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Antibacterial Activity of Propolis Ethanol Extract Against *Streptococcus mutans* as Influenced by Concentration, Temperature, pH and Cell Age

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ABSTRACT

In the present study, the ethanol extract of propolis (EEP) collected in Taiwan was prepared and assayed for the effects concentration, incubation temperature, pH and cell age on the antimicrobial activity against *Streptococcus mutans*, a dental cavity-causing oral pathogen. Additionally, cell leakage of *Str. mutans* in presence of EEP was also examined.

It was found EEP exerted bacteriostatic and bactericidal effects against *Str. mutans*, respectively, at concentrations of 1.875 and 3.75 μg/mL or more. At 37°C, *Str. mutans* was more sensitive to EEP than at 25°C while most resistant at 4°C. Cells of test organism were most susceptible to EEP at acid pH followed by neutral and alkaline pH. It was also noted that cells of *Str. mutans* in the stationary phase were more resistant, while cells in the mid-exponential phase were more susceptible to EEP. After exposure to EEP, a marked increase in the 260 nm absorbance for the supernatant of culture, was observed, indicating the release of UV-absorbing materials. Scanning electron micrographs also showed an increase in material with irregular shape on the surface of EEP-treated *Str. mutans* cells.

Key words: antibacterial activity, cell leakage, ethanol extract of propolis, *Streptococcus mutans*

INTRODUCTION

Propolis, a resinous substance, is derived from the plant resins which was collected by honeybees. Bees use it as a glue, general-purpose sealer and draught-extruder for beehives[(1)]. Antimicrobial activity of propolis has been reported by various investigators[(2-5)]. Researchers have noted that the antimicrobial activity of propolis reflects its composition, which may vary with the area and season[(3,5-8)]. It has also been reported that propolis, despite exerting antimicrobial activity on gram (+) bacteria, showed no activity on gram (–) bacteria[(9-10)]. In addition to antimicrobial activity, propolis has also been found to possess antioxidative and antiulcer activities. For these reasons, propolis is now considered as a useful ingredient for the applications in domestic goods, medicine, and food products.

Streptococcus mutans is the leading cause of dental cavities (tooth decay) worldwide and is considered to be the most cariogenic among the oral streptococci. It has been shown that there is a positive correlation between the number of *Str. mutans* in dental plaque and the occurance of dental cavities[(11-12)]. *Str. mutans* can colonize the tooth surface and initiate plaque formation through the synthesis of extracellular polysaccharides, mainly water-insoluble glucan from sucrose by using glucosyltransferase[(13)]. Park *et al.*[(4)] reported that ethanol extract of propolis (EEP) from various regions in Brazil inhibited both glucosyltransferase activity and the growth of *Str. mutans*. In addition, Koo *et al.*[(13)] observed that EEP exhibited *in vitro* antibacterial activity, the inhibition of cell adherence and the formation of water-insoluble glucan. Despite these findings, information concerning the factors that mediate the antimicrobial activity of propolis against *Str. mutans* is still lacking. Therefore, this study investigated the effects of concentration, incubation temperature, pH and cell age on the the susceptibility of *Str. mutans* to EEP. Besides, leakage of nucleic materials from cells of *Str. mutans* in presence of EEP was also examined.

MATERIALS AND METHODS

I. *Test Organism and Propolis*

Str. mutans BCRC 15256 was obtained from the Bioresource Collection and Research Center, Hsingchu, Taiwan. To activate the test organism before experimentation, *Str. mutans* was transferred twice successively in tryptic soy broth (TSB, Difco, Detroit, MI, USA) at 37°C for 24 hr. The inoculum was then prepared by inoculat-

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ing 0.1 mL of the activated culture into 10 mL of TSB and incubated at 37°C for 16 hr, except for the cell age experiments during which cells were cultured for 10, 16 and 30 hr to obtain the mid-exponential, late-exponential and stationary phase cells, respectively. These cultures were then properly diluted with sterile saline (0.85% NaCl) and used as inoculum in the susceptibility test.

The propolis was collected from beehives located in Nantou County, Taiwan in June and July of 2003. They were stored at -30°C before use. Twenty five grams of ground propolis was extracted by 250 mL of 80% ethanol by orbital shaking at 150 rpm at 25°C for 48 hr. The ethanol extract was then filtered through a Whatman #42 filter paper and restored to its original volume by adding 80% ethanol. Various concentrations of EEP solution were further made by diluting with the appropriate amounts of 80% ethanol based on the dry weight.

II. *Study on the Susceptibility of Str. mutans to EEP*

When the effect of EEP concentration was examined, 9.8 mL of TSB was mixed with 0.1 mL of the EEP solution of various concentrations EEP or 0.1 mL of 80% ethanol as control. They were then inoculated with 0.1 mL of the inoculum of *Str. mutans* BCRC 15256 to give an initial population of ca 10^4-10^5 CFU/mL. The viable population of *Str. mutans* was determined after 12 hr of incubation at 37°C.

To examine the effect of temperature, 98.0 mL of TSB was added to 1.0 mL of the EEP or 80% ethanol (control). This mixture was inoculated with 1.0 mL of the inoculum of *Str. mutans* to give an initial population of ca 10^5 CFU/mL and incubated at 4, 25 or 37°C for 12 hr. The viable population of the test organism in the samples taken at predetermined time intervals was determined.

To perform the experiment of pH effect, 98.0 mL of TSB of pH 5.0, 6.0, 7.1, 8.0 or 9.0 was added to EEP or 80% ethanol (control) and inoculated with *Str. mutans* and incubated at 37°C for 12 hr.

When the effect of cell age was examined, 98.0 mL of TSB was first mixed with 1.0 mL of EEP or 80% ethanol (control) and inoculated with 1.0 mL of the prepared inoculum containing mid-exponential, late-exponential or stationary phase cells of *Str. mutans*, which gave an initial population of ca 10^5 CFU/mL. They were then incubated at 37°C for 12 hr. The viable population in the samples taken after certain periods of incubation was also determined.

III. *Study on Cell Leakage*

Str. mutans BCRC 15256 cells in the late-exponential growth phase were recovered by centrifugation at 8,000 ×g for 15 min, washed with saline three times, and resuspended in saline solution with proper dilution. One milliliter of the cell suspension was combined with 98.0 mL of saline solution and 1.0 mL of 80% ethanol (control) or 1.0 mL of 80% ethanol containing EEP (200.0 μg/mL). They were then incubated at 37° C for 12 hr. At various time intervals, samples were taken to determine the viability and were centrifuged at 8,000 ×g for 15 min. Leakage of nucleic acid materials in supernatant was measured by the absorbance at 260 nm using a spectrophotometer.

IV. *Scanning Electron Microscopy (SEM)*

The method described by Fukushima *et al*.[14] was emoloyed to prepare samples for electron microscopy. The cell pellets samples were fixed in 2.5% glutaraldehyde in 0.1 M Tris buffer (pH 7.3) at 4°C for 1 hr. After being washed twice in a Tris buffer with 5% sucrose, they were post-fixed in 1.0% osmium tetroxide solution in the same buffer for 1 hr. The resultant osmium-treated cells were then washed with 0.1 M Tris buffer and dehydrated by successive extractions with 50, 70, 80, 90 and 95% ethanol, each for a period of 10 min, and finally with 100% ethanol for 15 min twice. They were then dried by the CO_2 critical-point drying technique, coated with gold and examined using a scanning electron microscope (Model JSM-6300, JEOL, Tokyo, Japan).

V. *Enumeration of Str. mutans*

To determine the viable population of *Str. mutans*, samples were first serially diluted with sterile saline. Viable counts were then made by pour-plating (1.0 mL) on tryptic soy agar (TSA, Difco, Detroit, MI, USA). The colonies were counted after 48-72 hr of incubation at 37°C.

VI. *Statistical Analysis*

In this study, each experiment was carried out in triplicate. The mean value and standard deviation were calculated from the data obtained. These results were then compared using the least significant difference (LSD) test[15].

RESULTS AND DISCUSSION

I. *Effect of Concentration*

The survival of *Str. mutans* in presence of various amounts of EEP is shown in Figure 1. In contrast to that observed in *Salmonella typhimurium* and *Escherichia coli*, but in accordance with that noted in *Staphylococcus aureus* and *Listeria monocytogenes*[5,16], propolis extract exhibited antibacterial activity against *Str. mutans*. The propolis extract, depending on the concentration tested, displayed bacteriostatic and bactericidal effects on *Str. mutans* in TSB. At a dosage of 3.75 μg/mL or more, propolis extract exerted a bactericidal effect on the test organism. No viable

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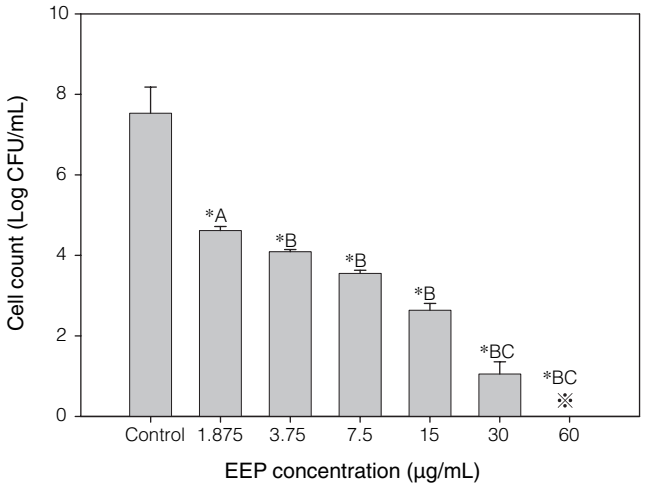

Figure 1. Survival of *Str. mutans* after 12hr of cultivation in TSB containing various amounts of EEP. *Str. mutans* was inoculated into TSB at an initial population of ca 10^4-10^5 CFU/mL. ※ indicates no viable cell detected. *The final population was significantly lower ($p < 0.05$) than that of the control by LSD test. (A) The final population was not significantly different ($p > 0.05$) with initial population by LSD. (B) The final population was significantly lower ($p < 0.05$) than initial population by LSD. (C) Survival cell was less than 0.1% of the initial population.

cell was detected in TSB containing 60 μg/mL of EEP after 12 hr of cultivation while propolis extract at a concentration of 1.875 μg/mL did not affect inhibite the growth of test organism. Testing in brain heart infusion broth with a similar level of initial population, a relatively high dosage of 3.75 μg/mL was required to exert the bacteriostatic effect on *L. monocytogenes*[17]. Therefore, *Str. mutans* seems to be more susceptible to EEP than *L. monocytogenes*.

Propolis collected in Taiwan contains flavones, flavonols, flavonones and isoflavones[18]. These constituents in propolis may contribute to the antimicrobial activity observed[19].

II. *Effect of Temperature*

The effect of propolis extract on the growth and survival of *Str. mutans* in TSB at different cultural temperatures is shown in Figure 2. Neither growth nor reduction in the viable population of *Str. mutans* was noted in TSB with or without EEP during 12 hr of incubation period at 4°C. However, when *Str. mutans* grew at 25 or 37°C in TSB, EEP showed bactericidal effect on *Str. mutans* during the incubation period. In general, the difference between the viable populations of test organism in the TSB with, and without EEP enhanced as the incubation period extended. This effect was more profound at 37°C. For example, the test organism in TSB without EEP showed a viable population of ca 7.8 log CFU/mL which is higher than that in TSB containing propolis extract by 2.6 log CFU/mL after 12 hr of

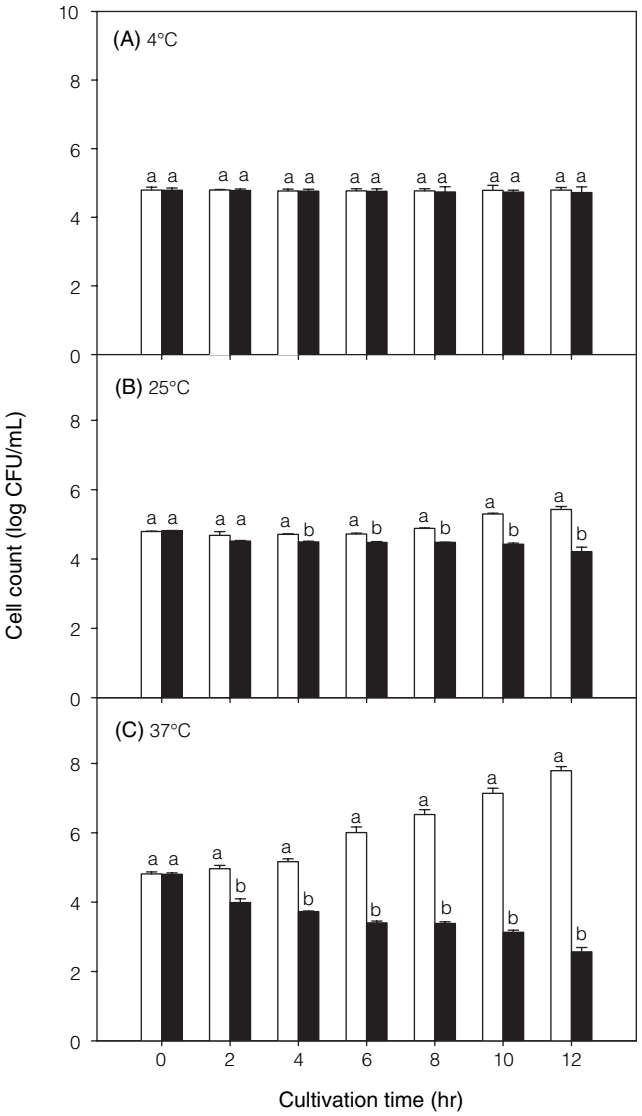

Figure 2. Susceptibility of *Str. mutans* to EEP at various cultivation temperatures. Viable cells of *Str. mutans* were determined after 12 hr of cultivation in TSB containing 15 μg/mL EEP (■) or without EEP (□). Each bars for the control or for the treated sample bearing different lowercase letters are significantly different by Duncan's multiple range test ($p < 0.05$).

incubation at 37°C. Meanwhile, the difference between the viable populations of the control and EEP-containing TSB was only 1.2 log CFU/mL when the test organism was incubated at 25°C. This finding is consistent with that observed on *Sta. aureus* and it is further suggested that *Str. mutans* is more susceptible to propolis extract at higher temperatures. The metabolic rate of *Str. mutans* is higher at 37°C than at other temperatures examined. Cells with higher metabolic rates are more susceptible to antimicrobials. Moreover, the reaction rate between the antimicrobial principles and microbial cells increased as the temperature was elevated[20]. These effects may all contribute to the phenomenon observed.

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III. *Effect of pH*

pH level is one of the most important factors concerning the activity of antimicrobial compounds[21]. The susceptibility of *Str. mutans* to the propolis extract at various pH levels is shown in Figure 3. At pH 6.0-9.0, *Str. mutans* showed various levels of growth in TSB without the propolis extract. On the other hand, the viable population of test organism decreased in TSB containing propolis extract, regardless of pH. Difference between the viable populations in control and EEP-containing medium varied with pH during the incubation period. After 4 hr of incubation, no viable cell of *Str. mutans* was detected in the medium with a pH of 5.0 or 6.0. It was also noted that, in EEP-containing TSB with a higher pH, a higher final viable population of *Str. mutans* was observed at the end of incubation. These results, similar to that observed on *Sta. aureus*[16], demonstrated that the antimicrobial activity exerted by the propolis extract against *Str. mutans* increased as the pH of the medium decreased.

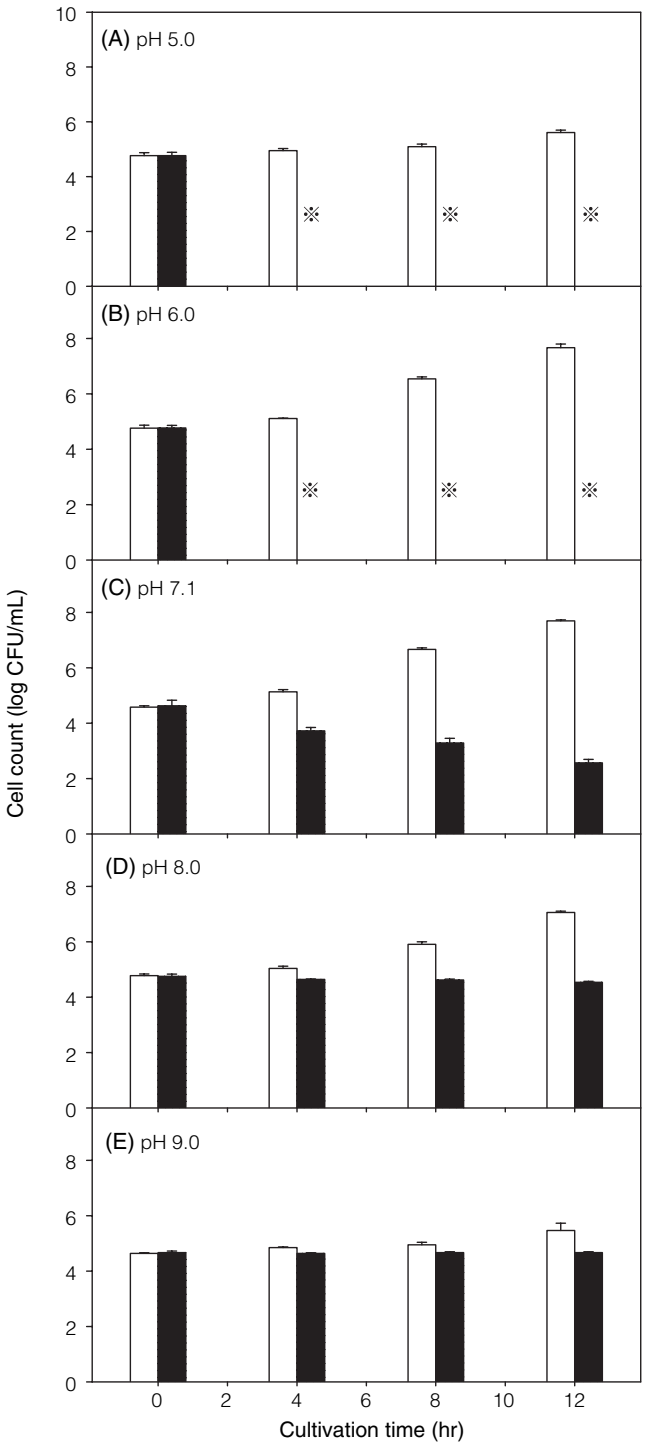

Figure 3. Susceptibility of *Str. mutans* to EEP at various cultivation pHs. Viable cells of *Str. mutans* were determined after 12 hr of cultivation in TSB containing 15 μg/mL EEP (■) or without EEP (□). ※ indicates no viable cell detected.

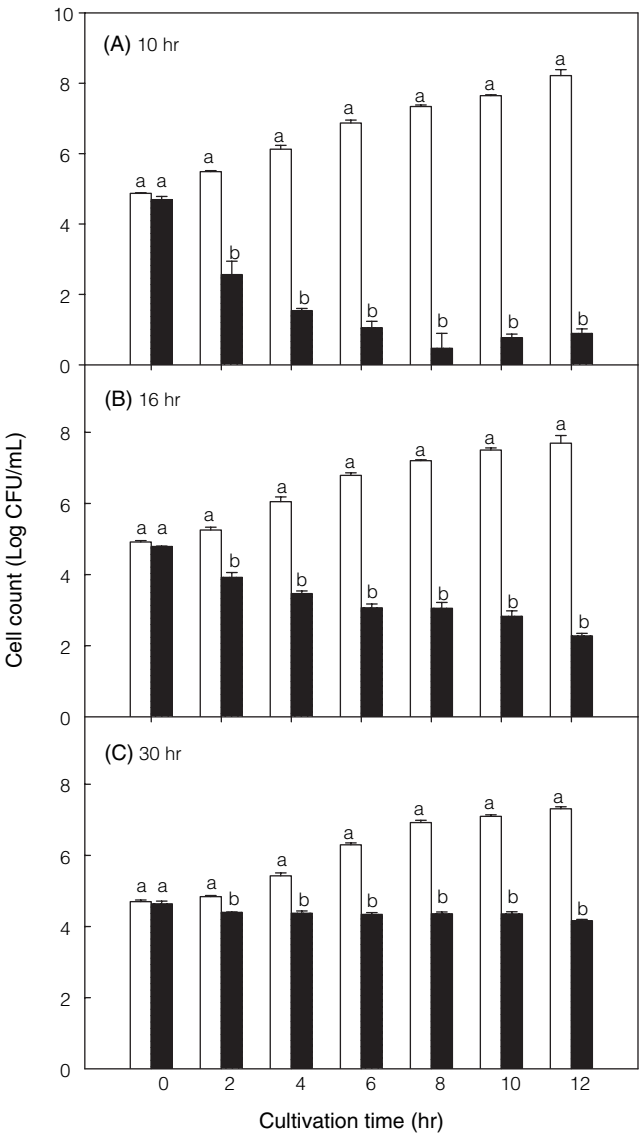

Figure 4. Effect of cell age on the susceptibility of *Str. mutans* to EEP. Viable cells of *Str. mutans* were determined after 12 hr of cultivation in TSB containing 15 μg/mL EEP (■) or without EEP (□). Each bars for the control or for the treated sample bearing different lowercase letters are significantly different by Duncan's multiple range test ($p < 0.05$).

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Bankova *et al.*[22] indicated that polyphenol compounds belong to the main components contributing to the biological activity of propolis. Zhu *et al.*[23] demonstrated that some of the polyphenols were extremely unstable in alkali condition, but relatively stable in acid condition. Therefore, it is suggested that the degradation of these phenolic compounds in alkaline condition, especially at pH 9.0, may thus diminish the antimicrobial activity of the propolis extract as observed at alkaline pH.

IV. *Effect of Cell Age*

Generally, microorganism, in log phase, the actively growing stage, are less resistant than those in the stationary phase[21]. Further, it has been suggested that the susceptibility of microorganisms at different cell ages to antimicrobials might vary with microorganism, test medium, and antimicrobials being examined[24-25].

The effect of cell age on the susceptibility of *Str. mutans* to propolis extract is shown in Figure 4. Regardless of cell age, viable population of test organism in TSB containing no propolis extract increased as the incubation time extended and reached to ca 7.3-8.2 log CFU/mL after 12-hr incubation. The viable population of *Str. mutans* in stationary phase did not change markedly, while that of test organisms in the mid-exponential and late-exponential phases decreased in the propolis extract-containing TSB during the incubation period (Figure 4A and B). Cells of *Str. mutans* in mid-exponential phase were the most susceptible to propolis extract. Relative to the viable population observed in the control TSB, cells in the mid-exponential phase showed the highest population reduction of 7.3 log CFU/mL as compared to that of 4.2 log CFU/mL with the cells in the late-exponential phase at the end of incubation period. These observations, in accordance with those observed on *L. monocytogenes*[26] shows that *Str. mutans* cells in the mid-exponential phase were most susceptible to propolis extract, followed by cells in the late-exponential and stationary phase.

V. *Effect of EEP on the Viability and Cell Leakage of Str. mutans in Saline Solution*

Similar to that observed in saline solution without EEP, the viability of test organism decreased, while the absorbance at 260 nm increased markedly, in the EEP-containing saline during the period of exposure (Figure 5).

Takaisi-Kikuni[27] reported that propolis inhibited the growth of *Str. agalactiae* by preventing cell division. They also indicated that propolis disorganized the cytoplasm, the cytoplasmic membrane and the cell walls. As shown in Figure 6, the scanning electron micrographs showed the appearance of materials with irregular shapes around the cell surface of *Str. mutans* after exposure to saline with or without EEP. These

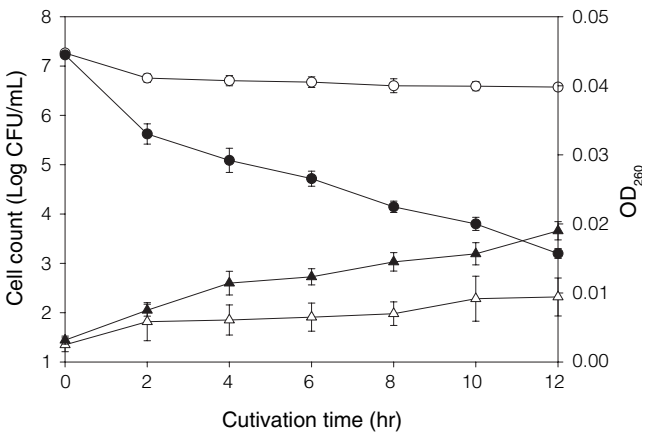

Figure 5. Effects of EEP on the viable cells and the leakage of 260-nm-absorbing materials from *Str. mutans*. The harvested *Str. mutans* cells (10^7 CFU/mL) were cultured with 0.85% sodium chloride solution containing 1.0 μg/mL EEP (solid symbols) or without EEP as control (open symbols). Cultures were incubated at 37°C. Viable cells, ● and ○; OD_{260}, ▲ and △.

materials increased as the exposure time extended and were more pronounced in the EEP-treated cells (Figure 6 D and E) than the control cells (Figure 6 A, B and C). This observation along with the marked increase of 260 nm absorbance (Figure 5) demonstrates that EEP caused the injury on the cell membrane of *Str. mutans* and resulted in cell leakage.

CONCLUSIONS

Based on the results obtained from the present study, propolis gathered in Taiwan possesses antimicrobial activity against *Str. mutans*. EEP caused the leakage of nucleic acid materials from cells of *Str. mutans*. *Str. mutans* in their mid-exponential phase, at 37°C and pH 5.0, were most susceptible to the EEP tested. These results indicated the possibility of using EEP as an active anti-*Str. mutans* ingredient in the dental cavities control medicines. Finally, factors mediating the susceptibility of *Str. mutans* to EPP as observed in this study should be further explored so that the most effective antimicrobial action of EPP can be employed in practical applications.

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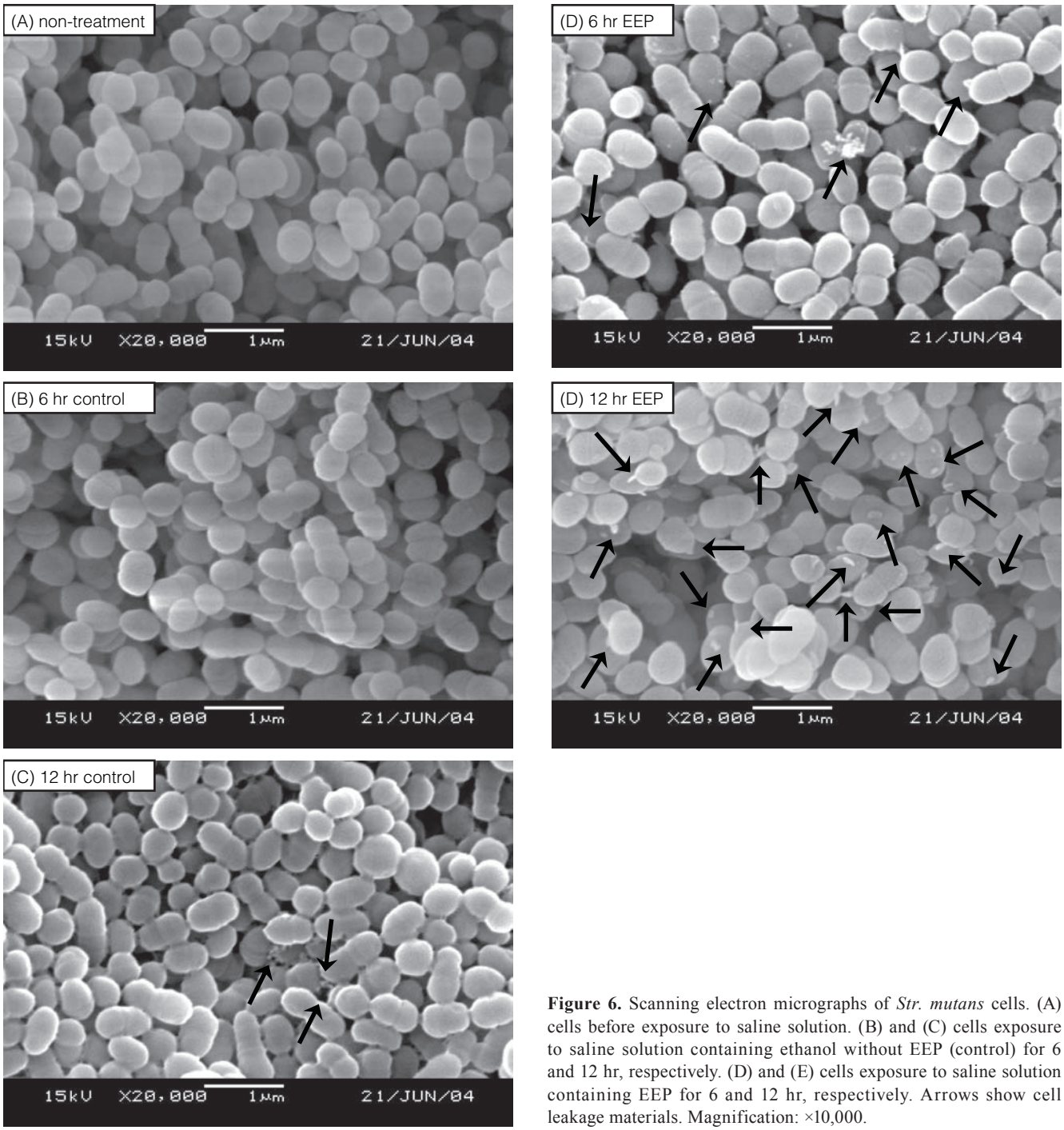

Figure 6. Scanning electron micrographs of *Str. mutans* cells. (A) cells before exposure to saline solution. (B) and (C) cells exposure to saline solution containing ethanol without EEP (control) for 6 and 12 hr, respectively. (D) and (E) cells exposure to saline solution containing EEP for 6 and 12 hr, respectively. Arrows show cell leakage materials. Magnification: ×10,000.

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