The *In Vitro* Study of Antimicrobial, Antioxidant, Anti-inflammatory Activities and Toxicity of Semi-purified *Anacardium occidentale* Leaf Extract

Sasiwan Lemso

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Oral Health Sciences

Prince of Songkla University

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This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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Candidate

ชื่อวิทยานิพนธ์ การศึกษาในห้องปฏิบัติการฤทธิ์ต้านเชื้อแบคทีเรีย ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านการอักเสบและความเป็นพิษของสารสกัดกึ่งบริสุทธิ์ใบมะม่วงหิมพานต์

ผู้เขียน นางสาวศศิวรรณ เหล็มโส๊ะ

สาขาวิชา วิทยาศาสตร์สุขภาพช่องปาก

ปีการศึกษา 2560

บทคัดย่อ

โรคปริทันต์เป็นโรคที่เกิดจากการติดเชื้อและกระบวนการอักเสบในช่องปากที่มีความชุกมากที่สุดในวัยผู้ใหญ่ ซึ่งเกิดจากความไม่สมดุลของการตอบสนองของร่างกายต่อเชื้อแบคทีเรีย มะม่วงหิมพานต์เป็นพืชที่พบมากทางภาคใต้ของไทย มีการศึกษาพบว่าสารสกัดจากใบของพืชชนิดนี้มีฤทธิ์ต้านเชื้อแบคทีเรียก่อโรคปริทันต์และมีฤทธิ์ต้านการอักเสบ หากทำให้สารสกัดบริสุทธิ์ขึ้นน่าจะทำให้ฤทธิ์เหล่านี้มีประสิทธิภาพที่ดีขึ้น

วัตถุประสงค์: เพื่อศึกษาฤทธิ์ต้านเชื้อแบคทีเรีย ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านการอักเสบและความเป็นพิษต่อเซลล์เนื้อเยื่อเกี่ยวพันเหงือกของสารสกัดกึ่งบริสุทธิ์จากใบมะม่วงหิมพานต์

วัสดุและวิธีการ: สารสกัดใบมะม่วงหิมพานต์ถูกแยกโดย Sephadex LH-20 column ได้สาร 5 ส่วน (ส่วนเอ บี ซี ดี และอี) จากนั้นนำสารไปคัดกรองการต้านเชื้อพอร์ไฟโรโมแนสจินจิวาลิส ซึ่งเป็นเชื้อก่อโรคปริทันต์ด้วยวิธี agar diffusion และเลือกสาร 2 ส่วนที่มีบริเวณการยับยั้ง (clear zone) กว้างที่สุดทดสอบหาความเข้มข้นต่ำสุดของสารที่สามารถยับยั้งการเจริญเติบโตของเชื้อแบคทีเรีย (เอ็มไอซี) ด้วยวิธี microdilution ทดสอบคุณสมบัติการทำลายอนุมูลอิสระดีพีพีเอช ทดสอบฤทธิ์ต้านการอักเสบต่อเซลล์เนื้อเยื่อเกี่ยวพันเหงือกที่กระตุ้นการอักเสบด้วยไลโปโพลีแซคคาไรด์ เข้มข้น 5 และ 10 ไมโครกรัมต่อมิลลิลิตร และใส่สารสกัดที่ เอ็มไอซี 2 และ 4 เท่าของเอ็มไอซี วัดระดับพรอสตาแกลนดินอี$_2$ ด้วยวิธี ELISA และทดสอบความเป็นพิษต่อเซลล์ด้วยวิธี MTT colorimetric assay

ผลการทดลอง: พบว่าสารสกัดกึ่งบริสุทธิ์ส่วนเอและส่วนซี มีบริเวณการยับยั้งกว้างที่สุด และมีค่าความเข้มข้นต่ำสุดของสารที่สามารถยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียที่ 1.25 และ 2.5 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ พบว่าค่าการต้านอนุมูลอิสระ (EC_{50}) ของสารส่วน

ซีดีกว่า กรดแอสคอร์บิก (ตัวแปรควบคุม) และสารสกัดส่วนเอ ที่ 1.33 ± 0.03, 2.17 ± 0.04, และ 17.33 ± 0.51 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ สารสกัดที่เอ็มไอซี 2 และ 4 เท่าของเอ็มไอซี ทั้งสองส่วนมีระดับ พรอสตาแกลนดินอี$_2$ น้อยกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติที่ $p < 0.05$ และมีอัตราการรอดชีวิตของเซลล์ไม่ต่างกับกลุ่มควบคุม

สรุป: สารสกัดกึ่งบริสุทธิ์ใบมะม่วงหิมพานต์ ส่วนเอและส่วนซี มีฤทธิ์ต้านเชื้อแบคทีเรียก่อโรคปริทันต์ ต้านอนุมูลอิสระ และต้านการอักเสบ แต่สารสกัดส่วนเอไม่เป็นพิษต่อเซลล์เนื้อเยื่อเกี่ยวพันเหงือกจึงมีโอกาสนำสารสกัดส่วนนี้ไปพัฒนาเพื่อรักษาโรคติดเชื้อและอักเสบในช่องปากเช่น โรคเหงือกอักเสบ และโรคปริทันต์อักเสบ

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Author	Miss Sasiwan Lemso
Major Program	Oral Health Sciences
Academic year	2017

Abstract

The most prevalence of infectious-inflammatory oral disease in adulthood, which caused by the imbalance of host-bacterial interaction, is periodontal diseases. *Anacardium occidentale*, A common plant in the southern part of Thailand. The previous study found antimicrobial and anti-inflammatory activities of the extract. So if the extract are more purified, it will make these effects more effective.

Objective: The aim of this *in vitro* study was to investigate antimicrobial, antioxidant, anti-inflammatory activities and toxicity of semi-purified *A. occidentale* leaf extract.

Material and methods: Using the Sephadex LH-20 column, the five semi-purified *A. occidentale* fractions were isolated (Fraction A, B, C, D, and E), and then were screened for antibacterial activity against *Porphyromonas gingivalis* by agar diffusion method. Two fractions that gave the widest clear zones were used to determine the minimal inhibitory concentration (MIC) by microdilution method, test their free radical scarvenging property by DPPH free radical. The anti-inflammatory activity was investigate with 5 and 10 µg/ml LPS treated HGFs at MIC, 2 and 4 times of MIC of fraction A and C extracts. PGE_2 levels were determined by ELISA. HGFs toxicity was investigated by MTT colorimetric assay.

Result: Semi-purified *A. occidentale* fractions A and C presented the widest clear zones and their MIC were 1.25 and 2.5 mg/ml, respectively. The free radical scavenging activity (EC_{50} value) of fraction C was significant stronger than ascorbic acid

(control) and fraction A at 1.33 ± 0.03, 2.17 ± 0.04, and 17.33 ± 0.51 μg/ml, respectively. MIC, 2 and 4 times of both fraction presented PGE_2 levels lower than control groups significantly at $p < 0.05$ and the survival rate of HGFs with the extract was not significantly different with the control.

Conclusion: The fraction A and C semi-purified *A. occidentale* leaf extract had antimicrobial antioxidant and anti-inflammatory activities. The fraction A extract was shown non-toxicity to HGFs, so it can be used this fraction as the alternative medicine to treat infectious-inflammatory oral diseases.

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Sasiwan Lemso

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LIST OF ABBREVIATIONS AND SYMBOLS

A. occidentale	*Anacardium occidentale*
S. anacardium	*Semecarpus anacardium*
P. gingivalis	*Porphyromonas gingivalis*
T. denticola	*Treponema denticola*
T. forsythia	*Tannerella forsythia*
A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
B. subtilis	*Basillus subtilis*
B. cereus	*Bacillus cereus*
P. aeruginosa	*Pseudomonas aeruginosa*
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. pyogenes	*Streptococcus pyogenes*
S. saprophyticus	*Staphylococcus saprophyticus*
S. sonnei	*Shigella sonnei*
E. coli	*Escherichia coli*
LPS	Lipopolysaccharide
TLR	Toll-like Receptor
IL	Interleukin
TNF	Tumor Necrotic Factor
PG	Prostaglandin
COX	Cyclooxygenase
ROS	Reactive Oxygen Species
O_2^-	Superoxide anion
H_2O_2	Hydrogen peroxide
$^{\bullet}OH$	Hydroxyl radical
CO_2	Carbon dioxide
HGFs	Human Gingival connective tissue Fibroblasts
MIC	Minimal Inhibitory Concentration

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

DMSO	Dimethyl sulfoxide
CFU	Colony Forming Unit
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	Effective Concentration
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
ELISA	Enzyme-linked Immunoabsobant Assay
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium Bromide
PBS	Phosphate Buffer Saline
BHT	Butylhydroxytoluene

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2. Lemso S, Wattanapiromsakul C, and Worapamorn W. The *in vitro* study of antibacterial activity against periodontopathic bacteria and antioxidant activity of semi-purified *Anacardium occidentale* leaf extract. The 28th TSU National Academic Conference 2018. "Research and Innovation for Social Stability, Prosperity and Sustainability"; 2018 May 8-9; BP Samila Beach Hotel, Songkhla, Thailand. Quintessence Publishing; 2018. p. 1252-1259.

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สถาบันวิจัยและพัฒนา
มหาวิทยาลัยทักษิณ วิทยาเขตพัทลุง
ต.บานพราว อ.ป่าพะยอม จ.พัทลุง 93210

15 พฤษภาคม 2561

เรื่อง ตอบรับการตีพิมพ์ผลงานวิจัย ในรายงานการประชุมวิชาการระดับชาติมหาวิทยาลัยทักษิณ (Proceedings)

เรียน นางสาวศศิวรรณ เหล็มโส๊ะ

ด้วยสถาบันวิจัยและพัฒนา มหาวิทยาลัยทักษิณ ร่วมกับสำนักงานคณะกรรมการวิจัยแห่งชาติ (วช.) สำนักงานคณะกรรมการการอุดมศึกษา (สกอ.) สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) สำนักงานพัฒนาการวิจัยการเกษตร (สวก.) สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยี (สวทช.) และสถาบันส่งเสริมการสอนวิทยาศาสตร์และเทคโนโลยี (สสวท.) **กำหนดจัดงานประชุมวิชาการระดับชาติมหาวิทยาลัยทักษิณ ครั้งที่ 28 ประจำปี 2561 ภายใต้หัวข้อ: งานวิจัยและนวัตกรรมเพื่อสังคมที่มั่นคง มั่งคั่ง และยั่งยืน (Research and Innovation for Social Stability, Prosperity and Sustainability) ในระหว่างวันที่ 8-9 พฤษภาคม 2561 ณ โรงแรมบีพี สมิหลา บีช อำเภอเมือง จังหวัดสงขลา** โดยมีวัตถุประสงค์เพื่อเป็นเวทีทางวิชาการให้กับนักวิจัยและนิสิตระดับบัณฑิตศึกษาได้พบปะแลกเปลี่ยนเรียนรู้และประสบการณ์ด้านการวิจัย ตลอดจนเป็นการสร้างเครือข่ายความร่วมมือด้านการวิจัย บัดนี้ คณะกรรมการฝ่ายวิชาการฯ ได้พิจารณาผลงานวิจัยเรียบร้อยแล้ว และขอแจ้งให้ทราบผลการพิจารณา ดังนี้

ผลงาน **การศึกษาฤทธิ์ต้านเชื้อแบคทีเรียและต้านอนุมูลอิสระของสารสกัดกึ่งบริสุทธิ์ใบมะม่วงหิมพานต์ในห้องปฏิบัติการ**

การตอบรับ ตอบรับการตีพิมพ์ในรายงานการประชุมวิชาการระดับชาติมหาวิทยาลัยทักษิณ (Proceedings) ครั้งที่ 28 ประจำปี 2561 และเผยแพร่ออนไลน์ทาง http://mis.sci.tsu.ac.th/tsuconference28/ ภายในวันที่ 14 มิถุนายน 2561

ทั้งนี้ ขอให้ท่านยืนยันการตีพิมพ์ผลงานวิจัยผ่านระบบออนไลน์ ที่เว็บไซต์การประชุมวิชาการระดับชาติฯ ตามรูปแบบที่กำหนดและปรับแก้ไขบทความวิจัย ตามข้อเสนอแนะของผู้ทรงคุณวุฒิ และจัดพิมพ์ให้ถูกต้องตามรูปแบบของการประชุมวิชาการระดับชาติฯ **ภายในวันที่ 26 พฤษภาคม 2561**

จึงเรียนมาเพื่อทราบและดำเนินการ

ขอแสดงความนับถือ

(อาจารย์ ดร.วันลภ ดิษสุวรรณ์)
ผู้อำนวยการสถาบันวิจัยและพัฒนา
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INTRODUCTION

Periodontal diseases, the most prevalence of infectious-inflammatory oral Diseases, [1,2,3] are caused by ecological shift of bacteria in dental biofilm that stimulates the host immune response.[4,5] The inflammatory processes of the diseases start at gingival epithelium and connective tissues known as gingivitis. If these processes are not stopped or received proper clearance, the pathogenesis continues and extends deep into the supporting connective tissue attachment and alveolar bone, the tissue destruction is called periodontitis.[6] Periodontitis has been shown to relate to some specific gram-negative anaerobic bacteria such as *Porphyromonas gingivalis* (*P. gingivalis*),[7,8] *Treponema denticola* (*T. denticola*), *Tannerella forsythia* (*T. forsythia*),[9] and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*).[10,11] Among these, *P. gingivalis* was found in subgingival biofilm of patients with chronic periodontitis up to 85.75%[8] and was reported to involve in the initiation and progression of the periodontal tissue destruction.[10] The outer membrane of all gram-negative bacteria consists of lipopolysaccharide (LPS).[12] LPS consists of a distal polysaccharide (O-antigen), a non-repeating oligosaccharide and a hydrophobic portion known as lipid A (endotoxin). Lipid A is the biological active part of LPS that is responsible to the innate immune system through the stimulation of toll-like receptor 4 (TLR4).[13] This process induces host cells to express pro-inflammatory cytokines such as IL-1, IL-6, IL-8, TNF-α [14,15] and subsequent prostaglandin E_2 (PGE_2) production.[16,17] Prostaglandins are arachidonic acid metabolites by cyclooxygenase (COX-1 and COX-2). While COX-1 is constitutive, COX-2 is stimulated by IL-1, TNF-α and LPS and it induces prostaglandin H_2 to convert to PGE_2.[18] PGE_2 is known to play the important role in inflammatory responses and alveolar bone resorption.[19] It is produced by the host cells such as fibroblasts and macrophages.[18] Patients with periodontitis have a high PGE_2 level and the level will be decreased after patients receive periodontal treatment.[20]

When infection occurs, neutrophils are the first line of defense against bacterial infection by oxygen-dependent (the respiratory or oxidative burst) and oxygen-

independent (lytic and proteolytic enzymes) mechanisms.[21] The oxidative burst causes for overproduction of reactive oxygen species (ROS), that is, neutrophils produce O_2^-, O_2^- can be transformed to hydrogen peroxide (H_2O_2) and H_2O_2 can be transformed to different derivatives such as hydroxyl radical ($^{\bullet}OH$).[22] The imbalance between oxidant and antioxidant activities causes oxidative stress leads to tissue destruction.[23] Patients with periodontitis were shown to reduce antioxidant capacity.[24,25] In contrast, after receiving periodontal treatment [26] or uptake antioxidant agents such as ascorbic acid,[27] alpha-tocopherol (vitamin E),[28] and coenzyme Q_{10},[29,30] the patients increased antioxidant levels.[31]

Figure 1 *Anacardium occidentale* (original picture)

Anacardium occidentale (A. occidentale), popularly known as the cashew, is easily found in the Southern part of Thailand. Many parts of the plant have been used for therapeutic purposes such as leaves and bark are used to treat diarrhea, thrush, ulcers,[32] diabetes mellitus,[33,34] and cancer [35] due to its antimicrobial,[36] antifungal,[37] anti-ulcer,[38,39] antihyperglycemic,[40,41] and antimutagenic effects.[42] The crude extract of cashew leave was shown to have antioxidant and anti-inflammatory properties.[43] In addition, Srisawat (2007) has shown that *A. occidentale* leaf extract had antimicrobial activity against *P. gingivalis, P. intermedia* and *A. actinomycetemcomitans*.[44] It also had a tendency to reduce PGE_2 and was non-toxicity on human gingival fibroblasts. To

enhance the efficacy of *A. occidentale* leaf extract, we aimed to purify the crude extract, and then test for their properties.

OBJECTIVES

The objectives of this study were:

1. To investigate the antimicrobial activity of semi-purified *A. occidentale* leaf extract against *Porphyromonas gingivalis* and determine their minimal inhibitory concentration (MIC)
2. To investigate the antioxidant activity of semi-purified *A. occidentale* leaf extract
3. To study the anti-inflammatory activity and toxicity of semi-purified *A. occidentale* leaf extract on human gingival connective tissue fibroblasts

MATERIALS AND METHODS

1. Preparation of semi-purified *A. occidentale* leaf extract

Cashew leaves were washed and dried at 50°C for 48 hours. The leaves weight 100 g were macerated with ethanol 300 ml. for 3 days. Filtrated supernatant was concentrated by rotary evaporation to obtain the crude extract 12.61 g. Using Sephadex LH-20 column,[45] the methanol dissolved crude extract was eluted by methanol. All fractions were analyzed by thin-layer chromatography [46] and pulled to give 5 main fractions (fraction A to E).

2. Screening the antibacterial activity against *P. gingivalis* and determination of minimal inhibitory concentration (MIC) of the semi-purified *A. occidentale* leaf extract

Five fractions of the semi-purified *A. occidentale* leaf extract dissolved in 20% dimethylsulfoxide (DMSO) were screened for antibacterial activity against *P. gingivalis* by agar diffusion method.[47] The 500 µl (1 x 10^8 CFU/ml) [48] of *P. gingivalis* W80 were added to each blood agar plates supplemented with 5 µg/ml hemin and 0.5 µg/ml vitamin K. The extracts were placed into a 6 mm. holes of agar plates and incubated at 37°C in anaerobic condition overnight. The solvent 20% DMSO was used as control. Two fractions that gave the widest inhibition zones were selected and further determined for their minimal inhibitory concentration (MIC) by microdilution method.[49] The agar diffusion and microdilution experiments had been done in separate triplicate tests. The data of clear zone width were shown as the mean ± standard deviation (S.D.).

3. Antioxidant activity

The antioxidant activity of the semi-purified *A. occidentale* leaf extracts, fraction A and fraction C, was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay.[50] Briefly, DPPH solution (6x10^{-5} M) was incubated with an equal volume of fraction A (1-400 µg/ml) and fraction C (0.2-20 µg/ml) in absolute ethanol for 30 minutes in the dark at room temperature. The reduction in the DPPH radical was measured by using 96 well microplates (PowerWaveX, Biotek) spectrophotometric absorbance at 517 nm. Ascorbic acid was used as a positive control.[50] The negative control was prepared

as above, but without the extract or ascorbic acid. All samples were tested in separate triplicate experiments.

DPPH radical scavenging activity was presented in term of % inhibition, which was calculated as follows:[51]

$$\% \text{ inhibition} = [(AC - AS)/AC] \times 100$$

Where AC was the absorbance of the control reaction (containing all reagents except the semi-purified *A. occidentale* leaf extract and ascorbic acid) and AS was the absorbance of the semi-purified *A. occidentale* leaf extract fraction A, fraction C and ascorbic acid. The effective concentration of sample required to scavenging DPPH by 50% (EC_{50} value) obtained by linear regression analysis of dose responds curve plotting between % inhibition and concentration. The EC_{50} values were shown as the mean ± S.D.

4. Human gingival connective tissue fibroblasts culture

Human gingival connective tissue fibroblasts (HGFs) culture was performed by using the method from the previous study with some modification.[52] Briefly, HGFs were derived from the explants of healthy gingiva from gingival surgery or gingival remnant from extracted sound tooth. Each biopsy was transported in sterile DMEM pH 7.2. The biopsy was washed extensively and cut into size 1x1 mm/piece, placed in a plastic culture dishes in DMEM pH 7.2 containing deactivated 10% FBS and 100 µg/ml penicillin-streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The media had been replaced every 2 days until confluence, HGFs were split 1:3 and this was denoted as the 1st passage. Cells from the same passage which between their 5th-10th passage were used for the experiments.

5. Anti-inflammatory activity

Semi-purified *A. occidentale* leaf extract fraction A and fraction C at their MIC, 2 times, and 4 times of MIC were evaluated for anti-inflammatory activities against PGE_2 on HGFs. Briefly, HGFs were added to each of 24 well culture plates at 5 x 10^4 cells in 1 ml of DMEM supplemented with 2% FBS and antibiotics, they were incubated at 37^{o}C in humidified atmosphere of 95% air and 5% CO_2 overnight. After that HGFs were treated

with 5 µg/ml and 10 µg/ml LPS of *Escherichia coli* (*E. coli*) (Sigma®, USA) and further incubated for 24 hrs before adding the studied extracts. After overnight incubation, all of the experiment supernatants were collected. The positive controls of these experiments were LPS treated HGFs without the extracts and the negative control was LPS untreated HGFs without the extracts. All samples were tested in separate triplicate experiments.

PGE_2 of the supernatant was measured by a commercial specific enzyme-linked immunoabsorbant assay (ELISA) kit (R&D system, USA). An ELISA reader (Ceres UV 900 HDi, Biotrak Instrument, USA) was used to measure the spectrophotometric absorbance at 450 nm.[53] The data were shown as the mean ± S.D.

6. Cell viability/Cytotoxicity

The cytotoxicity of HGFs was tested using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma®, USA), according to the method described by Verma et al. (2010) [54] and Stockert et al. (2012) [55] with slight modifications. Briefly, MTT solution was prepared at 5 mg/ml in DMEM just before use and filtered through a 0.22 µm filter for sterilization. After removing the supernatant from anti-inflammatory experiment, all of the well culture plates were replaced with MTT solution in DMEM. Cells were incubated for 4 hours at 37°C, after that the medium was removed and the cells culture plates were washed twice with phosphate buffer saline (PBS). The DMSO solution was added to dissolve formazan crystals and mixed to ensure complete solubilization. The absorbance was read at 570 nm with a microplate reader. All samples were tested in separate triplicate experiments. The cells survival rate was calculated as follows:

$$\text{Survival rate (\%)} = [(A_s - A_b)/(A_c - A_b)] \times 100$$

A_s was the absorbance of the test group reaction, A_c was the absorbance of the negative control reaction and A_b was the absorbance of the blank reaction. The data were shown as the mean ± S.D.

7. Statistical analysis

The mean values of all experiments (except MIC) were compared by using a one-way analysis of variance (ANOVA). The *p*-values were considered significant when $p < 0.05$. The MIC was analyzed by descriptive statistic.

RESULTS

1. Screening the antibacterial activity against *P. gingivalis* and determination of minimal inhibitory concentration (MIC) of the extract

The results from agar diffusion method showed that semi-purified *A. occidentale* leaf extract fraction A presented the widest zones of inhibition against *P. gingivalis*. The mean values of three repetitions, analyzed by one-way ANOVA, showed significantly different among the inhibition zone of each fraction of semi-purified *A. occidentale* leaf extract at $p < 0.05$.

The inhibition zones of semi-purified *A. occidentale* leaf extract are shown in table 1.

Table 1 Diameters of the zones of bacterial growth inhibition against *P. gingivalis* of semi-purified *A. occidentale* leaf extract fraction A to E

Fractions of *A. occidentale* leaf extract	Zones of inhibition (cm.) ± S.D.
A	2.72 ± 0.03*
B	1.02 ± 0.08*
C	1.95 ± 0.05*
D	1.33 ± 0.03*
E	1.58 ± 0.03*

The values are expressed as means of 3 repetitions and standard deviations (S.D.)
* means significantly different between each other fraction at $p < 0.05$, analyzed by one-way ANOVA

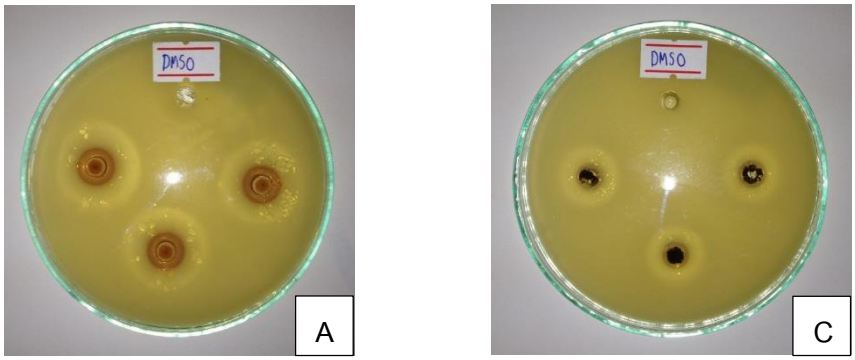

Figure 2 Inhibition zone against *P. gingivalis* of semi-purified *A. occidentale* leaf extract fraction A and fraction C

Fraction A and C that gave the widest inhibition zones (figure 2), subsequently determined for their minimal inhibitory concentrations (MIC) by microdilution method. The results from three repetitions showed their MIC at 1.25 mg/ml and 2.5 mg/ml, respectively.

2. Antioxidant activity (DPPH free radical scavenging activity)

The results of antioxidant activity of semi-purified *A. occidentale* leaf extract fraction A, fraction C and ascorbic acid showed that fraction C provided the highest potential, while fraction A provided the lowest potential. The mean values of three repetitions, analyzed by one-way ANOVA, showed significantly different at $p < 0.05$. EC_{50} value of ascorbic acid, semi-purified *A. occidentale* leaf extract fraction A and fraction C are shown in table 2.

Table 2 EC_{50} value of ascorbic acid, semi-purified *A. occidentale* leaf extract fraction A, and fraction C

Sample	EC_{50} value ± S.D. (µg/ml)
Ascorbic acid	2.20 ± 0.04*
Fraction A	16.88 ± 0.51*
Fraction C	1.29 ± 0.03*

The values are expressed as means of 3 repetitions and standard deviations (S.D.)

* means significantly different between each other sample at $p < 0.05$, analyzed by one-way ANOVA

3. Anti-inflammatory activity and toxicity

3.1 Anti-inflammatory activity

After 24 hrs stimulation, the 5 µg/ml and 10 µg/ml LPS treated HGFs (positive controls) showed statistical different in PGE_2 expression when compared to LPS untreated HGFs (negative control). However, there was not dose dependent of LPS stimulation in term of PGE_2 expression.

After 24 hrs incubation of LPS treated cells with the tests, the results showed that semi-purified *A. occidentale* leaf extract fraction A and C contained anti-inflammatory activities, which demonstrated by PGE_2 reduction, in all studied concentrations (one way ANOVA, significantly different at $p < 0.05$). At the same stimulated level of PGE_2, the fraction A seemed to have better anti-inflammatory activities than the fraction C both at 2.5 and 5 mg/ml, however, there was no significant difference at $p < 0.05$.

The anti-inflammatory activity, which demonstrated by PGE_2 level, of semi-purified *A. occidentale* leaf extract to LPS treated and untreated HGFs is shown in figure 3 and 4.

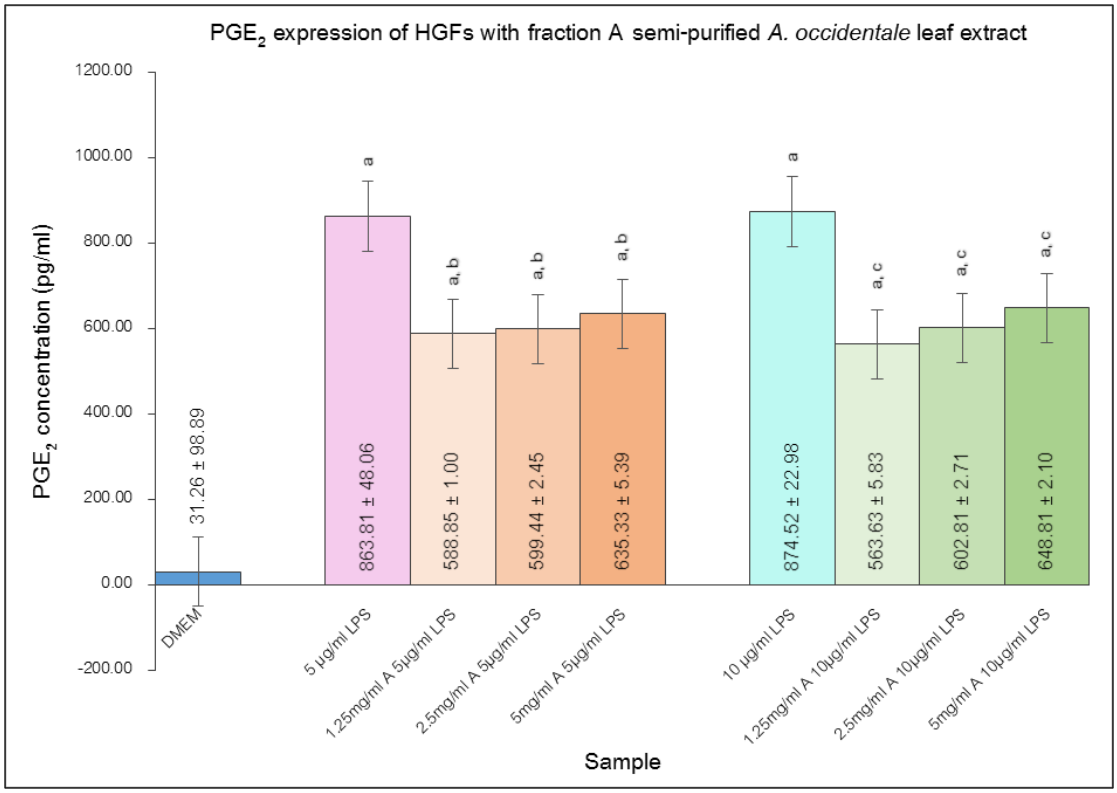

Figure 3 The anti-inflammatory activity graph shows PGE_2 expression of unstimulated HGFs (DMEM; negative control), stimulated with 5 µg/ml and 10 µg/ml LPS without the fraction A extract (positive controls), 5 µg/ml and 10 µg/ml LPS with the fraction A extract at MIC, 2 times, and 4 times of MIC. The values are expressed as means of 3 repetitions and standard deviations (S.D.).

[a] compared to the negative control group significantly different at $p < 0.05$.

[b] compared to 5 µg/ml LPS treated HGFs without the extract significantly different at $p < 0.05$.

[c] compared to 10 µg/ml LPS treated HGFs without the extract significantly different at $p < 0.05$.

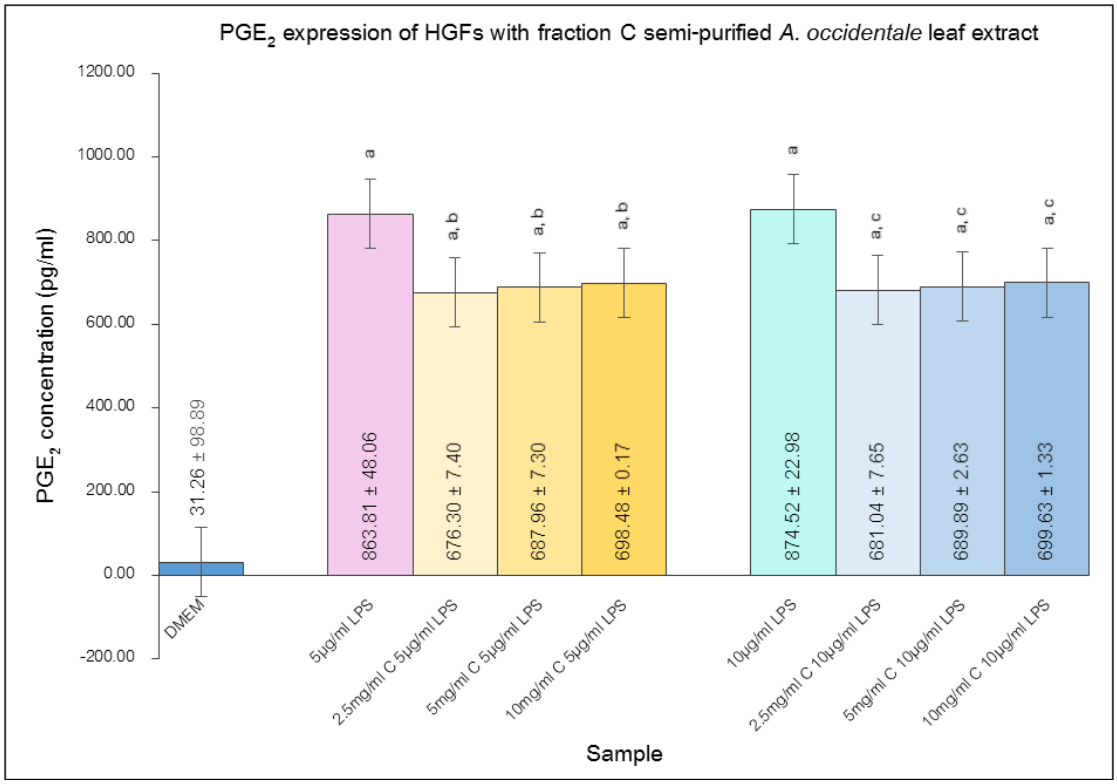

Figure 4 The anti-inflammatory activity graph shows PGE_2 expression of unstimulated HGFs (DMEM; negative control), stimulated with 5 µg/ml and 10 µg/ml LPS without the fraction C extract (positive controls), 5 µg/ml and 10 µg/ml LPS with the fraction C extract at MIC, 2 times, and 4 times of MIC. The values are expressed as means of 3 repetitions and standard deviations (S.D.).

[a] compared to the negative control group significantly different at $p < 0.05$.

[b] compared to 5 µg/ml LPS treated HGFs without the extract significantly different at $p < 0.05$.

[c] compared to 10 µg/ml LPS treated HGFs without the extract significantly different at $p < 0.05$.

3.2 Cell viability/cytotoxicity

The results from MTT assay showed that 1.25 and 2.5 mg/ml, but not 5 mg/ml, of semi-purified *A. occidentale* leaf extract fraction A presented a greater survival rate of HGFs than the controls significantly at $p < 0.05$. While all concentrations of fraction C semi-purified *A. occidentale* leaf extract presented a lower survival rate of HGFs than

the controls. At 2.5 and 5 mg/ml fraction C extracts presented cell survival rate lower than the controls significantly at $p < 0.05$, but at 10 mg/ml of the extract was not significant difference when compared to the control at $p < 0.05$. The results of 1.5625%, 6.25% and 12.5% DMSO, which were at the same concentration used to dissolve the tested extracts, also showed no significant difference in the cell survival rate of all concentration of DMSO when compared with the controls at $p < 0.05$. The cell viability of HGFs with semi-purified *A. occidentale* leaf extract and DMSO are shown with percentage of cell survival rate in figure 5.

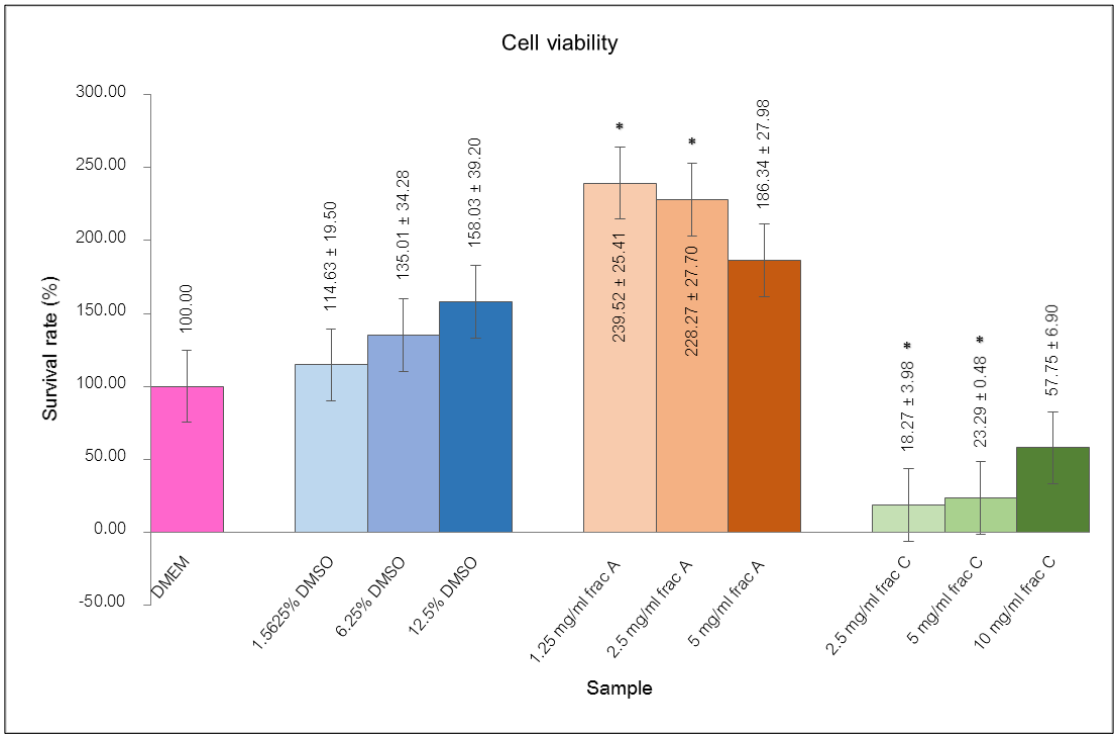

Figure 5 Cells viability graph shows cell survival rate (%) of DMEM (control), DMSO at the same concentration used to dissolve the fraction A and fraction C extracts, the fraction A and fraction C extract at MIC, 2 times, and 4 times of MIC. The values are expressed as means of 3 repetitions and standard deviations(S.D.). * compared to the control significantly different at $p < 0.05$.

DISCUSSION

Currently, people are widely interested in the use of medicinal plants. There have been ongoing researches to gain knowledge and applications of medicinal plants properties to use as an alternative medicine. Among these, many medicinal plants have been used in several forms to control local etiologic factors and in the treatment of periodontal diseases. For examples, *Acacia catechu* contained in dentifrice powder and *Aloe vera* contained in the mouth rinsing had effects on reducing dental biofilm and gingivitis,[56,57] topical gel containing tea tree oil was shown to reduce gingival inflammation,[58,59] as well as mouth rinse containing promegranate and chamomile extracts were shown to reduce gingival bleeding in patients with gingivitis. [60,61]

Srisawat (2007) studied antimicrobial, anti-inflammatory activities and toxicity of the crude extract of medicinal plants such as *A. occidentale* leaves and bark, *Syzygium cumini* leaves and bark, *Punica granatum* pericarp, and *Rhinacanthus nasutus* leaves. The antimicrobial activities were investigated against periodontopathic bacteria such as *P. gingivalis, P.intermedia* and *A.actinomycetemcomitans.* The study found that *A. occidentale* leaves and bark extracts were presented better MIC against *P. gingivalis,* at 1.56 and 0.48 mg/ml respectively, than the other studied plants, however, *A. occidentale* bark was shown toxicity to HGFs.[44] Moreover, the study of Anacardiaceae family including many parts of *Anacardium excelsum* (leaves, integument, flowers, seed and seed coat) showed its antimicrobial activity against gram positive bacteria such as *Basillus subtilis* (*B. subtilis*),[62] as well as *Semicarpus anacardium (S. anacardium)* nut extract had antimicrobial activity against gram positive and gram negative bacteria including *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*), respectively.[63] The other study of this family, *Schinus lentiscifolius* leaf extract acted as a broad spectrum of antimicrobial activity against gram positive bacteria including *B. subtilis, S. aureus, Staphylococcus epidermidis* (*S. epidermidis*), *Streptococcus pyogenes* (*S. pyogenes*) ,and *Staphylococcus saprophyticus* (*S.*

saprophyticus) and gram negative bacteria including *E. coli*, *P. aeruginosa*, and *Shigella sonnei* (*S. sonnei*).[64]

In this study, the *A. occidentale* leaves were extracted by absolute alcohol. Ojezele and Agunbiade (2013) had shown that the alcoholic extract presented chemical components (tannin, polyphenol, alkaloid, saponin, and oxalate) better than hot water and at room temperature water extract.[65] There are many reports regarding antimicrobial activity of medicinal plant extracts against gram-positive and gram-negative bacteria, for instance, *Mangifera indica* (mango) leaf extract, berry juices, *Maesa lanceolata,* and *Hypericum roeperianum.*[66,67,68] The antimicrobial activities may be related to the presence of tannin, phenol, and flavonoid in the studied plants.[69] Several plants contain phenolic compounds, which the hydroxyl groups on phenolic ring are related to microorganism toxicity by increasing hydroxylation.[65,70] The study of *Olea europpaea* L. (olive) leaf extract was shown phenolic compound and antimicrobial activity against gram positive bacteria including *Bacillus cereus* (*B. cereus*), *B. subtilis* and *S. aureus*, gram negative bacteria including *P. aeruginosa, E. coli* and *Klebsiella pneumonia*, and fungi (*Candida albicans* and *Cryptococcus neoformans*).[71] This point of view is supported by the study of antimicrobial activities against *S. aureus, P. aeruginosa* and *Enterococcus faecalis* of tannin-rich fractions from *Anacardium humile* (Anacardiaceae family, found in the Brazilian Savanna) leaf extract.[72] Moreover, the study of *Schinus terebinthifolius,* member of the same family, was shown to contain alkaloids and flavonoids and these acted as antimicrobial activity against *Agrobacterium tumefaciens* (gram negative bacteria).[73] Although this study did not aim to identify the composition of the extracts, there have been reported that *A. occidentale* leaves and bark contain tannin, polyphenol, alkaloid and saponin.[65] The antimicrobial activity of *A. occidentale* crude extract in the previous study and also in this study, therefore, may come from these compositions. The semi-purified *A. occidentale* leaf fraction A in this study is also shown the MIC against *P. gingivalis* lower than that of the crude extract from the previous study.[44]

Phenolic compound has ability to scavenge free radicals. Flavonoids are the large group of phenolic compound, act as antioxidant including suppression of ROS formation, inhibition of enzymes involved in ROS generation and lipid oxidation.[74] Junior et al, showed that leave of *A. occidentale* and *Myracrodruon urundeuva* contained total phenolic and exhibited free radical scavenging activity similar to butylhydroxytoluene (BHT).[75] In agreement to the study of *Olea ferruginea* Royle fruit extract was shown a high phenolic content [76] and *Tagetes erecta* flower extract was shown free radical scarvenging activity stronger than BHT.[77] Also supported by the study of Anacardiaceae family such as *Lannea barteri* stem bark and root extracts and *S. anacardium* leaf extract which contained polyphenols, flavonoids, and tannins presented free radical scarvenging activity similar to ascorbic acid.[78,79] The study of *A. occidentale* leaf, stem bark, and fruit extract which presented tannin, flavonoid, carotenoid, and total phenolic showed to exhibit free radical scavenging activity similar to ascorbic acid.[80] There was also the previous study reported that anacardic acid, the composition in cashew, inhibited the generation of superoxide and peroxidative activity.[43] The free radical scavenging activity of semi-purified extracts in this study, therefore, may due to the presence of anacardic acid, phenolic, tannin, and flavonoids. It is of interest to note that semi-purified extract fraction C provided stronger potential in free radical scavenging activity than ascorbic acid in this study.

Flavonoids are able to inhibit the expression of mediators in inflammatory processes such as cytokines, chemokines, and prostaglandins.[74] Also saponins may involve in the inhibition of inflammatory mediators such as histamine, serotonin, and prostaglandins.[81] These are supported by the study of flavonoid fraction isolated from *Butea monosperma* stem bark and saponin fraction from *Zizyphus lotus (L.)*, the flavonoid and saponin fractions were shown to reduce the inflammation in carrageenan-induced rat paw edema.[82,83] In addition, tannins from *Bacopa monnieri* (L.) was able to reduce the inflammation in carrageenan and histamine-induced rat paw edema.[84] Meanwhile, polyphenols extract from *Ilex latifolia* thumb was shown to inhibit the release of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) induced by 1 μg/ml LPS stimulated

macrophage cells RAW 264.7.[85] The study of anti-inflammatory activity of *S. anacardium* stem bark extract presented high potential of membrane stabilization heat induced hemolysis in human red blood cell and protein denaturation.[86] Souzo et al (2017), investigated anti-inflammatory property of *A. occidentale* leaf extract in macrophage cells RAW 264.7. The study found that the extract was able to inhibit the release of pro-inflammatory cytokines (IL-1β, TNF-α) induced by 1 μg/ml LPS stimulated macrophage cells RAW 264.7.[43] Srisawat (2007), investigated anti-inflammatory activity of *A. occidentale* leaf extract (at 3.125 and 31.25 mg/ml, 2 and 20 times of MIC) in HGFs treated with 1 μg/ml LPS. The study found that the extract had a tendency to reduce PGE_2.[44] This study shows that all of the studied concentration of fraction A and fraction C *A. occidentale* leaf extracts had PGE_2 level lower than the positive controls significant difference at $p < 0.05$, which demonstrates their anti-inflammatory activities. This anti-inflammatory property of the semi-purified *A. occidentale* leaf extract is better than the crude extract of this plant.

The investigation of cells viability in this study showed that all concentrations of the studied semi-purified *A. occidentale* leaf extracts, except at 2.5, 5 mg/ml of fraction C, had no effects on cell death. Meanwhile at the studied concentrations of DMSO, which was the same concentration used to dissolve in each extract, also showed no effects in cell viability. The previous study of crude *A. occidentale* leaf extract showed the cell survival at 3.125, 78.1, and 156.25 mg/ml for all time-point studied (12, 24, and 48 hours).[44] This means that *A. occidentale* leaf extracts, in crude or semi-purified fraction A form, are safe at their MIC, 2 times, and 4 times of MIC.

Taken together, the semi-purified *A. occidentale* leaf extracts fraction A and C showed antimicrobial property against *P.gingivalis* and had anti-inflammatory activities in term of PGE_2 reduction. The semi-purified *A. occidentale* leaf extract fraction A had no toxic to HGFs but provided weak antioxidant property. In the contrary, the semi-purified *A. occidentale* leaf extract fraction C provided strong antioxidant property but had toxicity to HGFs. Therefore, the semi-purified *A. occidentale* leaf extract fraction A

has the potential to use as an alternative medicine for the treatment of infectious-inflammatory oral diseases.

CONCLUSION

This study shows that the fraction A and C semi-purified *A. occidentale* leaf extracts have antimicrobial, antioxidant and anti-inflammatory activities. The fraction A extract is shown non-toxicity to HGFs. Therefore, the fraction A semi-purified *A. occidentale* leaf extract seems to have potential to use for alternative medicine in the prevention and/or treatment of infectious-inflammatory diseases such as gingivitis and periodontitis.

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APPENDICES

Appendix A

Submitted Manuscript

Lemso S, Wattanapiromsakul C, and Worapamorn W. Antimicrobial, antioxidant, anti-inflammatory activities and toxicity of semi-purified *Anacardium occidentale* leaf extract. ***Acad J Med Plant.*** Waiting for publication 2018.

Antimicrobial, antioxidant, anti-inflammatory activities and toxicity of semi-purified *Anacardium occidentale* leaf extract

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ABSTRACT

Periodontal diseases, the most prevalence of inflammatory oral diseases in adulthood, are caused by the imbalance of host-bacterial interaction. Many parts of cashew (*Anacardium occidentale*) plant have been used traditionally for therapeutic purposes such as to treat diarrhea, thrush, ulcers, toothache, gum problem and malaria. The crude extract of cashew leaves was shown to have antimicrobial, antioxidant and anti-inflammatory properties.

Objectives: The aims of this *in vitro* study were to investigate antimicrobial, antioxidant, anti-inflammatory activities and toxicity of semi-purified *A. occidentale* leaf extract.

Materials and methods: Using the Sephadex LH-20 column, the five semi-purified *A. occidentale* fractions were isolated (Fraction A to E), and screened for antimicrobial activity against *Porphyromonas gingivalis* by agar diffusion method. Two fractions that gave the widest clear zones, were further determined for minimal inhibitory concentration (MIC) by microdilution method, test their free radical scarvenging properties by DPPH free radical. The anti-inflammatory activity was investigated with 5 and 10 μg/ml LPS treated human gingival connective tissue fibroblasts (HGFs) at MIC, 2 and 4 times of MIC of the two fractions. PGE_2 levels were determined by ELISA. HGFs toxicity was investigated by MTT colorimetric assay.

Results: Semi-purified *A. occidentale* leaf extract fraction A and C presented the widest clear zones and their MIC were 1.25 and 2.5 mg/ml, respectively. The free radical scavenging activity (IC_{50} value) of fraction C was significant stronger than ascorbic acid (control) and fraction A at 1.29 ± 0.03, 2.20 ± 0.04, and 16.88 ± 0.51 µg/ml, respectively. Both fractions, at their MIC, 2 and 4 times of MIC, presented PGE_2 levels lower than the controls significantly at $p < 0.05$. The cell survival rate of HGFs with fraction A was higher than the controls and fraction C, respectively.

Conclusion: This study shows that the fraction A and C semi-purified *A. occidentale* leaf extracts have antimicrobial, antioxidant and anti-inflammatory activities. The extract fraction A is shown non-toxicity to HGFs. Therefore, the fraction A semi-purified *A. occidentale* leaf extract seems to have potential to use for alternative medicine in the prevention and/or treatment of inflammatory oral diseases such as gingivitis and periodontitis.

Keywords: *A. occidentale*, antimicrobial, *P. gingivalis*, antioxidant, anti-inflammatory, PGE_2

INTRODUCTION

Periodontal diseases, the most prevalence of inflammatory oral diseases in adulthood (World Health Organization, 2003; Frencken et al., 2017), are caused by ecological shift of bacteria in dental biofilm that stimulates the host immune response which can lead to loss of teeth (Johansson and Dahlen, 2018). The inflammatory processes start and locate at gingival epithelium and connective tissues known as gingivitis, if these processes are not stopped or received proper clearance, the pathogenesis continues and extends deep into the supporting periodontal ligament and alveolar bone, the tissue destruction is called periodontitis. Periodontitis has been shown to relate to specific gram-negative anaerobic bacteria such as *Porphyromonas gingivalis* (*P. gingivalis*) (Mysak et al., 2014; How et al., 2016) which was found in subgingival biofilm

of patients with chronic periodontitis up to 85.75% (How et al., 2016). Lipopolysaccharide (LPS) of gram-negative bacteria, through the stimulation of toll-like receptor 4 (TLR4) (Matsuura, 2013), induces host cells to express pro-inflammatory cytokines such as IL-1, IL-6, IL-8, TNF-α (Graves, 2008) and subsequent prostaglandin E_2 (PGE_2) production (Bage et al., 2011). PGE_2 is well known to play the important role in inflammatory responses and alveolar bone resorption (Asum, 2011). Patients with periodontitis have a high PGE_2 level and the level will be decreased after patients receive periodontal treatment (Kumar et al., 2013). Meanwhile, start at the beginning of innate defense against bacterial infection, upon phagocytic mechanism by neutrophils cause oxidative stress, the imbalance between oxidant and antioxidant activities. The overproduction of reactive oxygen species (singlet oxygen, superoxide ion, hydroxyl ion and hydrogen peroxide) leads to tissue destruction (Vincent et al., 2017). The previous studies reported that patients with periodontitis reduce antioxidant capacity (Zhang, 2016) and after receiving periodontal treatment (Yang et al., 2014) or uptake antioxidant agents such as ascorbic acid (Shimabukuro et al., 2015), alpha-tocopherol (vitamin E) (Hatipoglu et al., 2016), and coenzyme Q_{10} (Sale et al., 2014), the patients increased antioxidant levels (Thomas et al., 2014).

Anacardium occidentale (A. occidentale), commonly known as cashew, is a tropical nut tree found widely spread in South America, Asia and Africa. It is a member of genus Anacardium belonging to family Anacardiaceae (Dendena and Corsi, 2014). In traditional medicine, leaves are used to treat toothaches and gum problems and malaria. The bark is used to treat fevers, diabetes, detoxify snake bite as well as to get rid of intestinal parasites (Akinpelu, 2001). Many parts of the plant have been used for therapeutic purposes such as leaves and bark are used to treat diarrhea, thrush, ulcers (Brijyog et al., 2017), diabetes mellitus (Ruby et al., 2007), and cancer (Maity et al., 2015) due to their antimicrobial (Mustapha and Hafsat, 2007), antifungal (Kannan et al., 2009), anti-ulcer (Odo, 2017), antihyperglycemic (Ukwenya et al., 2012), and antimutagenic effects (Barcelos et al., 2007). The crude extract of cashew leaves was shown to have antioxidant and anti-inflammatory properties (Natalia et al., 2017). In addition, Srisawat et

al. (2005), have shown that the crude extract of *A. occidentale* leaves had antimicrobial activity against specific periodontopathic bacteria *P. gingivalis, Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans*. The extract provided a tendency to reduce PGE_2 and non-toxicity on human gingival fibroblasts. In light of a wider understanding of this medicinal plant and for utilizing the extract for preventive and therapeutic use, we aimed to purify the crude extract, and then test for its properties.

MATERIALS AND METHODS

Preparation of semi-purified *A. occidentale* leaf extract

Cashew leaves, collected in Songkhla province in Southern part of Thailand, were washed and oven-dried at 50°C for 48 hours. The grinded leaves weight 100 g were macerated with ethanol 300 ml. for 3 days. Filtrated supernatant was concentrated by rotary evaporation to obtain the crude extract 12.61 g. Using Sephadex LH-20 column (Amersham, 2002), the methanol dissolved-crude extract was eluted by methanol. All fractions were analyzed by thin-layer chromatography (Kumar et al., 2013) and pulled, according to the physical visibility, to give 5 main fractions (fraction A to E).

Screening the antimicrobial activity against *P. gingivalis* and determination of minimal inhibitory concentration (MIC) of the semi-purified *A. occidentale* leaf extract

Five fractions of the semi-purified *A. occidentale* leaf extract, each dissolved in 20% dimethylsulfoxide (DMSO), were screened for antimicrobial activity against *P. gingivalis* by agar diffusion method. The 500 µl (1×10^8 CFU/ml) of *P. gingivalis* W80 were added to each blood agar plates supplemented with 5 µg/ml hemin and 0.5 µg/ml vitamin K. Each extract was placed into a 6 mm hole of agar plates and incubated at 37°C in anaerobic condition overnight. The solvent 20% DMSO was used as control. Two fractions that gave the widest inhibition zones were selected and further determined for their minimal inhibitory concentration (MIC) by microdilution method. The agar diffusion and microdilution experiments had been done in separate triplicate tests. The data of clear zone width is shown as the mean ± standard deviation (S.D.).

Antioxidant activity

The antioxidant activity of the semi-purified *A. occidentale* leaf extracts, fraction A and fraction C, was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay. Briefly, DPPH solution ($6x10^{-5}$ M) was incubated with an equal volume of fraction A (1-400 µg/ml) and fraction C (0.2-20 µg/ml) in absolute ethanol for 30 minutes in the dark at room temperature. The reduction in the DPPH radical was measured by using 96 well microplates (PowerWaveX, Biotek) spectrophotometric absorbance at 517 nm. Ascorbic acid was used as a positive control. The negative control was prepared as above, but without the extract or ascorbic acid. All samples were tested in separate triplicate experiments. DPPH radical scavenging activity was presented in term of % inhibition, which was calculated as follow:

$$\% \text{ inhibition} = [(AC - AS)/AC] \times 100$$

Where AC was the absorbance of the control reaction (containing all reagents except the semi-purified *A. occidentale* leaf extract and ascorbic acid) and AS was the absorbance of the semi-purified *A. occidentale* leaf extract fraction A, fraction C and ascorbic acid. The effective concentration of sample required to scavenging DPPH by 50% (IC_{50} value) obtained by linear regression analysis of dose responds curve plotting between % inhibition and concentration. The IC_{50} values are shown as the mean ± S.D.

Human gingival connective tissue fibroblasts culture

Human gingival connective tissue fibroblasts (HGFs) culture was performed by using the method from the previous study with some modification. This experiment was under the approval of the Ethic committee, Faculty of Dentistry, Prince of Songkla University (REC project No. EC5505-22-L). Briefly, HGFs were derived from the explants of healthy gingiva from gingival surgery or gingival remnant from extracted sound tooth. Each biopsy was transported in sterile DMEM pH 7.2. The biopsy was washed extensively and cut into 1x1 mm/piece, placed in a plastic culture dishes in DMEM pH 7.2 containing deactivated 10% FBS and 100 µg/ml penicillin-streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The media had been replaced every 2 days until confluence, HGFs were split 1:3 and this was denoted as the 1st passage. Cells

from the same passage which between their 5^{th}-10^{th} passage were used for the experiments.

Anti-inflammatory activity

Semi-purified *A. occidentale* leaf extract fraction A and fraction C at their MIC, 2 times, and 4 times of MIC were evaluated for anti-inflammatory activities against PGE_2 on HGFs. Briefly, HGFs were added to each of 24 well culture plates at 5 x 10^4 cells in 1 ml of DMEM supplemented with 2% FBS and antibiotics, they were incubated at 37°C in humidified atmosphere of 95% air and 5% CO_2 overnight. After that HGFs were treated with 5 µg/ml and 10 µg/ml LPS of *Escherichia coli* (*E. coli*) (Sigma®, USA) and further incubated for 24 hrs before adding the studied extracts. After overnight incubation, all of the experiment supernatants were collected. The positive controls of these experiments were LPS treated HGFs without the extracts and the negative control was LPS untreated HGFs without the extracts. All samples were tested in separate triplicate experiments. PGE_2 of the supernatant was measured by a commercial specific enzyme-linked immunoabsorbant assay (ELISA) kit (R&D system, USA). An ELISA reader (Ceres UV 900 HDi, Biotrak Instrument, USA) was used to measure the spectrophotometric absorbance at 450 nm (Kumar et al., 2013). The data is shown as the mean ± S.D.

Cell viability/Cytotoxicity

The cytotoxicity of HGFs was tested using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma®, USA). Briefly, MTT solution was prepared at 5 mg/ml in DMEM just before use and filtered through a 0.22 µm filter for sterilization. After removing the supernatant from anti-inflammatory experiment, all of the well culture plates were replaced with MTT solution in DMEM. Cells were incubated for 4 hours at 37°C, after that the medium was removed and the cells culture plates were washed twice with phosphate buffer saline (PBS). The DMSO solution was added to dissolve formazan crystals and mixed to ensure complete solubilization. The absorbance was read at 570 nm with a microplate reader. All samples were tested in separate triplicate experiments. The cells survival rate was calculated as follow:

$$\text{Survival rate (\%)} = [(A_s - A_b)/(A_c - A_b)] \times 100$$

A_s was the absorbance of the test group reaction, A_c was the absorbance of the negative control reaction and A_b was the absorbance of the blank reaction. The data is shown as the mean ± S.D.

Statistical analysis

The mean values of all experiments (except MIC) were compared by using a one-way analysis of variance (ANOVA). The *p*-values were considered significant when $p < 0.05$. The MIC was analyzed by descriptive statistic.

RESULTS

Screening the antibacterial activity against *P. gingivalis* and determination of minimal inhibitory concentration (MIC) of the extract

The results from agar diffusion method showed that semi-purified *A. occidentale* leaf extract fraction A presented the widest zones of inhibition against *P. gingivalis*. The mean values of three repetitions, analyzed by one-way ANOVA, showed significantly different among the inhibition zone of each fraction of semi-purified *A. occidentale* leaf extract at $p < 0.05$. The inhibition zones of semi-purified *A. occidentale* leaf extract are shown in Table 1.

Fraction A and C that gave the widest inhibition zones, subsequently determined for their minimal inhibitory concentrations (MIC) by microdilution method. The results from three repetitions showed their MIC at 1.25 mg/ml and 2.5 mg/ml, respectively.

Antioxidant activity (DPPH free radical scavenging activity)

The results of antioxidant activity of semi-purified *A. occidentale* leaf extract fraction A, fraction C and ascorbic acid showed that fraction C provided the highest potential, while fraction A provided the lowest potential. The mean values of three repetitions, analyzed by one-way ANOVA, showed significantly different at $p < 0.05$. IC_{50} values of ascorbic acid, semi-purified *A. occidentale* leaf extract fraction A and fraction C are shown in Table 2.

Anti-inflammatory activity

After 24 hrs stimulation, the 5 µg/ml and 10 µg/ml LPS treated HGFs (positive controls) showed statistically different in PGE_2 expression when compared to LPS untreated HGFs (negative control). However, there was not dose dependent of LPS stimulation in term of PGE_2 expression. After 24 hrs incubation of LPS treated cells with the tests, the results showed that semi-purified *A. occidentale* leaf extract fraction A and C contained anti-inflammatory activities, which demonstrated by PGE_2 reduction, in all studied concentrations (one way ANOVA, significantly different at $p < 0.05$). At the same stimulated level of PGE_2, the fraction A seemed to have better anti-inflammatory activities than the fraction C both at 2.5 and 5 mg/ml, however, there was no significant difference at $p < 0.05$.

The anti-inflammatory activities, which demonstrated by PGE_2 level, of semi-purified *A. occidentale* leaf extracts to LPS treated and untreated HGFs are shown in Figure 1 and 2.

Cell viability/cytotoxicity

The results from MTT assay showed that 1.25 and 2.5 mg/ml, but not 5 mg/ml, of semi-purified *A. occidentale* leaf extract fraction A presented a greater survival rate of HGFs than the controls significantly at $p < 0.05$. While at 2.5 and 5 mg/ml of semi-purified *A. occidentale* leaf extract fraction C presented cell survival rate lower than the controls significantly at $p < 0.05$, but at 10 mg/ml was not significant difference. The results of 1.5625%, 6.25% and 12.5% DMSO, which were at the same concentrations used to dissolve the tested extracts, also showed no significant difference in the cell survival rate when compared to the controls at $p < 0.05$. The cell viability of HGFs with the tested extracts and DMSO is shown in Figure 3.

DISCUSSION

Currently, people are widely interested in the use of natural products, especially parts and products of plants, in a variety of medicinal, pharmaceutical, nutraceutical and cosmetic applications. Among these, many medicinal plants have been used in several

forms to control local etiologic factors and in the treatment of periodontal diseases. For example, dentifrice containing *Acacia catechu* and mouth rinse containing *Aloe vera* had effects on reducing dental biofilm and gingivitis (Kala et al., 2015; Vangipuram et al., 2016), topical gel containing tea tree oil reduced gingival inflammation (Soukoulis and Hirsch, 2004), as well as mouth rinse containing promegranate and chamomile extracts were shown to reduce gingival bleeding in patients with gingivitis (Batista et al., 2014). Our serial studies on mouthwash and dentifrice containing *A. occidentale* leaf extract and *Punica granatum* pericarp also demonstrated the reduction in dental biofilm deposition and gingivitis (manuscript in preparation). Towards standardization and understanding which aimed at enhancing their uses, the current effort includes detailed analysis of semi-purification *A. occidentale* leaf extract properties.

Srisawat (2007), studied antimicrobial, anti-inflammatory activities and toxicity of the crude extract of some medicinal plants (*A. occidentale* leaves and bark, *Syzygium cumini* leaves and bark, *Punica granatum* pericarp, and *Rhinacanthus nasutus* leaves). The study reported that *A. occidentale* leaf and bark extracts were presented better antimicrobial properties against *P. gingivalis,* at 1.56 and 0.48 mg/ml respectively, than the other studied plants, however, *A. occidentale* bark was shown toxicity to HGFs. Many previous studies of Anacardiaceae family also supported the plant antimicrobial activities including many parts of *Anacardium excelsum* (leaves, integument, flowers, seed and seed coat) showed their antimicrobial activities against gram positive bacteria such as *Basillus subtilis* (Celis et al., 2011), as well as *Semicarpus anacardium* nut extract had antimicrobial activity against gram positive and gram negative bacteria including *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*), respectively (Mohanta et al., 2007). Most of plants contain phenolic compounds, which the hydroxyl groups on phenolic ring are related to microorganism toxicity by increasing hydroxylation (Ojezele and Agunbiade, 2013; Hintz et al., 2015). As the members in Anacardiaceae family, like other plants, *A. occidentale* leaves contain polyphenols, tannin, alkaloid and saponin (Ojezele and Agunbiade, 2013). The antimicrobial activity of *A. occidentale* crude extract in the previous study and also in this study, therefore, may

come from these compositions. The semi-purified *A. occidentale* leaf extract fraction A was also shown the MIC (1.25 mg/ml) against *P. gingivalis* lower than that of the crude extract from the previous study (1.56 mg/ml) (Srisawat, 2007). The results from using Sephadex LH-20 column, we speculate that phenolic compounds in fraction A which contain the higher molecular weight than that in the fraction C (MIC 2.5 mg/ml) provides the better antimicrobial property. This point of view is supported by the study of antimicrobial activities against *S. aureus, P. aeruginosa* and *Enterococcus faecalis* of tannin-rich fractions from *Anacardium humile* (Anacardiaceae family, found in the Brazilian Savanna) leaf extract (Ferreira et al., 2012). These properties of tannins are based on their chemical structures having two or three phenolic hydroxyl groups on a phenyl ring, in a molecule of moderately large size.

Phenolic compounds contain ability to scavenge free radicals. Flavonoids are the large group of phenolic compounds; act as antioxidant including suppression of ROS formation, inhibition of enzymes involved in ROS generation and lipid oxidation (Kumar and Pandey, 2013). Junior et al, showed that leaves of *A. occidentale* contained total phenolic and exhibited free radical scavenging activity similar to butylated hydroxytoluene (BHT), the synthetic antioxidant (Junior et al., 2007). Also supported by the study of Anacardiaceae family such as *Lannea barteri* stem bark and root extracts and *Semecarpus anacardium* leaf extract which contained polyphenols, flavonoids, and tannins presented free radical scarvenging activity similar to ascorbic acid (Koné et al., 2011; Sheikh et al., 2016; Onuh et al., 2017). The free radical scavenging activity of semi-purified extracts in this study, therefore, may due to the presence of phenolic, tannins, and flavonoids. It is of interest to note that semi-purified extract fraction C, with lower molecular weight than fraction A, provided stronger potential in free radical scavenging activity than ascorbic acid and fraction A in this study. The antioxidant activity of compounds is due mainly to their redox properties, which allow them to act as reducing agents or hydrogen atom donors and that relate to the number and position, o -or p – position, of hydroxyl groups in the molecule (Castellano et al., 2012).

Flavonoids are able to inhibit the expression of mediators in inflammatory processes such as cytokines, chemokines, and prostaglandins (Kumar and Pandey, 2013). Also saponins may involve in the inhibition of inflammatory mediators such as histamine, serotonin, and prostaglandins (Desai et al., 2009). Souzo et al. (2017), investigated anti-inflammatory property of *A. occidentale* leaf extract in macrophage cells RAW 264.7. The study found that the extract was able to inhibit the release of pro-inflammatory cytokines (IL-1β, TNF-α) induced by 1 μg/ml LPS stimulated macrophage cells RAW 264.7. Srisawat (2007), investigated anti-inflammatory activity of *A. occidentale* leaf extract in HGFs treated with 1 μg/ml LPS. The study found that the extract had a tendency to reduce PGE_2. This study showed that all of the studied concentration of *A. occidentale* semi-purified leaf extracts fraction A and fraction C had PGE_2 levels lower than the positive controls significant difference at $p < 0.05$, which demonstrated their anti-inflammatory activities. The anti-inflammatory property of the semi-purified *A. occidentale* leaf extract was better than the crude extract.

The investigation of cells viability showed that all concentrations of the studied semi-purified *A. occidentale* leaf extracts, except at 2.5, 5 mg/ml of fraction C, had no effects on cell death. Meanwhile, at the studied concentrations of DMSO which was the same concentration used to dissolve in each extract, also showed no effects in cell viability. This means that toxicity of semi-purified fraction C, therefore, should be accounted by the extract itself. This point of view emphasizes the argument that isolated or synthesized active compounds may be toxic to human. In the other hand, the previous study of crude *A. occidentale* leaf extract showed the cell survival as high as 156.25 mg/ml for all time-point studied (12, 24, and 48 hrs) (Srisawat, 2007). This may imply that *A. occidentale* crude leaf extract is quite safe and in this study, at least, at 4 times MIC of the semi-purified fraction A.

Taken together, the semi-purified *A. occidentale* leaf extracts fraction A and C showed antimicrobial property against *P.gingivalis* and had anti-inflammatory activities in term of PGE_2 reduction. The semi-purified *A. occidentale* leaf extract fraction A had no toxic to HGFs but provided weak antioxidant property. In the contrary, the semi-purified

A. occidentale leaf extract fraction C provided strong antioxidant property but had toxicity to HGFs. Therefore, the semi-purified *A. occidentale* leaf extract fraction A has the potential to use as an alternative medicine for the treatment of infectious-inflammatory oral diseases. Within the limitation of the study, we concern that there are unaccounted factors, such as growing environment, growing season, climate, temperature, light, soil type, and solvent used which could affect phenolic contents in plants and contribute to their properties.

CONCLUSION

This study shows that the fraction A and C semi-purified *A. occidentale* leaf extracts have antimicrobial, antioxidant and anti-inflammatory activities. The extract fraction A is shown non-toxicity to HGFs. Therefore, the fraction A semi-purified *A. occidentale* leaf extract seems to have potential to use for alternative medicine in the prevention and/or treatment of infectious-inflammatory diseases such as gingivitis and periodontitis.

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Table 1 Diameters of the zones of bacterial growth inhibition against *P. gingivalis* of semi-purified *A. occidentale* leaf extract fraction A to E

Fractions of *A. occidentale* leaf extract	Zones of inhibition (cm.) ± S.D.
A	2.72 ± 0.03*
B	1.02 ± 0.08*
C	1.95 ± 0.05*
D	1.33 ± 0.03*
E	1.58 ± 0.03*
20% DMSO	0.6 ± 0.00

The values are expressed as means of 3 repetitions and standard deviations (S.D.)

* means significantly different between each other fraction at $p < 0.05$, analyzed by one-way ANOVA

Table 2 IC_{50} value of ascorbic acid, semi-purified *A. occidentale* leaf extract fraction A, and fraction C

Sample	IC_{50} value ± S.D. (μg/ml)
Ascorbic acid	2.20 ± 0.04*
Fraction A	16.88 ± 0.51*
Fraction C	1.29 ± 0.03*

The values are expressed as means of 3 repetitions and standard deviations (S.D.)

* means significantly different between each other sample at $p < 0.05$, analyzed by one-way ANOVA

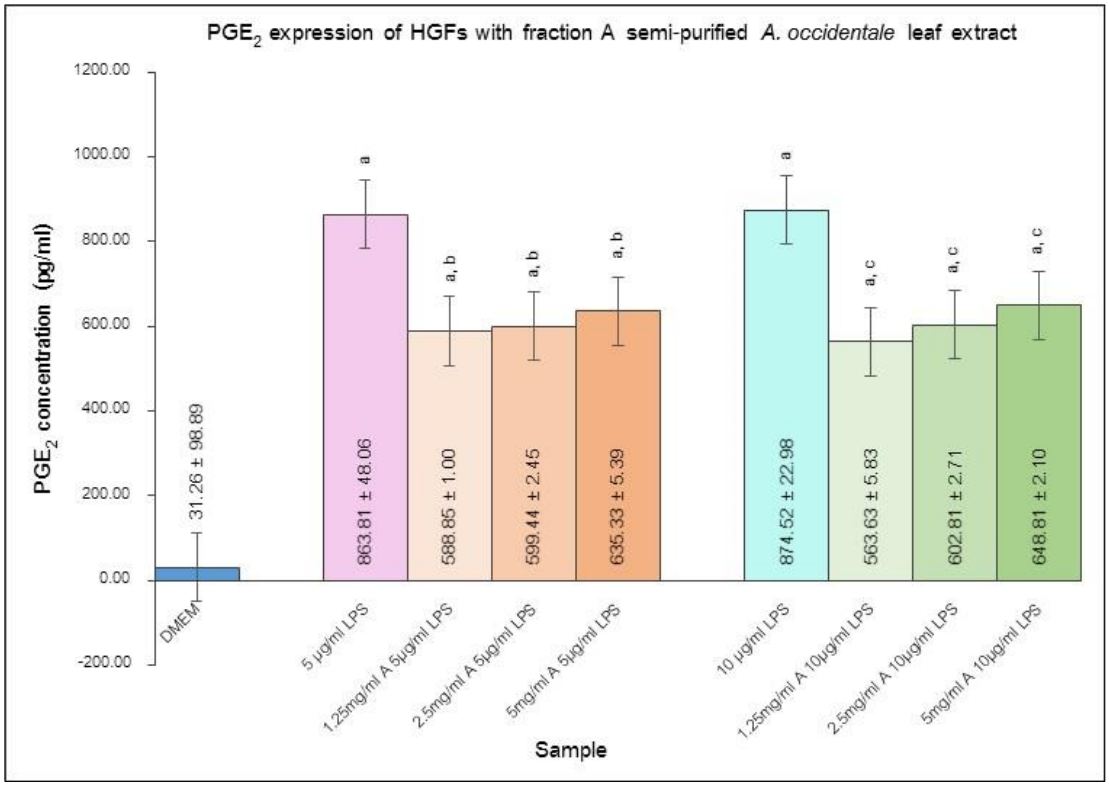

Figure 1 The anti-inflammatory activity graph shows PGE_2 expression of unstimulated HGFs (DMEM; negative control), stimulated with 5 µg/ml and 10 µg/ml LPS without the fraction A extract (positive controls), 5 µg/ml and 10 µg/ml LPS with the fraction A extract at MIC, 2 times, and 4 times of MIC.

The values are expressed as means of 3 repetitions and standard deviations (S.D.).

[a] compared to the negative control group significantly different at $p < 0.05$.

[b] compared to 5 µg/ml LPS treated HGFs without the extract significantly different at $p < 0.05$.

[c] compared to 10 µg/ml LPS treated HGFs without the extract significantly different at $p < 0.05$.

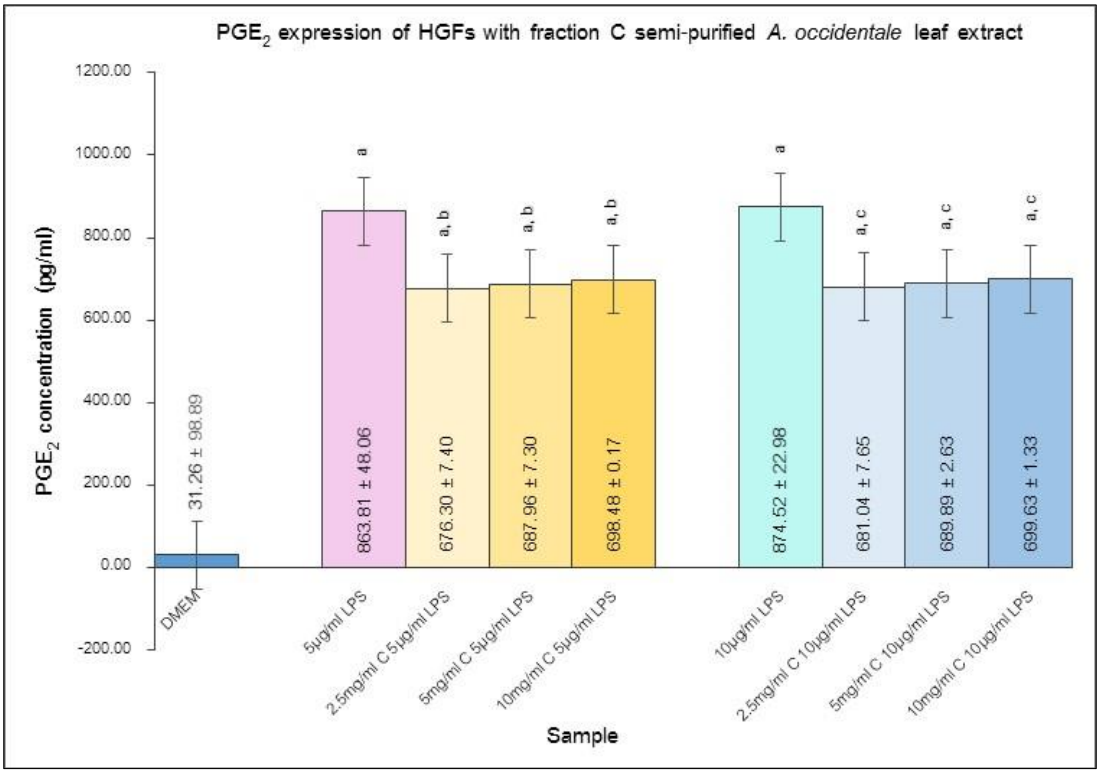

Figure 2 The anti-inflammatory activity graph shows PGE_2 expression of unstimulated HGFs (DMEM; negative control), stimulated with 5 µg/ml and 10 µg/ml LPS without the fraction C extract (positive controls), 5 µg/ml and 10 µg/ml LPS with the fraction C extract at MIC, 2 times, and 4 times of MIC.

The values are expressed as means of 3 repetitions and standard deviations (S.D.).

[a] compared to the negative control group significantly different at $p < 0.05$.

[b] compared to 5 µg/ml LPS treated HGFs without the extract significantly different at $p < 0.05$.

[c] compared to 10 µg/ml LPS treated HGFs without the extract significantly different at $p < 0.05$.

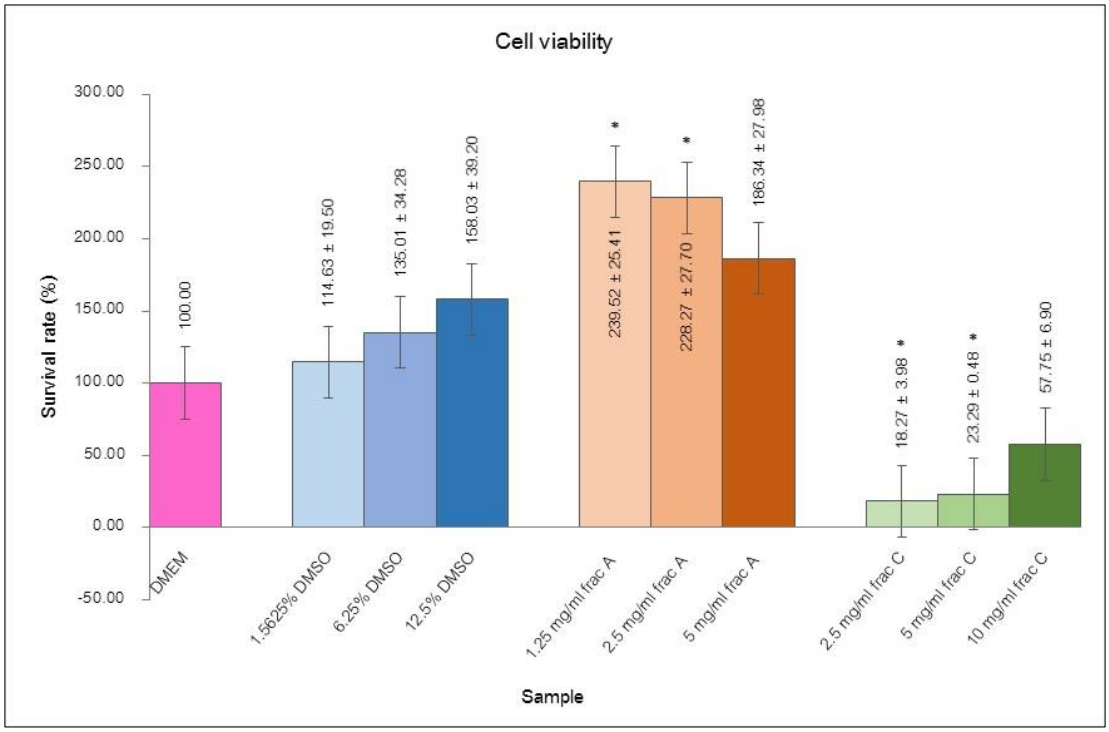

Figure 3 Cells viability graph shows cell survival rate (%) of DMEM (control), DMSO at the same concentration used to dissolve the fraction A and fraction C extracts, the fraction A and fraction C extract at MIC, 2 times, and 4 times of MIC.

The values are expressed as means of 3 repetitions and standard deviations (S.D.).

* compared to the control significantly different at $p < 0.05$.

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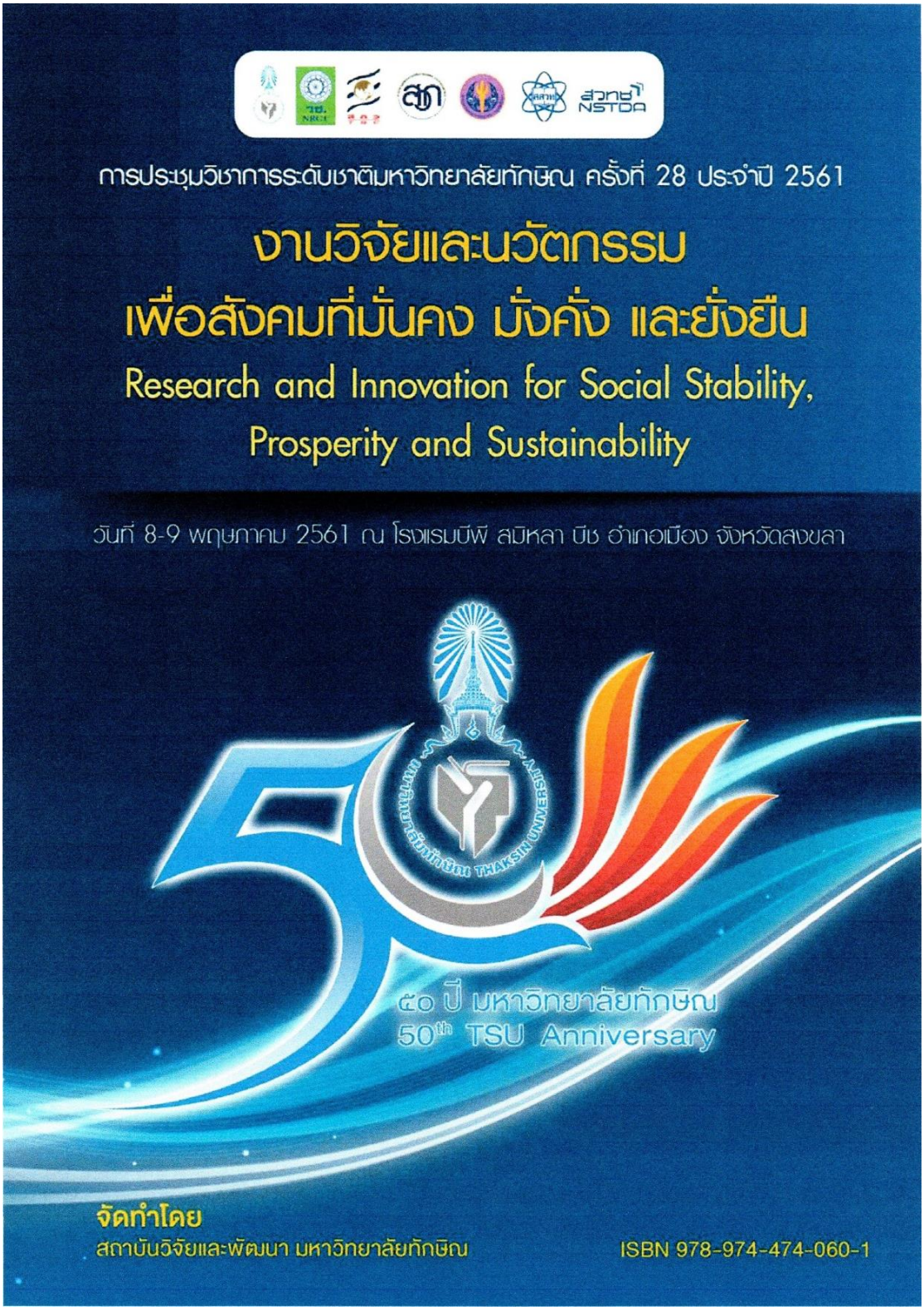
Appendix B

Proceeding

Lemso S, Wattanapiromsakul C, and Worapamorn W. The *in vitro* study of antibacterial activity against periodontopathic bacteria and antioxidant activity of semi-purified *Anacardium occidentale* leaf extract. The 28th TSU National Academic Conference 2018. "Research and Innovation for Social Stability, Prosperity and Sustainability"; 2018 May 8-9; BP Samila Beach Hotel, Songkhla, Thailand. Quintessence Publishing; 2018. p. 1252-1259.


การประชุมวิชาการระดับชาติมหาวิทยาลัยทักษิณ ครั้งที่ 28 ประจำปี 2561
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การนำเสนอผลงานวิจัยภาคโปสเตอร์
Poster Presentations

Session วิทยาศาสตร์สุขภาพ

Session การศึกษา

การศึกษาในห้องปฏิบัติการฤทธิ์ต้านเชื้อแบคทีเรียก่อโรคปริทันต์และฤทธิ์ต้านอนุมูลอิสระของสารสกัดกึ่งบริสุทธิ์ใบมะม่วงหิมพานต์

The *in vitro* study of antibacterial activity against periodontopathic bacteria and antioxidant activity of semi-purified *Anacardium occidentale* leaf extract

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บทคัดย่อ

โรคปริทันต์เป็นโรคในช่องปากที่เกิดจากการติดเชื้อและกระบวนการอักเสบที่มีความซุกมากที่สุดในวัยผู้ใหญ่ ซึ่งเกิดจากความไม่สมดุลของการตอบสนองของร่างกายต่อเชื้อแบคทีเรีย วัตถุประสงค์ของการทดลองนี้เพื่อศึกษาฤทธิ์ต้านเชื้อแบคทีเรียและฤทธิ์ต้านอนุมูลอิสระของสารสกัดกึ่งบริสุทธิ์จากใบมะม่วงหิมพานต์ สารสกัดใบมะม่วงหิมพานต์ถูกแยกโดย Sephadex LH-20 column ได้สาร 5 fraction (fraction A ถึง E) จากนั้นนำสารไปคัดกรองการต้านเชื้อ *Porphyromonas gingivalis* ซึ่งเป็นเชื้อก่อโรคปริทันต์ด้วยวิธี agar diffusion และเลือกสาร 2 fraction ที่มี clear zone กว้างที่สุดทดสอบหาความเข้มข้นต่ำสุดของสารที่สามารถยับยั้งการเติบโตของเชื้อแบคทีเรียด้วยวิธี microdilution รวมถึงทดสอบคุณสมบัติการทำลายอนุมูลอิสระดีพีพีเอช พบว่าสาร fraction A และ C มีระยะ clear zone กว้างที่สุด และมีค่าความเข้มข้นต่ำสุดของสารที่สามารถยับยั้งการเติบโตของเชื้อแบคทีเรียที่ 1.25 และ 2.5 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ พบว่าค่าการต้านอนุมูลอิสระ (EC_{50}) ของสาร fraction C ดีกว่า ascorbic acid (ตัวแปรควบคุม) และ fraction A ที่ 1.29 ± 0.03, 2.20 ± 0.04, และ 16.88 ± 0.51 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ การศึกษานี้แสดงให้เห็นว่าสารสกัดกึ่งบริสุทธิ์จากใบมะม่วงหิมพานต์มีฤทธิ์ต้านเชื้อแบคทีเรียและต้านอนุมูลอิสระ ซึ่งมีโอกาสนำไปพัฒนาเพื่อรักษาโรคติดเชื้อในอนาคต

คำสำคัญ: *A. occidentale* ความเข้มข้นต่ำสุด ต้านเชื้อแบคทีเรีย *P. gingivalis* ต้านอนุมูลอิสระ

Abstract

The most prevalence of infectious-inflammatory oral disease in adulthood which caused by the imbalance of host-bacterial interaction is periodontal diseases. The aim of this *in vitro* study was to investigate antibacterial and antioxidant activities of semi-purified *A. occidentale* leaf extract. Using the Sephadex LH-20 column, the five semi-purified *A. occidentale* fractions were isolated (Fraction A to E), and then were screened for antibacterial activity against *Porphyromonas gingivalis* by agar diffusion method. Two fractions that gave the widest clear zones were used to determine the minimal inhibitory concentration (MIC) by microdilution method as well as their free radical scarvenging property by DPPH free radical. The results showed that semi-purified *A. occidentale* fractions A and C presented the widest clear zones and their MIC were 1.25 and 2.5 mg/ml, respectively. The free radical scavenging activity (EC_{50} value) of fraction C was stronger than ascorbic acid (positive control) and fraction A at 1.29 ± 0.03, 2.20 ± 0.04, and 16.88 ± 0.51 µg/ml, respectively. This study demonstrated the antibacterial and antioxidant activities of semi-purified *A. occidentale* extracts. It is of interest to use these extracts as the alternative medicine.

Keywords: *A. occidentale*, MIC, antibacterial, *P. gingivalis*, antioxidant

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Introduction

Periodontal diseases, the most prevalence of infectious-inflammatory oral diseases [1, 2], are caused by ecological shift of bacteria in dental biofilm that stimulates the host immune response [3]. The inflammatory process of the diseases occur at gingiva is called gingivitis. If this process is not stopped or receives proper clearance, the pathogenesis continues to the supporting connective tissue attachment and alveolar bone, the tissue destruction is called periodontitis [4]. Periodontitis is related to specific gram-negative anaerobic bacteria such as *Porphyromonas gingivalis* (*P. gingivalis*) [5, 6, 7] and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) [7, 8].

When infection occurs, neutrophils are the first line of defense against bacterial infection by oxygen-dependent (the respiratory or oxidative burst) and oxygen-independent (lytic and proteolytic enzymes) mechanisms [9]. The oxidative burst causes for overproduction of reactive oxygen species (ROS), that is, neutrophils produce $O_2^{\cdot}$, $O_2^{\cdot}$ can be transformed to hydrogen peroxide (H_2O_2) and H_2O_2 can be transformed to different derivatives such as hydroxyl radical ($^{\cdot}OH$) [10]. The imbalance between oxidant and antioxidant activities causes oxidative stress leads to tissue destruction [11]. Patients with periodontitis were shown to reduce antioxidant capacity [12, 13] and increase of antioxidant level after periodontal treatment [14] or uptake antioxidant agent such as ascorbic acid [15] and coenzyme Q_{10} [16, 17].

Anacardium occidentale (A. occidentale), popularly known as the cashew, is easily found in the Southern part of Thailand. Many parts of the plant have been used for therapeutic purposes such as leaves and bark are used to treat diarrhea, thrush, ulcers [18], diabetes mellitus [19, 20], and cancer [21] due to its antimicrobial [22, 23], antifungal [24], anti-ulcer [25, 26], antihyperglycemic [27, 28], and antimutagenic effects [29]. In addition, Srisawat (2007) has shown that *A. occidentale* leaf extract had anti-inflammatory

effect and non-toxicity. The crude extracts of cashew leave were shown antioxidant and anti-inflammatory properties [30].

To enhance the efficacy of *A. occidentale* leaf extract, we aimed to purify the crude extract, and then test for their properties.

Objectives

This study aimed to investigate antibacterial and antioxidant activities of semi-purified *Anacardium occidentale* leaf extract

Material and Methods

Preparation of semi-purified *A. occidentale* leaf extract

Cashew leaves were washed and dried at 50°C, 48 hours. The leaves weight 100 g were macerated with ethanol 300 ml. Filtrated supernatant was concentrated by rotary evaporation to obtained crude extract 12.61 g. Dissolved crude extract in methanol and then load into saturated Sephadex LH-20 with methanol. After that, eluted it with methanol. Corrected all fractions to analyse by thin-layer chromatography and combine fractions to give 5 main fractions (fraction A to E).

Screening the antibacterial activity against *P. gingivalis* and determination of minimal inhibitory concentration (MIC) of the extract

All fractions of semi-purified extract were screened for antibacterial activity against *P. gingivalis* at 20 mg/ml by agar diffusion method [31] and two fractions of the extract were shown the widest inhibition zone were selected for experiments. The two fractions of the extracts were determined minimal inhibitory concentration (MIC) by using the microdilution method [32]. The fractions, were tested with agar diffusion method and microdilution method, were prepared in triplicate.

Antioxidant activity

The antioxidant activity of *A. occidentale* leaf extract fraction A and fraction C was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay. Briefly, DPPH solution ($6x10^{-5}$ M) in absolute ethanol was incubated with an equal volume of fraction A (1-400 µg/ml) and fraction C (0.2-20 µg/ml) of *A. occidentale* leaf extract, in absolute ethanol, 30 minutes in the dark room temperature. The reduction in the DPPH radical was measured by using 96 well microplates (PowerWaveX, Biotek) spectrophotometric absorbance at 517 nm. Ascorbic acid was used as a positive control. The negative control was prepared as above, but without the extract or ascorbic acid. All samples were prepared in triplicate.

DPPH radical scavenging activity was presented in term of % inhibition, which was calculated as follows:

$$\% \text{ inhibition} = [(AC - AS)/AC] \times 100$$

Where AC is the absorbance of the control reaction (containing all reagents except the semi-purified *A. occidentale* leaf extract and ascorbic acid) and AS is the absorbance of the fraction A, fraction C of *A. occidentale* leaves, and ascorbic acid. The effective concentration of sample required to scavenging DPPH by 50% (EC_{50} value) obtained by linear regression analysis of dose responds curve plotting between % inhibition and concentration.

Results and discussion

Screening the antibacterial activity against *P. gingivalis* and determination of minimal inhibitory concentration (MIC) of the extract

The semi-purified *A. occidentale* leaf extract fraction A to fraction E were shown in table 1, The fraction A and fraction C were presented wider zone of inhibition against *P. gingivalis* than other fractions.

Table 1 Inhibition zone of semi-purified *A. occidentale* leaf extract fraction A to fraction E

Fraction of *A. occidentale* leaf extract	Zone of inhibition (mm) ± S.D.
A	2.72 ± 0.03
B	1.02 ± 0.08
C	1.95 ± 0.05
D	1.33 ± 0.03
E	1.58 ± 0.03

Each data represented the mean ± S.D. (n = 3).

Table 2 MIC of semi-purified *A. occidentale* leaf extract fraction A and fraction C

Fraction of *A. occidentale* leaf extract	MIC (mg/ml)
A	1.25
C	2.5

These result (table 2) may be related to the presence of tannins, phenols, and flavonoids [33]. These compositions are phenolic compounds, presented in several plants, hydroxyl groups on the phenolic ring of the phenolic compounds are related to microorganism toxicity by increase of hydroxylation [34]. Support by the study of chemical components of the crude extract of *A. occidentale* leaves consist of tannin, polyphenol and saponin [35].

Antioxidant activity (DPPH free radical scavenging activity)

EC_{50} value of ascorbic acid, semi-purified *A. occidentale* leaf extract fraction A, and fraction C were shown in table 3

Table 3 EC_{50} value of ascorbic acid, semi-purified *A. occidentale* leaf extract fraction A, and fraction C

Sample	EC_{50} value ± S.D. (µg/ml)
Ascorbic acid	2.20 ± 0.04
Fraction A	16.88 ± 0.51
Fraction C	1.29 ± 0.03

Each data represented the mean ± S.D. (n = 3)

The free radical scavenging activity of semi-purified extract fraction C was stronger than ascorbic acid but semi-purified extract fraction A weaker than ascorbic acid. It may be due to the presence of anacardic acid, phenolic and flavonoids. The previous study was shown anacardic acid, was composition in cashew, inhibited generation of superoxide and peroxidative activity [30]. Phenolic compound has ability to scavenge free radicals. Flavonoids are the large group of phenolic compound, act as antioxidant including suppression of ROS formation, inhibition of enzymes involved in ROS generation and lipid oxidation [30, 36].

Conclusion

Fraction A and fraction C of semi-purified *A. occidentale* leaf extract displayed antimicrobial property against *P.gingivalis*. In addition, fraction C of the extract had strong antioxidant property. It had higher strength of DPPH radical scavenging activity than ascorbic acid and fraction A. Therefore, the semi-purified leaf extracts of this plant can use for alternative medicine to treat infectious-inflammatory diseases such as periodontitis.

Acknowledgement

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Appendix C

Ethical approval

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Documentary Proof of Ethical Clearance

Research Ethics Committee (REC)

Faculty of Dentistry, Prince of Songkla University

The Project Entitled The *in vitro* study of antibacterial activity against periodontopathic bacteria and toxicity on gingival connective tissue fibroblasts by semi-purified *Anacardium occidentale* leaf extract

REC Project No. : EC5505-22-L

Principal Investigator : Assist. Prof. Dr. Wilairat Worapamorn

Approved by Research Ethics Committee (REC), Faculty of Dentistry, Prince of Songkla University.

This is to certify that REC is in full Compliance with International Guidelines for Human Research Protection such as the Declaration of Helsinki, the Belmont Report, CIOMS Guidelines and the International Conference on Harmonization in Good Clinical Practice (ICH-GCP).

Date of Approval : 21 November 2012

(Asst. Prof. Dr. Srisurang Suttapreyasri)
Chairman of Research Ethics Committee

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(Asst. Prof. Dr. Potchanapond Graidist)

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ที่ ศธ 0521.1.03/ 1188

คณะทันตแพทยศาสตร์
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หนังสือฉบับนี้ให้ไว้เพื่อรับรองว่า

โครงการวิจัยเรื่อง "การศึกษาฤทธิ์ต้านเชื้อแบคทีเรียก่อโรคปริทันต์ และความเป็นพิษต่อเซลล์เนื้อเยื่อเกี่ยวพันเหงือกของสารสกัดกึ่งบริสุทธิ์จากใบมะม่วงหิมพานต์"

รหัสโครงการ EC5505-22-L

หัวหน้าโครงการ ผู้ช่วยศาสตราจารย์ ดร.ทพญ.วิไลรัตน์ วรภมร

สังกัดหน่วยงาน ภาควิชาทันตกรรมอนุรักษ์ คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

ได้ผ่านการพิจารณาและได้รับความเห็นชอบจากคณะกรรมการจริยธรรมในการวิจัย (Research Ethics Committee) ซึ่งเป็นคณะกรรมการพิจารณาศึกษาการวิจัยในคนของคณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นสากล ได้แก่ Declaration of Helsinki, the Belmont Report, CIOMS Guidelines และ the International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

ในคราวประชุมครั้งที่ 5/2555 **เมื่อวันที่** 17 สิงหาคม 2555

ให้ไว้ ณ วันที่ 21 ส.ค. 2555

(ผู้ช่วยศาสตราจารย์ ดร.ทพญ.ศรีสุรางค์ สุทธปริยาศรี)
ประธานคณะกรรมการจริยธรรมในการวิจัย

.....กรรมการ
(ผู้ช่วยศาสตราจารย์ ทพ.นพ.สุรพงษ์ วงศ์วัชรานนท์)

.....กรรมการ
(ผู้ช่วยศาสตราจารย์ ดร.พจนพร ไกรดิษฐ์)

.....กรรมการ
(ผู้ช่วยศาสตราจารย์ ดร.ทพญ.อังคณา เธียรมนตรี)

.....กรรมการ
(รองศาสตราจารย์ นพ.พรชัย สถิรปัญญา)

.....กรรมการ
(อาจารย์วศิน สุวรรณรัตน์)

.....กรรมการ
(อาจารย์ ดร. ทพญ. สุพัชรินทร์ พิวัฒน์)

.....กรรมการ
(อาจารย์ ทพ.กมลพันธ์ เนื่องศรี)

Appendix D

Invitation form (Translated)

ใบเชิญชวน

ขอเชิญเข้าร่วมโครงการวิจัยเรื่อง การศึกษาในห้องปฏิบัติการฤทธิ์ต้านเชื้อแบคทีเรีย ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านการอักเสบและความเป็นพิษของสารสกัดกึ่งบริสุทธิ์ใบมะม่วงหิมพานต์

เรียน ท่านผู้อ่านที่นับถือ

ข้าพเจ้า ทันตแพทย์หญิงศศิวรรณ เหล็มโส๊ะ กำลังศึกษาในหลักสูตรทันตแพทยศาสตรมหาบัณฑิต สาขาวิชาวิทยาศาสตร์สุขภาพช่องปาก คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ ใคร่ขอเล่าถึงโครงการวิจัยที่กำลังทำอยู่และขอเชิญชวนท่านเข้าร่วมโครงการนี้

โครงการวิจัยนี้ทำขึ้นเพื่อศึกษาฤทธิ์ต้านเชื้อแบคทีเรีย ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านการอักเสบต่อเซลล์เนื้อเยื่อเกี่ยวพันเหงือกและความเป็นพิษของสารสกัดกึ่งบริสุทธิ์ใบมะม่วงหิมพานต์ เพื่อนำองค์ความรู้ไปพัฒนาใช้ในมนุษย์ต่อไป

ในการวิจัยนี้ ผู้วิจัยจะทำการเพาะเลี้ยงเซลล์ที่ได้จากชิ้นเนื้อเหงือก จากฟันที่ถูกถอนตามแผนการรักษาทางทันตกรรมจัดฟัน หรือจากฟันคุดเพื่อศึกษาผลทางชีวโมเลกุลของเซลล์ที่เกิดขึ้นในห้องปฏิบัติการ โดยไม่มีการตัดชิ้นเนื้อเหงือกเพิ่ม ถ้าท่านตัดสินใจเข้าร่วมในโครงการนี้จะมีขั้นตอนของการวิจัยที่เกี่ยวข้องกับท่านคือ ผู้วิจัยจะขออนุญาตเก็บฟันรวมทั้งเนื้อเยื่อเหงือกที่ติดกับฟันที่ถูกถอนของท่านตามแผนการรักษาเดิมไปใช้ในการเพาะเลี้ยงเซลล์ในห้องปฏิบัติการ

สำหรับการเข้าร่วมในงานวิจัยนี้ ท่านจะไม่ได้รับการทดลองใดๆนอกเหนือไปจากการถอนฟันตามปกติ ทั้งนี้ ท่านจะไม่ได้รับความเสี่ยงใดๆจากการเข้าร่วมโครงการ แต่อาจจะมีความเสี่ยงที่เกิดภายหลังการถอนฟันตามแผนการรักษาเดิม ซึ่งอาจเกิดภาวะเลือดออก บวม หรือเกิดความเจ็บปวด ซึ่งท่านสามารถปฏิบัติตัวในเบื้องต้นด้วยการกัดผ้าก๊อซให้แน่น 30 – 60 นาทีเพื่อห้ามเลือด ใช้น้ำแข็งประคบเป็นระยะๆเพื่อลดอาการบวม และรับประทานยาแก้ปวดตามคำแนะนำของทันตแพทย์ผู้ทำการถอนฟัน หากเกิดภาวะแทรกซ้อนอื่นใด ให้ท่านติดต่อกลับมาที่คลินิก

ศัลยศาสตร์ช่องปากและแมกซิโลเฟเชียล โรงพยาบาลทันตกรรม คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์เพื่อรับการดูแลรักษาอย่างเหมาะสมต่อไป

ไม่ว่าท่านจะเข้าร่วมในโครงการวิจัยนี้หรือไม่ ท่านจะยังคงได้รับการรักษาที่ดีเช่นเดียวกับผู้ป่วยคนอื่น ๆ และถ้าท่านต้องการที่จะถอนตัวออกจากการศึกษานี้เมื่อใด ท่านก็สามารถกระทำได้อย่างอิสระ

หากท่านมีคำถามใด ๆ ก่อนที่จะตัดสินใจเข้าร่วมโครงการนี้ โปรดซักถามคณะผู้วิจัยได้อย่างเต็มที่

ขอขอบคุณเป็นอย่างสูง

ทพญ.ศศิวรรณ เหล็มโส๊ะ

หัวหน้าโครงการ

หมายเหตุ :- **กรุณาอ่านข้อความให้เข้าใจก่อนเซ็นชื่อยินยอมเข้าร่วมโครงการ**

Appendix E

Consent form (Translated)

แบบยินยอมเข้าร่วมการศึกษา

โครงการวิจัยเรื่องการศึกษาในห้องปฏิบัติการฤทธิ์ต้านเชื้อแบคทีเรีย ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ต้านการอักเสบและความเป็นพิษของสารสกัดกึ่งบริสุทธิ์ใบมะม่วงหิมพานต์

วันที่.....เดือน.....พ.ศ.....

ข้าพเจ้า.....อายุ.....ปี ข้าพเจ้า (หรือ) .....ผู้ปกครองของ นาย/นางสาว.....อายุ.....ปี ตำบล.....อำเภอ.....จังหวัด.....

ได้อ่าน/ได้รับคำอธิบายจากผู้วิจัยถึงขอบเขตของงานวิจัย วิธีการวิจัย วัตถุประสงค์ และอันตราย หรืออาการที่อาจเกิดขึ้นจากการวิจัยหรือจากยาที่ใช้ รวมทั้งประโยชน์ที่จะเกิดขึ้นจากการวิจัย อย่างละเอียดและมีความเข้าใจดีแล้ว

หากข้าพเจ้าได้รับผลข้างเคียงจากการวิจัย ข้าพเจ้าจะได้รับการดูแลโดยทันตแพทย์ผู้ทำการถอนฟันตามมาตรฐานการบริการของคลินิกศัลยศาสตร์ช่องปากและแมกซิโลเฟเชียล โรงพยาบาลทันตกรรม คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

โดยผู้รับผิดชอบโครงการวิจัยนี้คือ ทพญ.ศศิวรรณ เหล็มโส๊ะ สถานที่ติดต่อ ภาควิชาทันตกรรมอนุรักษ์ คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ เบอร์โทรศัพท์ 074-429877 หรือเมื่อมีปัญหาใด ๆ เกิดขึ้นเนื่องจากการทำวิจัยในเรื่องนี้ข้าพเจ้าสามารถร้องเรียนไปที่คณบดี คณะทันตแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อ.หาดใหญ่ จ.สงขลา 90112 โทรศัพท์ 074-287510

หากผู้วิจัยมีข้อมูลเพิ่มเติมทั้งด้านประโยชน์และโทษที่เกี่ยวข้องกับการวิจัยนี้ ผู้วิจัยจะแจ้งให้ข้าพเจ้าทราบอย่างรวดเร็วโดยไม่ปิดบัง

ข้าพเจ้ามีสิทธิที่จะของดการเข้าร่วมโครงการวิจัยโดยมิต้องแจ้งให้ทราบล่วงหน้าโดยการงดการเข้าร่วมการวิจัยนี้ จะไม่มีผลกระทบต่อการได้รับบริการหรือการรักษาที่ข้าพเจ้าจะได้รับแต่ประการใด

ผู้วิจัยรับรองว่าจะเก็บข้อมูลเฉพาะที่เกี่ยวกับตัวข้าพเจ้าเป็นความลับ จะไม่เปิดเผยข้อมูลหรือผลการวิจัยของข้าพเจ้าเป็นรายบุคคลต่อสาธารณชน จะเปิดเผยได้เฉพาะในรูปที่เป็นสรุปผลการวิจัย หรือการเปิดเผยข้อมูลต่อผู้มีหน้าที่ที่เกี่ยวข้องกับการสนับสนุนและกำกับดูแลการวิจัย

ข้าพเจ้าได้อ่าน/ได้รับการอธิบายข้อความข้างต้นแล้ว และมีความเข้าใจดีทุกประการ จึงได้ลงนามในใบยินยอมนี้ด้วยความเต็มใจโดยนักวิจัยได้ให้สำเนาแบบยินยอมที่ลงนามแล้วกับข้าพเจ้าเพื่อเก็บไว้เป็นหลักฐาน จำนวน 1 ชุด

ลงชื่อ.....ผู้ยินยอม

ลงชื่อ.....หัวหน้าโครงการ

ลงชื่อ.....พยาน

ลงชื่อ.....พยาน

<u>หรือ</u>ในกรณีผู้ถูกทดลองยังไม่บรรลุนิติภาวะ จะต้องได้รับการยินยอมจากผู้ปกครอง **<u>ให้ผู้เกี่ยวข้องเซ็นชื่อ ดังนี้</u>**

ลงชื่อ.....ผู้ยินยอม

ลงชื่อ.....บิดา/ผู้ใช้อำนาจปกครอง

ลงชื่อ.....มารดา

ลงชื่อ.....หัวหน้าโครงการ

ลงชื่อ.....พยาน

ลงชื่อ.....พยาน

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List of Paper and Proceeding

Lemso S, Wattanapiromsakul C, and Worapamorn W. Antimicrobial, antioxidant, anti-inflammatory activities and toxicity of semi-purified *Anacardium occidentale* leaf extract. ***Acad J Med Plant.***
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Lemso S, Wattanapiromsakul C, and Worapamorn W. The *in vitro* study of antibacterial activity against periodontopathic bacteria and antioxidant activity of semi-purified *Anacardium occidentale* leaf extract. The 28th TSU National Academic Conference 2018. "Research and Innovation for Social Stability, Prosperity and Sustainability"; 2018 May 8-9; BP Samila Beach Hotel, Songkhla, Thailand. Quintessence Publishing; 2018. p. 1252-1259.