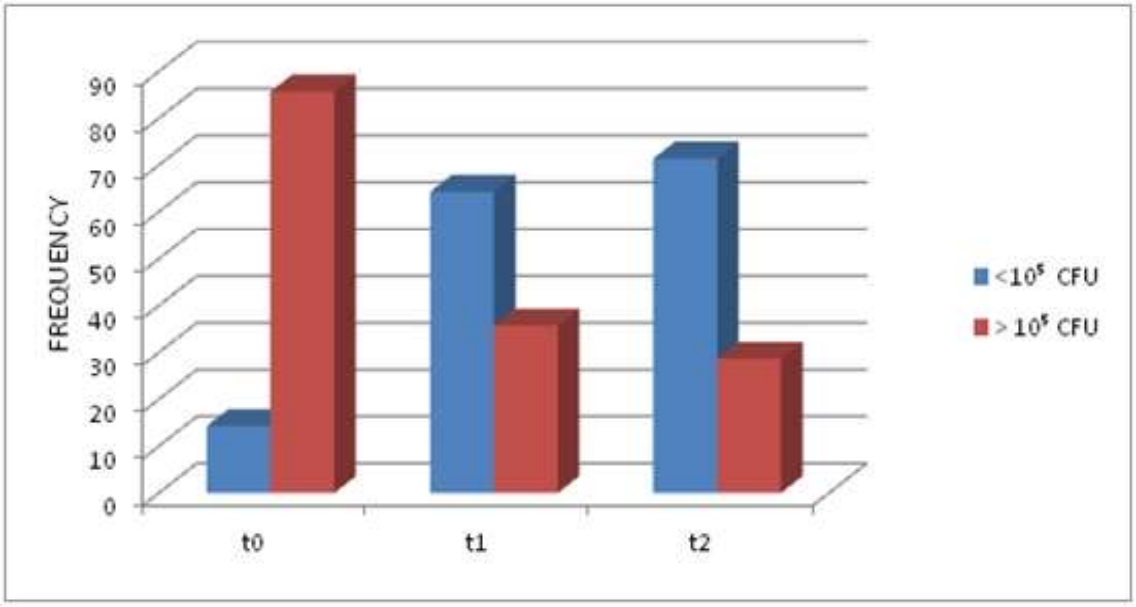

Figure 2: variation of *S. mutans* concentration (CFU/ml) at t0, t1, t2 (Group A).

The differences in CFU (CFU/ml) density of MS were statistically significant between t0 and t1 (p=0.012) and between t0 and t2 (p=0.005); between t1 and t2 they were not statistically significant.

Variations in *S. Mutans* density of the CFU (CFU/ml) at t0, t1, t2 for the control group (**B**) were represented in figure 3.

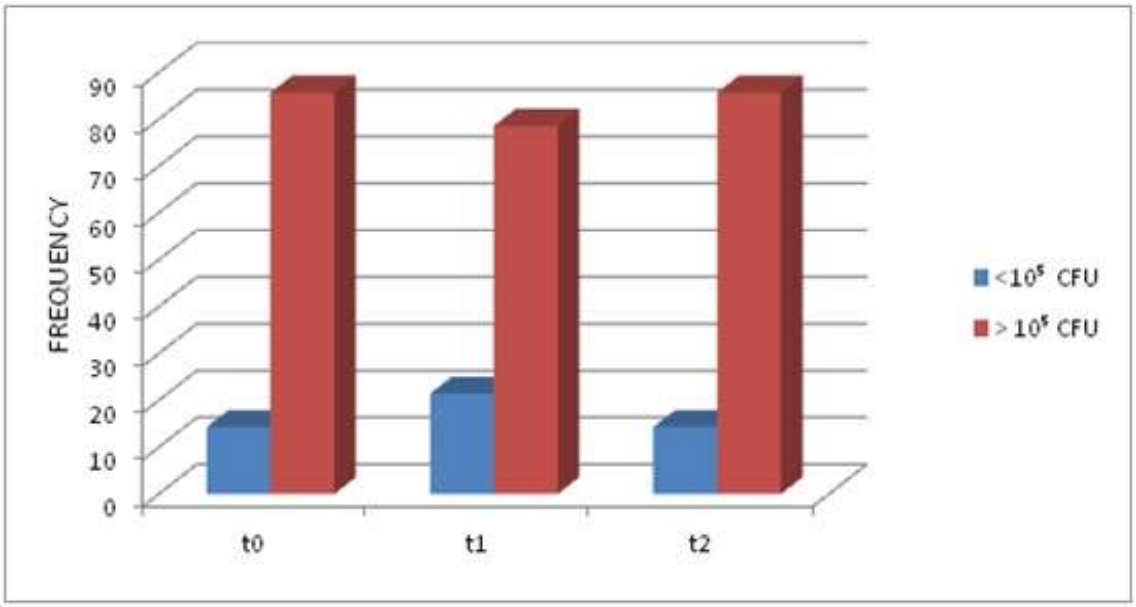

Figure 3: variation of *S. mutans* concentration (CFU/ml) at t0, t1, t2 (Group B).

The differences in CFU (CFU/ml) density of *S. mutans* between t0 and t1, t0 and t2, t1 and t2 were not statistically significant.

At t0 the differences in CFU (CFU/ml) density of *S. mutans* between groups A and B were not statistically significant, while at t1 and t2 the differences were statistically significant, respectively [t1: OR=0.15 (CI=0.28-0.81); t2: OR=0.06 (CI=0.01-0.44)].

DISCUSSION

We observed and report that about twenty vulnerary plants of Italian flora showed inhibitory activity against cariogenic bacteria. A good correlation was found between the speculative ranking system we adopted and the results of some specific bioassays: taking into account a cut-off value of 6 points, almost all the plants endowed of a measurable activity, presented a score above this boundary.

The species demonstrating an antimicrobial action belong to 13 families of vascular plants, not having any phylogenetic relationship. However, the most represented family was that of Lamiaceae, which includes many species with documented biocide activity [Sarac and Ugur, 2007].

All the bacterial strains tested revealed a higher sensitivity to ethanolic, followed by aqueous extracts. While the precise reasons of the higher activity displayed by ethanolic extracts are not clear to now, the possible presence of flavonoids and related compounds (very soluble in alcohols), may explain such property. Indeed, the flavonoids inhibitory action against cariogenic bacteria has been suspected since long time [Sato et al., 1996]. The aqueous extracts contain more polar compounds [Nikitina et al., 2007], are probably less effective against cariogenic bacteria, due to the strong hydrophobicity of their cell surfaces [Weiss et al., 1982]. To now, the minimal antimicrobial effect of extracts from hexane finds no clear explanation and definitely deserves further investigation. Interesting enough, we did not observed activity in extracts from plants as *Thymus vulgaris,* which, indeed, are known for their antimicrobial action [Panizzi et al., 1994].

A possible explanation for such discrepancy may reside in environmental factors and plant chemotypes [Hernandez et al., 2009] that can both strongly affect the amount of the active compounds produced by the plant.

Cotinus coggygria was the most active species among the plants selected for the screening. This plant is largely used in the Balkan and Anatolian regions to cure wounds, and reduce inflammations, as well as for the treatment of gastrointestinal and respiratory disorders. In Asiatic Countries *C. coggigrya*, is also known as a bactericide, and frequently administered against hepatitis and even anemia [Ajaib et al., 2010]. A relative of this species, *Rhus coriaria*, which grows in the Mediterranean Region, has demonstrated inhibitory properties towards *Streptococcus mutans* and *S. sanguinis*, common components of dental plaque [Babpour et al., 2009]. These authors attributed this effect to the presence of large amounts of tannins in the plant. Tannins can then generate smaller phenolics compounds (pyrogallol, catechol, ellagic acid) with known bactericidal actions. Similarly, *C. coggigrya* is very rich in phenolic compounds (i.e. 923.33 ± 14 μM, calculated as quercetin equivalents [Ivanova et al., 2005]), and displays a significant antimicrobial activity.

Helichrysum litoreum Guss, is a species endemic to Central-South Italy, Sicilia, andSardinia [Conti et al., 2006]. Preliminary research evidenced bactericidal activity of *H. litoreum* crude extracts, as also reported for other *Helichrysum* species [Guida et al., 1999]. In the species *H. compactum* the antimicrobial activity has been attributed to flavonoids and chemically related compound [Süzgeç et al., 2005]. The data obtained in the present study on the specific activity of *H. litoreum* extracts against *S. mutans* and the absence of cytotoxic effects are in agreement with the results previously reported for *H. italicum* by Nostro et al. [Nostro et al.,2004].

We have found that also *Phyllitis scolopendrium,* a fern belonging to Aspleniaceae, possesses significant activity towards cariogenic bacteria. The same holds true for the other Pteridophyta, *E. hymale*, a plant of Euro-Asiatic origin, but also diffused in the American continent. Indeed, recently it has been reported a specific activity of this species against *Staphylococcus aureus* [Canales et al., 2005], but, to date, this is the first report describing inhibitory activity of the plant against oral pathogen. The extracts of *Phyllitis scolopendrium* and *C. coggydria*, present a small but measurable effect on cell viability.

Another active species, *J. communis,* is already known for its antimicrobial properties: it has been shown that the essential oil from *J. communis* berries had definite inhibitory effect against Gram positive and Gram negative bacterial species [Pepeljnjak et al., 2005], and that the hexane extract of *J. communis* leaves was extremely effective against pathogenic multi-resistant bacteria [Sati and Joshi, 2010]. The extract is measurably citotoxic.

Interesting enough, two out of six active extracts have shown no statistically significant cytotoxicity on both keratinocyte-like transformed cells and normal gingival fibroblasts as the cells wellbeing was fully unaffected by their presence (even for extended time). At variance, two other extracts were endowed of slight growth-inhibitory properties and two were frankly cytotoxic (the reduction in cell viability in the latter cases was similar to that caused by the polychlorophenoxy phenol *Triclosan*, a widely used antibacterial and antifungal agent). The two non cytotoxic extracts, namely

H. litoreum and *E. hyemale*, displayed a different antimicrobial activity, the first being clearly more active.

The present in vivo study has shown that a regular daily rinsing with mouthwash containing *H. litoreum* ethanol extract could reduce on 50% of subjects the salivary levels of *S. mutans*, which are the most virulent cariogenic pathogens in the oral cavity. This is probably due to both inhibition of growth and adherence of *S. mutans* cells to teeth surfaces.

CONCLUSIONS

The results of this study on a large sample of vulnerary plants of Italian flora has identified a limited number of extracts that may find real application in the prevention of dental caries, as they work as effective weapons against all the major bacterial constituent of the plaque.

Further long-term studies in vivo involving more subjects are needed to clarify if this approach could represent an effective complementary strategy for reducing the severity of this illness.

Part 2

Antimicrobial properties of green tea extract against cariogenic microflora: an in vivo study

Abstract - The aim of the present study was to in vivo test the effectiveness of an experimental green tea extract in reducing the mutans streptococci and lactobacilli levels in saliva using a means of selective colture media.66 healthy patients with age range from 12 to 18 years were recruited and randomly divided into 2 Groups [Group A (n=33) and group B (n=33)]. Group A was asked to rinse the mouth with 40 ml of an experimental green tea extract, for 1 minute, 3 times a day for a week; Group B was asked to rinse with 40 ml of a placebo mouth-rinse.

Baseline (t0), four days (t1) and seven days (t2) saliva samples were obtained.

The counts of mutans streptococci and lactobacilli were investigate by chair-side kits.

Data were statistically processed. A regression binary logistic analysis was made. Statistical significance level was established at $p<0.05$. The treatment group showed a statistically significant reduction in colony counts of mutans streptococci and lactobacilli respect to the control group.

These findings showed the efficacy of a green tea extract on cariogenic oral flora, opening a promising avenue of clinical applications in the preparation of specific and natural anti-cariogenic remedies.

METHODS AND MATERIALS

Subjects and study design

The study population consisted of 66 volunteers, 36 female and 30 male, who were in good physical condition with an age range from 12 to 18 years.

The participants were recruited from the Department of Paediatric Dentistry of the University of Naples "Federico II", Italy.

They were selected with the following criteria. Inclusion criteria were: good general health (ASA I-II) and agreement to comply with study procedures. Exclusion criteria were: antibiotic treatment during the 14 days before starting the test, the use of an antibacterial mouth-rinse during the 12 hours before the test, presence of dental fixed orthodontic appliances and conditions that interfered with examination procedure (not cooperating subjects).

Participation was voluntary. Patients and their parents received verbal and written explanations of the experimental protocol and the study aims and written informed consent was signed by them prior to the start of the study. Permission was received from the appropriate authorities. The study protocol was in accordance with the Helsinki Declaration of Human Rights.

The subjects were randomly distributed into two groups of 33 (Group A and B) . All the subjects were submitted to a clinical examination carried out by two professionals, in the same room and using the same dental unit (so that all patients were examined under the same lighting conditions).

The presence of tooth decay was assessed by systematic evaluation of each subjects' caries

experience using the DMFT index (number of decayed, missing and filled teeth). In the Group A the mean DMFT value was 3.13 ± 1.76; in the Group B the mean DMFT value was 3.09 ± 1.89.

Experiment design –Group A

33 subjects were involved in this group. Prior to the start of the experiment, the subjects' salivary concentration of mutans streptococci and lactobacilli was calculated through a sample of saliva in order to establish the baseline levels (t0).

Means of selective colture media (CRT bacteria, Ivoclar Vivadent) were used for detection of the mutans streptococci and lactobacilli counts in saliva.

The test was conducted with the following way:

- Each subject chewed a enclosed paraffin pellet in order to stimulate the salivation;
- The saliva was collected in a sterile plastic container and then placed, using a pipette, on the blue mitis-salivarius-agar with bacitracin for determination of mutans streptococci and on the light culture medium, Rogosa agar, for determination of lactobacilli.
- A $NaHCO_3$ tablet was added to the container: this tablet was able to release CO_2 when it came into contact with moisture, creating favourable conditions for bacterial growth.
- The vial with each agar plate was marked with the name of the patient and the date using a waterproof pen;
- All the vials were placed upright in the incubator Cultura/Ivoclar Vivadent at 37 °C / 99 °F for 48 hours

After the collection of first sample, all participants of group A were instructed to rinse with 40 ml of an experimental mouth-rinse for 1 minute.

This procedure ought to be repeated three times a day (after breakfast, after lunch and before sleeping), after normal oral hygiene procedures, for seven days.

After the 4th and at the 7th days of treatment with mouth-rinse formulation, the salivary sample was re-collected and immediately incubated, in according to the step-by-step procedure above described, in order to calculate the density of the CFU (CFU/ml) of mutans streptococci and lactobacilli for each subject during (t1) and immediately after (t2) the treatment.

Therefore, a total of three saliva sample (t0, t1, t2) were taken for each individual.

During the 7-day experimental period no alterations were made to the subjects diet and oral hygiene procedures.

Experiment design-Group B

33 subjects were involved in this group. Prior to the start of the experiment, the subjects' salivary concentration of mutans streptococci and lactobacilli was calculated through a sample of saliva, in order to establish the baseline levels (t0) with the same procedure above mentioned for Group A.

After the first collection of sample, the participants of group B rinsed with 40 ml of a placebo mouth-rinse without polyphenolic substances, for 1 minute, 3 times a day (after breakfast, after lunch and before sleeping for seven days).

Subsequent saliva samples were obtained on 4th (t1) and 7th day (t2) after the beginning of the study.

During the 7-day experimental period no alterations were made to the subjects diet and oral hygiene procedures.

Mouth-rinse formulation

Two different mouth-rinse formulations were prepared:

1) Experimental (Group A)

   Experimental mouth-rinse was prepared with pulverized Camelia Sinensis leaves.

   For each rinsing, 1.6 g of pulverized leaves were suspended in 40 ml of distilled water at 100 °C for 3 minutes. After this procedure the mouth-rinse was kept at room temperature.

2) Placebo (Group B)

40 ml of distilled water were coloured with food dye.

Both mouth-rinses were put into hermetically sealed plastic bottles.

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

The mean stimulated saliva secretion rate was 1.41 ± 0.53 ml/minute for all subjects.

The CRT bacteria test results are expressed as a low (<10^5CFU) or a high (>10^5CFU) bacterial count.

Statistical analysis within group A

Variations in mutans streptococci and lactobacilli density of the CFU (CFU/ml) at t0, t1, t2 for the test group were summarized in figures 4 and 5, respectively.

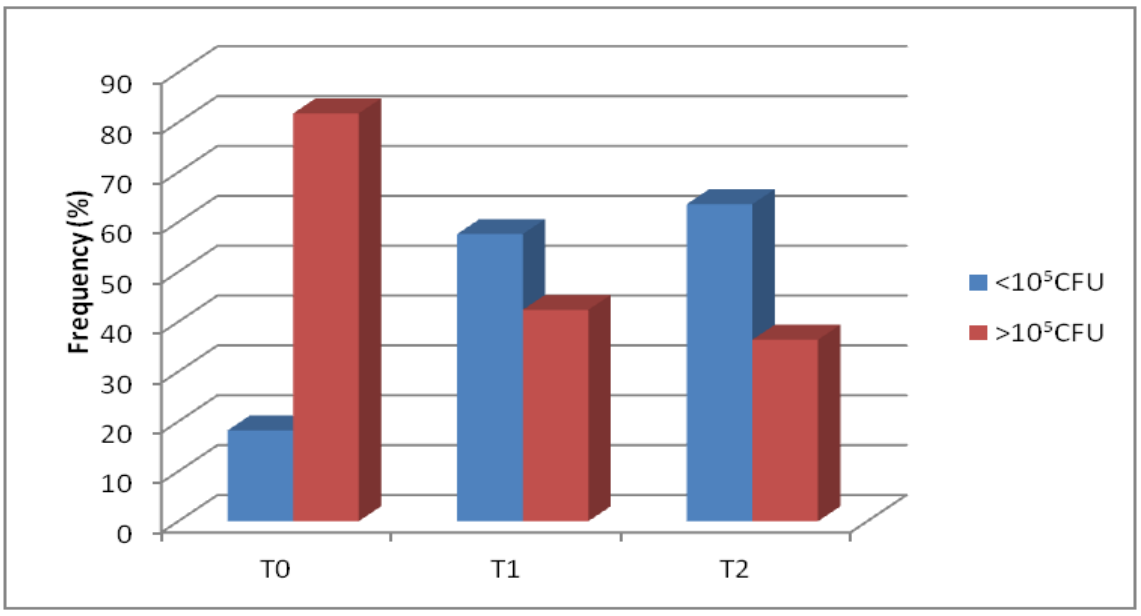

Figure 4: variation in streptococci mutans density of the CFU (CFU/ml) at t0, t1, t2 (Group A).

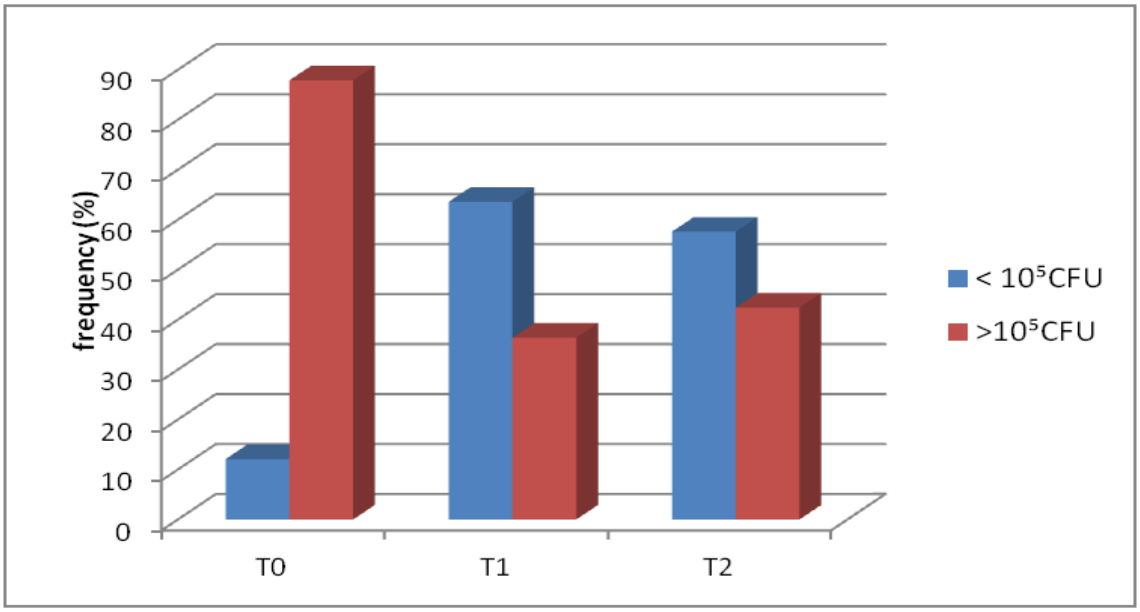

Figure 5: variation in lactobacilli density of the CFU (CFU/ml) at t0, t1, t2 (Group A).

The differences in CFU (CFU/ml) density of MS between t0 and t1 were statistically significant (p<0.001); between t0 and t2 they were statistically significant (p<0.001); between t1 and t2 they were not statistically significant.

The differences in CFU (CFU/ml) density of lactobacilli between t0 and t1 were statistically significant (p<0.001); between t1 and t2 they were not statistically significant (p<0.001); between t0 and t2 they were statistically significant (p<0.001).

Statistical analysis within group B

Variations in mutans streptococci and lactobacilli density of the CFU (CFU/ml) at t0, t1, t2 for the control group were represented in figures 6 and 7, respectively.

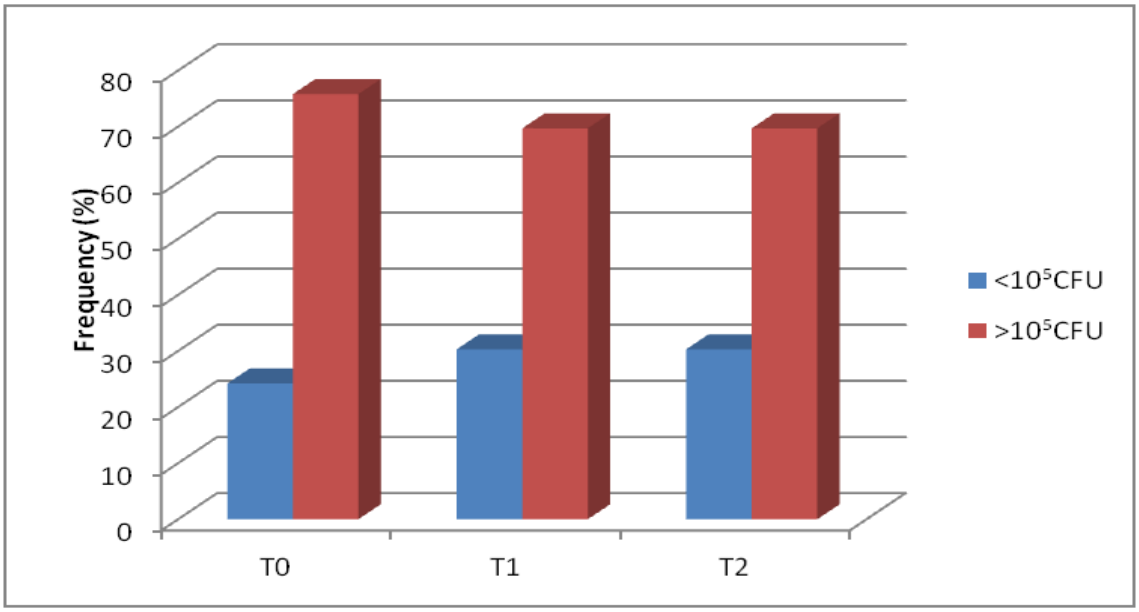

Figure 6: variation in streptococci mutans density of the CFU (CFU/ml) at t0, t1, t2 (Group B).

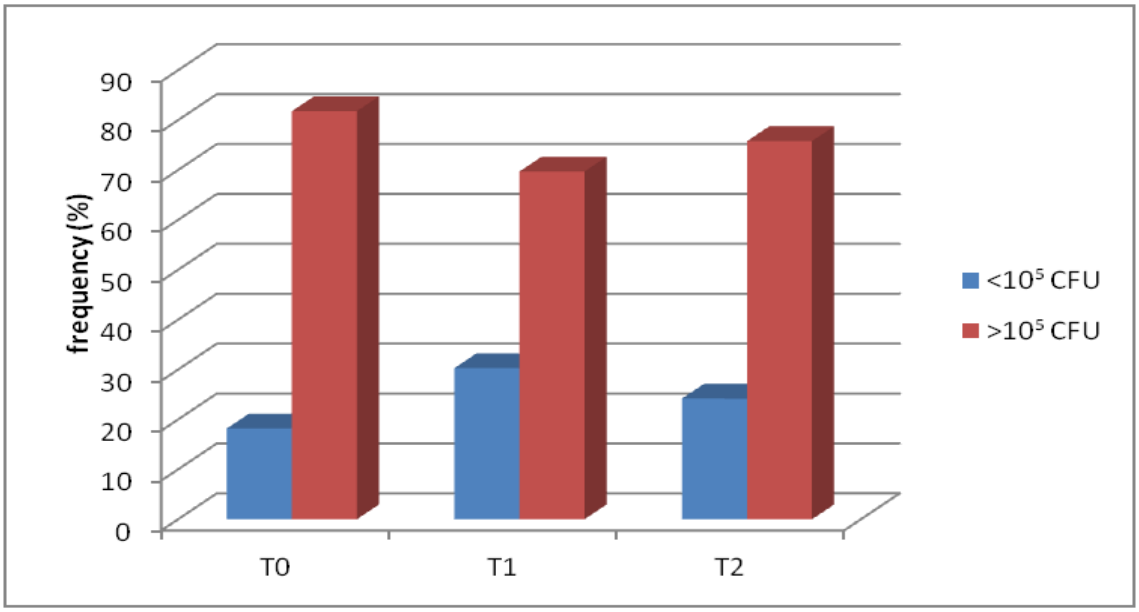

Figure 7: variation in lactobacilli density of the CFU (CFU/ml) at t0, t1, t2 (Group B).

The differences in CFU (CFU/ml) density of mutans streptococci between t0 and t1, t0 and t2, t1 and t2 were not statistically significant, respectively.

The differences in CFU (CFU/ml) density of lactobacilli between t0 and t1, t0 and t2, t1 and t2 were not statistically significant, respectively.

Statistical analysis between groups A and B

At t0 the differences in CFU (CFU/ml) density of mutans streptococci between groups A and B were not statistically significant, while at t1 and t2 the differences were statistically significant, respectively [t1: OR=3.12 (CI=1.13-8.60); t2: OR=4.2 (CI=1.44-11.23)].

At t0 the differences in CFU (CFU/ml) density of lactobacilli between groups A and B were not statistically significant, while at t1 and t2 the differences were statistically significant, respectively [t1: OR=4.02 (CI=1.44-11.23); t2: OR=4.24 (CI=1.47-12.16)].

DISCUSSION

The primary etiological dental caries agents are known to be several restricted strains of oral bacteria; thus, the majority of current commercial anti-plaque products are antimicrobial compounds, but many antibiotic and chemical bactericides currently used may disturb the bacterial flora of the oral cavity, resulting in induction and overgrowth of antibiotic-resistant bacteria and other opportunistic pathogens such as Candida albicans [Gunsolley., 2006].

So many researchers in the world have been searching for alternatives to prevent the occurrence of this process for example using natural substances, derived from food.

The analysis of the literature suggests that diet may influence dental decay experience in two ways: it may inhibit or it may promote the disease.

Far more is known about dietary factors which promote dental decay than those which inhibit it.

The studies carried out in these last decades have supported the anti-bacterial role of polyphenols but at the present time their potential use in the control of bacteria responsible of cariogenesis is still under scrutiny [Ferrazzano et al., 2009].

A relatively larger body of evidence has been accumulated on the effects of tea (particularly the green tea) on plaque formation, whereas the data on others polyphenols sources are at a preliminary stage, although in the case of cranberry numerous evidences have been accumulating [Bodet et al., 2008].

The present in vivo study has shown that the 60% of subjects, undergoing to green tea mouth-rinse, presented a significant lowering of mutans streptococci and the 42,4% of subject, undergoing to green tea mouth-rinse, presented a significant lowering of lactobacilli than the subjects undergoing to placebo mouth-rinse.

This is probably due to the antibacterial properties of polyphenols associated to the inhibition of adherence of bacterial cells to teeth surfaces.

In fact, the results from the present study on the in vivo tea extracts activity against oral microorganisms supported the hypothesis that tea polyphenols exert an anti-caries effect via an anti-microbial mode-of-action [Li JYet al., 2004].

Our findings reflected what was found in literature, where the association between use of specific foods and reduction of oral cariogenic bacteria has emerged [Hamilton-Miller, 2001].

Therefore, our study, demonstrating the in vivo effect of tea extracts on cariogenic bacteria, could open a promising avenue of applications, since they are relatively safe, have taste and odor largely appreciated and could be used at a reasonable cost in the preparation of specific anticariogenic remedies.

CONCLUSIONS

The present study demonstrated that daily consumption of green tea could reduce the salivary levels of mutans streptococci and lactobacilli, which are the most virulent cariogenic pathogens

in the oral cavity. This approach could be an alternative strategy for the prevention of dental caries.

More studies, particularly in vivo and in situ, are necessary to establish conclusive evidence for the effectiveness of polyphenols against dental caries with the aim of improving oral health; it is essential to determine the nature and distribution of these compounds in our diet and to better identify which of the hundred of existing polyphenols are likely to provide the greatest effects.

Since the evidence of therapeutic effects of dietary polyphenols continues to accumulate, it is becoming more and more important to understand the nature of in vivo absorption and metabolism.

Part 3

In vivo antimicrobial efficacy of Plantago Lanceolata extract mouth-rinse on salivary bacterial counts.

Abstact - The aim of the present study was to in vivo examine the antimicrobial short–term efficacy of an experimental mouth-rinse containing polyphenols in reducing *Streptococci mutans* and *Lactobacilli casei* levels in saliva using a means of selective colture media.

The study sample consisted of 44 adolescents divided into a study group (A) and a control group (B). Group A was instructed to rinse with an experimental mouth-rinse containing Plantago Lanceolata extract and Group B received a placebo mouth-rinse for a week.

Salivary counts of *S. mutans* and *L. casei* were estimated with a chair-side test at baseline, at the fourth and the seventh day of the experimental study.

Bacterial colonies were categorized as low ($<10^5$ colony forming unit (CFU)/ml of saliva) or high (> or = 10^5 CFU/ml).

In the test Group lower levels of salivary *S. mutans* were recorded after the conclusion of the experimental period respect to the baseline data, instead a slight decrease, not statistically significant, was observed after the treatment for *L. casei* counts. No significant differences were recorded between the baseline and the final samples both for *S. mutans* and *L. casei* counts in the control Group.

These findings suggest that Plantago Lanceolata extract could represent a natural anti-cariogenic agent by exhibiting antimicrobial properties against *S.mutans.*

METHODS AND MATERIALS

The experimental study was divided into two parts.

Primarily, the in vitro citotoxicity effect of Plantago lanceolata extract was evaluated with a XTT assay, followed by an in vivo evaluation of the antimicrobial properties.

XTT assay

The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active cells. This conversion only occurs in viable cells. The formazan dye formed is soluble in aqueous solutions and it is directly quantified using a scanning multi-well spectrophotometer (ELISA reader) that ensures a high degree of accuracy.

Cells, grown in a 96 well tissue culture plate, are incubated with the yellow XTT solution (final concentration 0.3 mg/ml) for 4–24 h. After this incubation period, orange formazan solution is formed, which is spectrophotometrically quantified using an ELISA plate reader.

An increase in number of living cells results in an increase in the overall activity of mitochondrial dehydrogenases in the sample.

Plantago lanceolata extracts are dried and resuspended in 40 µl of aqueous solution containing 0.1% DMSO (dimethyl sulfoxide). 5000 murine fibroblasts are grown in a 96 well tissue micro-

plates (Culture medium, e.g., DMEM containing 10% heat inactivated FCS (fetal calf serum), 2 mM Lglutamine, 0.55 mM L-arginine, 0.24 mM L-asparagine- monohydrat, 50 µM 2-mercaptoethanol, HTmedia supplement (1×), containing 0.1 mM hypoxanthine, and 16 µM thymidine and additionally supplement media with streptomycin was used) in a final volume of 100 µl culture medium per well, according to the media needs of the cells in a humidified atmosphere (e.g., 37°C, 5% CO_2) for an incubation period of 24 h.

After the incubation period for each well 0.5% plant extracts was added and then the micro-plates were incubated for 24 h in a humidified atmosphere (e.g., 37°C, 5% CO_2).

After the second incubation period, to each well was added 50 µl of 5 ml XTT labeling reagent with 0.1 ml electron coupling reagent (final XTT concentration 0.3mg/ml).

In vivo test

The sample consisted of forty-four adolescents (24 males and 20 females) attending to the Department of Paediatric Dentistry of the University of Naples "Federico II", Italy, ranging 12 to 18 years old.

The inclusion criteria are good general health (ASA I-II) and agreement to comply with study procedures. Oral status was determinate by clinical examination carried out by two professionals in the same room and using the same dental unit (so that all patients were examined under the same lighting conditions).

The exclusion criteria are use of antibiotics in the 14 days prior to the beginning of the study.

2 subjects of the 44 had used antibiotics for medical reasons in the 2-weeks before the study, and were excluded.

Furthermore, during the research period, two girls did not wish to continue their participation; therefore, the entire protocol was completed by 40 subjects.

Participation was voluntary. All parents, after receiving verbal and written explanations of the experimental protocol and the study aims, gave written informed consent.

Permission was received from the appropriate authorities. The protocol study was in accordance with the Helsinki Declaration of Human Rights and approved by the Ethical Committee of the School of Dentistry, University of Naples "Federico II", Italy.

A controlled random clinical study was carried out. The subjects were divided into 2 groups (Group A and Group B). Patients were randomly assigned to test and control groups using blocked randomization

from a computer-generated list.The group A was instructed to rinse with an experimental mouth-rinse containing P. lanceolata extracts for seven days and the group B received received the same regimen with a placebo solution.

Then salivary counts of *S. mutans* and *L. casei* were estimated with a chair-side test, containing two agar surfaces: the blue mitis-salivarius-agar with bacitracin was used to detect *mutans streptococci*, while the light culture medium, Rogosa agar, was used to evaluate *lactobacilli casei*, according to the manufacturer's instructions.

Salivary samples were taken from each patient in three stages and the levels of *S.mutans* and *L. casei* were determined at baseline (T0), at the fourth day of the experimental study (T1) and at the seventh day of the experimental period (T2).

No special dietary restrictions were imposed to the subjects and no tooth brushing was allowed for at least 1 h after having lunch and dinner.

Saliva was inoculated on a dip-slide with the selective media for *mutans streptococci* and *lactobacilli*. After adding a $NaHCO_3$ tablet to the tube, the dip-slides were immediately cultivated at 37°C for 48 hours. This tablet was able to release CO_2 when it came into contact with moisture, creating favourable conditions for bacterial growth.

The colonies were identified with the aid of a stereomicroscope with ×10 magnification and the density of the CFU (CFU/ml) was visually compared with the aid of a chart provided by the manufacturer.

Bacterial colonies were categorized as low (<10^5 colony forming unit (CFU)/ml of saliva) or high (> or = 10^5 CFU/ml).

a) Mouth-rinse preparation

Two different mouth-rinse formulations were prepared:

1) Experimental (Group A)

Experimental mouth-rinse was prepared with pulverized P. lanceolata leaves.

P. lanceolata was collected from the grassy areas of the Phlegrean territory (Italy).

The taxonomic identification of these plants was done by the Department of Biological Sciences / Section of Plant Biology, University of Naples "Federico II", Naples, Italy.

The leaves of Plantago were shade dried at 60 degree for 48 hours and then were ground to a fine powder. Two grams of powder were extracted using 20 ml of water, with centrifugation at 3000 r/min for 15 min, and then the supernatant was collected.

Solvent was then removed by evaporation [Özanet., 2007].

For each rinsing, 0.4 g of pulverized leaves was suspended in 10 ml of distilled water at 100 °C for 3 minutes. The preparation was left in infusion for 8 minutes then filtered and placed in containers of 10 ml. After this procedure the mouth-rinse was kept at room temperature.

2) Placebo (Group B)

Placebo mouth-rinse was prepared with distilled water colored with food dye.

Both mouth-rinses were put into hermetically sealed plastic bottles. The test and the control mouth-rinses, given in a randomized order, had a similar taste and consistency and were delivered in white bottles marked "A"or "B". The content was unknown to both the test and control group.

b) Experiment design –Group A

Twenty subjects were involved in this group. In the experimental period, after the collection of first saliva sample, all participants of group A were asked to rinse with 10 ml of the

experimental mouth-rinse for 60 seconds, three times a day (after breakfast, after lunch and before sleeping), after oral hygiene procedures, for seven days.

After the 4th and at the 7th days of treatment with mouth-rinse formulation, the salivary sample was re-collected and immediately incubated, in according to the step-by-step procedure above described, in order to calculate the density of the CFU (CFU/ml) of *streptococci mutans* and *Lactobacilli* for each subject during (T1) and immediately after (T2) the treatment.

All the subjects were, however, encouraged to maintain their normal oral hygiene habits.

c) Experiment design-Group B

20 subjects were involved in this group. After the first collection of saliva sample, the participants of group B were instructed to rinse with 10 ml of a placebo mouth-rinse without phenolic substances, for 1 minute, 3 times a day (after breakfast, after lunch and before sleeping) for seven days. Subsequent saliva samples were obtained on 4th (T1) and 7th day (T2) after the beginning of the study.

All the subjects were, however, encouraged to maintain their normal oral hygiene habits.

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

XTT assay

The cytotoxicity of plant extracts was evaluated by comparing the amount of soluble orange formazan of the fibroblasts cells in contact with the extract, with control cells without agents cultured in parallel using the same conditions with comparable media changes. The amount of soluble orange formazan salt formed in the sample is a direct expression of the number of cells

that remain viable and the presence of toxic substances is greatly enhanced by decreasing the activity of the enzyme.

XTT assay results are presented in figure 8.

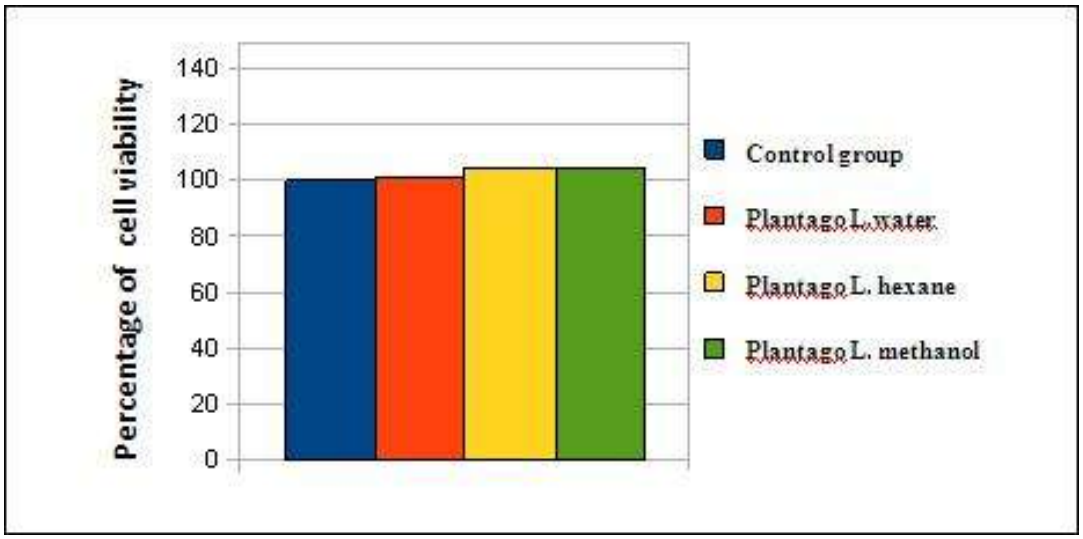

Figure 8: percentage of cell viability between cells treated with plant extracts and control cells.

On the basis of the made tests, plant extracts are not cytotoxic.

The amount of orange formazan salt produced by fibroblasts in contact with the extracts is almost comparable to that of the control group. In fact, while the viability of cells in the control group was 100%, the values range for cells treated with plant extracts were between 85% and 115% (Fig. 8).

In vivo test

Statistical analysis within the test group

Variations in *S. mutans* and *L. casei* density of the CFU (CFU/ml) at T0, T1, T2 for the test group were summarized in figures 9 and 10, respectively.

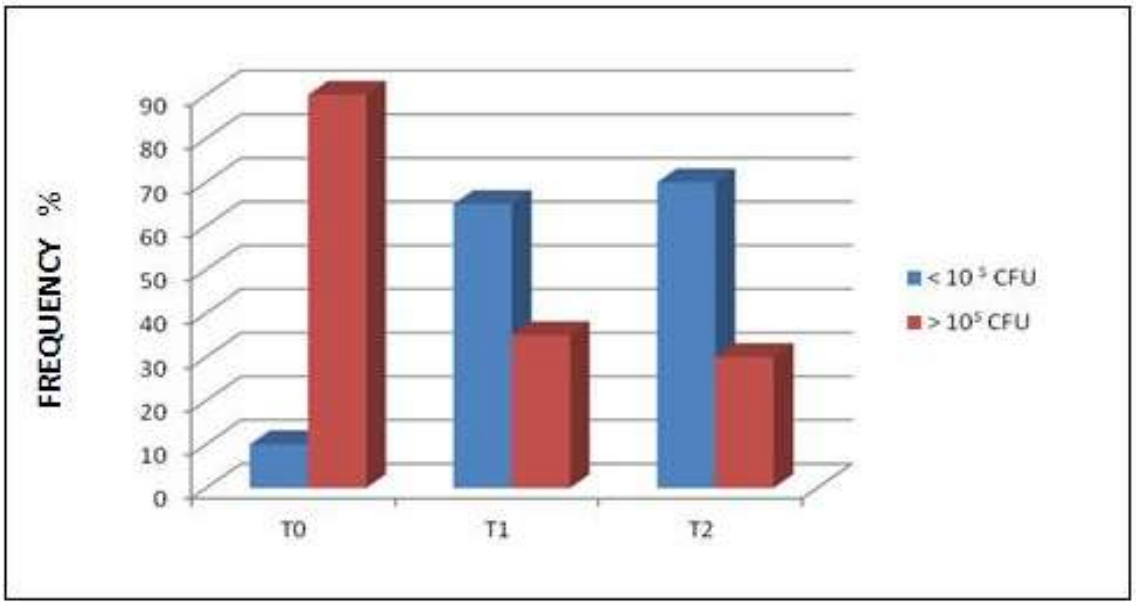

Figure 9: density of the *Streptococci mutans* CFU (CFU/ml) at T0, T1, T2 (test group).

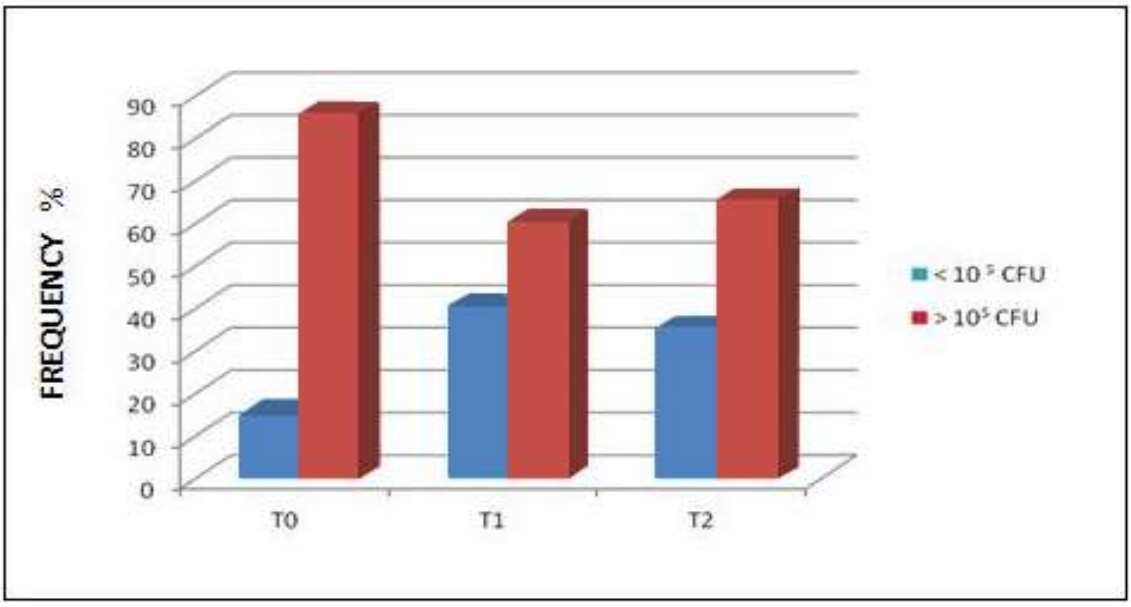

Figure 10: density of the *Lactobacilli casei* CFU (CFU/ml) at T0, T1, T2 (test group).

The differences in *S. mutans* CFU (CFU/ml) density between T0 and T1 and between T0 and T2 were statistically significant (both at $p<0.001$); between T1 and T2 they were not statistically significant.

The differences in *L. casei* CFU (CFU/ml) density between T0 and T1, T0 and T2, T1 and T2 were not statistically significant.

Statistical analysis within the control group

Variations in *S. mutans* and *L. casei* density of the CFU (CFU/ml) at T0, T1, T2 for the control group were represented in figures 4 and 5, respectively.

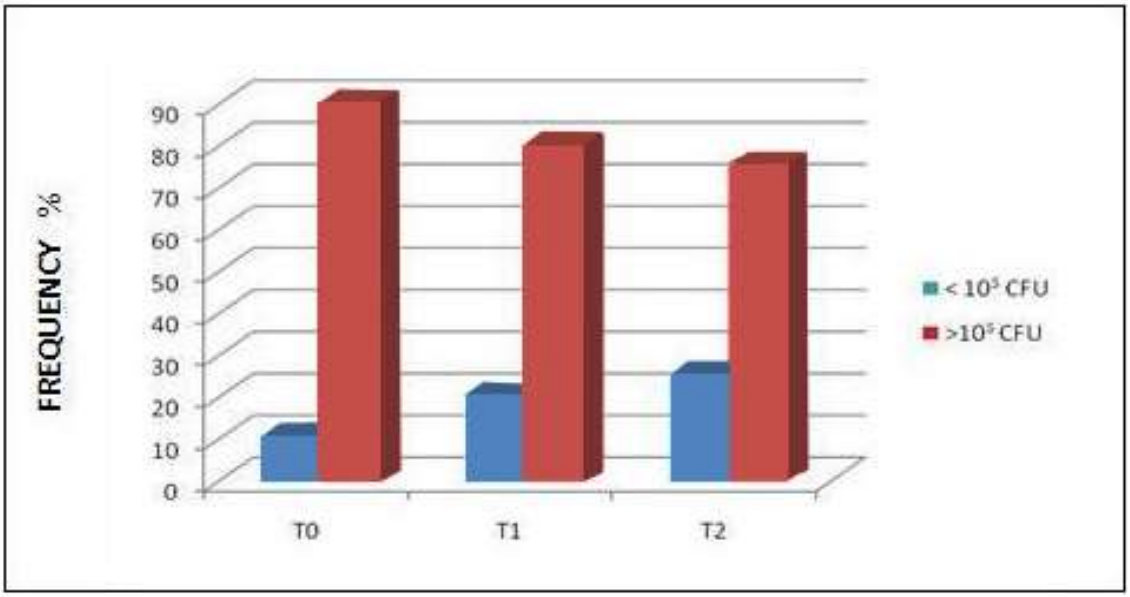

Figure 4: density of the *Streptococci mutans* CFU (CFU/ml) at T0, T1, T2 (control group).

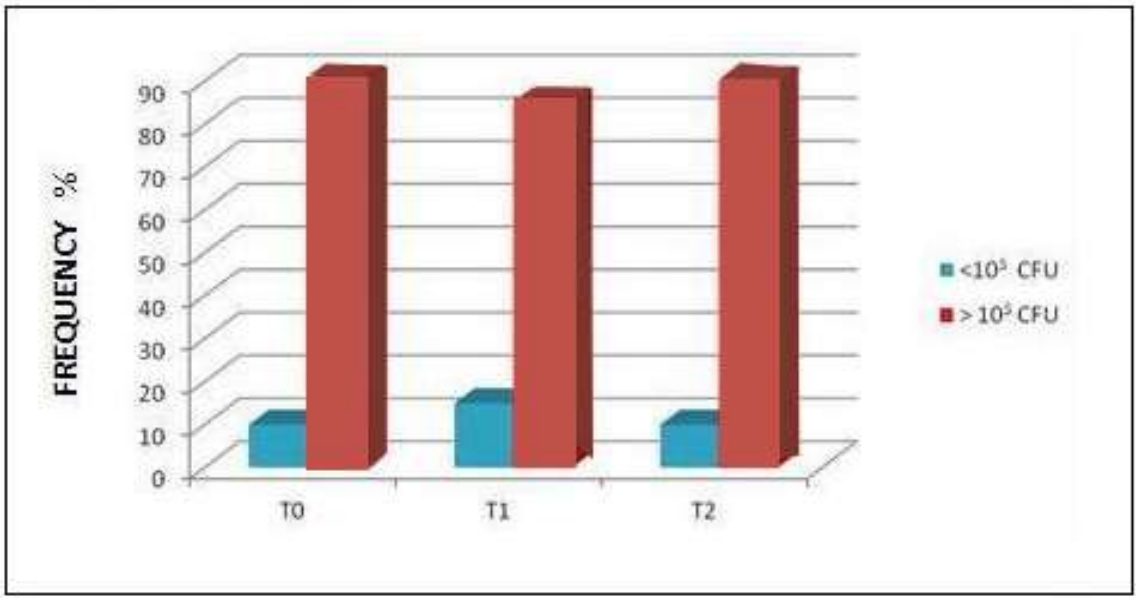

Figure 5: density of the *Lactobacilli casei* CFU (CFU/ml) at T0, T1, T2 (control group).

The differences in CFU (CFU/ml) density of *S. mutans* between T0 and T1, T0 and T2, T1 and T2 were not statistically significant, respectively.

The differences in CFU (CFU/ml) density of *Lactobacilli* between T0 and T1, T0 and T2, T1 and T2 were not statistically significant, respectively.

Statistical analysis between test and control groups

The differences in *S. mutans* CFU density between the test and control groups were not statistically significant at T0, but the differences between the test and control groups were statistically significant at T1and T2, respectively [T1: OR=0.095 (CI=0.021-0.440); T2: OR=0.048 (CI= 0.008-0.273)].

At T0, T1 and T2, the differences in *L. casei* CFU density between the test and control groups were not statistically significant, respectively.

DISCUSSION

The study was undertaken to investigate the short-term effect of mouth-rinse containing phenolic extracts from Plantago lanceolata on caries-associated microorganisms in saliva.

The novel approach used in the present research was the phenolic extracts utilized, since P. lanceolata mouth-rinse might represent a new option for the microbiological control of dental caries, being the active principle easily obtained, of low cost and showing beneficial effects.

Nowadays, given the incidence of oral disease, increased resistance by bacteria to antibiotics, adverse affects of some antibacterial agents currently used in dentistry and financial considerations in developing countries [Palombo, 2011], the utilization of new therapies for treatment of oral cavity diseases is of great importance. In fact, there is an urgent need for alternative prevention and treatment options that are safe, effective and economical.

On the basis of this consideration, P. lanceolata extract might be used as a further measure to reduce oral bacteria counts.

In fact, this in vivo study has shown that the subjects, undergoing to P. lanceolata mouth-rinse, presented a significant lowering of S*treptococci mutans* than the subjects undergoing to placebo mouth-rinse.

All subjects exhibited detectable levels of salivary *Streptococci mutans* and *Lactobacilli casei* at baseline and about over 90 per cent had levels $\geq 10^5$ CFU.

In the Group A with the experimental mouthrinse, the number of subjects with high *Streptococci mutans* count decreased from 90 to 30 per cent.

The high levels of *Streptococci mutans* did not decrease after use of Plantago mouthrinse in four of twenty studied subjects, possibly due to low compliance or a high sucrose intake, but other hypotheses, such as dissolution of the active principles, because polyphenols are highly unstable molecules that undergo oxidation, should not be discarded.

Regarding *L. casei*, a slight decrease in the density of the CFU (CFU/ml) was observed between the pre- and post -consumption samples in the test Group, although not statistically significant.

Apart from strain-specific differences, a possible explanation of poor reduction of *L. casei* respect *S. mutans* is that *S. mutans* normally grow on exposed surfaces, easy accessible for the

phenolic substrate, while *L. casei* are recovered in shed retentive areas with limited contact with the mouth-rinse.

No significant differences were observed between the baseline and the final samples both for *S. mutans* and *L. casei* counts in the control Group.

The reduction of the *Streptococci mutans* counts in the test Group respect to the control Group could be explained with the capability, in vitro, of phenolic substances present in the plant extract to inhibit the bacterial adherence to saliva-coated hydroxyapatite and/or inhibit the glucosyltransferases activity [Kashket et al., 1985; Ooshima et., 1993; Sakanaka et al.,1989; Smullenet al., 2007].

The adherence of bacterial cells to tooth surface is of great importance to the development of carious lesions, and the interference with some of the mechanisms of adherence can prevent dental caries. Furthermore, as suggested by Özan, phenolic substances present in plants extracts probably act on the microbial membrane or surface of the cell wall, causing structural and functional damage [Özan et al., 2007].

Researchers are currently interested in the promising perspectives that natural substances offer as alternatives for the control of caries disease in terms of antimicrobial response and lower associated risks.

The results of this study showed that daily usage of P. lanceolata mouth-wash for seven days decreased the *S. mutans* counts in saliva, instead a slight decrease, not statistically significant, was observed after the treatment for *Lactobacilli* counts in the test Group.

Further studies, particularly in vivo and in situ, are needed to establish conclusive evidence for the effectiveness of P. lanceolata extract against dental caries either alone or in combination with conventional therapies for improving oral health.

4) CONCLUSIONS

The results of in vitro study on a large sample of vulnerary plants of Italian flora has identified extracts that may find real application in the prevention of dental caries.

The in vivo study demonstrated that daily consumption of plants extract could reduce the salivary levels of mutans streptococci and lactobacilli, which are the most virulent cariogenic pathogens in the oral cavity.

These findings opening a promising avenue of clinical applications in the preparation of specific anti-cariogenic remedies

5) REFERENCES

Acs G, Lodolini G, Kaminski S, Cisneros GJ. Effect of nursing caries on body weight in a pediatric population. Pediatric Dentistry 1992;14(5):302-5.

Ajaib M, Khan Z, Khan N, Wahab M. Ethnobotanical studies on useful shrubs of Distric Kotli, Azad Jammu & Kashmir, Pakistan. Pak J Bot 2010;42(3):1407-1415.

Babpour E, Angaji SA, Angaji SM. Antimicrobial effects of four medicinal plants on dental plaque. J Med Plant Res 2009; 3(3):132-137.

Balz F, Jane VH. Antioxidant of tea polyphenols in vivo: evidence from animal studies. J Nutr 2003;133:3275S-3284S.

Biesalski HK, Dragsted LO, Elmadfa I, Grossklaus R, Müller M, Schrenk D, Walter P, Weber P. Bioactive compounds: definition and assessment of activity. Nutrition 2009;25(11-12):1202-5.

Birkeland JM, Haugejorden O, von der Fehr FR. Analyses of the caries decline and incidence among Norwegian adolescents 1985-2000. Acta Odontol Scand 2002;60(5):281-9.

Bodet C, Grenier D, Chandad F, Ofek I, Steinberg D, Weiss EI. Potential oral health benefits of cranberry. Crit Rev Food Sci Nutr 2008;48:672-80.

Brantner A, Grein E. Antibacterial activity of plant extracts used externally in traditional medicine. J Ethnopharmacol 1994;44(1):35-40.

Brighenti FL, Luppens SB, Delbem AC, Deng DM, Hoogenkamp MA, Gaetti-Jardim EJr et al. Effect of Psidium cattleianum leaf extract on Streptococcus mutans viability, protein expression and acid production. Caries Res 2008;42:148-54.

Brusick DJ. A critical review of the genetic toxicity of steviol and steviol glycosides. Food Chem Toxicol 2008;46 Suppl 7:S83-91.

Cabrera C, Artacho R, Giménez R. Beneficial effects of green tea--a review. J Am Coll Nutr 2006;25(2):79-99.

Campus G, Condò SG, Di Renzo G, Ferro R, Gatto R, Giuca MR et al. National Italian Guidelines for caries prevention in 0 to 12 years-old children. Eur J Paediatr Dent 2007;8(3):153-9.

Campus G, Solinas G, Matti M, Castiglia P, Strohmenger L. The Italian Study Group on Children Oral Health. Caries experience in 12 year-olds: the Italian National Pathfinder on children oral health. Caries Res 2006;40:330-32.

Canales M, Hernandez T, Caballero J, Romo de Vivar A, Avila G, Duran A et al. Informant consensus factor and antibacterial activity of medicinal plants used by the people of San Rafael, Coxcatlan, Puebla, Mexico. J Ethnopharmacol 2005; 97:429-439.

Ceunen S, Geuns JM. Steviol glycosides: chemical diversity, metabolism, and function. J Nat Prod 2013;76(6):1201-28.

Conti F, Alessandrini A, Bacchetta G. Updating of the checklist of the Italian vascular flora. Natura Vicentina 2006;10:5-74.

Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999;12(4):564-82.

Crossner CG, Claesson R, Johansson T. Presence of mutans streptococci and various types of lactobacilli in interdental spaces related to development of proximal carious lesions. Scand J Dent Res 1989;97(4):307-15.

Cypriano S, Hoffman RH, de Sousa Mda L, Wada RS. Dental caries experience in 12-year-old schoolchildren in southeastern Brazil. J. Appl. Oral Sci 2008;16(4):286-92.

Das S, Das AK, Murphy RA, Punwani IC, Nasution MP, Kinghorn AD. Evaluation of the cariogenic potential of the intense natural sweeteners stevioside and rebaudioside A. CariesRes 1992;26(5):363-6.

De Natale A, Pezzatti GB, Pollio A. Extending the temporal context of ethnobotanical database: the case-study of the Campania region (southern Italy). J Ethnobiol Ethnomed 2009;19:5-7.

Debnath M. Clonal propagation and antimicrobial activity of an endemic medicinal plant Stevia rebaudiana. J. Med. Plants Res 2008;2(2):045-051.

Dziedzic A, Kubina R, Wojtyczka RD, Kabała-Dzik A, Tanasiewicz M, Morawiec T. The antibacterial effect of ethanol extract of polish propolis on mutans streptococci and lactobacilli isolated from saliva. Evid Based Complement Alternat Med 2013.

Ferrazzano GF, Amato I, Ingenito A, De Natale A, Pollio A. Anti-cariogenic effects of polyphenols from plant stimulant beverages (cocoa, coffee, tea). Fitoterapia 2009;80(5):255-62.

Ferrazzano GF, Scaravilli MS, Ingenito A. Dental and periodontal health status in Campanian children and relation between caries experience and socio-economic behavioural factors. Eur J Paediatr Dent 2006;7(4):174-8.

Forrest RD. Early history of wound treatment. J R Soc Med 1982;75(3)198-205.

Freedman ML, Tanzer JM. Dissociation of plaque formation from glucan-induced agglutination in mutants of Streptococcus mutans. Infect Immun 1974;10(1):189-96.

Gardana C, Simonetti P, Canzi E, Zanchi R, Pietta P. Metabolism of stevioside and rebaudioside A from Stevia rebaudiana extracts by human microflora. J Agric Food Chem 2003;51(22):6618-22.

Geuns JMC, Augustijns P, Mols R, Buyse JG, Driessen B. Metabolism of stevioside in pigs and intestinal absorption characteristics of stevioside, rebaudioside A and steviol. Food Chem Toxicol 2003;41:1599-1607.

Giacaman RA, Campos P, Muñoz-Sandoval C, Castro RJ. Cariogenic potential of commercial sweeteners in an experimental biofilm caries model on enamel. Arch Oral Biol 2013;58(9):1116-22.

Guarrera P.M. Usi e tradizioni della flora italiana. Medicina popolare ed etnobotanica. Aracne Press, Rome 2006;1-432.

Guida M, Casoria P, Guarino C, Melluso G. Preliminary report on antimicrobial activity of Helichrysum litoreum Guss. Boll Chim Farm 1999;138(7):369-373.

Gunsolley JC. A meta-analysis of six-month studies of antiplaque and antigingivitis agents. J Am Dent Assoc 2006;137(12):1649-57.

Hamilton-Miller JM. Anti-cariogenic properties of tea (Camellia sinensis). J Med Microbiol 2001;50(4):299-302.

Hamissi J, Ramezani GH, Ghodousi A. Prevalence of dental caries among high school attendees in Qazvin, Iran. J Indian Soc Pedod Prev Dent 2008;26 Suppl2:S53-5.

Hattori M, Kusumoto IT, Namba T, Ishigami T, Hara Y. Effect of tea polyphenols on glucan synthesis by glucosyltransferase from Streptococcus mutans. Chem Pharm Bull 1990;38(3):717-20.

Hernandez T, Canales M, Duran A, Garcia A, Avila JG, Hernandez-Portilla L et al. Variation in the hexanic extract composition of Lippa graveolens in an arid zone from Mexico: environmental influence or true chemotypes? The Open Plant Science Journal 2009;3:29-34.

Hirasawa M, Takada K, Otake S. Inhibition of acid production in dental plaque bacteria by green tea catechins. Caries Res 2006;40:265-70.

Hobdell M, Petersen P.E, Clarkson J, Johnson N. Global goals for oral health 2020. Int Dent J 2003; 53:285-8.

Honkanen P. Consumer acceptance of (marine) functional food. Marine Functional Food 2009;1:141-154.

Houghtonn PJ, Hylands PJ, Mensah AY, Hensel A, Deters AM. In vitro tests and ethnopharmacological investigations: wound healing as an example. J Ethnopharmacol 2005;100(1-2):100-7.

Ikigai H, Nakae T, Hara Y, Shimamura T. Bactericidal catechins damage the lipid bilayer. Biochim Biophys Acta 1993;1147(1):132-6.

Ivanova I, Gerova D, Chervenkov T, Yankova T. Polyphenols and antioxidant capacity of Bulgarian medicinal plants. J Ethnoparmacol 2005; 96(1-2):145-150.

Jones C, Woods K, Whittle G, Worthington H, Taylor G. Sugar, drinks, deprivation and dental caries in 14-year-old children in the north west of England in 1995.Community Dent Health 1999;16(2):68-71.

Kashket S, Paolino VJ, Lewis DA, van Houte J. In-vitro inhibition of glucosyltransferase from the dental plaque bacterium Streptococcus mutans by common beverages and food extracts. Arch Oral Biol 1985;30(11-12):821-6.

Kashket S, Paolino VJ. Inhibition of salivary amylase by water-soluble extracts of tea. Arch Oral Biol 1988;33(11):845-6.

Kim HS. Do not put too much value on conventional medicines. Journal of Ethnopharmacology 2005;100(1-2):37-39.

Koo H, Rosalen PL, Cury JA, Park YK, Bowen WH. Effects of compounds found in propolis on Streptococcus mutans growth and on glucosyltransferase activity. Antimicrob Agents Chemother 2002;46(5):1302-9.

Koyama E, Kitazawa K, Ohori Y, Izawa O, Kakegawa K, Fujino A et al. In vitro metabolism of the glycosidic sweeteners, stevia mixture and enzymatically modified stevia in human intestinal microflora. Food Chem Toxicol 2003;41(3):359-74.

Krishnan R, Maru GB. 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.

Kutsch VK, Young DA. New directions in the etiology of dental caries disease. J Calif Dent Assoc 2011;39(10):716-21.

Lenander-Lumikari M, Loimaranta V. Saliva and dental caries. Adv Dent Res 2000;14:40-7.
Li JY, Zhan L, Barlow J, Lynch RJ, Zhou XD, Liu TJ. Effect of tea polyphenol on the demineralization and remineralization of enamel in vitro. Sichuan Da Xue Xue Bao Yi Xue Ban 2004;35(3):364-6.

Loesche WJ, Syed SA. The predominant cultivable flora of carious plaque and carious dentine. Caries Res 1973;7(3):201-16.

Loesche WJ. Role of Streptococcus mutans in human dental decay. Microbiol Rev 1986;50(4):353-80.

Luczaj W, Skrzydlewska E. Antioxidative properties of black tea. Prev Med 2005;40(6):910-8.

Marsh P. D. Microbial ecology of dental plaque and its significance in health and disease. Adv Dent Res 1994;8(2):263-71.

Marthaler TM. Changes in dental caries 1953-2003. Caries Res 2004;38(3):173-81.

Matsumoto M, Minami T, Sasaki H, Sobue S, Hamada S, Ooshima T. Inhibitory effects of oolong tea extract on caries-inducing properties of mutans streptococci. Caries Res 1999;33(6):441-5.

Mohd-Radzman NH, Ismail WI, Adam Z, Jaapar SS, Adam A. Potential Roles of Stevia rebaudiana Bertoni in Abrogating Insulin Resistance and Diabetes: A Review. Evid Based Complement Alternat Med 2013;2013:718049. Review.

Nikiforov AI, Eapen AK. A 90-day oral (dietary) toxicity study of rebaudioside A in Sprague-Dawley rats. Int J Toxicol 2008;27(1):65-80.

Nikitina VS, Kuz'mina Llu, Melent'ev AI, Shendel' GV. Antibacterial activity of polyphenolic compounds isolated from plants of Geraniaceae and Rosaceae families. Appl Biochem Microbiol 2007; 43(6):705-712.

Nostro A, Cannatelli MA, Crisafi G, Musolino AD, Procopio F, Alonzo V. Modifications of hydrophobicity, in vitro adherence and cellular aggregation of Streptococcus mutans by Helichrysum italicum extract. Lett Appl Microbiol 2004;38(5):423-7.

Ooshima T, Minami T, Aono W, Izumitani A, Sobue S, Fujiwara T et al. Oolong tea polyphenols inhibit experimental dental caries in SPF rats infected with mutans streptococci. Caries Res 1993; 27(2):124-129.

Otake S, Makimura M, Kuroki T, Nishihara Y, Hirasawa M. Anticaries effects of polyphenolic compounds from Japanese green tea. Caries Res 1991;25(6):438-43.

Özan F, Sümer Z, Polat ZA, Er K, Ozan U, Deger O. et al. Effect of mouthrinse containing propolis on oral microorganisms and human gingival fibroblasts. Eur J Dent 2007;1(4):195-201.

Palombo EA. Traditional medicinal plant xxtracts and natural products with activity against oral bacteria: potential application in the prevention and treatment of oral diseases. Evid Based Complement Alternat 2011;2011:680354.

Panizzi L, Flamini G, Cioni PL, Morelli I. Composition and antimicrobial properties of essentialoils of four Mediterranean Lamiaceae. J Ethnopharmacol 1993;39(3):167-70.

Pepeljnjak S, Kosalec I, Kalodera Z, Blazevic N. Antimicrobial activity of Juniper berry essential oil (Juniperus communis L., Cupressaceae). Acta Pharm 2005; 55(4):417-422.

Philippe RN, De Mey M, Anderson J, Ajikumar PK. Biotechnological production of natural zero-calorie sweeteners. Curr Opin Biotechnol 2014;3(26C):155-161.

Pieper K, Schulte AG. The decline in dental caries among 12-year-old children in Germany between 1994 and 2000. Community Dent Health 2004;21(3):199-206.

Sakanaka S, Kim M, Taniguchi M et al. Antibacterial substances in Japanese green tea extract against Streptococcus mutans, a cariogenic bacterium. Agric Biol Chem 1989;53:2307-2311.

Sang S, Lambert JD, Tian S, Hong J, Hou Z, Ryu JH et al. Enzymatic synthesis of tea theaflavin derivatives and their anti-inflammatory and cytotoxic activities. Bioorg Med Chem 2004;12(2):459-67.

Santhosh BP, Jethmalani P, Shashibhushan KK, Subba Reddy VV. Effect of casein phosphopeptide-amorphous calcium phosphate containing chewing gum on salivary concentration of calcium and phosphorus: an in-vivo study. J Indian Soc Pedod Prev Dent 2012;30(2):146-50.

Sarac N, Ugur S A. Antimicrobial activities and usage in folkloric medicine of some Lamiaceae species growing in Mucla, Turkey. Eur Asian J Biosc 2007;4:28-37.

Sati SC, Joshi S. Antimicrobial potential of leaf extracts of Juniperus communis L. from Kumanu Himalaya. Afr J Microbiol Res 2010;4(12):1291-1294.

Sato M, Fujiwara S, Tsuchiya H, Fujii T, Inuma M, Tosa H, Ohkawa Y. Flavones with antibacterial activity against cariogenic bacteria. J Ethnopharmacol 1996;54:171-176.

Scalbert A, Manach C, Morand C, Rémésy C, Jiménez L. Dietary polyphenols and the prevention of diseases. Crit Rev Food Sci Nutr 2005;45(4):287-306.

Seminario A, Broukal Z, Ivancaková R. Mutans streptococci and the development of dental plaque. Prague Med Rep 2005;106(4):349-58.

Shahidi F. Nutraceuticals and functional foods: Whole versus processed foods. Trends Food Sci Technol 2009;20:376-387.

Shivanna N, Naika M, Khanum F, Kaul VK. Antioxidant, anti-diabetic and renal protective properties of Stevia rebaudiana. J Diabetes Complications 2013;27(2):103-13.

Signoretto C, Burlacchini G, Bianchi F, Cavalleri G, Canepari P. Differences in microbiological composition of saliva and dental plaque in subjects with different drinking habits. New Microbiol 2006;29(4):293-302.

Lordan S, Ross RP, Stanton C. Marine Bioactives as Functional Food Ingredients: Potential to Reduce the Incidence of Chronic Diseases. Mar Drugs 2011;9(6):1056-100.

Siró I, Kápolna E, Kápolna B, Lugasi A. Functional food. Product development, marketing and consumer acceptance-a review. Appetite 2008;51(3)**:**456–67.

Smullen J, Koutsou GA, Foster HA, Zumbé A, Storey DM. The Antibacterial Activity of Plant Extracts Containing Polyphenols against Streptococcus mutans. Caries Res 2007;41(5):342-9.

Staat RH, Gawronski TH, Cressey DE, Harris RS, Folke LE. Effects of dietary sucrose levels on the quantity and microbial composition of human dental plaque. J Dent Res 1975;54(4):872-80.

Strauss S.The perfect sweetener? Technol. Rev 1995;98:18-20.

Sun J, Wang L, Waring MA, Wang C, Woodman KK, Sheil AG. Simple and reliable methods to assess hepatocyte viability in bioartificial liver support system matrices. Artif Organs 1997;21(5):408-413.

Süzgeç S, Meriçli AH, Houghton PJ, Cubukçu B. Flavonoids of Helichrysum compactum and their antioxidant and antibacterial activity. Fitoterapia 2005;76(2):269-272.

Taguri T, Tanaka T, Kouno I. Antimicrobial activity of 10 different plant polyphenols against bacteria causing food-borne disease. Biol Pharm Bull 2004;27(12):1965-9.

Tanzer JM, Freedman ML, Fitzgerald RJ, Larson RH. Diminished virulence of glucan synthesis-defective mutants of Streptococcus mutans. Infect Immun 1974;10(1):197-203.

Tinanoff N, Kanellis MJ, Vargas CM. Current understanding of the epidemiology mechanisms, and prevention of dental caries in preschool children. Pediatr Dent 2002;24(6):543-51.

Venugopal, V. Marine Habitat and Resources. In Marine Products for Healthcare: Functional and Bioactive Nutraceutical Compounds from the Ocean. Mar Drugs 2011;9(6):1056-1100.

Vogel G, Strohmenger L, Ferrari PA, Weinstein R, Carrassi A, Landenna P. Epidemiologic surveying of dental-periodontal disease in the compulsory school population of the city of Milan. Mondo Odontostomatol 1979;21(5):7-44.

Weiss E, Rosenberg M, Judes H, Rosenberg E. Cell-surface hydrophobicity of adherent oral bacteria. Curr Microbiol 1982;7(2):125-128.

Williams LD, Burdock GA. Genotoxicity studies on a high-purity rebaudioside A preparation. Food Chem Toxicol 2009;47(8):1831-6.

Wölwer-Rieck U. The leaves of Stevia rebaudiana (Bertoni), their constituents and the analyses thereof: a review. J Agric Food Chem 2012;60(4):886-95.

Xiao Y, Liu T, Zhan L, Zhou X. The effects of tea polyphenols on the adherence of cariogenic bacterium to the collagen in vitro. Hua Xi Kou Qiang Yi Xue Za Zhi 2000;18(5):340-2.

Yadav SK, Guleria P. Steviol glycosides from Stevia: biosynthesis pathway review and their application in foods and medicine. Crit Rev Food Sci Nutr 2012;52(11):988-98.

Yamane T, Nakatani H, Kikuoka N, Matsumoto H, Iwata Y, Kitao Y et al. Inhibitory effects and toxicity of green tea polyphenols for gastrointestinal carcinogenesis. Cancer 1996;77(8Suppl):1662-7.

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.

Table 1: Weighing and scoring of 72 vulnerary plants of Italian flora.

	Established vulnerary use	Used against oral affections	Tested antimicrobial activity	Species distribution	Final score	Inhibitory activity against bacteria of oral flora
Plantago major L. subsp. *major* (DSB52) Plantaginaceae	2	3	3	1	**9**	–
Rosmarinus officinalis L. (DSB56) Lamiaceae	3	2	3	1	**9**	–
Sambucus nigra L. (DSB58) Caprifoliaceae	2	3	3	1	**9**	+
Malva sylvestris L. subsp. *sylvestris* (DSB45) Malvaceae	2	3	2	1	**8**	–
Bellis perennis L. (DSB10) Asteraceae	2	2	2	1	7	++
Cupressus sempervirens L. (DSB20) Cupressaceae	1	3	2	1	7	++
Juniperus communis L. (DSB37) Cupressaceae	1	3	2	1	7	+++
Lavandula angustifolia Mill. subsp. *angustifolia* (DSB39) Lamiaceae	1	2	3	1	7	++
Mentha aquatica L. s.l. (DSB46) Lamiaceae	2	2	2	1	7	–
Thymus vulgaris L. s.l. (DSB69) Lamiaceae	1	3	3	0	7	++

Lamiaceae						
Arctium lappa L. (DSB5) Asteraceae	1	1	3	1	**6**	–
Ceterach officinarum Willd. s.l. (DSB17) Aspleniaceae	3	1	1	1	**6**	++
Cotinus coggygria Scop. (DSB19) Anacardiaceae	1	2	2	1	**6**	+++
Equisetum arvense L. s.l. (DSB25) Equisetaceae	1	2	2	1	**6**	–
Eucalyptus globulus Labill. (DSB29) Rutaceae	1	2	2	1	**6**	+
Gentiana lutea L. s.l. (DSB32) Gentianaceae	2	2	2	0	**6**	+
Helichrysum litoreum Guss (DSB34) Asteraceae	1	1	3	1	**6**	+++
Oxalis corniculata L. (DSB48) Oxalidaceae	1	1	3	1	**6**	–
Rumex crispus L. (DSB57) Polygonaceae	1	3	1	1	**6**	–
Verbena officinalis L. (DSB 72) Verbenaceae	1	2	2	1	**6**	+
Adiantum capillus-veneris L. (DSB2) Adiantaceae	0	1	3	1	5	–
Alliaria petiolata (M. Bieb.) Cavara et Grande (DSB4) Brassicaceae	3	1	0	1	5	–
Artemisia annua L. (DSB6) Asteraceae	0	2	2	1	5	–

Asteraceae *Asphodelus ramosus* L. subsp. *ramosus* (DSB7) Asphodelaceae	1	1	2	1	5	–
Calamintha nepeta (L.) Savi subsp. *nepeta* (DSB12) Lamiaceae	1	1	2	1	5	–
Calystegia silvatica (Kit.) Griseb. (DSB13) Convolvulaceae	3	1	0	1	5	–
Cynodon dactylon (L.) Pers. (DSB22) Poaceae	1	1	2	1	5	–
Cynoglossum creticum Mill. (DSB23) Boraginaceae	1	2	1	1	5	+
Equisetum telmateia Ehrh. (DSB27) Equisetaceae	1	1	2	1	5	–
Erigeron canadensis L. (DSB28) Asteraceae	2	1	1	1	5	–
Geranium purpureum Vill. (DSB33) Geraniaceae	2	1	1	1	5	–
Heliotropium europaeum L. (DSB35) Boraginaceae	1	2	1	1	5	–
Hypericum perforatum L. (DSB36) Clusiaceae	1	1	2	1	5	+
Laurus nobilis L. (DSB38) Lauraceae	1	1	2	1	5	–
Lavandula dentata L. (DSB40) Lamiaceae	0	2	2	1	5	+
Micromeria						

Micromeria juliana (L.) Benth. ex Rchb. (DSB47) Lamiaceae	1	1	2	1	5	–
Parietaria judaica L. (DSB49) Urticaceae	2	2	0	1	5	–
Rosa canina L. (DSB55) Rosaceae	1	2	1	1	5	+
Scrophularia nodosa L. (DSB59) Scrophulariaceae	1	1	2	1	5	–
Silene italica (L.) Pers. s.l. (DSB60) Caryophyllaceae	2	1	1	1	5	–
Sonchus asper (L.) Hill subsp. *glaucescens* (Jord.) Ball (DSB64) Asteraceae	2	1	1	1	5	–
Taraxacum officinale Weber (DSB67) Asteraceae	1	2	1	1	5	–
Verbascum thapsus L. subsp. *thapsus* (DSB73) Scrophulariaceae	1	2	2	0	5	–
Achillea millefolium L. subsp. *millefolium* (DSB1) Asteraceae	1	1	1	1	4	–
Asplenium onopteris L. (DSB8) Aspleniaceae	1	1	1	1	4	–
Asplenium trichomanes L. s.l. (DSB9) Aspleniaceae	1	1	1	1	4	–
Borago officinalis L.						

officinalis L. (DSB11) Boraginaceae	1	1	1	1	4	–
Capparis spinosa L. subsp. *rupestris* (Sm.) Nyman (DSB15) Capparaceae	1	1	1	1	4	–
Cichorium intybus L. s.l. (DSB18) Asteraceae	0	1	2	1	4	–
Equisetum hyemale L. (DSB26) Equisetaceae	0	0	3	1	4	+++
Fumaria capreolata L. subsp. *capreolata* (DSB30) Fumariaceae	1	1	1	1	4	–
Lavatera cretica L. (DSB41) Malvaceae	1	1	1	1	4	–
Ligustrum vulgare (DSB42) Oleaceae	1	1	1	1	4	–
Phyllitis scolopendrium (L.) Newman subsp. *scolopendrium* (DSB50) Aspleniaceae	1	1	1	1	4	++
Smilax aspera L. (DSB63) Smilacaceae	1	1	1	1	4	–
Stachys sylvatica L. (DSB65) Lamiaceae	0	1	2	1	4	–
Stellaria media (L.) Vill. s.l. (DSB66) Caryophyllaceae	1	1	1	1	4	–
Teucrium fruticans L. subsp. *fruticans* (DSB68) Lamiaceae	1	0	2	1	4	–
Urtica						

Urtica membranacea Poir. ex Savigny (DSB70) Urticaceae	1	1	1	1	4	–
Valeriana officinalis L. (DSB71) Valerianaceae	1	1	2	0	4	+
Viburnum tinus L. subsp. *tinus* (DSB74) Caprifoliaceae	1	1	1	1	4	–
Zea mays L. (DSB75) Poaceae	1	1	1	1	4	–
Centranthus ruber (L.) DC. subsp. *rubber* (DSB16) Valerianaceae	1	1	0	1	3	–
Cymbalaria muralis Gaertn., B. Mey. et Scherb s.l. (DSB21) Scrophulariaceae	1	0	1	1	3	–
Linaria vulgaris Mill. subsp. *vulgaris* (DSB43) Scrophulariaceae	1	1	0	1	3	–
Silene latifolia Poir. subsp. *alba* (Mill.) Greuter et Burdet (DSB61) Caryophyllaceae	0	1	1	1	3	–
Silene vulgaris (Moench) Garcke s.l (DSB62) Caryophyllaceae	0	1	1	1	3	–
Gentiana cruciata L. s.l. (DSB31) Gentianaceae	0	1	1	0	2	–
Robinia pseudoacacia L. (DSB54) Fabaceae	1	0	0	1	2	–

0.3%

Antibacterial activity is expressed as mean (±standard deviation) zones of inhibition (mm). A = 12.5 mg/mL, B = 25 mg/mL, C = 50 mg/mL. W = water, E = ethanol, H = hexane.

Table 2 – Antibacterial activity, expressed as mean (± standard deviation) zones of inhibition (mm), of plant extracts towards S. mutans, S. sobrinus, Actinomyces viscosus, and L. casei. A = 12.5 mg/mL B = 25 mg/mL C = 50 mg/mL. W = water, E = ethanol, H = hexane.

	E			9,3±0,4			10,1±0,3			9,3±0,4			
	H		10,8±0,4	11,8±0,4									
Equisetum hyemale	E			10±0			13±0					13,1±0,3	15,8±0,4
Juniperus communis	H		10,1±0,3	11,8±0,4	9±0	10,1±0,3	13,1±0,3	10±0	10,1±0,3	11,8±0,4			
Helichrysum litoreum	E				9±0	11,8±0,4	14±0	10,2±0,4	11,8±0,4	18±0			
Phyllitis scolopendrium	E				11,8±0,4	14,3±0,5	16±0				11,8±0,4	15±0	17±0
Bellis perennis	E	10,1±0,3	12±0	15,8±0,4									10,8±0,4
Ceterach officinarum	E		9,1±0,3	11,8±0,4									10±0
Thymus vulgaris	E		11,8±0,4	14±0						13,1±0,3			
Cupressus sempervirens	E									9,3±0,4			10,1±0,3
Lavandula angustifolia	E			11,8±0,4									12±0
Gentiana lutea	E							9±0	11,8±0,4	14,3±0,5			
Rosa canina	E					11,8±0,4	14,7±0,5						
Artemisia annua	E									10±0			
Cynoglossum creticum	E												13±0
Eucalyptus globulus	E			10±0									
Hypericum perforatum	W			10,8±0,4									
Lavandula dentata	E			13±0									
Sambucus nigra	E									11,8±0,4			
Valeriana officinalis	W			10,1±0,3									
Verbena officinalis	W			10,1±0,3									
Triclosan 0.3%		15±0			16±0			20±0			17±0		

Table 3: MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) of more effective plant extracts against S. mutans, S. sobrinus, A. viscosus, and L. casei.

		S. sobrinus		S. mutans		A. viscosus		L. casei	
		MIC90	MBC	MIC90	MBC	MIC90	MBC	MIC90	MBC
	W	12.5	50	50	100	50	100	50	100
Cotinus coggygria	M	25	100	50	100	25	100	100	100
	H	50	100	>100	>100	>100	>100	>100	>100
Equisetum hyemale	M	100	100	>100	>100	>100	>100	>100	>100
Juniperus communis	H	>100	>100	>100	>100	>100	>100	>100	>100
Helichrysum litoreum	M	>100	>100	5	25	25	50	>100	>100
Phyllitis scolopendrium	M	>100	>100	>100	>100	>100	>100	>100	>100

MIC and MBC are expressed in mg/ml. W = water, E = ethanol, H = hexane