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Topical application of *Achillea fragrantissima* methanolic extract and honey on full thickness wound of rats' model: Histological and Immune-histochemical studies

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This research aim to study efficacy of *Achillea* species methanolic extract alone and mixed with honey on enhancing wound healing in full thickness wound rats model. Histological and immune-histochemical studies used for evaluation skin tissue. Thirty adult male rats were used. Animals were divided into 6 groups, G1 (Control) unwounded painted with saline. G2 (Untreated+Wound(W)) full thickness wounded rats without any treatment. G3 (W+Mebo) painted with mebo cream. G4 (W+*Achillea*) full thickness wound treated by *Achillea* extract gel. G5 (W+Honey) wound treated with honey (1g/day). G6 (W+Mix) wound treated with honey and *Achillea*. Wound daily covered by previous medications and observed for healing or complications. Serum levels of inflammatory markers as interleukin (IL)-6, IL-10, tumor necrosis factor -α; antioxidants as glutathione, superoxide dismutase, catalase,and lipid peroxide were determined. Skin wound edge area was dissected at 21 days, staining by hematoxylin and eosin, Masson trichrome, alpha-smooth actin (α-SA),and vascular endothelial growth factor (VEGF) immunoexpression. Epithelial thickness, area density of α-SA, and VEGF was recorded. *Achillea* extracts or mixed with honey enhance wound healing by decreased inflammatory markers, oxidative stress, enhancing epithelization, neo-angiogenesis,and collagen maturation. Form this research can be concluded that application of *Achillea* extracts or mixed with honey for large full thickness wounds enhanced wound healing.

Keywords: *Achillea* methanolic extract, Full thickness wound, Growth factors, Honey, Immune-histochemistry, Rats.

INTRODUCTION

The largest body organ in animals is the skin, representing 20% of total body weight, covering the whole body, and is formed of an outer epidermal layer, epithelial layer and deep connective tissue dermis (Paulsen, 2010). Any damage or wound in skin can be referred to as cutaneous wound, because it is in fact an altered disruption of normal skin architecture and this might be simple just in the epithelial layer or go deep damaging the hypodermal layers like vessels, muscles, nerves and bone (Velnar et al. 2009). Healing of wounds normally passes through a number of stages, considered as biochemical and cellular processes for restoration of the damaged parts to their normal structures (Fronza et al. 2014).

Cutaneous wounds require extensive use of anti-inflammatory medications that are able to protect against infection and enhance tissue

repair (Thakur et al. 2011). Plants that are rich in flavonoids, tannins, volatile oils, sterols and triterpenes are well known for their healing promoting effects. Traditionally natural medicinal plants have been use as antiseptic and antipyretic materials. Volatile oil extracted from flowers is reputed for their activity as antimicrobial agents. Saudi Arabia areas are rich in natural plant species genetically important medicinal plants (Shahat et al. 2013). *Achillea fragrantissima* (*Asterales, Asteraceae*) is a wild growing plant in Northern area of Saudi Arabia (Elsharkawy et al. 2014). *Achillea* is a medicinal herb that has been used in popular medicine. *Achillea* species contains many bioactive materials with enhanced biological activity (Falconieri et al. 2011). *Achillea fragrantissima* essential oil was proved to exert antimicrobial,and antiviral activities (Zeedan et al. 2014). Few clinical uses for *Achillea fragrantissima* were described (Maswadeh et al., 2006). The genus *Achillea* was characterized by its astringent, anti-bleeding functions so it plays good role in wound healing (Akkol et al. 2011).

Honey was well known to accelerate cutaneous wound healing and this character is particularly beneficial in persons with large gapped wounds (Ghaderi and Afshar, 2004). Honey was found to be very effective in management wounds and ulcers (Yilmaz and Aygin, 2020). The adequacy of honey using as a dressing for infected wounds was due to its antibacterial actions (Molan, 2006), for promotion of clean healthy granulation tissue, and enhanced epithelial growth over the wound (Oryan et al. 2016). According to these characteristics it can be said that honey helps skin regeneration.

The present research was planned to study the topical efficacy of *Achillea* species methanolic extract in enhancing wound healing with scar free outcome, especially if mixed with high quality natural honey. Histological morphometric evaluation supported by immuno-histochemical staining of tissue promoting factors as well as inflammatory markers levels were used for evaluation.

MATERIALS AND METHODS

Plants: *Achillea* plant:

Achillea fragrantissima (*Asterales, Asteraceae*) plant was obtained from Northern region of Saudi Arabia (Arar) washed, dried and kept in air-tight container.

Plant preparation for topical application

Dried *Achillea* powder was subjected to extraction with methanol by maceration method at room temperature in pharmaceutical lab, King Fahd Medical Research Center (KFMRC). The supernatant was subjected to filtration and then evaporation to dryness. This dried extract was stored in sterile dark glass containers until used as topical medication. Honey of well- known source was used in a dose of (1g/ day) as previously reported by Ghaderi and Afshar (Ghaderi and Afshar, 2004). Mebo cream a commonly used topical medication used for wound healing was purchased from a local pharmacy (Gulf Pharmaceutical Industries, Ras Al Khalmah, U.A.E.). (Falconieri et al. 2011)

Animals and animal grouping

Ethical approval was obtained from KFMRC Committee for animal care before experimentation on animals. Thirty adult male rats (200 – 230g) used to create full thickness skin wound model. Animal acclimatization to lab condition was done for 3 days, (at 22 ±1ºC), providing them with standard animal pellets and free access for water *ad libitum*. Animals were randomized sorted into 6 groups (each 5 rats) as follows: G1 (Control) where a marked area was painted with normal saline. G2 (Untreated+W) full thickness wounded rats without any treatment. G3 (W+Mebo) was treated by well-known medicinal preparation (mebo) that is reported to enhance wound healing. G4 (W+*Achillea*) full thickness wound treated by gel made of *Achillea* methanolic extract in a plane base. G5 (W+Honey) wound treated with honey (1g/ day). G6 (W+Mix) wound treated with mixture of honey and *Achillea* extract.

Creation of full thickness wound

The animals were anesthetized with ether; hair on rat back skin (over lumbar vertebrae) was shaved with an electrical shaving machine. Animals were anesthetized before shaving taking to avoid any damage to skin surface. The wound area was marked by permanent marker (2X2 cm). Full thickness skin piece was removed by sharp scalpel and homeostasis ensured by sterile gauze. Analgesics were given in drinking water to ensure relieving pain (Falanga et al. 2007).

Topical treatment design

Wound was washed by sterile saline then daily covered by constant amount of the previous preparations for 21 days.

Wound healing evaluation

The wounded area in all animals was observed for healing criteria or any complications. Wound was photographed by digital camera with well-known scale to measure contraction rate (% wound contraction).

Inflammatory markers determination

At the end of the experiments, blood was collected into plain tubes from retro-orbital veins. Blood centrifugation at 3000 rpm for 15 min to separate serum. Interleukins (IL)-10, IL-6 and tumor necrosis factor (TNF)-α serum levels were assayed utilizing the commercial ELISA kits.

Antioxidants determination

Serum levels of oxidative stress markers as catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), and lipid peroxide (LPO) were determined by ELISA kits according to kits instructions..

Histological study

Skin wound edge area was dissected at 21 days, processed for paraffin sections, staining by hematoxylin and eosin, Masson trichrome, alpha-smooth actin (α-SA) and vascular endothelial growth factor (VEGF) immunoexpression (Bancroft and Gamble, 2008).

Morphometric study

Epithelial thickness and area density of α-SA and VEGF were recorded using pro-j image software connected to Cell Sense Standard camera (KFMRC).

Statistical analysis

The data were presented as mean +/- standard deviation (SD) and were analyzed by IBM SPSS Statistics for Windows, version 23 (IBM SPSS, IBM Corp., Armonk, N.Y., USA) and graph pad prism (version 5.0; Graph Pad software Inc. San Diego CA, California, USA). Shapiro – Wilk test was used to evaluate normal data distribution. Statistical comparisons between groups were done by One-Way analysis of variance (ANOVA) followed by least significant test (LSD). The results was statistically significant if *P*-values were <0.05.

RESULTS

Inflammatory markers analysis

Figure (2) showed differences in TNF-α, IL-6 and IL10 serum levels between control, wounded, mebo, *Achillea* extract, honey and mix treated rats. It was noticed that IL-6 and TNF-α levels were raised, while IL-10 was significantly decreased in G2 (Untreated+W) compared to control. The highest reduction in IL-6 and TNF-α serum levels were demonstrated in rats treated with honey, then *Achillea* extract *Achillea* + honey mix and mebo. For IL-10 serum level *Achillea* extract treated rats showed the highest level seconded by rats treated with mebo, then mix and honey respectively.

Antioxidants analysis

Figure (3) showed differences in GSH, SOD, CAT and LPO between control, untreated wound, mebo, *Achillea*, honey and mix groups. Rats treated with different materials (mebo, *Achillea* extract, honey and *Achillea* + honey mix) illustrated higher levels of GSH, SOD, and CAT compared to control, while in G2 (Untreated+W) demonstrated reduction in these antioxidants compared to control. The highest rise in GSH was in rats treated with (*Achillea* + honey mix) followed by *Achillea* extract, mebo and honey, while GSH level was the least in G2 (Untreated +W) (less than control). Nearly the same trend was observed in the levels of SOD and CAT, where in the four treatment groups (G3, G4, G5 and G6) the level of SOD was increased compared with control and G2 (Untreated+W). As for CAT levels there was significant increase in animals treated with the different materials compared with control and G2 (Untreated+W). There was also reduction in wounded untreated rats compared to control (unwounded untreated). On the other hand, LPO serum concentration was reduced under all treatments with mebo, *Achillea*, honey and *Achillea* + honey mix, while increased in G2 (Untreated+W) compared to control.

Morphometric evaluation of area density of positive stained structures by α-SA and VEGF

Figure (4) showed differences in area density of collagen and immune positive reaction using pro-image analyzer program. There was a high significant increase in density area of mature collagen by different treatments versus control. The highest increase was in honey treatment, then *Achillea* and mix, then mebo the least was in wounded untreated group.

Histopathological studies

Haematoxylin and Eosin stain

Figure (5) Photo taken for slides stained with

H&E help to show the general histological features of normal as well as wounded skin and treatment effects in restoring normal skin histological integrity. In G1 (Control) rats the skin of dorsal or back region is of thin type. It contains hairs, hair follicles and sebaceous glands. The outer epithelial layer or epidermis was formed of few layers of stratified squamous epithelium with thin keratin layer. The underlying connective tissue dermis is relatively thick and contains numerous hair follicles and sebaceous glands which were surrounded by dense connective tissue. In G2 (Untreated+W) there was wide gap and scar area between the edges of normal skin regions lacking epithelial layer. The floor of the wounded area showed numerous new capillaries. Immature poorly stained collagen among numerous which numerous nuclei of inflammatory cells could be seen. Lack of hair follicles and skin glands was observed. G3 (W+Mebo), there was a decrease in wound gap and scar tissue accompanied with proliferation and formation of thick epidermal layer from both sides of the wound edges. The underlying wounded area showed scattered regions of mature collagen, but still there were few inflammatory cells and newly formed blood capillaries. In G4 (W+*Achillea*) marked decreased in wounded area was observed, there was healthy surface epithelial layer. The underlying scar tissue was minimum compared to other groups. In G5 (W+Honey) there was decreases in wounded area and scar tissue with complete surface epithelization. The underlying wound area showed more scattered mature collagen and blood vessels with less inflammatory cells. In G6 (W+Mix) showed similar improvement to both G4 and G5 with a decreased in wound gap. There was also less scar tissues and few blood vessels and inflammatory cells.

Masson stain

Masson stain was used to provide more details regarding collagen content and maturation status based on color intensity of stained fibers. Figure (6) showed sections from all wounded skin (untreated versus treated) compared to control. In G1 (Control) skin collagen fibers were present as bundles randomly distributed in different directions among cutaneous hairs and glands and showed dark blue color by Masson stain. In G2 (Untreated + W) mature dark stained fibers were nearly absent and Masson lightly stains only thin fibers due to absence of mature collagen in wound floor. The wound showed lack of normal epithelization. In G3 (W+Mebo) healthy surface epithelium could be seen but scar area was still wide and collagen looked immature and lightly stained by Masson. In G4 (W+*Achillea*) showed marked approximation of wound edge and decreased in immature collagen area at wounded area compared to normal bordering region. In G5 (W+Honey) showed more approximating edges indicating good contraction, healthy epithelial surface and less area of immature dermal collagen. In G6 (W+Mix) showed epithelial surface in intact with unhealthy hyperplastic appearance, less immature collagen in wound area compared to untreated but more than other treated groups.

Alpha –smooth actin expression (α- SA)

Figure (7) detection of the non- muscular actin alpha –smooth actin expression (α- SA) after 21 days was carried out in all treatments. In G1 (Control), it was detected only in the walls of few blood capillaries most probably the surrounding pericytes. In G2 (Untreated + W) there was evident increase in α SA expressions in both walls of newly formed blood vessels and in numerous proliferating fibroblasts. In G3 (W+Mebo) α- SA increased in the proliferating dermal fibroblasts and newly formed blood capillaries. In G4 (W+*Achillea*) and G5 (W+Honey) and G6 (W+Mix), α- SA expression was markedly increased specially in mixture group.

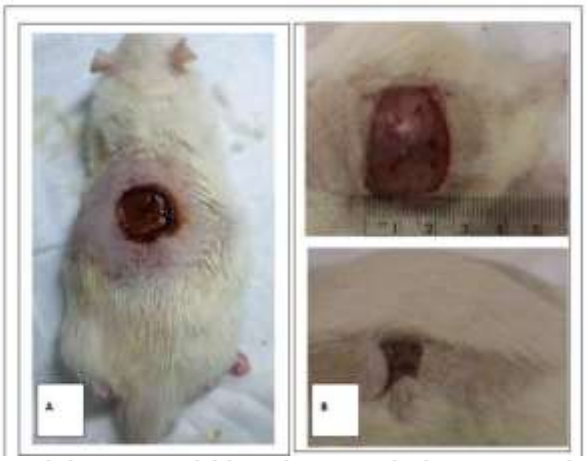

Figure 1: A. Initial excision wound (day 0). B. Healed contracted excision wound

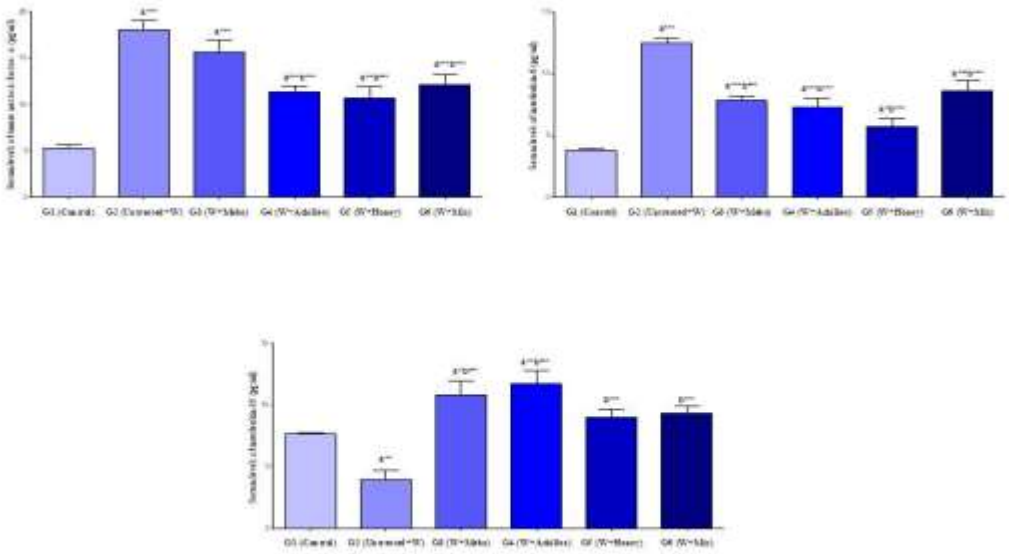

Figure 2: Comparison of serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10 of different studied groups at 21 days post wounding.

Data were represented as mean +/- standard error of mean (SE). a: Significance versus G1 (Control); b: significance versus G2 (Untreated+W). Significance was conducted as **P* <0.05, ***P* <0.01and ****P* <0.001

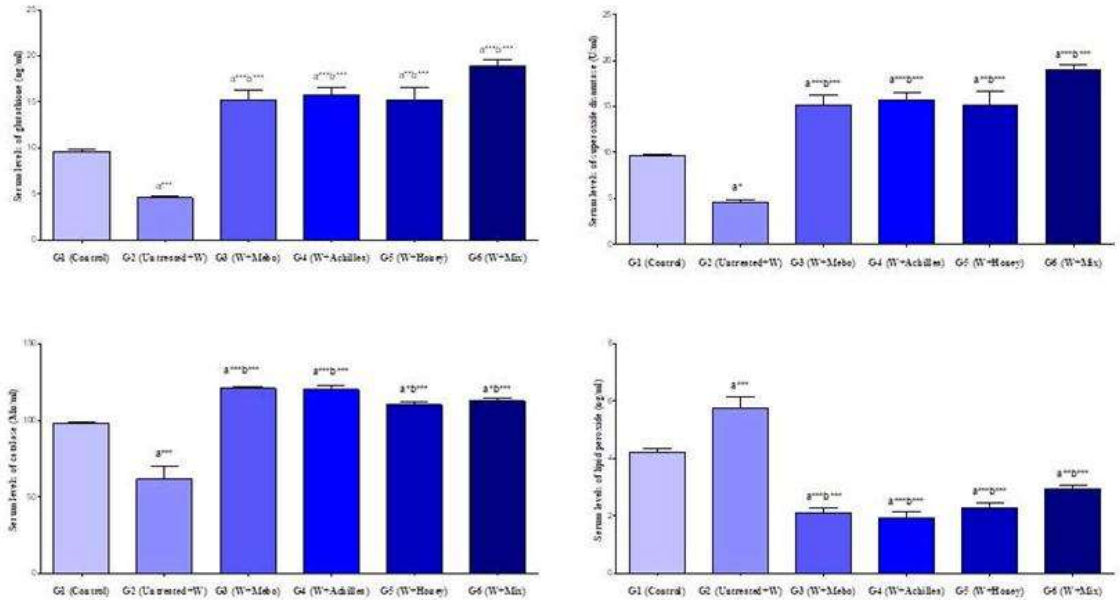

Figure 3: Comparison of serum levels of superoxide dismutase (SOD), glutathione (GSH), catalase (CAT) and lipid peroxide (LPO) serum levels in different studied groups at 21 days post wounding.
Data were represented as mean +/- standard error of mean (SE). a: Significance versus G1 (Control); b: significance versus G2 (Untreated+W). Significance was conducted as **P* <0.05, ***P* <0.01and ****P* <0.001.

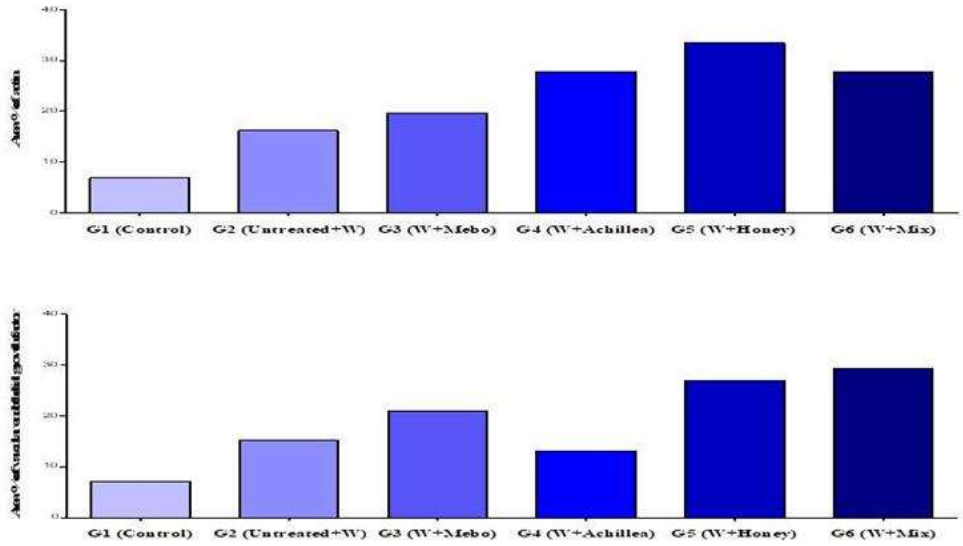

Figure 4: Comparison of area density of positive stained structures by alpha- smooth actin (α-SA) and vascular endothelial growth factor (VEGF) in different studied groups in all days (21) of wound healing.
Data were represented as mean +/- standard error of mean (SE). a: Significance versus G1 (Control); b: significance versus G2 (Untreated+W). Significance was conducted as **P* <0.05, ***P* <0.01and ****P* <0.001.

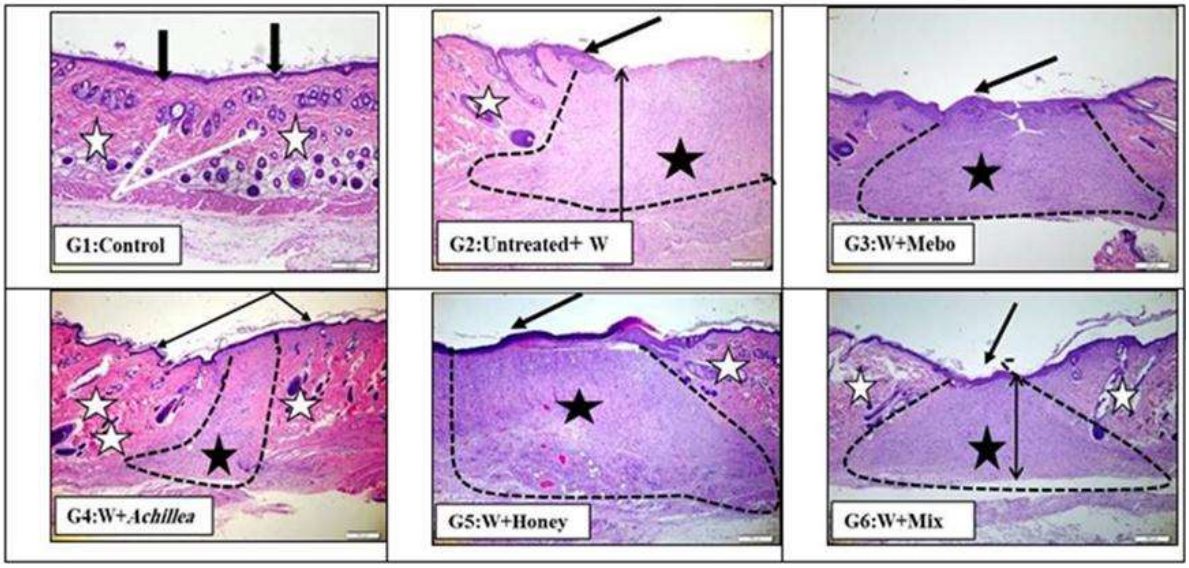

Figure 5: Sections of rat back skin (21 days post wounding) stained by (H&E X 40) to show control and healed skin of all groups:

G1 (Control) showed thin epidermis (thick black arrows), deep dermis with well-stained collagen (white stars) surrounded complex hair follicles and their sebaceous glands (white arrows). G2 (Untreated+W) showed wide gap and scar area (dotted outline) free of hair follicles or glands with immature lightly stained collagen (white star), with more mature collagen (black star) compared to normal skin at wound edge; notice the hyperplastic epidermal growth (black arrow). G3 (W + Mebo) showed thick epidermal surface (arrow), larger scar tissue (dotted outline) with more mature collagen (black star than wounded skin). G4 (W+*Achillea*) showed marked narrowing of scar area (dotted outline), more mature collagen (black star), and glands with immature lightly stained collagen (white star). Normal thin epidermis at both wound edges (arrows). G5 (W+Honey) showed normal epithelial regeneration (surface arrow), glands stained collagen (white star), but still wide scar area with more mature collagen fiber formation (black star and dotted outline). G6 (W+Mix) showed epithelization (arrow) with less scar area (black star).

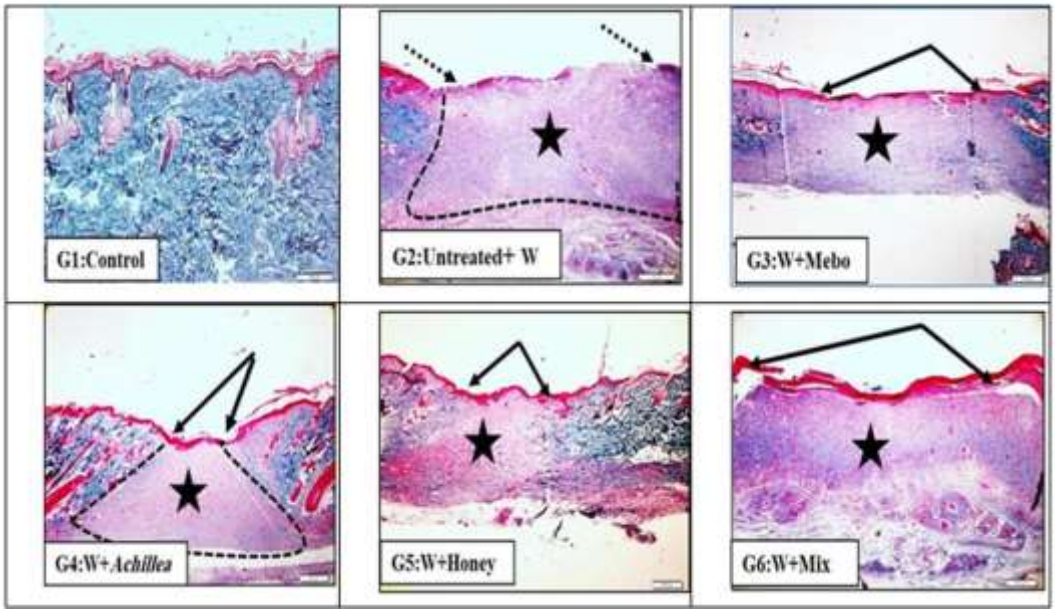

Figure 6 : Sections of control, wounded and different treated groups rat dorsal skin stained by

Masson (X 40) for collagen. G1 (control unwounded skin) normal mature collagen fibers distribution stained dark blue in rat skin dermis. G2 (Untreated+W) showed pale blue staining due to absence of mature collagen in skin wound floor (star). Notice lack of normal epithelization (dotted arrows). G3 (W+ Mebo): showed contracted wound with approximating wound edge (black arrows) with intact epithelial surface. Less immature collagen in the dermis of wound area (star).
G4 (W+*Achillea*): showed marked approximation of wound edge (black arrows) and decreased in immature collagen area at wounded area (star) compared to normal bordering region (dotted arrows). G5 (W+Honey): showed more approximating edges (black arrows) indicating good contraction, health epithelial surface and less area of immature dermal collagen (star). G6 (W+Mix): showed epithelial surface with unhealthy hyperplastic appearance (black arrows), less immature collagen in wound area (star) compared to non- treated but more than other treated groups.

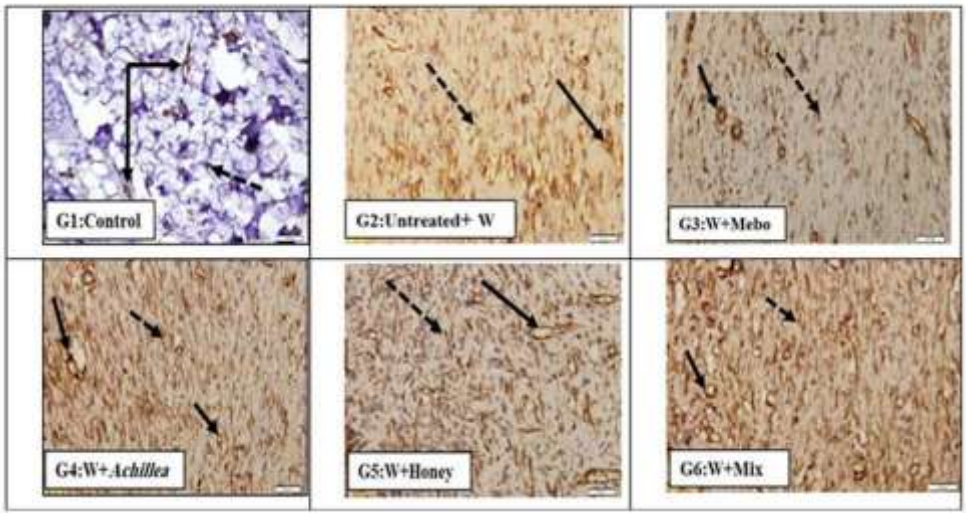

Figure 7: Sections of rat skin treated (21 days) stained for alpha –smooth actin expression (α- SA) immune-expression (X 40). G1 (Control): showed weakness of alpha smooth expression in the walls of few blood capillaries (black arrow) and absence in dermal fibroblast (dotted arrows). G2 (Untreated+W): showed moderate expression of α- SA in dermal fibroblast (dotted arrows) and blood capillary walls (black arrow). G3 (W+Mebo): showed increased expression of α- SA in the proliferating derm al fibroblasts (dotted arrow) and newly formed blood capillaries (black arrow). G4 (W+*Achillea*): showed also more increased expression of α - SA in dermal fibroblast (dotted arrow) and blood capillaries (black arrow) compared to mebo group . G5 (W+Honey): showed more increased expression of α- SA in dermal fibroblast (dotted arrow) and blood capillaries (black arrow) compared to mebo group. G6 (W+Mix): showed the most evident increase of α- SA expression in dermal (dotted arrow) and blood capillaries (black arrow) compared to other treated groups.

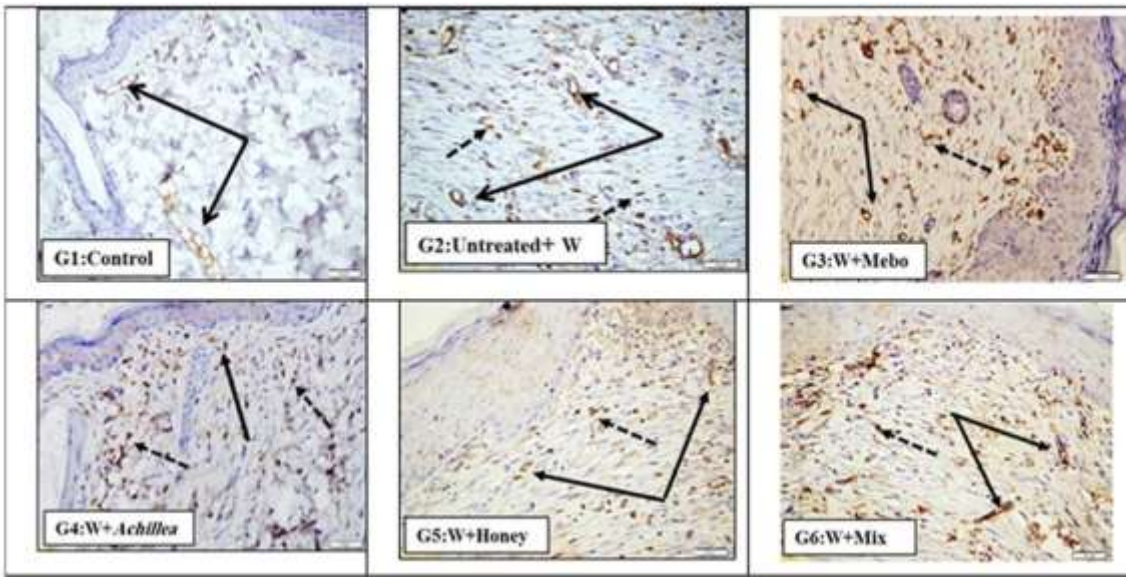

Figure 8: Sections from rat skin immune-stained for vascular endothelial growth factor (VEGF) (X 40) (21 days). G1 (Control): showed moderate VEGF expression in the few blood capillaries in skin dermis (Black arrows). G2 (Untreated+W): showed increased immuno-positive reaction for VEGF in the numerous newly formed capillaries (black arrows) and dermal fibroblasts (dotted arrows). G3 (W+Mebo): showed apparent decrease in blood vessels (black arrows) expressing positive VEGF marker, numerous fibroblasts showed positive reaction (dotted arrows). G4 (W+*Achillea*): showed tiny blood capillaries with mild VEGF immune-positive reaction with numerous fibroblasts showed high positive expression (dotted arrows). G5 (W+Honey): showing marked decrease in the blood vessels (black arrows) expressing positive VEGF marker, still fibroblasts (dotted arrows) exhibits positive reaction. G6 (W+Mix): showed decreased blood vessels expressing VEGF immune-positive reaction (black arrows) with numerous fibroblasts showed high positive expression (dotted arrows).

Vascular endothelial growth factor stain

VEGF use for detecting reactive endothelial cells in biological tissue, in this study it was observed that VEGF expression Figure (8): In G1 (Control) few capillaries expressed faint VEGF. In G2 (Untreated+W) showed increased immune-positive reaction for VEGF in the numerous newly formed capillaries and dermal fibroblasts. In G3 (W+Mebo) an apparent decreased in blood vessels expressing positive VEGF marker was observed while numerous fibroblasts showed positive reaction. In G4 (W+*Achillea*): few tiny blood capillaries showed mild VEGF immune positive reaction with numerous fibroblasts with high positive expression. In G5 (W+Honey): showed marked decrease in the blood vessels expressing positive VEGF marker. Still fibroblasts exhibited positive reaction. In G6 (W+Mix): a decrease in blood vessels expressing VEGF immune positive reaction was observed with numerous fibroblasts showed high positive expression.

DISCUSSION

Any breakdown in the normal anatomical structure of the skin, or damage or disruption is referred to as cutaneous wound. Wound healing is a series of biochemical and cellular processes of restoration of the damaged cellular structures and tissue layers and returning it to its normal state (Fronza et al. 2014). Wound healing passes through four overlapping phases, hemostasis, inflammation, proliferation, and remodeling phases (Norouzi et al. 2015).Wound damage repairing passes through different phases, the initial infection phase, distribution and movement of epithelial cells, formation of granulation tissue and wound contraction (Shanbhag et al. 2010).

The results recorded in this research suggested that treatment with mebo, *Achillea* extract, honey, *Achillea*-honey mix, inhibited productions of IL-6 and TNF-α in wounded treated rats compared to untreated rats, with honey attained the significantly higher reduction of IL-6 and TNF-α serum levels followed by *Achillea* + Honey Mix and *Achillea*. Meanwhile, serum levels of interleukin IL-10 were significantly elevated in the treated wounded rats versus wound untreated

rats and the highest level was in *Achillea* extract treatment, followed by mebo. This was in agreement with Horton (Horton, 2003) who found that values of pro-inflammatory markers, IL-1β and TNF-α increased in injured cells. TNF-α might have lowered vascular endothelial cells activity and affected endothelial permeability by activating tyrosine kinases (Samarghandian et al., 2017). TNF-α worked on the endothelial cells for adhesion of intracellular adhesion molecule 1 (ICAM-1) that related to endothelial–monocyte interactions in early thermogenesis. TNF-α induction to ICAM-1 resulted in initiation of adhesion of neutrophils to endothelial cells, and this would also resulted in enhancement of cascade of other inflammatory mediators (Hashizume and Mihara, 2012). TNF-α stimulates adhesion of leukocyte endothelial and tissue infiltration by initiating endothelial cells, resulting in local inflammation (Jia and He, 2016).

In addition, the results indicated that treating wounds with mebo, and *Archillea* extract. honey and *Archillea* + Honey Mix had enhanced activities and increased serum levels of antioxidant enzymes GSH, SOD,and CAT in wounded rats compared to control. SOD was known to modify oxidation state by removing free superoxide radicals. Catalase break down toxic hydrogen peroxide to its components (oxygen and water) (Mohammad et al. 2011). Mebo, *Achillea* extract, honey and mix treated rats, had been acted through decrease in free radical generation that resulted in restoration the catalytic enzymes SOD, GSH and CAT activities and forming antioxidant defense, as suggested by (Funk et al. 2012) in his study on effects of saffron extract on injured rats.(Sachdeva et al., 2012) revealed that aqueous extract of saffron improves injury in rats by strengthening antioxidant defense system. Lipid peroxidation marker level was decreased in treated rats versus untreated wounded animals, the highest reduction was under *Achillea* extract treatment, followed by *Achillea* + Honey Mix. In this respect, (Samarghandian et al. 2017) demonstrated that injured untreated rats showed significant increase in total lipids and malondialdehyde (MDA) levels compared to the control group, where injured rats treated with Saffron extract undergone reduction in the serum values of total lipids and MDA during the experimental period. Research studies reported increase in lipid peroxidation activity and a reduction in level of SOD due to oxidative stress (Toyokuni, 2008). Saffron was found to scavenge radicals that inhibited lipid peroxidation in wounds, and also could decrease lipid peroxidation (Papandreou et al. 2011).

The present results also showed absence of mature collagen in G2 (Untreated + W) while unwounded group have normal mature collagen distribution. Formation of mature collagen and myofibroblasts are considered the most important factors, which help, wound contraction and restore normal skin structure after epithelization. Reduction in collagen and fibroblast deposition delays wound healing (Wicke et al., 2000). (Sarkar et al., 2018) suggested that honey is capable of restoring back collagen to normal in wounded rats through collagen deposition. Collagen is an important connective tissue fibers connecting element and is found in excess in many tissues especially skin tissue (Gelse et al. 2003), with vital role in wound healing process (Roh and Lyle, 2006; Yamaguchi and Yoshikawa, 2001). The enhanced of collagen formation in skin wounds of animals treated with different elements (using Masson stain) is attributed to active fibroblast proliferation as was documented by (Muskhelishvili et al. 2003).

Considering the histopathological studies, hematoxylin and eosin stained sections from untreated wounded rats showed lack of hair follicles and skin glands, wide gaped and scar area between the edges of skin wounds. Under treatment with mebo wound gap and scar tissue started decreasing. In G_4, G_5,and G_6 treated wounds there was gradual decrease in wounded area, and scar tissue, appearance of healthy surface and mature epithelial layer with more scattered mature collagen, blood vessels and less inflammatory cells. Staining with Masson stain revealed absence of mature collagen and of normal epithelium in untreated wounded animals (control- unwounded), while G_3 (W+Mebo) showed features of contraction in wounded area and less immature collagen. G_4 (W+*Achillea*) showed marked contraction of wounded area, and decrease in immature collagen area. Treatment G_5 (W+Honey) showed also good contraction in wounded area, healthy epithelial surface and less area of immature dermal collagen, and G_6 (W+Mix) showed less immature collagen in wounded area compared to G2 (Untreated+W) but more than in other treatments. This observation in collagen formation and maturation in the wounded areas after treatment with *Achillea* extract, honey, mebo and mix can be attributed to the efficient activity of fibroblasts as was suggested by (Muskhelishvili et al. 2003).

Using actin-stain G2 (Untreated+W) had

undergone moderate alpha smooth actin (α-SA) expression in fibroblasts and blood capillaries. In actin stain showed the non- muscular actin alpha –smooth actin expression (α-SA) after 21 days was carried out in all treatments increased in G3 (W+Mebo) in proliferating dermal fibroblasts and newly formed blood capillaries and in G4 (W+*Achillea*) and G5 (W+Honey) and G6 (W+Mix). Alpha –smooth actin expression was markedly increased specially effect on mixture group. In G2 (Untreated + W), there was evident of increased in actin expressions in both walls of newly formed blood vessels and in the numerous proliferating fibroblasts. The present results coincide with (Zhou et al. 2013) who found rise in collagen synthesis rate up to 480% and 860% in the wounded muscle after 2 and 7 days respectively after surgery compared to undamaged area of the same muscle, and collagen content raised by up 100% after 2 and 7 days.

VEGF has an important action in wound healing (Barrientos et al. 2008).In this study, VEGF serum levels increased under *Achillea* + Honey, Mix, ~~then~~ Honey, ~~then~~ Mebo, ~~then~~ untreated wound and *Achillea*. Levels of VEGF increased in the blood vessels in early stages of wounding and decreased later after 24 days in wounded treated by (Fronza et al. 2014). (Barrientos et al. 2008) showed that wound healing is a complex process that regulated by numerous factors including VEGF.

The present study suggested VEGF level was decreased because well-wound healing efficiency depend on normal effective neovascularization to provide required essential nutrients and oxygen for repaired tissue. In agreement, (Fronza et al., 2014) reported that VEGF was expressed in wounded area within 24H increased at 2–3 days and then declined on the following stages. (Savari et al., 2019) reported high VEGF gene expression in the first week with a decrease in the third week in the phenytoin-treated group versus control group. And the results indicate significant relationship between these two genes (VEGF and transforming growth factor-β) and the rate of wound healing in rats. (Lin et al., 2017) emphasized the important role played by VEGF in diabetic wound healing delay of healing upon reduction. In study carried out by (Omar et al. 2020) on two groups of patients to test magnitude of IL-6, Matrix metallopeptidase 9 (MMP-9) and VEGF. The results showed significant differences between IL-6 and VEGF ($P<0.05$), on week four and week six for IL-6 whereas VEGF revealed significant changes ($P<0.05$) the first and third days and week four and six, while VEGF was increased

CONCLUSION

Achillea extract gave significant improvements in all measured parameters in this study and was even better than honey in some cases and better than mebo in nearly all cases. Such conclusions were based on increase in level of interleukin IL-10, CAT, GSH and reduction of values of IL-6, TNF-α and LPO, as well as the improvement of collagen in wounds healing. *Achillea* extract alone or mixed with honey can efficiently enhance wound healing and regulates the process of tissue repair. It could be advised to be included in dressing of wounds with large defects to enhance both epithelization and wound contraction especially if mixed with honey. More work is recommended on such natural substances for use in clinical trials..

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

TMAlS designed and performleed the animal experiments, analyzed the results and draft the manuscript. The author read and approved the final version of manuscript for publication.

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