



Preliminary Phytochemical Wound Healing Activity of Hibiscus Cannabinusl Seed Aqueous and Ethanolic Extracts

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ABSTRACT

The present study was carried out to evaluate preliminary phytochemicals and wound healing activity of hibiscus cannabinus seed of ethanolic aqueous extract. In this study we observed that percentage yield of ethanolic extract was 8.5% w/w and the extract was subjected to TLC analysis and also carried out the estimation of total phenolic and flavonolic content. In this study we observed that no toxic symptoms like behavioral changes, locomotion, convulsions etc. were observed. Also, we calculated the IC_{50} values. For the conducting wound healing activity we utilized excision and incision wound models.

Keywords: Hisbiscus cannabinus L. seed, locomotion, TLC analysis, IC_{50} values, incision and excision wound models.

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INTRODUCTION

Plants have been mostly exploited from the day, when humans realized their effectiveness and versatility as medicines. There is a genuine expectation in developing countries that their drug problems can be alleviated through a sensible scientific exploitation medicinal plants some of which have been used for generation by indigenous populations. Then there is the worldwide green revolution that is reflected in the belief that herbal remedies are safer and less damaging to the human body than synthetic drugs. [Aggarwal V.S. and Ghosh B. Drug plants of India (Root drugs) Kalyani publishers, New Delhi (1985)].

Traditional systems followed in ancient civilization have been passed on through generations. Systems like Ayurveda, Siddha, Chinese system, Tibetan system indigenous African medicine utilized plant source to maximum. [P13K/Ak signaling pathways in fibroblast and stimulates cell proliferation and migration, cellular signaling; 2005: 17(12):1486-1494].

One major criterion for the selection of a plant for such study is traditional healer's claim for its therapeutic usefulness.[Aravindsaklani and Samuel k. Kutty. plant derived compounds in clinical trails. Drug discovery today (2008)]

The number of species of higher plants on the planet is estimated between 370,000 and 500,000. All higher plants elaborate chemical secondary metabolites that are of potential medical interest. Therefore, the determination of the criteria for selecting plants for phototherapeutic investigation is perhaps as important an exercise as the investigation itself. Selection is mainly based on traditional usage, chemical composition and screening for a specific biological activity. [Bartkova, J.; Gron, B.;Dabelsteen, E.;Bartek, J. (2003). "Cell-cycle regulatory proteins in human wound healing". *Archievs of Oral Biology* 48 (2)125-132].

MATERIALS AND METHOD

Preparation of Extraction

The *Hibiscus cannabinus* seeds were collected and dried for 20 days under shade and powdered by using mechanical grinder. A weighed quantity of powder (500 gm) was passed into sieve no. 40 and subjected to aqueous and Ethanolic extraction (maceration) with distilled water and 90% ethanol and kept at room temperature for 7 days with occasional stirring. The extract was filtered. The aqueous and Ethanolic extracts were concentrated in water bath (kokate; sofawora 1993).

Materials used

Powdered plant material, Magnetic stirrer Hotplate, What man filter paper, Solvents, Ethanol, Distilled water.

Phytochemical Analysis

We have done phytochemical tests and found compounds such as carbohydrates, saponins, tannins, flavonoids, phenols, proteins and amino acids in aqueous extract. Where as in ethanolic extract alkaloids, phytosterols, terpenoids were identified (table.no:1). In addition, we used Thin layer chromatography (TLC) technique (table.no:2). We estimated the total phenol and flavonol content. Pharmacological activity can be determined by the acute toxicity studies and In-vitro anti oxidant activity with the scavenging of ABTS radical action. Therefore the present study was planned to evaluate the wound healing activity of *Hibiscus cannabinus* using Incision and Excision wound models in rats. We prepared 5%w/w *Cardiospermum helicacabum* ointment, its composition mention in (table no-3). In excision we measure the epithelialization period and estimate the protein content where as in Incision we determine the wound breaking strength. We selected the animals and followed the experiments were approved by CPCSEA and the IEC(table no-4).

Experimental design

i) Excision model: (n=6)

Group I: Control, treated with ointment base,

Group II: Test, treated with 5% w/w ointment leaf extract,

Group III: Standard, treated with 5 % w/w Povidone iodine ointment

ii) Incision model: (n=6)

Group I: Control, treated with ointment base,

Group II: Test treated with 5% w/w ointment leaf extract,

Group III: Standard, treated with 5 % w/w Povidone iodine ointment.

Statistical analysis: The results were expressed as Mean \pm SEM. The data were analyzed by one-way analysis of variance (ANOVA) followed by Turkey multiple comparisons test and $p < 0.01^*$, 0.001^{**} was considered significant.

Table: 3 Formula for preparation of ointment

Ingredients	Quantity (gm)
White bees wax	10gm
Hard paraffin	15gm
Cetosteryl alcohol	25gm
White soft paraffin	450gm
<i>Hibiscus cannabinus</i> extract	2.5gm

Table: 4 specification laboratory animals

Strain	Albino wistar rats
Age	4-5 Month
Gender	Either sex
Body Weight	150-250g

Phytochemical Evaluation

Percentage yield of ethanolic extract

Total amount of crude drug used = 200 gm

Amount obtained as ethanolic extract = 17 gm

Percentage yield = 8.5% W/W

Table: 1 Preliminary phytochemical studies

Sl. No.	Phytochemical constituents	Ethanolic Extract	Aqueous Extract
1.	Alkaloids	+	-
2	Carbohydrates	+	+
3	Glycosides	-	-
4	Phytosterols	+	-
5	Fixed oil and fats	-	-
6	Saponins	+	+
7	Tannins	+	+
8	Phenols	+	+
9	Proteins and amino acids	+	+
10	Gum and Mucilage	-	-
11	Flavonoids	+	+
12	Terpenoids	+	-

(+) Present (-) Absent

Thin layer chromatography

The extract was subjected to TLC analysis. Different spots were identified by using iodine chamber and the R_f values are correspondingly calculated.

Table 2: Contents used in chromatograph

Extract	Mobile phase	Spraying Reagent	R_f values
Ethanolic	Chloroform : Benzene (5 :1)	Iodine Chamber	Blue : 0.90 Green : 0.53 Yellow : 0.28
Ethanolic	Chloroform : Benzene (4 :1)	Iodine Chamber	Blue : 0.90 Green : 0.84
Ethanolic	Chloroform : Benzene (4 :2)	Iodine Chamber	Blue : 0.96 Green : 0.18 Yellow : 0.47 Rose : 0.36

Ethanolic	Chloroform : Benzene (5 :3)	Iodine Chamber	Blue	: 0.99
			Green	: 0.18
			Yellow	: 0.28
			Rose	: 0.83
Ethanolic	Chloroform : Benzene (5 :2)	Iodine Chamber	Blue	: 0.95
			Green	: 0.28
			Yellow	: 0.41

Estimation of total phenolic and flavonol content

Total phenol and flavonol content of the extracts were determined by using the Folin-Ciocalteu and aluminum chloride methods. Ethanolic and aqueous extracts, total phenolic content were found to be 134.50 ± 5.54 and 36.70 ± 1.13 mg GAE/g, respectively and total flavonol content were found to be 33.07 ± 0.68 and 16.20 ± 0.42 mg RE/g, respectively.

Acute toxicity studies

On the basis of toxicity study, it was observed that the Ethanolic and aqueous extracts of *Hibiscus cannabinus* were nontoxic and did not induce death at the highest single dose, 2000 mg/kg body weight. No toxic symptoms like behavioral changes, locomotion, convulsions etc., were observed.

In-Vitro Antioxidant Activity

Ethanolic extract exhibited potent *in vitro* antioxidant activity in scavenging of ABTS radical cation with IC₅₀ value, 5.37 ± 0.20 µg/ml. The result was found to be high value when compared to standard drug (ascorbic acid). Aqueous extract showed moderate antioxidant activity with IC₅₀ value 56.03 ± 0.1 µg/ml when compared to standard drug.

In hydrogen peroxide method, Ethanolic extract showed potent activity with IC₅₀ value, 25.89 ± 0.14 µg/ml as compared to the standard Rutin whereas aqueous extract has shown less activity with IC₅₀ value, 167.14 ± 0.33 µg/ml.

In the alkaline DMSO method, since IC₅₀ values were found to be greater than 1000 µg/ml, both the extracts were found to be inactive.

Table 5: In-vitro antioxidant activity of the *Hibiscus cannabinus* is extracts

Extracts/ Standards	IC ₅₀ values ± SEM (µg/ml)		
	ABTS	H ₂ O ₂	Alkaline DMSO
Ethanolic extract	5.37 ± 0.20	25.89 ± 0.14	> 1000
Aqueous extract	56.03 ± 0.15	167.14 ± 0.33	> 1000
Ascorbic acid	11.95 ± 0.06		34.24 ± 0.92
Rutin		36.00 ± 0.58	

Average of six determinations, mean \pm SEM.

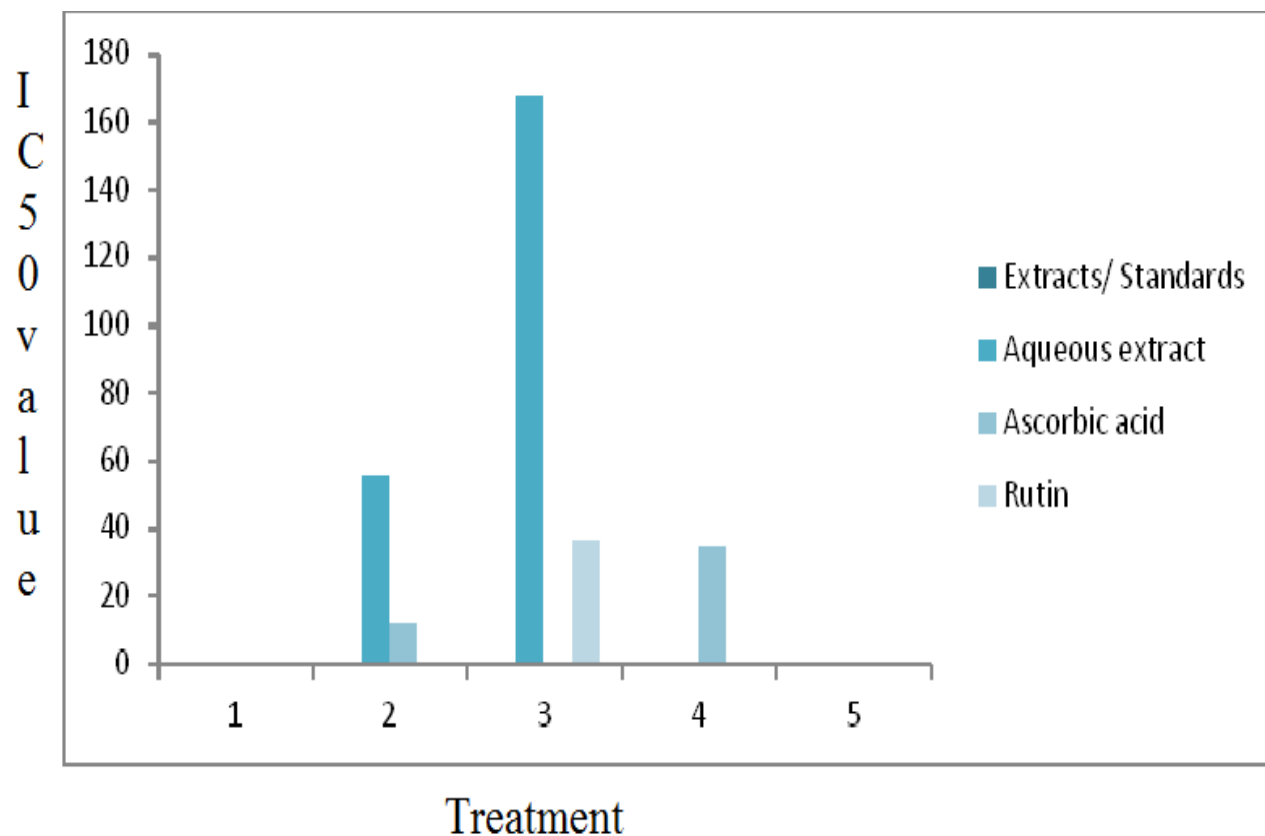


Figure 7: In-vitro Antioxidant Activity

PHARMACOLOGICAL EVALUATION

Excision wound model

i) Determination of percentage of wound contraction:

Table 6 Effect of *Hibiscus cannabinus* leaf extract on excision wound model (%wound closure).

S.NO	0 th day	4 th day	8 th day	16 th day
CONTROL	514.12 \pm 0.76	411.26 \pm 3.34	402.36 \pm 1.16	359.81 \pm 0.62
STANDARD	511.62 \pm 1.52	326.62 \pm 1.45	134.67 \pm 0.89	15.37 \pm 0.91
EXTRACT (100 mg)	507.00 \pm 2.86	465.67 \pm 1.85	276.27 \pm 0.83	72.02 \pm 0.72
EXTRACT (200 mg)	509.82 \pm 1.62	442.36 \pm 1.32	259.27 \pm 0.62	52.27 \pm 0.94

Values are expressed as Mean \pm S.E.M.* $p < 0.01$ significant, ** $p < 0.001$ highly significant, when compared to control.

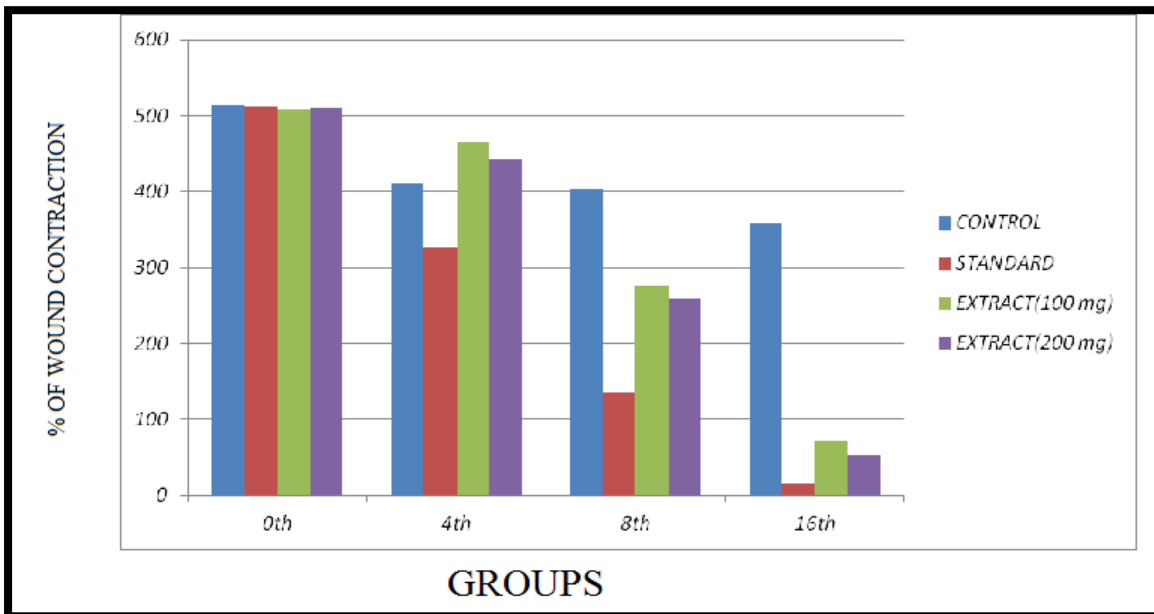


Figure 8: Percentage of wound contraction

Table 7 Epthelialisation period

GROUPS	Period of Epthelialisation
Group (Control)	26.26 ± 0.40
Group II (Extract)	23.17 ± 0.54*
Group III (Standard)	20.03 ± 0.39**

Values are expressed as Mean ± S.E.M.* p<0.01 significant, **p<0.001 highly significant, when compared to control.

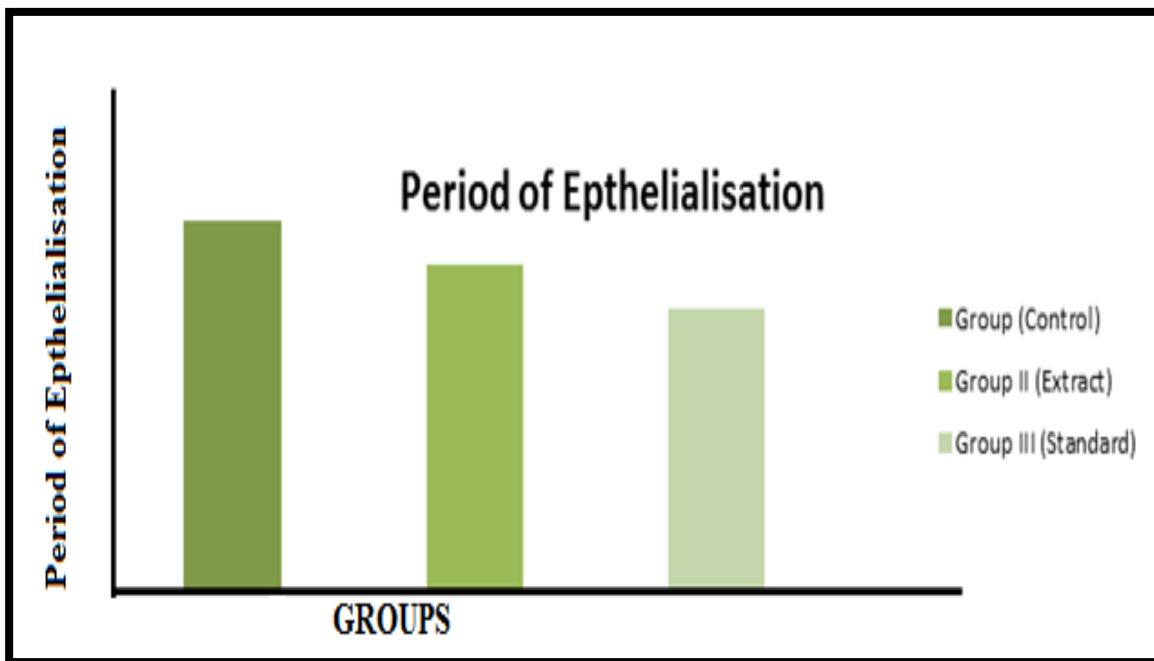


Figure 9: Time period of epithelialization

iii) Estimation of protein content:

Table 8 Effect of *Cardiospermum halicacabum (L)* ointment on biochemical parameter protein estimation.

Groups	Protein content after days		
	4 th Day	8 th Day	16 th Day
Group(Control)	2.04 ± 0.2	6.85 ± 0.57	10.41 ± 3.22
Group II (Extract)	4.23 ± 1.01*	8.60 ± 2.09	13.66 ± 3.10*
Group III (Standard)	8.18 ± 0.06**	13.65 ± 1.42**	15.95 ± 2.71**

Values are expressed as Mean ± S.E.M.* p<0.01 significant, **p<0.001 highly significant, when compared to control.

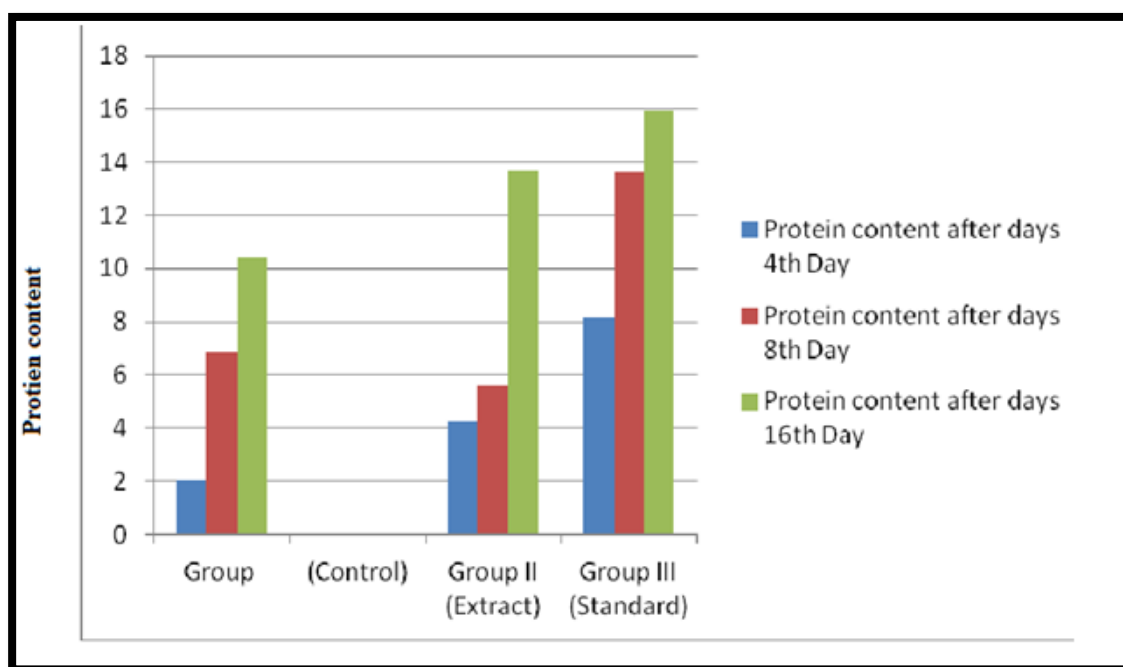


Figure 10: Estimation of protein content

Incision wound model:

Table 9 Effect of leaves extract of *hibiscus cannabinus L* incision wound (breaking strength in grams).

Groups	Incision wound breaking strength (g)
Group (Control)	263.17 ± 31.9
Group (Extract)	435.17 ± 34.64**
Group III (Standard)	421 ± 81.14*

Values are expressed as Mean ± S.E.M.* p<0.01 significant, **p<0.001 highly significant, when compared to control.

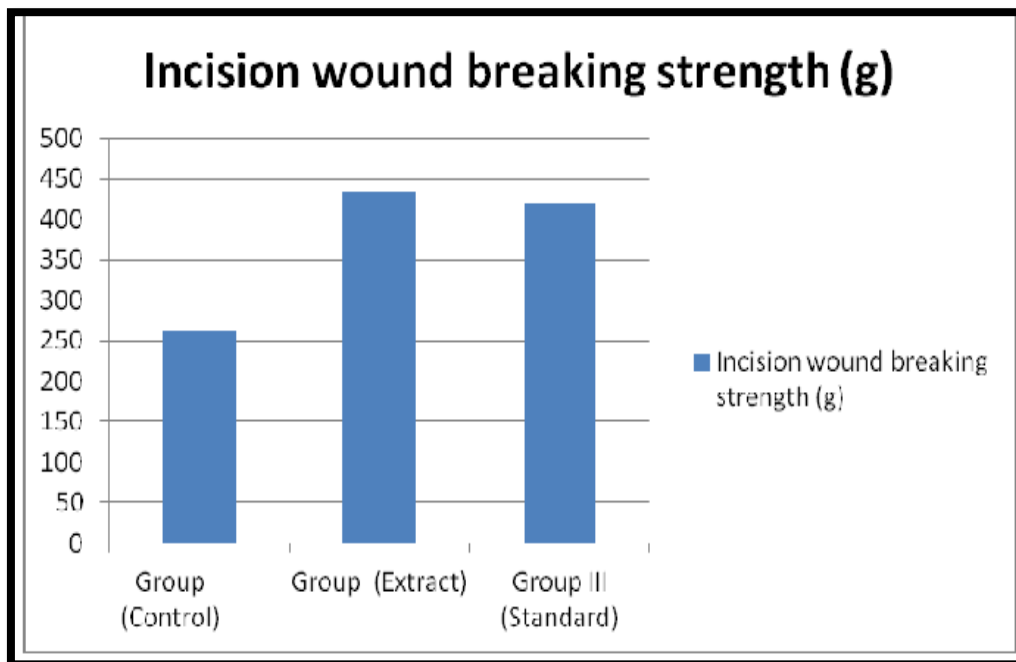


Figure 11: incision wound breaking strength in gram



Figure 12: Excision Wound Model on Zero Day

Image no 4 : control on 16th dayImage no 5 test on 16th dayImage no 6: standard on 16th day**Figure 13: Excision Wound Model on sixteenth day**Image no 7 : control on 0th dayImage no 8 test on 0th dayImage no 9: standard on 0th day**Figure 14: Incision Wound Model on zero day**

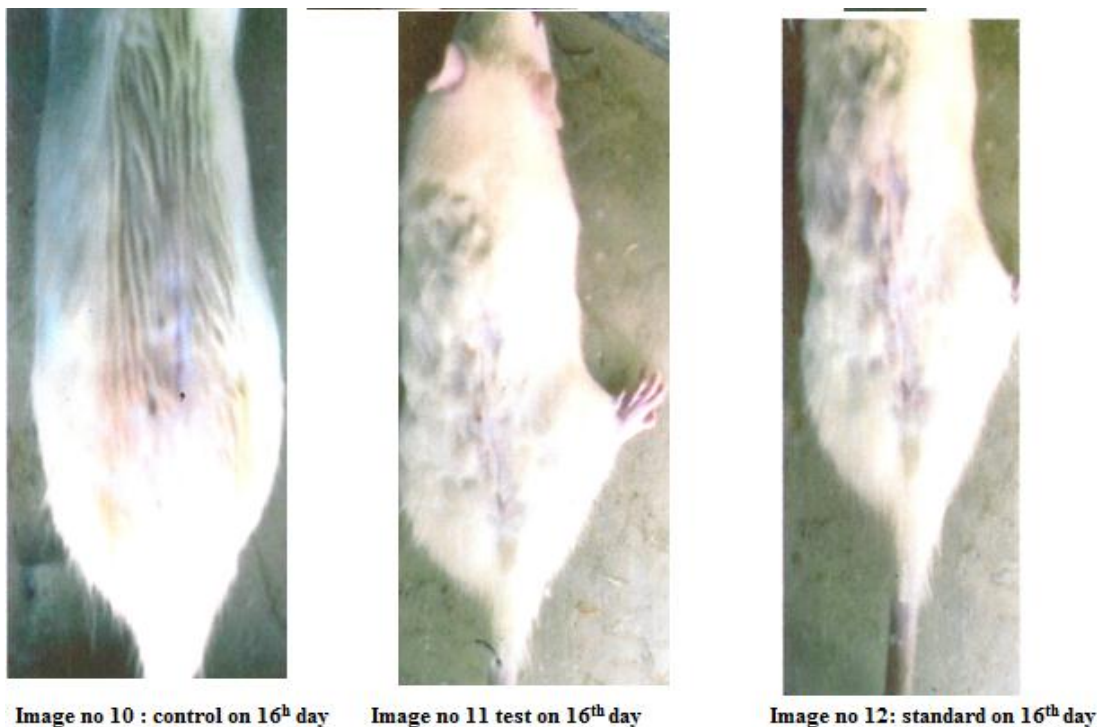


Figure 15: Incision Wound Model on Sixteenth day

RESULTS AND DISCUSSION

Hibiscus cannabinus is a large woody shrub with many-branched. The ethanol extract of *Hibiscus cannabinus* seeds showed strong by inhibiting ABTS radical action and hydrogen peroxide scavenging activities when compared with standards such as ascorbic acid and Rutin. In addition, the Ethanolic extract found to contain a noticeable amount of total phenols, which play a major role in controlling antioxidants.

Flavonoids reduce inflammation and promote circulation and inhibit allergic reactions. Phenols can scavenge a wide range of reactive oxygen and nitrogen because of their scavenging activity owing to the presence of hydroxyl group. These results provide strong evidence that the Ethanolic extract of *Hibiscus cannabinus* is seeds have wound healing activity due to the presence of flavonoids and phenol.

The topical application of *Hibiscus cannabinus* is ointment increased the percentage of wound contraction and this indicates rapid epithelization and collage nation. Wound healing process consists of different phases such as granulation, collage nation, collagen maturation and scar maturation which are concurrent but independent of each other. It is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as close as possible to its normal state. Wound contracture is a process, commencing in the fibroblastic stage whereby

the area of the wound undergoes shrinkage. Collagen, the major component which strengthens and supports extracellular tissue is composed of amino acids, hydroxylproline, which has been used as a biochemical marker for tissue collagen (Kumar R, 2006)

Hence in this investigation to models were used to assess the effect of the Ethanolic extract of *Hibiscus cannabinus* seeds. The result of the present investigation showed that a *Hibiscus cannabinus* possess a definite pro healing action. The Ethanolic leaf extract was screened for wound healing activity. Table 7 shows the results of the wound healing activity of extract ointment formulations by excision method.

The results were expressed as mean percentage closure of excision wound area. The studies on excision wound healing model reveal that the test group showed a decrease in wound area from 1st day to 16th day. Ointment prepared from Ethanolic leaf extract has shown significant wound healing activity, which was comparable to that of standard. However, the rate of contraction is less when compared to standard. On 16th day complete healing of wound was observed with standard marketed ointment and ointment of Ethanolic leaf extract produced 90.98% healing of wound as compared to control. The control (ointment base) has shown 76.86% healing.

In similar manner percentage protein concentration increased. The observed increase in protein (Table 9), an important constituent of extracellular matrix in the treated animals confirmed that the extract had positive effects towards cellular proliferation, granulation tissue formation and epithelization. The increase in protein content in the treated group is predominantly due to enhanced collagen synthesis. The importance of protein in wound healing has been appreciated for a long time for the simple reason that the ultimate result of most repairs in the higher vertebrates is the formation of scar tissue composed of collagenous fibers.

The Ethanolic extract treated group had sharp increase in protein content from day 4 to 16. This is comparable to the standard povidone iodine used. The present investigation demonstrated that Ethanolic extract of *Hibiscus cannabinus* seeds has properties that render it capability in promoting wound – healing activity compared with standard, treatment, control.

Table 9 shows the results of the wound healing activity of extract ointment formulations by incision method. The results were expressed as mean breaking strength of incision wound area. The studies on incision wound healing model reveal that the test group showed high breaking strength in wound area from 1st day to 10th day. Ointment prepared from Ethanolic leaf extract has shown significant wound healing activity, which was comparable to that of standard marketed preparation. The rate of breaking strength is more when compared to standard. On 10th day complete healing of wound was observed with standard marketed ointment, and ointment of

Ethanollic leaf extract produced 435.17 g healing of wound as compared to control. The control (ointment base) has shown 263.17 g healing.

CONCLUSION

Considering the rich diversity of plants, it is expected that screening and scientific evaluation of plant extracts for their wound healing activity may provide new antiseptic and ant microbial substances, hence in the present investigation the wound healing activity of hibiscus cannabinus seeds of both aqueous and ethaonolichas been demonstrated for the first time against wound healing by two methods. Thus this plants can be utilized as an alternative source of useful drugs. Further studies are needed with this plant to isolate, characterize and elucidate the structure of the bioactive compounds of this plant for industrial drug formulation.

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