SCREENING OF ALCOHOLIC EXTRACT OF *MUSSAENDA FRONDOSA* LEAF FOR WOUND-HEALING AND ANTIBACTERIAL ACTIVITIES IN ALBINO RATS

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Summary

Mussaenda frondosa L has been used to treat a wide assortment of diseases. The objective of our study was to evaluate the wound-healing and antibacterial activities of leaves extracts of *Mussaenda frondosa* in albino rats. Wound healing activity was determined in rats, after administration (200mg $kg^{-1}day^{-1}$) of the alcoholic extract of *Mussaenda frondosa* leaves, using excision, incision and dead space wound models. The animals were divided into two groups of 6 each in all the models. In the excision model, group I animals were orally treated with carboxymethyl cellulose as placebo control, group II received oral administration of alcoholic extract of *Mussaenda frondosa* at a dose of 200mg/kg body weight/day. In an incision and dead space model group I animals were given 2% gum acacia orally as placebo control and group II received alcoholic extract of *Mussaenda frondosa* leaves at a dose of 200mg $kg^{-1}day^{-1}$.

Healing was assessed by the rate of wound contraction, period of epithelization, tensile strength (skin breaking strength), granulation tissue weight, and hydroxyproline content. Antibacterial activity of the leaves extracts against four microorganisms was also assessed. The extract of *Mussaenda frondosa* leaves significantly increased the wound breaking strength in the incision wound model compared with controls (P<0.001). The extract treated wounds were found to epithelialize faster, and the rates of wound concentration was significantly increased in comparison to control wounds (P<0.001), Wet and dry granulation tissue weights, and hydroxyproline content in a dead space model increased significantly (p<0.05). Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and Staphylococcus albus have shown sensitivity to *Mussaenda frondosa*. Increased wound concentration and tensile strength, augmented hydroxyproline content along with antibacterial activity support the use of *Mussaenda frondosa* in the topical management of wound healing.
Key words: Alcoholic extract, *Mussaenda frondosa,* Wound healing.

Introduction

The therapeutic efficacies of many indigenous plants, for various diseases have been described by traditional herbal medicine practitioners[1]. Natural products are a source of synthetic and traditional herbal medicine. They are still the primary health care system in some parts of the world[2]. The past decade has been seen considerable change in opinion regarding ethnopharmacological therapeutic applications. The presence of various life sustaining constituents in plants has urged scientists to examine these plants with a view to determine potential wound healing properties.

Wound healing is the process of repair that follows injury to the skin and other soft tissues. Following injury, an inflammatory response occurs and the cells below the dermis (the deepest skin) begin to increase collagen (connective tissue) production. Later, the epithelial tissue (the outer skin layer) is regenerated. There are three stages to

the process of wound healing: inflammation, proliferation and remodeling. The proliferative phase is characterized by angiogenesis, collagen deposition, epithelialisation and wound contraction. Angiogenesis involves new blood vessel growth from endothelial cells. In fibrioplasia and granulation tissue formation, fibroblasts exert collagen and fibronectin to form new, provisional extracellular matrix. Subsequently epithelial cells crawl across the wound bed to cover to and the wound is contracted by myofibroblasts, which grip the wound edges and undergo contraction using a mechanism similar to that in smooth muscle cells.

Mussaenda frondosa L (Rubiaceae) commonly known as Nagavalli and pink Mussaenda. It's found throughout India and has historically been used to treat a wide assortment of diseases. Plant is known by various names in different languages as Bedina in Hindi, Bhutkasi in Marathi, and Billothi in Kannada. Whole plant is used as Astringent, expectorant, anti-inflammatory, cardiotonic, cough bronchitis, fever, wound, jaundice. Traditionally *Mussaenda frondosa* Linn is reported to possess number of medicinal properties[3,4] Traditionally leaves are used in the treatment of jaundice, asthma, hyperacidity, fever, ulcers, leprosy, diuretic, *wound*, swells[5], antimicrobial[6], diuretic activity[7] , Hepatoprotective activity[8], fever and cough[9]. Hypolipidemic effect of Methanolic extract of *Mussaenda frondosa linn*. Leaves in high fat diet fed rats was investigated.[10]

Methods

Plant material and extract preparation

The leaves of *Mussaenda frondosa* L were collected from Jamboti forest in July 2009 and identified by the plant taxonomist and curator, Indian Council of Medicinal Research (ICMR), Belgaum, Karnataka, India. A voucher specimen was also deposited at the herbarium (specimen number: RMRC-484). The fresh leaves were shade dried and ground into powder using electric blender. The fine powder (100 g) was then passed through sieve number 40 and suspended in 200ml of ethanol for 20 hours at room temperature using continuous extraction (soxhlet apparatus). The mixture was filtered using fine muslin cloth followed by filter paper (Whatman

No: 1).The filtrate was placed in water bath to dry at 40^0C and the clear residue was used for the study. The extract was subjected to preliminary phytochemical tests.

Animals

The study was approved by the Institutional Ethics Committee for animal's experimentation (Resolution No.01 dated 21/12/2009), Maratha Mandal's College of Pharmacy, Belgaum.

Healthy young albino rats of either sex weighing 200-220 g were used for the study. They were individually housed and maintained on normal food and water *ad libitum*. Animals were periodically weighed before and after the experiment. The rats were anaesthetized prior to and during the infliction of the experimental wounds. The surgical interventions were carried out under sterile conditions using light ether anesthesia (120mg/kg body weight). Animals were closely observed for any infection were separated and excluded from the study and replaced.

An acute toxicity study was conducted for the extract by the stair-case method[11]. The animals were administered with increasing doses of (1, 2, 4 and 8 g/kg body weight).

Wound- healing activity

Excision, incision and dead space wound models were used to evaluate the wound –healing activity of *Mussaenda frondosa*.

Excision wound model

Animals were anaesthetized prior to and during creation of the wounds. The rats were inflicted with excision wounds as described by Morton and Malon[12]. The dorsal fur of the animals was shaved with an electric clipper and the anticipated area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of 2.5 cm (circular area=300mm^2) in length and 0.2

cm depth was created along the markings using toothed forceps, a surgical blade and pointed scissors. The entire wound was left open[13]. The animals were divided into two groups of 6 each. Group I animals were orally treated with carboxymethyl cellulose (200mg/kg/day) as a placebo control. Group II animals were orally treated with alcoholic extract of *Mussaenda frondosa* at a dose of 200mg/kg/day till complete epithelization. The wound closure was assessed by tracing the wound on days 1, 5 and 18 post wounding using transparency paper and a permanent marker. The wound areas were measured using a graph paper. Number of days required for falling of eschar without any residual raw wound gave the period of epithelization.

Incision wound model

As the above model rats were anesthetized 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 on the back as described by Ehrlich and Hunt et al.[14]. After the incisions, surgical sutures were applied to the parted skin at intervals of one centimeter. The wounds were left undressed. The rats were given the alcoholic leaves extract of *Mussaenda frondosa* orally at a dose of 200mg $kg^{-1}day^{-1}$. The controls were given 2% gum acacia. The sutures were removed on the 8^{th} post wound day and the treatment was continued. The skin breaking strength was measured on the 10^{th} day by the method described by Lee [15].

Dead space wound model

Dead space wounds were inflicted by implanting two sterile cotton pellets (10 mg), one on either side of in the lumbar region on the ventral surface of each rat. On the 10^{th} 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 60^0C for 12 hours, and weighed, and the weight was recorded. To the dried tissue added 5 ml of 6 N HCl and kept at 110^0C for 24 hours. The neutralized acid hydrolysate of the dry tissue was used for the determination of hydroxproline[16].

Determination of wound breaking strength

The anaesthetized animal was secured to the table, and a line was drawn on either side of the wound 3 mm 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 gradual increase in weight, pulling apart the wound edges. As the wound just opened up, addition of the weight was stopped and the weights added was noted as a measure of breaking strength in grams. Three readings were recorded for a given incision wound, and the procedure was repeated on the contralateral wound. The mean reading for the group was taken as an individual value of breaking strength. The mean value gives the breaking strength for a given group.

Estimation of Hydroxyproline

Hydroxyproline present in the acid hydrolysate of granulation tissue oxidized by sodium peroxide in the presence of copper sulphate, when complexed with para-dimethyl-aminobenzldehyde, develops a pink color that was measured at 540 nm using colorimetry.

Phytochemical screening methods [19]

Test for flavanoids: The presence of flavanoids was determined using 1% aluminum chloride solution in methanol, concentrated HCl, magnesium turnings and potassium hydroxide solution.

Test for saponins: Boiled 200 mg of extracts with 5 ml water for two minutes. Mixture was cooled and mixed vigorously and left it for three minutes. The formation frothing indicates the presence of saponins.

Test for Steroids: Extract was treated with few drops of acetic anhydride, boiled and cooled, concentrated sulphuric acid was added from the side of the test tube; showed brown ring at the junction of two layers and the upper layer turned green which showed the presence of sterols.

Test for Glycosides:

Test I:

Extract 200 mg of the drug by warming in a test tube with 5 ml of dilute (10%) sulphuric acid on a water bath at 100^{o}C for two minutes, centrifuge or filter, pipette out supernatant or filtrate. Neutralize the acid extract with 5% solution of Sodium hydroxide (noting the volume of NaoH added). Add 0.1 ml of Fehling's solution A and B until alkaline (test with pH paper) and heat on a water bath for 2 minutes. Note the quantity of red precipitate formed and compare with that formed in Test II.

Test II:

Extract 200 mg of the drug using 5 ml of water instead of sulphuric acid and boil on water bath. After boiling add equal volume of water to the volume of NaoH used in the above test. Add 0.1 ml of Fehling's A and B until alkaline (red litmus changes to blue) and heat on water bath for two minutes. Note the quantity of the red precipitate formed.

Compare the precipitates of Test II with Test I. If the precipitate in Test-II is greater than in Test-I, then Glycoside may be present. Since Test I represent the amount of free reducing sugar already present in the crude drug, whereas Test-II represents the Glycoside after acid hydrolysis.

The thin layer chromatography of the alcoholic extract was done by using the following mobile phase as solvent system.

Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:26)

Antibacterial activity

Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and *Staphylococcus albus* were the organisms tested. The bacterial strains were obtained from fresh colonies *Mussaenda frondosa*. The bacterial strains were obtained from the fresh colonies grown on broth media at 37^{0}C for 18 hours. The sensitivity testing was done using Agar diffusion method. Known volume of bacterial suspension

was transferred to each microplate well. Ten microliters of *Mussaenda frondosa* extracts dissolved in deionised water (200µg/ml) was added to the microplate wells and inoculated at 35-37^0 C for 18-20 hours. Results were analyzed visually on the basis of turbid zone of inhibition. [+ = bacterium colonies deposited in the bottom of the well, ++ = turbidity with bacterium colonies being deposited, +++ = light turbidity, and ++++ = total growth inhibition.

Statistical analysis

Results, expressed as mean ± SD were evaluated using Students t-test and significance was set at $p<0.05$.

Results

In acute toxicity studies, the rats of either sex were fed with increasing doses (1, 2, 4 and 8 g/Kg body weight) of alcohol extract for 16 days. The doses up to 4 g/Kg body weight did not produce any signs of toxicity and mortality. The animals were physically active and were consuming food and water in a regular way. We did not notice any abnormal behavior even with a dose of 8 g/Kg body weight.

The significant increase in the wound healing activity was observed in the animals treated with the *Mussaenda frondosa* extracts compared with those who received the placebo control treatments. Table 1 shows the effect of the alcoholic extract of *Mussaenda frondosa* leaves administered orally at a dose of 200mg $kg^{-1}day^{-1}$ for 10 days on wound healing activity in rats inflicted with incision wound. In the incision wound model, a significant increase in the wound breaking strength (293.92±1.58) was observed in alcoholic extract treated animals when compared to controls. The wound breaking strength (280.92 ±1.25). In the excision wound model, *Mussaenda frondosa* treated animals showed a significant reduction in the wound area ($p<0.01$) and epithelization period (Table 2). In the dead space model (Table 3), the alcoholic extract treated animals showed significantly increased levels of Hydroxyproline content ($p<0.05$) as compared with the control group of animals. A

significant increase was observed in the weight ($p<0.001$) of the granulation tissue in the animals treated with the extract.

The quantitative tests used to identify phytochemical constituents of the *Mussaenda frondosa* leaves extracts showed the presence of Flavanoids, Steroids, Saponins and Glycosides .The thin layer chromatography studies of alcoholic extract of *Mussaenda frondosa* exhibited 02 spots using Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:26) as mobile phase and Rf value was found to be 0.8.

Table 1: Wound healing effect of *Mussaenda frondosa* in Incision wound model

Group	Dose (Oral) mg/kg body weight	Wound Breaking strength(g)
Control	1ml of 2% Gum Acacia	144.60±0.63
Alcohol Extract	200	293.92±1.58**

N=6, Values are expressed as mean ± SD *p<0.05 and **p<0.01 vs. control (t-test)

Table 2: Wound healing effect of *Mussaenda frondosa* in Excision wound model

Group	Mean± SEM of % of wound closure on				Epithelization Time (Days)	Scar Area (mm^2)
	4^{th} Day	8^{th} Day	12^{th} Day	16^{th} Day		
Control	15.60 ±1.75	28.16 ± 0.12	42.65 ± 0.58	59.58 ± 1.14	30.12 ± 0.02	25.86 ± 0.02
Alcohol Extract	25.43 ±0.75**	75.17 ± 0.14**	86.17 ± 1.07**	97.95 ± 0.49***	17.80 ± 0.84**	9.06 ± 0.80***

N=6, Values are expressed as mean ± SD *p<0.05, **p<0.01, ***p<0.001 vs. control. (*t*-test).

Table 3: Wound healing effect of *Mussaenda frondosa* in Dead space wound model

Group	Dose (Oral) mg/kg body weight	Wound Breaking Strength (g)	Granuloma tissue dry weight (mg/100g)	Hydroxproline (µg/100mg)
Control	1ml of 2% Gum Acacia	164.60 ±3.89	32.06 ±0.70	1365.00 ±9.46
Alcohol Extract	200	320.66 ±1.45***	52.80 ±0.49***	2914.00 ±8.40***

N=6, Values are expressed as mean ± SD *p<0.05 and **p<0.01, ***p<0.001 vs. control. (*t*-test).

Table 4: Antibacterial activity of *Mussaenda frondosa* extracts

Bacteria	Turbidity pattern in Experimental Alcoholic Extract
Escherichia coli	+++
Pseudomonas aeruginosa	+++
Staphylococcus aureus	+++
Staphylococcus albus	+++

+ = Bacterium colonies deposited in the bottom of the well.
++ = Turbidity with bacterium colonies being deposited.
+++ = Light turbidity.
++++ = Total growth inhibition.

Discussion

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its natural state. Wound contracture is a process that occurs throughout the healing process, commencing in the fibroblastic stage whereby the area of wound undergoes shrinkage.

It has three phases; inflammatory, proliferative and maturational and is dependent upon the type and extent of damage, the general state of the host's health and the ability of the tissue to repair. The inflammatory phase is characterized by hemostasis and inflammation, followed by epithelization, angiogenesis and collagen deposition in the proliferative phase. In the maturational phase, the final phase of wound healing undergoes contraction resulting in a smaller amount of apparent scar tissue.

Granulation tissue formed in the final part of the proliferative phase is primarily composed of fibroblasts, collagen, edema, and small blood vessels. The increase in dry granulation tissue weight in the test treated animals suggests high protein content. The alcoholic extract of *Mussaenda frondosa* demonstrated a significant increase in the hydroxyproline content of granulation tissue indicating increased collagen turnover. Collagen, the major component which strengthens and supports extra cellular tissue is composed of the amino acid, hydroxyproline, which has been used as a biochemical marker for tissue collagen.[18]

The preliminary phytochemical analysis of the leaves extract of *Mussaenda frondosa* showed the presence of Flavonoids, Saponins, Glycosides and steroids. Any of the observed phytochemical constituent present in the *Mussaenda frondosa* may be responsible for the wound healing activity. Recent studies have shown that phytochemical constituents like Flavonoids [19] are known to promote the wound healing process mainly due to their astringent and antimicrobial properties, which appear to be responsible for wound contraction and increased rate of epithelization.

The wound healing property of *Mussaenda frondosa* may be attributed to the phytoconstituents present in the plant, and the quicker process of wound healing could be a function of either the individual or the additive effects of the phytoconstituents.

Conclusion

The present study has demonstrated that the alcoholic extract of *Mussaenda frondosa* leaves has properties that render it capable of promoting accelerated wound healing activity compared to placebo controls. Wound contraction, increased tensile strength, increased hydroxyproline content and antibacterial activity support further evaluation of *Mussaenda frondosa* for the management of wounds.

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