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In vivo Angiogenic Activity of Dichloromethane Extracts of *Aloe vera* Gel

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The angiogenic activity of *Aloe vera* (*Aloe barbadensis*), known as a good healing plant, was investigated. We have extracted and fractionated dichloromethane extract (G1M1D1) and methanol soluble fraction of dichloromethane extract (G1M1D1M1) which contain low-molecular weight substances of *Aloe vera* gel. G1M1D1 and G1M1D1M1 fractions induced a radially arranged, spoke-wheel-like vasculature in chick embryo chorioallantoic membrane (CAM) assay. The angiogenic activity was dose-dependent and the angiogenic pattern in the CAM assay was very similar to that of phorbol 12-myristate-13-acetate (PMA) used as a positive contol. The modified CAM assay, a simple and accurate quantitating method, was used to quantitate the angiogenic activity of G1M1D1M1 fraction. Application of G1M1D1M1 fraction (100 μg/egg) resulted in much more intense angiogenesis than in contol while slightly less intense angiogenesis than in PMA (100 ng/egg).

Key words : Angiogenesis, Chorioallantoic membrane (CAM) assay, Modified CAM assay, Phorbol 12-myristate-13-acetate (PMA)

INTRODUCTION

Aloe plants have been used medicinally for centuries. Among them, *Aloe barbadensis*, commonly called *Aloe vera*, has been one of the most used healing plants in the history of mankind. A number of pharmaceutical publications eulogized the ability of the *Aloe vera* gel to promote the healing of burns and other cutaneous injuries and ulcers (Klein and Penneys, 1988; Lushbaugh and Hale, 1953). Recently, it has been reported that *Aloe vera* gel has improved wound healing in a dose-dependent fashion, reduced edema and pain (Davis *et al.*, 1986; Davis *et al.*, 1987). In wound healing, angiogenesis is an essential process (Folkman, 1986; Thompson *et al.*, 1991). Angiogenesis is the growth of new capillaries from pre-existing capillaries and post-capillary venules (Bischoff, 1995; Folkman and Klagsbrun, 1987), and is required to furnish the new tissue with oxygen and metabolites and to dispose of the waste products of metabolism. When angiogenesis is impaired, as in the aged or in the irradiated tissues, wound healing is retarded or unsuccessful (Phillips *et al.*, 1991; Thompson *et al.*, 1992). Thus, we suppose that *Aloe vera* gel may contain the angiogenic component. The aim of this study is to investigate the angiogenic activity of *Aloe vera* gel extracts.

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MATERIALS AND METHODS

Materials

Lyophilized *Aloe vera* gel (Gl) was kindly provided by Namyang Aloe Co. Ltd.. Phorbol-12-myristate-13-acetate (PMA) was purchased from Sigma Chemical Co.. Thermanox coverslip was purchased from Nunc Inc. and fat emulsion (10%) was purchased from Korea Green Cross Corp.. All other chemicals were of reagent grade.

Extraction and fractionation of *Aloe vera* gel

Lyophilized *Aloe vera* gel (G1) was refluxed with methanol three times for three hours. The methanol layer was filtered and concentrated by rotary vacuum evaporator (G1M1). Fractionation of *Aloe vera* gel was conducted as shown in Scheme 1. The methanol extract (G1M1) was fractionated with dichloromethane, filterd and concentrated (G1M1D1).

Aloe vera gel (G1)

↓ extracted with hot methanol 3 times filtered

methanol layer

↓ evaporated *in vaccuo*

methanol extract (G1M1)

↓ suspended in water extracted with dichloromethane

dichloromethane layer

↓ evaporated *in vaccuo*

dichloromethane extract (G1M1D1)

↓ refractionation with hexane/90% methanol

90% methanol layer

↓ evaporated *in vaccuo*

90% methanol extract (G1M1D1M1)

Scheme 1. Fractionation procedure of *Aloe vera* gel.

The dichloromethane extract (G1M1D1) was further extracted with hexane/90% methanol and the 90% methanol layer was evaporated (G1M1D1M1).

Chick embryo chorioallantoic membrane (CAM) assay

The fertilized chick eggs used in this study were kept in a humidified incubator at 37°C. After three day's incubation, about 2 ml of ablumin was aspirated from eggs with an 18-gauge hypodermic needle through the small hole at the narrow end of the eggs. At 4-day chick egg, the shell covering the air sac was punched out and removed by forceps, and the shell membrane on the floor of the air sac was peeled away. At 9-day chick embryo, a sample-loaded thermanox coverslip was applied to the CAM surface. The embryos were returned to the incubator. Three days later, about 1 ml of 10% fat emulsion was injected into the CAM and is was observed with a microscope. Data on the number of positive CAM were analyzed by means of Student's t test with $P<0.05$ as the level of significance.

Modified CAM assay

Incubation of fertilized chick eggs at 37°C

↓

After incubation for three days, albumin is aspirated

↓

At 4-day chick eggs, shell covering the air sac is punched out and shell membrane is peeled away

↓

Formation of collagen suspension

↓

Incubation for 20-25 min. at 37°C

↓

At 9-day chick embryo, collagen gel-loaded bottom mesh is applied to the CAM surface

↓

Covering with top mesh and incubation at 37°C

↓

Observation from day 3 to 7 after implantation

Scheme 2. Protocol of the modified CAM assay

The modified CAM assay was recently developed to quantitate the angiogenic activity. Until 4th day, the procedure was same as the CAM assay illustrated in Scheme 2. At 9th day, the sample was mixed with type I collagen suspension. Type I collagen suspension was composed of the following ratio: 400 μl type I collagen (previously adjusted to pH 7.4)+20 mg sucralfate (aluminum sucrose octasulfate)+200 μl of 1 mg BSA/ml saline(Nguyen *et al.*, 1994). A 40 μl aliquot of the above suspension was deposited onto a piece of mesh (Spectra, 300/50, 8×8 mm). The mesh had been previously cut into the desired dimensions and then autoclaved. The sample was allowed to gel on top of the flat end of a 10 mm diameter teflon rod mounted within a 100 mm petri dish(Crum *et al.*, 1985). A plastic cover was placed over the petri dish, which was then put in an incubator at 37°C with 65-70% humidity for 20-25 min. In order to avoid a tight seal and to allow adequate air exchange, the plastic cover opened slightly. Subsequently, the collagen gel was transferred onto the CAM of a 9-day-old chick embryo with sterile fine forceps. A smaller piece of mesh (4×4mm) was then placed on top of the collagen gel. The eggs were returned to the incubator and observed from day 3 to 7 after implantation with a microscope. The stimulation of angiogenesis in response to the sample was expressed as the percentage of the squares in the top mesh which contained blood vessels.

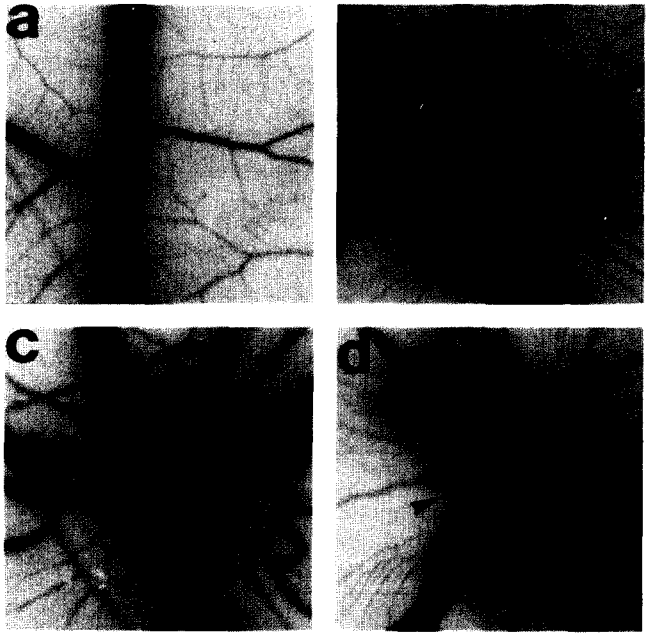

Fig. 1. Photographs of CAM after three day-treatment with PMA and *Aloe vera* gel extracts. Blood vessels were made visible by injection of 10% fat emulsion (white color). Arrowheads indicate spoke-wheel-like vascular pattren. Control(empty coverslip) had no effect on the vascular pattern. (a) control (b) PMA, 60 ng/egg (c) G1M1D1, 250 μg/egg (d) G1M1D1M1, 100 μg/egg Magnification: x12.

Table I. Angiogenic activity of *Aloe vera* gel extracts by CAM assay

Tested compound	Dose (μg/egg)	Total test No. of CAM	No. of positive	% positive	*P value
control (empty coverslip)		50	2	4	
PMA	60 (ng/egg)	50	38	76	<0.001
G1M1D1	50	50	30	60	<0.001
	125	50	37	74	<0.001
	250	50	43	86	<0.001
	500	50	47	96	<0.001
G1M1D1M1	50	50	36	72	<0.001
	100	50	41	82	<0.001
	200	50	45	90	<0.001
	400	50	49	93	<0.001

*; data on the no. of positive CAM treated with samples were compared with the no. of positive CAM treated with empty cover slip by means of Student's *t* test.

RESULTS

Angiogenic activity of dichloromethane extracts of *Aloe vera* gel in the chick embryo chorioallantoic membrane(CAM) assay

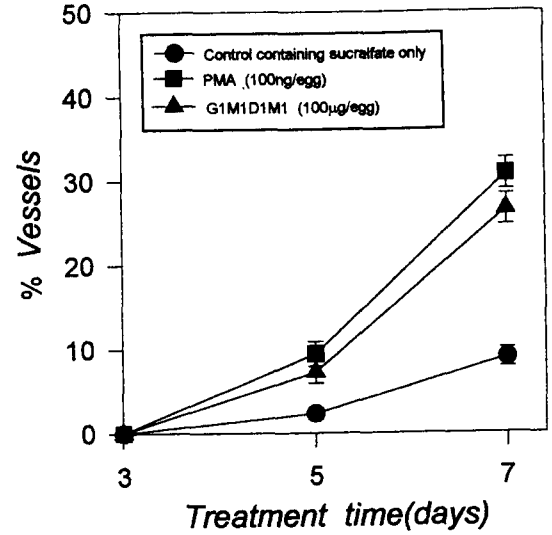

Fig. 2. Quantitation of the angiogenic activity by the modified CAM assay. The angiogenic responses were observed with a microscope (x30) and expressed as the percentage of the squares in the top mesh which contained blood vessels.

Preparation of dichloromethane extract (G1M1D1) and methanol soluble fraction of dichloromethane extract (G1M1D1M1) was shown in Scheme 1. To examine the angiogenic activity of G1M1D1 and G1M 1D1M1 fractions, chick embryo CAM was used. The sample-impregnated coverslip was used as a local delivery system. Application of an empty coverslip (control) to the CAM for three days resulted in no change in the normal blood vessel pattern (Fig. 1a). Treatment of G1M1D1 (250 μg/egg) and G1M1D1M1 (100 μg/egg) for the same period of time resulted in a marked angiogenic effect, spoke-wheel-like pattern (Fig. 1c,d). It was similar to the effect of PMA used as a positive control (Fig. 1b). As shown in Table I, the angiogenic activity of G1M1D1 and G1M1D1M1 fractions was dose-dependent and statistically significant ($p<0.001$).

Quantitation of the angiogenic activity by the modified CAM assay

The angiogenic activity of G1M1D1M1 fraction was quantitated by using the modified CAM assay. Type I collagen gel containing sucralfate alone was used as a control. The angiogenic response was observed from day 3 to 7 after implantation of collagen gel on CAM. There was no response until day 3 after implantation. After day 4, new capillary blood vessels traversed two layers of mesh separated by approximately 1mm. The angiogenic activity of the sample was quantitated as the percentage of the squares in the top mesh which contained blood vessels. As shown in Fig. 2, G1M1D1M1 fraction (100 μg/egg) el-

icited much higher angiogenic response than that of control while it was slightly lower angiogenic response than that of PMA (100 ng/egg).

DISCUSSION

Aloe vera gel has been known to have a good wound healing effect (Davis *et al.*, 1987). Its healing effect may be associated with angiogenesis which is critical for the succesful healing of wounds. We have extracted and fractionated G1M1D1 and G1M1D1M1 fractions containing low-molecular weight substances from *Aloe vera* gel (Scheme 1). The angiogenic activity of *Aloe vera* gel extracts was evaluated by the CAM assay. G1M1D1 and G1M1D1M1 fractions stimulated angiogenesis in a dose-dependent manner (Table I). The pattern of vasculature was similar to that of PMA known as an angiogenic activator. The chick embryo chorioallantoic membrane (CAM) is one of the most widely used systems for studying the process of angiogenesis (Ausprunk *et al.*, 1975; Lobb *et al.*, 1985), but it is qualitative at best and serves mainly to test a given substance for its ability to induce or to inhibit angiogenesis. The modified CAM assay has been recently developed as a method of quantitating angiogenic activities (Nguyen *et al.*, 1994). A major advantage of this method is the ability to compare the potency of different angiogenesis regulators. As shown in Fig. 2, application of G1M1D1M1 (100 μg/egg) resulted in much more intense angiogenesis than in control while slightly less intense angiogenesis than in PMA (100 ng/egg). Sucralfate used as a control providing a white background induced a low level of background angiogenesis.

As the results of the present study, dichloromethane extract (G1M1D1) and methanol soluble fraction of dichloromethane extract (G1M1D1M1) of *Aloe vera* gel stimulated angiogenesis judged by the CAM assay and the modified CAM assay. Thus, it could be suggested that the wound healig effect of *Aloe vera* gel would be related to its angiogenic activity. In the future, it will be necessary to investigate the angiogenic mechanism of *Aloe vera* gel extracts. In addition, further studies on the effect of *Aloe vera* gel extracts on the gene expression related to angiogenesis and the further purification of *Aloe vera* gel extracts will be required for the development of a potent wound healing drug.

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