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Der Pharmacia Lettre, 2015, 7 (12):296-305
(http://scholarsresearchlibrary.com/archive.html)

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ISSN 0975-5071
USA CODEN: DPLEB4

Pharmacological evaluation of honey, Daruhaldi and Shatdhautghrut on wound healing activity in incision model in rats

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ABSTRACT

Aim of present investigation was to evaluate wound healing activity of Honey, Daruhaldi and Shatdhautghrut singly and in combination on experimentalexcision wound healing activity in rats. Incisions wounds were used to study the topical effect of Honey, Daruhaldi and Shatdhautghrut singly and in combination for 20 days (once a day) on various biochemical parameters. Honey + Daruhaldicombination treatment showed significant increase (p <0.01) in tensile strength, hydroxyproline content and total protein level as compared to vehicle control rats. Daruhaldi showed more significant (p <0.01) comparable effect in increasingtensile strength, hydroxyproline content and total protein level as compared to vehicle control rats as well as Honey + Shatdhaut or Honey orShatdhaut. When compared with vehicle control rats, topical administration of Honey + Shatdhaut or Honey orShatdhaut showed significant increase (p <0.05) in tensile strength, hydroxyproline content and total protein level. In conclusion, oral administration of honey, Daruhaldi and Shata-dhauta-ghrita as well as their combinations promotes faster wound healing by acting simultaneously favorably on various processes of wound healing such as collagen synthesis and epithelization. The presence of flavonoid moities in honey and Daruhaldi combination showed better wound healing potential than Honey &Daruhaldi alone.

Keywords: Daruhaldi, Honey, Hydroxyproline, Incision wound, Shatdhautghrut, Tensile strength.

INTRODUCTION

Wounds are inescapableparts of life and that may arise due to any agent that induces stress & injury[1-4]. Wound healingis an intricate process in which involve an arrays of biochemical and cellular process. Healing of wound is necessary for survival and it represents an attempt to maintain normal anatomical structure and function[5].In normalskin, the epidermis (outermost layer) and dermis (inner or deeperlayer) exists in steady-state equilibrium, forming a protectivebarrier against the external environment[6, 7].

The healing of wound involves the complex cascade of event starts from the moment of injury and continues for varying periods of time depending on the severity of wounding[8, 9]. It is comprised of three phases: initial inflammatory phase consisting of the establishment of homeostasis and inflammation)whereas secondary proliferative phase consisting of granulation, contraction and epithelialization and finally the remodeling phase which ultimately determines the strength and appearance of the healed tissue [10]. It forms anintricate network of blood cells, cytokines and growth factors which ultimately leads to the restoration to the normal condition of the injured skin or tissue[11].

Management of under healing of wounds is a complicated and expensive program and research on drugs that increase wound healing is a developing area in modern biomedical sciences[12-15]. The central dogma for the optimal wound healing includes decrease tissue damage, removal of nonviable tissue, maximizing tissue perfusion oxygenation and proper nutrition [16]. In various conditions such as severe burns, trauma, diabetic decubitus and venus stasis ulcers, there is a need of rapid and proper healing [17]. Hence there is a need for rapid wound healing treatment with minimal pain, discomfort and scarring to the patients within the physiologic environment that is favorable to tissue repair and regeneration [18].

In the wake of advances in the pharmaceutical drug discovery the availability of agents having capacity of stimulating the process of wound repair is still limited [19]. Moreover, defining the particular wound treatment within the increasing number of patients with wounds is another challenging task. Existing therapies for the management of wound has high cost and also possesses unwanted side effects [20, 21]. Hence, there is a need for the rational pro-healing agents that can promote healing with minimal side effect and thereby reduce the cost of hospitalization and save the patient from amputation or other severe complications.

Incision wound model is well established and reproducible animal modelthat is used regularly in preclinical studies of drugs in wound healing [22-25]. The multitude of cellular events that occurs in animals including cell proliferation, cell migration, contraction and extracellular matrix degradation and synthesis, etc. have grate clinical relevance in wound healing process [11].

An array of drugs ranging from simple non-expensive analgesics to complex and expensive chemotherapeutic agents administered in the management of wound affect healing either positively or negatively [26]. Aspirin, indomethacin, cytotoxic agents and immunosuppressant have been proved experimentally to affect healing negatively[27-29].The treatment of wounds with traditional medicine is locally available at low cost with the advantage of local knowledge of indigenous treatments [30].Severaldrugs obtained from plant sources are known to increase the healingof different types of wounds[31, 32].

According to Ayurveda, the various plants and other traditional ayurvedic formulation are useful in wounds healing.Honey has been reported to be effective in gastrointestinal disorders [33, 34], in healing of wounds and burns [35, 36], as an anti-microbial and antifungal agent [37, 38]. Daruhaldi have been used in ethno medicine and in many Ayurvedicpreparation for several medicinal properties including antibacterial, antiperiodic, antidiarrhoeal, ophthalmic and in skindiseases[39, 40].Shatdhautghrut is an Ayurvedic preparation which is a 100 times washed clarified butterfat that has beed used for management of conditions like burns, chicken pox, scars, herpes, leprosy and other skin diseases [41].

In view of the above the present study was planned to study the therapeutic potential of Honey (H), Daruhaldi (DH) and Shatdhautghrut (SDG) as well as their combination i.e. Honey + Daruhaldi (H + DH) and Honey + Shatdhautghrut (H + SDG) on experimentally induced incision wounds in rats.

MATERIALS AND METHODS

2.1. Material:

Standard Agmark brand of Honey (H), water based paste of Daruhaldi (DH) and Shatdhautghrut (SDG) were purchased from VatsalAyurvedic, Nasik.

2.2. Animals:

Healthy adult male Sprague-Dowley rats (150-200 g) were obtained from the D. Y. Patil Medical College, Pune (India). The animals were housed in groups of 7 in solid bottom polypropylene cages. They were maintained at 24^0C ± 1^0C, with relative humidity of 45-55% and 12:12 h dark/light cycle. The animals were acclimatized for a period of two weeks and were kept under pathogen free conditions. The animals had free access to standard pellet chow (Chakan Oil Mills, Sangli) throughout the experimental protocol. The animals were provided with filtered water. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Padmashree Dr. D. Y. Patil Medical College, Pune.

2.3. Chemicals:
Ketamine manufactured by Aqua Fine InjectaPvt Ltd., Pune was purchased from local Chemist. Anesthetic ether, ethanol, formalin, sodium hydroxide, chloroform, ether, hydrochloric acid and conc. Sulphuric acid were purchased from S.D. Fine Chemicals, Mumbai, India.

2.4. Incision wound model:[42]
All the animals were anaesthetized with ketamine and the back hair of the rats were shaved by using a shaving machine. Six centimeter long, two linear-paravertebral incisions were made with a sterile surgical blade through the full thickness of the skin at the distance of 1.5 cm from the midline of each side of the vertebral column. The wounds were closed with three surgical interrupted sutures of 1 cm apart. All the sutures used in the experiments were non-absorbable braided non-capillary and siliconized. The study comprised six different groups of six animals in each groups as follows and the ointment was tropically applied once in a day. The sutures were removed on 8^{th} post wound day. The skin breaking strength, total protein and hydroxyproline content of the wounds were measured on 10^{th} day.

Group I - Control animals: did not receive any cream or drug treatment.
Group II - Honey (H) treated animals: received treatment with Honey (1 ml)
Group III- Daruhaldi (DH) treated animals: received treatment with Daruhaldi(1 ml)
Group IV-Shatdhautghrut (SDG) treated animals: received treatment with Shatdhautghrut(1 ml)
Group V - Honey + Daruhaldi (H + DH) treated animals: received treatment with combination of Honey + Daruhaldi (H + DH) (1:1)(1 ml)
Group VI - Honey + Shatdhautghrut (H + SDG) treated animals: received treatment with combination of Honey + Shatdhautghrut (H + SDG) (1:1) (1 ml)
Group VII - Povidon iodide (PI) treated animals: received treatment with Povidon iodide (1 ml)

2.4.1. Measurement of tensile strength:[43]
On the 10^{th} day the animals were sacrificed and there tensile strength was measured as follows:
After sacrificing the animals after anaesthesia, sutures were gently pulled out. Both wound areas from each animal were removed carefully. Wound stripes of equal size (width) were then cut using a knife in which two blades were fixed at a fixed distance. Both ends of each strip were fixed with the help of a pair of steel clips. One clip allowed hanging on a stand and a polyethylene bottle was then allowed to fill with water gradually till the wound strip was broken at the site of wound. The amount of water required to break the wound was noted and expressed as tensile strength of wound in gm.

2.4.2. Estimation of Collagen (Hydroxyproline Content):[44-46]
Wound tissues were analyzed for hydroxyproline content, which is basic constituent of collagen. The collagen composed of amino acid (hydroxyproline) is the major component of extra-cellular tissue, which gives strength and support. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of hydroxyproline hence can be used as a biochemical marker for tissue collagen and an index for collagen turnover. For preparation of protein hydrolysate, 50 mg of tissue sample in 1.0 ml of 6.0N HCl was weighed and sealed in screw-capped glass tube. The tubes were autoclaved at 151.056 kg/ cm^2 for 3 h. The hydrolysate was neutralized to pH 7.0 and brought to the appropriate volume (filtered if necessary). Test tubes marked as sample, standard and blank were taken. One milliliter of test sample was added to test tubes marked as sample, 1.0 ml of DM water to test tubes marked as blank and 1.0 ml standard solutions to test tubes marked as standard. One milliliter of 0.01M copper sulphate solution was added to all the test tubes followed by the addition of 1.0 ml of 2.5N sodium hydroxide and 1.0 ml of 6% hydrogen peroxide. The solutions were occasionally mixed for 5 min and then kept for 5 min in a water bath at 80^0C. Tubes were chilled in ice-cold water bath and 4.0 ml of 3.0N sulphuric acid was added with agitation. Two milliliters of p-(dimethylamino) benzaldehyde was then added and heated in water bath at temperature 70^0C for 15 min. The absorbance was measured at 540 nm. The concentration of the sample was calculated as:

$$\text{Concentration of the sample} = \frac{\text{OD of the sample}}{\text{OD of sta...}} \times \text{Concentration of standard}$$

2.5. Data and statistical analysis:

All the results were expressed as mean ± S.E.M. Data analysis was performed using GraphPad Prism 5.0 software (GraphPad, San Diego, USA). Statistical comparisons were made between drug-treated groups and disease control animals. Data of tensile strength and hydroxyproline content was analyzed using one-way ANOVA; Dunnett's multiple range test was applied for post hoc analysis. A value of p <0.05 was considered to be statistically significant.

RESULTS

3.1. Effect of treatment of Honey, Daruhaldi and Shatdhautghrut on incision wound healing:

A regular wound healing pattern was observed in honey alone (0C), shatdhautghrut alone (0E) and Honey + Shatdhautghrut combination (0G) treated rats. The optimal wound healing was evidenced indaruhaldi treated rats (0D). The complete wound healing was observed in the povidone iodide(0B)andHoney + Daruhaldi combination (0F) treated rats.In vehicle control rats, the wound closure was delayed (0A).

3.2. Effect of treatment of Honey, Daruhaldi and Shatdhautghrut on tensile strength:

Tensile strength in vehicle control rats was 158.33 ±16.17 gm/cm^{2}on right side of the wound whereas on the left side it was 181.50 ± 12.12 gm/cm^2. Rats treated with the honeyshowed significant increased (p <0.05) in the tensile strength(328.16 ± 30.57gm/cm^{2}on right side and337.16 ± 30.19 gm/cm^2 on left side) on both side as compared to vehicle control rats. Shatdhautghrut treated rats also showed significant increased (p <0.05)in the tensile strength (281.16 ± 37.08 gm/cm^2 on right sideand 273.50 ± 31.42 gm/cm^2 on left side) as compared to vehicle control rats. Tensile strength on the right side of wound in the daruhaldi treated rats was 582.33 ± 42.96 gm/cm^2 whereas 555.16 ± 31.90 gm/cm^2 was the tensile strength on the left side of the wound which was found significantly increased (p <0.001 and p <0.01 respectively) as compared to vehicle control rats.Honey and Shatdhautghrut combination treated rats showed a significant increase (p <0.05)in the tensile strength (373.33 ± 25.11 gm/cm^2 on right side and 386.66 ± 28.97 gm/cm^2 on left side) as compared to vehicle control rats. When compared with the vehicle control rats the tensile strength in the honey and daruhaldi combination treated rats was significantly increased (644.50 ± 28.39 gm/cm^{2}on right side and 666.66 ± 16.66 gm/cm^2, p <0.001). Povidone Iodide treated rats showed significant increased (p <0.001) in the tensile strength (687.16 ± 35.39 gm/cm^2 on right side of wound and 726.00 ± 39.12 gm/cm^2 on left side of wound) as compared to vehicle control rats (Figure 2).

3.3. Effect of treatment of Honey, Daruhaldi and Shatdhautghrut on hydroxyproline content:

The hydroxyproline level in the vehicle control rats was 4.09 ± 0.20 X10^{3}µg/gm of tissue. Treatment with Shatdhautghrut showed significant increased in the hydroxyproline level 5.19 ± 0.26 X10^{3}µg/gm of tissue as compared to vehicle control rats. Hydroxyproline level in the honey treated rats was 5.36 ± 0.22 X10^{3}µg/gm of tissue which was significantly higher (p <0.01) than vehicle control rats. 6.82 ± 0.22 X10^{3}µg/gm of tissue was the hydroxyproline level of the Daruhalditreated rats which was significantly increased as (p <0.001) compared to vehicle control rats. Honey and Shatdhautghrut combination treated rats also showed significant increased (p <0.001) in the hydroxyproline level (6.42 ± 0.24 X10^{3}µg/gm of tissue) as compared to vehicle control rats. When compared with vehicle control rats,Honey and Daruhaldicombinationtreated rats showedsignificant increased in thehydroxyproline level (7.99 ± 0.27 X10^{3}µg/gm of tissue, p <0.001) (Figure 3).

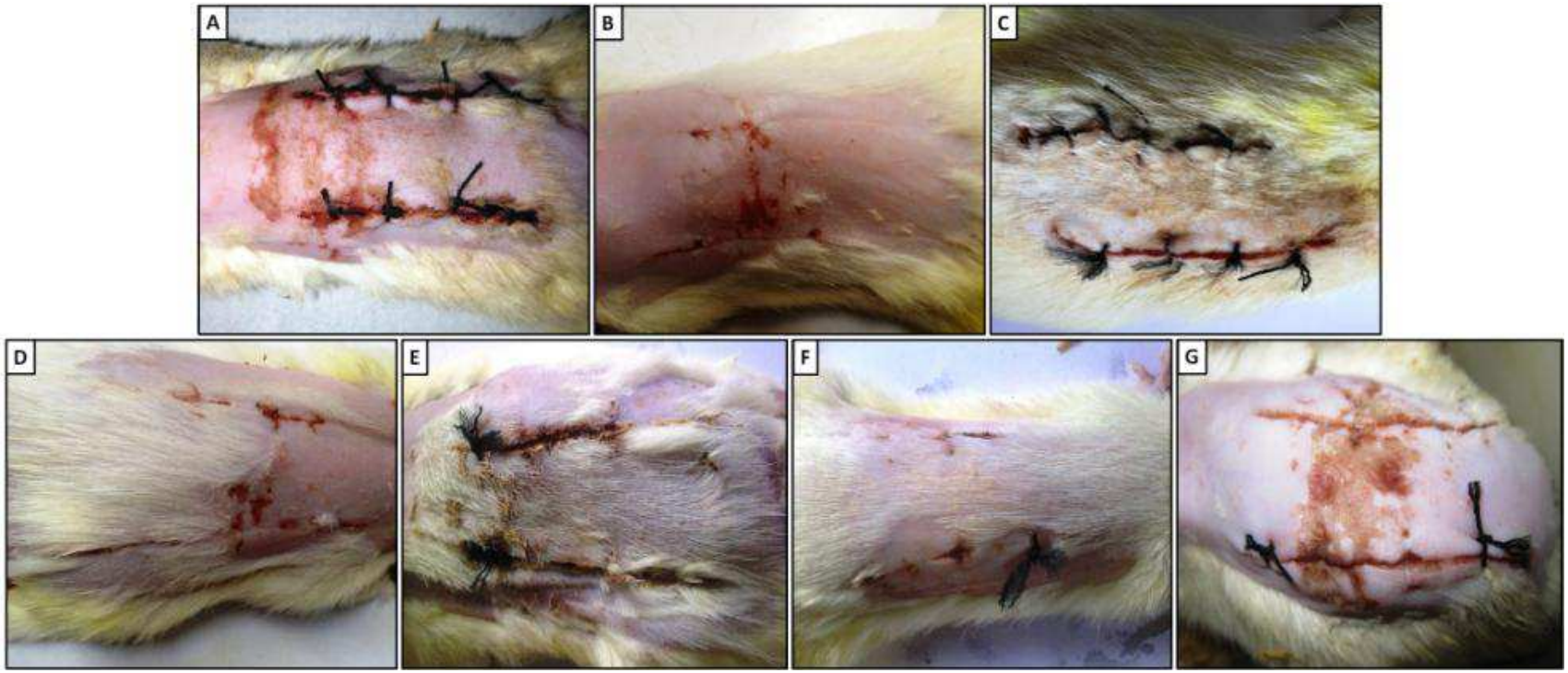

Figure 1. Photographs of rats showing effect of treatment of Honey, Daruhaldi and Shatdhautghrut on incision wound. Representative rats photograph of vehicle control (A), Povidone Iodide (B), Honey (C), Daruhaldi (D), Shatdhautghrut (E), Honey + Daruhaldi (F) and Honey + Shatdhautghrut (G)

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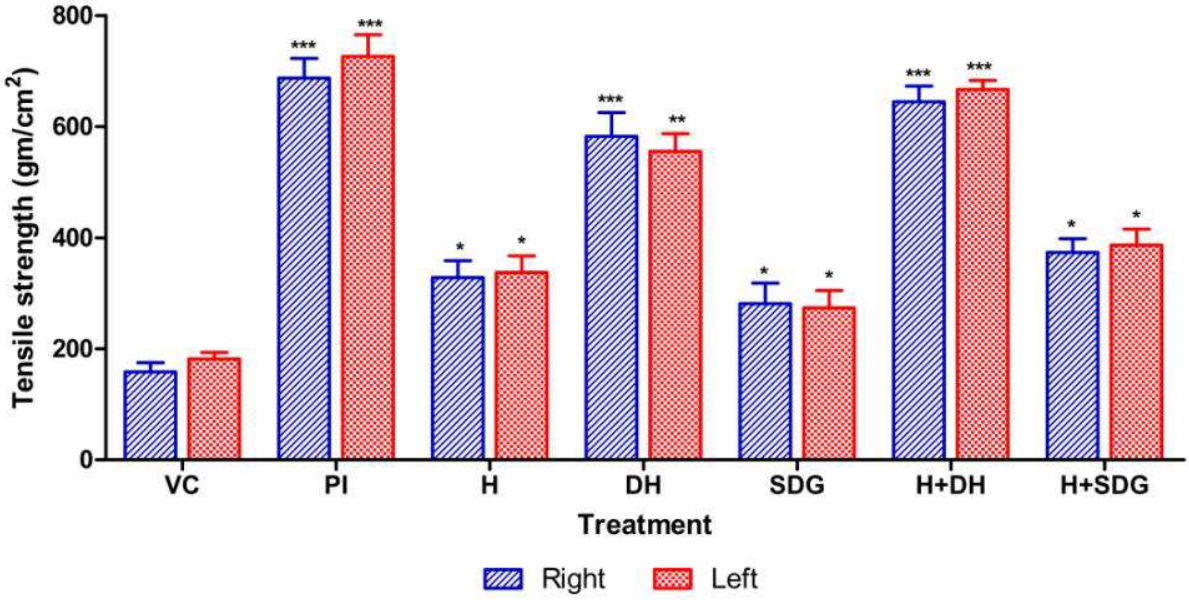

Figure 2. Effect of treatment of Honey, Daruhaldi and Shatdhautghrut on tensile strength in rats
*Data are expressed as mean ±S.E.M. from six rats and analyze by one Way ANOVA followed by Dunnett's test. *p <0.05, **p <0.01, ***p <0.001 as compared to vehicle control group rats*

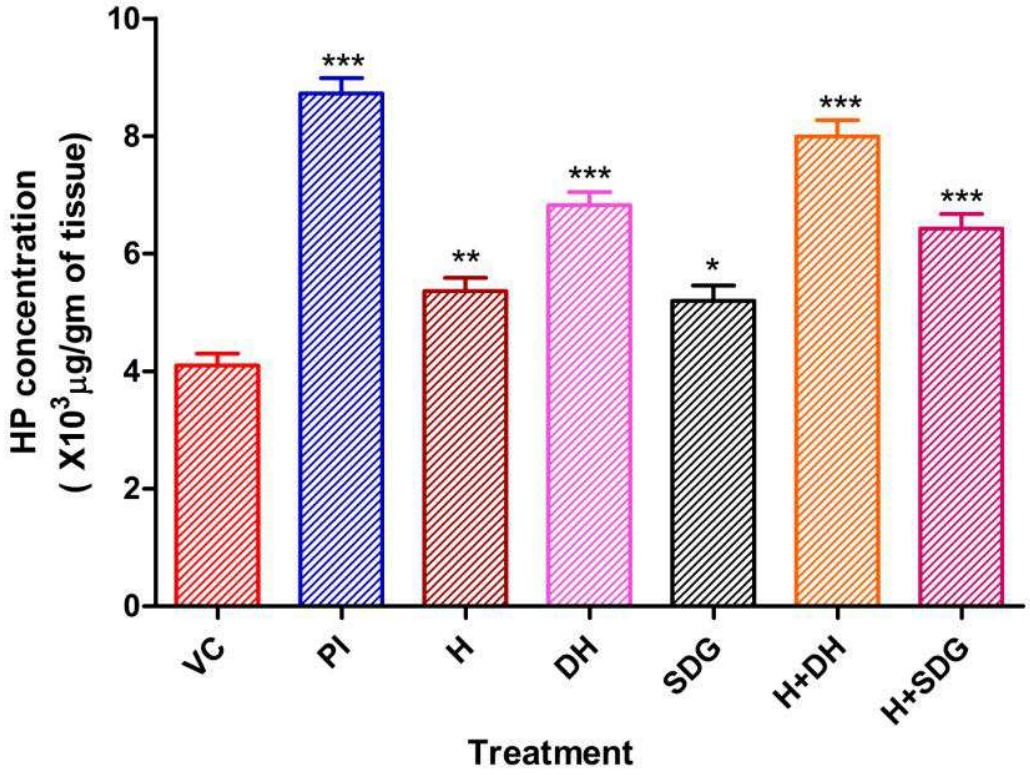

Figure 3. Effect of treatment of Honey, Daruhaldi and Shatdhautghrut on hydroxyproline content in rats
*Data are expressed as mean ±S.E.M. from six rats and analyze by one Way ANOVA followed by Dunnett's test. *p <0.05, **p <0.01, ***p <0.001 as compared to vehicle control group rats*

3.4. Effect of treatment of Honey, Daruhaldi and Shatdhautghrut on total protein level:

The total protein level in the vehicle control rats was 42.88 ± 2.18mg/gm of tissue. Total protein level in the honey treated rats was 59.17 ± 2.17mg/gm of tissue which was significantly higher (p <0.01) than vehicle control rats. 69.80 ± 0.83mg/gm of tissue was the total protein level of the Daruhalditreated rats which was significantly increased as (p <0.001) compared to vehicle control rats. Honey and Shatdhautghrut combination treated rats also showed significant increased (p <0.001) in the total protein level (62.40 ± 2.16mg/gm of tissue) as compared to vehicle control rats. When compared with vehicle control rats, Honey and Daruhaldicombinationtreated rats showedsignificant increased in thetotal protein level (68.17 ± 4.63mg/gm of tissue, p <0.001). Treatment with Shatdhautghrut failed to produce significant increased in the hydroxyproline level (50.45 ± 1.52 mg/gm of tissue as compared to vehicle control rats(Figure 4).

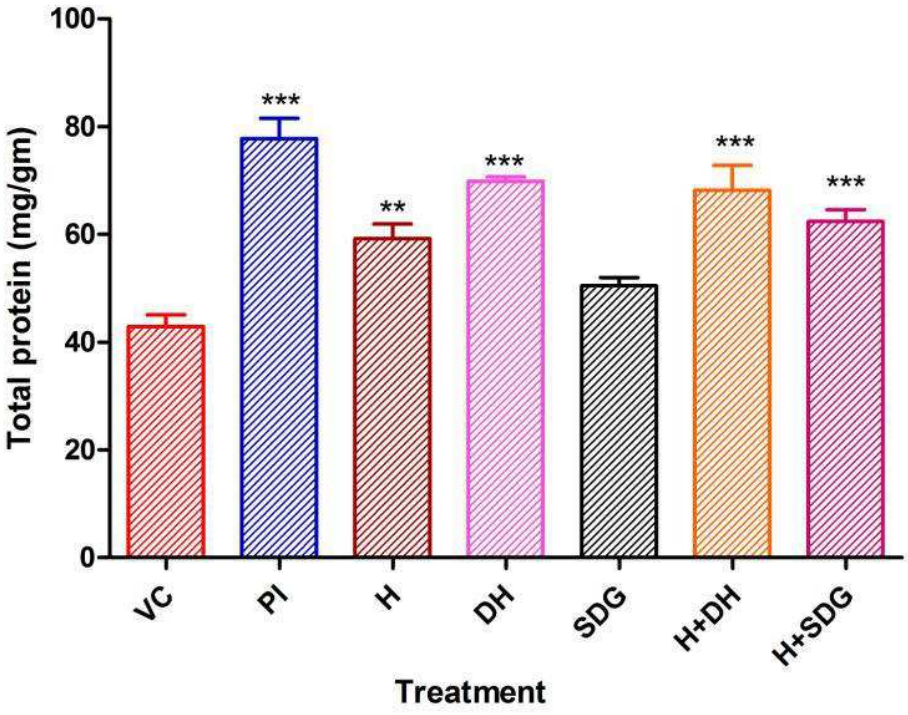

Figure 4. Effect of treatment of Honey, Daruhaldi and Shatdhautghrut on total protein level in rats

*Data are expressed as mean ±S.E.M. from six rats and analyze by one Way ANOVA followed by Dunnett's test. *p <0.05, **p <0.01, ***p <0.001 as compared to vehicle control group rats*

DISCUSSION

Wound is a rupture in the normal tissue continuum, resulting in a variety of cellular and molecular sequelae. Healing of wound is a complex and dynamic process that results in restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state[47].Decreased tissue damage, debriding non-viable tissue, increasing tissue perfusion and oxygenation with proper nutrition and moist wound healing environment have been recommended for optimal wound healing[23, 24].

Granulation, collagen maturation and scar formation are some of the many phases of wound healing which run concurrently, but independent of each other.The inflammatory phase played vital role in wound healing and it is characterized by hemostasis and inflammation, whereasproliferative phaseis characterized by epithelization, angiogenesis and collagen deposition[2, 42]. Final phase of wound healing is maturational phasein whichscar tissue formed. The granulation tissue of the wound is primarily composed of fibroblast, collagen, edema and small new blood vessel[48].

Tensile strength represents the force per unit of cross-sectional area that is required for breaking down the wound. It's a hallmark for the intra- and intermolecular cross linking of collagen fibers within the newly deposited collage in subdermal area [49]. Skin tensile strength is determined by the amount and quality of synthesized collagen, as well as degradation of preformed collagen. Collagen is the family of protein which provide structural support and it is the main component of tissue such as fibrous and cartilage. It has been well documented that collagen is an important biomarker of wound healing process which provide support and strength [50-52]. It also plays an important role in homeostasis and in epithelialization in the later stages of wound healing. In wound healing process, the growth of healing tissues depends upon the collagen synthesis which can be directly determined by the concentration of hydroxyproline. Thus, higher concentration of hydroxyproline reflects increased collagen turnover and indicates faster rate of wound healing [53, 54]. Increased collagen maturation via elevated cross linking of collagen fibers resulted in increase in breaking strength of wound.Enhanced healing activity has been attributed to increased collagen formation and angiogenesis [53, 54]. Angiogenesis in granulation tissues improves blood supplementation to the wound site, thus providing nutrients and oxygen essential for the healing process [55].In the present investigation treatment with honey in combination with Daruhaldisignificantly increasedhydroxyproline level and thereby increased tensile strength in incision wound model. It has been reported that that honey increase collagen synthesis during wound healing[56, 57]. Results of are in accordance with the findings of previous workers[56, 57].

In developing countries, the presence of poor hygienic conditions and microbial infections affect the management of wound and delay wound healing [58]. *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa, Streptococcus pneumoniae, and Klebsiella pneumoniae* are some prominent organisms that affect the wound healing [59]. If a wound becomes infected, the acute phase of inflammation becomes pronounced leading to further production of tissues oxidants which damage cellular membranes, DNA, proteins, lipid and extracellular matrix[60, 61]. It the present investigation vehicle control animals showed the significant decreased in the level of total protein whereas treatment with honey, Daruhaldi and Shata-dhauta-ghrita as well as their combinations significantly increased the level of total protein.

Flavonoids are known to inhibit cellnecrosis and thus improving vascularity[62-74]. Hence, any drug that containsflavonoids is believed to increase the viability of collagenfibrils by increasing the strength of collagen fibres, increasingthe circulation, preventing the cell damage and by promoting theDNA synthesis [75, 76]. Flavonoids are also known topromote the wound healing process mainly due to their astringentand antimicrobial property, which seems to be responsible for woundcontraction[77].It has been well documented that honey as well as Daruhaldi is a rich source of flavonoids[78-80]. The presence of the flavonoids may attributes to wound healing potential of honey and Daruhaldi.

So we conclude that oral administration of honey, Daruhaldi and Shata-dhauta-ghrita as well as their combinations promotes faster wound healing by acting simultaneously favorably on various processes of wound healing such as collagen synthesis, wound contraction, epithelization.The presence of flavonoid moities in honey andDaruhaldi combination showed better wound healing potential than Honey &Daruhaldi alone.

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