Research Article

Preclinical evaluation of *Alpinia galanga* rhizomes for wound healing activity with reducing oxidative status

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Abstract

Objective: The wound healing is a complex dynamic process consists of four integrated and overlapping phases: hemostasis, inflammation, proliferation and tissue remodeling. These phases and their biophysiological functions must occur in the proper sequence and continue for a specific duration at an optimal intensity. The aim of present study was to evaluate wound healing properties of *Alpinia galanga* rhizomes used in traditional medicine. **Materials and methods**: The qualitative chemical analysis was performed to detect the presence of different chemical constituents. The petroleum ether extract and ethanolic extract of the rhizomes were formulated as ointment (2% and 5%w/w) and were investigated for wound healing activity by using incision and dead space wound models. Different biochemical parameters i.e. hydroxylproline content and protein content in the skin tissues were determined. **Results**: Phytochemical screening reveals the presence of steroids, glycosides, flavonoids, carbohydrates and proteins in ethanol extract. Ethanol extract was shows significantly ($P < 0.01$) faster wound healing up to days 9th day. Various antioxidants enzymes and nonenzymatic substances found increased in healed tissue. The histopathological findings also support with increased fibroblasts throughout the healed tissue. **Conclusion:** Results were concluded that the ethanolic extract from rhizomes of *A. galanga* exhibited a definite wound healing effect which can be correlated its antioxidant property.

***Keywords*:** *Alpinia galanga*, wound healing, incision, dead space wound

Introduction

Wound healing is a multifaceted physiological process that requires a sequence of steps, each consist several factors for completion. The sequential phases of healing process are inflammation, proliferation and migration of connective tissue cells, production of extracellular matrix including collagen synthesis, epithelial cells migration and proliferation leading to neovascularization of wounded tissue.

Alpinia galanga Linn. (Zinziberaceae) consists of dried rhizome, a perennial herb native to Indonesia, and become naturalized in many parts of India. Its roots are tuberous, aromatic, leaves are oblong-lanceolate, acute margins, white sheath long ligule rounded. It contains essential oil (0.4 %) – (-pinene, limonene, -terpineol, linalool, terpinen-4-ol, eugenol and 1, 8-cineol) (Chopra et al., 1956). It also contains quercetin, kaempferol, kaempferide, quercetin 3-methyl ether, galangin, 1-acetoxyeugenol acetate; galangal A and B, and galanolactone as major chemical constituents. Bioassay guided separation, yielded three new linked neolignans, galanganal, galanganols screened for nitric oxide production inhibitory action (Morikawa et al., 2005) and Nuclear factor-kappa B activation was suppressed by 1-acetoxychavicol acetate. *A. galanga* is traditionally used to treat dyspepsia, fevers, urinary incontinence, halitosis, and in throat infections (Nadkarni, 1976). It is also useful in respiratory troubles. One study demonstrated that an alcoholic extract of the rhizome induced hypothermia, rheumatism and catarrhal infections in mice (Satyavati et al., 1976). Number of compounds have been reported for biological activities i.e. terpenyl ester (2-endo-70 hydroxy-1,8-cineole) for antimicrobial and antibacterial activity (Ogiso and Kobayashi, 1974; Miyazawa and Hashimoto, 2002) essential oils for antifungal activity, hypoglycemic activity (Akhtar et al., 2002) and in vitro cholinesterase enzyme inhibition. The objective of present study was to screen petroleum ether and ethanolic extracts of rhizomes

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for wound healing activity (Khattak et al., 2005).

Material and methods

Plant materials and preparation of extracts

The rhizomes of *A. galanga* were collected from campus of college, village Kukrikheda, Jabalpur. The plant, materials were identified (Specimen No. AG-14B) in the Department of Botany, JNKV, Jabalpur (M.P.). The rhizomes were dried in shade, powdered moderately and pass through sieve No. 10. The powdered plant material (200 gm) were successively extracted in a soxhlet apparatus with petroleum ether (60-80 °C), and ethyl alcohol (95%). The extract obtained with each solvent was weighed to a constant weight and tested for identification of different chemical constituents. The phytochemical screeing of both extracts were performed qualitatively for detection of different phytoconstituents. Both extracts were subjected to ointment formulation at 2% and 5% w/w by using Simple ointment base.

Pharmacological screening for wound healing

Animal groups

Wistar albino rats (160-180 g) were selected for wound healing study. The rats were acclimatized to the laboratory environment for about 7 day's period prior experiment. Six animals were taken in each group for study. Group I was referred as control group, while Group II and III referred as treated groups received ethanol extract 2% w/w and 5% w/w ointment, respectively. Group IV referred as reference group that received Povidone-Iodine ointment. The animal experimentation was preformed according to permission of Institutional animal ethical Committee (Registration No. GRKIST(P)/406/02/IAEC/16A).

Incision wound model

The selected animals were anaesthetized before wound creation and a 1.5 cm long incision was made through the skin at dorsal portion of rat skin. No local or systemic antimicrobials were used throughout the experiment. The both edges of wound kept together and stitched with black silk surgical thread and a curved needle used for stitching (Hemalata et al., 2001). Both wound edges were tightened for good closure of the wound and after stitching, wound was left undressed. All extracts and reference drug ointment were applied daily up to 9 days; upto wounds were healed completely. The sutures were removed on the 9th day and tensile strength of cured wound skin was measured using Tensiometer.

Dead space wound method

This model is used for the study of granuloma tissue. Animals were anaesthetized by light ether and an implantation of polypropylene tube (2.0 x 0.5), by making longitudinal incision in the lumber region on the dorsal surface. On the 9th post-wounding day, granuloma tissue formed on an implanted tube was dissected out carefully. Granuloma tissue was dried (60^{o}C) and stored in 10% formalin for the biochemical parameters and histological study (Shirwaiker et. al, 2003).

Tensile strength measurement

The tensile strength indicates the degree of wound healing. For the measurement of tensile strength, an instrument is known as Tensiometer, was used which is designed by Kuwano method (Kuwano et al., 1994). The different extracts ointment and reference were topically applied daily up to 9 days. The tissue samples on day 9th were taken from each group. The tensile strength of treated wounds was compared with control and reference ointment treated groups. Increased tensile strength indicates the better wound healing stimulated by applied topical extracts.

Biochemical estimations

Wound tissues were analyzed for hydroxyproline content, a basic constituent of collagen. Tissues were dried in a hot air oven at 60-70 ^{0}C to constant weight and hydrolysed in 6 N HCl at 130 ^{0}C for 4 hour in sealed tubes. The hydrolysate was neutralized to pH 7 then subjected to Chloramine-T oxidation for 20 minutes (Woessner, 1961). The reaction was terminated by addition of 0.4M perchloric acid and developed color with Ehlrich reagent at 60 ^{0}C was read at 557 nm in UV (Agilant Technology) Spectrophotometer.

To estimate tissue protein, tissue lysate was treated with a mixture of sodium tartrate, copper sulphate and sodium carbonate. This was left to stand for 10 minutes and then treated with Folin-Ciocalteau reagent that resulted in a bluish color in 20-30 minutes. The absorbance was measured using standard Spectrophotometry techniques 660 nm (Lowry et al., 1951).

Antioxidant status

The granuloma tissue collected from wound sites were tested for antioxidants assay. Superoxide dismutase (SOD) was assayed (Misra and Fridovich, 1972) based on the inhibition of epinephrine autoxidation by the enzyme. Reduced glutathione (GSH) level was determined by the method of Moron (Moron et al., 1979). Catalase was estimated following the breakdown of hydrogen peroxide (Beers and Sizer, 1952). Skin homogenates were immediately precipitated with 0.1 ml of 25% TCA and removed after centrifugation. Free-SH groups were assayed in a total 3 ml volume by the addition of 2 ml of 0.6 mM DTNB and 0.9 ml 0.2 mM sodium phosphate buffer (pH 8.0) to 0.1 ml of the supernatant and the absorbance was read at 412 nm using UV spectrophotometer.

Histological study

The microscopic examinations of wound tissues collected

from different animal groups was examine in terms of epithelial proliferation, granulation tissue formation and newly formed capillaries. Wound tissue sample from control, sample treated and reference group were collected and store in 10% formalin. After usual processing 6 μm thick sections were cut and stained with haematoxylin and eosin (McManus and Mowry, 1965). Sections were qualitatively observed under light microscope for fibroblast proliferation, collagen maturation and epithelialization.

Statistical analysis

Pharmacological data were represented as the mean ±S.D. for six rats and data were evaluated using the Dunnett test. Values of P <0.01 were considered to be statistically significant.

Results

Phytochemical studies

The percent yields of petroleum ether extract and ethanol extract were found 6.25% w/w and 4.58%w/w respectively. The petroleum ether extract gives positive test of steroids while ethanol extract showed presence of alkaloids, glycosides, flavonoids and proteins.

Pharmacological screening for wound healing

Wound healing activity was investigated on incision and dead space wound models. The tensile strength, biochemical parameters and histopathological changes were measured in healed skin on 9th day. The results of tensile strength on day 9th were shown in table 1. The tensile strength of skin tissues collected from the animals group treated with EE (5% w/w) ointment was observed significant improvement when compared with control group and reference ointment group.

Table 1. Effect of different extracts of *A. galanga* on tensile strength of incision wound in rats

Groups	Tensile strength (gm/cm^2)
Control	425.4±32.45
Petroleum ether extract (2% w/w)	536.1±35.27
Petroleum ether extract (5% w/w)	547.2±34.22
Ethanol extract (2% w/w)	724.6±45.57*
Ethanol extract (5% w/w)	785.4±47.34*
Reference ointment	812.6±52.61*

n = 6 albino rats per group, value represents Mean S.D. *P< 0.01, when compared each treated group with control group

The hydroxyproline content and protein content were determined as higher significantly in the group treated with EE 2% and 5% w/w ointment. The hydroxyproline content of ethanol extract treated group of animals were found 18.43±0.82 and 21.82±1.27 respectively for 2% and 5% w/w ointment which were significantly higher than the control group.

The protein content of 2% and 5% w/w ointment treated groups were showed higher than the control group and comparable to the reference ointment group (Table 2). The higher protein content of treated animals suggests that ethanolic extract through an unknown mechanism, stimulate cellular proliferation.

The antioxidant enzymes levels in granuloma tissue were found increased during wound healing process. SOD and CAT activity in granulation tissue were found to be significantly increased in case of rats treated with alcoholic extract when compared with control (Table 3). GSH concentration in granulation tissue was significantly increased in rats treated with extracts, when compared to control.

Table 2. Effect of different extracts of *A. galanga* on various biochemical parameters from dead space wound method in rats

Animal Groups	Hydroxyproline content (mg/g tissues)	Protein content (mg/g tissues)
Control	11.24±0.27	38.71±2.17
Petroleum ether extract (2% w/w)	12.36±0.31	39.14±2.31
Petroleum ether extract (5% w/w)	12.94±0.61	41.72±2.61
Ethanol extract (2% w/w)	18.43±0.82*	72.65±5.24*
Ethanol extract (5% w/w)	21.82±1.27*	81.42±6.52*
Reference ointment	22.41±1.34*	88.67±7.38*

n = 6 albino rats per group, value represents Mean S.D. *P< 0.01, when compared each treated group with control group in respective parameters

Histopathological studies were also supported the healing potential of ethanol extract in dose dependent manner. Ethanol extract treated group as well as reference group of animals was found to be covered by new epidermal layers. The proliferation of collagen, fibrous tissue and blood capillaries was observed. Large number of fibroblast was clearly observed in the dermis and the process of epidermal regeneration is complete. The control group presented edema, monocyte cells and cellular necrosis that were not observed in treated group.

Table 3. Effect of different extracts of *A. galanga* on various antioxidants levels from dead space wound skin in rats

Animal groups	Enzymatic study		
	SOD(µg/50mg tissue)	CAT(µmol/50 mg tissue)	GSH(µmol/50 mg tissue)
Control	14.63±0.44	12.36±0.85	24.26±1.75
Petroleum ether extract (2% w/w)	15.63±0.54	14.28±0.69	23.75±1.64
Petroleum ether extract (5% w/w)	16.37±0.52	17.67±1.08	26.34±2.14
Ethanol extract (2% w/w)	26.52±1.83*	28.46±2.56*	31.50±2.52
Ethanol extract (5% w/w)	27.83±1.63*	30.76±2.75*	37.86±3.01
Reference ointment	29.38±1.99*	31.46±3.42	39.42±3.75*

n = 6 albino rats per group, value represents Mean S.D. *P< 0.01, when compared each treated group with control group in respective parameters

Discussion and conclusion

The phytochemical studies of *A. galanga* rhizome revealed the presence of alkaloids, steroids, flavonoids, carbohydrates, proteins, and glycosides. rhizome of the plant already reported as antoxidant effect (Jain et al., 2011).The aim of this work was to evaluate healing property with antioxidant effect of *A. galanga* rhizomes. A significant increase in the tensile strength and protein content were compared to control group and also found comparable to reference ointment. Collagen is a major component of the extracellular matrix and ultimately contributes to wound strength. Hydroxyproline its peptides produced after breakdown of collagen. Determination of hydroxyproline amount therefore has been used as an index of collagen turnover. The improved hydroxyproline content of the wounded tissues has indicated faster collagen turnover leading to rapid healing with concurrent increase in the tensile strength of the treated wounds. The flavonoids may significantly improve the antioxidant enzyme activities (CAT, SOD and GSH) to maintain the balance of the antioxidant inside the body (Chen et al., 2009). *Alpinia galanga* contains quercetin, kaempferol, isorhamnetin and quercetin 3-methyl ether. The glycoside of quercetin reported (Khattak et al., 2005).

There is abundance of facts to suggest that improved production of reactive oxygen species, lipid peroxidation and ineffective scavenging play a crucial role in various skin lesions and in modulation of fibroblast proliferation (Murrell et al, 1990). Cutaneous wounding causes a depression in the overall antioxidant condition making it more vulnerable to oxygen radical attack (Shukla et al, 1997). All these finding indicate that in wound healing, antioxidants may play an important role. Flavonoids may help to prevent injury caused by free radicals. One way is the direct scavenging of free radicals. Flavonoids also support to increase collagen synthesis, promote the cross-linking of collagen, accelerate the conversion of soluble collagen to insoluble collagen, and inhibit the catabolism of soluble collagen. In conclusion, our findings suggest that *A. galanga* rhizomes have a potential effect in enhancing the wound healing process with antioxidant mechanism.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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