ORIGINAL ARTICLE

Anti-inflammatory activity and chemical constituents of red limestone

Thidarat Duangyod[1,2], Narawadee Rujanapan[2], Sorraya Champakam[1,2], Rawiwan Charoensup[1,2]

[1]Department of Applied Thai Traditional Medicine, School of Integrative Medicine, Mae Fah Luang University, [2]Medicinal Plants Innovation Center of Mae Fah Luang University, Mae Fah Luang University, Chiang Rai, Thailand

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ABSTRACT

Red limestone is a mixture of turmeric (*Curcuma longa* L.) powder and limestone which is made from burning shells at high temperature. The yellow mixture turns to red color or deep orange because of the reaction between turmeric and calcium carbonate in limestone. Red limestone is traditionally used to treat many diseases such as abscess, cut wound and insect bite. The purpose of this study was to investigate anti-inflammatory activity and chemical constituents of red limestone. The chemical analysis of red limestone extract by liquid chromatography with tandem mass spectrometry revealed that red limestone consisted of alpha-turmerone and curcumanolide B as major components. These compounds were related with the chemical constituents in *C. longa* extract which is a main ingredient of red limestone. However, curcuminoids were not detected in red limestone extract. Cytotoxicity of red limestone extract was investigated. Macrophage cell lines (RAW 264.7) and human keratinocyte cell lines (HaCaT cells) were investigated cell viability using MTT assay. Red limestone extract was nontoxic to normal cells such as macrophage cells and human keratinocyte cells. Moreover, the inflammatory activity was detected nitric oxide (NO) secretion in RAW 264.7 cells. The result showed that the extracts inhibited NO in dose-dependent manner and IC_{50} was found to be 102.42 µg/ml. It suggested that red limestone extract had a potential for anti-inflammatory activity.

Key words: Anti-inflammatory activity, chemical constituent, *Curcuma longa* L, red limestone

INTRODUCTION

Red limestone commonly known as Poon-daeng in Thai is a mixture of turmeric (*Curcuma longa* L.) powder and limestone. It is a part of the betel chewing culture in Thai elderly. Moreover, limestone solution is traditionally used in a few Thai recipes. In Thai traditional medicine, red limestone is used in various therapeutic applications such as wound healing and anti-inflammatory. The limestone is made from burning shells at high temperature and it contains 95-99% of calcium carbonate.[1] The major chemical constituents of *C. longa* L. are cucuminoids that consist of curcumin, bis-demeth oxycurcumin, and demethoxycurcumin. Previous reports revealed that *C. longa* showed good anti-inflammatory activity.[2-6] However, there is no report about red limestone. Therefore, this present study was attempted to investigate chemical

Address for correspondence:
Dr. Thidarat Duangyod,
Department of Applied Thai Traditional Medicine, School of Integrative Medicine, Mae Fah Luang University, Chiang Rai 57100, Thailand.
Medicinal Plants Innovation Center of Mae Fah Luang University, Mae Fah Luang University, Chiang Rai 57100, Thailand.
E-mail: thidarat.dua@mfu.ac.th

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constituents and anti-inflammatory activity of red limestone in Thailand.

MATERIALS AND METHODS

Sample collection

C. longa rhizome were collected from Chiang Rai province, Thailand, in December 2018 and authenticated by Charoensup, R. The voucher specimen was deposited at Medicinal Plants Innovation Center of Mae Fah Luang University with voucher specimen number MPIC0135.

Anadara granosa L. shells were collected from Surat Thani province, Thailand, in January 2019. The specimen was deposited at Medicinal Plants Innovation Center of Mae Fah Luang University.

Limestone preparation

Anadara granosa shells were burned at 500°C for 5 h and then grinded to provide limestone.

Red limestone preparation

Dried powder of *C. longa* rhizome was mixed with limestone and then added DI water to provide red limestone.

Extraction

Dried powder of *C. longa* rhizome was extracted with DI water for 6 h. The mixture was filtered and then freeze-dried to provide water extract.

Limestone was extracted with DI water for 6 h. The mixture was filtered and then freeze-dried to provide water extract.

Red limestone was extracted with DI water for 6 h. The mixture was filtered and then freeze-dried to provide water extract.

Liquid chromatography quadrupole time-of-flight mass spectrometer analysis

Preparation of sample

One milligram of *C. longa* extract or red limestone extract was mixed with liquid chromatography–mass spectrometry grade methanol (1 ml) and then diluted to the concentration of 1 µl/ml.

Chromatographic condition

The chemical constituent of *C. longa* and red limestone extracts was analyzed by liquid chromatography/time-of-flight mass spectrometer (Agilent 6500 Series LC Q-TOF System). Chromatographic separation was accomplished with a Zorbax eclip plus C-18 column (2.1 mm × 50 mm, 1.7 µm, Agilent Technologies, USA). A gradient elution of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was performed at a flow rate of 200 µl/min. Total run time was 26 min. The gradient program was started at 5% B for a minute and then it was linearly increased to 17% B within 10 min. After 3 min, it was increased to 100% B within 20 min and the eluent composition was maintained for 2 min before it was decreased to 5% B over 2 min. The filtered sample solution through a 0.22 µm PTFE membrane was analyzed in a volume of 1 µl. The gas temperature was 350 ºC and gas flow was of 13 l/min. Full scan mass spectra were acquired over the mass-to-charge ratio (m/z) from 100 to 1000 amu in positive and negative ion mode. The nebulizer was 45 psig. The data analysis was performed by using Agilent Mass Hunter B.08.00 software (qualitative navigator, qualitative workflows) and PCDL database. Peak identification was evaluated by comparing the retention time, fragmentation patterns and mass spectra with references compounds from mass spectra library.

Curcuminoids analysis by high performance liquid chromatography

The determination of curcuminoids contents in *C. longa* extract and red limestone extract were performed by high performance liquid chromatography (HPLC) analysis.

Chromatographic conditions

HPLC analysis was performed on an Agilent Technology HPLC 1260 infinity II. The chromatographic separation was accomplished with an InfinityLab Poroshell 120 EC-C18 column (4.6 mm × 150 mm, 4.0 µm) at 25°C. Two mobile were used including water containing 2% acetic acid (A) and acetonitrile (B). The isocratic elution was performed with a flow rate of 1 ml/min. The elution was set at 40% B for 30 min. Before analysis, the filtered (0.45 µm nylon membrane) mobile phases were degassed using an ultrasonic bath for 30 min. The injection volume was 10 µl. Detection wavelength was 425 nm.

Preparation of standard solution

One milligram of standard curcuminoids was dissolved in 1 ml of methanol (HPLC grade). Then the filtered stock solution through a 0.45 µm PTFE membrane was dissolved in HPLC grade methanol to give concentrations of 0.25–1.0 µg/ml. The calibration curves of curcuminoids were fitted by linear regression.

Determination of cytotoxicity using MTT assay

MTT assay was evaluated to measure cell viability. RAW 264.7 and HaCaT cells were used in this assay. Briefly, the cells were seeded at 4×10^4 cells/well in 96 well plates. Then they were incubated overnight at 37°C and 5% CO_2. After that the cells were treated with 5 different concentrations of sample extracts (6.25, 12.5, 25, 50 and 100 µg/ml) for 24 h. After, 24 h, the cells were washed with phosphate buffer saline. Then, 0.5 mM MTT reagent was added into the cells and incubated for 4 h. The cell viability was measured at 570 nm with EZ read 400 microplate reader.

Determination of inflammatory in RAW 264.7 cells using nitric oxide assay

The anti-inflammatory was detected nitric oxide (NO) secretion in RAW 264.7 cells according to the method

modified by Suthiphasilp *et al*.[7] Briefly, the cells were seeded at 4×10^4 cells/well in 96 well plates and incubated overnight at 37°C with 5% CO_2. To induce cells inflammation, 1 µl of lipopolysaccharides was added into the cells and incubated for 1 h. After that the cells were treated with 5 nontoxic concentrations of the sample extracts and incubated. After 24 h, 100 µl of Griess reagent was added into the samples and then incubated for 10 min. The determination of NO was measured at 570 nm with EZ read 400 microplate reader. In addition, the results were presented as IC_{50} which calculated by GraphPad Prism 6.0 software.

RESULTS AND DISCUSSION

Liquid chromatography quadrupole time-of-flight mass spectrometer analysis

The chemical compositions of *C. longa* extract presented in Table 1 and Figure 1 demonstrated that the extract consisted of alpha-turmerone (31.31%), xanthorrhizol (12.72%), 12-oxabicyclo [9.1.0] dodeca-3,7-diene, 1,5,5,8-tetramethyl (10.45%), curcumenol (8.71%), curcumanolide B (8.41%), p-methylacetophenone (7.52%), curculonone D (5.18%), curcumin (3.99%), demethoxycurcumin (1.83%), zedoaronediol (1.21%), and bisdemethoxycurcumin (1.10%), respectively. The result suggested that alpha-turmerone was a main component of *C. longa* which was in accordance with previous studies.[8-11]

The chemical compositions of red limestone presented in Table 2 and Figure 2 demonstrated that the red limestone extract consisted of alpha-turmerone (25.61%), curcumanolide B (16.47%), Jioglutin E (15.80%), Torilolone (12.20%), respectively. Alpha-turmerone and curcumanolide B were related with the chemical constituents in *C. longa* extract which was the main component of red limestone.

Curcuminoids content

Quantitative analysis of curcuminoids in *C. longa* extract and red limestone extract was performed by HPLC analysis. There were 3 derivatives of curcuminoids including bis-demethoxycurcumin, demethoxycurcumin, and curcumin. The derivatives identification was performed by comparing the ultraviolet spectrum and retention time of each peak in the sample with the standard compound. The contents of bis-demethoxycurcumin, demethoxycurcumin, and curcumin were analyzed by comparing the peak area of each compound in the sample with the calibration curve of each compound.

Figure 3 and Table 3 reveal that total curcuminoids in *C. longa* extract was found to be 1.981 mg/g of crude extract. Bis-demethoxycurcumin, demethoxycurcumin, and curcumin were of 0.790, 0.464, and 0.727 mg/g of crude extract, respectively. However, curcuminoids were not detected in red limestone extract as shown in Figure 4.

Cytotoxicity

RAW 264.7 and HaCaT cells were investigated cell viability using MTT assay. The cells were treated with five different

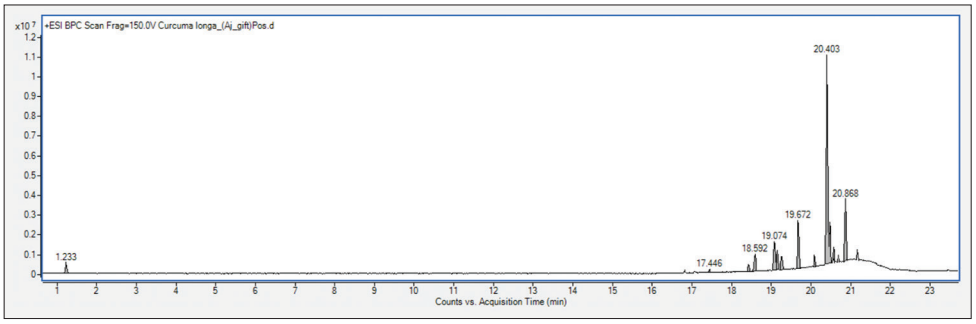

Figure 1: Liquid chromatography quadrupole time-of-flight mass spectrometer chromatogram of *Curcuma longa* extract

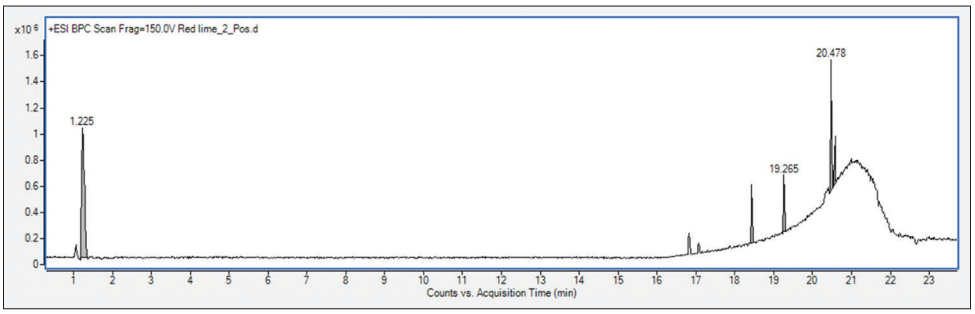

Figure 2: Liquid chromatography quadrupole time-of-flight mass spectrometer chromatogram of red limestone extract

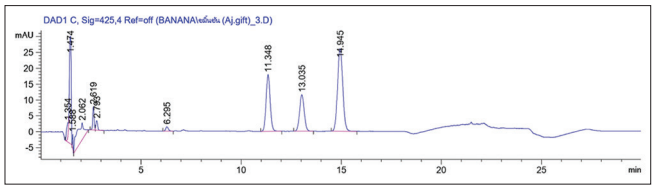

Figure 3: High performance liquid chromatography chromatogram of *Curcuma longa* extract

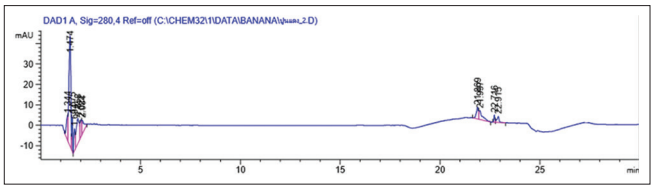

Figure 4: High performance liquid chromatography chromatogram of red limestone extract

Table 1: The chemical compositions of *Curcuma longa* extract

RT	m/z (estimated)	m/z (expected)	Chemical formula	Error PPM	Major MS-MS fragments	Name of compounds	Percentage area
17.44	252.1728	252.1725	$C_{15}H_{24}O_3$	1.18	191, 153	zedoaronediol	1.21
18.59	254.1879	254.1881	$C_{15}H_{26}O_3$	−0.78	198, 147	curculonone D	5.18
18.79	308.1039	308.1048	$C_{19}H_{16}O_4$	−2.92	291, 215, 147	bisdemethoxycurcumin	1.10
18.92	338.1159	338.1154	$C_{20}H_{18}O_5$	1.47	321, 177, 147	demethoxycurcumin	1.83
19.06	368.1253	368.1259	$C_{21}H_{20}O_6$	−1.62	337, 271, 215, 177, 149	Curcumin	3.99
19.07	234.1623	234.1619	$C_{15}H_{22}O_2$	0.42	217, 149	curcumanolide B	8.41
19.27	134.0729	134.0731	$C_9H_{10}O$	−1.49	117, 93	p-methylacetophenone	7.52
19.67	234.1614	234.1619	$C_{15}H_{22}O_2$	−2.13	191	Curcumenol	8.71
20.40	218.1673	218.1670	$C_{15}H_{22}O$	1.37	201, 177, 135, 83	alpha-turmerone	31.31
20.68	220.1833	220.1827	$C_{15}H_{24}O$	2.72	203, 185, 163	12-oxabicyclo [9.1.0] dodeca-3,7-diene, 1,5,5,8-tetramethyl	10.45
20.86	218.1681	218.1670	$C_{15}H_{22}O$	0.0	136, 121	Xanthorrhizol	12.72

MS: Mass spectrometer, RT: Retention time, PPM: Parts-per million

Table 2: The chemical compositions of red limestone extract

RT	m/z (estimated)	m/z (expected)	Chemical formula	Error PPM	Major MS-MS fragments	Name of compounds	Percentage area
16.82	252.1730	252.1725	$C_{15}H_{24}O_3$	1.98	236, 222, 181	Torilolone	12.20
17.07	232.1310	232.1310	$C_{11}H_{20}O_5$	0.00	216, 164, 163,	Jioglutin E	15.80
18.43	234.1626	234.1619	$C_{15}H_{22}O_2$	2.98	218, 204, 194, 147	curcumanolide B	16.47
20.47	218.1673	218.1670	$C_{15}H_{22}O$	1.37	201, 177, 135, 83	alpha-turmerone	25.61

MS: Mass spectrometer, RT: Retention time, PPM: Parts-per million

Table 3: Curcuminoids content in *Curcuma longa* extract

Sample name	Curcuminoids content (mg/g)			
	Bis-demethoxycurcumin (1)	Demethoxycurcumin (2)	Curcumin (3)	Total curcuminoids (1-3)
Curcuma longa extract	0.790	0.464	0.727	1.981

Table 4: RAW 264.7 cells viability after treated with sample extracts

Sample (100 μg/ml)	Cells viability (%)
Red limestone extract	90.62±0.94
Curcuma longa extract	87.33±0.64
Indomethacin	89.35±0.52

Table 5: HaCaT cells viability after treated with sample extracts

Sample (100 μg/ml)	Cells viability (%)
Red limestone extract	94.26±2.44
Curcuma longa extract	90.59±2.09
Limestone extract	99.54±0.91

concentrations of *C. longa* extract, limestone extract and red limestone extract. The treatment results revealed that viability of the cells was slightly decreased. The cell viability was reduced <15% [Tables 4 and 5]. Therefore, the results suggested that the sample extracts were nontoxic to RAW 264.7 and HaCaT cells.

Determination of inflammatory in RAW 264.7 cells using nitric oxide assay

The inflammatory was detected NO secretion in RAW 264.7 cells. The cells were investigated anti-inflammatory with *C. longa* extract, limestone extract, and red limestone extract treatment. The results showed that the extracts decreased NO in dose-dependent manner. In addition, IC_{50} of *C. longa* extract and red limestone extract were

found to be 66.53 and 102.42 µg/ml respectively whereas IC_{50} of indomethacin was 39.81 µg/ml. However, the IC_{50} of limestone cannot determine. A previous study showed that alpha-turmerone exhibits anti-inflammatory effect.[5,8] Therefore, the anti-inflammatory activity of red limestone extract might be due to alpha-turmerone content.

CONCLUSION

The anti-inflammatory as well as chemical constituents of red limestone were shown here for the first time. The results found that the extract affect to anti-inflammatory and safety. Furthermore, red limestone extract does not have cytotoxic effects in HaCaT cells and RAW 264.7 cells at its anti-inflammatory dose. Red limestone may have therapeutic potential for product development.

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Conflicts of interest

There are no conflicts of interest.

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