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Original Article

PRELIMINARY PHARMACOLOGICAL SCREENING OF *MICROCOCCA MERCURIALI* (L.) BENTH. LEAVES EXTRACTS FOR WOUND HEALING ACTIVITY

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ABSTRACT

Objective: Wound healing is a complex physiological process that requires a series of steps, each with several factors to come to completion. The objective of present study was to screen different extracts of *Micrococca mercurialis* (L.) Benth. leaves for wound healing activity using incision and excision wound model in experimental animals.

Methods: Different extracts of *Micrococca mercurialis* leaves were obtained by extraction with petroleum ether, chloroform, acetone, ethyl acetate, methanol and water. All these extracts were tested for detection of phytochemical constituents using different chemical test qualitatively. Preliminary wound healing activity was tested using incision and excision wound models and observed tensile strength of wound tissue in incision wound model. Wound contraction and biochemical estimation from wound tissue were observed in excision wound model.

Results: Results of present study was confirmed that methanol extract of *Micrococca mercurialis* leaves was showed higher tensile strength significantly ($P<0.05$) in comparison to the control group of animals. Hydroxyproline content and protein content were found significant ($P<0.05$) greater than the other extracts and standard marketed formulation (Povidone-Iodine ointment).

Conclusion: In conclusion, the potent wound healing effect of *Micrococca mercurialis* leaves extract may be attributed by the presence of phenolic and flavonoids components in the extract. Further detail study is required to explore the mechanism behind the healing effect and specific component responsible for activity.

Keywords: Wound healing, *Micrococca mercurialis,* Hydroxyproline, Povidone-iodine ointment, Flavonoids

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INTRODUCTION

Wound is a break in the skin especially in epidermis. Cuts or scalps usually cause wounds; symptoms at wound or injury include swelling, stiffness, tenderness, discoloration skin tightness, scabbing, itching and scar formation. Chronic wounds affect a large number of patients and seriously reduce their quality of life. Current estimates indicate that nearly 6 million people suffer from chronic wounds worldwide [1]. In the past, not much attention was devoted to chronic wounds, as the federal governments, the private sector, and the medical community was slow to focus on this growing health care problem. But with the rising elderly population and the increasing incidence of diabetes, the care of these wounds is fast becoming a billion-dollar business. Treatments that exist today, however, are often expensive and often not reimbursed through medical insurance. 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 [2].

Micrococca mercurialis (L.) Benth (Family: Euphorbiaceae) leaves is used in the treatment of various disorders like arthritic pain, constipation, antioxidant, liver disorder, skin diseases etc. This plant also has been used by tribal peoples for the management of arthritis [3]. Methanol extract of *Micrococca mercurialis* leaves has been reported to possess alkaloids, flavonoids, reducing sugars, gums, tannins and saponins. Leaf extract has potent antibacterial against different human pathogenic bacteria like *Bacillus subtilis, Escherichia coli* and *Enterococcus faecalis* [4]. *Micrococca mercurialis* has been documented in traditional healing practices, especially to cure sores, rheumatic pain, and constipation. Chemical analysis and biological assay of medicinal plants are the important factors for identification of novel bioactive phytochemical and drug discovery [5]. Objective of present study was to study for wound healing activity of *Micrococca mercurialis* leaves extracts using incision and excision wound models in experimental animals.

MATERIALS AND METHODS

Collection and authentication of plant materials

The plant materials were identified in botany department, Saifia Science College, Bhopal (M. P.). The leaves of *Micrococca mercurialis* were collected and authenticated in Department of Botany, Saifia College of Science Bhopal. The plant materials were dried in shade, powdered moderately and subjected to extraction using different solvents.

Extraction and phytochemical screening of different extracts

The dried powdered leaves of *Micrococca mercurialis* were extracted in soxhlet extractor using different solvents up to complete extraction with each solvent successively. The powdered crude drugs (500 g) of each were successively extracted in a soxhlet apparatus with petroleum ether (60-80°C), chloroform, acetone, 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. After ethyl acetate extraction the marc obtained was dried and macerated with water for 48 h. The liquid extracts were collected in a tared conical flask. The solvent removed by distillation. Last traces of solvent were removed under vaccum on evaporator. The extract obtained with each solvent was weighed to a constant weight and percentage was calculated.

Qualitative analysis of different extracts

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. The dried extracts of each crude drug were subjected for following chemical test for different chemical constituents [6].

Preparation and administration of test samples

The petroleum ether, chloroform, ethyl acetate, methanolic and aqueous extracts of *Micrococca mercurialis* leaves were suspended with 0.5% Carboxymethyl cellulose and applied topically twice daily.

Preliminary pharmacological screening of different extracts for wound healing activity

Preliminary pharmacological screening was performed to study the effect of different extracts of *Micrococca mercurialis* leaves on incision and excision wounds.

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 *Micrococca mercurialis* leaves (PEMM) twice daily (0.5 g) topically

Group III: Received chloroform extract of *Micrococca mercurialis* leaves (CEMM) twice daily (0.5 g) topically

Group IV: Received acetone extract of *Micrococca mercurialis* leaves (AEMM) twice daily (0.5 g) topically

Group V: Received ethyl acetate extract of *Micrococca mercurialis* leaves (EEMM) twice daily (0.5 g) topically

Group VI: Received methanol extract of *Micrococca mercurialis* leaves (MEMM) twice daily (0.5 g) topically

Group VII: Received water (aqueous) extract of *Micrococca mercurialis* leaves (WEMM) 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 d; when wounds were healed thoroughly, the sutures were removed on the 9th d and tensile strength of cured wound skin was measured using Tensiometer [7].

Tensile strength measurement

Tensile strength is the resistance to breaking under tension. It indicates how much the repaired tissue resists to breaking under tension and may indicate in part the repaired tissue. The newly repaired tissue, including scar was used to measure the tensile strength using Tensiometer [8]. 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 tensile strength increment indicates better wound healing stimulation by the applied medicines [9].

Table 1: Effect of *Micrococca mercurialis* leaves extracts on tensile strength of different animal groups in incision wound model

Animal groups	Tensile strength (g/cm^2)
Group I: Control (0.5% CMC)	456.46±14.38
Group II: PEMM	535.24±15.54
Group III: CEMM	562.27±18.88
Group IV: AEMM	529.55±17.24
Group V: EEMM	543.21±16.25
Group VI: MEMM	748.36±24.33*
Group VII: WEMM	582.30±17.52
Group VIII: Standard (Povidone-Iodine ointment)	720.27±23.76*

Where, PEMM: petroleum ether extract of *Micrococca mercurialis*; CEMM: chloroform extract of *Micrococca mercurialis;* AEMM: acetone extract of *Micrococca mercurialis;* EEMM: ethyl acetate extract of *Micrococca mercurialis;* MEMM: methanol extract of *Micrococca mercurialis,* WEMM: Water (aqueous) extract of *Micrococca mercurialis*; n = 6 albino rats per group, value represents mean±SD. 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 mm^2 full-thickness layer of skin from a predetermined area. The wound was left undressed to the open environment [10]. 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.

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

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

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 [11]. The samples were sealed in small pyrex text tubes and hydrolyzed for 3 h at 130^{oc}. 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 neutralization by 2.5N NaOH. 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 1 ml 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 a 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 mixture was left to stand for 10 min and then treated with Folin-Ciocalteau reagent that resulted in a bluish color in 20-30 min. The absorbance was measured in UV (Shimadzu, Japan) Spectrophotometer at 650 nm [12].

Table 2: Effect of *Micrococca mercurialis* leaves 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)	6.24±0.07	11.92±0.20	14.52±0.28	21.77±1.76	30.61±1.75	38.27±1.38	46.05±1.63	55.12±2.10	64.37±2.76	76.24±3.13	25
PEMM	8.65±0.08	14.08±0.31	18.64±0.26	28.27±1.63	36.31±1.45	47.67±1.48	58.17±2.03	67.94±2.55	72.32±3.21	80.62±3.42	22
CEMM	7.52±0.06	15.46±0.24	17.61±0.34	29.76±1.85	34.20±1.64	46.28±1.62	55.60±2.10	65.31±2.77	71.99±3.64	77.37±3.62	23
AEMM	9.58±0.09	18.42±0.32	20.37±1.05	28.61±1.62	35.22±1.70	44.96±1.90	58.27±2.13	68.10±3.10	75.91±3.28	83.21±3.77	21
EEMM	10.21±0.17	17.26±0.75	21.36±0.84	30.28±1.17	37.20±1.62	46.29±2.04	61.81±2.84	66.72±3.15	77.16±3.64	85.10±4.15	22
MEMM	11.54±0.13	21.75±0.25	28.09±1.60	36.74±1.47	48.25±1.64	62.38±2.07	78.61±2.88	92.48±3.88	100.0	100.0	18
WEMM	9.56±0.10	18.72±0.16	22.34±1.25	29.66±1.86	38.17±1.72	47.62±1.67	56.25±2.31	66.27±2.65	78.38±3.65	84.20±3.49	21
Standard (Povidone-Iodine ointment)	10.27±0.15	17.64±0.34	27.86±1.62	38.61±1.67	45.28±1.99	59.63±2.03	75.44±2.44	82.62±3.82	93.27±4.20	100.0	19

Where, PEMM: petroleum ether extract of *Micrococca mercurialis*; CEMM: chloroform extract of *Micrococca mercurialis;* AEMM: acetone extract of *Micrococca mercurialis;* EEMM: ethyl acetate extract of *Micrococca mercurialis;* MEMM: methanol extract of *Micrococca mercurialis,* WEMM: Water (aqueous) extract of *Micrococca mercurialis*; n = 6 albino rats per group, value represents mean±SD. P<0.05, was considered significant when compared each treated group with control group

Table 3: Effect of *Micrococca mercurialis* leaves 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)	34.86±1.53	45.63±2.10
PEMM	42.33±2.75	55.28±2.88
CEMM	41.02±1.58	56.61±3.01
AEMM	38.21±1.94	52.74±3.12
EEMM	42.18±2.05	57.20±2.57
MEMM	88.67±3.44*	95.45±4.10*
WEMM	51.75±2.75	50.41±2.57
Standard (Povidone-Iodine ointment)	81.36±3.51*	87.24±3.66*

Where, PEMM: petroleum ether extract of *Micrococca mercurialis*; CEMM: chloroform extract of *Micrococca mercurialis;* AEMM: acetone extract of *Micrococca mercurialis;* EEMM: ethyl acetate extract of *Micrococca mercurialis;* MEMM: methanol extract of *Micrococca mercurialis,* WEMM: Water (aqueous) extract of *Micrococca mercurialis*; n = 6 albino rats per group, value represents mean±SD. 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 Dunnets' post hoc test. P<0.05 were considered significant. Statistical Package for Social Scientist (SPSS) Version 22.0 software was used for all statistical analysis.

RESULTS AND DISCUSSION

Phytochemical screening of different extracts of *Micrococca mercurialis* leaves was confirmed the presence of glycosides, terpenoids, phenols, flavonoids, tannins and steroids. Preliminary screening of different extracts of *Micrococca mercurialis* leaves were carried out on incision and excision wound models in experimental animals. The tensile strength of wound tissues was measured in incision method, while excision method was used to study the effect of extracts on wound area, wound closure time and biochemical parameters in healed skin.

Effect of different extracts of *Micrococca mercurialis* leaves 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. The tensile strength of the animals treated with MEMM (748.36±24.33) and Standard (720.27±23.76) was significantly greater than that of control group of animals (456.46±14.38). While no significant increase in tensile strength was observed in groups treated with PEMM, CEMM, AEMM, EEMM and WEMM.

Effect of different extracts of *Micrococca mercurialis* leaves 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. Thetreatment with *Micrococca mercurialis* extracts, on days 4 to 10, MEMM and Standard (Povidone-Iodine ointment) treated animals groups exhibited significant increase in the percent wound contractions as compared to control group. The other extracts PEMM, CEMM, AEMM, EEMM and WEMM treated animals showed non-significant increase in the percentage of wound contraction from days 2 to 18. On days 18, the wound of MEMM and standard group of treatment were found 100% and 93.27±4.20 percentage of wound contraction. That means MEMM treated wound was healed completely and standard group of animals wound was healed in 20 d of treatment. The other extracts PEMM, CEMM, AEMM, EEMM and WEMM treated groups of animal were not found complete healing up to 20 d (table 2). On day 18 no scars were observed in animal treated with MEMM and reference ointment, 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 (88.67±3.44) and standard group (81.36±3.51) of treatment when compared with the control group of animals. The hydroxyproline content of PEMM, CEMM, AEMM, EEMM and WEMM treated group does not showed significant increase in comparison to the control group of animals. The hydroxyproline content in MEMM and standard treated group of animals were found 88.67±3.44 and 81.36±3.51 respectively which were significantly higher than the control group 34.86±1.53 (table 3).

The protein content of wound tissues indicates the level of protein synthesis and cellular proliferation. By the treatment with the *Micrococca mercurialis* extracts, the protein content of wound tissue treated by MEMM and standard group were recorded significantly higher than the control group, that confirm the cellular proliferation greater than the control group. Other extract PEMM, CEMM, AEMM, EEMM and WEMM treated group of animal's wound tissues does not showed significant greater than the control group.

Previous study reported that ethanol extracts of whole plant of *Micrococca mercurialis* has been showed potent antioxidant, free radical scavenging and antibacterial potential [13]. Antioxidant and antibacterial activities of *Micrococca mercurialis* indirectly involved to promote wound healing in our previous reports [13, 14]. In present study, wound healing effect was evidenced by a significant increase in hydroxyproline and protein content which was a reflection of increased collagen levels. This indicated improved collagen maturation by increased cross-linking through increased tensile strength after treatment with ethanol extract.

Collagen plays a role in haemostasis and in providing strength and integrity to the wound matrix. It is also essential for re-epithelialization and cell–cell and cell–matrix interactions [15]. As the wound heals, collagen molecules are synthesized and deposited at the wound site. These molecules become cross-linked to form fibers. The strength of the repaired wound tissue is a result of remodeling of collagen and formation of stable intra and inter-molecular cross linking. These results may imply that flavonoids, as well as flavonoids containing extracts [16] are able to increase collagen synthesis and possibly aid in formation of cross linkages as the collagen matures. The protein content of granulation tissue is said to be an indication of protein synthesis and cell proliferation levels. If the protein contents of treated wounds are greater than the control group, it implies that the treatment stimulates cell proliferation.

CONCLUSION

In conclusion, results of present study suggested that ethanol extract of *Micrococca mercurialis* showed good wound healing activity. This may be due to presence of phenolic and flavonoids components in the ethanol extract. The healing effect may be related to its antioxidant and antibacterial activities reported by other researchers. The detail study of ethanol extract of *Micrococca mercurialis* needed to explore the mechanism and specific constituent involved behind the potent healing effect of extract.

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AUTHORS CONTRIBUTIONS

Ms. Renu Singh was performed experimental work and make draft of manuscript. Dr. Santram Lodhi formatted and edited language of the manuscript and approve for final submission.

CONFLICT OF INTERESTS

Declared none

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