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In vivo study wound healing potential (incision) of herbal formulation

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ABSTRACT

Lygodium flexuosum is a climbing fern it is the sole genus in the family Lygodiaceae, though it is included in the family Schizaceae by some botanist. It occurs on mangrove and had tree dominated habitat sub division is petridophyte and had life form of cryptophytes category. It is commonly epiphytically grows on moss covered tree trunks, branches a lithophytes on shady boulders along with moss and in Thailand its habitat is in abundance. The present study has demonstrated that an ethanol extract of *Lygodium flexuosum* leaves extract has properties that render it capable of promoting accelerated wound healing activity compared with the controls. Wound contraction increased tensile strength, increased hydroxyproline content. It is used in external applications for rheumatism, sprains, scabies, eczema and cut wounds, they are reported to be particularly useful for carbuncles, reduce inflammation and acts as panacea for wounds, treat ulcer, various respiratory diseases, general disorders, muscles sprains. In pharmacological studies Wound healing activity was done and it was observed that topically administered drugs are effective in faster wound contraction due to the larger availability at the wound site. A significant increase in wound contraction was seen in both doses of ELF compared to control. Hence it appears that ELF has prohealing effect.

Key Words: Wound healing, *Lygodium flexuosum*, Lygodiaceae.

MATERIALS AND METHODS

Collection and Authentication of Plant Material

The plant of *Lygodium flexuosum* was collected in October 2010 from Hathinala region (M.P) near Maharashtra border and authenticated by Tariq Hussain, Scientist-in-charge, Raw material Herbarium and Museum, NBRI Lucknow and a voucher specimen was also deposited (specimen number: 97871) for further references. The leaves were separated, washed under

running tap water; air dried under shade, coarsely powdered and kept in airtight container until further use.

Microscopical Characters

It showed the presence of covering uniseriate multicellular, blunt tip non glandular type of trichomes, vascular bundles, palisade cells and anomocytic stomata. It consist of a single layer of epidermis, which consists of small tangentially elongated rectangular cells with brownish, thick-outer walls with a thin layer of cuticle. Collenchyma are Spongy cells that are spaciously arranged and irregularly shaped and it is lacunar type. Collateral type Vascular bundles in which Xylem and phloem aligned along a radius.

Trichomes are non glandular, Calcium oxalate crystals are found scattered and in small groups, they are irregular prismatic and show considerable variation in size,vessels are annular and thickened, starch grains are ovoid and fairly small; they also show a rounded or cleft shaped helium and it also showed the presence of pollen grains.

Powder Microscopy

The well-dried homogenous, free of dirt and foreign matter drug samples of leaves were subjected to grinding. The powder so obtained was sieved through No. 180 (W.H.O, 1992)]. The powders were kept in air tight glass containers for further studies. Each time, a small quantity of the powder was taken on the slide and after treatment with respective dehydrating, fixing and staining agents, was observed under the microscope.

In powder microscopy it was found that vessels are annular and thickened, trichomes are non glandular multicellular it also shows the presence of pollen grains, calcium oxalate crystals and starch grains which are irregular ovoid, the fragments of cork, which are present to a greater extent; the cells are polygonal in surface view and have thin, lignified walls; the outer layers are filled with granular contents.

Procedure

To supplement the characterization of powdered drug, particularly in the light of chromatographic separation and identification, the powders were treated with different acids. In most of the cases these were observed definite colour variations under the ordinary and ultraviolet light (365nm and 254nm). Observations are therefore recorded as given in Table no 2.

Table 2: Fluorescence Analysis of the leaves Powder of *Lygodium flexuosum*.

TREATMENT	LIGHT	U.V 254	U.V 366
Powder as such	Green	Dark green	Black
Powder + Conc H_2SO_4	Yellowish green	Brown	Black
Powder + dil.HCL	Yellow	Bluish Brown	Blue
Powder + Ammonia	Brownish Green	Dark Green	Black
Powder + Lead acetate	Dark Green	Bluish Black	Blue
Powder + Cobalt chloride	Pink	Brown	Purple
Powder + Ferric Chloride	Yellow	Light Green	Black
Powder + Copper sulfate	Green	Purple	Light Blue
Powder + Iodine	Yellowish Orange	Brown	Black
Powder +Ammonium Thiocynate	Green	Greenish Black	Dark Green
Powder + Nitric acid	Orange	Blue	Blue
Powder + Sodium Hydroxide	Dark Green	Green	Brownish Black
Powder+Ammonium Molybadate	Green	Purple	Bluish Black
Powder +Magnesium Sulfate	Green	Dark Green	Purple
Powder+ Water	Green	Blue	Dark Green

Successive solvent extraction

The coarse powder of leaves of "*Lygodium flexuosum*" drug was successively extracted separately beginning with non-polar and gradually proceeding to polar solvents using soxhlet apparatus except in aqueous extraction. All the extracts were dried in a vacuum evaporator and weighed to constant weight.

Extraction

Flow chart 1: Successive extraction

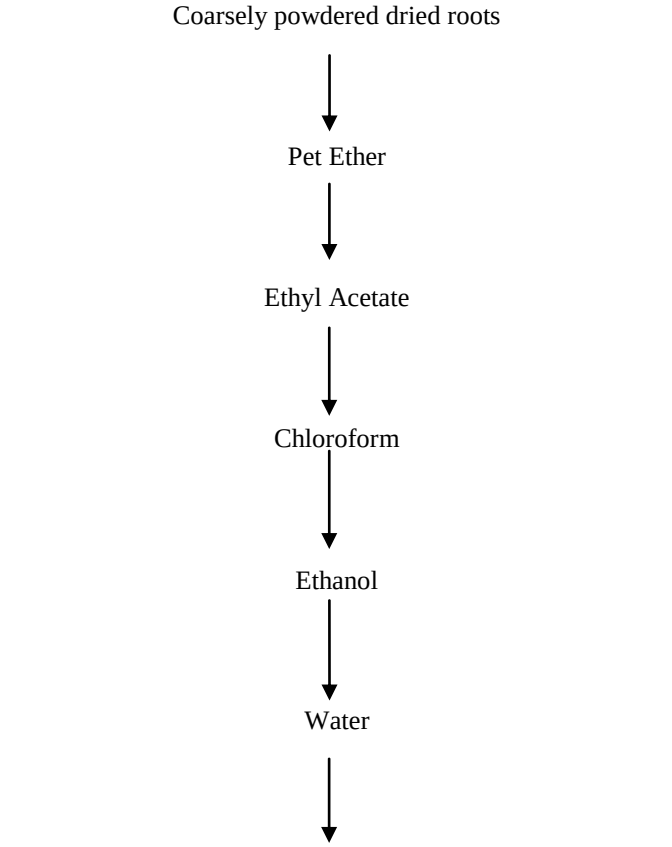

Table 3 Preliminary Phytochemical Screening of Different Extracts of *Lygodium flexuosum* (Organic elements)

S.no	Tests	Pet. Ether	Chloroform	Ethyl acetate	Alcohol	Water
1	Alkaloids	–	–	–	+	–
2	Glycoside	–	–	–	+	–
3	Carbohydrates	–	–	+	+	–
4	Fixed oils and fats	+	+	–	–	–
5	Saponins	–	–	–	+	+
6	Phenolic comp. and tannins	–	–	–	–	–
7	Proteins and amino acids	–	–	+	–	–
8	Flavanoids	–	–	–	+	–
9	Steroid	–	–	+	+	+

(-) Not present, (+) present

Table 4 Inorganic Constituents of Root Powder of *Lygodium flexuosum*.

Elements	Results
Calcium	_
Magnesium	_
Sodium	_
Potassium	_
Iron	_
Sulphate	_
Phosphate	+
Chloride	+
Carbonate	_
Nitrates	_

(-) Not present, (+) present

Formulation of the ointment

Three batches of the ointment were prepared and used in the study. Batches contain varying concentration of the extract 100mg extract, 200mg extract and the blank control ointment was prepared with neither the extract nor the standard drug. In preparation of hydrophilic ointment of *Lygodium flexuosum* stearyl alcohol and white petrolatum were melted together at about 75^0c. the other agents including extracts in different concentration were dissolved in purified water are added with stirring until the mixture congeals. Sodium laurly sulphats were act as emulsifying agents with stearyl alcohols and white petrolatum comprising the oleaginous phase of emulsion and the other ingredients aqueous aqueous phase. At last methyl and propyl paraben were added which is preservative.

Table 5: Preparation of medicated with ethanolic extracts of *Lygodium flexuosum*

S.no	Ingredients	Concentration (%w/w)
1	Extract	5
2	Sodium laurly sulphate	1
3	Propylene glycol	12.5
4	Stearyl alcohol	25
5	White petrolatum	25
6	Methyl paraben	1 drop
7	Purified water	31.5

Homogenecity

All the developed ointments were tested for homogensity by visual inspection. They were tested for their appearance with no lump.

Acute skin irritation

This test was performed on albino rats and weighing between 150-200g. the animals were given standard animal feed and had free access to water. The total mass was separated into four groups, each batch containing five animals. Dorsal hairs at the back of the rats were removed one day prior to the commencement of the study and kept individually in cages to avoid contact with the other rats. Two groups of each were used for control and standard irritant. Other two groups were used as test. The 50 mg of the each formulation of different concentration were applied over one square centimeter area of whole and abrades skin to different animals. Aqueous solution of 0.8% formalin was used as standard irritant. The animals

were observed for seven days for any signs of edema and erythema.

Rate of penetration

the rate of penetration of solid dosage form is crucial in the onset and duration of action of the drug. Weighed quantity of the penetration should be applied over the selected area of the skin for a definite period of time. Then the preparation was left over is collected and weighed. The difference between the initial and final weights of the preparation gives the amount of preparation penetrated through the skin and this when divided by the time period of application gives the rate of penetration of the preparation and it was found .02 mg. hence it gives the positive results

MATERIAL AND METHODS

Plant material and extract preparation

The leaves of *Lygodium flexuosum* were collected in October 2010 from Hathinala region (M.P) near Maharashtra border and authenticated by Dr. Tariq Hussain, Scientist-in-charge, Raw material Herbarium and Museum, NBRI Lucknow and a voucher specimen was also deposited (specimen number: 97871). Leaves of L. flexuosum were shade dried and powdered. An aliquot of powdered leaves (250 g) was submitted to successive solvent extractions with the help of soxhlet apparatus separately with petroleum ether, ethyl acetate, chloroform, ethyl alcohol and water. The extracts were evaporated to dryness in rota evaporator. Yields of each extracts were 6.8% for petroleum ether, 5.3% for chloroform, 5.3% for ethyl acetate, 24.0% for ethyl alcohol and 16.5% for water.

Animal

Wister albino rats weighing 180-220gm of either sex were procured from the animal house of University and kept in room at 27 ± 2^0C, and relative humidity 44-56%, light and dark cycles of 12 hrs each, during the experiments. Animals were provided with standard rodent diet and ad libitum. Animals were periodically weighed before and after the experiment. The rats were anaesthetized prior to and during infliction of the experimental wounds. The surgical interventions were carried out under sterile conditions using diethyl ether. Animals were closely observed for any infection and those which showed signs of infection were separated and excluded from the study and replaced. All the chemicals used were of the analytical grade from standard companies and the water used was always the double distilled water. A standard orogastric cannula was used for oral drug administration.

Preliminary phytochemical studies

All the extracts obtained were subjected to preliminary qualitative tests for various plant constituents by suitable chemical tests (Trease et al., 1987).

Acute toxicity study

ELF at the dose level 2000mg/kg body weight of the animals was used for acute toxiciy in accordance to Organization for Economic Cooperation Development (OECD) guideline 423. Three female rats, each sequentially dosed at intervals of 48 hrs, were used for the test. Once daily cage side observations included changes in skin, fur, eyes, mucous membrane (nasal), autonomic (salivation, lacrimation, perspiration, piloerection, urinary incontinence and defecation) and central nervous system (drowsiness, gait, tremors and convulsions) changes. Mortality, if any, was determined over a period of 2 weeks**.**

Selection of doses

For assessment of excision wound healing activity extracts were formulated in ointment by using simple ointment BP as base. 5% and 10% (w/w) ELF ointments were applied where 500mg and 1gm of ELF were incorporated separately; in 10gms of simple ointment base BP. 0.5 g of each of ELF ointment and 0.2% nitrofurazone ointment was applied once daily to treat different groups of animals, respectively.

For the assessment of wound healing activity by incision wound model and dead space wound model, three dose level were chosen in such a way that, middle dose was approximately one-tenth of the maximum dose during acute toxicity studies, and a low dose, which was 50% of the one-tenth dose, and a high dose, which was twice that of one-tenth dose (100, 200, and 400mg/kg).

In vivo wound healing activity

Excision, incision and dead space wound models were used to evaluate the wound-healing activity of ELF.

Incision wound model

As the above model rats were anaesthetized prior to and during creation of the wound. The dorsal fur of the animals was shaved with an electric clipper. A longitudinal paravertebral incision, six centimeters in length was made through the skin and cutaneous muscle was made through the skin and cutaneous muscle on the back as described by the Ehrlich and Hunt et.al, 1976. After the incision, surgical sutures were applied to the parted skin at intervals of one centimeter. The wound were left undressed. The drugs were given orally to different groups, control and treated with ELF (100, 200 and 400mg/kg) of the body weight of the rats orally and the standard groups rats were treated with 0.2% nitrofurazone ointment tropically. The sutures were removed on the 8th post wound day and the treatment was continued. The skin-breaking strength was measured on the 10thday.

Dead space wound model

The animals were divided into 5 groups containing 6 each. Group 1 served as the control. Group 2, 3 and Group 4 were treated orally with ELF (suspended in 1% w/v CMC) of 100 mg/kg, 200mg/kg, 400mg/kg respectively. Group5 animals were treated with 0.2% nitrofurazone ointment topically. Dead space wound model were inflicted by implanting two sterilized cotton pellets (10mg), one on either side of in the lumbar region on the ventral surface of each rat. On the 10th post wounding day, the granulation tissue formed on the implanted cotton pellet was carefully removed. The wet weight of the granulation tissue was noted. These granulation tissues were dried at 60^{0}C for 12 hours, and weighed, and the weight was recorded. To the dried tissues added 5ml 6N HCl and kept at 110^{0}C for 24hours. The neutralized acid hydrolysate of the dry tissue was used for the determination of hydroxyproline (Neuman et al., 1950). Hydroxyproline present in the acid hydrolysate of granulation oxidized by sodium peroxide in the presence of copper sulfate, when complexed with para-dimethylaminobenzaldehyde, develops a pink color that was measured at 540nm.

Determination of wound breaking strength

The anesthetized animals were secured to the table, and a line was drawn on either side of the wound 3mm away from the line. This line was gripped using forceps one at each end opposed to each other. One of the forceps was supported firmly, whereas the other was connected to a freely suspended light weight metal plate. Weight was added slowly and the gradual increases in weight, pulling apart the wound edges. As the wound just opened up, addition of weight was stopped and the weights added was noted as a measure of breaking strength in grams (Shivhare et al., 2010).

RESULTS

Preliminary phytochemical studies

Qualitative phytochemical analysis of ELF revealed the presence of flavonoids, tannins, glycosides and alkaloids as major active constituents which were confirmed by suitable chemical tests.

Acute toxicity study

In the present study, single dose oral administration of ELF in female rats at 2000mg/kg had no effect on mortality, clinical signs, body weight change or gross observation. Therefore, no acute toxicity was found in rats treated with ELF and the approximate lethal doses for rats were determined to be higher than 2000mg/kg.

Table 1: Wound healing effect in Excision wound model

Parameters	**Wound Area (mm^2) on**		
	Day 1	**Day 5**	**Day 10**
ointment	371.16	350.41	197.16
4% ELF ointment	367.16	234.16	77.33
5% ELF ointment	340.33	145.33	18.33
Standard	307.33	114.00	12.50

Incision wound model

Table 7.2 compares the tensile strength of the healing skin treated with control, different dosage of EFL for 10 days.

The rats of control group had the minimum strength (460gm). The tensile strength of the tissue of treated rats

with 200mg/kg and 400mg/kg ELF was more or less similar but substantially higher in treated than control rats. The tensile strength of wound treated with standard nitrofurazone ointment was highest (564gm), but difference between this and those treated with ELF dosage were not statistically significant. It may be inferred that, 0.2% (w/w) nitrofurazone, 200mg/kg and 400mg/kg ELF exert more or less same results on the tensile strength of the healing tissue. This observation confirms that the crude extract of ELF possesses excellent wound healing property so far as tensile strength of wound healing tissue is concerned.

DISCUSSION AND CONCLUSION

Discussion

Lygodium flexuosum is a climbing fern it is the sole genus in the family Lygodiaceae, though it is included in the family Schizaceae by some botanist. It occurs on mangrove and had tree dominated habitat sub division is petridophyte and had life form of cryptophytes category. It is commonly epiphytically grows on moss covered tree trunks, branches a lithophytes on shady boulders along with moss and in Thailand its habitat is in abundance. It is used in external applications for rheumatism, sprains, scabies, eczema and cut wounds, they are reported to be particularly useful for carbuncles, reduce inflammation and acts as panacea for wounds, treat ulcer, various respiratory diseases, general disorders, muscles sprains. In India it is found in Dehradun, Kumaon, Shahjanpur, Gorakhpur, throughout the plains in Bengal up to 5000 feet, both the sides of Madras state up to 4000 feet and Kerela it is also found in Madhya Pradesh in regions like Annupur, Bastar, Betul, Bilaspur, Chhindwara, Damoh, Gwalior, Hoshangabad, Indore, Khandwa, Mandla, Raigarh, Raipur, Sidhi, Shivpuri and Panna. Lygodium is the climbing fern it is the genus of about 40 species native to tropical region across the world with a few temperate species in eastern Asia and eastern North America. Four species are recorded in India which are *L.circinatum, L.flexuosum, L.japonicum, L.microphyllum*. Traditionally *Lygodium flexuosum* was used as a medicine in many ways that I have studied in the literature survey for treating various diseases, so this plant has been selected for studies.

In pharmacological studies Wound healing activity was done and it was observed that topically administered drugs are effective in faster wound contraction due to the larger availability at the wound site. A significant increase in wound contraction was seen in both doses of ELF compared to control. Hence it appears that ELF has prohealing effect as evidenced by the above findings.

In incision wound, the increase in tensile strength of treated wounds may be due to the increase in both remodeling of collagen, and the formation of stable intra- and intermolecular crosslinks.

The collagen molecules synthesized are laid down at the wound site and become crosslinked to form fibres. Since incision wound treated with 4, 5% (w/w) SP showed greater tensile strength, it may be speculated that it not only increases collagen synthesis per cell, but also aids in cross-linking of the protein. The present study has demonstrated that an ethanol extract of *Lygodium flexuosum* leaves extract has properties that render it capable of promoting accelerated wound healing activity compared with the controls. Wound contraction increased tensile strength, increased hydroxyproline content.

Wound contraction is the process of mobilizing healthy skin surrounding the wound to cover the denuded area and involves complex and superbly orchesterated interactions of cells, extracellular matrix and cytokines. This centripetal movement of wound margin is believed to be due to the activity of myofibroblast. Since ELF enhanced wound contraction, it would have either enhanced contractile property of myofibroblasts or increased the number of myofibroblasts recruited into the wound area. Granulation, collagen maturation and scar formation are some of the many phases of wound healing which run concurrently, but independent of each other. Topically administered drugs are effective in faster wound contraction due to the larger availability at the wound site. A significant increase in wound contraction was seen in both doses of ELF compared to control. Hence it appears that ELF has prohealing effect as evidenced by the above findings. In incision wound, the increase in tensile strength of treated wounds may be due to the increase in both remodeling of collagen, and the formation of stable intra- and intermolecular cross links. The collagen molecules synthesized are laid down at the wound site and become crosslinked to form fibres. Since incision wound treated with 4, 5% (w/w) SP showed greater tensile strength, it may be speculated that it not only increases collagen synthesis per cell, but also aids in cross-linking of the protein.

The granulation tissue of the wound is primarily composed of edema, fibroblast, collagen and new blood

vessels. The mesenchymal cells of the wound area adjust themselves into fibroblast then begin migrating into the wound gap together with the fibrin strands. The collagen is the main constituent of extra cellular tissue, which is responsible for support and strength. Free hydroxyproline and its peptides are released with collapse of collagen. Thus, measurement of the hydroxyproline could be used as an indicator for collagen turnover. Furthermore, increase in dry tissue also indicates the presence of elevated protein content .

CONCLUSION

The present study has demonstrated that an ethanol extract of *Lygodium flexuosum* leaves extract has properties that render it capable of promoting accelerated wound healing activity compared with the controls. Wound contraction increased tensile strength, increased hydroxyproline content.

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