

Evaluation of the wound-healing activity and anti-inflammatory activity of aqueous extracts from *Acorus calamus* L.

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Abstract: In folklore medicine, *Acorus calamus* has been used as a wound-healing agent for thousands of years; however, there have been few scientific reports on this activity so far. Now, we explored deeply the wound-healing effect of aqueous extracts from the fresh roots and rhizomes of *A. calamus in vivo*, as well as anti-inflammatory activity *in vitro*, so as to provide scientific evidence for the traditional application. The wound-healing effect was determined by the image analysis techniques and the histological analysis in the excisional wounding test, and the anti-inflammatory activity was evaluated by the real-time RT-PCR techniques in the lipopolysaccharide-induced RAW 264.7 cells test. Aqueous extracts, administered topically at the dose range from twice to thrice in a day, could enhance significantly the rate of skin wound-healing. Moreover, the extracts could effectively inhibit the mRNA expressions of inflammatory mediators induced by lipopolysaccharide in RAW 264.7 cells. These results showed significantly the wound-healing activity of aqueous extracts in the animal model of excise wound healing, and anti-inflammatory activity *in vitro*.

Keywords: *Acorus calamus*, aqueous extracts, wound-healing activity, anti-inflammatory activity.

INTRODUCTION

Acorus calamus L., a perennial semi-aquatic plant, belongs to the Araceae family. This plant distributes widely in the North Temperate Zone and many tropic countries, such as India, Pakistan, China and Korea (Kumari *et al.*, 2010). The aqueous extracts from rhizomes and roots of *A. calamus* have been utilized as an effective agent for wound-healing for thousands of years in folklore medicine (Jain *et al.*, 2010). In China, Dengzhou Area in Shan-dong Province in particular, the native peasants were often found to treat the skin injure with the aqueous extracts (semi-solid state) from the fresh root and rhizome of this plant (Jiang, 1983).

Recently, it has been reported that the large quantity of chemical constituents has been isolated from the roots and rhizomes of *A. calamus*, including acorenone, β -asarone, calamendiol, α -calacorene and shyobunone (Raina *et al.*, 2002). These compounds have diverse biological activities, such as anti-diabetic activity, antioxidant, sedation and anti-microbial activity (Kim *et al.*, 2011). Interestingly, although *A. calamus* is useful for wound-healing in folklore medicine, there have been few reports on this activity so far.

Moreover, it's well known that skin tissue injury is in concomitant with inflammation in most situations (Sugihara *et al.*, 2000; DiPietro and Burn, 2010). Therefore, we would explored further the wound-healing effect of the aqueous extracts (AE) on the excisional

wound healing model in present study, as well as anti-inflammatory activity *in vitro*, so as to provide scientific evidence for the traditional application.

MATERIALS AND METHODS

Plant material

A. calamus was collected in the South Lake Area (Shenyang) in 2010, and was identified in the Shenyang Pharmaceutical University. The voucher specimen (# 66) was preserved at the Herbarium of that university.

Animals

Each group was composed of 20 ICR mice (20 \pm 2g, equal numbers of male and female). They were housed at 20 ~ 22 in a 12 h light /12 h dark cycle (light on at 6:30 am) and were free access to tap water and standard pellet diet. The whole experimental protocols were consented by the Animal Care and Use Committee of Shenyang North Hospital.

The sample preparation

The fresh roots and rhizomes of *A. calamus* (5.0 kg) were extracted with distilled water for 3 h. AE (256.7 g) was obtained in rotary evaporator at 85. The semisolid mass of AE (50 g) mixed homogeneously with ointment base in the concentration of 20% (w/w).

Protocols

The doses of AE referred to the utilization principles of this plant in the folk medicine and the results from the

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preliminary experiment. AE was administered topically at the dose range from once to thrice in a day in the excisional wounding test. The vehicle group was administered with the ointment base.

Wound-healing activity

Each mouse was anesthetized with the mixture of xylazine (10 mg/kg) with ketamine (80 mg/kg) by the way of intraperitoneal injection. Then, the shaved hip was wiped with a sufficient amount of 70% EtOH. The final localization of two wounds was marked before starting to excise the skin areas.

The skin of hip was lifted back with forceps and incised with a first and careful cut using the scissors. Lifting up the skin would ensure that the incision could move through the panniculus carnosus. Following the first cut, the partially removed skin area was hold with forceps, and the excision was completed with two to three additional cuts. The shape of wound was almost round whose diameter was 0.8 cm (Dipietro and Burns, 2010).

Wounds area measurement

The measurement of wound area was taken based on the digital photographs of the mouse's wounds using the image analysis program (Image-Pro Plus 6.0). The same laboratory technician took the digital pictures on the days 1, 3, 5, 7 and 13 post-wounding, respectively.

Histological Analysis

Five microns thick sections of the wound samples, which were collected on the day 7 and 13 post-wounding and were embedded in paraffin, were stained with hematoxylin and eosin (H&E) and Masson. For histological evaluation, slides were observed under 200× or 400× magnifications in a blind manner (Wu *et al.*, 2012).

Anti-inflammatory activity

Cells culture

The murine macrophage cell line RAW264.7 was purchased from American Type Culture Collection (ATCC, TIB-71). Cells were maintained in complete RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Sigma, USA) at 37° and in the 5% CO₂ humidified atmosphere. Cells were subcultured in plastic dishes every 4 days at a dilution of 1 : 6 using 0.25% trypsin and 0.02% EDTA in phosphate buffered saline (PBS).

Cytotoxicity test

AE was dissolved in dimethyl sulfoxide (DMSO) and diluted with RPMI-1640 (final concentration of DMSO was less than 0.01%). The dilution of RPMI-1640 contained DMSO (less than 0.01%) was used as negative control.

RAW264.7 cells were planted on 96-well plates with 2×10⁴ cells per well and cultured overnight. Then cells

were incubated with various concentrations of AE (5~160 µg/ml) for 24 h. Thereafter 0.5 mg/mL MTT was added into each well and cells were incubated for another 4 h to test the cell viability. The medium was then discarded and 100 µL DMSO was added. Finally the absorbance was detected at 490 nm using a microplate reader.

Total RNA extraction

RAW264.7 cells were seeded into 6-well plates with 5×10⁵ cells per well. After cells adhered to the plate, the medium was removed. Then, the fresh medium containing 3 ml of lipopolysaccharide (LPS) (100 ng/ml) with/without different concentrations of samples (0.1, 1 and 10 µg/ml) was added. After incubated for 10 h, total RNA of cells was extracted with RNAiso Plus (Takara) (Guo *et al.*, 2011).

Real-time RT-PCR detection

As the template, total RNA (~ 1 µg) was utilized in the first strand cDNA synthesis with PrimeScript RT-PCR Kit (Takara). The ABI 7300 sequence detection system was applied in the test. RT-PCR Primers of TNF-α, IL-1β, IL-6, iNOS and GAPDH were shown in table 1. The reactive program of PCR was firstly 95°C for 30 s, then, 40 cycles of 95°C for 5 s, finally 60°C for 30 s. Dissociation was performed according to a melting program. All the reactions were performed three times in parallel.

STATISTICAL ANALYSIS

These results were shown as the mean ± S.E.M and were analyzed by ANOVA with SPSS software (version 11.0).

RESULTS

Effects of AE on the excision wound model

Measurement of wounds area

AE, administered topically at the dose range from twice to thrice in a day, could enhance significantly the rate of wound-healing on the day 3, 5, 7 and 13 post-wounding, in comparison with vehicle group (table 2).

Histological analysis

Photographs of tissue sections from the vehicle group or AE group were exhibited in fig. 1. In summary, in the process of wound-healing, there were less inflammatory cells and more collagen bundles, hair follicles and fibroblasts in the granulation tissue of the wound samples from the AE group than those in the wound samples from the vehicle group. Moreover, the blood vessels density in the granulation tissue from the AE group was obviously higher than that from the vehicle group.

Effects of AE on the LPS-induced RAW 264.7 cells

MTT assay

The result showed that AE (≤10 µg/ml) couldn't exhibit the cytotoxicity, in comparison with the vehicle group (fig. 2A).

Effects of AE on mRNA expressions of inflammatory mediators

AE (10 µg/ml) could inhibit effectively LPS-stimulated mRNA expressions of TNF-α, IL-6, IL-1β and iNOS in the RAW 264.7 cells (fig. 2 B-E).

DISCUSSION

It's well known that wound-healing started instantly while the skin was subjected to injury or trauma. This process was composed of three complicated and well-organized phases, that is, the inflammation phase, the proliferation phase and the maturation phase (Singer and Clark, 1999). In the animal model of excisional wounding, mice were sacrificed at day 3, 5, 7, and 13 post-wounding, which was considered the desired experimental time points, to remove the wounded tissues, as these time points reflect central time points of repair including inflammation, keratinocyte migration and proliferation, and the formation of new stroma (D 1-7) as well as the end point of the acute healing process (D 13) (DiPietro and Burn, 2010). In this work, these data showed AE, administered topically at the dose range from twice to thrice in a day,

could enhance significantly the rate of skin-wound healing *in vivo* by the image analysis techniques, in comparison with the vehicle group. In addition, those results from the granulation tissue sections had also revealed further that the rate of tissue regeneration was much quicker in the treated group: 1) The well-organized bundles of collagen in the granulation tissue from the AE group indicated the effects of AE on the proliferation of fibroblast and the formation of extracellular matrix in the process of wound-healing. 2) The blood vessel density in the granulation tissue from the AE group exhibited the significant angiogenesis activity of AE in the proliferative stage of wound-healing. As we known, collagen bundles could contribute to wound-healing by building the structural framework for cambium (Mahmood *et al.*, 2009). In addition, angiogenesis was conducive to transporting the required nutrients for the tissue regeneration by improving the blood flow in the process of wound-healing (Wu *et al.*, 2012). 3) More hair follicles in the granulation tissue demonstrated that AE could contribute to the re-epithelialization and weak the forming of scar.

Table 1: Primers and probes used in this study

Gene Name	Sequence	
TNF-a	F	5'-CTCCAGAACATCTTGGAA -3'
	R	5'-GAGTGAAAGGGACAGAAC -3'
	P	5'-(FAM) AAAGCAAGCAGCCAACCAGG (Eclipse)-3'
IL-1β	F	5'-TACCCAAAGAAGAAGATG -3'
	R	5'-CCCATACTTTAGGAAGAC -3'
	P	5'-(FAM) TCAATTATGTCCTGACCACTGTTGT (Eclipse)-3'
IL-6	F	5'-CCAGAAACCGCTATGAAG -3'
	R	5'-AGTGGTATAGACAGGTCTG -3'
	P	5'-(FAM) TATCCTCTGTGAAGTCTCCTCTCCG (Eclipse)-3'
iNOS	F	5'-GACTGAGCTGTTAGAGAC -3'
	R	5'-GGTAGGATTTGACTTTGAAG -3'
	P	5'-(FAM) CTCACGCTTGGGTCTTGTTCAC (Eclipse)-3'
GAPDH	F	5'-CAATGTGTCCGTCGTGGATCT -3'
	R	5'-GTCCTCAGTGTAGCCCAAGATG -3'
	P	5'-(FAM) CGTGCCGCCTGGAGAAACCTGCC(Eclipse)-3'

Table 2: Effects of AE on the excision wounds model

	Area of Wounds (mm ²)				
	Day 1	Day 3	Day 5	Day 7	Day 13
Vehicle Group	0.27±0.019 (100%)	0.22±0.016 (81%)	0.16±0.016 (59%)	0.086±0.0055 (31%)	0.020±0.0024 (7%)
Aqueous Extract (o.d.)	0.28±0.022 (100%)	0.20±0.028 (73%)	0.13±0.018 (45%)	0.076±0.015 (27%)	0.014±0.0093 (5%)
Aqueous Extract (b.i.d.)	0.28±0.013 (100%)	0.18±0.0084 (65%)*	0.11±0.0073 (39%)*	0.048±0.0044 (17%)*	0.0060±0.0022 (2%)*
Aqueous Extract (t.i.d.)	0.28±0.014 (100%)	0.17±0.014 (60%)*	0.091±0.011 (33%)*	0.037±0.0049 (13%)*	0.0031±0.0012 (1%)*

Each group represents the mean ± S.E.M. (n=20). Asterisks indicated significant difference from control. *P<0.05, **P<0.01, ***P<0.001 (One-Way ANOVA Test). Percentage of wounds-healing was shown in parentheses

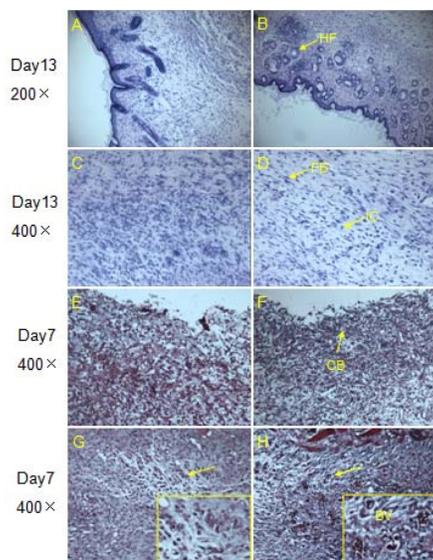


Fig. 1: Histological figure from wound tissue on day 7 and day 13 post-wounding under 200× or 400× magnifications (slide A, B, C and D were stained with H&E, slide E, F, G and H were stained with Masson). The extract group showed granulation tissue with more hair follicles (B, HF) and fibroblast (D, FB), more well-organized bands of collagen (F, CB), the larger number of

blood vessels (H, BV), and less inflammatory cells (D, IC). The control group showed granulation tissue contains less hair follicles (A), fibroblast (C), collagen (E), and the small number of blood vessels (G), more inflammatory cells (C).

Several lines of literature have indicated that inflammation in the process of wound-healing played an important role in activating the inflammatory cytokines and chemokines and in recruiting macrophages, which could enhance rate of wound-healing (Keylock *et al.*, 2008). However, prolonged inflammatory interaction in the injurious tissue could also delay the rate of wound-healing (Sasidharan *et al.*, 2012). Therefore, it's essential for wound-healing to inhibit properly inflammation in the wound. The dose of AE, which showed no cytotoxicity, could decrease significantly the mRNA expressions of TNF- α , IL-1 β , IL-6 and iNOS in LPS-induced RAW246.7 cells. These results indicated that AE might inhibit effectively the extra inflammation induced by the uncontrolled infection in trauma. In addition, the expression of iNOS could be up-regulated by inflammatory stimuli, leading to catalyzing promptly the synthesis of NO, in turn; the larger quantities of peroxynitrite were produced. Therefore, suppression of

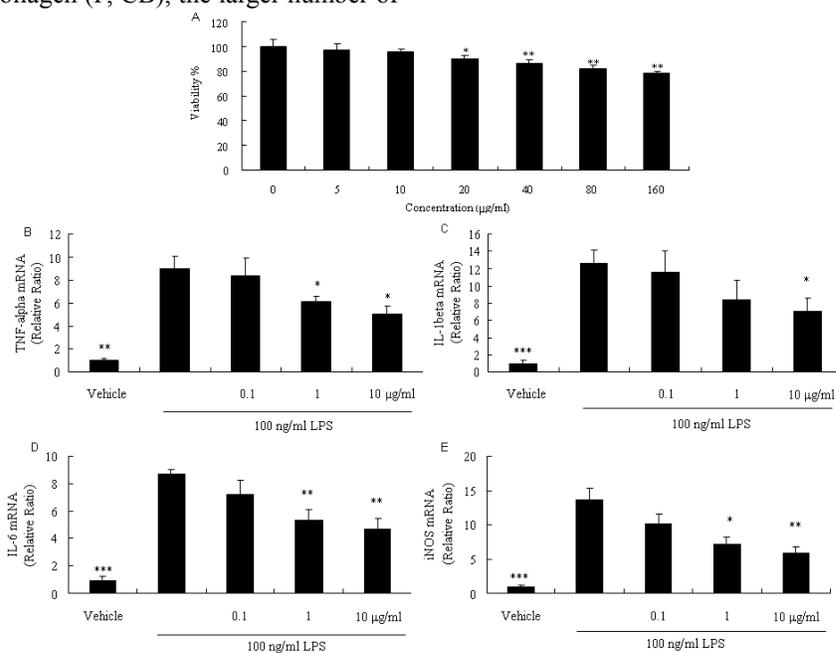


Fig. 2(A): RAW264.7 cells were incubated with various concentrations of AE (5 ~ 160 $\mu\text{g/ml}$) for 24 h. Thereafter MTT solution was added to cell cultures and incubated for 4 h. Cell viability measured at different concentrations of AE was compared using one-way analysis of variance (ANOVA). Proliferation of the cells exposed to AE was compared to the negative control, * $p < 0.05$, ** $p < 0.01$ was considered statistically significant. Each column represented the mean \pm S.E.M (n=3).

(B-E) Effect of AE (0.1, 1 and 10 $\mu\text{g/ml}$) on the mRNA expressions of TNF- α , IL-1 β , IL-6 and iNOS induced by LPS in RAW264.7 cells. RAW264.7 cells were seeded into 6-well plates and incubated overnight. Then medium was removed, and the fresh medium containing 3 ml of LPS (100 ng/ml) with or without different concentrations of samples (0.1, 1 and 10 $\mu\text{g/ml}$) was added, and cells were further incubated for 10 h. Each column represented the mean \pm S.E.M (n=3). Asterisks indicated significant difference from the LPS group. * $p < 0.05$. ** $p < 0.01$ and *** $p < 0.001$ (ANOVA followed by Dunnett's test)

the expression of iNOS might also inhibit potentially the oxidative stress which was a negative factor to wound-healing.

CONCLUSION

These results have demonstrated significantly the wound healing activity of AE from the fresh roots and rhizomes of *A. calamus* in the animal model of excise wound healing, and anti-inflammatory activity *in vitro*.

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