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# Screening of *Alstonia scholaris* Linn. R. Br., for wound healing activity

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## SUMMARY

*Alstonia scholaris* (Family: Apocynaceae) has been indicated for the treatment of various diseases, one among which is wound healing. The purpose of this study is to investigate the wound healing effect and antioxidant role of *Alstonia scholaris* leaves in animal models. The ethanol and aqueous extracts of *Alstonia scholaris* (EEAS, AEAS respectively) were tested against excision, incision and dead space wound models to evaluate the wound healing activity. In excision wound model, treatment was continued till the complete healing of wound, in incision and dead space wound models, the treatment was continued for 10 days. For topical application, 5% w/w ointment of EEAS and AEAS were prepared in 2% sodium alginate. For oral administration, EEAS was suspended in distilled water using Tween 80 and AEAS was dissolved in distilled water. The wound healing was assessed by the rate of wound contraction, period of epithelialisation, skin breaking strength, granulation strength, dry granulation tissue weight, hydroxyproline, collagen and histopathology of granulation tissue. Malondialdehyde level was also estimated to evaluate the extent of lipid peroxidation. AEAS and EEAS significantly promoted wound healing activity in all the wound models studied. Increase in the rate of wound contraction, skin breaking strength, granulation strength, dry granulation tissue weight, hydroxyproline and collagen, decrease in the period for epithelialisation and increased collagenation in histopathological section were observed with EEAS and AEAS treated groups. EEAS and AEAS also significantly decreased the levels of lipid peroxidation. The present study is suggestive that EEAS and AEAS promote wound healing activity.

**Key words:** *Alstonia scholaris;* Wound contraction; Incision wounds; Deadspace wounds

## INTRODUCTION

*Alstonia scholaris* Linn. R. Br., belonging to family Apocynaceae is native to India growing wild throughout in deciduous, evergreen forests and even in plains. Bark of *Alstonia scholaris* possess spectrum of pharmacological activity, ranging from bitter, astringent, thermogenic, laxative, antipyretic, anthelmintic to galactogoguic to cardiotonic properties, therefore used in fever, malarial fever, abdominal disorder, dyspepsia, leprosy, skin diseases, asthma, bronchitis, cardiopathy etc., (Nadkarni, 1976; Kirtikar and Basu, 2002). An antimalarial Ayurvedic preparation containing *Alstonia scholaris* is marketed Ayush-64 (Versha *et al*, 2003). Folklore use include application of milky juice of leaves on wounds, ulcers, and for rheumatic pain, as well mixed with

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oil and applied for earache (Nadkarni, 1976).

Extracts of *Alstonia scholaris* is reported to possess several pharmacological activities of interest that include pronounced antiplasmodial activity of methanolic extract of plant (Keawpradub *et al*, 1999), antimutagenic effect (Lim-Sylianco, 1990), immunostimulatory effect of bark extract in low dose and inhibition of delayed hypersensitivity reaction in higher dose of aqueous extract (Iwo, 2000), hepatoprotective against $CCl_4$, β-D galactosamine, acetaminophen and ethanol induced hepatotoxicity (Lin *et al*, 1996) and a promising anticancer activity against sarcoma - 180 by echitamine, an indole alkaloid extracted from bark (Saraswathi *et al*, 1998; 1999).

Tribals of Yercaud in Tamil Nadu, India, apply leaf juice and exudates on wounds to promote the healing. The current study was carried out to explore the scientific basis for such use. Aqueous and ethanolic extracts of *Alstonia scholaris* were investigated for their wound healing activity as well for its antioxidant status.

## MATERIALS AND METHODS

**Plant material and extraction**

The leaves of *Alstonia scholaris* (Family: Apocynaceae) were collected in the month of September - October 2003 from hills of Savanthwadi, Maharashtra, India. The plant material was taxonomically identified by the Botany Survey of India (BSI), Pune and the voucher specimen AS-1 is retained in herbarium of BSI, Pune for future reference. The dried powdered leaves (500 g) were defatted using petroleum ether and subjected to subsequent extraction in a Soxhlet apparatus by using ethanol and water. The solvents were removed from the respective extracts under reduced pressure to obtain a semisolid mass and vacuum dried to yield solid residues (5.24% w/w ethanol extract and 4.12% w/w aqueous extract). The extracts showed positive tests for alkaloids, tannins, saponins, glycosides, triterpenoids and flavonoids.

**Chemicals and Reagents**

Chemicals and reagents used for the study were of high grade procured from approved organizations.

**Animals**

Male Albino Wistar rats weighing between 150 - 200 g were used for the present study. The animals were maintained under standard environmental conditions and were fed with standard pellet diet and water *ad libitum*. The study was approved by Institutional Animal Ethics Committee (Reg. No. 626/02/a/CPCSEA). CPCSEA guidelines were adhered to during the maintenance and experiment.

**Acute toxicity studies**

Acute toxicity study was carried out for both the extracts (OECD guidelines, 2001). The extract suspended in water in the dose of 2 g/kg body weight was orally administered to overnight-fasted, healthy Wistar Albio rats (n = 3). The animals were observed continuously for 24 h for mortality.

**Extract formulation**

Two types of drug formulations were prepared from each extracts. For topical administration, 5% w/w ointments were prepared in 2% sodium alginate. For oral administration, 50 mg/ml suspension of EEAS in 2% Tween 80 and 50 mg/ml solution of AEAS in distilled water was prepared.

**Excision wound**

Rats were inflicted with excision wounds (Morton and Malone, 1972) under light ether anaesthesia. A circular wound of about 500 sq. mm was made on depilated ethanol sterilized dorsal thoracic region of the rats. The animals were divided into four groups, six in each. The animals of group I were left untreated and considered as the control, group II served as reference standard and treated with 1 % w/w framycetin sulphate cream (FSC), animals of group III and IV were treated with 50 mg of ointment prepared from EEAS and AEAS respectively. The ointment was topically applied once a day, starting

from the day of the operation, till complete epithelialisation. The parameters studied were wound closure and epithelialisation time. The wounds were traced on $mm^2$ graph paper on days 0, 4, 8, 12, 16 and 20 and thereafter on alternate days until healing was complete. The regenerated tissue was collected on these days and collagen content was estimated (Vijaya Ramesh and Sehgal, 1992). The percentage of wound closure was calculated using the formula,

$$\% \text{ Wound closure} = 1 - \frac{\text{Area of wound on corresponding day}}{\text{Area of wound on day zero}} \times 100$$

The period of epithelialisation was calculated as the number of days required for falling of the dead tissues remnants of the wound without any residual raw wound. The blood was collected on day 20 by retroorbital puncture and plasma lipid peroxide levels were estimated by using thiobarbituric acid (el Hafidi and G. Banos, 1997).

**Incision wound**

In incision wound model, 6 cm long paravertebral incisions were made through the full thickness of the skin on either side of the vertebral column of the rat (Ehrlich and Hunt, 1968). The wounds were closed with interrupted sutures of 1 cm apart. The animals were divided into four groups, six animals in each. The animals of group I were left untreated and considered as the control, the group II served as reference standard and received 1 % w/w FSC, animals in groups III and IV were treated with 50 mg of ointment of EEAS and AEAS respectively. The ointment was topically applied once a day. The sutures were removed on the post wound day 8. The skin breaking strength of the wounds was measured on day 10 (Lee, 1968). Simultaneously, blood was withdrawn on day 10 and plasma lipid peroxide levels were estimated (el Hafidi and Banos, 1997).

**Dead space wound**

Under light ether anaesthesia, dead space wounds were created by subcutaneous implantation of sterilized cylindrical grass piths (2.5 cm × 0.3 cm), one on either side of the dorsal paravertebral surface of the rat (Turner, 1965). The animals were divided into three groups, six in each. Group I served as the control, which received Tween 80 (2%, 2 ml/kg, p.o), group II and III received suspension of EEAS and solution of EEAS respectively (200 mg/kg, p.o). The granulation tissues formed on the grass piths were excised on the post wounding day 10 and breaking strength was measured. Simultaneously, granulation tissue harvested was subjected to hydroxyproline estimation (Woessner, 1961) and histopathological study to evaluate the effect of the extracts on collagen formation.

**Estimation of Malondialdehyde (MDA) in blood**

Plasma (0.75 ml) was mixed with 3 ml of reagent [75 mg Thiobarbituric acid (TBA), 15 g Trichloro acetic acid (T.C.A) and 2.08 ml 0.2 N HCl in 100 ml water] and kept in boiling water bath for 15 min, cooled, centrifuged for 10 min at 10,000 rpm. Absorbance of the supernatant is read against the blank at 535 nm.

MDA nanomoles/100 ml of blood

$$= \frac{\text{Absorbance of test} \times \text{Total volume (3.75 ml)}}{\text{Nanomolar extinction Coefficient } (1.56 \times 10^5) \times \text{sample volume (0.75 ml)}}$$

= Absorbance of test × 3205.

**Statistical analysis**

The data were subjected to ANOVA followed by Dunnet's multiple comparison test. The values of $P < 0.05$ were considered statistically significant.

## RESULTS

As suggested by OECD guidelines, the test animals were observed individually, after dosing at least once during the first 30 min, periodically during

the first 24 h with special attention during first 4 h. The test animals did not exhibit any visible change and survived beyond recommended duration of observation. Hence extracts, EEAS and AEAS were safe up to 2 g/kg. Dose of 200 mg/kg was selected (1/10$^{th}$ of 2 g/kg) for further experimentation (OECD guidelines, 2001).

Significant promotion of wound healing activity was observed in both AEAS and EEAS in all the three wound models; excision, incision and dead space wounds.

The excision wound heals by a combined process of wound contraction and epithelialisation and the wound contraction is indicative of the myofibroblast (Gabbiani *et al*, 1971). In the present study, the mean percentage closure was calculated on the 4, 8, 12, 16, 20 post wounding days as shown in Fig. 1. The EEAS and AEAS treated groups showed faster epithelialisation and contraction of wound than the control (Fig. 1). The collagen content in EEAS and AEAS treated groups were found to be higher than the control group (Fig. 2).

Collagen synthesis is maximum in a healing wound in about 1 to 3 weeks functioning for the rapid increase in breaking strength (Patil and Kulkarni, 1985). By second week, wound will have

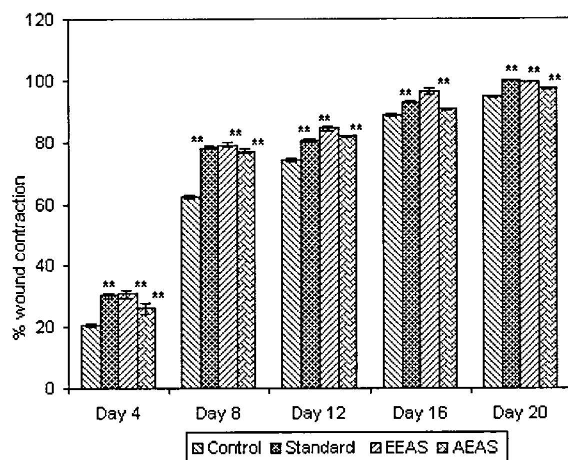



**Fig. 1.** Effect of *Alstonia scholaris* Linn. on excision wounds All values are represented as mean ± S.E.M. (n = 6). ANOVA followed by Dunnet's '*t*' test. $^*P < 0.05$, $^{**}P < 0.01$ compared to control.

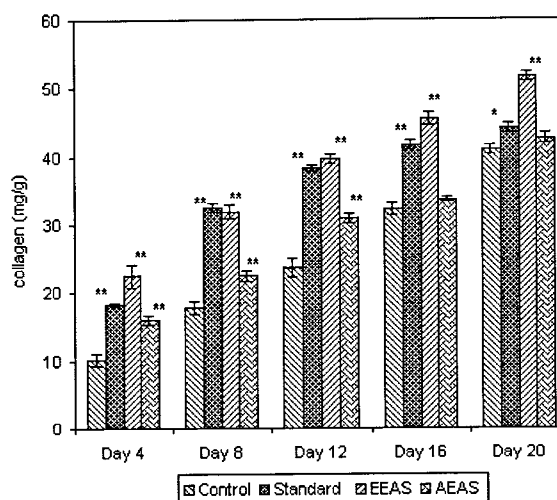



**Fig. 2.** Effect of *Alstonia scholaris* Linn. on collagen content of the granuloma tissue.
All values are represented as mean ± S.E.M. (n = 6) ANOVA followed by Dunnet's '*t*' test. $^*P < 0.05$, $^{**}P < 0.01$ when compared to control.

the greatest mass of collagen, which is often described as 'healing ride' and the wound strength at this time corresponds to its collagen content (Madden and Peacock, 1968). In the present study EEAS and AEAS treated animals showed significant ($P < 0.01$, $P < 0.05$ respectively) increase in breaking strength (421.6 ± 17.30 g and 328.4 ± 15.54 g respectively) when compared to the control (276.4 ± 8.074 g). The mean breaking strength was also significant ($P < 0.01$) in animals treated with standard drug FSC (494.42 ± 4.30 g). EEAS and AEAS showed significant ($P < 0.001$) decrease in mean blood MDA levels when compared to control group (Table 1) in incision and excision wound models.

In dead space wound model, histological studies of the granulation tissue of the control group of animals showed greater aggregation of macrophages with few collagen fibers (Fig. 3). In the case of AEAS treated animal groups, moderate collagen deposition, macrophages and fibroblasts were noticed (Fig. 4) whereas the EEAS treated group evidenced significant increase in collagen deposition, lesser macrophages and fibroblasts (Fig. 5). Compared to the control

H & E stain, Magnification 100. C – collagen, M – macrophages, B – Blood vessels.

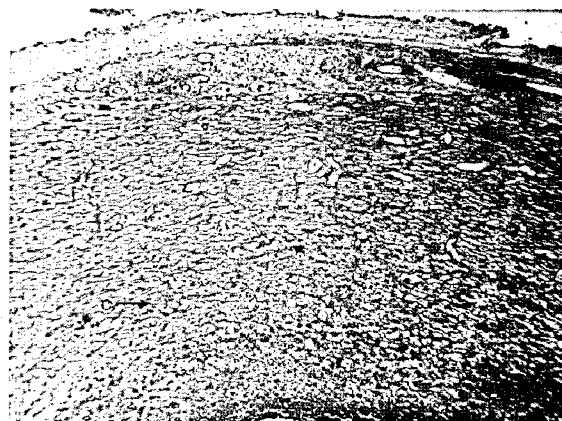
**Fig. 3** Histopathological section of granulation tissue of control animal.

H & E stain, magnification 100. C – collagen.

**Fig. 4.** Histopathological section of granulation tissue of the aqueous extract of *Alstonia scholaris* Linn. (AEAS) treated animal.

group of animals, EEAS treated animals showed significant increase in dry weight of granulation tissue and breaking strength followed by AEAS treated group (Table 1).

Collagen contains large molecules of glycine, proline and hydroxyproline, hence the hydroxyproline content is indicative of collagen formation (Smith, 1985). Estimation of hydroxyproline content in the granulation tissue revealed that the EEAS treated group had significantly high hydroxyproline content



H & E stain, Magnification 450. C – collagen.

**Fig. 5.** Histopathological section of granulation tissue of the ethanol extract of *Alstonia scholaris* Linn. (EEAS) treated animal.

followed by the AEAS treated group. However the control group showed less hydroxyproline content.

## DISCUSSION

Wound is a disruption of anatomic or functional continuity and regeneration, a normal response to injury that helps restore continuity of living tissue which is possible due to the synthesis of connective tissue matrix. Collagen, a major protein of extracellular matrix is one that ultimately contributes to wound strength (Schilling, 1968).

Formation of a precursor polypeptide - proline and lysine residue is the first step in collagen synthesis and larger the amount of hydroxyproline indicates more collagen is being synthesized at the site of cellular injury. Data represented in Table 1 and Figs. 2 - 5 reveals the collagen and hydroxyproline content of the granulation tissue in the animals treated with EEAS and AEAS which were significantly higher compared to control group.

Increase in tensile strength facilitates healing and in the animals treated with EEAS and AEAS, the collagen appears to increase with time in parallel to tensile strength (Smith, 1985). Increased breaking strength of granulation tissue of EEAS and AEAS

**Table 1.** Effect of *Alstonia scholaris* Linn. on Excision, incision and dead space wounds

| Parameter | Control | Standard | EEAS treated | AEAS treated |
|---|---|---|---|---|
| Excision wounds | | | | |
| Epithelialisation period (days) | 24.5 ± 0.84 | 17.5 ± 0.84** | 15.5 ± 0.84** | 17.84 ± 0.408** |
| MDA levels | 171.82 ± 5.48 | 144.6 ± 3.34** | 132.68 ± 6.84** | 142.84 ± 2.62** |
| Incision wounds | | | | |
| Tensile strength (g) | 276.30 ± 8.06 | 440.66 ± 2.58** | 421.6 ± 17.30** | 328.30 ± 15.54* |
| MDA levels (nmol/100 ml) | 186.54 ± 2.84 | 148.4 ± 5.24** | 126.56 ± 6.82** | 138.76 ± 5.92** |
| Dead space wounds | | | | |
| Breaking strength (g) | 287.4 ± 11.23 | - | 378.4 ± 19.12** | 312.6 ± 5.372 |
| Granuloma weight (g/100 g b.w) | 35.18 ± 2.96 | - | 47.48 ± 2.08** | 45.28 ± 1.90* |
| Hydroxyproline content (g) | 6.2 ± 0.10 | - | 8.92 ± 0.14** | 6.92 ± 0.22* |

All values are represented as mean ± S.E.M. (n = 6). ANOVA followed by Dunnet's '*t*' test. *$P < 0.05$, **$P < 0.01$ when compared to control.

treated animals indicates the enhanced collagen maturation of increased cross linking and increase in dry granulation tissue weight indicating the presence of higher protein content (Azad, 2002).

Elimination of reactive oxygen species is an important strategy to improve wound healing (Soneja *et al*, 2005) and decrease in lipid peroxide levels observed in EEAS and AEAS treated group is suggestive of improved wound healing.

Preliminary phytochemical analysis (Harborne, 1998) of AEAS and EEAS revealed the presence of flavanoids, saponins, tannins, glycosides. EEAS was positive for triterpenoids in addition. Flavanoids are known to reduce lipid peroxidation by preventing /slowing onset of cell necrosis as well by improving vascularity and drugs that inhibits lipid peroxdiation is also belived to increase viability of collagen fibers by increasing the strength of collagen fibers, the circulation, preventing the cell damage and by promoting DNA synthesis (Getie *et al*, 2002). Tannins (Ya *et al*, 1988), flavanoids (Tsuchiya *et al*, 1996) and terpenoids (Scortichini and Pia Rossi, 1991) have been reported to promote wound healing due to its astringent and antimicrobial property, which may be contributing to wound contraction and increase rate of epithelialisation. Antioxidant property of flavanoids and terpenoids may also be contributing to wound healing (Bastianetto *et al*, 2000).

Thus, accelerated wound healing activity in EEAS and AEAS may be due to phytoconstituents, acting individually or an additive effect. Further studies are focused on fractionation, purification and identification of active compound(s) of these extracts and its role in wound healing.

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