ANTI-INFLAMMATORY ACTIVITY OF *QUERCUS INFECTORIA*, *GLYCYRRHIZA URALENSIS*, *KAEMPFERIA GALANGA* AND *COPTIS CHINENSIS*, THE MAIN COMPONENTS OF THAI HERBAL REMEDIES FOR APHTHOUS ULCER

Nuntana Aroonrerk[1] and Narisa Kamkaen[2,*]

[1]Faculty of Dentistry, Srinakharinwirot University, Wattana, Bangkok 10110, Thailand
[2,*]Faculty of Pharmacy, Srinakharinwirot University, Ongkharak, Nakhon-Nayok, 26120, Thailand

ABSTRACT: Four medicinal plant powders (*Quercus infectoria*, *Kaempferia galanga*, *Coptis chinensis* and *Glycyrrhiza uralensis*) and one traditional Thai herbal recipe for aphthous ulcer were selected for the anti-inflammatory activity *in vitro* and skin irritation test *in vivo*. Among four plant powders, *K. galanga, C. chinensis*, and *G. uralensis* were inhibited IL-6 production with IC_{50} value of 0.04 ± 0.01, 0.07 ± 0.01 and 0.08 ± 0.01 μg/ml, respectively. *Q. infectoria* had lower anti-IL-6 activity with IC_{50} value of 0.31 ± 0.02 μg/ml. Those four plants also had anti-PGE_2 activities but lower than the aphthous powder and aphthous gel. Whereas a traditional herbal recipe had significantly higher anti-inflammatory activity than each of the isolated plants by inhibit IL-6 and PGE_2 production with IC_{50} value of 0.04 ± 0.01 and 0.08 ± 0.01 μg/ml, respectively. The anti-inflammatory activities were significantly higher than prednisolone and the COX-2 inhibitor. The plant powders and the herbal recipe had any growth inhibitory effect on the human gingival fibroblast cells even at the highest concentration. No sign of irritation was observed during the dermal irritation test with all experiments. Finally, the efficacy of anti-inflammation and safety of the medicinal plants and traditional Thai herbal remedy for aphthous ulcer treatment were evaluated by the scientific method.

Keywords: *Quercus infectoria*, *Kaempferia galanga*, *Coptis chinensis*, *Glycyrrhiza uralensis*, anti-inflammatory, interleukin, prostaglandin, skin irritation test

INTRODUCTION: Recurrent aphthous ulceration (RAU) is the most common oral disease affecting 5-20% of general population in world wide[1]. It seems to be mediated principally by the immune system. Ongoing inflammation in the oral tissues may be facilitated by expression of inflammatory cytokines including IL-1β, a key mediator of various immunological and inflammatory phenomena[2,3]. IL-1β can stimulate the expression of IL-6 and prostaglandin E_2 (PGE_2) from human gingival fibroblasts[4-6]. Inhibition in the release of these mediators is a potential strategy to control inflammation; therefore, we have determined the effect of herbal on IL-1β-inducing IL-6 and PGE_2 production.

Quercus infectoria Olivier ("Ben Ka Nee" in Thai; Fagaceae) The galls have a great medicinal value and have pharmacologically been deciphered to be astringent, anti-diabetic, anti-tremor, local anesthetic, antipyretic and anti-Parkinson. The constituents of galls comprise a large amount of tannins: gallic acid, syringic acid, ellagic acid, β-sitosterol, amentoflavone, hexamethyl ether, iso-cryptomerin, methyl betulate, methyl oleanate, hexagalloyl glucose. In Asian countries, the galls have been used for centuries for treating inflammatory diseases. Gargle of hot water extract of galls is very effective against inflamed tonsils, while direct application of boiled and bruised galls on skin effectively cures any swelling or inflammation. The application of powdered galls in the form of ointment also cures hemorrhoids caused by inflammation of the skin[7].

Glycyrrhiza uralensis Fisch. ("Cha Em Jean" in Thai; Leguminosae) In the traditional system of chinese medicine, the roots and rhizomes have been in clinical use for centuries. The main constituents are glycyrrhizin and glycyrrhizic acid. Licorice roots have many pharmacological effects including anti-ulcer, antipyretic, antimicrobial, antiviral, anti-inflammation, antidepressant, anti-oxidant activities, expectorant, diuretic, laxative, sedative, and menopausal complaints[8].

Kaempferia galanga Linn. ("Proh hom" in Thai; Zingiberaceae) The rhizomes of the plant, which contains essential oils, have been used in a

*To whom correspondence should be addressed.
E-mail: narisa@swu.ac.th,
Tel. +66 2260 1012, Fax. +66 2258 4006

decoction or powder for indigestion, cold, pectoral and abdominal pains, headache and toothache. Its alcoholic maceration has also been applied as liniment for rheumatism. In Chinese medicine, the rhizomes have been used as an aromatic stomachic, and also as incense. The constituents of this rhizome have included cineol, borneol, 3-carene, camphene, kaempferol, kaempferide, cinnamaldehyde, *p*-methoxycinnaamic acid, ethyl cinnamate, and ethyl *p*-methoxycinnamate. The methanol extract showed larvicidal activity against the second stage larva of dog roundworm; *Toxocara canis* and possessed an effective amebicidal for three species of *Acanthamoeba*[9].

Coptis chinensis Franch ("Oung Ney" in Thai; Ranunculaceae) Rhizome of *C. chinensis* has been used in traditional Chinese medicine for clearing away heat and depriving dampness for treatment of diarrhea, dysentery and jaundice, and purging the ethnic fire and clearing away toxic material for the cases of seasonal febrile diseases, carbuncle, sore throat. The major active ingredient of the herb, berberine, is an isoquinoline derivative alkaloid and has many pharmacological effects[10].

We studied anti-inflammatory activities of *Q. infectoria*, *K. galanga*, *C. chinensis* and *G. uralensis* which are the main components of Thai herbal remedies for aphthous ulcer in order to validate some of the ethnopharmacological claims in this particular disease condition.

MATERIALS AND METHODS:

Plant materials

The dried and pulverized galls of *Q. infectoria*, rhizomes of *K. galanga*, roots of *G. uralensis*, and roots of *C. chinensis* were kindly provided from Khaolaor Bhaesaj Ltd., Partn, Thailand. The voucher specimens were deposited in the Pharmacognosy Department Herbarium, at the Faculty of Pharmacy, Srinakharinwirot University, Thailand.

Formulation of Aphthous powder and gel

Aphthous powder was kindly provided from the manufacturer, Khaolaor Bhaesaj Ltd., Part, Thailand. "Ya-Kwad-Samarn-Lin Khaolaor Bhaesaj" has been popularly known in Thailand more than 70 years and is still the best seller of the manufacturer. The mixed powder (650 g) consisted of 150 g of galls of *Q. infectoria*, 25 g of roots of *G. uralensis*, 110 g of rhizomes of *K. galanga*, and 25 g of roots of *C. chinensis.* Aphthous gel was developed in Faculty of Pharmacy, Srinakharinwirot University by using carbopol 934P as the gellingagent and consisted of 10%w/w of aphthous powder.

Preparation of plant powders

The galls of *Q. infectoria*, rhizomes of *K. galanga*, roots of *G. uralensis*, and roots of *C. chinensis* were washed thoroughly, then chopped into small pieces, dried in the hot air oven at 50 °C and ground to powder, passed through sieve with mesh no. 80 and kept in the well-closed desiccators.

The plant materials and preparations were dissolved in dimethylsulfoxide (DMSO) at the stock concentration of 10 mg/ml for *in vitro* assays. The final concentration of DMSO had never exceeded 0.001%.

Study of plant extracts on *in vitro* models of inflammation

Cell culture

Gingival tissues were obtained from a healthy person at the time of clinical crown lengthening. Primary cultures of human gingival fibroblast were prepared from gingival explants according to previously described protocols[11]. Cells at passage three to six were used for all experiments.

Experimental protocol

HGF, were seeded at 1 x 10^5 cells per ml in 96 well plates (100 μl/well) in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% FBS (Seromed, Biochrom, Berlin, Germany), 1X antibiotic-antimycotic mixture (Gibco) in humidified 5% CO_2 in the air at 37°C. The medium was subsequently removed and each well was washed once with serum-free DMEM. Immediately before each experiment, fresh serum-free medium containing IL-1β (Sigma, St. Louis, MO, USA) was added. To test the inhibition of various herbal extracts on the effect of IL-1β, the anti-inflammatory steroid prednisolone (Sigma, 1-Dehydrocortisol; MW 360.5) was added 30 min before the addition of IL-1β. Experiments with herbal extracts were added for 24 h. The final concentration of DMSO was 0.1% in DMEM. The culture media were harvested at the indicated times and stored at -20°C before analysis for PGE_2 and IL-6 content using an ELISA method. In

determination of effective concentration of each compound, IC_{50} is measured and reported as the concentration required for 50% inhibition of the control medium.

Commercial PGE_2 kits

For detection of PGE_2 levels, culture supernatants were assay by PGE_2 Biotrak (EIA) system (Amersham Pharmacia Biotech, Buckinghamshire, UK). The final PGE_2 concentration was calculated by subtracting the baseline from the assayed supernatant value.

ELISA for IL-6 determination

To detect cellular production of IL-6, enzyme linked immunosorbent assays (ELISA) was used by the method of Shirai[12].

Cytotoxicity assay

The effects of the herbal extracts and control media (DMEM or DMEM-DMSO) on fibroblast viability were determined by SRB proliferation activity assay as described by Skehan[13].

Study of plant extracts on rabbit dermal irritation

Animals

Six healthy Albino rabbits (weight: 1500 ± 500 g each) were purchased from the National Laboratory Animal Centre, Mahidol University (Nakhon-pathom province, Thailand). Rabbits, identified by ear tattoo, were individually housed in suspended cages with temperature of 25 ± 2 °C, humidity: 60 ± 5%, for 1 week before the experiment.

Ethical authorization

The experimental protocol has been approved by the ethics committee of Faculty of pharmacy, Srinakharinwirot university before the experiment.

Experimental protocol

Primary irritation to the skin is measured by a patch-test technique on the intact skin of the albino rabbit in accordance with the guidelines of the Consumer Product Safety Commission, Title 16, Chapter II, Part 1500 as described previously [14]. Briefly, the backs of the rabbits were clipped free of fur with an electric clipper at least 4 h before application of the sample. Introduction under a square patch of surgical gauze measuring 1 inch by 1 inch and two single layers thick, 0.5 g of the test semisolid substances in gel base were applied on the back of the animals. The animals are immobilized with patches secured in place by adhesive tape. The entire trunk of the animal is then wrapped with a rubberized cloth, for the 24-h period of exposure. This material aids in maintaining the test patches in position and retards the evaporation of volatile substances. After 24 h of exposure, the patches are removed and the resulting reactions are evaluated on the basis of the designated values for erythema and edema with the FHSA–recommended Draize scoring criteria. Readings are again made at the end of a total of 72 h (48 h after the first reading). The Primary Irritation Index (P.I.I.) of the test article was calculated following test completion. As defined in CFR16, Chapter II, Part 1500, and a material producing a P.I.I. score of greater than or equal to 5.00 would be considered positive; the material would be considered a primary irritant to the skin.

Statistics

All values are expressed as mean±S.D. Statistical significance of the difference was assessed by Student's *t*-test. Values of $p<0.05$ were considered as significant.

RESULTS:

Effect of plant extract on *in vitro* models of inflammation

All of the plant extracts investigated appeared to have no direct toxic effects on gingival fibroblast since there was no reduction in cell numbers when compared with HGF exposed to medium or medium with DMSO. The results of anti-IL-6 and anti-PGE_2 production of the IL-1β-induced HGF are reported in Table 1 and 2. The *K. galanga*, *C. chinensis* and *G. uralensis* exhibited good anti-IL-6 activity with the IC_{50} value of 0.04 ± 0.01, 0.07 ± 0.01 and 0.08 ± 0.01 μg/ml respectively. *Q. infectoria* exhibited moderate anti-IL-6 activity, with the IC_{50} value of 0.31 ± 0.02 μg/ml. Statistical analysis showed that the anti-inflammatory activity of *Q. infectoria* was significantly different from the corticosteroid (prednisolone).

Since aphthous powder exhibited high anti-IL-6 activity, with the IC_{50} value of 0.04 ± 0.01 μg/ml, it was likely that this was the synergistic effect of those three active plant powders. Whereas the three active plant powders in aphthous gel were exhibited weak activities (0.62 ± 0.01). This may be due to the inefficient release of the active

Table 1 Effect of plant extracts, aphthous powder, gel and prednisolone on IL-6 production

Treatment	IC_{50} (μg/ml)[b]	% viability
DMEM	Inactive	100.0
DMEM-DMSO	Inactive	100.0
Prednisolone	0.11 ± 0.04	101.0 ± 2.2
Quercus infectoria	0.31 ± 0.02[a]	112.4 ± 1.0
Glycyrrhiza uralensis	0.08 ± 0.01	113.2 ± 1.7
Kaempferia galanga	0.04 ± 0.01	100.7 ± 1.9
Coptis chinensis	0.07 ± 0.01	109.1 ± 2.6
Aphthous powder	0.04 ± 0.01	102.0 ± 3.0
Aphthous gel	0.62 ± 0.01	117.0 ± 1.8

Each point represents the mean ± S.D. [a]$P < 0.05$ *vs* prednisolone. [b]The concentration of substances required to effect IL-6 production with 50% inhibition.

Table 2 Effect of plant extracts, aphthous powder, gel and COX-II inhibitor on PGE_2 production

Treatment	IC_{50} (μg/ml)[c]	% viability
DMEM	Inactive	100.0
DMEM-DMSO	Inactive	100.0
COX-2 inhibitor (NS398)	1.82 ± 0.13	101.0 ± 1.2
Quercus infectoria	1.25 ± 0.06[a]	100.3 ± 1.0
Glycyrrhiza uralensis	1.25 ± 0.04[a]	101.9 ± 1.1
Kaempferia galanga	1.21 ± 0.05[a]	95.7 ± 2.9
Coptis chinensis	2.48 ± 0.07[a]	109.1 ± 2.6
Aphthous powder	0.08 ± 0.01[b]	102.0 ± 2.0
Aphthous gel	0.62 ± 0.01[b]	117.0 ± 1.8

Each point represents the mean ± S.D. [a]$P < 0.05$, [b]$P < 0.01$ *vs* COX-2 inhibitor. [c]The concentration of substances required to effect PGE_2 production with 50% inhibition.

ingredient from these extracts in gel base into the cell suspension of gingival fibroblasts.

The effect of the plant extracts, the aphthous powder and the aphthous gel to inhibit PGE_2 production were also evaluated (Table 2). All plant extracts except *C. chinensis* had higher inhibitory effect than the COX-2 inhibitor (NS-398). Due to the activity of these plant extracts, the aphthous powder and the aphthous gel scored high active in the tests with IC_{50} value of 0.08 ± 0.01 and 0.62 ± 0.01 respectively. Statistical analysis showed that the anti-inflammatory activities of these plant extracts, aphthous powder and aphthous gel were significantly different from the COX-2 inhibitor (NS-398).

Effect of plant extract on rabbit dermal irritation

Dermal application test of 0.5 g of single herb and aphthous powder triturated in gel base caused no serious signs of erythema and edema on the intact sites. Individual results of dermal scoring appear in Table 3. All rabbits had slight erythema after 24 h and some had very slight erythema after 72 h. Some rabbits had slight edema after 24 h and few had very slight edema after 72 h. The primary irritation index (P.I.I.) is calculated, based on the sum of the scored reactions divided 24 (two scoring intervals multiplied by two test parameters multiplied six rabbits. P.I.I. of *Q. infectoria*, *G. uralensis*, *K. galanga*, *C. chinensis* in gel base were 0.375, 0.625, 0.667and 0.792, respectively; Irritation barely perceptible were observed on the skin of the rabbits. Whereas P.I.I. the aphthous gel and gel base were 1.125 and 0.917 respectively; Slight irritation were observed on the skin of the rabbits.

DISCUSSION: *Q. infectoria*, *G. uralensis*, *K. galanga* and *C. chinensis* are four medicinal plants consisting of traditional Thai herbal remedy for aphthous ulcer. In this study, we investigated the role of those plants on mitogen-activated protein kinase (MAPK) activation. All plants demonstrated an inhibitory effect on MAPK activation, IL-1β and COX-2 expression, IL-1β and PGE_2 synthesis production by human gingival fibroblast (HGF).

Among four plant powders, *K. galanga* had stronger inhibited IL-6 production while *Q. infectoria* had weaker anti-IL-6 activity than the others. Those four plants also had anti-PGE_2 activities but lower than the aphthous powder and aphthous gel. Whereas a traditional herbal recipe had significantly higher anti-inflammatory activity than each of the single plants by inhibit IL-6 and PGE_2 production. The anti-inflammatory activities were significantly higher than predni-solone and the COX-2 inhibitor. It is demonstrated that the plant powders and the herbal recipe had any growth inhibitory effect on the human gingival fibroblast cells even at the highest concentration.

The correlation of the active chemical constituents and the pharmacological activity has been confirmed by the previous and this present studies. Due to the active constituents of galls of *Q. infectoria* comprise a large amount of tannins, the activity of the *Q. infectoria* in the aphthous powder and aphthous gel could inhibit the production of the inflammatory mediators such as IL-6 and PGE_2. *Q. infectoria* effectively inhibited edema produced by serotonin, histamine and PGE_2, which suggested that the anti-inflammatory activity of this plant is possibly mediated by either

Table 3 Results of primary skin irritation test from six rabbits observed at 24 and 72 h

Sample	Reaction	24 h						72 h						Primary Irritation Index
		1	2	3	4	5	6	1	2	3	4	5	6	
Q. infectoria	Erythema	1	1	1	1	2	1	0	0	0	0	1	1	0.375
	Edema	0	0	0	0	0	0	0	0	0	0	0	0	
G. uralensis	Erythema	2	2	2	1	3	2	0	0	1	0	1	1	0.625
	Edema	0	0	0	0	0	0	0	0	0	0	0	0	
K. galanga	Erythema	1	1	3	3	2	1	0	0	1	0	1	1	0.667
	Edema	0	0	0	2	0	0	0	0	0	0	0	0	
C. chinensis	Erythema	1	2	3	3	3	1	0	1	0	0	2	1	0.792
	Edema	0	0	0	2	0	0	0	0	0	0	0	0	
Aphthous gel	Erythema	3	3	3	4	3	3	1	0	0	0	0	1	1.125
	Edema	0	0	0	2	0	4	0	0	0	0	0	0	
Gel base	Erythema	3	2	3	4	3	1	0	1	2	0	0	1	0.917
	Edema	0	0	0	2	0	0	0	0	0	0	0	0	

For erythema: 0 = No erythema, 1 = Very slight erythema (barely perceptible), 2 = Well-defined erythema, 3 = Moderate to severe erythema, 4 = Severe erythema (beet redness) to slight eschar formations (injuries in depth)
For edema: 0 = No edema, 1 = Very slight edema (barely perceptible), 2 = Slight edema (edges of area well defined by definite raising), 3 = Moderate edema (raised approximately 1 millimeter), 4 = Severe edema (raised more than 1 millimeter and extending beyond the area of exposure)
Evaluation of primary irritation index: 0.00 No irritation, 0.04 – 0.99 Irritation barely perceptible, 1.00 – 1.99 Slight irritation, 2.00 – 2.99 Mild irritation, 3.00 – 5.99 Moderate irritation, 6.00 – 8.00 Severe irritation

inhibiting the synthesis, release or action of these mediators[7]. The major constituents of licorice root are triterpenoid saponin glycyrrhizin, a mixture of potassium and calcium salts of glycyrrhizic acid[15-17]. Licorice was also reported to act as an *in vivo* antioxidant and inhibit the initiation and promotion of lipid peroxidation[18,19]. The constituents of this rhizome of *K. galanga* have included a large amount of steroid and terpenoid, its alcoholic maceration has also been applied as liniment for rheumatism[20,21]. In addition, the biologically active constituent of *C. chinensis* extract was characterized as the isoquinoline alkaloid, berberine which has many pharmacological effects including activation of the aryl hydrocarbon receptor[22], insulin sensitizing and insulinotropic action[23], inhibition of arylamine *N*-acetyltransferase activity[24], inhibition of HIF -1 alpha expression as a potent anti-angiogenic agent[25], anxiolytic effect by acting at 5-HT receptors[26], anti-inflammatory and anti-cancer by inhibiting basal and 12-otetradecanoylphorbol-13-acetate mediated PGE_2 level and COX-2 expression[27], and lowering blood cholesterol, and especially LDL-cholesterol[28].

However, a dermal irritation test must be performed to ensure human safety in case of substance exposure. Animals have been used to assess dermal irritation by the observation of visible changes ranging from erythema and edema to corrosion and ulceration. Information that derives from tests for dermal irritation serves to identify the possible risk to the population who use and are exposed to substances such as aphthous gel. In the present study, the treatment of four single herbs and aphthous gel caused no serious signs of irritation. It produced 'barely irritation' (index 0.04-0.99) and 'slight irritation' (index 1.00-1.99). This finding indicates that four medicinal plants and one herbal recipe for aphthous ulcer can be categorized as a "non-irritant".

CONCLUSION: From these overall results, we can conclude that the topical preparation both in terms of aphthous powder and gel containing the four main components of Traditional Thai herbal remedies for aphthous ulcer possesses anti-inflammatory effect which can be useful for the treatment of local inflammation in humans without skin irritation effect.

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