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Harsha Kumar
College of Pharmacy, Dr. A. P. J. Abdul Kalam University, Village, Post, Indore Bypass Rd, Arandia, Jhalariya, Madhya Pradesh, India

Sourabh Jain
College of Pharmacy, Dr. A. P. J. Abdul Kalam University, Village, Post, Indore Bypass Rd, Arandia, Jhalariya, Madhya Pradesh, India

Karunakar Shukla
College of Pharmacy, Dr. A. P. J. Abdul Kalam University, Village, Post, Indore Bypass Rd, Arandia, Jhalariya, Madhya Pradesh, India

Corresponding Author:
Sourabh Jain
College of Pharmacy, Dr. A. P. J. Abdul Kalam University, Village, Post, Indore Bypass Rd, Arandia, Jhalariya, Madhya Pradesh, India

Evaluation of alfalfa (*Medicago sativa*) leaves for wound healing activity

Harsha Kumar, Sourabh Jain and Karunakar Shukla

Abstract
Medicago sativa (*M. sativa*) has a long tradition of use as Ayurvedic and homoeopathic medicine in central nervous system disorders. The plant has been reported to have antioxidant, anti-inflammatory and antidiabetic effects. The present study was carried out to evaluate the effect of hydroalcoholic extract of *M. sativa* on experimentally induced wounds in rats and compare the effects observed with a known wound healing agent, *Aloe vera*. The models selected were excision wound, incision wound, burn wound and dead space wound. A suitable gel formulation was selected for the application using cellophane membrane penetration. In the excision wound and burn wound models, animals treated with *M. sativa* leaf extract showed significant reduction in period of epithelisation and wound contraction 50%. In the incision wound model, a significant increase in the breaking strength was observed. *M. sativa* leaf extract treatment orally produced a significant increase in the breaking strength, dry weight and hydroxyproline content of the granulation tissue in dead space wound. It was concluded that *M. sativa* leaf extract applied topically (5% and 10% gel formulation) or administered orally (250 mg and 500mg/kg body weight) possesses wound healing activity.

Keywords: *Medicago sativa*, wound healing, excision wound, incision wound, burn wound, dead space wound

Introduction
A wound is an injury to a part of the body, especially one in which a break is made in the skin. There are various types of wounds, including an incised wound, lacerated wound, abrasion, contusion, ulcer, and burn wound [1]. Wound healing, or wound repair, is an intricate process in which the skin (or another organ tissue) repairs itself after injury [2]. The process of wound healing occurs in different phases such as coagulation, epithelization, granulation, collagenation, and tissue remodeling. The healing cascade is activated when platelets come into contact with exposed collagen leading to platelet aggregation and the release of clotting factors resulting in the deposition of a fibrin clot at the site of injury. The fibrin clot serves as a provisional matrix and sets the stage for the subsequent events of healing [3]. Inflammatory cells also arrive along with the platelets at the injury site providing key signals known as growth factors [4]. The fibroblast is the connective tissue cell responsible for collagen deposition required to repair the tissue injury [5]. Collagen accounts for 30% of the total protein in the human body [6]. In normal tissues, collagen provides strength, integrity, and structure. When tissues are disrupted following injury, collagen is required to repair and restore normal structure and function. In India, there has been interesting in the potential of the medicinal plant for the development of drugs with wound healing properties as taught in a popular form of Indian medicine known as Ayurveda [7]. Recent studies with other plant extracts have shown that phytochemical constituents such as flavonoids [8] and triterpenoids [9] are known to promote the wound healing process mainly due to their astringent and antimicrobial properties, which appear to be responsible for wound contraction and increased rate of epithelization. Based on the information furnished in the literature, the main effects of the active constituents of the plant extracts toward wound healing are phytochemical constituents contributing to antimicrobial activity, phytochemical constituents working as antioxidants, active components having enhanced mitogenic activity (contributing to increased cell proliferation), angiogenesis, enhanced collagen production, and increased DNA synthesis. Several drugs obtained from plant sources are known to increase healing in different types of wounds. *M. sativa*, also called alfalfa or lucerne, is a plant from the pea family Fabaceae cultivated in many countries as an important forage crop. *M. sativa* was reported phytochemicaly as it contains tannin, flavonoids, digestive enzymes, alkaloids, coumarins, phytosterols, phytoestrogens, triterpenes and saponins. The reduction of cholesterol absorption and atherosclerotic plaque formation in the arteries due to the ingestion of *M. sativa* was reported by several clinical and animal

studies. More over its benefits in convalescence, cardiovascular complaints, diabetes and debility and also used as a tonic during anemia and after blood loss [10]. Tannins have been reported to be bacteriostatic or bactericidal against S. aureus [11]. *M. sativa* was reported to have selective toxicity in dog cancer cells grown in vitro and anti-tumor activity against certain types of leukemia cells in mice [10]. However, there is no scientific report for confirmation of their wound healing activity. Thus, the present study was undertaken to ascertain the effect of hydroalcoholic extract of *M. sativa* leaves on experimentally induced wounds in rats.

Materials and Methods

Plant material

The plant *M. sativa* leaves was collected from garden of Dr A.P.J. Abdul Kalam University, Indore and was authenticated by Dr. Karunakar Shukla, Professor & Head, Department of Pharmacognosy, College of Pharmacy, Dr. APJ Abdul Kalam University Indore M.P. and Voucher specimen No. APJAKU/COP/2019/037 was obtained.

Chemicals and reagents

Aloe Vera (standard drug) gel formulation was purchased from local market of Indore (M.P). All other chemicals used in this study were obtained from SRL Pvt. Ltd. (Mumbai, India), Hi Media Laboratories Pvt. Ltd. (Mumbai, India), SD Fine-Chem. Ltd. (Mumbai, India). Sigma-Aldrich, Chemicals Pvt. Ltd., India. All other chemicals and solvents used were of analytical grade available commercially.

Animals

Male Wister Albino rats with a weight of 250-275 gm were used for the study in different models. All rats were kept at room temperature of 20 ^{0}C in the central Animal house of the Pinnacle Biomedical research Institute (PBRI), Bhopal. The animal house was well maintained in hygienic conditions. The rats were housed in groups of four in polyethylene cages (32x24x16 cm). They were maintained on Standard diet-pellets (Ashirvad Industries, Chandigarh) and water *ad libitum*. All experiments were performed as per the directives of the institutional Animal Ethics Committee (PBRI/IAEC/PN-19038b). Cleaning and sanitizing work was carried out every other day. The rice husk was supplied as litter material, which was changed every day. The cages were kept clean and all experiments were carried out between 10 and 6 pm.

Extraction process

The powder form of leaves of *M. sativa* defatted by petroleum ether to remove fatty or fatty acid content at room temperature. When the solvent become concentrated, the petroleum ether content were filtered through muslin cloths after the defatting process residue again macerate with hydro-alcoholic (ethanol) solvent in equal ratio (1:1) or 50% both at room temperature. The bottle was kept at room temperature and allowed to stand for several 8-11 days with occasional shaking and stirring. When the solvent become concentrated, the liquid hydroalcoholic contents were filtered through cotton and then through filter paper (Whatman Fitter Paper No. 1). Finally, a highly concentrated hydroalcoholic extract was of *M. sativa* obtained [12].

Phytochemical investigation

Preliminary photochemical screening was carried out for qualitative identification of phytochemical constituents employing standard methods. *M. sativa* extracts were subjected to preliminary phytochemical screening to identify the various phyto-constituents present in them i.e. steroids, triterpenoids, flavonoids, carbohydrates, alkaloids, terpenoids, glycosides, saponins and tannins [13].

Acute toxicity study and dose selection

The acute toxic class method set out in this Guideline is a stepwise procedure with the use of 3 animals of a single sex per step. Depending on the mortality and/or the moribund status of the animals, on average 2-4 steps may be necessary to allow judgement on the acute toxicity of the test substance. The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of a single sex (normally females). Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step, i.e.; no further testing is needed, dosing of three additional animals, with the same dose and, dosing of three additional animals at the next higher or the next lower dose level. Three animals are used for each step. The dose level to be used as the starting dose is selected from one of four fixed levels, 5, 50, 300 and 2000 mg/kg body weight [14].

Selection of dose, gel base and treatment period

The HPMC gel formulation has been prepared for topical administration. *M. sativa* (5%) in HPMC (7.5%) is used as low dose and *M. sativa* (10%) in HPMC (7.5%) is used as high dose for topical use in excision, incision and burn wound model and *M. sativa* 250 mg/Kg body wt and 500mg/Kg body wt were taken as an oral dose in the dead space wound model. The treatment period was considered 10 days for incision and dead space wound model and the treatment period was considered till scar falling of wound in case of excision and burn wound model [15].

Wound Healing Models

Excision wound model

Wister male albino rat weighing between 250-275 gm body weigh were divided into four groups, each group consisting of 6 rats and each animal kept separately under laboratory condition. They had free access to commercial pallet diet and ad libitum.

Group I: Control group: animal of this group received 7.5% HPMC gel (2% tween 80)

Group II: Standard group: animal of this group received *Aloe Vera* (90%) gel formulation

Group III: Test group: animal of this group received *M. sativa* (5%) in HPMC (7.5%) gel formulation

Group IV: Test group: animal of this group received *M. sativa* (10%) in HPMC (7.5%) gel formulation.

The animals were anesthetized using ketamine (100 mg/kg, im) and xylazine (16 mg/kg, im). An impression was made on the dorsal thoracic region 1 cm away from vertebral column and 5 cm away from ear on the anaesthetized rat. The particular skin area was shaved one day prior to the experiment. The skin of impressed area was excised to the full thickness to obtain a wound area of about 500 mm^2. Haemostasis was achieved by blotting the wound with cotton

swab soaked in normal saline. Wound area was measured by tracing the wound on a millimeter scale graph paper on predetermined days i.e., 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 days post-wounding. The wound contraction-50% (days) was determined by plotting the wound area Vs days on a graph paper. Falling of scab leaving no raw wound behind was taken as end point of complete epithelization and the days required for this was taken as period of epithelization [16, 17].

Incision wound model

Para vertebral straight incision of 6 cm length was made through the entire thickness of the skin, on either side of the vertebral column with the help of a sharp scalpel. After complete haemostasis, the wounds were closed by means of interrupted sutures placed at approximately 1 cm apart. Animals were treated daily with drugs, as mentioned above under excision wound model from 0th day to 9th post-wounding day. The wound breaking strength was estimated on 10th day by continuous, constant water flow technique [18].

Burn wound model

Partial thickness burn wounds were inflicted on overnight-starved animals under ketamine (100 mg/kg, im) and xylazine (16 mg/kg, im) anesthesia by pouring hot molten wax (2 g) at 80 ^{0}C. The wax was poured on the shaven back of the animal through a cylinder of 300 mm2 circular opening. The wax was allowed to remain on the skin till it gets solidified. Immediately after the injury and on subsequent days, the drugs or base was applied topically as mentioned above [19].

Dead space wound model

Wister male albino rat weighing between 250-275 gm body weigh were divided into three groups, each group consisting of 6 rats and each animal kept separately under laboratory condition. They had free access to commercial pallet diet and *ad libitum.*

Group I: Control group: animal of this group received (2% tween 80 solution)

Group II: Test group: animal; of this group received *M. sativa* (250mg/kg rbw, p.o.)

Group III: Test group: animal; of this group received *M. sativa* 500 mg/kg rbw, p.o.)

This type of wound was created by the subcutaneous implantation of a 2.5 x 0.5 cm polypropylene tube in the dorsal side wood region. The animals received the drug from day 0 to 9 after the day of the wound, on day 10 after the wound, the granulation tissue collected in the implanted tube was carefully dissected together with the tube. The tubular granulation was cut along its length to obtain a granulation tissue sheet. Tear resistance was measured as described in the incision wound model. The pieces of granulation fabric were collected, dried at 60 °C for 24 hours, to obtain a constant weight and weighed. After noticing the dry weight of the granulation tissues, they were used to determine the hydroxyproline content [20, 21].

Determination of hydroxyproline

The granulation mass, which had dried at 600 ^{0}C for about 24 hours was weight and placed in a selected tube containing 10 ml of 6 N HCl. Heating the sealed tubes at 110 ^{0}C for 24 hours hydrolyzed the tissues. The hydrolysate was cooled and excess of acid was neutralized with 10N NaOH using methyl red as indicator. The volume of neutral hydrolysate was made up to 20ml with distilled water. From this 0.1 ml was used to estimate hydroxyproline.

Hydroxyproline estimation

0.1 ml of hydrolysate sample was pipetted out into clean test tubes, volume made upto 0.5 ml with distilled water. From the stock solution of standard hydroxyproline 1.6 ml was taken and diluted up to 100 ml. from this 0.5 (8µg) was pipetted out into a clean test tube. To this 1 ml each of 2.5 N NaOH, 0.01 M $CuSO_4$ and 6% H_2O_2 were added. Immediately, the tubes were placed in a water bath at 800 ^{0}C for 16 minutes and then cooled for 5 minutes [22]. To this 2 ml of freshly prepared 5% solution of para-dimethylamino-benzaldehyde in n-propanol, and 4 ml of 3N H_2SO_4 were added. Test tubes were once again placed in a hot water bath at 80 ^{0}C for 15 minutes and then cooled for 5 minutes. The optical density (O.D.) of the pink colour of these test samples were compared to that of standard hydroxyproline of known concentration samples at 540 nm using

Skin Irritation Study

Ratings corresponding to the following definitions were derived from data obtained from the test methods as described in 16 CFR 1500.41 and/or NAS Publication 1138, and categories of toxicity as described in 16 CFR 1500.3.The rabbit was shaved the skin in three different position of dorsal side, each about 500 mm^2. The rabbit was kept in rabbit holder and the 1st area was kept as control, to which vehicle was applied. 2nd area was applied with *M. sativa* gel (5%) and the 3rd area treated with *M. sativa* gel (10%). After 4 hour the skin was observed and compares to control the score was given.

Score

Practically non-irritating: The undiluted product causes no noticeable irritation, or causes slight inflammation (edema and erythema skin reaction values of 0 or 1) of intact or abraded skin of rabbits during the study period. Primary irritation index was 0-1.9.

Moderately irritating: The undiluted product causes well-defined inflammation (edema and erythema skin reaction values of 2) during the study period. Primary irritation index was 2-4.9.

Primary skin irritant: The undiluted product causes moderate to severe inflammation (edema and erythema skin reaction values of 3 or 4) of the intact or abraded skin of rabbits during the study period. Primary Irritation Index of 5 or more.

Corrosive: The undiluted product causes visible destruction or irreversible alterations of the tissue structure at the site of contact on intact or abraded skin of rabbits during the study period.

Statistical analysis

Results are expressed as mean ± SEM. The differences between experimental groups were compared using one-way Analysis of Variance (ANOVA) followed by Bonferroni's test. The results were considered statistically significant when $P<0.05$.

Results

The preliminary phytochemical investigation of the hydroalcoholic extracts of the medicinal plant *M. sativa* revealed the presence of carbohydrate, tannin, anthraquinone glycoside, and protein. *M. sativa* hydroalcoholic extract up to a dose of 2000 mg/kg body weight did not show any mortality. Hence 1/4th and 1/8th of this dose i.e. 500 mg/kg and 250mg/kg body weight of *M. sativa* were used for wound healing activity. A significant decrease in period of epithelization was observed after *M. sativa* (5%) and *M. sativa* (10%). Treatment with *Aloe vera* (slandered) also significantly reduced period of epithelization as compared with control group. At the same time *M. sativa* (5%) and *M. sativa* (10%) and *Aloe vera* also decreased the wound contraction (50%) as compared with control. Comparative analysis revealed that *M. sativa* (5%), *M. sativa* (10%) and *Aloe vera* had almost equal wound healing activity Table 1. The breaking strength of 10 day wound was significantly increased in all treatment groups when compared to control Table 2. Both *M. sativa* (5%) and *M. sativa* (10%) gel applied topically shorten the period of epithelization significantly when compared with control. *Aloe vera* gel applied topically shortens the period of epithelization and all the three also decreased the wound contraction (50%) significantly as compared with control Table 3. The breaking strength of 10 days old granulation tissue was significantly promoted by *M. sativa* (250mg/kg) and *M. sativa* (500mg/kg).The dry tissue weight also significantly increased in *M. sativa* (250mg/kg) and *M. sativa* (500mg/kg) when compared with control group. The hydroxyproline content was significantly more *M. sativa*. High dose treated animal compared to *M. sativa* low dose treated animal. From the above findings it is clear that *M. sativa* promotes the breaking strength by promoting the wound collagen content Table 4. Low dose does not showed any severe type of irritation, there was no evidence of showing any noticeable inflammation but slight redness was observed in case of *M. sativa* low dose gel formulation. On the other hand high dose of *M. sativa* showed well-defined redness and moderate type of inflammation Table 5.

Table 1: Effect of *M. sativa* on period of epithelization and wound contraction in excision wound model

Parameter studied	Epithelization period (days)	Wound contraction wc-50% (days)
Control (7.5% HPMC gel)	22.40 + 0.342	9.24 + 0.512
Aloe Vera gel (90%)	16.24 + 0.725***	7.42+ 0.545**
M. sativa (5%) in 7.5% HPMC gel	14.35 + 0.335***	6.13 + 0.364***
M. sativa (10%) in 7.5% HPMC gel	17.75 + 0.412***	5.07 + 0.360***

All values are mean ± SEM, n=6, ** p<0.01, *** p<0.001 vs. control

Table 2: Effect of *M. sativa* on breaking strength in incision wound model

Parameter studied	Breaking strength
Control (7.5% HPMC gel)	292.35 + 6.333
Aloe Vera gel (90%)	387.75 + 14.456***
M. sativa (5%)	402 + 11.035***
M. sativa (10%)	378.85 + 5.555***

All values are mean ± SEM, n=6, *** p<0.001 vs. control

Table 3: Effect of *M. sativa* on period of epithelization and wound contraction in burn wound model

Parameter studied	Epithelization period (days)	Wound contraction wc-50% (days)
Control (7.5% HPMC gel)	21.25 + 0.750	8.14 + 0.333
Aloe Vera gel (90%)	14.72 + 0.541***	6.14 + 0.245***
M. sativa (5%)in 7.5% HPMC gel	13.65 + 0.632***	5.23 + 0.331***
M. sativa (10%)in 7.5% HPMC gel	12.45 + 0.574***	4.98 + 0.175***

All values are mean ± SEM, n=6 , *** p<0.001 vs. control

Table 4: Effect of *M. sativa* on breaking strength, dry tissue weight and hydroxyproline content in dead space wound model

Parameter studied	Breaking strength	Dry tissue weight	Conc. of hydroxyproline
Control	345.15 + 18.741	91.425 + 2.626	2878.24 + 139.53
M. sativa 250mg/kg rbw	589.52 + 16.352***	192.188+ 5.820***	5989.61 + 189.50***
M. sativa 500mg/kg rbw	625.42 + 15.240***	188.224 + 4.946***	7588.20 + 283.25***+++

All values are mean ± SEM, n=6, *** p<0.001 vs. control $^{+++}$ p<0.001 vs. low dose

Table 5: Results of skin irritation study

Group	Sign	Score
Control	–	0
M. sativa (5%)	Not noticeable inflammation but slight redness	0.6
M. sativa (10%)	Well defined redness and moderate type of inflammation	4.2

Discussion

The present study was conducted to assess whether *M. sativa* leaf extract could promote wound healing in wounds experimentally produced in rats. This observation confirms the use of *M. sativa* leaves in folk medicine for wound treatment. The study was designed to study the influence of the hydroalcoholic extract of *M. sativa* on three main stages of wound healing, namely collagenization, wound contraction and epithelialization. In the present study, the extract applied topically or orally promoted resistance to wound rupture and the epithelialization period. Collagenization, wound contraction and epithelialization are crucial phases of wound

healing. The phase of inflammation, macrophage, fibroplasia and collagenization is intimately related. Therefore, an intervention in any of these phases by the drugs could lead to the promotion or depression of the healing phase of collagenization. Growth hormone promotes the healing process by increasing epithelial cell proliferation and cell collagen formation. Collagen is the family of proteins, which provides structural support and is the main component of the tissue, such as fibrous tissue and cartilage. The collage is synthesized by a complex biochemical mechanism of the ribosome. Collagen synthesis is stimulated by various growth factors [23]. It is also known that growth hormone promotes fibroblast proliferation [24] and fibroblast proliferation is the granulation tissue. In the dead space wound model, treatment with *M. sativa* increased the weight of the granuloma and the resistance to breakage. Therefore, it can be assumed that the medicinal activity of *M. sativa* could be due to direct or indirect influence on the release of growth hormone. *M. sativa* contains tannin, which is used in the medical field as an anti-inflammatory agent and is also used locally for burns. Lipid peroxidation is an important process of different types of injuries, such as burns, wounds and skin ulcers. Any drug that inhibits lipid peroxidation is believed to increase the vitality of collagen fibrils, increasing the resistance of collagen fibers by increasing circulation, preventing cell damage and promoting DNA synthesis [25]. Antioxidants, such as metronidazole, vitamin C and vitamin E, have been shown to promote wound contraction and epithelialization. The antioxidant property of *M. sativa* leaves, conferred by the presence of high amounts of tannins, may be responsible for the curative action of the extract in wound models. The study of skin irritation in rabbit skin has shown that the high concentration drug produces irritation in the skin, while it does not show any serious irritation if applied at low concentration in the skin. This suggests that the drug may contain some chemical components, which not produce irritation at low doses, but at high concentrations can cause irritation and navigate in the healing activity of *M. sativa*. This explains why the low dose of *M. sativa* is more active than the high dose when it is applied topically, but not when it is administered orally. Science *M. sativa* is more powerful than *Aloe Vera* in excision, incision, burns and wounds in dead space. The isolation of the active ingredient from the extract can lead to the development of a wound healing agent that can become a promising agent not only in the open wound but also in the ulcer of the leg, in the skin graft and in the severe burn type.

Conclusion

A preliminary phytochemical investigation was carried out on *M. sativa* and it was found that it contained carbohydrates, tannins, anthraquinone glycosides and proteins. Two doses of *M. sativa* (250 mg/kg and 500 mg/kg) were selected based on toxicity studies; there was no mortality up to 2000 mg/kg. Therefore, 1/8 and 1/4 of the toxicity dose was selected for oral dose preparation. Two different doses of *M. sativa* (5% and 10%) were selected for the preparation of the gel formulation, and the particular gel base was selected after conducting the penetration study through a cellophane membrane. The hydroalcoholic extract of *M. sativa* has shown an important wound healing activity in the pattern of excision, incision, burn and wound in dead space, which is comparable to the commercialized formulation of the *Aloe vera* gel.

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