

EVALUATION OF ANTI-BACTERIAL AND WOUND HEALING ACTIVITY OF THE FRUITS
OF *AMORPHA FRUTICOSA* L.

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Abstract

As the traditional Chinese medicine, the fresh fruits of *Amorpha fruticosa* L. were applied for the treatment of carbuncle, eczema and burn (Das et al., 2007). However, little is known about the functional roles of the fruits of *Amorpha fruticosa* L. during wound healing progress. In the present study, we evaluated both antimicrobial potential against a wide range of microorganisms and wound healing activity of the seven compounds isolated from the fruits of *Amorpha fruticosa* L. *in vitro* and *in vivo*. Our results showed that compounds I (6a,12a-dehydroamorphin), V (dehydrosermundone) and VI (tephrosin) isolated from the fruits of *Amorpha fruticosa* L. performed dominant antimicrobial potential against microorganisms. Moreover, these compounds significantly enhanced fibroblasts proliferation and migration, leading to promotion of wound healing. Thus, it could be possible for the therapeutic utilization of *Amorpha fruticosa* L. for wound healing in the future.

Key words: *Amorpha fruticosa* L. ;anti-bacterial; wound healing

Introduction

The use of plant makes it possible to double our life span in the 20th century and the natural products from plants have been an overwhelming success in modern society. *Amorpha fruticosa* L. is a shrub plant belonging to the genus *Amorpha* in Leguminosae family (Lee et al.,2006). There are about twenty-five species plants in genus *Amorpha* in the whole world. Only the species *fruticosa* had been introduced to be planted in China and had a wide distribution in Liaoning Province of China. The therapeutic utilization of *Amorpha fruticosa* L. for wound healing could date back to the beginning of civilization (Das et al., 2007). As the Traditional Chinese Medicine theories described, the fresh fruits of *Amorpha fruticosa* L. were applied for the treatment of carbuncle, eczema and burn (Das et al.,2007). In the China, Record of Natural Resources of Chinese Materia Medica, the effectiveness of dried fruits of *Amorpha fruticosa* L. was applied as paste externally twice or thrice a day for wound-healing purpose (Liu et al.,2005).

Wound healing is a complex, integrated series of biochemical, cellular and physiological processes (Martin, 1997). The purpose of repairing events is to resist pathogens invasion, establish the integrity of the damaged tissue and reconstruct physiological function of skin. The healing process in full-thickness skin wounds includes inflammation, wound contraction, development of granulation tissue, scar formation and tissue remodeling (Singer and Clark, 1999) which are concurrent but independent to each other. During wound reparation progress, a critical step is the proliferation and migration of the resident

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epidermal keratinocytes and dermal cells from the wound edge and surrounding epidermis to the wound bed (Babu and Wells, 2001; Eming et al., 2007). The dermal cells, including dermal fibroblasts and dermal microvascular endothelial cells, perform important function in this reparation process. Topical application of compounds with enhancement of dermal cells proliferation and migration is to improve significantly wound healing (Pan et al., 2010). In previous study, we reported the isolation from the PE, AcOEt and n-BuOH of the fruits of *Amorpha fruticosa* L. Seven compounds were obtained and their structures were elucidated (Wang et al., 2010), and we evaluated both antimicrobial potential against a wide range of microorganisms and wound healing activity of the seven compounds isolated from the fruits of *Amorpha fruticosa* L. *in vitro* and *in vivo*.

Experimental Section

Plant

The fruits of *Amorpha fruticosa* L. were collected from Dalian City of Liaoning province, China in July. It was authenticated by vice Prof. Yun-peng Diao, college of pharmacy, Dalian Medical University and a voucher specimen has been deposited in pharmacognosy laboratory under specimen number AF0017.

Cells, animals and microorganisms

The mouse fibroblast cell line (L929) was obtained from Chinese Academy of Sciences and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100µg/ml streptomycin. All cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C. Healthy, male, Sprague-Dawley rats, weighing 200-220g, were supplied by the Animal Experimental Center of Dalian Medical University and housed one per cage for a week before the study in a room with controlled environment (12-h light/dark cycle, 23±2°C and relative humidity 70%). They were also allowed free access to standard laboratory diet and water. All experimental procedures were approved by the Animal Research Ethics Committee of Dalian Medical University, Dalian, China. The Bacterial (*Bacillus subtilis*, *Bacillus cereulences*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella aeruginosa*) were got from China Center for Type Culture Collection.

Wound healing test in vitro

MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) viability assay was performed as previously described with the following modification (Do et al., 2008). Briefly, cells were seeded into 96-well plate with 0.6×10^4 cells/well. After 24 h attachment, various concentrations (1–1000µg/ml) of seven compounds were applied to the cells in serum-free RPMI 1640, and incubation was extended for another 48 h. Then the cells were washed with PBS and 200 µl MTT (0.5 mg/ml) was added to each well and further incubated for 4 h. The MTT solution was carefully removed by aspirating, and the formazan product was dissolved in 150 µl DMSO. Absorbance was measured at 570nm on a microplate reader (Bio-TEK Instrument, Winooski, VT).

Transwell assay

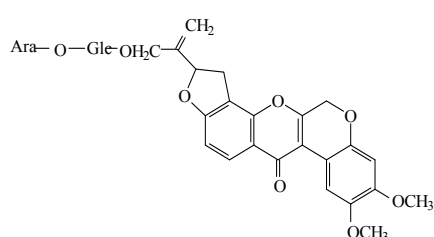
The migration activity of fibroblasts was performed using 24-well transwell chamber with 8.0µm pore polycarbonate filter inserts (Costar, Cambridge, MA, USA). Cells (3×10^4 cells/well) suspended in serum-free RPMI 1640 containing 0.1% BSA were overlaid in the upper chamber of each transwell. In each lower chamber, 500µl of serum-free RPMI 1640 containing 0.1% BSA in the presence or absence of compounds was added. Then the inserts were incubated at 37°C in a humidified

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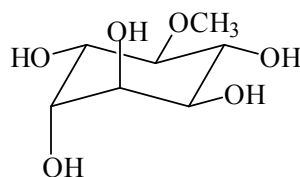
atmosphere containing 5% CO₂ for 24h. The cells that had not penetrated the filters were removed using cotton swabs. The migrated cells attached to the bottom side were fixed in 100% methanol for 10 min and stained with Wright–Giemsa. The cells migrating the lower surface of the filter in five microscopic fields of 400× magnification were counted in each filter. Triplicate samples were acquired and the data were expressed as the average cell number of 15 fields.

Structures of seven compounds

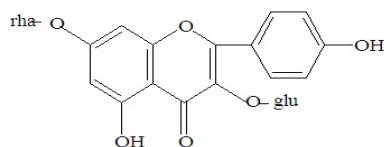
The compounds were named as 6a,12a-dehydroamorphin(I), D-3-O-methyl-chiro-inositol(II), Kaempferol-3-glucoside(III),7,2',4',5'-tetromethoxy-isoflavone(IV),dehydrosermundone(V),tephrosin(VI),7,4'-dimethoxyisoflavone(VII). Their structures are showed as followed:



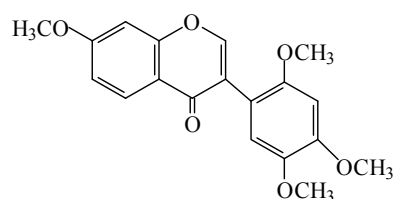
(I)



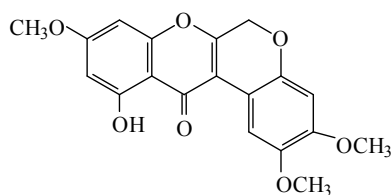
(II)



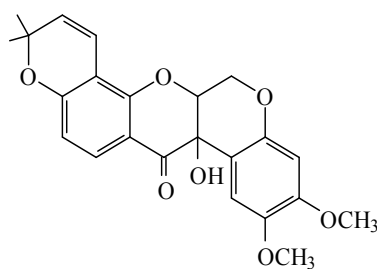
(III)



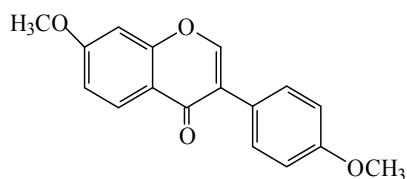
(IV)



(V)



(VI)



(VII)

Wound healing test in vivo

Drug formulations

Based on preliminary screening, dose-response study of seven compounds isolated from the fruits of *Amorpha fruticosa* L. were performed to determine the optimal concentration range. For the present study of wound healing evaluation *in vivo*, seven 10%(w/w) ointment were prepared, with 5 mg of every compound formulated with 50 mg of Vaseline.

Excision wounds model

A total of 54 rats were randomly divided into nine groups of six animals each. The animals were anesthetized by an intra-peritoneal injection of 10% chloral hydrate (0.3ml/100g), and the dorsal surface of the rat was shaved and the underlying skin was cleaned with povidone iodine. An excision wound was created on the dorsal aspect of the rats by cutting away a 500mm² full thickness of skin. The seven experimental groups received isolated compounds' ointment. The control group received the vehicle alone. The positive group received recombinant human epidermal growth factor (rhEGF) ointment. All drugs were applied topically once a day until the wounds completely healed. The wounds were measured every other day by placing a transparent paper over the wound and tracing it out. The parameters studied were percentage wound contraction calculated using the following formula: percent wound contraction = (original wound area - unhealed area)/original wound area ×100%.

Incision wounds model

A total of 54 rats were randomly divided into nine groups of six animals each. After anesthesia, disinfection and skin preparation, a paravertebral long incision of 4 cm length was made through full thickness of the skin at a distance about 1.5 cm from the middle on right side of the back. The wounds were closed with interrupted sutures of 0.5cm apart using sterile surgical thread (No. 000) and a curved needle (No. 11) (Udupa et al.,1995). The nine groups were treated with the same manner as the excision model for 9 days. On day 9, sutures were removed and the tensile strength of healed wounds was measured with a tensiometer and calculated using the following formula: tensile strength = breaking strength/ cross sectional area of skin.

Determination of the hydroxyproline content

On the day 22 after excision wounds creation, the healed wound tissues were excised and analyzed for hydroxyproline content, which is a basic constituent of collagen. Tissues were dried in a hot air oven at 60°C to constant weight and were hydrolysed in 6N HCl at 130 °C for 4 h in sealed tubes. The hydrolysate was neutralised to pH 7.0 and was subjected to Chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4M perchloric acid and colour was developed with the help of Ehrlich reagent at 60°C (Woessner, 1961) and measured at 560 nm using a spectrophotometer.

Histological examination

The excised tissue of excision wounds was fixed in 10% neutral buffered formalin, dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. Five-micron-thick sections, including the epidermis, the dermis, and the subcutaneous panniculus carnosus muscle, were mounted on glass slides, dewaxed, rehydrated in distilled water, and stained with hematoxylin and eosin(HE). All subsequent analyses were performed by an experienced pathologist without knowledge

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of the previous treatment to evaluate the degree of re-epithelization, granulation tissue formation and collagen organization in the wound

Antimicrobial activity

Minimal Inhibitory concentration (MIC) determination

Minimum inhibition concentration assay was performed in nutrient broth containing 0.05% phenol red and supplemented with 10% glucose (NBGP). All the test compounds were initially dissolved in DMSO and the solution obtained was added to NBGP to a final concentration of 5000 µg/ml for each crude extract. This was serially diluted by twofold, to obtain concentration ranging from 5000 µg/ml to 4.9 µg/ml. One hundred microlitres of each concentration was added to a 96-well microplate containing 95 µl of NBGP and 5 µl of standard inoculum, the appropriate inoculum size for standard MIC was 10⁵ CFU/ml. The final concentration of DMSO in the well was less than 1%. The negative control well was composed of 95 µl of NBGP and 5 µl of the standard inoculum. Chloramphenicol was taken as the positive control and the concentration was diluted from 64 µg/ml to 0.063 µg/ml. The plates were covered with a sterile plate sealer, then agitated to mix the content of the wells using a plate shaker and incubated at 37 °C for 24 h. The assay was repeated twice and microbial growth was determined by observing the chromatic change in the wells (red when there is no growth and yellow when there is growth). The lowest concentration demonstrating no color change in the well was considered as the MIC (Dash et al., 2001).

Minimum bactericidal concentrations (MBC)

For the determination of MBC, a portion of liquid (5 µl) from each well that showed no change in color was plated on agar plate and incubated at 37°C for 24 h. The lowest concentrations that yielded no growth after this sub-culture were taken as the MBC (Srinivas et al., 2008).

Results

Wound healing activity in vitro

Cell viability of fibroblast

MTT cell viability assay was performed in response to different concentrations of the seven compounds. The result demonstrated that at lower concentrations (1 and 10 µg/ml), no clear effect was seen with all compounds. At higher concentrations (100 and 1000 µg/ml), only compound I and V increased cell viability ($p < 0.05$), but the cell viability was no significant difference with the concentration of 100 µg/ml and 1000 µg/ml ($p > 0.05$) (Figure 1). So 100 µg/ml of seven compounds was used in the following experiments.

Cell migration of fibroblast

Transwell assay is a commonly used model for analyzing the molecular mechanism underlying cell migration. With this migration assay, the fibroblasts were cultured in serum-free media with 1% BSA for 24 h in the presence or absence of seven different compounds. The results showed that compound I and V significantly induced cell migration at 100 µg/ml compared with control group and other five compounds ($p < 0.05$), and there is no difference within the above two compounds ($p > 0.05$) (Figure 2).

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Wound healing activity in vivo

The percent wound contraction of excision wounds

Starting from day 4 to day 16 after wound creation, the wound contraction of compound I, V and positive control group was found to be significant compared with negative control group and other compounds ($p < 0.05$). On day 18, wounds in compounds I, V and positive control treated group healed completely. Wounds in other five compounds and negative control treated group did not heal until day 22 after wound creation (Table 1).

Tensile strength of incision wounds and hydroxyproline content of excision wounds

The tensile strength and hydroxyproline content in compound I and positive control treated group was the highest, and significantly higher than negative control treated group ($p < 0.01$). The above two healing parameters in compound V was also higher than negative control treated group ($p < 0.05$). In contrast, the difference between other four compounds and negative control treated group was not found to be statistically significant (Table 2).

Histological examination

When the excision wound healed, the tissue was excised to evaluate the healing quality under light microscope. The progression of new epithelium, granulation tissue formation and collagen organization related to wound healing in compound I, V and positive control group were better compared to compound II, III, IV, VI, VII and negative group. Wounds in compound I, V and positive control group demonstrated that the collagen fiber was more matured with few capillaries, robust fusiform fibrocytes and regenerative folliculus and sebaceous glands. In contrast, in compound II, III, IV, VI, VII and negative control group, there was more granulation tissue within the wound bed showing poorly-organized capillary, many fibroblast, moderate collagen and no regenerative folliculus or sebaceous glands. In addition, compared with compound II, III, IV, VI, VII and negative group, a thicker continuous epithelial line covering the whole wound bed was observed in compound I, V and positive control group (Figure 3).

Antimicrobial activity

Among the seven compounds (I - VII) from the fruits of *Amorpha fruticosa* L, compound I, V and VI exhibited higher inhibitory effect towards all the pathogens than other four compounds. The MIC and MBC of these compounds against pathogens was summarized in Table 1. The results showed that *Bacillus subtilis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were most sensitive to compound VI. *Bacillus cereulences* was most sensitive to compound I and VI equally. *Escherichia coli* was most sensitive to compound V, and *Klebsiella aeruginosa* was most sensitive to compound I respectively (Table 3).

Discussion

Wound healing involving interactions of multiple cell types, various growth factors, their mediators, and the extracellular matrix proteins is a complicated biological process. Physiological healing of wound takes place by itself and does not require much other intervention, but some risk factors such as the defect of local blood supply and infection could delay the healing process (Keeling et al., 2010; Leaper, 2010). Many natural products based on plant have been found to have therapeutic effects as promoters of wound healing (Woollard et al., 2007; Skórkowska-Telichowska et al., 2010). *Amorpha fruticosa* L. leaves and fruits have been used for treating wounds in traditional medicine. In previous study of our laboratory, the compounds isolated from *Amorpha fruticosa* L. were mainly rotenoids, which have demonstrated many pharmacological

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activities, such as antitumor, antiviral and antibacterial action, inhibition of phosphodiesterase, hepatoprotection, fish poison and insecticide activity (Petkov et al., 1983; Li et al., 1993). The results of present study using wound healing experiments in animal models, biochemical analysis of wound biopsy, and *in vitro* studies showed that three different compounds isolated from the fruits of *Amorpha fruticosa* L. have antibacterial activity and promote the healing of wounds.

Table 1. The percent wound contraction of excision wounds at different time point

Group	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Compound I	17.9±1.0	36.2±1.8*	43.5±2.0*	62.6±2.1*	77.3±1.8*	86.1±2.4*
Compound II	16.0±1.1	30.2±1.3	37.6±2.2	57.4±1.6	64.8±1.9	76.3±1.8
Compound III	16.5±1.3	31.0±1.1	39.3±2.9	56.8±2.0	63.3±1.6	76.1±2.6
Compound IV	15.7±1.5	32.1±2.0	39.5±1.9	56.1±2.2	64.1±1.4	77.0±1.6
Compound V	16.8±1.0	35.7±1.6*	42.4±2.4*	60.7±3.1*	76.9±3.1*	85.2±2.3*
Compound VI	16.2±0.9	31.9±1.0	38.0±2.1	58.3±2.5	65.8±2.3	75.2±2.1
Compound VII	17.3±1.0	31.1±1.3	38.3±3.0	54.9±2.5	63.5±1.5	75.9±1.6
Positive control	18.3±1.4	39.3±1.5*	45.2±2.3*	63.7±1.4*	77.4±2.6*	86.6±2.7*
Negative control	16.2±1.1	31.5±2.1	38.2±1.7	55.5±2.0	63.6±1.9	75.7±2.1
Group	Day 14	Day 16	Day 18	Day 20	Day 22	
Compound I	90.7±2.0*	96.8±1.9*	100*	100*	100	
Compound II	82.4±1.4	87.5±1.9	92.5±2.1	96.3±2.1	100	
Compound III	83.7±1.5	88.5±1.9	91.7±2.0	96.9±1.1	100	
Compound IV	84.3±3.1	87.9±1.6	92.8±2.1	96.3±1.4	100	
Compound V	90.5±2.0*	96.1±1.1*	100*	100*	100	
Compound VI	84.9±2.3	89.1±2.7	93.5±3.1	97.0±1.4	100	
Compound VII	82.9±2.6	87.0±2.6	93.0±1.2	96.5±1.3	100	
Positive control	92.1±1.9*	97.3±1.1*	100*	100*	100	
Negative control	83.4±1.5	87.2±3.1	92.2±2.3	95.4±1.8	100	

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Values are mean ± S.D.

*P<0.05 as compared to negative control group.

Table 2: Tensile strength of incision wounds and hydroxyproline content of excision wounds

	Tensile strength(g/cm ²)	Hydroxyproline(mg/g tissue)
Compound I	547.3±7.9 [#]	80.3±4.4 [#]
Compound II	431.5±8.3	63.1±5.1
Compound III	440.3±7.4	58.4±3.7
Compound IV	451.8±6.0	63.6±4.9
Compound V	500.2±8.9*	70.7±4.2*
Compound VI	442.0±8.1	63.2±5.9
Compound VII	449.8±6.4	59.3±4.7
positive control	568.1±8.3 [#]	84.6±5.3 [#]
Negative control	436.5±7.6	60.2±4.1

Values are mean ± S.D.

[#]P<0.01 as compared to negative control group

*P<0.05 as compared to negative control group

Table 3: MIC and MBC of compounds from *Amorpha fruticosa* L against different bacteria (µg/ml)

compounds	<i>Bacillus subtilis</i>		<i>Bacillus cereulences</i>		<i>Staphylococcus aureus</i>	
	MIC	MBC	MIC	MBC	MIC	MBC
I	156.3	312.5	156.3	312.5	156.3	156.3
II	—	—	—	—	—	—
III	2500	2500	1250	2500	—	—
IV	1250	1250	1250	2500	5000	5000
V	156.3	312.5	312.5	312.5	78.2	156.3
VI	156.3	156.3	156.3	312.5	78.2	78.2
VII	2500	2500	5000	5000	2500	2500
Chlormycetin	1	2	1	2	2	4

<i>Escherichia coli</i>		<i>Pseudomonas aeruginosa</i>		<i>Klebsiella aeruginosa</i>	
MIC	MBC	MIC	MBC	MIC	MBC
312.5	312.5	312.5	312.5	156.3	312.5
—	—	—	—	—	—
—	—	—	—	—	—
2500	2500	5000	5000	2500	2500
156.3	156.3	312.5	312.5	312.5	312.5
156.3	312.5	156.3	156.3	312.5	312.5
1250	2500	—	—	2500	2500
0.5	1	1	2	1	2

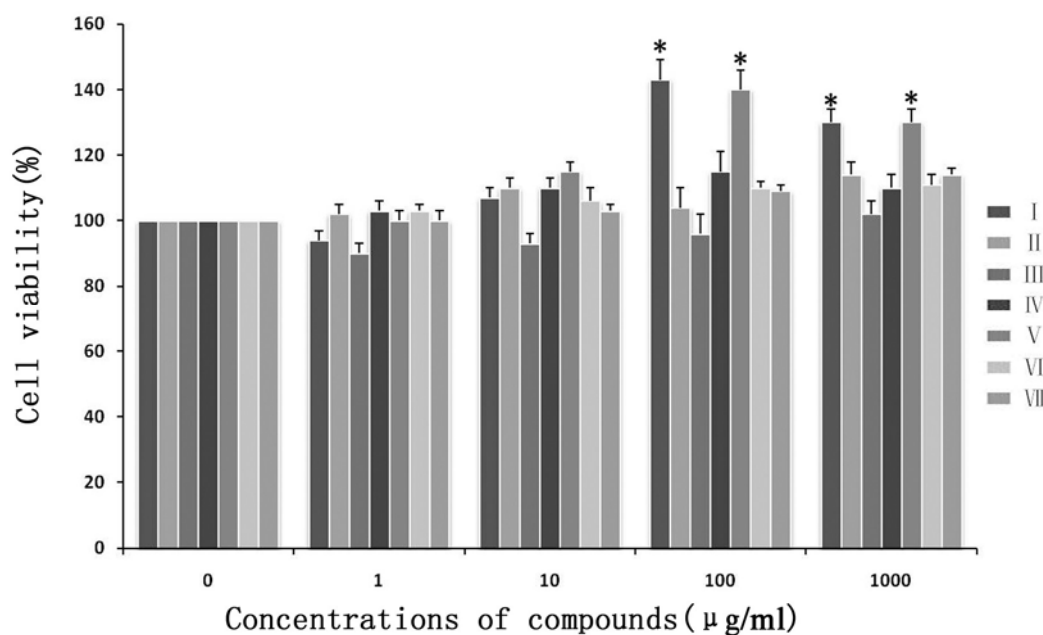


Figure 1: Viability of fibroblasts after different compounds treatment measured with MTT assay. Cells were exposed to gradually increased concentration of different compounds. The results are represented as a percentage of absorbance relative to control cells (100%). * Differ ($P < 0.05$) from the control

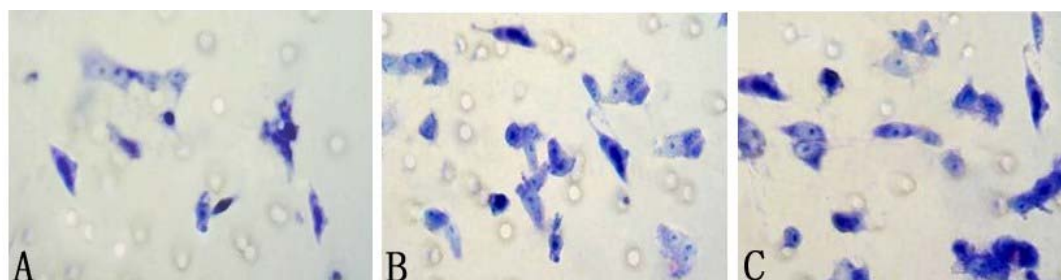


Figure 2: Effect of different compounds on fibroblast migration activity in transwell assay. After treated with compound I (B) and V (C), the fibroblasts demonstrated significant capability of migration, compared with absence of any compounds (A).

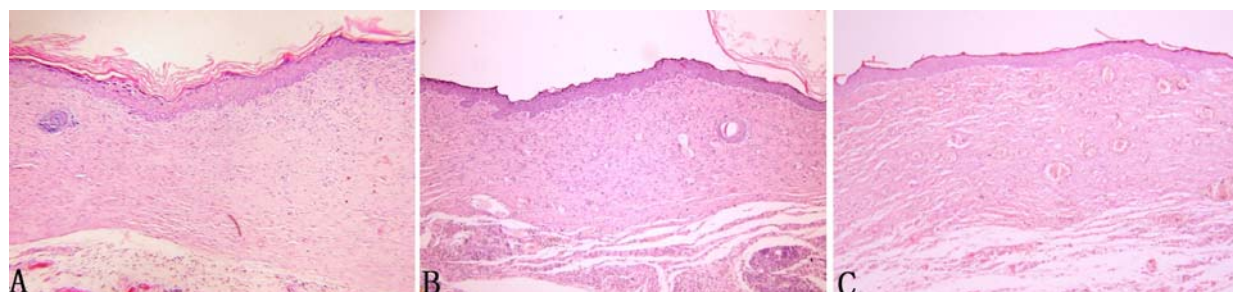


Figure 3: Histological compare in the character of excision wound healing (100× magnification). Wounds of all groups demonstrated a continuous epithelial line covering the whole wound bed. However, the epithelium was thicker in compared with negative control group. Moreover, in compound I (A) and V (B) group, the granulation was matured showing capillary vertically oriented, robust fusiform fibrocytes and moderate well-arranged collagen. In contrast, in negative control group (C), the granulation tissue was un-matured with capillary poorly-organized, many fibroblast and slight collagen formation.

Fibroblast is a major cell type participating in wound healing process which is responsible for the synthesis, deposition, and remodeling of the extracellular matrix (Hinz,2010). During wound repair, fibroblasts initially proliferate *in situ*, then migrate from the wound edges to the wound site, proliferate again, and subsequently produce collagen (Werner et al.,2007). Collagen is a crucial constituent of extracellular matrix and the amount of hydroxyproline is a reflection of concentration of collagen (Bhattacharjee and Bansal, 2005). Therefore, the more hydroxyproline represents the faster rate of collagen synthesis corresponding to enhanced wound healing. In present study, according to the results of MTT assay and transwell assay which is widely used for detection of cell proliferation and migration activity *in vitro*, compound I, V and VI isolated from the fruits of *Amorpha fruticosa* L. demonstrated significantly enhanced effect on fibroblasts in proliferation and migration, thus promoting wound healing. And this finding was further confirmed by biochemical assay for hydroxyproline content *in vivo*, which means collagen synthesis was improved after topical application of related compounds, so that the amount of collagen increasing was one of the reasons for the wound healing promotion.

When wound healing starts, it is prone to be attacked by bacteria. It is well known that infections may delay wound healing and cause failure of healing, even wound deterioration (Guo and Dipietro,2010). Microbial pathogens delay wound healing through several mechanisms, including persistent production of inflammatory mediators, metabolic wastes and toxins; causation of tissue hypoxia, the granulation tissue hemorrhagic and fragile; reduction of fibroblast number and collagen production; continuous activation of neutrophils, which produce cytolytic enzymes and free oxygen radicals, and competition with host cells for nutrients and oxygen necessary for wound healing (Macri and Clark,2009). Thus, keeping the wound axenic or maintaining a low bio-burden is a crucial aim in wound healing. In the present study, the inhibitory effects of seven compounds isolated from the fruits of *Amorpha fruticosa* L on six strains referring to Gram positive and Gram negative bacteria growth were evaluated. As a result of rotenoids structure, the compounds I, V and VI demonstrated a more significant antibacterial effect comparing with other four compounds. To our knowledge, in aerobic organisms the rotenone-insensitive enzymes provide at least two vitally important functions. First, they can reduce NADH produced by multiple dehydrogenases, thus not maintaining and controlling general oxidative metabolism. Second, they also never serve as the energy-transducing devices accumulating free energy from the redox gap between NADH/NAD⁺ and the quinone pair as the proton-motive force (PMF) across the coupling membranes. By observing the chemical structures of compound I, V and VI, the slight difference of antibacterial activity may mainly come from the 12a-OH which connected with 12a-C (Fang and Beattie, 2002;Grivennikova et al., 2007). Therefore, the compounds I, V and VI may account for the commendable activity of *Amorpha fruticosa* L against the test pathogens, and the topical application of the three compounds on wounds would prevent microbes from invading the susceptible state.

Acknowledgements

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