Comparing the healing effect of *Lotus Corniculatus* Hydroethanolic Extract and phenytoin cream 1% on the rat’s skin wound:

A Morphometrical and Histopathological Study

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ABSTRACT

**Aim:** The aim of this study was to evaluate *Lotus corniculatus* L. hydro-ethanolic extract healing effect on skin wound in male rat compared with phenytoin cream 1%, and complementary histological study on the healing process in the understudy groups.

**Method:** After induction of anesthesia in 80 male Wistar rats, using 2*1shablon and Surgical blade a full-thickness rectangular wound with an area of 2cm² was made behind their head and the skin was removed. Male Wistar rats were divided into 8 groups of ten including: the control (A), phenytoin 1% (B), *Lotus corniculatus* hydroethanolic extract 10% (C), *Lotus corniculatus* hydroethanolic extract 20% (D), *Lotus corniculatus* hydroethanolic extract 40% (E), phenytoin cream 1% + *Lotus corniculatus* hydroethanolic extract 10% (F), phenytoin cream 1% + *Lotus corniculatus* hydroethanolic extract 20% (G) and phenytoin cream 1% + *Lotus corniculatus* hydroethanolic extract 40% (H). Wound measurement with digital image analysis and medication prescription was performed for 21 days. In microscopic studies, biopsy was performed from wound healing site on the days 3, 7, 14, and 21. Considering histopathologic factors healing outcome for all groups were determined. The results were analyzed using SPSS 18 software.

**Results:** The results showed that based on treatment period; histometric findings, the mean of the healed wound area in groups H (phenytoin cream 1% + *Lotus corniculatus* hydroethanolic extract) and E (*Lotus corniculatus* hydroethanolic extract 40%) was lower than other groups (p<0.01). Also based on the histopathological results, the outcome of healing in these groups was better than the other groups.

**Conclusion:** Considering the anti-inflammatory, anti-microbial and healing effects of *Lotus corniculatus* hydroethanolic extract compared with phenytoin cream 1%, it can be claimed that its components are more effective in the healing of full-thickness skin wounds.

**KEY WORDS:** Wound healing, *Lotus corniculatus*, Phenytoin cream, Rat, Skin.

1. INTRODUCTION

Wound healing in a shorter time and with fewer complications is one of the main goals of medical science (Adzick, 1997). Wounds has been the most important issue in the surgery for a long time and of particular interest to medical and physiology researchers. For this reason, many studies and various methods have been conducted on wound healing (Rao and Kummara, 1988; William, 1979; Barnett and Varley, 1987). The use of chemical drugs, herbal, homeopathic and physical methods such as laser therapy and other methods are common. The aim of all these methods is fast, safe, and at the same time low cost treatment of wounds (Barnett and Varley, 1987; Cohen and Diegeimann, 1999). A study of the literature shows that due to individual differences and the different nature of superficial wounds, none of the chemicals and herbal methods that have been introduced could be recommended as an effective method (Khaksari, 2000). Of the studies which have been conducted in this field the effect of topical antiseptics could be pointed out. Based on those studies, topical antiseptics could slow wound epithelialization. According to recent studies, topical antibiotics, which are used to reduce wound infection can cause contact dermatitis. Also, studies have shown that laser irradiation on the leukocytes on one hand increases the phagocyte activity and on the other hand increase secretion of mediators (Nowrouzian, 2009). Of course exposure to high levels of radiation, particularly in the 4 to 48 hours after wounding, not only delays the healing, but also prepares the infected wound to infection (Silver, 1953). The use of other topical materials to accelerate healing has been continued from the past to the present. The use of butter and honey has been common in Egypt. The use of bitter almonds and sugar cane, which its leaves contain sucrose and cannot be metabolized except in the intestine is recommended as well. Therefore, while there is no sugar to grow bacteria, excess water is removed from the wound site and stimulates the production of granulation tissue. Also, using angiotensin analogues accelerate healing with minimal scarring and if in the process of restructuring their systemic administration continue leads to regeneration of skin appendages even in the center of the wound (Rodgers, 2003).
The importance of wound healing should be considered because if open skin wounds left untreated, it may lead to local infection and ultimately cancer (Ahmadi, 2014). On the other hand the lack of effective existing therapies also has adverse side effects and has limited use. Therefore, efforts are continued in this regard (Adzick, 1997; Khaksari, 2000).

One of the best ways that can help us achieve this aim is to use the pure biological materials (Islor, 1991). Nowadays attitude toward the study of Physio-pharmacological effect of herbal extracts has increased for the following reasons; fewer side effects, lower economic costs, many active compounds found in the plants, development of industries related to the cultivation of herbal medicine to prevent the outflow of foreign currency out of the country, creation of useful work, especially the World Health Organization recommendation to use herbal medicine (Taghizadeh-Jahed, 2008; Lazareva, 2002; Sylvester, 2000; Sobizewska and Szmigielski, 1997).

Given the lack of introducing an effective medication for wound healing, it is necessary to study the effects of medicinal plants on wound healing (Khaksari 2000). Reviewing Iranian and Islamic Traditional Medical literature, herbs are named as effective ingredients in healing wounds, including Calendula officinalis, onions and ivy leaf which are considered as a dermal ointments.

*Lotus Corniculatus* is from Fabaceae family. This plant is found in southern parts of Iran, Dena Mountain, Azerbaijan, Alvand Mountain and other regions (Yousaf 2010). Studies have indicated some *Lotus Corniculatus* effects, including; antibacterial, anticoagulant, anti-inflammatory and anti-parasite effects (Koelzer, 2009; Min, 2003; Ramirez, 2004; Molan, 2001). This plant as a dermal ointment has a special position in traditional medicine, nomads, farmers and horticulturists in Hamedan province. Considering that so far no effective medicine has been introduced for wound healing (Pereira, 2009), the aim of this study is to evaluate the healing effect of *Lotus Corniculatus* extract on rat wound skin also comparing it with a chemical drug, phenytoin which is useful in skin wound treatment.

2. MATERIALS AND METHODS

**Animals:** A total of 80 male Wistar rats with weight ranges 250 ± 40 g were used in this study. Rats were fed with pellet food and water. They were kept in a climate-controlled room in separate cages. Ethical principles to use laboratory animals in biomedical research were considered at all stages of the present study.

**Collecting plants, extraction and preparing ointment:** *Lotus corniculatus* was collected at the end of April from orchards around Hamadan; it was identified by reliable experts. The plant was dried in the room (shades) using electric grinding and sieving a powder was prepared from aerial parts of the plant (Koelzer, 2009). The powder was poured into the beaker for extraction using maceration method; the ratio of 1 to 8 of 96% ethanol was added and was kept in the refrigerator for a month. In order to better extraction during this period, the beakers were placed in the ultrasound device (Bandelin Sonorex) 2 times for 15 minutes (Min, 2003). After a month, the contents of the beaker were filtered through paper filter and funnel glass. Then it was condensed using the rotary (Evaporator Rotation Model lab tech, Ev 311). It was poured into the Petri dish to dry. Then it was placed under the hood (Koelzer, 2009). After 4 days the dry extract was obtained. Dry extract was mixed up with Euserin ointment to formulate 10, 20 and 40 percentage ointments (Rezaei, 2008).

**Method of creating trauma:** Anesthesia was induced with ketamine 10% and Xylazine 2% injection. The mice were placed ventrally on the surgery table, dorsal surface of the mice were scrubbed and prepared. A full-thickness rectangular wound was created in the middle of the back, between the shoulders with an area of 2 × 2 cm using sterile surgical blade. The day of surgery was considered as the zero day. The depth of the wound was including the dermis and hypodermis (Rezaei, 2008; Hafezi, 2009). After creating trauma, the wound surface was coated with ointment. Then, animals were kept in their cage marked according to the kind of treatment (Wanda, 2004).

**The experimental Groups:** After creating trauma, mice were randomly divided into 8 groups. There were 10 rats in each group (7 mice to morphometric study and 3 mice for histopathology sampling) and the groups included: (A), phenytoin 1% (B), *Lotus corniculatus* hydroethanolic extract 10% (C), *Lotus corniculatus* hydroethanolic extract 20% (D), *Lotus corniculatus* hydroethanolic extract 40% (E), phenytoin cream1% + *Lotus corniculatus* hydroethanolic extract 10% (F), phenytoin cream1% + *Lotus corniculatus* hydroethanolic extract 20% (G) and phenytoin cream 1% + *Lotus corniculatus* hydroethanolic extract 40% (H). Ointment was administered topically for 21 days to the wound so that the whole wound cavity was filled.

**Sampling and preparing histological sections:** On the days 3, 7, 14 and 21 (the final day of the experiment), a mouse was selected randomly from each group. Then, full thickness tissue samples from wound healing site with the healthy skin around were removed by the use of scissors and scalpel (Taghizadeh-Jahed, 2008). Tissue anesthesia was performed using Ketamine and Xylazine. After fixation, samples were under tissue passage, then molding and 5 micrometers thin sections were prepared from samples. Sections were prepared using histological staining with hematoxylin and eosin staining (Taghizadeh-Jahed, 2008; Derakhshanfar, 2010). Overall from eight experimental groups, 32 mold tissues were prepared during days 3, 7, 14 and 21.
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In order to histopathologic study of microscopic sections a conventional microscope (Olympus-cx31) with 40×, 100× and 400× magnification was used for the images. In order to count the number of fibroblasts and vascular buds of granulation tissue in square millimeters, digital images were made from tissue sections with image analysis software, Scion Image. After calibration using calibrate slides, images were analyzed from different aspects. To determine the desired level (square mm) grid was drawn on the picture. Then, fibroblasts and vascular buds were counted and other components were examined (Derakhshanfar, 2010).

Statistical analysis: Data were analyzed using SPSS version 18. The results were examined as Mean ± SEM. Two-way ANOVA and post hoc Bonferroni test were used to compare the groups. The mean difference as p<0.05 was considered significant.

3. RESULTS

Morphometric Results: As can be seen in Figure 1, the size of the wound area in the control group had increased in the first three days. In the next few days the wound got smaller slowly. In the next twenty-first days the wound was not treated fully (Fig -A 3). In the group treated with phenytoin 1% the wound surface in the first and second days was increased significantly, and in the following days slowly the wound got smaller, so that in the twenty-first day also the wound was not treated fully, but it was better than the control group (Fig -D3). In the group treated with the Lotus corniculatus hydroethanolic extract 10%, the wound surface increased in the early days, wound area declined in the following days (Fig -B3). Finally, on the seventeenth day, the wound was healed and on the eighteenth day the wound completely disappeared. These results compared to the control group and the group treated with phenytoin 1% showed no significant difference (p>0.05) (Figure 1).

In the group treated with extract 20%, the wound surface on the first and second days was not significant compared with the control group; but in the next days, the wound surface group began to fall, with a relatively higher rate than the control group, so that on the fourteenth day the wound surface was very small, finally, on the fifteenth day the wound was healed (p<0.05). In the group treated with extract 40%, the wound surface in the days after creating trauma had the lowest initial increase compared with the control group. In the following days, the wound surface was decreased quicker than the control group and the group treated with the extract 10% and 20% (p<0.01). In the group treated with a combination of 10% extract and phenytoin 1%, the wound surface increase was observed in the early days. On subsequent days, the wound surface compared to the control group begin to decline slowly. The wound size reduction in this group compared with the control group was not statistically significant (Figure 1). In the group treated with a combination of 20% extract and phenytoin, the wound surface showed less increase in the early days than the control group and in the following days, it was reduced rapidly; so that on the seventh day, the wound surface reduction was significant and finally on the fifteenth day the wound disappeared. In the group treated with combined extract 40% and phenytoin, the wound surface was increased slightly in the early days. In the following days the wound surface with great speed compared to the control group begins to decline (Figure C-11).

In this study the wound size during the first three days after the creation in all groups was increased. The mean increase in the control group was higher of all groups and in the group treated with the extract 40% it was lower than other groups (p<0.01). The greatest extent of the wound was related to the control group which did not receive any treatment. The lowest level of the wound was related to the group treated with an extract of 40% (high concentration of the extract) (Figure 1).

Histopathological Results

After studying the microscopic sections and counting fibroblasts and vascular buds in the center of granulation tissue in the control and experimental groups it was found that the cellular changes in the area of wound healing in these groups is like the general trend of healing, scilicet inflammation, duplication and contraction. Though in terms of time to reach any of these steps there were differences between different groups.

Comparing the number of fibroblasts and vascular buds of granulation tissue in the control and experimental groups indicates that using Lotus corniculatus hydroethanolic extract alone and also in combination with phenytoin can increase the speed of wound healing (Figs. B2 and C-2), because the maximum density of fibroblasts in groups E, H, G, D, C and F is shorter than groups A and B (Figure A-2). On the other hand, almost the time to reach the maximum density of fibroblasts and also the fibroblast density depend on the dose of extract; so that, by increasing the concentration of active ingredient of the plant fibroblast density was increased significantly (Figure C-2).

DISCUSSION

Herbs have paramount history and credibility and are considered as a valuable treasure in Pharmaceutical Sciences. Using medicinal plant extracts in skin wound healing has been common in traditional medicine since
ancient times. *Lotus corniculatus* hydroethanolic extract in addition to various properties, accelerate the wound healing process of the skin. Anti-inflammatory effect of *Lotus corniculatus* hydroethanolic has been reported in 2009 by Koelzer. Based on the results of this study, the ointment made from *Lotus corniculatus* hydroethanolic extracts showed good anti-inflammatory effect in the early days. It is likely that a part of ointment healing effect is related to its anti-inflammatory effect that has been effective in the wound healing in the inflammatory phase. Min in 2003 reported that anti-inflammatory effects of *Lotus corniculatus* hydroethanolic extract (Molan, 2001). According to the studies of BYL (1992), wound inflammation phase adjustment is accelerated in wound healing. According to Khaksari (2000) and Koelzer (2009) reports, *Lotus corniculatus* hydroethanolic due to anti-inflammatory properties caused by inhibition of leukocyte and reducing wound secretions, as well as inhibition of proinflammatory enzymes and medications such as IL-1B (interleukin-1 beta), ADA (adenosine-deaminase), MPO (myeloperoxidase) by modulating the inflammation phase accelerate wound healing. The results of the present study are consistent with the results of the above mentioned studies. Chopra (1986), and Dalmarco (2010), indicated that increasing blood supply and oxygenating the wound site; by dilating the vessels is another wound healing factor. Min (2003), reported that *Lotus corniculatus* hydroethanolic has an antispasmodic activity. It seems that this property helps wound healing and also shorten the healing time significantly.

Khaksari (2000), reported that preventing wound infection accelerate wound healing: also, topical antibiotics accelerate wound healing by infection control. Ashrafi (2010), reported the antimicrobial effect of *Lotus corniculatus* hydroethanolic. Based on these reports, it could be said that *Lotus corniculatus* hydroethanolic accelerate the healing process due to its antimicrobial properties, (Restrepoa, 2004).

Dill (1997), have reported increasing effects of phenytoin on muscle tissue fibroblasts and tissue growth factors. Song and Cheng (1997), pointed out the increasing number of macrophages in the healing wounds treated with phenytoin. According to Modaggh and Salchians (1989) studies, topical use of phenytoin increase tensile strength of wounds, new blood vessels and synthesis of collagen and fibroblasts filtration. Comparing the groups treated with *Lotus corniculatus* hydroethanolic extract and phenytoin it became apparent that wound healing was much better and faster in the groups that had received the *Lotus corniculatus* hydroethanolic extract locally than the group treated with phenytoin. So it is likely that the mechanisms that accelerate wound healing in phenytoin are considered more powerful for *Lotus corniculatus* hydroethanolic.

In the group treated with *Lotus corniculatus* hydroethanolic 10%, the mean wound area was lower than the control group, but the mean wound area of the group treated with *Lotus corniculatus* hydroethanolic 10% and phenytoin was lesser than the control group. As a result, based on observations and statistical analysis, the combination of Phenytoin and *Lotus corniculatus* hydroethanolic extract showed no significant effect on wound healing at this level. Perhaps the reason is decreasing the concentration of phenytoin active ingredient using at the same time with *Lotus corniculatus* hydroethanolic extract and also the presence of preservatives in phenytoin.

This study showed that the mean wound area of mice that received *Lotus corniculatus* hydroethanolic extract 20% was higher than the mean wound area of mice that received a combination of *Lotus corniculatus* hydroethanolic extract 20% and phenytoin 1%. Perhaps the reason is the strengthening effect of phenytoin and *Lotus corniculatus* hydroethanolic extract on each other having a synergistic healing effect. This effect in the group who received the extract 40% combined with phenytoin was statistically significant.

According to the results, by increasing the concentration, wound healing and shrinkage rate increased. Studies by Tsegahun (2006), on *Lotus corniculatus* hydroethanolic determined that the plant contained a lot of anthocyanins, flavonoids, sterols, alkaloids and tannins. Based on Dicko (2006), reports tannins are able to perform many biological reactions. It is likely that accelerating skin wound healing in rats is due to the active ingredients of the plant. Hemingway and Karchesyijj (1989), reported the healing effects of tannins. According to Taghizadeh Jahed (2008), inhibiting the production of free radicals leads to faster dermal wound healing. Since *Lotus corniculatus* hydroethanolic extract has tannins and antioxidant properties (Dalmarco, 2010) perhaps its healing effects on skin wound healing is due to the presence of tannins and antioxidant properties of the plant. However, to clarify the mechanism, further studies are required. We hope that these questions be answered in the future studies.

4. CONCLUSION

The findings of this study showed that the use of *Lotus corniculatus* hydroethanolic with different concentrations can affect the cellular changes of wound healing time dependently. By faster angiogenesis in granulation tissue as well as faster and more proliferation of fibroblasts and subsequently faster and more production of collagen and other extracellular matrix components the plant improves the overall situation of wound healing. Therefore the present study confirmed the results of previous studies on *Lotus corniculatus* hydroethanolic. It is also based on its method and results showed that this extract morphometrically and histopathologically after topical application on the skin open wounds may relieve inflammation and accelerates the healing process.
Figure 1. Full thickness skin wound in the back and between the shoulders of experimental mice in the control group.

Chart 1. Wound healing process in the study groups within 21 days of the experiment compared to the control group. Two-way ANOVA statistical method was used. Data are presented as Mean ± SEM and significant difference between groups as * P <0.05 and ** P <0.01 (n=7).

Figure 2. Microscopic view of the healing site on the seventh day, A) treated with Lotus corniculatus hydroethanolic extract 10%, B) treated with Lotus corniculatus hydroethanolic extract 20%, C) treated with Lotus corniculatus hydroethanolic extract 40% + phenytoin 1%. 1- Fibroblasts, 2- Vascular bud, 3- Extracellular matrix, 4- Inflammatory cells and macrophages (H & E × 400).

Figure 3. The status of the wound on the fourteenth day: A) control group, B) treated with Lotus corniculatus hydroethanolic extract 10%, C) treated with Lotus corniculatus hydroethanolic extract 40% + phenytoin 1%, D) treated with phenytoin 1%.
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