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Original Article

In vitro evaluation of the antibacterial and anti-inflammation activities of *Clausena lansium* (Lour.) Skeels

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Abstract

Crude extracts of twigs and roots of *Clausena lansium* (Lour.) Skeels were separated and purified using repeated silica gel column chromatography to yield 12 compounds identified as xanthotoxol (1), imperatorin (2), heraclenol (3), heraclenin (4), wampetin (5), indicolactonediol (6), murrayanine (7), *O*-demethylmurrayanine (8), indizoline (9), 3-formyl-6-methoxycarbazole (10), lansine (11) and glycozolidal (12). All pure compounds were tested for their antibacterial and anti-inflammation activities using disc diffusion method and enzyme-linked immunosorbent assay (ELISA), respectively. At a concentration of 50 µg/mL, three carbazole alkaloid components (compounds 10, 11, and 12) demonstrated moderate antibacterial activity against the periodontopathic bacteria, *Porphyromonas gingivalis*. Compound 10 and the crude extract of twig revealed impressive anti-inflammation potency. From these results, selected carbazole alkaloid compounds from *Clausena lansium* might be potential raw products in generating new anti-inflammation and antibacterial agent used as an adjunctive medication in treating periodontal disease.

Keywords: *Clausena lansium*, carbazole alkaloid, antibacterial, anti-inflammation, periodontal disease

1. Introduction

The emergence of antibiotic resistance and undesirable effects due to the use of synthetic chemicals stress the importance of finding alternative agents to treat various diseases. *In vitro* phytochemical studies of higher plants reveal that these plants are the source of hundreds of thousands of diverse chemical compounds with different biological activities (Balandrin *et al.*,1985). This might be strong evidence supporting the successful treatment of various diseases using edible plants (Raskin *et al.*,2002). Periodontal disease, a chronic inflammatory disease, is considered a major oral problem since fifteen percent of the global population is suffering from its severe form. The accumulation of various bacterial species, bacterial toxins, and inflammatory cytokines in the site of periodontal inflammation, accounting for the disease initiation, render the importance of the usage of antibacterial and anti-inflammation drugs to slow and/or halt the disease progression. *Clausena lansium* (Lour.)

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Skeels known in Thai as "Mafai jean" is an indigenous plant in the Southeast Asia region. It is a slow growing, strongly scented evergreen plant belonging to the *Rutaceae* family and can reach 3 to 8 meters height. Previous studies of its bioactivities showed that compounds isolated from its parts had antioxidant, anti-inflammation, antifungal, hepatoprotective, and anticancer activities (Adebajo *et al.,*2009; Ng *et al.,*2003; Prasad *et al*.,2009). Therefore, it is of interest to investigate the antibacterial and anti-inflammation activities of the crude extract from twig and isolated compounds from twig and root of *Clausena lansium* (Lour.) Skeels.

2. Materials and Methods

2.1 Plant materials

Twigs and roots of *Clausena lansium* (Lour.) Skeels were collected from Muang District, Nan Province, Thailand. The identification of the plant was performed by one of the author (S.L.) through comparison with a voucher specimen no. QBG 25077 in the herbarium collection of Queen Sirikit Botanic Garden, Mae Rim, Chiang Mai, Thailand.

2.2 Extraction and Isolation

All pure compounds, including xanthotoxol(1), imperatorin (2), heraclenol (3), heraclenin (4), wampetin (5), indicolactonediol (6), murrayanine (7), *O*-demethylmurrayanine (8), indizoline (9), 3-formyl-6-methoxycarbazole (10), lansine (11) and glycozolidal (12), were isolated from the twigs (compound 1-7) and roots (compound 8-12) of *C. lansium* as described previously (Maneerat *et al*., 2010a; 2010b; 2012) (Figure 1). Each of the isolated compounds and crude extract from twig were dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, U.S.A.) to obtain the solutions of 1 mg/mL (w/v) concentration. The stock solutions were kept at -20°C.

2.3 Cell line and Chemicals

The monocyte cell line U937 was kindly provided by the Siriraj Medical School, Thailand. Human gingival fibroblast (HGF) cell line (ATCC CRL-2014) was purchased from ATCC (U.S.A.). 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, U.S.A.). Fetal bovine serum (FBS), Trypan blue, 3-(4,5dimethylthiazol-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, U.S.A.). Dexamethasone (DM) was purchased from APP Pharmaceuticals, LLC (Schaumberg, IL, U.S.A.). ELISA kit was purchased from Thermo Scientific (Rockford, IL, U.S.A.).

2.4 Cell culture

The HGF cells and U937 cells were maintained in DMEM and RPMI 1640, respectively. Each medium was supplemented with 100 U/mL of penicillin, 100 g/mL of streptomycin and 10% fetal bovine serum. Cells were grown at 37°C and 5% CO_2 in humidified air.

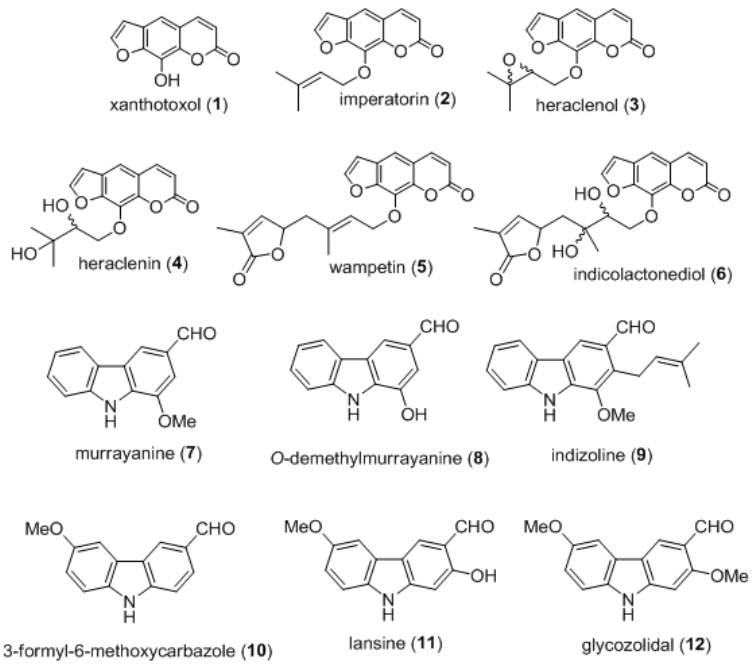

Figure 1. The chemical structures of isolated compounds

2.5 Cytotoxic activity

Stock solutions of plant extracts were diluted on the day of the experiment using appropriate culture medium to the concentration ranging from 10 - 100 µg/mL. The 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 using the MTT method (Mosman, 1983) with minor modifications (Kuvatanasuchati *et al.*, 2011).

Briefly, the cell were seeded in 96 well-plates (200 µl/well at a density of 1×10^5 cells/ml) incubated at 37°C in 5% CO_2 atmosphere for 24 h. At 24 h, cells were washed with sterile phosphate buffer (PBS) 100µl and treated with 100 µl of plant extract solution at various concentrations (five wells for each treatment) then incubated at 37°C in 5% CO_2 atmosphere for another 24 h. The plate was washed twice with sterile PBS100 µl of MTT solution (0.5 mg/ml) added to each well and incubated in the dark at 37°C for another 2 hours. The formazan crystals were solubilized with 100 µl of DMSO and left for 30 minutes at room temperature. The absorbance was read at 540 nm by a microplate reader (Model series UV 900 Hdi, U.S.A.).Three separate experiments were performed.

2.6 Microorganisms

The antibacterial activities of all isolated compounds were determined against *Porphyromonas gingivalis strain* ATCC 33277(*Pg* ATCC 33277). The medium used for the activation of the microorganisms was Brain heart infusion broth (BHI). Brucella (Oxoid®, U.K.) was used as cultivation media. The microorganisms were inoculated into BHI and incubated at 37°C in 5% CO_2 atmosphere (Anaerobic system; Forma Scientific, Inc.) for 7 days. The bacterial suspension was then diluted with BHI broth medium to obtain approximately 1×10^8cfu/mL. This working concentration obtained transmittance comparable to 0.5 McFarland turbidity standard using spectrophotometer at 540 nm.

2.7 Antibacterial activity

The growth inhibition tests were performed using agar diffusion technique. One hundred microlitre (µL) of BHI-suspended microorganism was distributed on the agar medium (25 mL/plate) using small-size glass beads. Once the agar surface was dried, paper discs 6 mm in diameter (Whatman International, U.K.) soaked with 10 µL of the isolated compound solution (10µg/mL) on each side of the disc, were placed on the agar surface. Chlorhexidine 2% and DMSO were used as positive and negative control respectively. Each plate was incubated in anaerobic chamber at 37°C in 5% CO_2 atmosphere for 7 days. All tests were performed in triplicate and the antibacterial activity was expressed as the diameters (mean±SE) of inhibition zone (mm) produced by the isolated compound.

2.8 Anti-inflammation activity

2.8.1 Control establishment

There were three kinds of control groups as follows: positive control consisting of 0.5 µg/mL dexamethasone (DM) and 0.5 µg/mL lipopolysaccharides (LPS) from *E. coli*; cell stimulated by LPS from *E. coli* (0.5 µg/mL) without any intervention was used as blank; and cell incubated by DMEM or RPMI1640 medium was regarded as normal group.

2.8.2 Cellular model establishment and intervention

Before LPS treatment, the cells (400 µL/well at concentration of $1x10^5$ cell/mL) were inoculated into 24 microwell plates. Then later, after the cells had adhered to the bottom of the well (24 hours for HGF and 48 hours for U937), the cell supernatants were disposed and prepared non-cytotoxic concentrations of plant fractions were added (400 µL/well). Two hours later, 1 µg/mL of LPS from *E. coli* (400 µL/well) was added into each well. After stimulation for 24 h, the supernatants were harvested and were kept at -80°C until further use.

2.8.3 ELISA assay of TNF-α

The assay consists of four incubations and three wash cycles. The harvested supernatants were added into designated wells of coated 96-well microplate containing sample diluents. Plate was covered and incubated for 1 h at room temperature then washed three times with deionized water. The biotinylated antibody was then added followed by the streptavidin horseradish peroxidase. After each substrate was added, the plate was covered and left for 30 minutes at room temperature then washed. Lastly TMB substrate was added and incubated in the dark for 30 minutes at room temperature. Absorbance was measured by microplate reader (Model series UV 900 Hdi, U.S.A.) at 450 and 550 nm.

Calculation of the relative absorbance units and the TNF-α concentration (pg/ml) for each sample, as well as construction of the standard curve of recombinant mouse TNF-α calibration curves, were performed as described in the instruction manual. Each sample was measured in duplicate and concentrations were derived from the standard curve.

3. Results

3.1 Cytotoxic activity

The cytotoxicity of isolated compounds from *Clausena lansium* (Lour.) Skeels extract on both cell types was shown in Table 1. The sensitivity of both cells to all compounds tested was in the same range. It was found that the IC_{50} of all compounds tested with HGF was in the range of 29.38 to 128.62 µg/mL and 38.19 to 103.04 µg/mL for

Table 1. *In vitro* cytotoxicity of isolated compounds from *C. lansium* against human gingival fibroblasts and monocytes (U937)

Compounds	IC_{50} (μg/mL)	
	HGF	U937
1	106.61±0.29	80.36±0.20
2	74.48±0.23	58.77±0.40
3	123.05±0.26	95.76±0.30
4	123.28±0.28	96.35±0.16
5	109.42±0.29	83.09±0.01
6	128.62±0.06	103.04±0.67
7	38.36±0.17	38.19±0.07
8	29.38±0.58	39.97±0.06
9	Nil	Nil
10	70.20±0.15	56.55±0.16
11	79.01±0.22	61.61±0.16
12	120.86±0.38	97.74±0.13

monocytes U937. Only compound 9 showed toxicity effect on both cells.

3.2 Antibacterial activity

Only some carbazole alkaloids (compounds 10, 11, and 12) demonstrated antibacterial activity against *P. gingivalis* but the potency was lower than that of 2% chlorhexidine digluconate (positive control) (Table 2). Compound 11 gave the highest antibacterial activity with the diameter of inhibition zone of 20 mm. Compounds 10 and 12 demonstrated the same growth inhibition efficacy but were less effective than compound 11. Other compounds showed the same result as negative control (DMSO).

3.3 Anti-inflammatory activity

After incubation with monocytes followed by LPS stimulation, almost all components ineffectively inhibited the release of cytokine except 3-formyl-6-methoxycarbazole (compound 10) and the crude extract from twigs. At a concentration of 50 μg/mL, compound 10 showed a TNFα suppression capacity comparable to the positive control (dexamethasone) while the crude extract from twigs demonstrated exceptional result. Other compounds seemed to increase the expression of this pro-inflammatory cytokines (Table 3).

4. Discussion

According to previous phytochemical studies, carbazole alkaloids from plants in the family *Rutaceae* expressed antimicrobial activity (Arbab*et al.*, 2012; Chakraborty *et al.*, 1995; Rahman and Gray, 2005; Wu and Furukawa, 1982). It was demonstrated in our study that several carbazole alkaloids from *C. lansium* possessed a moderate growth inhibition activity on a gram-negative periodontopathic bacteria, *P. gingivalis*. The chemical structure of all carbazole alkaloids with antibacterial activity (compounds 10, 11, and 12) was occupied by the formyl group and methoxy group at the position C-3 and position C-6, respectively. Since the potency of the three active carbazole alkaloids showed differently, the ring substituents might be responsible for the bioactivity of the pure compounds. Evidently, additional aromatic hydroxyl group promoted the antibacterial activity of some plants (Kim *et al.,* 2012). The same phenomenon was

Table 2. Antibacterial activity of isolated compounds in paper disc method against *Porphyromonas gingivalis*

Compounds	Diameters of inhibition zone (mm)
1	0
2	0
3	0
4	0
5	0
6	0
7	0
8	0
10	14±0.62
11	20±0.94
12	12±1.16
CHX 2%	30±0
DMSO	0

CHX 2% Chlorhexidine digluconate 2%
DMSO Dimethyl sulfoxide

Table 3. Effects of isolated compounds from *C. lansium* on TNF-á release

Compounds	TNFα (pg/mL)
1	1795.5
2	1847.15
3	1763.73
4	1776.35
5	1787.03
6	1842.68
7	1876.35
8	1355.93
10	378.15
11	1444.38
12	1810.23
Crude extract of the twig	17.7
LPS	1098.45
DMSO	144.53
Dexa	343.78

found in this study since carbazole alkaloids with an additional hydroxyl group (-OH) on C-2 (compound 11) possessed the most potent antibacterial activity. Though furanocoumarins demonstrated antimicrobial activities against various strains of gram-negative bacteria (Dongfack *et al.,* 2012), our study did not reveal the same result wherein there was no such antibacterial activity as determined by the inhibition zone.

As tumor necrosis factor-α (TNF-α) mediates the production of many other cytokines during inflammation (Amiot *et al.,* 1997), in this study, the *in vitro* model of macrophage-mediated inflammatory event was chosen to assess the effect of crude extract and pure compounds from *C. lansium* on the production of TNF-α. Tested coumarins and almost all carbazole alkaloids showed no inhibitory effects on TNF-α production. Nevertheless, compound 10 showed comparable efficacy to dexamethasone and most interestingly the crude extract from twigs was highly effective in inhibiting LPS-induced monocyte secretion of TNF-α. This result may indicate that there might be some other chemical constituents which express anti-inflammation activity in this plant.

It is evident that the possible physiological properties of compounds are likely to depend on chemical structure which requires suitable substitution at a suitable position (Kumar *et al.,* 2012; Singh *et al.,* 2013). This might be the explanation for the difference in the potency of the studied compounds which possessed different side chain on the aromatic ring.

The antibacterial and anti-inflammation activities of the carbazole alkaloids from *C. lansium* described in this study seem to justify the ethnobotanical use of this plant which might have a potential for the development of a drug for the treatment of inflammation. Due to their anti-periodontopathogen activity, these compounds may be of interest for use in the treatment of periodontal diseases. The molecular structures of the natural product compounds, to some extent, might be responsible for their biological activities and further investigation is recommended at least in elucidating cellular mechanisms with a view of standardizing them.

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