

Experimental Medicine

Wound Healing Profile of Areca Catechu Extracts on Different Wound Models in Wistar Rats

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ABSTRACT

Objective: To study the wound healing effect of extracts of *areca catechu* i.e., alkaloid of areca, polyphenols of areca, a combination of both and synthetic arecoline hydrobromide.

Design: Experimental study

Setting: Kasturba Medical College, Manipal, Karnataka State, India

Material and Methods: Twelve-week-old healthy male wistar rats weighing 150-200 g of three wound models were used in this study. In the incision wound model, the skin tensile strength was measured by using the continuous water flow technique on the tenth day. In the excision wound model, wound contraction and period of epithelization was measured. In the dead space wound

model, the harvested granulation tissue was assessed for dry weight, tensile strength, hydroxyproline content and histopathological studies.

Results: The arecoline alkaloid, polyphenol of areca and the combined formulation enhanced the breaking strength in incision wound model. All the extracts increased the wound contraction on the 4th and 16th day and the period of epithelization. In the dead space model, only the areca alkaloid fraction enhanced the tensile strength of granulation tissue.

Conclusion: Our study showed that the alkaloid of areca and polyphenols of areca could be used to enhance the healing of burn wounds, leg ulcers and skin graft surgery.

KEY WORDS: alkaloid of areca, *Areca catechu*, polyphenol of areca, wound healing

INTRODUCTION

The betel nut palm *Areca catechu* Linn, is a common masticant and appetizer. Betel nut chewing is an established practice among Indians for the past 2000 years. Its cultivation is concentrated in the southwestern and northeastern regions of India and it is extensively cultivated in the states of Karnataka, Kerala, Assam and West Bengal. More than ten million people are dependent on this crop for their livelihood. Seeds of areca contain catechin, tannins (15%), gallic acid, fat, gum and alkaloids like arecoline (0.07%), arecaine (1%). Arecaidine and guvacoline, guvacine and choline are present in trace amount.

Among these, arecoline is the most important alkaloid. The dried nuts have stimulant, astringent and vermifuge properties. Pharmacological actions of arecoline resemble that of muscarine and pilocarpine^[1]. The awareness regarding submucous fibrosis, a premalignant condition that is characterized by excessive collagen production by mucosal

fibroblasts has brought a major blow to the use of arecanut as masticator. Harvey *et al* showed that arecoline was responsible for the stimulatory effect on the fibroblasts in habitual betel nut chewers^[2], leading to a fall in the price for arecanut in market, provoking a search for other medicinal properties of areca. Scutt *et al* reported that polyphenols of areca stabilizes collagen by human and bacterial collagenases in a concentration dependent manner thus promoting the development of submucosal fibrosis following damage to the oral epithelium^[3]. This provided us an insight that when areca catechu extracts are used for short duration, it may enhance wound healing by proliferation of collagen.

SUBJECTS AND METHODS

Animal care and Handling:

The animal care and handling was done according to the guidelines set by the Indian National Science Academy, New Delhi, India.

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Twelve-week-old healthy male wistar rats bred locally in the animal house of Kasturba Medical College Manipal, weighing 150-200 g were selected for the study. They were housed under controlled conditions of temperature ($23 \pm 2^\circ\text{C}$), humidity ($50 \pm 5\%$) and 10-14 hr of light and dark cycles respectively. The animals were housed individually in polypropylene cages containing sterile paddy husk (procured locally) as bedding throughout the experiment and had free access to sterile food (animal chow) and water *ad libitum*. The study was undertaken after obtaining the approval of institutional animal ethical committee.

Extraction of arecoline: This was done by the general titration method of AOAC. Areca powder (-10 g) was transferred to a separating funnel with about 20 ml water and 1 ml of dilute sulfuric acid ($\text{H}_2\text{SO}_4 : \text{H}_2\text{O} = 1:9$). The solution was extracted with 25 ml portions of chloroform three times. Approximately 1 ml of ammonia solution was added and again extracted with 25 ml portions of chloroform four times. Alkalinity of the solution was ensured by checking the pH of the solution and adding more ammonia solution, if required. The chloroform layer was drained into a conical flask; the extraction was repeated till all the alkaloids were extracted into the chloroform. The chloroform was evaporated. A portion of it was used for quantification, by dissolving in excess of 0.02 N H_2SO_4 and titrating against 0.02 N NaOH using methyl red as indicator.

Estimation of arecoline: The normal contents ranged between 0.03 - 0.24%. It was detected on silica G TLC, using standard arecoline hydrobromide (Sigma chemicals, USA) as reference, and Dragendorff reagent - for color development.

Extraction and detection of polyphenols (tannins): Extraction of phenols: Areca powder (10 g) was left to stand in 100 ml of 80% methanol overnight. The solution was filtered and the process was repeated two more times. All the filtrates were pooled and the methanol removed by evaporation. The phenol obtained was dissolved in 200 ml water and quantitated using the method of Malik and Singh^[4] and ranged between 23-48 mg /g dry weight. A solution of 0.1 mg/ml was used for the animal studies.

Treatment schedule: Five groups (n = 6) of animals were taken for incision, excision and dead space wound models separately.

Group I received double distilled water; Group II was administered 0.5 mg/100 g body wt of the alkaloid extract of areca; Group III was administered 0.5 mg/100g body wt of the polyphenol

Table 1: Wound breaking strength in incision wound model

Groups (n)	Wound breaking strength (g) Mean \pm SE
Control	244.16 \pm 14.92
Alkaloid of areca	293.16 \pm 16.23*
Polyphenols of areca	347.83 \pm 22.32*
Alkaloid & Polyphenols of areca	339.25 \pm 17.47*
Arecoline hydrobromide	264.16 \pm 16.5

n=6, *p < 0.05 vs. Control

extract of areca; Group IV was administered 0.5 mg/100g body wt of a combination of the alkaloid and polyphenol extracts of areca; Group V was administered 0.5 mg/100g body wt of synthetic arecoline hydrobromide (Sigma chemicals, USA).

All compounds were administered per orally. In incision and dead space wound model, all extracts were given from day zero to 9th day in the above-mentioned dosage. In the excision wound model, extracts were administered till the wound was completely healed (about 21 days).

Wound models:

All wounding procedures were carried out under light ether anesthesia. In the present study, no animal showed visible signs of infection.

1. Incision wound: On the depilated backs of the animals, two paravertebral incisions 6 cm in length were made, cutting through the full thickness of the skin. Interrupted sutures, 1 cm apart, were placed to approximate the cut edges of the skin^[5]. The sutures were removed on the 7th post wound day and breaking strength was measured on the 10th day by continuous water flow technique of Lee^[6].

2. Excision wound: An excision wound was inflicted by cutting away 500 mm² full thickness of a pre-determined area on the depilated back of the rat. Epithelization period was noted as the number of days after wounding, required for the eschar to fall off leaving no raw wound behind. Wound contraction rate was monitored by planimetric measurement of the wound area on alternate days. This was achieved by tracing the wound on a graph paper. Reduction in the wound area was expressed as percentage of the original wound size^[7].

3. Dead space wound: Dead space wounds were created through a small transverse incision made on the lumbar region^[8]. A polypropylene tube (2.5 x 0.5 cm) was implanted subcutaneously beneath the dorsal paravertebral lumbar skin. The day of the wound creation was considered as day zero. Granulation tissue formed on the polypropylene

Table 2: Percentage of wound contraction at different time intervals and period of epithelization in excision wound model.

Groups	4 th day Mean ± SE	8 th day Mean ± SE	12 th day Mean ± SE	16 th day Mean±SE	Period of epithelization Days Mean ± SE
Control	5.23 ± 7.7	53.69 ± 3.10	81.73 ± 3.96	94.76 ± 1.44	21.33 ± 0.42
Alkaloids of areca	25.61 ± 12.45*	61.13 ± 2.30	87.1 ± 2.41	99.37 ± 2.9'	17 ± 0.45'
Polyphenols of areca	50.15 ± 4.15*	65.19 ± 2.37	92.67 ± 2.37	99.21 ± 0.36*	17 ± 0.45"
Alkaloids & Polyphenols of areca	23.85 ± 9.7	65.09 ± 4.79	88.67 ± 2.36	99.86 ± 0.17*	16 ± 0.52"
Arecoline hydrobromide	36.08 ± 6.14*	62.82 ± 3.93	92.90 ± 3.93	99.82 ± 0.18*	16.33 ± 0.33 ^b

p < 0.05 vs. Control, F = 2.79

*p < 0.01 vs. Control, F = 4.092, One way ANOVA followed by Bon -Ferroni test.

p < 0.05 vs. Control

^bp < 0.01 vs. Control, One way ANOVA

G-I (water control group) (p < 0.05) (Table 1). This shows that polyphenols of areca and alkaloid can be used for incised wounds.

Table 3: Effect of various extracts of areca on dry weight, tensile strength and hydroxyproline content of granulation tissue in dead space wound model.

Groups	Dry Weight (mg) Mean ± SE	Tensile Strength (g) Mean ± SE	Hydroxyproline (mg/g of tissue) Mean ± SE
Control	56 ± 3.93.	286.67 ± 11.45	24.52 ± 7.25
Alkaloids of areca	71.83 ± 21.08	416.67 ± 21.4a	23.26 ± 10.81
Polyphenols of areca	79.16 ± 18.47	303.3 ± 12.02	17.40 ± 1.28
Alkaloids & Polyphenols of areca	72.66 ± 24.08	263 ± 22.03	2.85 ± 0.79
Arecoline hydrobromide	57.66 ± 10.12	271.67 ± 17.21	2.39 ± 0.1

p < 0.05 vs. Control, F = 3.05, One way ANOVA followed by Bon -Ferroni test

tube was harvested by careful dissection on day 10 and the tensile strength of the granulation tissue was measured. A piece of this tissue was preserved in 10% formaldehyde and sent for histopathological examination. The remaining granulation tissue was dried in an oven at 60 °C overnight and the dry weight was noted. Acid hydrosylate of the dry tissue was used for the determination of the hydroxyproline content¹⁴. The standardization was done for hydroxyproline using the Neumann and Logan method¹⁵ and it followed Beers law for the range of 12 - 18 µg of hydroxyproline. The recovery obtained was 95% using 4 and 12 µg of hydroxyproline. •

Statistical Analysis: Statistical analysis was done using one way analysis of variance (ANOVA) followed by Bon-Ferroni test wherever required using SPSS package. Significance was noted at p-value < 0.05.

RESULTS

Incision Wound model - Wound breaking strength: The G-III (polyphenol) and G-IV (alkaloid + polyphenol) along with Group II (alkaloid), in that order, showed statistically significant improvement in the wound breaking strength when compared to

Excision Wound model - Period of Epithelization: All the test compounds showed statistically significant hastening of epithelization *i.e.*, control versus G-II & G-III (p < 0.05), control versus G-IV & G-V (p < 0.01) (Table 2). This again shows that both the areca extracts and the synthetic arecoline are effective in healing excision type of wounds such as abrasions. No compound can be claimed superior as there was no significant difference among the test groups.

Percentage of Wound Contraction: The percentage of wound contraction in all the test groups were significant statistically when compared to control on the 4th day (p < 0.05, F = 2.79) (Table 2). On the 4th day the percentage of wound contraction ranged from a low of 5.23 ± 7.7 in control to 50.15 ± 4.15 in the polyphenol extract. There was no statistical significance in the percentage of wound contraction during the 8th and 12th days. But on 16th day there was a statistically significant difference in percentage of wound contraction (p < 0.01, F = 4.092) between the control and the test groups. So the test compounds could be used to hasten healing in excision wounds like abrasions.

Dead Space Wound Model - Breaking Strength: There was a significant increase in the breaking strength of granulation tissue in G-II when compared to control (p < 0.05) but no statistically significant increase in G-III was observed (Table 3). Histopathology studies revealed good connective tissue response in Group II & Group III with decreased inflammatory component, but it was not very different from Group I. The healing process was very mild in the Groups-IV and V (Figs. 1-5). There was no significant change in the hydroxyproline content of the granulation tissue of G-II and G-III when compared to G-I (Table 3). The dry weight of the granulation tissue was not significantly increased in any of the test groups. However, the alkaloid (G- II) and polyphenol

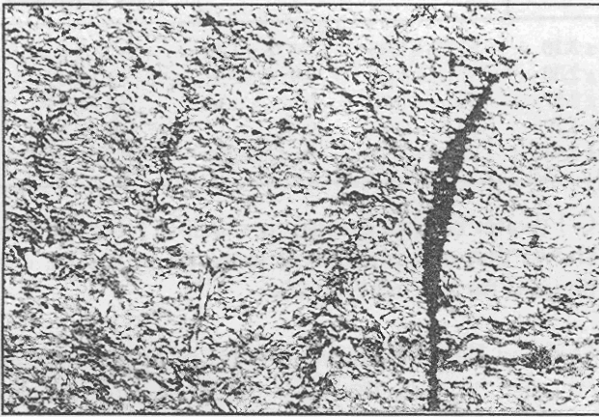


Fig. 1: Reveals deposition of collagen in wavy manner with mild inflammatory response with few proliferating fibroblasts (40 X, H&E) (G-I)

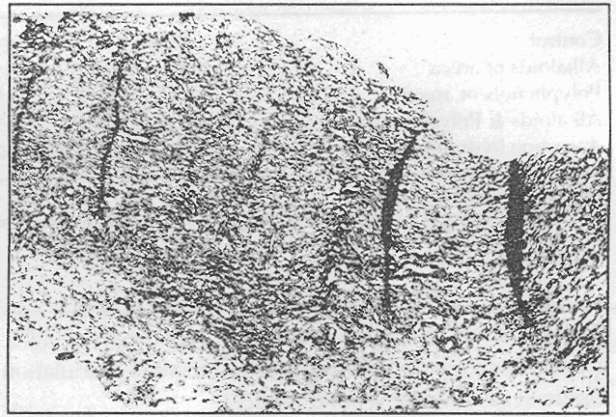


Fig. 2: Reveals an increased density of collagen fiber with mild inflammatory response that is more around the implant (40 X, H&E) (G-II)



Fig. 3: Retention of inflammatory exudates around the implant with evidence of fibrillar stroma and with mild inflammatory response with few proliferating fibroblasts and neovascularization (40 X, H&E) (G-III)

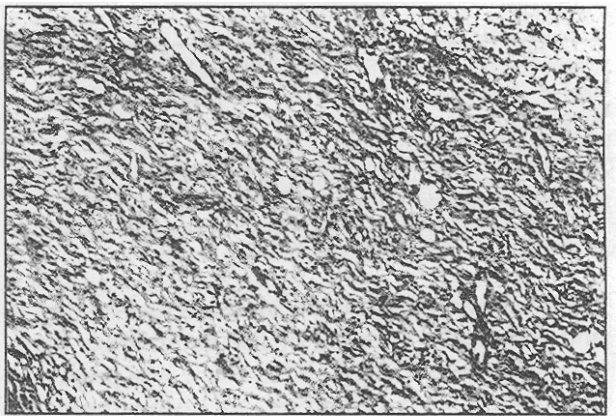


Fig. 4: Shows retention of inflammatory exudates and fibrillar stroma with numerous anastomosing vascular channels, mild inflammatory response and proliferating fibroblasts (40 X, H&E) (G-IV)

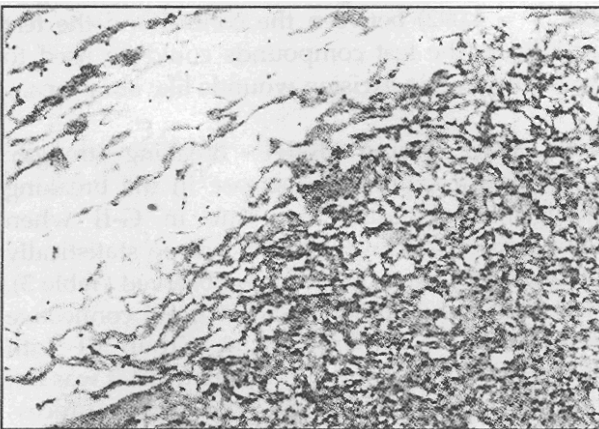


Fig. 5: Shows fibrillar stroma with mild to moderate inflammatory cell response around the implant with neovascularization (40 X, H&E) (G-V)

treated tissues (G- III) had higher dry weight when compared to the other two groups (Table 3).

DISCUSSION

It is a well-accepted fact that wounds in most tissues heal by repair, by laying down non-specific connective tissue^[10]. More than 50% of connective tissue is made up of collagen in case of sutured wounds. In excised wounds, since the edges are not in contact with each other contraction and

epithelization are necessary for the repair process. Hence laying down and weaving of the collagen material into the healing wound is an important feature. So it is understandable that substances that influence the collagen turnover or maturation enhance the process of wound healing. Collagen is a fibrous protein component of the connective tissue and provides a structural framework to the tissue^[11,12] consisting of hydroxyproline, hydroxylysine and glycine as principle constituents among which hydroxyproline is considered a specific amino - acid. Hence its estimation in the granulation tissue may throw light on the maturation and healing process^[13].

In our study, polyphenols and alkaloid fractions have enhanced the healing of incision wounds by increasing the breaking strength of the wounds. The polyphenol fraction especially seems more effective, as is obvious from Table 1, where the treatment having a combination of both the alkaloid and polyphenol fractions also has a relatively high wound breaking strength. A marginal increase in wound breaking strength was seen in alkaloid and synthetic arecoline treatments. All the test groups enhanced the wound healing by

hastening the period of epithelization and wound contraction on 4th and 16th days when compared to the control group; here again the polyphenol fraction had a head start. In the case of the dead space wound model, only the arecoline alkaloids showed significant and polyphenol fractions showed insignificant increase in the breaking strength of the granulation tissue compared to the control group and only polyphenol fraction had increased the dry weight of the granulation tissue. From the above findings, we have confirmed the wound healing effects of alkaloid and polyphenol fractions of areca in incision and excision wound models only.

Reportedly the alkaloid fraction of areca enhances the collagen production and hence wound healing¹¹. Similarly polyphenols of areca have been stated to promote wound healing of incision and dead space wounds and the period of epithelization in the excision wounds¹². But contrary to the above two studies, there was no increase in the hydroxyproline content of granulation tissue in arecoline and polyphenol treatments (infact, there was a decrease) and insignificant change in wound breaking strength of the granulation tissue with polyphenol treatment of the dead space wound model. Histopathological examination also revealed no marked alteration in the healing pattern in these treatments compared to control. Hence the effect of these extractions in healing of surgical wounds is questionable. Clinically increased wound strength is an important aspect in healing of surgical wounds. Scar weakness can lead to wound dehiscence and incisional hernia. Drugs like NSAIDs, steroids and antineoplastics have adversely affected healing in the peri-surgical period^{16,14,15}. Studies have quoted that local or systemic application of areca and polyphenols of areca extracts can prevent such an eventuality¹³. Thus the conclusion drawn by the previous authors *i.e.*, possible use of these in the healing process of surgical wounds is suspect¹³. We have to reconsider the effect of alkaloid and polyphenol fractions of areca in the healing of wounds of the dead space model.

However, we can assume that alkaloids and polyphenols of areca can be tested in the treatment

of leg ulcers, extensive burns, healing of donor area in skin graft surgery as this can lead to quicker coverage of epithelial layers as found in the healing of incision and excision wound. These need further careful clinical evaluation.

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