Antimicrobial and Antioxidant Activities of Polyphenols against *Streptococcus mutans*

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ABSTRACT

Natural polyphenols, gallic acid, tannic acid, quercetin and salicylic acid, were investigated for their antimicrobial and antioxidant activities against *Streptococcus mutans*. Ascorbic acid, well known for its antimicrobial and antioxidant activities, was used as a criterion for the polyphenols. The antimicrobial effect was assessed using the plate dilution assay and the minimum inhibitory concentration (MIC) of each polyphenol was then determined from the antimicrobial activity results. Salicylic acid was the weakest antimicrobial with the highest MIC (3.8 mg/mL), and tannic acid was the strongest antimicrobial with the lowest MIC of 0.4 mg/mL. Antioxidant capacities were evaluated using the DMPD and ABTS decolorizing assays. These polyphenols show high antimicrobial activity and inoxidizability. Antioxidant activity for quercetin according to the DMPD method was inconclusive because it had color interference with the DMPD radicals. Although some conflicting results were observed between the DMPD and ABTS methods, the polyphenols with high antioxidant capacities still showed high antimicrobial activities, which suggest that the antioxidant capacity attributes to the antimicrobial effects.

Keywords: ABTS method, DMPD method, MIC

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INTRODUCTION

Plant polyphenols have attracted great attention for their biochemical and microbial effects over the past years. These secondary plant metabolites are naturally present in most edible fruits and vegetables, and therefore are common in the everyday diet of many people. It is widely known that diets containing an abundance of fruits and vegetables could be protective against a variety of diseases, particularly cardiovascular disease and cancer. The primary characteristic thought to provide the protection in fruits and vegetables are the antioxidant capacities of the polyphenols.[1] For example, flavonoids are well known to scavenge the free radicals and in addition are toxic to microorganisms.[2,3] Gallic acid reported as a free radical scavenger plays an important role in the prevention of malignant transformation and development of cancer.[4-6] These polyphenols are also well acclaimed for their antimicrobial activity.[7] For example, anacardic acid found in cashew nut shell liquid (CNSL) has been investigated for its antimicrobial activities against *Streptococcus mutans* and *Staphylococcus aureus*.[8,9]

It is useful to study the effects and mechanisms of the antioxidant and antimicrobial activities of polyphenols for their wide applications in food supplements, health-care, and therapeutic fields. The results obtained in this study will help determine which and how much polyphenols should be used (or avoided) for antimicrobial, antioxidant, or both effects. One of the hypotheses in this study is that polyphenols' antimicrobial effect is caused or facilitated by the significant electrical charge change, e.g. redox potential, through the free radical scavenging activity of the antioxidants.[10-12] This hypothesis will be examined by measuring the two activities of several polyphenols and investigate correlations between the two.

To determine the antimicrobial and antioxidant activities of these polyphenols, first the antimicrobial effect of polyphenols was assessed against *S. mutans* by the minimum inhibitory concentration (MIC) assay using the plate dilution technique. *S. mutans*, Gram-positive bacteria, was chosen because the antimicrobial effect of natural polyphenols on it was seldom performed in antimicrobial research compared to on other Gram-negative bacteria such as *Pseudomonas aeruginosa*. *S. mutans* is a dental plaque-forming bacterium, and antimicrobial effect of dietary polyphenols and its relation to antioxidant activity have not been studied much. Antioxidant capacities of the polyphenols were determined by the DMPD (N,N-dimethyl-p-phenyldiamine dihydrochloride)

method and the ABTS (2,2´-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) decolorizing assay.

The results for the antioxidant capacity were investigated to determine which analytical method should be used for different polyphenols. For the investigation of antimicrobial and antioxidant activities, quercetin, gallic acid, tannic acid, and salicylic acid were selected as model polyphenols because of their commercial availability in food and pharmaceutical industries. In this work, ascorbic acid was used as a control as it is a strong antimicrobial and antioxidant agent.

MATERIALS AND METHODS

Bacterial Strain

The strain used was *Streptococcus mutans* ATCC 25175, a Gram-positive bacterium (American Type Culture Collection, Manassas, VA, USA).

Materials

S. mutans was cultured in yeast nutrient broth (YN) essentially free of sucrose. The broth contained 0.2% sodium chloride, 0.4% potassium phosphate, 0.2% sodium phosphate, 0.1% magnesium sulfate, 0.25% glucose and 1% yeast extract. Glucose was autoclaved separately and combined with the broth aseptically. All materials were autoclaved for 20 min at 120 °C before use. Quercetin was obtained from MP Biomedicals (Aurora, OH, USA). Other polyphenols, DMPD, ABTS, Trolox and ferric chloride were obtained from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO, Fisher Scientific, Fair Lawn, NY, USA) was used as a solvent for the polyphenols. Spectrophotometric measurements were recorded by using an UV-vis Shimadzu 1700 (Japan) apparatus. Chemical structures of the phenolic compounds used are illustrated in Figure 1.

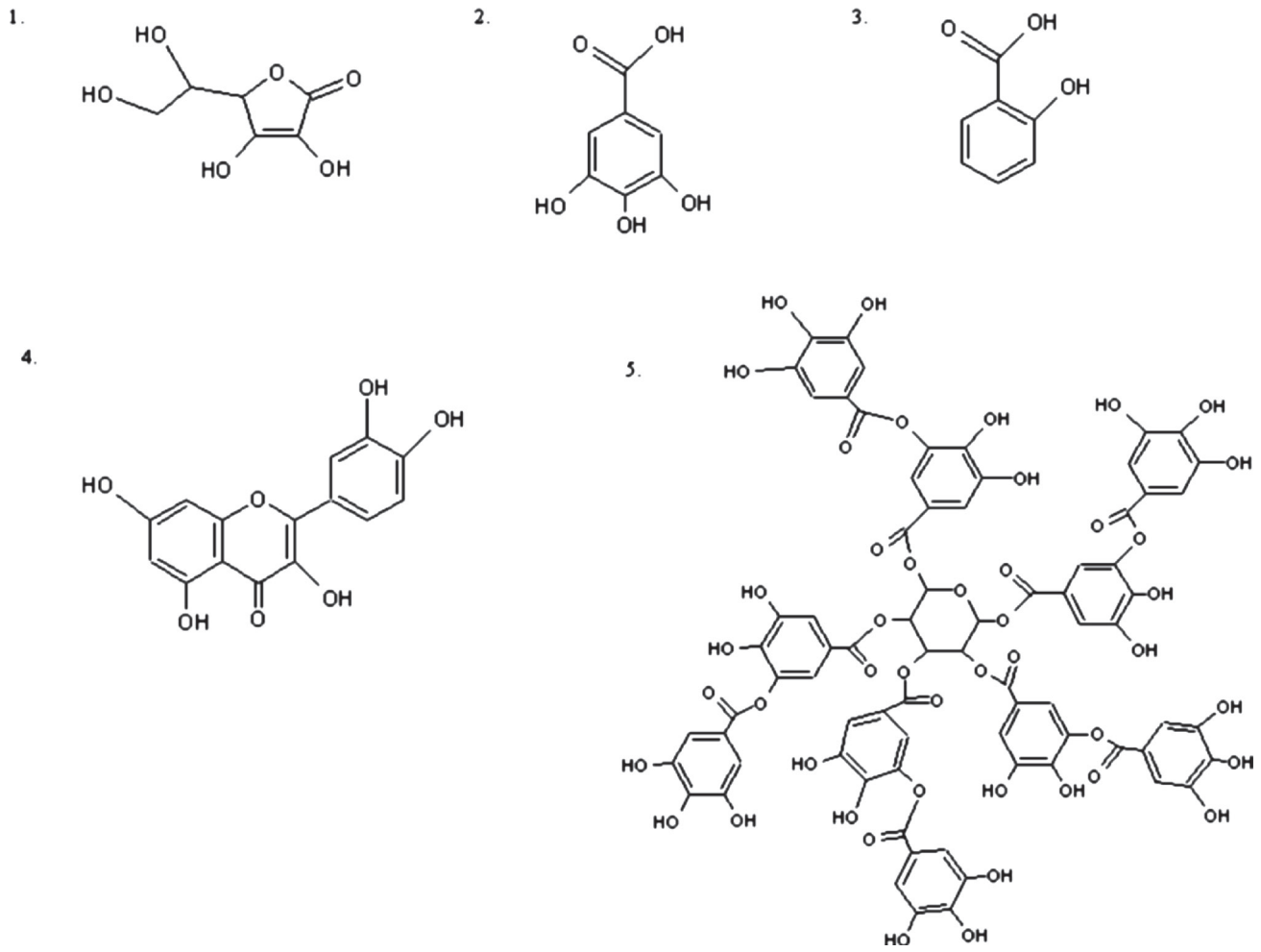

Figure 1. The structures of the polyphenolic compounds used in this study. 1. ascorbic acid, 2. gallic acid, 3. salicylic acid, 4. quercetin, 5. tannic acid.

Determination of Antimicrobial Activity (Plate Dilution Assay)

A broth dilution method was performed to assess the MIC's.[9,13-16] Briefly, *S. mutans* was grown overnight in YN with glucose. After 16 hours 0.1 mL of the stationary phase culture from the broth was transferred to a culture tube that contained 15 mL of the broth. The natural polyphenols were dissolved in DMSO at different concentrations resulting in 1% DMSO when added to the sterile medium. The solution of 1% DMSO concentration and polyphenols in the desired concentrations were tested for their effects on the growth of *S. mutans*.

When the growth in the culture tubes reached the stationary phase, 0.1 mL of the various polyphenols with different concentrations were added to different culture tubes. At regular intervals, 0.1 mL of the solution from each culture tube was serial-diluted and plated on the yeast nutrient agar plates. The plates were incubated at 37 °C and colonies were counted after 24-48 hrs. The antimicrobial assay was done in triplicates for all the samples, and averages of the results were taken.

Determination of Antioxidant Activity

As mentioned earlier, the antioxidant capacity of each polyphenol was measured at its MIC to see if there is a relationship between antimicrobial activity and antioxidant capacity. We used two different methods to measure the antioxidant activities of the different polyphenols.

DMPD/$FeCl_3$ Spectrophotometric Method

We used the DMPD method as it guarantees a very stable end point in contrast to the ABTS method.[17] The standard protocol developed by Fogliano *et al.* (1999) [18] was followed. Briefly, a 100 mM $DMPD^+$ solution was prepared by dissolving 0.209 g of DMPD in 10 mL of deionized water; 1 mL of this mixture was added to 100 mL of a 0.1 M acetate buffer, at pH 5.25; the purple colored radical cation was then created by the addition of 0.2 mL of a 0.05 M ferric chloride solution to the acetate buffer. One mL of the final $DMPD^+$ solution was placed in a quartz cuvette, and the absorbance was taken at 505 nm.

When the stable $DMPD^+$ solution was achieved, with optical density in the proper range, 0.1 mL of various antioxidant compounds was added to quartz cuvettes. After 10 minutes the absorbance was taken again, to measure the color change observed after the addition of the antioxidant compound. The resultant change in absorption was recorded as the percentage inhibition of the radical cation solution. In the presence of an antioxidant that can donate a hydrogen atom to the $DMPD^+$ molecule, the $DMPD^+$ solution changes from purple to a clear solution. Antioxidant capacities of the polyphenols were expressed as TEAC (Trolox equivalent).[19]

ABTS Decolorization Assay

Measurements of the antioxidant capacities of polyphenols were carried out as described by Jiménez *et al.* (2008).[20] The $ABTS^+$ radical cation was generated by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulphate and by allowing the mixture to remain in the dark at room temperature for 12-16 hours before use. The $ABTS^+$ solution (2 days stable) was diluted with methanol to an absorbance of 0.70 ± 0.02 at 658 nm.

The effect of each antioxidant on the $ABTS^+$ radicals was estimated according to the procedure in Brand Williams *et al.* (1995)[21] described for the DPPH· radicals and modified by Sánchez-Moreno *et al.* (2002).[22] An aliquot of DMSO (0.1 mL) solution containing the different polyphenols was added to 3.9 mL of $ABTS^+$ in methanol prepared daily. Absorbance at 658 nm was measured using a spectrophotometer. The reaction was monitored for 6 minutes. The absorbance was taken and the percentage of inhibition was calculated for each polyphenol. Methanolic solutions of the known Trolox concentrations were used for calibration.

RESULTS AND DISCUSSION

Antimicrobial activity measurement

The antimicrobial activity data provided below has been published in our previous paper.[23] The data has been included in this work, because of its importance in this study. We conducted a plate dilution assay in order to assess the antimicrobial activity of polyphenols against *S. mutans*, and the minimum concentration of polyphenols required to kill the bacterial cells was investigated by using various concentrations of the polyphenols. The bacterial cell counts in the presence of different polyphenols checked at regular time intervals are depicted in Figure 2. The arrow in Figure 2 represents the time of addition of the polyphenols to the culture. MIC as mentioned earlier is the minimum concentration of polyphenols required to kill the cells. It can be clearly seen in Figure 2 when the polyphenols were added to

the stationary phase attained (after 16 hours of inoculation), the cells started dying.

The experimental MIC values obtained for gallic acid, salicylic acid and tannic acid against *S. mutans* in our work were close to the values reported by other researchers.[8,13,24] Tannic acid was the strongest antimicrobial against *S. mutans* as it exhibited strong antimicrobial activities at a very low MIC of 0.4 mg/mL, while the other polyphenols used in this study were comparatively less effective with MIC values ranging from 1.5-3.8 mg/mL. As previous research has suggested, the strong antimicrobial activity of tannic acid may be attributed to the presence of ester linkage between gallic acid and glucose in its structure.[25] Salicylic acid exhibited the weakest antimicrobial activity with a very high MIC concentration of 3.8 mg/mL.

Comparison between ABTS$^+$ and DMPD$^+$ Assays

The antioxidant capacities of polyphenols are reported to be attributed to various mechanisms; among these are preventing the chain initiation, binding the transition metal ions, decomposing peroxides, scavenging free radicals, and preventing continued hydrogen abstraction.[26] Polyphenolic compounds are very important plant constituents because of their free-radical scavenging ability that is due to their hydroxyl groups.[27] Antioxidants, under proper conditions, may protect important molecules from free radicals' attack and consequently reduce the risks of health problems such as accelerated aging, cancer and heart disease.[28-30] To more accurately measure the antioxidant capacities of the polyphenols, two different methods were used in our study, ABTS and DMPD methods.

As the ABTS method is one of the most widely used assays by the food industry and agricultural researchers to measure the antioxidant capacities of foods,[31] we compared the results obtained by this assay and the DMPD$^+$ assay for the same samples, to see whether the antioxidants show a similar trend towards these two radicals, or a quite different one.

In Figure 3, each bar graph represents the color of the DMPD radical cation in the presence of the different polyphenols. A high percentage of inhibition represents high antioxidant capacity. The results showed ascorbic acid to be the strongest antioxidant with a 99% inhibition of the DMPD free radicals among the polyphenols used in our study. These results clearly indicate that ascorbic acid is a very good source of natural antioxidants. Gallic acid, tannic acid and salicylic acid exhibited significant radical scavenging effects with 62%, 49% and 44%

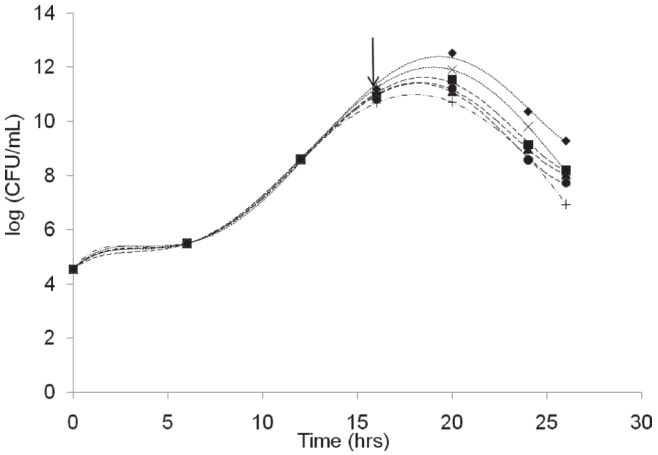

Figure 2. Antimicrobial activities of polyphenols against *S. mutans*; control (♦), ascorbic acid (■), gallic acid (▲), salicylic acid (×) acid, tannic acid (+) and quercetin (●).

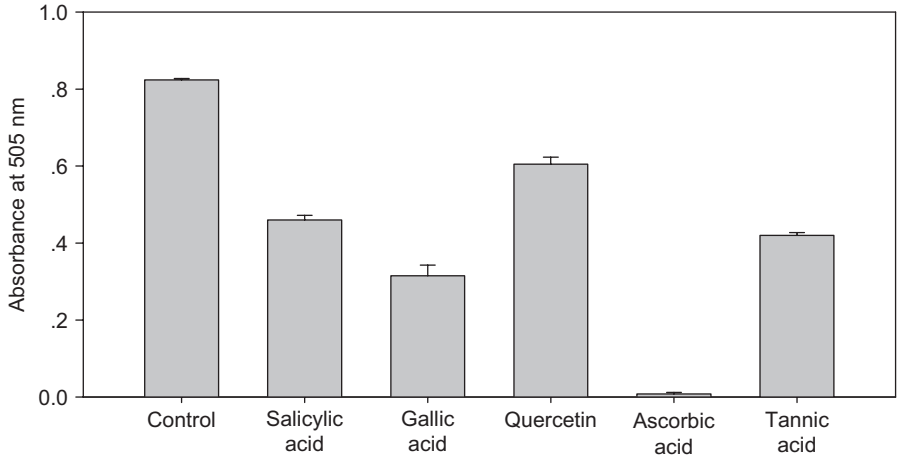

Figure 3. Changes in the absorbance of the DMPD radical after the addition of different polyphenols.

inhibitions of the free radicals when compared to quercetin (27%). Normally upon the addition of an antioxidant compound, the color of the $DMPD^+$ solution changes from purple to a clear solution. However, in our experiment for quercetin, the color of the DMPD solution changed to a yellow solution, which disqualified justifiable comparison with other polyphenols. Due to the interfering color change of quercetin in the DMPD solution, the antioxidant capacity of quercetin with the DMPD method was not used for comparison with other results.

The ABTS assay is based on the inhibition by an antioxidant of the absorbance of the radical cation ($ABTS^+$). As shown in Figure 4, ascorbic acid, gallic acid, tannic acid and quercetin were all very strong antioxidants when compared to salicylic acid. It was very difficult to rank the antioxidant capacities of all these polyphenols because the % of inhibition results was very close to each other. Ascorbic acid, a well-known antioxidant was used as a standard to compare the antioxidant activity of polyphenols. Gallic acid, quercetin and ascorbic acid showed close to 99% inhibition and tannic acid showed 96% inhibition of the ABTS radical. According to the ABTS assay, salicylic acid turned out to be the weakest antioxidant in comparison with the other polyphenols as it was able to scavenge only 3.5% of the ABTS free radicals.

The results for both the DMPD and ABTS assays were expressed in Trolox equivalents as shown in Table 1.[32] The results revealed that ascorbic acid has the highest antioxidant capacity with the highest Trolox concentrations in the both assays. Gallic acid came next to ascorbic acid in the both methods. The trends were similar for ascorbic acid, gallic acid and tannic acid in the both assays. Again, however, antioxidant capacity of quercetin was inconclusive because it had color interference on the DMPD radical giving a totally contradictory result when compared with the ABTS assay result. For comparison, antioxidant capacity of polyphenols by the ABTS method and antimicrobial effect expressed by MICs are plotted in Figure 5. Antimicrobial activities of the polyphenols are represented in terms of the inverse of MIC, and plotted on the left-side y-axis. The higher the bar, the higher is the antimicrobial activity. Antioxidant capacities are plotted on the right-side y-axis. Although the order of the antioxidant capacities does not exactly match with that of the antimicrobial effects of the polyphenols, it is clear

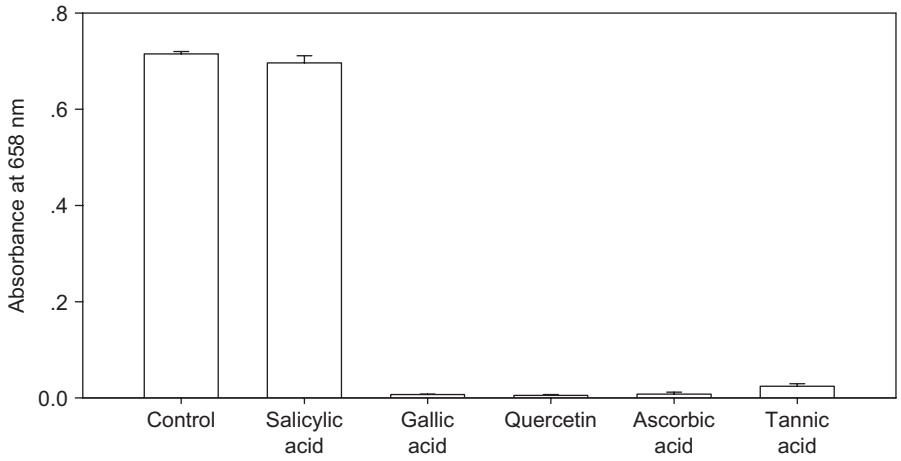

Figure 4. Changes in the absorbance of the ABTS radical after the addition of different polyphenols.

Table 1. Results of the DMPD and ABTS assays expressed as Trolox equivalents

Sample	TEAC (DMPD Method) Trolox Equivalent Conc., μM	TEAC (ABTS Method) Trolox Equivalent Conc., μM
Ascorbic acid	887.88 (± 5.36)	2390.71 (± 14.18)
Gallic acid	473.79 (± 36.47)	2394.26 (± 3.57)
Quercetin	82.45 (± 27.79)	2400 (± 5.47)
Salicylic acid	278.08 (± 18.83)	–15.82 (± 35.54)
Tannic acid	332.11 (± 7.03)	2333.7 (± 17.81)

that all the strong antimicrobials showed high antioxidant capacities.

Statistical Analysis

The results were obtained from three independent experiments and averaged. A t-test was done to statistically compare the mean values of the antioxidant activity of each individual polyphenol with the control. As shown in Table 2, the statistical analysis allowed us to submit significant differences for the antioxidant activity of polyphenols using the DMPD method ($p < 0.001$). However with the ABTS method, there was a statistically significant difference for all the polyphenols ($p < 0.001$) except salicylic acid. As described earlier, salicylic acid ($p = 0.108$) was the weakest antioxidant showing negligible antioxidant activity in our study using the ABTS method.

Antimicrobial and Antioxidant activities of polyphenols

As shown in Table 3 and Figure 5, a clear trend was observed between the antimicrobial and antioxidant activities of the polyphenols. We used the inverse of MIC as the antimicrobial activity of polyphenols in Figure 5. Tannic acid, gallic acid and quercetin showed very good antimicrobial activities as well as good antioxidant activities. However salicylic acid was the weakest polyphenol used in our study with very weak antimicrobial and antioxidant activities. This was a clear indication that the antioxidant capability of the polyphenols was involved in antimicrobial mechanisms.[33] Quercetin, however, could not be properly measured for its antioxidant capacity by the DMPD method. Tannic acid can be used as an effective antimicrobial agent (MIC: 0.4 mg/mL). Tannic acid, due to its strong antimicrobial activity, also has been commonly used to treat burns caused by incendiary bombs, mustard gas or lewisite.[34] Therefore, it is recommended to carefully control the concentration of tannic acid, for example, when enhancing the antioxidant effect while reducing the cell killing effect.

Ascorbic acid, though being lower than tannic acid in antimicrobial activity (MIC: 2.0), showed very good antioxidant capacity (2390.71 ± 14.18 μM Trolox equiv. by ABTS) compared to tannic acid (2333.7 ± 17.81 μM), that can play a major role in maintaining the proper functioning of the immune system by neutralizing

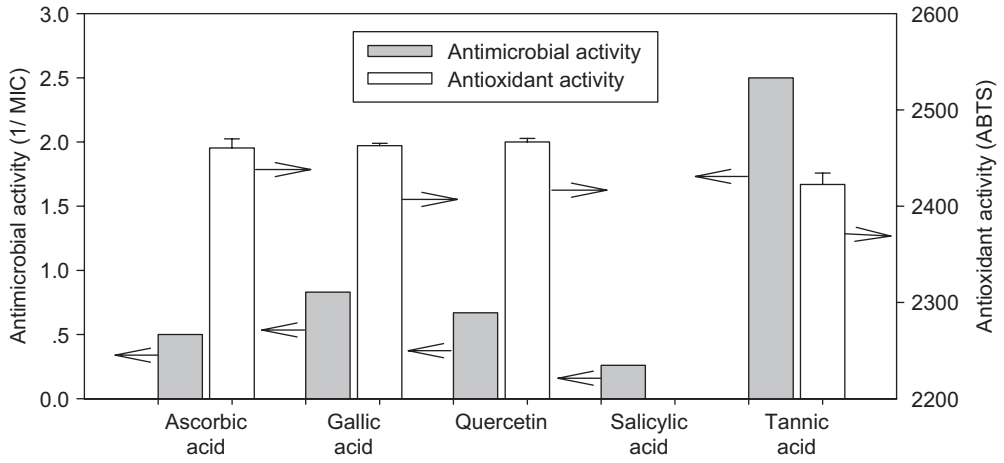

Figure 5. Comparison of the antioxidant capacities and antimicrobial activities of the polyphenols.

Table 2. Statistical Analysis for ABTS and DMPD methods

Sample	DMPD		ABTS	
	t value	Statistical significance	t value	Statistical significance
Salicylic acid	50.378	Yes	2.064	No
Gallic acid	31.399	Yes	240.96	Yes
Quercetin	20.621	Yes	235.076	Yes
Ascorbic acid	265.411	Yes	191.244	Yes
Tannic acid	89.726	Yes	166.69	Yes

Table 3. Antimicrobial effects and antioxidant capacities of polyphenols against *S.mutans*

Sample	MIC (mg/mL)	TEAC (DMPD Method)	TEAC (ABTS Method)
		Trolox Equivalent Conc., μM	Trolox Equivalent Conc., μM
Ascorbic acid	>2.0	887.88 ± 5.36	2390.71 ± 14.18
Gallic acid	>1.2	473.79 ± 36.47	2394.26 ± 3.57
Quercetin	>1.5	82.45 ± 27.79	2400 ± 5.47
Salicylic acid	>3.8	278.08 ± 18.83	–15.82 ± 35.54
Tannic acid	>0.4	332.11 ± 7.03	2333.7 ± 17.81

potentially damaging free radicals. Salicylic acid showed a weak antimicrobial effect and also a weak antioxidant capacity. Nevertheless, it is well known for its frequent usage as a commercial cosmetic preservative.[3]

CONCLUSIONS

The polyphenols that we used in our work showed very good antimicrobial and antioxidant activities. Antimicrobial tests showed that tannic acid killed the cells at the lowest concentration among the tested samples (i.e., the strongest antimicrobial). However, tannic acid was not of the highest antioxidant activity. Therefore, when increasing the tannic acid concentration to enhance the antioxidant activity, it is required to be carefully controlled not to harm healthy cells. The other tested compounds didn't show a direct correlation between antimicrobial and antioxidant activities. However it was clear that all the strong antimicrobials have high antioxidant effect. Consequently there may be some contribution of the antioxidant capacities of polyphenols to their antimicrobial mechanisms. Antioxidant activity may play an important role in polyphenols' antimicrobial effects. A further study is necessary to better understand how the rapid electrochemical changes of polyphenols affect antimicrobial activities during free radical scavenging. The antioxidant activity results from ABTS method were used to compare with antimicrobial activities because the DMPD method produced an inconclusive result for quercetin.

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