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RESEARCH ARTICLE

EFFECT OF CISSUS QUADRANGULARIS ON FRACTURE HEALING : AN ANIMAL STUDY

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Abstract

Traditionally Cissusquadrangularis has been used as a medicinal plant since antiquity. Cissus has been used in various Ayurvedic classical medicines to heal broken bones and injured ligaments and tendons. It is being used very commonly & without much clinical evidences of level-1. The said drug is being widely used by orthopaedic surgeons. The current study was done on 30 healthy male albino rabbits after clearance from IAEC. The animals were divided into two groups viz. control and study group. Fibula of left leg of all the animals were fractured surgically. The study groups received oral CissusQuadrangularis in solution. Fracture healing of study group was compared with the control group radiologically and histopathologically to establish the role of CissusQuadrangularis on fracture healing.

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Introduction:-

The biology of fracture healing is a complex biological process that follows specific regenerative patterns and involves changes in the expression of several thousand genes.

In animal models (rat, rabbit, mouse) the peak of soft callus formation occurs 7–9 days post trauma with a peak in both type II pro-collagen and proteoglycan core protein extracellular markers (2). At the same time, an intramembranous ossification response occurs subperiostally directly adjacent to the distal and proximal ends of the fracture, generating a hard callus that ultimately provides the fracture with a semi-rigid structure which allows weight bearing (3).

Cissus Qudrangularis

It is a perennial plant of grape family. It is also known as veldt grape, devil's backbone, adamant creeper, asthisamharaka, hadjod and pirandai.

It is probably native to India, Sri Lanka and Bangladesh, but is also found in Africa, Arabia and Southeast Asia. It has been imported to Brazil and the southern United States.

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Cissus has been used in various Ayurvedic classical medicines to heal broken bones and injured ligaments and tendons. In Siddha medicine it is considered a tonic and analgesic, and is believed to help heal broken bones, thus its name *asthisamharaka* (that which prevents the destruction of bones). The Garo tribe of Bangladesh have used *C. quadrangularis* as a medicinal plant for bone fracture.

C. quadrangularis has been studied for its effects in a rat model for osteoporosis(4).

C. quadrangularis has been studied in animal models of bone fracture(1).

Materials And Methods:-

Present study was conducted in department of Orthopaedics Government Medical College Jammu during the period from July 2016 to November 2017 on 30 albino rabbits weighing 1-2 kgs, to study the effect of *Cissusquadrangularis* on fracture healing.

1: **Animals:** In the present study, 30 healthy male albino rabbits of 1-2 kg weight and around 2 yrs of age were taken as experimental animals. (FIG 1)

The rabbits were procured from the central animal house of the department of pharmacology, GMC, Jammu.

Experiments were performed on the rabbits after taking clearance from the IAEC proposal vide Number: IEC/Thesis/Research/T5B/2016/307 dated 29-10-2016.

All the CPCSEA guidelines were followed.

All the animals were kept in standard cages (FIG 2) and maintained under standard laboratory conditions (temperature 25±2°C with 12 hrs light/12 hrs dark cycle) with free access to standard diet and water throughout the study.

2: **Drugs:** *Cissusquadrangularis* was procured from the market & 10mg/kg drug was given to study group as dissolved solution.

Experimental design:

Thirty rabbits, taken as experimental animals, were divided by randomization into two groups of fifteen rabbits each and each group was as follows:

Group I(n=15) :- served as control and received normal saline.

Group II(n=15) :- served as study group and received 10 mg/kg of *Cissusquadrangularis* orally once daily for 4 weeks.

Methodology:-

Healthy male albino rabbits between 1-2 kgs were taken for study.

The animals were divided in two groups (I & II) as follows:-

Group I = Control 15 animals

Group II = Study 15 animals

The animals were kept in iron cages in a room (Fig. 2). They were fed with standard diet. The body weight of rabbits were recorded before the onset of experiment. The animals of Group I were not given *Cissusquadrangularis*.

Group II were given 10mg/kg solution of *Cissusquadrangularis* orally.

Preparation Of Drug solution:

Drug solution was prepared by dissolving *Cissusquadrangularis* in normal saline before oral administration.

Administration of the drug:

Cissusquadrangularis solution was administered to the rabbits with a syringe/cannula.

Anaesthesia, surgical fracture of fibula of rabbit and its dissection:

After anaesthesia with Inj. Ketamine 35mg/kg i/m and Inj. Xylazine 5mg/kg i/m.

Under all aseptic precautions part was prepared (Fig. 3,4,5 &6).

A small longitudinal skin incision was given over lateral aspect of middle of leg (Fig.7&8). Soft tissue dissected. Fibula exposed (Fig. 9). A transverse fracture of middle 1/3 of fibula done with help of bone cutter (Fig. 10). Skin closed with 3-0 ethilon (Fig. 11). ASDs done (Fig. 12), Analgesics (Inj. Meloxicam 0.2mg/kg i/m B.D.) + antibiotics (Inj. Ampicillin 25mg/kg i/m B.D.) were given for one week post surgery. Radiographs were taken.

Oral *Cissusquadrangularis* administered to the study group daily for 4 weeks. Regular ASDs done.

Radiographs of all animals were taken on day 0. (Fig. 13&14)

Radiographs of 8 animals from each group were taken at 2 weeks (Fig. 15&16) & the segment of fibula of these animals were dissected for histological assessment (Fig. 19,20,21&22). The remaining animals (7 in each group) were still under study and the animals from study group were administered with the drug under study for two more weeks.

The remaining animals' radiographs were taken at 4 weeks (Fig. 17&18) and the segment of their fibula dissected for histological assessment. (Fig 23&24).

Preparation of tissue for microscopy:

1. The specimens were fixed in neutral buffered 10% formalin solution for 24 hrs.
2. Samples were decalcified by using 5% nitric acid, whenever required.
3. Microtomy: Rotatory microtome used to obtain 3-5 μ m thick sections.
4. Deparaffinization: Slides were incubated at 37°C for 24hrs.
5. Hydration was done using ethanol in descending order.
6. Slides were stained with Haematoxylin & Eosin.
7. Mounting was done in DPX (Distyrene & Plasticizer & Xylene).
8. Special stains were employed wherever required.

Measurement of fracture healing:

Parameters:

1. **Radiological:** Radiographs taken serially at 0, 2 & 4 weeks to see the callus formation and fracture union.
2. **Histological:** By using modified Allen's grading system.

Allen's Fracture Healing Scoring System

Healing Staging	Score
Non union (fibrous tissues)	0
Incomplete cartilage union (cartilage with some fibrous tissues)	1
Complete cartilage union (entirely cartilage)	2
Incomplete bony union with phase of ossification (predominantly cartilage with some trabecular bone)	3
Incomplete bony union with intermediate phase of ossification (equal amounts of cartilage & trabecular bone)	4
Incomplete bony union with late phase of ossification (predominantly trabecular bone with some cartilage)	5
Complete bony union (entirely bone)	6

Results:-

Changes in each group observed & compared on radiographs & histological examination (By Allen's fracture healing scoring system).

Radiographic comparison of two groups: (Table 1)

No gross difference was seen on plain radiographs between both the groups at 2 weeks. Both the groups showed similar callus formation. (Fig. 15&16).

However at 4 weeks radiographs of 3 out of 7 samples of study group showed complete union and 4 out of 7 showed incomplete union whereas all the samples of control group showed incomplete union. (Fig. 17&18).

Table 1:- Radiological Comparison of findings in two groups:

		Control (n=15)	Study (n=15)	
2 weeks (n=8)	No. of animals showing fracture union	0(0%)	0(0%)	p>0.05
4 weeks (n=7)	No. of animals showing fracture union	0(0%)	3(42.8%)	p<0.05(0.0180)

Chi square= 5.600

Degree of freedom is 1

Two tailed p value is 0.0180 which is significant in favor of study group.

The data is shown in n(%age), Two Tailed Chi Square Fischer's Exact Test was applied and $p < 0.05$ was considered significant.

Histological comparison of two groups:(Table 2 & Table 3)

Observations in Group I (Control):

At 2 weeks:(Fig. 25)

4 out of 8 samples showed only fibrous tissue, 4 out of 8 samples showed cartilage with some fibrous tissue.

At 4 weeks:(Fig 27)

3 out of 7 samples showed entirely cartilage, 4 out of 7 samples showed cartilage with some trabecular bone

Observations in Group II (Study):

At 2 weeks:(Fig 26)

4 out of 8 samples showed cartilage with some fibrous tissue, 3 samples showed only cartilage, 1 sample showed cartilage with some trabecular bone.

At 4 weeks:(Fig 28)

3 out of 7 samples showed cartilage with some trabecular bone, 4 samples showed equal amount of trabecular bone & cartilage.

Comparison of Histological Scoring of Study Group with that of Control group in Rabbits (According to Allen's Fracture Healing Scoring System):

Table 2:- Comparison of Mean (SD) in Allen's Fracture Healing Scoring system at 2weeks between two Groups:

	N	Mean	SD	SEM
Control	8	0.5	0.5345	0.189
Study	8	1.625	0.744	0.263
Statistical Inference	Mann- Whitney U z-2.72; p-0.006; HS			

The data is shown in mean \pm SEM, Mann-Whitney U Test was applied and $p < 0.05$ was considered significant; $p < 0.01$ was considered highly significant(HS).So results of the present study are highly significant statistically at 2 weeks in favor of the said drug.

Table 3:- Comparison of Mean (SD) in Allen's Fracture Healing Scoring system at 4weeks between two Groups:

The data is shown in mean \pm SEM, Mann-Whitney U Test was applied and $p < 0.05$ was considered significant; $p < 0.01$ was considered highly significant.

As per Mann-Whitney U, p at 4 weeks is 0.01 which is also highly significant as $p < 0.05$. So results of present study are also highly significant at 4 weeks in favor of the drug under study.

*SD=Standard Deviation, SEM=Standard Error of Mean.

As per Fisher's Exact Test:

Table 4:- At 2 weekscomparison of control & study group according to Allen's fracture scoring.

	N	Mean	SD	SEM
Control	7	2.571	0.5345	0.202
Study	7	3.571	0.5345	0.202
Statistical Inference	Mann-Whitney U z-2.5;p-0.01;HS			

Score (Allen's)	Control	Study
0 or 1	8	4
>1	0	4

The Fisher's Exact Test statistic value at 2 weeks is **0.0385**. The result is significant at $p < 0.05$.

Table 5:- At 4 weeks comparison of control & study group according to Allen's fracture scoring.

Score (Allen's)	Control	Study
2 or <2	3	0
>2	4	7

The Fisher's Exact Test statistic value at 4 weeks is **0.1923**. The result is not significant at $p < 0.05$.

The results at 2 weeks are significant clinically as well as statistically. The results at 4 weeks are significant clinically but not significant statistically due to small sample size as per Fisher's Exact Test.

Discussion:-

Cissus Quadrangularis has been used as a medicinal plant since antiquity. It has been used in various Ayurvedic classical medicines to heal broken bones and injured ligaments and tendons. In Siddha medicine it is considered a tonic and analgesic, and is believed to help heal broken bones.

Deka D K et al (1994) in their while evaluating the effect of methanol extract of cissusquadrangularis on experimentally fracture radius and ulna of dog revealed that cissusquadrangularis treated animals record faster initiation on healing process than the control animals both on radiological and histological examination. The results are in accordance with current study.

An increase in osteoblastic activity in cissusquadrangularis treated animals during fracture repair has been reported by Udupa & Prasad (1963) while evaluating effect of cissusquadrangularis on healing of cortisone induced fracture. The results of this study are in the line with the findings of the current study.

Banu J et al (2012) similarly in their study concluded that cissusquadrangularis effectively inhibits bone loss in the callus and cortical bones of femur and proximal tibia in mice thereby suggesting the role of cissusquadrangularis in fracture healing like our study.

The study of Siddaram et al (2012) in a clinical evaluation to evaluate the effect of cissusquadrangularis in a preliminary study on 30 registered clinically diagnosed colle's fracture evaluated the fracture healing effect of cissusquadrangularis, suggested highly significant fracture healing effect on signs, symptoms and callus formation.

Singh V et al (2012) in a clinical trial based on radiological and clinical observations observed that the cissusquadrangularis cause considerable reduction in healing time of fracture and recorded clinical and radiological parameters in the form of fracture healing.

Brahmkshatriya H R et al, (2016) while undertaking a clinical evaluation of cissusquadrangularis as osteogenic agent in maxillofacial fractures in a pilot study suggested that cissusquadrangularis helps in reducing pain, swelling and fracture mobility and accelerates the healing of fractured jaw bone.

Summary And Conclusion:-

1. On radiographic comparison between study and control groups, there was not any gross difference in callus formation at 2 weeks. However, at 4 weeks 3 animals out of 7 in the study group showed complete union of the fracture unlike controls. The results were significantly better in favor of animals treated with the test drug with p value < 0.05 .
2. The mean Allen's Fracture Healing Score in both the groups in 2 weeks treated animals and 4 weeks treated animals showed highly significant scores in comparison to the control group respectively with p value < 0.01 thereby indicating a better and early callus formation histo-pathologically in favor of cissusquadrangularis treated animals in comparison of controls.
3. On grading the histological Allen's fracture healing score, better signs of histological fracture healing were seen in study group with 50% animals from the study group showed > 1 score at 2 weeks and all the animals showed

>2 score at 4 weeks. Thereby indicating with increase in duration of treatment will have significant impact on histological fracture healing.

4. The results were proved and further substantiated by radiological and histological findings.
5. No mortality or any apparent sign of acute or sub-acute toxicity was observed in the animals treated with *Cissusquadrangularis*. Although toxicity study was out of preview from the current study.

The current study established both radiological and histological evidences by showing signs of callus formation as well as fracture union in small animals treated with *Cissusquadrangularis* in comparison to control. Further the study suggested that with increase in duration of therapy the rate of healing and quality of callus formation was better in comparison with healthy controls.

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Fig. 1: Experimental Albino Male Rabbits



Fig. 2: Experimental Male Albino Rabbits in Iron Cage



Fig. 3: Preparation of the Part



Fig. 4: Albino Rabbits with Surgical Instruments



Fig. 5: Shaved (Prepared) Part of Albino Rabbit



Fig. 6: Part Painted with Povidone Iodine



Fig. 7: Skin Incision being given with No. 15 Surgical Blade



Fig. 8: Skin Incision over Lateral Aspect of Leg



Fig. 9: Soft Tissue Dissected and Fibula Exposed



Fig. 10: Fibula Cut Experimentally on Day 0



Fig. 11: Wound Closed in Layers



Fig. 12: Antiseptic Dressing Done

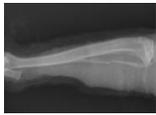


Fig. 13: Plain Radiograph of Control Group on Day 0 Showing Fracture of Fibula



Fig. 14: Plain Radiograph Of Study Group on Day 0 Showing Fracture Of Fibula



Fig. 15: Plain Radiograph of control Group at 2 weeks showing no Signs of Union



Fig. 16: Plain Radiograph of Study Group at 2 Weeks Showing no Signs of Union

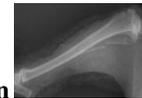


Fig. 17: Plain Radiograph of Control Group at 4 Weeks Showing Incomplete Union at Fracture Site



Fig. 18: Plain Radiograph of Study Group at 4 Weeks showing Complete Union of Bone



Fig. 19: Minimal Callus Formation in Control group at 2 Weeks



Fig. 20: Segment of Fibula taken from Control Group at 2 Weeks for Histological Examination



Fig. 21: Callus Formation in Study Group at 2 Weeks



Fig. 22: Segment of Fibula taken from Study Group for Histological Examination at 2 Weeks



Fig. 23: Complete Bony Union in Study Group at 4 Weeks



Fig. 24: Segment of Fibula taken for Histological Examination at 4 weeks in Study Group

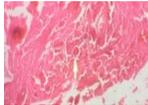


Fig. 25: H&E Stained Section of Control at 2 Weeks Show Haematoma and Fibrous Tissue Only (Allen's Fracture Healing Score-0) (H&E STAIN x 400)



Fig. 26: H&E Stained Section of Study at 2 Weeks Show Evidence of Cartilage Formation with Some Fibrous Tissue (Allen's Fracture Healing Score-1) (H&E STAIN x 400)



Fig. 27: H&E Stained Section of Control Group at 4 Weeks Showing Mostly Cartilage (Allen's Fracture Healing Score-2) (H&E x 400)



Fig. 28: H&E Stained Section of Study Group at 4 Weeks Showing Cartilage with Trabecular Bone (Allen's Fracture Healing Score-4) (H&E STAIN x 400)