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Effects of *Alchemilla vulgaris* and Glycerine on Epithelial and Myofibroblast Cell Growth and Cutaneous Lesion Healing in Rats

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Wound-healing properties have been suggested for *Alchemilla vulgaris*. Since epithelial and myofibroblast cell growth is required for wound healing, the effects of *A. vulgaris* on cell growth were investigated in Chang liver and Madin Darby Bovine Kidney (MDBK) epithelial cell lines and rat aortic myofibroblast cultures. Putative healing properties were investigated on dorsal circular 8 mm excisional skin lesions in adult male rats. Cell numbers increased with 0.1–1% *A. vulgaris*, attaining 21.3 ± 2.1%, 15.5 ± 2.25% and 10.6 ± 0.6% in MDBK, myofibroblast and Chang liver cells, respectively ($p < 0.005$). No morphological changes or cytotoxicity were noted. In rats *A. vulgaris* (3%)-treated lesions were significantly decreased in diameter by 10.0 ± 0.7% ($p < 0.005$) after 2 days of treatment. On day 3 of treatment, the lesion diameter was significantly reduced by 15.9 ± 1.1% in glycerine vehicle-treated rats compared with distilled water ($p < 0.005$), whereas that in *A. vulgaris*-treated rats was reduced further by 23.2 ± 1.4% ($p < 0.005$). Glycerine alone significantly reduced the lesion diameter between days 3 and 5 but complete healing occurred a day earlier in *A. vulgaris*-treated rats. The results demonstrate wound-healing properties of *A. vulgaris* associated with promitotic activity in epithelial cells and myofibroblasts. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: Alchemilla vulgaris; glycerine; proliferation; wound healing; skin lesion.

INTRODUCTION

Alchemilla vulgaris (Lady's Mantle) is commonly known for its astringent and antiinflammatory properties and is traditionally used to treat ulcers, eczema and skin rashes. Similarly to other members of the Rosaceae family, *A. vulgaris* contains polyphenols to which the main pharmacological activities of the plant have been attributed (Fraisse *et al.*, 1999). Inhibition of intercellular matrix degrading proteases (Jonadet *et al.*, 1986; Lamaison *et al.*, 1990), antioxidant (Filipek, 1992) and angioprotective activities (Jonadet *et al.*, 1986) have been reported for *A. vulgaris*. The plant contains tannins composed of some gallic and mostly ellagic acid, flavonoids, the most abundant being quercetin, along with others, including luteolin and proanthocyanidins (Fraisse *et al.*, 1999; Lamaison *et al.*, 1990; Heikel, 1995; Lamaison *et al.*, 1991). Putative wound-healing properties have been suggested for the main active ingredients in *A. vulgaris* (Ashoori *et al.*, 1994; Gomathi *et al.*, 2003; Jimenez *et al.*, 2006; Skaper *et al.*, 1997).

Cell growth is well known to play a key role in the closure and healing of wounds (Kirsner and Eaglstein, 1993; Moulin *et al.*, 2000; Singer and Clark, 1999). The rapidity of wound healing on the skin or on the mucous membrane depends upon constant competition between the growth of bacterial cells which retard healing and the growth of fibroblast and epithelial cells which close the wound (Singer and Clark, 1999; Baum and Arpey, 2005; Hunt *et al.*, 2000). Proliferation of myofibroblasts is an important feature of wound healing, since the newly formed cells occupy space in the wound, leading to *de novo* extracellular matrix deposition and neovascularization (Baum and Arpey, 2005; Amadeu *et al.*, 2003; Phan *et al.*, 2000) which are typical features of granulation tissue formation (Singer and Clark, 1999). Epithelial cell proliferation seals the wound and restores skin barrier function (Singer and Clark, 1999; Baum and Arpey, 2005), stopping microbial proliferation and preventing the degradation of collagen and elastin fibres which constitute the intercellular matrix (Singer and Clark, 1999). However, it is not known whether *A. vulgaris* can affect wound healing or the associated myofibroblast and epithelial cell growth.

The aim of the present study was therefore twofold: (i) to study the putative effects of *A. vulgaris* on epithelial and myofibroblast cell growth *in vitro*, and (ii) to investigate whether *A. vulgaris* could affect wound healing in a conventional *in vivo* rat model of cutaneous excisional lesions (Dorsett-Martin, 2004; Gupta *et al.*, 2005).

MATERIALS AND METHODS

In vitro studies

Chang liver and MDBK epithelial cells. Chang liver human and MDBK (Madin-Darby Bovine Kidney) epithelial cell lines were purchased from American Type Culture Collection (ATCC, USA). Cells were maintained as monolayers in minimum essential medium

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(MEM) supplemented with 10% fetal calf serum (FCS; growth medium). Complete and confluent cell monolayers were dispersed with trypsin EDTA (0.02%, Gibco, USA), cells were resuspended in growth medium (10 mL), pelleted by centrifugation (22 × **g** for 5 min at 4 °C) and seeded (0.1 million cells/mL in growth medium) in 96-well tissue culture microplates. During the exponential growth phase in the first 24 h, fresh growth medium (100 μL) with 10% serum was added to each well. These cells were then used to conduct *in vitro* experiments.

Primary smooth muscle cell myofibroblast cultures. Cells were prepared according to the method of Shrivastava *et al.* (1993) using rat adult aortic smooth muscle cells obtained from male Wistar rats (280–300 g, Iffa-Credo, France) isolated by enzymatic digestion. Cells were seeded in 25 cm^2 tissue culture flasks (Corning Glassworks, USA) in DMEM containing 10% FCS. After 48 h incubation at 37 °C in a 5% CO_2 in air atmosphere, the growth medium was renewed. Following 6–8 days incubation, complete and confluent monolayers were dispersed by washing with trypsin (0.25%, Gibco, USA) and were subcultured in 75 cm^2 plastic tissue culture flasks and finally in 96-well plates (Corning Glassworks, USA). Confluent monolayers between 3 and 5 passages become myofibroblastic (Shrivastava *et al.*, 1993).

Experimental protocol. The test product was diluted in culture medium to obtain the final required concentration. The growth medium was removed from the corresponding well and was replaced by medium containing the appropriate concentration of test product. Control cultures were treated identically with medium containing no test product.

After 72 h in culture, the cells were dispersed with trypsin-EDTA and live cell counts were performed with standard MTT coloration. Experiments were conducted in triplicate and repeated at least twice. The results are expressed as % change in cell number compared with the corresponding untreated control cultures.

In vivo study

An *in vivo* study was carried out after obtaining the agreement of the institute's ethics committee. Three groups of 10 adult male Sprague-Dawley rats (160–220 g, Iffa-Credo, France) were employed following an acclimatization period of 7 days with free access to food and water.

Experimental protocol. The protocol employed was similar to that described by Gupta *et al.* (2005). The back was shaved and three circular cutaneous excisional lesions were created on each side of the back by gently rotating a biopsy punch (a pencil-like metal tube with a cutting edge) under light ether anesthesia. Initially, each lesion measured approximately 8 mm in diameter. The lesion depth was carefully controlled so as to create only superficial injury with an identical depth without penetrating the hypodermic skin layer. Two hours after producing the lesions, the injury site was cleaned with a sterile cotton swab and test products were applied as follows: Group 1: *A. vulgaris* (3%) in glycerine; Group 2: Glycerine (100%) vehicle; Group 3: Distilled water (sterile); 0.2 mL of test product or vehicle was spread homogeneously from a 1 mL sterile syringe on each of the three ipsilateral lesions per rat. The three remaining contralateral lesions per animal remained untreated, serving as internal controls. The lesioned area of the back was then covered with a fresh 8-layered sterile bandage which was secured by adhesive tape. This treatment procedure was repeated once a day for 7 consecutive days.

Parameters measured. Lesion diameter (mm) was measured with a transparent ruler, aided by a magnifying glass and values were meaned for the three ipsilateral lesions per rat.

***A. vulgaris* extract.** A hydroglycerinated fluid extract of *Alchemilla vulgaris* was manufactured by Laboratoires Biosphere-99, Les Martres de Veyre, France, under GMP requirements using the aerial parts of the plant. One batch of *A. vulgaris* extract was employed which contained tannins (2.67%), of which 3.8% was ellagic acid; the flavonoids quercetin (0.67%) and luteolin (0.0069%), determined by HPLC analysis according to Fraisse *et al.* (1999) and the European Pharmacopoeia V monograph. The *A. vulgaris* extract was a limpid solution with a characteristic odour and a dark copper-type colour. The batch employed (no. B 3765) was released by the manufacturer following standard pharmaceutical quality control procedures, including verification of absence of bacterial contamination.

Data analysis

For *in vitro* studies, mean percent changes in cell numbers compared with controls are presented as mean ± SEM. For the *in vivo* study, mean scores were calculated in each treatment group by adding the mean lesion diameters per day of the ten rats. In each case, statistical analysis was performed with one-way ANOVA with the paired Student's *t*-test *post-hoc*.

RESULTS

Effects of *A. vulgaris* on *in vitro* cell proliferation

The effects of *A. vulgaris* on the proliferation of MDBK and Chang liver epithelial cells in culture are shown in Fig. 1. Concentration-dependent, statistically significant increases in cell numbers were observed, attaining 21.3 ± 2.1%, 15.5 ± 2.2% and 10.6 ± 0.6% increases in MDBK, aortic myofibroblasts and Chang liver cells with 1% *A. vulgaris*, respectively ($p < 0.005$ compared with untreated controls in each case; Fig. 1).

No significant changes in cell morphology were noted in any of the three cell types, or in any of the treatment groups, indicating the absence of cytotoxicity. Concentrations below 0.1% *A. vulgaris* or glycerine alone (up to 2% maximum employable concentration) had no effect on proliferation in MDBK, myofibroblasts or Chang liver cells (data not shown).

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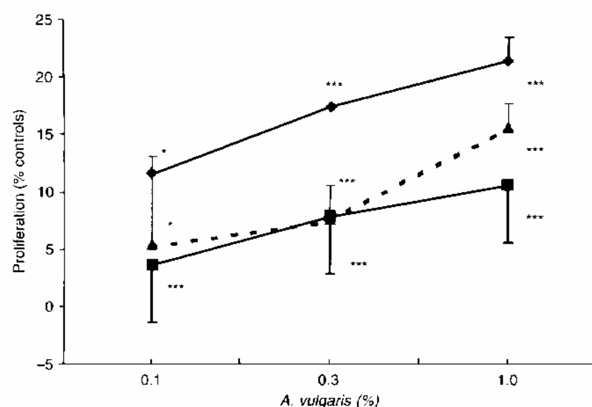

Figure 1. Effects of *A. vulgaris* (0.1%, 0.3% and 1.0%) on proliferation of rat aortic myofibroblasts (filled triangles on dotted line), Chang liver epithelial cells (filled squares), and MDBK epithelial cells (filled diamonds) following 72 h in culture. Data are mean ± SEM of percent changes compared with controls (untreated cells). * $p < 0.05$; *** $p < 0.005$.

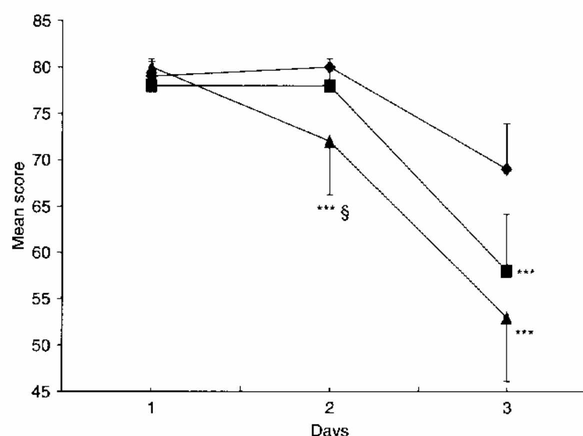

Figure 2. Evolution of superficial dorsal cutaneous lesion diameter in adult male rats during the first 3 days of healing. Lesion diameter is presented as mean score for all animals ($n = 10$) per treatment group per day. Distilled water-treated rats (filled diamonds), glycerine vehicle (filled squares) and *A. vulgaris* (3%; filled triangles). Treatment was once a day topical application of a few drops. See Materials and Methods for further details. Data are presented as mean ± SEM. *** $p < 0.005$ compared with water; § $p < 0.005$ compared with glycerine vehicle.

Effects of *A. vulgaris* on cutaneous wound healing in rats

The putative healing properties of *A. vulgaris* (3%) on superficial excisional dorsal skin lesions in rats compared with those of its vehicle (glycerine) and distilled water controls in the first three days of healing are shown in Fig. 2. The mean lesion diameter in each group over the 7 day treatment period is shown in Table 1.

No significant differences in lesion diameter were noted between treatment groups on day 1 (Fig. 2, Table 1), but healing was faster in the *A. vulgaris*-treated group already from day 2. In distilled water and glycerine-treated lesions, lesion size was not statistically significantly different after 2 days of treatment (Fig. 2, Table 1), whereas *A. vulgaris*-treated lesions were significantly decreased in diameter from 8.0 ± 0.1 to 7.2 ± 0.6 mm (10.0 ± 0.7% reduction; $p < 0.005$ compared with glycerine or water; Fig. 2, Table 1). On day 3 of treatment, lesion diameter was statistically significantly reduced by 15.9 ± 1.1% in glycerine-treated rats compared with distilled water ($p < 0.005$; Fig. 2, Table 1), whereas the lesion size in *A. vulgaris*-treated rats was reduced even further by 23.2 ± 1.4% ($p < 0.005$; Fig. 2, Table 1), but was not statistically significantly different to glycerine-treated lesions (Fig. 2, Table 1). Glycerine alone significantly reduced lesion diameter between days 3 and 5 (Table 1).

Accelerated wound healing in *A. vulgaris*-treated animals is illustrated in Table 1, in which complete healing, as assessed by mean lesion diameter less than 1 mm, was obtained at day 6 compared with day 7 in the other groups (Table 1). The lesion diameter in the *A. vulgaris* treatment group was statistically significantly smaller than that in distilled water from day 2 up to the end of the study period (Table 1).

DISCUSSION

Putative wound-healing properties have been suggested for the main active ingredients of *A. vulgaris* (Fraisse *et al.*, 1999; Ashoori *et al.*, 1994; Gomathi *et al.*, 2003; Jimenez *et al.*, 2006; Skaper *et al.*, 1997). A major feature of the healing process is the growth of myofibroblasts into the wound space and epithelial cells to seal the wound (Singer and Clark, 1999; Baum and Arpey, 2005; Amadeu *et al.*, 2003; Phan *et al.*, 2000), but it is not known whether *A. vulgaris* can affect cell growth. Therefore the effects of a standard *A. vulgaris* extract were studied on epithelial and myofibroblast

Table 1. Lesion diameter (mm) evolution. Effects of distilled water (control), glycerine vehicle and *A. vulgaris* (3%) in glycerine. Lesions were created on day 1. Treatments were once a day topical applications. Lesion diameter was determined as explained in the methods section. Each group consisted of 10 animals. Data are presented as mean ± SEM. Note accelerated healing on days 2 to 3, and complete lesion healing on day 6 in *A. vulgaris*-treated group compared with other groups. [a] $p < 0.05$ compared with distilled water; [b] $p < 0.05$ compared with glycerine

	Mean lesion diameter (mm)						
Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Distilled water	7.9 ± 0.2	8.0 ± 0.1	6.9 ± 0.5	5.1 ± 0.5	3.7 ± 0.7	1.4 ± 0.7	0.2 ± 0.3
Glycerine	7.8 ± 0.3	7.8 ± 0.3	5.8 ± 0.6[a]	4.0 ± 0.7[a]	3.2 ± 1.0[a]	1.6 ± 0.7	0.2 ± 0.3
A. vulgaris 3%	8.0 ± 0.1	7.2 ± 0.6[a,b]	5.3 ± 0.7[a]	3.5 ± 0.6[a]	2.8 ± 0.6[a]	0.6 ± 0.7[a,b]	0.1 ± 0.1

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cell growth *in vitro*, and on wound healing in an *in vivo* rat model of cutaneous lesions (Dorsett-Martin, 2004; Gupta *et al.*, 2005).

A. vulgaris (0.1%–1%) concentration dependently and markedly enhanced the proliferation of the Chang human liver and MDBK epithelial cell lines and rat aortic myofibroblast primary cultures. Increases of 10%–20% cell proliferation were observed with *A. vulgaris* (1%) depending upon the cell type. To our knowledge, such effects have not been described previously for the plant. Importantly, no cytotoxic effects or induced morphological changes were noted in cells exposed to the plant extract, indicating that enhancement of proliferation occurred at relatively low, non-cytotoxic concentrations. The precise mode of action of *A. vulgaris* in enhancing cell proliferation *in vitro* and probably *in vivo* is not yet clearly understood although the protease-neutralizing activity of the plant extract (Jonadet *et al.*, 1986) could play a major role by reducing extracellular matrix destruction. The presence of *A. vulgaris* in the cell culture medium could favour the deposition of newly formed extracellular matrix during the preconfluent growth phase by preventing its breakdown by proteases (Jonadet *et al.*, 1986; Lamaison *et al.*, 1991). In addition, free radical scavenging, antioxidant activity of *A. vulgaris* (Filipek, 1992) may also be involved and cannot be excluded.

A. vulgaris (3%) in glycerine exhibited wound healing effects in a conventional rat model of cutaneous excisional lesions. *A. vulgaris*-treated lesions were significantly reduced in size (10%) by the second day of treatment whereas distilled water or glycerine vehicle had no effect. These results suggest cutaneous wound-healing properties of *A. vulgaris*, which have not been reported previously to our knowledge. The present data suggest that the cellular proliferation-enhancing properties of *A. vulgaris* observed *in vitro* are likely to play a prominent role in accelerating the skin regeneration process *in vivo*. A reduction in wound area of 10%–15% per week represents normal clinical healing (Attinger *et al.*, 2006), but since the wound is largest during the first few days, most of the discomfort and pain is manifested at this time. Novel therapies may therefore be directed to speed up the healing process, particularly during the first 2–3 days of wound formation (Kirsner and Eaglstein, 1993; Hunt *et al.*, 2000).

The rapidity of wound healing on the skin or on the mucous membrane depends upon competition between the proliferation of bacterial cells which retard healing and the growth of fibroblast and epithelial cells which close the wound (Singer and Clark, 1999; Baum and Arpey, 2005; Hunt *et al.*, 2000). Proliferation of myofibroblasts is an important feature of wound healing, since the newly formed cells occupy space in the wound, leading to *de novo* deposition of extracellular matrix and neovascularization, which are associated with granulation tissue formation (Singer and Clark, 1999). Granulation tissue myofibroblasts develop several ultrastructural and biochemical features of smooth muscle cells, including the presence of microfilament bundles and the expression of alpha-actin (Desmoulière, 1995). It has been suggested that the main role of myofibroblasts in wound healing is the synthesis of extracellular matrix (Moulin *et al.*, 2000). Since smooth muscle cells in culture have similar properties to myofibroblasts, they can be considered to be a useful model of wound healing (Desmoulière, 1995; Moulin *et al.*, 1998; Gabbiani, 1994). Wound healing also involves epithelial cell proliferation to seal the wound and restore skin barrier function (Singer and Clark, 1999; Baum and Arpey, 2005), stopping microbial proliferation and preventing the degradation of collagen and elastin fibres which constitute the intercellular matrix (Singer and Clark, 1999). The speed of healing is therefore proportional to the speed of cell growth inside the lesion and collagen production by myofibroblasts (Singer and Clark, 1999; Amadeu *et al.*, 2003). The presence of extracellular matrix degrading enzymes such as trypsin, chymase and elastase (Huttunen and Harvima, 2005) significantly retard the healing process, since the *de novo* deposition of extracellular matrix may be impaired (Huttunen and Harvima, 2005). As mentioned earlier as a possible mechanism for enhancing *in vitro* cell growth, inhibition of extracellular matrix proteases by *A. vulgaris* (Jonadet *et al.*, 1986) could also play a significant role in accelerating cutaneous wound healing.

Potential wound-healing properties have been suggested for the main active principles contained in *A. vulgaris*. Thus, prevention of dermal enzyme degradation, cutaneous lipid peroxidation and enhanced wound healing properties have been described for ellagic acid (Ashoori *et al.*, 1994; Jimenez *et al.*, 2006), the main tannin found in *A. vulgaris* (Fraisse *et al.*, 1999; Heikel, 1995), as confirmed in our extract, and polyphenolic compounds containing gallic acid and catechin tannins (Murthy *et al.*, 2004). Antiinflammatory (Morikawa *et al.*, 2003; Rotelli *et al.*, 2003) and antioxidant activity favouring cutaneous wound healing (Gomathi *et al.*, 2003; Skaper *et al.*, 1997) have been described for quercetin, the principal flavonoid found in *A. vulgaris* (Fraisse *et al.*, 1999; Lamaison *et al.*, 1991; Shih *et al.*, 2004), which was present in the extract employed in the present study. Antiinflammatory activity has also been described for luteolin (Gutierrez-Venegas *et al.*, 2006; Hougee *et al.*, 2005), although only trace amounts were detected in the *A. vulgaris* extract employed.

No scientific evidence of antimicrobial properties of *A. vulgaris* has been reported to our knowledge, although bacteriostatic activity has been claimed for ellagic acid (Akiyama *et al.*, 2001; Lu *et al.*, 2005). Nevertheless, the plant's healing properties are unlikely to be mediated principally by removal of microorganisms from wounds. Indeed, cell culture medium does not normally contain any viable bacteria, again supporting the notion that the cell growth-enhancing properties of *A. vulgaris* observed on the two major cell types involved in the wound healing process are mediated independently of any antibacterial or antiseptic activities. The use of glycerine as vehicle is also of interest, since it is traditionally employed to store biological material such as skin grafts. Indeed, glycerine alone accelerated wound healing between days 3 and 5. Hypertonic glycerine solution may create an osmotic gradient which favours plasma exudation from inside the wound along its osmotic gradient, thus extruding contaminating bacteria, and consequently favouring healing. In addition, possible antibacterial activities of glycerine (Smith *et al.*, 1986; Svanberg and Birkhed, 1991; van Baare *et al.*, 1998) could also play a role. The healing properties of *A. vulgaris* and glycerine therefore appear to be complementary and additive.

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In summary, *A. vulgaris* (0.1%–1%) enhanced proliferation of cultured Chang liver and MDBK epithelial cells and rat aortic myofibroblasts *in vitro*. Healing of superficial dorsal skin lesions in adult male rats was accelerated between days 3 and 5 when treated with glycerine alone, whereas the association of *A. vulgaris* (3%) and glycerine enhanced healing from day 2 with complete healing occurring a day earlier. The results demonstrate for the first time the cutaneous wound-healing properties of *A. vulgaris* which may be associated with promitotic activity in epithelial cells and myofibroblasts.

Associating *A. vulgaris* with glycerine (Shrivastava, 1999) could provide a safe and attractive novel solution to ameliorate the early stages of the wound healing process. Further studies in this direction are warranted.

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