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Application of Plant Extracts for the Prevention of Dental Erosion: An in situ/in vitro Study

Marie-Theres Weber[a] Matthias Hannig[b] Sandra Pötschke[a] Franziska Höhne[a] Christian Hannig[a]

[a]Clinic of Operative and Pediatric Dentistry, Medical Faculty Carl Gustav Carus, TU Dresden, Dresden, and [b]Clinic of Operative Dentistry, Periodontology and Preventive Dentistry, University Hospital, Saarland University, Homburg/Saar, Germany

Key Words
Erosion · In situ study · Oregano · *Origanum* · Pellicle · Plant extracts · Polyphenols · Ribes · *Ribes nigrum* leaves

Abstract
Objectives: Antiadherent and antibacterial effects of certain plant extracts have been proven to be beneficial in preventive dentistry. In the present in situ/in vitro crossover study, the impact of plant extracts rich in polyphenols on the erosion-protective properties of the in situ pellicle was evaluated. ***Methods:*** Individual splints were prepared for 12 subjects for intraoral exposure of bovine enamel specimens. Following formation of a 1-min pellicle, watery plant extracts (leaves of the wild form of *Ribes nigrum*, the wild form of *Origanum* as well as a combination of both) were administered for 10 min in situ. Alternatively, a mouth rinse with fluorides (Elmex Kariesschutz) was performed for 1 min. After further oral exposure for 19/28 min, respectively, slabs were removed and incubated with HCl in vitro over 120 s (pH 2, 2.3, 3). The resulting calcium and phosphate release was quantified photometrically. Slabs with and without a 30-min in situ pellicle served as controls. The modification of pellicle ultrastructure was evaluated by transmission electron microscopy (TEM). ***Results:*** Plant extracts modulated the erosion-protective properties of the native in situ pellicle in all test groups in a pH-dependent manner. The combination of *R. nigrum* leaves and *Origanum* enhanced the protective properties of the pellicle at all pH values; the administration of this preparation was comparable, yet superior, to the effect of the fluoridated mouth rinse. TEM images indicated that rinsing with *R. nigrum* leaves*/Origanum* yielded a distinctly thicker and more electron-dense pellicle. ***Conclusion:*** The combination of certain plant extracts offers a novel approach to the complementary prevention of dental erosion.

Dental erosion shows a high prevalence in several epidemiological studies [Larsen et al., 2005; Jaeggi and Lussi, 2006; Cheng et al., 2009; Taji and Seow, 2010]. The direct contact with extrinsic and intrinsic acids without bacterial involvement causes a slowly proceeding loss of dental hard substance [Lussi et al., 2011; Hannig and Hannig, 2014].

Different stages of tooth erosion from initial tooth surface alterations in the enamel up to 50% of tooth surface loss can be detected [Featherstone and Lussi, 2006]. A main reason for the increasing prevalence of dental erosion is the extensive consumption of acidic beverages and

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E-Mail karger@karger.com
www.karger.com/cre

Christian Hannig
Clinic of Operative and Pediatric Dentistry
Medical Faculty Carl Gustav Carus, TU Dresden
Fetscherstrasse 74, DE–01307 Dresden (Germany)
E-Mail christian.hannig@uniklinikum-dresden.de

foodstuffs [Lussi et al., 2002]. The intake of sugar-containing acidic soft drinks but also of 100% fruit and vegetable juices in correlation with a healthy diet leads to a rapid increase in dental erosion [Lussi et al., 2004; Ehlen et al., 2008]. Other factors such as eating and drinking habits (long duration, high frequency), medication (e.g. tranquilizer, antihistamines, antiemetics), as well as diseases (reflux esophagitis, bulimia nervosa, chronic alcohol abuse) can cause and modulate dental erosion in the oral cavity [Zero, 1996; Lussi et al., 2004; Zero and Lussi, 2005; Moazzez and Bartlett, 2014]. This illustrates the high clinical relevance of strategies and preparations to retard and to prevent dental erosion. Different types of fluoride dentifrices and mouth rinses are well established for this purpose [Sundaram and Bartlett, 2001; Bartlett, 2009], but there is still a strong demand for additive and alternative biological and biomimetic approaches.

Besides the buffering effects of saliva and the clearance of acids in the oral cavity, the interactions at the tooth surface have to be considered, especially the omnipresent pellicle [Hannig and Hannig, 2014]. This physiological coating is formed almost instantaneously on all solid substrata in the oral cavity. Pellicle formation is driven by physicochemical interactions of the oral fluids and especially of salivary protein aggregates – so-called micelle-like globular structures and heterotype complexes – with the respective surfaces [Vitkov et al., 2004; Hannig and Joiner, 2006]. The initial pellicle is of high tenacity and is visible as an electron-dense basal layer by transmission electron microscopy (TEM) [Hannig and Joiner, 2006]. Further adsorption of proteins leads to the formation of the outer less electron-dense pellicle layers of more globular and granular structure [Hannig and Joiner, 2006]. Typical structural and functional components of the pellicle are proteins such as proline-rich proteins, histatins, amylase, lysozyme, and lactoferrin as well as glycoproteins like mucins [Lendenmann et al., 2000; Hannig et al., 2005b; Hannig and Joiner, 2006]. Recent proteomic studies of the pellicle demonstrated that it contains much more proteins and peptides of unknown function than assumed before [Siqueira et al., 2012; Lee et al., 2013]. The pellicle layer contains many protective and antibacterial components and serves as a lubricant [Hannig and Joiner, 2006]. Furthermore, it acts as a barrier against erosive noxae, but is semipermeable and, therefore, its protective effect is limited [Hannig et al., 2007, 2009a, 2012; Hannig and Hannig, 2014]. Pellicle formation is of high selectivity, and the sustainable immobilization of protective ions and molecules at the tooth surface is rather difficult [Hannig and Joiner, 2006].

It has been postulated that edible oils are suitable for this purpose, but recent in situ experiments indicated lacking efficacy [Hannig et al., 2012; Kensche et al., 2013b]. Rinses with edible oils impaired the ultrastructure of the pellicle and erosive mineral loss was not hampered [Hannig et al., 2012; Kensche et al., 2013b]. However, other plant compounds such as polyphenols seem to be beneficial for the protective properties of the pellicle [Joiner et al., 2003, 2004, 2006; Hannig and Joiner, 2006; Hannig et al., 2011]. Possibly tanning or denaturing effects enhance the tenacity of the pellicle and reduce the permeability for acidic noxae [Hannig et al., 2011].

There are numerous promising medicinal plants, teas and culinary herbs with potential impact on oral health due to their high content in polyphenols and other secondary plant compounds such as green tea, black tea, *Cistus incanus*, thyme, oregano or marjoram [Nakatani, 2000]. Two plants native to central Europe, *Ribes nigrum* and *Origanum vulgare,* were considered in the present study [Opara and Chohan, 2014].

The well-known spice oregano, *O. vulgare*, belongs to the mint family Lamiaceae. It is a perennial plant and native to the Eurasian and Mediterranean region [Spiridon et al., 2011]. Abundant components are monoterpenoids and monoterpenes, carvacrol, and in a region-dependent manner thymol. The polyphenols, tannins and anthocyanins vary considerably depending on the type and origin of the respective plant. Chlorogenic acid, protocatechuic acid and neochlorogenic acid as well as apigenin derivates are detectable; there are also kaempferol derivates, quercetin glucosides, rosmarinic acid and luteolin derivatives [Dambolena et al., 2010; El Babili et al., 2011; Spiridon et al., 2011; Tair et al., 2014; Zhang et al., 2014].

It has high antioxidant activity, which is attributed to the high content of phenolic acids and flavonoids [Spiridon et al., 2011; Zhang et al., 2014]. Antibacterial activity has been shown against Gram-positive and Gram-negative bacteria such as *Escherichia coli, Pseudomonas aeruginosa* and *Listeria monocytogenes.* Teas and ointments are adopted for the treatment of disorders of the gastrointestinal tract, respiratory tract, and nervous system in folk medicine [Spiridon et al., 2011; Vasko et al., 2014; Zhang et al., 2014].

Black currant leaves, ribis nigri folium, belong to the main plant *R. nigrum,* which is counted among the family Grossulariaceae. The plant is native to middle, Northern and Eastern Europe, Eastern Siberia, Mongolia as well as Chinese provinces [Gopalan et al., 2012].

The leaves contain flavonoids (primarily camphor oil, quercetin as well as myrecetin and isorhamnetin), proanthocyanidins (catechin, epicatechin), lignoids (chicanine, ribesins named after the plant), anthocyanins (delphinidin, cyanidin), vitamin C, caffeic acid, chlorogenic acid, protocatechuic acid, triglycerides with linoleic acid and traces of essential oils [Tabart et al., 2011; Vagiri et al., 2012; Sasaki et al., 2013; Butnariu, 2014; Liu et al., 2014].

In folk medicine, *R. nigrum* leaves are used for inflammation, rheumatic diseases, disorders of the respiratory tract, influenza, and others [Gopalan et al., 2012; Ehrhardt et al., 2013; Haasbach et al., 2014]. The beneficial properties are also attributed to the anti-inflammatory and antioxidative compounds, especially the polyphenols. Also antiviral and antibacterial effects have been described [Ehrhardt et al., 2013; Haasbach et al., 2014]. In contrast to the seeds, especially the leaves contain high amounts of polyphenols [Tabart et al., 2011].

The aim of the present in situ/in vitro study was to evaluate whether the application of watery plant extracts gained from oregano and/or ribes has an effect on the protective properties of the pellicle layer against erosive mineral loss. The respective extracts are available as oral health care preparations in chemist's shops and in pharmacies (table 1, Kremo 058®, Ladania 067®, Teutopharma GmbH/Dr. Pandalis Gruppe, Glandorf, Germany) [Haasbach et al., 2014]. All experiments were based on an established model for the determination of calcium and phosphate release [Hannig et al., 2005a, 2008a, 2012]. Furthermore, the modification of pellicle ultrastructure was visualized by TEM.

Table 1. Overview of rinsing solutions

Fluoridated mouthwash	Elmex Kariesschutz GABA GmbH, Lörrach, Germany	250 ppm amine fluoride
R. nigrum leaves, fluid	Ladania 067®, Teutopharma GmbH/Dr. Pandalis Gruppe, Glandorf, Germany	1.9223 g/ 100 ml
Oregano, fluid	Teutopharma GmbH/Dr. Pandalis Gruppe, Glandorf, Germany	3.1766 g/ 100 ml
R. nigrum leaves and oregano, fluid	Kremo 058®, Teutopharma GmbH/Dr. Pandalis Gruppe, Glandorf, Germany	2.7836 g/ 100 ml

Methods

Subjects

Twelve volunteers, members of the laboratory staff and dental students (age 22–26), participated in this study. Visual oral examination was carried out by an experienced dentist. The volunteers were of good periodontal health and showed no signs of caries or dental erosion. Physiological salivary flow rate was determined during oral examination. Furthermore, the anamnesis revealed no abuse of alcohol, tobacco or other drugs. Informed written consent had been given by the volunteers for participation in this study. The study design was reviewed and approved by the Ethics Committee of the Medical Faculty, TU Dresden, Germany (Vote EK 147052013).

Specimen Preparation

Bovine incisors from 2-year-old cattle were used to prepare enamel slabs with a diameter of 5 mm. With a trepanning drill 2–3 samples were cut from each bovine incisor. In order to expose just the enamel side, the slabs were etched at all sites except for the outer enamel surface for 30 s with 37% phosphoric acid (Scotchbond Universal Etchant, 3M ESPE, Neuss, Germany) and treated for 25 s with Optibond Primer (OptiBond FL, Kerr, Karlsruhe, Germany). Afterwards, Optibond Adhesive was applied 3 times onto the specimens followed by light-curing in a halogen light furnace for 30 s each time. In order to remove possible sealer residues, the unsealed enamel surfaces were ground flat and polished under water cooling (grit 1,200, 4,000) [Hannig et al., 2007]. Hereby, approximately 200 µm of the enamel was removed. Enamel slabs with structural alterations of the enamel were excluded from the study. To clear the enamel surfaces from the smear layer, the specimens were sonicated for 2–3 min with sodium hypochlorite (3%) and were washed twice in distilled water for 5 min using again an ultrasonic bath (24.5 kHz). Following this, the slabs were disinfected in denatured ethanol (70%) for 10 min (ultrasonication) [Hannig et al., 2009c, 2012]. Conclusively, the specimens were washed and finally stored in distilled water for 24 h at 2°C to induce the formation of a hydration layer surrounding each enamel slab [Fu et al., 2004; Hannig et al., 2009c, 2012].

Formation of in situ Pellicle

To generate in situ pellicles, individual maxillary wire/polymethyl methacrylate splints were customized for the volunteers by a dental technician [Hannig et al., 2013; Kensche et al., 2013a]. At the buccal sides of the splints, cavities were prepared in the regions 14–16 and 24–26. For one intraoral rinsing procedure, a number of 6 enamel slabs was placed on a subject's splint with polyvinyl siloxane impression material (Aquasil, Denstply DeTrey, Konstanz, Germany) so that only the enamel surfaces were exposed to the oral cavity. In the later in vitro experiments two samples each were incubated in HCl at pH 2.0, 2.3 and 3.0, respectively (fig. 1).

The testing period took place from 7 to 12 a.m. The volunteers were instructed not to eat and to brush their teeth without toothpaste 2 h beforehand. To allow initial pellicle formation on the specimen surfaces, the splints were worn intraorally for 1 min. Afterwards, the volunteers had to follow a certain regime where the enamel specimens were exposed to the oral cavity in total for 30 min. They either rinsed with a solution obtained from a wild form of *Origanum*, from the leaves of a wild form of *R. nigrum*, or from *Origanum/R. nigrum* leaves (both wild forms; table 1, Kremo 058®, Ladania 067®, Teutopharma GmbH/Dr. Pandalis Gruppe) for 10

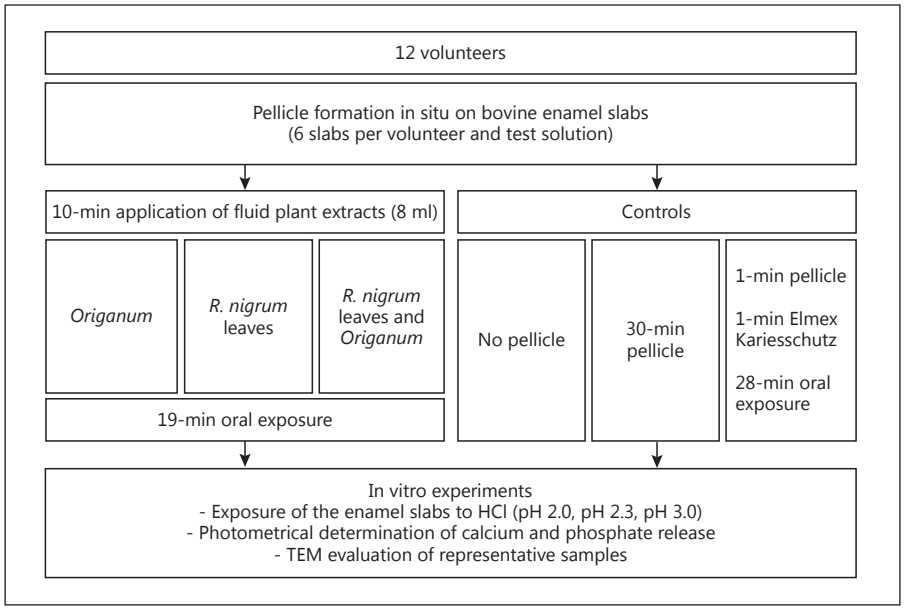

Fig. 1. Flow chart of the experiments.

min and then wore the splints for another 19 min. All plant extracts were provided by Teutopharma GmbH/Dr. Pandalis Gruppe in a standardized quality. Elmex Kariesschutz (GABA GmbH, Lörrach, Germany) served as a control with which the volunteers had to rinse for 1 min and then wore the splints for another 28 min (table 1). The pH of the dissolved tablets and liquid plant extracts ranged between 4.8 and 5.4. Enamel slabs with a physiological 30-min pellicle and no additional rinsing as well as native enamel slabs that were not worn intraorally served as controls.

After intraoral exposure, the slabs were quickly removed from the splints and were rinsed with running water for 5 s. It was a crossover design study, all participants used all test solutions in a randomized order. There was a washout period of at least 48 h between the different experiments.

In vitro Erosion and Determination of Calcium and Phosphate Release

For the performance of the in vitro erosion, the enamel slabs were embedded in polyvinyl siloxane impression material at the bottom of a 2-ml Eppendorf cup exposing only the enamel surface. The in vitro erosion was performed as described previously in detail [Hannig et al., 2012]. In brief, the enamel samples were incubated in 1,000 µl hydrochloric acid (HCl) of pH 2, 2.3 or 3 to provide an excess of acid and to maintain a constant pH. By continuous pumping with a 100-µl pipette, the acid was moved (1 surge of the pipette per second). Every 15 s, 100 µl of the acid was removed for photometric analysis and replaced by 100 µl to ensure a constant availability of fresh acid reacting with the enamel slabs. A number of 8 samples were obtained at varying incubation times. From each of these samples, 10 µl were obtained 3 times for a triple determination of every incubation step. The slabs were assigned to the different erosive challenges in a randomized manner.

Photometric Determination of Calcium and Phosphate Release

Release of calcium and phosphate ions from the enamel slabs was induced by incubation in HCl. The mineral dissolution was determined photometrically by measuring the calcium and phosphate release in double assays using the Arsenazo III method (Fluitest®, Ca-A-II, Analyticon, Lichtenfels, Germany) and the malachite green assay [Attin et al., 2005a, b; Hannig et al., 2005a, 2012].

Calcium reacts with Arsenazo III in an acidic solution to form a blue purple complex. The resulting intensity of the blue purple complex is proportional to the calcium concentration in the solution. The blue purple complex absorbs light at $\lambda = 650$ nm according to standard curves. The reagent to determine the calcium concentration was composed of 100 mM imidazole buffer (pH 6.5) and 0.12 mM Arsenazo III. A volume of 10 µl of the mineral dissolution sample was pipetted to 100 µl Arsenazo III reagent and blended thoroughly. The extinction was measured subsequently [Attin et al., 2005a; Hannig et al., 2012].

Phosphate reacts with malachite green and forms a colored complex which can be determined photometrically at $\lambda = 650$ nm. For the test reagent 0.045 mg of malachite green dissolved in 100 ml distilled water was mixed with 12.69 g of ammonium molybdate solubilized in 300 ml HCl (4 M). Afterwards, the test reagent was stirred for 30 min and filtered (pore size 0.22 µm). Again, 10 µl of the sample were pipetted to 200 µl of the malachite reagent. After 15 min, the absorption could be determined [Attin et al., 2005b].

In the Arsenazo III assay as well as the malachite green assay, two samples were measured for each specimen and the average absorption was calculated. The release of calcium and phosphate was calculated based on the mean photometric absorption values for the specimens and their surface areas (5 mm in diameter).

Transmission Electron Microscopy

Furthermore, in situ pellicle samples were gained for TEM analysis in order to visualize the influence of the plant extracts on the ultrastructure of the pellicle. The in situ experiments were carried out as described above. Some of the samples were incubated in HCl for 60 s after the intraoral exposure. Afterwards, the slabs were transferred to preparation for TEM. In a first step, the enamel slabs were fixed in glutaraldehyde for 2 h (2.5% glutaraldehyde,

1.5% formaldehyde in phosphate buffer, pH 7.4). The samples were then washed 5 times in phosphate buffer. Postfixation for visualization of organic structures took place in 1% osmium tetroxide for 2 h. The specimens were dehydrated in a series of increasing alcohol concentrations and embedded in Araldite M (Serva, Darmstadt, Germany). The dentine was removed from the samples with a diamond bur and the samples were decalcified in 1 M HCl. Re-embedding was performed with Araldite. Ultrathin sections of the pellicle samples were cut in series with an ultramicrotome (Ultracut E, Reichert, Bensheim, Germany) using a diamond knife. The ultrathin sections were mounted on mesh grids (Plano, Wetzlar, Germany) and contrasted with uranyl acetate and lead citrate. TEM investigation took place at 3,000- to 50,000-fold magnification in a TEM TECNAI 12 Biotwin (FEI, Eindhoven, The Netherlands) [Hannig et al., 2012].

Energy-Dispersive X-Ray Spectroscopy

In order to detect fluoride in the pellicle-covered enamel surfaces, energy-dispersive X-ray spectroscopy of pellicle-coated enamel slabs with and without application of the fluoridated mouth rinse was performed (n = 6 each). In line with the main experiments (fig. 1), application of the fluoride-based mouth rinse was performed, and after 30-min in situ exposure the fluoride content of the surface was measured. Samples with a 30-min pellicle without fluoride rinsing served as controls. The elemental analysis was conducted by scanning electron microscopy/energy-dispersive X-ray spectroscopy in an ESEM XL 30 FEG (FEI) at a magnification of ×10,000.

Statistics

The data of this in situ/in vitro study were evaluated by the Mann-Whitney U test using SigmaPlot software. Statistical analysis was performed considering the results from the 24 enamel slabs of the 12 subjects in each subgroup.

Results

Calcium and Phosphate Release

For all specimens linear kinetics of calcium and phosphate release were detected at pH 2, 2.3 and 3. Mineral loss was strongly dependent on the pH value. Formation of a physiological pellicle layer diminished mineral loss significantly (reduction of calcium release at pH 2: 17%, pH 2.3: 26%, pH 3: 22%; phosphate release at pH 2: 25%, pH 2.3: 16%, pH 3: 32%). As expected, this effect was enhanced by the application of a fluoride-containing mouth rinse (Elmex Kariesschutz, gold standard). The kinetics of calcium and phosphate release are depicted for pH 3 exemplarily in figures 2 and 3. Thereby also the protective effect of the pellicle was strongly dependent on pH value. Statistical evaluation was performed for the cumulative mineral loss over 120 s as depicted in figures 4 and 5, a significant impact of some rinsing procedures was observed (Mann-Whitney U test).

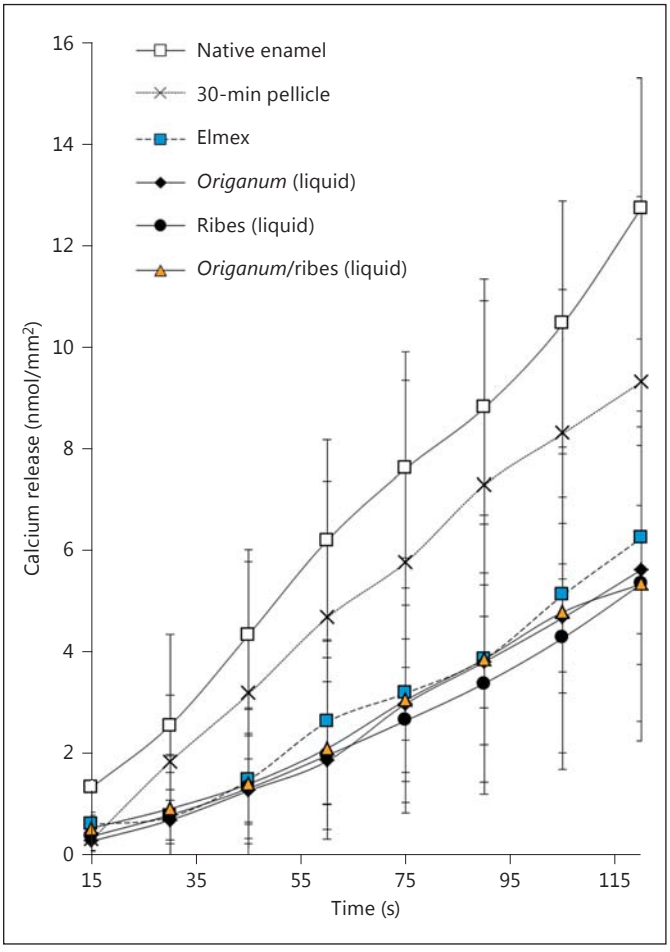

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Fig. 2. Kinetics of calcium release from enamel slabs at pH 3 in vitro during short-term incubation in HCl with and without the application of plant extracts in situ. After 1-min pellicle formation, mouth rinses with 8 ml of a plant extract were performed for 10 min. Subsequently, the enamel slabs stayed exposed to the oral cavity for another 19 min. Additionally, a 1-min mouth rinse with Elmex Kariesschutz and a 28-min oral exposure as well as enamel slabs with an acquired 30-min pellicle and without a pellicle (native enamel) served as controls; n = 24 samples per subgroup (2 enamel samples from each subject), mean ± SD.

Calcium and phosphate release was modulated by all plant extracts tested. However, if the combination of *Origanum* and *R. nigrum* leaves was used in situ, the lowest mineral loss was recorded (reduction of calcium loss by the liquid extract as compared with controls without pellicle at pH 2: 36%, pH 2.3: 43%, pH 3: 50%; phosphate at pH 2: 28%, pH 2.3: 32%, pH 3: 41%). The protective effect of the simultaneous use of these two plant extracts in situ was equivalent or even better as compared with the gold standard (fluoride-based mouth rinse, Elmex Kariesschutz).

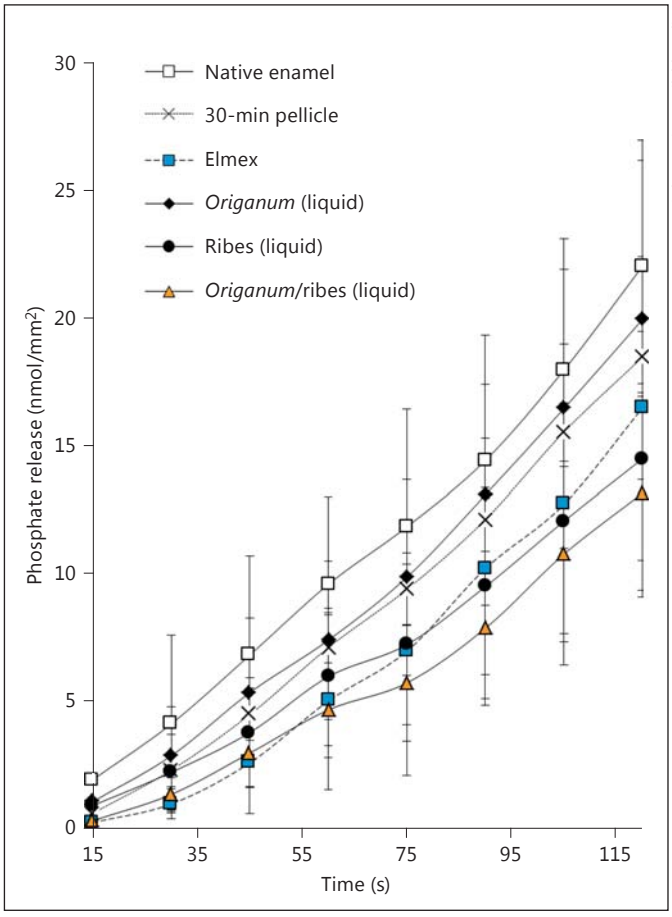

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Fig. 3. Kinetics of phosphate release from enamel slabs at pH 3 in vitro during short-term incubation in HCl with and without the application of plant extracts in situ. After 1-min pellicle formation, mouth rinses with 8 ml of a plant extract were performed for 10 min. Subsequently, the enamel slabs stayed exposed to the oral cavity for another 19 min. Additionally, a 1-min mouth rinse with Elmex Kariesschutz and a 28-min oral exposure as well as enamel slabs with an acquired 30-min pellicle and without a pellicle (native enamel) served as controls; n = 24 samples per subgroup (2 enamel samples from each subject), mean ± SD.

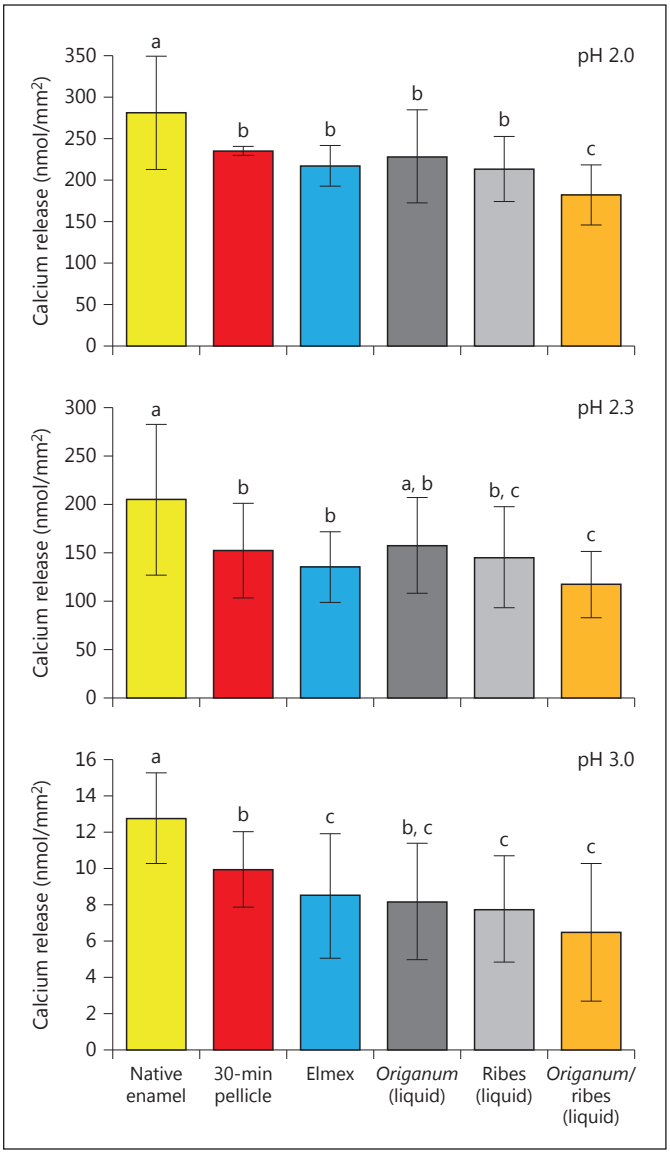

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Fig. 4. Cumulative calcium release over 120 s (pH 2.0/2.3/3.0); n = 24 samples per subgroup, mean ± SD. Data significantly different from each other are marked with different letters; statistical evaluation was carried out within one pH level (U test, Mann-Whitney).

In this context it is noteworthy that the single-plant extracts even had converse effects. *Origanum* yielded higher phosphate release than observed with physiological pellicles (all pH values). Pure ribes led to an increased calcium release at pH 2.0.

Transmission Electron Microscopy

In order to evaluate the impact of the plant extracts on the tenacity and on the ultrastructure of the pellicle, TEM imaging was carried out before and after exposure of the specimens to HCl.

As expected, the native pellicle in controls was of a fine granular structure and showed an electron-dense basal layer (fig. 6). The pellicle after application of the fluoridated mouth rinse looked similar. All plant extracts modified the ultrastructure of the pellicle layer. The liquid extracts had no effect on the underlying enamel and caused a thickened and more electron-dense pellicle layer (fig. 6).

In good accordance with the measurement of calcium and phosphate release, the physiological pellicle offered

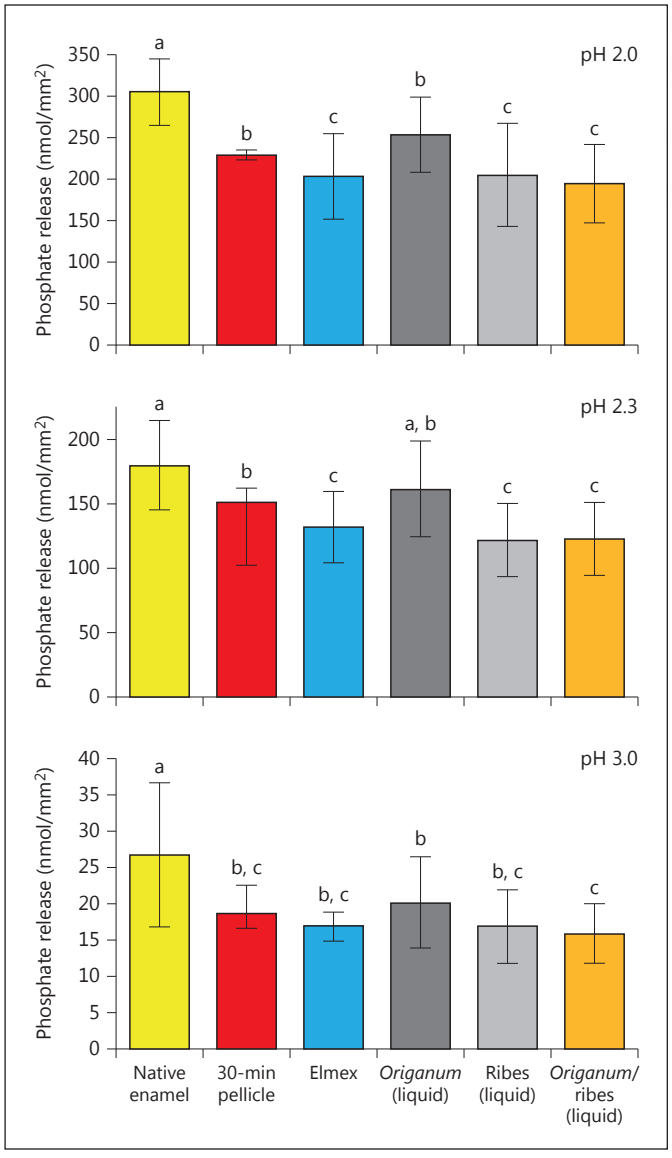

Fig. 5. Cumulative phosphate release over 120 s (pH 2.0/2.3/3.0); n = 24 samples per subgroup, mean ± SD. Data significantly different from each other are marked with different letters; statistical evaluation was carried out within one pH level (U test, Mann-Whitney).

limited protection against HCl and was degraded considerably. After the application of the fluoridated mouth rinse the pellicle was of higher rigidity. Also the pellicles modified by plant extracts were of higher tenacity than controls in an acidic milieu. This applied especially to *R. nigrum* liquid and the mixture *Origanum/R. nigrum* liquid (fig. 6).

Energy-Dispersive X-Ray Spectroscopy

After the application of the fluoride-based mouth rinse, the fluoride content of the pellicle-covered enamel surface amounted to 0.88 ± 0.13 wt%. Without the mouth rinse, the fluoride content of a pellicle-coated enamel slab was significantly lower (p = 0.026) and amounted to 0.40 ± 0.40 wt%.

Discussion

To the best of the authors' knowledge the adoption of *Origanum* and *R. nigrum* leaves in preventive dentistry has never been investigated before. The general impact of plant extracts on dental erosion has also been investigated rather sparsely. Most studies on these compounds focus on the oral biofilm; many of them are pure in vitro studies [Percival et al., 2006; Hannig et al., 2008b, 2009b; Tomczyk et al., 2013; Furiga et al., 2014; Giacaman et al., 2014; Girardot et al., 2014; Kong et al., 2014; Riihinen et al., 2014]. The present experimental in situ study found for the first time that a combination of wild *Origanum* and wild *R. nigrum* leaves has the potential to improve the protective properties of the pellicle against erosive noxae.

This effect can be attributed to the thicker and more electron-dense ultrastructure of the pellicle after rising with the mixture of plant extracts. Possibly the secondary plant compounds aggregate the salivary proteins, facilitating their adsorption to the enamel surface [Hannig and Hannig, 2014]. It is to be expected that hydrophilic compounds such as polyphenols dominate in watery extracts.

Furthermore, tanning and denaturing may take place, yielding a more electron-dense and potentially less permeable pellicle layer [Joiner et al., 2003, 2004, 2006; Hannig and Joiner, 2006]. However, also other components of the watery extracts could contribute to these obviously beneficial effects. Potentially also traces of lipophilic compounds may be relevant (fig. 6).

The adopted methodology does not completely mirror the clinical situation and especially not the repeated application of the different extracts. This has to be investigated in a further controlled clinical trial. Yet, the in situ/in vitro model allows investigating immediate effects of mouth rinses on the functional properties and ultrastructure of the pellicle. In a previous study it was used to evaluate the effects of edible oils on the in situ pellicle considering its protective properties against acidic noxae [Hannig et al., 2012]. The adopted photometric assays are

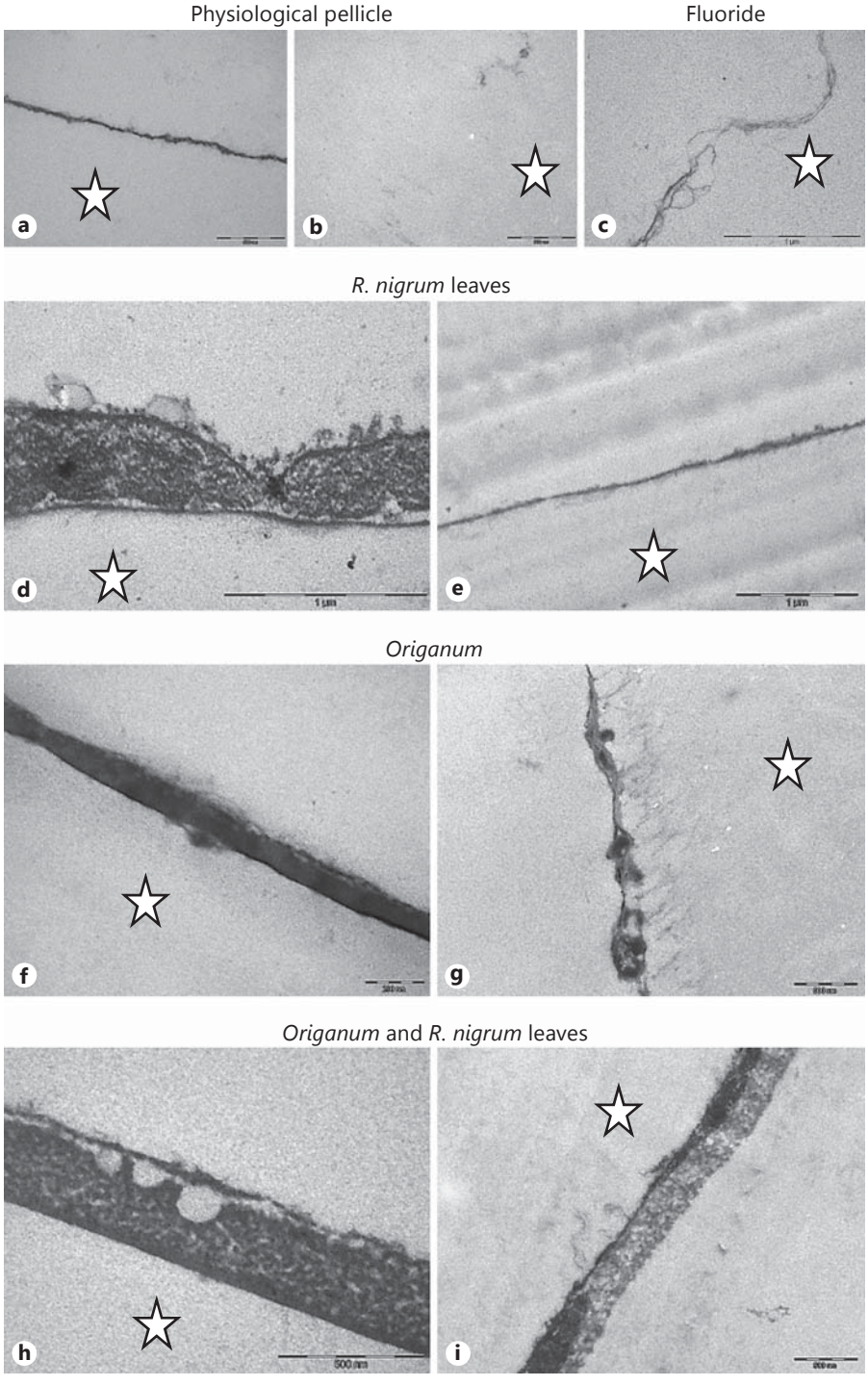

Fig. 6. TEM evaluation of representative pellicle samples before (**a**, **c**, **d**, **f**, **h**) and after incubation in HCl, pH 2.3 for 1 min (**b**, **e**, **g**, **i**). The physiological pellicle was of a fine granular structure covering an electron-dense basal layer (**a**). The fluoridated mouth rinse had no effect on pellicle ultrastructure (**c**); incubation of the physiological pellicle in HCl destroyed it nearly completely (**b**). Application of the plant extracts (**d**, **f**, **h**) yielded considerable layer formation. These layers were of higher tenacity during incubation in HCl; this applied especially to the mixture of *Origanum* and *R. nigrum* leaves (**i**). Please note the vesicular structures that might indicate the inclusion of lipophilic components (**h**). Original magnification: ×30,000. Enamel was removed during sample preparation. The former enamel side is marked with stars.

of very high sensitivity [Attin et al., 2005a, b]. Furthermore, the in vitro exposure to HCl is restricted to very short time spans of clinical relevance mimicking the clinical situation. The solutions were used after 1 min of pellicle formation to simulate the application of the mouth rinses briefly after toothbrushing.

As in many previous studies with a similar design, HCl was adopted [Hannig et al., 2005a, 2012]. This acid is

completely dissociated and does not interfere with the photometric assays [Hannig et al., 2005a]. Furthermore, this setup mimics erosive challenges due to vomiting. Organic acids such as citric acid may form complexes with calcium, which hampers the accuracy of the measurement [Hannig et al., 2005a; Lussi and Jaeggi, 2006]. A fluoride-based mouth rinse served as a gold standard; its application yielded a slight fluoride accumulation at the surface of the pellicle-coated enamel. This confirms the protective effect of fluorides. The slightly increased level of fluoride detected in the pellicle-covered enamel specimens after rinsing with Elmex Kariesschutz might be either explained by interaction of the fluoride with the hydroxyapatite of the enamel surface or by incorporation in the pellicle binding to proteins, respectively. The extracts have the taste of a flavorsome herbal tea, and their application may be of interest to patients who prefer a healthy and ecological lifestyle. These patients often consume considerable amounts of fruits and acidic juices but sometimes reject fluorides despite their well-known efficacy in preventing erosive mineral loss.

It is to be expected that the plant extracts could have staining effects, which has to be evaluated. However, this would mean extrinsic stains removable by dental prophylaxis. During the experiments no staining of the teeth or the enamel samples was observed, and the extracts had no pronounced astringent effect, as reported by the volunteers.

Further studies are needed to evaluate the effect of the tested extracts on dentin erosion. It can be postulated that the polyphenolic compounds have a beneficial impact as they have the potential to inhibit proteolytic enzymes in the oral fluids as well as matrix metalloproteinases originating from the dentine itself. Polyphenols such as punicalagin have the potential to inhibit collagen degeneration [Jean-Gilles et al., 2013]. Tea extracts of green tea, white tea, oolong tea, and black tea rich in polyphenols were shown to inhibit the activity of host and bacterial proteases and the catalytic activity of matrix metalloproteinase-9 [Zhao et al., 2013]. It has been shown previously that Cistus tea inhibits peroxidase immobilized in the in situ pellicle [Hannig et al., 2008b]. Accordingly, polyphenols from plant extracts might also have an effect on proteolytic enzymes in the pellicle layer. This could enhance the tenacity of the pellicle; the presence of immobilized polyphenols in the pellicle could also hamper its degradation by proteolytic enzymes in the oral fluid.

The present study is based intentionally on whole-plant extracts and not on the application of single components. This corresponds to the clinical application of the preparations available to the patients. It also mirrors in part the preparation of tea by the patients from dried herbage. However, it is necessary to analyze the composition of the adopted plant extracts qualitatively and quantitatively by HPLC and mass spectrometry in order to identify the components that are relevant for the observed protective effects. It may be hypothesized that no single components but the interaction of all constituents is responsible for the beneficial modification of the pellicle. In this context it is noteworthy that the mixture of oregano and ribes yielded the best results, which were superior to those of single extracts. This applies to the erosive mineral loss as well as to the modification of pellicle ultrastructure. It might be postulated that the interaction of certain components from oregano and ribes leads to the more electron-dense pellicle structure. In general polyphenols have tanning and denaturing properties. It seems that the mixture of the two plant extracts allows optimal tanning of the pellicle as well as protein aggregation.

Besides dental erosion, periodontitis and caries – both induced by bacterial biofilms – are still the most relevant challenges in dentistry. It still needs to be investigated whether the plant extracts have antibacterial properties on cariogenic bacteria or protract oral biofilm formation. Also, anti-inflammatory effects require further research. Nonetheless, the combination of certain liquid plant extracts gained from wild *R. nigrum* leaves and wild oregano seems to be an interesting and relevant biological and ecological approach to the prevention of dental erosion and requires further research.

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Author Contributions

Marie-Theres Weber: literature research, writing of the Introduction and most parts of the paper.

Matthias Hannig: electron microscopic imaging, interpretation and discussion of the TEM results.

Sandra Pötschke: laboratory work, determination of calcium and phosphate release, statistics.

Franziska Höhne: acquisition and screening of the subjects, sample preparation, laboratory work, determination of calcium and phosphate release, coordination of the in situ experiments.

Christian Hannig: interpretation of the data, coordination and planning of the research project, revision of the manuscript, interpretation of the data, writing of the manuscript.

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The sponsor had no influence on the evaluation of the data or on the manuscript.

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