Research Article

Evaluation of altered ground matrix and matrix metalloproeinase (MMP'S) in wound healing with Aloe vera extract

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Abstract

Background: Aloe vera is known for its anti-tumor, anti-inflammatory, skin protection, anti-diabetic, anti-bacterial, anti-viral, antiseptic, and wound healing properties. This study was undertaken to evaluate the wound healing properties of Aloe vera (Aloe barbadensis) on cutaneous wound healing in dead space wounds created in rats. **Materials and methods:** Thirty rats were randomly divided into two equal groups (control and experimental). Wound created surgically were treated with Aloe vera aqueous extract through orally. **Results:** Increases in the level of hydroxyproline cross linking and collagen maturation enzyme lysyl oxidase were observed indicating definite prohealing action. In addition significant increase in the levelof antioxidants such as catalase, glutathione peroxidase and decrease in oxidative biomarker Malondialdhyde (MDA) and matrix destryoing enzyme MMP-2 and 9 were noted in the Aloe vera treated group. **Conclusion:** Aqueous extract of Aloe vera promotes wound healing by wound contraction and accelerates healing significantlyby increase in collagen and antioxidants enzymes.

***Keywords*:** Wound healing, hydroxyproline, antioxidant enzymes, Aloe vera

Introduction

Wound is the disruption of functional and anatomic continuity of living tissue produced by physical, chemical, electrical or microbial insults to the tissue. The mechanism of tissue healing is a complex biological process that involves a perfect and coordinated cascade of cellular and molecular events promoting tissue reconstitution (Hunt et al., 2000). Despiterecent advances in sciences and availability of wide variety therapieswound healing disorders continued to bedetrimental to causes diseases and even deaths (Honnegowda et al., 2016). The application of phytotherapeutic agents has also shown to be highly effective in the healing of wounds and burns (Rao et al., 1991). Aloevera (L.) (Liliaceae), popularly called aloe, is widely known for its therapeutic effects and has been used as a medicine since ancient times (Reynolds et al., 1999). Two fractions can be extracted from its leaves: an exudate and a mucilaginous gel. Transparent gel-like portion (mucilage) originates from the leaf parenchyma and has been used for the treatment of burns and wounds for its healing properties (Dorneles et al., 2003). This mucilage consists of biologically active molecules that act on fibroblasts during the formation of cicatricial tissue, stimulating the deposition of collagen fibers in the extracellular matrix (Chithra et al., 1998). Reynolds and Dweck (Choi, et al., 2002) investigated the biological activities of various Aloe species and observed that the whole gel extract of Aloe vera presents various pharmacological properties which promoting healing of wound, burns byactivate macrophages, stimulate T lymphocytes. It has been reported that Av gel has angiogenic effects and causes new vessel formation in cingulated cortex and septal areas in gerbil brain after ischaemia/reperfusion injury (Choi et al., 2002) and Av leaf gel was found to contain Vitamin C, E and aminoacids which are essential for wound healing (Davis, et

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al.,1994). In contrast to the reported positive effects of Av, some researchers have stated that the topical application of Av does not seem to enhance healing of the damaged skin, thereby still conflict and contradiction prevails regarding the wound healing efficacy Av (Surjushe et al., 2008; Vogler et al., 1999). In the present study, we investigated the effects of topical application of an *Aloe vera* gel on the healing of burn wounds induced in Wistar rats.

Materials and methods

The present study was under taken in department of pharmacology, Kannur Medical College, Kannur. Institutional Ethics Committee reviewed and approved the study protocol. Thirty mature wistar rats, weighing 150-200 g were housed in individual cages with controlled light, temperature and humidity. The rats were fed with pellet rodent diet and water ad libitum.

Wound model

The animals were anesthetized by intra muscular injection of ketamine hydrochloride (40 mg/kg). An excision wound of size 4 cm2 was made by cutting out a 2×2 cm piece of skin from the shaved area on the dorsal paravertebral lumbar skin.

Preparation of herbal extract

Full size mature leaves were cut from the plant and the rind removed (Chithra P et al.,1998) dried at room temperature without exposure to direct sunlight. After adding a small quantity of water, the leaves were then ground in a blender and centrifuged at 15,000 g to remove the fibers and then filtered through filter papers (Whatman® qualitative filter paper, Grade 1, Sigma-Aldrich, Bengaluru). The filtered aqueous extract was stored at +4 °C and allowed to thaw to room temperature before few hours to use.

Experimental protocol

After wound creation, the animals were randomly divided into two equal groups; Controls received 2 ml of 1% carboxymethyl cellulose orally, while the test group received 2 ml of the drug (150 mg/kg body weight) dissolved in 1% carboxymethyl cellulose through the same route, once daily, for a period of 10 days. On the 11th post-operative day,the granuloma tissue formed on the dead space wound was excisedstored at -20°C until subjected to biochemical analysis.

Preparation of tissue for biochemical parameters

Preparation of tissue for Hydroxyproline estimation

The granulation tissues were dried at 60°C for 24 hr. Dissolved in 6NHCl in boiling water bath for 24 hr for hydrolysis. The hydrolysate cooled and neutralised by 10N NaOH using phenolphthalein/ Methyl red as an indicator. The volume of neutral hydrolysate was diluted to a concentration of 20 mg/ml of dried granulation tissue in the final hydrolysate with distilled water. The hydrolysate was used for the estimation of hydroxyproline.

Preparation of tissue homogenate for antioxidant assay

A granuloma tissue was homogenized using 0.02 M potassium-phosphate buffer, pH 7.6 (1:10 w/v). The tissue homogenate was centrifuged at 6000 rpm in a cold centrifuge. The clear supernatant was used for all the assays.

Biochemical parameters studied

Hydroxyproline

The neutralized acid hydrolysate of the dry tissue was used for the determination of Hydroxyproline. The reaction mixture contains 0.05M copper sulphate, 2.5N sodium hydroxide, 6% hydrogen peroxide, 3N sulphuric acid, 5% p-Dimethylaminobenzaldehyde using L-Hydroxyproline as standard. The absorbance was measured at 540nm and expressed in µg/mg dry tissue weight (Neuman et al., 1950).

Total Protein

Total Protein content of the tissue homogenate was estimated by the method of Lowry et al. The absorbance was measured at 540nm and expressed in mg/g of tissue. Standards were treated similarly using Bovine serum albumin (BSA) at concentrations of 0, 20, 40, 60, 80, and 100µg/ml in 0.1M phosphate buffer at pH 7.4 (Lowry et al., 1951).

Extraction and estimation of lysyl oxidase from granuloma tissue

In this case the granuloma tissue homogenate was prepared by homogenising the tissue in 0.02 M phosphate buffer, pH 8.2 containing 1.5 M urea (1:10w/v). This cold centrifuged at 15000 rpm for 20 min. The supernatant served as a source of lysyl oxidase estimation.

Lysyl oxidase activity was assayed spectrofluorometrically, using a monoamine as substrate (Trackman et al., 1979) and adaptation of the peroxidase coupled assay (Trackman et al., 1981; Udupa et al., 1991).

Glutathione peroxidase (GPx)

GPx activity was determined by using cumene hydro-peroxide as substrate. The standard assay mixture contained in 1.0ml final volume: 50 mmol/l tris buffer, pH 7.4; 0.14 mmol/l β nictoinamide adenine dinulciated phosphate (reduced form, NADPH); 1.0 mmol/l glutathione; 1.0 kU/l glutathione reductase; 0.5 mmol/l

cumene hydrogen peroxide; and a rate limiting amount of glutathione peroxidease (tissue homogenate supernatant fraction). For each determination a non enzymatic glutathione peroxidase activity; the value obtained was subtracted from the rate observed in the presence of glutathione peroxidase activity. One unit of glutathione peroxidase activity is expressed as the amount of enzyme required to oxidize 1.0 µmol/min of NADPH under the assay conditions (Paglia et al., 1967).

Estimation of catalase activity

Exactly 0.2 ml of the diluted homogenate (1:500)was added to the reaction mixture, which consisted of 0.2 ml of 0.05 M sodium phosphate buffer and 1.2 mM H202. After 25 minutes of incubation at room temperature, the reaction was arrested by the addition of 2.5 ml of peroxide reagent containing peroxide and chromogen system. The absorption was measured at 500 nm. A blank without H^20^2 and control with 1 mM sodium azide and catalase enzyme were run simultaneously (Cohen et al., 1970).

Lipid peroxidation

Malondialdhyde (MDA) a measure of lipid peroxidation was measured spectrophotometrically in homogenate, according to the method of Ohkawa et al. (1979). Where MDA determined by using 1ml of Trichloroacetic acid (TCA) 10% and 1 ml of thiobarbituric acid (TBA) 0.67% and were then heated in a boiling water bath at 100^0c for 30 min. Mixture was cooled under tap water and centrifugation at 12000 rpm for 10 min, Thiobarbituric acid reactive substances (TBARS) were determined by the absorbance at 535 nm and expressed as nmole/mg protein (Ohkawa et al., 1979).

Matrix metalloproteinase-2 and -9

A total of 100 mg of tissue was homogenized in 1 mL of ice-cold lysis buffer. Subsequently, homogenates were centrifuged at 3,000 g for 5 min at 4°C, and supernatants were stored at -80°C until use. MMP-2 and MMP-9 were measured using prefabricated ELISA kits, according to manufacturer protocol (R and D Systems, Uscn Life Science Inc. USA). Plates were read at 450 nm and 540 nm and concentrations were calculated using a 4-point standard curve and expressed as ng/mg of protein (Young et al., 1994).

Statistical analysis

Data expressed as Mean ± S.Dand were evaluated by student's 't' test. Values of $p<0.005$ were considered statistically significant.

Results

The effects of the Aloe veraon wound healing are depicted in Table 1. Significant increase in the ground substance hydroxyproline (collagen fiber) ($p< 0.05$) was noticed with the animal treated with extract of Av leaves. The Lysyl oxidase activity was increased in the test group ($p< 0.05$) when compared with the control group. The antioxidant enzymes catalase, glutathione were also increased. The increase in catalase level was significantly higher ($p< 0.010$) than the glutathione peroxidase enzyme. Oxidative stress marker malondialdhyde (MDA) and MMP-2 and 9 were significantly decreased in test group than compare to control ($p< 0.05$). Protein content in the granuloma tissue was increased too.

Discussion

Normal wound healing may be arbitrarily divided into a sequence of four time-dependent phases: (i) coagulation and haemostasis; (ii) inflammation; (iii) fibroblast proliferation and secretion of mucopolysaccharides; and (iv) wound

Table 1. Levels of hydroxyproline and antioxidant parameters of control and test group

Parameters	Control	Drug-treated	P value
Hydroxyproline (µg/mg of dry weight of tissue)	61.7±11.7	97.6±17.2	0.013
Total protein (mg/g of wet weight of tissue)	10.2±2.9	22.0±5.0	0.003
Lysyl oxidase (SFU/ml)	1875± 124	3798± 240	0.038
GPx (µMoles NADPH oxidized/min /mg protein)	261.0±64.1	343.1±78.6	0.048
Catalase (µMoles H202 degraded/min/ g tissue)	61.77x 103± 56	168.45x 103± 48	0.010
MDA (nmole/mg protein)	19.3±6.66	6.6±3.7	0.002
MMP-2 (ng/mg tissue protein)	0.92 ± 0.52	0.36 ± 0.49	0.030
MMP-9 (ng/mg tissue protein)	0.96 ± 0.22	0.40 ± 0.32	0.045

Values are expressed in mean ±SD; SFU- Syncytia-forming units per milliliter (SFU/ml)
MMP: Matrix metalloproteinase, MDA: Malondialdehyde, SD: Standard deviation

remodelling with scar tissue formation (Young et al., 1994). Several studies have reported the antioxidant properties of Aloe vera gel and found to contain Vitamin C, Vitamin E and aminoacids which are essential for wound healing (Hashemi et al., 2015). Carbohydrates Constituents present in Aloe vera gel such as Polysaccharides, mannose accelerate multifunctional cytokine TGF-1 which enhances granulation tissue formation in wound healing (Atiba et al., 2011). It has been reported that Av increases the collagen content of the granulation tissue. Hydroxyproline, an amino acid present in collagen is a biochemical marker for tissue collagen (Honnegowda et al., 2016). The present study reported that Aloe vera gel has a profound stimulatory effect on ground substance by enhancing the level of collagen (hydroxyproline) which not only confers strength and integrity to the tissue matrix but also plays an important role in homeostasis and epithelialization in wound healing (Daburkar et al., 2014). The increased tensile strength was attributed to the cross-linking and maturation of collagen, resulting in better alignment of the collagen bundles in the healing wound (Kumar et al., 2013). The present study shows that extract of Aloe vera stimulated the enzyme lysyl oxidase, which is crucial for cross- linking and maturation of collagen. Wounding initiates inflammation, which in turn stimulates the production of free radicals by phagocytes and inhibits myofibroblasts thereby retarding the healing process (Birben et al., 2012). MDA is oxidative biomarker produced byattack of free radical on membrane lipoproteins and polyunsaturated fatty acid (Honnegowda et al., 2016). Delayed healing of diabetic wounds is characterized by an increase in matrix metalloproteinases (MMPs), a decrease in the tissue inhibitors of metalloproteinases (TIMPs) (Liu et al., 2009). In the present study significant decreases in the concentrations of MMP-2 and MMP-9 were observed in the aloe vera treated group vs control group.

Conclusion

Aloe vera increased the antioxidant enzymes, thereby affording protection to the developing fibroblasts against oxidativefree radicals.It is yet prove whether the drug stimulates the antioxidant enzymes synthesis at gene level or increases its half life period; the increased protein synthesis in the granuloma tissue does hint the possibility of increased enzyme synthesis.

Conflict of interest

Declared none

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References

Atiba A, Ueno H, Uzuka Y. 2011. The effect of aloe vera oral administration on cutaneous wound healing in type 2 diabetic rats. Journal of Veterinary Medical Science, 73(5):583-9

Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O, 2012. Oxidative Stress and Antioxidant Defense. World Allergy Organization Journal, 5:9-19.

Chithra P, Sajithlal BG. Chandrakasan G 1998. Influence of Aloe vera on the healing of dermal wounds in diabetic rats. Journal of Ethnopharmacology, 59:195-201.

Choi S, Kim KW, Choi JS, Han ST, Park YI, Lee SK, Kim JS, Chung MH. 2002. Angiogenic activity of ß-sitosterol in the ischaemia/reperfusion-damaged brain of Mongolian gerbil. Planta Medica, 68:330-335.

Cohen G, Dembiec D, Marcus J. 1970. Measurement of catalase in tissue extract. Annals of. Biochemistry, 34:3038-3045.

Daburkar M, Lohar V, Rathore AS, Bhutada P, Tangadpaliwar S. 2014. An in vivo and in vitro investigation of the effect of Aloe vera gel ethanolic extract using animal model with diabetic foot ulcer. Journal of Pharmacy & Bioallied Sciences, 6:205–212.

Davis RH, Donato JJ, Hartman GM, Haas RC. 1994. Anti-inflammatory and wound healing activity of a growth substance in Aloe vera. Journal of the American Podiatric Medical Association, 84: 77-81.

Dorneles D, Wouk AF, Pontarolo R, Oliveira AB. 2003. Evaluation of aloe vera linné healing properties in rabbits skin wounds. Visão Acad, 4(1):39-46.

Hashemi SA, Madani SA, Abediankenari S. 2015. The Review on Properties of Aloe Vera in Healing of Cutaneous Wounds. BioMed Research International, 714216.

Honnegowda TM, Kumar P, PadmanabhaUdupa EG, Sharan A, Singh R, Prasad HK. 2016. A comparative study to evaluate the effect of limited access dressing (LAD) on burn wound healing. International Wound Journal, 13: 791-8.

Honnegowda TM, PadmanabhaUdupa EG, Rao P, Kumar P, Singh R. 2016. Superficial Burn Wound Healing with Intermittent Negative Pressure Wound Therapy Under Limited Access and Conventional Dressings. World Journal of Plastic Surgergery, 5:265-273.

Hunt TK, Hopf H, Hussain Z. 2000. Physiology of wound healing. Advances in Skin & Wound Care, 13:6-11.

Kumar P, Honnegowda TM, Udupa P, Rao P, Saran A, Bhandari S. 2013. The role of nitric oxide in chronic and acute wound healing. Journal of Society for Wound Care and Research 6:14-23.

Liu Y, Min D, Bolton T, Nubé V, Twigg SM, Yue DK, McLennan SV. 2009. Increased matrix metalloproteinase-9 predicts poor wound healing in diabetic foot ulcers. Diabetes Care, 32:117-9.

Lowry OH, Rosebrough MH, Farr L, Randell RJ. 1951. Protein measurement with the folin-phenol reagent.

Journal Biological Chemistry, 93: 265–75.

Nayak S, Udupa L, Udupa S, 2003. Altered antioxidant enzyme profile in wound healing. Indian Journal Clinical Biochemistry, 18:75-79.

Neuman R, Logan M. 1950. The determination of collagen and elastin in the tissues. Journal Biological Chemistry, 186:549-56.

Ohkawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Annals of Biochemistry, 95: 351–58.

Paglia DE, Valentine WN. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. Journal of Laboratory and Clinical Medicine, 70:158-169.

Rao SG, Udupa AL, Udupa SL, Rao PGM, Rao G, Kulkarni DR. 1991. Calendula and Hypericum: two homeopathic drugs promoting wound healing in rats. Fitoterapia, 62(6):508-10.

Reynolds T, Dweck AC. 1999. Aloe vera leaf gel: a review update. Journal of Ethnoparmacology, 68(1):3-37.

Surjushe A, Vasani R, Saple DG. 2008. Aloe vera: a short review. Indian Journal Dermatology, 53:163–166.

Trackman PC, Kagan HM. 1979. Nonpeptidylamine inhibitors are substrates of lysyl oxidase. Journal of Biological Chemistry, 254:7831-36.

Trackman PC, Zoski CG, Kagan HM. 1981. Development of a peroxidase coupled flourometric assay for lysyl oxidase. Annals of Biochemistry, 113: 336-342.

Udupa S L, Udupa AL, Kulkarni DR. 1991. Influence of Tridaxprocumbens on lysyl oxidase activity and wound healing. Planta Medica, 57: 325-327.

Vogler BK, Ernst E. 1999. Aloe vera: a systematic review of its clinical effectiveness. British Journal of General Practice 49: 823–28.

Young PK, Grinnell F. 1994. Metalloproteinase activation cascade after burn injury: a longitudinal analysis of the human wound environment. Journal of Investigative Dermatology, 103:660-4.