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Full Length Research Paper

Cytotoxic and anti-inflammatory activity of some Thai medicinal plants

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In order to search for new anti-inflammatory agent for use as an adjunctive treatment for periodontal diseases, we have studied crude extracts from plants of family *Rutaceae, Asteraceae* and *Maliaceae* which have been claimed to possess anti-microbial and anti-inflammatory activities. The cytotoxic effect of these plant crude extracts on human gingival fibroblasts (HGFs) and monocytes was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Then, a non-cytotoxic dose was chosen for each plant extract and used to determine the anti-inflammatory activity. The enzyme linked to immunosorbent assay was used to assay tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) released from cells treated with bacterial lipopolysaccharides (LPS). The results showed that the half maximal inhibitory concentration (IC_{50}) of plant extracts for HGFs were in the range of 308.12 to 1168.66 µg/ml while the IC_{50} for macrophages were in the range of 54.6 to 90.92 µg/ml. Stem bark of *Cratoxylum formosum* demonstrated a comparable inhibitory activity to dexamethasone (DM) on TNF-α production by both cells, but showed detectable reduction of IL-1β only in macrophages. Other plant extracts showed no significant difference for reduction of inflammatory cytokines from macrophages. Moreover, leaf and branch of *Murraya paniculata* showed greater inhibitory activity than DM on the production of TNF-α from stimulated HGFs. Therefore, these plant extracts may be good candidates for further drug development for adjuctive treatment of periodontal diseases.

Key words: Cytotoxic effect, anti-inflammatory activity, Rutaceae, Asteraceae, Maliaceae.

INTRODUCTION

Chronic periodontal inflammation in periodontal disease is caused by various noxious stimuli (soluble enzymes, toxins and metabolic products) produced and released into the periodontium by periodontopathic bacteria (Siqueira and Rocus, 2007). However, treatment targeted to eradicate bacterial contamination often does not successfully cure the disease since residual bacteria remain in periodontal tissues (Cugini et al., 2000). Moreover, certain factors may indirectly cause inflammatory, for example many mediators [interleukin-1beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α)] released by host cells. Lipopolysaccharides (LPS) and other bacterial products, may also initiate or attenuate inflammation of surrounding tissues (Bainbridge and Darveau, 2001; Yuzyilmaz et al., 1995). At present, adjunct use of anti-inflammatory drugs in addition to mechanical bacterial eradication often leads to improved periodontal treatment (Salvi and Lang, 2005).

Previous studies revealed that crude extracts from plants of the family Rutaceae, Asteraceae and Maliaceae showed antimicrobial activity against periodontopathic bacteria (Rodanant et al., 2010; Kuvatanasuchati et al., 2011). Moreover, phytochemical analysis of plant parts showed the presence of xanthones (Duan et al., 2010),

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triterpenoids (Siddiqui et al., 2004), flavonoids (Suksamrarn et al., 2004) and coumarins (Jiwajinda et al., 2000) which possess anti-inflammatory activities, which may be useful as an adjunctive treatment of periodontal disease (Korkina et al., 2011; Tanus-Rangel et al., 2010). Though medicinal plants have played major roles as sources of pharmacologically active substances, this is unlikely to lead to drug development unless their mechanisms of action are known. It is widely known that cytokines play an important role in regulating cell growth and survival of cells, as well as regulate the inflammatory process leading to failure in treatment or attenuation of the severity of disease. Human gingival fibroblasts (HGFs) are major cells found in periodontal tissue and have been reported to produce pro-inflammatory cytokines, after treatment with bacterial LPS (Wendell and Stein, 2001). In addition to monocytes and macrophages, HGF cells have been used as a model for the study of periodontal diseases, since they may also play important roles in periodontal diseases. In this study, the anti-inflammatory activities of crude extracts from several plant families have been investigated in order to find new adjunct materials for treatment of periodontal diseases. This involved *in vitro* models using HGF and monocyte cell lines, stimulated by bacterial LPS. Production of TNF-α and IL-1β from these cells was studied in the presence and absence of crude extracts from these plants families at pharmacologically non-cytotoxic concentrations *in vitro* using an enzyme linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Cell lines and Chemicals

The monocyte cell line U937 was kindly provided by Siriraj Medical School. HGF cell line (ATCC CRL-2014) was purchased from ATCC (USA). Growth media (RPMI 1640 containing 2 mM L-glutamine and Dulbecco's Modified Eagle's Medium (DMEM)) and antibiotic-antimycotic solution were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS), Tryphan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), cell culture grade dimethylsulphoxide (DMSO), LPS (*Esterichia coli* 026:B6) and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma-Aldrich Co. (St. Louise, MO, USA). Dexamethasone (DM) was purchased from APP Pharmaceuticals, LLC (Schaumberg, IL, USA). ELISA kits were purchased from Thermo Scientific (Rockford, IL, USA).

Plant materials

Murraya paniculata (Mp)*, Azadirachta indica* var. *siamensis* (Ai) and *Chromolaena odorata* (Cd) were identified by Prof. Dr. Apichart Suksamrarn (Faculty of Science, Ramkhamhaeng University). Voucher specimens were deposited at Ramkhamhaeng University. *Cratoxylum formosum* ssp. *pruniflorum (*Cf*)* was identified by Asst. Prof. Dr. Sprat laphookhieo (Faculty of Science, Mae-Fah Luang University) and voucher specimens were deposited at the Prince of Songkla University and at Queen Sirikit Botanic Garden, Mae Rim District, Chiang Mai Province, respectively.

Extraction process

The air-dried powdered plant materials were extracted using the following organic solvents: ethyl acetate (EtOAc) for the leaves and branches of *M. paniculata*; chloroform ($CHCl_3$) for the leaves of *A. indica* var. *siamensis*; dichloromethane (CH_2Cl_2) for the leaves of *C. odorata*; methanol (MeOH) and partitioning with CH_2Cl_2 for the stem bark of *C. formosum*. The solutions were filtered and the solvents were removed under vacuum at 40°C using a rotary evaporator to yield crude extracts. *M. paniculata, A. indica, C. odorata* were provided by Prof. Dr. Apichart Suksamrarn; *C. formosum* was provided by Asst. Prof. Dr. Surat laphookhiew. Stock solutions of 100 mg/ml (w/v) were obtained by dissolving each crude extract in DMSO and were kept at -20°C until use.

Cell culture

Monocyte cell line (U937) was grown in RPMI 1640 medium and HGF cell line (ATCC CRL- 2014) was grown in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. For anti-inflammation study, U937 cells were further treated with PMA (1 µg/ml) for 48 h to differentiate into macrophages before commencing the experiment. The cells were grown under the atmosphere of 5% CO_2 in humidified air at 37°C.

Cytotoxic activity

Stock solutions of plant extracts were diluted on the day of experiment using appropriate culture medium. Dilutions of 20 to 1000 µg/ml were prepared, and final concentration of DMSO in each sample did not exceed 1% v/v (Prayong et al., 2008). The cytotoxic activity of the extracts were tested with HGF and U937 cells by using the MTT method (Mosmann, 1983) with minor modifications (Kuvatanasuchati et al., 2011). Three separate experiments were performed.

Cytokines assay using ELISA

To investigate the inhibitory effect of the crude extracts on TNF-α and IL-1β protein secretion from LPS-stimulated HGF cells and macrophage cells, cells were pretreated with crude extracts (at the concentration that gave cell viability of 80% or more) for 2 h at 37°C, followed by LPS (1 µg/ml) treatment for 24 h at 37°C, and the cell culture medium was collected. Two control groups were also performed: (1) positive control consisting of 0.5 µg/ml DM and 0.5 µg/ml LPS, (2) negative control comprising cells incubated in 1% DMSO in RPMI 1640 and 0.5 µg/ml LPS. For assaying purposes, the cell supernatants were used for measuring the levels of TNF-α and IL-1β proteins using ELISA kit according to the manufacturer's instructions. ELISA results were recorded using microplate reader (Model series UV 900 Hdi, USA) at wavelengths of 450 and 550 nm. Calculation of the relative absorbance units and the TNF-α and IL-1β concentration (pg/ml) for each sample, as well as construction of the standard curve of recombinant mouse TNF-α and IL-1β calibration curves, were performed as described in the instruction manual. Each sample was measured in duplicate and concentrations were derived from the standard curve.

RESULTS

Cytotoxic activity

The concentrations of extracts giving 50% inhibition (IC_{50}) were less for monocyte cells than HGFs as shown in Table 1. Thus, IC_{50} of plant extracts for HGF were in the range of 308.12 to 1168.66 µg/ml while the IC_{50} for U937 were in the range of 54.6 to 90.92 µg/ml. Table 2 shows the concentration of crude extracts which gave more than 80% viability of HGF cells and U937 cells, which were selected for use in the anti-inflammation study.

Inhibition of pro-inflammatory cytokines production of HGF

The effects of plant extracts on TNF-α production of HGF cells, stimulated by LPS, is shown in Figure 1. Thus, stem bark extract of *C. formosum* showed similar levels of inhibition compared to the standard medication (DM), while leaf and branch extract of *M. paniculata* showed even greater inhibitory effect than DM. On the other hand, the extract from leaves of *A. indica* and *C. odorata* showed a stimulatory effect on TNF-α production. LPS stimulated HGF cells and did not secrete any detectable amount of IL-1β, and crude extracts also did not exert any effect on their production.

Inhibition of pro-inflammatory cytokines production of monocytes

In monocytes, all crude extracts showed an inhibitory effect on the production of pro-inflammatory cytokines namely, TNF-α and IL-1β Figure 2. Crude extract from stem bark extract of *C. formosum* could inhibit TNF-α and IL-1β comparable to DM. Other extracts also showed inhibitory effect on the production of these cytokines from macrophages, but to a lesser extent than stem bark extract of *C. formosum*.

Note: Mp (leaf): *M. paniculata* (leaf); Mp (branch): *M. paniculata* (branch); Ai: *A. indica* (leaf); Co: *C. odorata* (leaf); Cf: *C. formosum* (stem bark); LPS: Lipopolysaccharide as negative control; Dexa: DMe as positive control (standard drug).

DISCUSSION

This paper has explored the anti-inflammatory effects of crude extracts from plants in the Rutaceae, Asteraceae and Maliaceae family. Although, plants in these families have been used on inflammation related diseases, there has been little research on their chemistry and pharmacology. As in other inflammatory processes, periodontitis is attenuated via upregulation of a variety of pro-inflammatory cytokines and chemokines. Therefore, study on the inhibition of this process may help in finding new materials for treatment of periodontal diseases.

LPS play a key role in the destruction of periodontal tissue through the production of pro-inflammatory mediators, such as TNF-α (Drouet et al., 1991), IL-1β (Dinarello et al., 1993) and prostaglandin (Agarwal et al., 1995). Macrophages have been used to demonstrate over-expression of the inflammatory mediators involved in many inflammation related diseases, such as rheumatoid arthritis, atherosclerosis, chronic hepatitis, and periodontitis (Wilkinson et al., 2010; Maciejewska et al., 2009). In this study we also used LPS stimulated HGFs to demonstrate the production of pro-inflammatory mediators since they are the most abundant cells in the gingiva (Imatani et al., 2001). These cytokines are essential for initiating the inflammation process leading to tissue destruction. They can amplify the degree of inflammation, induce tissue destruction and reduce the capacity to repair damaged tissue by stimulating the production of other mediators (Bascones et al., 2004; Preshaw and Taylor, 2011). The reduction of these cytokines may therefore, reflect the anti-inflammatory activity of medicinal plant extracts. DM, a potent drug for treatment of periodontal diseases, could inhibit the production of these cytokines and was used as a positive control in this study (Ozaki et al., 2010).

Table 1. IC_{50} of HGF and U937 cells after direct exposure to crude extracts for 24 h.

Crude extract	IC_{50} (µg/ml)	
	HGF cells	U937 cells
M. paniculata (leaf)	865.24	78.26
M. paniculata (branch)	1168.66	54.6
A. indica (leaf)	498.51	90.92
C. odorata (leaf)	461.59	64.07
C. formosum (stem bark)	308.12	68.72

Table 2. Concentrations of crude extracts which gave 80% viability or more which were used for the anti-inflammation experiments.

Crude extract	Concentration (µg/ml)	
	HGF cells	U937 cells
M. paniculata (leaf)	250	50
M. paniculata (branch)	500	50
A. indica (leaf)	125	25
C. odorata (leaf)	125	25
C. formsum (stem bark)	250	25

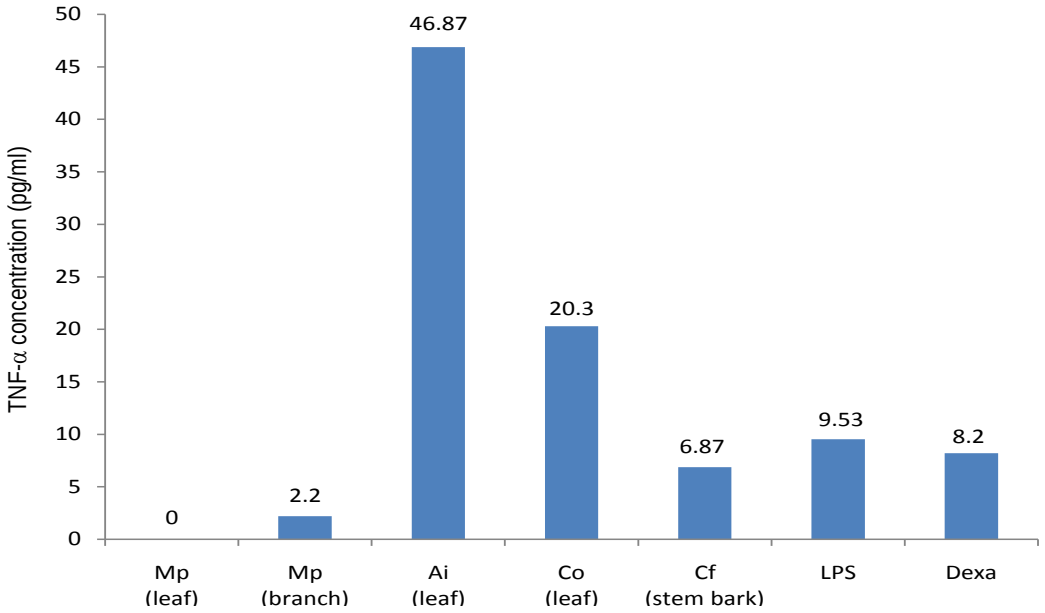

Figure 1. Effect of plant extracts on production of TNF-α from human gingival fibroblasts. Mp (leaf), *Murraya paniculata* (leaf); Mp (branch), *Murraya paniculata* (branch); Ai, *Azadirachta indica* (leaf); Co, *Chromalaena odorata* (leaf); Cf, *Crataxylum formosum* (stem bark); LPS, lipopolysaccharide as negative control; Dexa, dexamethasone as positive control (standard drug).

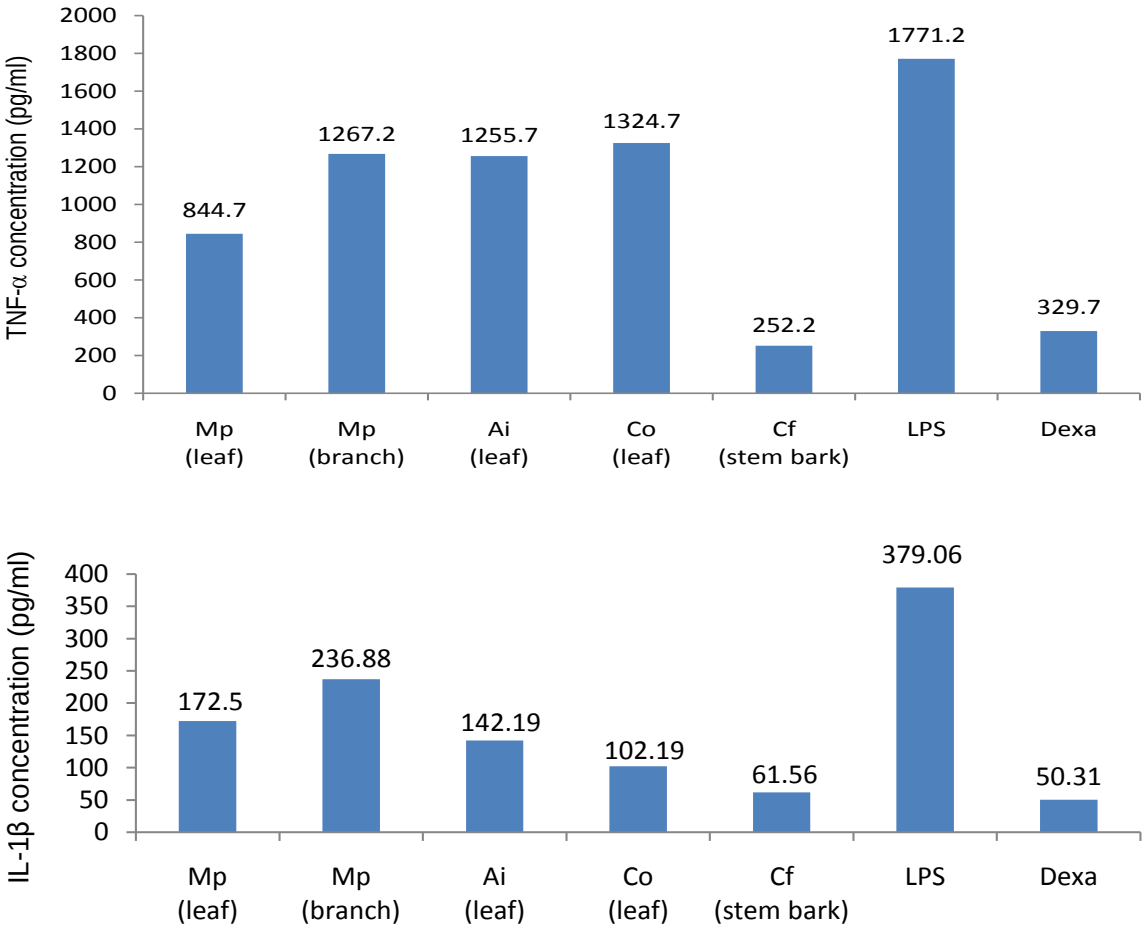

Figure 2. Effect of plant extracts on production of pro-inflammatory cytokines from macrophages.

M. paniculata, A. indica, C. odorata and *C. formosum* Dyer are commonly found in Thailand. They have been widely used as traditional medicine in East/Southeast Asia. However, very few reports were concerning its usage as an adjunctive medication for periodontal diseases. The extract of *M. paniculata* was reported to contain various alkaloids, coumarins, and flavones (Jiwajinda et al., 2000). It also contained anti-bacterial and anti-fungal activities (Rodanant et al., 2010; Phongpaichit et al., 2005). The anti-inflammatory activity was reported in rat models (Narkhede, 2012). Results from this study showed that its extracts showed inhibition of cytokine production from stimulated HGFs, but no such inhibition in macrophages. Therefore extracts of *M. paniculata* may be useful for treatment of inflammation in the oral cavity, as well as for periodontal diseases.

A. indica is an edible plant which is widely found in Thailand and has been used as an insecticide. There are several reports showing that this plant extract can inhibit growth of cancer cells and can be used for treatment of diabetes and hypertension. The plant also contained high levels of anti-oxidant (Tejesvi et al., 2008). *A. indica* mouthrinse has also been reported to be as effective as chlorhexidine in reducing periodontal indices in a clinical study (Chatterjee et al., 2011). Such effects were not found in the present cell culture study. Therefore, the effectiveness of such mouthrinse may be due to the antibacterial activity of the extracts. *C. odorata* has been widely use in Thailand as hemostatic, anti-inflammation drug, and for treatment of intestine diseases and wounds. Previous studies showed that this plant contained several oils, fatty acids, flavonoids, and alkaloids which have anti-oxidation, anti-bacterial, anti-cancer, and anti-inflammation activities (Suksamrarn et al., 2004). However, our study indicates no significant difference in TNF-α production from both stimulated HGFs and macrophages. *C. formosum* Dyer (Teaw) is an indigenous Thai vegetable that is traditionally consumed as fresh leaves in Thailand. It has interested Thai researchers due to its constituents which have high potential for drug usage, such as anti-caries and anti-oxidation (Suddhasthira et al., 2006). This plant extract was also found to be able to inhibit periodontopathic bacteria (Kuvatanasuchati et al., 2011). Our study showed that the extract could reduce the release of cytokines, namely TNF-α from stimulated HGFs and all cytokines from macrophages, this has not previously been reported. Therefore this plant extract may have potential use for treatment for periodontal disease, as well as caries prevention.

In conclusion, down-regulation of pro-inflammatory cytokines from inflammatory cells has been found after treatment with some Thai medicinal plant extracts. Thus, there may be chemical constituents with anti-inflammation activity in these plant extracts, which may have potential use for drug development. Among all plants tested, *C. formosum* Dyer showed most promising results. Further study using purified compounds and elucidation of the chemical structure of substances exerting anti-inflammation activity might be useful in identifying natural agents for the development of new products for treating periodontal diseases.

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