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Screening Wound-Healing Potential of Different *Aloe vera* L. Germplasms at the Cellular Level

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ABSTRACT

Aloe vera L. germplasms were tested for their ability to heal wounds following proliferation and migration of murine L929 fibroblast cells employing MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and an *in vitro* scratch assay. All the germplasms were capable of promoting cell proliferation and migration. A positive correlation was observed in proliferation and migration of cells among the aloe germplasms independent of treatment concentration. Of the tested germplasms, TN exhibited maximum while RJN showed minimumwound-healing potential. This ability of *A. vera* to stimulate fibroblast proliferation and migration might help skin regeneration and repair. The *in vitro* scratch assay employed in the present study for the first time documents its efficiency in evaluating wound-healing potential following fibroblast migration.

Keywords: aloe, cell migration, fibroblasts, MTT assay, scratch assay

Abbreviations: DMEM, Dulbecco's modified minimal essential medium; **DMRT**, Duncan's multiple range test; **FBS**, fetal bovine serum; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PBS**, phosphate buffer saline

INTRODUCTION

Wound healing is the process of repair and regeneration of dermis and epidermis that follows injury to the skin and other tissues of the body. It involves inflammation, cell proliferation and contraction of collagen lattice formation (Kumar *et al*. 2007; Reddy *et al*. 2008). A large number of plants have been screened for the evaluation of their wound-healing potential using various model systems (Graham *et al.* 1984). The most commonly used model systems are excision, incision and dead space where higher breaking strength and higher hydroxyproline content are obtained in the treated groups with a decrease in surface area of wound. However, evaluation of wound-healing activity of plant extracts is meager at the cellular level. Fibroblast cell cultures have been proposed as a method for the investigation of wound-healing activity (Liang *et al.* 2007; Ozturk *et al.* 2007). Fibroblasts are the major source of extra cellular connective tissue matrix and migration of fibroblasts are considered to be vital in rapid and effective repair of damaged skin. The *in vitro* scratch assay is an easy and low-cost method to measure fibroblasts migration *in vitro* (Davis *et al.* 1989). It is based on the observation that, upon a creation of an artificial gap or scratch, on a confluent cell monolayer, the cells on the edge will move toward the opening and close the gap gradually by cell-to-cell contacts.

Aloe vera L., belonging to the family Liliaceae, is a perennial succulent plant. The plant has been known traditionally as the 'healing plant' and is used in a variety of cosmetic product formulations. The mucilaginous gel of this plant has been shown to have wound-healing (Rodriguez-Bigas *et al.* 1988), burn-healing (Davis *et al.* 1992), anti-inflammation (Strickland *et al.* 1994) and immunostimulatory properties (Davis *et al.* 1991). The stimulatory effect of aloe whole extracts or components on wound healing have been reported in several instances using *in vivo* models (Heggers *et al.* 1996; Yagi *et al.* 1997; Thomas *et al.* 1998). However, there have been contradictory reports on the wound-healing effects of aloe gel components. Acemannan isolated from aloe did not show a significant wound-healing effect, whereas mannose 6-phosphate and a glycoprotein fraction stimulated proliferation in mice fibroblasts and human keratinocytes, respectively (Davis *et al.* 1994; Choi *et al.* 2001).

In this study, our objective was to evaluate the wound-healing potential of aloe gel from different germplasms following proliferation and migration of fibroblasts using MTT and *in vitro* scratch assays.

MATERIALS AND METHODS

Plant materials

Germplasms of *Aloe vera* L. (*A. barbadensis* Miller) were collected from different agro-climatic zones of India (**Table 1**). The plant material was identified by Prof. G.G. Maity, Taxonomist, University of Kalyani, and was grown in the Agricultural and Food Engineering Departmental farm of IIT Kharagpur. The five germplasms designated and studied are S24, RM, TN, OR and RJN.

Table 1 Categorization of *Aloe vera* L. germplasms with their place of collection and agroclimatic zones.

Aloe germplasms	Place of collection	Agroclimatic zones of India
S 24	South 24 Pgs, West Bengal	Lower Gangetic Plains
TN	Tatanagar, Jharkand	Middle Gangetic Plains
OR	Sundargarh, Orissa	Eastern Plateau
RJN	Chittorgarh, Rajastan	Western Dry Region
RM	Raja Mundry, Andhra Pradesh	East Coast Plains

Gel extraction

The mucilaginous gel was removed from the leaves (10 g) of 10-12 month old plants, homogenized with phosphate buffer saline

Received: 17 March, 2009. Accepted: 31 March, 2009.

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(PBS, pH 7.4) and 95% ethanol (3:1) and centrifuged at 10,000 rpm for 15 min. The supernatant was lyophilized with a yield of 2.5, 2.1, 0.9, 1.8 and 1.5 g, respectively, for S24, RM, TN, OR and RJN.

Murine L929 fibroblasts culture

For cell culture, a Murine L929 fibroblast cell line obtained from National Centre for Cell Science, Pune was passaged in Dulbecco's modified minimal essential medium (Gibco, USA) supplemented with non-essential amino acids and 10% fetal bovine serum (DMEM-FBS). Fibroblasts were cultured at 37°C in an atmosphere of 95% air and 5% CO_2. Sub-culturing was done by exposure to trypsin (Takashima *et al.* 1999).

MTT cell proliferation assay

The MTT (Loba Chemie, India) cell proliferation assay was performed based on a method previously described with little modifications (Torre *et al.* 2006). Briefly, 180 µl of 3×10^5 cells/ml was added to each well of a 96-well culture plate (Tarson, India) and incubated overnight at 37°C and 5% CO_2. A sample (20 µl each of various concentrations as indicated in **Table 2**) was then added into each well and the culture was incubated for three days at 37°C and 5% CO_2. Phosphate buffer solution (PBS; Sigma, India) was used as control. The percent proliferation was calculated as (O.D. of sample/O.D. of control × 100) considering 100% proliferation in control.

In vitro scratch assay for cell migration

The scratch assay was performed following the method of Liang *et al.* (2007). L929 fibroblast cells were cultured to confluence in a 96-well culture plate and the culture medium was drained away. A scratch (0.5 mm in length) was made with the help of a 10 µl micropipette tip by moving back and forth once against the top of the culture. The wells were washed three times with PBS to remove cellular debris. The scratch was photomicrographed with the help of a phase-contrast inverted microscope (Olympus, model no. CKX41) fitted with a digital camera (Olympus). Aloe sample (20 µl each of various concentrations as shown in **Table 3**) was added to the culture, and the control culture received PBS. Fibroblast migration was photographed 24 hr after creation of the scratch. For image analysis image J software (http://rsbweb.nih.gov./ij/) from the National Institute of Health (NIH, USA) was used. The pixel length of 2022.32 as displayed in the set scale window was calibrated into micrometers by using a haemocytometer. This calibration was made uniform for all images under analysis. Two parallel lines were drawn at the edges of the scratch and the distance between them was measured by drawing a line between two points perpendicular to each other. Three distance values were taken for each image. The values obtained were transferred to a data window using the "measure" option. This process was repeated for each image taken at 0 and at 24 hr. The distance cells migrated on addition of samples was then compared with the control and a migration % was calculated. For each concentration three wells were used for analysis.

Statistical analysis

Statistical significance was analyzed by two-way ANOVA followed by Duncan's Multiple Range Tests (DMRT) at $P \leq 0.05$ using MSTATC software package. Percentage data were subjected to arcsine transformation before analysis.

RESULTS AND DISCUSSION

Proliferation of L929 fibroblast cells was observed for all the germplasms in a dose-independent manner. At 25 µg/ml there was maximum proliferation of 137.15% in TN, whereas 50 µg/ml was found to stimulate maximum proliferation ranging from 125.57 to 129.64% in the remaining germplasms (**Table 2**). A significant difference in the percentage proliferation was noted at 25 µg/ml with TN and RJN.

At the onset of the *in vitro* scratch assay, there were few or no cells within the scratch area. Migration of L929 fibroblast cells was prominent after 24 h of incubation in all the five germplasms (**Fig. 1, Table 3**). The migration of fibroblasts in the scratch area is likely to be a combined effect of absolute cellular migration, proliferation and cell death (Ozturk *et al.* 2007). The treatment concentration influenced the fibroblast migration significantly. Migration was stimulated at lower concentrations up to 25 µg/ml, whereas a slower rate of migration was evident with 50 and 75 µg/ml. The maximum migration of 142.24 µm was observed in RM with a 19.7% increase in cell migration compared to the control at 25 µg/ml. A similar trend was observed in TN except at 10 µg/ml.

Table 2 Effect of aloe gel from various germplasms on % cell proliferation.

Germplasms \ Conc. µg/ml	PBS 0	10	25	50	75
	100				
TN		103.16 a	137.15 a	128.12 a	120.48 a
S24		101.02 a	119.34 ab	126.97 a	125.19 a
OR		101.27 a	119.97 ab	125.57 a	116.16 a
RJN		100.51 a	110.56 b	125.83 a	121.25 a
RM		101.01 a	126.08 ab	129.64 a	125.32 a

The values in each column in different superscripts are significantly different from each other at *P* = 0.05 following Duncan's Multiple Range Test.

Table 3 Effect of aloe gel obtained from various germplasms of *Aloe vera* L. on L929 fibroblast migration in distance (µm) and in percentage (indicated in parentheses). Values are expressed as mean ± SD.

Germplasms \ Conc. µg/ml	PBS 0	10	25	50	75
	53 ± 4.7 (7.34 ± 0.67)				
TN		140.04 ± 15.88 a (19.4 ± 2.19 a)	98.92 ± 21.34 b (13.7 ± 2.95 b)	84.56 ± 18.79 b (11.69 ± 2.6 b)	84.31 ± 19.35 a (11.68 ± 2.6 a)
S24		94.84 ± 1.99 b (13.3 ± 0.28 b)	115.50 ± 4.55 ab (15.99 ± 2.01 ab)	88.03 ± 3.06 b (12.19 ± 0.42 b)	80.91 ± 0.98 a (11.2 ± 0.13 a)
OR		112.03 ± 11.29 ab (15.52 ± 1.56 ab)	101.96 ± 12.98 ab (14.12 ± 2.95 ab)	84.27 ± 17.56 b (11.67 ± 2.43 b)	77.59 ± 1.89 a (10.75 ± 0.26 a)
RJN		122.57 ± 7.61 ab (16.98 ± 1.05 ab)	110.69 ± 6.02 ab (15.33 ± 0.83 ab)	95.64 ± 3.11 ab (13.25 ± 0.43 ab)	68.53 ± 4.7 a (9.49 ± 0.65 a)
RM		111.17 ± 30 ab (15.39 ± 4.29 ab)	142.24 ± 18 a (19.7 ± 2.63 a)	133.98 ± 17 a (18.55 ± 2.39 a)	93.26 ± 6 a (12.92 ± 0.98 a)

The values in each column in different superscripts are significantly different from each other at *P* = 0.05 following Duncan's Multiple Range Test.

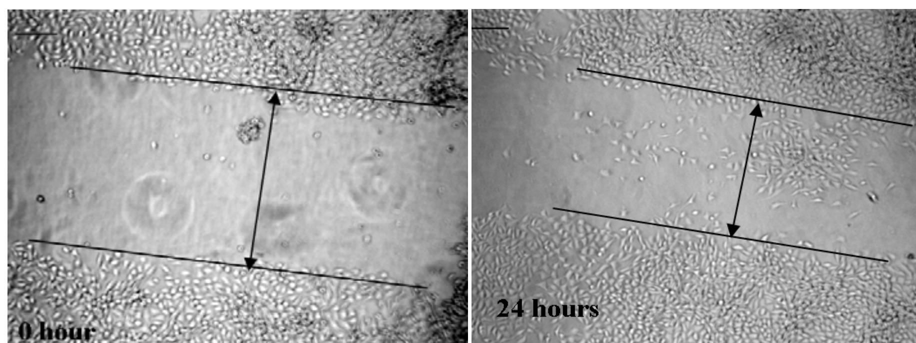

Fig. 1 Micrographs showing fibroblast migration at 25 µg/ml of aloe extract of the germplasm RM. Scale bar: 150 µm.

In the present work, the wound-healing potential of aloe gel from five different germplasms was documented with proliferation and migration of fibroblasts adopting two distinct methods. A positive relationship was observed in proliferation and migration of cells among the aloe germplasms independent of treatment concentration. These findings indicate the presence of stimulatory factors such as glycoproteins, responsible for cell proliferation and migration in all the germplasms but in a varied manner. Among the germplasms, the wound-healing potential was evidently maximum with TN and minimum with RJN. Interestingly, an opposite trend was observed with UV-opacity potential of aloe germplasms, where maximum UV-filtering capacity was noted in RJN and the minimum was found in TN (Shyam Kumar *et al.* 2009). Such differential behavior necessitates the screening of germplasms to identify the source plants for optimum production of phyto-constituents with specific biological potential. Collagen and proteoglycans synthesis and epidermis formation suggesting wound-healing potential have been reported earlier with glycoprotein fractions and mannose-6-phosphate isolated form aloe (Davis *et al.* 1994; Yagi *et al.* 1997). The *in vitro* scratch assay employed in the present study for the first time could be an inexpensive and convenient method to evaluate the wound-healing potential of medicinal plants following fibroblast migration.

ACKNOWLEDGEMENTS

The work was supported by grants (SP/SO/PS-28/2003) from the Department of Science and Technology, New Delhi, India.

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