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Research Article

INVITRO WOUND HEALING ACTIVITY OF *URENA LOBATA* USING CAM ASSAY

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ABSTRACT

The plant Urena lobata reported for use in the traditional system of medicine for treating various diseases. The study presented was an attempt to screen the hydro alcohol extract of the aerial parts for wound healing potency using chick embryo wound model developed as part of the study. As evident from the experimental data the hydro alcohol extract showed good dose-dependent healing potency. The hydro alcohol extract at 500 µg concentration showed increased wound contraction by 50% compared to the negative control model in the chick embryo chorioallantoic membrane excision wound model. Statistically the results were significant when compared to the control groups ($p<0.05$). The extract was found to have better angiogenic properties from neovascularization studies.

KEYWORDS: *Angiogenesis, Urena lobata, Chick embryo, Chorioallantoic membrane, Wound healing.*

INTRODUCTION

The general repair response of the body immediately after the disruption of skin integrity is Wound healing. It has five interconnected and overlapping phases; haemostasis and inflammation, neovascularization, granulation, re-epithelization and remodeling [1]. In chronic wounds with a prevalence of 4 per 1000 population [2] the treatment is sometimes problematic. Because of their low availability, high cost, and various detrimental side effects [3] wound healing drugs currently in use are not affordable and effective. Therefore, medicinal plant derived drugs is under great demand due to a common belief that they are safe, reliable, clinically effective, low cost, globally competitive and better tolerated by patients [4]. Since ancient times, based on empirical observations without any scientific knowledge for the treatment of wounds, cuts, and burns [5] human beings have been using many plant resources. Tannins, triterpenoids and alkaloids have been found to affect one or more phases of wound healing process [3, 6] which are biologically active compounds found in plants. *Urena lobata* which is commonly known as Caeser weed is an annual in sub tropic and perennial in the tropics.These species include various clinically important constituents including flavonoids, saponins, flavonoid glycosides [7]. Various parts of the *Urena* species have been indicated as alternative remedies for the treatment of several diseases. The important member of the genus, *U.lobata*, has been introduced as antioxidant and cytotoxic activities [8], hepatoprotective and antidiabetic activity [9], besides use in diarrhea [10]. Furthermore, other *Urena* species have been used for the neurological disorders [11], Apart from its antibacterial activity, *U.lobata* is used externally for treatment of wounds, cuts and skin disorders traditionally. In spite of its wide use over a long period of time, there have been no attempts to study the molecular mechanism of *U.lobata* wound healing properties. Therefore, the aim of this study was to investigate the possible wound healing properties of hydro alcoholic extract of *U.lobata* using *in vitro* wound healing assays.

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METHODOLOGY

Plant material:

Aerial parts of the plant *Urena lobata* were collected from Thirunelveli district, Tamil Nadu, India in the month of February 2014. The plant material was identified and authenticated by botanist, Dr. P. Jayaraman at Plant Anatomy Research Center (PARC), Chennai. The plant materials were shade dried in open air and ground to a coarse powder.

Preparation of plant extracts:

Fifty grams of the powder was extracted with water: ethanol (1:1) and was filtered through eight layers of muslin cloth. The procedures were repeated twice with 250 ml of ethanol and water each. The pooled extracts were concentrated by evaporation. The residue was stored in stock vials for further use.

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Embryo collection:

Fertilized white shell eggs were purchased from Tamilnadu Veterinary and Animal Science University, Madhavaram Milk Colony, Chennai. The outer surface of the embryos were cleaned with 75% ethanol and incubated at 37°C throughout the study.

Preparation of saturated filter disk for wound assay:

Whatman No. 1 filter paper was purchased from Millipore. Small disks were generated using a standard 5 mm hole puncher, sterilized by autoclaving and stored for further use. The pre-sterilized filter disks were saturated with different concentrations of the crude extract, from 100-500µg/ml, and the control solutions. Diclofenac sodium (50µg/ml) in 4% ethanol and sterile saline were used as positive and negative controls respectively [12].

Wound assay:

All dissection tools used in the assay were sterilized using 75% ethanol before use. The embryos were incubated for 11 days to allow good maturation of the chorioallantoic membrane. On day 12 of incubation the outer shell was wiped with 75% ethanol to sterilize the surface. Under aseptic conditions a tiny hole was made carefully in the egg shell with a needle and a small window of the shell was cracked open exposing the opaque inner shell membrane (Figure 1). About 0.5-1 ml sterile saline was added to the inner shell membrane to make it translucent. This layer was then peeled to visualize the CAM layer. The CAM layer was pulled gently by using sterile forceps and an excision wound of approximately 3 mm diameter was created in the CAM layer by using a small dissecting scissor. The drug saturated discs were then placed on the CAM of the embryos labeled with the corresponding concentrations and controls. The window on the egg shell was covered with para film and the eggs were returned to the incubator. Measurements of wound closure were made on alternative days up to day 5 of observation post wounding.

The wound closure was measured as wound contraction percentage (WC %) by using the formula [13].

$$WC\ \% = \frac{\text{Initial wound size} - \text{Specific day wound size}}{\text{Initial wound size}} \times 100$$

Angiogenesis assay:

The vascular network forms well in the developing embryo by 7 days of incubation. On day 8 of incubation the outer shell was wiped with 75% ethanol to sterilize the surface. Under aseptic conditions a tiny hole was made carefully in the egg shell with a needle and a small window of the shell was cracked open. The CAM layer was exposed as above. The drug saturated disks were then placed carefully on the CAM vasculature of the embryos labeled with the corresponding concentrations and controls and the egg shell window closed with parafilm before incubation. Morphometric evaluation of the blood vessels on the CAM was done on alternative days of observation in terms of number of new vessels and thickness. The results were photographed on each observation and recorded (Figure 2).

Statistical analysis:

The collected data were analyzed with SPSS 16.0 version. The values are presented as mean with Standard Deviation. To find the significance with positive controls, One-Sample Wilcoxon Signed Rank Test was used. In the above statistical tool the probability value $p<0.05$ was considered as significant level.

RESULTS

The results showed that % wound closure was dose-dependent with the maximum being at 500 µg/mL concentration. At 500 µg/ml concentration the hydro alcoholic extracts showed 51% wound contraction compared to the positive control which showed 79.21 % and the negative control which showed no significant wound closure (Table 1).

Angiogenesis was morphometrically analyzed and tabulated by counting the number of blood vessels in various treatments. The extract promoted an increase in number of blood vessels compared to saline control. The hydro alcoholic extract was more angiogenic in terms of increase in number and thickness of blood vessels than the aqueous extract.

Table No. 1: ID Values presented are mean with standard deviation where n=6; p<0.05 was considered statistically significant compared to the control group

Treatment	ID (mm)	% WC
Positive control (Diclofenac Sodium 50 µg/ml**)**	0.49± 0.02	79.21
Negative Control (Saline**)**	3.0± 0.009	0.0
Hydro alcoholic extract		
100 µg/ml	2.71± 0.08	9.56
200 µg/ml	2.62 ± 0.04	10.12
300 µg/ml	2.29 ± 0.20	12.32
400 µg/ml	2.0 ± 0.12	32.21
500 µg/ml	1.29 ± 0.15	51. 02
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500 µg/ml	1.29 ± 0.15	51. 02

(ID –Internal diameter in mm, WC-wound contraction in percentage)

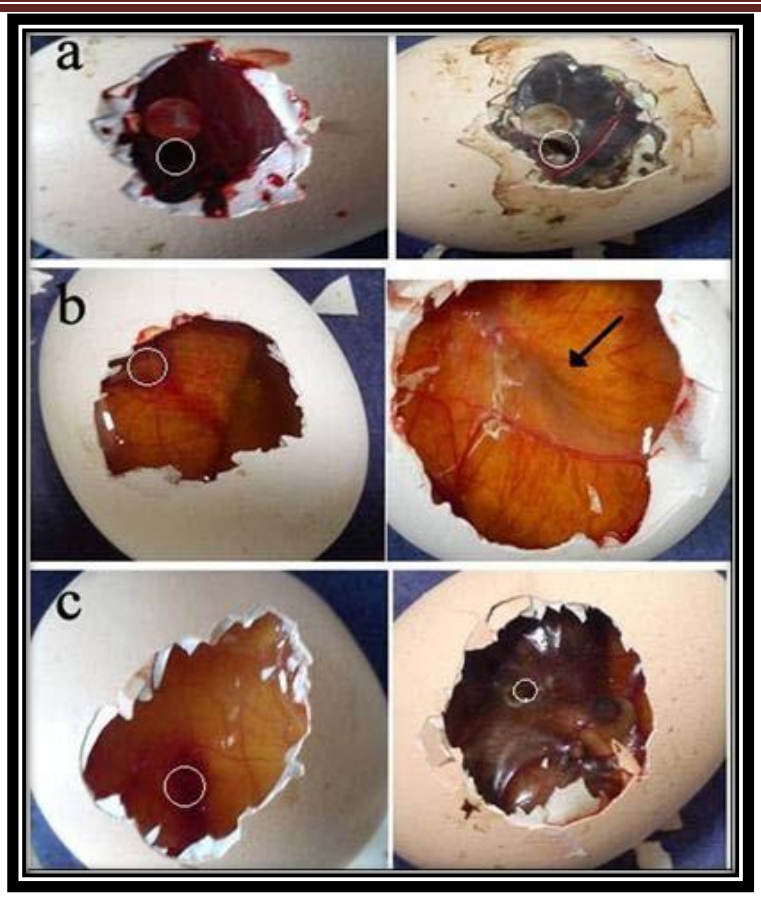

Fig. 1: Treatment of the established wound model
(a) Negative control (saline); (b) Positive control (Diclofenac sodium-50 µg/ml) and (c) Treatment with the ethanol extract (500 µg/ml).

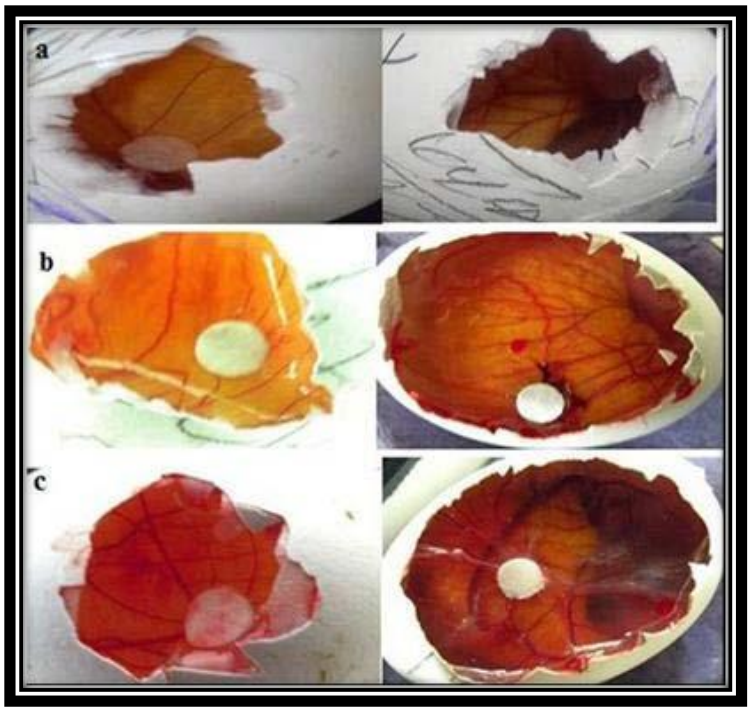

Fig. 2: Neovascularization observed on the CAM layer of the treated models.
(a) Positive control (Diclofenac sodium-50 µg/ml); (b) Negative control (saline) and (c) Ethanol extract treatment (500 µg/ml)

DISCUSSION

Through the results we found that the potency of the drug for wound healing increases with increasing concentrations, both in the form of angiogenesis and wound contraction which was comparable with positive control Diclofenac sodium (50µg/ml).The presence of phenolic compounds, saponins, steroids, tannins and falvonoids was observed through preliminary phytochemical screening with the hydro alcoholic extract. Falvonoids have proved that they possess anti-inflammatory activity. Inflammation being the first phase of wound repair mechanism has to be immediately controlled when the actual healing begins. Since the extract has the anti-inflammatory activity, it may accelerate the healing process. The compounds like terpenoids, tannins, saponins, steroids have proved antimicrobial activity which eliminates the heavy microbial load at the wound site and avoid prolonged inflammation. Vascular endothelial growth will be unregulated by the compounds like Phenolic and tannins which enhances the angiogenesis and wound contraction [14, 15].

CONCLUSION

The established alternative to the *in-vivo* animal wound models for screening of wound healing herbal extracts is found to be chick embryo wound model .The wound healing potency for the extracts may be based on the wound contraction and angiogenic traits. The hydro alcoholic extract of the *Urena lobata* was found to possess excellent wound healing potency which was dose-dependent. The healing potency of the plant was evident from the phytoconstituents. Further molecular mechanism behind the activity could be determined using the established model with the knowledge of the target protein or other factors involved in the complex healing pathway.

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