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Full Length Research Paper

In vitro effect of aqueous plant extracts on antioxidant parameters in saliva samples

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It is very common in Turkey to consume *Eruca sativa, Ocimum basilicum* L. and/or *Petroselinum crispum* either alone or in salads. These plants and also *Cotinus coggygria* Scop., are among the medicinal plants that have some concurrent qualifications such as antioxidant and antidiabetic effects. Saliva composition is an important factor to maintain oral health. The aim of this study was to determine, *in vitro*, the effect of aqueous extracts of these plants on glutathione level, superoxide dismutase and tissue factor activity in saliva samples. This study was performed on saliva samples obtained from clinically healthy subjects whose caries status and oral hygiene were similar. The aqueous extract was added to give final concentration of 1 mg of extract per ml of saliva sample and then it was incubated at 37°C for 1 h. Before and after incubation with aqueous extract, the saliva sample was examined for pH, buffering capacity, glutathione, tissue factor and superoxide dismutase activity. The saliva samples were also evaluated cytologically. *In vitro* incubation with aqueous extracts did not alter pH and protein levels of the saliva samples. All extracts affected saliva samples differently. These aqueous extracts may be of great interest for future studies with respect to treatment of oral diseases.

Key words: Aqueous plant extract, saliva, antioxidant parameters, *in vitro*.

INTRODUCTION

Various medicinal qualifications have been described to natural herbs. Medicinal plants constitute the main source of new pharmaceuticals and healthcare products, including medications for ethnoveterinary medicine. It is very common in Turkey to consume *Eruca sativa, Ocimum basilicum* L. and/or *Petroselinum crispum* either alone or in salads. These plants and also *Cotinus coggygria* Scop. are among the medicinal plants that have some concurrent qualifications such as antioxidant effects (Sacan et al., 2008; Kaurinovic et al., 2011; Dorman et al., 2011; Nićiforović et al., 2010).

Leaves of *E. sativa* Mill. (Brassicaceae) are used, especially, in salads or as a food ingredient, and have a characteristic horseradish-like odour and acidic taste (Cerny et al., 1996). The leaves are increasingly eaten by humans either alone or as part of mixed salads and are also used in herbal remedies (Yaniv et al.,1998). *E. sativa* Mill. is used as diuretic in traditional medicine (Mahran et al., 1991). *O. basilicum* L. (Labiaceae) is an important commercial plant, widely cultivated in many countries and is used in many traditional medicines (Tarchhoune et al., 2010). *O. basilicum* has been used as a potent antiseptic, preservative, slight sedative, digestive regulator, and diuretic (El-Beshbishy and Bahashwan, 2012). Numerous laboratory studies have shown various effects of *Ocimum* species, including bactericidal, anti-inflammatory, antioxidative, antiulcer, antidiarrhoeal, hypoglycemic, radiation protection, chemopreventive, and nervous system stimulation (Singh, 1999; Uma Devi et al., 2000).

Parsley (*P. crispum)* is a widely cultivated light green biennial herb of the Apiaceae (Umbelliferae) family, which is widely used for its organoleptic quality and essential

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oil. A cursory search through the scientific literature reveals that parsley extracts possess variety of biological activities, including protection against diabetes induced oxidative damage, antibiotic, antihypertensive, anti-oxidant, antitumorigenic, diuretic, enzyme inducing, cardiac or renal diseases, gastroprotective and platelet-normalizing, etc (Dorman et al., 2011).

C. coggygria Scop. (Anacardiaceae) is a shrubby tree commonly known as the smoke tree. It grows mainly in South and Central Europe, South Russia, Crimea, Caucasia, Latakia, and Turkey (Davis, 1982). The leaves are used as infusion in folk medicine for its antiseptic, anti-inflammatory, antimicrobial, antihaemorragic, para-dontosis, wound-healing features, and againts diarrhoea (Ivanova et al., 2005; Staniċ et al., 2009). The leaves and young branches from naturally growing trees are also utilized in producing an essential oil with terpenic odour to be used in perfumery in various countries (Demirci et al., 2003).

Saliva is a secretion which consists of 99% water, small quantities of various organic and inorganic compounds, bacteria, epithelial cells and gingival fluid. Saliva composition is an important factor for maintaining the oral health. Saliva acts as a cleansing solution, an ion reservoir, a lubricant, and a buffer. Saliva also has other protective functions on the ecological balance in the oral cavity via: debridement/lavage; aggregation and reduced adherence by both immunological and non-immunological means; and direct antibacterial activity. Saliva also possesses anti-fungal and anti-viral systems. It is effective in maintaining pH in the oral cavity and it contributes to the regulation of plaque pH (Edgar and Higham, 1995; Fenoll-Palomares et al., 2004). In addition to its other host-protective qualifications, saliva constitutes a first line of defense against free radical-mediated oxidative stress, since the process of mastication and digestion of ingested foods promote a variety of reactions. The antioxidant defense system of saliva includes various molecules and enzymes. Glutathione (GSH) and super-oxide dismutase (SOD) are among these antioxidants. GSH is a tripeptide containing a SH group. It has been shown that the levels of GSH in saliva are altered by different factors. SOD, a zinc-attached enzyme that catalyzes the conversion of superoxide anion to hydrogen peroxide, exists in a few isoenzymes in saliva and has a secondary antioxidant role (Battino et al., 2002).

Tissue factor (TF) (thromboplastin or Factor III) in saliva is thought to supply the hemostasis when injury takes place in the mouth, and it facilitates the barrier function of buccal mucosa (Zacharski and Rosenstein, 1979). TF initiates the extrinsic coagulation system and is a component of cell membrane (Mann et al., 1995; Bachli, 2000; Roberts et al., 2001). Like various tissues and body fluids, saliva has also been known to have TF activity (Zacharski and Rosenstein, 1979; Bachli, 2000; Yarat et al., 2004). 78% of TFa of saliva is attributed to to the cells in the saliva (Zacharski and Rosenstein, 1979). The physiopathological importance of TF in human saliva is not clearly known. The aim of this study was to determine, *in vitro*, the effect of aqueous extracts of *E. sativa, O. basilicum* L., *P. crispum,* and *C. coggygria* Scop. on salivary pH, buffering capacity, GSH level, SOD, and TFa. The saliva samples were also evaluated cytologically.

MATERIALS AND METHODS

Preparation of plant extracts

Plant leaves were collected in Istanbul, Turkey and identified by Prof. Dr. Kerim Alpınar, Faculty of Pharmacy, Istanbul University. Plants materials were washed with water and dried at room temperature. The dried plants were stored in -20°C until used. Dried leaves (50 g) were extracted by adding 500 ml of distilled water and boiling for 30 min. The extracts were then filtered and lyophilized. The extracts were kept at -20°C.

Subjects

The study was performed on saliva samples obtained from clinically healthy subjects (3 boys and 7 girls) whose number of decayed, missing and filled teeth (DMF-T) index (World Health Organization 1987), oral hygiene, and salivary flow rates were comparingly similar. Their ages were between 26 to 28 years. All subjects were instructed to refrain from smoking, eating, drinking and tooth brushing for 12 h prior to the saliva collection. Subjects who did not meet inclusion criteria and/or did not sign an informed consent were excluded from the study.

Collection of saliva samples

Fasting unstimulated whole saliva was collected at the same time of the day (between 08:30 and 11:00 h) for period of two days by the same researcher in all cases. The mouth was rinsed with distilled water before saliva collection. Subsequently, saliva was allowed to accumulate on the floor of the mouth and the subjects were instructed to spit out into the test tubes. Each saliva collection period was 20 min long. Immediatly after collection, saliva volume was measured and then salivary flow rate was calculated as ml/min.

Incubation of saliva samples with plant extracts and salivary analysis

The aqueous extract was added to give final concentration of 1 mg of extract per ml of saliva sample and then it was incubated at 37°C for 1 h. Before and after incubation with and without aqueous extract, the saliva sample was examined for pH, buffering capacity, total protein TP, GSH, tissue factor activity (TFa), and superoxide dismutase(SOD) activity.

Salivary pH was directly measured with pH paper (pH indicator strip, Merck, pH = 2.0 to 9.0). Salivary buffering capacity was measured by Ericson's method (Ericson, 1959). 200 µl of saliva was mixed with 600 µl HCl (0.0033 M). After 10 min, pH of the mixture was measured with pH paper, immediately. Based on the color change of the indicator paper strip, the pH was assessed in comparison with a color chart. The corresponding value was taken as the pH of the mixture. Total protein (TP) level was determined by the method of Lowry et al. (1951).

Bovine serum albumin was used as a standard, and absorbance evaluated at 500 nm. TP level was expressed as mg%. GSH was determined by the spectrephotometric method using Ellman's reagent (Beutler, 1975), the results were expressed as µM%.

TFa of saliva sample was evaluated according to Quick's one stage method using normal plasma (Ingram and Hills, 1976). This was performed by mixing 0.1 ml saliva with 0.1 ml of plasma, with the clotting reaction being started on addition of 0.02 M $CaCl_2$. All reagents were in the reaction temperature (37°C) before adding the mixture. TFa was expressed as seconds, shortened clot formation time shows increase in TFa.

SOD activity in saliva sample was measured according to the previously described method (Mylorie et al., 1986). Briefly, measurements were performed in cuvettes containing 2.8 ml and 50 mM potassium phosphate (pH = 7.8) with 0.1 mM EDTA, 0.2 mM riboflavin in 10 mM potassium phosphate (pH 7.5), 0.1 ml of 6 mM o-dianisidin, and sample. Cuvettes with all their components were illuminated with 20-W Slylvania Grow Lux fluorescent tubes that were placed 5 cm above and to one side of cuvettes maintaining a temperature of 37°C. Absorbance was measured at 460 nm and the results were expressed in U/ml SOD.

The saliva samples were also evaluated cytologically (Atay and Topalidis, 1994). Furthermore, for the cytological examination, one drop of saliva was dried over a slide. The slides were kept first in concentrated May Grünwalde solution for 7 min and then in Giemsa solution which was diluted 1/20 with distilled water, for 13 min. The slides were washed with distilled water for 1 min and left to dry at room temperature. Dried slides were observed under light microscope (Atay and Topalidis, 1994). Epithelial cells, keratinized epithelial cells and the density of bacteria were evaluated in the areas where the cells were homogeneously distributed.

Statistical analysis

Graph-pad statistical program was used for the evaluation of data. The significance in the statistical analyses was assessed using paired Student's t-test and ANOVA between groups. $p < 0.05$ were considered to be significant.

RESULTS

The mean levels of DMF-T index and salivary flow rate were 4.1 and 0.50 ml/min, respectively. Oral hygiene was also similar between subjects. One hour of incubation without plant extracts at 37°C did not change salivary parameters significantly (Figure 1a to e; $p > 0.05$) except SOD activity, which was significantly decreased ($p < 0.05$) at the end of 1 h (Figure 1f). *In vitro* incubation with aqueous extracts did not change protein levels of the saliva samples significantly ($p_{ANOVA} > 0.05$) (Figure 2c); however, it changed all other parameters significantly ($p_{ANOVA} < 0.05$) (Figure 2).

pH

The pH of saliva samples incubated with *P. crispum* was significantly lower than untreated saliva ($p < 0.05$) and *O. basilicum* L. treated saliva ($p < 0.01$). The pH of saliva samples incubated with *C. coggygria* Scop. was significantly lower than *O. basilicum* L. treated saliva ($p < 0.05$) (Figure 2a).

Buffering capacity

The buffering capacities of *P. crispum* treated saliva samples and *C. coggygria* Scop. treated saliva samples were significantly higher than untreated saliva samples ($p < 0.001$) and higher than *E.* sativa or *O. basilicum* L. treated saliva samples ($p < 0.001$) (Figure 2b).

Tissue factor activity (TFa)

The TFa of saliva samples incubated with *C. coggygria* Scop. was significantly higher than *E. sativa* treated saliva samples ($p < 0.05$) (Figure 2d).

GSH levels and SOD activities

All extracts caused an increase in GSH levels and a decrease in SOD activity. The GSH levels of saliva samples incubated with *C. coggygria* Scop. were significantly higher than untreated saliva samples ($p < 0.05$) (Figure 2e). The SOD activities of saliva samples incubated with *O. basilicum* L.*, P. crispum* or *C. coggygria* Scop. were significantly lower than untreated saliva samples and *E. sativa* treated saliva samples ($p < 0.001$) (Figure 2f).

Cytologic examination

Samples incubated with the *C. coggygria* Scop. extract were different from the others in terms of basophilic staining of the background and the cells. Cytological examination of the salivary imprint samples (before and after 1 h of incubation) from the untreated saliva sample revealed no difference in the keratinized epithelial cells and the density of bacteria. There was no difference in the density of epithelial and keratinized epithelial cells in all of the salivary imprint samples, bacterial aggregation was increased in the saliva samples that were incubated with the *E. sativa* and *P. crispum* extracts. Bacterial aggregation in the samples incubated with *O. basilicum* L. extract was reduced when compared to the samples incubated with *E. sativa* and *P. crispum* extracts. In the samples incubated with *C. coggygria* Scop. extract, there was decreased number of bacteria and almost no aggregation were seen (Table 1).

DISCUSSION

Herbs have been used for centuries to prevent and control diseases. Herbal extracts are effective because

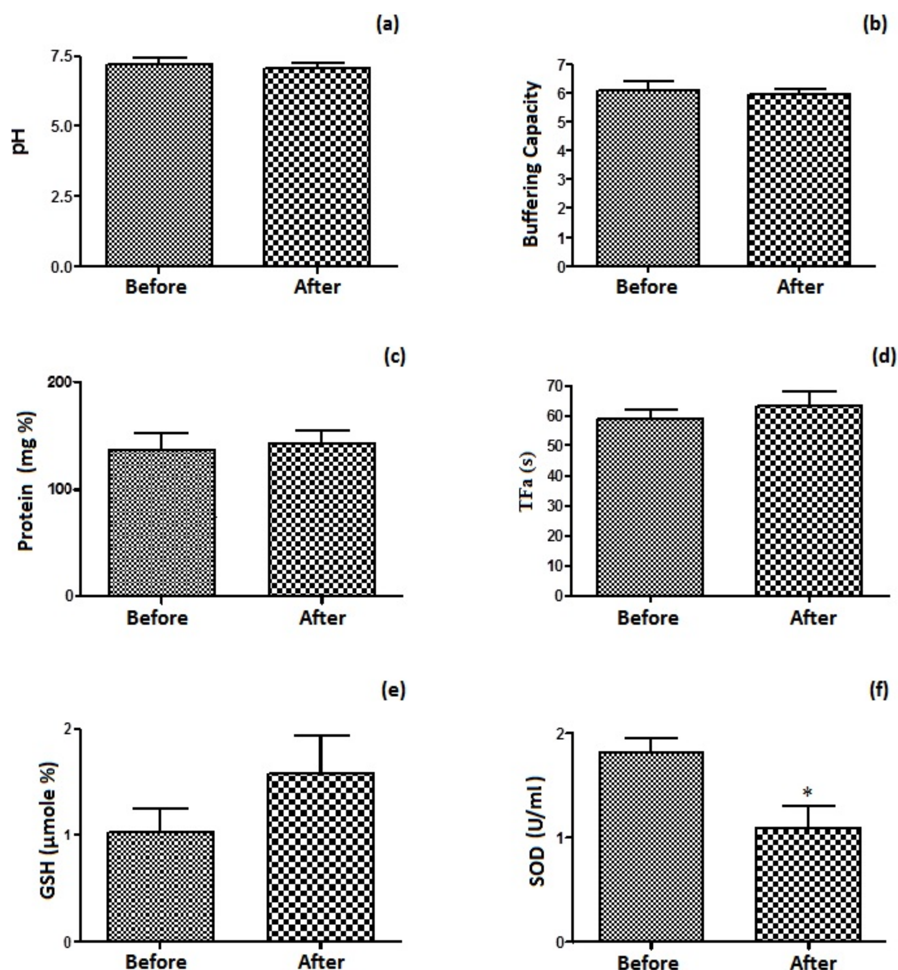

Figure 1: Saliva parameters before and after 1h of incubation at 37°C. Values are given mean ± standart error. (a) Comparison of pH values ($p>0.05$); (b) comparison of buffer capacities ($p>0.05$); (c) comparison of protein values ($p>0.05$); (d) comparison of TFa ($p>0.05$); (e) comparison of GSH levels ($p>0.05$); (f) comparison of SOD activity (*$p<0.05$). TFa: Tissue factor activity (lengthening of seconds is a manifestation of decreased TFa), GSH: Glutathione, SOD: Superoxide dismutase

they interact with specific chemical receptors within the body and are, in a pharmacodynamic sense, drugs themselves. By using herbal medicines, patients have averted the many side effects that generally come with conventional medicines, but this does not mean that side effects do not occur. Now, pharmaceuticals are called conventional, and herbs are labeled as the 'alternative'. The biggest challenge and problem is lack of information about the effect of herbs in oral tissues, mechanism of effect, and side effects (Taheri et al., 2011). Recent research suggests that some herbal extracts have potential beneficial effects in oral diseases (Meisser et al., 2012).

One of the most important universal health problems is oral disease. There is a well-known association between oral infections and microbial activities. More than 750 species of bacteria dwell in the oral cavity and a number of these are associated with oral diseases. Resistance by pathogenic bacteria to currently used antibiotics and chemotherapeutics has increased the global requirement for alternative safe, efficacious and cost-effective treatment options and products for such infections, particularly in developing countries. Traditional plants and natural products can treat bacterial infections. Even though natural products are not inevitably safer than synthetic antibiotics, health care professionals should recognize the value of herbal antibiotics (Borhan-Mojabi et al., 2012).

In the last few years, antimicrobial features have been reported in wide range of plant extracts and natural products attempting to discover new chemical classes of antibiotics, that could resolve the problems such as the appearance of undesirable side effects and emergence of previously uncommon infections (Feres et al., 2005; Alviano et al., 2008; Haffajee et al., 2008; Zheng et al., 2009). Garlic is a plant with antimicrobial effects, and

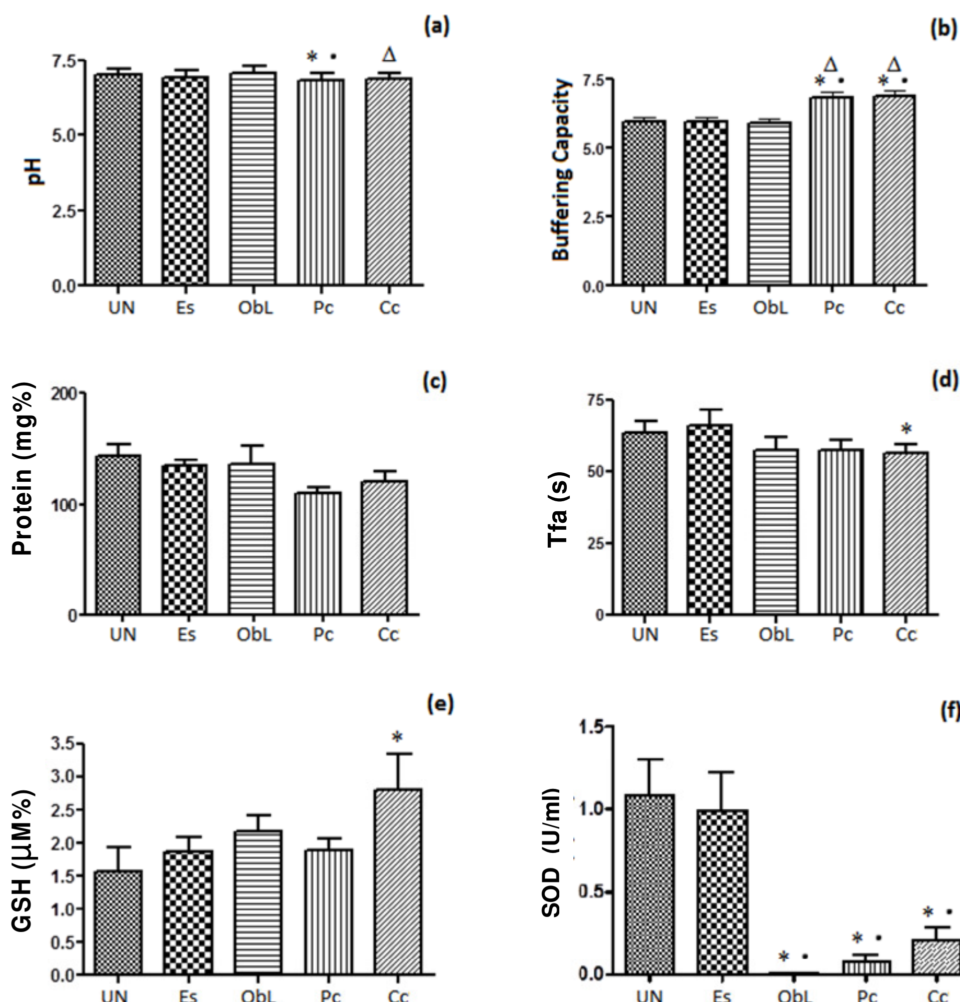

Figure 2. Salivary parameters after 1 h incubation with plant extrats. Values are given mean ± standart error. (a) pH values comparison after incubation with plant extracts ($p_{ANOVA} < 0.0001$; *p < 0.05 significantly different from UN; •p < 0.01 significantly different from ObL; Δp < 0.05 significantly different from ObL); (b) Buffer capacities comparison after incubation with plant extracts ($p_{ANOVA} < 0.0001$; *p < 0.001 significantly different from UN; •p <0.001 significantly different from Es; Δp < 0.001 significantly different from ObL); (c) Protein values comparison after incubation with plant extracts ($p_{ANOVA} > 0.1$); (d) Tissue factor activities comparison after incubation with plant extracts ($p_{ANOVA} < 0.05$; *p < 0.05 significantly different from Es); (e) GSH levels comparison after incubation with plant extracts ($p_{ANOVA} < 0.05$; *p < 0.05 significantly different from UN); (f) SOD activity levels comparison after incubation with plant extracts ($p_{ANOVA} < 0.0001$; *p < 0,001 significantly different from UN; •p < 0.001 significantly different from Es). UN: Untreated saliva (incubate without plant extracts), Es: *Eruca sativa*, ObL: *Ocimum basilicum* L., Pc: P*etroselinum. crispum*, Cc: *Cotinus coggygria* Scop. TFa: Tissue factor activity (lengthening of seconds is a manifestation of decreased TFa), GSH: Glutathione, SOD: superoxide dismutase.

different concentrations of garlic extract can decrease oral microorganisms. Borhan-Mojabi et al. (2012) have evaluated the effectiveness of different concentrations of garlic extract in an oral salivary microbial population. According to findings, garlic extract is effective in the reduction of an oral microbial population. It may be useful as an alternative product and new treatment modality with fewer side effects.

Several antiseptic agents including chlorhexidine, cetyl pyridinium chloride, fluorides, and phenol derivatives have been used widely in dentistry to inhibit bacterial growth (Subramaniam and Nandan, 2011; Malhotra et al., 2011). However, these substances have several adverse effects such as vomiting, diarrhea, and teeth staining. When the antimicrobial effectiveness of an herbal mouthrinse was compared with an essential oil and a chlorhexidine mouthrinse, it was found that the herbal rinse was more effective than the essential oil rinse in

Table 1. Cytological evaluation of saliva imprint samples.

Parameter	Epithelial (%)	Bacterium (%)	Bacterium aggregation (%)
Untreated saliva sample			
Untreated saliva (n = 10)	1 (70)	2 (30)	1 (100)
(before incubation)	2 (30)	3 (70)	1 (100)
Untreated saliva	1 (70)	2 (30)	1 (100)
(After 1 h incubation)	2 (30)	3 (70)	1 (100)
Saliva samples treated with plant extracts			
Incubation with *Eruca sativa*	1 (70)	2 (30)	3 (100)
	2 (30)	3 (70)	3 (100)
Incubation with *Ocimum basilicum* L.	1 (70)	2 (30)	2 (100)
	2 (30)	3 (70)	2 (100)
Incubation with *Petroselinum crispum*	1 (70)	2 (30)	3 (100)
	2 (30)	3 (70)	3 (100)
Incubation with *Cotinus coggygria* Scop.	1 (70)	1 (30)	-
	2 (30)	1 (70)	-

Epithelial cells; 1:low, 2: high. Bacteria and bacteria aggregation; 1: low, 2: moderate, 3: high.

inhibiting the growth of oral bacteria *in vitro* (Haffajee et al., 2008). Propolis showed significant antimicrobial quality in saliva samples from periodontically healthy and diseased subjects, and this suggests that this substance may be used therapeutically in the future to inhibit oral microbial growth (Feres et al., 2005).

Willerhausen et al. (1991) examined the efficacy of plant-derived active substances in a combined application of toothpaste and mouth rinse on dental plaque and the pH values of the saliva. A significant reduction of plaque accumulation and bleeding signs under herbal extracts in contrast to the placebo preparation was found. The salivary pH values were found to be shifted to the alkaline range in the herbal extract group, whereas the placebo product showed the opposite effect. Their data demonstrated the usefulness of herbal extracts as an adjunct in the treatment of periodontal disease and in a routine preventive regimen.

Licorice, which is the name of the roots and stolons of Glycyrrhiza species and its potential beneficial effects in common oro-dental diseases have been investigated and the potential beneficial effects of licorice and its constituents for preventing/treating oro-dental diseases were found (Messier et al., 2012). In this study, we have not examined the antimicrobial effects of plant extracts. However, it has been reported that *O. basilicum* L. and *C. coggygria* Scop. have antimicrobial effects (Singh, 1999; Uma Devi et al., 2000; Singh et al., 2007; Stanić, 2009).

The normal pH of saliva is 6 to 7, meaning that it is slightly acidic. The pH in salivary flow can range from 5.3 (low flow) to 7.8 (peak flow). Major salivary glands contribute most of the secretion volume and electrolyte content to saliva, whereas minor salivary glands contribute little secretion volume and most of the blood-group substances (Humphery and Williamson, 2001). Although pH values obtained by incubation with plant extracts in the present study were significantly different, this is not to be considered as having clinical importance.

The decrease in salivary buffering capacity is one of the secondary factors which affect caries formation (Edgar and O'Mullane, 1996). Incubation with *P. crispum* and *C. coggygria* Scop. caused an increase in salivary buffering capacity in the present study. These extracts may be effective in preventing caries formation. We also expected saliva protein levels to decrease, however these decreases were not statistically different. The plant extracts used in this study are rich in flavonoids, phenolic compounds (tannins), coumarins, etc. (Sacan et al., 2008; Gadi et al., 2009; Dorman et al., 2011; Tunali et al., 2011).

The preventive and therapeutic effects of antioxidants have been drawing great deal of attention in recent times. Saliva levels of Glutathione (GSH) and the activities of SOD and catalase, are essential for maintaining cell integrity. It is known that deficiency or depletion of GSH and other antioxidant enzymes cause damage to macromolecules or to membrane lipids when there is consistent formation of oxygen free radicals. The role of antioxidant mechanism is therefore specific for removing harmful oxidants or reactive oxygen species (ROS) as soon as they are formed, or for repairing the damages caused by ROS *in vivo*. Numerous antioxidants have been tried and tested both by systemic administration and as mouthwashes. These include synthetic products

like vitamins, natural products like wine, and green tea. As described previously, naturally occurring substances in higher plants have antioxidant activity, which are of a great application in the control of chronic disorders caused by oxygen-containing free radicals (Nagler and Dayan, 2006; Mallery et al., 2007; Alviano et al., 2008; Weiner et al., 2008).

Furthermore, periodontal diseases proved to be associated with an imbalance between oxidants and antioxidants, due to both an increase in free radical production and a defect in the total antioxidant activity of saliva. Oral peroxidase, the pivotal enzyme in the salivary antioxidant system, seems to be of paramount importance in the oral defense mechanism, especially against the attack of free radicals related to cigarette smoke and the evolution of oral cancer (Reznick et al., 2003). In the present study, all the plant extracts caused an increase in GSH levels and a decrease in SOD activity. However, the only significant result was related to *C. coggygria* Scop. extract. The GSH levels of saliva samples incubated with *C. coggygria* Scop. were significantly higher than those of untreated saliva samples. The SOD activities of saliva samples incubated with *O. basilicum* L., *P. crispum,* and *C. coggygria* Scop. were significantly lower than those of untreated saliva samples and those of saliva samples incubated with *E. sativa.*

The effectiveness of alternative strategies for controlling bacterial growth is extremely important nowadays. The oral hygiene has a direct effect on oral health, and is based on mouth rinse as a corrective treatment and on reduction by daily tooth brushing and frequent dental cleaning or prohylaxis. However, there is very little pertinent literature about the use of cleaning solution and in fact, dentists are inclined to choose a solution without knowing the effect it may cause on the resident oral microbiota. Increasing attention is also being given to the use of tannins as antimicrobial agents, for example in prevention of dental caries. Sometimes, undesirable side effects and emergence of previously uncommon infections may be present. For example, it has been demonstrated that the hydroxyl radical is formed in the human oral cavity during betel quid chewing, common in South-East Asia, and is probably implicated in the genetic damage that has been observed in oral epithelial cells of chewers (Nair et al., 1995).

In this study, bacterial aggregation was increased in the saliva samples that were incubated with the *E. sativa* and *P. crispum extracts.* Bacterial aggregation in the samples incubated with *O. basilicum* L. extract was reduced when compared to the samples incubated with *E. sativa* and *P. crispum* extracts. In the samples incubated with *C. coggygria* Scop. extract, decreased number of bacteria and almost no aggregation were seen. Furthermore, TFa was increased in samples incubated with *O. basilicum* L., *P. crispum and C.coggygria* scop. extracts. However, such increase was not significant. This increase may indicate preventive effects towards bleedings. In a literature, it has been reported that *C. coggygria* Scop is an antihemorrhagic plant (Stanić et al., 2009).

Conclusion

C. coggygria Scop. seems to have superior properties than the other plant extracts, because it increased salivary buffering capacity, prevented bacterial aggregation, and upgraded GSH levels. Herbs may be good alternatives to current treatments for oral health problems, but it is clear that we need more research. Additional tests, experimental models, and the pharmacological applicability, are required before considering these plants extracts as promising compounds. These extracts may be of great interest for future studies about treatment of oral diseases.

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