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In vitro growth stimulatory and in vivo wound healing studies on cycloartane-type saponins of *Astragalus* genus

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Dedicated to Prof. İhsan Çalış on the occasion of his 60th birthday.

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ABSTRACT

Aim of the study: The present study was undertaken to evaluate the wound healing effects of the four chief saponins of *Astragalus* species [cycloastragenol (CA), astragaloside IV (AG), cyclocephaloside I (CCI) and cyclocanthoside E (CCE)].
Material and methods: Effects of cell viability and proliferation of the isolated compounds were evaluated by the MTT assay on human keratinocyte. The wound healing activity was studied by using in vitro wound healing, proliferation and migration scratch assay. In order to see in vivo effectiveness of the compounds, an animal study with Sprague–Dawley male rats at the age of 12 weeks was carried out, and then the main histological outcomes were investigated to observe reepithelization, neovascularization, and presence of inflammatory cells, granulation tissue amount and maturation.
Results: All the compounds increased both fibroblast proliferation and migration, but the effects were much superior for CA at 1 ng/ml concentration. Among the compounds, based on the histological findings, 5% CA preparation was found to be the most remarkable in vivo wound healing agent showing greater cell density, more regularly organized dermis and more newly formed blood vessels.
Conclusion: Results of this study indicate that the cycloartane-type saponins are the principal constituents responsible for wound healing activities of the roots of *Astragalus* species substantiating its use in traditional medicine.

1. Introduction

The World Health Organization estimates that 80% of the world's population still relies on plant-based medicines for their primary health care, and it is estimated that the third most common reason for medical visits in the developing world is for skin disorders, especially wounds. Wound care can be traced back to early civilizations and many of these treatments were based on the use of herbal remedies (Ryan and Cherry, 1996).

Astragalus L., the largest genus in the family Leguminosae, is represented by 380 species in the flora of Turkey (Davis, 1970). The roots of *Astragalus* species represent a very old and well-known drug in Traditional Chinese Medicine for its usage as an antiperspirant, tonic and diuretic. It has also been used in the treatment of diabetes mellitus, nephritis, leukemia and uterine cancer (Tang and Eisenbrand, 1992).

In the district of Anatolia, located in South Eastern Turkey, an aqueous extract of the roots of *Astragalus* is traditionally used against leukemia and for its wound-healing properties. Known biologically active constituents of *Astragalus* roots represent two major classes of chemical compounds, polysaccharides and cycloartane-type saponins (Tang and Eisenbrand, 1992).

Cycloartanes occupy a special position among low molecular bioregulators because they are produced by photosynthesizing organisms only, and one from the initial representatives of this range, cycloartenol serves as key link in the biosynthesis of different phytosterols. In this connection, cycloartenol and its weakly polar derivatives widespread in the plant kingdom. In the main, the plants of *Astragalus* genera proved to be the richest source of this class of compounds.

Up to now, about four hundred cycloartane-type saponins were determined and one hundred and sixty of them were isolated from *Astragalus* genus (Mamedova and Isaev, 2004).

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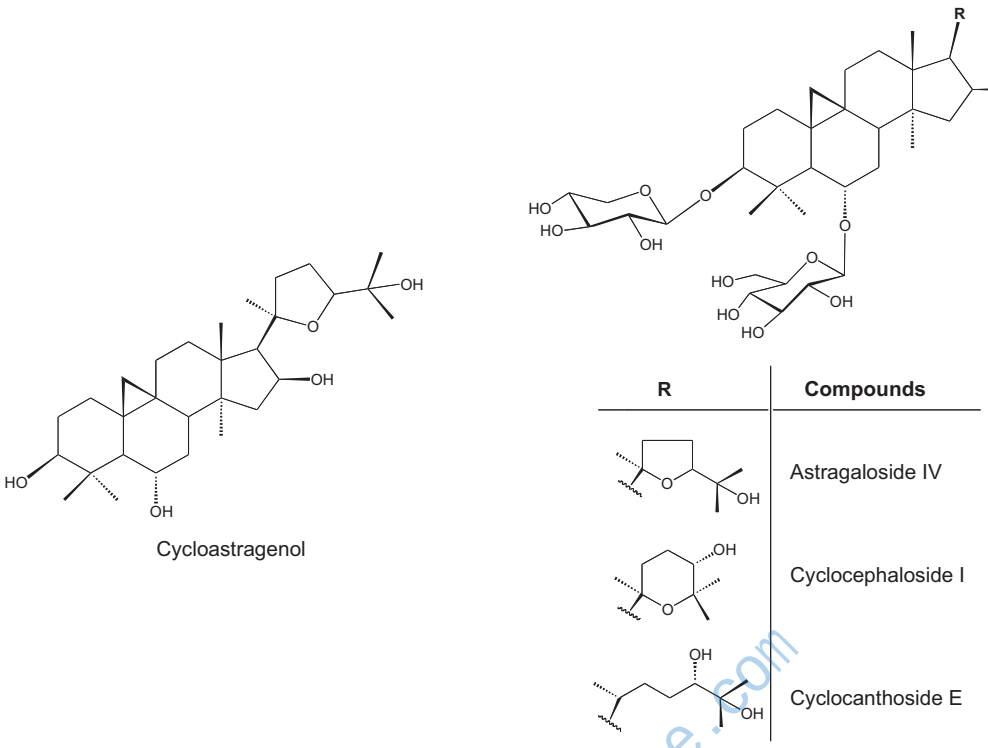

Fig. 1. Structure of the compounds.

Cycloartanes from *Astragalus* genus are found to possess cardiotonic, hypocholesteremic, anti-depressive and antiblastic actions as well as immunomodulatory activity (Calis et al., 1997; Bedir et al., 2000; Mamedova and Isaev, 2004). Especially semi-synthetic glycosides demonstrated strong cardiotonic activity and had several advantages over cardenolides owing to lack of toxicity and cumulative effects (Mamedova and Isaev, 2004).

Our earlier investigations of Turkish *Astragalus* species resulted in the isolation of over sixty cycloartane glycosides including five different aglycones (Bedir et al., 1998a,b, 1999a,b, 2001a,b; Polat et al., 2009, 2010; Horo et al., 2010).

Based on the traditional claims that *Astragalus* has several important therapeutic properties, including wound healing and immune modulation (Calis et al., 1997; Bedir et al., 2000; Yeşilada et al., 2005), our team has decided to focus on *Astragalus* as a lead to discover new wound healing agents. However, whole water extracts of *Astragalus* are used, and the relationship between the various components and their effects has not been well elucidated. Therefore, in *Astragalus* research, it is important to isolate single components with biological activity, to examine these effects, and to elucidate their functional mechanism. Taking into account the results of our comprehensive studies and preliminary screenings in addition to recent progress in the literature, we have chosen four cycloartane type saponins that are present in Turkish *Astragalus* species as major chemical entities (astragaloside IV, cycloastragenol, cyclocephaloside I and cyclocanthoside E) (Fig. 1).

2. Materials and methods

2.1. Pure saponins

All of the molecules (+98% purity) were purchased from Bionorm Natural Products (Ankara Asfaltı, No. 359, D:18, Naldöken, Bornova-İzmir, Turkey).

2.2. Cell viability and proliferation assays

Human keratinocyte (coded HS2) obtained from Animal Cell Culture Collection (HUKUK, Sap Institute, Ankara, Turkey) were produced with DMEM-HAM's/F12 (Cat. No. T 481-01, Biochrom, Germany) medium supplemented with 10% fetal calf serum (FCS, Biochrom, Germany) and were incubated at 37 °C with 5% CO_2 in humidified atmosphere.

The compounds dissolved in DMSO to a final concentration of 100 μg/ml (1000×). Subsequent dilutions were made in culture medium. The same proportion of DMSO/culture medium was added to the controls. The final DMSO content was never above 0.1%. Cells in exponential growth phase were placed in 96 well plates so as to make 6000 cells/wells. After 24 h of incubation and adding sample solutions in concentrations ranging from 0.0001 to 100 ng/ml in each well, respectively, they were incubated for 72 h. Groups were treated with 0.1% DMSO as negative control and with EGF (Sigma, USA, E4127, Epidermal Growth Factor from murine submaxillary gland cell culture tested) (10 ng/ml) as positive control.

Cell proliferation was determined by adding 0.5 μg/ml per well, prepared as a sterile stock solution of 5 mg/ml in Dulbecco's-phosphate buffered saline (DPBS, Gibco, USA), diluted 1:10 with medium prior to use. Medium was removed 4 h later and blue formazan crystals dissolved in 200 μl of 100% dimethylsulfoxide (DMSO, Sigma) per well. Quantities of blue formazan product were measured at 570–690 nm using UV–visible microplate reader spectrophotometer (Molecular Devices, Versamax, Tunable Microplate Reader, USA). For human keratinocyte, strong correlations between numbers of cells present and amounts of MTT formazan product were observed. The data were obtained from three independent assays, using three wells for each assay.

2.3. In vitro wound healing, proliferation and migration method

Cells were placed in 24 well plates so as to make 200.000 cells/well and were cultured until covering the surface

Table 1
The scoring system used for histological examination.

Criteria	Score			
	0	1	2	3
Reepithelization	None	Partial	Complete, but immature or thin	Complete and mature
Neovascularization	None	Up to 5 vessels/HMF	6–10 vessels/HMF	>10 vessels/HMF
Granulation tissue amount	None	Scant	Moderate	Abundant
Granulation tissue maturation	Immature	Mild maturation	Moderate maturation	Fully matured
Inflammatory cells	None	Scant	Moderate	Abundant

completely. In a circular zone of 5 mm diameter cell layer, making use of a sterile Teflon bar that removes cells, a wound was formed by scratching carefully (Arikan et al., 2007). After the formation of the wound, cell debris was removed by discarding the medium and washing the wells 4 times with DPBS. Medium containing concentrations of sample solutions ranging from 0.0001 to 10 ng/ml was added to the cultures in which the wound models were formed. As negative control groups DMEM-HAM's/F-12 medium with 10% FCS, DMEM-HAM's/F-12 medium without 10% FCS and Hank's balanced salt solution (HBSS, Biochrom, Germany) were used. For the positive control group DMEM-HAM's/F-12 supplemented with 10 ng/ml of epidermal growth factor (EGF, Sigma, USA) was used. At the end of 72 h of incubation at 37 °C with 5% CO_2, the cells were fixed with 4% paraformaldehyde and stained with Giemsa and/or by hematoxyline–eosine (HE). Healing in the wound zone was photographed. After transferring the pictures to a computer, the number of cells that were formed as a result of migration and proliferation were determined.

2.4. Animal model and surgical procedure

The experimental protocol was approved by the Institutional Committee on the Care and Use of Laboratory Animals, Hacettepe University, Ankara, Turkey. The study was permitted by the Institutional Animal Ethics Committee. A total of 21 Sprague–Dawley male rats (outbreed stock from own breeding colony) aged 12 weeks were used in this study. The rats weighed 250–300 g at the beginning of experiment. They were socially housed in Eurostandard type IV polycarbon cages ($w \times h \times d$ = 380 mm × 200 mm × 590 mm) with standard rat food pellets (Korkutelim Ltd., Turkey) and water available ad libitum. The colony room was maintained at a temperature of 21 ± 20 °C, a relative humidity range of 40–50% and on a 12-h-light/12-h-dark cycle. The rats were randomly assigned to three groups, each consisting of 7, according to time points. All surgical procedures were performed under general anesthesia by i.p. injection of 90 mg/kg-bw ketamine hydrochloride (Ketalar, Eczacıbaşı Ilaç A.Ş., Istanbul, Turkey) and 10 mg/kg-bw xylazine (Alfazyne, Alfasan International B.V., Woegen, Holland). The hair coat of the dorsal area was removed with an electrical shaver and the skin was disinfected with 70% alcohol solution. Six circular full-thickness skin wounds (=8 mm) were created using a sterile biopsy punch (Shukla et al., 1999; Rezende et al., 2007).

Everyday, test materials were applied to the wounds on each animal. Each wound was kept as one group. The groups are seen in Fig. 5.

2.5. Histological examination

The wound specimens including full thickness skin layers (epidermis, dermis, and hypodermis) and the underlying muscle layer were fixed in 10% buffered formaldehyde and processed according to the routine light microscope tissue processing methods, and the processed tissues were embedded in paraffin. 5 μm tissue sections stained with HE were examined and photographed by Leica image analyzing system (Leica, Germany).

All specimens were evaluated individually by two histologists who were blinded to the drug type and the time from wounding. The main histological criteria were reepithelization, neovascularization, presence of inflammatory cells, amount of granulation tissue and maturation (Table 1). The scoring system was modified from the one used by Abramov et al. (2007).

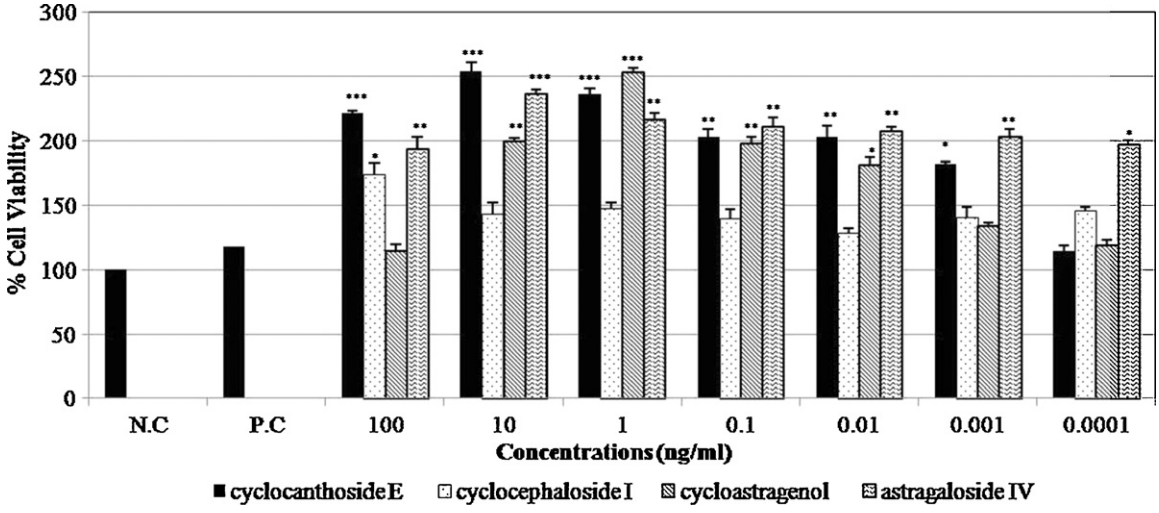

Fig. 2. Cell viability and proliferation effects of the compounds. "N.C" [cell + medium + 0.1% DMSO)] denotes negative control whereas "P.C" stands for positive control (EGF, 10 ng/ml). The data were obtained from three independent assays using three wells for each assay (mean ± SE). The resulting data were subjected to two-tailed paired *t*-test for statistical significance. $^{*}p < 0.04$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the control.

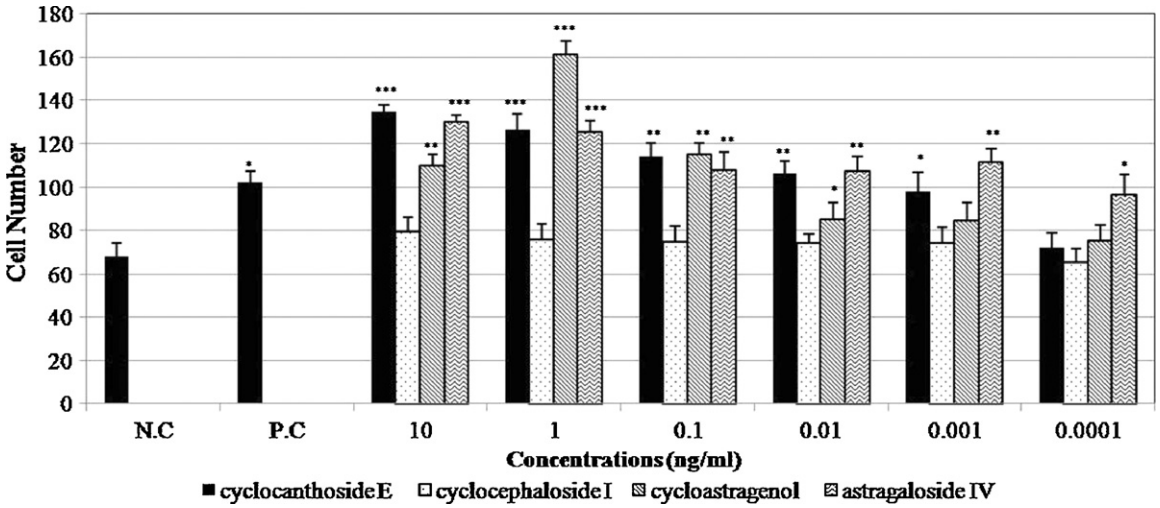

Fig. 3. Wound healing, cell proliferation and migration effects of the compounds. As negative control group (NC) DMEM-HAM's/F-12 medium with 0.1% DMSO, and as the positive control group (PC) 10 ng/ml of EGF were used. The data were obtained from three independent assays using three wells for each assay (mean ± SE). The resulting data were subjected to two-tailed paired *t*-test for statistical significance. $^{*}p < 0.04$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the control.

2.6. Statistical analysis

The resulting data were subjected to two-tailed paired *t*-test for statistical significance. *p* value < 0.05 was considered significant.

3. Results and discussion

For assessment of in vitro wound healing effect of the molecules in terms of cell viability and proliferation, human keratinocyte were treated for 72 h with doses ranging from 0.0001 to 100 ng/ml and EGF (10 ng/ml) as a positive control (Figs. 2 and 3). Cycloastragenol (CA) showed the best dose dependent stimulation of cell growth which in return at the end of 72 h, revealed cell viability rates ranging from 100% to 254% on human keratinocyte at 1 ng/ml concentration. These values were also high for cyclocanthoside E (CCE) at 10 ng/ml concentration, revealing cell viability rates ranging from 100% to 254%. Cell viability rate for astragaloside IV (AG) was 237% at 10 ng/ml concentration at the end of 72 h, whereas less effective cell viability rate was obtained for cyclocephaloside I (CCI) as 174% at 100 ng/ml concentration. Additionally, cell viability for CA, CCE and AG decreased in a dose dependent manner between 1 and 0.0001 ng/ml concentrations (254% → 119% for CA,

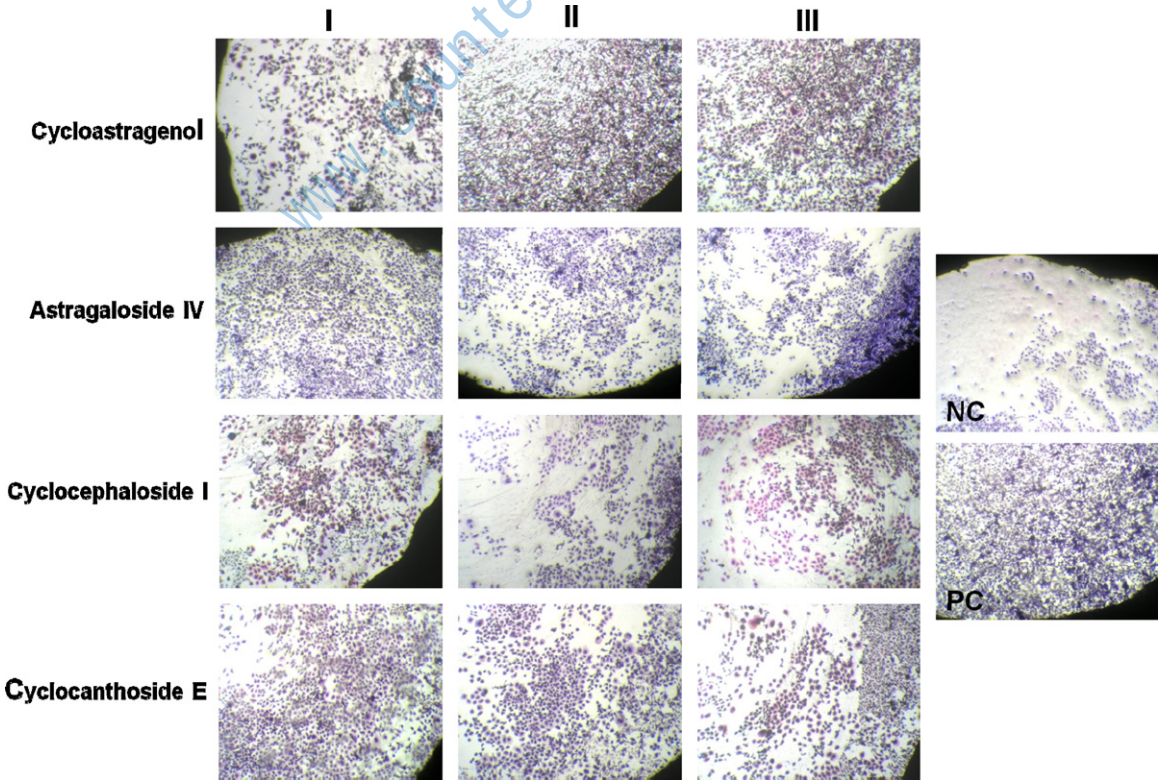

Fig. 4. Morphological observation of the wounded edge of the primary human skin fibroblast cell cultures. Confluent cultures of primary human keratinocytes cells were wounded and incubated for 72 h in presence of the compounds at doses ranging from 0.0001 to 10 ng/ml. (I) 10 ng/ml; (II) 1 ng/ml; (III) 0.1 ng/ml. Negative control (DMEM-HAM's/F-12 with 0.1% DMSO); PC, positive control (EGF, 10 ng/ml) (40×).

237% → 115% for CCE, and 216% → 197% for AG). Cell viability of the EGF (10 ng/ml) exhibited an increasing pattern ranging from 100% to 118% at the end of 72 h. Based on these results, we suggest that CA, CCE and AG are much more potent in activating the growth of human keratinocyte compared to CCI and the positive control.

In order to assess in vitro wound healing and migration effects, the samples and the positive control were analyzed particularly with the wound healing model explained above. Migration was monitored in relation to the closure of a denuded area scratched in a confluent monolayer. Medium, containing concentrations of the sample solutions ranging from 0.0001 to 10 ng/ml was added to the cultures in which the wounds were formed. As a positive control group, EGF (10 ng/ml) was used. The number of cells formed as a result of migration and proliferation was determined with a random measurement of quantity in the wound site (Figs. 4 and 5). Except the CA, all of the compounds generally showed a similar activation profile in terms of quantity of cells where a dose dependent increase in cell proliferation was observed at the end of 72 h. CA displayed an increase in cell proliferation pattern 110 (±5) → 161 (±6) at concentrations between 10 and 1 ng/ml, followed by a decrease in cell proliferation 75 (±6) → 115 (±5) at concentrations between 0.0001 and 0.1 ng/ml at the end of 72 h. However, CA showed the best growth stimulation at a dose of 1 ng/ml which increased the number of cells from 68 (±6) to 161.4 (±6) at the end of 72 h of treatment. As for EGF (10 ng/ml), the number of cells counted

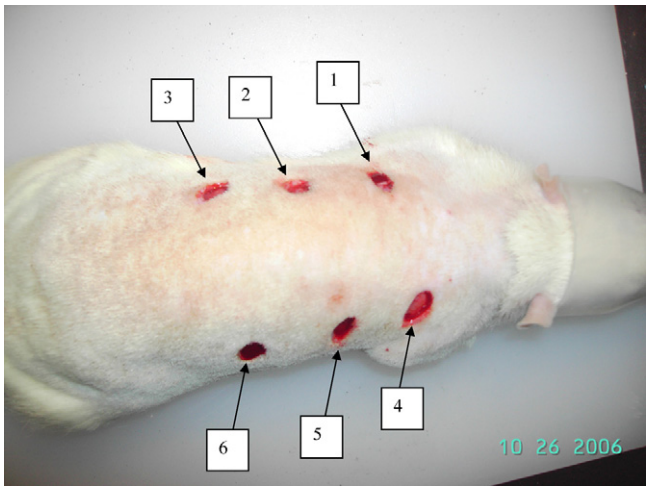

Fig. 5. Groups of the test materials. Six full-thickness skin defects were made on the back of the rats. 1 – Placebo gel, 2 – 5% cycloastragenol, 3 – 1.25% cycloastragenol, 4 – 2.5% astragaloside IV, 5 – 10% astragaloside IV, 6 – 2.5% cyclocanthoside E. Every day, the rats were examined, and the length and width of the lesions were measured. To evaluate the efficiency of the materials, specimens encompassing the whole area were removed under general anesthesia on 3rd, 7th and 14th days after the operation. Specimens were fixed in formaldehyde for histological examination.

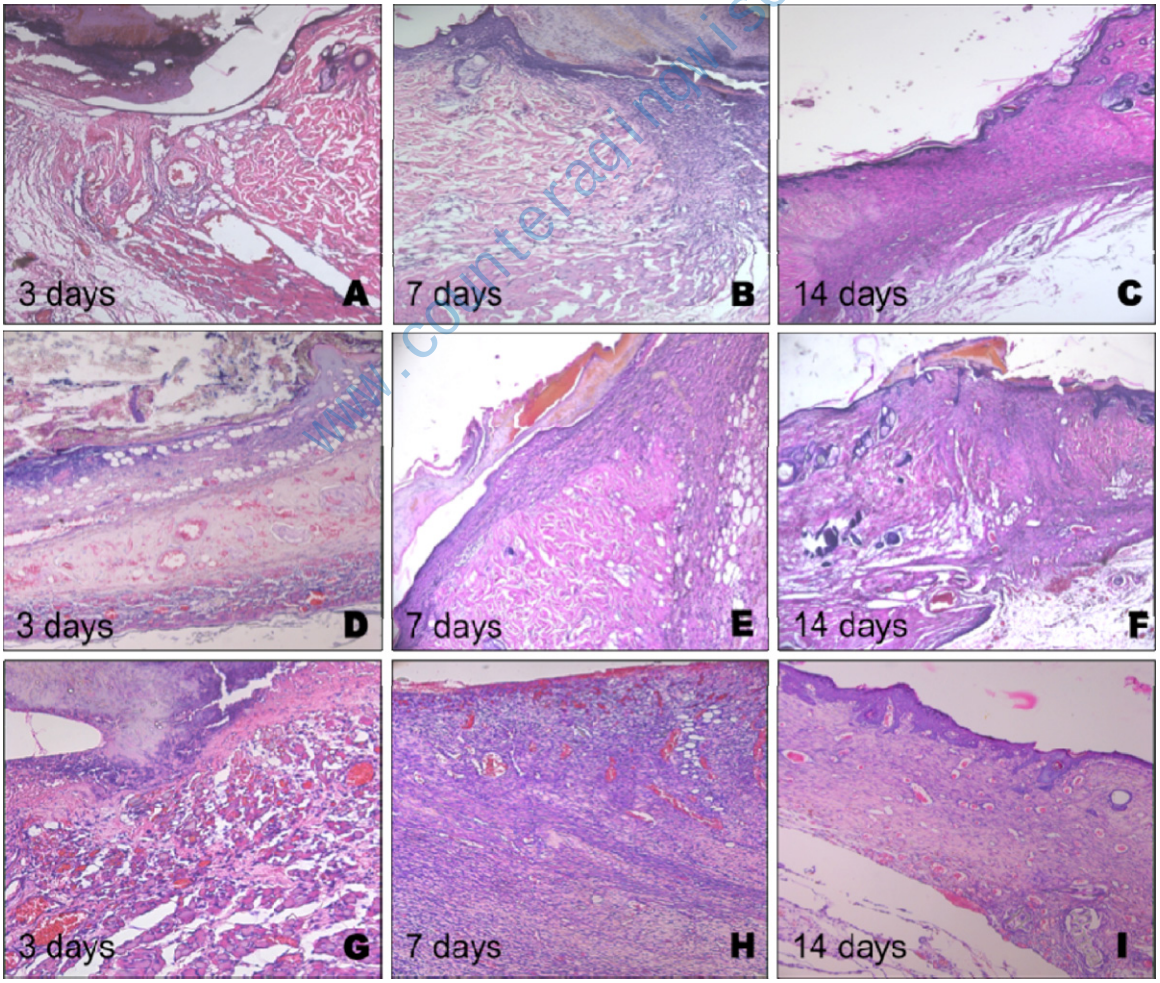

Fig. 6. In placebo gel group, crute over the wound region extending deep to edematous muscles (A), reepithelization at the edges of the granulation tissue (B), completed epithelization over the scar tissue composed of densely oriented collagen fibers (C) are seen. In 2.5% AG gel group, granulation tissue observed to develop under the crute (D), thick collagen fibers are seen under the granulation tissue (E), no epithelium in the center of the wound region (F). In 10% AG gel group, granulation tissue with inflammatory cells (G), epithelization over the granulation tissue and stasis in the blood vessels reaching to surface of the wound (H) and epithelization is completed (I) (A–I: HE 100×).

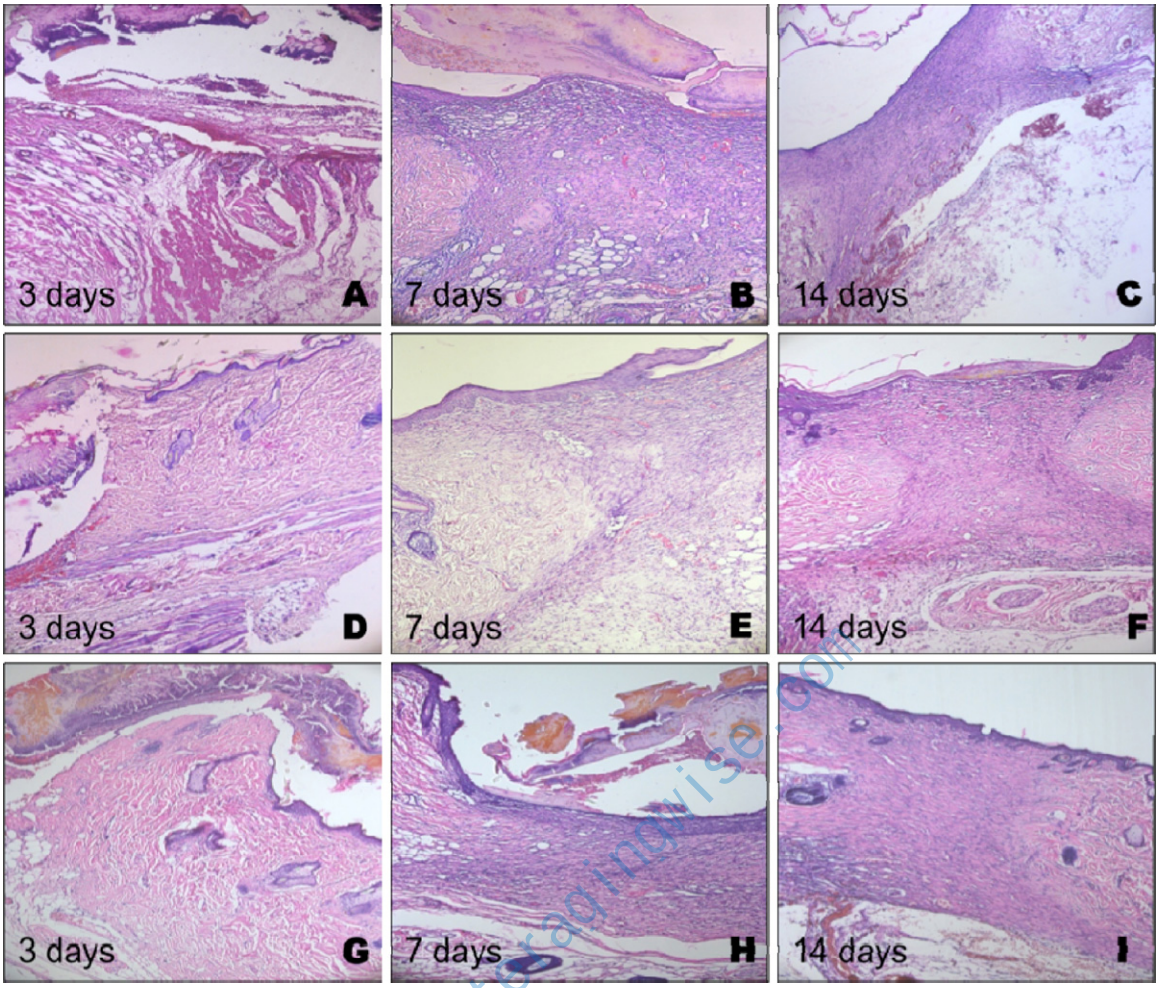

Fig. 7. In 1.25% CA group; granulation tissue over the swollen muscles (A), epithelization at the edges of the wound with blood vessels reaching the surface (B), epithelization is completed over the scar tissue (C). In 5% CA group; well organized granulation tissue is present with inflammation in the muscle layer (D), stratified squamous epithelium is covering the surface of the granulation tissue (E) and scar tissue composed of irregular dense connective tissue is seen (F). In 2.5% CCE group; large wound extending deep to the muscle layer (G), epithelization at the edges of the wound and collagen formation underneath the crute (H), thin stratified squamous epithelium over the well organized scar tissue (I). (A–I: HE 100×).

was found to be 102 (±5) as a result of the same treatment. For 10 ng/ml treatment, CCE and AG also exhibited potent activity [68 (±6) → 134.6 (±3) and 68 (±6) → 130 (±3), respectively]. CCI was less active with the numbers of 66 (±6) → 79 (±7) at concentrations between 0.0001 and 10 ng/ml at the end of the experiment.

In regards to the histological outcomes from in vivo experiments (Fig. 5), in the placebo applied groups; on the 3rd day after the operation, there was a wound extending deep to muscle layer where the cells were edematous. The wound region was covered with a thick crute. There was stasis in the dilated blood vessels (Fig. 6A). On the 7th day, epithelization was observed starting from the edges of the granulation tissue formed on the wound region. Dilated blood vessels with stasis were seen underneath and in the granulation tissue. Thick coarse collagen fibers accumulated in the wound region under the granulation tissue. The underlying muscle cells were still edematous and swollen (Fig. 6B). On the 14th day, a thin epidermis covered the wound region. A scar tissue composed of densely oriented collagen fibers was extending to the normal dermis on each side of the wound region (Fig. 6C).

In 2.5% AG gel applied groups; on the 3rd day as in the placebo group, the wound was extending deep to the edematous muscle layer. Dilatation and stasis was observed in all blood vessels. Granulation tissue is observed to develop under the crute over the wound (Fig. 6D). On the 7th day, epithelium began to grow under the crute over the granulation tissue. Beneath the granulation tissue thick collagen fibers were observed to fill the dermis region (Fig. 6E). On the 14th day, a thin epithelium with small papilla at some regions was observed to cover the wound surface except two of the animals in which the center of the wound region were still without epithelium. Scar tissue with densely packed collagen fibers was extending to deeper regions. Edema of the muscle cells was still present in some regions of the muscle layer (Fig. 6F).

In 10% AG gel applied groups; on the 3rd day as in the other groups, large wound region was covered with a thick crute under which granulation tissue with many inflammatory cell started to form. Dilated blood vessels with stasis were observed and edematous muscle cells were seen in the underlying muscle tissue (Fig. 6G). On the 7th day, epithelization and inflammation in the granulation tissue was ongoing, and blood vessels were reaching to the surface and edema in the muscle layer was still present (Fig. 6H). On the 14th day, compared to the first two groups, healing was better. The surface was completely covered with epithelium. Compared to other groups, less scar tissue formation was noted with thick and almost normally arranged collagen fibers (Fig. 6I).

In 1.25% CA gel applied groups; on the 3rd day, deep wound region was reaching to the muscle layer as in the other groups. Granulation tissue formation was observed below crute layer. Muscles were swollen and inflammation was present (Fig. 7A). On the

7th day, the wound region was filled with collagen fibers, and epithelization was starting from the edges. Blood vessels were reaching to the surface. On the surface, the thick crute layer was still present (Fig. 7B). On the 14th day; epithelization was complete but papillae were not significant. Scar tissue was observed at the upper layer of dermis (Fig. 7C).

In 5% CA gel applied groups; on the 3rd day, a thick crute layer was covering the wound. Under the wound, granulation tissue formation was significant. The muscle layer was edematous under the granulation tissue (Fig. 7D). On the 7th day, epidermis covering the wound region was intact. The blood vessels were reaching to the epidermis (Fig. 7E). On the 14th day, a small and less dense scar tissue was observed under the intact epithelium. The wound region was observed considerably smaller compared to other groups (Fig. 7F).

In 2.5% CCE gel applied groups; on the 3rd day, a large wound extending to the edematous muscle layer was observed. Granulation tissue with dilated blood vessels and stasis began to form (Fig. 7G). On the 7th day, epithelization starting from the edges was observed under the crute. Granulation tissue was still present. Thick collagen fibers were noted under the granulation tissue (Fig. 7H). On the 14th day, epithelization was complete with papillae in some regions. Scar tissue was observed reaching to the deep dermis (Fig. 7I).

In summary, 5% CA gel group have a substantial effect on removal of histological signs of tissue damage in experimentally created rat skin lesions followed by 10% AG, 2.5% CA, 2.5% CCE, placebo and 2.5% AG.

4. Conclusion

The results presented here indicate that cycloartane type saponins of *Astragalus* genus are capable of promoting wound healing based on proliferation and migration in scratch assay, proliferation in MTT assay, and in vivo wound model study.

Although all the compounds increased both fibroblast proliferation and migration, the effects are most prominent for cycloastragenol (CA), astragaloside IV (AG) and cyclocanthoside E (CCE). The effect on migration was outstanding for CA at 1 ng/ml, whereas AG and CCE had their highest activities at 10 ng/ml. Parallel to abovementioned results, the highest proliferation rates at 10 ng/ml for AG and CCE, and 1 ng/ml for CA were observed in MTT assay.

We have also shown that the topical treatments of *Astragalus* cycloartanes improve healing of subsequently induced abrasion skin wounds in rats compared to the control. At the end of 14 days treatment period, 5% CA preparation was found to be the most remarkable in the treated skin. Histological findings also showed that the CA treated group had a greater cell density, more regularly organized dermis (linear alignment) and more newly formed blood vessels compared to the other groups.

In conclusion, the present study substantiates traditional use of *Astragalus* preparations in South East Anatolia for wound healing, for cycloartane type-saponins tested here are the major constituents of *Astragalus* species. Further studies are warranted to understand the mechanism of action of the remarkable wound healing agents CA, AG and CE.

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