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TESI

"ANTI-MICROBIAL EFFECTS OF PHENOLIC EXTRACTS ON

CARIOGENIC ORAL BACTERIA: EXPERIMENTAL STUDY"

Coordinatore   **Candidata**
Prof. Sandro Rengo   **Dott.ssa Ivana Amato**

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SCIENTIFIC BACKGROUND

Today, polyphenols occupy a unique place in science as the only class of bioactive natural products that the general public is aware of has certainly heard about as a consequence of their presence in plant-derived foods and beverages and their inclusion in the formulations of well-marketed cosmetic [Grollier et al., 2009; Bernaert and Allegaert, 2009; Rao et al., 2010] and para-pharmaceutical products [Vercauteren, 2009; Jiang, 2009].

Polyphenols constitute one of the most common and widespread groups of substances in flowering plants, occurring in all vegetative organs as well as in flowers and fruits. They are considered secondary metabolites involved in the chemical defence of plants against predators and in plant-plant interference. Several thousand plant polyphenols are known, encompassing a wide variety of molecules that contain at least one aromatic ring with one or more hydroxyl groups in addition to other substituents. The biological properties of polyphenols include antioxidant [Luczaj and Skrzydlewska, 2005], anticancer [Krishnan and Maru, 2004], and anti-inflammatory [Sang et al., 2004] effects.

Emerging findings suggest a variety of potential mechanisms of action by which polyphenols may prevent disease, such as the inhibition of bacterial replication enzymes, the induction of apoptosis in tumour cells, the stimulation of monocytes/macrophages to produce cytokines, and the stimulation of myeloperoxidase-dependent iodination of neutrophils [Sakagami et al., 1999]. The antimicrobial effects of polyphenols have also been widely reported for their ability to inactivate bacterial toxins, and there is an increasing interest in this topic because plant phenols could represent a source of new anti-infective agents against antibiotic-

resistant human pathogens. Today, dental caries are still one of the most common diseases in the world. The results of multi-variable modelling support the hypothesis that bacterial infection is important in the aetiology of dental caries [Milgrom et al., 2000]. The central role of the mutans streptococci in the initiation of caries on smooth surfaces and fissures of crowns of teeth suggests their role in the induction of root-surface caries [Tanzer et al., 2001].

Classification of polyphenols

The empirical classification of plant polyphenols as molecules having a "tanning" action led to their being referred to in the early literature as "vegetable tannins". Haslam proposed the first comprehensive definition of the term "polyphenol", attributing it exclusively to water-soluble phenolic compounds having molecular masses of 500 to 3000-4000 Da and possessing 12 to 16 phenolic hydroxyl groups and 5 to 7 aromatic rings per 1000 Da [Haslam et al., 1992]. The original definition of "polyphenols" has broadened considerably over the years to include many much simpler phenolic structures. They encompass several classes of structurally-diverse entities that are essentially all biogenerated through either the shikimate/phenylpropanoid or the "polyketide" acetate/malonate secondary metabolic pathways, or both [Manitto, 1981].

Fig. 1: Polyphenols classification
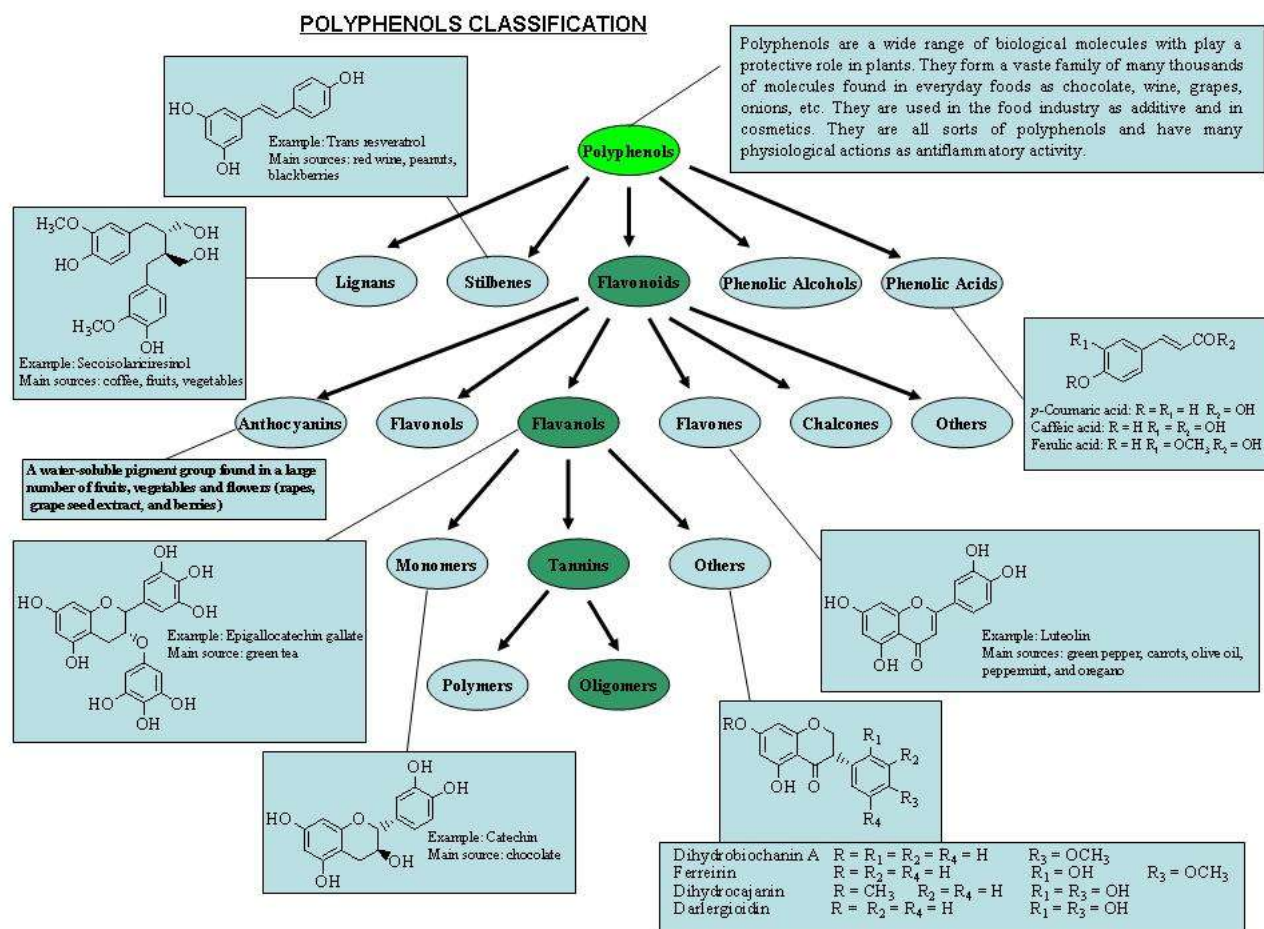
Some members of this huge class of natural products (> 8000 structures), usually bearing two mono- to trihydroxyphenyl units, can serve as precursors to oligo- and polymeric phenolic systems. The general phenylpropanoid metabolism furnishes a series of hydroxycinnamic acids (C_6-C_3) differing from one another by the number of hydroxy and methoxy groups on their phenyl units (i.e., ferulic acid, caffeic acid). These monophenolic carboxylic acids are often found esterified to polyols. Through hydration, esterification, and phenolic oxidative coupling reactions, caffeic acid also gives rise to oligomeric structures. The phenylpropanoid/acetate hybrid metabolic pathway leads to another important class of phenolic substances, the polyhydroxystilbenes (C_6-C_2-C_6). The most famous example of this class is the

phytoalexin *trans*-resveratrol (i.e., 3,5,4'-trihydroxy-*trans*-stilbene), which has been the centre of much scientific attention and media exposure following its biological evaluation as a cancer chemopreventative and its detection in red wine [Hu et al., 2010; Llorach et al., 2010; Figueira and Resveratrol, 2010]. Such phenolic systems featuring a conjugated carbon–carbon bond in their side-chains are particularly prone to undergo oligomerisation events via coupling of delocalised phenoxy radicals generated by one-electron oxidation reactions. Much like the hydroxycinnamic acids, esters and alcohols that are converted into lignan/neolignan dimers (C_6-C_3)$_2$ and plant cell wall lignin polymers ((C_6-C_3)n) by such oxidative coupling processes, resveratrol and its hydroxystilbenoid analogues can react in the same manner to furnish polyphenolic oligomers. The presence of more than one hydroxyl group on a benzene ring or other arene systems does not make them "polyphenols". Catechol, resorcinol, and pyrogallol are all di- and trihydroxylated benzene (C_6) derivatives, but they are still defined as "phenols" according to the IUPAC official nomenclature rules of chemical compounds. Many monophenolics are often called "polyphenols" by the cosmetic and para-pharmaceutical industries, but they cannot be classified as such by any scientifically accepted definition. The meaning of the chemical term "phenol" includes both the arene ring and its hydroxyl substituent(s), and the term "polyphenol" should be confined, in a strict chemical sense, to structures bearing at least two phenolic moieties, independently of the number of hydroxyl groups that they each bear. Moreover, many natural products of various biosynthetic origins do not contain more than one phenolic unit. It is, for example, the case for many alkaloids derived from the amino acids phenylalanine and tyrosine. The term "polyphenol" should be used to define compounds exclusively derived from the

shikimate/phenylpropanoid and/or the polyketide pathways, featuring more than one phenolic unit and deprived of nitrogen-based functions. Polyphenols can be classified into several categories: the flavonoids are obtained by the lengthening of the side chain of cinnamic acids by the addition of one or more C_2 units, typically resulting in mixed biosynthesis metabolites with important biological properties. In particular, these polyphenolic compounds have 15-carbon skeletons, represented as the C_6-C_3-C_6 system. The flavonoids are 1,3-diarylpropanes, isoflavonoids are 1,2-diarylpropanes, and neoflavonoids are 1,1-diarylpropanes. The term "flavonoid" was first used by Geismann and Hinreiner in 1952 for the classification of those compounds whose structure is correlated to the 2-phenil-chroman heterocyclic system (flavan) [Geissman and Hinreiner, 1952]. Their skeleton is made up of two benzene rings with a chain of three carbon atoms of a γ-pirone system. Thus, the several flavonoidic compound classes differ in the oxidation states of their heterocyclic systems. Single constituent flavonoids of every class are mainly distinguished by the number and the stereochemistry of the hydroxyl groups and/or methoxyls on the two benzene rings and/or the heterocyclic system. These replacements are found in defined positions of flavonoids, such that they indicate a different biogenetic origin for two aromatic rings, A and B. In many cases, then, the flavonoidic compounds have been isolated, such as glycosides, one or more hydroxyl groups are joined with a hemiacetalic bound, generally through the C-1 carbon and with a bond of type β, to one or more sugars. Flavonoids are fundamentally important for ecological role as pigment in flowers and fruits. Flavonoids are important for plants' ecological roles in that they are the pigments that give colour to fruits and flowers, thereby attracting pollinators. The coumarins are typical

metabolites of higher plants formed from cinnamic acids through a very simple pathway. The lignans comprise a group of natural compounds with carbon skeletons derived from two phenylpropane units joined together by at least one carbon-carbon bond between the two central β-carbons of the C_3 chains (lignans) or by bonds other than the ββ'-carbon-carbon bond (neolignans). Compounds formed by shortening the phenylpropane skeleton can be divided into three groups: the C_6-C_2 compounds, used by plants in the biosynthesis of alkaloids, the C_6-C_1 compounds, such as benzoic acids and their variously oxygenated derivatives usually found as glycosides or as esters, and the C_6 compounds, which are simple polyphenols rarely found in higher plants [Geissman and Hinreiner, 1952].

Antibacterial activity of plant Polyphenols

Phenolic compounds have diverse defensive functions in plants, such as cell wall strengthening and repair (lignin and suberin) [Korkina et al., 2007] and antimicrobial and antifungal activities. Some phenols are phytoanticipins, compounds with a defensive role that are not synthesised in response to a pathogen attack but rather are constitutively present in plant cells [Osbourn, 1996]. Constituent phenolics occur on the surface of plants or in the cytoplasmic fraction of the epidermal cells, where they act as a deterrent to pathogens. In contrast, phenolic phytoalexins are secreted by wounded plants or in response to incompatible pathogens [Bhattacharya et al., 2010]. The induced defence response includes cell death and the formation of a lesion that limits the growth of the pathogen. Cells around the lesion accumulate phenols and other antibiotic compounds [Lattanzio, 2006]. Phenolics act on pathogen cells by generating hydrogen peroxide [Arakawa et al., 2004] and by altering the permeability of the microbial membrane [Tamba et al., 2007]. Microbes stressed by exposure to

polyphenols upregulate proteins related to defensive mechanisms, which protect cells while simultaneously downregulating various metabolic and biosynthetic proteins involved, for example, in amino acid and protein synthesis as well as phospholipid, carbon, and energy metabolism [Cho et al., 2007]. Moreover, polyphenols have been reported to interfere with bacterial quorum sensing, i.e., the production of small signal molecules by bacterial cells that trigger the exponential growth of a bacterial population [Hubert et al., 2003].

A large body of evidence indicates that many plants used as folk remedies contain high concentrations of polyphenolic compounds [Murphy, 1999]. Plants from a wide range of angiosperm families show antibacterial activity. In one study, 35 of 146 seed extracts inhibited microbial growth, and the biocidal activity of the seed extracts correlated with their polyphenol content. Plants from more than 20 different families, including Asteraceae, Fabaceae, Poaceae, Lythraceae, Onagraceae, Polygonaceae, Primulaceae, and Verbenaceae showed bactericidal action [Borchardt et al., 2008]. Members of the Geraniaceae and Rosaceae families are also rich in polyphenolic compounds with antimicrobial activity [Nikitina et al., 2007], and *Cydonia oblonga* Miller, a member of the latter family, was found to be an important source of polyphenols that are active against bacteria growth [Fattouch et al., 2007]. Polyphenols with relevant biocidal activity have been isolated from members of other plant families: Taguri et al. isolated castalagin and protodelphinidin flavenoids that are fundamentally important for ecological role as pigments in flowers and fruits, from *Castanea crenata* Siebold & Zucc (Fagaceae) and *Elaeocarpus sylvestris* (Lour.) Poir. var. *ellipticus* (Elaeocarpaceae), respectively, and found them to be effective against different bacterial strains [Taguri et al., 2004].

Pathogenesis of dental caries

Dental caries is a common chronic disease, arising from the interplay between oral flora, teeth and dietary factors. Dietary carbohydrates, mainly mono- and disaccharides, are absorbed into dental plaque and broken down into organic acids by the micro-organisms present in dense concentrations. The acids produced cause the equilibrium between remineralisation and demineralisation to shift in favour of demineralisation, thus initiating carious lesions.

Several strains of oral streptococci are capable of initiating the formation of dental plaque, which plays an important role in the development of caries and also of periodontal disease in humans [Freedman and Tanzer, 1974]. The major aetiological players are thought to be the two α-haemolytic streptococci, *Streptococcus mutans* and *Streptococcus sobrinus*, which are potent cariogenics, although several other types of bacteria (notably lactobacilli and actinomyces) may also be involved.

S. mutans produces three types of glucosyltransferase (GTFB, GTFC, and GTFD) and synthesises an adherent, water-insoluble glucan from appropriate carbohydrate substrates, most favourably, sucrose at low pH values, which causes the organisms to adhere firmly to the tooth surface [Edwardsson, 1968]. The adherent glucan also contributes to the formation of dental plaque, in which the accumulation of acids leads to localised decalcification of the enamel surface.

The carbohydrate substrates can become available either directly (as sugar ingested in food or drink) or be derived from dietary starch by the action of bacterial or salivary amylases, or both.

Polyphenols have been shown in many studies, both in animals and in humans, to interfere specifically with each of the processes described [Lee et al., 2004].

Anti-cariogenic action of phenols

A variety of compounds capable of controlling dental caries have been extensively surveyed; however, only limited numbers of compounds from natural products are available because of effectiveness, stability, odour, taste, and economic feasibility. The effects of phenols have been surveyed through both *in vitro* studies investigating the effect of polyphenols against mutans streptococci and *in vivo* studies in animals and humans.

In vitro studies

Studies on the activities of phenolic compounds toward cariogenic bacteria can be divided based on the chemical structure of the compound under study (Fig. 2-3-4 and Table 1). Few studies deal with the anti-streptococcal action of simple phenols. Xanthorrhizol (XTZ), isolated from *Curcuma xanthorrhiza* Roxb., has been reported to possess antibacterial activity against several oral pathogens, and it has shown to have rapid bactericidal activity against *S. mutans* [Rukayadi and Hwang, 2006]. The activity of XTZ in removing *S. mutans* biofilm was dependent on its concentration and exposure time as well as the growth phase of the biofilm. A concentration of 5 μmol l^{-1} of XTZ completely inhibited biofilm formation by *S. mutans* at the adherent phase of growth, whereas 50 μmol l^{-1} of XTZ removed 76% of the biofilm at the plateau accumulated phase after a 60-min exposure. Another simple phenol, bakuchiol, isolated from *Psoralea corylifolia* L, showed inhibitory activity against *S. mutans* [Katsura et al., 2001]. Yanti et al reported anti-biofilm activity of macelignan, isolated by nutmeg (*Myrisica fragrans* Houtt.) against oral bacteria including *S.mutans*, *S. sanguinis* and *Actinomyces viscosus* [Yanti et al., 2008]. This study demonstrated that macelignan activity at 10μg/mL for a 30 min exposure time

could remove more than half of each single oral biofilm formed by *S.mutans, S.sanguinis* and *A. viscosus* at the plateau accumulated phase (24h).

From an ethanol extract of *Alcea longipedicellata* (Malvaceae) malvidin-3,5-diglucoside (malvin) was identified as the principal constituent which was responsible for antibacterial activity. 0.1% malvin could inhibit strongly acid producing ability of *S. mutans* and was about 60% effective in inhibiting bacterial adherence [Esmeelian et al., 2007]. Kuwanon G , isolated from methanol extract of root bark of *Morus alba* L. showed bactericidial action at the concentration 20 μg/ml in 1 min. against *S. mutans* and other cariogenic bacteria as *S. sobrinus*, *S. sanguinis* and *Porpyromonas gengivalis* [Park et al., 2003].

The activity of crude ethanol extract from *Piper cubeba* seeds, the purified compounds (-)-cubebin and its semi-synthetic derivatives were evaluated against oral pathogens. The crude ethanol extract was more active against *S. salivarium* (MIC value of 80μg/ml) and purified compounds and semisynthetic derivaties displayed MIC values ranging from 0.20mM for *S. mitis* to 0.32mM for *S. mutans* [Silva et al., 2007].

The active flavonoid compound, quercetin-3-O-α-L-arabino-pyranoside (guaijaverin) isolated from *Psidium guajava* L. demonstrated high potential antiplaque agent by inhibiting the growth of the *S. mutans* [Prabu et al., 2006].

Magnolol and honokiol isolated from extracts of *Magnolia* sp. bark have a phenyl-propanoid dimer structure and are active against the cariogenic bacterium *S. mutans* (MIC 6.3 mg/ml) [Namba et al., 19821].

Fig. 2: Chemical structures of active polyphenols. 1 Xanthorrhizol, 2 Bakuchiol, 3 Macelignan, 4 Malvin,   5 Kuwanon G, 6 (-)-Cubebin, , 8 Magnolol, 9 Honokiol, 10 Dihydrobiochanin A,   11 Ferreirin, 12 Dihydrocajanin, 13 Dalbergioidin

8 R = H   R_1 = OH
9 R   OH R_1   H

10   R = R_1 = R_2 = R_4 = H   R_3 = OCH_3
11   R = R_2 = R_4 = H   R_1 = OH   R_3 = OCH_3
12   R = CH_3   R_2 = R_4 = H   R_1 = R_3 = OH
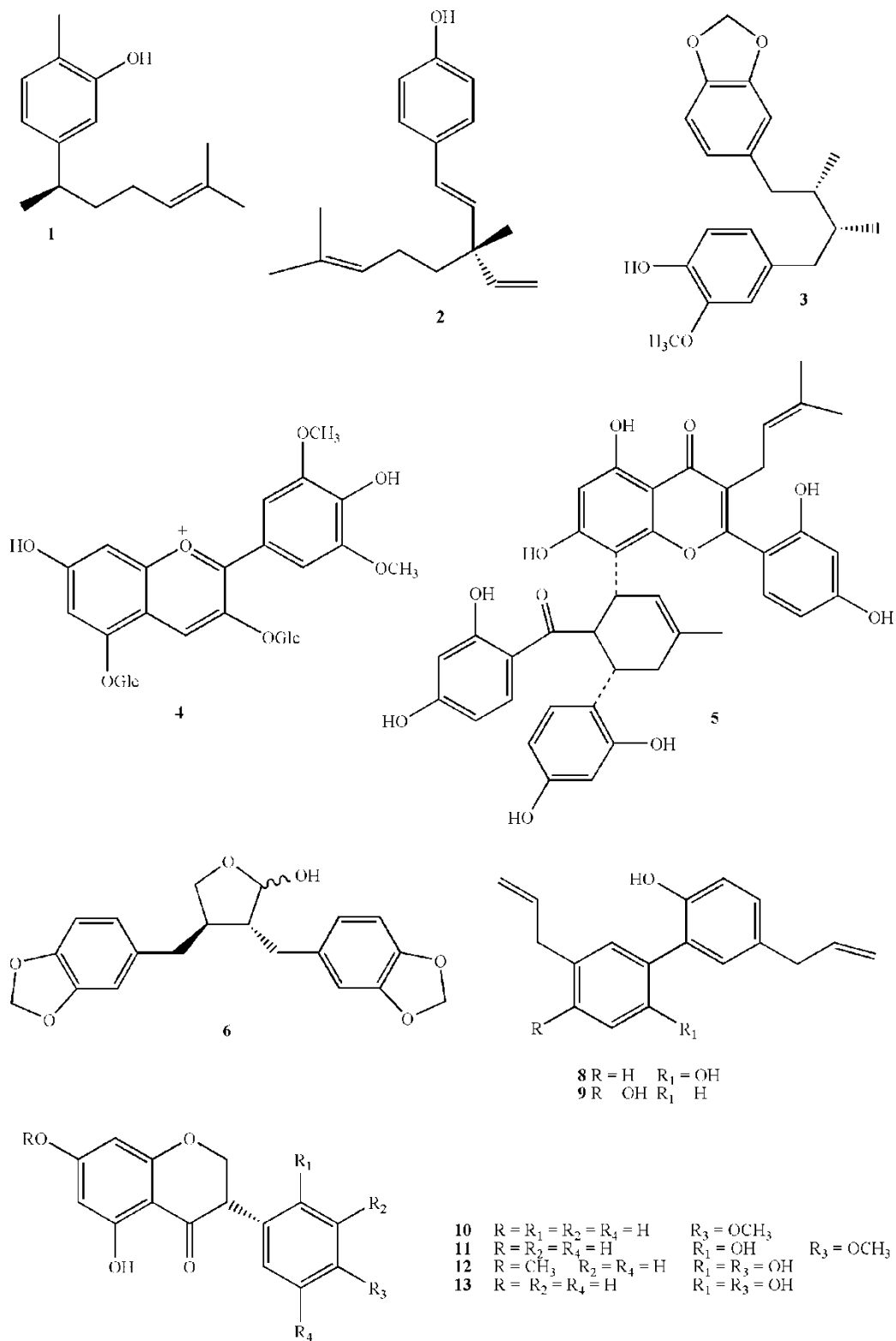13   R = R_2 = R_4 = H   R_1 = R_3 = OH
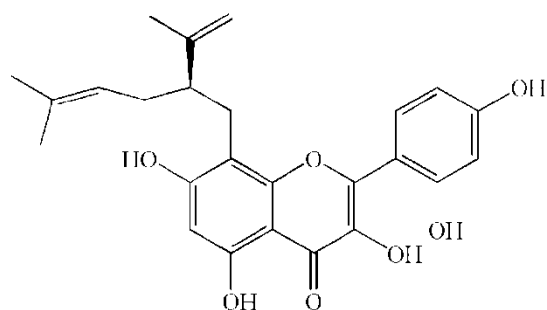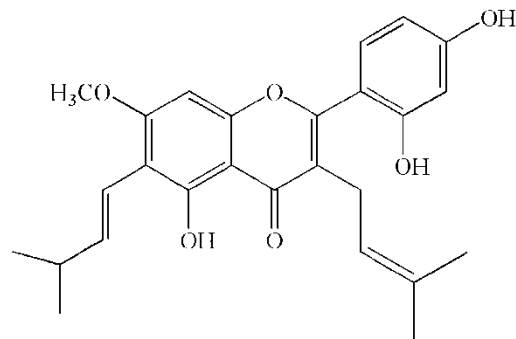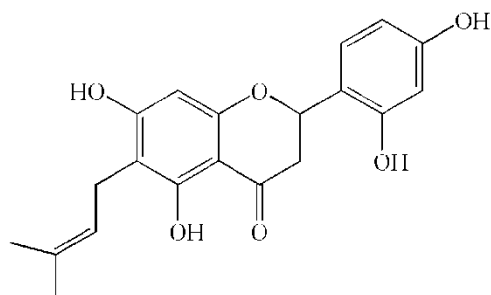
13

Fig. 3: Chemical structures of active polyphenols. 7 Guaijaverin, 14 Lavandulylflavanone, 15 Artocarpin, 16 Artocarpesin, 17 Erycristagallin, 18 Luteolin, 19 Quercetin, 22 Quercetin-3-arabinofuranoside, 23 Myricetin
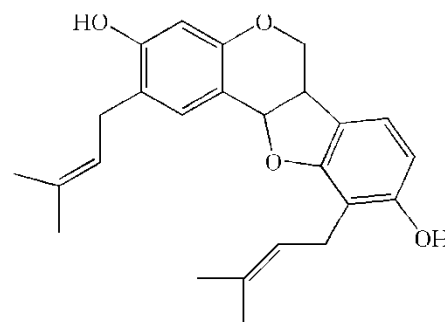
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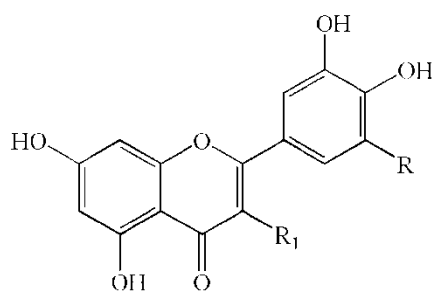
7   R = H R_1 = Arabinopyranoside
18 R = H R_1 = H
19 R = H R_1 = OH
22 R = H R_1 = Arabinofuranoside
23 R = OH R_1 = OH
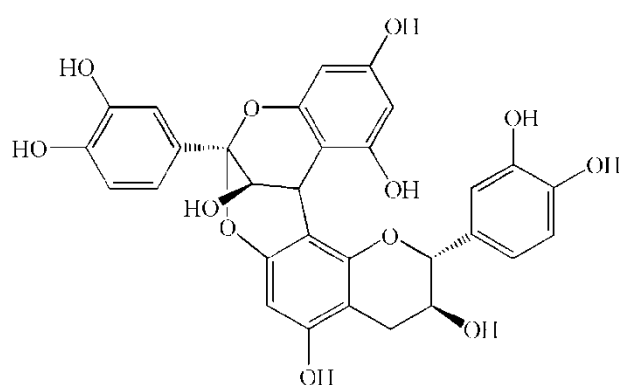
24

Fig. 4: Chemical structures of active polyphenols. 25 Theaflavin, 26 Theaphlavin monogallate A, 27 Theaphlavin monogallate B, 28 Theaphlavin digallate, 29 Epigallocathechin gallate

25 R = R$_1$ = H

26 R = [structure]   R$_1$ = H

27 R = H   R$_1$ = [structure]

28 R = R$_1$ = [structure]

29
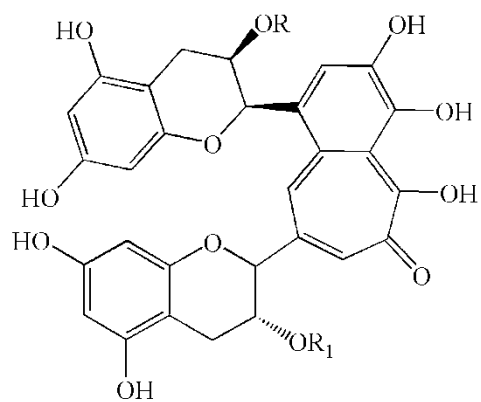
Table 1: Activity of plant phenolics against *Streptococcus mutans*.

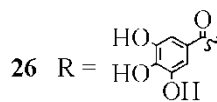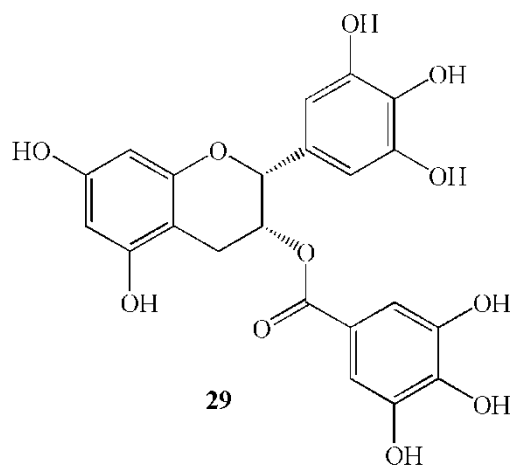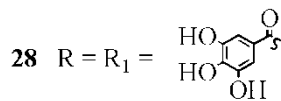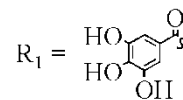|	Name	Mol. weight	Plant name	Part of the plant	Activity against *S. mutans*	Ref.
1	Xanthorrhizol	218.3	*Curcuma xanthorrihiza*	rhizome	5 mmol l⁻¹ inhibit biofilm formation	34
2	Bakuchiol	256.4	*Psoralea corylifolia* L.	seeds	20 µg/ml prevented growth	35
3	Macelignan	328.4	*Myristica fragrans* Houtt.	seeds	10µ/ml for 30' exposure remove	36
4	Malvin	655.2	*Alcea longipedicellata*	flowers	M.I.C. 0.16 mg/ml for	37
5	Kuwanon G	692.7	*Morus alba* L.	Root bark	M.I.C. 8µg/ml	38
6	(-)-Cubebin	356.4	*Piper cubeba* L.	seeds	M.I.C. 0.32mM	39
7	Guaijaverin		*Psidium guaiava* L.	leaves	M.I.C. 4mg/ml	40
8	Magnolol	266.3	*Magnolia officinalis*	bark	0.32 mg/ml reduced by 87.3%	41
9	Honokiol	266.3	*Magnolia officinalis*	bark	0.32 mg/ml reduced by 58.1%	41
10	Dihydrobiochanin A	286.3	*Swartzia polyphylla* DC	heartwood	M.I.C. 50 µg/ml	50
11	Ferreirin	302.3	*Swartzia polyphylla* DC	heartwood	M.I.C. 50 µg/ml	50
12	Dihydrocajanin	302.3	*Swartzia polyphylla* DC	heartwood	M.I.C. 100 µg/ml	50
13	Dalbergioidin	288.3	*Swartzia polyphylla* DC	heartwood	M.I.C. 100 µg/ml	50
14	Lavandulylflavanone	438.5	*Sophora exigua* Craigg	heartwood	Growth inhibition in the range 1.56-	51
15	Artocarpin	436.5	*Artocarpus heterophyllus*	heartwood	M.I.C. 6.25 µg/ml	52
16	Artocarpesin	354.4	*Artocarpus heterophyllus*	heartwood	M.I.C. 6.25 µg/ml	52
17	Erycristagallin	392.5	*Erythrina variegata* L.	root	M.I.C. 6.25 µg/ml	53
18	Luteolin	286.2	*Perilla frutescens*	seeds	M.I.C. 50-100 µg/ml (on	54
19	Quercetin	302.2	Commercial source	-	Inhibition of adhesive glucan	58
20	Proanthocyanidins	/	*Humulus lupulus* L.	bracts	0.01%, HBP (Hop Bracts	65
21	Tannins	/	*Areca catechu* L.	nut	50% of growth inhibition	68
22	Quercetin-3-arabinofuranoside	434.3	*Vaccinium macrocarpon*	fruit	21-41% Inhibition of GTF activity at	68
23	Myricetin	318.0	*Vaccinium macrocarpon*	fruit	15-28 % Inhibition of GTF	68
24	Procyanidin A2	576.1	*Vaccinium macrocarpon*	fruit	21-41 % Inhibition of GTF	68
25	Theaflavin	564.1	*Camellia sinensis* L.	leaves	Inhibition of GTF activity in the	71
26	Theaphlavin monogallate A	716.3	*Camellia sinensis* L.	leaves	Inhibition of GTF activity in the	71
27	Theaphlavin monogallate B	716.3	*Camellia sinensis* L.	leaves	Inhibition of GTF activity in the	71
28	Theaphlavin digallate	868.1	*Camellia sinensis* L.	leaves	Inhibition of GTF activity in the	71
29	Epigallocathechin gallate	458.4	*Camellia sinensis* L.	leaves	167 mg/l caused 91% growth	71

(M.I.C. = Minimum Inhibition Concentration. GTF =Glucosyltransferases)

There is a large body of evidence supporting the inhibition of cariogenic bacteria by larger phenolic compounds, which are considered the "true" polyphenols. Research on this subject can be divided into two groups: a) studies on fractions of plant extracts containing high concentrations of polyphenols, without the identification of individual compounds occurring in the extracts and b) reports of the antibacterial activity of specific polyphenols.

The first group includes some early studies, such as that performed by Stralfors, who carried out a series of experiments using the hamster caries model and cocoa products. He demonstrated that the addition of 20 g whole cocoa powder/kg to a cariogenic diet that contained 50 g sucrose/kg reduced the amount of caries by 42% [Stralfors, 1966]. Subsequently, phenolic substances were suggested to be responsible for the observed anti-caries effect of cocoa powder [Kashket et al., 1985], probably due to their inhibition of the synthesis of water-insoluble glucans [Ito et al., 2003].

Onion extracts have been reported to act on *Streptococcus mutans* and *S. sobrinus* as well as *Porphyromonas gingivalis* and *Prevotella intermedia,* which are considered to be the main causal bacteria of adult periodontitis [Kim, 1997]. Although no active components of the onion extracts were identified, onion is among the richest sources of flavonoids and contributes significantly to the overall dietary intake of flavonoids [Slimestad et al., 2007].

An *in vitro* study demonstrated that the tea polyphenol (TP) has no effect on de/remineralisation of enamel blocks, but it exerts an anti-caries effect via an anti-microbial mode-of-action [Li et al., 2004].

Smullen et al. have shown that extracts from unfermented cocoa, green tea, and red grape seeds, all with a high polyphenol content, are effective against *S. mutans* and reduce its adherence to glass [Smullen et al., 2007]. Moreover, grape seed extracts inhibit the growth of anaerobic bacteria, such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum*, associated with periodontal diseases [Furiga et al., 2009]. There are numerous reports of the anti-streptococcal action of flavonoids. Three known isoflavanones, dihydrobiochanin A, ferreirin and darlbergioidin, and one new isoflavanone, 5,2',4'-trihydroxy-7-methoxyisoflavanone (dihydrocajanin), which was isolated from *Swartzia polyphylla* DC heartwood, had potent activity against cariogenic bacteria [Osawa et al., 1992]. A lavandulylflavone isolated from *Sophora exigua* Craig completely inhibited the growth of oral bacteria, including primary cariogenic mutans streptococci, other oral streptococci, actinomycetes, and lactobacilli, at concentrations of 1.56 to 6.25 micrograms/ml [Tsuchiya et al., 1994]. Isoprenylflavones from *Artocarpus heterophyllus* showed antibacterial activity against cariogenic bacteria [Sato et al., 1996]. Sato et al. reported that erycristagallin from *Erythrina variegata* showed a high antibacterial activity against mutans streptococci, other oral streptococci, actinomycetes, and lactobacilli [Sato et al., 2003].

In recent years, polyphenols from some edible plants have attracted attention as potential sources of agents capable of controlling the growth of oral bacteria. Extracts from *Perilla frutescens* var. *japonica* seeds have shown inhibitory activity against oral cariogenic Streptococci and periodontopathic *Porphyromonas gingivalis*. *Perilla* seed polyphenols were isolated and their activity was tested. The flavonoid

luteolin was the phenol that was most active against bacterial growth [Yammamoto and Ogawa, 2002].

Sunphenon is a mixture of flavonols isolated from leaves of *Camellia sinensis*. The major components of this mixture are (+)-Catechin, (+)-Gallocathechin, (-)-Epicathechin, (-)-Epicathechin gallate, (-)-Epigallocathechin, and (-)-Epigallocathechin gallate [Juneia et al., 2000]. The addition of Sunphenon to *S. mutans* JC-2 (c) decreased cell viability; multiple applications of Sunphenon caused the death of cells, and the maximum effect was seen with treatment of 60 and 90 minutes. In vivo studies on rats infected with *S. mutans* JC-2 confirmed the anti-cariogenic activity of Sunphenon. Furthermore, supplying drinking water containing 0.1% Sunphenon reduced the incidence of caries in *S. mutans*-infected animals [Saito, 1990].

Inhibition of adherence

The adherence of bacterial cells to tooth surfaces is of great importance to the development of carious lesions, and interference with some of the mechanisms of adherence can prevent the formation of carious lesions [Featherstone, 2000]. 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 [Tamba et al., 2007]. One of the first studies on this topic reported that quercetin, in the range 12,5-50 mg/ml, was effective in preventing adhesive glucan formation by *S. mutans* strains [Ito et al., 1984].

A chromatographically isolated oolong tea polyphenol (OTF6) may inhibit bacterial adherence to tooth surfaces by reducing the hydrophobicity of mutans streptococci [Matsumoto et al., 1999].

An *in vitro* study demonstrated that when *S. mutans* JC-2 (c) was pretreated with Sunphenon, its cellular attachment to a saliva-treated hydroxyapatite surface was significantly reduced [Saito, 1990].

Barley coffee (BC) interferes with *Streptococcus mutans* adsorption to hydroxyapatite. A low-molecular-mass (<1,000 Da) fraction containing polyphenols, zinc, and fluoride ions and a high-molecular-mass (>1,000 kDa) melanoidin fraction displayed strong anti-adhesive properties towards *S. mutans* [Stauder et al., 2010]. A cocoa polyphenol pentamer (the most active component from MIC studies) significantly reduced biofilm formation and acid production by *S. mutans* and *S. sanguinis* [Percival et al., 2006].

Inhibition of glucosyl transferase and amylase

The enzymatic activity of glucosyl transferase from *Streptococcus mutans* is inhibited by plant polyphenols.

Apple polyphenols extracted from immature fruits markedly reduced the synthesis of water-soluble glucans by glycosyltransferases (GTF) of *S. mutans* and *S. sobrinus* but did not inhibit salivary α-amylase activity. GTF inhibitors from apples are high-molecular-weight phenols with a chemical structure similar to catechin-based oligomeric forms and/or gallate-ester compounds [Yanagida et al., 2000]. Procyanidins from betel nuts (the seed of *Areca catechu* L.) were the major inhibitors of glucosyltransferase from *S. mutans* [Surarit and Koontongkaew, 1988]. A high-molecular-weight polyphenol of *Humulus lupulus* L. (HBP) inhibited the cellular adherence of *S. mutans* MT8148 (serotype C) and *S. sobrinus* ATCC 33478 (serotype g) at much lower concentrations than those needed for the polyphenols extracted from oolong tea or green tea leaves. Furthermore, HBP also inhibited the

action of glucosyltransferase, which was involved in the synthesis of water-insoluble glucan, but did not suppress the growth or acid production of the bacteria [Tagashira et al., 1997]. *H. lupulus* polyphenols significantly reduced the growth of *S. mutans* compared to the control. After an 18-hour incubation, HBP at 0.1% and 0.5% significantly reduced lactic acid production, and HBP at 0.01%, 0.1%, and 0.5% also suppressed water-insoluble glucan production [Yaegaki et al., 2008]. The polyphenols from bracts of *H. lupulus* were purified by countercurrent chromatography (CCC). The most potent cavity-prevention activity was found in a very hydrophilic fraction, whose major components were high-molecular-weight substances, probably proanthocyanidins, consisting of approximately 22 catechin units in their structures [Kurumatani et al., 2005].

Grape and pomace phenolic extracts inhibited GTF of *S. mutans* at concentrations of 62.5 µl/ml. These extracts had qualitative and quantitative differences in their phenolic content but similar activity toward *S. mutans* GTF [Timothe et al., 2007].

Extracts of flavonols (FLAV) and proanthocyanidins (PAC) from American cranberry (*Vaccinium macrocarpon* Ait.), alone or in combination, inhibited the surface-adsorbed glucosyltransferase and F-ATPase activities as well as acid production by *S. mutans* cells [Duarte et al., 2006]. Flavonols and proanthocyanidins moderately inhibited the activity of surface-adsorbed GTF and disrupted acid production by *S. mutans* cells without killing them. The combination of three flavonoids, quercetin-3-arabinofuranoside, myricetin, and procyanidin, displayed pronounced biological effects on *S. mutans*, suggesting that the bactericidal activity could be the result of synergistic effects of flavonoids occurring in cranberry extracts [Gregoire et al., 2007]. A subsequent study by Yarnanaka-Omada et al. has

confirmed that cranberry polyphenols are effective against hydrophobicity, biofilm formation, and bacterial growth of *S. mutans* [Yamanaka-Okada et al., 2008].

Extracts of oolong tea and its chromatographically isolated polyphenolic compound inhibited insoluble glucan synthesis from sucrose by the GTases of *Streptococcus mutans* MT8148R and *S. sobrinus* 6715 [Ooshima et al., 1993]. Moreover, both extracts caused a decrease in the cell-surface hydrophobicity and aggregation of *S. mutans*, *S. oralis*, S. *sanguinis*, and *S. gordonii* [Matsumoto et al., 1999]. Among the flavonoids isolated from tea infusions, theaflavin and its mono- and digallates were strong inhibitors of the synthesis of adherent water-insoluble glucans from sucrose catalysed by a glucosyltrasferase (GTF); (+)-Catechin, (-)-Epicatechin, and their enantiomers were moderately active, and galloyl esters of (-)-Epicatechin, (-)-Epigallocatechin, and (-)-Gallocatechin showed increased inhibitory activities [Hattori et al., 1990].

In vivo studies

Research in the field of dental caries using human subjects has been restricted for a number of reasons. First, dental decay is a disease of slow progression. Indeed, it has been estimated that a new lesion in a permanent tooth takes between 18 and 60 months to become clinically detectable [Parfitt, 1956]. Second, once established, a lesion is irreversible, thus experimental induction of caries is wholly unethical. Third, because of the length of the study period, it is quite impossible to obtain dietary histories and even less possible to control dietary intake. Fourth, perhaps most importantly, diet is but one of a large group of secondary factors, many of which may still be unknown, that contribute to an individual's experience of this multifactorial disease.

For these reasons, most of the research on dental caries and diet has been carried out in animals, the rat model being by far the most common. Because of the dental and other obvious differences between humans and rats, the application of these animal findings to humans must be carried out with great caution. Clearly, this problem has greatly restricted the rate of progress in our knowledge and understanding of the precise role of dietary factors in relation to dental decay.

However, the number of human studies investigating the protective effects of polyphenols has rapidly increased over the last decade.

The administration of oolong tea extract and the isolated polyphenol compound in the diet and drinking water resulted in significant reductions in caries development and plaque accumulation in the rats infected with mutans streptococci. A study on black tea has determined the effects of a standardised black tea extract (BTE) on caries formation in inbred hamsters that were fed regular and cariogenic diets. The frequent intake of black tea significantly decreased caries formation, even in the presence of sugars in the diet [Linke and LeGeros, 2003].

A clinical test to evaluate the effect of a mouthwash containing 0.1% *H. lupulus* bract polyphenols (HBP) on dental plaque regrowth over three days has shown that the HBP mouthwash was effective in reducing dental plaque regrowth (total plaque reduction of 25.4% compared with the placebo), and it lowered the number of mutans streptococci [Shinada et al., 2007].

The possible protective effect of cocoa on dental caries is also receiving increasing attention, but previously published data concerning the anticariogenic effects of constituents of chocolate are conflicting. An early study indicated that a high-sucrose diet was equally cariogenic in the presence or absence of cocoa bean ash [Wynn et

al., 1960], while the incorporation of cocoa powder or chocolate into hamster diets was reported to reduce caries [Stralfors, 1976]. Another in vivo study showed that the cariogenic potential indices (CPI) of chocolate with high cocoa levels was less than 40% that of sucrose (10% w/v) and also lower than that of chocolate containing low cocoa levels [Verakaki and Duggal, 2003]. The anticariogenic effects of polyphenols isolated from cocoa have not been studied yet. Recently, the ground husks of cocoa beans, which are a product of cocoa manufacturing that have a high polyphenol content, were used to prepare a mouthwash for children. The regular use of this mouthwash gave a 20.9% reduction in mutans streptococci counts and was even more effective in decreasing plaque scores [Srikanth et al., 2008].

EXPERIMENTAL STUDY

AIM

The intent of this research program was to determine a new way in caries prevention. The score was the evaluation of anti-caries effects of bio-active molecules derived from plants, in order to elaborate a new preventive methodology. The under discussion molecules were polyphenols.

The present study was divided in 3 part.

Part 1: In vitro evaluation of the anti-microbial effects of phenolic extracts on cariogenic oral bacteria;

Part 2: Evaluation of citotoxicity effect and antimicrobial properties of Camelia sinensis extract against cariogenic microflora;

Part 3: Evaluation of citotoxicity effect and antibacterial power of Plantago lanceolata extract against oral cariogenic microflora.

The final score could be the creation of a new preventive methodology against dental caries. In fact, on the basis of the results obtained from this research, the protection against tooth decay could be done in different ways. First of all, people could protect their self simply by consumption of substances containing polyphenols , then these active principles could be exploited by nutritional and pharmacological industries by adding these bio-molecules both in food and in commercial products used for oral hygiene (mouth-rinse, toothpaste).

PART 1

IN VITRO EVALUATION OF THE ANTI-MICROBIAL EFFECTS OF PHENOLIC EXTRACTS ON CARIOGENIC ORAL BACTERIA

MATERIALS AND METHOD

1. Plant material

79 plants were selected and collected for this study:

Sonchus asper	Calystegia selvatica
Fumaria capriolata	Asplenium anopteris
Stellaria media	Urtica membranacea
Plantago major	Adiantum capillus-veneris
Bellis perennis	Arctium lappa
Plantago lanceolata	Asplenium trichomanes
Rumex (crispus)	Taraxacum officinale
Sambucus nigra	Geranium purpuresces
Silene italica	Alliaria petiolata
Lolium rigidum	Zea mays
Laurus nobilis	Scrophularia nodosa
Thymus vulgaris	Rosmarinus officinalis
Ceterach officina rum	Equisetum telmateia

Lavandula sp	Equisetum hyemalis
Lavandula angustifoli	Eucalyptus sp.
Phyllitis scolopendris	Teucrium frutican
Cupressus sempervirens	Oxalis corniculata
Rhamnus alpinus	Valeriana officinalis
Achillea millefolium	Stachys sylvestris
Lavatera cretica	Borago officinalis
Dittrichia viscosa	Ranunculus millefoliatus
Hypericum perforatum	Cymbalaria muralis
Juniperus communis	Equisetum Arvense
Artemisia annua	Acrimonia Eupatorium
Linaria purpurea	Asphodelus ramosus
Verbascum Thapsus	Silene vulgaris
Centranthus ruber	Thymaelea tartonraira
Viburnum tinus	Cotinus coggygra
Micrometria Juliana	Calaminta nepeta
Smilax aspera	Cynodum dactylon
Verbena officinalis	Rappacia spinosa
Ligustrum	Linaria vulgaris
Helichrysum	Cynoglossum
Rosa canina	Genziana lutea
Kixia commutata	Robinia pseudoacacia
Robinia pseudoacacia	Menta acquatica

Cichorium intibus	Heliotropium europaeum
Malva silvestris	Paritaria Judaica
Conyza canadensis	Camelia Sinensis
Silene Alba	

The Department of Biological Sciences / Section of Plant Biology, University of Naples "Federico II"- Naples, Italy, was used as reference point for taxonomic identification of plants.

Collected plant materials were air-dried under shade at room temperature and then ground with an electric grinder into fine powders which were stored into containers at -20°C.

2. Plant extracts

Dried powdered plant materials were extracted with different solvent (methanol, hexane and water) for comparative analyses. Two grams of each plant sample were mixed with 20 ml of each solvent. The mixtures were left overnight on a mechanical shaker at 150 rpm for 24 h at room temperature and then filtered through Whatman No. 1 filter using B¨uchner funnel. The extracts were further concentrated to dryness under reduced pressure at 37° C, using a B¨uchi rotary evaporator. The yields from the different extracts were weighed, recorded and dissolved in 100% of dimethyl sulphoxide (DMSO) in the first experimental phase and in 10% of DMSO in the second one. The samples were then stored at 4° C and further used for antibacterial tests.

3. Antibacterial assays

3.1 Bacterial strains

In an early experimental phase the microorganisms used in the determination of antibacterial activities of 79 different plant extracts were: *S. sobrinus, S. bovis, S. mitis, S. viridians* from clinical specimens obtained from "Diagnostic unit of microbiology of the University Federico II of Naples", Italy.

Different bacterial strains were maintained on nutrient agar and subcultures were freshly prepared before use. Bacterial cultures were prepared by transferring one colony into a tube containing 8 ml nutrient broth and grown overnight at 37 °C for 24h. The turbidity of the culture was adjusted with sterile saline solution to match 0.5 Mc Farland standard.

Successively, in the second experimental phase, the anti-microbial activity of the 45 most active plant extracts were evaluated.

S. mutans (ATCC 25175) and *L. casei* (ATCC 393) obtained from American Type Culture Collection (ATCC; Rockville, MD, USA) were used. Bacterial strains culture were prepared as above described and grown at 37° C in 5% CO_2 atmosphere for 48h.

3.2. Agar-well diffusion assay

The antibacterial tests were performed using agar-well diffusion assay (Perez et al., 1990). Bacterial strains of standardized cultures were evenly spread onto the surface of the trypticase soy agar with 5% sheep blood plates using sterile swab sticks.

In the first experimental phase three wells (8mm diameter) were made in each plate. 100 microliters of methanol, hexane and water plant extracts were added in each

well. 100 microliters of DMSO per well were used as a negative control. The agar

plates were then covered with lids and incubated at 37 ºC for 18 h.

In the second experimental phase three wells were punched into agar medium and

100 µl of methanol, hexane and water plant extracts were added in each well at

concentration of 1.25mg, 2.5mg and 5mg. The agar plates were incubated at 37 ºC in

5% CO_2 atmosphere for 48h.

The plates were observed for the presence of inhibition of bacterial growth that was

indicated by a clear zone around the wells. The size of the zones of inhibition was

measured and the antibacterial activity was expressed in terms of the average

diameter of the zone inhibition in millimeters. The absence of a zone inhibition was

interpreted as the absence of activity.

4. Statistical analysis

For data on agar diffusion assays, ANOVA was used to test the effects of bacteria

within extracts on zone of inhibition using software SPSS10.

RESULTS

The extracts from 77 of the 79 plant species studied inhibited the growth of all/or some tested microorganisms.

Only two plants, "Asplenium anopteris" and "Plantago major", did not show any antimicrobial activity against the tested organisms.

In particular, the 71.18% of tested plant extracts had bactericidal effect against *Streptococcus sobrinus*, while the 28.2% of extracts didn't show antibacterial effect (Fig. 5 and Table 2).
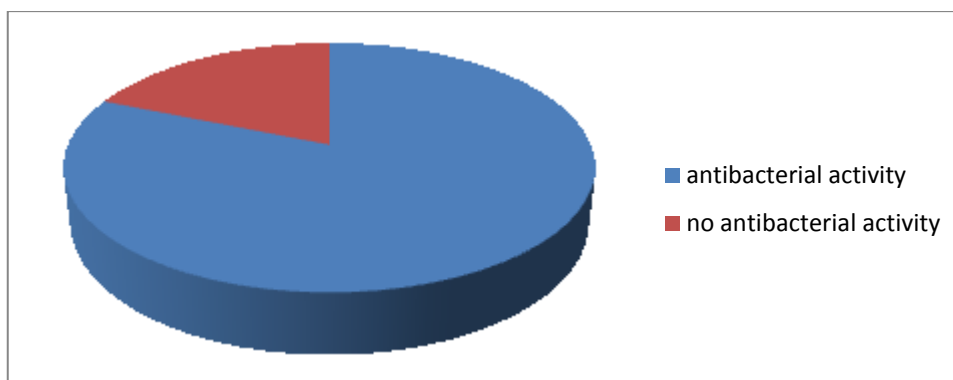

Fig. 5: Evaluation antibacterial activity against *Streptococcus sobrinus*.

	Frequency	Percent	Valid percent	Cumulative percent
antibacterial activity	168	71,2	71,2	71,2
no antibacterial activity	68	28,8	28,8	100
total	236	100	100	

Table 2: Evaluation antibacterial activity against *Streptococcus sobrinus*.

The 88,1% of extracts exhibited antibacterial activity against *Streptococcus viridians*, the remaining 11.9%, instead, didn't show any antimicrobial activity on this strain (Fig. 6 and Table 3).
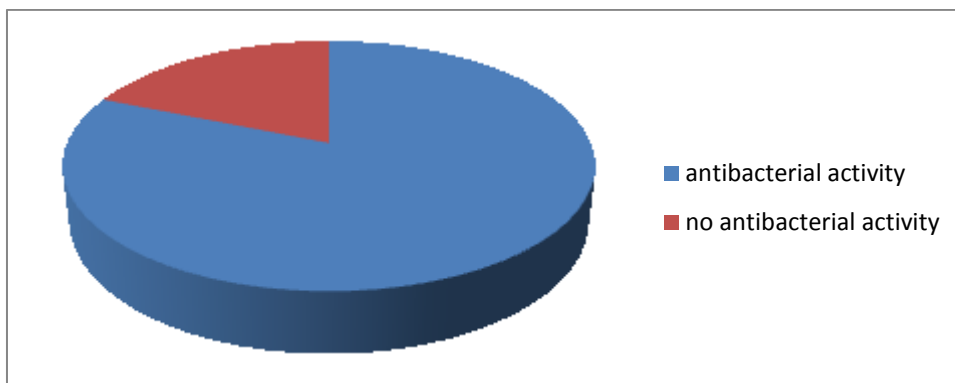
Fig. 6: Evaluation antibacterial activity against *Streptococcus viridans.*

	Frequency	Percent	Valid percent	Cumulative percent
antibacterial activity	208	88,1	88,1	88,1
no antibacterial activity	28	11,9	11,9	100
total	236	100	100	

Table 3: Evaluation antibacterial activity against *Streptococcus viridans.*

The antibacterial action on the *Streptococcus bovis* occurred only from the 11,86% of the extracts. The 88.9% of the substances was not able to inhibit the bacterial growth of this strain (Fig. 7 and Table 4).
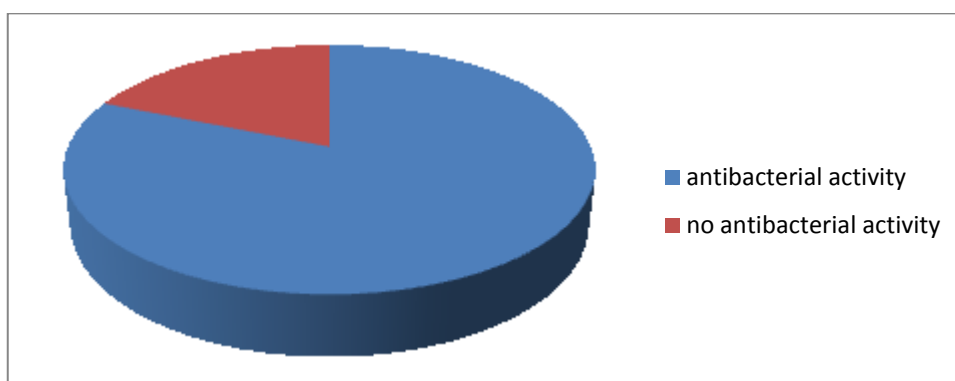

Fig. 7: Evaluation antibacterial activity against *Streptococcus bovis.*

	Frequency	Percent	Valid percent	Cumulative percent
antibacterial activity	28	11,9	11,9	11,9
no antibacterial activity	208	88,1	88,1	100
total	236	100	100	

Table 4: Evaluation antibacterial activity against *Streptococcus bovis.*

Finally, the *Streptococcus mitis* was inhibited by the 81,3% of the extracts, only 18.7% of the plant species tested had not antibacterial effect on this microorganism (Fig. 8 and Table 5).
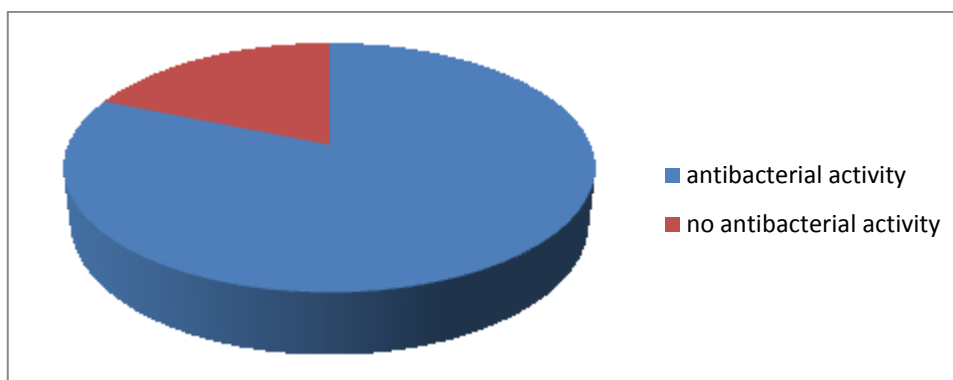
Fig. 8: Evaluation antibacterial activity against *Streptococcus mitis.*

	Frequency	Percent	Valid percent	Cumulative percent
antibacterial activity	192	81,4	81,4	81,4
no antibacterial activity	44	18,6	18,6	100
total	236	100	100	

Table 5: Evaluation antibacterial activity against *Streptococcus mitis.*

Furthermore, it was evaluated if there were significant differences in the anti-bacterial activity between the different solvents (water, hexane and methanol) used. Initially, the difference in the activity between water and hexane extracts was evaluated.

For the *Streptococcus sobrinus*, there were significant differences (P < 0.01) in the activity between the hexane and water extract (Table 6).

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
SOBRINUS	Between Groups	2,285	1	2,285	10,437	,002
	Within Groups	34,152	156	,219		
	Total	36,437	157			
VIRIDANS	Between Groups	,405	1	,405	3,168	,077
	Within Groups	19,949	156	,128		
	Total	20,354	157			
BOVIS	Between Groups	6,329E-03	1	,01	,204	,652
	Within Groups	4,835	156	,03		
	Total	4,842	157			
MITIS	Between Groups	,310	1	,310	1,796	,182
	Within Groups	26,937	156	,173		
	Total	27,247	157			

Table 6: ANOVA analysis of the difference in the antimicrobial activity between water and hexane extracts.

For *Streptococcus viridans, Streptococcus bovis* and *Streptococcus mitis* there were no significant differences (P < 0.01) in activity between the hexane and water extract (Table 6).

Then the antimicrobial activity of the hexane and methanol extract was compared. The results showed that there was no statistically significant difference in activity between the hexane or methanol extract against all tested microorganisms (Table 7).

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
SOBRINUS	Between Groups	,388	1	,388	2,521	,114
	Within Groups	23,879	155	,154		
	Total	24,268	156			
VIRIDANS	Between Groups	,10	1	,10	1,384	,241
	Within Groups	10,985	155	,07		
	Total	11,083	156			
MITIS	Between Groups	,150	1	,150	1,194	,276
	Within Groups	19,481	155	,126		
	Total	19,631	156			

Table 7: ANOVA analysis of the difference in the antimicrobial activity between hexane and methanol extracts.

Then the difference in activity between the methanol and water extract was analyze (Table 8).

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
SOBRINUS	Between Groups	4,537	1	4,537	24,107	,000
	Within Groups	29,170	155	,188		
	Total	33,707	156			
VIRIDANS	Between Groups	,898	1	,898	8,407	,004
	Within Groups	16,554	155	,107		
	Total	17,452	156			
BOVIS	Between Groups	2,852	1	2,852	24,331	,000
	Within Groups	18,167	155	,117		
	Total	21,019	156			
MITIS	Between Groups	,923	1	,923	6,099	,015
	Within Groups	23,308	154	,151		
	Total	24,231	155			

Table 8: ANOVA analysis of the difference in the antimicrobial activity between water and methanol extracts.

There were significant differences (P < 0.01) in the activity between the methanol and water extracts.

In the second experimental phase, of the 45 tested plant extracts the most active were 11.

In particular five methanol extracts were active only against *Streptococcus mutans* (Table 9).

	Streptococcus mutans		
	1mg/100µl	2.5mg/100µl	5mg/100µl
Plantago lanceolata	11	14	16
Equisetum hyemalis	X	13	15
Phyllitis scolopendris	12	14	16
Helichrysum	X	X	12
Camelia sinensis	12	15	18

Table 9: Inhibition of bacterial growth in mm.

Eight methanol extracts were active only against *Lactobacillus casei* (Table 10).

	Lactobacillus casei		
	1mg/100µl	2.5mg/100µl	5mg/100µl
Bellis perennis	X	X	11
Plantago lanceolata	10	13	14
Equisetum hyemalis	X	13	16
Lavandula angustifoli	X	X	12
Phyllitis scolopendris	12	15	17
Cupressus sempervirens	X	X	10
Acrimonia eupatorium	X	X	11
Camelia sinensis	11	13	15

Table 10: Inhibition of bacterial growth in mm.

One water extract were active both on *S.mutans* and on *L.casei* (Table 11).

	Streptococcus mutans		
	1mg/100µl	2.5mg/100µl	5mg/100µl
Cotinus coggyra	X	9	10
	Lactobacillus casei		
	1mg/100µl	2.5mg/100µl	5mg/100µl
Cotinus coggyra	X	12	17

Table 11: Inhibition of bacterial growth in mm.

One hexane extract was active only against *S.mutans* (Table 12).

	Streptococcus mutans		
	1mg/100µl	2.5mg/100µl	5mg/100µl
Juniperus communis	9	10	13

Table 12: Inhibition of bacterial growth in mm.

One hexane extract was active only against *L.casei* (Table 13).

	Lactobacillus casei		
	1mg/100µl	2.5mg/100µl	5mg/100µl
Helichrysum	X	X	13

Table 13: Inhibition of bacterial growth in mm.

DISCUSSION

The emergence of multi-drug resistance in human and animal pathogenic bacteria as well as undesirable side effects of certain antibiotics has triggered immense interest in the search for new antimicrobial drugs of plant origin.

In the present study extracts of 80 plants were tested against some of the cariogenic oral bacteria.

The antimicrobial activity of the extracts was evaluated by agar wells diffusion assay and quantitatively assessed by the presence or absence of inhibition zone.

The results of screening of the first phase are encouraging as out of the 80 plants, 78 extracts showed antibacterial activity against one or more test bacteria.

The *Streptococcus sobrinus* was found to be the most susceptible bacteria to all tested extracts obtained from different plants. While the *Streptococcus bovis* was found to be the least sensitive compared to the other test bacteria.

The difference in potency may be due to the different sensitivity of the test strains.

Furthermore, the successful prediction of botanical compounds from plant material may be largely dependent on the type of solvent used in the extraction procedure.

In the present study, the plant extracts in methanol provided more consistent antimicrobial activity as compared to those extracted in water or in hexane. It is probably because various organic compounds can be leached more in this solvent.

The second phase has showed that 11 extracts were the most active against *S.mutans* and *L.casei.*

In this phase the plant extracts were dissolved in a lower concentration of DMSO to make the extracts not harmul for the human fibroblasts and furthermore the plant extracts were utilized at a standardized concentration in order to find an optimal

concentration able to determine an antibacterial effect for a consequential in vivo experimental phase.

The results obtained in this study appeared to confirm the antibacterial potential of the plants investigated, this may be a good reason to encourage the promotion of these plants as rich source of anti-bacterial agents.

Further pharmacological evaluation of refined extracts are needed before they can be used as therapeutic antimicrobials.

PART 2

EVALUATION OF CITOXICITY EFFECT AND ANTIMICROBIAL PROPERTIES OF GREEN TEA EXTRACT AGAINST CARIOGENIC MICROFLORA

MATERIALS AND METHOD

For the second part of the experimental study we have evaluated the in vitro citotoxicity effect of green tea extract 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 is directly quantified using a scanning multiwell spectrophotometer (ELISA reader). This 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.

Camelia sinensis extracts were 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 microplates (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% CO2) for an incubation period of 24 h.

After the incubation period for each well was added 0.5% plant extracts prepared as described above and then it was incubated the microplate for 24 h in a humidified atmosphere (e.g., 37°C, 5% CO2).

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 0.3 mg/ml).

In vivo test

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 Group A the mean DMFT value was 3.13 ± 1.76; in 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

XTT assay

The cytotoxicity of plant extracts were evaluated by comparing the amount of soluble orange formazan of the fibroblasts cell in contact with the extract, with a 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.

On the basis of the made tests, plant extracts are not cytotoxic for any of the three extractions. 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%, those of cells treated with plant extracts was in the values range between 85% and 115 % (Fig. 9).
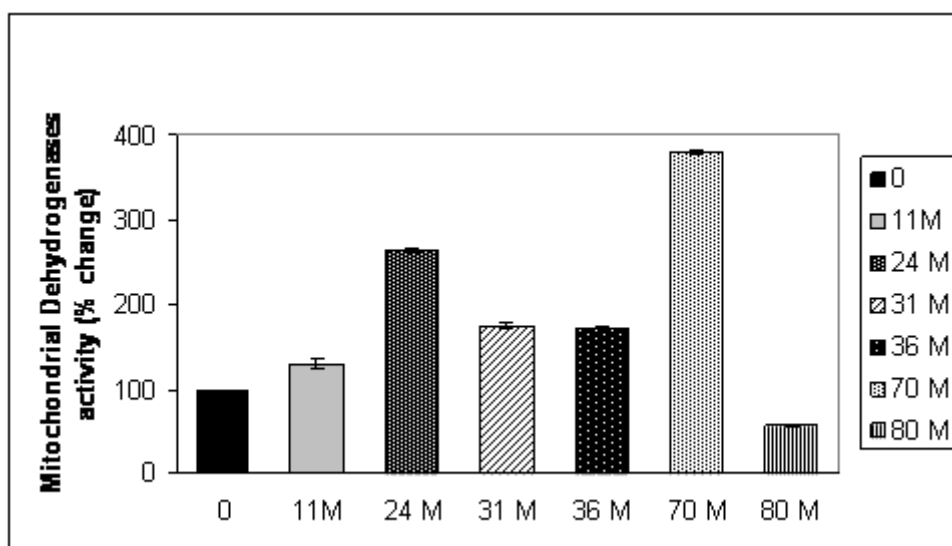

Fig. 9: Citoxicity assay of plant extracts.

In vivo test

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 ($<10^5$CFU) or a high ($>10^5$CFU)

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 10 and 11, respectively.
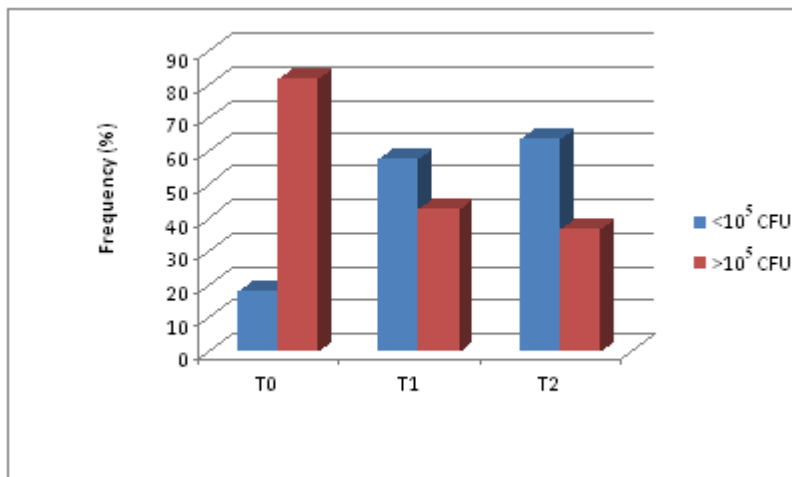

Fig. 10: variation in streptococci mutans density of the CFU (CFU/ml) at t0, t1, t2 (Group A).
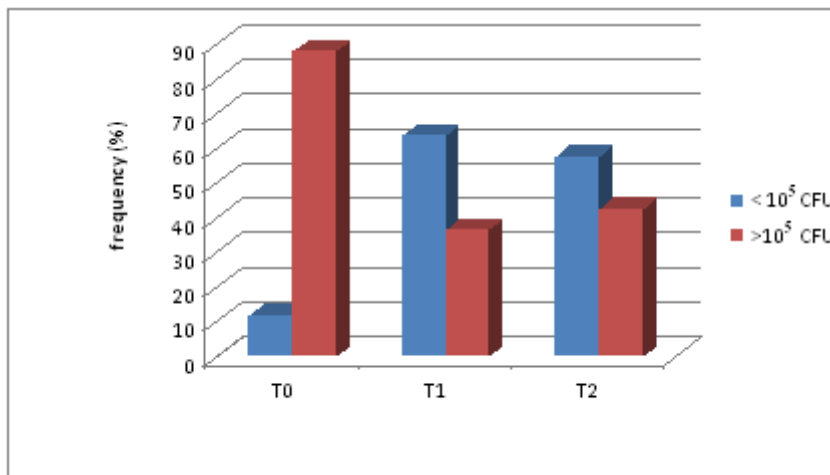

Fig. 11: variation in lactobacilli density of the CFU (CFU/ml) at t0, t1, t2 (Group A).

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 12 and 13, respectively.
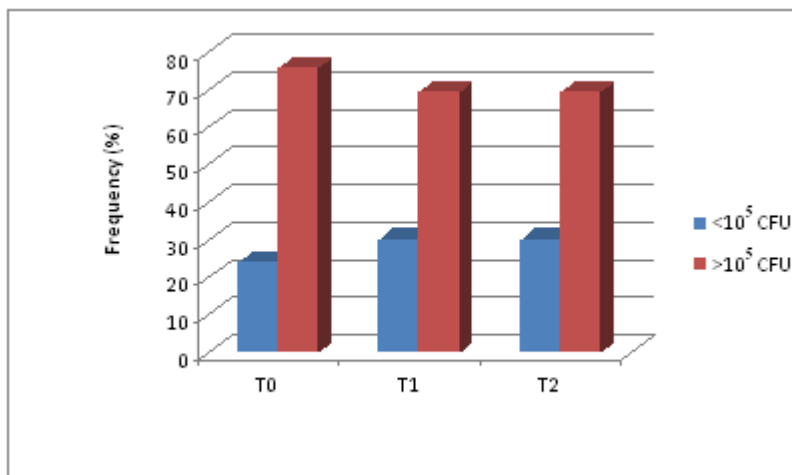

Fig. 12: variation in streptococci mutans density of the CFU (CFU/ml) at t0, t1, t2 (Group B).
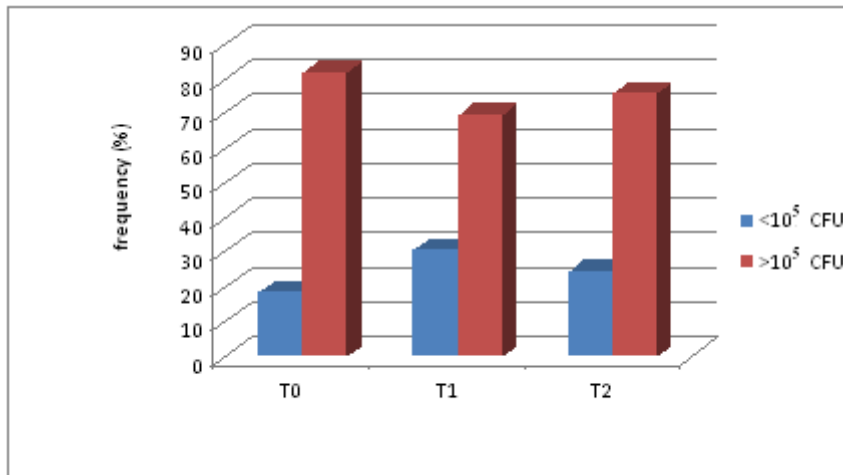
Fig. 13: variation in lactobacilli density of the CFU (CFU/ml) at t0, t1, t2 (Group B).

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)] (tab. 14).

| | Sig. | OR | 95,0%C.I. | |
			Lower	Upper
T_0	.548	.694	.211	2.283
T_1	.028	3.121	1.133	8.603
T_2	.008	4.025	1.442	11.238

Table 14: Statistical analysis between test and control groups for mutans streptococci CFU (CFU/ml) density

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)] (tab. 15).

	Sig.	OR	95,0%C.I.	
			Lower	Upper
T_0	.495	.621	.158	2.441
T_1	.008	4.025	1.442	11.238
T_2	.007	4.241	1.479	12.165

Table 15: Statistical analysis between test and control groups for lactobacilli CFU (CFU/ml) density

DISCUSSION

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 et 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.

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

EVALUATION OF CITOTOXICITY EFFECT AND ANTIBACTERIAL POWER OF PLANTAGO LANCEOLATA EXTRACT AGAINST ORAL CARIOGENIC MICROFLORA

MATERIALS AND METHOD

For the second part of the experimental study we have evaluated the in vitro citotoxicity effect of Plantago Lanceolata extract 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 is directly quantified using a scanning multiwell 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 microplates (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% CO2) for an incubation period of 24 h.

After the incubation period for each well was added 0.5% plant extracts and then the microplate were incubated for 24 h in a humidified atmosphere (e.g., 37°C, 5% CO2).

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 0.3 mg/ml).

In vivo test

The study was carried out in the Department of Paediatric Dentistry of the University of Naples "Federico II", Italy.

The sample consisted of 44 adolescents (24 males and 20 females) attending to the Unit of Pediatric Dentistry, ranging 12 to 18 years old.

All of the subjects were good general health (ASA I-II) and not taking sweetened oral medications at their time of entry into the study. Health and medication status was determined 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 44 subjects were selected with the following inclusion criteria: absence of dental cavities and periodontitis, no report of use of antibiotics in the 3 months prior to the beginning of the study and were agreement to comply with study procedures.

Also habitual consumers of xylitol chewing gums within 4 weeks were not included.

Two subjects used antibiotics for medical reasons in the 30-day period 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. After parents and patients had been given verbal and written explanations of the experimental protocol and the study aims, written informed consent was signed prior to the start of the study. Permission was received from the appropriate authorities.

A controlled random clinical essay was carried out. The subjects were divided into 2 groups (Group A and Group B). The group A were instructed to rinse with an experimental mouthrinse containing Plantago Lanceolata extracts and the group B received a placebo mouthrinse.

Then salivary counts of *mutans Streptococci* and *Lactobacilli* were estimated with a chair-side test (CRT, Ivoclar Vivadent AG, Schaan, Liechtenstein) according to the manufacturer's instructions.

Salivary samples were taken from each patient in three stages and the levels of MS and lactobacilli were determined at baseline (T0), at the third day of the experimental study (T1) and immediately after the conclusion of the experimental period (T2).

The collecting of the samples of saliva was carried out in the first hours of the day, with the patient in fasting, in a relaxed posture stimulating the salivary production by masticating a capsule of paraffin for 1 minute and the saliva was collected directly into a graded test tube.

Saliva was inoculated on a dip-slide with selective agar 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.

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.

Counts were classified according to the manufacturer's instructions, that is: level 1 : <100,000 colony forming units (CFU) and level 2: ≥100,000 CFU.

Mouthrinse preparation

Two different mouth-rinse formulations were prepared:

1)   Experimental (Group A)

Experimental mouth-rinse was prepared with pulverized Plantago Lanceolata leaves.

Plantago Lanceolata were collected from the grassy areas of the Phlegrean territory from April to August, 2010.

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

The seeds of Plantago were shade dried at 60 degree for 48 hours and then were ground to a fine powder. Two grams of powder was extracted using 20 ml of water, with centrifugation at 3000 r/min for 15 min, and then the supernatant was collected. Solvent were then removed by evaporation [Moazedi et al., 2007].

For each rinsing, 0.4 g of pulverized leaves were 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)

10 ml of distilled water were coloured with food dye.

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

The test and the control mouthrinse, 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 either the test subjects.

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 $p < 0.05$.

Experiment design –Group A

20 subjects were involved in this group. In the experimental period, after the collection of first 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 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.

All the subjects were, however, encouraged to maintain their normal oral hygiene habits and to continue to brush their teeth.

Experiment design-Group B

20 subjects were involved in this group. After the first collection of 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 and to continue to brush their teeth.

RESULTS

XTT assay

The cytotoxicity of plant extracts were evaluated by comparing the amount of soluble orange formazan of the fibroblasts cell in contact with the extract, with a 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.

On the basis of the made tests, plant extracts are not cytotoxic for any of the three extractions. 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%, those of cells treated with plant extracts was in the values range between 85% and 115 % (Figure 14).
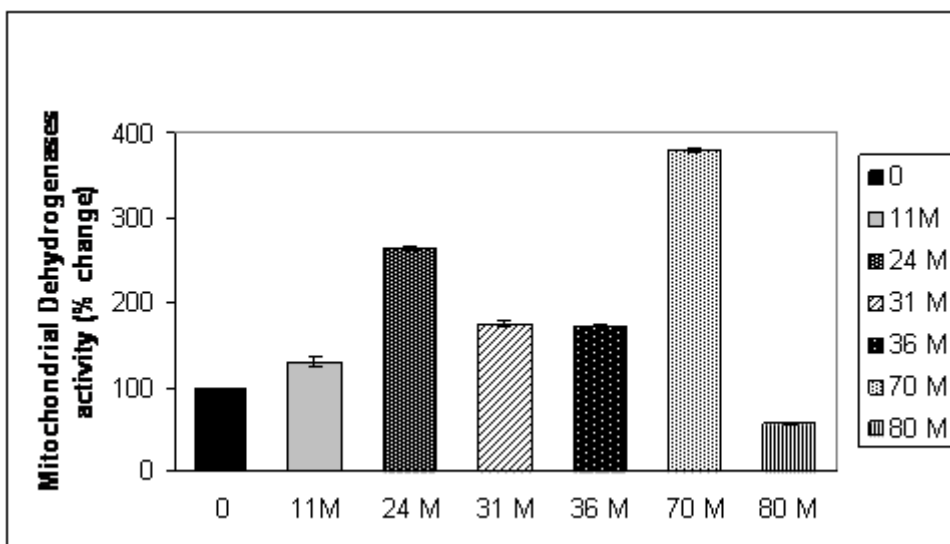

Fig. 14: Citoxicity assay of plant extracts

In vivo test

Statistical analysis within the test group

Variations in streptococci mutans and lactobacilli density of the CFU (CFU/ml) at t0,

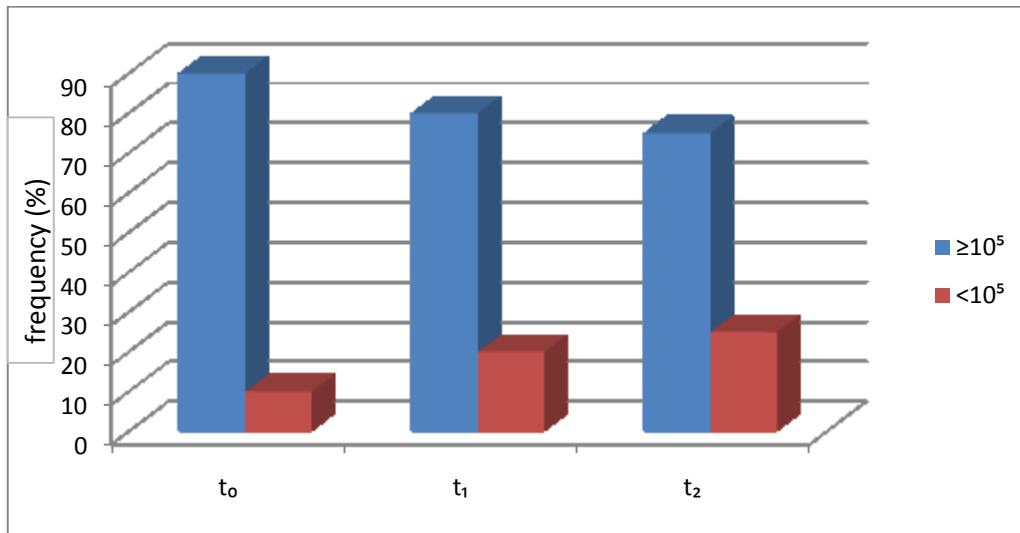t1, t2 for the test group were summarized in figures 15 and 16, respectively.

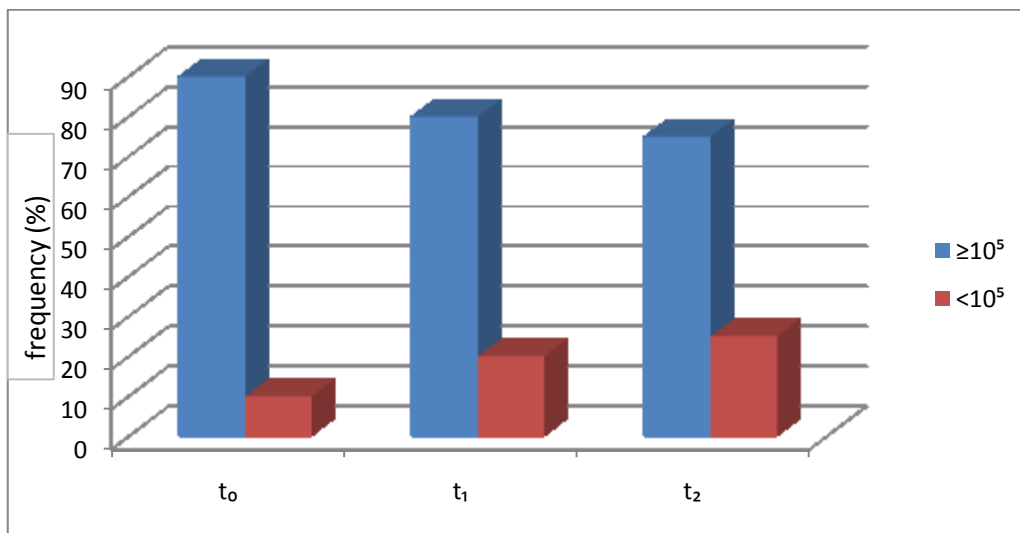**Fig. 15**: variation in streptococci mutans density of the CFU (CFU/ml) at t0, t1, t2 (test group).

Fig. 16: variation in lactobacilli density of the CFU (CFU/ml) at t0, t1, t2 (test group).

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

Statistical analysis within the control group

Variations in streptococci mutans and lactobacilli density of the CFU (CFU/ml) at t0, t1, t2 for the control group were represented in figures 17 and 18, respectively.
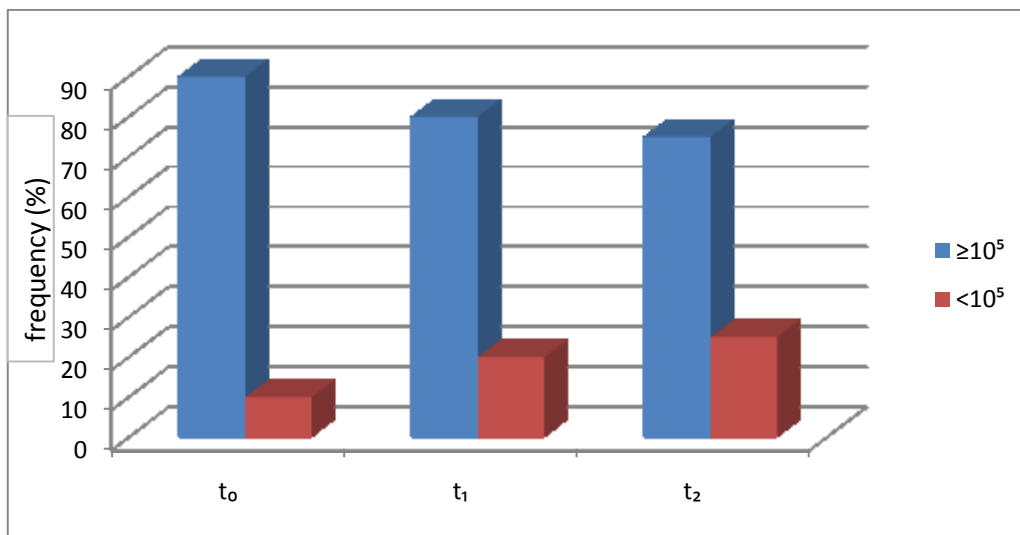

Fig. 17: variation in streptococci mutans density of the CFU (CFU/ml) at t0, t1, t2 (control group).
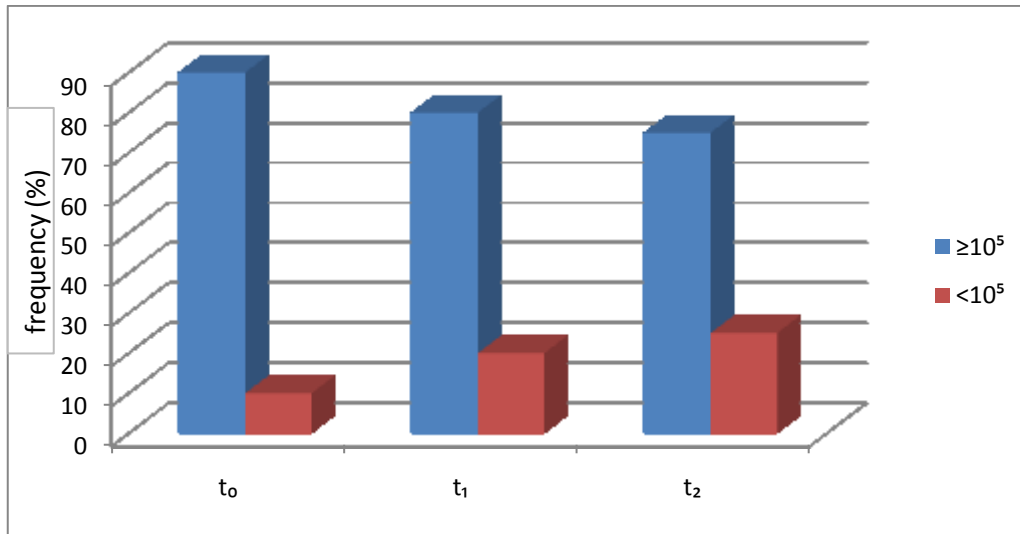
Fig. 18: variation in lactobacilli density of the CFU (CFU/ml) at t0, t1, t2 (control group).

The differences in CFU (CFU/ml) density of streptococci 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

At t0 the differences in CFU (CFU/ml) density of streptococci mutans between test and control groups were not statistically significant, while at t1 and t2 the differences were statistically significant, respectively [t1: OR=7.0 (CI=1.7-28.17); t2: OR=9.0 (CI=12.15-37.6)] (tab. 16).

	Sig.	OR	95,0%C.I.	
			Lower	Upper
T_0	.635	1.588	.236	10.704
T_1	.174	2.667	.648	10.971
T_2	.047	4.635	1.023	20.993

Table 16: Statistical analysis between test and control groups for mutans streptococci CFU (CFU/ml) density.

At t0, t1 and t2 the differences in CFU (CFU/ml) density of lactobacilli between test and control groups were not statistically significant, respectively (tab. 17).

	Sig.	OR	95,0%C.I.	
			Lower	Upper
T_0	.635	1.588	.236	10.704
T_1	.174	2.667	.648	10.971
T_2	.047	4.635	1.023	20.993

Table 17: Statistical analysis between test and control groups for lactobacilli CFU (CFU/ml) density

DISCUSSION

The study was undertaken to investigate the effect of mouthrinse containing phenolic extracts on caries-associated microorganisms in saliva. The novel approach used in the present research was the in vivo experimental design and the phenolic extracts utilized.

Furthermore, plantago mouthrinse taste was commonly agreed by adolescents and no compliance problems were experienced.

All subjects exhibited detectable levels of salivary mutans streptococci and lactobacilli at baseline and about over 80 per cent had levels $\geq 10^5$ CFU.

In the Group A with the experimental mouthrinse , the number of subjects with high mutans streptococci count decreased from 80 to 20 per cent. 15 subjects exhibited decreased scores, 4 had unchanged scores, while one displayed an increased score. In the control group, 2 subjects displayed a decreased score while 18 had an unchanged score.

The high levels of MS did not decrease after use of Plantago mouthrinse in one of ten studied subjects, possibly due to presence of Plantago resistant strains, but other hypotheses, such as a low compliance or a high sucrose intake leading to high glucose/fructose levels in saliva, should not be discarded.

Regarding salivary lactobacilli, no statistically significant changes were observed between the pre- and post -consumption samples, either in the test group and in the control group. In the test group, 13 subjects had unchanged scores, 1 showed an increase and 6 a decrease.

In the control group 17 subjects had unchanged scores and 3 showed a decrease.

The reason for the demonstrated difference between the reduction seen in the streptococci and the lack of reduction in lactobacilli is not clear. Apart from strain-specific differences, a possible explanation is that mutans streptococci normally grow on exposed surfaces, easy accessible for the phenolic substrate, while lactobacilli are recovered in shed retentive areas with limited contact with the mouthrinse.

The results showed that daily usage of plantago mouthrinse for 7 days decreased the mutans streptococci counts in saliva.

A significant reduction in the colony count of mutans streptococci was observed during the first 4 days compared with the baseline count, while no significant decrease in the count of total streptococci or lactobacilli was found during the other 3 days.

A statistically significant ($P < 0.05$) reduction of salivary mutans streptococci was registered after 1 week consumption of the test mouthrinse, while no alterations were found in the control group.

This study shows the changes in salivary bacterial levels that occur in the oral environment after usage of a phenolic extracts mouthrinse on 40 adolescents.

Several strains of oral streptococci are capable of initiating the formation of dental plaque, which plays an important role in the development of caries and periodontal disease in humans [Freedman, Tanzer, 1974]. The use of antimicrobial agents in the oral cavity may reduce caries risk by reducing salivary levels of main cariogenic oral becteria.

In fact the effect of concentrated antimicrobial agents, such as 1% chlorhexidine gel, on oral bacteria salivary levels can be observed after a couple of applications [Decker et al., 2008] , but the use of chlorhexidine in high concentrations (1% or 2% gel), or

other forms of application, such as varnishes, requires professional procedures. Otherwise, the results from this study showed that the use of Plantago mouthrinse is also able to reduce oral baceria levels with a procedure easily performed by all the subject.

Our exploratory study indicate that phenolic mouthrinse could be a treatment strategy that holds promise for the treatment of adolescents with high-risk caries.

If successful, the phenolic home-care intervention may be a cost-effective alternative to a professional topical programme for dental caries prevention.

Further studies are needed to clarify if this approach is an alternative strategy for the prevention of dental caries.

CONCLUSIONS

Caries are still considered one of the main problems of public health, and the primary etiological agents of this oral disease are known to be several restricted strains of oral bacteria; thus, the majority of current commercial antiplaque products are antimicrobial compounds, but many antibiotic and chemical bactericides currently used to prevent bacterial infection disturb the bacterial flora of the oral cavity and digestive tract [Gunsolley, 2006].

The studies carried out in recent decades have supported the antibacterial role of polyphenols, but their potential use in the control of bacteria responsible of cariogenesis is still under scrutiny. More studies, particularly in vivo and in situ, are necessary to clarify the effectiveness and the clinical applications of these compounds. Further research on the anticariogenic activity of polyphenols could open a promising avenue of applications because these compounds largely occur in flowering plants and could be used at a reasonable cost in the preparation of specific remedies. Flavonoids seems to be particularly promising anticariogenic molecules, but studies on the structure and activity of these compounds, as well as on their synergistic/antagonistic effects, are still required.

An analysis of the literature suggest that diet may influence decay by either inhibiting or promoting disease. Far more is known about the dietary factors that promote dental decay than those that inhibit it. A relatively large body of evidence has been accumulated on the effects of polyphenols occurring in foods and beverages [Petty and Scully, 2009; Ferrazzano et al., 2009].

To establish conclusive evidence for the effectiveness of polyphenols in caries prevention, it is essential to determine the nature and distribution of these compounds

in our diet and to better identify which of the hundreds of existing polyphenols are likely to provide the greatest effects. Furthermore, it is crucial to understand the factors involved in the release of polyphenols from foods in which they are contained, the extent of their absorption, and their fate in the organism. These properties that collectively describe a compound's "bioavailability", a term originally used in pharmacology to define the concept of the "rate and extent to which a drug reaches its site of action" [Stahl et al., 2002].

The health effects of polyphenols depend both on their intake and their bioavailability. The concept of bioavailability integrates several variables, including intestinal absorption, metabolism by the microflora, intestinal and hepatic metabolism, the nature of circulating metabolites, binding to albumin, cellular uptake, accumulation in tissues, and biliary and urinary excretion. The main difficulty is integrating all this information and relating it to health effects at the organ level.

Since the evidence for the therapeutic effects of dietary polyphenols continues to accumulate, it is becoming more and more important to understand the nature of their absorption and metabolism *in vivo*. Moreover, the identification and measurement of the physiologic polyphenol metabolites represents a key prerequisite for the understanding of the role of dietary polyphenols in human health.

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