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In vitro and in vivo wound healing activity of asiaticoside isolated from *Centella asiatica*

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Abstract

The activity of asiaticoside, isolated from *Centella asiatica*, has been studied in normal as well as delayed-type wound healing. In guinea pig punch wounds topical applications of 0.2% solution of asiaticoside produced 56% increase in hydroxyproline, 57% increase in tensile strength, increased collagen content and better epithelisation. In streptozotocin diabetic rats, where healing is delayed, topical application of 0.4% solution of asiaticoside over punch wounds increased hydroxyproline content, tensile strength, collagen content and epithelisation thereby facilitating the healing. Asiaticoside was active by the oral route also at 1 mg/kg dose in the guinea pig punch wound model. It promoted angiogenesis in the chick chorioallantoic membrane model at 40 μg/disk concentration. These results indicate that asiaticoside exhibits significant wound healing activity in normal as well as delayed healing models and is the main active constituent of *Centella asiatica*.

Keywords: *Centella asiatica*; Wound healing; Angiogenesis; Asiaticoside

1. Introduction

Cutaneous injury is characterised by fibroplasia, angiogenesis and re-epithelisation and involves the migration and proliferation of cells such as fibroblasts, endothelial cells and epithelial cells, deposition of connective tissue and contraction of the wound (Clark, 1991). These steps are

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orchestrated in a controlled manner by a variety of bioactive molecules like growth factors, cytokines, their receptors and matrix molecules. Such a controlled phenomenon can be disrupted in diseases like diabetes, immunocompromised persons, ischaemia etc. thus leading to the development of a chronic wound. Prolonged or incomplete wound healing is then a troublesome complication (Ingold, 1993).

Efforts are being made all over the world to discover agents that can promote healing and thereby reduce the cost of hospitalisation and save the patient from amputation or other severe complications. We have recently reported wound healing activity of the latex of *Euphorbia neriifolia* (Rasik et al., 1996). The latex is, however, difficult to obtain and to use. We have been, therefore, investigating other plants used for this purpose in the Indian traditional systems of medicine. *Centella asiatica* (Hindi name Brahma-manduki, Family Umbelliferae), a small herb, occupies an important place in the indigenous system of medicine as a tonic in skin diseases and leprosy (Chopra et al., 1956). In addition, it has been shown to promote fibroblast proliferation and collagen synthesis (Maquart et al., 1990) and to have antiulcer activity (Yoshinori et al., 1982). Rosen et al. (1967) have also reported wound-healing activity of the plant. In primary screening an ethanolic extract of the plant showed significant wound healing activity. Repeated chromatography of the saponin mixture led to the isolation of two pure saponins identified as asiaticoside (Fig. 1) and madecassoside. Asiaticoside showed a promising wound healing activity whereas madecassoside was found to be inactive (personal observation). Boiteau and Batsimamanga (1950) have reported the use of asiaticoside in healing experimental or refractory wounds, but a detailed study does not appear to have been undertaken. Detailed evaluation of the wound healing activity of asiaticoside has now been carried out using in vivo and in vitro models.

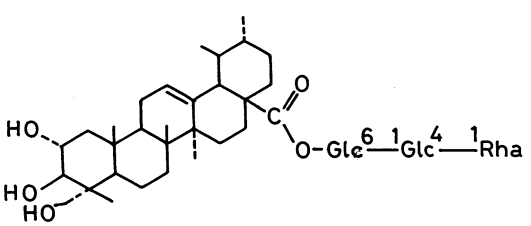

Fig. 1. Structure of asiaticoside.

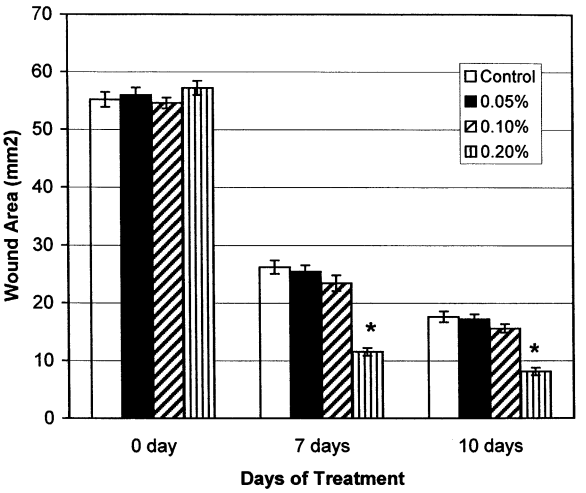

Fig. 2. Area (mm^2) of guinea pig wounds at 0, 7 and 10 days after topical treatment with different doses of asiaticoside (0.05, 0.1 and 0.2%). Values are mean ± S.E. for eight different observations. * $P < 0.01$ as compared to the same day control.

2. Materials and methods

2.1. Preparation of asiaticoside dosage forms

Different concentrations of solution in sterile saline were prepared for in vivo studies. Methylcellulose disks were loaded with 20–80 μg asiaticoside for in vitro tests. A gum acacia suspension in water was used for oral administration.

2.2. Models for wound healing activity

2.2.1. In vivo models

2.2.1.1. Normal animals. Guinea pigs (male, 300–325) were used in the study. Four cutaneous (full thickness, completely transdermal) circular wounds of 8 mm diameter were made on the pre-shaved, sterile (wiped with 70% alcohol) dorsal surface of the animal with the help of a biopsy

Table 1
Effect of topical asiaticoside treatment for 7 days on selected markers of wound healing in guinea pig punch wound models

Asiaticoside (% concentration)	DNA (mg/g tissue)	Protein (mg/g tissue)	Hydroxyproline (mg/g tissue)	Tensile strength (N/cm^2)
Vehicle control	6.54 ± 1.02	121.6 ± 6.92	70.4 ± 2.61	8.24 ± 0.232
0.05	3.47 ± 0.44	114.6 ± 2.52	83.6 ± 3.80**	8.86 ± 0.340
0.1	4.50 ± 0.68	114.7 ± 5.64	90.0 ± 3.02*	9.15 ± 0.643
0.2	9.79 ± 0.71**	116.0 ± 3.49	110.2 ± 3.44*	12.91 ± 0.936***

Values are mean $\pm$ S.E. ($n = 8$ animals).
* $P < 0.001$
** $P < 0.02$.
*** $P < 0.05$ as compared to vehicle control.

punch (Acuderm, Louderole, USA). All surgical procedures were carried out under thiopentone sodium (25 mg/kg, i.p.) anaesthesia. Animals were allowed to recover and were housed individually in metallic cages containing autoclaved paper cuttings. They received food and water ad libitum.

Asiaticoside solution (20 μl/wound) was applied topically in concentrations of 0.05%, 0.1%, 0.2% twice daily for 7 days. The control group received an equal amount of vehicle. In another series of experiments asiaticoside (0.5, 1.0 or 10 mg/kg) was given orally for 7 days. Controls received the vehicle alone.

2.2.1.2. Diabetic animals. Sprague Dawley male rats (150–180 g) were made diabetic by being given a single injection of streptozotocin (STZ) prepared in citrate buffer (0.1 M, pH 4.5) (50 mg/kg, i.p.) after overnight fasting. Blood was drawn from the orbital plexus 24 h after the injection and the glucose level was estimated using Glucometer (Ames, Bayer Diagnostic, India). Wounds were made on the rats showing elevated blood glucose (more than 250 mg/dl) using the same method as described for the guinea pig. In the diabetic animals, 0.2 and 0.4% concentrations of asiaticoside were applied topically with the treatment schedule as in the case of guinea pigs. Blood glucose levels were estimated at the time of creation of the wounds as well as at excision of wounds to check whether the plant has got any antidiabetic activity per se.

2.2.2. In vitro model

2.2.2.1. Chick chorioallantoic membrane (CAM) model. This model was used to assess the angiogenic activity of asiaticoside (Lobb et al., 1985). Nine day-old fertilised chick eggs were selected and a small window of 1.0 cm^2 made in the shell. A small hole was drilled at the air space and air was sucked out using a rubber bulb, as a result of which the membrane fell. The window was opened and a sterile disk of methylcellulose loaded with different amounts of asiaticoside was placed in at the junction of two big vessels. The window was resealed by tape and the eggs were incubated at 37°C in a well-humidified chamber for 72 h. The eggs were then opened. New vessel formation was observed and compared with that in eggs containing disks without asiaticoside.

2.3. Wound tissue excision

Wound tissue excision on the 7th day after making the wound was also done using biopsy punch. This procedure excises only the newly formed (regenerated) tissues in the wounded area, thus avoiding contamination from surrounding tissue. The animals were anaesthetised before excision as described in the preceding section. Detailed procedure for the creation of the wound and its excision of has been described in another publication (Shukla et al., 1997).

Table 2
Quantitative histopathologic findings of 7 day wound of normal guinea pigs after topical application of asiaticoside

Treatment	Congestion	Oedema	Infiltration of		Necrosis	Proliferation of fibroblasts	Angiogenesis	Epithelisation
			PMNLs	Monocytes				
Vehicle control	+++	+++	++	+++	+++	++	++	−
Asiaticoside 0.2%	+	+	+	++	+	+++	+++	+

+, Slight; ++, moderate; +++, marked; ++++, extensive; −, absent.

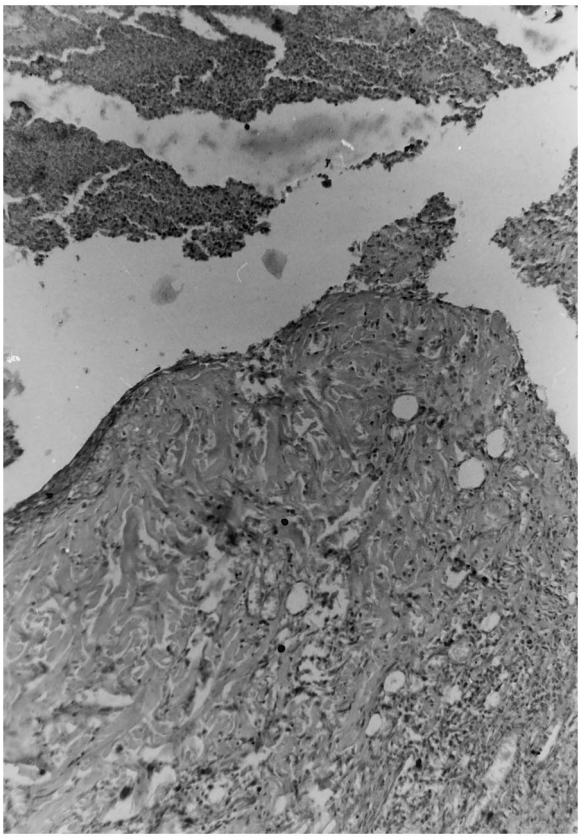

Fig. 3. Skin plug of normal guinea pig (vehicle alone) at 7-day post wounding showing presence of necrotic debris along with inflammatory cells above the denuded epidermis while marked oedema and congestion in dermis. Hematoxylin and eosin, × 108.

2.4. Assessment of healing

In each animal study eight animals/group were taken. From each animal, two wounds were pooled to make one sample for hydroxyproline and one each was used for tensile strength and histology.

2.4.1. Area of wound

The surface area of healing (7 and 10th day) wound was measured by tracing the boundary of still open wound on semi-transparent paper and calculation of area was done by using a graph paper.

2.4.2. Tensile strength

On the 7th day after creating the wound the animals were anaesthetised. Healing tissue along with normal skin at two ends was excised for tensile strength measurement using Tensile Testing Machine TKG-20 (from Fine Testing Machines, Miraz, India). Strips of 8 mm width and 20 mm length were cut out from the excised tissue in treated and control animals and were loaded between the upper and lower holder of the machine in such a way that the effective load bearing size was 8 × 8 mm with the wound remaining in the centre. The total breaking load is measured in Newtons and the tensile strength was calculated by the following equation:

Tensile strength

= Total breaking load/Cross-sectional area

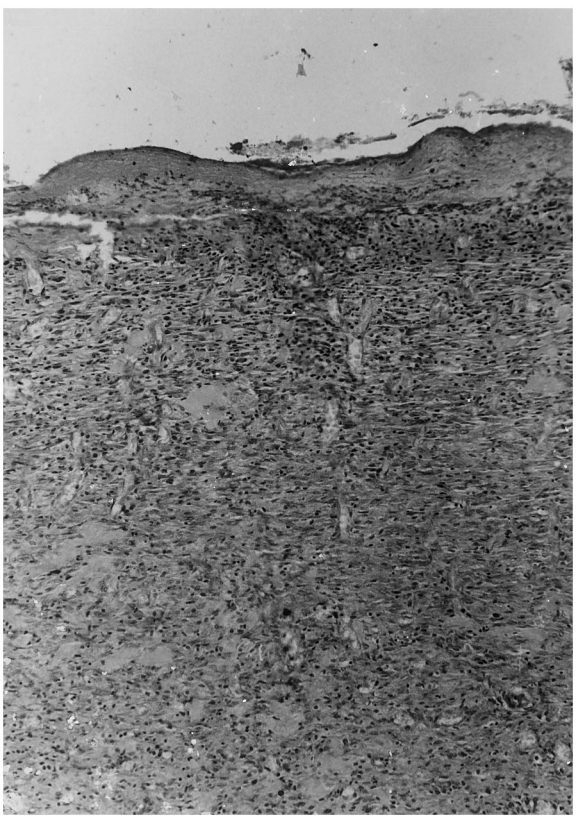

Fig. 4. Skin plug of normal guinea pig at 7-day post wounding which received daily topical application of 0.2% asiaticoside from *Centella asiatica*. Evidence of epithelisation with dermal proliferation of fibroblast and mononuclears. Hematoxylin and eosin, × 108.

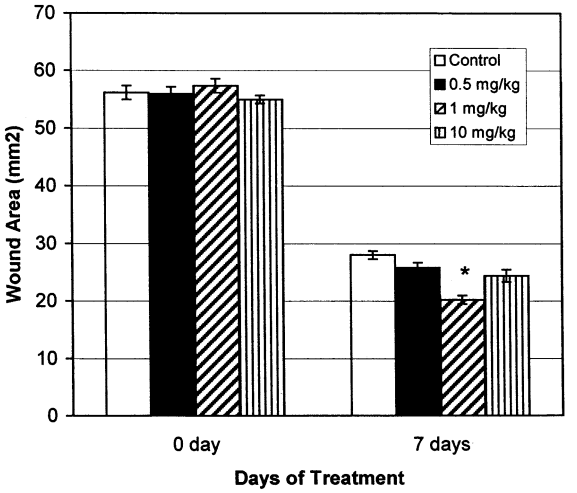

Fig. 5. Area (mm^2) of guinea pig wounds at 0 and 7 days after oral treatment with different doses of asiaticoside (0.5, 1.0 and 10 mg/kg). Values are mean ± S.E. for eight different observations. * $P < 0.05$ as compared to the same day control.

2.4.3. Histopathological studies

Wound tissue specimens from treated and untreated rats were collected in 10% buffered formalin and after the usual processing 6 μm-thick sections were cut and stained with haematoxylin and eosin (McManus and Mowry, 1965). Sections were qualitatively assessed under the light microscope and graded in respect of congestion, oedema, infiltration of polymorphonuclear leukocytes and monocytes, necrosis, fibroblast proliferation, collagen formation, angiogenesis and epithelisation.

Table 3
Effect of oral asiaticoside treatment for 7 days on hydroxyproline content and tensile strength of the wound in guinea pig punch wound model

Asiaticoside (mg/kg)	Hydroxyproline (mg/g tissue)	Tensile strength (N/cm^2)
Vehicle control	32 ± 4.32	6.29 ± 0.443
0.5	35.5 ± 3.27	6.82 ± 0.512
1	56.9 ± 6.02**	14.00 ± 0.173*
10	35.7 ± 3.15	5.99 ± 0.432

Values are mean ± S.E. ($n = 8$ animals).
* $P < 0.001$ and ** $P < 0.01$ as compared to vehicle control.

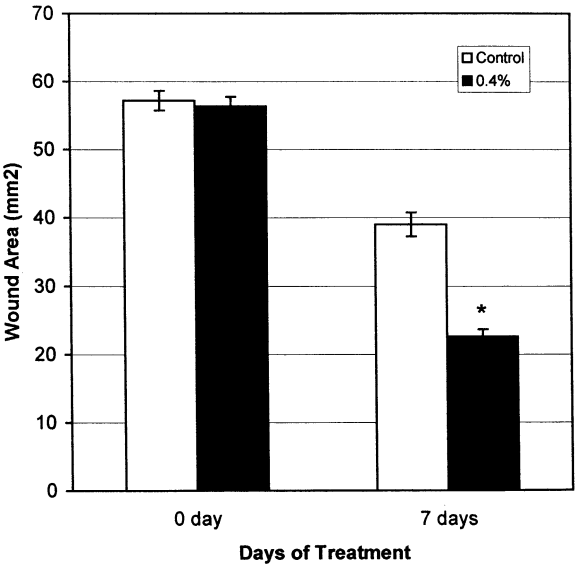

Fig. 6. Area (mm^2) of diabetic rat wounds at 0 and 7 days after topical treatment with asiaticoside (0.4%). Values are mean ± S.E. for eight different observations. * $P < 0.05$ as compared to diabetic vehicle control of the same day.

2.4.4. Collagen estimation

Wound tissues were analysed for hydroxyproline content, which is a basic constituent of collagen. Tissues were dried in a hot air oven at 60–70°C to constant weight and were hydrolysed in 6 N HCl at 130°C for 4 h in sealed tubes. The hydrolysate was neutralised to pH 7.0 and was subjected to chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4 M perchloric acid and colour was developed with the help of Ehrlich reagent at 60°C (Woessner, 1961) and measured at 557 nm using a Pye Unicam spectrophotometer.

Table 4
Effect of topical asiaticoside treatment for 7 days on hydroxyproline content and tensile strength of the wound of diabetic rats

Asiaticoside (% concentration)	Hydroxyproline (mg/g tissue)	Tensile strength (N/cm^2)
Control	53.8 ± 4.9	6.82 ± 0.050
0.2%	62.7 ± 4.50	6.97 ± 0.071
0.4%	79.8 ± 6.2**	11.35 ± 0.103*

Values are mean ± S.E. ($n = 8$ animals).
* $P < 0.001$ and ** $P < 0.01$ as compared to vehicle control.

Table 5
Quantitative histopathologic findings of 7 day wound of diabetic rats after topical application of asiaticoside

Treatment	Congestion	Oedema	Infiltration of		Necrosis	Proliferation of fibroblasts	Angiogenesis	Epithelisation
			PMNLs	Monocytes				
Vehicle control	+++	++++	+++	+++	++	++	−	
Asiaticoside 0.2%	+	+	+	+	+	+++	++	++
Asiaticoside 0.4%	+	+	−	+	+	+++	++	++

+, Slight; ++, moderate; +++, marked; ++++, extensive; −, absent.

2.4.5. DNA and protein estimation

In some animals, 4th day wound tissues were analysed for DNA and protein content using the methods of Burton (1956) and Lowry et al. (1951), respectively.

2.5. Statistical evaluation

Data were evaluated statistically using Student's *t*-test. *P* values less than 0.05 were considered to be significant.

3. Results

3.1. Normal healing

In control guinea pigs and rats excision type of wounds take 14–16 days to heal completely and the healing pattern is also similar as far the above mentioned parameters are concerned. In diabetic rats similar wounds take 30 days or more to heal if no treatment is given.

3.2. Effect of asiaticoside on wound healing in guinea pigs

3.2.1. Topical application

3.2.1.1. Area. Areas of wound in 0.2% asiaticoside treated wounds decreased by 56 and 54% on 7th and 10th day post wounding, respectively, as compared to vehicle treated controls of the same day. Lower concentrations (0.05 and 0.1%) were found to have no significant effect on wound area (Fig. 2).

3.2.1.2. Tensile strength. The wounds treated with 0.2% solutions of asiaticoside showed significant increase in tensile strength (57%, $P < 0.05$) as compared to the vehicle control (Table 1). Lower concentrations (0.05 and 0.1%) were found to be ineffective.

3.2.1.3. Collagen content. A dose-dependent increase in hydroxyproline levels up to a concentration of 0.2% was found (Table 1).

3.2.1.4. DNA and protein content. Asiaticoside produced no change in protein content in any of the concentrations tested. The DNA content, however, was found to increase significantly with 0.2% concentration of the compound (Table 1).

3.2.1.5. Histopathological observations. Treatment of guinea pig wounds with 0.2% asiaticoside solution led to reduced congestion, oedema, polymorphonuclear leukocytes (PMNLs) and mononuclear leukocyte infiltration and necrosis. However, enhanced fibroblast proliferation, angiogenesis and epithelisation were observed (Table 2 and Figs. 3 and 4).

3.2.2. Oral administration

3.2.2.1. Area. The effect of oral administration was followed up to the 7th day post wounding only. The dose of 1 mg/kg produced significant reduction in wound area (28%) as compared to vehicle treated controls. However, 0.5 and 10 mg/kg doses were found to have no effect on wound area (Fig. 5).

3.2.2.2. Tensile strength. An oral dose of 1 mg/kg produced a significant increase in tensile strength as compared to control. Other doses were found to have no effect on tensile strength (Table 3).

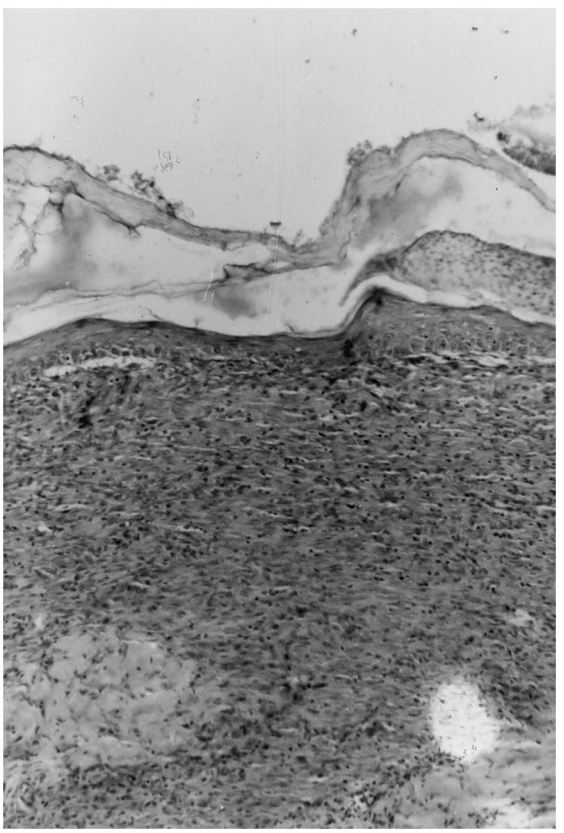

Fig. 7. Skin plug of diabetic rat at 7-day post wounding which received daily topical application of 0.4% asiaticoside from *Centella asiatica*. Prominent epithelisation with increased fibroblastic reaction in the dermis along with mononuclears. Hematoxylin and eosin, × 108.

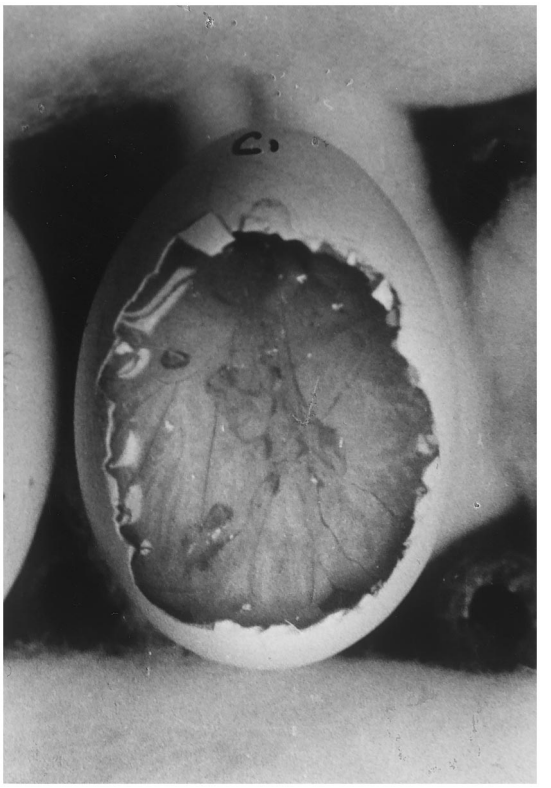

Fig. 8. Chorioallantoic membranes of chick egg (12-day-old) showing vessel formation without asiaticoside treatment. Eggs were loaded only with sterile methylcellulose disks.

3.2.2.3. Collagen content. In collagen content also the 1 mg/kg dose produced a significant (76%, $P < 0.01$) increase, whereas other doses were ineffective (Table 3).

3.3. Effect of asiaticoside on wounds in diabetic rats

3.3.1. Area

Topical application of 0.4% asiaticoside solution to wounds resulted in healing (visual appearance) comparable to that of non-diabetic rat wounds. In diabetic animals 0.4% asiaticoside produced 42% reduction in wound area as compared to vehicle treated diabetic controls (Fig. 6).

3.3.2. Tensile strength

In diabetic animals only the 0.4% solution of asiaticoside produced a significant increase (66%,

$P < 0.001$) in tensile strength over untreated diabetic controls (Table 4).

3.3.3. Collagen content

The effect was similar to the effect on tensile strength. Hydroxyproline contents were found to be increased significantly by 0.4% asiaticoside application (Table 4).

3.3.4. Histopathological observations

In diabetic rat wounds application of 0.4% asiaticoside solution reduced tissue necrosis, infiltration of PMNLs and mononuclear leukocytes. Oedema and congestion were also reduced. Fibroblast proliferation was better than in the controls and epithelisation was enhanced severalfold in the animals treated with both concentrations of asiaticoside (Table 5 and Fig. 7). Since the normal skin of rat and guinea pig was quite similar histologically, only a photograph of normal guinea pig skin has been provided for comparison.

Topical asiaticoside treatment was found to have no effect on the rat blood glucose level.

3.4. Effect of asiaticoside on angiogenesis

Methylcellulose disks containing either 20, 40 or 80 μg compound were applied to different chorioallantoic membranes and after 72 h of incubation, eggs were observed for new vessel formation. All the concentrations promoted angiogenesis but the maximum effect was observed with disk containing 40-μg asiaticoside (Figs. 8 and 9).

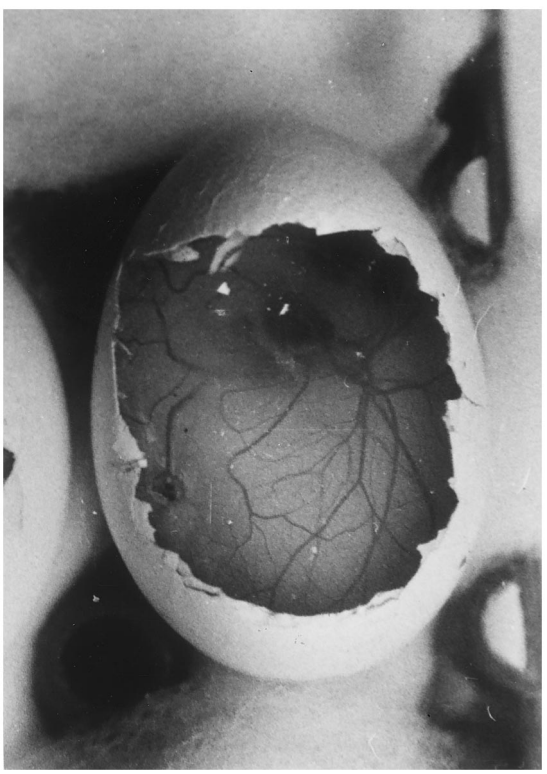

Fig. 9. Chorioallantoic membrane of chick eggs (12-day-old) showing increased number of vessels after treatment with 40-μg asiaticoside impregnated in methylcellulose disks.

4. Discussion and conclusion

Healing is a physiological process and does not normally require much help but still wounds cause discomfort and are prone to infection and other complications. Therefore, use of agents expediting healing is indicated. Further, some diseases like diabetes, immunocompromised conditions, ischaemia and conditions like malnourishment, ageing, local infection, local tissue damage due to burn or gun shot wounds lead to delay in healing. Such conditions specially require the use of agents, which can facilitate healing. Asiaticoside isolated from the plant *Centella asiatica*, has been, therefore, studied for its wound healing activity in normal as well as in diabetic animals. The selection of the plant was based on its traditional medicinal use and reported pharmacological activities such as promotion of fibroblast proliferation (Veechai et al., 1984) and stimulation of collagen synthesis (Maquart et al., 1990). In the present study, topical application of asiaticoside in normal as well as in diabetic animals or its oral administration in normal animals significantly enhanced the rate of wound healing as assessed by increase in collagen synthesis and tensile strength of the wound tissues. Maquart et al. (1990) have also reported elevated collagen synthesis in fibroblasts by asiaticoside in vitro. Histological findings also showed enhanced prolif-

eration of fibroblasts thereby supporting the bio chemical results. Epithelisation was also remarkably better in treated normal and diabetic animals as compared to vehicle treated controls. The hydroxyproline levels of wound tissues obtained from controls treated with normal saline topically were higher as compared to that of untreated controls in the oral treatment group. This effect could possibly be attributed to topical application of saline. The observation of an unaltered total protein content in spite of enhanced collagen protein in case of treated wound tissues may be explained on the basis of specificity of these two methods for different amino acids.

Angiogenesis plays an important role in wound healing and newly formed blood vessels comprise 60% of the repair tissue. Neovascularization helps hypoxic wounds to attain the normoxic conditions (Ehrlich et al., 1972). Asiaticoside promoted angiogenesis in both in vitro and in vivo models as indicated by histological studies and new vessel formation in CAM model. The increased vessel growth can facilitate both the extent and direction of fibroplasia. Improved angiogenesis, therefore, would be contributing significantly to wound healing activity of asiaticoside.

It is now well accepted that several local growth factors help in the wound healing process. It is possible that asiaticoside may have a growth factor like activity or has the ability to stimulate the expression of growth factors like the basic fibroblast growth factor (bFGF). Basic FGF has the broadest range of target cells, including all those involved in wound healing viz. endothelial cells, fibroblasts, myoblasts etc. (Schweigerer, 1988). Since pro-inflammatory cytokines have been implicated to stimulate the synthesis of platelet activating factors by the recruited monocytes, which in turn induce several angiogenic factors and chemokines (Lupia et al. 1996). More in depth studies would therefore be needed to delineate the likely beneficial properties of this agent in wound healing at the pro-inflammatory cytokine level. Studies to assess the effect of asiaticoside on selected growth factors are in progress and results will be published later.

In conclusion, this study confirms the promising wound healing activity of asiaticoside in normal as well as in diabetic animals and warrants more detailed experimental and clinical studies. It also provides a rationale for the use of Centella asiatica preparations in the Indian traditional system of medicine to promote wound healing.

Acknowledgements

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