

# The Antioxidant Effect of Fermented Papaya Preparation in the Oral Cavity

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Oxidative stress has been recognized to play important roles in various diseases, including of the oral cavity. However, nutritional supplementation of antioxidants to ameliorate the consequences of oxidative stress is debatable. One caveat is that oxidative status is often measured under non-physiological conditions. Here, we investigated the antioxidant potential of fermented papaya preparation (FPP), a product of yeast fermentation of *Carica papaya* Linn, under conditions that prevail in the oral cavity. Employing highly sensitive luminol-dependent chemiluminescence assays, we show that its antioxidant capacity was augmented by saliva (up to 20-fold,  $p < 0.0001$ , at 10 mg) and its components (mucin, albumin) as well as by red blood cells (RBC) and microorganisms present in the normal and pathological environment of the oral cavity. Polyphenols are major plant antioxidants. Using the Folin–Ciocalteu's assay, a very low amount of phenols was measured in FPP suspended in a salt solution. However, its suspension in saliva, albumin, mucin or RBC produced up to sixfold increase,  $p < 0.001$ , compared with the sum of polyphenols assayed separately. The results suggested that these enhancing effects were due to the solubilization of antioxidant polyphenols in FPP by saliva proteins and the binding to RBC and microorganisms, thus increasing their availability and activity. Copyright © 2015 John Wiley & Sons, Ltd.

**Keywords:** Antioxidants; oral cavity; saliva; *Carica papaya*; chemiluminescence; polyphenols; mucin.

## INTRODUCTION

Reactive oxygen species (ROS) play important physiological functions such as signal transduction and antibacterial protection (Holmstrom and Finkel, 2014), but in excess, can also be injurious to various cells (Avery, 2011). Therefore, normally, ROS levels are tightly balanced by antioxidant systems that regulate the rate of ROS generation and scavenge excess ROS. However, under certain pathological conditions, the generation of ROS may exceed the oxidant scavenging abilities (OSA) of the antioxidative systems, resulting in oxidative stress (Amer *et al.*, 2008).

Oxidative stress has been recognized to play important roles in the pathophysiology of various diseases, but the ability of supplementation of antioxidants to ameliorate the consequences of oxidative stress is still a dilemma (Sies, 2010). For example, it has been shown that supplementation of vitamin C and  $\beta$ -carotene had failed to significantly affect the incidence of cardiovascular disorders and that excessive amounts of vitamin E resulted in an increase in an all-cause mortality (Miller *et al.*, 2005).

Over the centuries, numerous plants and their extracts have been used to treat a large variety of maladies (Sofowora *et al.*, 2013). Some of their beneficial effect has been assumed to be related to their antioxidant action (Lee *et al.*, 2013). One such botanical source that has been reported to possess therapeutic effects is the papaya fruit (Webman *et al.*, 1989) and its fermentation

products (Aruoma *et al.*, 2010). Fermented papaya preparation (FPP) is a product of yeast fermentation of *Carica papaya* Linn. Studies in chronic and degenerative disease conditions (such as thalassemia, cirrhosis, diabetes and aging) and sport performances have shown that FPP favorably modulates immunological, hematological, inflammatory, vascular and oxidative stress damage parameters (Aruoma *et al.*, 2010).

We have studied the antioxidant properties of FPP on blood cells in various hematological diseases, such as thalassemia (Amer *et al.*, 2008), hereditary spherocytosis (Ghoti *et al.*, 2010a) and paroxysmal nocturnal hemoglobinuria (Ghoti *et al.*, 2010b). *In vitro* and *in vivo* (in both patients and experimental animals) studies showed that FPP ameliorates the oxidative stress of red blood cells (RBC), granulocytes and platelets.

Antioxidants, including FPP, are usually taken orally and thus start to exert their effects already in the oral cavity. The health of the oral cavity depends to a large extent on its oxidative conditions (Canakci *et al.*, 2005). The latter are affected by ROS generated by the epithelial cells covering the oral cavity as well as by invading bacteria and migrating blood neutrophils (Ginsburg *et al.*, 2013). Antioxidative components of the saliva such as uric acid, ascorbate,  $\alpha$ -tocopherol and reduced glutathione counteract the effect of the oxidants in the oral cavity (Liskmann *et al.*, 2007). In the present study, we investigated the antioxidant effect of FPP under conditions that may exist in a healthy and an inflamed oral cavity. Employing two highly sensitive luminol-dependent chemiluminescence (LDLC) assays, we showed that the OSA of FPP is dose-dependently augmented by saliva and its components (mucin, albumin) as well as by RBC and microorganisms in the normal and pathological flora.

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## MATERIALS AND METHODS

**Fermented papaya preparation.** A product of yeast fermentation of *Carica papaya* Linn. was supplied by Osato Research Institute, Gifu, Japan. The composition of its principal components has been previously reported (Fibach *et al.*, 2010). Unless otherwise stated, the material was dissolved in water.

**Saliva.** Collected from healthy adults by the spitting method (Navazesh, 1993), was subjected to centrifugation for 10 min at 13 000 cpm in an Eppendorf centrifuge, and the supernatant fluid was kept on ice and used on the same day.

**Red blood cells.** Heparinized blood samples were obtained from consented adult donors at the Blood Bank of Hadassah Hospital, Jerusalem. The cells were washed three times and suspended in normal saline and were kept on ice until being used on the same day.

**Microorganisms.** *Candida albicans* (ATCC 20092) obtained from the Department of Microbiology, Hadassah Hospital, Jerusalem, Israel was cultivated overnight on brain heart infusion agar plates, and washed suspensions were adjusted to approximately  $10^9$  cells/mL as previously described (Ginsburg *et al.*, 2013).

The study was carried out in accordance with the Helsinki's convention guidelines and was approved by the Hadassah Institutional Committee for Human Experimentation (# 0313-1).

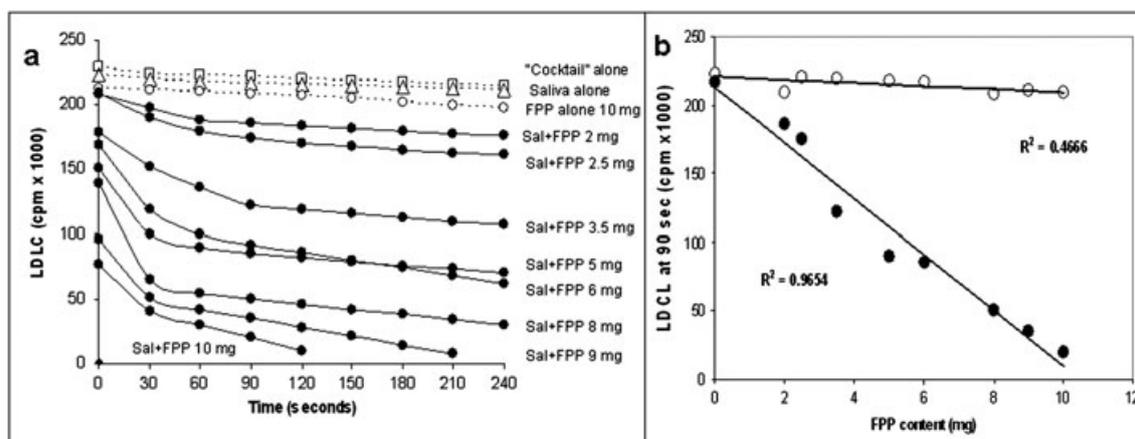
**Quantification of oxidant scavenging abilities by luminol-dependent chemiluminescence assays.** The tested materials, suspended in 800  $\mu$ L of Hank's balanced salt solution (pH 7.4, Biological Industries, Beit-Haemek, Israel), were added to two LDCL cocktails:

A. The 'H<sub>2</sub>O<sub>2</sub> cocktail' (Ginsburg *et al.*, 2004b) is composed of luminol (10  $\mu$ M), sodium selenite (2 mM), H<sub>2</sub>O<sub>2</sub> (1 mM) and CoCl<sub>2</sub>·6H<sub>2</sub>O (10  $\mu$ M). The cocktail generates an immediate flux of light, which remains constant (Fig. 1a) because of peroxide and hydroxyl radicals. B. The 'SIN-1 cocktail' (Ginsburg *et al.*, 2004a) containing morpholino sydononimine (SIN-1) (10  $\mu$ M) instead of H<sub>2</sub>O<sub>2</sub>. This cocktail generates a time-dependent light flux that becomes constant after about 2 min (Fig. 3a) because of the generation of peroxyxynitrite resulting from the interaction of nitric oxide and superoxide. In both assays, light quenching, measured as counts per minutes by a Lumac 2500 Luminometer (Landgraaf, The Netherlands) and compared with the 'cocktails' alone, indicated scavenging activities of the tested materials.

**Quantification of total polyphenols by the Folin-Ciocalteu's reagent.** The assay was performed as previously described (Singleton *et al.*, 1999). In short, the tested materials were added in 800  $\mu$ L normal saline. Five min later, 50  $\mu$ L of the Folin-Ciocalteu's cocktail (a mixture of phosphomolybdate and phosphotungstate) was added, followed 1 min later by 150  $\mu$ L of a 25% solution of sodium carbonate. After an additional 5 min at room temperature, the reaction mixtures were centrifuged at 2000 rpm for 5 min, and the blue color developed in the supernatant was read spectrophotometrically at 760 nm. A standard graph was prepared based on gallic acid equivalents.

## RESULTS

The OSA of FPP and saliva, each one alone and in combination, measured by the H<sub>2</sub>O<sub>2</sub>-LDCL assay is presented in Fig. 1a. The time-dependent LDCL quenching indicates the ability of the tested materials to decompose H<sub>2</sub>O<sub>2</sub> and hydroxyl radical generated by the cocktail. The cocktail alone showed no decrease in



**Figure 1.** Antioxidant activity of fermented papaya preparation (FPP) in the absence and presence of saliva. Saliva alone (Sal, 15  $\mu$ L,  $\Delta$ ), FPP alone (10 mg,  $\circ$ ) and combinations of saliva and increasing amounts of FPP ( $\bullet$ ) were assayed by the H<sub>2</sub>O<sub>2</sub>-luminol-dependent chemiluminescence (LDCL) as described in Materials and Methods. (a) Kinetics of LDCL (measured every 30 s). The results of one out of four experiments are shown. The time-dependent light quenching (as compared with the 'cocktail' alone) indicates antioxidant activity. (b) The effect of FPP concentrations, with saliva ( $\bullet$ ) and without saliva ( $\circ$ ), on the LDCL after 90 s incubation (means  $\pm$  standard deviation of four experiments). The results show a linear dose-response antioxidant activity. In the presence of saliva, FPP showed higher antioxidant activity, starting at 2.5 mg (four times,  $p < 0.05$ ) up to 20 times,  $p < 0.0001$  at 10 mg, in the presence of saliva compared with FPP alone.

luminescence with time. Saliva (at 15  $\mu\text{L}$ ), in agreement with a previous report (Ginsburg *et al.*, 2013), and FPP (up to 10 mg), each one alone, had a very modest effect, while combinations of FPP (2–10 mg) and saliva (15  $\mu\text{L}$ ) had a significant quenching effect, indicating the ability of FPP in saliva to decompose  $\text{H}_2\text{O}_2$  and hydroxyl radicals generated by the cocktail. Fig. 1b shows the effect of different amounts of FPP in saliva (15  $\mu\text{L}$ ) on the luminescence after 90 s incubation with the cocktail. The results show a linear dose–response antioxidant activity. In the presence of saliva, FPP showed higher antioxidant activity, starting at 2.5 mg (four times,  $p < 0.05$ ) up to 20 times ( $p < 0.0001$ ) at 10 mg, in the presence of saliva compared with FPP alone.

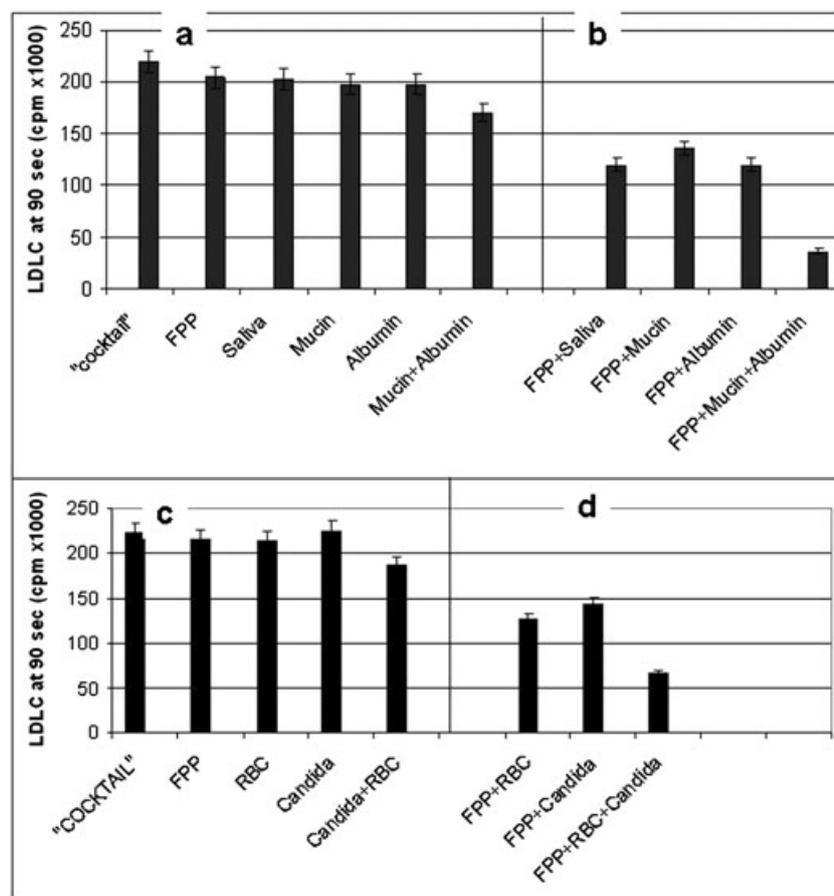
We next studied the effect of mucin and albumin – the major components of saliva. Fig. 2a shows the enhancing effect of either gastric mucin or human/bovine albumin (both from Sigma, St. Luis, MO), and the synergistic effect ( $p < 0.001$ ) of both proteins when added together, on the OSA of FPP. RBC and bacteria are often present in the oral cavity. We therefore studied their influence as well. The results show (Fig. 2b) that RBC ( $4 \times 10^6$ ) and *C. albicans* ( $10^8$ ) as well as their combination significantly ( $p < 0.001$ ) increased the OSA of FPP.

We further studied the scavenging effect of peroxynitrite resulting from the interaction of nitric oxide and superoxide using the SIN-1–LDCL assay. The

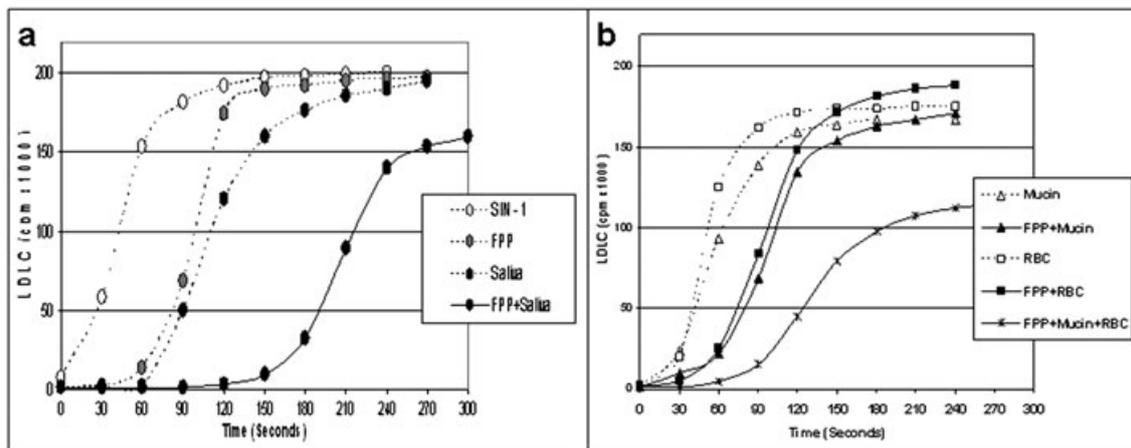
results (Fig. 3a and b) show that while FPP, saliva, mucin and RBC, each one alone, had only a mild effect, their combinations produced a significant ( $p < 0.001$ ) quenching effect of the LDLC.

Polyphenols are major components of the antioxidant capacity of plant products. Using the Folin–Ciocalteu's assay, a very low amount of measurable phenol groups was found in FPP suspended in a salt solution (Fig. 4). However, when the FPP was suspended in a salt solution containing saliva (15  $\mu\text{L}/\text{mL}$ ), albumin (100  $\mu\text{g}$ ), mucin (10  $\mu\text{g}$ ) or RBC ( $4 \times 10^6$ ), significant ( $p < 0.001$ ) increases were noted in its polyphenol content. Addition of both albumin and RBC (or mucin and RBC, not shown) to FPP produced a maximal effect (6  $\times$  fold increase compared with the sum of the components assayed separately). These results suggest that constituents of saliva, such as mucin and albumin, and RBC contribute to the solubility and the availability of antioxidant polyphenols of FPP and thereby increase their activity.

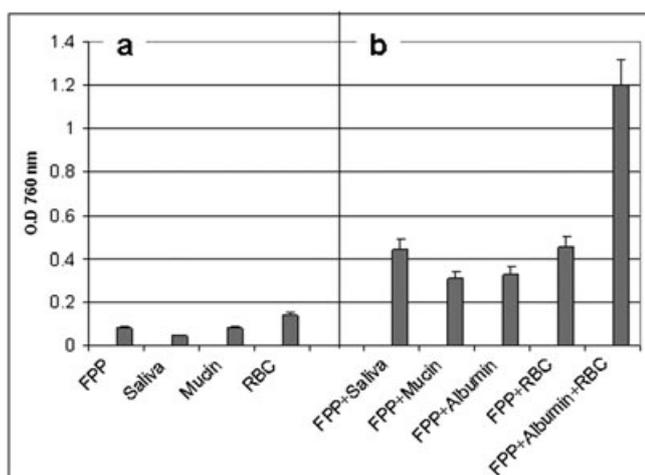
We have previously shown that polyphenols in the blood stream tend to bind to RBC surfaces (Koren *et al.*, 2010). When washed RBC incubated with FPP were pelleted and washed, most of their antioxidant activity ( $82 \pm 7\%$ ,  $N=4$ ) and polyphenol content ( $76 \pm 5\%$ ,  $N=4$ ) were found in the pellet. These results suggest that phenol groups became irreversibly bound to RBC surfaces, thereby augmenting their antioxidant activity.



**Figure 2.** Antioxidant activity of fermented papaya preparation (FPP) alone or in the presence of mucin and albumin (a–b) as well as red blood cells (RBC) and *C. albicans* (c–d). FPP (10 mg), mucin (10  $\mu\text{g}$ ), albumin (100  $\mu\text{g}$ ), RBC ( $4 \times 10^6$ ) and *Candida* ( $4 \times 10^8$ ) cells, each one alone or in combinations, were tested for their antioxidant activity by the  $\text{H}_2\text{O}_2$ –luminol-dependent chemiluminescence (LDCL) assay as described in Materials and Methods. The results are presented as counts per minute of LDCL measured after 90 s incubation (means  $\pm$  standard deviation of four experiments). Significant higher antioxidant activities (indicated by decreases in LDLC compared with the 'cocktail' alone) were observed when FPP was mixed with saliva, mucin, albumin (b), RBC or *Candida* (d) compared with FPP alone (a and c) ( $p < 0.01$ ).



**Figure 3.** Antioxidant activity of fermented papaya preparation (FPP) in the presence of saliva (a), mucin and red blood cells (RBC) (b). FPP (10 mg), saliva (15  $\mu$ L), mucin (10  $\mu$ g) and RBC ( $4 \times 10^6$ ), each one alone or in combinations, were tested for their antioxidant activity by the SIM-1–luminol-dependent chemiluminescence (LDCL) assay as described in Materials and Methods. LDCL was measured every 30 s. The results of one out of four experiments, presented as counts per minute of the LDCL, are shown. Statistical analysis of the results at 90 s of four experiments indicated significant higher antioxidant activities (indicated by decreases in LDLC) when FPP was mixed with saliva ( $p < 0.001$ ), mucin or RBC ( $p < 0.05$ ) or both mucin and RBC ( $p < 0.01$ ), compared with FPP alone.



**Figure 4.** The phenol content of fermented papaya preparation (FPP) in the presence of saliva, mucin, albumin and red blood cells (RBC). The polyphenol content of FPP (10 mg), saliva (10  $\mu$ L), mucin (10  $\mu$ g), albumin (100  $\mu$ g) and RBC ( $4 \times 10^6$ ), separately (a) and in combinations (b), were measured by the Folin–Ciocalteu's assay. The results, presented as optical density at 760 nm (means  $\pm$  standard deviation of four experiments), show a significant ( $p < 0.001$ ) increase (at least  $3 \times$  fold) in measurable polyphenols in FPP following addition of saliva, mucin, albumin or RBC, and a six-fold increase after addition of both albumin and RBC.

## DISCUSSION

In the present study, we studied FPP, a product of yeast fermentation of *Carica papaya* Linn, for its antioxidant potential under conditions that might prevail in the oral cavity. Employing two highly sensitive LDCL assays, we showed that the ability of FPP to scavenge ROS was markedly augmented by saliva and its components, mucin and albumin, as well as by RBC and microorganisms, the numbers of which can increase significantly under inflammatory conditions. The results suggested that this enhancing effect was due to the ability of saliva, RBC or microorganisms to increase the solubility and availability of polyphenols in FPP as antioxidants.

We have previously suggested that the antioxidant status in the oral cavity might be affected by interactions among antioxidants of the saliva, the oral microbial flora, blood elements extravasated from injured capillaries and polyphenols from nutrients (Ginsburg *et al.*, 2013). The antioxidants may counteract toxic oxidants that include peroxides, thiocyanous acid (Grisham and Ryan, 1990) and oxidants generated by activated neutrophils (Battino *et al.*, 1999) and bacteria (Okahashi *et al.*, 2014), which accumulate in infectious sites in the oral cavity. The main antioxidants of saliva are low molecular-weight agents, such as uric acid, ascorbate,  $\alpha$ -tocopherol and reduced glutathione (Sculley and Langley-Evans, 2002). In addition, saliva and its components, such as mucin and albumin, also markedly enhanced the OSA of diet-derived polyphenols and of complexes formed among RBC, platelets, bacteria and polyphenols (Ginsburg *et al.*, 2013).

We have previously demonstrated that nutritional polyphenols fail to demonstrate OSA in aqueous solutions because of poor solubility. The binding of polyphenols to proteins and cell surfaces increases their accessibility and their activity as antioxidants (Koren *et al.*, 2010). Therefore, the OSA of antioxidants should be always measured in their natural environment – in the oral cavity in the presence of saliva, RBC and microorganisms. Likewise, measuring OSA in the plasma does not reveal the true OSA in the whole blood (Ginsburg *et al.*, 2011).

The results presented here suggest that antioxidants, such as polyphenols present in FPP, may reveal their full OSA potential in the oral cavity only when assayed under conditions that prevail under normal and pathological states. In the presence of saliva, RBC and microorganisms, FPP demonstrated a potent OSA activity, which suggests that it may reduce the oxidative status of the oral cavity and thereby contribute to its health. It should be noted, however, that the effect of polyphenols in the oral cavity may be a double-edged sword: By scavenging ROS, they may reduce the oxidative damage, but on the other hand, may reduce the bacteriolytic effect of oxidants, such as  $H_2O_2$  generated by catalase-negative cariogenic streptococci, especially when grown on D-glucose (Okahashi *et al.*, 2014). Current work in our laboratory focuses on interactions of

polyphenols with planktonic bacteria and semi-planktonic flocks of the oral biofilm.

Interestingly, Marotta *et al.* have reported on a pilot study that tested the effect of FPP on some parameters relevant to common seasonal respiratory tract infections (Marotta *et al.*, 2012). The results showed that a one-month treatment with FPP increased the rate of salivary secretion, its IgA and lysozyme contents as well as all phase II enzymes and SOD gene expression tested in nasal lavage cells.

Another group investigated the protective effects and the mechanism of action of FPP on stress-induced acute gastric mucosal lesion in rats. A six-hour exposure to stress resulted in splinter hemorrhages and mucosal lesions in the stomach, which was accompanied by an increase in lipid peroxidation and a decrease in SOD-like activity in the plasma and the gastric mucosa as well as increased myeloperoxidase activity and Nuclear factor-kappa B activation in the gastric mucosa. Oral administration of FPP to rats reduced all the aforementioned changes, suggesting that FPP might provide protection against stress-induced acute gastric mucosal lesions through its antioxidative and antiinflammatory properties (Murakami *et al.*, 2012).

It was also shown that the contents of some saccharides increased significantly when FPP was mixed with saliva (Nishida, 2010). Specifically, the maltooligosaccharides, maltose and maltotriose, which are considered to improve enteral environment immunity, increased significantly. The author suggested that these changes in sugars might be related to the protective effect of FPP in the oral cavity.

We have also shown that part of the antioxidative effect of FPP is related to its ability to bind and remove

(chelate) free iron from cells and their environment (Prus and Fibach, 2012). Free, non-bound (labile) iron is redox-active and participates in biochemical reactions (the Fenton reaction) leading to the generation of ROS. Indeed, the antioxidant ability of polyphenols, such as those found in nutrients, has been attributed to both radical-scavenging (Hanasaki *et al.*, 1994) and iron-chelating mechanisms (Melidou *et al.*, 2005). Two pathways have been suggested for the protective effect of polyphenols against iron-mediated damage: They bind to Fe<sup>2+</sup>, preventing it from reacting with H<sub>2</sub>O<sub>2</sub> in the Fenton reaction, and oxidation of the Fe<sup>2+</sup>-polyphenol complex that occurs in the presence of ·O<sup>2-</sup> to generate a Fe<sup>3+</sup> complex (Perron *et al.*, 2010) that cannot participate in the Fenton reaction. FPP has been shown to protect against damage to DNA (induction of single and double strand breaks) and proteins (albumin) caused by combined treatment with ferric nitrilotriacetate and H<sub>2</sub>O<sub>2</sub>, suggesting a mechanism involving hydroxyl scavenging (Rimbach *et al.*, 2000).

The findings of this and previous reports (Ginsburg *et al.*, 2011; Koren *et al.*, 2010) that polyphenols act in concert with other components present in the normal/pathological environment, suggest that the potential beneficial influence of polyphenols in FPP should be tested in preclinical studies under conditions that prevail *in vivo* and that controlled clinical trials are warranted.

### Conflict of Interest

The authors have declared that there is no conflict of interest.

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