

Pharmacological Investigation of *Enicostema axillare* (Lam.) A. Raynal Extracts for Wound Healing Activity

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Abstract

Present research work was aimed to study preliminary wound healing activity of different extracts of aerial parts of *Enicostema axillare* using incision and excision wound models in experimental animals. Aerial parts of *Enicostema axillare* was extracted successively with petroleum ether, chloroform, acetone, ethyl acetate, methanol and aqueous solvents and obtained respective extracts. Each extract was tested qualitatively for detection of phytochemicals present in extracts using various chemical tests. Pharmacological screening of all extracts were performed using incision and excision wound models in experimental animals. Wound healing effect was observed by measurement of tensile strength of wound tissue of animals from incision model. Wound contraction measurement and biochemical estimation of wound tissue was performed in excision wound model. Results of present study was confirmed that methanol extract of *Enicostema axillare* was showed significant ($P < 0.05$) increase in tensile strength of wound tissue on 9th day. Higher percentage of wound contraction was observed with methanol extract between day 8-16th and complete healing was observed on 18th day. This fast healing also supported by significant ($P < 0.05$) increase in hydroxyproline and protein content of wound tissue in excision model. Effect was compared with the marketed formulation (Povidone-Iodine ointment). In conclusion, healing effect of methanol extract of *Enicostema axillare* may be contributed by phenolic compounds, flavonoids and glycosides present in the extract.

Key words: Wound healing, *Enicostema axillare*, hydroxyproline, Povidone-Iodine ointment, flavonoids.

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INTRODUCTION

Wound healing is a complex physiological process that requires a series of steps, each with several factors to come to completion. The sequential phases of healing process are inflammation, proliferation and migration of connective tissue cells, production of extracellular matrix including collagen synthesis, epithelial cells migration and proliferation leading to neovascularization of wounded tissue (Tortora and Grabowski, 1993).

Current efforts in wound healing research are directed toward developing new methods for promoting wound closure to be used alongside the traditional approaches of debridement and infection control. It should be borne in mind that the new product should be easy to manufacture and clinically efficacious, and regulatory bodies should permit the product to be marketed with reimbursement from medical insurance (Shukla *et al.*, 2005). With the evolution of wound care technologies, a wide range of materials have been developed for therapeutic intervention. Wound dressings are primarily passive materials designed to protect the

wound, maintain moisture, and prevent infection. In contrast, scaffolds are bioactive constructs that support cell adhesion, proliferation, and tissue regeneration, often mimicking the extracellular matrix. Clarifying these roles is crucial for understanding how various biomaterials contribute differently to wound healing outcomes (Bogadi *et al.*, 2025).

Enicostema axillare (Lam.) A. Raynal (Family: Gentianaceae) is commonly known as the Indian whitehead, has a long history of medicinal use in India and some parts of Africa, including South Africa. The plant is used in folk medicine to treat diabetes mellitus, rheumatism, abdominal ulcers, hernia, swelling, itching and insect poisoning, anti-inflammatory, hypoglycaemic and anticancer activities have been reported (Leelaprakash and Mohandass, 2010). Qualitative phytochemical analysis the presence of various phytochemicals like alkaloids, glycosides, tannins, carbohydrates, proteins and amino acids, saponins and flavonoids (Leelaprakash and Mohandass, 2012).

On the basis of previous research and traditional claim, the present study was aimed to evaluate preliminary wound healing effect of different extracts of *Enicostema axillare* (Lam.) A. Raynal (aerial parts) using incision and excision wound models.

MATERIAL AND METHODS

Collection and authentication of plant materials

Aerial parts of *Enicostema axillare* was collected from around the RKDF University campus and identified in botany department, Saifia Science College, Bhopal (M.P.). The plant materials were dried in shade, powdered moderately and stored for further processing.

Extraction and phytochemical screening of different extracts

The dried powdered aerial parts of *Enicostema axillare* were extracted in 266 Soxhlet extractor using different solvents up to complete extraction with each solvent. The powdered crude drug (500 g) of aerial parts of *Enicostema axillare* successively extracted in a 266 Soxhlet apparatus with petroleum ether (60-80°C), chloroform, ethyl acetate and finally with water by maceration process. The completion of extract was confirmed by evaporating a few drops of the extract on the watch glass and ensuring that no residue remained after evaporating the solvent. The marc was dried in a hot air oven below 40°C before being extracted with the next solvent. Last traces of solvent were removed under vacuum on evaporator. The extract obtained with each solvent was weighed to a constant weight and percentage w/w was calculated (Paeck and Tracey, 1955; Hoover, 1970). Different extracts were subjected to various qualitative analysis to detect the presence of chemical constituents such as alkaloids, glycosides, carbohydrates, saponins, tannins and phenolic compounds, flavonoids, proteins and amino acids (Vikram *et al.*, 2024; Kokate *et al.*, 2002).

Preliminary pharmacological screening of different extracts for wound healing activity

Preliminary pharmacological screening was performed to study the effect of different extracts of *Enicostema axillare* (aerial parts) on incision and excision wounds in experimental animals.

The petroleum ether, chloroform, acetone, ethyl acetate, methanol and aqueous extracts of *Enicostema axillare* (aerial parts) were applied topically twice daily. Each extract was suspended with 0.5% Carboxymethyl cellulose and applied topically.

Animal protocol

For the incision and excision wound model, Wistar albino rats (150-200 g) were selected. The rats were used after acclimatization to the laboratory environment for about 7 day's period prior to experiment. Six animals were taken in each group for study.

Group I: Control group received only 0.5% CMC twice daily topically

Group II: Received petroleum ether extract of *Enicostema axillare* (PEEA) twice daily (0.5 g) topically

Group III: Received chloroform extract of *Enicostema axillare* (CEEA) twice daily (0.5 g) topically

Group IV: Received acetone extract of *Enicostema axillare* (AEEA) twice daily (0.5 g) topically

Group V: Received ethyl acetate extract of *Enicostema axillare* (EAEA) twice daily (0.5 g) topically

Group VI: Received methanol extract of *Enicostema axillare* (MEEA) twice daily (0.5 g) topically

Group VII: Received water (aqueous) extract of *Enicostema axillare* (WEEA) twice daily (0.5g) topically

Group VIII: Standard group received Povidone-Iodine ointment twice daily

Incision wound model

All animals were anaesthetized before wound creation and a 1.5 cm long incision was made through the skin at dorsal portion of rat skin. No local or systemic antimicrobials were used throughout the experiment. The both edges of wound kept together and stitched with black silk surgical thread (No. 000) and a curved needle (No. 22) was used for stitching. Both wound edges were tightened for good closure of the wound and after stitching, wound was left undressed. All extracts and standard drug were applied daily up to 9 days; when wounds were healed thoroughly the sutures were removed on the 9th day and tensile strength of cured wound skin was measured using Tensiometer (Lodhi and Vadnere, 2017; Hemalata *et al.*, 2001).

Tensile strength measurement

Tensile strength is the resistance to breaking under tension. The newly repaired tissue including scar was used to measure the tensile strength. The instrument used for measurement is called 'Tensiometer' (Rashed *et al.*, 2003). Before testing, the animals were anaesthetized with ether in an open mask. One day before measurement of tensile strength, the sutures were removed from the stitched wounds of rats after recovery. The animal was then placed on a stack of paper towels on the middle of the board. The amount of the towels could be adjusted so that the wound was on the same level of the tips of the posts. The clamps were then carefully clamped on the skin of opposite sides of the wound at a distance of 0.5 cm. away from the wound. The longer piece of fishing line was placed on the pulley and the position of the board was adjusted so that the polyethylene bottle was freely hanging in the air, water was added to the polyethylene bottle at a rapid but constant rate by siphon from a large reservoir (20 L bottle) until the wound began to open up. The amount of water in the polyethylene bottle was weighed and considered to as the tensile strength of the wound. The tensile strength increment indicates better wound healing stimulation by the applied medicines (Kuвано *et al.*, 1994).

Table 1: Effect of *Enicostema axillare* (aerial parts), extracts on tensile strength of different animal groups in incision wound model

Group No.	Animal Groups	Tensile strength (g/cm ²)
Group I	Control (0.5% CMC)	484.34±14.75
Group II	PEEA	512.75±15.45
Group III	CEEA	585.16±17.44
Group IV	AEEA	588.74±17.67
Group V	EAEA	571.28±16.51
Group VI	MEEA	884.75±22.18*
Group VII	WEEA	502.86±16.48
Group VIII	Standard (Povidone-Iodine ointment)	824.10±25.55*

Where, PEEA: petroleum ether extract of *Enicostema axillare*; CEEA: chloroform extract of *Enicostema axillare*; AEEA: acetone extract of *Enicostema axillare*; EAEA: ethyl acetate extract of *Enicostema axillare*; MEEA: methanol extract of *Enicostema axillare*; WEEA: water (aqueous) extract of *Enicostema axillare*; n = 6 albino rats per group, value represents Mean ± S.D. *P< 0.05, was considered significant when compared each treated group with control group

Excision wound model

All animals in each group were anaesthetized by the open mask method with anesthetic ether before wound creation. One excision wound was inflicted by cutting away a 500 mm² full thickness of skin from a predetermined area. The wound was left undressed to the open environment (Rajoo *et al.*, 2021; Phulmogare *et al.*, 2024). The Standard group received marketed formulation Poviz (Povidone-Iodine Ointment USP; zenith Drugs Pvt Ltd, India). In this model wound contraction and wound closure time was monitored. Wound contraction was measured as percent contraction in each two days after wound formation. Small skin samples were collected and biochemical estimation (hydroxyproline estimation and protein estimation) was performed.

Wound contraction and epithelization time measurement

The contraction of individual wound of control and treated animals were periodically measured using transparent graph sheet and rate of healing calculated and expressed as percentage contraction. Wound contraction was measured in each two days interval (Lodhi *et al.*, 2016).

The following formula was used to calculate percentage of wound contraction:

$$\text{Percent wound contraction} = \frac{\text{healed area}}{\text{total area}} \times 100$$

Table 2: Effect of *Enicostema axillare* (aerial parts), extracts on wound contraction area of different animal groups in excision wound model in rats

Animal groups	Post wounding days (Percent wound contraction)										Epithelialization period
	2	4	6	8	10	12	14	16	18	20	
Control (0.5% CMC)	5.34±0.05	8.51±0.17	11.62±0.13	17.40±0.28	22.39±1.06	38.16±1.42	47.25±1.48	54.06±2.13	61.81±2.16	68.27±3.66	26
PEEA	7.26±0.07	12.08±0.67	18.30±0.77	25.04±1.35	32.22±1.34	40.01±1.88	49.71±1.93	56.11±2.07	63.52±2.37	71.32±3.15	24
CEEA	7.61±0.24	13.13±0.82	21.74±1.01	29.55±1.30	37.05±1.91	42.31±1.78	51.28±1.83	60.42±2.17	66.43±2.51	72.55±3.85	25
AEEA	8.46±0.31	13.88±0.43	20.39±0.88	28.43±1.28	36.11±1.67	44.19±1.64	50.75±1.27	57.16±1.93	64.47±2.66	70.64±3.42	24

Animal groups	Post wounding days (Percent wound contraction)										Epithelialization period
	2	4	6	8	10	12	14	16	18	20	
EAEA	8.87±0.08	14.12±0.27	21.62±0.62	30.68±1.42	38.55±1.81	45.28±2.34	51.27±2.60	61.21±2.81	65.20±2.76	77.29±3.27	24
MEEA	12.07±0.61	24.17±1.06	36.42±1.35	45.08±2.05	56.25±2.10	67.75±2.77	78.23±3.41	87.24±3.15*	100.0*	100.0	18
WEEA	10.71±0.82	18.10±0.94	26.41±1.72	32.08±1.33	39.42±1.59	43.22±1.69	52.42±2.30	62.75±2.31	73.28±3.11	81.29±3.22	22
Standard (Povidone-Iodine ointment)	10.67±0.64	22.62±1.05	31.85±1.88	42.16±1.88	55.28±2.07	63.42±2.08	71.08±3.05	80.79±3.05*	88.34±3.65*	100.0	20

Where, PEEA: petroleum ether extract of *Enicostema axillare*; CEEA: chloroform extract of *Enicostema axillare*; AEEA: acetone extract of *Enicostema axillare*; EAEA: ethyl acetate extract of *Enicostema axillare*; MEEA: methanol extract of *Enicostema axillare*; WEEA: water (aqueous) extract of *Enicostema axillare*; n = 6 albino rats per group, value represents Mean ± S.D. *P< 0.05, was considered significant when compared each treated group with control group

Hydroxyproline measurement

The method of Woessner (1961) was used for the quantitative determination of hydroxyproline in tissue material containing as little as one part of hydroxyproline in 4000 parts of amino acids. This method has been applied to study of hydroxyproline distribution in cell particulates, tissue fluid and purified plant and animal proteins. Following reagents were used for estimation of hydroxyproline in the tissues (Lodhi *et al.*, 2011; Woessner, 1961).

Small samples of tissues protein were hydrolyzed without preliminary purification by adding HCL to a final concentration of 6N. The samples were sealed in small pyrex test tubes and hydrolyzed for 3 hrs at 130°C. The tubes were opened and the contents are decanted into a graduate cylinder or volumetric flask. Several drops of 0.02% methyl red indicator was added, followed by the theoretical amount of 2.5N NaOH required for neutralization. A final adjustment was made with dilute HCL and NaOH until the indicator turns slightly yellow corresponding to pH 6-7. The samples were prepared as above and 2.0-ml portions containing hydroxyproline was placed in test tubes. Hydroxyproline oxidation was initiated by adding 1 ml Chloramine T to

each tube in a predetermined sequence. The tube contents are mixed by shaking a few times and allowed to stand for 20 min at room temperature. The Chloramine T was then destroyed by adding 1ml of Perchloric acid to each tube in the same order as before. The contents are mixed and followed allowed to stands for 5 min. Finally, 1 ml of p-dimethylaminobenzaldehyde solution was added and the mixture was shaken until no schlieren can be seen. The tubes were placed in 60 °C water bath for 20 min then cooled in tap water for 5 min. The developed color was stable for at least one hr. The absorbance of the solution was determined using UV visible spectrophotometer (Shimadzu, Japan) at 557 nm. The hydroxyproline content was determined directly from the standard curve.

Protein estimation

The measurement of protein with copper and the Folin reagent was used. The tissue lysate was treated with a mixture of sodium tartrate, copper sulphate and sodium carbonate. This was left to stand for 10 minutes and then treated with Folin-Ciocalteu reagent that resulted in a bluish color in 20-30 minutes. The absorbance was measured in UV (Shimadzu, Japan) Spectrophotometer at 650 nm (Lowry *et al.*, 1951).

Table 3: Effect of *Enicostema axillare* (aerial parts) extracts on hydroxyproline and protein content of tissues of different animal groups in excision wound model

Animal Groups	Hydroxyproline content (mg/g tissues)	Protein content (mg/g tissues)
Control (0.5% CMC)	28.10±0.73	41.36±1.03
PEEA	35.27±1.05	43.18±1.56
CEEA	38.61±1.18	45.20±1.81
AEEA	41.13±1.57	48.05±1.27
EAEA	42.61±1.88	47.13±1.69
MEEA	71.55±2.75*	81.24±3.07*
WEEA	46.28±1.30	50.17±2.05
Standard (Povidone-Iodine ointment)	70.28±2.68*	76.24±3.12*

Where, PEEA: petroleum ether extract of *Enicostema axillare*; CEEA: chloroform extract of *Enicostema axillare*; AEEA: acetone extract of *Enicostema axillare*; EAEA: ethyl acetate extract of *Enicostema axillare*; MEEA: methanol extract of *Enicostema axillare*; WEEA: water (aqueous) extract of *Enicostema axillare*; n = 6 albino rats per group, value represents Mean ± S.D. *P< 0.05, was considered significant when compared each treated group with control group

Statistical Analysis

All data were expressed as mean ± SD. The significance of differences between treated groups was determined using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. P-values < 0.05 were considered significant. Statistical Package for Social Scientist (SPSS) Version 22.0 software was used for all statistical analysis.

RESULTS AND DISCUSSION

Preliminary screening of different extracts of *Enicostema axillare* (aerial parts) were carried out on incision and excision wound models. The tensile strength of wound tissues was measured in incision method, while excision method was used to study the effect of fractions on wound area, wound closure time, biochemical parameters in healed skin tissues.

Effect of different extracts of *Enicostema axillare* on incision wound

The tensile strength indicates how much the repaired tissue resists to breaking under tension and may indicate in part the quality of repaired tissue. The results of the measurement of tensile strength on day 9th were shown in table 1. Treatment with *Enicostema axillare* extracts, the significant (p<0.05) increase in tensile strength was observed with the MEEA (884.75±22.18) and Standard (Povidone-Iodine ointment) (824.10±25.55) treated animals, while control group of animals showed 484.34±14.75 g/cm². While no significant increase in tensile strength was observed in groups treated with PEEA, CEEA, AEEA, EAEA and WEEA.

Effect of different extracts of *Enicostema axillare* on excision wound

The wound contraction percentage was determined at every two days interval of post wounding days. The wound margins were traced and measured to calculate the non-healed area which was then subtracted from the original wound area to obtain the healed area. Wound contraction on different days is shown in table 2. The wound contraction percentage was determined from

the first time on second day after application of homogenized each extract and marketed formulation as Standard (Povidone-Iodine ointment). This was carryout at two days intervals for duration of three weeks. With the treatment of *Enicostema axillare* extracts, MEEA and Standard treated group of animals showed significant increase in percentage wound contraction from day 2 to 14 and healed completely on 18th day. Standard group was showed complete healing at 20 days. Other extracts PEEA, CEEA, AEEA, EAEA and WEEA treated animals showed lowest percentage of wound contraction as compare to other treated group. On day 18 no scars were observed in animal treated with MEEA and standard group, which was an indication for complete healing.

The breakdown of collagen liberates free hydroxyproline and its peptide. Measurement of this hydroxyproline therefore has been used as an index of collagen turnover. The hydroxyproline content was determined on day 18 in small tissue specimen collected from each group of animals. The hydroxyproline level was found significant increases in group treated with MEMM, MEEA and standard group of treatment when compared with the control group of animals.

In case of *Enicostema axillare* extracts, the hydroxyproline content of MEEA and standard group treated were found 71.55±2.75 and 70.28±2.68 respectively which were significantly higher than the control group (Table 3). Other extracts PEEA, CEEA, AEEA, EAEA and WEEA does not showed significant increase in hydroxyproline level.

The protein content of wound tissues indicates the level of protein synthesis and cellular proliferation. In case of *Enicostema axillare* extracts, MEEA (81.24±3.07) and standard group (76.24±3.12) treated wound tissues were observed significant (p<0.05) increase in protein content in comparison to the control group (41.36±1.03) of animals (Table 3). The higher protein content of treated animals suggest that MEEA and standard treated group through an unknown

mechanism, stimulate cellular proliferation. Other extracts of *Enicostema axillare* PEEA, CEEA, AEEA, EAEA and WEEA not showed significant increase in protein content of the wound tissues.

The positive healing effect of *Enicostema axillare* may be due to presence of various chemical constituents already reported by various researches. *Enicostema axillare* contains 8% swertiamarin, a secoiridoid glycoside as the major bioactive component (Tana *et al.*, 2010; Saravanan *et al.*, 2014). The number of flavonoids has been identified in *Enicostema axillare* including genkwanin, swertisin, apigenin, myricetin, isovitexin and saponarin (Adetunji *et al.*, 2023). Some flavonols e.g. catechin, galangin, quercetin and kaemferol are also found in the plant (Retnam and Britto, 2003; Adetunji *et al.*, 2023).

CONCLUSION

Results of the present study was confirmed that methanol extracts of *Enicostema axillare* (aerial parts) was showed significant increase in tensile strength of wound tissue. Significant increase in hydroxyproline and protein content of healed tissue was the consideration of fast healing by the methanol extract of *Enicostema axillare*. These results may be related to the flavonoids present in the methanol extract. Further detail study is required to explore the mechanism of healing effect and study of individual phytoconstituent responsible for wound healing effect.

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