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Original Article

Investigation of the effect of some plant aqueous extracts on calcium phosphate precipitation as a simulation of initial dental calculus formation *in vitro*

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ABSTRACT

Background and Aims: Calcium phosphate is most of the inorganic content of dental calculus. Therefore, knowing or controlling the precipitation mechanism of calcium phosphate is very important for the inhibition of dental calculus formation at the beginning. Plants have been known to be excellent sources of many nutritional and phytochemical content. The aim of this study is to investigate the effects of *Petroselinum crispum*, *Eruca vesicaria ssp. sativa, Beta vulgaris* L.var.*cicla*, *Rumex cristatus DC*. and *Cotinus coggygria Scop*. aqueous extracts on calcium phosphate precipitation, which is thought to reflect the onset of dental calculus formation *in vitro*.
Methods: The optical density (OD) increases first with the calcium phosphate nucleation and when the balance is reached, the optical density decreases gradually when the nuclei begin to aggregate and precipitate. The OD change was monitored by recording the absorbance at 620 nm.
Results: The effect on the calcium phosphate precipitation varied differently among the 5 types of aqueous extracts. The smoke tree (*Cotinus coggygria Scop*.) extract activated calcium phosphate precipitation while all others inhibited precipitation.
Conclusion: These results suggest that some types of plant aqueous extracts may have protective potential against dental calculus initially and, therefore they may be used in toothpastes or in mouthwashes.

Keywords: Dental calculus, calcium phosphate precipitation, plant aqueous extract

INTRODUCTION

Among oral and dental health problems, dental calculus has an increasing prevalence due to changes in the diet and nutritional habits of societies (Akar, 2014) and causes many oral problems, such as noneaesthetic appearance and bad breath. Although dental calculus is not directly responsible for the occurrence of health problems such as diabetes, urinary stone formation, and cardiovascular disease, it is a secondary factor in disease progression (Batool et al., 2018, Clarke, 2015).

Dental calculus is a mineralized bacterial plaque which is a hard and calcified deposit with a bacterial plaque layer clustered on natural teeth and restorations (Moolya et al., 2010). It is composed of various inorganic components, mainly calcium phosphate

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compound, and organic matrix (Jin & Yip, 2002; White, 1997). Many factors such as diet, especially alkaline foods and sugars, saliva pH, composition and bacterial load, age, sex, race, gender, tobacco use, presence of systemic diseases, drugs used, oral hygiene practices and socioeconomic status affect dental calculus formation (Akçalı & Lang, 2018). Bacterial endotoxins damage the gum and periodontal tissue (Hidaka, Nishimura, Nakajima, & Liu, 1996). Knowing and controlling the factors affecting calcium phosphate precipitation is very important for the inhibition of initial dental calculus formation (Tarasevich, Chusuei, & Alloro, 2003). The use of plants has also become widespread in dental and oral health, with secondary metabolites and a wide range of biological activities (Gulfraz, Sadıq, Tarıq, Imran, & Qureshi, 2011).

Petroselinum crispum (parsley, Apiaceae) has an antihistaminic, antiseptic, hepatogenic, hypotensive, and plasma calcium-enhancing effect (Hazim, Al-Daraji, Al-Mashadani, Al-Hassani, & Mirza, 2012). It contains high amounts of apigenin, ascorbic acid, eugenol, carotenoids, flavonoids, coumarins, phenylpropanoids, phthalates, furano coumarins and tocopherol components (Ajmera, Kalani, & Sharma, 2019; Pápay, Kállai-Szabó, Ludányi, Klebovich, & Antal, 2016; Tunali et al., 1999). *Eruca vesicaria ssp. sativa* (garden rocket) belongs to the cabbage *Brassicaceae* family. It is known as a diuretic, antiulcer, antithrombotic, antioxidant, anticancer, antiplatelet and anti-inflammatory (Taviano et al., 2018 and 2017; Gulfraz et al., 2011; Sacan, Orak, & Yanardag, 2008). It contains important secondary metabolites such as flavonoids, alkaloids, tannins, phenols, saponins and ascorbic acid. Its essential oils contain high concentrations of an especially antibacterially effective erucic acid (Sarwar Alam, Kaur, Jabbar, Javed, & Athar, 2007). *Beta vulgaris* L. var.*cicla* (chard) is from the family *Chenopodiaceae* and is a vegetable rich in vitamins A, B, C, calcium, iron, and phosphorus. In its structure, it contains fatty acids such as palmitic acid, citric acid, oleic acid, linoleic acid, and phospholipids, glycolipids, polysaccharides, saponin, pectin, and flavonoids (Tunali, 2020; Mzoughi et al., 2019). It has antidiabetic, antioxidant, antitumor, antimicrobial, hepatoprotective, antiseptic, and anti-acetylcholinesterase activity (Sacan & Yanardag, 2010; Bolkent, Yanardağ, Tabakoğlu-Oğuz, & Ozsoy-Saçan, 2000; Mzoughi et al., 2019). *Rumex crispus* (curled dock) *Polygonaceae* is a plant rich in anthracene derivatives from the blackgrain family. It also contains tannins, flavonoids, and naphthalene derivatives. It has antioxidant and antimicrobial effects (Idris, Wintola, & Afolayan, 2019; Demir, Bozkurt, Onur, Kay, & Somer, 2017; Coruh, Gormez, Ercisli, & Sengul, 2008). *Cotinus coggygria* (smoke tree) is a plant belonging to the Anacardiaceae family, generally known as "smoke tree". It has been shown by *in vivo* and *in vitro* studies that it has many activities such as antioxidant, antibacterial, antifungal, antiviral, hepatoprotective, and anti-inflammatory (Matić, Stanić, Mihailović, & Bogojević, 2016). It has been reported that the *Cotinus coggygria*, which has a high flavonoid content, has cytotoxic effects on bacteria and shows inhibitory properties against the common components of the dental plaque, *S. mutans* and *S. sanguinis* (Ferrazzano et al., 2013; Wang, Wang, Du, Fei, & To, 2016).

In this study, the effects of five plant aqueous extracts (*Petroselinum crispum*, *Eruca vesicaria ssp. sativa*, *Beta vulgaris* L.var.*cicla*, *Rumex cristatus DC*. and *Cotinus coggygria Scop.)* on the mechanism of nucleation and aggregation of calcium phosphate precipitation which is thought to reflect initial dental calculus formation *in vitro,* was evaluated.

MATERIALS AND METHODS

Preparation of plant aqueous extracts

Plant extracts were prepared at the Department of Chemistry, Faculty of Engineering, Istanbul University-Cerrahpasa. *Petroselinum crispum, Beta vulgaris* L.var.*cicla* were identified by Prof. Dr.Neriman Ozhatay. *Rumex cristatus DC.,* and *Eruca sativa* were identified by Prof. Dr. Kerim Alpinar. *Cotinus coggygria* was identified by Prof. Dr. Sukran Kultur, Faculty of Pharmacy, Istanbul University. The plant materials were washed with water and dried at room temperature. The dried plants were stored at -20°C until used. Dried leaves (50 g) were extracted by adding 500 mL of distilled water and boiling for 8 hours. The extracts were then filtered and lyophilized. Then, were kept at -20°C. When used, the extracts were dissolved in distilled water to obtain a saturated solution.

Screening of calcium phosphate precipitation

To obtain the standard curve for nucleation and aggregation of calcium phosphate precipitates were obtained by recording the change in optical density at 620 nm at two-minute intervals for approximately 30 minutes using the RT-9000 Semi-auto Chemistry Analyzer instrument, after mixing equal volumes of calcium chloride dihydrate (4 mM) and trisodium phosphate (10 mM) solutions at 37^{o}C. Final concentrations of calcium and phosphate ions were about 2mM and 5 mM respectively. They are between physiological salivary concentrations. The optical density increases first with the calcium phosphate nucleation, when the balance is reached, the optical density decreases gradually when the nuclei begin to aggregate and precipitate (Selmi Cepis, Akyuz, & Yarat, 2020).

The effects of plant aqueous extract on calcium phosphate precipitation were examined by the addition of 50 µL of an aqueous extract into the mixture of calcium and phosphate ions. Each sample was studied five times. At the end of the experiment, charts drawn between time and optical density (absorbance) and the effects of each aqueous extract on calcium phosphate precipitation were evaluated statistically.

Statistics

The Statistical Program for the Social Sciences (SPSS) (16.0, Windows) was used in the statistical evaluation of the data obtained as a result of the study and $p<0.05$ was considered statistically significant. The consistency of continuous variables to normal distribution was investigated using the Kolmogorov-Smirnov test. Student's t test was used in two independent group comparisons for normally distributed variables. Mann Whitney U test was used in comparison of two independent groups for non-normally distributed variables. Values are given as mean ± standard deviation (SD).

RESULTS

The standard curve for precipitation of calcium phosphate: Time-course measurements of optic density obtained by 2 mM of calcium and 5 mM of phosphate solutions at 620 nm were illustrated in Figure 1 (Selmi Cepis, Akyuz, & Yarat, 2020).

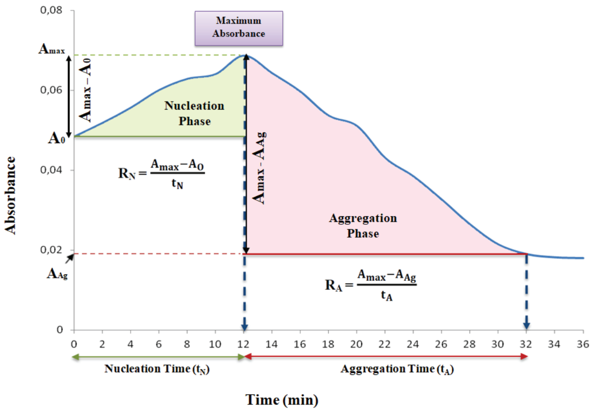

Figure 1. The standard curve for nucleation and aggregation phases of the formation of calcium phosphate precipitate (Selmi Cepis, Akyuz, & Yarat, 2020). After mixing the calcium and phosphate solutions, the increase in optical density over time reflects the rate of formation of new nuclei and the increase in the number of calcium phosphate nuclei. When the optical density reaches the maximum value, the formation of calcium phosphate nuclei is complete. This phase is called the nucleation phase. Nucleation rate (R_N) is the ratio of the difference between the maximum absorbance value and the original absorbance value to the time taken to reach the maximum absorbance value (t_N). Optical density decreases gradually over time. The decrease in the optical density reflects the reduction of the number of nuclei dispersed in the solution due to the aggregation of calcium phosphate nucleis. This is called the aggregation phase. R_A is the ratio of difference between the maximum absorbance value and the absorbance value where the optical density no longer decreases to the time taken to decrease the maximum absorbance (t_A).

Effects of plants aqueous extracts in calcium phosphate precipitation: The effects of *Petroselinum crispum*, *Eruca vesicaria ssp. sativa*, *Beta vulgaris* L.var.*cicla*, *Rumex cristatus DC.* and *Cotinus coggygria Scop.* aqueous extracts are shown in the Figure 2. Compared to a standard curve, all extracts except *Cotinus coggygria* prolonged nucleation time, decarsed the nucleation rate significantly. *Cotinus coggygria* and *Rumex crispus.* shortened aggregation time significantly. All extracts increased aggregation rate significantly compared to a standard curve. The increase in the aggregation rate for *Cotinus coggygria* was significantly more than those of other extracts (Table 1 and Figure 2).

DISCUSSION

Physiologically, normal Ca^{2+} ion concentration in saliva has been reported to be 1.03–3.6 mM and PO_4^{3-} ion concentration 4.5-6 mM. Any imbalance in these concentrations bring various problems in terms of dental and oral health. The most important of these is dental calculus formation. Calcium phosphate supersaturation, which is realized by increasing the concentration of $Ca^{2+}$and PO_4^{3-} ions in saliva, is a thermodynamic driving factor for dental calculus formation (Morta, Mante, Rasa, & Gintaras, 2018). Therefore, to obtain a standard curve, which nucleation and aggregation phases of calcium phosphate precipitation can be monitored in a short time interval and in a good manner, preliminary experiments were carried out with different concentrations of these two ions. As a result, for final concentration in the mixture, 2 mM calcium and 5 mM phosphate ion concentrations, which are in the interval of salivary physiologic concentaration, were found suitable to obtain a standard curve of calcium phosphate precipitation. To examine the effect of plant aqueous extracts, this standard curve was used for comparison in the present study.

In their study, Hidaka & Oishi (2007), examined the effect of nutritional components on dental calculus formation, and used both calcium and phosphate ion final concentrations of 3 mM,

Table 1. The effects of plant aqueous extracts on the nucleation and aggregation phases of calcium phosphate precipitation.

	Standard (Calsium phospathe without extracts)	*Petroselinium crispum*	*Eruca vesicaria ssp. Sativa*	*Beta vulgaris* L. Var. *Cicla*	*Rumex cristatus DC.*	*Cotinus coggygria Scop.*
Nucleation rate (R_N) (x10^{-3}/min)	1.66±0.69	1.42±0.29	1.04±0.11	1.36±0.61	1,10±0,71	3,14±0,89$^{*\#\#b}$
Aggregation rate (RA) (x10^{-3}/ min)	2.68±0.35	3.36±0.38^{*a}	3.90±0.83^{*a}	0.72±0,25^{**b}	3,92±0,29^{**b}	5,46±1,34$^{**\#\#b}$
Nucleation time (t_N) (min)	12.80±1.78	40.0±4.24^{**b}	60.0±3.16$^{**\varepsilon\varepsilon b}$	17,20±3,03^{*a}	37,6±0,89^{**b}	12,40±1,67
Aggregation time (t_A) (min)	21.20±2.04	22.40±3.20	20.40±2.94	25,60±3,44	14,4±0,80^{**b}	17,60±1,49^{*a}

Values were given as mean ± standard deviation; *p<0.05, ** p<0.01: Significantly different from standard; $^{\#\#}$p<0.01: Significantly different from *Petroselinum crispum*, *Eruca vesicaria ssp. sativa*, *Beta vulgaris* L. var.*cicla, Rumex crispus;* $^{\varepsilon\varepsilon}$p<0.01: Significantly different from *Petroselinum crispum*, *Beta vulgaris* L. var.*cicla*, *Rumex crispus;. Cotinus coggygria* ^{a}Student t test, ^{b}Mann Whitney U test

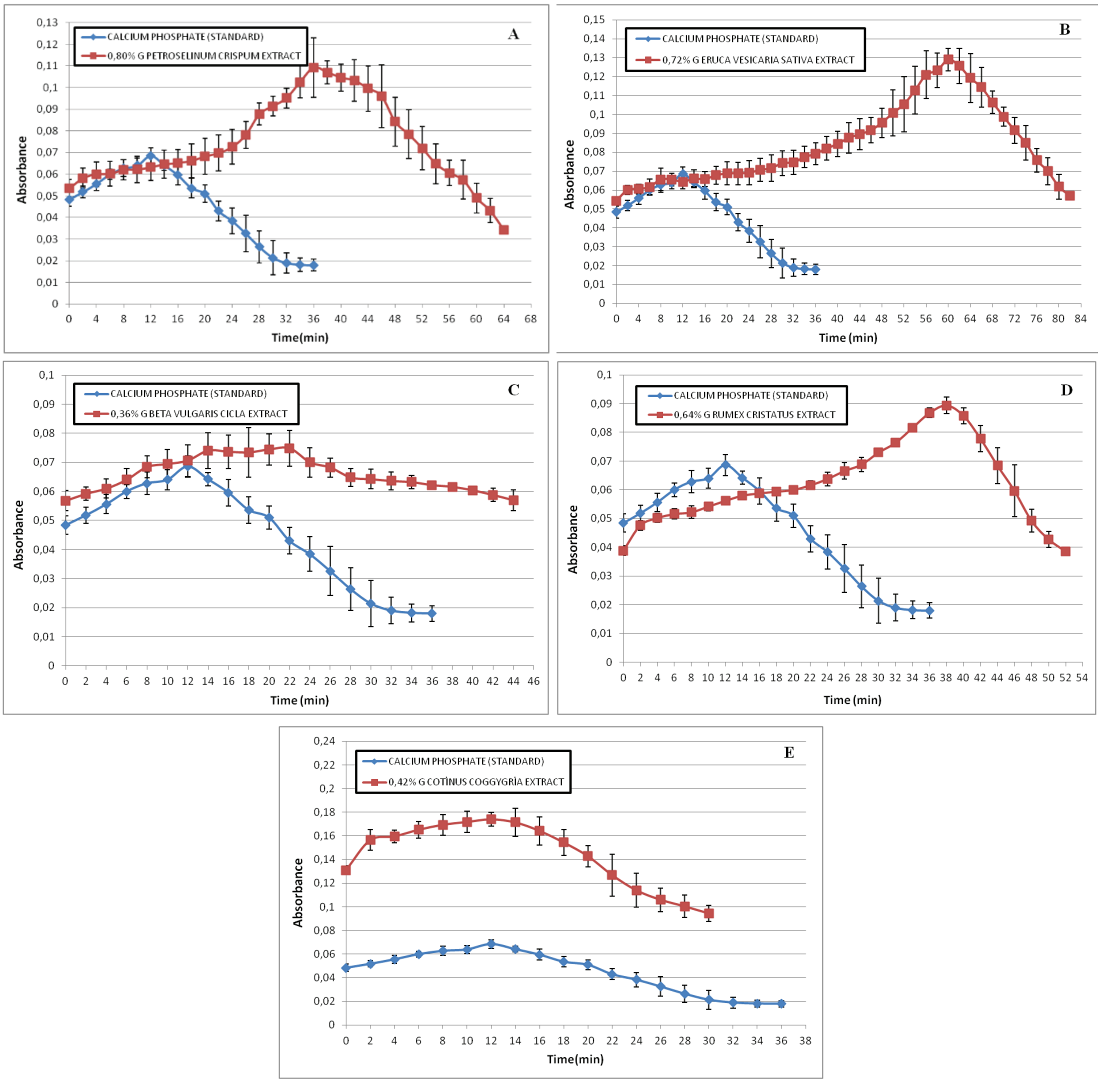

Figure 2. The effect of (A) *Petroselinum crispum,* (B) *Eruca vesicaria ssp. sativa*, (C) *Beta vulgaris* L. var.*cicla*, (D) *Rumex crispus,* (E) *Cotinus coggygria,aqueous* extracts on calcium phosphate precipitation.

unlike our study. However, the concentrations they used did not fully reflect saliva conditions.

Plant extracts have an important place in modern medicine due to their chemical and medicinal ingredients. As their metabolites have a wide range of biological activities, the use of plants has also become widespread in dental and oral health (Sener & Kilic, 2019; Chandra Shekar et al., 2015; Gulfraz et al., 2011). It has been suggested that some plant extracts can be effective in the formation of calculus in different rates because of their phenolic components such as flavonoids, tannins, and coumarins (Ohtani & Nishimura 2020; Ben Lagha, Dudonné, Desjardins, & Grenier, 2015; Weber, Hannig, Pötschke, Höhne, & Hannig, 2015; Ferrazzano et al., 2011). In studies conducted with high polyphenol content, plants have an inhibitory effect on calcium phosphate precipitation. Hidaka, Okamoto, Ishiyama, & Hashimoto (2008) reported that propolis and honey varieties known to contain flavonoids have an inhibitory effect on calcium phosphate precipitation, and that their inhibitory effects increase with increasing flavonoid content. Similar results were obtained in studies with green tea, *R. rhizoma* plant, and various Kampo plants with high polyphenol content (Torki et al., 2018; Anushya & Freeda, 2017; Hidaka et al., 1996). In the present study, *Petroselinum crispum, Eruca vesicaria ssp. sativa*, *Beta vulgaris* L. var.*cicla* and *Rumex crispus* were shown to inhibit calcium phosphate precipitation.

However, the *Cotinus coggygria* aqueous extract showed an activator effect on calcium phosphate precipitation. It has been reported that *Cotinus coggygria* with a high flavonoid content has cytotoxic effects on bacteria, and shows inhibitory properties against the common components of dental plaque such as *S. mutans* and *S. sanguinis* (Ferrazzano et al., 2013; Wang et al., 2016). The contrasting effect of *Cotinus coggygria*, rich in polyphenols and expected to prevent calcium phosphate precipitation, may have arisen from its antibacterial effects and dye components.

Moreover, polyphenols in plants have been reported to inhibit the glucosyl transferase activity of *Streptococcus mutans* (Veloz et al., 2016; Guven & Erkan, 2015; Smullen, Finney, Storey, & Foster, 2012). For this reason, polyphenols are considered as important substances for oral hygiene in terms of dental caries and dental calculus formations (Dash, Singh, Gupta, Panwar, & Ramisetty, 2014). Its mechanisms of action is based on their affinity for proteins. When approached from this perspective, they bind to amylase and glycosyl transferase enzymes and they inactivate those enzymes.

CONCLUSION

The results of our study showed that *Petroselinum crispum*, *Rumex cristatus DC, Beta vulgaris* L.var.*cicla,* and *Eruca sativa* aqueous extracts can be effective at the beginning on the calcium phosphate precipitation which may reflect on dental calculus formation. In the limitations of our study, any effect of *Cotinus coggygria Scop*. was found on precipitation. Nevertheless, more study needs to be done. Our study is the first attempt to apply the plant aqueous extracts to the area of oral health, such as their potential effects on calcium phosphate precitation at the beginning of dental calculus formation. Furthermore our study also highlights that more plants can be examined in this way.

In our study, we examined the effects of plant extracts separately. Our aim was to determine the impact of each separately. Further studies may be done to see what kind of result can be obtained by mixing two or more of the plant extracts having synergic effects. This may be especially important in the process of producing more effective products to prevent dental calculus precipitation.

Ethics Committee Approval: Not necessary for this study.

Informed Consent: Written consent was obtained from the participants.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study: A.Y., S.A.; Data Acquisition: B.S.Ç., A.Y.; Data Analysis/Interpretation: A.Y., B.S.Ç., S.A., Ö.S., R.Y.; Drafting Manuscript: B.S.Ç., A.Y.; Critical Revision of Manuscript: S.A., Ö.S., R.Y.; Final Approval and Accountability: A.Y., B.S.Ç., S.A., Ö.S., R.Y

Conflict of Interest: The authors have no conflict of interest to declare.

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