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Anti-Inflammatory Activity of Selected Edible Herbs and Spices on Cultured Human Gingival Fibroblasts

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Abstract: The aims of the study were to determine the *in vitro* cytotoxicity and anti-inflammatory activities of common edible herbs and spices on cultured human gingival fibroblasts (HGFs). *Piper betle* L. (betel leaf), *P.sarmentosum* Roxb. (wild betel / kadok leaf), *P.nigrum* L. (black pepper seed), *Eugenia caryophyllata* L. (clove bud) and *Cinnamomum zeylanicum* Blume (cinnamon bark). Essential oils were extracted using steam distillation technique and analysed using gas chromatography (GC) and gas chromatography – mass spectrometry (GC-MS). The HGFs were exposed to essential oils at 5 - 0.04 µg/mL in less than 1% dimethylsulfoxide and the number of viable cells was counted to assess cytotoxicity effect. Anti-inflammatory action was determined via the inhibitory action of Interleukin-6 (IL-6), a major pro-inflammatory cytokine in the periodontal tissue inflammation. Treatment of fibroblasts with essential oils resulted in > 70% cell viability. The oils from black pepper seed, clove bud and cinnamon bark showed dose-dependent inhibitory action on IL-6 on cultured bacterial Lipopolysaccharide (LPS)-induced human gingival fibroblasts. Of all the oils, cinnamon bark oil showed the most prominent action comparable to acetylsalicylic acid. *Conclusion:* Essential oils of selected herbs and spices retained compatibility with gingival fibroblasts in culture and showed inhibitory activity on IL-6 released by LPS-induced HGFs. These findings suggest therapeutic potential for application of assay in the management of periodontal disease.

Keywords: Anti-inflammatory, periodontal, Piperaceae, Eugenia caryophyllata, Cinammomun zeylanicum.

INTRODUCTION

Periodontal disease remains as one of the common oral diseases worldwide and in Malaysia despite improvements and advancement in the oral health service [1, 2] Bacterial-infected mouth inevitably causes inflammation of the tooth-supporting tissues, i.e. periodontal tissues, in a disease-prone individual. With this knowledge, disease management prioritise elimination of infective pathogens and inhibition of inflammation of the periodontium. However, the control of inflammation may take time and can be affected by many factors including access for treatment. Availability of over-the-counter and easy-to-apply anti-inflammatory agents that are evidence-supported would benefit the patients before they seek treatment at the dental clinic and may reduce the symptoms.

With current advancement of drug discovery research and safe traditional practices, medicinal herbs have been increasingly being studied for potential benefits including antibacterial and anti-inflammatory actions for use in dentistry [3-6]. Nevertheless, information is still scarce on the anti-inflammatory effect of common edible herbs and spices, namely:

- *Piper betle* L (betel, sireh), of *Piperaceae* family
- *Piper sarmentosum* Roxb. (wild betel, kadok)
- *Piper nigrum* L. (black pepper)
- *Eugenia caryophyllata* L. (clove, cengkih), *Myrtaceae* family
- *Cinnamomum zeylanicum* Blume (cinnamon, kayu manis), *Lauraceae* family on oral and periodontal tissues particularly.

The aims of this study were to determine (i) the cytotoxicity effect of selected herbal essential oils on cultured human gingival fibroblasts (HGFs) and (ii) the inhibitory effect of herbs on Interleukin-6 (IL-6) as the pro-inflammatory biomarker released by lipopolysaccharide (LPS)-induced HGFs.

MATERIALS AND METHODS

Plant Materials and Sample Preparation

Leaves of *P.betle* and *P.sarmentosum* were cleaned and air-dried in a ventilated room away from direct sunlight and with temperature between 26-30°C for 2 weeks. Leaves were then cut into small pieces while the spices (*P.nigrum* seeds, *E.caryphyllata* buds and *C.zeylanicum* tree bark) were grounded coarsely. Approximately 150g of coarsely grounded herbal material were used for extraction of essential oils using the hydro-distillation technique for 8

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hours. The oily layers were separated and dried using anhydrous magnesium sulphate, purified through a modified wool filter and finally stored at 4°C in air-tight bottles until use [7]. Moisture content was measured separately using the Dean's stalk and done in triplicates.

Analyses of the Essential Oils

Gas Chromatography (GC) analysis was used for identification of the essential oils components. The oil samples were dissolved in ethyl acetate and 0.1 µL of each samples were injected in split mode into a Shimadzu GC2000 GC equipped with flame ionization detector (FID) detector and DB-5 column (1 µm thickness, 30.0 m length, 0.25 mm diameter). Nitrogen was the carrier gas used with a flow rate of 1.0 mL/min. The temperature of injector and detector were maintained at 250 °C. Initial temperature of the oven was programmed at 75 °C for the initial 10 min, then 3°C/min to 230°C for 5 min. Total program time was 73.33 min [8]. Subsequently, the essential oils were 'spiked' using the combined chromatography technique [9]. Using the same protocol as before, each essential oil sample was injected with a mixture of standard hydrocarbon series of C_8 – C_{21}. Location of each carbon series were identified based on the retention time of carbon series ran in the chromatogram earlier.

The essential oils were also examined using a Shimadzu QP5050A Gas Chromatography-Mass Spectrometry (GC-MS) System, with HP-5MS column and held at stationary phase under controlled condition. The initial temperature of oven was set at 75°C for 5 min, then maintained at 250°C for 10 min. Helium was used as the carrier gas at 1.2 mL/min air flow. Injector and detector temperature were maintained at 240 – 280 °C.

Qualitative Identification of Essential Oils Components

All components of the essential oils were identified by comparing their retention indices based on Kováts index calculation [10] and GC-MS database library. Results were tabulated and compared.

Human Gingival Fibroblast (HGF) Culture

Primary cell culture explants of human gingival fibroblasts (HGF) were obtained from sound tooth extracted for orthodontics purposes. This protocol was in accordance to the requirements issued by the Research Ethics Committee of Universiti Kebangsaan Malaysia (UKM1.5.3.5/244/NF-04-09) and modified from previous reported studies [11, 12]. Gingival tissues were scrapped off from the tooth surface using sterile blade and cultured in 6-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% Penicillin-Streptomycin and 1% fungizone (Amphotericin B). The explant was incubated in humidified atmosphere with 5% CO_2 at 37°C for 2 weeks. Growth medium was changed every 3 days. Once cells became >75% confluent, cells were sub-cultured, stored and/or tested for assay. Cells passages between 3-10 were used in this study.

Cell Viability Assay

The viability of HGFs was determined using alamarBlue® cell proliferation assay and techniques were modified from Unlu *et al*., 2010 [13]. Briefly, cells in serum-free growth medium were seeded at a density of 1 x 10^5 cells/well into 96-well microtitre plates for 24h. Main stocks of essential oils were dissolved in 100% dimethylsulfoxide (DMSO) at 500 mg/mL and then diluted in two-fold series to obtain eight serial dilutions. Each series were later mixed with serum-free medium to produce working series with concentrations ranging from 10 – 0.08 µg/mL and dispensed into well plates. For each 100 µL/well of cell suspension, 100 µL oil samples were added to give final concentrations of 5, 2.5, 1.25, 0.63, 0.31, 0.13, 0.08 and 0.04 µg/mL in the reaction wells in triplicate copies. In addition, 20 µL alamarBlue® solution (Invitrogen Corp., U.S.A) was added to each well as a reagent for detecting cell viability. Wells with serum-free medium were referred as the blank wells, wells with oils samples as test wells and those seeded with HGFs in serum-free medium only were the negative control. Plates were incubated at 37°C with 5% CO_2 supply for 24h. Following incubation, absorbance was measured at 570nm (OD_{570}) using a platereader (Thermo Scientific® Varioskan Flash Multimode Reader) the mean OD_{570} for each set of wells were calculated. The assay was repeated twice. Subsequently, the percentage of cell viability was calculated using the following formula:

Percentage of cell viability = $\frac{\text{mean } OD_{570} \text{ (test wells – blank wells)}}{\text{mean } OD_{570} \text{ (control wells – blank wells)}} \times 100\%$

Effective Inhibitory Concentration (IC)

The percentage of cell growth inhibition was calculated (% Inhibition = 100 – viability) and a dose-response curve was generated. Effectively, the half maximal inhibitory concentration, IC_{50}, and 95% confidence interval were automatically calculated for each of oil samples by a non-linear regression analysis using the GraphPad Prism version-6 software (2012 GraphPad Software, Inc.).

Induction and Treatment of HGFs

For the anti-inflammatory assay modified from Zdařilová *et al*. (2009) [13], HGFs in serum-free growth medium were seeded at 1 x 10^5 cells/well in 96-well microtitre plates for 24h. Overnight-cultured HGFs were then induced with 1µg/mL lipopolysaccharides from *Escherichia coli* 055:B5 (Sigma-Aldrich, U.S.A). After 4h of incubation, the LPS-induced HGFs were then washed with PBS twice before exposed to oil sample mixtures in serum-free media. The final concentration of essential oils used for this assay was 1, 5, 10 and 25 µg/mL. Medium without any mixture of essential oils (0 µg/mL) was referred as the negative control while acetylsalicylic acid (Aspirin) was used as the positive control. Cells were incubated further for 4h and the supernatant for each of the series were collected in micro-tubes and kept at -20°C.

Quantification of IL-6

The effect of *E.caryophyllata* and *C.zeylanicum* oils on IL-6 production by LPS-induced HGFs were determined using Enzyme-Linked Immunosorbent Assay (ELISA) kits according to the manufacturer's instructions (Invitrogen Corp., U.S.A) for detectable range between 500 – 2 pg/mL. Briefly, culture supernatant was added to each well in duplicate copies and incubated for 2 h in room temperature

Table 1. Constituents of Essential Oils.

	***Pb* Leaf Oil**	***Ps* Leaf Oil**	***Pn* Seed Oil**	***Ec* Flower Bud Oil**	***Ec* Bark Oil**
Total compound identified	72	74	111	22	39
Total amount identified (%)	98.01	91.99	91.62	98.69	95.67
Major constituents identified (relative amount in %)	β- bourbonene (71.06) [a] Chavicol (4.16) [a,b] piperitone (4.16) [a,b] *E*- piperitol (1.54) [a,b] *Z*-β- farnesene (1.24) [a,b] *Z*- calamenene (1.03) [a] γ- cadinene (1.00) [a] δ- cadinene (1.00) [a]	Caryophyllene oxide (26.22) [a,b] α- bisabolene (26.22) [a,b] *Z*-α- bisabolene (11.54) [a,b] Aromadendrene (7.68) [a,b] δ- cadinene (2.90) [a] *E*- asarone (2.68) [a,b] Methyl eugenol (2.36) [a,b] α- muurolene (2.26) [a] α- copaene (2.08) [a,b] *Z*- calamene (1.08) [a] *Z*- pinane (1.04) [a]	Limonene (20.48) [a,b] *Z*-β- ocimene (18.85) [a,b] α- humulene (13.72) [a,b] β- pinene (11.16) [a,b] α- pinene (5.92) [a,b] Sabinene (3.54) [a,b] Cineole <1,4-> (2.53) [a] Myrcene (2.33) [a,b] 1,8- cineole (1.66) [a,b] β- elemene (1.55) [a,b]	*E*- methyl cinnamate (71.97) [a,b] Eugenol acetate <dihydro-> (12.63) [a] *E*- isoeugenol (11.72) [a,b] γ- muurolene (1.17) [a,b]	*E*- cinnamaldehyde (82.51) [a] α- pinene (1.40) [a,b] *Z*-β- ocimene (1.27) [a,b] γ- terpineol (1.09) [a,b]

Percentages of yield were calculated based on the concentrations obtained on GC column DB5 and retention index based on calculation of Kováts index; a = constituents identified by GC, b = constituents identified by GC-MS and database.

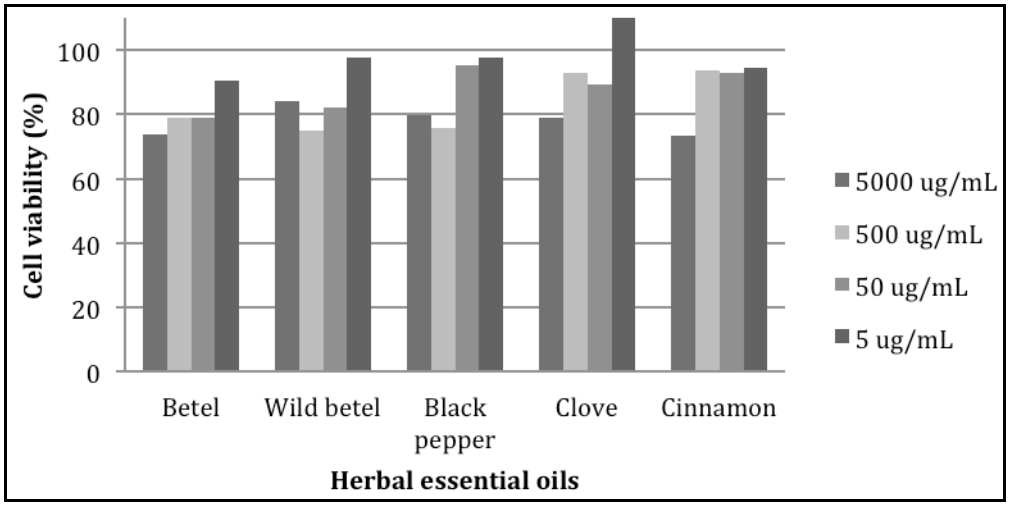

Fig. (1). Viability of HGFs after 24h exposure to essential oils at various concentrations.

with specific biotinylated antibodies against the cytokines, followed by 30 min incubation with streptavidin-horseradish peroxidase. The plates were washed four times with phosphate buffer saline (PBS) between each incubation step. Stabilized chromogen was added in each well and incubated for further 30 min at room temperature and in the dark. Stop solution was added to each well and absorbance was measured at 450 nm wavelength using a platereader (Thermo Scientific® Varioskan Flash Multimode Reader). The amount of cytokine in each well was calculated using purified cytokine provided in the kit. A four parametric logistic curve fit for absorbance of the standard (OD_{450}) against the standard's mean concentrations (pg/mL) was generated using the curve fitting software (Thermo Scientific SkanIt®) and finally the concentrations of IL-6 in the test samples were determined.

RESULTS AND DISCUSSION

The analyses of essential oils in this study identified approximately 91 – 98% of constituents in all oils tested. Total compounds identified were varied from 22 - 111 compounds (Table **1**). Major constituents for each oil that were more than 1% are as listed. These findings were in concordance with those previously reported for *P.betle* [14-17], *P.sarmentosum* [18-20], and *P.nigrum* [21], *E.caryophyllata* [22, 23] and *C.zeylanicum* [8, 12].

The use of essential oils on cultured HGFs showed high cell viability (mean $\geq$ 70%) within 24h treatment (Fig. **1**). This suggests that all the essential oils are safe to be used on HGFs.

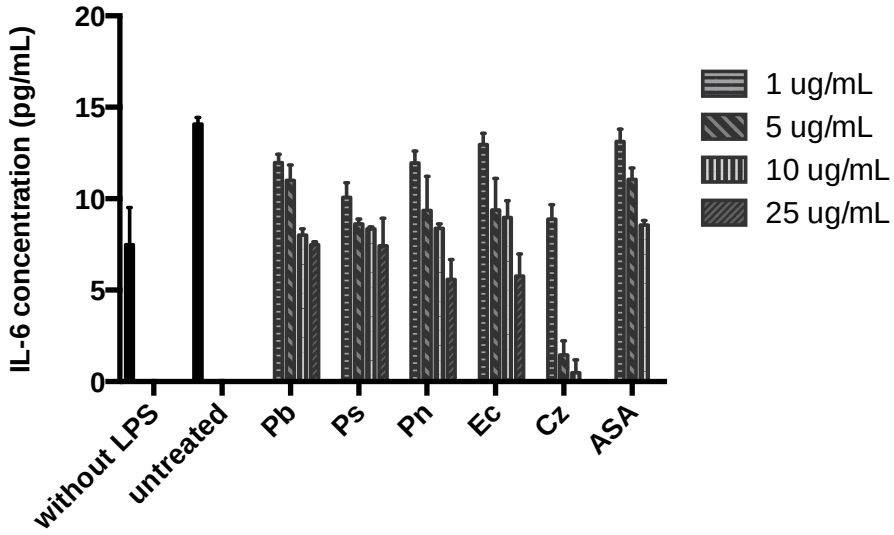

Fig. (2). Concentration of IL-6 produced by LPS-induced HGFs after 4h treatment with essential oils.

Table 2. Effectiveness of Essential Oils in Inhibition IL-6 Production by LPS-Induced HGFs

Essential Oil	IC_{50} Value	95% CI
Piper betle	27.03	13.55 – 53.94
Piper sarmentosum	30.78	11.98 – 79.07
Piper nigrum	14.78	9.23 – 23.68
Eugenia caryophyllata	16.43	10.00 – 27.00
Cinnamomum zeylanicum	1.38	1.18 – 1.61
Acetylsalicylic acid (positive control)	10.54	8.35 – 13.30

Values were calculated using GraphPad Prism (v6) software. CI = confidence interval.
IC_{50} concentration of oils that effectively inhibit 50% of the IL-6 production by cells, measured as mean µg/mL.

Following 4h treatment to various concentrations of essential oils, the LPS-induced HGFs showed reduction in production of IL-6. Inhibitory action of all the essential oils and aspirin on IL-6 produced by treated LPS-induced HGFs was observed to be dose-dependent (Fig. **2**).

Of all the essential oils used in the study, cinnamon bark oil showed the most prominent action compared to acetylsalicylic acid with the least IC_{50} value (Table **2**). The other oils tested showed higher IC_{50} on HGFs compared to control, with values >10 µg/mL. The oils from the spices i.e. cinnamon, clove and black pepper seeds were found to be more effective in inhibiting IL-6 production compared to the oils from edible leaves of betel and wild betel.

The *in-vitro* anti-inflammatory activity in this study supported findings from previous studies [12, 24]. More importantly, the relatively low cytotoxicity on cultured HGFs suggests that these oils are safe for use. In addition, the inhibition of IL-6 production by LPS-induced HGFs suggests beneficial property for future use in treating inflammed periodontal tissue.

CONCLUSION

Essential oils from edible herbs and spices namely betel leaf (*P.betle*), wild betel leaf (*P.sarmentosum*), black pepper seed (*P.nigrum*), clove bud (*E.caryophyllata*) and cinnamon bark (*C.zeylanicum*) showed low cytotoxicity on cultured HGFs and inhibited IL-6 released by lipopolysaccharide (LPS)-induced HGFs.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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