Original Article

Pharmacological evaluation of ameliorative effect of aqueous extract of *Cucumis sativus* L. fruit formulation on wound healing in Wistar rats

Abstract

Aim: The aim of present investigation was to formulate and evaluate the ameliorative effect of aqueous extract of *Cucumis sativus* L. fruit cream formulation on experimentally induced wounds in rats. **Materials and Methods:** The cream was formulated using soft white paraffin base containing 2.5%, 5%, and 10% w/w of aqueous extract of of *Cucumis sativus* L. fruit. Excision wounds of size 300 mm^2 and 2 mm depth were used for the study of rate of contraction of wound and epithelization. All the three formulations were evaluated for various pharmaceutical parameters such as pH, viscosity, spreadability, and acute skin irritation study. Epithelialization period, wound contraction, scar width, and histopathological evaluation parameters were used for pharmacological evaluation of wound healing activity of the formulation. **Statistical Analysis:** All the results were expressed as mean±SEM. Data analysis was performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). Statistical comparisons were made between drug-treated groups and disease control animals. Data of disease activity index were analyzed using one-way analysis of variance; Dunnett's multiple range test was applied for *post hoc* analysis, whereas data of wound area and percent wound contraction were analyzed using two-way repeated analysis of variance, Bonferroni's multiple range test was applied for *post hoc* analysis. A value of $P<0.05$ was considered to be statistically significant. **Results:** Cream formulation of AECS when applied topically did not show any sign and symptoms of skin irritation. The treatment with aqueous extract of *C. sativus* fruit cream formulation (2.5%, 5%, and 10% w/w) resulted in significance decrease ($P<0.05$, $P<0.001$, and $P<0.001$, respectively) in wound area, epithelization period, and scar width, whereas rate of wound contraction significance increased ($P<0.001$ respectively) when compared with control group animals. **Conclusion:** The present investigation demonstrates that by virtue of its antioxidant property and presence of the flavanoids content in *C. sativus* may responsible for wound contraction and elevated rate of epithelization in wound healing in laboratory animals.

Key words:
Cream, Cucumis sativus aqueous extract, excision wounds, framycine sulfate cream, period of epithelialization, wound contraction, wound healing

Introduction

Induction of the stress or injury by means of agents such as complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants results in the generation of the wounds and that are inescapable part of human life.[1] Because of poor hygienic condition, wound infection is becomes the most ordinary disease in developing countries.[2] Generation and maintenance of normal anatomical cellular structure, i.e., dermal and epidermal tissue and function for the purpose of the survival is known as the healing process. Generation of the trauma is an initial step in the wound formation, whereas it ends with the formation of scar.[3] Wound healing is a complex biological

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phenomenon that involves the various phase processes such as coagulation, epithelization, granulation, collegenation, and tissue remodeling of the extracellular matrix.[4,5] Growth factor generated at the site of injury when inflammatory cells combine with the platelets plays vital role in process of wound healing.[6] Also, fibroblast that causes collagen deposition is necessary for repair of tissue injury.[7] Collagen accounts for structural strengthening and maintenance of integrity of normal tissue in the human body.[8]

Decreasing damage of tissue, removal of the nonviable tissue, increasing the rate of tissue perfusion and oxygenation, and providing the appropriate and specific tissue nutrition are the fundamental theories for increasing wound healing process rate.[9] The wide range of medicinal plants has been reported which affect various phases of wound healing process.[10,11] An array of drugs including simple non-expensive analgesics as well as complex and expensive chemotherapeutic agents has been administered in the treatment of wound, but they are associated with one or more side effect.[12] Vitamins such as A, C, and E as well as zinc, arginine, glutamine, and glucosamine have been identified as nutritional cofactors which help in the process of tissue repair.[13]

The cucumber (*Cucumis sativus*) is a widely cultivated plant in the gourd family Cucurbitaceae. *C. sativus* have been evaluated for a wide spectrum of activity including diuretic,[14] antihypergycemic,[15] antioxidant,[16] amylolytic,[17] anticancer,[18] and analgesic[19] using various *in vitro* and *in vivo* models. Hence, the present investigation was designed to formulate and evaluate the ameliorative effect of aqueous extract of *C. sativus* fruit cream formulation on experimentally induced wounds in rats.

Materials and Methods

Collection of plant material

The fruits of *C. sativus* L. were collected from rural areas of Pune district, Maharashtra, in the month of December 2010. Authentication of plant was carried out by P.G. Diwakar, Joint Director, Botanical Survey of India, Pune. The voucher specimen number was MIPCUSA1.

Preparation of extract

The plant material was cut into small pieces and was macerated in 6 L of water for about 48 hours with occasional shaking. Macerate was decanted and filtered through cloth and then through filter paper to obtain a clear extract. Macerates were pooled and collected in trays and evaporated to dryness at 30–35°C to obtain dried extract. The yield was found to be 5.5%. Solution of AECS was prepared by using distilled water as vehicle.

Preliminary phytochemical screening

Phytochemicals analysis of the extract was performed for the identification of the phytochemicals such as alkaloid, flavonoids, steroid, and phenols according to the methods described by Khandelwal *et al*.[20] and Kokate *et al*. [21]

Animals

Healthy adult male Swiss albino mice (20–30 g) and male Wistar rats (230–250 g) were obtained from the National Institute of Biological Sciences, Pune, India. The animals were housed in groups of six in solid bottom polypropylene cages. They were maintained at 24°C±1°C, with relative humidity of 45–55% and 12:12-hour dark/light cycle. The animals were acclimatized for a period of 2 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 pharmacology and acute toxicity protocols were approved by the Institutional Animal Ethics Committee.

Acute toxicity testing

Acute oral toxicity in Swiss albino mice was performed according to OECD guidelines using AOT 425 software. Graded doses of the *C. sativus* were dissolved in distilled water and were administered orally, and the animals were observed for 2 weeks following administration. Body weight, food consumption, fluid intake, and psycho-motor activities were recorded daily.

Chemicals

Anesthetic ether, ethanol, formalin, sodium hydroxide, chloroform, ether, hydrochloric acid, and conc. sulfuric acid were purchased from S.D. Fine Chemicals, Mumbai, India.

Cream preparation for topical application

An AECS fruit was used for the preparation of the cream for topical application.[22] A 2.5% (w/w), 5% (w/w), and 10% (w/w) of extract cream was formulated using soft white paraffin base.

Physical evaluations

Preliminary evaluation of formulations at different concentrations was carried out as follows:

pH

The pH of various formulations was determined by using Digital pH meter. One gram of cream was dissolved in 100 ml of distilled water and stored for 2 hours. pH measurement of each formulation was performed in triplicate.[23,24]

Viscosity

The viscosity measurement was carried out with Brookfield Viscometer in triplicate,[25] and viscosity values were expressed as mean±standard deviation.

Spreadability

Spreadability is a term expressed to denote the extent of area to which the cream and gel readily spread on application to skin or affected part[26] and it is expressed in terms of times

in seconds taken by two slides to slip off from cream and placed in between the slides under the direction of certain load.[27]

Acute skin irritation study

The primary skin irritation test was performed on albino rats and weighing about 150–200 g. The animals were maintained on standard animal feed and had free access to water *ad libitum*. The animals were kept under standard laboratory condition. The total mass was divided into four batches, each batch containing six animals. Two batches of each were used for control and test. Dorsal hairs at the back of the rats were clipped off 1 day before the commencement of the study. Animals showing normal skin texture were housed individually in cages with copography meshes to avoid contact with the bedding; 50 mg of the each formulation of different concentrations were applied over 1 cm^2 area of intact and abraded skin to different animals. Aqueous solution of 0.8% formalin was applied as standard irritant. The animals were observed for 7 days for any signs of edema and erythema.[28,29]

Extrudability

The formulations were filled in the collapsible tubes, and extrudability was determined in terms of weight in grams required to extrude a 0.5 cm ribbon of cream for 10 seconds.[25,30]

External characters of developed cream formulations were also identified, such as color, odor, smoothness, and grittiness.

Excision wound model

Excision wounds were used for the study of rate of contraction of wound and epithelization. Animals were anaesthetized with 80 mg/kg dose of ketamine (i.p.) and the back hairs of the animals were depilated by shaving. 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. Excision wounds sized 300 mm^2 and 2 mm depth were made by cutting out layer of skin from the shaven area.[31-35] Hemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. The entire wound was left open. The study comprised six different groups of six animals in each groups as follows and the treatment was done topically in all the cases:

- Group I - Normal animals: Did not receive either surgery or injury for wound formation
- Group II - Control animals: Did not receive any cream or drug treatment.
- Group III - FSC-treated animals: Received treatment with Framycine sulfate cream (1% w/w)
- Group IV - Drug-treated animals: Received treatment with AECS cream (2.5% w/w)
- Group V - Drug-treated animals: Received treatment with AECS cream (5% w/w)
- Group VI - Drug-treated animals: Received treatment with AECS cream (10% w/w).

Measurement of wound area

The progressive changes in wound area were monitored by a camera (Fuji, S20 Pro, Japan) on predetermined days, i.e., 2, 4, 8, 12, 16, and 20. Later on, wound area was measured by tracing the wound on a millimeter scale graph paper.[36]

Calculation of wound contraction

Wound contraction was calculated as percentage of the reduction in original wound area size. It was calculated by using the following formula:

Percentage wound contraction =

$$\frac{\text{Initial area of wound} - \text{N}^{\text{th}}\text{day area of wound}}{\text{Initial area of wound}} \times 100$$

Determination of period of epithelization

Falling of scab leaving no raw wound behind was taken as end point of complete epithelization, and the days required for this were taken as period of epithelization.

Measurement of wound index

Wound index was measured daily with an arbitrary scoring system[37] [Table 1].

Histopathology

A specimen sample of tissue was isolated from the skin of each group of rat collected at the end of the experiment to evaluate the histopathological alterations.[38] Samples were fixed in 10% buffered formalin, processed, and blocked with paraffin and then sectioned at 5 μm thickness and stained with hematoxylin and eosin (H and E), Photomicrographs were captured at a magnification of ×100. Sections were analyzed and scored as mild (+), moderate (++), and severe (+++) for epidermal or dermal remodeling. Re-pithelization or ulcus in epidermis; fibroblast proliferation, mononuclear, and/or polymorphonuclear cells, neovascularization, and collagen depositions in dermis were analyzed to score the epidermal or dermal remodeling. At the end of the examination, all the wound healing processes were combined and staged for wound healing phases as inflammation, proliferation, and remodeling in all groups.

Table 1: An arbitrary scoring system for measurement of wound healing index

Gross changes	Wound index
Complete healing of wounds	0
Incomplete but healthy healing	1
Delayed but healthy healing	2
Healing has not yet been started but the environment is healthy	3
Formation of pus evidence of necrosis	4
Total	10

Statistical analysis

All the results were expressed as mean±SEM. Data analysis was performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). Statistical comparisons were made between drug-treated groups and disease control animals. Data of disease activity index were analyzed using one-way analysis of variance; Dunnett's multiple range test was applied for *post hoc* analysis, whereas data of wound area and percent wound contraction were analyzed using two-way repeated analysis of variance, Bonferroni's multiple range test was applied for *post hoc* analysis. A value of $P<0.05$ was considered to be statistically significant.

Result

Acute toxicity testing

Acute toxicity studies of the AECS show no signs and symptoms such as restlessness, respiratory distress, diarrhea, convulsions, and coma and it was found safe up to 5000 mg/kg.

Preliminary phytochemical screening

The aqueous extract of *C. sativus* L. fruit was screened for various chemical tests as per the reported methods and was found to contain alkaloids, steroids, flavonoids, and polyphenols [Table 2].

Physicochemical evaluations of different formulation of cream

The pH of the AECS cream formulation was 6.52–6.62, it lie in normal pH range of human skin, whereas the viscosity was 16.40 cps which indicated that as the torque and shear stress increases. The spreadability time of cream was 25 seconds. Spreadability of test formulations was compared with that of marketed formulation that is FSC. The readings indicate they are nearly same in the terms of applicability or spreading capacity. The viscosity of the formulation decreases as the spreadability increases. Acute skin irritation study of aqueous extract of *C. sativus* L. fruit cream formulation did not produce any skin irritation, i.e., erythema and edema for about a week when applied over the skin. Test formulations also showed good homogeneity and extrudability [Table 3].

Pharmacological evaluation of wound healing activity

Effect of AECS cream on wound area

The wound area (mm^2) in all animal groups was measured on days 0, 2, 4, 8, 12, 16, and 20. The wound area in control group was 281.86±5.44, 269.91±6.76, 265.41±5.02, 256.86±5.54, 251.30±9.0, 243.55±7.51, and 244.6±7.54 mm^2 on respective days. The treatment with AECS cream formulation (2.5%, 5%, and 10% w/w) resulted in significance decreased ($P<0.05$, $P<0.001$, and $P<0.001$, respectively) in wound area when compared with control group animals. The wound area in AECS cream formulation (2.5% w/w) treated animal on 16th and 20th day was 217.70±3.62 and 214.68±3.21 mm^2 respectively. The wound area in AECS cream formulation (5% and 10%w/w) treated animal on 4th, 8th, 12th, 16th, and 20th day was 242.52±8.15, 200.88±6.78, 164.93±8.023, 109.9±8.19, 89.05±4.90 mm^2, and 225.45±7.16, 165.10±10.52, 116.69±7.72, 57.82±7.51, 33.35±4.37 mm^2, respectively [Figures 1 and 2].

Effect of AECS cream on rate of wound contraction

The wound contraction rate was not altered significantly in control and AECS cream formulation (2.5% w/w) treated animal

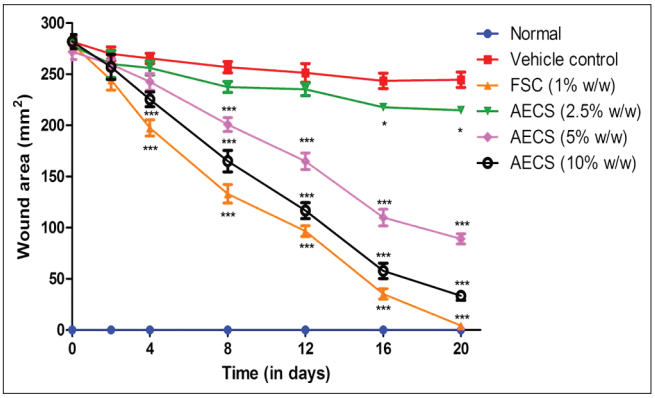

Figure 1: Effect of AECS cream formulation treatment on wound area (mm^2) in rats. Data are expressed as mean±SEM from six rats and analyzed by two-way analysis of variance followed by Bonferroni's test. *$P<0.05$, **$P<0.01$, ***$P<0.001$ when compared with control group animals

Table 2: Preliminary phytochemical constituents present in aqueous extract of *Cucumis sativus* L. fruit

Phytoconstituents	Present/absent
Alkaloids	
Mayer's reagent	+
Wagner's reagent	+
Dragendorff's reagent	–
Tannin	+
Saponin	–
Flavonoids	+
Steroids	+
Cardiac glycosides	+
Carbohydrate	+
Terpenoid	–

+ - Present; – - Absent

Table 3: Physicochemical evaluations of different formulation of cream

Parameter	AECS (2.5%)	AECS (5%)	AECS (10%)
pH	6.62	6.58	6.55
Viscosity (cps), mean±SD	16.30±100	16.40±50	16.25±75
Spreadability time (sec)	24	24	25
Acute skin irritation study	No skin irritation	No skin irritation	No skin irritation

on days 0, 2, 4, 8, 12, 16, and 20. AECS cream formulation (5% and 10% w/w) treated animals resulted in significantly increased ($P<0.001$, respectively) rate of wound contraction on 8th, 12th, 16th, and 20th day when compared with control group animals. The wound contraction rate in AECS cream formulation (5% and 10% w/w) treated animals was 25.75±3.57, 38.94±3.95, 59.32±3.28, 67.18±1.69 and 41.43±3.53, 58.38±3.24, 79.30±2.86, 88.01±1.81, respectively [Figures 2 and 3].

Effect of AECS cream on period of epithelization

The epithelization period in the control animals was 23.17±0.40 days. Treatment with AECS cream formulation (5% and 10% w/w) resulted in significant decrease in ($P<0.05$ and $P<0.001$) epithelization period (20.50±0.67 and 15.83±0.47 days, respectively) when compared with control group animals [Figure 4].

Effect of AECS cream on wound index

In control animals, wound index was 3.16±0.30, whereas in AECS cream formulation (5% and 10% w/w) treated animals it was 1.83±0.30 and 1.33±0.33, respectively. Treatment with AECS cream formulation (5% and 10% w/w) resulted in significant decrease ($P<0.05$ and $P<0.01$) in wound index when compared with control group animals [Table 4].

Effect of AECS cream on scar formation

In control animals, scar width was 144.4±5.16 μm, whereas in AECS cream formulation (5% and 10% w/w) treated animals it was 120.0±6.14 and 95.43±4.37 μm, respectively. Treatment with AECS cream formulation (5% and 10% w/w) resulted in significant decreased ($P<0.01$ and $P<0.001$) in scar width when compared with control group animals [Table 5].

Histopathological examination

Inflammation, proliferation, and remodeling are the various phases in wound healing processes that were observed during the experimental period. Delayed wound healing processes were recorded in control groups. Aggregation of macrophages with poor collagenation was observed in histological examination of control group animals. Treatment with AECS cream formulation resulted in decreased inflammation, increasing the rate of tissue perfusion and proliferation as well as remodeling, along with reepithelization. The reduced macrophages and increased collagen fibers with low scar formation were observed in the AECS cream formulation-treated animals [Table 6 and Figure 5].

Discussion

The aim of the present investigation was formulation and pharmacological evaluation of ameliorative effect of AECS fruit cream formulation on experimentally induced wounds in rats. Depending on the ordinary health status as well as type and degree of damage, the tissue repairing ability also plays an important role in the wound healing. Fibroblast, collagen, and new blood vessels are the primary component of granulation tissue of the wound.[39] The wound healing is a multifaceted process involving various stages including wound contraction, granuloma formation, collagen maturation, and scar formation. The most important event that occurred during the wound healing process is migration of myofibroblast by incorporation of mesenchymal cells into wound gap with the fibrin strands.[9,40]

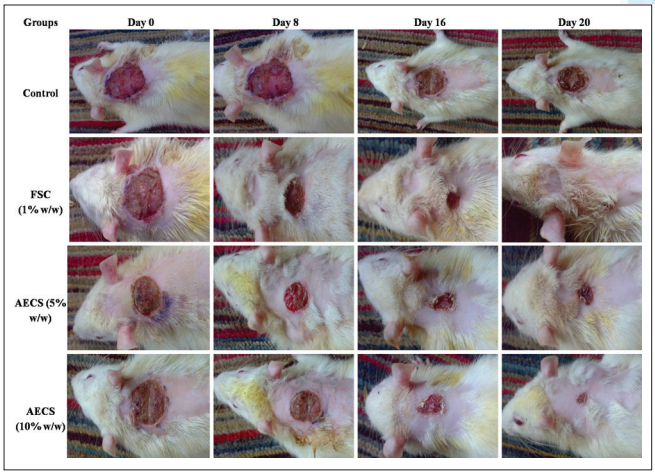

Figure 2: Photographs of rats showing various phases of wound healing

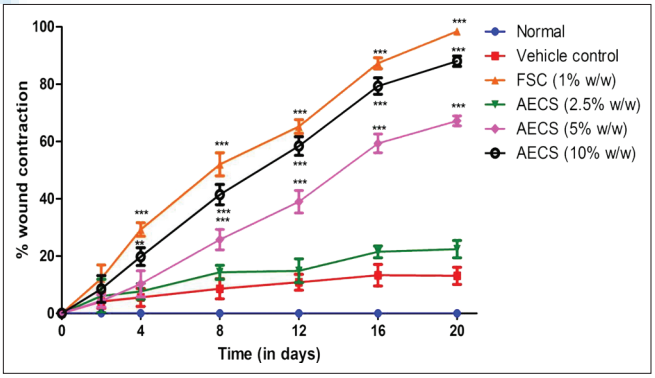

Figure 3: Effect of AECS cream formulation treatment on rate of wound contraction in rats. Data are expressed as mean±SEM from six rats and analyzed by two-way analysis of variance followed by Bonferroni's test. *$P<0.05$, **$P<0.01$, ***$P<0.001$ when compared with control group animals

Table 4: Effect of AECS cream formulation treatment on wound index in rats

Control	FSC (1% w/w)	AECS (2.5% w/w)	AECS (5% w/w)	AECS (10% w/w)
3.16±0.30	1.00±0.36***	2.83±0.30	1.83±0.30*	1.33±0.33**

Data are expressed as mean±SEM from six rats and analyzed by one-way analysis of variance followed by Dunnett's test. *$P<0.05$, **$P<0.01$, ***$P<0.001$ when compared with control group animals

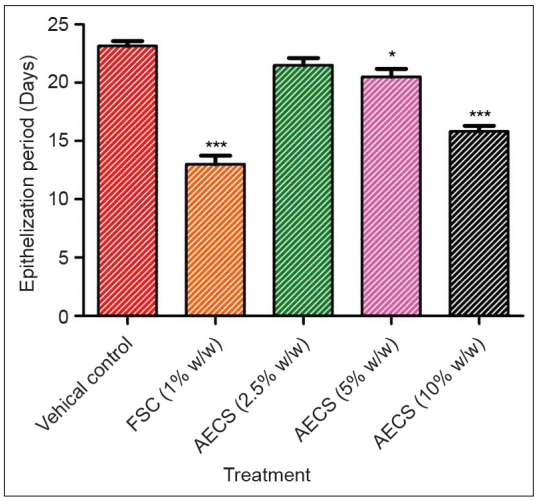

Figure 4: Effect of AECS cream formulation treatment on period of epithelization in rats. Data are expressed as mean±SEM from six rats and analyzed by one-way analysis of variance followed by Dunnett's test. *$P<0.05$, **$P<0.01$, ***$P<0.001$ when compared with control group animals

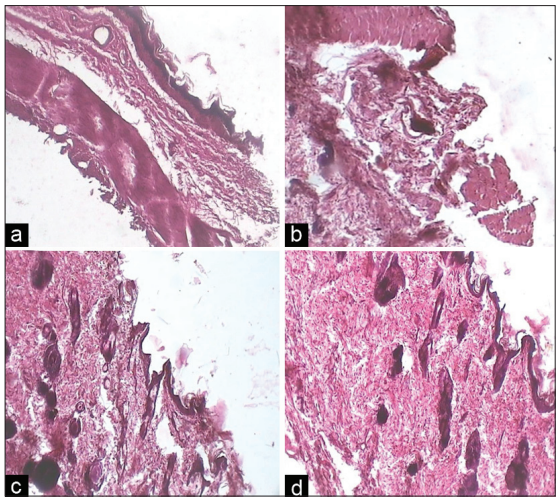

Figure 5: Photomicrographs of sections of skin from rats stained with H and E. Skin microscopic image of (a) normal rat, (b) wound control rat, (c) FSC (1% w/w) treated rat, and (d) AECS cream formulation (10% w/w) treated rat. Images (×100 magnification) are typical and representative of each study group

Table 5: Effect of AECS cream formulation treatment on scar width in rats

Vehicle	FSC (1% w/w)	AECS (2.5% w/w)	AECS (5% w/w)	AECS (10% w/w)
144.4±5.16	72.07±3.00***	137.7±4.05	120.0±6.14**	95.43±4.37***

Data are expressed as mean±SEM from six rats and analyzed by one-way analysis of variance followed by Dunnett's test. *$P<0.05$, **$P<0.01$, ***$P<0.001$ when compared with control group animals

Table 6: Wound healing processes and healing phases of the control, FSC (1% w/w) and AECS cream formulation (2.5%, 5% and 10% w/w) treated animals

Histopathological observations	Control	FSC (1% w/w)	AECS (2.5% w/w)	AECS (5% w/w)	AECS (10% w/w)
Scar	+ + +	+	+ + +	–	–
Ulcus	+ + +	-–	+ +	+	–
Reepithelization	–	+ + +	–	+	+ +
fibroblast proliferation	+ + +	+	+ + +	+	+
mononuclear cells	+ +	–	+ +	–	–
Polymorphonuclear cells	+ + +	–	+ + +	+	–
Neovascularization	+ + +	–	+ +	+	+
Inflammation phase	+ + +	+	+ + +	+	–
Proliferation phase	+ +	+ +	+ +	+	+ +
Remodeling phase	–	+ + +	–	+	+ +

In the present study, topical treatment of AECS cream formulation significantly increased the wound healing rate. By virtue of its antioxidant property[16] and presence of the flavanoids, content[41] in *C. sativus* may be responsible for wound contraction and elevated rate of epithelization in wound healing. It may act either by increasing the myofibroblasts contractile property or causes enhancement of myofibroblasts number that was incorporated into mesenchymal cells of wound area. It can be hypothesized that *C. sativus* causes enhancement of epithelialization via its proliferation enhancement or by increasing the viability of epithelial cells.

It is reported that oxidative stress plays a vital in role in acute and chronic inflammatory conditions such as wound healing.[42] Phytochemical constituents such as flavanoids and triterpenoids, which possess antioxidant and free radical scavenging activity[43-45] along with antimicrobial and astringent properties,[46] are responsible for the contraction of wound and enhancement of epithelization which would have resulted in increasing wound healing rate.

The present investigation provides pharmacological credence to the ethnobotanical claims of *C. sativus* mentioned in the traditional Indian system of medicine.

However, fractionation of *C. sativus* needs to be carried out to determine the isolated bioactive moieties responsible for healing effect of wounds in laboratory animal.

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