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Original article

Evaluation of wound healing activity of Hibiscus *Cannabinus* Linn Leaves Extract

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ABSTRACT

Introduction: Hibiscus *Cannabinus* Linn. is medicinal herbs have been known from millennia and are highly recognized all over the word as a rich source of therapeutic agents for prevention of diseases. The plant material is used in traditional medicines for various treatments. Therefore, recent research in the developed countries is focused on herbal formulation for wound healing activities of various wound models.

Material and Method: The Macroscopic, Microscopic, Microchemical examinations of leave part was carried out for the authentication of the samples. The extraction was successively done with various solvents viz. aqueous, Hydro-alcoholic and Ethanolic in Soxhlet extractor. The pharmacological screening for wound healing activity was carried with excision, incision and burn wound models.

Result: The Pharmacological screening of wound healing activity of various extracts like Aqueous, Hydro alcoholic and ethanolic extract was carried out and was found to be potent and safe.

Conclusion: The closure and percentage wound area contraction was taken into account; it was observed that there was a marked contraction in the wounded area. HAHC showed improved healing property than EEHC and AEHC as compared to standard.

Keywords: Hibiscus *Cannabinus* Linn, Pharmacological screening, Excision, Incision, Burn wound models.

INTRODUCTION:

Hibiscus *Cannabinus* Linn; (Malvaceae) is a large bushy shrub or small tree; about 8-12 ft in height. It is cultivated in Indians gardens as an decorative plant [1]. It's reveals that Hibiscus *cannabinus* leaves possess wound healing pharmacological activity and therapeutic properties [2]. Literature Survey reveals it contains the chemical constituents as a glucosides saponins, lignans, phytosterol, flavonoids, polyphenols, tannins, steroids, alkaloids, essential oils, such a cannabiscitrin, cannabiscetin and anthocyanin glycosides. Alph-terpenyl acetate, citral, p-tolualdehyde, n-dotriacosane, n-triacontane, n-tetraacosane, n- pentacosane, n-hentriacontane and β-sitosterol have been isolated from the leaves of the plant [3]. Wound healing is a biological process that is entered by trauma and often terminated by scar formation. The process of wound healing occurs in different phases such as coagulation, epithelization, granulation, collagen formation and tissue remolding. Animal wound healing models are important biological tools to understand basic process of tissue repair and to develop and validate strategies for treatment of wounds. The process of wound healing also gets affected by other disease such as diabetics, neoplasm, microbial may also interfere. Although animal wound healing models are imperfect observation of wound healing processes in human beings and its clinical challenges, these models continue to be crucial tools for the development of new strategies and approaches for therapy of wound healing [4].

MATERIALS AND METHODS:

Plant Material: The plant was authenticated by Mrs. Priyanka A. Ingle, Scientist 'C' by comparing morphological features. The herbarium of the plant specimen was deposited at Ministry of Environment, Forest & Climate change/Botanical Survey of India/

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Western Regional Center, Koregaon road, Pune (Ref. No. BSI/WRC/100-1/Tech./2019/50 Dated 22nd Oct. 2019).

Extraction: Dried and coarsely powdered leaves part of Hibiscus *Cannabinus Linn* was subjected to successive solvent extraction in Soxhlet extractor using aqueous, hydroalcoholic and ethanolic as solvent. All the extracts were vacuum dried to produce Aqueous (15.80%) hydroalcoholic (17.00%), ethanolic (13.60%) respectively [5].

Animals: Wistar Rat (Both male and female) weighing 150-200 gm were housed under standard laboratory conditions. The animals were purchased from National Institute of bioscience, Pune and were housed under 12 hrs day and night conditions for acclimatization up to one week. The animal had free access to rat food pellet (purchased from Prashant Enterprises, Pune) the ethical committee of the institute approved the protocol of the study.

Drugs and Chemicals: The following drugs and chemicals were used Ether (PCL, India), ethanol AR (PCL, India), Ketamine hydrochloride (PCL, India).

PHARMACOLOGICAL STUDIES:

Experimental design and drug treatment: The wistar rats of either sex were divided into 11 groups with six animals in each group. Standard and other group were administered with drugs for a period of 16 days as per experimental design. For oral administration of Carboxy methyl cellulose (CMC; 0.5% w/v, p.o.) was used as a suspending agent, whereas ointment base was used for topical administration. Animals were fasted for 18 hrs (with free access to water *ad libitum*) prior to the commencement of the experiment [6].

Group I: animals (ointment base treated group) received 1 g topical administration of the ointment base devoid of extract.
Group II: animals (VTC 10%) received 1 g topical administration of vitamin C 10% w/w
Group III: animals (HAHC 5%) received 1 g topical administration of the HAHC ointment (5% w/w).
Group IV: animals (HAHC 10%) received 1 g topical administration of the HAHC ointment (10% w/w).
Group V: animals (HAHC 20%) received 1 g topical administration of the HAHC ointment (20% w/w).
Group VI: animals (EEHC 5%) received 1 g topical administration of the EEHC ointment (5% w/w).
Group VII: animals (EEHC10%) received 1 g topical administration of the EEHC ointment (10% w/w).
Group VIII: animals (EEHC 20%) received 1 g topical administration of the EEHC ointment (20% w/w).
Group IX: animals (AEHC 5%) received 1 g topical administration of the AEHC ointment (5% w/w).
Group X: animals (AEHC 10%) received 1 g topical administration of the AEHC ointment (10% w/w).
Group XI: animals (AEHC 20%) received 1 g topical administration of the AEHC ointment (20% w/w).

Excision wound model: The animals were divided into 11 groups, six animals in each group (n¼6) and were anaesthetized by open mask method with 3–5% light ether anesthesia, before the formation of the wound. The particular skin area was shaved 1 day prior to the experiment. An excision wound was exposed by cutting away the skin measuring 300 mm^2 full thickness and 2 mm depth from a predetermined shaved area. The wounds were left undressed to the open environment and the animals were carefully observed for any infection. Those animals that showed any sign of infection were separated, excluded from the study and replaced. Haemostasis was achieved by blotting the wound with a cotton swab soaked in normal saline solution. The wounded animals were kept separately in different cages. Wound area was measured without delay by placing a transparent tracing paper over the wound and tracing it out. The tracing paper was then placed on a $1mm^2$ graph sheet and traced out. The wound area was measured out on respective days (0^{th}, 4^{th}, 8^{th}, 12^{th} and 16^{th}day) and the percentage wound contraction was calculated by formula given below [7-8].

% Wound contraction = (Wound area on day '0'- Wound area on day 'n'/ Wound area on day '0') X 100

The period of epithelialization was calculated as the number of days required for falling off of the dead tissue remnants without any residual raw wound [9].

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Incision wound model: All the animals were grouped in the similarly as per the excision wound model described above. The animals were anaesthetized with ketamine hydrochloride (100 mg/kg) prior to and during the formation of experimental wounds. The vertical hairs of the animals was properly shaved and a paravertebral long incision of 4 cm length was made through the skin at a distance of about 1.5 cm from the midline on each side of the depilated back of the animals. After the incision, the wounded skin was closed by means of interrupted sutures at intervals of 0.5 cm using surgical thread (No. 000) and curved needle (No. 11). The wounds were then left undressed. Sutures were removed on 7th post-wounding day and the treatment was continued. The wound breaking strength was measured on the 10th day. The breaking strength is defined as the strength of a healing wound which is measured by the amount of force required to disrupt it [10-12].

$$\text{Wound tensile strength} = \frac{\text{Breaking strength (g)}}{\text{Cross section area of skin (mm}^2\text{)}}$$

Burn wound model: In this model, all the animals were grouped in the similarly as per the excision wound model described above. The animals were anaesthetized with ketamine hydrochloride (100 mg/kg) prior to and during the creation of experimental wounds. Burn wounds were created on dorsal part of shaved rat's skin surface using concentrated sulphuric acid, exposed for 10 s. After 24 hrs, dead tissues were excised using sterile surgical blade through a template designed to produce a third degree burn. All groups were treated same as in excision model. In this model, wound contraction and epithelialization period was monitored [13-14].

RESULTS:

In excision wounded model, group treated with simple ointment base (topical) showed no significant difference ($p > 0.05$) in the wound contraction area when compared to the positive control group (untreated group). However, treatment of the animals treated with HAHC 5%, 10%, 20% and vitamin C 10% w/w ointment topically on day 4, day 8, day 12 and day 16 depicted a dose dependant and significant ($p<0.05$) reduction in the wounded area when compared to their respective untreated control group (ointment base control). Table no.1.

Table no.1 Evaluation of in vivo wound healing activity of *Hibiscus cannabinus* (L.) extract on excision wound model in rats:

Sr. no.	Group (n=6)	Closure of excision wound area mm^2 (original wound area 300 mm^2)					Epithalization Period (days)
		Day 0	Day 4	Day 8	Day 12	Day 16	
1	Control	303.50±1.54	210.00±0.57	141.00±1.06	80.66±1.83	18.66±0.95	22.50±0.42
2	Standard	309.17±1.47 ns	161.83±1.35**	71.16±0.90**	7.66±0.88**	7.14±0.21**	12.50±0.43**
3	HAHC 5 %	310.83±1.66 ns	200.17±1.40*	110.50±1.23*	4.50±1.25*	14.12±0.12*	16.00±0.57*
4	HAHC 10%	312.83±1.38 ns	184.17±3.44*	130.00±1.39*	13.00±0.63*	12.85±0.12*	14.50±0.76*
5	HAHC 20 %	314.17±1.72 ns	172.00±1.50*	120.33±1.17*	.66±0.66**	8.10±0.21**	13.50±0.75**
6	EEHC 5 %	314.67±1.76 ns	173.17±1.49*	120.67±1.11*	2.66±0.49*	12.50±0.11*	14.83±0.47*
7	EEHC 10%	314.83±1.74 ns	172.33±1.64*	120.33±0.95*	3.33±0.66*	13.20±0.23*	15.00±0.36*
8	EEHC 20 %	314.67±1.72 ns	172.34±1.22*	120.50±0.88*	2.83±0.40*	12.14±0.02*	14.50±0.22*
9	AEHC 5 %	314.33±1.62 ns	172.67±1.52*	120.50±0.88*	3.33±0.88*	13.10±0.14	14.66±0.33*
10	AEHC 10 %	315.00±1.65 ns	172.33±1.58*	121.00±1.31*	3.83±0.65*	13.22±0.17*	14.50±0.42*
11	AEHC 20 %	315.33±1.38 ns	172.67±1.47*	120.67±1.20*	2.66±0.71*	12.10±0.12*	15.00±0.36*

HEHC-Hydroalcoholic Extract Hibiscus *Cannabinus*, EEHC-Ethanolic Extract Hibiscus *Cannabinus,* AEHC-Aqueous Extract Hibiscus *Cannabinus*.

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NS - non significant, * P<0.05, **P<0.01 Values are Mean ± SEM, n=6, when compared with Control by using one way ANOVA followed by Dunnette's multiple comparison test.
In incision wounded model, rats treated topically (VTC 10% w/w, HAHC 5% w/w, 10% w/w and 20% w/w) showed significant (p<0.05) increase in the tensile strength expressed in terms of gram.
The percentage increase in tensile strength is represented in Table 2, signifying that the topically treated rats showed significant activity as compared to the untreated groups.

Table 2: Evaluation of in vivo wound healing activity of *Hibiscus cannabinus* (L.) extract on incision wound model in rats:

Sr. no.	Group (n=6)	Effect of topical treatment on the tensile strength of incision wound model	
		Tensile strength in gram	% Increase in tensile strength
1	Control	303.50±1.54	-
2	Standard	417.00±2.42**	37.62
3	HAHC 5 %	351.50±2.46*	15.84
4	HAHC 10%	380.83±1.24**	25.41
5	HAHC 20 %	414.50±1.82**	36.63
6	EEHC 5 %	314.17±1.72*	3.50
7	EEHC 10%	351.33±3.66*	15.84
8	EEHC 20 %	381.00 ±1.48**	25.74
9	AEHC 5 %	309.17±1.47*	1.98
10	AEHC 10 %	310.83±1.66*	2.31
11	AEHC 20 %	312.83±1.38*	2.97

* P< 0.05, **P<0.01 Values are Mean ± SEM, n=6, when compared with Control by using one way ANOVA followed by Dunnette's multiple comparison test.

In burn wounded model, group treated with simple ointment base (topical) showed no significant difference (p>0.05) in the wound contraction area when compared to the positive control group (untreated group). However, treatment of the animals treated with HAHC 5%, 10%, 20% and vitamin C 10% ointment topically on day 4, day 8, day 12 and day 16 depicted a significant (p<0.05) reduction in the wounded area when compared to their respective untreated control group (ointment base control). However, when the closure and percentage wound contraction was taken into account, it was observed that there was a marked contraction in the wounded area from day 12 to day 16, with HAHC 5%,10% & 20% and VTC 10% showing improved healing property. The rate of epithelialization (in days) was also examined where the rats treated topically with HAHC 5%, 10% & 20%, showed dose dependant and significant (p<0.05) reformation of epithelial cells as compared to other tested groups. The wounded area (in mm^2), percentage wound contraction and epithelialization period are represented in Table no.3.

Table 3: Evaluation of in vivo wound healing activity of *Hibiscus cannabinus* (L.) extract on burn wound model in rats

Sr. no.	Group (n=6)	Closure of excision wound area mm2 (original wound area 300 mm2)					Epithelization Period (days)
		Day 0	Day 4	Day 8	Day 12	Day 16	
1	Control	302.83±1.01	210.33±0.42	140.00±0.68	78.33±1.76	18.16±1.01	22.16±0g.47
2	Standard	308.00±1.39 ns	161.00±1.06**	71.16±0.94**	7.66±0.71**	7.10±0.12	12.83±0.40**
3	HAHC 5 %	310.50±1.72 ns	198.83 ±1.40*	10.50±1.83*	13.33±0.76*	13.05±0.15*	15.50±0.88*
4	HAHC 10%	312.33±1.66 ns	185.00 ±2.88*	28.33±1.33*	12.33±0.42*	12.10±0.01	13.66±0.66*
5	HAHC 20 %	313.33±1.62 ns	71.17±1.19*	119.83±1.13*	7.83±0.60**	7.01±0.10**	13.16±0.60**
6	EEHC 5 %	314.17±1.81ns	174.00±1.36*	21.17±1.24*	14.16±0.60*	14.01±0.10	15.16±0.30*
7	EEHC 10%	314.33±1.74 ns	173.00±1.59*	20.83±1.04*	13.83±0.47*	13.02±0.24*	15.33±0.33*
8	EEHC 20 %	315.67±1.30 ns	172.83±1.13*	21.00±0.96*	13.16±0.40*	13.11±0.23*	14.83±0.30*
9	AEHC 5 %	313.67±1.14 ns	173.83±1.55*	21.17±1.04*	14.97±0.57*	14.25±0.57*	15.16±0.47*
10	AEHC 10 %	314.33±1.70 ns	173.17±1.55*	121.67±1.38*	14.33±0.55*	14.02±0.24*	14.83±0.30*
11	AEHC 20 %	315.00±1.12	72.83±1.42*	121.00±1.21*	13.00±0.73*	12.84±0.11*	15.16±0.40*

NS- non significant, * P< 0.05, **P<0.01 Values are Mean ± SEM, n=6, when compared with Control by using one way ANOVA followed by Dunnette's multiple comparison test.

DISCUSSION:

However, when the closure and percentage wound area contraction was taken into account, it was observed that there was a marked contraction in the wounded area from day 12 to day 16, with HAHC 5%, 10% & 20% and VTC 10% showed improved healing property. The rate of epithelialization (in days) was also examined where the rats treated topically with HAHC 5%, 10% & 20%, showed significant (p< 0.05) reformation of epithelial cells as compared to other tested groups[7-9]. The wounded area (in mm^2), percentage wound contraction and epithelialization period are represented in Table no.1. The promotion of wound healing activity is also well gazed by its tensile strength of the incision wound. Generally wound-healing agents have the properties to enhance the deposition of collagen content, which provides strength to the tissues and forms cross-linkages between collagen fibers. The tensile strength of the extract treated group was showed significant (p<0.05) increase in the tensile strength expressed in terms of gram. The closure and percentage increase in tensile strength is represented in Table 2. It was signifying that the topically treated rats showed significant activity as compared to the untreated groups [10-12].

The effect of topical administration of the extract ointment treated group and control treated group on burn wound model. Group treated with simple ointment base (topical) showed no significant difference (p>0.05) in the wound contraction area when compared to the positive control group (untreated group). However, treatment of the animals treated with HAHC 5%, 10%, 20% and vitamin C 10% ointment topically on day 4, day 8, day 12 and day 16 depicted a significant (p<0.05) reduction in the wounded area when compared to their respective untreated control group (ointment base control). However, when the closure and percentage wound contraction was taken into account, it was observed that there was a marked contraction in the wounded area from day 12 to day 16, with HAHC 5%, 10% & 20% and VTC 10% showing improved healing property. The rate of epithelialization (in days) was also examined where the rats treated topically with HAHC 5%, 10% & 20%, showed significant (p<0.05) reformation of epithelial cells as compared to other tested groups [13-14]. The wounded area (in mm^2), percentage wound contraction and epithelialization period are represented in Table no.3

CONCLUSION:

From the present study we conclude that the closure and percentage wound area contraction was taken into account, it was observed that there was a marked contraction in the wounded area from day 12 to day 16, with HAHC 5%, 10% & 20% and VTC 10% showing improved healing property. In excision,

incision and burn wound model, HAHC 5%, 10% & 20 % ointment showed potent wound healing activity as compare to other wound healing ointment like EEHC & AEHC respectively. The healing effects seemed to be due to decreased free radical generated tissue damage, promoting effects on antioxidant status, faster collagen deposition, and other connective tissue constituent formation. The above effect may be due to presence of phytoconstituents like flavonoids, phytosterols, tannin, alkaloid etc.

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