

ANALYSIS OF THE EFFECT OF *LAKSHA GUGGULU* (AN AYURVEDIC FORMULATION) ON FRACTURE HEALING IN A RAT MODEL

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Abstract: *Objectives:* *Laksha Guggulu*, an Ayurvedic formulation advocated to enhance the fracture healing in ancient Indian texts, was selected to validate its effect on fracture healing on certain scientific parameters like Radiological, Serological and Histopathological studies in an experimental rat model. *Methods:* Eighteen Wistar rats (*Rattus norvegicus*) were taken to create identical fractures by a transverse osteotomy of Radial bone of their left fore limb under Ketamine and Xylazine anesthesia (intraperitoneally). Intact ulna acted as an internal splint hence, no other external or internal support to the fractures was given. Post operatively injectable antibiotic (Gentamicin) was given. These osteotomised rats were divided into three groups of six each. Group I rats were given 13.5mg/100gm body weight of the trial drug (*Laksha Guggulu*) twice daily through oral route. Group II rats were given half of the Group I dose of the trial drug whereas in Group III, no drug was given (Control group). The drug was given orally with Cow's milk. Total duration of trial was of four weeks. Healing was assessed Radiologically, Biochemically and Histopathologically. *Results:* Assessment of results was done according to pre-designed protocol and data analysis done statistically. Histopathological and Serological studies revealed significant results in Group I animals as compared to Group II and Group III. Radiologically the difference between the groups was not detectable. *Conclusion:* *Laksha Guggulu* has got a definite role in the enhancement of fracture healing by forming early and improved quality of the callus. However, further studies on large sample size, with more advanced investigations like Histomorphometry, Bone turnover markers and Micro CT scanning are required.

Keywords: Fracture healing, Histopathology, *Laksha Guggulu*, Rat model.

Introduction

A cursory glance of the available medical treatise reveals that Ayurveda has spread enough thought to the care of injured (*Sushruta Samhita*). Treatment of skeletal injuries has been given prime importance. As without proper treatment these injuries can disable a person for rest of life. Since Vedic period, surgeons are pondering over skeletal injuries and are trying to overcome their complications. *Acharya Sushruta* has described detailed etiology, classification, management and prognosis of bone and joint injuries. He has also described various measures including drugs to hasten the healing process.

For the management of fracture, reduction and immobilization are universally required and to be done accordingly (*Sushruta Samhita*). But attention also be paid to avoid or minimize the forthcoming complications due to fracture itself or immobilization. Reduction in fracture healing time, when achieved will reduce complications.

The healing is a natural process and it occurs spontaneously. But sometimes, healing is delayed or bone ends fail to unite because of factors like improper immobilization, deficiency conditions, reduced blood supply to the fractured part, severe soft tissue damage (**Abdelhamid H. Elgazzae, 2004**), smoking (**Mara L. Schenker**

et al., 2013), endocrinal disorders and continuous nerve irritation (Henery Turner, 1936).

To overcome this problem and to minimize the healing period, various drugs or formulations for local, oral use have been advocated in Ayurvedic literature (*Sushruta Samhita*, Chakradatta and Yogratnakara). These drugs are claimed to enhance fracture healing process. *Laksha Guggulu* (Yogratnakara) is one of them which is advocated to promote fracture healing.

Laksha Guggulu is a classical Ayurvedic formulation containing *Laksha Churna* (Shellac powder), *Asthishrinkhla* (*Cissus quadrangularis* Linn.), *Arjun Twak Churna* (Bark powder of *Terminalia arjuna*), *Ashwagandha Churna* (Root powder of *Withania somnifera*), *Nagbala Mool Churna* (Root powder of *Grewia hirsuta*) and *Guggulu* (gum resin of *Commiphora mukul*). Among these ingredients *Cissus quadrangularis* Linn. is of utmost importance. *Cissus quadrangularis* alone is also used as a single drug for promoting fracture healing since ancient times. Its fracture healing potential has also been scientifically validated earlier by Udupa, 1962; Udupa and Prasad, 1964; Chopra *et al.*, 1976.

In this study, the scientific evaluation of *Laksha Guggulu* formulation, as fracture healing drug in a rat model on parameters like radiography, serum alkaline phosphatase and histopathology of fracture callus was planned.

Materials and Methods

Method of Drug Preparation

Well identified ingredients were taken in equal quantity and fine powder of each was prepared separately except *Guggulu*. All these powdered contents were mixed properly. Further, purified *Guggulu* of equal weight was added and mixed. *Laksha Guggulu* was prepared in the College Pharmacy as per the Ayurvedic Formulary of India (AFI).

Experimental Animals

Eighteen Wistar rats (*Rattus norvegicus*) of either sex more than three months of age,

weighing between 150-250 gm were procured from reliable source. Animals were fed on commercially available standard balanced rat feed. All the animals were acclimatized to laboratory conditions for one week prior to the trial. They were maintained at 12 hrs light cycle with room temperature at $15^{\circ}\text{C} \pm 5^{\circ}\text{C}$ in well ventilated animal house.

Experimental Protocol

For osteotomy purpose, rats were shifted to operation theatre of the department and all the operative works were done under aseptic conditions. Rats were anaesthetized with inj. Ketamine (60mg/kg) and inj. Xylazine (8mg/kg body weight) intraperitoneally.

A cranio-medial incision was applied over the left fore-limb and open transverse osteotomy of left radial bone was done. As only radial bone of fore limb was osteotomised (fractured) and ulna acted as a splint. Hence, no external splint was applied. Post operative antibiotic (Inj. Gentamicin by I. M. route 12 hourly) for seventy-two hours of post operative period was given. All doses including antibiotic and trial drug were calculated by using conversion factor i.e. a dose for a rat of 200gm weight = $0.018 \times$ Human adult dose.

After creating similar fractures on similar bones in all 18 animals, they were divided into three groups (six animals in each group). To identify each animal they were marked on tail with code number. The cages were also labeled as group I, group II and group III respectively.

Group I: This group was fed with *Laksha Guggulu* dissolved in 1ml of cow's milk in dose of 13.5 mg per 100gm body weight. The drug was given through intragastric route 12 hourly with the help of soft baby feeding tube of No. 10 size.

Group II: Second group was fed with half of the dose of group I, dissolved in 1ml of cow's milk, given through intragastric route 12 hourly with the help of soft baby feeding tube of No. 10 size.

Group III: Only 1ml of cow's milk was given through intragastric route 12 hourly. This group was kept as a control group.

Total duration of trial was Four weeks.

Investigations used were:

1. X-rays of operated limb.
2. Serum alkaline phosphatase study.
3. Histopathology of the callus.

As the operated limb needed to be disarticulated for histopathology, so euthanasia of the animals was inevitable and done according to international guidelines. Inhalation agent Halothane was used in high doses for euthanasia.

After euthanizing, blood samples were collected from all the animals, directly from heart for serum alkaline phosphatase study. Cranio-caudal and lateral radiographs of osteotomised animal limbs of all the three groups were taken for radiological assessment. Operated limbs were disarticulated and histopathological study of the callus done.

The segments were placed in buffered neutral 10% formalin for three days, followed by decalcification with Stewart and Gooding fluid. Specimens were processed for making paraffin blocks and 5-6 micro meter thick tissue sections were stained with Hematoxyline Eosin (H & E) and Masson's trichrome stains. An eleven point scale based on the amount of fibrous tissue, cartilaginous tissue and woven bone in callus formation was used to evaluate the degree of healing process.

Assessment Criteria

Radiographic Assessment

Cranio-caudal and lateral radiographs were graded on a **six point union score** (Sano *et al.* 1999).

Zero: Sharp or sclerotic line seen throughout.

One: Sharp or sclerotic line in more than 75% of diameter.

Two: A well defined osteotomy line extending in both projections.

Three: Same as two but in one projection only.

Four: Osteotomy faintly seen.

Five: Osteotomy not seen.

Histopathological Assessment

It was done on the following Eleven Grade scale (Huddleston *et al.*, 2000)

- i. All fibrous tissue.
- ii. More fibrous tissue than cartilage.
- iii. Fibrous and cartilaginous tissue in equal proportion.
- iv. Evidence of fibrous tissue with more cartilaginous tissue than woven bone.
- v. Evidence of fibrous tissue with equal cartilage and woven bone.
- vi. Evidence of fibrous tissue with more woven bone than cartilage.
- vii. Cartilaginous tissue and woven bone in almost equal proportion.
- viii. Less cartilage and more woven bone.
- ix. Entirely woven bone.
- x. Woven bone and some mature bone.
- xi. Lamellar (mature) bone.

Serum Alkaline Phosphatase Assessment

Blood samples drawn directly from heart were allowed to clot, centrifuged and serum collected. The serum samples analyzed for serum alkaline phosphatase value with semi auto analyzer.

Results

All the data subjected to statistical analysis (Tables 1-9). All the values were expressed as Mean \pm SD. The differences were compared using Students 't' test. The *p* values <0.05 were considered significant.

Table 1. Radiographic Assessment of Experimental Animals at the end of trial i.e. after 4 weeks (Gradation on Six point union scale).

S. No.	Group		
	G-I	G-II	G-III
1	4	3	5
2	3	5	2
3	3	3	4
4	4	3	2
5	4	4	2
6	5	3	2
Means	3.83	3.5	2.83

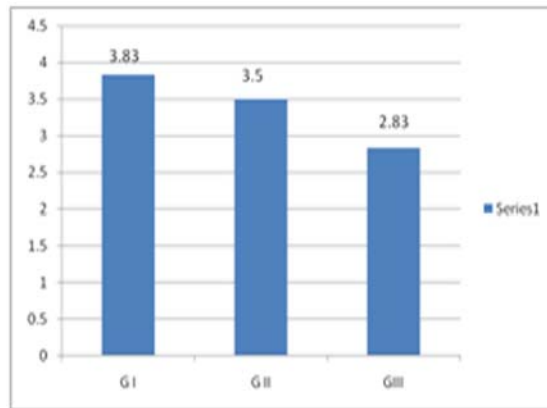


Figure 1. Graphical presentation of mean of Radiographic Gradation of all Groups.

Table 2. Comparison of groups for Radiographic assessment at the end of 4 weeks.

S. No.	Group.	Number	Mean	S.D.	SE _M
1	I	6	3.83	0.97	0.3960
2	II	6	3.5	0.8366	0.3416
3	III	6	2.83	1.328	0.542

Table 3. Inter group Comparison

Group	Group	't' value	D.F.	P value
I	II	0.629	10	P> 0.05
I	III	1.041	10	P> 0.05
II	III	1.047	10	P> 0.05

Table 4. Histopathological Assessment of Calluses - after 4 weeks.^s

S. No.	G-I	G-II	G-III
1	6	-*	4
2	6	6	-*
3	6	4	4
4	6	-*	4
5	5	5	-*
6	5	6	5
Means	5.66	5.25	4.25

* Indicates that sample couldn't be obtained for study

^s Gradation on 11 point Histopathological Assessment Scale

Discussion

Present study is an effort to assess the effect of *Laksha Guggulu* in fracture healing in a rat model. Statistical analysis of the results obtained shows that *Laksha Guggulu* enhances

Table 5. Comparison of Histopathological assessment of Callus at end of 4 weeks.

S. No.	Group	n	Mean	S.D.	SE _M
1	I	6	5.66	0.5164	0.2108
2	II	4	5.25	0.9573	0.4786
3	III	4	4.25	0.5	0.25

Table 6. Inter Group Comparison

Group	Group	't' value	D.F.	P value
I	II	0.888	8	P>0.05
I	III	4.36	8	P<0.01
II	III	1.852	6	P>0.05

Table 7. Alkaline phosphatase values of experimental animals after 4weeks (all values are in u/l).

S. No.	Group-I	Group-II	Group-III (Control)
1	270	319	117.42
2	390.4	230.7	186.6
3	420.2	162.2	252.3
4	396.4	270	172.08
5	170	180	144.16
6	370	300	137.2
Means	336.16	243.65	168.29

Table 8. Comparison of Groups for Serum Alkaline Phosphatase values of all Groups after 4 weeks.

No.	G	n	Mean	S.D.	SE _M
1	I	6	336.1667	96.7109	39.48
2	II	6	243.65	63.875	26.078
3	III	6	168.293	48.049	19.616

Table 9. Inter Group Comparison

Group	Group	't' value	D.F.	P value
1	2	1.95	10	P<0.05
1	3	3.81	10	P< 0.01
2	3	2.31	10	P<0.05

the fracture healing process. The comparison of Group-I with other groups shows statistical significant results (**Tables 1-9**).

In radiographic assessment the results obtained are non significant in all groups in comparison to the control group. Inter group

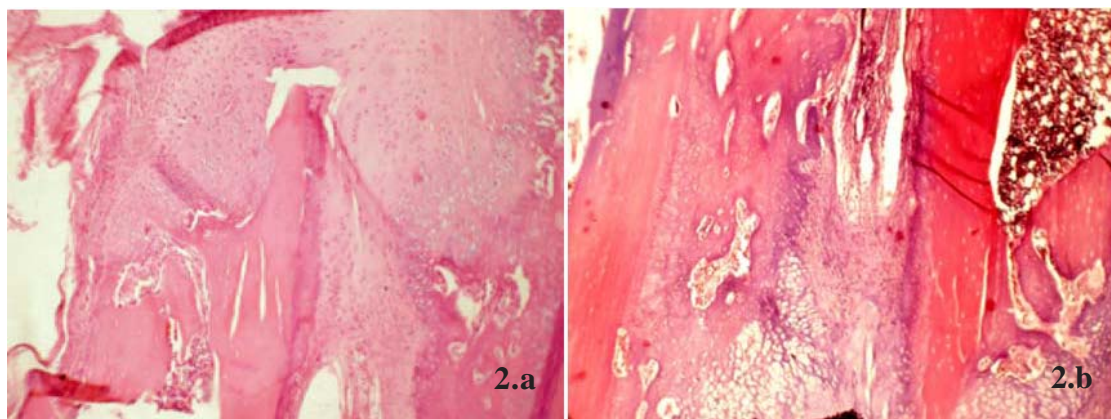


Figure 2 (a, b) Showing more woven bone than cartilage

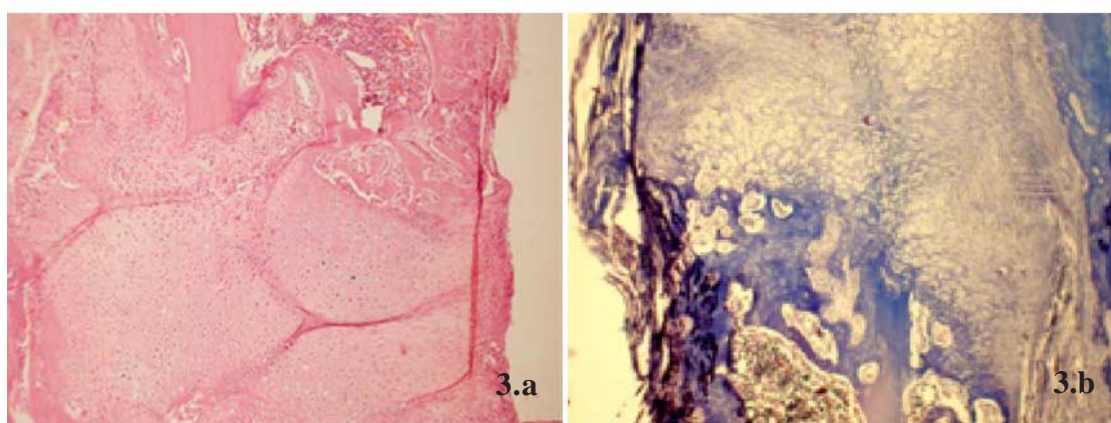


Figure 3 (a, b) Showing equal woven bone and cartilage.

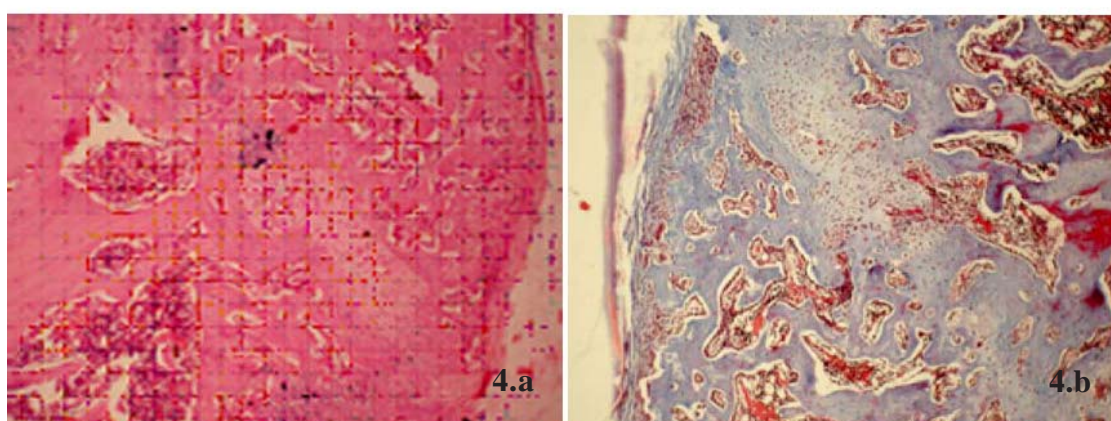


Figure 4 (a, b) Showing fibrous tissue with more cartilage and less woven bone.

comparison also shows no significant results (Tables 1-3, Fig. 1). The cause of non significant results might be poorly defined shadow of callus on radiographs. It is hypothesized that the callus so formed has not casted its shadow very well. The analysis of serum alkaline phosphatase value and histopathology of calluses proved the efficacy of appropriate dose of *Laksha Guggulu* on bone healing.

The histopathological study (conducted at College of Veterinary and Animal Sciences Palampur, H.P.) of all groups reveals that the calluses of group-I were containing more part of woven bone (immature bone) than cartilage and fibrous tissue (Fig. 2 a,b). Group-II specimens revealed equal amount of cartilage and woven bone (Fig. 3 a,b). Whereas, group-III showed more amount of fibrous and cartilaginous tissue than woven bone (Fig. 4 a,b). Statistically results were also significant in Group-I than Groups II and III (Tables 4-6, Fig. 5). Hence, it is clear that the drug *Laksha Guggulu* accelerates fracture healing.

The serum alkaline phosphatase is composed of a group of iso-enzymes which originate from liver, bone and to a minor extent from the intestine and placenta. It functions principally at the site of absorption, deposition and excretion of calcium and also phosphorus. In bones it is concentrated at the main points of ossification (i.e. the epiphyseal line and the sub-periosteal area). During the active bone destruction (as in fracture) a compensatory stimulation of osteoblasts to replace bone is reflected in an increased intra cellular content of alkaline phosphatase and increased levels in blood stream. During fracture repair, osteoblasts try to fill up the gap and cause increased serum alkaline phosphatase value. The increased serum alkaline phosphatase is also found in patients with certain diseases like Paget's disease, hyperparathyroidism, rickets, osteoblastic osteogenic sarcoma and diseases of liver. Because, animals used were healthy and screened out for any disease, so the possibility of alkaline phosphatase rise due to any such pathology was ruled out. Hence, the

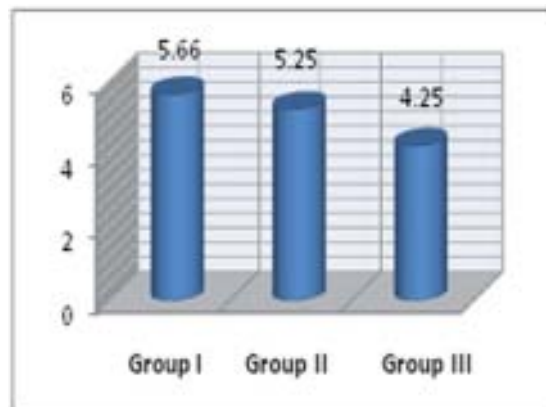


Fig. 5. Diagram of Mean of Histopathological Assessment of Callus as per 11 scale grading.

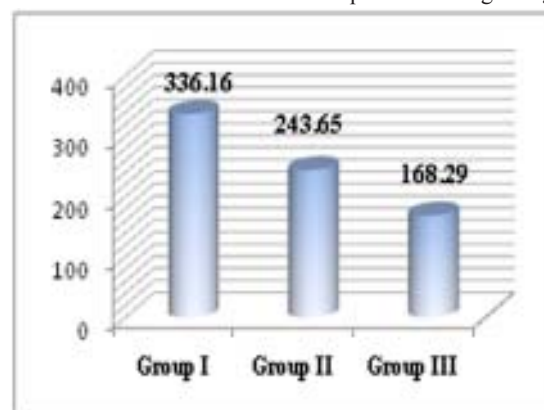


Fig. 6. Diagram of Mean of Alkaline Phosphatase values.

increase in serum alkaline phosphatase value in group-I was due to *Laksha Guggulu*. The results found were statistically highly significant (Tables 7-9, Fig. 6).

Formulation *Laksha Guggulu* contains *Guggulu* as a main ingredient. *Guggulu* is gum resin of a shrub named *Commiphora mukul* Engl. This resin is having anti-inflammatory, analgesic (Jain and Gupta 2006) as well as bone healing properties (Bhavaprakasha). Another important ingredient of this formulation is *Cissus quadrangularis* which has been advocated to promote the fracture healing in Ayurvedic classics (Bhava Prakasha). This plant is found to contain vitamins and steroid like various versatile constituents such as flavonoids,

triterpenoids, vitamin C, stilbene derivatives and many others. (Adsenya *et al.*, 1999). Out of these ascorbic acid, triterpene, α -sitosterol, ketosteroids, triterpenoids and calcium were found to have specific effect on the bone healing (Chopra *et al.*, 1975, 1976; Udupa and Prasad 1963 and 1964). *Cissus quadrangularis* also contains anti-oxidant and anti microbial properties (Murthy *et al.*, 2003; Deka and Lohan 1994). *Arjuna* (*Terminalia arjuna* Roxb.) however, is having cardio-protective properties but the methanol extract also contains analgesic and anti-inflammatory properties (Moulissha Biswas *et al.*, 2011). Studies have also revealed bone healing potential of this plant (Singh H., 1992). *Ashwagandha* (*Withania somnifera* (L) Dunal) is mainly an immunomodulator and immunoprotective drug but is also having analgesic properties (Sahni and Srivastava 2011) and some good effect on bone healing (Jaiswal *et al.*, 2004). *Grewia hirsuta* is a general and nervine tonic (Bhava Prakasha) whereas *Laksha* (Shellac powder) is also having healing effect upon *Kshata* (tissue injuries) (Bhava Prakasha). On the basis of the properties of individual ingredients of the formulation under study (*Laksha Guggulu*) it can be well assumed that this should be helpful in enhancing the bone healing process. This study also revealed that *Laksha Guggulu* potentiates the bone healing process in rats.

Conclusion

On the basis of this experimental work it can be concluded that the drug *Laksha Guggulu* enhanced the fracture healing process and can be used as an adjuvant therapy to promote the fracture healing. However, further studies on large sample size, with more advanced investigations like histomorphometry, Bone turnover markers and Micro CT scanning are required.

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