

# Evaluation of wound healing activity of baicalein-7-O- $\beta$ -D-glucuronide isolated from *Leucas aspera*

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## ABSTRACT

*Leucas aspera* (Willd.) Linn. (Lamiaceae), commonly known as "Thumbai," is distributed throughout India. This study includes the isolation and characterization of flavonoids present in *Leucas aspera* flowers were compared wound healing activity with standard soframycin ointment. The plant materials were extracted with 95% methanol, petroleum ether, ethyl acetate, and were subjected to column and TLC chromatographic separation analysis. The chemical constituents isolated from the flowers of *L. aspera* were characterized based on chemical tests and spectral analysis such as UV and NMR spectroscopy. The structure of the isolated compound was confirmed as baicalein-7-O-  $\beta$ -D-glucuronide (baicalin). It has been investigated for wound healing activity by applying on the albino rats. A slow rate of healing was observed earlier which turns to very rapid on the 12<sup>th</sup> day. The histopathological examination provided additional evidence for the experimental wound healing studies. Protease is a biochemical marker and pH measured to support the wound healing activity. We conclude that the baicalin isolated with the flowers of *L. aspera* has better wound healing activity.

## INTRODUCTION

A review of literature has revealed that plant metabolites such as alkaloids, flavonoids, glycosides, etc. play an important role in many of activities including wound healing, cardio-tonic, analgesic, anti-inflammatory, anti-oxidant, and antimicrobial activity (Noor Fathima *et al.*, 2011). Wounds can be described as any damage or break of the skin or underlying tissues. Medical treatment of wounds includes administration of drugs either locally or systematically in an attempt to aid wound repair (Savanth and Shah, 1998). The classical system of Indian medicine especially Ayurveda, Siddha and Unani employ a large number of medicinal plants for treatment of skin disease, which includes cut wounds and burns.

There are four recognized components to the wound healing process such as inflammation, destruction, proliferation; maturation. Protease activity is a normal recognized part of this process. The proteases assist in the removal of damaged tissue especially the extracellular matrix (ECM), the scaffold in which new blood vessels grow and upon which granulation tissue is

formed. Proteases are produced by either activated inflammatory, cells such as neutrophils and macrophages - these are referred to as endogenous proteases include collagens, gelatinase and elastase (Gibson *et al.*, 2009; Walker and Bowler, 2007). Following wounding, the protease levels peak at day three and reduce at day five (Nwomeh *et al.*, 1998).

Protease action and activity is pH- dependant and non-healing wounds generally have a pH level of 8. If the pH level is reduced to a more acidic level (approximately 4) the protease activity is reduced by approximately 80% (Greener *et al.*, 2005). *Leucas aspera* (Willd.) Linn. (Lamiaceae) commonly known as 'Thumbai' is distributed throughout India on wastelands and roadsides. The leaves are said to be useful in chronic rheumatism. The juice is applied in psoriasis and other chronic skin eruptions. The whole plant is used for analgesic-antipyretic, anti-rheumatic, anti-inflammatory, and anti-bacterial treatments and is shown to have anti-oxidant activity (Reddy *et al.*, 1993; Sadhu *et al.*, 2003).

*Leucas aspera* is a traditional medicine with multiple health related benefits. However, the medicinal values of the plant pertaining to wound has not yet been reported. Therefore, the aim of treating a wound is either to shorten the time required for healing or to accelerate the wound healing process.

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## MATERIALS AND METHODS

### Plant material

The plants were selected on their wide medicinal uses in the traditional literature. Fresh flowers (3 kg) of *L. aspera* were collected during the month of October to March 2012 from Ariyalur district in Tamil Nadu, India and authenticated by Dr. N. Ramakrishnan, Head and Associate Professor and voucher specimens (GACBOT-113) were deposited at the Herbarium of the Department of Botany, Government Arts College (Autonomous), Kumbakonam, Bharathidasan University, India. The plant materials were extracted with 95% methanol, petroleum ether and ethyl acetate (Sigma Aldrich Co, India).

### Preliminary phytochemical screening

Collected extracts underwent various chemical tests for the preliminary determination of phytoconstituents (Wagner *et al.*, 1984; Harborne, 1998). The ethyl acetate extract transferred to small vial and kept at 4°C for further analysis of wound healing activity using excision wound model.

### Isolation of flavonoid

The preliminary phytochemical screening showed the presence of alkaloid, flavonoid, tannin, saponin and glycosides. Total 87 g of dried extract was found which was then re-dissolved in methanol. The methanol extract was subjected to column chromatography on silica gel to yield several sub - fractions. The column was eluted with organic solvents in increasing order of polarities petroleum ether and ethyl acetate and then washed with ethyl acetate and methanol in the ratio 9:1. Each step was repeated thrice to ensure complete extraction. Petroleum ether fraction was rejected due to its being rich in fatty substances, whereas ethyl acetate fraction was washed with distilled water to neutrality, dried *in vacuo* and analyzed for bound flavonoid. The excess of solvent was removed using rotatory flash evaporator. The yellow precipitate was separated and redissolved in methanol and recrystallized.

The crystallization was performed four more times in methanol at -4°C. The yellow solid crystals (24.6 g) had a melting point 203 - 208°C thus obtained were found to be a pure compound by thin-layer chromatographic (20 × 20 cm plates precoated with silica gel, Sigma Aldrich Co., India) analysis using three solvent systems (benzene/ methanol/acetic acid, 20:2:1; methanol/ethyl acetate/water, 7:2:1; butanol/water/acetic acid, 4:1:1) and only one single spot was visualized under ultraviolet (UV) light, indicating that it was a pure compound and responds to usual flavonoid color tests.

The compound produced green color with alc. Fe<sup>3+</sup> and orange red color precipitate with lead acetate. It dissolved in alkali and NH<sub>3</sub> with a yellow color and changed to dark brown. The compound answered Wilson's boric acid, Molisch and Gibb's tests, and at the same time it won't respond to Horhammer-Hansal tests. This clearly indicates that this fraction contains flavonoid. The chemical constituents isolated were characterized based on

chemical tests, R<sub>f</sub> value and spectral analysis such as UV were recorded using UV- Visible Spectrophotometer Lambda 35 from Perkin Elmer and NMR (125 MHz, CDCl<sub>3</sub>) spectral data that were recorded on a Bruker AMX 400 NMR spectrometer. Chemical shifts were referenced to the respective residual solvent peaks and the values were recorded in δ. Optical rotations were measured using a JASCO DIP-180 digital spectropolarimeter.

### Gel formulation

The ethyl acetate extract thus obtained was evaporated at 40°C to dryness in a rotary evaporator in vacuum. The product thus obtained was used for gel formulation. 1 g of the isolated compound was made up to 100 g using Vaseline.

### Wound healing activity

#### Animals

Healthy albino rats (Wistar strain) of either sex and of approximately the same age, weighing about 200-250 g were used for the study. They were fed with standard diet and water *ad libitum*. They were individually housed, maintained in polypropylene cages under standard conditions. Before starting the experiment on animals, the experimental protocol was subjected to the scrutiny of the Institutional Animal Ethics Committee (IAEC), Bharathidasan University, Trichirappalli, Tamilnadu, India (Approval No. BDU/IAEC/2011/31/29.03.2011).

#### Excision wound model

Excision wound model was employed to study the rate of wound contraction and the time required for full epithelization of the wounds (Nayak *et al.*, 2007). The animals were divided into four groups of eight rats.

*Group I:* No treatment and served as controlled.

*Group II:* Test group with wound and treated with isolated baicalin (100 mg per day).

*Group III:* Test group with wound and treated with isolated baicalin (200 mg per day).

*Group IV:* Test group with standard drug ointment (Soframycin).

*Group V:* Test group with crude extract.

Animals were anaesthetized with slight vapor inhalation of diethyl ether at the pre-determined area and the back of rat was shaved. The excision wound, a circular piece of full thickness sized approximately 500 mm<sup>2</sup> and 2 mm depth, were made by cutting out the skin from the shaved area. The treatment was done topically in all the cases. The isolated baicalin was applied at a dose of 100 and 200 mg per day for sixteen days. The progressive changes in wound area were monitored by a camera every fourth day. The size of the wound was also measured and recorded on a one mm<sup>2</sup> graph paper every 4 day until complete wound healing. Wound contraction was calculated as percentage of the reduction in wound area (Werner *et al.*, 1994). Percentage of wound contraction = [(Initial wound area - Specific day wound area) / Initial wound area] x 100.

### Statistical Analysis

The experimental results were expressed as multiple comparisons of Mean  $\pm$  SEM were carried out by one way analysis of variance (ANOVA) followed by Dunnet Multiple Comparisons Test and statistical significance was defined as  $P < 0.05$ .

### Histopathological examination

Small pieces of the tissue were isolated from the healed skin of each group of rate for the histopathological examination (Sadaf *et al.*, 2006). Samples were fixed in 10% buffered formalin, processed and blocked with paraffin wax. Serial sections of 5  $\mu$ m were prepared, and Light microscopic study of H&E and Masson's trichrome stained tissues for routine histopathological evaluation. Masson's trichrome stain was used to determine degree of collagenization. All slides were examined in a blinded manner by surgical pathologist.

### Detection of elevated protease activity and pH in the wound bed

Elevated protease levels are detected as soon as possible to prevent a wound ending up in a static state of permanent inflammation. However, there are no clinically visible signs that can specifically identify elevated protease levels in a wound bed. Protease action and activity could be directly pH-dependant. The most frequently used instrument is a glass top electrode attached to a meter when a probe is used it is first calibrated in pH 4 and 7 and/or 9 buffers. The probe is rinsed in deionized water and then placed flat against the wound for 30 seconds and the result is displayed on the meter.

## RESULTS AND DISCUSSION

### Chemical constituents

The flower extract of *L. aspera* was subjected to adsorption chromatographic separation analysis (column chromatography and TLC) to isolate flavonoid. Structure of the compound was identified by spectral data analysis and confirmed by comparing the data with the published literature as baicalin (m.p. 203-208°C). Its melting points were compared using pure chemical of baicalin (95%) purchased from Sigma Aldrich Co (India) as external standard and found to have the same values. We have referred for these flavonoid extracted from *O. indicum* (Kumar *et al.*, 2007; Shah *et al.*, 1936). The  $R_f$  value of this compound was compared with pure baicalin (Sigma Aldrich Co., India) and found to have same value. The chemical constituents isolated were characterized by spectral analysis such as UV and NMR spectroscopy.

The UV spectrum of the compound showed two absorption peaks at 315 nm and 278 nm showing a flavone skeleton. The flavone oxygenated in A - ring, but not in the B - ring, tends to give spectra in methanol with an expected band II and a weak band I. The sodium methoxide spectrum indicates the absence of 4'-OH group. The  $AlCl_3$ -HCl spectra of the aglycone show two absorption peaks indicating a free 5 - OH group. A bathochromic shift of 23 nm is an evidence for the 6 - oxygenation

pattern along with the free 5 - OH group. A bathochromic shift of +33 nm on the addition of sodium acetate showed that the 7<sup>th</sup> position is not at all free. This was evidenced by the sodium acetate spectrum of its aglycone a smaller shift of only +3 nm on the addition of NaOAc/  $H_3BO_3$ , showed that A - ring has an *O*-dihydroxyl grouping. Analysis of  $^1H$  and  $^{13}C$ -NMR data (Table 1) revealed that the aromatic signals are close to those reported for baicalein-7-*O*-  $\beta$ -D-glucuronide (baicalin).

**Table 1:**  $^{13}C$  NMR and  $^1H$  NMR spectroscopic data for baicalin.

$^{13}C$ NMR ( $[^2H_6]$ DMSO, $\delta$ in ppm, J in Hz)		$^1H$ NMR ( $C_2H_5O_2$ H, TMS, $\delta$ in ppm, J in Hz)	
carbon	Baicalin	H	$\delta_H$
1 <sup>I</sup>	130.6	2 <sup>I</sup>	7.6 – 7.9 (2H, d)
1 <sup>II</sup>	101.0	3	6.75 (1H, s)
2	163.0	3 <sup>I</sup>	7.3 – 7.5 (3H, m)
2 <sup>I</sup>	127.2	4 <sup>I</sup>	7.3 – 7.5 (3H, m)
2 <sup>II</sup>	74.0	5	12.10 (1H, s)
3	105.4	5 <sup>I</sup>	7.3 – 7.5 (3H, m)
3 <sup>I</sup>	129.1	6	8.25 (1H, s)
3 <sup>II</sup>	76.8	6 <sup>I</sup>	7.6 – 7.9 (2H, d)
4	181.4	8	6.80 (1H, s)
4 <sup>I</sup>	133.0	Anomeric H	5.10 (1H, tri)
4 <sup>II</sup>	70.6		
5	146.2		
5 <sup>I</sup>	129.1		
5 <sup>II</sup>	76.0		
6	131.4		
6 <sup>I</sup>	127.2		
6 <sup>II</sup>	60.14		
7	148.9		
8	94.1		
9	151.3		
10	106.8		

In the  $^1H$ -NMR spectrum of the A-ring protons at C-6 and C-8 appear separately at  $\delta$  8.25 and  $\delta$  6.80 ppm respectively. The 5-OH proton resonates at  $\delta$  12.10 ppm. The signal at  $\delta$  7.9 ppm corresponds to the protons at C-2<sup>I</sup> and C-6<sup>I</sup>. The protons at C-3<sup>I</sup>, C-4<sup>I</sup> and C-5<sup>I</sup> appear at  $\delta$  7.3 - 7.5 ppm. The  $^{13}C$  NMR data indicated that were 21 carbons in this structure, 15 of which were typical for a flavone skeleton and the others were assigned to glycoside. The sugar signals  $^{13}C$ -NMR,  $\delta$  ppm 101.0, 76.8, 76.0, 74.0, 70.6 and 60.14 are comparable with those reported for *O*-glucoside (Li *et al.*, 2003). The  $^1H$  NMR spectrum showed the presence of anomeric proton signal at  $\delta$  ppm 5.10 (1H, tri) indicated the presence of *O*- linked sugar. It was proved that the sugar moiety bonded to hydroxyl group at C-7 of the aglycone as deduced from the correlation between the anomeric proton at  $\delta$  5.10 ppm and the C-7 at  $\delta$  148.9 ppm. In crude plant extracts, flavonoids are often present as *O*- or *C*-glycosides. The *O*-glycosides have sugar constituents bonded to a hydroxyl group of the aglycone, whereas the *C*-glycosides have sugar constituents bonded to a carbon of the flavonoid aglycone (Lin *et al.*, 1993).

The flavonoid from the ethyl acetate fraction can be characterized as baicalin (Figure 1). Its optical rotation was  $[\alpha]_{25/D}$ :  $-86^\circ$  ( $c = 1$  in DMSO). By comparing all the above mentioned physical and chemical part of evidences the flavonoid obtained from *L. aspera* flowers has been characterized as the baicalin.

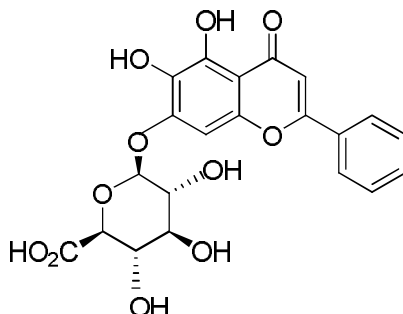
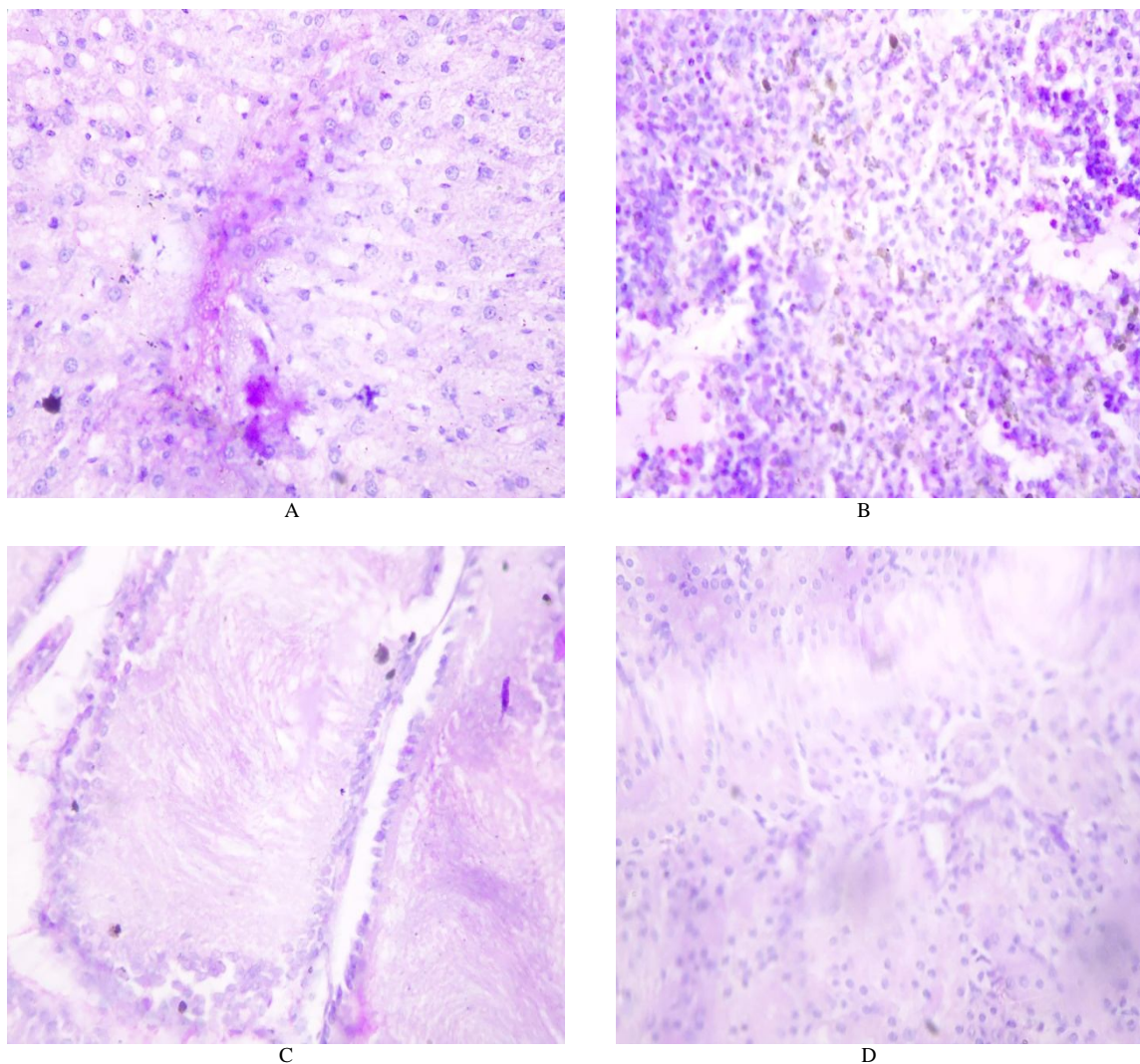
**Table. 2:** Effect of drug on excision wound [wound area (mm<sup>2</sup>)]

Oral treatment	Wound contraction (mm <sup>2</sup> /rat) (M±SD)				
	0 day	4 <sup>th</sup> day	8 <sup>th</sup> day	12 <sup>th</sup> day	16 <sup>th</sup> day
Group I	385.83±1.61	322.00±3.00	272.30±1.57	128.14±1.03	102.46±2.72
Group II	374.87±2.58	305.67±1.53	206.87±2.10	107.02±2.31	31.18±1.71
Group III	369.10±3.44	252.80±2.70	145.11±3.01	58.45±1.71	3.50±1.50
Group IV	334.43±3.88	260.43±1.51	160.10±6.42	80.23±1.36	10.93±2.52
Group V	382.54±1.68	310.24±2.10	234.32±4.20	118.14±1.83	57.08±1.84

Values are expressed by mean ± stranded deviation (M±SD)

One-way ANOVA (Dunnets method) Means for groups in homogeneous subsets are displayed.

Subset for alpha = 0.05 level

**Fig. 1:** baicalin - 7 - O -  $\beta$ -D-glucuronide (baicalin).**Fig. 2:** Histopathological studies of the healed tissues excised on the 12<sup>th</sup> day. Tissue stained with Hematoxylin and Eosin Stain (HE): (a) Control group (b) Standard drug treated group (c) 100 mg baicalin treated group (d) 200 mg baicalin treated group.

### Wound healing activity

The part of wound healing involves a variety of process such as inflammation, cell proliferation and contraction of the collagen lattice formed (Bodekar and Hughes, 1998). The present investigation revealed that the baicalin extracted from of *L. aspera* is investigated for its wound healing activity by comparing it with the standard soframycin ointment. Albino rats were used as animal models where a control is used for deciding the healing activity. The clinical observations on wound healing were as follows:

The studies on excision wound model reveals that all the four groups showed decreased wound area from day to day while complete wound closure and epithelization was observed on 16<sup>th</sup> day of wound induction compared with day zero. The mean percentage closure of wound area was calculated on the 4, 8, 12, and 16 post wounding days. All readings are found to be statistically significant and comparable with control (Table 2).

The period of epithelialization  $128.14 \pm 1.03$  was found to be high in control group which was untreated and take more days in healing while in standard group it was significantly reduced to  $80.23 \pm 1.36^{**}$  ( $P < 0.01$ ). The period of epithelialisation of test ointment of (100mg) and (200mg) baicalin was found to be  $107.02 \pm 2.31^*$  ( $P < 0.05$ ) and  $58.45 \pm 1.71^{**}$  ( $P < 0.01$ ) respectively. From the experiment data, the 200mg baicalin ointment (Group III) was more potent than 100mg baicalin ointment (Group II). On 12<sup>th</sup> day the standard and baicalin drug treated animals were showed significantly greater wound closure as compared to control animals. However the crude methanolic extract showed mild wound healing activity. It may be attributed to the fact that the plant extract being in crude form contains a smaller concentration of bioactive compounds.

The histopathological examination provided additional evidence for the experimental wound healing studies (Figure 2) which was based on the contraction value of wound area. A few macrophages and lymphocytes were seen beneath the newly formed epidermis and seen fibrous tissue which filled the defect at the dermis. This study revealed a significant increase in epithelialization in drug treated group and also granuloma showed increase in both the number of proliferating capillaries and amount of fibroblastic collagenous connective tissue, when compared with granuloma of the control animal.

Wound healing involves regeneration of specialized cells by proliferation of surviving cells and connective tissue response characterized by the formation of granulation tissue. It is also characterized by hemostasis, epithelialization and remodeling of the extracellular matrix (Whaley and Burt, 1996). Epithelialization, which is the process of epithelial renewal after injury, involves the proliferation and migration of epithelial cells towards the center of the wound while wound contraction is largely due to the action of fibroblasts (Cotran *et al.*, 1994; Mohan, 2005). Thus, the effect of baicalin on wound contraction and epithelialization suggest it may enhance epithelial cells migration and proliferation, as well as the formation, migration and action of fibroblasts, it may also stimulate processes associated with tissue regeneration. Also regulation of inflammation and oxidation are

important in the process of wound healing. In previous studies baicalin exhibits anti-inflammatory activity by binding to chemokines (Li *et al.*, 2000) and significant anti-oxidant activity (Shieh *et al.*, 2000), which would help to prevent oxidative damage and promote the healing process. Elevated protease activity is a biochemical marker for predicting poor wound healing in acute and chronic wounds. Proteases and pH are considered to play an important role in wound healing. Monitoring surface pH may provide a method of 'measuring' the condition of wound bed and ultimately aid in determining the wounds response to treatment (Georgina, 2007; Greener *et al.*, 2005). The readings are taken on 4<sup>th</sup> day, all the four groups becomes basic around pH 8 it will be reduced to neutral (pH 6-7) on 8<sup>th</sup> day and then moves to acidic (pH 4-5) on 12<sup>th</sup> post wounding day. It is important to state that results obtained are of surface pH and not tissue pH. In addition to the effects on protease activity and oxygen release, other effects of lowering the pH to a more acidic environment are to reduce the toxicity of bacterial end products such as ammonia, enhancing the destruction of abnormal collagen in the wound bed, increased macrophages and fibroblast activity (Molan, 2002; Greener *et al.*, 2005).

### CONCLUSION

Based on the results obtained in the present investigation, we conclude that the baicalin isolated from flowers of *L. aspera* has better wound healing activity. The histopathological examination provided additional evidence for the experimental wound healing studies which was based on the contraction value of wound area. It may be attributed to anti-oxidant and anti-inflammatory activity, additionally it can be a good therapeutic agent for accelerating the wound healing process.

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