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Effect of pH on *Galla chinensis* extract's stability and anti-caries properties *in vitro*

Xuelian Huang [a,b,d], *Lei Cheng* [a,b], *R.A.M. Exterkate* [d], *Mingdong Liu* [c], *Xuedong Zhou* [a,b], *Jiyao Li* [a,b,*], *J.M. ten Cate* [d]

[a] *State Key Laboratory of Oral Diseases, Sichuan University, Chengdu, China*
[b] *West China School of Stomatology, Sichuan University, Chengdu, China*
[c] *Department of Analytical Toxicology, West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu, China*
[d] *Department of Cariology Endodontology Pedodontology, Academic Centre for Dentistry Amsterdam (ACTA), University of Amsterdam and Free University Amsterdam, Amsterdam, The Netherlands*

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ABSTRACT

Objectives: Considering that *Galla chinensis* extract (GCE) solution has a low pH, which might dissolve dental enamel, we investigated the effects of elevation of pH on GCE stability, and on its anti-caries properties.

Designs: Stability of GCE solutions, either in H_2O (pH less than 4.0) or when buffered at pH 5.5, 7.0 and 10.0, was assessed from UV–VIS spectra. Inhibition of enamel demineralization was determined in a pH-cycling set up, comprising treatments with either GCE solutions or negative control buffers and acid and neutral buffer immersions. Demineralization was assessed by calcium in the acetate buffers. To determine antimicrobial properties, polymicrobial biofilms were formed after saliva inoculation on glass surfaces which were treated after 48 h. Treatment output parameters were lactic acid formation and viability, the latter by colony forming unit (CFU) counts.

Results: At pH 7.0 and higher GCE solutions changed colour and absorption spectra in UV–VIS, indicative of chemical changes. Regarding enamel demineralization, significant inhibitions ($P < 0.05$) were found for all GCE treatments when compared with corresponding controls. In polymicrobial biofilms, GCE reduced the acid production, compared with the negative controls ($P < 0.05$). However, this difference was only significant at the lower pH values.

Conclusions: GCE solutions were unstable under neutral and alkaline conditions. pH did not significantly influence the inhibiting effect of GCE on enamel demineralization. However, GCE was not effective on polymicrobial biofilms at alkaline pH (8.5). To avoid enamel damage due to acidic treatment, GCE solutions should be used at about pH 5.5.

* *Corresponding author at*: Department of Operative Dentistry and Endodontics, West China College of Stomatology, No. 14, Unit 3, Renmin Nan Road, Chengdu City 610041, Sichuan Province, China. Tel.: +86 28 85501439; fax: +86 28 85582167.
E-mail address: jiyao_li@126.com (J. Li).

Abbreviations: GCE, *Galla chinensis* extracts; TP, total polyphenol; FC assay, Folin-Ciocalteu assay; GAE, gallic acid equivalent; SMH, surface microhardness; KHN, Knoop hardness number; AAS, atomic absorption spectrophotometry; CDR, calcium depletion rate; CFU, colony forming unit.

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1. Introduction

Galla chinensis, a traditional natural Chinese medicine, is rich in hydrolysable tannins and possesses a wide range of biological activities.[1,2] In recent years, our research group has obtained *Galla Chinensis* extract (GCE), which contains substantial quantities of polyphenols (*e.g.* gallotannin, gallic acid).[3] Both in *in vitro* and *in vivo* experiments GCE was shown to inhibit growth and metabolism of caries pathogens, and also to enhance remineralization and inhibit demineralization of enamel.[3–11] The latter was considered the primary mode of action of GCE as anti-caries agent. Other types of polyphenols, such as those occurring in cocoa, coffee, tea, oat hull, hop bract, mainly contain condensed tannins. Their anti-caries mode of action primarily concerns the physiology of caries pathogens.[2,12] Although GCE is a promising agent for effective caries prevention, in aqueous solution it is generally too acidic; for example, the pH of a 4000 ppm GCE solution is lower than pH 4.0. Normally, the pH of the oral fluids is approximately neutral (pH of individual unstimulated saliva varies between 5.8 and 8.0[13]). A pH below about 5.5 could give rise to demineralization of enamel and a lower pH cause dental erosion.[14,15] Therefore, it is suggested to increase the pH of GCE solutions to make them feasible as anti-caries agent.[6]

However, increasing the pH of GCE solutions created various problems: (1) When the pH was increased to 7.0, the solution initially turned light-brown but then rapidly became dark-brown, similar to green tea catechins in Krebs–Ringer bicarbonate buffer.[16] Such dark-brown solutions would stain enamel, and are therefore not suitable for clinical use. (2) At higher pH some of the polyphenol molecules in GCE could break down and lose their efficacy.[16–20] Based on these observations, the following questions should be addressed: could GCE be applied at neutral pH? Will the colour change, indicative of instability, have a negative impact on GCE's anti-caries properties?

The inhibition of both the demineralization of enamel and the growth and fermentation of bacteria in biofilms on teeth are effective strategies in the prevention of dental caries. *In vitro*, the former can be tested in pH-cycling experiments with the rate of demineralization generally evaluated by assessment of mineral loss.[15] The latter can be evaluated in saliva induced polymicrobial biofilms model.[4,21]

The aim of the current study was to analyze the stability of GCE solutions at different pH and to subsequently measure the effects of GCE at different pH on enamel demineralization and on polymicrobial biofilms *in vitro*. The rationale was to determine the optimal (effective and safe) pH-condition for prospective anti-caries applications of GCE.

2. Materials and methods

2.1. *G. chinensis sample*

GCE was extracted as described in previous studies.[3–11] In brief, *G. chinensis* was dried at 60 °C for 3 days, powdered, double extracted with distilled water, dissolved in ethanol and subsequently GCE was recovered by evaporation of the ethanol.

2.2. *Determination of total phenol content*

Total phenols (TP) was determined by Folin-Ciocalteu (FC) assay and expressed as gallic acid equivalents (GAE) based on a gallic acid calibration curve. FC assay was performed in accordance with the method of Singleton and Rossi,[22] which was previously modified to determine the total phenols of *G. chinensis* extracts.[23] Each sample was performed in triplicate. Absorbance values were recorded on a Perkin–Elmer Lambda 35 UV–VIS spectrophotometer (Perkin Elmer, Norwalk, CT, USA).

2.3. *Determination of tannin content*

Tannin content was determined using the method described by Tian,[23,24] calculated as the difference of total phenols of samples before and after precipitation with casein with application of FC assay. Tannin content was also determined as GAE. Each sample was performed in triplicate.

2.4. *Spectrophotometric analysis*

Stability of GCE in H_2O and in pH 5.5, 7.0 and 10.0 buffers was studied from UV–VIS spectra, which were measured at wavelengths ranging from 200 to 700 nm on a UV-1700 spectrophotometer (Shimadzu, Tokyo, Japan). Sodium dihydrogen phosphate and NaOH were mixed in various ratios to prepare buffers at concentration of 0.01 M and at pH 5.5, 7.0, 10.0. The stock solutions (4.0 mg/mL GCE in H_2O or in buffers) were diluted with H_2O or one of the buffers to the concentration of 10 μg/mL and UV–VIS absorption spectra were measured immediately and 1 h, 6 h, 24 h, and 48 h after pH adjustment. In addition, an aliquot of GCE stored for 24 h at pH 10.0 was titrated to pH 7.0 with concentrated HCl, and additional spectra were recorded immediately, and after 2 h, 24 h, 48 h. All samples were analyzed in triplicate.

2.5. *Determination of inhibition of bovine enamel demineralization*

Bovine enamel specimens were prepared and the baseline surface microhardness (SMH) was determined as in previous studies.[5,7,8] Ninety specimens were selected from a batch of 420 specimens, each with baseline SMH values between 388.7 and 436.1 Knoop hardness number (KHN). The specimens were randomly allocated to one of the following 9 treatment groups (each $n = 10$): 1000 ppm NaF solution (positive control), 4000 ppm GCE in H_2O and in pH 5.5, 7.0, 10.0 buffers and each of these buffers without GCE (negative control). All treatment solutions were stored at room temperature for 24 h before performing the pH-cycling experiment.

Next, specimens were pH-cycled following the procedure described in a previous study.[8,25] In brief, after immersion of the specimens in one of the treatment solutions for 5 min, the blocks were placed in an acidic buffer (50 mmol/L acetic acid, 1.5 mmol/L potassium dihydrogen orthophosphate, pH 5.0) for