Review

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Natural Products in Caries Research: Current (Limited) Knowledge, Challenges and Future Perspective

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Antimicrobials · Biofilms · Dental caries · Extracellular matrix · Glucosyltransferases · Natural products

Abstract
Dental caries is the most prevalent and costly oral infectious disease worldwide. Virulent biofilms firmly attached to tooth surfaces are prime biological factors associated with this disease. The formation of an exopolysaccharide-rich biofilm matrix, acidification of the milieu and persistent low pH at the tooth-biofilm interface are major controlling virulence factors that modulate dental caries pathogenesis. Each one offers a selective therapeutic target for prevention. Although fluoride, delivered in various modalities, remains the mainstay for the prevention of caries, additional approaches are required to enhance its effectiveness. Available antiplaque approaches are based on the use of broad-spectrum microbicidal agents, e.g. chlorhexidine. Natural products offer a rich source of structurally diverse substances with a wide range of biological activities, which could be useful for the development of alternative or adjunctive anticaries therapies. However, it is a challenging approach owing to complex chemistry and isolation procedures to derive active compounds from natural products. Furthermore, most of the studies have been focused on the general inhibitory effects on glucan synthesis as well as on bacterial metabolism and growth, often employing methods that do not address the pathophysiological aspects of the disease (e.g. bacteria in biofilms) and the length of exposure/retention in the mouth. Thus, the true value of natural products in caries prevention and/or their exact mechanisms of action remain largely unknown. Nevertheless, natural substances potentially active against virulent properties of cariogenic organisms have been identified. This review focuses on gaps in the current knowledge and presents a model for investigating the use of natural products in anticaries chemotherapy.

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The use of natural products to prevent and/or treat oral maladies dates back several thousand years both in Western and Eastern societies. For example, a large number of recipes for mouthwashes and dentifrices can be

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found in the Ebers Papyrus, which are composed of natural substances [Hirschfeld, 1939]. In addition, chewing stick, introduced by the Babylonians in 5000 B.C., and a wide range of traditional medicinal plants continue to be used among many African and Asian communities [Jagtap and Karkera, 2000; Pai et al., 2004; Song et al., 2007]. The term 'natural products' is usually associated with secondary metabolites produced by an organism, which in most cases function as defense mechanisms against herbivores, microorganisms, insects and competing plants [Singer et al., 2003]. Plants produce a diverse array of more than 100,000 secondary metabolites, and estimates of the total number in plants exceed 500,000 [Singer et al., 2003]. Secondary metabolites can be classified on the basis of composition (e.g. whether or not they contain nitrogen), the pathway by which they are synthesized, or chemical structure (e.g. the presence of rings with or without sugar moieties) [Chinou, 2008]. A simple classification includes three main groups: (i) phenolic compounds, which are made from simple sugars, containing benzene rings, hydrogen and oxygen; (ii) terpenoids, which are made from mevalonic acid and composed almost entirely of carbon and hydrogen; and (iii) alkaloids, which are nitrogen-containing compounds [Chinou, 2008]. These chemical groups have been associated with biological effects of natural products, which may include antimicrobial, antioxidant, and anticancer activities [Newman and Cragg, 2007]. In this review, we will focus on natural products that harbor such bioactive molecules, which may exert anticaries properties.

Natural products have been used as a major source of innovative and effective therapeutic agents throughout human history, offering a diverse range of structurally distinctive bioactive molecules. Of the 1,184 new chemical entities introduced between 1981 and 2006, about half (48%) were natural products, semisynthetic natural product analogues or synthetic compounds based on natural-product pharmacophores [Newman and Cragg, 2007]. More importantly, over 70% of the therapeutic agents developed between 1981 and 2006 for infectious diseases (both bacterial and fungal) were derived from natural products [Newman and Cragg, 2007]. Naturally occurring compounds possess structures that have high chemical diversity and biochemical specificity, as well as other molecular properties that make them attractive candidates for drug discovery and development [Koehn and Carter, 2005]. Yet, studies using natural products to prevent or treat oral diseases such as dental caries have received minimal attention compared with other fields in medicine. Furthermore, many published studies are lacking critical information such as chemical characterization of the natural extracts used, and/or utilize experimental approaches that are poorly designed (see also the *Caries Research* policy on papers concerning anticaries effects of natural products).

Influence of Natural Products on the Pathophysiology of Dental Caries

Despite the widespread use of different sources of fluoride, dental caries continues to be the single most prevalent and costly oral infectious disease worldwide [National Institutes of Health, 2001; Marsh, 2003; Dye et al., 2007]. Virulent biofilms that are tightly adherent to oral surfaces are a primary cause of infectious diseases in the mouth, including dental caries [Bowen and Koo, 2011]. Dental caries results from the interactions of specific bacteria and their metabolic/virulence products with salivary constituents and dietary carbohydrates that occur on the susceptible tooth surface. The formation of the extracellular polysaccharide (EPS)-rich biofilm matrix, acidification of the milieu and the maintenance of a low-pH environment at the tooth-biofilm interface are major controlling virulence factors that modulate dental caries pathogenesis. Biofilms formed in vivo are comprised of mixed flora, although mutans streptococci are recognized as the primary producers of the EPS-rich matrix. *Streptococcus mutans* plays a key role in the development of virulent biofilms, although additional microorganisms may be also involved in the pathogenesis of the disease [Beighton, 2005]. This bacterium (i) effectively utilizes dietary sucrose (and possibly starch) to rapidly synthesize EPS through the activity of glucosyltransferases (Gtfs) and a fructosyltransferase that are adsorbed to saliva-coated tooth enamel surfaces, (ii) adheres tenaciously to glucan-coated surfaces, and (iii) is both acidogenic and acid tolerant [Bowen, 2002; Quivey et al., 2000]. Thus, *S. mutans* thrives in the complex oral microbiome and effectively modulates the transition from nonpathogenic to cariogenic biofilms.

Cariogenic biofilms develop following initial microbial attachment to and further accumulation on the tooth surface, which is predominantly mediated by sucrose-dependent mechanisms. The EPS is mainly comprised of glucans, which are synthesized by Gtfs present in saliva, in the acquired pellicle (primarily GtfC), and those adsorbed on bacterial surfaces (mostly GtfB) in the presence of sucrose. The glucans formed in situ provide (i) avid binding sites for colonization by *S. mutans*

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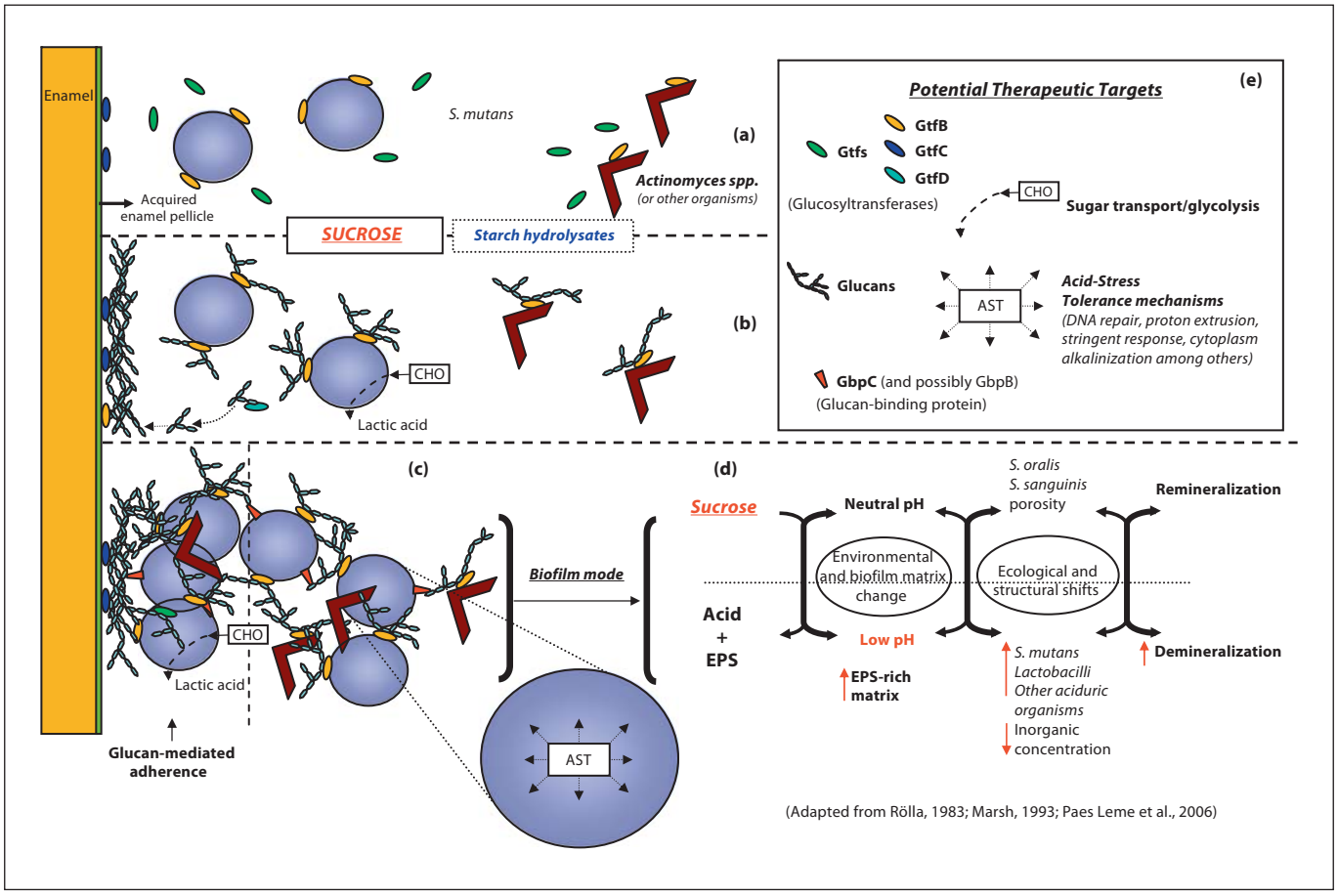

Fig. 1. Cariogenic biofilm formation (**a–d**) and potential targets (**e**) for disruption by natural products. **a** The Gtfs secreted by *S. mutans* are incorporated into pellicle (particularly GtfC) and adsorb on bacterial surfaces (mainly GtfB), including microorganisms that do not produce Gtfs (e.g. *Actinomyces* spp.). **b** Surface-adsorbed GtfB and GtfC rapidly utilize dietary sucrose (and starch hydrolysates) to synthesize insoluble and soluble glucans in situ; the soluble glucans formed by GtfD could serve as primers for GtfB enhancing overall synthesis of EPS. The Gtfs adsorbed onto enamel and microbial surfaces provide in situ an insoluble matrix for dental plaque-biofilm. Concomitantly, dietary carbohydrates (CHO) are metabolized into acids by acidogenic/aciduric organisms (e.g. *S. mutans*). **c** The glucan molecules provide avid binding sites on surfaces for *S. mutans* and other microorganisms mediating tight bacterial clustering and adherence to the tooth enamel (through glucan-glucan and glucan-Gbp interactions). Furthermore, Gtf-adsorbed bacteria de facto become glucan producers binding to tooth and microbial surfaces by the same mechanisms. This model could explain the rapid formation and accumulation of highly cohesive-adherent plaque in the presence of sucrose (and possibly starch) even if the number of *S. mutans* is relatively low. After the establishment of a glucan-rich biofilm matrix, ecological pressure (e.g. pH) will determine which bacteria may survive and dominate within plaque under frequent sucrose (or other fermentable carbohydrate) exposure. **d** If biofilm remains on tooth surfaces with frequent consumption of high-carbohydrate diet (especially sucrose), the amount of EPS and extent of acidification of the matrix will be increased. Such conditions cause biochemical, ecological and structural changes favoring the survival and dominance of highly acid stress-tolerant organisms in cohesive and firmly attached biofilms. The low-pH environment at the tooth-biofilm interface results in enhanced demineralization of enamel.

and other acidogenic/aciduric organisms, and (ii) a matrix that holds the microbial cells together to form structurally cohesive cell clusters known as microcolonies [Bowen and Koo, 2011] (fig. 1). If these biofilms are not removed from the tooth surface and are frequently exposed to dietary carbohydrates (especially sucrose), *S. mutans* (and other acidogenic and aciduric bacteria) within the biofilm community will metabolize sucrose to organic acids and synthesize polysaccharides in situ. The elevated amounts of EPS enhance bacterial adher-

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ence and biofilm cohesiveness, shelter matrix-encased bacteria from environmental assaults (e.g. antimicrobials), improve the stability of the structure and affect the diffusion properties of the biofilm matrix [Paes Leme et al., 2006; Bowen and Koo, 2011]. Furthermore, insoluble EPS provides a framework for the establishment of tightly adherent three-dimensional biofilm structures [Koo et al., 2010b]. Conversely, soluble glucans, fructans and intracellular polysaccharides serve as short-term storage compounds that can be metabolized to increase overall acid production. Acidification of the biofilm matrix affords a competitive ecological advantage to acid stress-tolerant and acidogenic flora, such as *S. mutans* [Quivey et al., 2000; Marsh, 2003; Lemos and Burne, 2008]. The low-pH environment subsequently created at the tooth-biofilm interface results in demineralization of the enamel. Clearly, exopolysaccharides, acidification of the biofilm matrix as well as acid tolerance mechanisms are critical for the development of cariogenic biofilms, and thus could be attractive and specific targets for precise and highly selective chemotherapeutic strategies.

Despite an increased understanding of the biological and physicochemical processes involved in the pathogenesis of dental caries, the vast majority of the chemical agents aiming at controlling dental biofilm formation are broad-spectrum nonspecific microbicidal agents. Chlorhexidine and triclosan are the most commonly used and clinically tested agents for antiplaque therapies [Axelsson, 1993; Eley, 1999]. There are several review articles that examine the advantages and disadvantages of these agents [Eley, 1999; Twetman, 2004], which will not be discussed in this review.

We have attempted to provide a brief summary of the current, albeit limited, state of knowledge concerning the potential biological effects of natural products on three relevant categories: (i) antimicrobial activity, specifically the inhibition of growth and metabolism of acidogenic and aciduric organisms, (ii) inhibition of exopolysaccharide synthesis, and (iii) inhibition of bacterial adherence.

Antimicrobial Activity

Inhibitors of Bacterial Growth

The majority of the studies conducted thus far have centered on investigating the effects of natural products on the growth of cariogenic microorganisms, primarily mutans streptococci (table 1). The focus on the development of bacteriostatic or bactericidal therapies is not surprising as dental caries is an infectious disease and there is an established and successful history of discovery of novel antibiotics as a result of natural product screening. Most of these compounds are derived from secondary metabolites of plant origin, including phenolic acids, anthraquinones, flavonoids, stilbenes, tannins, terpenoids and alkaloids, all of which are effective inhibitors of the growth of other pathogenic microorganisms [Wen et al., 2004]. Compounds targeting bacterial viability are typically aiming at bacterial eradication via one or more of the following modalities [Cowan, 1999]: (i) disruption of cell wall biosynthesis and/or cell membrane permeability, (ii) complexing with surface-adsorbed components, (iii) inhibiting protein synthesis or nucleic acid metabolism, and/or (iv) inhibition of enzyme activity through oxidation of other, often uncharacterized, interactions with bacterial proteins. Unfortunately, all of the modalities are generally not selective for specific species and possess broad-spectrum antibacterial effects. Table 1 lists several natural extracts and their putative active compounds which show bacteriostatic or bactericidal effects as determined using classical assays such as minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) for batch or plate culture planktonic cells.

Camellia sinensis (used to make green tea and oolong tea) has been widely studied for its antibacterial activities against caries-related bacteria, using both in vitro and in vivo models [Otake et al., 1991; Shapiro et al., 1994; Shapiro and Guggenheim, 1995; Ooshima et al., 1998]. Specific catechins from green tea have been associated with antibacterial activity against *S. mutans* and *Streptococcus sobrinus* [Sakanaka et al., 1989]. This inhibitory effect appears to be related to the presence of three hydroxy moieties at 3′, 4′ and 5′ on the B ring of the catechin and epicatechin molecular structure [Sakanaka et al., 1989]. However, the exact mechanisms of action and their putative target(s) remain to be elucidated.

Essential oils have also been extensively studied for antimicrobial activity against caries-related bacteria. Essential oils derived from plants are typically a complex mixture of approximately 20–60 compounds that are in solution at various concentrations. Overall, the main chemical group is primarily composed of terpenoids, followed by aromatic and aliphatic constituents [Bakkali et al., 2008]. Thymol and eugenol inhibit the growth of a wide range of oral microorganisms including mutans streptococci [Shapiro et al., 1994; Shapiro and Guggenheim, 1995]. In general, essential oils would appear to affect bacterial viability by compromising the integrity of the bacterial membrane. For example, thymol induces the rapid efflux of intracellular bacterial components, which likely results

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Table 1. Biological activities of natural products with potential use as alternative or adjunctive anticaries chemotherapy

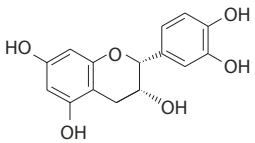
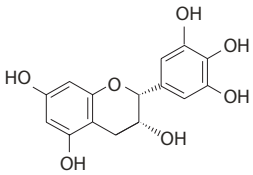
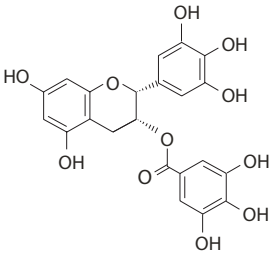
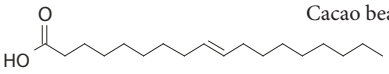
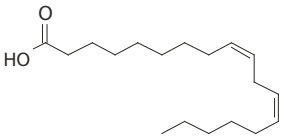
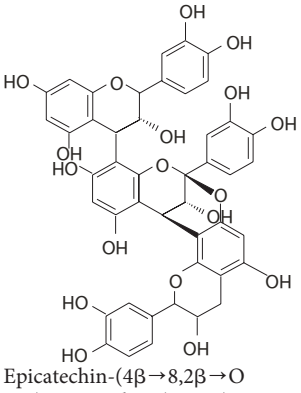
Putative active constituents	Examples of chemical structure of main putative active constituents	Source of natural products	Biological activity	References
Catechin-based polyphenols		Black tea (*Camellia sinensis*)	– Inhibitory effects on Gtf activity – Reduction in caries development in rats and hamsters infected with *S. mutans*	Hattori et al., 1990; Touyz and Amsel, 2001; Linke and LeGeros, 2003
	Epicatechin 	Green tea (*C. sinensis*)	– Antimicrobial activity against planktonic cells of *S. mutans* – Inhibitory effects on Gtf activity and *S. mutans* adherence – Reduction in caries development in rats infected with *S. mutans* – Inhibitory effects on acid production in human dental plaque	Sakanaka et al., 1989; Hattori et al., 1990; Otake et al., 1991; Hirasawa et al., 2006
	Epigallocatechin 	Oolong tea (*C. sinensis*)	– Antimicrobial activity against planktonic cells of mutans streptococci – Inhibitory effects on Gtf activity and mutans streptococci adherence – Reduction in cellular hydrophobicity and induction of aggregation of mutans streptococci – Reduction in plaque accumulation and caries development in rats infected with *S. mutans* or *Streptococcus sobrinus* – Inhibitory effects on human plaque accumulation	Nakahara et al., 1993; Ooshima et al., 1993, 1994, 1998; Matsumoto et al., 1999; Sasaki et al., 2004
	Epigallocatechin gallate			
Oleic acid, linoleic acid, epicatechin polymer	 Oleic acid	Cacao bean husk	– Antimicrobial activity against planktonic cells of mutans streptococci – Inhibitory effects on water-insoluble glucan synthesis, adherence, acid production by mutans streptococci – Reduction in plaque accumulation and caries development in rats infected with *S. mutans* or *S. sobrinus* – Reduction in human plaque accumulation and salivary mutans streptococci counts	Ooshima et al., 2000; Osawa et al., 2001; Matsumoto et al., 2004
	 Linoleic acid			
Proanthocyanidins, phenolic acids, flavonols	 Epicatechin-(4β→8,2β→O →7)-epicatechin-(4β→8) epicatechin	Cranberry (*Vaccinium macrocarpon*)	– Antimicrobial activity against biofilm cells of mutans streptococci – Disruption of acidogenic/aciduric properties of planktonic and biofilm cells of *S. mutans* – Inhibitory effects on Gtf activity and adherence by mutans streptococci – Reduction of formation of *S. mutans* biofilms and EPS content – Enhanced detachment of bacterial cells from biofilms of *S. mutans* – Reduction in caries development in rats infected with *S. mutans*	Steinberg et al., 2004; Yamanaka et al., 2004; Duarte et al., 2006a; Gregoire et al., 2007; Koo et al., 2010a

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Table 1 (continued)

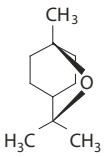
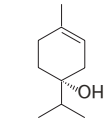
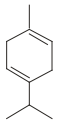
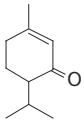
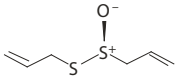
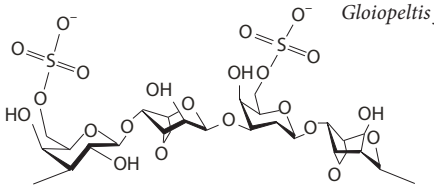
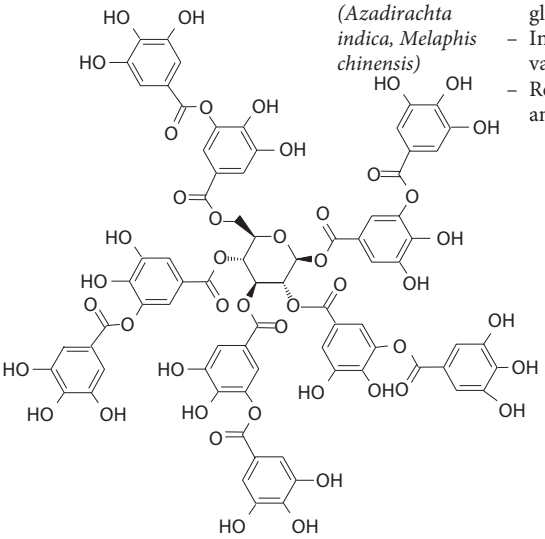
Putative active constituents	Examples of chemical structure of main putative active constituents	Source of natural products	Biological activity	References
Cineole, terpinen-4-ol	 Cineole  Terpinen-4-ol	Essential oil from *Melaleuca alternifolia*	– Antimicrobial activity against planktonic cells of mutans streptococci – Reduction in human salivary mutans streptococci counts	Groppo et al., 2002; Hammer et al., 2003; Takarada et al., 2004
Terpinene, piperitenone oxide, piperitone, pinene	 Terpinene  Piperitone	Essential oils from *Mentha piperita* and *Rosmarinus officinalis*	– Antimicrobial activity against planktonic cells of mutans streptococci – Inhibitory effects on human plaque formation	Takarada et al., 2004; Rasooli et al., 2008
Allicin	 Allicin	Garlic *(Allium sativum)*	– Antimicrobial activity against planktonic cells of various oral pathogens including mutans streptococci – Reduction in salivary mutans streptococci counts	Groppo et al., 2002; Bakri and Douglas, 2005
Funoran	 Funoran	*Gloiopeltis furcata*	– Inhibition of mutans streptococci adherence and desorption effects – Reduction in dental plaque and caries development in rats infected with *S. sobrinus* – Reduction in human dental plaque formation	Saeki et al., 1996; Sato et al., 1998
Gallotannins	 Gallotannin	Neem *(Azadirachta indica, Melaphis chinensis)*	– Inhibitory effects on water-insoluble glucan synthesis by mutans streptococci – Induction of bacterial aggregation of various oral streptococci – Reduction in human dental plaque formation and salivary mutans streptococci counts	Wolinsky et al., 1996; Pai et al., 2004; Wu-Yuan et al., 1988

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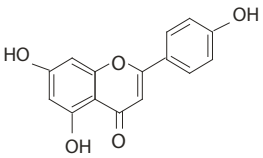
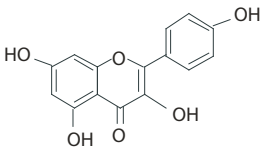
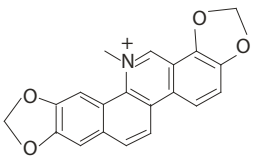
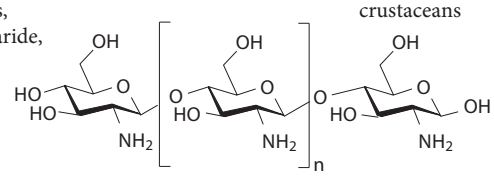
Table 1 (continued)

Putative active constituents	Examples of chemical structure of main putative active constituents	Source of natural products	Biological activity	References
Apigenin, kaempferol, *tt*-farnesol	 Apigenin  Kaempferol	Propolis	– Antimicrobial activity against planktonic and biofilm cells of *S. mutans* – Disruption of acidogenic/aciduric properties of planktonic and biofilm cells of *S. mutans* – Inhibitory effects on Gtf activity and *gtfBCD* gene expression – Reduction of formation of *S. mutans* biofilms and EPS content – Reduction in caries development in rats infected with *S. mutans* or *S. sobrinus* – Reduction in human dental plaque accumulation and its insoluble EPS content	Ikeno et al., 1991; Koo et al., 2000, 2002a, b, 2003, 2005
Sanguinarine	 Sanguinarine	*Sanguinaria canadensis*	– Antimicrobial activity against planktonic cells of various oral pathogens including *S. mutans* – Without effect on human salivary bacterial counts	Dzink and Socransky, 1985; Godowski, 1989; Hoover and To, 1990; Cai et al., 1994
Low-molecular-weight chitosans, chitooligosaccharide, water-soluble chitosans	 Chitosan	Shells of crustaceans	– Antimicrobial activity against planktonic and biofilm cells of *S. mutans* – Inhibitory effects on *S. mutans* adherence – Reduction in bacterial viability in human dental plaque and salivary mutans streptococci counts	Tarsi et al., 1997; Choi et al., 2001; Fujiwara et al., 2004; Bae et al., 2006; Hayashi et al., 2007; Busscher et al., 2008
Lenthionine, disulfide derivative, oligosaccharides	Lenthionine	Shiitake *(Lentinus edodes)*	– Antimicrobial activity against planktonic cells of mutans streptococci – Reduction in biofilm formation and water-insoluble glucan synthesis by mutans streptococci – Reduction in caries development in rats infected with *S. mutans*	Hirasawa et al., 1999; Shouji et al., 2000
Polyphenols	Unknown	Oat hulls	– Growth inhibition of *Lactobacillus acidophilus* – Reduction in caries development in rats	Vogel et al., 1962; Stookey and McDonald, 1974
Polyphenols	Unknown	Hop bracts	– Inhibitory effects on water-insoluble glucan synthesis by mutans streptococci – Reduction in human dental plaque formation	Tagashira et al., 1997; Shinada et al., 2007
Unknown	Unknown	Miswak *(Salvadora persica)*	– Antimicrobial activity against planktonic cells – Reduction of salivary bacterial level	Darout et al., 2002; Almas and Al-Zeid, 2004

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from the perforation of the cellular membrane [Shapiro and Guggenheim, 1995].

Propolis, a natural beehive product, and cacao bean husk extracts have also shown significant antibacterial activity against *S. mutans* and/or *S. sobrinus* in vitro [Ikeno et al., 1991; Park et al., 1998; Ooshima et al., 2000; Osawa et al., 2001]. Specific flavanones (pinocembrin), dihydroflavonols (pinobanksin-3-acetate) and terpenoids (*tt*-farnesol) were associated with the bioactivity of propolis [Koo et al., 2002c]. *tt*-Farnesol has been demonstrated to be particularly effective against both planktonic and biofilm-associated *S. mutans* cells [Koo et al., 2003]. Oleic and linoleic acids, a monounsaturated ω–9 fatty acid and an unsaturated ω–6 fatty acid, respectively, were identified as the main bactericidal agents in cacao extract [Osawa et al., 2001]. The antibacterial activity of *tt*-farnesol, oleic and linoleic acids may be related to their lipophilic characteristics and membranotropic effects, which likely damage the integrity of the cell membrane [Cowan, 1999; Osawa et al., 2001; Inoue et al., 2004] and increase proton permeability [Jeon et al., 2009].

There are many other reports in the literature concerning the antimicrobial activities that various plant extracts may have against cariogenic bacteria, although the majority of these studies provide limited or incomplete information due to the lack of chemical characterization of the extracts. However, there are a few exceptions. For example, Wu-Yuan et al. [1988] and Li et al. [1997] have identified gallotannins from *Melaphis chinensis* and triterpenes (ceanothic acid and ceanothetric acid) from *Ceanothus americanus* as antimicrobial agents that harbor activity against mutans streptococci. Furthermore, a chemically characterized extract of *Galla chinensis* (containing gallic acid and methyl gallate) has been demonstrated to impede the growth of *S. mutans* and other caries-related organisms, including *Lactobacillus rhamnosus* and *Actinomyces naeslundii*, within biofilms [Xie et al., 2008]. Recently, Greenberg et al. [2008] established that naturally occurring phenolic compounds generally display antibacterial activity by disrupting the membrane lipid-protein interface as nonionic surface-active agents.

Inhibitors of Acid Production and Acidurance

Several studies have highlighted a range of natural products that inhibit acid production in cariogenic streptococci (table 1). However, these inhibitory effects appear to be related to a reduction in bacterial growth rather than the specific inhibition of the glycolytic activity and/or aciduricity of the cariogenic organisms. Due to the limitations of the experimental procedures, compounds with specific activities were not identified (see Problems and Limitations section).

Nevertheless, recent studies have shown that some of the agents affect the acidogenicity of *S. mutans* by targeting multiple mechanisms, sometimes simultaneously, including (i) disruption of the membrane proton motive force, (ii) enzymatic activity and expression of specific enzymes related to sugar transport, (iii) glycolysis, and/or (iv) general metabolism. For example, 7-epiclusianone (a prenylated benzophenone isolated from *Rheedia gardneriana*), *tt*-farnesol and some cranberry flavonoids (e.g. myricetin, procyanidin A2 and A-type oligomers) have been reported to increase the proton permeability of *S. mutans* cells, causing cytoplasmic acidification, and thereby inhibiting the acid-sensitive intracellular glycolytic enzymes [Gregoire et al., 2007; Murata et al., 2008; Koo et al., 2010a]. Combining flavonoids (apigenin or myricetin) with *tt*-farnesol also disrupts the accumulation of intracellular iodophilic polysaccharides (IPS) by *S. mutans* in biofilms [Koo et al., 2003; Jeon et al., 2009]. IPS (a glycogen-like storage polymer) provides an endogenous source of carbohydrates for *S. mutans* and other cariogenic organisms, which can be metabolized when exogenous fermentable substrates have been depleted in the oral cavity, thus lowering the fasting pH in the plaque matrix. More importantly, IPS production has also been associated with dental caries development in both animals and humans [Loesche and Henry, 1967; Tanzer et al., 1976; Spatafora et al., 1995]. Recently, a crude extract of *Psidium cattleianum* and epigallocatechin gallate (from green tea) have been shown to disrupt the expression and activity of specific enzymes involved in the glycolytic pathway of *S. mutans*, including lactate dehydrogenase [Hirasawa et al., 2006; Brighenti et al., 2008]. Although these observations are promising, additional studies are needed to elucidate the mechanisms by which these agents impair the acidogenicity and aciduricity of cariogenic streptococci and lactobacilli.

Inhibition of Exopolysaccharide Synthesis

One approach to reducing the incidence of dental caries is the discovery and development of therapeutic agents that may prevent the formation of the plaque EPS-rich matrix. EPS is largely comprised of glucans synthesized by microbial Gtfs, which use dietary sucrose as their primary substrate, and Gtfs are proven virulence factors implicated in the pathogenesis of dental caries [Paes Leme et al., 2006; Bowen and Koo, 2011]. Therefore, we will focus on the activity and inhibition of the Gtfs for the pur-

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poses of this review. *S. mutans,* a key contributor to EPS synthesis, produces at least three distinct Gtfs – GtfB, C and D – which synthesize water-insoluble glucans rich in α-1,3-linked glucose (GtfB and C) and water-soluble glucans with mostly α-1,6-linked glucose (GtfC and D) [Loesche, 1986]. Each of the Gtfs and their polysaccharide products have been implicated in various roles in plaque biofilm formation and dental caries pathogenesis, particularly GtfB and GtfC [Tanzer et al., 1985; Yamashita et al., 1993; Venkitaraman et al., 1995; Tamesada et al., 2004; Koo et al., 2010b; Bowen and Koo, 2011]. These enzymes are found in solution in saliva as well as bound to the salivary pellicle formed on the tooth surface or to microbial surfaces in their active forms. These enzymes can even be found bound to non-Gtf-producing oral bacteria [McCabe and Donkersloot, 1977; Rölla et al., 1983; Schilling and Bowen, 1988; Venkitaraman et al., 1995; Vacca-Smith and Bowen, 1998].

The glucans in the pellicle, which are produced by surface-adsorbed Gtfs (primarily GtfC followed by GtfB), provide avid binding sites that promote adherence of larger numbers of *S. mutans* and *S. sobrinus* as these organisms bind with greater adhesion strength to the in situ glucans than to saliva-coated apatitic (sHA) surfaces [Schilling and Bowen, 1992; Venkitaraman et al., 1995; Cross et al., 2007]. The binding of these organisms to glucans formed in situ is mediated by the presence of cell-associated Gtf enzymes and non-Gtf glucan-binding proteins (Gbps) such as GbpC [Banas and Vickerman, 2003] (fig. 1). Furthermore, it is apparent that glucan synthesized in situ by Gtfs in pellicle provides enhanced binding for several oral microorganisms [Bowen and Koo, 2011]. Concomitantly, glucan formed by Gtf adsorbed on the surface of microbial cells (primarily GtfB) enhances binding between bacterial cells, thus promoting the initial clustering of cells and the subsequent formation of microcolonies [Koo et al., 2010b]. These glucan-mediated processes increase the accumulation of cells tightly adherent to the surface, and enhance the cohesiveness and mechanical stability of the biofilms, allowing them to persist on tooth enamel for prolonged periods [Bowen and Koo, 2011]. Thus, the Gtfs (particularly GtfB and GtfC), in addition to Gbps, may be ideal targets for therapeutic intervention aiming at preventing plaque formation and dental caries.

As shown in table 1, the polyphenolic compounds have been extensively studied because of their ability to reduce glucan synthesis, although most studies use crude preparations of Gtfs in solution. Among these compounds, various polyphenols from the leaves of *C. sinensis* (extracts of green or oolong tea), propolis, cacao bean husk, cranberry and often traditional plants *(Serindeia warnecki, Azadirachta indica, R. gardneriana* and *M. chinensis)* exhibit inhibitory effects against glucan synthesis, particularly water-insoluble glucan. The biologically active compounds range from low-molecular-weight (LMW) flavonoids (e.g. in propolis), benzophenones, epigallocatechin/epicatechin gallate (e.g. in green tea) over smaller proanthocyanidin oligomers (e.g. in cranberries) to high-molecular-weight (HMW) polymeric polyphenols (e.g. in oolong tea, cacao and traditional medicinal plants) [Wolinsky and Sote, 1984; Wu-Yuan et al., 1988; Hattori et al., 1990; Otake et al., 1991; Nakahara et al., 1993; Hamada et al., 1996; Wolinsky et al., 1996; Hamilton-Miller, 2001; Osawa et al., 2001; Koo et al., 2002a, b, 2010a–c; Matsumoto et al., 2003; Murata et al., 2008].

According to Yanagida et al. [2000], the HMW plant polyphenols that display strong anti-Gtf activity have a common structural feature that is shared with catechin-based oligomeric forms (condensed tannins) and/or gallate ester form compounds (hydrolysable tannins). HMW polymeric proanthocyanidins (PAC), primarily composed of epicatechin units linked by C-4β and C-8 bonds, were identified as the major biologically active compounds in cocoa extracts [Osawa et al., 2001]. Furthermore, it appears that these inhibitory effects are enhanced with increasing polymerization of monomeric polyphenol [Hamada et al., 1996], which may be attributed to nonspecific binding and precipitation of the enzymes in solution (e.g. gallotannins) [Wu-Yuan et al., 1988]. However, these HMW compounds may have site-specific actions, as reported by Matsumoto et al. [2003], whose research has demonstrated that oolong tea fractions rich in polymeric polyphenols noncompetitively reduce glucan synthesis by targeting the glucan-binding domain of *S. mutans*-derived GtfB and GtfD in solution.

Furthermore, the type of interflavan linkage may also play a critical role in the anti-Gtf activity of polyphenols. Cranberry PAC dimers containing the A-type double interflavan bond (which confers structural rigidity to the molecule) display significant inhibitory activity [Gregoire et al., 2007; Koo et al., 2010a]. The B-type dimers derived from apple have a single linkage and have not been demonstrated to possess any such activity [Yanagida et al., 2000]. On the other hand, LMW apigenin and 7-epiclusianione appear to be effective noncompetitive inhibitors of GtfB and GtfC [Koo et al., 2002c; Murata et al., 2008]. The position of hydroxyl (OH) groups and the presence of a double bond between C_2 and C_3 in the flavone or flavonol molecular structure may provide a site

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for nucleophilic addition, which could disrupt the enzymatic activity of Gtfs [Koo et al., 2002c].

The effects of natural products on surface-adsorbed Gtfs have received limited attention despite their clinical importance (see Problems and Limitations section). Ethanolic extracts of *Apis mellifera* propolis (a resinous beehive product) and *R. gardneriana* displayed inhibitory effects against surface-adsorbed GtfB and GtfC, which was attributed to the presence of LMW inhibitors such as flavonols (e.g. kaempferol and myricetin), flavones (e.g. apigenin) and benzophenones (e.g. 7-epiclusianione) [Koo et al., 2002c; Murata et al., 2008]. It has recently been shown that specific A-type proanthocyanidin oligomers (from dimers to dodecamers) in an aqueous extract of cranberry effectively diminished the synthesis of insoluble glucans by surface-adsorbed GtfB [Gregoire et al., 2007; Koo et al., 2010a]. The presence of an A-type interflavan linkage and a degree of polymerization that ranged from 4 to 12 was considered optimal for the effective inhibition of the surface-adsorbed enzyme [Gregoire et al., 2007; Koo et al., 2010a]. In addition, the presence of apigenin (and other bioactive flavonoids) also decreased the expression of *gtfB* and *gtfC* (but not *gtfD*) in *S. mutans* growing planktonically or as a biofilm, all without affecting bacterial growth or culture pH [Koo et al., 2006]. However, little is known about the exact molecular mechanisms by which these naturally occurring molecules inhibit enzyme activity and *gtf* gene expression.

Inhibition of Bacterial Adherence

As shown in table 1, some natural products also inhibit bacterial adherence to hard surfaces (glass or hydroxyapatite) and/or cause bacterial aggregation. HMW polyphenols (from green tea, oolong tea, grape, cranberry and pine bark), chitosans and 1-deoxynojirimycin (isolated from *Morus alba*) have been shown to inhibit sucrose-dependent (EPS-mediated) and independent (pellicle-mediated) adherence of mutans streptococci [Tarsi et al., 1997; Steinberg et al., 2004; Koo et al., 2005; Furiga et al., 2008; Islam et al., 2008]. However, most of these studies were conducted when test agents were mixed with planktonic organisms, making data interpretation difficult due to overlapping biological activities (e.g. effects on growth, aggregation and Gtf activity). In general, the inhibition of sucrose-dependent adherence is associated with a reduction in water-insoluble glucan synthesis, which could decrease the accumulation of cariogenic bacteria and further biofilm development [Koo et al., 2005; Furiga et al., 2008; Islam et al., 2008].

Problems and Limitations

Antimicrobial Activity

Despite early efforts to identify the putative antimicrobial agents from natural products, the precise mechanisms of action and effectiveness are still largely unknown owing to the limitations of the assays used to measure inhibition of growth (e.g. MIC and MBC determination). Such approaches to assess the antimicrobial activity of a possible anticaries agent are problematic for a number of reasons. First, bacteria in the oral cavity would never be exposed to a static (constant) concentration of an exogenously introduced antimicrobial agent over a 24-hour period. Unless a compound has an outstanding substantivity, microorganisms in the oral cavity are unlikely to be exposed to high concentrations of an antimicrobial for more than 30 s to a couple of minutes. Second, bacteria in classical MIC/MBC assays are in suspension, whereas the oral bacteria associated with caries are enmeshed in the plaque biofilm matrix. There is a plethora of evidence showing that biofilm cells are more resistant to antimicrobials than cells in suspension. Thus, describing antibacterial activity against planktonic oral bacteria with constant exposure to an agent at a high concentration over a 24-hour period is hardly relevant to how oral bacteria would respond in the mouth. Nevertheless, determination of MIC and MBC can still be useful when employed in high-throughput bioscreening techniques.

Despite incomplete information concerning their mechanisms of action and a lack of studies to evaluate treatment in a more clinically relevant biofilm model system, many natural biocidal agents have been incorporated in oral health care products. For example, extracts of miswak, tea tree oils, essential oils, peppermint and green tea have been added to mouthrinses and toothpastes to enhance their overall antimicrobial activities [Allaker and Douglas, 2009]. Furthermore, the biological relevance of agents displaying effects on bacterium growth needs to be interpreted with caution because antimicrobial activity does not necessarily correlate with clinical antiplaque effects, and there is no direct association with cariostatic activity [Scheie, 1989; Twetman, 2004].

Inhibition of EPS Synthesis

Following recognition of the importance of Gtfs in the pathogenesis of dental caries, specific inhibition of a proven virulence factor became an attractive and potentially highly selective approach to preventing plaque-related diseases. However, much of the work in this area has focused largely on enzymatic activity in solution using

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crude preparations, all without regard to the importance of the enzymes in the pellicle and kinetic differences among individual Gtfs. The development of dental caries can be attributed to events that occur at the tooth pellicle-plaque interface, and enzymatically active Gtfs are present in the pellicle and on bacterial surfaces. The identification of inhibitors that target these adsorbed enzymes is an attractive possibility for the development of new anticaries therapies. It is also noteworthy that surface-adsorbed Gtfs display an increased resistance to the most common inhibiting agents compared with solution phase enzymes, which includes the currently available antiplaque mouthrinses [Vacca-Smith and Bowen, 1998; Wunder and Bowen, 1999; Koo et al., 2002a]. The reason for this phenomenon is unclear, although it may be related to conformational changes that these enzymes undergo during adsorption, which can alter their enzymatic kinetics and physicochemical properties [Schilling and Bowen, 1988; Venkitaraman et al., 1995; Bowen and Koo, 2011].

Clearly, agents that inhibit enzymes in solution may be without effect when the enzymes are adsorbed to a surface. Our studies have shown that many natural products and their bioactive constituents display partial inhibition of GtfB and GtfC in solution, but only a few are potent inhibitors (>90% inhibition), and even fewer disrupt the activity of surface-adsorbed enzymes. In general, we have observed that effective surface-adsorbed GtfB and GtfC inhibitors also disrupt the EPS content in biofilms, reducing the biomass and modulating cariogenicity in vivo [Koo et al., 2003, 2005, 2010a; Koo and Jeon, 2009]. Thus, we would recommend that inhibitors of Gtfs should at minimum be as effective in inactivating surface-adsorbed enzymes as they are in inhibiting these enzymes in solution before any conclusions can be drawn concerning the disruptive effects a natural product may have on glucan synthesis.

In vivo and Clinical Studies

Despite evidence that several natural products may have the potential to modulate the pathogenesis of dental caries, few studies have actually been conducted in vivo (e.g. using rodent models of dental caries) and even fewer have been evaluated at the clinical level (table 1). Here, we review some of the available, although limited, in vivo and clinical data. Some studies have shown that extracts of green tea or oolong tea enriched with polyphenols significantly reduce plaque accumulation and/or the development of carious lesions on the sulcal and/or smooth tooth surfaces of rats infected with *S. mutans* or *S. sobrinus* when delivered by drinking water or diet [Rosen et al., 1984; Otake et al., 1991; Ooshima et al., 1993]. The cariostatic effects of these agents may be related to the presence of (–)-epigallocatechin, (–)-epigallocatechin gallate and HMW polymeric polyphenols which display antibacterial and/or anti-Gtf activity [Otake et al., 1991; Ooshima et al., 1993].

Topical applications of chemically characterized propolis extracts have also been shown to be highly effective in reducing the incidence and severity of smooth surface and sulcal caries in rats [Ikeno et al., 1991; Koo et al., 1999; Hayacibara et al., 2005; Duarte et al., 2006b]. However, the cariostatic effects of propolis are highly variable depending on its chemical composition and geographical origin [Koo et al., 1999]. The biological activity is associated in part with the presence of flavonoids and terpenoids, such as apigenin and *tt*-farnesol, that display anti-GtfB/GtfC activity and inhibit acidogenicity and/or aciduricity in *S. mutans* [Koo et al., 2002c, 2003; Koo and Jeon, 2009]. Furthermore, apigenin and *tt*-farnesol (alone or in combination) significantly reduce caries development in rats with minimal effects on the population of viable cells in the plaque [Koo et al., 2003]. Topical applications of the PAC-rich fractions of cranberry extracts were recently demonstrated as having cariostatic effects in vivo. This may be attributed to the inhibition of bacterial glycolysis and glucan-mediated processes involved in biofilm formation, which appears to be exacted by specific bioactive A-type dimers and oligomers present in the PAC extract [Koo et al., 2010a].

Clinical studies evaluating anticaries effects are virtually nonexistent. However, some studies have demonstrated inhibitory effects on salivary levels of cariogenic bacteria (mutans streptococci and lactobacilli) and on plaque accumulation [Koo et al., 2002a; Matsumoto et al., 2004; Pai et al., 2004; Menezes et al., 2006]. For example, a mouthrinse containing propolis extracts (3% w/v) effectively reduced the accumulation and insoluble polysaccharide content of dental plaque (44.7 and 61.7% reduction, respectively, vs. placebo mouthrinse) [Koo et al., 2002a]. In the same context, oolong tea-based mouthwashes (0.05% w/v extract) reduced the mean plaque index by 21% [Ooshima et al., 1994]. Despite limited investigations in vivo, the available evidence indicates that there is potential for the discovery of novel and effective anticaries/antiplaque therapy using natural products. There is a clear need to conduct additional studies to evaluate the clinical efficacy and safety of these substances [Groppo et al., 2008].

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Challenges in Investigating Anticaries Effects of Natural Products

Contemporary drug discovery is based on rapid screening of synthetic or naturally occurring molecules for their ability to affect specific pharmacological target(s), often using high-throughput screening, as reviewed in Koehn and Carter [2005] and Schmidt et al. [2007]. The complexity of crude or semipure natural extracts and the chemical nature of the components found in them are major challenges in discovering new bioactives. Furthermore, several rounds of purification and additional bioassays are required for the identification and isolation of the active components of an exceptionally complex matrix, which is laborious, technically challenging and time-consuming. Then, there is the need for structural elucidation and molecular formula determination, which is technically feasible using the current spectroscopic techniques, albeit difficult. Purification is a rate-limiting step because separation methods must be designed to yield sufficient quantities of the pure bioactive molecule from the original natural source. The latter highlights one of the fundamental concerns in natural product drug discovery: the limited availability of chemically characterized natural supplies.

Furthermore, the chemical composition of the naturally derived products depends on geographic location, seasonal effects and biological diversity. For example, there is a diverse range of Brazilian propolis, a beehive product from *A. mellifera* honeybees, as more than 13 types of propolis have been classified based on physicochemical characteristics, botanical origin and bioactivity. This variation was found to be associated with a number of factors, including (i) the biodiversity in Brazil, which is at least partially dictated by the geographical location, (ii) seasonal changes that affect plant growth and availability to the honeybees, and (iii) genetic variation in *A. mellifera* bees [Park et al., 1998, 2002; Bankova, 2005; Salatino et al., 2005; Silva et al., 2008; Castro et al., 2009a].

Biofilm Research

The effects of the natural products on bacterial growth, metabolism, adherence and polysaccharide synthesis clearly indicate that such agents would have an effect on initial formation and further accumulation of biofilm on apatitic surfaces. Surprisingly, very few studies have examined the effects of such products on biofilms related to caries such as those formed by mutans streptococci or other biofilm-forming cariogenic microorganisms [Koo et al., 2003, 2005, 2010a; Xie et al., 2008; Islam et al., 2009; Jeon et al., 2009; Cheng et al., 2011]. Thus, without further in vitro assessment of the antibiofilm activity of such agents in a more clinically relevant (brief exposure) model system, it is not safe to postulate their anticaries potential.

A Suggested Model for Investigating the Use of Natural Products in Caries Research

Many studies on natural products in the field of caries research have offered incomplete information because they lack basic chemical characterizations of the extracts or their compositional profiles and/or identification of putative active compound(s). Unfortunately, most biological assays commonly used are focused on the growth and metabolism of planktonic cells, and these cells are exposed for prolonged and inappropriate periods of time, considering biofilm development and treatment regimens in the context of dental caries disease.

It is evident that research involving natural products is complex. In conducting such studies, it is important to consider whether there is (i) a reliable and reproducible source of collection of and purification from the natural product, (ii) expertise in analytical and medicinal chemistry, and (iii) use of clinically relevant and feasible models for bioactivity evaluation. Therefore, a stepwise research plan in a multidisciplinary fashion is required, which may involve at least 6 major steps or phases: (1) discovery – finding natural sources and conducting bioscreens; (2) characterization of bioactives – identification, isolation and chemicostructural elucidation; (3) validation of biological activity – in vitro and in vivo studies using clinically relevant models as well as determination of mechanisms of action; (4) toxicology studies; (5) clinical studies of efficacy and safety (approval by FDA in the USA, or similar entities elsewhere); and (6) product development and commercialization. In the case of multicomponent botanical therapeutics (MCBT), which is a mixture of interacting compounds produced by plants, the extract should be chemically characterized and standardized before being evaluated using in vitro and in vivo models [Schmidt et al., 2007].

Here, we suggest a research model for initial evaluation (steps 1–3) of natural products in caries research based on our more than 15 years of experience. Steps 4–6 will not be discussed here as these require the highly specialized technical expertise of medicinal chemists, clinical scientists and toxicologists, which may also include industrial partnerships. A comprehensive program ranging from basic laboratory studies to in vivo investigations in ani-

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mals is offered, which could aid in the development of new chemotherapeutic anticaries approaches for clinical testing. Our suggested strategy is diagramed in figure 2.

Step 1: Discovery Phase[1]

Finding Natural Sources

Natural products can be selected and obtained via a number of approaches including but not limited to: (i) natural substances used in traditional medicine, (ii) systematic collection of a biodiverse set of natural products (plants, microbes or marine organisms), and/or (iii) selection of species of plants or other organisms (microbes and marine) based on their phylogenetic relationship to those known to produce bioactive compound(s) [Jones and Kinghorn, 2006].

The local micro- and macroenvironment, plant diversity, climate, geographic location and even seasonal variation (e.g. time of collection) significantly influence the content of bioactive compounds in natural products, which can dramatically influence the biological activity and potency of the samples [Sforcin et al., 2000; Bankova, 2005; Castro et al., 2007; Schmidt et al., 2007]. Finding a reliable, reproducible and unadulterated natural product source is a major hurdle both in medicine and dentistry. Thus, it is critical to work closely with local or native plant taxonomists and research groups to obtain representative samples according to collection period and site as well as identification of the plants or other natural sources. After collection, the samples should be stored at –20 or –80°C to prevent any degradation or enzymatic changes. The natural products must be precisely identified and the authenticity must be confirmed by a taxonomist. Dried specimens should also be available at any time as reference specimens that represent the natural products that were collected and used for the preparation of extracts [Kaufman et al., 1999].

Once a potential bioactive natural product is identified, the source material (e.g. plant or fruits) could be cultivated under defined environmental conditions to control climate and nutrition. DNA fingerprinting may also be used to evaluate and produce highly standardized active extracts. For example, optimized and chemically characterized extracts of *Artemisia dracunculus* (Russian tarragon) and *Vaccinium macrocarpon* (American cranberry) have been successfully obtained using such cultivation processes [Schmidt et al., 2007; Koo et al., 2010a].

[1] This step may not always be included in research studies using natural products since previously characterized bioactive substances may be readily available for testing.

Biological Screening

The initial screening of natural substances should be simple and designed to rapidly evaluate the potential bioactivity of the whole or crude extracts. Usually water, ethanol or an aqueous ethanol-based solvent is used to prepare plant or plant-derived extracts (e.g. propolis). The selection of biological targets is critical to the design of medium to high-throughput screens, which should be technically feasible, reproducible and cost effective. In order to identify natural products with potential anticaries properties, we would focus on three assays: (i) extracellular glucan synthesis by GtfB and GtfC in solution and adsorbed onto sHA surfaces, (ii) glycolytic pH drop assays for *S. mutans* or other cariogenic organisms to test acidogenicity and aciduricity, and (iii) bacterial growth rate over time using high-throughput systems such as the Bioscreen C. These assays are simple and affordable and can be rapidly performed with multiple replicates, all the while addressing established virulence traits associated with the pathogenesis of dental caries. The bioscreen step would assist in the identification of pharmacologically active natural products that could be further characterized through fractionation and purification steps.

Step 2: Bioactive Characterization Phase

The chemical composition of a bioactive crude extract can be examined using standard spectroscopic procedures such as high-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS). Promising extracts could then be subjected to a bioassay-guided fractionation and purification process, which allows for localization of the bioactivity and target-directed isolation of the compounds [Koehn and Carter, 2005]. Although new technologies are available for online bioassays with real-time fractionation and simultaneous identification/structure determination, they are still costly and demand special expertise for the immobilization of targets.

Identification, Isolation and Chemicostructural Elucidation

The selected active extract can be fractionated by several methods using chemical gradients based on polarity and column chromatography. Analysis of the chemical composition of the crude extract can provide some guidance on the selection of solvents, using polarity and relative biohazard ranking as a guide. We have used dry (e.g. silica gel) or liquid (e.g. Sephadex LH-20) chromatographic columns for the fractionation of various natural

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products from plant to food extracts [Hayacibara et al., 2005; Duarte et al., 2006a; Koo et al., 2010b]. The fractionation step allows for the initial separation of the various compounds from the crude mixture, and subsequent identification of the most biologically active fraction [Gunatilaka et al., 2001].

The active fraction can then be subjected to a series of chromatographic-spectroscopic methods including semipreparative HPLC to isolate and purify individual molecules (both known and unknown) that are present in the semipure mixture. Concomitantly, the isolated compounds need to be identified and structurally characterized, and their purity should be assessed. The following methods may be used to identify and determine the chemical structures based on chromatography (HPLC and GC), molecular weight (MS, X-ray, infrared and ultraviolet) and physical characteristics (nuclear magnetic resonance, NMR) among others (see details in Koehn and Carter [2005] and Castro et al. [2009a]). Advances in NMR, MS, HPLC and matrix-assisted laser desorption/ionization time-of-flight technologies and the ability to use them in tandem with one another (e.g. HPLC-NMR-MS) greatly enhances the isolation, analysis and structural determination of the compounds from complex natural product extracts. Although the determination of complex structures is technically challenging, it is no longer a major impediment to the contemporary drug discovery process. For example, individual LMW and HMW flavonoids can be isolated and characterized from complex mixtures, such as in cranberry extracts, in as little as a few weeks [Gregoire et al., 2007; Koo et al., 2010a]. Nevertheless, the choice of which one should be used depends on individual factors including the advantages and disadvantages of each method and the physicochemical characteristics of the natural substance(s). Subsequently, partnership with analytical chemists may be required [Koehn and Carter, 2005; Verpoorte et al., 2005; Castro et al., 2009a]. Thus, a complex and multidisciplinary approach is required, and will not be discussed further in this review.

Each isolated compound and the selected fraction should be subjected to dose-response studies using the assays described in the bioscreening step. We would draw particular attention to surface-adsorbed GtfB/C activity and acidogenicity/aciduricity using glycolytic pH drop assays. It would be expected that the isolated compound is more potent than the semipure fraction or the original crude extract. However, individual compounds frequently are not as effective as the active fractions or crude extract. This occurs because the biological activity of the natural whole product is a result of the activity of synergistic or additive interactions of different compounds in the mixture rather than of a single highly active molecule [Castro et al., 2009b]. Thus, at this stage a decision should be made about whether further analysis will be conducted using the individual compound (single-molecule type) or the semipure chemically characterized mixture (multicomponent botanical therapeutics (MCBT) type).

Step 3: Biological Activity Validation Phase

The biological activity of selected natural agents should be confirmed by assays that take into account the pathophysiological aspects of caries disease (fig. 1), using clinically relevant, feasible and reproducible models. Laboratory studies should focus primarily on biofilms formed on hydroxyapatite (a surrogate for tooth enamel) or enamel surfaces that are placed in a vertical position and coated with a salivary pellicle [Koo et al., 2005, 2010b]. The test agents should be topically applied for a relatively short period of time (30–60 s) and then ideally would be removed from the system, allowing for a more tightly controlled substance exposure time, as these conditions would be most similar to those experienced in vivo (e.g. brief exposure of dental plaque to exogenously introduced mouthrinses) [Guggenheim et al., 2001; Koo et al., 2003]. Shear forces may also be applied to dislodge loosely bound bacteria and/or excess agents after treatment. This could be simply accomplished by swirling and dip-washing the biofilms during culture medium changes and after topical applications [Guggenheim et al., 2001; Koo et al., 2003]. Also, the presence of saliva in the system is particularly important, considering that salivary proteins, especially proline-rich proteins, are known to bind polyphenols [Bennick, 2002].

We suggest a three-stage process which utilizes in vitro mono- and mixed-species biofilms and an in vivo (rodent) model of dental caries to further evaluate the biological activity of the selected agents and to identify potential new anticaries therapies.

Stage 1

Initially, the previously selected active substances (pure individual compounds or fractions) should be tested using the simplest model system: monospecies biofilms. These biofilms are formed on sHA disks or enamel slabs placed vertically in 24-well plates [Koo et al., 2005, 2010b; Lemos et al., 2010; Klein et al., 2011]. A monospecies biofilm system does not reflect the complex microbial community found in coronal dental plaque, although this model incorporates specific elements associated with the formation of cariogenic biofilms (e.g. the presence of

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a glucan-rich matrix and culture of a proven virulent organism such as *S. mutans* UA159). This biofilm system is simple and cost effective, allowing for multiple replicates of a single experiment to minimize variability and allow for straightforward topical applications of the agent. Furthermore, biofilms using a single organism are advantageous as they allow for determination of the mechanisms of action of selected test agents and their overall effect on microbial physiology and the transcriptional responses to treatment. This model can be used to focus on glucan-mediated and other metabolic processes involved in biofilm matrix formation and acidogenicity.

In this system, biofilms are exposed to the selected agents twice daily (60-second exposure, each time followed by removal of the excess agent) until the end of the experimental period (usually 5 days). After treatments, the biofilms are either homogenized by sonication or kept intact for multiple biochemical assays. Homogenized biofilm suspensions can be used for (i) determination of biomass (dry weight), (ii) determination of the viable populations, (iii) total protein, and (iv) total polysaccharide content (e.g. EPS glucans and fructans, and IPS) [Koo et al., 2003]. The physiological response of intact biofilms can be analyzed using glycolytic pH drop, acid-killing, proton permeability and F-ATPase activity assays [Koo et al., 2003; Duarte et al., 2006a; Gregoire et al., 2007; Jeon et al., 2009; Lemos et al., 2010], while the spatial distribution/ratio of EPS to bacteria biomass can be assessed using confocal fluorescence imaging [Xiao and Koo, 2009; Koo et al., 2010b]. The surface of attachment (sHA or enamel slabs) can be examined for demineralization and structural changes using established techniques such as determination of microhardness, laser-induced fluorescence and surface topography analysis [Zero, 1995; Aires et al., 2006]. Furthermore, the persistence of biological effects of topically applied agents after brief exposure (and removal of excess agent) may also be an indicator of substantivity, which is critical for developing novel chemotherapeutic approaches against oral diseases such as dental caries [Brecx, 1997]. Studies using a monospecies biofilm model yield valuable information that can be applied to complex ecological interactions, which may be evaluated using a multispecies biofilm method in vitro (stage 2) and in a rodent model of dental caries in vivo (stage 3). This step can be used to identify and select agents and their optimal concentrations which may be most effective in disrupting the formation of biofilms by mechanisms that target the major virulence attributes of a cariogenic organism (e.g. glucan production, acidogenicity and aciduricity).

Stage 2

In this phase, the effects of agents selected from stage 1 are evaluated using a multispecies biofilm model that follows the ecological plaque hypothesis [Marsh, 2003]. Our multispecies biofilm model was designed to mimic the formation of cariogenic biofilms in a simple and reproducible system that allows for the short-term exposure of replicate biofilms to selected substances (see details in Koo et al. [2010b]). In this model, we can examine whether test agents affect the EPS matrix development, acidogenicity and ecological interactions of *S. mutans* in the presence of other relevant oral species such as early colonizers including non-mutans streptococci and *Actinomyces* spp. Biofilm development can be assessed before and after changing a key environmental factor: the introduction and persistent availability of sucrose. This can be accomplished by altering the concentration and the availability of sucrose provided in the culture media. In addition, all the bioassays and methods described for the monospecies model can be employed here as well. This comprehensive analysis can provide a detailed evaluation of the effectiveness of selected agents, as well as identify potential anticaries candidates based on their ability to: (i) reduce biofilm biomass, (ii) inhibit the formation and establishment of the EPS matrix, (iii) impair acid stress survival mechanisms of *S. mutans* or other cariogenic organisms, (iv) disrupt the formation of an aciduric environment within the mixed microbial community of the biofilm, and/or (v) reduce demineralization of the apatitic/enamel surface. At this point, following completion of stages 1 and 2, the candidate(s) can be selected based on potency and the type of bioactive [single molecule(s) or MCBT], and can then be validated in vivo (stage 3). There are many other multispecies biofilm models [Fontana et al., 1996; Guggenheim et al., 2001; Wong and Sissons, 2001; Tang et al., 2003] including those using an even more complex, mixed microflora (including artificial mouth systems), which can of course be used in this step and may provide additional relevant information about ecological changes to the biofilms and other in vitro parameters, as discussed above.

Stage 3

In the final stage, we suggest evaluating the efficacy of agents using an established rodent model of dental caries [Bowen et al., 1988; Koo et al., 1999]. This in vivo model simulates more precisely the variables encountered in the oral cavity, such as exposure to salivary and cellular components as well as hydrodynamic and abrasive forces. The principles of bacterial adhesion (*Streptococcus* and *Acti-*

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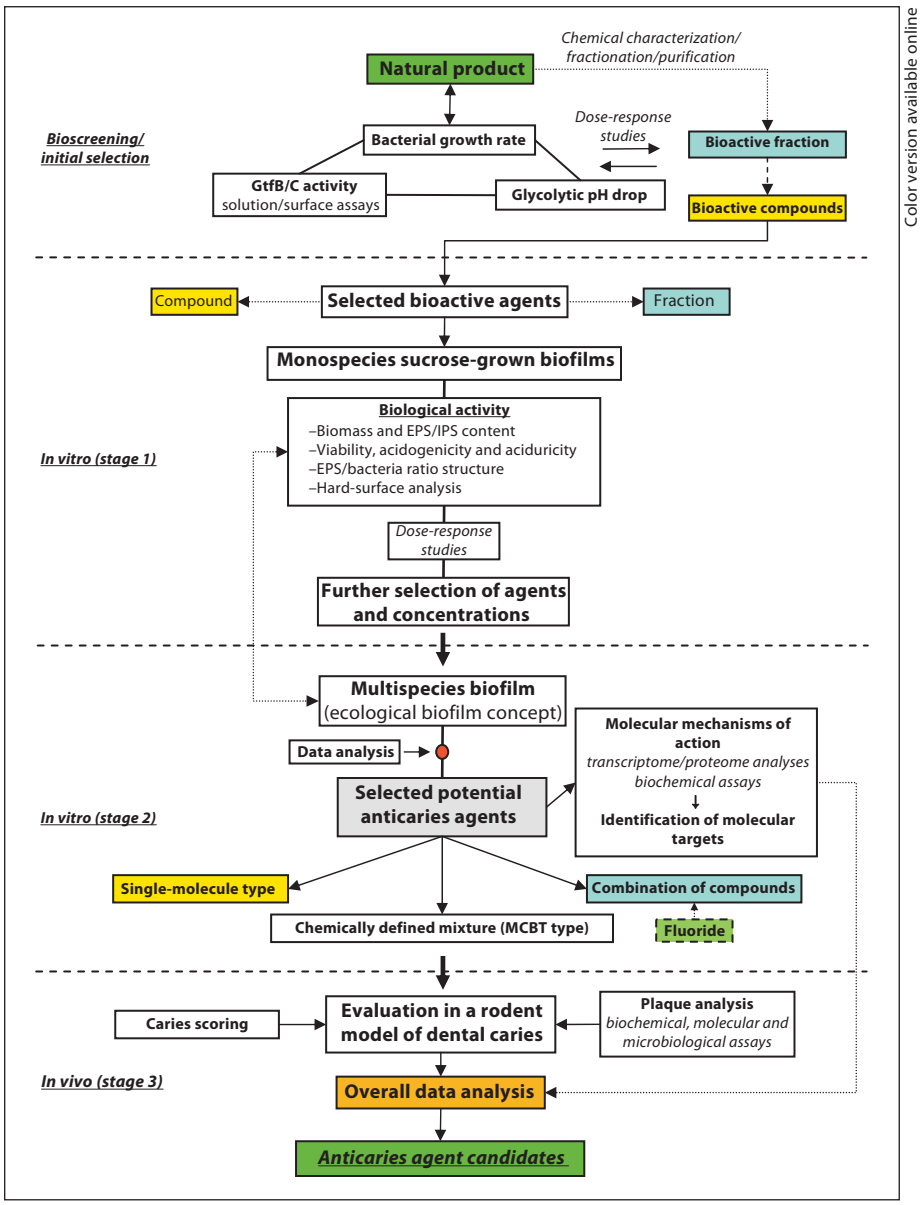

Fig. 2. Overview of the Suggested Model for Discovery of Naturally Occurring Anti-Caries Candidates.

nomyces spp.) to HA surfaces coated with rat saliva are similar to those observed with human saliva coated HA surfaces [Kopec and Bowen, 1995]. Our rat model allows the formation of cariogenic dental plaque on the tooth enamel surface of molars when the animals are provided a sucrose-rich diet (as would occur in humans). In addition, the rat harbors a complex and mixed oral flora even when infected with *S. mutans* or other cariogenic streptococci. More importantly, the model allows the precise examination of the incidence and severity of carious lesions on smooth, sulcal and interproximal surfaces simultaneously (and dynamically). We believe that there is no other model that can simulate such a range of variables found in the oral cavity and at the same time assess the complexity of the development of carious lesions in such a variety of surfaces. Properly designed and conducted studies using a rat caries model, despite its limitations, remain ethically sound and the most comprehensive and reproducible way of evaluating therapeutic agents preclinically. In alignment with stages 1 and 2, the test agents may be applied topically twice daily, and the impact of treatment may be assessed via the following: (i) determination of total aerobic and anaerobic cell populations recovered from animals' plaque; (ii) determination of

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S. mutans (or other cariogenic strains of interest); (iii) the ratios of these populations, and (iv) caries scoring according to Larson's modification of the Keyes system [Larson, 1981]. The scoring system assesses the incidence of dental caries on smooth, sulcal and interproximal surfaces, and the degree of severity of these carious lesions. The data may serve as an indicator of the efficacy of treatment under given experimental conditions which are designed to reflect the conditions likely to be experienced in vivo in the human mouth (e.g. brief exposure and clearance of the agents in the oral cavity). In this design, the animals are fed a sucrose-rich diet, which leads to development of carious lesions similar to that in humans. Thus, we can compare the abilities of various agents to prevent or reduce the initiation and progression of the disease by including positive controls known to be clinically effective in preventing dental caries (fluoride at 250 or 1,100 ppm) and plaque formation (chlorhexidine). Although our goal is to identify anticaries agents that are more effective than fluoride, we are also interested in agents that could be used as potential adjuvants to fluoride or other therapies (see later). We have already implemented this stepwise protocol to identify and evaluate specific bioactive molecules from cranberry and propolis, displaying potential anticaries effects [Koo et al., 2002c, 2003, 2005, 2010a; Duarte et al., 2006a; Gregoire et al., 2007].

The in vivo model may also allow for initial evaluation of the potential toxic effects of topical applications of test agents by monitoring daily food intake and weight gain, and by performing histopathological evaluations of major organs and mucosal surfaces. Although limited, this information may be helpful in designing future toxicological studies, including toxicopharmacokinetics, genotoxicity and carcinogenicity. Traditional in vitro studies to measure cytotoxicity and mutagenicity assays under high-throughput conditions may be combined with functional genomics and computational biology to enhance the ability to predict toxic effects of chemical agents [Committee on Toxicity Testing and Assessment of Environmental Agents, 2007]. However, such studies require a team of experts that can carefully select and perform assays.

Determination of Mechanisms of Action and Combination with Fluoride

Once a potential candidate is selected, it is critical to elucidate the molecular mechanisms of action by which these compounds disrupt the ability of cariogenic bacteria to form EPS and acids, and survive stress and an acidic environment. Understanding these molecular mechanisms may allow for the enhancement of their therapeutic effects. We suggest characterizing the impact of the selected agents on the expression of genes and their encoded proteins required for (i) EPS-rich matrix synthesis, (ii) acid tolerance response/stress survival mechanisms, (iii) enzymatic activity of Gtfs, (iv) sugar uptake systems, and (v) glycolytic enzymes, which are likely to modulate the pathogenesis of caries disease. We are in the process of developing protocols that may be used to determine the molecular mechanisms of action using a combination of standard biochemical assays and whole transcriptome/proteome profiling technologies (e.g. microarrays, RNA-Seq and MudPIT). Some of these protocols have recently been combined in an analytical toolbox for biofilm analyses [Klein et al., 2011]. This approach could then be used to identify the precise pharmacological targets of such agents in order to improve their effectiveness. Agents could then be tailored, including fluoride or other bioactive agents, to impact separate and/or complementary targets associated with EPS-rich matrix formation as well as acidogenicity and aciduricity, which may provide maximum therapeutic activity.

Fluoride in various preparations is arguably the mainstay of caries prevention. However, current applications of fluoride (i) offer incomplete protection against caries, and (ii) do not effectively address the infectious aspect of the disease. Nevertheless, fluoride does to some extent impede insoluble glucan synthesis, acid production and acid tolerance of cariogenic streptococci at micromolar levels found in the plaque matrix [Marquis et al., 2003; van Loveren, 2004]. Thus, it is promising that a combination of natural agent(s) acting in concert with fluoride may enhance the overall inhibitory effects that fluoride exerts on the virulence attributes of cariogenic bacteria. This may represent a superior approach to the prevention and treatment of dental caries, as demonstrated and reviewed by Koo et al. [2005, 2008].

Conclusions and Future Directions

Natural products remain a largely unexplored source of effective antibiofilm molecules of potentially low toxicity that could be used in alternative or adjunctive anticaries therapies. The emergence of powerful new analytical technologies and advances in '-omics'-based knowledge and in (bio)synthetic chemistry has opened the door to a new era in the development of novel antiplaque/anticaries therapies using naturally occurring agents. It is currently possible to obtain large quantities of purified protein targets for inhibitor screening (e.g. GtfB and

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GtfC). Furthermore, new separation and structure determination technologies have increased the plausibility of screening mixtures of structurally complex molecules. More importantly, advances in plant genomics, metabolic engineering and biosynthetic chemistry can now provide reproducible and optimized sources for the isolation and/or production of bioactive agents. These natural molecules can also be used as a template for the generation of synthetic and semisynthetic ligands using in silico approaches [Clardy and Walsh, 2004; Koehn and Carter, 2005; Dürig et al., 2010]. Although natural products from traditional medicine or plant-derived foods are generally not expected to have acute toxicological effects, comprehensive safety studies, especially following brief-exposure topical application of these agents, need to be evaluated prior to clinical trials [Groppo et al., 2008; Wu et al., 2008]. In addition, innovative delivery systems and improved solubilization methods would greatly enhance the applicability of natural products as anticaries agents.

Finally, natural agents can be used alone as single molecules, as MCBT and/or in combination with other agents for enhanced therapeutic effects. The use of natural agents to enhance the cariostatic properties of fluoride (without increasing the concentration of exposure) could be a promising and potent novel anticaries strategy [Koo and Jeon, 2009]. Clearly, there is great potential for the discovery of therapeutically relevant compounds from nature. However, careful experimental designs using multidisciplinary approaches in conjunction with the standardization and characterization of natural products are critical for the successful development of novel and useful anticaries chemotherapy. The implementation of standardized and stepwise cross-disciplinary approaches, as outlined here, would help to advance this field of investigation in caries research.

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Disclosure Statement

The authors declare no conflict of interest is correlated to this paper.

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