

Effect of an Extract of *Agave americana* on Wound Healing Model in Experimental Animals

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ABSTRACT

Background: Medicinal plants have immense potential since ancient time based on error and trial method. *Agave americana* has been known since antiquity, as century plant or American aloe. The leaves contain steroidal saponins, isoflavones and coumarins. It has long been used on the wound. **Objectives:** This study was designed to evaluate the wound healing activity of the extract of *Agave americana*. **Materials and Methods:** *Agave americana* leaves extract is collected and the extract is done by percolation method. The wound is induced in experimental animals by excision and incision method under ketamine. The extract of *Agave americana* and standard, Soframycin is applied on the wound. The wound closure was measured at regular intervals of time to see the percentage of wound closure; epithelialization time and tensile strength are monitored to see the effect of the extract and its comparison with the standard. **Results:** The extract of *Agave americana* showed improved in wound healing from Day 4 onward as compared with control in excision method. The rate of epithelialization for 10% HEAA is almost comparable to standard, Soframycin ointment. In incision method, mice treated with 10% ointment of *Agave americana* leaf extract showed significant ($p < 0.05$) increase in tensile

strength as compared with control. **Conclusion:** Wound healing activity of hydroalcoholic extract of *Agave americana* is seen in graded dose. The plant contains flavonoids, tetratriacontanol and homoisoflavanoids which due to its anti-oxidant and antibacterial activities help in wound healing. The plant also contains genins which help in reducing the inflammatory process. Thus *Agave americana* leaves need to be used in higher doses to have a better understanding of the mechanism of wound healing.

Key words: *Agave americana*, wound healing, soframycin, flavonoids, anti-inflammatory, anti-bacterial

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INTRODUCTION

“Herb” is a plant valued since ancient times for its medicinal, savory and aromatic qualities. It contains various natural substances that are capable of producing physiological and pharmacological effects on the body. It is one of the oldest medicinal cares for human since 4000-5000 BC.^[1] It is one of the integral parts of modern culture. Apart from its medicinal use, it also provides with food, shelter and other necessities of life. It played an important role in maintaining human health and improving the quality of human life for thousands of years. Herbs contain a variety of chemical substance that has an important action on the human body. Herbal plant is a major component in all traditional medicine and it is a common element of conventional sciences like Ayurveda, Unani, Homeopathic, Rasa siddha, Traditional oriental, Naturopathic, and Native American Indian Medicine. Medicinal plants have immense potential due to the databases collected through the use of reverse pharmacology since ancient time based on error and trial method. Hence, there has been a great focus on plant research all over the world. Hence, there has been a surge in the use of herbal drugs for various diseases.^[2]

Despite such intensive research on various herbal medicines, many species of plants are still left unexplored. Therefore plants named *Agave americana* is chosen for the present study. *Agave americana* has been known since antiquity, as century plant or American aloe.^[3] The different parts of *Agave americana* have been found useful in a variety of symptoms and disorders. The leaves contain steroidal saponins, isoflavones and coumarins.^[4,5] It has long been used on the wound.^[6,7] Also use in the treatment of diarrhea, dysentery, etc.^[7,8] It is antiseptic, diaphoretic, diuretic and laxative. The leaves are used in mucosal inflammation, digestive disorders, and base for the plaque.^[8] It also has antimicrobial properties.^[8] In Ayurveda the plant is also used for the treatment of rheumatoid arthritis, sciatica.^[9] The root is diaphoretic

and diuretic.^[4] It is used in the treatment of syphilis.^[7]

From the perusal of literature, it appears that the different important pharmacological parameters of *Agave americana* have been less investigated and can be scientifically proved by reverse pharmacology. Therefore, it was found of interest to evaluate these properties of extract of leaves of *Agave americana* in experimental models.

MATERIALS AND METHODS

Animals

The present study was conducted in experimental animals i.e., Albino Wistar rats and Swiss albino mice. The animals were caged in polyvinyl wire mesh cages in the animal room of Department of Pharmacology. Healthy Swiss Albino mice of either sex weighing between 22-30 grams were chosen for the excision and incision models of wound healing. They were maintained under standard laboratory condition (12 hour light and dark cycle) and temperature ($22^{\circ}\text{C} \pm 3^{\circ}\text{C}$), humidity ($60 \pm 10\%$) with access to food and water ad libitum according to OECD guidelines, revised draft guidelines 425 and by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.^[10] The animals were allowed to adapt to the new surrounding by giving rest of one week before subjecting them to experimentation.

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Cite this article as: Misra AK, Varma SK. Effect of an Extract of *Agave americana* on Wound Healing Model in Experimental Animals. J Basic Clin Pharma 2017;8:45-48.

Plant extract and preparation

The *Agave americana* leaves were collected from the college garden and authenticated by an expert in Botany. The leaves were shade dried, powdered and stored in an airtight container for study. The powders (leaves) were macerated for 24 hours in 70% w/w ethanol. The hydro-alcoholic extracts were obtained by percolation using 70% w/w ethanol as a solvent. The fresh solution was prepared by dissolving extract in distilled water before each experiment. The yield of leaf extract by percolation is around 15% and 20% respectively. For oral administration, *Agave americana* leaves extract was used which was prepared by dissolving the extract in distilled water before each experiment. Whereas for topical application *Agave americana* leaves extract was mixed with simple ointment as mention below.^[11] Simple ointment (100 gram) is made by mixing soft paraffin (85 gram), hard paraffin (10 gram) and lanolin/wool fat (5 gram). 5% (w/w) ointment is made by simple ointment (95 gram) and *Agave americana* leaves extract (5 gram), and 10% (w/w) ointment is made by simple ointment (90 gram) and *Agave americana* leaves extract (10 gram).

Drugs and chemicals

In wound healing model, Soframycin 30 gm (Aventis Pharma Limited, Goa), simple ointment and silk (3-0) (Centenial Surgical Suture Limited Thane, Mumbai) are used.

Ethical clearance

Ethical clearance was taken from Institutional Research Ethics Committee and Institutional Animal Ethics Committee (IAEC) before commencement of the study.

Acute toxicity study

Acute oral toxicity study of the test extract of the *Agave americana* was carried out as per the guidelines set by Organization for Economic Co-operation and Development (OECD/OCED), revised draft guidelines 425 and by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Healthy adult albino Wistar rats were used for this study. The animals should be kept as per requirement in the OECD guidelines. Animals used for the test were fasted overnight prior to testing. The dose was calculated according to the body weight of each animal.^[10]

The drug was initially administered (2000 mg/kg body weight) orally to one animal. If the first test animal survived, four more animals were administered with the drug sequentially. Animals were observed individually during the first 30 min after the dosing, periodically during the first 24 hours (special attention was given during the first 4 hours), and thereafter daily for a total of 14 days. Since no animal died, median lethal dose LD₅₀ of test drug can be taken as greater than 2000 mg/kg.^[10]

The animals were observed for mortality as well as changes in clinical signs. These include changes in skin, eyes and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, and somatomotor and behavior pattern. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.^[10]

Accordingly, the test doses of the extract were selected by trial and error. Pilot study was done using the various doses of the test drugs i.e., 1/80th (25 mg/kg), 1/40th (50 mg/kg), 1/20th (100 mg/kg), 1/10th (200 mg/kg) and 1/5th (400 mg/kg) of 2000 mg/kg. The doses 100 mg/kg, 200 mg/kg and 400 mg/kg were selected as working doses for all the experiments in the present study.^[10]

Assessment of wound healing activity

Wound healing activity of hydroalcoholic extract of *Agave americana*

leaves was studied in the following two models: i) Excision model and ii) Incision model.

Excision wound model

The albino Swiss mice of either sex were divided into 4 groups, 6 animals in each group (total 24 animals).^[11]

Group 1- Control-simple ointment locally once daily

Group 2- HEAA (5% ointment) locally once daily

Group 3- HEAA (10% ointment) locally once daily

Group 4- Standard drug Soframycin ointment locally once daily

The animals were anesthetized by using Ketamine (80 mg/kg i.m).^[12] A round seal of an impression of 2.5 cm diameter was made on the dorsal thoracic region 1 cm away from vertebral column and 3 cm away from the ear on the anaesthetized rat. The particular skin area was shaved one day prior to the experiment. Full thickness skin from the demarcated area was excised to get a wound approximately measuring 10 mm diameter on the shaved and cleaned skin. Haemostasis was achieved by blotting the wound with the cotton swab soaked in normal saline. Animals received the drugs from day of wounding to 21st postoperative day. Wound contraction rate was monitored in six animals from each group on 1st, 4th, 7th, 10th, 13th and 16th post wound day starting from day 1 by planimetric measurement. The wound tracings were then transferred to a 1 mm² graph paper, to determine the wound area. Wound contraction was calculated as a percentage of the original wound size. Epithelialization period was monitored by noting the number of days required for eschar to fall away, leaving no raw wound behind. The percentage of wound contraction was recorded.

$$\text{Wound contraction} = \frac{\text{Area on day zero} - \text{area on day of measurement}}{\text{Area on day zero}} \times 100$$

Incision wound model

The albino Swiss mice of either sex were divided into 4 groups, 6 animals in each group (total 24 animals).^[11]

Group 1- Control- Simple ointment locally once daily

Group 2- HEAA (5% ointment) locally once daily

Group 3- HEAA (10% ointment) locally once daily

Group 4- Standard drug Soframycin ointment locally once daily

The animals were anesthetized by using Ketamine (80 mg/kg i.m).^[12] The back of the rats were shaved. Paravertebral straight incisions of 3 cm were made through the entire thickness of the skin at the distance of about 1.5 cm from midline on the depilated back of mice. No local or systemic antimicrobials were used throughout the experiment. Wounds were closed with intermittent sutures, 1 cm apart, with black silk thread (no. 3) and curved needle (no. 11). After stitching, the wound was left undressed then simple ointment base, extract ointment and standard ointment were applied daily from 1 days to 9th post- wounding days; sutures were removed on day 7. On the 10th day post-wound, sutures were removed and the wound breaking strength was estimated on the 10th day by continuous, constant water flow technique as describe by Lee. The breaking strength was expressed as minimum weight of water necessary to bring about gapping of the area.

Statistical analysis

All the results were expressed as Mean ± Standard Error Mean (SEM). The differences between experimental groups were compared by one-way Analysis of Variance (ANOVA) followed by Tukey's post hoc test, using SPSS computer software (version 15.0). A *p*-value<0.05 was considered as statistically significant.

RESULTS

Acute toxicity studies

A preliminary toxicity study was designed to demonstrate the appropriate safe dosage range of extract to be used for subsequent experiments. Acute toxicity studies conducted revealed that the administration of the hydroalcoholic extract of *A. Americana* leaves (up to a dose of 2000 mg/kg) did not produce any adverse effects and significant change in the behavior of the animals. No death was observed up to the dose of 2000 mg/kg body weight and the experimental animals were physically active. It indicated that the median lethal dose (LD₅₀) could be greater than 2000 mg/kg body weight.

Wound healing-Excision method

In the excision wound healing model, the decrease in wound area in control group measured on day 1, 4, 7, 10, 13 and 16 in 94.83 ± 0.8724, 89.83 ± 0.6009, 70.00 ± 0.8944, 50.33 ± 1.430, 25.17 ± 1.400 and 12.33 ± 0.7601, respectively but there was a significant decrease in wound contraction started from day 4. The animals treated with 5% HEAA extract showed an increase in the percentage of wound contraction when compared with control animals from day 4 but was becomes significant (p<0.05) from day 10. Whereas 10% HEAA leaves showed a reduction in the wound from Day 4 onwards (p<0.05) with wound area of 81.00 ± 0.9661 in comparison with control. On comparison of animals receiving 5% and 10% of *Agave americana* leaf extract showed by using one-way ANOVA followed by Tukey's post hoc test, there was no significant difference in wound area between the groups on the measured respective days. The rate of epithelialization for 10% HEAA is almost comparable to standard, Soframycin ointment [Table 1].

Incision method

In incision wound model, the breaking strength of ten-day old incision wound was measured. The mean breaking strength of wound in control group was 110.0 ± 0.8944 gram. Although the mean breaking strength in the group treated with 5% ointment of *Agave americana* leaf was increased to 123.2 ± 2.762 gram extract showed significant (p<0.05) increase in tensile strength as compared to control. The animals that received 10% ointment of *Agave americana* leaf extract demonstrated a mean breaking strength of 135.7 ± 2.261 gram showed significant (p<0.05) increase in tensile strength as compared with control [Table 2]. On comparison of animals receiving 5% and 10% of *Agave americana* leaf extract showed by using one-way ANOVA followed by Tukey's

post hoc test, it showed a significant difference (p<0.05) between the two groups.

DISCUSSION

Wound healing is a complex, natural and dynamic process of restoring dermal and cellular structures as closely as possible to its normal and original state.^[13] In the event of any injury, a set of overlapping events takes place to repair the damage.^[14] Wound healing has been discussed into phases which include the inflammatory, proliferative and the maturational or remodeling phases.^[15] The process of wound healing depends upon the type and extent of damage occurred and also it depends on the general health of the host and its ability to repair. After the injury, a haemostatic process takes place which prevents further blood loss and provides the initiation for other phases namely inflammation, proliferation, and remodeling.^[16] In the inflammatory phase of healing, neutrophils and macrophages are attracted into the injured tissue and they locate and phagocytize, kill and digest microorganisms and eliminate wound debris.^[17] Inflammatory phase is followed by angiogenesis, collagen deposition, granulation tissue formation and epithelialization in the proliferative phase.^[18] In the maturation phase, the wound undergoes contraction by a combination of all the process and resulting in a smaller amount of apparent scar tissue.^[19]

In the inflammation phase of healing, neutrophils and macrophages eliminate wound debris through their characteristic 'respiratory burst' activity and phagocytosis. At higher concentrations, radical oxygen species (ROS) can cause damage to cellular membranes, DNA, proteins and lipids and thus impede the healing process.^[20] Thus a compound or a plant extract which possesses antioxidant potentials and antimicrobial activity can produce a potential therapeutic agent for wound healing.

Wound contracture begins 4 to 5 days after injury. It helps to close the wound gap between dermal edges and reducing the wound surface area. It is due to the action of myofibroblasts. Granulation tissue of the wound is a tissue formed in the inflammatory phase of wound healing. It is composed of blood vessels, macrophages, and fibroblast with a loose connective tissue matrix. The cells contract the wound and produce a large amount of ECM components. Collagen is a major constituent in the remodelling phase. It composed of amino acid (hydroxyproline) which gives strength and support. Breakdown of collagen liberates free hydroxyproline and its peptides; measurement of the hydroxyproline could be used as an index for collagen turnover.^[21]

Table 1: Wound Healing activity of hydroethanolic extract of *Agave americana* by excision method on mice.

Group	Post wounding days wound area (mm ²) mean ± SEM						Period of epithelialisation
	(Percentage of wound Contraction)						
	Day 1	Day 4	Day 7	Day 10	Day 13	Day 16	
Control	94.83	89.83	70.00	50.33	25.17	12.33	18.5
Simple Ointment	± 0.8724 (0.0)	± 0.6009 ^{αβ} (5.27)	± 0.8944 ^{αβ} (26.18)	± 1.430 ^{αβ} (46.92)	± 1.400 ^{αβ} (73.45)	± 0.7601 ^{αβ} (86.99)	
5% HEAA	97.17 ± 0.6009 (0.0)	84.67 ± 1.382* (12.86)	67.00 ± 0.9309 (31.04)	33.83 ± 0.9098* ^β (65.18)	15.17 ± 0.7923* (84.38)	8.167 ± 0.3073* (91.60)	17.5
10% HEAA	95.33 ± 1.022 (0.0)	81.00 ± 0.9661* (15.03)	51.33 ± 0.8028* (46.15)	25.67 ± 1.358* ^α (73.07)	13.00 ± 0.7303* (86.36)	6.000 ± 1.000* (93.70)	15
Soframycin	94.50 ± 0.4282 (0.0)	80.00 ± 0.7303* ^{αβ} (15.34)	41.83 ± 1.014* ^{αβ} (55.73)	19.33 ± 0.5578* ^{αβ} (79.54)	8.833 ± 0.4773* ^{αβ} (90.65)	0.0 ± 0.0* ^{αβ} (100)	14.16

Table 2: Wound Healing activity of hydroethanolic extract of *Agave americana* by incision method on mice

Group	Dose (mg/kg)	Tensile strength (gm)
Control	Simple Ointment	110.0 ± 0.8944 ^{αβ}
HEAA	5% Ointment	123.2 ± 2.762 ^{*β}
HEAA	10% Ointment	135.7 ± 2.261 ^{*α}
Standard	Soframycin	165.7 ± 3.621 ^{*αβ}

Note: Number of animals n=6. Inter-group comparisons were made by using one way ANOVA followed by Tukey's post hoc test. Results are expressed in Mean ± SEM: *p<0.05- significant as compared to control, ^αp<0.05- significant as compared to 5% HEAA and ^βp<0.05- significant as compared to 10% HEAA. HEAA-Hydroalcoholic extract of *Agave americana* leaves

In the excision wound model, 5% ointment of HEAA leaves extract showed a significant reduction in wound area from Day 4 onwards (p<0.05) as compare to control. On the other hand, 10% ointment of HEAA leaves extract showed the reduction in wound area on Day 4 onward (p<0.05) which was highly significant as compared to control [Table 1].

In the incision wound model, animals treated with 5% ointment and 10% ointment of HEAA leaves showed a significant increase in tensile strength as compared to control (p<0.05) [Table 2].

Agave americana leaves were reported to have flavonoids which have antioxidant activities in the body.^[22] On the other hand, the plant also contains tetratriacontanol and homoisoflavanoids derivatives which attribute to its antibacterial activities.^[4] The plant also contains genins (total steroidal saponin, hacogenin, and tigogenin) which play a major role in the inflammatory process.^[9] It is due to its major constituents, it has a role in wound healing processes.^[23]

In the inflammatory process of wound healing, as free radicals are produced which attract different inflammatory mediators and which are responsible for tissue damage and reduce the anti-oxidant in the body. Flavonoids of the plant, *Agave americana* may thus contribute an additive effect to the endogenous anti-oxidants and may inhibit the eicosanoid biosynthesis. Flavonoids and their derivatives are known to decrease lipid peroxidation by improving vascularity and also by slowing cell necrosis. It decreases the formation of the inflammatory metabolites and prevents further damage. Thus flavonoids by virtue of its free radical scavenging, antioxidant and anti-inflammatory properties help in healing of wounds.^[24] Tetratriacontanol and homoisoflavanoids derivatives in the plant may provide additional benefit in the inflammatory phase of wound healing due to its antimicrobial activities. On the other hand, genins may also help in the inflammatory phase of wound healing.

It can be concluded that the phytochemical constituents present in *Agave americana* such as flavonoids, genins, tetratriacontanol, homoisoflavanoids, etc.^[25] may be responsible for its wound healing activity.

CONCLUSION

Herbal medicines have been used for many centuries and quoted in ancient literature to be of use for various illnesses. They have immense potential due to better availability, affordable cost, and fewer side effects. There is intensive research on various herbal medicines but many species of plants are still left unexplored. *Agave americana* plant has been known since ages for various indications but the medicinal values are less investigated. Hence the present study was planned to assess the various medicinal properties of *Agave americana* leaves.

It was found that the leaves of *Agave americana* contain various constituents which are known for its various pharmacological actions. The hydroalcoholic extract of *Agave americana* leaves (HEAA) mostly contain flavanoids, coumarins, flavanone, hacogenin, kammogenin, steroidal saponins, tetratriacontanol and homoisoflavanoids.

Wound healing activity of HEAA is seen in graded dose. 10% HEAA has wound healing activity almost comparable to the standard, Soframycin. In HEAA, flavonoids due to its anti-oxidant activities help in reducing inflammatory mediators, increase vascularity and decrease cell necrosis. The plant also contains tetratriacontanol and homoisoflavanoids derivatives which help in wound healing due to its antibacterial activities. The plant also contains genins which help in reducing the inflammatory process.

To conclude, the extract of *Agave americana* leaves demonstrated significant wound healing on experimental animals in this study. These activities may be attributable to the various phytoconstituents of *Agave americana* such as flavonoids, genins, saponin, tetratriacontanol and homoisoflavanoids. Thus, *Agave americana* leaves need to be used in higher doses to have a better understanding of the mechanism of wound healing effects. Thus, *Agave americana* leaves need to be explored intensively so that it can be used to treat various disorders.

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