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Original Article

Wound healing activity of *Curcuma zedoaroides*

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Abstract

Curcuma zedoaroides rhizomes have been used in Thai folk medicine as antidote and wound care for king cobra bite wound. The inhibitory effect of *C. zedoaroides* extract and its fractions on inflammation were detected by reduction of nitric oxide release using RAW264.7 cells. The improvement capabilities on wound healing were determined on fibroblast L929 cells proliferation and migration assays. The results showed that crude EtOH extract, $CHCl_3$ and hexane fractions inhibited NO release with IC_{50} values of 14.0, 12.4 and 14.6 μg/ml, respectively. The $CHCl_3$ and EtOAc fractions significantly increased L929 cells proliferation, enhanced fibroblast cells migration (100%) on day 3 and scavenged DPPH with IC_{50} of 40.9 and 7.2 μg/ml, respectively. Only the $CHCl_3$ fraction showed marked effect against carrageenan-induced rat paw edema (IC_{50} = 272.4 mg/kg). From the present study, both *in vitro* and *in vivo* models support the traditional use of *C. zedoaroides*.

Keywords: *Curcuma zedoaroides,* anti-inflammation, wound healing effect

1. Introduction

Curcuma zedoaroides A. Chaveerach & T. Tanee called "Waan Phaya Ngu Tuamia" in Thai belongs to the Zingiberaceae family. Its morphology was clarified and illustrated by Chaveerach and coworker in 2008 (Chaveerach *et al*., 2008). The plant height ranges from 70 to 130 cm, leaf blades are 17×62 cm in length with green color. The primary rhizome of *C. zedoaroides* ranges from 8-12×2-6 cm, while secondary rhizome is 6-10×2-3 cm. Its rhizomes are pale yellow which is different from those of *C. zedoaria* Roscoe (deep yellow). The native villagers of King cobra village in the northeastern part of Thailand (Ban Khok Sa-Nga, Khon Kaen province, Thailand) use *C. zedoaroides* rhizomes as snake bite antidote by thoroughly pounding the rhizomes and mixing with Thai white spirit, and taking the mixture orally. The residue is then applied to the snake bite wound. Phytochemical compounds exerting antagonistic activity against King cobra venom were isolated and identified as labdane-type diterpenes, especially labdane dialdehyde, which were found to have high yield (79% w/w) and showed good activity (83% response for muscle contraction at concentration of 10 μM). The antivenom activity of this plant rhizomes was proven by showing good protecting effect on neuromuscular transmission of rat phrenic nerve-hemidiaphragm (Lattmann *et al*., 2010; Salama *et al*., 2012) but the biological effects on wound healing property have not been clarified. The principal idea for management of snake bite to

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focus on envenomation antidote; however, there are incidences of secondary infection and necrosis of the bite wound. The complex proteins found in snake venom are serine proteinases, phospholipase A_2 and metalloprotease enzyme, which play influential roles in the inflammatory process (Kang *et al*., 2011). Damage of tissue, destruction of blood and lymphatic vessels, and necrosis are progress rapidly. Treatment with corticosteroids on the bite wound is not beneficial to reduce tissue damage (Pochanugool *et al*., 1998). The mixture of both aerobic and anaerobic bacterial species in snake oral cavity results in a poor prognosis of infected wounds after snake bite (Theakston *et al*., 1990; Shek *et al*., 2009; Abrahamian and Goldstein, 2011). The anti-inflammatory, antimicrobial and wound healing activities of *C. zedoaroides* were therefore investigated.

2. Materials and Methods

2.1 Drugs and chemicals

Analytical grade solvents such as chloroform, methanol, ethyl acetate, etc., were obtained from RCI Labscan, Bangkok, Thailand. Luria-Bertani (LB) agar and broth were bought from Difco™ (Difco Laboratories Inc., Maryland, USA.), Mueller–Hinton (MH) agar and broth from Oxoid (Oxoid Ltd., Hampshire, England). 3-(4,5-Dimethyl-2-thiazolyl) -2,5-diphenyl-2*H*-tetrazolium bromide (MTT), penicillin streptomycin (Pen Strep), 0.25% trypsin EDTA, Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco® (*Life Technologies*, Paisley, *Scotland*). Roswell Park Memorial Institute (RPMI) 1640 Medium, fetal bovine serum (FBS), lipopolysaccharide (LPS, from *Escherichia coli*), caffeic acid phenethylester (CAPE), L-nitroarginine (L-NA), indomethacin, carrageenan type 4, Tween 80, propylene glycol, dimethyl sulfoxide (DMSO), vancomycin and other chemicals were bought from Sigma Aldrich (Sigma Aldrich, Missouri, USA).

2.2 Plant material preparation and extraction

C. zedoaroides rhizomes were bought from Ban Khok Sa-Nga (King Cobra village), Nam Pong district, Khon Kaen province, Thailand, on February 2013. The plant was identified by Prof. Dr. Arunrat Chaveerach, Khon Kaen University, Thailand. The voucher specimen number SKP 201032601 is kept at the Herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. Fresh rhizomes (13.3 kg) were cleaned, cut and dried at 60°C in a hot air oven for three days. Crude ethanol extract was then achieved by reflux of the coarse pulverized dried rhizomes (2.4 kg) with 95% ethanol (3x6 L) and the solvent evaporated under reduced pressure (232.8 g; yield 9.4% w/w). Consecutive partition of the ethanol extract (137 g) in increasing order of polarity to group the phytochemical compounds was performed. Four fractions of extract including hexane (55.2 g; yield 40.3% w/w), chloroform (30.7 g; yield 22.5% w/w), ethyl acetate (13.1 g; yield 9.6% w/w) and water fractions (37.8 g; yield 27.7% w/w) were stored in air-tight bottles and kept under 4°C until use.

2.3 Cell cultures

Murine macrophage cell line, RAW264.7 cells (American Type Culture Collection, ATCC No. TIB-71) were cultivated in RPMI-1640 medium and mouse fibroblast L929 cells (Chinese Academy of Preventive Medical Sciences, Beijing, China) were cultured in DMEM medium. Both media were supplemented with sodium bicarbonate, 10% FBS and 1% penicillin/streptomycin in 75 cm^2 flasks. All cells were maintained in humidified air containing 5% CO_2 at 37°C. The cells were reaped with 0.25% trypsin-EDTA, followed by centrifugation at 1,000 rpm for 5 min and the supernatant discarded. Fresh media was replaced to suspend the cells for further experiment.

2.4 Experimental animals

Male Wistar rats (180-220 g) and both male and female of Swiss albino mice (30-40 g) were provided by the Southern Laboratory Animal Facility of the Prince of Songkla University, Hat Yai, Songkhla, Thailand. The animals were housed in plastic cages with sawdust bedding, controlled temperature (25±2°C), artifact light dark cycle (12/12 h) and food and water *ad libitum*. The study protocols were followed the Organization for Economic Cooperation and Development (OECD) Guidance Document on Humane Endpoints in order to decrease exhaustive suffering of animals used (OECD, 2000). Procedure documentation was reviewed and approved by the Animal Ethics Committees (MOE 0521.11/599), Prince of Songkla University, Hat Yai, Songkhla, Thailand.

2.5 Evaluation on anti-inflammatory activity

2.5.1 Determination of inhibitory effect on nitric oxide (NO) production from LPS-stimulated RAW264.7 cells

The protocol described by Tewtrakul and Subhadhirasakul (2008) was modified for this experiment. Briefly, RAW264.7 cells were harvested and seeded in 96-well plate and were then incubated for 1 h. The medium was replaced with fresh medium for normal group, the medium containing 1 μg/ml of LPS together with 1% DMSO in RPMI medium for control, 1 μg/ml of LPS together with various concentrations of plant extract or fractions (3-100 μg/ml) for test groups and LPS together with standard inhibitors (3-100 μM) including CAPE (NF-κB inhibitor), L-NA (NO synthase inhibitor) and indomethacin (COX inhibitor) for positive controls. After 24 h of incubation, the accumulation of nitrite in the culture supernatant was detected by Griess reagent at wavelength 570 nm. Inhibition (%) was calculated using the following equation. IC_{50} values of each sample were determined graphically (n=4):

Inhibition (%) = [(A-C) - (B-C)/ (A-C)] × 100
A: LPS (+), sample (-); B: LPS (+), sample (+);
C: LPS (-), sample (-)

2.5.2 Determination of inhibitory effect on carrageenan-induced rat hind paw edema

The method of carrageenan-induced rat hind paw edema described by Winter *et al.* (1962) was followed in this study. The rats were divided for control (co-solvent 10 ml/kg) and treatment groups. The treated groups were divided into standard drug group (indomethacin 10 mg/kg), and test groups. Test samples (EtOH extract, hexane and $CHCl_3$ fractions) were chosen from those with high potency capability to reduce NO release from RAW264.7 cells and were prepared into three concentrations (30, 100, 300 mg/kg). Volume of rat paws was measured and calculated at baseline and various time points. The percentage of inhibition was calculated and expressed as IC_{50} values for each treatment (n=6).

$$\text{Inhibition (\%)} = [((V_t - V_o)_{control} - (V_t - V_o)_{treated}) / (V_t - V_o)_{control}] \times 100$$

V_t = paw volume after carrageenan injection at any time point
V_o = paw volume at base line

2.6 Evaluation on safety

2.6.1 Determination of safety *in vitro*: MTT assay

A simple colorimetric method for measurement of viability cells was originally described by Mosmann in 1983. Briefly, MTT solution (10 µl, 5 mg/ml in PBS) was added into the wells and incubated at 37°C for 4 h. Acidified isopropanol solution (0.04N HCl in isopropanol) was replaced into the wells and thoroughly mixed to dissolve the crystal product into a colored solution. The absorbance of this purple solution was measured at wavelength 570 nm. Optimal densities of treated cells were compared with untreated control cells. Cytotoxicity of samples was considered at lower than 80% cell viability when compared to that of the untreated control group.

2.6.2 Determination of safety *in vivo*: Acute toxicity test on mice

Up and down method (UDPfin7) guideline, which is based on the procedure of Bruce (1985) as adopted by the American Society for Testing and Materials (ASTM) in 1987 and revised in 1990, was applied (National Institute of Environmental Health Sciences: SAP meeting, 2001). Both male and female mice between 8 to 12 weeks old (30-40 g body weight) were included as a model for the test. The limit test was achieved in a single dose of 2,000 mg/kg and the main test was designed to initiate at 175 mg/kg and multiplicative viz 3.2. Any abnormality signs such as tremor, convulsion, salivation, hyperactivity, lethargy, diarrhea, loss of righting reflex, hyper- or suppressed respiration were observed carefully at the earliest 8 h and then at 24 h in finally until day 14 of sample administration.

2.7 Evaluation of wound healing properties

2.7.1 Determination of antimicrobial activity

Broth microdilution method outlined by the Clinical and Laboratory Standards Institute (CLSI, 2012) was modified for the study. Two culture strains of *Staphylococcus aureus* (ATCC no. 29213) and *Escherichia coli* (ATCC no. 25922) were assigned as targets of antimicrobial approach of plant extract and fractions. The MIC was recorded and MBC was defined at the lowest concentration of sample that completely killed the microorganism.

2.7.2 Determination of antioxidant activity: DPPH scavenging assay

DPPH (1,1-diphenyl-2-picrylhydrazylradical) free radical scavenging experiment was modified from the method described by Jitsanong and coworkers (2011). Briefly, 10 µg/ml of test samples was prepared in 100% DMSO as stock solution. Fresh dilutions of stock solution and standard antioxidants, butylated hydroxytoluene (BHT) and quercetin, with absolute ethanol into the maximum concentration of 200 µg/ml were prepared. The percentage of scavenging activity of samples or standards was calculated by the following equation and 50% of effective concentration IC_{50} were estimated graphically (n=4).

$$\text{Inhibition (\%)} = [(A-C)-(B-C)/ (A-C)] \times 100$$

A: absorbance of control;
B: absorbance of sample or standard;
C: absorbance of blank

2.7.3 Determination on proliferation of L929 fibroblast cells

Proliferation of L929 cells was determined using the modified method described by Sudsai and coworkers (2013). L929 cells were seeded into 96 well plates. The plates were incubated for 24 h, after which the medium was removed and then restored with fresh media together with DMSO (at the highest concentration as use for the test sample, 0.6% DMSO) for the control group and with various concentrations of test samples (1-30 µg/ml) for the sample group. After incubation of separately plate for 24, 48 and 72 h, the MTT solution (10 µl, 5 mg/ml in PBS) was added into wells and incubated at 37°C for 4 h. The blue formazan crystals were dissolved with 0.04N HCl in isopropanol. Optimal density at wavelength 570 nm was examined.

2.7.4 Determination of improvement on cells migration: Scratch assay

Migration of L929 cells was performed using the modified method described by Sudsai and coworkers (2013). Briefly, L929 cells were seeded into 24-well plates. After 24 h incubation, each well was vertically scratched using a sterile pipette tip and cellular debris was gently washed with PBS. Subsequently, fresh media together with DMSO (0.6% DMSO, for control group) or test samples (1-30 μg/ml) were filled into each well. The plate was then incubated at 37°C with 5% CO_2 until the end of the experiment. Photographs of the scratch line were taken at a 4x magnication using a microphotograph (Olympus CK2, Japan) at 0 (before treat), 24, 48 and 72h, respectively. Percentage of immigration at any time point was calculated by the cell distance value of each to compare with that obtained from before treatment (0 h). Increasing of the percentage of narrow gap was considered to indicate cell migration.

2.8 Statistic analysis

Data of the half maximal of inhibitory (IC_{50}) and lethal (LC_{50}) concentration were analyzed using Microsoft Excel program. The best fit trend line of dose-response curve was selected at R^2 values more than 0.900 and then the values were exhibited by an equation. Statistical significance was analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's test. A p value less than 0.05 at 90% of confident interval was signified.

3. Results and Discussion

3.1 Evaluation on inflammatory activity

3.1.1 Inhibitory effect on NO production from LPS-stimulated RAW264.7 cells

The activation by LPS on macrophage cell surfaces is initiated the downstream cascade activation of transcription factor, nuclear factor-kappa B (NF-κB) and leads ultimately to expression of many pro-inflammatory cytokines and proteins related to the inflammatory process such as COX-2 (Nadjar *et al*., 2005; Charalambous *et al*., 2009) and iNOS (Aktan, 2004). Excessive productions of NO by iNOS concurrent with the presence of superoxide anion lead to the formation of peroxynitrite ($ONOO^-$) that causes cell damage (Salvemini *et al*., 1996; Mayer *et al*., 1997; Zhou *et al*., 2006; Tewtrakul *et al*., 2009; Murphy, 2011). In addition, NO has been demonstrated to activate transcriptional factor, NF-κB, which plays an important key role in the expression of several pro-inflammatory cytokines and enzymes such as phospholipase A_2, and COX (Salvemini *et al*., 1996). Thus the inhibition on NO production was challenged to reduce excessive inflammation. The standard inhibitors, *C. zedoaroides* extract and its fractions exhibited their inhibition on NO release from LPS-stimulated RAW264.7 cells (Table 1). CAPE and indomethacin presented potent inhibitory effects with IC_{50} of 2.4 and 6.1 μg/ml, respectively. The effect of $CHCl_3$ fraction was demonstrated at IC_{50} of 12.4 μg/ml, which was more active than L-NA (13.6 μg/ml), followed by crude EtOH extract (IC_{50} = 14.0 μg/ml) and hexane fraction (IC_{50} = 14.6 μg/ml). Less potent effects of ethyl acetate and water fractions were at IC_{50} of 59.5 and 80.7 μg/ml, respectively.

3.1.2 Inhibitory effect on carrageenan-induced rat hind paw edema

The change of paw volume by carrageenan-induced rat hind paw edema was measured. Subcutaneous injections of 1% w/v of carrageenan into rat hind paw caused of biphasic responses, namely accumulation of cellular migration and exudates. The initial phase of edema (0-1 h) involves the release of histamine, 5-hydroxytryptamine (5-HT) and bradykinin (Di Rosa *et al*., 1971; Salvemini *et al*., 1996). Degranulation of histamine and serotonin causes arteriolar dilation and increases venous permeability. The release of kinins leads to an amplification of the inflammatory responses such as vasodilatation, edema, smooth muscle contraction, pain, and hyperalgesia by stimulating local tissue cells and inflammatory cells to generate additional mediators including prostanoids, cytokines especially TNF-α and interleukins, NO and tachykinins (Duchene *et al*., 2007; Murphy, 2011). This first phase extends to 2 h after carrageenan injection. The secondary phase is the accumulated releasing of prostaglandins, and also increases expression of inducible COX-2 and iNOS in the area of injection. Prostaglandins together with NO exert more amplification of inflammatory response (Salvemini *et al*., 1996). *C. zedoaroides* EtOH extract, hexane and $CHCl_3$ fractions had revealed potential effects on anti-inflammation in *in vitro* experiments, thus the investigation on anti-inflammation using animal model was then conducted. As shown in Table 2, their inhibitory effects were compared with standard drug indomethacin. The effect of indomethacin (10 mg/kg)

Table 1. Inhibitory effects of *Curcuma zedoaroides* extract and its fractions on NO production and cytotoxicity in RAW264.7 cells.

Test sample	IC_{50} (μg/ml)	LC_{50} (μg/ml)
EtOH extract	14.0±0.1	>100
Hexane fraction	14.6±1.1	>100
$CHCl_3$ fraction	12.4±4.5	>100[a]
EtOAc fraction	59.5±2.7	>100
Water fraction	80.7±1.3	>100
CAPE	2.4±0.3	11.8[a]
Indomethacin	6.1±0.7	>100
L-NA	13.6±3.0	>100

[a]cytotoxic effect at 100 μg/ml.
Values express as mean ± S.E.M (N=4).

Table 2. Inhibitory effects of *Curcuma zedoaroides* extract and its fractions on carrageenan-induced rat paw edema at 2 h and 5 h.

Sample	Dosage (mg/kg)	2 h after carrageenan injection		5 h after carrageenan injection	
		Inhibition (%)	IC_{50} (mg/kg)	Inhibition (%)	IC_{50} (mg/kg)
Control	10[a]	0.0 ± 4.3	-	0.0 ± 3.6	-
Indomethacin	10	42.7 ± 2.8*	-	58.78 ± 2.0*	-
EtOH extract	30	3.5 ± 3.1	> 300	-0.8 ± 2.2*	> 300
	100	15.1 ± 4.2		23.1 ± 3.1	
	300	18.4 ± 2.5*		35.2 ± 2.1*	
Hexane fraction	30	8.5 ± 3.6	> 300	3.6 ± 2.3*	> 300
	100	12.0 ± 3.8		14.2 ± 3.0	
	300	21.5 ± 3.5		29.0 ± 3.0	
$CHCl_3$ fraction	30	7.9 ± 3.4	> 300	8.9 ± 3.8	272.4
	100	18.4 ± 2.6		21.4 ± 3.2	
	300	31.0 ± 3.0*		54.6 ± 2.1*	

[a]dose given in ml/kg. Values express as mean ± S.E.M (N=6).
*Significantly different from control at p value < 0.05.

was shown at both 2h and 5h after carrageenan injection (42.7% and 58.8%, respectively), whereas at 2h after carrageenan injection, the highest concentration (300 mg/kg) of crude EtOH extract, hexane and $CHCl_3$ fractions showed inhibition on paw edema at 18.4, 21.5 and 31.0%, respectively. At 5h later, the volume of paws was reduced by the $CHCl_3$ fraction (54.6%), EtOH extract (35.2%) and hexane fraction (29.0%), respectively. The $CHCl_3$ fraction reached the half maximum inhibitory activity at an IC_{50} value of 272.4 mg/kg.

3.2 Evaluation on safety

3.2.1 *In vitro*: Cytotoxic effect on LPS-stimulated RAW264.7 cells

The cytotoxicity of *C. zedoaroides* EtOH extract and fractions on LPS-stimulated RAW264.7 cells was carried out using MTT assay based on viability of cells. The non-cytotoxic effect of treated RAW264.7 cells was considered in all treatments, except for CAPE that was toxic at LC_{50} of 11.8 µg/ml (Table 1). Therefore, it is suggested that crude EtOH extract and its fractions at concentration of 100 mg/ml are safe for use at the cellular level.

3.2.2 *In vivo*: Acute toxicity in mice

Single order dose of 2,000 mg/kg of *C. zedoaroides* EtOH extract and fractions was administered to five of both male and female of Swiss albino mice as described in the UDPfin7 guideline. The result showed that they were alive, active and healthy without any sign of toxicity or abnormal behaviors throughout the testing period (14 days). Survival and health of all mice implies the safety of short-term use of *C. zedoaroides* extract and its fractions.

3.3 Evaluation on wound healing properties

Secondary bacterial infection of injured tissue leads to the persistence of inflammation, higher production of reactive oxygen species and delays the healing process or excessive inflammation, eventually, becoming a chronic inflammatory response (Murphy, 2011). The chemical attractants like chemokines and insoluble substrates of the extracellular matrix released at the site of injury chemotactically recruit many cells for tissue regeneration and repair. Cell migration, cell proliferation, together with extracellular matrix organization and remodeling are the three fundamental processes of wound healing (Sephel and Woodward, 2011). The active compounds that promote wound healing effect through antimicrobial capability, antioxidant activity, stimulation of cell proliferation and enhancement of cell migration were further investigated.

3.3.1 Antimicrobial activity

The incidence of organ distortion, especially of the distal extremity, or sepsis after venomous snake bite in Thailand has been reported. It is caused by bacterial infection right after the snake bite. There are reports of no microbial strain growth in 50% of rattlesnake venom samples and it has been suggested that the antimicrobial activity of snake venom could protect the bite wound from any pathogen and bacterial infection (Goldstein *et al*., 1979; Abrahamian *et al*., 2011). However, normal flora in the snake oral cavity and proteolytic enzyme in snake venom cause tissue damage,

cellulitis and necrosis. The devitalized tissues are then easily attacked by bacteria. Garg and coworkers (2009) have reported the two most common bacterial strains found in snake bite-associated wound infections are *Staphylococcus aureus* (32%) and *Escherichia coli* (15%). The MIC/MBC of *C. zedoaroides* extract and fractions compared with vancomycin and the aminoglycoside, gentamicin, using broth microdilution assay were determined. The results indicated that antimicrobial activity of vancomycin against *Staphylococcus aureus* and gentamicin against *Escherichia coli* were at MIC/MBC of 0.5/1 and 0.25/0.5µg/ml, respectively. However, the plant rhizomes extract and its fractions showed mild antimicrobial activity (Table 3). Thus, the application of the plant rhizomes with white spirit for topical applying on snake bite wound should be considered together with rigorous cleansing and fresh dressing.

3.3.2 Antioxidant activity

The increasing number of PMNs in elimination process of inflammation can cause increased oxygen consumption and production of reactive oxygen species (ROS). Extreme oxidative stress causes damage of the surrounding tissue, fibroblasts and many other cells (Houghton *et al*., 2005). The compound with low energy of bond dissociation such as OH atom and/or low ionization potential with competence to donate electron to the radical molecules was the purposes to find out for scavenging activity. The ability to oxidize the free radical from biomolecules can prevent the oxidative damage that is involved in the development of cancer, aging, infection, etc. (Wright *et al*., 2001). The compounds such as phenolic derivatives with OH substituted at the ortho position and suitable to form intramolecular hydrogen bonding, leading to good antioxidant activity better than the substitution at para and meta positions (Bendary *et al*., 2013). The DPPH radical scavenging assay is a rapid and simple method for preliminary screening of plant antioxidant efficacy (Clarke *et al*., 2013). Compounds that scavenge radicals or hydrogen donors will trap the odd electron of DPPH nitrogen free radical molecules to form the reduced DPPH-H and result in the change of DPPH from purple to yellow. Antioxidant activity of the standard agents (quercetin and BHT), *C. zedoaroides* extract and fractions is shown in Table 4. Good scavenging activities were found in quercetin and BHT with IC_{50} value of 1.8 and 24.3 µg/ml, respectively. The IC_{50} values of EtOAc and $CHCl_3$ fractions were at 7.2 and 40.9 µg/ml, respectively, whereas the crude EtOH extract and other fractions exhibited mild effect (IC_{50} = 97.6 to >200 µg/ml). The efficacy of DPPH assay indicates that EtOAc and $CHCl_3$ fractions could be used as antioxidant by interaction with the extracellular radicals.

3.3.3 Enhancement effect on fibroblast L929 cells proliferation

Fibroblasts differentiation, proliferation and migration toward the site of injury was activated by several growth factors, chemokines and matrix degradable product (Schreier *et al*., 1993; McDougall *et al*., 2006; Sephel and Woodward, 2011). The enhancement effect on fibroblast cells proliferation was achieved by the MTT method. Increasing cell viability of *C. zedoaroides* extract and its fractions (1, 3, 10 and 30 mg/ml) is presented in Table 5. On day 1 after incuba-

Table 3. The MIC and MBC values of *Curcuma zedoaroides* extract and its fractions against *Staphylococcus aureus* and *Escherichia coli*.

Sample	MIC/MBC (µg/ml)	
	Staphylococcus aureus	*Escherichia coli*
EtOH extract	1,024/1,024	1,024/>1,024
Hexane fraction	1,024/1,024	1,024/>1,024
$CHCl_3$ fraction	1,024/1,024	>1,024/NA
EtOAc fraction	1,024/1,024	>1,024/NA
Water fraction	1,024/>1,024	1,024/>1,024
Vancomycin	0.5/1	-
Gentamicin	-	0.25/0.5

NA, not applicable; N=3

Table 4. Antioxidant activity of *Curcuma zedoaroides* extract and its fractions on DPPH scavenging assay.

Sample	%inhibition at various concentrations (µg/ml)							IC_{50} (µg/ml)
	1.56	6.25	12.5	25	50	100	200	
EtOH extract	-	-	11.4 ± 4.3**	17.9 ± 2.6*	31.6 ± 4.7**	48.9 ± 5.8**	69.4 ± 4.4**	97.6
Hexane fraction	-	-	15.3 ± 2.6*	18.4 ± 2.7*	24.7 ± 3.7**	30.9 ± 4.0**	43.9 ± 3.5**	>200
$CHCl_3$ fraction	11.5 ± 0.4	16.2 ± 1.5	26.4 ± 0.7	37.9 ± 0.8	50.0 ± 1.5	71.8 ± 2.7	76.3 ± 0.9	40.9
EtOAc fraction	17.1 ± 0.7	35.8 ± 0.6	59.8 ± 0.4	88.5 ± 0.7	90.3 ± 0.3*	89.6 ± 0.2**	86.7 ± 0.4	7.2
Water fraction	-	-	10.3 ± 3.1**	11.1 ± 2.9*	19.7 ± 2.5*	22.8 ± 4.7**	21.6 ± 4.5**	>200
Quercetin	42.2 ± 2.6**	86.3 ± 0.4	86.3 ± 0.2	87.3 ± 0.1*	86.2 ± 1.3	87.5 ± 0.1*	88.6 ± 0.4	1.8
BHT	5.9 ± 0.1*	21.8 ± 0.1*	35.9 ± 0.7	52.7 ± 0.1*	68.8 ± 1.4	79.5 ± 0.4	83.4 ± 0.7	24.3

Values express as mean ± S.E.M (N=4).
*, **Significantly different from control at *p value* < 0.05 and < 0.01, respectively.

Table 5. Enhancement effect of *Curcuma zedoaroides* extract and its fractions on proliferation of mouse fibroblast L929 cells.

Sample	Concentration (μg/ml)	%Viability of L929 cells at various time points (day)		
		1	2	3
EtOH extract	1	99.7±9.2	106.9±5.0*	117.9±6.6**
	3	98.6±9.3	108.2±6.3**	97.9±1.7
	10	117.5±9.5	93.6±3.5	87.7±0.8
	30	104.8±4.8	98.4±7.3**	85.0±2.8
Hexane fraction	1	90.8±4.8	107.4±6.8**	92.8±2.0
	3	93.1±4.3	107.1±9.8**	95.5±7.9
	10	106.6±9.2	103.3±7.8**	97.7±9.0
	30	113.8±2.0	94.0±6.7**	97.0±7.2
$CHCl_3$ fraction	1	120.5±5.4	108.7±4.1*	120.7±7.0
	3	105.0±6.2	113.3±7.9**	109.1±9.9
	10	90.4±6.3	102.4±7.5**	107.5±8.4
	30	101.2±5.2	110.6±6.4**	115.9±6.6
EtOAc fraction	1	95.7±5.3	94.9±3.1*	119.9±6.8*
	3	95.5±4.0	97.1±4.5*	118.9±5.9
	10	97.8±6.2	84.9±2.7	118.7±5.3**
	30	111.3±4.7	81.3±2.8	119.6±6.2*
Water fraction	1	109.3±8.3	97.1±6.7*	104.0±6.2
	3	107.0±9.1*	140.1±2.5**	116.7±4.8
	10	78.6±7.2	103.8±3.7*	100.1±5.0
	30	96.3±6.9	108.8±4.4*	97.7±1.9

The viability of L929 cells of the control group at various time points (day) were assigned as 100%. Values express as mean ± S.E.M (N=4). *,** Significantly different from control at p value < 0.05 and < 0.01, respectively.

tion of test samples, cell viability was observed at high concentration (30 μg/ml) of EtOH extract (104.8% cell viability), hexane (113.8%) and EtOAc (111.3%) fractions and almost of all concentrations of $CHCl_3$ fraction (90.4-120.5%), whereas the water fraction was signified at low concentrations of 1 and 3 μg/ml with 109.3 and 107.0%, respectively. Increasing proliferative effect on day 2 was mostly observed from the $CHCl_3$ fraction (102.4-113.3%). On day 3, decreases in vital cells were detected at high concentration (30 μg/ml) of EtOH extract (85.0%) and hexane fraction (97.0%), whereas $CHCl_3$ and EtOAc fractions exhibited good cell viability at 120.7-115.9% and 119.9-119.6%, respectively. Hence, the enhancement of cell viability of all fractions was further determined for fibroblast cell migration.

3.3.4 Improvement effect on fibroblast cells migration

The vertical line on monolayer of fibroblast cells was drawn to imitate the gap of dermal wound. At the site of injury, oviform of fibroblasts stretched their extremity and degraded the brin to form collagen and other matrix protein (McDougall *et al*., 2006; Sephel and Woodward, 2011) leading to reconstitution of the manifold connective tissue elements. In this experiment, the progression of cell proliferation and migration were demonstrated as early 12 h after laceration. Narrow margin of both border lines of gap present cells migration toward the cell-cell interaction was observed on day 0, 1, 2 and 3. On day 2, the cells treated with EtOAc fraction (30 μg/ml) showed meaningful cell migration at 75.8% (Table 6 and Figure 1). Complete cell junction (100% migration) on the stimulation of $CHCl_3$ (10 μg/ml), EtOAc (30 μg/ml), and water fractions (3 μg/ml) were significantly observed on day 3. Although the EtOH extract (1 μg/ml) and hexane fraction (3 μg/ml) were not examined for the cell-cell contact, they expressed marked migration on day 3 (87.9% and 67.5%, respectively) and also showed higher activity than that of the control group (49.5%). The results indicate that *C. zedoaroides* extract, $CHCl_3$, EtOAc and water fractions are suitable for using in traditional remedy to promote wound healing. However, the $CHCl_3$ fraction possessed the most potent wound healing property through fibroblast proliferation (Table 5) and fibroblast migration (Table 6), which are consistent with the result on NO inhibitory effect (Table 1) and carrageenan-induced rat paw edema (Table 2).

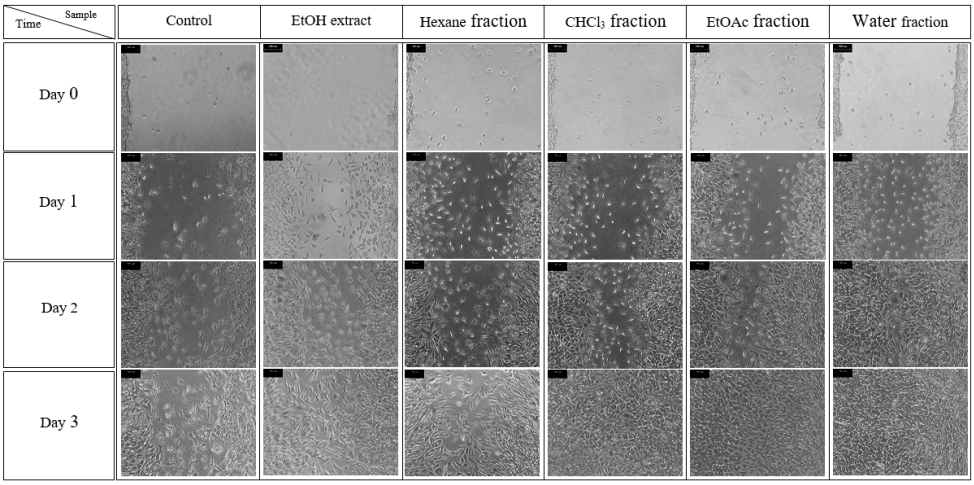

Figure 1. Migration of L929 cells of *Curcuma zedoaroides* extract and its fractions. The photographs were captured at 4 x magnifications at day 0 to day 3 after treated with EtOH extract (1 μg/ml); hexane fraction (3 μg/ml); $CHCl_3$ fraction (10 μg/ml); EtOAc fraction (30 μg/ml) and water fraction (3 μg/ml).

Table 6. Effect of *Curcuma zedoaroides* extract and its fractions on mouse fibroblast L929 cells migration.

Sample	Dose (μg/ml)	Length of L929 cells (μm)				% Migration		
		Day 0	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Control	-	794.4 ± 9.7	645.9 ± 9.6	535.6 ± 1.3	401.8 ± 8.7	19.2 ± 1.5	33.2 ± 1.2	49.5 ± 1.2
EtOH extract	1	746.2 ± 2.6	476.5 ± 11.3	321.4 ± 9.5	90.5 ± 2.6*	36.2 ± 0.9	57.1 ± 0.8	87.9 ± 0.3*
Hexane fraction	3	820.0 ± 15.1	565.3 ± 8.0	379.9 ± 11.5	268.3 ± 8.7	30.8 ± 1.0	53.4 ± 1.6	67.5 ± 0.5
$CHCl_3$ fraction	10	809 .0 ± 5.4	592.8 ± 7.0	293.5 ± 7.5	0.0 ± 0.0**	26.6 ± 0.8	63.8 ± 0.8	100.0 ± 0.0**
EtOAc fraction	30	804.1 ± 9.5	391.4 ± 4.2	195.5 ± 5.7	0.0 ± 0.0**	51.2 ± 0.8	75.8 ± 0.4*	100.0 ± 0.0**
Water fraction	3	805.2 ± 7.3	409.7 ± 13.5	87.3 ± 9.2	0.0 ± 0.0**	48.8 ± 1.0	89.5 ± 1.0	100.0 ± 0.0**

Values express as mean ± S.E.M (N=4)
*,** Significantly different from control at p value < 0.05 and <0.01, respectively.

4. Conclusions

The biological properties found in this study support the traditional use of *C. zedoaroides* for wound care. The $CHCl_3$ fraction showed appreciable anti-inflammatory activity both in *in vitro* and *in vivo* experiments. Inhibition by *C. zedoaroides* of NO release from RAW264.7 cells and reduction of rat paw edema at both 2 and 5 h compared with the control group imply the inhibitory effects of this plant on several mediators. The EtOH extract of *C. zedoaroides* and its fractions expressed their property on fibroblast L929 proliferation, migration, and DPPH radical scavenging effect. Especially, the $CHCl_3$ and EtOAc fractions showed the advantage of improvement of wound regeneration and wound repair, nevertheless, they also exhibited mild effect against *Staphylococcus aureus* and *Escherichia coli* infection. The isolation of active compounds responsible for wound healing property from the $CHCl_3$ fraction of *C. zedoaroides* and investigation of the plausible mechanism study are now in progress. Moreover, the NMR spectrum showed that the isolated compounds from this fraction are diterpene and sesquiterpenes, which are now under structure elucidation.

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