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Use of Biochemical Parameters in Radiologically Proved Fracture Healing Property of *Arjuna Terminalia*, in Rats.

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ABSTRACT

Plant extracts are attractive sources of new drugs. Except few studies, no extensive investigations have been made for improving this inherent biological phenomenon of bone healing. It has been mentioned in Sarangdhar samhita & by Vagbhata that *Arjuna terminalia* belongs to nyagrodadhi group possessing fracture healing property. No scientific studies have been conducted so far to confirm or refute these long standing claims. The research question was formulated as “whether *Arjuna terminalia* induces favorable response in bone healing as judged through a systemic effect upon biochemical parameters [alkaline phosphatase, phosphate, calcium] & to compare its relative efficacy with Normal saline treated group assisted with closed reduction. In our study ethanolic extract of *Arjuna terminalia* Linn is evaluated for its fracture healing property in experimentally fractured tibia (3-point bending method) of rats. *Arjuna terminalia* treated animals revealed faster initiation of healing process than the control group (Normal saline) on biochemical examination. Healing was almost complete within 4 weeks of fracture in the *Arjuna terminalia* treated animals & remained incomplete in the control group. The results indicate that the ethanolic extract of *Arjuna terminalia* produce beneficial effect in fracture healing & support the claims of its traditional wages as traditional bone healer.

Keywords: Ethanolic extract, 3-point bending method.

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INTRODUCTION

Musculoskeletal injuries have existed throughout human history [1]. The word “fracture” is defined as a break in the continuity of the bone [1]. Classical experiments & researches conducted by John Hunter, Hallar, Duhamel, & their successors during the 17th to the 19th centuries led to the discovery of “reparative osteogenesis” – a concept that indicated the healing of broken bones by formation of new bone [1]. Bone is a very active tissue & any injury to it will induce a series of biological & biochemical changes [2].

The prolonged period is required for healing of fractures. This entails much suffering & economic loss to the patient. Therefore, it has all along been the endeavour of scientists to find out certain agents which may hasten healing & thereby shorten the duration of convalescence [3]. There is lack of any proved drug that would hasten bone healing. World Health Organization has reported that majority of Indian population tries Ayurvedic/herbal/traditional medicines first [4]. Except few studies, no extensive investigations have been made for improving this inherent biological phenomenon of bone healing. Plant extracts are attractive sources of new drugs & have been shown to produce promising results. Hence, the research question was formulated as “whether the herb *Arjuna terminalia* induces favorable response in bone healing as judged through a systemic effect upon biochemical parameters [alkaline phosphatase, phosphate, total calcium] in experimental animals (Rats) & to compare its relative efficacy with Normal saline treated group assisted with closed reduction method.

Arjuna terminalia[family *Combretaceae*] is one such Ayurvedic remedy that has been mentioned in much ancient Indian medicinal literature including Charaka Samhita & Astang Hridayam [5]. The plant found in plenty throughout Indo sub Himalayan tracts [5] It is a commonly occurring medicinal plant growing as a 20-30m high tree. Chemical constituents of different classes such as hydrolysable tannins, triterpenoid acids & their glycosides, flavonoids, phenolics, phytosterols, were reported from stem & bark portion of *Arjuna terminalia* species [6].

The bark paste of *Arjuna terminalia* plant is being successfully used for the treatment of fractured bone of animals as well as human being. The decoction of the bark is used therapeutically to relieve the pain & inflammation [6].

Vagbhata places it in the vellantaradi & nyagrodhadi groups indicated in urinary disorders & broken bones, respectively. According to the Sarangadhara Samhita, *Arjuna terminalia* belongs to the nyagrodhadi group specific for fractures, ulcers, uterine & urological complaints, skin diseases etc. *Arjuna terminalia* is extensively used to treat osteoporosis & other bone related disorders as it improves the synthesis & secretion of female hormones.[8]

MATERIALS AND METHODS

The study was conducted in Pharmacology Department of Jawaharlal Nehru Medical College, Sawangi, Wardha. The research protocol was approved by the Institutional Animal Ethical Committee. Bark of *Arjuna terminalia* purchased from local herbarium “Shri Shail Herbarium, Nagpur”. The plant was identified & authenticated (**Herbarium sheet**) as *Arjuna terminalia* by Botany Department, R. T. M. University Nagpur. The study was conducted using 16 Wistar Albino Rats, of either sex weighing 150-200g purchased from Institute of Pharmaceutical Education & Research Borgaon (Meghe), Wardha. The preliminary phytochemical studies done at Pharmacognosy Department, R.T.M.Nagpur University. It shows the presence of Tannins, Saponins and Carbohydrates in the extracts.

Method of Extraction-

Bark of *Arjuna terminalia* which was in dried form was coarsely powered. 4 kg of powered *Arjuna terminalia* soaked in 6 L of ethanol for 48 h, the extract was filtered & allowed to dry under fan. The last traces of solvent were removed under vacuum drier & the brown powdered mass obtained which was pulverized & sieved for further use. [6] For administration solution of *Arjuna terminalia* prepared daily by mixing extract with sugar water. [9]

Animals were acclimatized for 8 days in the laboratory before experiment. Animals were kept on standard nutritional & environmental condition in separate cages. They were fed with standard laboratory chow & provided with water ad libitum.

Clinical orthopedic evaluation of every rat was done before surgery and dorso-palmar radiographic views of the left tibia were obtained (40 kV/32mAs and 0.26 s). [10]

Creating tibia fracture

Animals were anaesthetized with Phenobarbitone 60mg/kg, IP & Ketamine [Anikate, 40 mg/kg, IP]. Then closed transverse fracture of the mid – diaphysis of left tibia were created in both groups by three point bending method. [11] These fractured limbs were stabilized with splints after reduction (Closed reduction method). Animals were allowed to move after recovering from anesthesia.

Animals were divided into 2 groups of eight animals each.

- Group I: Control group - Normal saline (2 ml/kg) orally, bid, for 30 days.
- Group II: *Arjuna terminalia* group. *Arjuna terminalia* extract with sugar water orally (500 mg/kg body weight) bid, for 30 days.

The required amount of powdered extract of *Arjuna terminalia* measured as per the dose/bodyweight (500mg/kg) of rats used as fresh solution prepared with sugar & water (2ml)

Pre and Post-operative biochemical evaluation

The animals were evaluated clinically daily to determine general condition and lameness. Lameness was evaluated by observing each animal moving freely in the cage. Biochemical estimation in serum was made before fracture induction, immediately after fracture induction & at 2nd & 4th week in the post operative period. Approximately 2ml of blood was collected from retro-orbital plexus with the capillary tubes in the clean bulbs & then transferred into the clean dry test tubes for centrifugation to separate the serum. The rats included in our study were fed with the same food throughout the trial & blood samples were obtained only in the morning to eliminate circadian influence.

Biochemical estimation done by following methods

Alkaline phosphatase - pNPP (p-Nitrophenylphosphate) Kinetic method

Inorganic phosphate - UV Molybdate method

Calcium– Modified Arsenazo method.

Statistical evaluation

The data was subjected to statistical evaluation by using SPSS 13.0 One way ANOVA.

RESULTS

In our previous study we found great results of *Arjuna terminalia* in fracture healing in radiological evaluation. In 3rd week the x-ray showed greater amount of calcification of callus in *Arjuna terminalia* treated group in which one could hardly see a gap at the fracture site, fragments could not be elicited whereas in Normal Saline group some gap was still visible. In 4th week in the skiagram there was an evidence of union in *Arjuna terminalia* treated group. Almost complete bridging of the fracture ends with extensive bony deposition is seen in *Arjuna terminalia* treated group compared to that of Normal saline group.

Results of biochemical parameters evaluation go in pace with the results of radiological evaluation & convincingly demonstrated that the herb *Arjuna terminalia* has definite influence in the rate of fracture healing.

Biochemical studies reveals such changes in serum values of calcium, phosphorus & alkaline phosphatase of the *Arjuna terminalia* treated rats which suggests that the primary locus of action of *Arjuna terminalia* may be in the region of bone regeneration itself.

Biochemical Parameters

Serum calcium & inorganic phosphate are the markers of bone resorption while alkaline phosphatase is the marker of bone formation. [12]

Table 1: Biochemical parameters 24 hours, 2nd week & 4th week after fracture

Parameters	Group	Normal (Baseline) values Mean	24 hours after fracture Mean	Std. Deviation	2 nd week after fracture Mean	Std. Deviation	4 th week after fracture Mean	Std. Deviation
Calcium	Normal saline	9.4	8.6000	.2757	8.5000	.3784	10.2000	1.0043
	Arjuna terminalia	9.7	8.4167	.3764	8.5833	.3764	9.5750	.5560
Phosphorus	Normal saline	7.3	8.3000	.3847	5.2000	.5450	4.3000	.7557
	Arjuna terminalia	7.6	8.1167	.5193	5.4833	.4401	4.1000	.2944
Alkaline Phosphatase	Normal saline	242	252.5000	14.8020	359.0000	112.6745	290.0000	110.1895
	Arjuna terminalia	231	238.1667	12.6082	345.3333	110.6159	278.7500	134.9849

Dynamics of serum bone markers in rats after fracture (n=6). Data is presented as mean± SD

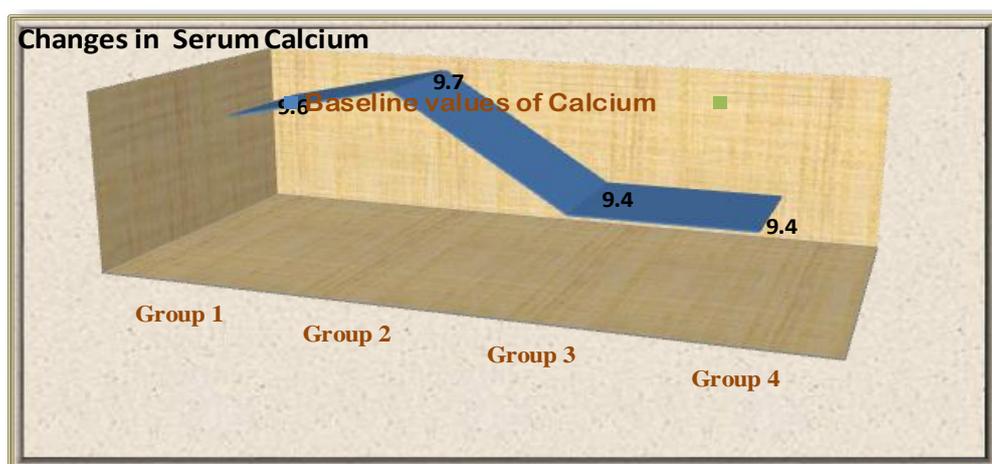


Figure 1

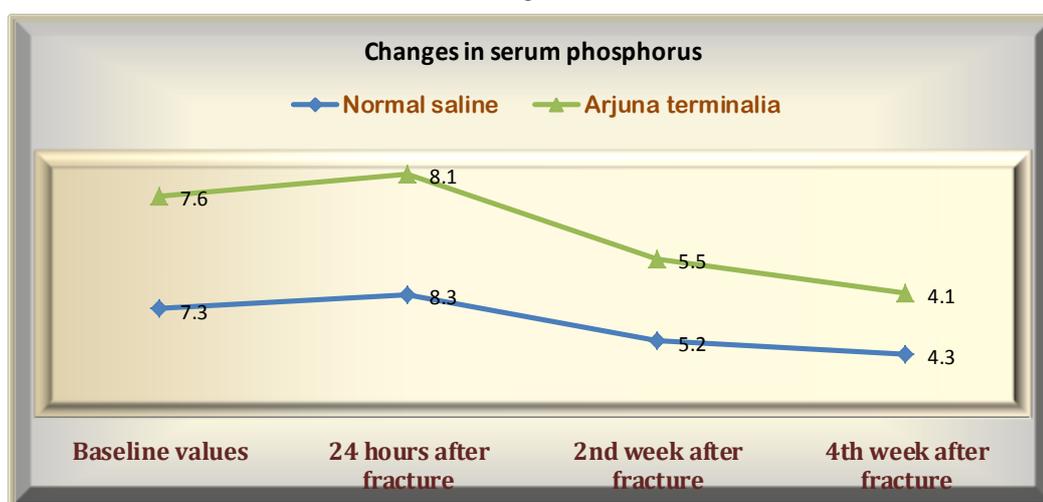


Figure 2

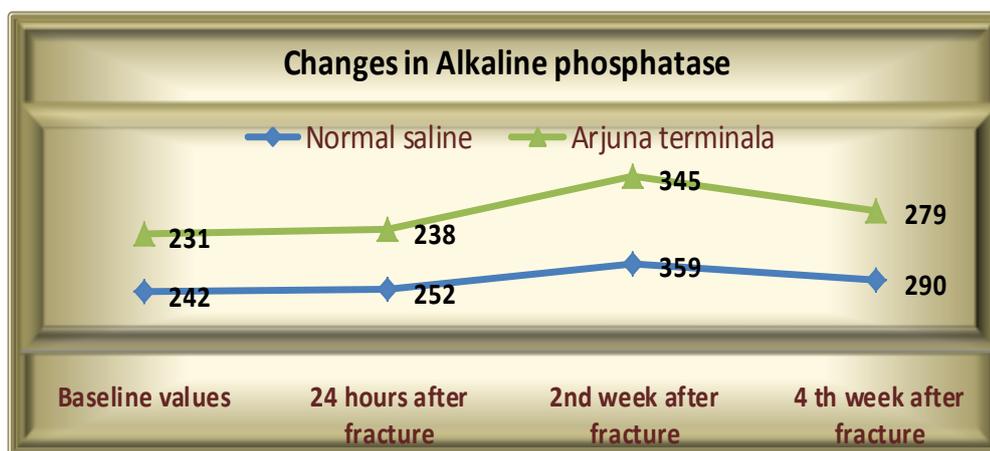


Figure 3

There is a significant drop in serum calcium, 24 hrs. after fracture while phosphorus & alkaline phosphatase, both increases significantly [$p < 0.001$] in both groups, Normal saline, Arjuna terminalia.

While during 2nd week of fracture healing calcium remains unchanged or slightly increased & Phosphorus is decreased while alkaline phosphatase is increased in both groups. During fourth week of fracture healing calcium & alkaline phosphatase are increased, while phosphorus is decreased in both groups.

Alkaline phosphatase, the enzyme secreted by the osteoblasts, accelerates the process of mineralization. An increase in alkaline phosphatase level during fracture healing has been recorded in the present study. The enzyme activity was remained at a high level from its 0 hour value at later weeks.

Early rise in serum inorganic phosphate corresponds to the necrotic disintegration of the cells at the site of fracture, thereafter it decreases which results in corresponding increase in calcium.

Drop in serum calcium favours the resorption of bone spicules resulting from the trauma. By 2nd week after fracture there is a rise in serum calcium level bringing about a state of super saturation of the body fluids favourable for the deposition of bone salts at the site of fracture healing. Calcium then returns to the normal levels or slightly rose. In this phase new bony trabeculae replace the cartilage.

The statistical analysis of data was done using SPSS 13.0. One-way ANOVA was used to study the significant difference in calcium; phosphorus & alkaline phosphatase in two different groups [*Arjuna terminalia* & Normal saline] & comparison was made by using Post-Hoc Test.

DISCUSSION

Instances are not rare in the past to illustrate that the use of medicinal herbs by the indigenous medical practitioners has had rationality. Many a time's detailed investigation had revealed not only useful information but had led to the findings of many newer synthetic substances of more potent action. [6]As mentioned by Singh DV et al 2004, tribal healers used the bark paste of *Arjuna terminalia* plant successfully for the treatment of fractured bone of animals as well as human being. [6] *Arjuna terminalia* when given with honey or sugar known to promote union of fractures. [13] Further it was tried for internal use by way of drinking decoction prepared by boiling the fresh herb in milk. This indicated that the active principle might be a fat soluble substance. [3] Hence we prepared alcoholic extract for use in animals.

In our previous study the callus formed around the fracture of the rats treated with *Arjuna terminalia* was comparatively denser than the cortex of the intact bone as evident from radiographs taken during 4th week. The beneficial effects of *Arjuna terminalia* seem to be significant as promoter of the bone healing.

Bone markers are used to determine effects of medications on bone formation, bone resorption or both. Serum calcium & inorganic phosphate are the markers of bone resorption while alkaline phosphatase is the marker of bone formation. [12] There is constant remodeling of the skeleton; the osteoclastic bone

resorption & osteoblastic bone formation are tightly coupled. If the calcium & phosphate concentrations in the circulation & extracellular fluid are adequate, calcium-phosphate crystals precipitate into the new osteoid, causing it to harden & mature. Osteocytes then develop from mature osteoblasts & reside in cervices within bone tissue & act as mechanoreceptor that sense developing areas of skeletal stress & then orchestrate local bone remodeling. Antiresorptive agents inhibit osteoclastic bone resorption while anabolic agents stimulate osteoblastic bone formation. [12]Antiresorptive agents increase bone mass by inhibiting bone resorption without initially affecting bone formation. Calcium being antiresorptive preventing bone loss. Serum biochemical markers of bone turnover are often used to assess effects of various medications on fracture healing. Since bone healing is characterized by a close interrelationship of bone resorption & bone formation, hence we chose biochemical markers that straddle both processes together with the primary macro elements that build the inorganic bone matrix.

The role of alkaline phosphatase, inorganic phosphate & calcium in fracture healing has been studied by several investigators, Udupa & Prasad, 1963.

In our study the biochemical evaluation reveals such difference in serum values of alkaline phosphatase, inorganic phosphate & calcium in *Arjuna terminalia* treated group suggesting that the primary locus of action of *Arjuna terminalia* may be in the region of bone regeneration itself. Our results agree with those of Chopra et al 1975. [14]

It could be stated that within a period of one month the markers of bone resorption (calcium, phosphate) were altered whereas the markers of bone formation (alkaline phosphatase) showed a tendency towards increase.

We recorded increased alkaline phosphatase level during fracture healing period in both groups but definitely more in *Arjuna terminalia* treated group. The enzyme activity remained at a high level even at the fourth week of fracture healing. These results are in resemblance to the observations recorded by Chopra et al (1975) & Mathur (1967). [14] Alkaline phosphatase is involved in bone formation & healing of fracture. [15, 16] The enzyme secreted by the osteoblasts accelerates the process of mineralization either by increasing the local concentration of inorganic phosphate or activating the collagen fibers to induce deposition of calcium salts. [17] Large concentration of alkaline phosphatase indicates active bone formation. Therefore in the early stage of fracture healing greater these changes, more rapid will be the healing.

An increase in the concentration of alkaline phosphatase possibly through enhanced stimulation of osteoblasts &/or physico-chemical changes in the collagen fibers might induce a more propitious milieu for mineralization. [14] This however needs to be explored by histochemical & bio-physical studies.

In our study serum inorganic phosphate was significantly increased 24 hour after fracture & then returned to normal level with a temporary depression below the normal level about 15 days after fracture. The early rise in phosphorus level in the blood corresponds to the necrotic disintegration of the cells at the site of fracture. This is followed by drop in serum calcium level which favours the resorption of bone spicules resulting from the trauma. [18].The rise in the level of phosphorus in the blood of rats 24 hr after fracture & the pronounced drop in serum Calcium might be expected to stimulate the secretion of parathyroid hormone. [19] This observation agrees with those reported by Chopra et al 1975 & Tornblom 1949.

Phosphate metabolism plays an important role in bone mineralization. Serum alkaline phosphatase hydrolyses organic compounds, viz, glycerophosphates & liberates inorganic phosphate. The early increase in phosphate ions disturbs the equilibrium between calcium & phosphorus & induces deposition of salts. [20]

We recorded decline in the level of serum calcium in both groups, 24 hr after fracture & 2nd week after fracture. However the decline was slightly higher in *Arjuna terminalia* treated group. A low level of serum calcium in the early stages of fracture healing was reported by Soliman FA et al (1964). Further the calcium of callus was stated to be derived from the serum calcium. [21]The decreased in the level of serum calcium to a greater extent in the *Arjuna terminalia* treated group may be due to faster healing process with more mobilization of calcium from serum in the formation of callus during fracture repair as reported by Udupa & Prasad (1963). Udupa & Prasad (1976) suggested the probable pathway of action to be through the anterior pituitary followed by adrenal, testes & liver. [22] However, the possible involvement of the thyroid gland also

cannot be ruled out which probably caused parafollicular or “C” cells to release more calcitonin & thereby a decrease in serum calcium level as observed in the present study since calcitonin increases the osteoblastic activity. [23]

In our study two weeks after fracture there is a pronounced rise in serum calcium level, bringing about a state of super saturation of the body fluids favorable for the deposition of bone salts at the site of healing. At this stage new callus sweep towards the break from the osteogenic layer of the periosteum & new bony trabeculae replace the cartilage until the trabeculae from each fragment meet & join. [18]. This however needs to be explored by histo-pathological & histo-chemical studies.

CONCLUSION

The finding assessed on the basis of biochemical parameters showed that ethanolic extract of the plant *Arjuna terminalia* had a definite fracture healing effect. The observed effect could be due to presence of tannin & saponins & tripenoid contents of *Arjuna terminalia* which have definite action on bone regeneration & calcium, phosphorus & alkaline phosphatase metabolism which also plays important role in osteoblastic activity. Detailed studies on the active constituents are needed which might provide new insight in fracture healing promoter drugs.

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