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Flavonoid Glucosides Are Hydrolyzed and Thus Activated in the Oral Cavity in Humans²

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Abstract

Increasing epidemiological evidence supports the view that dietary flavonoids have protective roles in oral diseases, including cancer. However, the dietary forms of flavonoids, the flavonoid glycosides, must first be hydrolyzed to the aglycones, which is thought to occur mainly in the intestine. In the present study we tested whether this hydrolytic activity occurs in the oral cavity. Saliva was collected from human subjects, incubated with flavonoid glycosides, and analyzed for aglycone formation by HPLC. When quercetin 4'-glucoside or genistein 7-glucoside was incubated with human saliva, hydrolysis to quercetin and genistein, respectively, was detected within minutes. Studies of additional flavonoid glycosides demonstrated that glucose conjugates were rapidly hydrolyzed, but not conjugates with other sugars, i.e., rutin, quercitrin, and naringin. In a limited study of 17 subjects, the interindividual variability in the hydrolysis of genistein 7-glucoside was >20-fold. This supports the contention that salivary hydrolysis of certain flavonoid glycosides may be important in some individuals but not in others. Support for a bacterial contribution to this hydrolysis was obtained from the inhibitory effect of antibacterials *in vivo* and *in vitro* and from experiments with subcultured oral bacterial colonies. However, cytosol isolated from oral epithelial cells was also capable of effective hydrolysis. Dietary flavonoid glycosides may thus be hydrolyzed in the oral cavity by both bacteria and shedded epithelial cells to deliver the biologically active aglycones at the surface of the epithelial cells. The aglycones quercetin and genistein both potently inhibited proliferation of oral cancer cells. The large interindividual variability in this hydrolytic activity may be a factor that should be taken into consideration in future studies.

Keywords

flavonoid glycosides; salivary hydrolysis; β -glucosidase; oral cancer

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Epidemiological studies have clearly demonstrated a protective role of fruits and vegetables in oral cancers, presumably mediated by their content of polyphenols, particularly flavonoids (1–3). Numerous mechanisms for these effects have been suggested, mainly based on in vitro and cellular studies (4–6). The dietary sources of flavonoids, except for the tea flavonoids (7), are flavonoid glycosides, which in most cases first must undergo hydrolysis to their aglycones to be able to produce effects. Still, serious questions remain regarding how these dietary components gain access to proposed cellular sites of action in the human body. For the tea flavonoids, which are gallic acid esters rather than glycosides, the access to oral epithelial cells may be less complex, as very recently noted (7).

In the past, it was strongly believed that flavonoid glycosides could not be absorbed per se but only after hydrolysis by the bacterial flora in the lower part of the intestine (8,9). In 1995 researchers proposed that flavonoid glycosides can be absorbed intact, presumably via the sodium-dependent glucose transporter SGLT1 (10). Although this was later confirmed (11), it was also shown that many glycosides are not absorbed due to efficient efflux transport by multidrug resistance-associated protein-2 (12). Other studies suggested that hydrolysis of flavonoid glycosides can occur in the small intestine (13), maybe by the broad-specific enterocyte β -glucosidase (14) and/or the lactase phloridzin hydrolase (15). Once this hydrolysis occurs, the aglycones formed are efficiently absorbed, although the bioavailability may be extremely low due to extensive presystemic metabolism (16). Thus, the potential protective effects of dietary flavonoids against cancers of the oral cavity are not understood.

Saliva has been suggested to be able to hydrolyze flavonoid glycosides (17–19), but it has never been considered an important factor. In the present study we attempted to establish the importance of salivary hydrolysis, using as substrates 4 different glycosides of quercetin, i.e., quercitrin (the 3-rhamnoside), rutin (the 3-rhamnoglucoside), isoquercitrin (the 3-glucoside), and spiraeoside (the 4'-glucoside), which previously have been shown to be hydrolyzed by β -glucosidases of human (14,15) and/or bacterial origin (9). We also tested genistin (genistein 7-glucoside), naringin (naringenin 7-rhamnoglucoside), and phloridzin (phloretin 2'-glucoside) as substrates, all commonly present in the human diet.

MATERIALS AND METHODS

Materials.

Genistin, genistein, naringin, phloridzin, quercitrin (quercetin 3-rhamnoside), rutin (quercetin 3-rhamnoglucoside), quercetin, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT)⁴ were obtained from Sigma; spiraeoside (quercetin 4'-glucoside) and isoquercitrin (quercetin 3-glucoside) were purchased from Indofine Chemicals. Colgate manufactured Chlorhexidine Gluconate Oral Rinse (PerioGard), and Listerine[®] was distributed by Warner-Lambert Consumer Health Care. Dimethyl sulfoxide (DMSO), glacial acetic acid, and methanol were purchased from Fisher Scientific; trifluoroacetic acid was obtained from Aldrich Chemical Company. Fetal bovine serum was produced by Atlanta Biologicals, and other cell culture medium components were obtained from Cellgro Mediatech, Fisher Scientific.

Collection of human saliva.

The study was approved by the Institutional Review Board for Human Research. The limited population study was conducted using 17 volunteers recruited from 1 high school science class (ages 15–17 y). Other saliva samples were from adult subjects (23–64 y). Unstimulated saliva (2 mL) was collected in the morning with the subject abstaining from toothbrushing since the previous evening. In some adult subjects saliva was collected before and after brushing (no toothpaste) or before and after rinsing with an antibacterial mouthrinse (chlorhexidine or Listerine) for 30 s. In the latter experiments, saliva was collected at 6 min and 1, 2, 6, and 24 h after the mouthrinse.

Flavonoid hydrolysis by saliva.

The saliva (2 mL) was diluted 1:1 with distilled water and shaken vigorously to reduce viscosity. In some experiments, diluted saliva was centrifuged at $10,000 \times g$ and filtered with 1- and $0.2\text{-}\mu\text{m}$ filters to remove oral cells and bacteria, respectively. Other saliva samples (1 mL) were incubated on a shaking water bath at 37°C for 24 h with an equal volume of DMEM (containing 10% fetal bovine serum) with or without 200,000 U/L penicillin and 0.2 g/L streptomycin prior to incubation with flavonoid glycoside.

Flavonoid glycosides dissolved in DMSO (final concentration $< 0.1\%$) were added to two 1-mL aliquots of the saliva mixture to a final flavonoid concentration of 25 or $50\ \mu\text{mol/L}$. After vigorous shaking and vortexing, argon was added to the samples before incubation for specified times at 37°C . The pH of the saliva samples was 6.7 ± 0.2 (mean \pm SEM) before and 6.4 ± 0.1 after incubation. An equal volume of methanol was added to the samples following incubation. The samples were centrifuged at $16,000 \times g$ for 2 min and the supernatant was analyzed by HPLC with flavonoid-specific UV detection.

Flavonoid hydrolysis by cultured oral bacteria.

A cotton swab was held in the mouth of 1 volunteer whose saliva had been shown previously to have a high level of flavonoid hydrolysis. The swab was streaked on blood agar and chocolate agar plates and incubated overnight. Eight different-looking common bacterial colonies were subcultured twice to ensure a homogeneous population. One plate for each colony was used for heavy inoculation of fresh plates. Twenty-four hours later, the bacterial colonies were removed with a cotton swab to tubes of sterile broth, vortexed, and rocked at room temperature for 1 h. Aliquots (0.5 mL) of the bacterial suspensions as well as broth (negative control) were incubated with $50\ \mu\text{mol/L}$ genistin for 1 h at 37°C on a shaking water bath. The samples were analyzed as above. The experiment was done twice with separate inoculates.

Flavonoid hydrolysis by SCC-9 oral squamous carcinoma cell cytosol.

Oral squamous carcinoma SCC-9 cells obtained from the American Type Culture Collection were cultured in F-12/DMEM containing glutamine and HEPES, fetal calf serum (10%), 100,000 U/L penicillin, 0.1 g/L streptomycin, and hydrocortisone (0.2 g/L). SCC-9 cytosol was prepared by harvesting the cells at confluency. The cell pellet was resuspended in buffer containing protease inhibitors and was then sonified and centrifuged at $100,000 \times g$ for 15 min at 7°C . The cytosol (supernatant) was stored at -80°C . Aliquots of cytosol

were mixed with 0.1 mol/L acetate buffer (pH 6) to mimic the pH of the oral environment. Quercetin 4'-glucoside was added to a final concentration of 25 $\mu\text{mol/L}$, and the samples were incubated at 37°C for 1 h. After the addition of an equal volume of methanol and centrifugation, the samples were subjected to HPLC analysis as described below.

HPLC.

All flavonoid glycosides and their respective aglycones were detected by reverse phase HPLC of 200- μL samples on a Millennium HPLC system with a Symmetry C18 column (3.9 \times 150 mm) and a Model 996 photodiode array detector, using slight modifications of previous studies (20,21). The flow rate was 0.9 mL/min. A mobile phase consisting of 35% methanol and 5% acetic acid with UV detection at 370 nm was used for the quercetin glycosides and at 260 nm for genistin and naringin. Phloridzin hydrolysis was analyzed using a mobile phase of 45% methanol and 0.3% trifluoroacetic acid with detection at 283 nm. Quantitation was done by peak area measurements in comparison with standard curves for each of the flavonoid glycosides and aglycones. The detection limits were adequate for all experiments. The recoveries were estimated to exceed 90% for all compounds tested, as previously noted (20,21).

Cell proliferation assay.

SCC-9 cells were seeded in 96 wells at a density of 5000 cells/well and cultured as described above. On d 2–4 after plating, fresh medium including 0.1–200 $\mu\text{mol/L}$ flavonoid or DMSO (0.25%, v:v) was added. On d 5 the medium was aspirated and the cells were incubated with MTT in buffer (0.5 g/L) for 3 h before the addition of 0.1 mol/L HCl and 10% Triton X-100 in 2-propanol (Sigma protocol) to lyse the cells and dissolve the formazan crystals. The absorbance was read with a plate reader at 570 nm with 690-nm background subtraction. This assay measures the conversion of MTT to blue formazan by mitochondrial dehydrogenases in living cells.

Statistics.

Data are means \pm SEM. The statistical significance of differences between 2 treatments was evaluated using a 2-tailed unpaired Student *t* test with a significant level of $P < 0.05$. In experiments with multiple treatments, ANOVA with Dunnett's posttest (InStat) or 2-way ANOVA with Bonferroni's posttest (Prism) was used.

RESULTS

The hydrolysis of the flavonoid glycosides to their respective aglycones by saliva was examined using HPLC separation with flavonoid-specific UV detection. This was shown for quercetin 4'-glucoside (spiraeoside), one of the most important flavonoid glycosides in the human diet, present in large amounts in onions, for example (22). Both the glucoside and its aglycone quercetin had excellent chromatographic properties, and there was little interference from other salivary components at the 370-nm wavelength used. The hydrolytic reaction was linear with time for at least 3 h. There was no evidence of other products formed under these conditions. The reactions were carried out under argon to avoid

degradation of any of the aglycones formed. For a given saliva sample, the reproducibility of the reaction was acceptable with a CV of about 15%.

The hydrolysis of all 7 of the flavonoid glycosides studied was examined using saliva from 1 subject in replicate analyses (Table 1). For estimation of the rates of hydrolysis, the percentage conversion to the aglycones, taking into account the molar extinction coefficients, was determined after 2-h incubations at 37°C. Four of the glycosides were hydrolyzed efficiently, spiraeoside, phloridzin, genistin, and isoquercitrin, with spiraeoside being by far the best substrate. On the other hand, rutin was hydrolyzed very little, and quercitrin and naringin were not hydrolyzed at all. The same pattern was seen with saliva from 3 other subjects, although the interindividual variability in the hydrolysis rate was large (see below).

We next examined the hydrolysis of one flavonoid glucoside in 17 healthy student volunteers. For this purpose we used genistin, i.e., the 7-glucoside of genistein, because of its superior stability under the trial conditions used. The purpose of this trial was to determine the interindividual variability in hydrolysis rate. Variability was extremely large (Fig. 1). One subject (No. 7) showed no hydrolysis at all [minimum detectable amount 1 $\mu\text{mol}/(\text{L saliva h})$] and 4 subjects (Nos. 1, 3, 5, and 8) had barely detectable levels of hydrolysis. On the other hand, 3 subjects (Nos. 12, 14, and 17) had very high hydrolysis rates, consuming virtually all of the genistin substrate. The only known phenotypes among the subjects that were examined, i.e., age, race, sex, and regular use of mouthwash, were not correlated with hydrolysis rate in this limited number of subjects.

To examine the effect of antibacterial mouthwashes, we performed time course studies in several adults after they rinsed with chlorhexidine and Listerine (Fig. 2). Chlorhexidine efficiently inhibited the hydrolysis of genistin and this inhibition persisted for at least 6 h. Listerine had a modest effect, persisting for only 2 h. These effects were consistent with the antibacterial potencies of these agents (23). The findings with the antibacterial agents strongly indicated that the hydrolysis was due to the oral bacterial flora. Centrifugation ($10,000 \times g$) and filtration (1- and 0.2- μm filters) of the saliva also removed >90% of the enzymatic activity, indicating that the activity was related to a cellular source rather than to soluble enzymes. To further determine the importance of the bacterial flora, we compared the hydrolysis of genistin to genistein by raw saliva and raw saliva preincubated with cell culture medium (without antibiotics) for 24 h. There was a dramatic increase consistent with the anticipated bacterial growth in cultured saliva (Fig. 3). In the presence of penicillin and streptomycin, this increase was abolished (Fig. 3). To further confirm the importance of bacteria in the hydrolysis of flavonoid glycosides, blood agar and chocolate agar plates were inoculated with saliva from 1 volunteer who had previously been shown to have high salivary hydrolytic activity. Eight different oral bacterial colonies subcultured to homogeneity were tested for their ability to hydrolyze genistin. Two of the cultures showed a high level of activity, about 25% hydrolysis after a 1-h incubation with 25 $\mu\text{mol}/\text{L}$ genistin, whereas the others were totally inactive. The experiment was repeated with the same results. The 2 active strains were classified as gram-positive cocci in tetrads and gram-negative diplococci, both common nonpathogenic oral bacteria.

These observations, although highly supportive of a bacterial source for the β -glucosidase activity, did not rule out a contribution from shedded oral epithelial cells. When saliva samples were examined by microscopy, they all contained variable numbers of scaly oral epithelial cells, about half of which appeared to be intact. To examine the effect of these cells on hydrolysis, 3 subjects brushed their cheeks, gums, and tongue with a toothbrush before providing saliva samples, an established procedure to collect loosely attached epithelial cells. Microscopy confirmed a substantial increase in cell numbers. The quercetin 4'-glucoside to quercetin hydrolysis in these samples increased substantially compared to samples obtained before brushing ($P < 0.05$; $n = 3$), consistent with a contribution by shed epithelial cells. The aglycone/glucoside ratios after a 30-min incubation were 0.12 ± 0.03 before brushing vs. 0.55 ± 0.17 after brushing and after a 60-min incubation were 0.33 ± 0.08 vs. 4.42 ± 0.34 . To further test the hydrolytic activity of oral epithelial cells without the confounding presence of oral bacteria we used cytosols prepared from oral squamous carcinoma SCC-9 cells (24). Hydrolysis of quercetin 4'-glucoside to quercetin by this cytosol was efficient (Fig. 4). The reaction was clearly saturable over the 5–100 $\mu\text{mol/L}$ quercetin 4'-glucoside concentration range. Nonlinear regression analysis of the data yielded an apparent K_m of 34 $\mu\text{mol/L}$ and a V_{max} of 64 $\text{nmol}/(\text{h mg protein})$.

To obtain a quantitative estimate of the effectiveness of the oral hydrolysis of flavonoid glycosides irrespective of mechanisms, we conducted experiments in situ. Thus, 15 mL of a 10 $\mu\text{mol/L}$ solution of quercetin-4'-glucoside was held in the mouth of 1 volunteer for 5 min, and glucoside and aglycone quercetin content were assayed by HPLC. In 3 experiments, a consistent 57–63% of the glucoside disappeared during this short time period, taking into account volume changes. Also, 17–24% of the glucoside was hydrolyzed to quercetin.

To determine the potential biological importance of the collective findings, we examined the antiproliferative effects of quercetin and genistein, the 2 flavonoids demonstrating efficient salivary formation from their precursor glucosides, on the oral squamous carcinoma SCC-9 cells using the MTT assay and a wide range of flavonoid concentrations (0.1–200 $\mu\text{mol/L}$) (Fig. 5). Both flavonoids produced potent inhibition with a minimum effective concentration (MEC) of 5 and 10 $\mu\text{mol/L}$ for quercetin and genistein, respectively. Thus, the aglycones formed in the oral cavity may have anticancer and antibacterial (3) effects or more generally scavenge hydrogen peroxide and other reactive oxygen species (19).

DISCUSSION

Although several previous studies have demonstrated that saliva can hydrolyze certain flavonoid glycosides (17–19), those studies were limited in scope. From the present study it appears that this hydrolysis is limited to glucose conjugates, because other glycosides, such as the rhamnosides (quercitrin) and rhamnoglucosides (rutin and naringin), either were hydrolyzed very slowly or were resistant to salivary hydrolysis. Interestingly, these latter types of glycosides are easily hydrolyzed by the human fecal flora (9). Quercetin 4'-glucoside in particular, but also genistin, i.e., genistein 7-glucoside, can be rapidly hydrolyzed by saliva. These are 2 of the more abundant flavonoids in the human diet (22,25). Thus, the formation of quercetin and genistein from their dietary sources may be

rapid enough in certain individuals to be important also in vivo. Holding a solution of quercetin 4'-glucoside in the mouth for 5 min resulted in a 60% loss of glucoside. About 20% of that loss could be accounted for as quercetin in the saliva, with the remaining 40% being absorbed by the oral epithelial cells, presumably as quercetin.

Still, whether this oral hydrolysis will be of importance for local effects on the oral epithelium, considering the relatively short residence time of most foods in the oral cavity, is difficult to assess. Our study of 17 high school students demonstrated a remarkable interindividual variability in hydrolysis rate. These observations suggest that oral hydrolysis may be quantitatively important in some individuals but not in others. Such variability may have a genetic origin, but could also involve environmental factors.

Hydrolysis of flavonoid glucosides by β -glucosidases in the oral cavity is also likely to be influenced by the food matrix in which they are contained. Thus, if contained in a liquid form, they will obviously have greater access to the enzymes than if contained in a solid form requiring extensive chewing. Even so, it should be clear that the hydrolytic activity will start in the oral cavity and continue through the passage of the food throughout the aerodigestive tract.

The mechanism of hydrolysis was presumably through β -glucosidases, but the source of these enzymes was difficult to pinpoint. Bacterial flora in the oral cavity play a role. This can be deduced from the effectiveness of the antibacterial agents chlorhexidine and Listerine in inhibiting the salivary hydrolysis and is also strongly supported by the experiments using cell culture techniques, either for the whole saliva or for subcultured oral bacterial colonies. However, cytosols from cultured oral epithelial cells also displayed a high rate of hydrolysis and the saliva from all subjects, in particular after brushing the cheeks, gums, and tongue, contained high numbers of epithelial cells. It is interesting to note that the cytosolic hydrolysis of quercetin 4'-glucoside to quercetin proceeded with an apparent K_m of 34 $\mu\text{mol/L}$ and a V_{max} of 64 nmol/(h mg protein), very similar to the hydrolysis by cytosol from human liver and small intestine (14), implying the involvement of the same β -glucosidase enzyme. Clearly, more studies will be needed to better determine the contribution of bacterial and human epithelial β -glucosidases.

Finally, it was of great interest to note that both quercetin and genistein were able to inhibit proliferation of the oral squamous carcinoma SCC-9 cells. This occurred with an MEC of 5–10 $\mu\text{mol/L}$ for these flavonoids, which should be achievable in the oral cavity after consumption of a diet containing these flavonoids. The mechanisms of these effects are likely different. Quercetin inhibits PI3-kinase (26), whereas genistein inhibits tyrosine kinases (26). A mixture of flavonoids with different mechanistic properties may be an advantage in cancer prevention.

In conclusion, flavonoid glucosides were hydrolyzed to their aglycones in the oral cavity. The β -glucosidase enzymes responsible were derived both from bacteria and shedded oral epithelial cells. Two of the flavonoid aglycones studied, quercetin and genistein, showed potent inhibition of oral cancer cell proliferation. The ability of some individuals but not

others to hydrolyze these protective dietary flavonoids in the oral cavity should be an important consideration in future studies.

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Abbreviations used:

DMSO	dimethyl sulfoxide
MEC	minimum effective concentration
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

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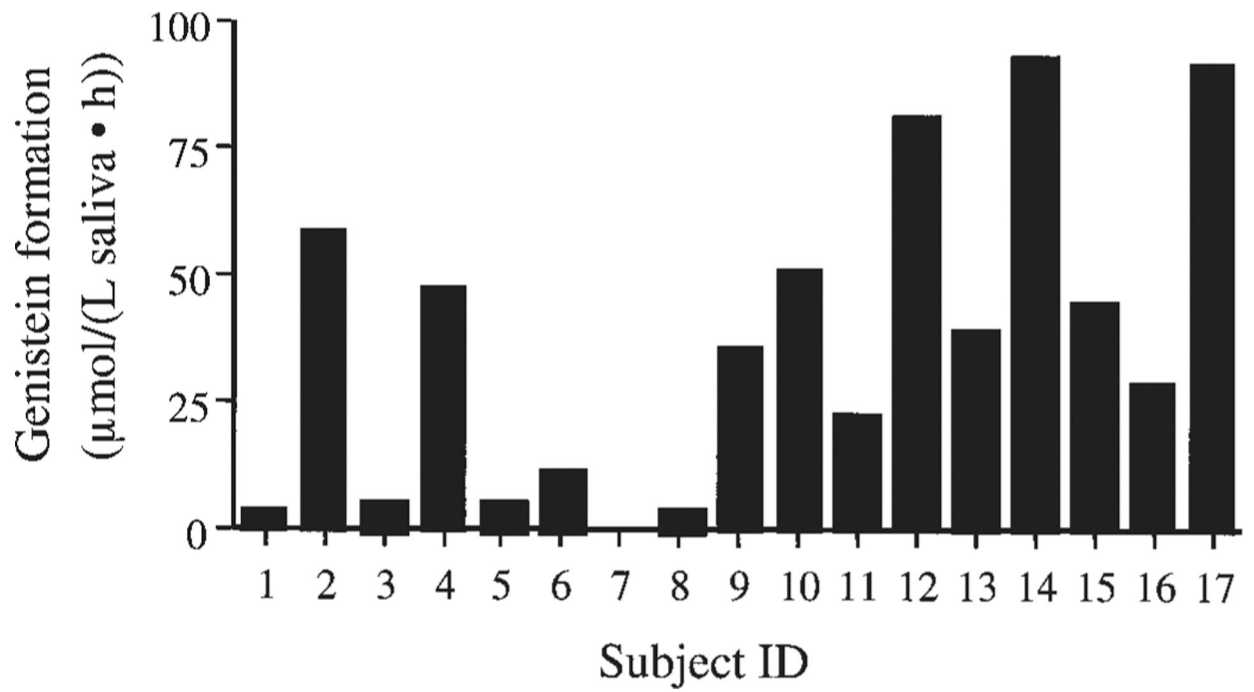


FIGURE 1. Genistin hydrolysis to genistein in the saliva from a group of young, healthy human subjects. Saliva was incubated for 1 h with 25 µmol/L genistin.

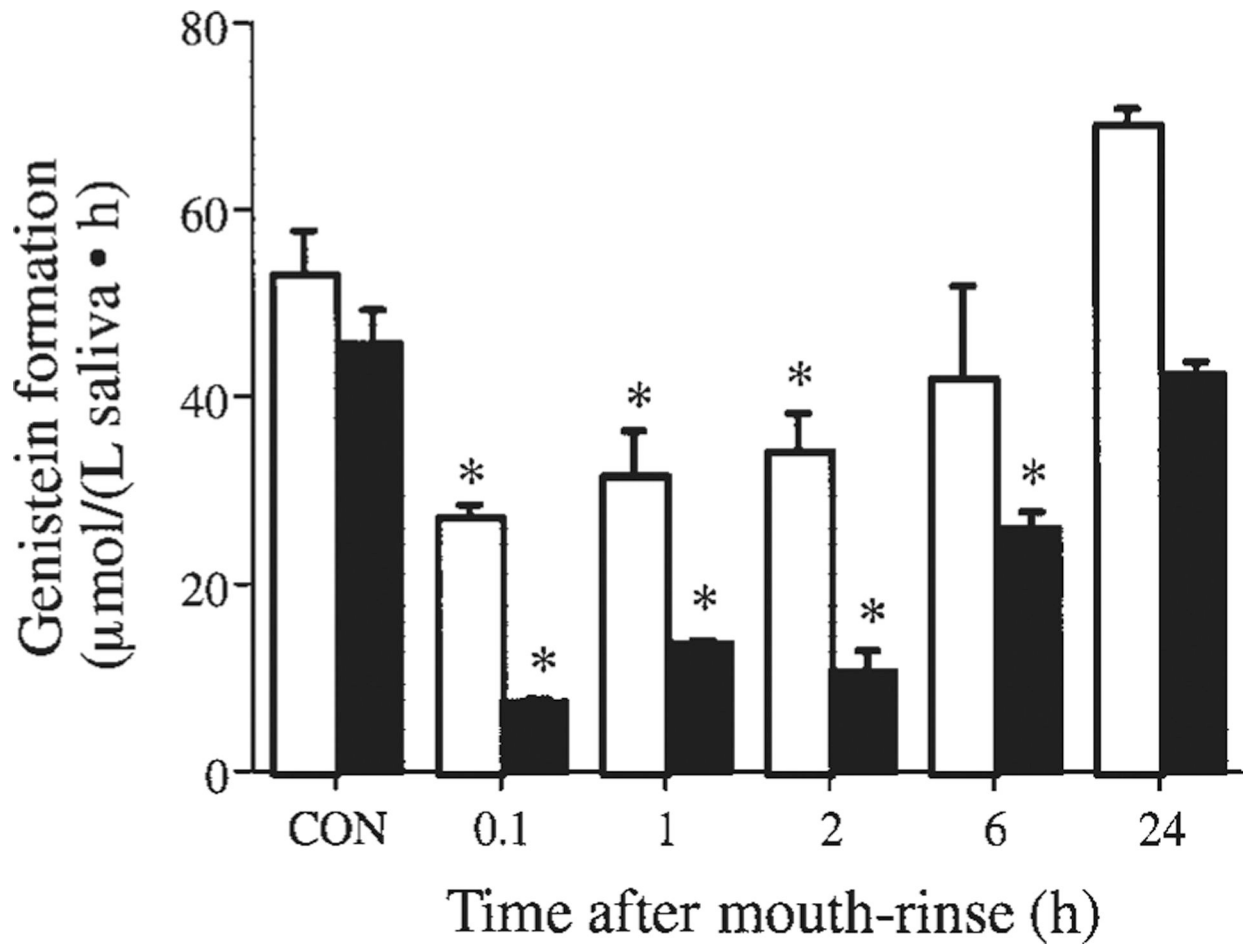


FIGURE 2.

Inhibitory effect of chlorhexidine (■) and Listerine (□) on the salivary hydrolysis of genistin to genistein after a 30-s mouthrinse followed by a brief rinse with tap water. Human saliva samples were collected before (CON) and at various times after the mouthrinses and incubated with $25 \mu\text{mol}/\text{L}$ genistin for 1 h. Values are means \pm SEM, $n = 3-6$. *Different from CON, $P < 0.05$.

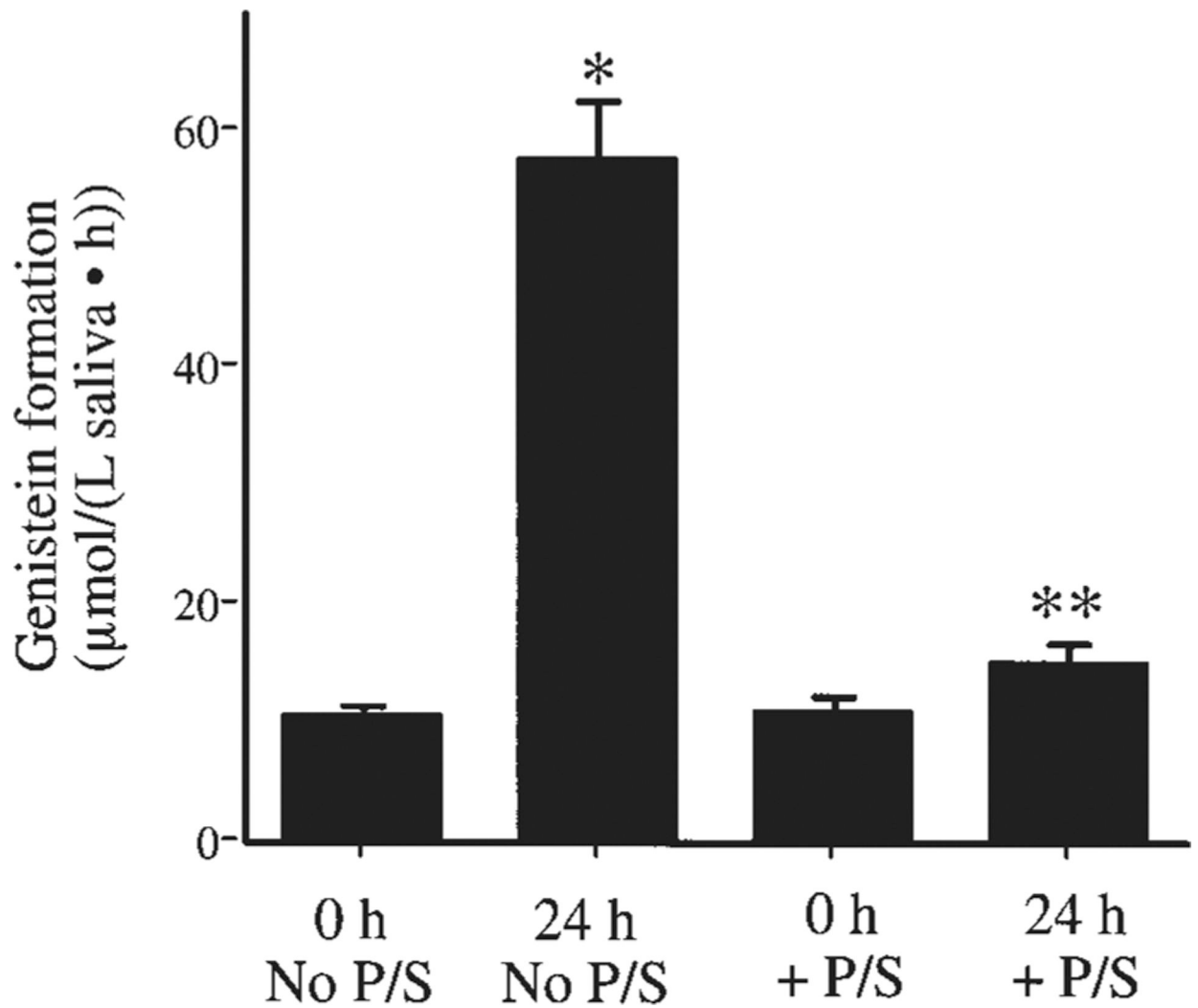


FIGURE 3.

Effect of preincubating saliva from 1 human volunteer with cell culture medium for 0 or 24 h in the absence and presence of penicillin and streptomycin (P/S) on the hydrolysis rate of 50 $\mu\text{mol}/\text{L}$ genistin (1-h incubation). Values are means \pm SEM, $n = 6$. *Different from 0 h, no P/S, $P < 0.001$; **Different from 24 h, no P/S, $P < 0.001$.

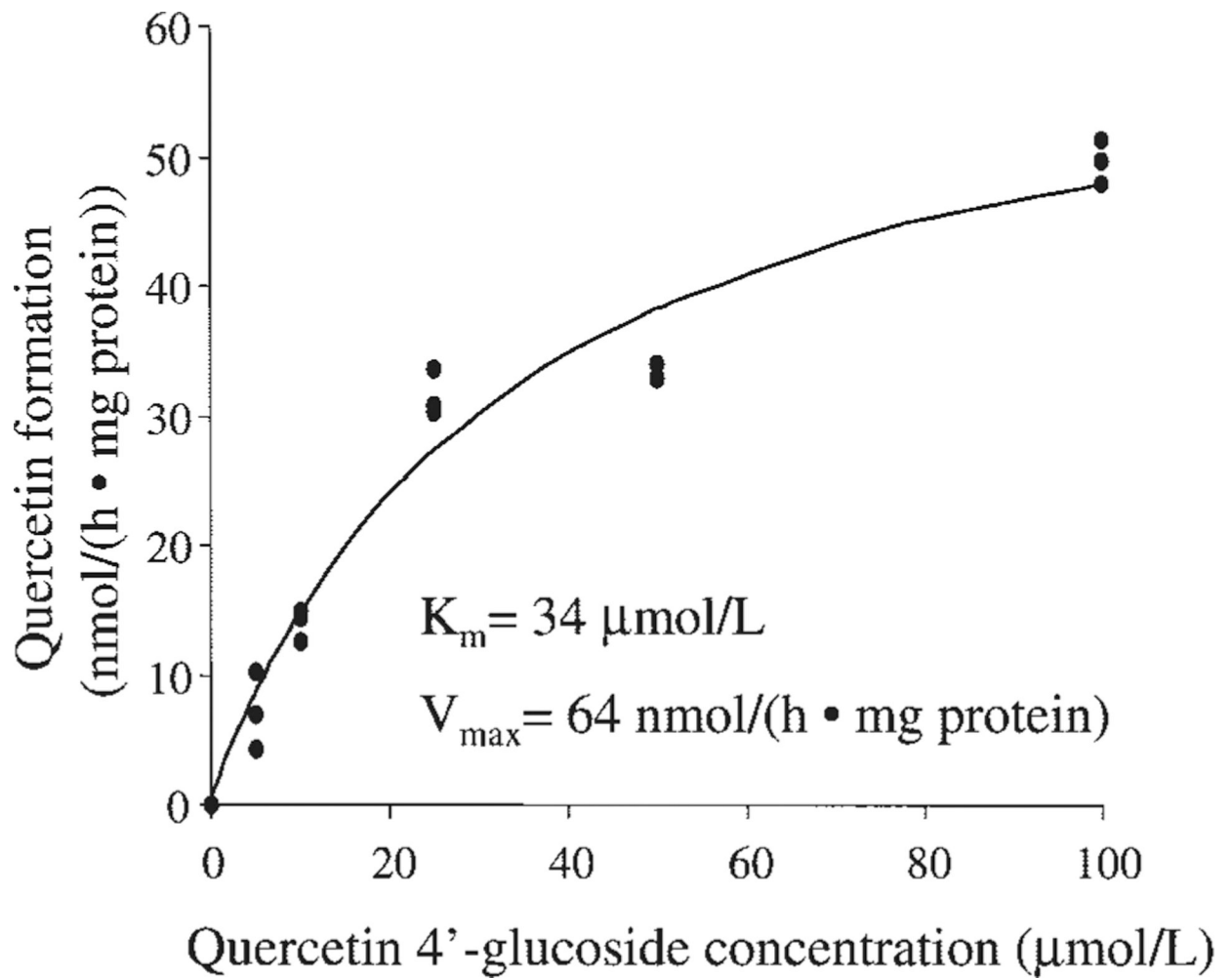


FIGURE 4. Michaelis-Menten kinetics of the hydrolysis of quercetin 4'-glucoside to quercetin by cytosol from human oral epithelial SCC-9 cells. The incubation time was 1 h.

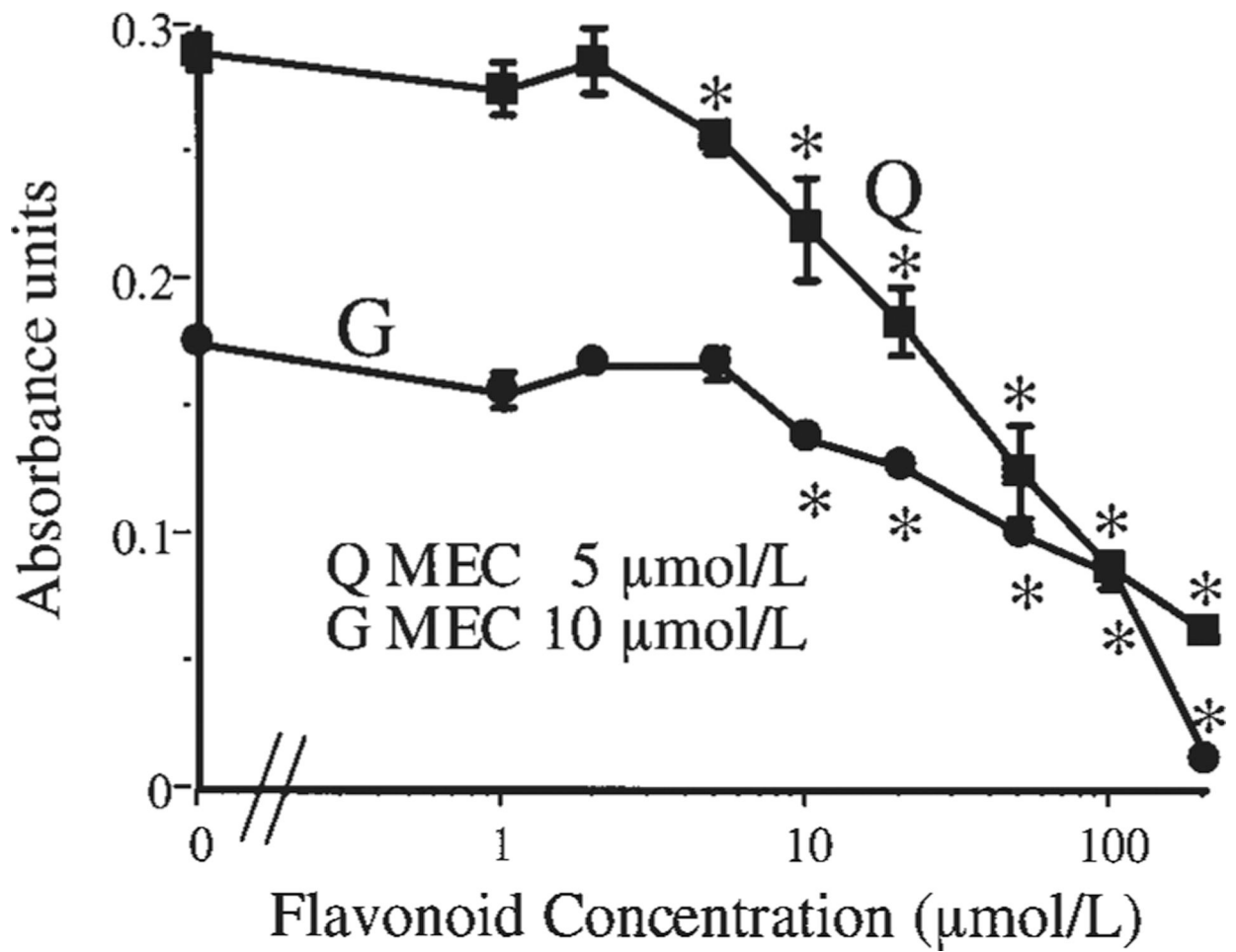


FIGURE 5.

Antiproliferative effects of quercetin (Q) and genistein (G) in human oral squamous carcinoma SCC-9 cells. MEC, minimum effective concentration. Values are means \pm SEM, $n = 12-18$. *Different from the flavonoid-free ($0 \mu\text{mol/L}$) DMSO control; $P < 0.05$.

TABLE 1Hydrolysis of flavonoid glycosides by human saliva^{1,2}

Flavonoid glycoside	Aglycone formation % of added glycoside
Spiraeoside (quercetin 4'-glucoside)	85.6 ± 1.3
Phloridzin (phloretin 2'-glucoside)	67.8 ± 2.9
Genistin (genistein 7-glucoside)	44.3 ± 4.3
Isoquercetin (quercetin 3-glucoside)	40.5 ± 6.6
Rutin (quercetin 3-rhamnoglucoside)	9.0 ± 0.6
Quercitrin (quercetin 3-rhamnoside)	0
Naringin (naringenin 7-rhamnoglucoside)	0

¹Values are means ± SEM, *n* = 3–4.

²Saliva from 1 volunteer was incubated for 2 h at 37°C with 25 μmol/L flavonoid glycosides and analyzed by HPLC for the aglycone products and the parent flavonoid glycosides.