จุฬาลงกรณ์มหาวิทยาลัย
ทุนวิจัย
กองทุนรัชดาภิเษกสมโภช

รายงานวิจัย

การศึกษาฤทธิ์ต้านเชื้อแบคทีเรียที่ทำให้เกิดโรคปริทันต์อักเสบความเป็นพิษต่อเซลล์ที่ได้จากเนื้อเยื่อเหงือกของคนปกติและฤทธิ์ต้านการอักเสบของสารสกัดจากเปลือกมังคุด

โดย

กิตติ ต.รุ่งเรือง

ตุลาคม 2549

จุฬาลงกรณ์มหาวิทยาลัย

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รายงานผลการวิจัย

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โดย

อ.ทพ.ดร.กิตติ ต.รุ่งเรือง

26 ตุลาคม 2549

กิตติกรรมประกาศ

ผู้วิจัยขอขอบคุณ ทพญ. พิราภรณ์ วิเชียรโรจน์ ภญ. วรทัย แซ่ตัน นายมานพ ปะจันทบุตร และนายนพดล เอี่ยมสอาด ในการจัดเตรียมวัสดุ สารเคมี และในการทำการทดลอง และขอขอบคุณ ผศ.ทพญ. รังสินี มหานนท์ ที่ให้คำแนะนำในงานวิจัยนี้ นอกจากนี้ผู้วิจัยขอขอบคุณ Dr. Schifferle (Department of Periodontics and Endodontics, State University of New York at Buffalo, NY, USA) ที่ให้ *P. gingivalis* Lipopolysaccharide มาใช้ในการทดลอง

ชื่อโครงการวิจัย การศึกษาฤทธิ์ต้านเชื้อแบคทีเรียที่ทำให้เกิดโรคปริทันต์อักเสบ ความเป็นพิษต่อเซลล์ที่ได้จากเนื้อเยื่อเหงือกของคนปกติ และฤทธิ์ต้านการอักเสบของสารสกัดจากเปลือกมังคุด

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เดือนและปีที่ทำวิจัยเสร็จ 30 ธันวาคม 2548

บทคัดย่อ

เป็นที่ทราบกันดีว่าสารสกัดจากเปลือกมังคุดมีคุณสมบัติทางยาหลายอย่าง เช่น ฤทธิ์ต้านแบคทีเรียและเชื้อรา และฤทธิ์ต้านการอักเสบ โรคปริทันต์อักเสบเกิดจากการติดเชื้อแบคทีเรียทำให้เกิดการอักเสบเรื้อรังและการทำลายของอวัยวะปริทันต์ ดังนั้นสารสกัดดังกล่าวอาจพัฒนามาใช้รักษาโรคนี้ได้ วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาความเป็นพิษต่อเซลล์ไฟโบรบลาสต์ที่ได้จากเนื้อเยื่อเหงือกของคนปกติ ฤทธิ์ต้านเชื้อแบคทีเรียที่ทำให้เกิดโรคปริทันต์อักเสบ และฤทธิ์ต้านการอักเสบของสารสกัดจากเปลือกมังคุด การศึกษาความเป็นพิษต่อเซลล์ทำโดยใช้กล้องจุลทรรศน์แบบหัวกลับ และการวิเคราะห์ด้วยเอ็มทีที (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT assay) โดยพบว่าเซลล์ไม่ได้รับอันตรายเมื่อได้รับสารสกัดที่ความเข้มข้นไม่เกิน 200 μg/ml เป็นเวลาถึง 48 ชั่วโมง สารสกัดจากเปลือกมังคุดมีฤทธิ์ต้านเชื้อ *P. gingivalis* แต่ไม่มีผลต่อเชื้อ *A. actinomycetemcomitans* โดยค่าความเข้มข้นต่ำสุดที่มีผลยับยั้งเชื้อ *P. gingivalis* คือ 20 μg/ml ขณะที่ค่าความเข้มข้นต่ำสุดที่มีผลในการฆ่าเชื้อคือ 40 μg/ml การวัดฤทธิ์ต้านการอักเสบของสารสกัดจากเปลือกมังคุด ทำโดยวัดการหลั่งพรอสตาแกลนดินอีสอง (prostaglandin E_2) จากเซลล์โมโนไซต์ (monocyte) ที่ได้รับการกระตุ้นด้วยไลโปพอลิแซ็กคาไรด์ (lipopolysaccharide) จากเชื้อ *P. gingivalis* โดยพบว่าสารสกัดสามารถยับยั้งการหลั่งพลอสตาแกลนดินอีสองได้ โดยมีฤทธิ์มากขึ้นตามความเข้มข้นที่เพิ่มขึ้นและมีฤทธิ์มากที่สุดที่ความเข้มข้น 10 μg/ml การศึกษานี้แสดงให้เห็นว่าสารสกัดจากเปลือกมังคุดมีคุณสมบัติที่อาจนำมาพัฒนาเพื่อใช้ในการรักษาโรคปริทันต์อักเสบได้

Project Title	Antimicrobial activity against periodontopathic bacteria, cell toxicity against gingival fibroblasts and anti-inflammatory effect of crude extract from mangosteen pericarp
Name of the Investigator	Kitti Torrungruang
Year	December 30, 2005

Abstract

Extract from mangosteen pericarp has demonstrated various pharmacological activities including anti-bacterial, anti-inflammatory and anti-fungal. It may have potential for the treatment of periodontal disease, which is a chronic inflammatory disease caused by anaerobic bacteria. The aim of this study was 1) to investigate the toxicity of mangosteen extract to human gingival fibroblast, 2) to examine the anti-bacterial activity of the extract against periodontopathic bacteria including *P. gingivalis* and *A. actinomycetemcomitans*, and 3) to examine the inhibitory effect of the extract on PGE_2 production in lipopolysaccharide (LPS)-activated peripheral blood monocytes. The changes in cell viability were observed by inverted phase contrast microscopy and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The extract was not toxic when exposed to fibroblasts for up to 48 hours at the concentration of 200 μg/ml or less. The extract exhibited anti-bacterial activity against *P. gingivalis*, but not *A. actinomycetemcomitans*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *P. gingivalis* were 20 and 40 μg/ml, respectively. The anti-inflammatory activity of the extract was determined by measuring PGE_2 production with enzyme-linked immunosorbent assay (ELISA). The extract significantly inhibited LPS-induced PGE_2 production in a dose-dependent manner. Its inhibitory effect reached maximal level at 10 μg/ml. These results suggest that the extract from mangosteen pericarp may be beneficial for periodontal treatment.

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Introduction

Periodontal disease is a chronic inflammatory disease caused by a group of anaerobic bacteria in dental plaque. Bacteria that have been implicated in the etiology of periodontal disease include *Porphyromonas gingivalis*, *Tannerella forsythia* (formerly *Bacteroides forsythus*) and *Actinobacillus actinomycetemcomitans*.[1] One of the virulence factors common to these periodontopathic bacteria is lipopolysaccharide (LPS). It has been known to interact with host immune cells and non-immune cells, leading to the release of inflammatory mediators such as prostaglandin E_2 (PGE_2), interleukin-1β, interleukin-6 and tumor necrosis factor-α.[2] Among these, PGE_2 secreted by monocytes/macrophages plays a major role in periodontal destruction. Therefore, elimination of periodontal pathogens and/or suppression of PGE_2 production is a crucial step for management of periodontal disease.

Garcinia mangostana Linn., commonly known as mangosteen, is a fruit tree found in Southeast Asia and South India. It has been used in Thai traditional medicine for treatment of diarrhea, skin infection and chronic wound.[3] Extract from its pericarp have demonstrated various biological activities including anti-bacterial,[4, 5] anti-fungal,[4, 6] anti-inflammatory,[7-9] and anti-allergy activities.[8, 10] Phytochemical studies have shown that its active components belong to a group of xanthone derivatives such as α-, β- and γ-mangostin, gartinin, 1- and 3-isomangostin, etc [11]. For anti-bacterial activity, the extract was active against a wide variety of gram-positive and gram-negative microorganisms including *Staphylococcus aereus* (both normal and methicillin-resistant), *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Enterococcus* species, *Mycobacterium tuberculosis* and *Propionibacterium acnes*.[4, 5, 12-16] Among the xanthones found in the mangosteen extract, α-mangostin possesses the most potent anti-bacterial activity.[4, 5, 12-14] The anti-inflammatory activity of the extract is exerted through inhibition of PGE_2 production. A short-term treatment (10 minutes) with mangosteen extract inhibited Ca^{2+} ionophore-induced PGE_2 release in C6 rat glioma cells.[7, 8] A long-term treatment (18 hours) also decreased spontaneous PGE_2 synthesis in these cells.[9]

Mangosteen pericarp extract has demonstrated low toxicity when given orally or applied topically. Xanthones isolated from mangosteen pericarps were not toxic to rats when given orally at a dose of 100 mg/kg body weight/day for up to 7 days.[17] Another study examined the hepatotoxicity of α-mangostin, a major component of the extract, when administered orally to rats at a high dose (1.5 g/kg body weight). It was found that after 12 hours, increases in serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) activities were much less than those of paracetamol given at the same dose.[18] In human clinical trials, 1.5% α-mangostin cream was locally applied on skin of patients with chronic ulcers for up to 3 weeks. No local irritation or side effects were observed.[19, 20]

From its various biological activities and low toxicity, mangosteen extract may have potential for periodontal therapy. However, its anti-bacterial action on periodontal pathogens and its anti-inflammatory action on LPS-stimulated human monocytes have never been demonstrated. Therefore, the objectives of this study were to determine 1) the *in vitro* cytotoxic effects of mangosteen extract in human gingival fibroblasts, 2) the MIC and MBC and kinetics of killing of mangosteen extract against *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* in comparison with α-mangostin and chlorhexidine, a commonly used antiseptic, and 3) the effect of the mangosteen pericarp extract on the PGE_2 production from LPS-activated human peripheral blood monocytes.

Materials and methods

1. Preparation of mangosteen extract and α-mangostin

Pericarps of mangosteen were collected from Thewate market in Bangkok in July 2003. Crude extract and purified α-mangostin were prepared as previously described.[21, 22] Briefly, dried and ground pericarps were macerated in hexane for 24 hours to remove non-polar substances. The resulting marc was subsequently macerated in ethyl acetate for 24 hours. The ethyl acetate extract was then recrystallized, and ground into powder. The yield of mangosteen extract from the dried pericarp was approximately 3% (w/w).

To obtain α-mangostin, the crude extract was chromatographed on silica gel eluted with hexane followed by a stepwise addition of ethyl acetate. Thin layer chromatography was used to confirm the purity of each isolate. The selected fraction was further identified as α-mangostin by using mass spectrometry, nuclear magnetic resonance spectroscopy and a Gallenkamp melting point apparatus. The yield of α-mangostin from the dried pericarp was approximately 0.4% (w/w).

2. Cytotoxicity test

Gingival fibroblast cell culture

Primary gingival fibroblasts were isolated from gingival biopsies taken during surgical removal of impacted third molars of healthy subjects between 18 and 30 years of age. Informed consent was obtained from the donors prior to the operation. The protocol was reviewed and approved by the Ethical Review Committee of the Faculty of Medicine at Chulalongkorn University.

The gingival tissues were finely minced and placed into tissue culture dishes in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizone (Gibco-Invitrogen, Carlsbad, CA, USA). After incubation for several days at 37 °C in a humidified atmosphere of 5% carbon dioxide in air, fibroblasts migrated out of the tissue pieces and started proliferating. When the cells became confluent, they were

subcultured and maintained under the same conditions. Cells utilized in the experiments were from the fourth passage to the seventh passage.

Cytotoxicity test

Gingival fibroblasts were seeded at a density of 5 x 10^4 cells/well in 24-well plates until they reached 70-80% confluence. Mangosteen extract was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and added to the fresh cell culture medium to obtain the final concentrations of 100, 200, 400, 800 or 1,600 μg/ml. The cells were then exposed to the medium containing extract for 24 or 48 hours. Cells treated with extract-free medium served as a control. DMSO was kept at a final concentration of 0.5% (w/v),[23] which had no significant effect on cell growth (data not shown). After incubation for the specified times, cytotoxicity was assessed by altered cell morphology and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Morphological analysis

To observe morphological changes, the cells were viewed under an inverted phase contrast microscope (Olympus CK2, Olympus Optical Co., Ltd, Tokyo, Japan). Photomicrographs were taken at 200x magnification.

MTT assay

The effect of mangosteen extract on cell viability was determined using the MTT colorimetric assay.[24] MTT (Sigma-Aldrich, St. Louis, MO, USA) was prepared as 5 mg/ml solution in phosphate buffered saline (PBS) and filtered through a 0.22-μm filter (Millipore, Bedford, MA, USA). Prior to use, it was diluted to a final concentration of 0.5 mg/ml. The cell culture medium was replaced with MTT solution, and the cells were further incubated for 4 hours at 37 °C. The MTT solution was also added to some wells in the absence of cells to use as a blank. At the end of incubation period, the MTT solution was removed, and DMSO was added to solubilize the colored products. The optical density (OD) was quantified at an absorbance of 570 nm using a micro-plate reader (Zenyth 200rt, Anthos Labtec Instruments GmbH, Salzburg, Austria). The OD values were corrected for the blank

$$OD_{570} \text{ (sample – blank)}$$
$$OD_{570} \text{ (control – blank)}$$

(background absorbance), and the percentage of cell viability in relation to the control group was calculated as follows:

$$\% \text{ Cell viability} = \frac{\text{OD of treated group}}{\text{OD of control group}} \times 100$$

Each assay was repeated four times.

3. Anti-bacterial activity

Bacterial culture

Bacterial strains used in this study were *P. gingivalis* ATCC 53978 (W50) and *A. actinomycetemcomitans* ATCC 43718 (Y4). *A. actinomycetemcomitans* was cultured in an incubator containing 5-7% carbon dioxide at 37°C. *P. gingivalis* was grown anaerobically in a GasPak system (BBL Microbiology Systems, Cockeysville, MD, USA) at 37°C.

Growth on liquid media: Both bacteria were cultured in trypticase soy broth (BBL Microbiology Systems). The broth for *P. gingivalis* was supplemented with 5% fetal bovine serum (Life Technology, Paisley, Scotland), 5 mg/l hemin (Sigma Chemical Co., St. Louis, MO, USA) and 0.1 mg/l vitamin K (Atlantic Laboratories, Corp., Ltd., Bangkok, Thailand).

Growth on solid media: *P. gingivalis* was grown on Brucella blood agar (BBL Microbiology Systems) supplemented with 5% human whole blood, 5 mg/l hemin and 0.1 mg/l vitamin K. *A. actinomycetemcomitans* was grown on brain heart infusion agar (Difco Laboratories, Detroit, MI, USA).

Identifying MIC

MIC was determined by a broth dilution method. Mangosteen extract or α-mangostin was dissolved in dimethy sulfoxide (DMSO), and subsequent two-fold serial dilutions were performed in the culture medium. Chlorhexidine was used as a positive control, and was serially diluted in a similar fashion. Medium without extract served as a control for bacterial growth. Each tube was inoculated with bacteria obtained during the log phase of growth. The initial density of bacteria was approximately 2×10^6 colony forming units (CFU)/ml for *A. actinomycetemcomitans*, and approximately 5×10^7 CFU/ml for *P. gingivalis*. After 24-h incubation, MIC was recorded as the lowest

concentration that limited the turbidity of the broth to <0.05 at the absorbance of 600 nm. To rule out the effect of DMSO on bacterial growth, media containing serially diluted DMSO were also tested in a similar fashion as the mangosteen extract.

Identifying MBC

MBC was determined by comparing the number of remaining viable bacteria with the initial number of bacteria. All tubes from the MIC experiments that showed no visible turbidity were serially diluted and spread onto agar plates for viable cell counting. The plates were incubated for 48 hours for *A. actinomycetemcomitans* and 72-96 hours for *P. gingivalis*. MBC was then recorded as the lowest concentration that killed at least 99.99% of the initial number of bacteria. All MIC and MBC experiments were repeated three times.

Time-kill kinetics

Time-kill kinetics was determined by the number of remaining viable bacteria at varying time after exposed to the mangosteen extract at the concentrations of two or four times of MBC. After exposed to the extract for 5, 15 and 30 minutes, all samples were diluted at least 10 folds to arrest anti-bacterial activity and reduce carry over. The suspensions were then transferred onto agar plates for viable cell counting. The control broth without extract was served as a control for bacterial growth at each time point. The number of remaining viable bacteria was reported as $\log_{10}$ CFU/ml. Anti-bacterial activity was expressed in term of $\log_{10}$ reduction in CFU/ml, which was calculated as follows:

$$\log_{10} \text{reduction in CFU/ml} = \log_{10} \left[\frac{\text{CFU/ml in control broth}}{\text{CFU/ml in mangosteen extract}} \right]$$

Time-kill curve was plotted as the $\log_{10}$ reduction in CFU/ml against time. The time-kill kinetics of the extract was also compared to that of chlorhexidine at the same concentration. The sensitivity limit of detection was 10^3 CFU/ml. All assays were performed four to five times.

4. Anti-inflammatory activity

Isolation of human peripheral blood monocytes

Human peripheral blood monocytes were isolated using OptiPrep density-gradient medium (Axis-Shield PoC AS, Oslo, Norway) according to the method modified from Graziani-Bowering and her colleagues.[25] Informed consent was obtained from all subjects. The protocol was reviewed and approved by the Ethical Review Committee of the Faculty of Medicine at Chulalongkorn University.

Peripheral blood from healthy subjects aged 18 to 30 years old were collected in a tube containing ethylenediaminetetraacetic acid (EDTA). OptiPrep (density 1.32 g/ml) was diluted with Hepes-buffered saline (HBS) containing 1 mM EDTA and 0.5% bovine serum albumin to obtain 1.078 and 1.068 g/ml solution. The collected blood was mixed with OptiPrep at a ratio of 2.5:1. The mixture was then overlaid with 1.078 g/ml solution, followed by 1.068 g/ml solution and HBS. The tube was centrifuged at 700 ×g for 30 minutes at 4°C with a swinging bucket rotor. The monocytes that floated to the top of the 1.068 g/ml layer were collected. The cells were then resuspended in RPMI 1640 medium (Cambrex, Rockland, Maine, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco-Invitrogen, Carlsbad, CA, USA).

PGE_2 production assay

Highly purified LPS from *P. gingivalis* strain 381 was a generous gift from Dr. Schifferle (Department of Periodontics and Endodontics, State University of New York at Buffalo, NY, USA), and the method of preparation was previously described.[26] Mangosteen extract was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and further diluted in the cell culture medium to obtain the final concentrations of 10, 5, 2.5 and 1.25 μg/ml. Peripheral blood monocytes were seeded at the density of 4×10^5 cells/well in 96-well plates, and allowed to attach for 2 hours. The cells were then stimulated with 1 μg/ml LPS in the presence or absence of the extract. Cells received neither LPS nor extract served as a control. LPS-stimulated cells were also treated with DMSO alone to rule out its effect on PGE_2 production. After incubation at 37°C with 5% CO_2 for 24 hours, supernatant was collected for measurements of PGE_2 levels. The samples were stored at –20°C until use. A competitive enzyme-linked immunosorbent assay (ELISA) was

performed using a commercially available kit (R&D Systems, Inc, Minneapolis, MN, USA). The detection limit was <13.4 pg/ml. The experiments were repeated four times.

5. Statistical analysis

All statistical computations were performed by SPSS (version 10.0; SPSS Inc., Chicago, IL) software. Differences among groups at each time point were analyzed by one-way analysis of variance (ANOVA). Dunnett's or Tukey's test was used for post hoc analysis. The chosen level of significance was $P < 0.05$.

Results

1. Cytotoxicity test

Morphological analysis

After 24-hour incubation, gingival fibroblasts cultured in the control group (extract-free) were spindle-shaped with long cytoplasmic processes and oval-shaped nuclei, and were arranged parallel to one another (Fig. 1A). In the groups treated with 100 and 200 μg/ml mangosteen extract, no changes in cell morphology were observed (Fig. 1C). When the extract concentration increased to 400 μg/ml, cell shape and cell adherence appeared normal, but numerous vacuoles were observed in the cytoplasm (Fig. 1E). At 800 μg/ml, the cells showed the loss of their normal spindle shape conformation. They were rounded up and slightly detached from the plates (Fig. 1G). The changes were more evident in the group treated with 1,600 μg/ml extract (Fig. 1I).

After incubation for 48 hours, fibroblasts in the control group became more confluent (Fig. 1B). In the groups treated with 100 and 200 μg/ml mangosteen extract, the cells remained no change as compared to the control group (Fig. 1D). When the extract concentration increased to 400 μg/ml, some cells started losing their normal spindle shape conformation (Fig. 1F). At 800 μg/ml, the cells were rounded up and detached from the plates (Fig. 1H). In the group treated with 1,600 μg/ml extract, the normal cell morphology was completely lost, and most cells were detached from the plates (Fig. 1J).

MTT assay

Cells treated with 100 and 200 μg/ml mangosteen extract for 24 hours demonstrated slightly higher % cell viability, but was not significantly different from the control group. The extract at a concentration of 400 μg/ml had no significant effect on the cell viability. At the concentrations of 800 and 1,600 μg/ml, cell viability significantly decreased to 37% and 19%, respectively (Fig. 2A).

When treated with 100 and 200 μg/ml mangosteen extract for 48 hours, cell viability remained no significantly different from the control group. However, the extract at a concentration of 400 μg/ml significantly decreased cell viability to 58%. At the concentrations of 800 and 1,600 μg/ml, cell viability further decreased to 23% and 8% of the control group, respectively (Fig. 2B).

2. Anti-bacterial activity

MIC and MBC of mangosteen extract

The results demonstrated that the mangosteen pericarp extract was active against *P. gingivalis*, but not *A. actinomycetemcomitans*. The MIC and MBC of the extract against *P. gingivalis* were 20 and 40 μg/ml, respectively. However, the extract at a concentration as high as 640 μg/ml did not affect the growth of *A. actinomycetemcomitans*. Its anti-bacterial activity was not due to DMSO since DMSO alone did not have a significant effect on the growth of bacteria (data not shown).

The MIC and MBC values of α-mangostin were the same as those of the extract. However, chlorhexidine, a positive control used in this study, was strongly active against all tested organisms. Its MIC and MBC against both *P. gingivalis* and *A. actinomycetemcomitans* were 1.25 and 2.5 μg/ml, respectively.

Time-kill assays

The mangosteen extract at the concentrations of 80 (2x MBC) and 160 (4x MBC) μg/ml were used to study time-kill kinetics of *P. gingivalis* (Table 1). At 5 minutes, the group treated with the extract at 2x MBC showed a slight decrease in viable cell count, while the extract at 4x MBC decreased viable cell count by one order of magnitude. Only the latter group reached statistical significance. At 15 minutes, the group treated with the extract at 2x MBC showed a significant decrease in viable cell count by almost two orders, while the extract at 4x MBC completely killed the bacteria. At 30 minutes or longer, the extract at both concentrations completely killed the bacteria. The time-kill kinetics of chlorhexidine was similar to that of the extract at the same concentrations. However, the number of remaining viable bacteria was about half an order of magnitude less than that of the extract.

The time-kill curve was plotted as the $\log_{10}$ reduction in CFU/ml of *P. gingivalis* against time (Figure 3). Treatment with the extract at 2x MBC decreased the viable organisms to an undetectable level in 30 minutes, while the extract at 4x MBC completely killed the bacteria in 15 minutes. The anti-bacterial activity of the extract was not significantly different from that of chlorhexidine when compared at the same concentration and at the same time point ($P > 0.05$).

3. Anti-inflammatory activity

The PGE_2 levels of the control group were below the detection limit of the assay. When cells were treated with *P. gingivalis* LPS, PGE_2 was produced at a high level. LPS-activated cells were treated with varying concentrations of mangosteen pericarp extract as shown in Figure 4. At the concentration of 1.25 μg/ml, PGE_2 level was decreased to 80.28% of the LPS group, but the difference was not statistically significant. When the extract concentration was increased to 2.5 μg/ml, PGE_2 production was significantly decreased to 60.45% of the LPS group. At 5 and 10 μg/ml, PGE_2 levels were further decreased to 38.28% and 18.13%, respectively. Increasing the extract concentrations to more than 10 μg/ml did not result in a further increase in PGE_2 inhibition (data not shown).

Discussion

The cytotoxicity test provides useful information in choosing a drug dose that gives the maximal therapeutic response, with the least toxicity. MTT assay and morphological analysis demonstrated that the mangosteen pericarp extract used in this study was not toxic to human gingival fibroblasts at the concentrations of 200 μg/ml or less. At the concentration of 400 μg/ml, the extract did not affect the MTT measurement at 24 hours, but the number of viable cells was significantly declined at 48 hours. However, alteration in cell morphology was observed earlier at 24 hours. At the concentrations of 800 μg/ml and higher, both MTT assay and morphological analysis showed some degrees of cell toxicity. The cell viability was decreased with increasing extract concentrations and increasing exposure time.

The extract from mangosteen pericarp has been known for its inhibitory activity against bacterial pathogens that caused skin infection, diarrhea, tuberculosis and acne.[4, 5, 12-15] In this study, the extract exhibited strong anti-bacterial activity against periodontopathic *P. gingivalis*. Its MIC and MBC were 20 and 40 μg/ml, respectively. These values were higher than those of chlorhexidine. However, Its MIC was comparable or lower than those of extract from other known medicinal plants such as sanguinarine (MIC = 8 μg/ml), or green tea catechins (MIC = 1,000 μg/ml).[27, 28]

Among xanthone derivatives isolated from mangosteen pericarp, α-mangostin has the strongest anti-bacterial activity. Its MIC values were in the range of 1 to 50 μg/ml.[4, 5, 12-15] The mangosteen extract used in this study contained approximately 80% α-mangostin,[21, 22] and its MIC and MBC were found to be equivalent to that of α-mangostin. Therefore, it seemed likely that the anti-bacterial activity of the extract is due to α-mangostin. However, other components of the extract have not yet been identified, and may partly contribute to its activity.

The MBC of mangosteen extract was not greater than two times the MIC, suggesting that it acted bactericidally against this organism. To determine the rates at which bacteria were killed, the time-kill assays were performed for *P. gingivalis*. At the

concentration equivalent to 2x MBC, it reduced the viable counts by almost two orders of magnitude in 15 minutes and completely killed the bacteria within 30 minutes. When the extract concentration was increased to 4x MBC, the viability of the bacteria was completely lost within 15 minutes. These results indicate that the extract possesses strong bactericidal activity against *P. gingivalis*.

Chlorhexidine, a commonly used antiseptic, was used as a positive control in this study. The minimum concentration of chlorhexidine that showed inhibitory activity against *P. gingivalis* was lower than that of the mangosteen extract. However, when used at the concentrations of 2 and 4 times the MBC of the extract, their anti-bacterial activity was not significantly different. It should be noted that the concentrations used in the latter study were at 32x and 64x MBC of chlorhexidine. It could be speculated that with increasing concentrations, the anti-bacterial activity of chlorhexidine increased with slower rate than that of the extract, thus their activity reached the same level at the concentrations used in the time-kill kinetic study.

The extract from mangosteen pericarp has been reported to exert anti-inflammatory activity. A short-term treatment (10 minutes) of rat glioma cells with γ-mangostin or crude extract reduced Ca^{2+} ionophore-induced PGE_2 release from rat glioma cells.[7, 8] An 18-hour treatment also inhibited spontaneous PGE_2 release in a concentration-dependent manner.[9] In this study, the mangosteen crude extract inhibited LPS-induced PGE_2 production in human peripheral blood monocytes. Its inhibitory effect was increased with increasing concentrations and reached a maximal level at 10 μg/ml.

Previous studies have demonstrated that the components in mangosteen extract that inhibited PGE_2 synthesis was α- and γ-mangostin.[7, 8] For short-term treatment, the 50% inhibitory concentration (IC_{50}) of γ-mangostin was 5 μM (2 μg/ml).[7] For long-term treatment, the IC_{50} value was 2 μM (0.8 μg/ml).[9] When crude extract using 100%, 70% and 40% ethanol and water were compared, the 40% ethanol extract of mangosteen had the highest inhibitory activity with the IC_{50} value of approximately 8 μg/ml.[8] This extract contained 10% α-mangostin and 12% γ-mangostin. The extract used in this study contained approximately 80% α-mangostin. Its IC_{50} value

was approximately 3 μg/ml. Because of the differences in cell types, PGE_2 stimulants and methods of PGE_2 assay, it is difficult to compare the results between studies. However, the inhibitory activity of the extract used in this study seemed to be consistent with those of previous studies.

Cyclooxygenase (COX) is a key enzyme in PGE_2 biosynthesis, and exists in two isoforms, constitutive (COX-1) and inducible (COX-2). In vitro enzyme assay experiments have demonstrated that α- and γ-mangostin suppressed PGE_2 synthesis by directly inhibiting the activities of both COX-1 and COX-2.[7, 8] Nuclear factor-κB (NF-κB) is a central regulator of the transcription of several inflammatory cytokines and PGE_2. It participates in the transcriptional regulation of COX-2 gene induced by LPS. Inhibitor-κB kinase (IKK) catalyzes inhibitor-κB phosphorylation followed its degradation and the subsequent nuclear translocation of NF-κB, leading a stimulation of NF-κB-mediated transcription. γ-Mangostin has been shown to inhibit IKK activity, which subsequently prevented NF-κB-mediated transcription of COX-2 gene.[9] Therefore, mangosteen extract appeared to inhibit PGE_2 synthesis by blocking COX enzymes at both transcription level and protein level.

Conclusion

Cytotoxicity tests demonstrated that the mangosteen pericarp extract used in this study was not toxic to human gingival fibroblasts at the concentrations of 200 μg/ml or less. It was effective against periodontopathic *P. gingivalis*, at the MIC of 20 μg/ml and the MBC of 40 μg/ml. Its bactericidal activity was comparable to that of chlorhexidine. In addition, it possessed an anti-inflammatory activity by inhibiting of LPS-induced PGE_2 synthesis in human monocytes. These results suggest that the mangosteen pericarp extract may be useful for the treatment of periodontal disease.

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Table 1. The number of remaining viable cells (log_{10} CFU/ml) of *P. gingivalis* after exposed to mangosteen extract or chlorhexidine for 5, 15 and 30 minutes. The results are presented as means ± standard deviations of four to five independent experiments.

time (minutes)	control	Concentration 80 μg/ml		Concentration 160 μg/ml	
		mangosteen	chlorhexidine	mangosteen	chlorhexidine
5	6.40±0.10	6.12±0.30	5.85±0.33	5.21±0.31∗	4.68±0.74∗
15	6.71±0.31	4.97±0.31∗	4.34±0.67∗	0∗	0∗
30	6.91± 0.15	0∗	0∗	0∗	0∗

∗P < 0.01, compared with the control group at the same time point.

Figure legends

Figure 1. Representative photomicrographs showing the morphological changes of human gingival fibroblasts in response to mangosteen pericarp extract. The cells were exposed to the extract at concentrations of 0 (A and B), 200 (C and D), 400 (E and F), 800 (G and H) or 1,600 μg/ml (I and J). The incubation period was 24 (A, C, E, G and I) or 48 hours (B, D, F, H and J). Original magnification x200.

Figure 2. The percentage of cell viability as determined by MTT assay. Human gingival fibroblasts were exposed to mangosteen pericarp extract at varying concentrations for 24 (A) or 48 hours (B). Data are expressed as means ± SE of four independent experiments. ∗Indicates the significant difference from the control at $P < 0.0001$.

Figure 3. Time-kill curve plotted as the $\log_{10}$ reduction in CFU/ml (means ± standard deviations) of *P. gingivalis* against time. The bacteria were treated with mangosteen pericarp extract at 2X and 4X MBC compared to chlorhexidine at the same concentrations.

Figure 4. Effect of mangosteen pericarp extract on LPS-induced PGE_2 release from peripheral blood monocytes. Data are expressed as means ± standard deviations of four independent experiments. ∗Indicates the significant difference from LPS-activated cells without the extract at $P < 0.01$.

Figure 1

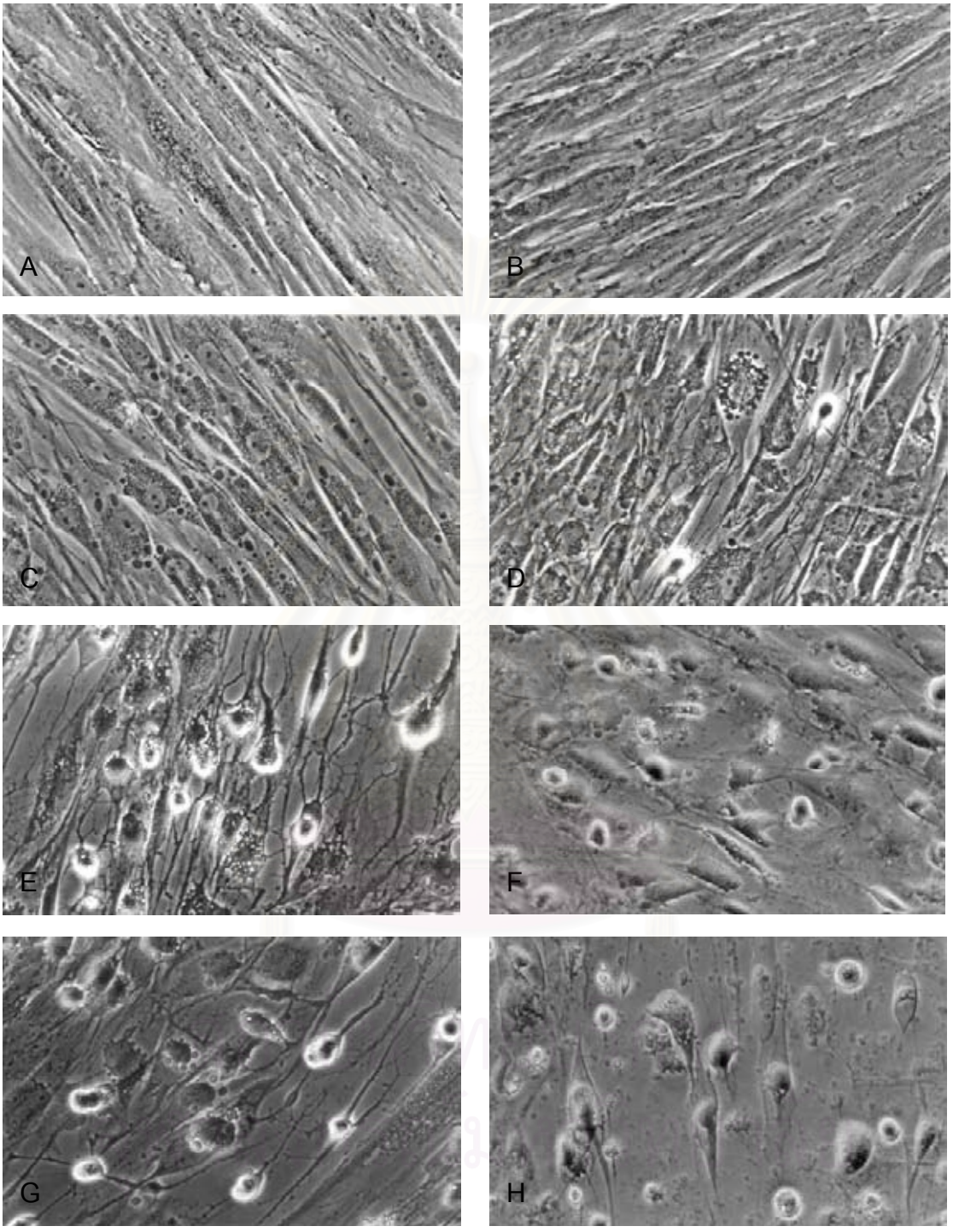
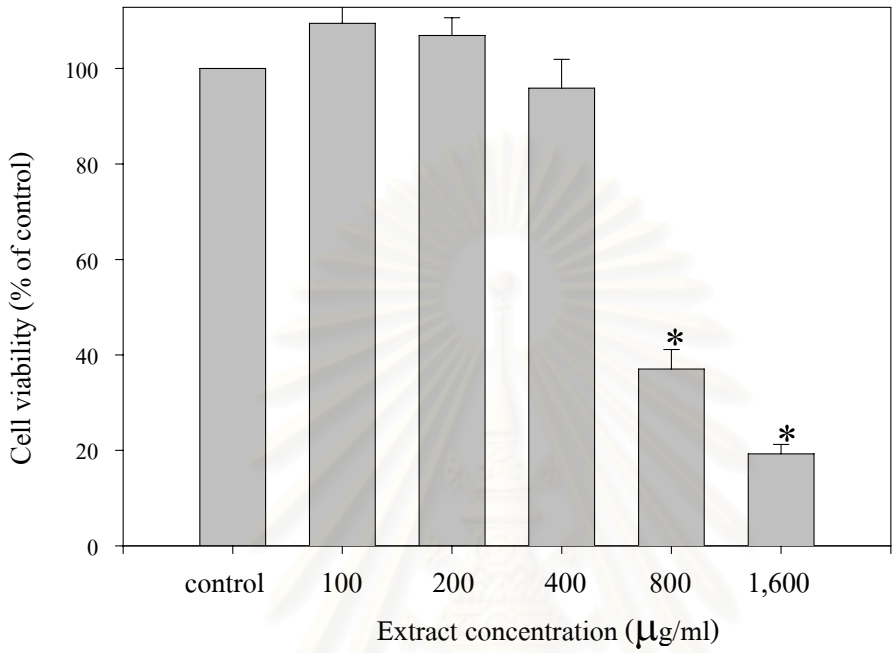


Figure 2

A.


Cell viability (% of control)
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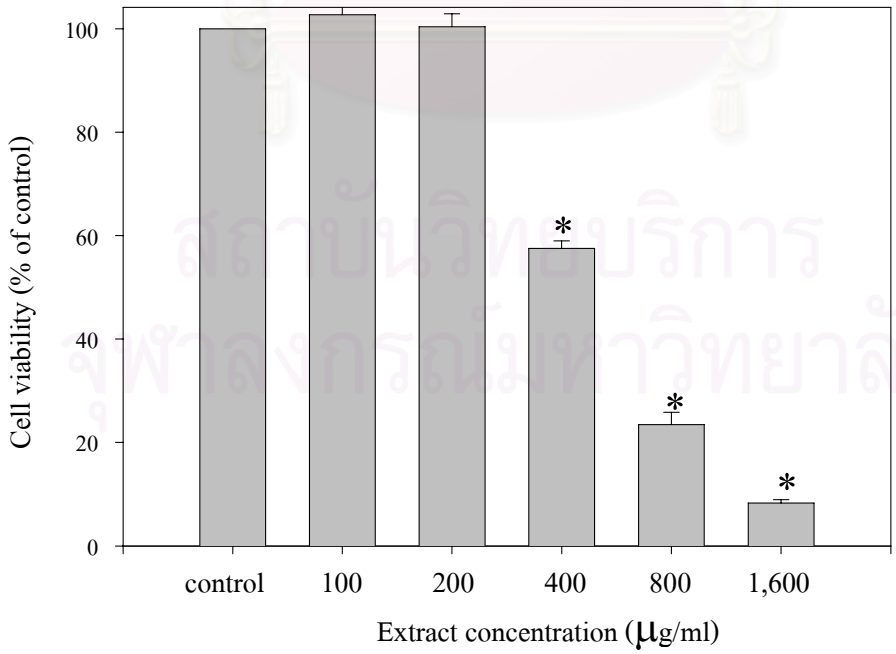
20
40
60
80
100
control
100
200
400
800
1,600
Extract concentration (μg/ml)
*
*

B.


Cell viability (% of control)
0
20
40
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80
100
control
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200
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800
1,600
Extract concentration (μg/ml)
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*
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Figure 3

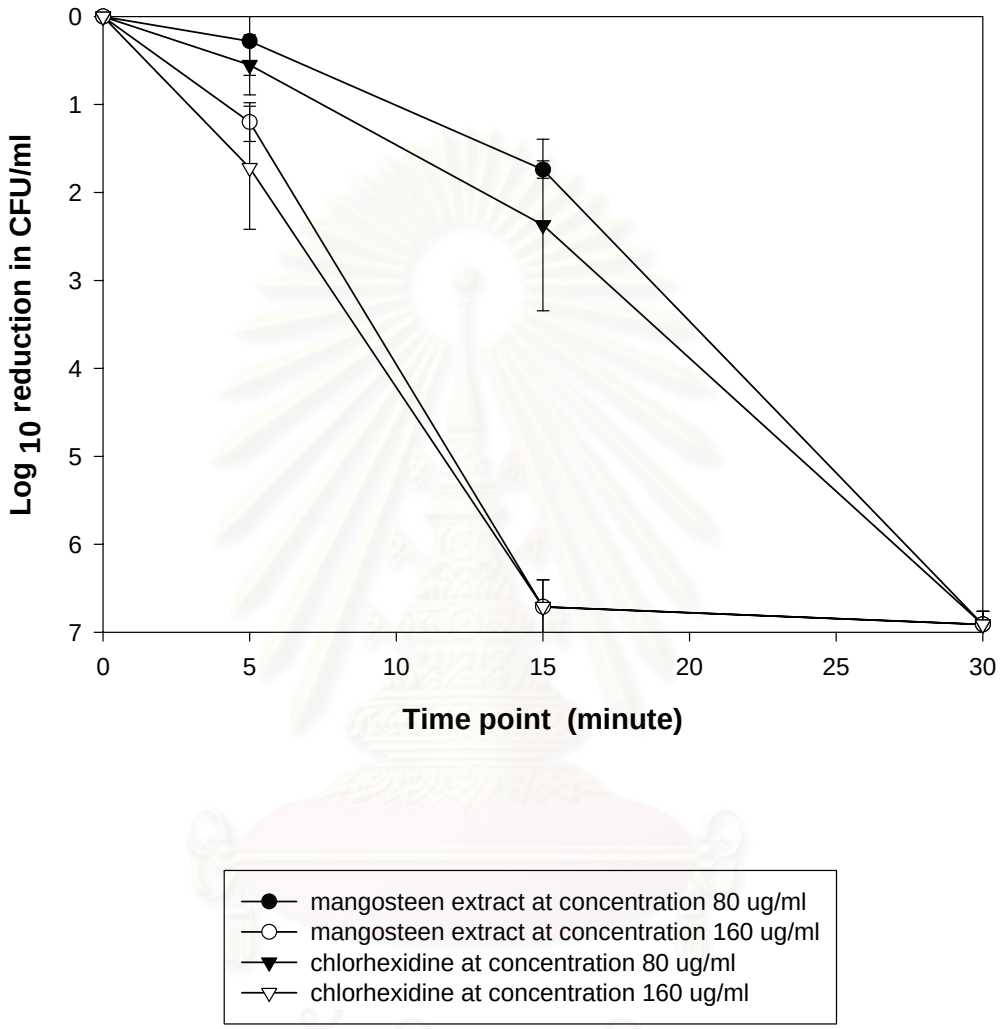

Figure 4