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# PRECLINICAL EVALUATION OF *MARSILEA QUADRIFOLIA* LEAVES FOR ITS WOUND HEALING ACTIVITY

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#### ABSTRACT

Marsilea quadrifolia is widely used for the treatment of varieties of skin diseases in the Ayurvedic medicine system, as well as it is also used as folk medicine in wound management. But still there was no scientific data available on wound healing activity of Marsilea quadrifolia. So, we had attempted to investigate this activity. The purpose of this study was to screen the wound healing properties of a hydro-alcoholic extract of Marsilea quadrifolia leaves. In this research study, the hydroalcoholic extract of Marsilea quadrifolia leaves was obtained by percolation method. Two doses of Marsilea *quadrifolia* (2.5% and 5%) suitable gel formulation was prepared with the help of HPMC as a gel base to apply topically. Evaluation of the wound healing activity was done by "Excision, and Incision wound" models, also we performed skin irritation study. In the excision wound model, rats treated with Marsilea quadrifolia leaves extract showed a significant improvement in the epithelization time and 50% wound contraction. As well as, the breaking strength of rats treated with Marsilea quadrifolia leaves extract increased significantly in an incision wound model as compared to the control group. Marsilea quadrifolia leaves extract gel formulations shown likely similar activities as compared to synthetic standard/marketed povidone-iodine as well as herbal standard Aloe vera gel formulation. Marsilea quadrifolia does not produce any irritation in the skin So, it can be concluded that Marsilea quadrifolia leaves extract (2.5 % and 5% gel formulation) has potent wound healing activity.

## Keywords: *Marsilea quadrifolia*, Phytochemicals, Wound healing, Skin irritation study, Excision wound model, Incision wound model

#### **INTRODUCTION:**

Wound healing is the process of repairing the skin and other soft tissues after an injury. An inflammatory response happens after an injury, and the cells below the injury become inflamed. The dermis (the deepest layer of the skin) begins to produce more collagen, synthesis of connective tissue. The epithelial tissue (outer skin) will then be regenerates [1]. The process of wound healing is separated into four different overlapping stages: hemostasis, inflammation or defensive, proliferation, and maturation [2, 3]. Herbal medicine in these days has opened a broad way in medical science due to their phytochemical constituents, therapeutic activities and cheaper price. It is estimated that about 1-10% of plants present on earth are utilized as food by both the humans as well as wildlife and a large number of them are being used as medicinal agents in humans and wildlife [4]. One of the herbs that is widely used in herbal medicine is common sushni (Marsilea quadrifolia), from the family Marsileaceae, native to the Eastern and southern Asia. Sushni is mainly produced in South India, Japan, North America, China, and Central and Southern Europe. Some studies have shown the presence of several phytochemical compounds in the leaves such as marsilin (1triacontanol-cerotate), hentriacotan-6-ol, methylamine, 3-hydroxy-triacontan-11-one, flavonol-O-mono diglycoside, C-&

Glucoflavones, Beta-sitosterol, marsileagenin A, and C-glucosyl xanthones. It contains various phytoconstituents such as alkaloids, tannins, flavonoids, saponins, carbohydrates, proteins steroids, terpenoids, and phenolic compounds **[5, 6]**.

As an edible herb Marsilea quadrifolia shows different activities like Antioxidant, Hypoglycemic, Anticonvulsant, Antibacterial, Antifungal, Anti - Stress, Analgesic, Anti-Psychopharmacological inflammatory, activity. In the folklore medical practice in different countries, Marsilea quadrifolia and its leaves and petioles are used in the treatment of hypertension, sleep difficulties, headaches coughs as well as leg and and migraine. muscular convulsions, Throughout verities pharmacological and phytochemical studies, the Marsilea quadrifolia shows anti-microbial, antioxidant activity, analgesic and antiinflammatory activity as well as anti-stress activity [7]. It has been already established that plant showing anti-microbial, antioxidant activity, analgesic and antiinflammatory activity, exerts possible healing activity towards wound [8]. In this regard, Leaves of Marsilea quadrifolia has been selected to find out its wound healing activity in preclinical evaluation.

**MATERIALS AND METHODS:** 

Collection and preparation of leaves extract: [9] The leaves of Marsilea quadrifolia were collected from the local area of Chakdaha, West Bengal, India. Fresh tender leaves were collected, washed with clean and fresh water for removing the dust particles followed by shade drying. Dried leaves were subjected for extraction. The extraction process of Marsilea quadrifolia leaves were done with 70% ethanol by using cold percolation technique for consecutive 48 hours. The extract has been collected and kept for evaporation under vacuum evaporator to get the constant weight. After that the Marsilea quadrifolia leaves extract was collected and stored under refrigeration at 4°C.

## **Experimental Animals:**

Albino wistar male rats weighing around 200-250 gm and Rabbit weighing around 1.5kg were used for the study. The animals were purchased from M/S Saha Enterprise (authorised animal breeder). They had provided with commercial food pallets and tap water ad libitum. The animals were kept hygienically in separate polypropylene cage all experiments were conducted and between 10 am to 6 pm. The rats and rabbits were housed in CPCSEA approved N.S.C.B.I.P (Netaji Subhas Chandra Bose Institute of Pharmacy) animal house (approval no:1502/PO/a/11/CPCSEA) under standard laboratory conditions with 12-hour light/12-hour dark cycles and a room temperature of 22±2°C.

### **Drugs and chemicals:**

Ethanol (Dr. S.C. Deb's Dilution), Distilled water, Povidone iodine ointment (Betadine 10% Ointment, Win-Medicare Pvt Ltd), Xylazine (Indian Immunologicals), Ketamine (Ciron Drugs & Pharmaceuticals Pvt Ltd), Hydroxypropyl methylcellulose (Loba Chemie Pvt Ltd), Methylene blue, *Aloe vera* gel (Himalaya).

## Preliminary phytochemical investigation of the extract: [9]

The Marsilea quadrifolia leaves hydroalcoholic extract was subjected to phytochemical analysis of various phytoconstituents Alkaloids, such as Carbohydrates, Flavonoids, Glycosides, Saponins, Steroids, Phenolic acids, and Proteins.

Gel preparation and Dose selection: [10] Wound-healing properties of different herbal drugs have been studied in a variety of topical carriers, including gels, ointments, and creams. There is a bias in the vehicle selection towards gel. This is preferable due to the gel's aqueous composition, which should mix better and faster with the wound's aqueous exudates. In addition, the phytochemical extract employed in the various experiments contains aqueous, hydroalcoholic, and organic components. As a result, the lipophilic element of the extract would be difficult to separate from lipophilic ointments or creams, making the gel the logical choice. With the better drug flow and minimal retention expected from a polymeric gel-based vehicle. Therefore, we selected hydroxypropyl methylcellulose (HPMC) as a gel base.

A topical HPMC gel formulation has been prepared. For topical usage in an excision, incision wound model, *Marsilea quadrifolia* (2.5%) in HPMC (7.5%) is used as a low dose while *Marsilea quadrifolia* (5%) in HPMC (7.5%) is used as a high dose. For incision wound models, the treatment duration was 10 days, while for excision wound models, the treatment period was till the scar fell off the wound.

#### **Skin Irritation Study: [11]**

The rabbit's skin was shaved in two distinct dorsal positions, each about 500 mm<sup>2</sup>. The rabbit was housed in a rabbit holder, and the first area served as a control for the vehicle application. The second area was treated with *Marsilea quadrifolia* gel (2.5%). After 4 hours, the skin was examined and a score was selected depending on the comparison to the control.

#### Wound Healing Models:

Wound healing activity was evaluated through two models viz. excision wound model as well as incision wound model.

#### **Excision Wound Model:** [12-17]

Male wistar male albino rats weighing 200-250 grams was taken. The animals were split into five groups, each with six animals. Prior to the formation of the wounds and during the operation, animals were anaesthetized by using xylazine(13mg/kg) along with Ketamine (87mg/kg ip.). Excision wounds were produced on the rats, The animals' fur in the lumber section in either side was cleaned with the help of an electric clipper, and a circular stainless-steel stencil (500mm<sup>2</sup>) was used to define the predicted area of the wound to be produced on the backs of the animals with the help of methylene blue. Using forceps (toothed), surgical scissors, a full thickness excision wound of 500 mm<sup>2</sup>, 0.2 cm depth was produced along the markings. The wound was left completely exposed.

Group I: Control group: animals of this group were not received any treatment, only7.5% HPMC gel was provided twice a day.

Group II: Standard group: animals of this group were received povidone-iodine ointment USP 10%w/w (Betadine Ointment 10%) twice a day.

Group III: Herbal standard group: animals of this group were received Aloe vera gel, twice a day.

Group IV: Test group: animals of this group were received *Marsilea quadrifolia* 2.5% in 7.5% HPMC gel till complete epithelialization, twice a day.

Group V: Test group: animals of this group were received *Marsilea quadrifolia* 5% in 7.5% HPMC gel till complete epithelialization, twice a day. The contraction of wound was determined by tracing the wound with cellophane paper and a permanent marker at days 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22 after wounding. On a millimeter scale graph paper, the wound regions were noted and quantified. Given the duration of epithelialization, the required number of days for eschar to fall without any remaining raw wound was determined, and recorded.

Incision Wound Model: [14, 15, 18, 19, 20] Male wistar male albino rats weighing 200-250 grams were split into five groups in the same way as in the excision wound model. Rats were anaesthetized before and during the wound formation, much like in the previous model. The animals' dorsal fur was shaved with using an electric clipper. As per to Ehrlich and Hunt, a 6 cm long straight paravertebral incision was made on the skin and sub-cutaneous muscle on the back. The wound was closed with interrupted sutures put at equidistance spots about 1 cm apart after full haemostasis. Animals were given their proposed drug every day from the 0th to the 9th post-wounding day, on the 10th post-wounding day, the wound breaking strength test was conducted utilising a continuous, constant water flow approach. Allis forceps was firmly put on both flank of the incision wound on adjacent normal skin, 3 mm distant from the wound border. On one side, the forceps was connected to a fixed metal rod, while on the other, the forceps was tied to a thread passing over a pulley was attached with a light weight plastic container. The container kept suspending which was connected with a constant water flow technique (100 drops/ min). As soon as the wound began to gap, the addition of weights was halted, and the weights was lifted to prevent further tissue damage. The weights required to create gapping was recorded.

#### **Statistical Analysis:**

The results are presented as mean $\pm$ S.E.M. The differences between experimental groups were evaluated using one-way ANOVA (control vs. treatment), followed by Dunnett's test, and were accepted statistically significant when P<0.05.

#### **RESULTS AND DISCUSSION:**

**Preliminary phytochemical investigation:** The hydroalcoholic extract of *Marsilea quadrifolia* leaves were imperiled for phytochemical investigation and the following observations were found (**Table 1**).

#### Skin irritation test:

250 mg of hydroalcoholic extract of *Marsilea quadrifolia* was gel formulated by using HPMC and applied to the dorsal skin of the rabbit in the lumber section and observed for 4 hours, and found no irritation or colour changes in the open skin. From the above findings it can be concluded that *Marsilea quadrifolia* does not produce any irritation in the skin or it can be stated as

nonirritant /safe for topical application (Table 2, Figure 1).

## **Excision Wound Model:**

This project was designed to evaluate the wound healing effect of *Marsilea quadrifolia* leaves on the basis of its high popularity in different countries of Asia and Europe as a drug to heal many kinds of skin ailment including wounds. The leaves were extracted by using 70% ethanol and the yield was gel formulated by using 7.5% HPMC.

Two different wound model namely excision and incision wound were created on the predesigned 5 treatment groups. The first group was kept as untreated and received the HPMC gel omitting any active ingredients. Exceptionally in the present study, two groups were kept as standard I and standard II. Standard I gp animals were treated with Povidone Iodine, which is a broad-spectrum antimicrobials but here is a predicament to compare the herbal formulation with a synthetic standard. It drives this project to incorporate the second standard which is herbal and having proven wound healing properties. By keeping this logic in mind Aloe vera has been incorporated as a herbal standard (Standard II).

The hydroalcoholic extract of *Marsilea quadrifolia* was formulated as gel formulation by using HPMC to obtain two different formulations as 2.5% *Marsilea quadrifolia* in 7.5% HPMC and 5% Marsilea quadrifolia in 7.5% HPMC (Table

## **3, Figure 2)**.

Both the formulations of *Marsilea quadrifolia* has shown significant decrease in the period of epithelialization while compared with the untreated control. both the standard has once again proven its efficacy as per as the same parameter concern (Figure 3).

50% wound contraction is an important parameter which can help to determine quality of epithelialization, i.e. the rate of healing. Faster rate of healing can presume the faster proliferation of the damaged cells. In this investigation both standard groups as well as both *Marsilea quadrifolia* group has shown significant decrease in wound contraction 50% while treated with the untreated control (Figure 4).

## **Incision Wound Model:**

In the other hand *Marsilea quadrifolia* has shown significant wound healing effect in incision wound model. Two different doses of *Marsilea quadrifolia*, Aloe vera and povidone iodine all the groups has shown significant increase in breaking strength while compared with the untreated control, which directly indicate that the two formulations of *Marsilea quadrifolia* has promoted the collagenation comparable with the effect of povidone iodine and somehow better than herbal standard *Aloe vera* (**Table 4, Figure 5**).

Phytochemicals	Test Names	Results
Flavonoids	Lead Acetate Test	Positive
	Zinc Hydrochloric Acid Test	Positive
Proteins and Amino Acid Test	Biuret Test	Positive
	Millon's Test	Positive
Alkaloids	Mayer's Test	Positive
	Dragendroff's Test	Positive
Saponins	Froth Formation Test	Positive
	Haemolysis Test	Positive
Steroids	Liebermann-Burchard Test	Positive
	Salkowski Test	Positive
Tannins	Gelatin Test	Positive
	Lead Acetate Test	Positive
Phenol	Litmus Test	Positive
	Bromine Water Test	Positive
Carbohydrates	Barfoed's Test	Positive
Terpenoids	Salkowski Test	Positive

Table 1: Results of Preliminary	phytochemical investigation
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#### Table 2: Effect of M.Q. on skin irritation study

Group	Sign	Score
Control	_	
M.Q. 2.5%	No noticeable inflammation and redness	0

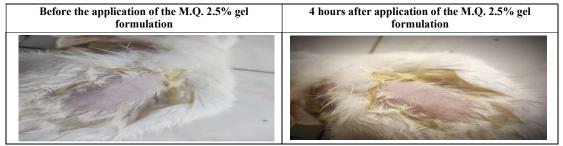
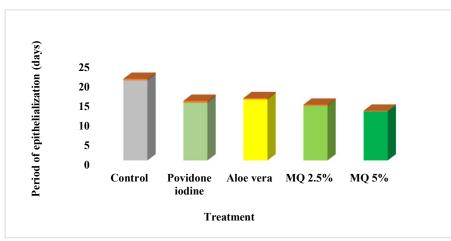


Figure 1: Image of skin irritation study

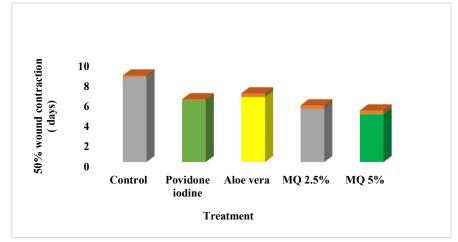
#### Table 3: Effect of M.Q. on period of epithelialization and 50% wound contraction in excision wound model

Treatment	Period of epithelialization (days)	50% Wound contraction (days)
Control	20.332±0.421	8.412±0.176
(7.5% HPMC gel)		
Aloe vera Gel	15.503±0.341***	6.452±0.364***
Povidone Iodine	14.661±0.494***	6.105±0.123***
M.Q (2.5%) in 7.5%	13.866±0.307***	5.258±0.372***
HPMC gel		
M.Q (5%) in 7.5%	12.434±0.213***	4.703±0.415***
HPMC gel		

All values are mean ±SEM, n=6, \*\*\* p<0.001 vs. control



All values are mean ±SEM, n=6 Figure 2: Effect of M.Q. on period of epithelialization



All values are mean ±SEM, n=6 Figure 3: Effect of M.Q. on 50%wound contraction in excision wound model

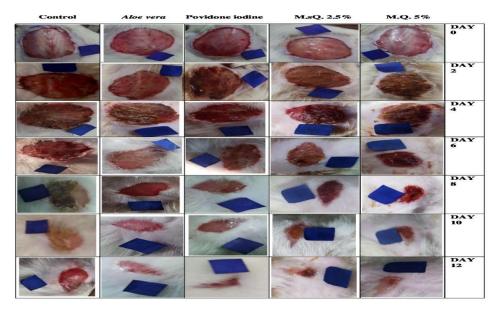


Figure 4: Image of healing in excision wound model

Treatment	Breaking strength
Control (7.5% HPMC gel)	277.33±4.81
Aloe vera Gel	393.54±5.65***
Povidone Iodine	426.78±6.95***
M.Q (2.5%) in 7.5% HPMC gel	478.52±8.71***
M.Q (5%) in 7.5% HPMC gel	667.5±5.52***

All values are mean ±SEM, n=6, \*\*\* p<0.001 vs. control

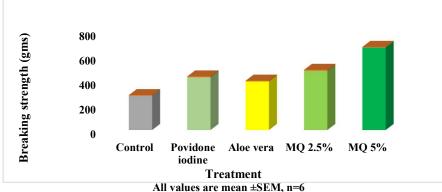


Figure 5: Effect of M.Q. on breaking strength in incision wound model

In the incision wound model the breaking strength of the collagen fiber is used to determine by the continuous water fall technique. The drug used to have antioxidant properties obvious to show the progression of collagen fiber by decreeing the superoxide load in the wound area.

#### **General outcome:**

Through chemical investigation we came to know *Marsilea quadrifolia* has steroids, alkaloids, tannin and protein and carbohydrate as chemical constituents. Some of these chemical constituents also may responsible for the healing activities of *Marsilea quadrifolia*.

Steroids exerts anti-inflammatory activity by inhibiting the action of phospholipase A2, phospholipase A2 critical the production of inflammatory compounds [21]. Alkaloids contain nitrogen atom in its structure generally exert anti-inflammatory, antimicrobial and antioxidant activity **[22]**. Tannins are established for its antioxidant activity, and the drug used to show antioxidant activity definite to show wound healing activity in different extent.

Protein is known as building blocker of our body. Amino acids are the structural unit of protein. Amino acid may accelerate the healing procedure by hastening the proliferation of the cell. Amino acid also help to reinforce the maturation of eschar.

Flavonoids may play an important role in the processes that contribute to faster wound healing **[23]**. The eicosanoid biosynthesis used to inhibit by the endogenous antioxidants, stimulated by flavonoids. By this the inflammatory metabolites gets depleted and shows prominent antiinflammatory activity [24].

Marsilea quadrifolia has proven antiantioxidant inflammatory, and antimicrobial activities. Any drug that already has anti-inflammatory, antimicrobial, as well as antioxidant activity is expected to have a healing effect [25, 26]. The antioxidant activity may be due to the presence of alkaloids in it. The presence of alkaloids steroid, tannin and may responsible for its anti-inflammatory activity. As its already established that tannin is the most active constituent of a herb, responsible for its wound healing effect. By all the above findings it is clear that, Marsilea quadrifolia has shown potent wound healing effect may be due to the presence of its chemical constituents, tannin, alkaloids, steroid and protein and at the same time makes Marsilea quadrifolia an unique herb.

#### **CONCLUSION:**

The purpose of the present study was to evaluate whether the phytochemicals in Marsilea quadrifolia leaves would work on improving wound healing in albino wistar rats having experimentally conceived wounds. Phytochemical screening of revealed that contains carbohydrates, flavonoids, tannins, alkaloid, steroids, phenolic acid, saponin and protein. The hydroalcoholic extract of MQ leaves that was obtained by percolation method, was

used in two different doses of MQ (2.5%, 5%) gel formulation by using HPMC as gel base, which was used for in vivo wound healing study on albino wister rats. Hydroalcoholic extract of Marsilea quadrifolia leaves demonstrated remarkable wound healing activity in excision as well as incision wound models. which is comparable to the povidone-iodine ointment as well as Aloe vera gel formulation. The findings also support the use of Marsilea quadrifolia in folk medicine for wound management.

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