Full Length Research Paper

Evaluation of *Ellagic acid* on the activities of oral bacteria with the use of adenosine triphosphate (ATP) bioluminescence assay

Wings TY Loo[1,2]*, LJ Jin[1], Mary NB Cheung[3] and Louis WC Chow[2,4]

[1]Faculty of Dentistry, University of Hong Kong, China.
[2]UNIMED Medical Institute and Organization for Oncology and Translational Research, Hong Kong, China.
[3]Keenlink Dental Clinic, Hong Kong, China.
[4]Clinical Trial Centre, Li Ka Shing Faculty of Medicine, University of Hong Kong, China.

Ellagic acid, a natural herb extract from *Galla Chinensis* in traditional Chinese medicine, shows antimicrobial activity to certain bacteria. The present study evaluated the effect of *Ellagic acid* on the growth of oral bacteria as well as their generation of water-insoluble glucan and adhesion to saliva-coated hydroxyapatite (S-HA) beads. *Streptococcus mutans* ATCC 25175, *Streptococcus sanguis* ATCC 10556, *Streptococcus salivarius* ATCC 25975, *Actinomyces naeslundii* ATCC 12104, *Actinomyces viscosus* ATCC 15987, *Lactobacillus rhamnosus* ATCC 53103, *Porphyromonas gingivalis* ATCC 33277 and *Bacteroides forsythus* ATCC 43037 were the bacterial cell lines used in this study. Antibacterial activity of *Ellagic acid* was determined by using adenosine triphosphate (ATP) bioluminescence assay at various concentrations from 0.125 to 8 mg/ml. Anthrone method was used to evaluate the level of water-insoluble glucan generated by oral bacteria. The numbers of 3H-thymidine labeled bacteria attached to S-HA was counted by scintillation counting method. Sprague Dawley rats were orally fed with 0.5mg/mL ellagic acid for 28 days and their behaviours and excretions were monitored. Ellagic acid reduced bacterial metabolic rates and inhibited the growth of the tested bacterial strains. The water-insoluble glucan generated by *S. mutans* and its adhesion to S-HA were reduced. Ellagic acid demonstrated no toxicity in animals fed for 28 days. Ellagic acid might be a promising compound for the development of antimicrobial agents against oral pathogens in human, thereby reducing the incidence of dental caries.

Key words: Ellagic acid*, Galla chinensis,* adenosine triphosphate bioluminescence assay*,* oral bacteria.

INTRODUCTION

Dental caries is the most common of all oral diseases. The main cause of dental caries was attributed to oral biofilm, also known as dental plaque, a film of micro-organisms sticking to the tooth surface (Marsh, 1992). Cariogenic bacteria can colonize the acquired pellicle on the tooth surface and initiate biofilm formation by their ability to synthesize extracellular polysaccharides from sucrose (Gibbons and van Houte, 1975). The synthesis of extracellular polysaccharides, especially water-insoluble glucan is essential for the adherence of *Streptococcus mutans* and other oral microorganisms to the tooth surface. The extracellular matrix acts as a barrier preventing the diffusion of acids produced by bacteria. The acid accumulates *in situ* and consequently decalcifies minerals in the enamel, which results in dental caries (Marsh, 1994).

A wide variety of sources have been explored in the search for effective anti-plaque agents (Loe and Schiott, 1970; Southard et al., 1984, Wennstrom and Lindhe, 1985). A few recent studies have focused on the anti-

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*Corresponding author: E- mail: tyloo@hku.hk. Tel.: 00852-28610286. Fax: 00852-28611386.

Abbreviations: S-HA, Saliva-coated hydroxyapatite; **MIC,** minimal inhibitory concentrations; **ATP,** adenosine triphosphate; **TPY,** tryptone-peptone-yeast; **SD,** Sprague-Dawley; **CMP,** scintillation counting.
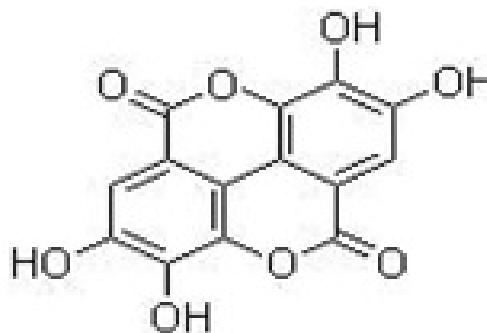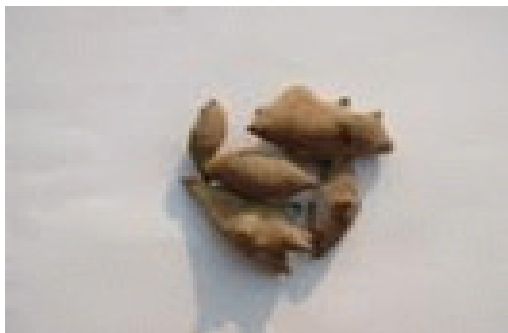
Figure 1. Appearance of raw *gala chinensis* and chemical structure of ellagic acid. The structural formula is $C_{14} H_6 O_8$, its molecular weight is 228.25 and the extract presents gray-brown powder meticulous.

microbial activity against selected oral pathogens from natural sources. Natural products have been used for thousands of years in folk medicine, they were believed to be the new source of antimicrobial agents. Extract of the native American plant, *Ceanothus americanus*, showed growth-inhibitory effects against *S. mutans*, *Actinomyces viscosus*, and *Porphyromonas gingivalis* (Li et al., 1997). *Bakuchiol,* isolated from the seeds and leaves of *Psoralea corylifolia* Linn, a tree native to China with various uses in traditional Oriental medicine, has been demonstrated to possess antimicrobial activities against some oral microorganisms *in vitro*, with minimal inhibitory concentrations (MIC) ranging from 1 to 4 µg/ml (Harumi et al., 2001). Extracts of propolis showed antimicrobial activity against some oral microorganisms (Koo et al., 2000; Koo et al., 2002). Among the natural herbs used in the traditional Chinese medical prescription, ellagic acid has been used to cure the pain caused by dental caries, but there is no direct scientific evidence for the use of ellagic acid to prevent dental caries. Ellagic acid is the gall produced mainly by parasitic aphids of *Melaphis chinensis* (Bell) Baker on the leaf of *Rhus chinensis Mill*, *Rhus potaninii Maxim*, or *Rhus phunjabensis Stew* var sinica (Diels) *Rehd* (*Fam. Anacardiaceae*). According to its form the drug is divided into "Dubei" and "Jiaobei". Native to Eastern Asia, *Galla Chinensis* (*Essence-ellagic* acid) is grown on lowland, hills and mountains in China, Japan, Indochina, Java, Malaysia, Sumatra, etc. The structure of ellagic acid is as shown in Figure 1. Ellagic acid has been shown to be a potent antioxidant and protective against cell death (Hwang et al., 2009; Turk et al., 2010). Lots of research has determined the effects of ellagic acid regarding its anti-cancer properties as it induces cell apoptosis (Edderkaoui et al., 2008; Bell and Hawthorne 2008).

Adenosine triphosphate (ATP) bioluminescence assay has become widely used when measuring the viable cell numbers in culture. It is a reproducible, practicable and promising method for predicting and assessing the response of oral microorganisms to anti-plaque agents that can also be used for pre-therapeutic drug testing. ATP provides energy in all living cells and is degraded by

ATPases when the cell dies (Erecinska and Wilson, 1982). Thus, measuring the level of ATP is a good indicator of cell viability. In the presence of ATP, magnesium and oxygen, the luciferin is catalyzed by the luciferase enzyme, which is obtained from firefly. This gives a luminescent signal, and this light intensity emitted is directly proportional to the ATP concentration (Sykes, 2009). The purpose of the present study is to evaluate the anti-bacterial effect of ellagic acid.

MATERIALS AND METHODS

Bacterial strains and preparation

The following 8 strains of oral bacteria were purchased from American Type Culture Collection, namely *Streptococcus mutans* ATCC 25175, *Streptococcus sanguis* ATCC 10556, *Streptococcus salivarius* ATCC 25975, *Actinomyces naeslundii* ATCC 12104, *Actinomyces viscosus* ATCC 15987, *Lactobacillus rhamnosus* ATCC 53103, *Porphyromonas gingivalis* ATCC 33277 and *Bacteroides forsythus* ATCC 43037 (ATCC , USA). The bacteria were inoculated into tryptone-peptone-yeast (TPY) culture medium in test tubes and grown to stationary phase for 18 hours at 37°C (80% N_2, 20% CO_2). The cultured bacteria were centrifuged and the concentration of the bacteria turbidity was adjusted to 1.0 (ABS=1.0) at 540 nm.

Preparation of ellagic acid powder from *G. chinensis* extract

Ellagic acid was purchased from Medichem Specialties Co., Ltd. (Xian, China). It was extracted from 100% pure natural *Chinese Gall* using the ultra-low-temperature squeezing technology so that the composition and the activity of ellagic acid are preserved. The purity of ellagic acid was more than 90% from the ellagic acid extract

Animal study

The animal study was approved by the ethic committee of the University of Hong Kong, China. Sprague-Dawley (SD) rats were bred and maintained in the University of Hong Kong animal unit. SD rats were housed under constant environmental conditions (photo-period, temperature, air humidity, food). At the weight of 135 g, 80 female and 90 male rats were fed with 0.5 mg of ellagic acid extracts in 1 ml distilled water daily. For the control group, 80 rats were given the same amount of distilled water instead. The rats

were monitored by the same technician every day in terms of their behaviors and excretions, and they were weighed once a week throughout a 28-day observation period (Chow et al., 2001).

Susceptibility testing

In accordance with the methods described by the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (1990), the MICs of ellagic acid for each bacterial strain were determined. Aliquots of 10 µl of the diluted cultures of each oral microorganism were inoculated in TPY agar growth medium containing 1% (wt/vol) glucose and extract of ellagic acid at final concentrations of 8, 4, 2, 1, 0.5, 0.25, 0.125 mg/ml. The numbers of colonies present were counted manually after 48 h of incubation at 37°C (80% N_2, 20% CO_2).

ATP bioluminescence assay

The bacteria were inoculated into TPY agar growth medium containing 1% (wt/vol) glucose and extract of ellagic acid at final concen-trations of 8, 4, 2, 1, 0.5, 0.25, and 0.125mg/ml.

After 48 h, the cell lysis buffer was added into collection tubes with the bacteria. In preparation for the luciferase reagent of the ATP bioluminescence assay, 10 ml dilution buffer was added to the luciferase reagent supplied by the kit. Cell lysis was performed by a sonicator (Sonics & Materials Inc. Danbury, CT. USA) at a pulse of 30 per minute with 20% amplitude. A 10-s binding period was done by amalgamation of 50 ul of both samples and luciferase reagent. The absorbance value of the samples was read by TD-20/20 Luminometer (Turner Designs, CA. USA) at 420 nm wavelength. The kit provided a standard ATP for conversion of samples' optical densities to bioluminescence (RLU, Relative Light Units). All experiments were done in triplicate. The results were compiled using statistical package for the social sciences (SPSS) program. Values of $p < 0.05$ were considered to be statistically significant.

Determination of percentage inhibition of ellagic acid on bacteria

The percentage inhibition (%INH) was calculated as:

(%INH = [(CL – Test)/CL] x 100%

Where, CL = control without ellagic acid; Test = samples with ellagic acid.

Ellagic acid inhibition on the water-insoluble glucan generation

Aliquots of 1ml of the diluted cultures of bacteria were cultured in 10ml of fresh TPY broth containing 1% (wt/vol) glucose and extract of ellagic acid at the mentioned concentrations for each bacterial strain in sterile glass bottles and incubated at 37°C (80% N_2, 20% CO_2) for 24 h. The cultures were centrifuged and the sedimentation was gently washed with saline followed by 0.4mol/L NaOH. The supernatant fluid was collected for future or further analysis. The specimens were analyzed by the anthrone method (Trevelyan and Harrison, 1956), using glucose as a standard.

Assays for adhesion to saliva-coated hydroxyapatite (S-HA) surface

The adherence of bacteria was assayed by a modification of the method described by Clark et al. (1978). Whole saliva was collected

from one donor into a container chilled over ice. The saliva was clarified by centrifugation and the supernatant was collected and stored at 4°C. The extract of ellagic acid was added into buffered KCl (2 mM potassium phosphate containing 5 mM KCl and 4 mM $CaCl_2$ at pH 6.0). The bacterial strains were inoculated into 4 ml TPY broth supplemented with [H^3-TdR] (Shanghai Research Institute of Atomic Energy, China) at concentration of 10 µ Ci/ml and grown to stationary phase for 18 h at 37°C (80% N_2, 20% CO_2). The cultures of each bacterial strain were centrifuged, washed and resuspended (Zhou et al., 2007)

4 mg of Spheroid HA beads (BDH Chemicals, Poole, UK) were placed in polypropylene tubes and soaked in 200 µl buffered KCl over night before use. The HA beads were incubated in 100 µl clarified whole saliva (positive control and test groups) or 100 µl buffered KCl (negative control) for one h at room temperature in an apparatus which continuously inverted the tubes ten times a min. The beads were then washed twice with buffered KCl. 100 µl of bovine serum albumin (BSA) (Sigma, USA) were added to each tube and continuously inverted for half an hour. 100 µl test solution was added to the test group and 100 µl buffered KCl to the control groups, followed by inversion for 1 h.

Samples of 100 µl of H^3-labeled bacteria in buffer KCl were added to each tube. Triplicates were assayed for each bacterial suspension that had been grown in the presence or absence (control) of ellagic acid. After incubation for 1.5 h at room temperature, the beads were washed with buffered KCl for 3 times. The beads were dried and transferred to scintillation vials. The numbers of cells bound were measured by scintillation counting (CMP). The adhesion values from three replicates for each tubes of S-HA pre-incubated with or without ellagic acid (control) were presented as a percentage of the original suspension that adhered to 4 mg S-HA. The adhesion inhibition rates were calculated from the formula:

Adhesion inhibition rate = [1- ($CMP_{test\ group}$ - $CMP_{negative\ control\ group}$)/($CMP_{positive\ control\ group}$ - $CMP_{negative\ control\ group}$)]*100%

RESULT

Animal study

No abonormality was observed in the feces and urine of the SD rats in the test group. There was no significant difference on the body weight of the SD rats (186.5 g, ±: 1.23) fed with ellagic acid when compared with the control group (190.4 g, ±: 2.21).

MIC of ellagic acid for cariogenic bacteria

The MICs of ellagic acid extracts to the tested bacterial strains are shown in Figure 2. The *S. mutans* and *S. salivarius* were more susceptible to the ellagic acid than the others.

Ellagic acid reduced bacterial activities as measured by ATP bioluminescence assay

The ATP bioluminescence assay measured the metabolic rate of various bacterial strains treated with ellagic acid. Statistically significant difference was observed at all
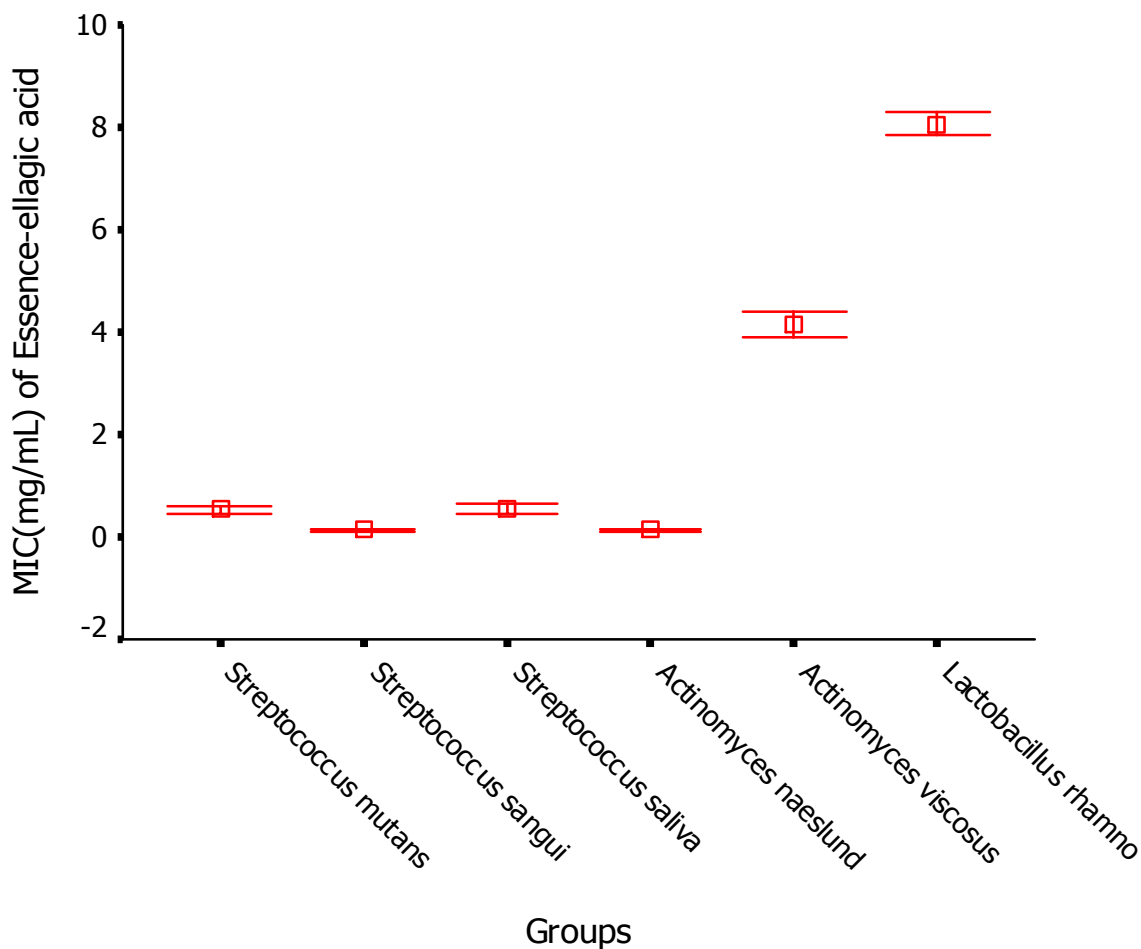
Figure 2. The minimal inhibitory concentrations of ellagic acid applied to bacterial strains: *Streptococcus mutans* ATCC 25175, *Streptococcus sanguis* ATCC 10556, *Streptococcus salivarius* ATCC 25975, *Actinomyces naeslundii* ATCC 12104, *Actinomyces viscosus* ATCC 15987, *Lactobacillus rhamnosus* ATCC 53103.

concentrations. The inhibition rates were more than 50% for 0. 25 to 8 mg/ml.

Inhibitory effect on water-insoluble glucan generation

The generation of water-insoluble glucan of *S. mutans* was significantly reduced by ellagic acid. The optical density reading determined by the Anthrone method for the control group of *S. mutans* was 4.38. The readings for ellagic acid at concentrations 0.125, 0.25, 0.5 and 1 mg/ml were 3.32, 2.88, 2.27 and 1.25, respectively. Statistically significant difference from the control group was observed for the above concentrations (P<0.05). 100% inhibition was observed for concentrations 2, 4, 8 mg/ml. However, no statistically significant difference was seen for the other bacterial strains.

Bacterial adherence to S-HA

The adhesion of *S. mutans* cells to S-HA was significantly

inhibited by pretreating the S-HA with the ellagic acid. The number of cells bound to the bead for the control group was 2457. Those for ellagic acid at concentrations 0.5, 1, 2 and 4 mg/ml were 1545, 1309, 544 and 396, respectively. Statistically significant difference was observed for the above concentrations (P<0.05) and the adhesion inhibition rate was 100%. Again, no statistically significant difference from the control group was seen for the other bacterial strains although the number of cells bound to the beads was less when pre-treated with ellagic acid.

DISCUSSION

Dental caries is one of the most common oral disease worldwide. It is clear that cariogenic microorganisms, especially *S. mutans* plays essential role in the patho-genesis of dental caries. It is involved in the initiation of almost all carious lesions in enamel (Samaranayake, 1996). The prevention of caries focused on antimicrobial agents against cariogenic bacteria. This is achieved by

inhibiting adhesion of cariogenic microorganisms on the tooth surface, and *S. mutans* from forming water-insoluble glucan (Samaranayake, 1996). Chemical agents such as fluoride and chlorhexindine, which have been used to prevent dental caries for several decades, were associated with some side effects such as staining of teeth and fluorosis. Thus, there is no perfect antimicrobial agent to prevent dental caries until now.

The use of natural products has been one of the most successful strategies for the discovery of new drugs (Harvey, 2000). According to Harvey, the access to biodiversity is fundamental to expanding the range of natural products to be used in the search for new drugs (Harvey, 1999). Extensive screening for biologically active compounds from natural sources with these effects has been performed. *Propolis* was reported to have the ability to reduce dental caries in rats (Ikeno et al., 1991). Tea polyphenols inhibited the growth of *S. mutans* as well as the production of insoluble glucans by glucosyltransferases (Nakahara et al., 1993). The methanol extract of the native American plant, *C. americanus,* demonstrated antimicrobial activity against selected oral pathogens (Li et al., 1997).

In the present study, we evaluated the anticariogenic activities of ellagic acid against some cariogenic microorganisms *in vitro* and demonstrated that ellagic acid had inhibitory effects against the tested bacterial strains with MICs ranging from 0.125 to 8 mg/ml (Figure 2). Not only can ellagic acid inhibit cariogenic bacterial growth, but can also inhibits the water-insoluble glucan production of *S. mutans* significantly at MIC. Furthermore, the amount of adhered *S. mutans* to pretreated S-HA with ellagic acid was significantly less than the control. It was, however, noted that *S. mutans* seemed to be more responsive to ellagic acid than the other bacterial strains tested in this study.

The mechanisms for the observed anticariogenic effect are at present not completely understood. The main component of ellagic acid is tannin, which is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution, a property known as astringency. Plant polyphenols have been proved to have many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumor activity, and a wide range of anti-infective actions (Haslam, 1996). One of tannin's molecular actions is to complex with proteins through so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Stern et al., 1996). Thus, the antimicrobial action of ellagic acid may be related to its ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and complex with polysaccharide. There is evidence for direct inactivation of microorganisms: low tannin concentrations modify the morphology of germ tubes of *Crinipellis perniciosa*. Tannins in plants inhibit insect growth and disrupt digestive events in ruminant animals. According to the ultra-low-temperature squeezing

technology, extraction of ellagic acid showed strong inhibitory activity against *Pseudomonas*, *Aeromonas, Alealigenes, Flavobacterium, Vibrio, Photobacterium, Moraxella* and *Chromobacterium* in another study (Li et al., 2003). Polyphenols showed biphasic actions, acting as antioxidants at lower doses, but acting as pro-oxidants at higher doses. The development and progression of oral diseases might be modified by these natural products directly. On the other hand, factors such as interaction with saliva, proline-rich proteins, antioxidants, metals and dental materials may also contribute but renders further research (Sakagami et al., 1999). ATP bioluminescent assay has been used as an additional test to show that ellagic acid suppressed activities in all strains of bacteria among the range of concentrations used in this study. The number of viable cells is quantified by using an enzyme cocktail to link the ATP with luciferase, so that the light emission is proportional to the ATP concentration. In addition it is able to screen a large-scale of samples in a short time. It does not require the replication of bacteria, therefore it has been widely used such as in anticancer drugs screenings (Garewal et al., 1986; Kangas et al., 1984; Sevin et al., 1988) and microbes detection (Mackett et al., 1982; Selan et al., 1992). Therefore, ellagic acid might be a promising compound for the development of antimicrobial agents against oral pathogens in humans. Ellagic acid is potentially useful when applied to candy and chewing gum or oral hygiene regimens such as mouth rinse. The mechanism of the antimicrobial activity of ellagic acid on its caries inhibition ability will be under investigation in our future studies.

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