Evaluation of Wound Healing Potency of *Echinochloa colona* using *In Vivo* and *In Vitro* Methods.

Vijay S. Borkar*

Research Scholar M. Pharm. Padmavathi College of Pharmacy,
Dharmapuri (TN) India.
Email address: vijayborkar79@gmail.com
Mobile no :09975505734

Dr. K Senthil Kumaran. Ph.D.

Prof. and Head Department of Pharmaceutics,
K.K. College of Pharmacy, Chennai (TN)

Dr. KL Senthil Kumar. Ph.D.

Prof. and Principal, Padmavathi College of Pharmacy,
Dharmapuri (TN) India.

Abstract

Purpose: The present study was designed with the purpose of evaluation of various fractions obtained from the plant *Echinochloa colona* for its wound healing activity using *in-vivo and in-vitro* models.

Methods: The whole plant was extracted with ethanol using soxhlet apparatus by continuous hot extraction method. The ethanolic extract was then fractionated using various polarity solvents and evaluated for wound healing activity using *in-vivo* guinea pig punch wound model and *in vitro* wound assay and chick chorioallantoic membrane model.

Results: The chloroform, ethyl acetate and ethanol residues were evaluated for wound healing activity and shown potency towards wound healing.

Conclusion: The chloroform, ethyl acetate and ethanol residues were evaluated for wound healing activity. Among the three fractions, chloroform has shown enhanced wound healing activity in guinea pigs. It promoted wound contraction and angiogenesis at 200 µg/ml and 40 mg/disk concentration in chick chorioallantoic membrane model respectively. These results indicated that chloroform fraction of the plant in the present study exhibits significant wound healing activity evaluated by *in vivo* and *in vitro* models.

Keywords: *Echinochloa colona, wound* healing, punch wound, angiogenesis, chick chorioallantoic membrane.

1. Introduction

The process of wound healing starts with different phases such as coagulation, epithelization, granulation, collagen formation and tissue remodeling. Each and every phase of it has its own contribution in wound healing process. Angiogenesis is an elementary process affecting blood vessel formation and essential in reproduction, development, and wound repair [1, 2].

The applicability of measurement of wound area, tensile strength, collagen estimation and histopathological studies is well known in various *in vivo* animal models. In addition to this angiogenesis research is being studied into three major areas namely diagnostic, wound healing and inhibition of angiogenesis in neoplasia. It has been already proved that topically and orally applied basic fibroblast growth factor accelerates angiogenesis in chronic wounds and duodenal ulcers respectively, which are helpful in the process of wound healing [3, 4]. Therefore great efforts are being made to understand the mechanisms of angiogenesis, and to develop agents that have angiogenic activity which will be helpful in accelerating in wound healing process. The main aim of wound treatment is to achieve a rapid closure of the wound and formation of new blood vessels which will progress the process of wound healing. The rapid closure of wound and formation of new blood vessels can be achieved by wound contraction and angiogenesis respectively. The wound contraction is an important feature in healing which helps to close the wound by decreasing the gap between its dermal edges and reducing the wound surface area [5].

The plant in present study is *Echinochloa colona* (Poaceae) a terrestrial, tufted and erect grass commonly known as Jungle rice in India. This species propagates mostly by seeds and widely distributed in tropics and subtropics, including South and Southeast Asia and tropical Africa. Leaves are alternate spiral, sessile linear, more than 2 cm long/wide, apex acute, base clasping, parallel-veined. It is Annual, culms geniculately ascending, or

decumbent; 10-100 cm long. *Echinochloa colona* already has proved its antioxidant potential and three bioactive constituents have been isolated from chloroform fraction of ethanol extract [6]. In relation to its antioxidant property, our present study included *in vivo* guinea pig punch wound model and *in vitro* wound assay and chick chorioallantoic membrane model to evaluate wound healing potential of plant.

2. Materials and methods

2.1 Plant material

The plant *Echinochloa colona* was collected from Dharmapuri, Tamilnadu, India and was authenticated from Botanical survey of India. The whole plant material was cleaned to remove earthy matters with the aid of water and then dried under shade avoiding direct drying in sunrays.

2.2 Extraction of plant

The shade dried whole plant was coarsely powdered and extracted with ethanol using soxhlet apparatus for 72 hrs. The ethanol extract was then concentrated using rotary evaporator and fractioned successively using petroleum ether, hexane, chloroform, ethyl acetate and ethanol to obtain various fractions. The phytochemical analysis for the presence of bioactive constituents and powdered drug analysis for the determination of ash values and extractive values was performed [6]. The chloroform, ethyl acetate and ethanol residues were formulated as 1 % fraction mixed with ointment base for *in vivo* studies and 25, 50, 75 and 100 µg/ml for wound assay.

2.3 Wound healing activity

***In vivo* guinea pig punch wound model** [7]

Animals: Healthy male guinea pigs weighing 300– 325 mg were selected for the study. Animals were divided into 5 groups, consisting 2 animals in each group. **Group I-**Vehicle control treated with ointment base. **Group II-** Standard treated with Povidone- Iodine ointment. **Group III, IV and V-** Treated (1 %) topically with chloroform, ethyl acetate and ethanol fraction mixed with ointment base for 7 days.

The dorsal surface of the animals was shaved; sterilized (70% alcohol) and four cutaneous circular wounds of full thickness, completely transdermal of 8 mm diameter were made with the help of a biopsy punch. Thiopentone sodium (25 mg: kg, i. p.) was used to produce anesthesia to carry out all surgical procedures. Animals were allowed to recover and were housed in standard experimental conditions supplied with standard diet.

Assessment of healing

The wound healing was assessed by measuring area of wound, estimation of collagen in terms of hydroxyproline and measurement of tensile strength in healing tissue.

Area of wound

The healing wound was measured for the surface area on 7^{th} and 10^{th} day by tracing the boundary of wound which is still open using semi-transparent paper and calculation of area was done using a graph paper. The effect of various fractions of *Echinochloa colona* on punch wound model in terms of wound area is shown in (Table 1).

Collagen estimation

Collagen was estimated on 7^{th} day by determining hydroxyproline content of wound tissues, which is a basic constituent of collagen. Tissues were dried in a hot air oven at 60–70^{o}C to constant weight and were hydrolyzed in 6 N HCl at 130^{o}C for 4 h in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4 M perchloric acid and color was developed with the help of Ehrlich reagent at 60^{o}C and measured at 557 nm using UV spectrophotometer.

Tensile strength

After the completion of treatment of wounds in the animals, on the 7^{th} day the animals were anaesthetized and healing tissue along with normal skin at two ends was pooled for tensile strength measurement (Tensile Testing Machine). The excised tissues from treated and control animals were cut out (8 mm width and 20 mm length) and loaded between the upper and lower holder of the machine. Effect of various fractions of *Echinochloa colona* for 7 days on collagen (hydroxyproline content) and tensile strength is shown in (Table 2). The total breaking load was measured in Newtons and the tensile strength was calculated by using following equation:

Tensile strength= Total breaking load/Cross-sectional area

The experimental protocols were permitted and approved by the Institutional Animal Ethical Committee (IAEC); number CPCSEA /PCP/IAEC/PHD/119/12. The experimental animals were treated as per the guideline of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

***In vitro* wound assay** [8]

All dissections in the assay were performed using sterilized tools with 75% ethanol. The 14 embryos were selected and incubated for 11 days so that good maturation of the chorioallantoic membrane is occurred. On 12th day of incubation, the outer shell was wiped with 75% ethanol to sterilize the surface. All the 14 embryos were divided into 5 groups containing standard Diclofenac sodium; treated chloroform, ethyl acetate and ethanol residues and control without treatment. All the eggs were kept 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 to expose the inner shell membrane. 0.5-1 ml of sterile saline solution was transferred to the inner shell membrane to make it translucent then the layer was peeled to visualize the chorioallantoic membrane (CAM) which was then pulled 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 discs saturated with chloroform, ethyl acetate and ethanol residues along with standard drug were then placed on the CAM of the embryos labeled with the corresponding concentrations and controls. The window of the egg shell was covered with para film and then all eggs were incubated. Wound closure was measured on alternative days of 5 day observation period after wounding. The wound closure was measured as wound contraction percentage (WC %) by using following formula [9]. Measurement of internal diameter and percentage wound contraction of various fractions are tabulated in (Table 3).

$$\text{Percent Wound Contraction} = \frac{\text{Initial wound size} - \text{Specific day wound size}}{\text{Initial wound size}} \times 100$$

Chick chorioallantoic membrane model [10]

Chick chorioallantoic membrane (CAM) model was used to assess the angiogenic activity of various fractions from the plant. Nine day-old fertilised chick eggs were selected for the study and were incubated at 37°C and 80% relative humidity condition in an incubator. The shells of the eggs were cleaned with 70% ethanol to avoid infections. After 72 h a small window of 1.0 cm^2 was made in the shell. The membrane was fell by drilling a small hole at the air space and air was removed out using a rubber bulb. The window was opened and a sterile disk of methylcellulose loaded with different concentrations (10–40 mg) of chloroform, ethyl acetate and ethanol residues was placed in at the junction of two big vessels. The window was then resealed by tape and the eggs were incubated at 37^{o}C in a in an incubator for 72 h. The eggs were then opened. New vessel formation was observed and compared with that in eggs containing disks without test compounds as shown in (Fig 1). The effect of various fractions from the plant compared to the control on chick embryo chorioallantoic models is presented graphically (Fig 2, 3 and 4).

2.4 Statistical analysis

Data were evaluated statistically by Dunnett multiple comparison test. *P* values less than 0.05 were considered to be significant.

3. Results and discussion

The various fractions from *Echinochloa colona* were evaluated for wound healing activity using *in vivo* guinea pig punch wound model and *in vitro* wound assay and chick chorioallantoic membrane model.

***In vivo* guinea pig punch wound model**: In this model the wound healing activity was assessed as decrease in wound area (mm^2), estimation of hydroxyproline content (Mg/g tissue) a major determinant of collagen content and tensile strength (N/cm^2) of the healing tissue. Chloroform fraction was able to decrease wound area by 14.8 mm^2 as compare to vehicle control 30.6 mm^2 and standard 8.5 mm^2 measured on 10th day. There was 85.87 % and 83.37 % increase in hydroxyproline content and tensile strength with chloroform treatment. The above results obtained indicated better wound healing activity of chloroform fraction as compare to control and standard.

***In vitro* wound assay:** The assessment criteria for this method were to calculate percentage wound contraction by measuring internal diameter of the wound in mm. The percentage wound contraction for chloroform, ethyl acetate and ethanol residue was 40.67, 26.47 and 30.61 at 200 µg/ml concentration respectively.

Chick chorioallantoic membrane model: Chloroform, ethyl acetate and ethanol residue was able to form 14, 1 and 1 new blood vessels as compared to control, which indicates better angiogenic activity of chloroform fraction comparing other two where there was decrease in angiogenic activity upon increase in concentration.

4. Conclusion

The chloroform, ethyl acetate and ethanol fractions were obtained from ethanol extract of *Echinochloa colona* and screened for wound healing activity using *in vivo and in vitro* methods. All these fractions shown wound healing potential but chloroform fraction has shown good wound healing activity comparing other two. In conclusion *Echinochloa colona* may compose of principal source of presumed bio active compounds which are able to increase the content of collagen, tensile strength and angiogenesis to cause effective wound healing activity.

5. Acknowledgements

Authors are thankful to the Principal, Padmavathi College of Pharmacy, Dharmapuri (TN) for providing necessary laboratory facility to complete the work.

References

[1] P. Carmeliet. Angiogenesis in life, disease and medicine. Nature, 2005, 438:932-936.
[2] R.H. Adams, K. Alitalo. Molecular regulation of angiogenesis and lymphangiogenesis. Nat Rev Mol Cell Biol, 2007, 8:464-478.
[3] M.C. Robson, L.G. Phillips, W.T. Lawrence, J.B. Bishop, J.S. Youngerman, P.G. Hayward, *et al*. The safety and effect of topically applied recombinant basic fibroblast growth factor on the healing of chronic pressure sores. Ann Surg, 1992, 216:401–408.
[4] J. Folkman, S. Szabo, M. Stovroff, P. McNeil, W. Li, Y. Shing. Deodenal ulcer: discovery of a new mechanism and development of angiogenic therapy that accelerates healing. Ann Surg, 1991, 214: 414-427.
[5] I.A. Darby, T.D. Hewitson. Fibroblast differentiation in wound healing and fibrosis. Int Rev Cytol, 2007, 257:143.
[6] V.S. Borkar, K. Senthil Kumaran, K.L. Senthil Kumar, H. H. Gangurde, M.A. Chordia. Antioxidant effect and characterization of bioactive constituents isolated from *Echinochloa colona* (Poaceae). World J Pharmaceut Res, 2015, 4: 1652-1661.
[7] A. Shukla, A.M. Rasik, G.K. Jain, R. Shankar, D.K. Kulshrestha, B.N. Dhawan. *In vitro* and *in vivo* wound healing activity of asiaticoside isolated from *Centella asiatica.* Journal Ethnopharmacol, 1999, 65: 1–11.
[8] S. Chris, M. David, G.S. Dwayne. Angiogenesis assay in the chick CAM. Methods in Molecular Biology, 2005, 294:123-136.
[9] L. Vaidyanathan, D. Thanikachalam, T.S. Lokeswari, Sivaswamy. Evaluation of Wound Healing Potency of C*assia auriculata* Flower Extracts Using Chick Embryo Wound Model. Int J Pharm Sci Rev Res, 2014, 27: 222-227.
[10] N. Narendra, P. Gaurav, D. Lokesh, K. Naveen. Wound healing activity of latex of *Calotropis gigantea*, Int J Pharm Pharm Sci, 2009, 1: 34-35.

Table 1: Effect of various fractions of *Echinochloa colona* on punch wound model (Wound Area in mm^2).

Day	**Vehicle control**	**Standard**	**Chloroform**	**Ethyl acetate**	**Ethanol**
0	57.5	52.5	56.3	55.7	54.8
7	28.3	12.3	18.5	22.6	24.7
10	30.6	8.5	14.8	20.3	22.2

Table 2: Effect of various fractions of *Echinochloa colona* for 7 days on collagen (hydroxyproline content) and tensile strength.

Treatment (Concentration 1 %)	**Hydroxyproline (mg/g tissue)**	**Tensile strength (N/cm^2)**
Vehicle control	52.13± 2.61	09.13± 2.31
Standard	72.23± 1.41	13.23± 1.31
Chloroform	62.03± 1.32	11.03± 1.61
Ethyl acetate	43.33± 2.11	10.43± 1.41
Ethanol	36.43± 1.61	09.63± 1.42

Values are mean9S.E. (*n* =2 animals). * *P* 0.001 and ** *P* 0.01 as compared to vehicle control.

Table 3: Measurement of internal diameter and percentage wound contraction with various fractions from *Echinochloa colona.*

Treatment	ID (mm)	% WC
Standard		
50 µg/ml	0.5±0.03*	85.33
Control (saline)	3±0.02*	00.00
Chloroform		
50 µg/ml	2.4±0.11*	17.33
100 µg/ml	2.1±0.08*	21.67
150 µg/ml	1.6±0.16*	26.34
200 µg/ml	1.1±0.10	40.67
Ethyl acetate		
50 µg/ml	2.7±0.12*	14.33
100 µg/ml	2.3±0.08*	17.67
150 µg/ml	1.9±0.16*	24.33
200 µg/ml	1.6±0.10	26.47
Ethanol		
50 µg/ml	2.6±0.11*	16.13
100 µg/ml	2.5±0.07*	18.17
150 µg/ml	1.9±0.11*	22.31
200 µg/ml	1.5±0.10	30.61

ID Values presented are mean with standard deviation where n=6; $p<0.05$ was considered statistically significant compared to the control group.

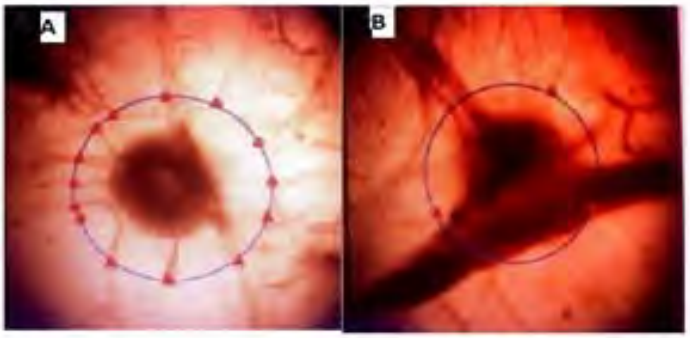

Fig- 1 : Chick embryo chorioallantoic membrane model treated with various fractions from *Echinochloa colona* at 40 mg/disk. Chloroform (A) and untreated control (B).

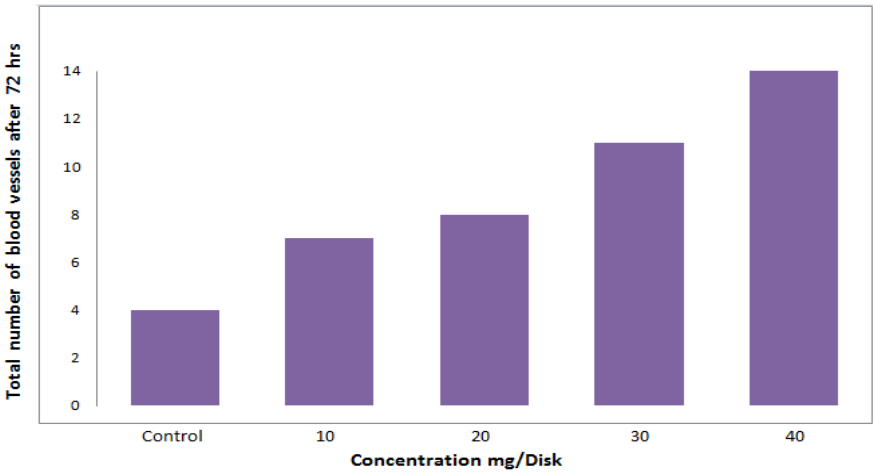

Fig 2 : Chick embryo chorioallantoin models treated with different concentration of Chloroform fraction from *Echinochloa colona*.

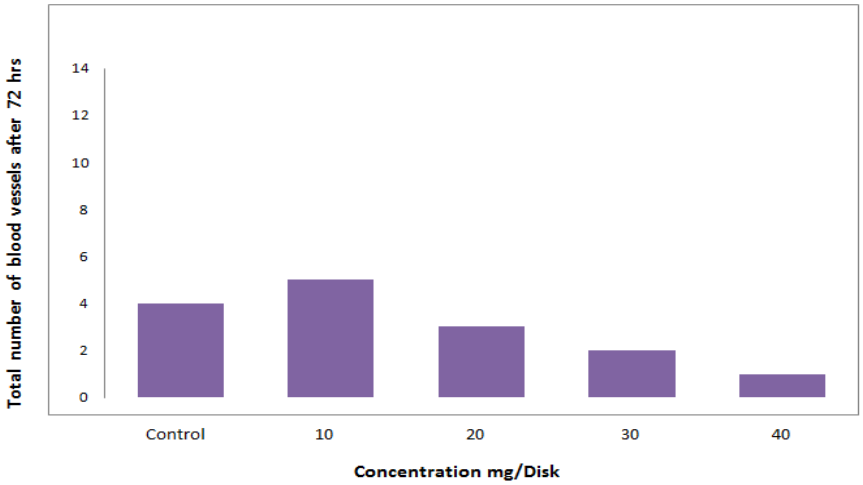

Fig 3 : Chick embryo chorioallantoin models treated with different concentration of Ethyl acetate fraction from *Echinochloa colona*.

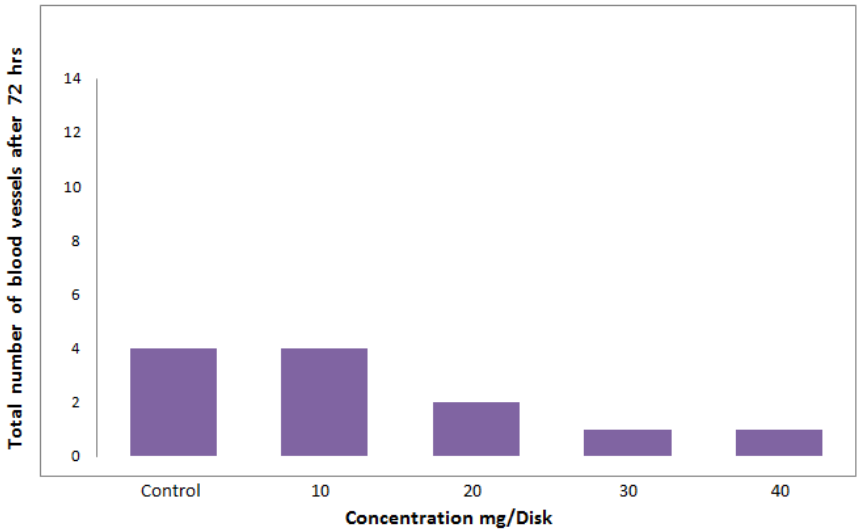

Fig 4 : Chick embryo chorioallantoin models treated with different concentration of Ethanol recedue from *Echinochloa colona*.