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Evaluations of healing potential of ethanol extract from *Macrothelypteris torresiana* (Gaudich) aerial parts

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Abstract

The aim of the present study is to evaluate healing potential of 5% and 10% (w/w) ethanol extract from *Macrothelypteris torresiana* aerial parts (EEMTAP) in simple ointment (family thelypteridaceae). In excision wound model the ethanol extract from *M. torresiana* aerial parts showed significant increase in percentage wound closure and increased rate of epithelization. In incision wound model, EEMTAP treated animals showed increase in breaking strength when compared with control group. Similarly, the efficacy of treatment of chemical and thermal burn injuries in rats showed significant reduction in the burn wound area when compared with control group animals. These results showed that the burn wounds of untreated groups were contaminated, as they were not repaired, while those of treated groups by silver sulfadiazine and EEMTAP restored the normal architecture more rapidly and the percentage wound closure time was significantly reduced. In dead space wound model the ethanol extract treated animals (200 mg/kg, p.o.) showed significant increase in both wet and dry weight of granulation tissue. Estimation of hydroxyproline content in the granulation tissue revealed that the animal groups treated with EEMTAP had high hydroxyproline content as against the control group. The histological studies of the wound tissues revealed that the granulation tissue of the control group of animals showed more aggregation of macrophages with few collagen fibers, persistent inflammation, oedema and with chronic inflammation. In the case of povidone iodine treated animal possess significant increase in collagen deposition showing lesser macrophages with granulation tissue formation and lesser fibroblasts. In the case of 5% (w/w) EEMTAP treated animals revealed that moderate collagen deposition, macrophages and fibroblasts were noticed and the wounds treated with 10% (w/w) EEMTAP in simple ointment showing significant increased collagenation, few macrophages and capillaries.

Keywords: *Macrothelypteris torresiana*, Excision and incision wound model, Dead space wound model, Histological studies, Chemical and thermal burn, Povidone-iodine, Silver sulfadiazine, Simple ointment.

Introduction

Macrothelypteris torresiana (Gaudich), syn. *Lastrea torresiana* Moore (family: thelypteridaceae) is a species of fern which is native to tropical and subtropical region. It is a robust fern with a short creeping rhizome [1,2]. In traditional medicine *M. torresiana* leaves and roots have a wide range of reputed medicinal application. The aerial parts are used for granulation, healing and reducing odor in chronic skin ulcer by the tribes of Pakistan, India and China. It is also used in Chinese folk medicine for the treatment of oedema for patient suffering from kidney problems [3]. Only few phytochemical and pharmacological properties have been reported on this plant like renoprotective potential of *M. torresiana* via ameliorating oxidative stress and proinflammatory [3]. *In vitro* and *in vivo* antitumor activities were reported by Huang *et al.,* 2010 [4]. A novel flavonoid was isolated from the root and the structure was identified as 5,7-dihydroxy-2-(1,2-isopropyldioxy-4-oxocyclohex-5-enyl)-chromen-4-one [5], along with four known flavonoids like protoapigenin, apigenin, kaempferol and quercetin were reported from the aerial parts [6].

Literature available from all possible scientific sources revealed very little research work on this selected medicinal plant. So, the present study is investigated to explore healing potential of ethanol extract from *Macrothelypteris torresiana* aerial parts (EEMTAP).

Materials and Methods

Plant Material

The aerial parts was collected from in and around East Godavari dist., Andhra Pradesh, India and authenticated by Dr. K. Madhava Chetty, Professor, Dept. of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. A voucher specimen has been kept in our research laboratory for further reference. The collected materials were washed with water and shade dried for one week and then the dried aerial parts were pulverized by using a mechanical grinder to obtain a coarse powder.

Preparation of the extract

The powdered plant material (500 g) was extracted with 1.5 litres ethanol (90% v/v) for 48 hrs using Soxhlet extractor. The extract obtained was evaporated under vacuum to remove the solvent completely and concentrated to obtain a dark greenish semisolid residue (10.68 g). Preliminary phytochemical studies were performed on the extract using standard procedures [7,8].

Animals

Swiss albino mice (20-25 g) and Wistar albino rats (150–250 g) of either sex were maintained in the animal house at GITAM institute of pharmacy, GITAM University, Visakhapatnam, Andhra Pradesh under standard environmental condition of temperature (250C) and light/dark cycles (12/12h). All experimental protocols were approved by the Institutional Animal Ethics committee of GITAM Institute of Pharmacy, Visakhapatnam, Andhra Pradesh, India (Regd. No.1287/ac/09/CPCSEA and protocol No: IAEC/GIP-1307/B Pharm/IP/SM-HV/11/2012-13). Experiments were performed according to the guide for the care and use of laboratory animals.

Acute toxicity study

The acute toxicity studies were conducted over mice as per OECD guidelines 423 [9], where the limit test dose of 2000 mg/kg, p.o., used. Observations were made and recorded continuously for the first 4 h for any behavioral changes. They were then kept under observation up to 14 days after drug administration to find out the mortality if any. One-tenth of the maximum tolerated dose of EEMTAP (200 mg/kg, body weight, p.o.) was selected for dead space wound model study.

Experimental design for excision and incision wound healing model

The wound healing experiment was performed using the ethanol extract from *Macrothelypteris torresiana* aerial parts (EEMTAP). The rats were divided into four groups each consisting of six rats: group I: treated with simple ointment I.P. (vehicle), group II: treated with povidone-iodine ointment 5% w/w [Cipladine; mfg by: Jeps Pharmaceuticals, Sirmour-173025 (H.P.), Batch No: JMI 92] was used as reference standard for the activity comparison, groups III and IV were treated with 5% and 10% w/w EEMTAP in simple ointment I.P., respectively. The ointments were applied topically onto the wound surfaces twice daily after cleansing with 0.9% saline solution for 15 days. Wound scar tissues were cut on the 16th day post wounding for histopathology studies and breaking strength determination.

Excision wound healing model (Normal wounds)

The wound healing experiment was performed according to the method described by Morton and Malone [10] and suggested by Kamath *et al.* [11]. The rats were anaesthetized with an intramuscular dose of 50 mg/kg body weight of ketamine hydrochloride. Then dorsal fur of the rats was shaved using razor blades; the shaved area was then cleaned with ethanol (70%v/v) 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 circular area of 500 mm^2 and 2 mm depth was created along the markings using toothed forceps, scalpel and pointed scissors. Hemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. The entire wound was left open [12]. All surgical procedures were performed under aseptic conditions. The wounds were left untreated for a period of 24 h. The extracts and reference drugs were applied topically at the wound site twice a day. The wound closure rate was assessed by tracing the wound on days 5, 10 and 15 post wounding days using transparent paper and a permanent marker. The wound areas recorded were measured using graph paper [13]. The percentage of wound healing was calculated for final analysis of results [14]. The period of epithelialization was calculated as the number of days required for falling of the dead tissue remnants without any residual raw wound. The results are tabulated in Table 1.

Histological investigations

Wound tissues were cut on the 16th day post wounding from all the groups and fixed in Bouin's solution. The fixed tissues were dehydrated through increasing grades of ethanol and embedded in paraffin wax. The tissues were then cut to 5μm sections with a rotary microtome, deparaffinised, mounted on clean glass slides and stained with haematoxylin and eosin. The glass slides were then

observed under the microscope for histopathology changes [15, 16].

Incision Wound Model (Determination of breaking strength of healed wound tissues)

The breaking strengths of the wounds were determined according to the method described by *Krishna et al.,* [17]. A longitudinal paravertebral incision of 6 cm long was made through the skin and cutaneous tissue on the back. After the incision, the parted skin was sutured 1 cm apart using a surgical thread and curved needle. The wounds were left undressed. The extract and reference drugs were topically applied to the wound twice a day. The sutures were removed on 8th post wound day and continued the application of the extract. The wound breaking strength was measured on the 10th day after the last application [18].

Dead space wound model

Dead space wounds were created by implanting two pre weighed sterilized polypropylene tube (2.5 cm length and 0.25 diameters) beneath the dorsal paravertebral skin of the anaesthetized rats [16]. The animals were divided into two groups of six Wistar rats in each group. Group I serve as control, which received only plain drinking water (3 ml/kg, p.o.) and group II received EEMTAP orally in water at a dose of 200 mg/kg, body weight daily. On the 10th post wounding day, the granulation tissue formed on the implanted tubes was carefully detached from surface of the tubes. The wet weight of the granulation tissue collected was noted. The tissue samples were dried at 60^0 C for 12 hrs and weighed to determine the dry granulation tissue. Simultaneously, the dried tissue was added to 1 ml 6 M HCl and kept at 110^0 C for 24 hrs. The neutralized acid hydrolysate of dry tissue was used for the determination of hydroxyproline [19]. The results are tabulated in Table 3.

Evaluation of EEMTAP on chemical and thermal burn injuries in rats

The hairs of the dorsal skin were shaved before burn induction. Two models of burn injuries were used. The rats were divided into four groups each consisting of six male rats for both the models. The first by inducing a chemical injury by spreading few drops of concentrated hydrochloric acid over the shaved skin on 7 x 7 cm area [20] and the second model by inducing thermal injury, burn wound on dorsal skin of the rat were induced by pressing of metal rod with 2.5 cm diameter for 10 sec., which were preheated in boiling water and produced second degree burn [21]. Then wound area dressing with sterile gauge and animals were housed separately after complete recovery from anesthesia. Then the topical treatment of burns were carried out in the following manner. Drugs were applied twice daily. The control groups (group I) for both the models were left without treatment, while positive control for both the models (group II) was treated with silver sulfadiazine (1% w/w) and test groups (group III and IV) for both the models was dressed with 5% w/w and 10% w/w EEMTAP in simple ointment I.P., in a similar manner twice a day upon the burn. The wound closure rate was assessed on days 5, 10, 12, and 15 post wounding days using transparent paper and a permanent marker. The percentage of wound healing was calculated for final analysis of results. The results are tabulated in Table 4 and 5.

Statistical Analysis

The data obtained in the studies were subjected to one way of analysis of variance (ANOVA) for determining the significant difference. The inter group significance was analyzed using Dunnet's t-test. A p-value <0.05 was considered to be significant. All the values were expressed as Mean ± SEM.

Results

Preliminary phytochemical screening the ethanol extract from *Macrothelypteris torresiana* aerial parts (EEMTAP) contains flavonoids, saponins, proteins, reducing sugar, tannins, phlobatannins and phenolic compounds.

Acute oral toxicity studies of EEMTAP were carried out as per the OECD guidelines, draft guidelines 423. However, there was no mortality or morbidity observed in animals through the 14-day period following single oral administration. Morphological characteristics (fur, skin, eyes and nose) appeared normal. No tremors, convulsion, salivation, diarrhea, lethargy or unusual behaviors such as self mutilation, walking backward and so forth were observed; gait and posture, reactivity to handling or sensory stimuli, grip strength were all normal. There was no significant difference in body weights between control and treatment groups. Food and water intake showed daily fluctuations within the range of control animals. Evaluations of healing potential of ethanol extract from *Macrothelypteris torresiana* aerial parts possess significant promotion of wound healing activities were observed in the entire wound models such as excision, incision, dead space wound models, chemical and thermal burn injuries in rats.

In excision wound model (normal wounds), the mean percentage of wound contraction was calculated on 5, 10 and 15 post wounding days. The results of the progress of the wound healing by 5% and 10% w/w of EEMTAP in

simple ointment, povidone iodine (Standard drug) and simple ointment (control group) were shown in Table 1. Percentage wound contraction of the extract was significantly greater than control group. EEMTAP in simple ointment (5% and 10% w/w) treated groups (Group II and II) showed significant wound healing from the fifth days onwards which was comparable to that of the standard drug (group II treated with 5% povidone iodine). The percentage of wound closure was 95.23±2.62 in the case of standard drug povidone iodine on 15th day of treatment, whereas the ethanol extract at 5% and 10% (w/w) in simple ointment demonstrated 85.17±3.9 and 98.51±2.08 percentage of wound closure. The percentage of wound contraction was much more with the 10% w/w EEMTAP in simple ointment treated group which was almost better than that of povidone iodine treated group. The epithelization of wound with 10% (w/w) extract ointment was found to be earlier as compare to control and povidone iodine treated group. Epithelization period 5% and 10% (w/w) EEMTAP in simple ointment was 19.05±2.03 and 16.04±1.03 days, whereas 16.63±1.23 days for the standards drug treated group and the control groups animals it took more than 23.86±2.01 days.

The histological studies of the wound tissues revealed that the granulation tissue of the control group of animals showed more aggregation of macrophages with few collagen fibers, persistent inflammation and oedema, evident of chronic inflammation. In the case of povidone iodine treated animal possess significant increase in collagen deposition showing lesser macrophages with granulation tissue formation and lesser fibroblasts was observed. In the case of 5% (w/w) EEMTAP in simple ointment treated animals revealed that moderate collagen deposition, macrophages and fibroblasts were noticed and the histopathology of wounds treated with 10% (w/w) EEMTAP in simple ointment showing significant increased collagenation, few macrophages and capillaries. Appreciable angiogenesis and granulation tissue formation. Evidence of hair follicle and re-epithelialisation. Photographs were taken from each slide and presented in Figure. 1. The breaking strength of incision wound model was increased in drug treated groups to significant extent ($P<0.01$). The 5% and 10% (w/w) EEMTAP in simple ointment treated animals showed increase in breaking (241.03±3.05 and 398.48±4.33), respectively when compared with control group (108.26±5.28). The mean breaking strength was also significant in animals treated with standard drug povidone iodine (363.44±2.11), whereas 10% (w/w) EEMTAP in simple ointment treated group showed increased in breaking strength than the standard drug (Table 2).

In dead space wound model the ethanol extract from *M. torresiana* treated animals (200 mg/kg, p.o.) showed significant increase in both wet and dry weight of granulation tissue. Estimation of hydroxyproline content in the granulation tissue revealed that the animal groups treated with EEMTAP had high hydroxyproline content (2316.07±51.66) as against the control group (1438.23±32.06). Results are depicted in Table 3.

Contracting ability of burn wounds in both chemically and direct heat induced results are depicted in Table 4 and 5. The percentage wound closures of the control groups were bigger than those of treated groups, in addition to their contamination and inflammation. The efficacy of treatment of chemical burn injuries in rats showed significant reduction in the burn wound area when compared with control group animals. Silver sulfadiazine and 10% (w/w) EEMTAP significant reduction from 5th days of treatments, whereas 5% (w/w) EEMTAP possess significant reduction from 10th days onwards in both the models. The percentages of wound closure for chemical burn injuries was 91.65±1.04 in the case of standard drug silver sulfadiazine on 15th days of treatment, whereas the ethanol extract at 5% and 10% (w/w) in simple ointment demonstrated 64.33±3.28 and 88.66±0.22 percentage of wound closure, and for control group provide 51.91±2.01. Similarly in case of thermal burn injuries in rats the percentages of wound closure after 15th day of treatments for control group (Group I), silver sulfadiazine (Group II), 5% and 10% (w/w) EEMTAP (Group III and IV) were 61.09±1.22, 96.03±0.88, 71.63±2.08 and 92.33±1.08. Simultaneously, in the study period for control groups animals and group III treated with 5% (w/w) EEMTAP we found that there was not any sign of hair growth on the burn region for both the models, whereas the group II which was treated with silver sulfadiazine and group IV treated with 10% (w/w) EEMTAP the hair growth gradually started from day 12.

Table 1: Percentage wound contraction of ethanol extract from *M. torresiana* aerial parts (Excision Wound Model)

Treatment (Topically)	Percentage (%) wound closure			Period of epithelialization (No. of days)
	5th day	10th day	15th day	
Group I: Control (simple ointment)	12.45±2.08	34.05±3.08	68.7±1.87	23.86±2.01
Group II: Povidone-iodine (5% w/w)	38.60±0.96**	78.36±1.16**	95.23±2.62**	16.63±1.23**
Group III: EEMTAP (5% w/w)	21.15±3.02*	62.32±3.09**	85.17±3.9*	19.05±2.03*
Group IV: EEMTAP (10% w/w)	42.33±2.06**	86.48±3.08**	98.51±2.08**	16.04±1.03**

Values are expressed as mean ± S.E. (n = 6). All columns are significant using ANOVA.
* P<0.05, ** P<0.01 when compared to control; Dunnet's t-test.

Table 2: Effect of ethanol extract from *M. torresiana* aerial parts on wound breaking strength (Incision Wound Model)

Groups	Treatment (Topically)	Breaking strength (g)
I	Control (simple ointment)	108.26±5.28
II	Povidone-iodine (5% w/w)	363.44±2.11**
III	EEMTAP (5% w/w)	241.03±3.05**
IV	EEMTAP (10% w/w)	398.48±4.33**

Values are expressed as mean ± S.E. (n = 6). All columns are significant using ANOVA.
* P<0.05, ** P<0.01 when compared to control; Dunnet's t-test.

Table 3: Effect of ethanol extract from *M. torresiana* aerial parts on wound breaking strength (Incision Wound Model)

Groups	Treatment	Wet tissue weight (mg)	Dry tissue weight (mg)	Hydroxyproline (mg/100 g dry tissue)
I	Control (3 ml/kg, p.o.)	87.22±3.08	37.41±1.22	1438.23±32.06
II	EEMTAP (200 mg/kg, p.o.)	108.48±2.33**	65.83±4.21**	2316.07±51.66**

Values are expressed as mean ± S.E. (n = 6). All columns are significant using ANOVA.
* P<0.05, ** P<0.01 when compared to control; Dunnet's t-test.

Table 4: Percentage wound contraction on chemical burn injuries in rats.

Treatments	Percentage (%) wound closure		
	5th day	10th day	15th day
Group I: Control (simple ointment)	3.05±0.98	17.08± 2.47	51.91±2.01
Group II: Silver sulfadiazine (1% w/w)	25.83±1.22**	68.23±2.54**	91.65±1.04**
Group III: EEMTAP (5% w/w)	10.03±1.09	35.02±2.09**	64.33±3.28*
Group IV: EEMTAP (10% w/w)	18.22±0.88**	61.01±2.83**	88.66±0.22**

Values are expressed as mean ± S.E. (n = 6). All columns are significant using ANOVA.
* P<0.05, ** P<0.01 when compared to control; Dunnet's t-test.

Table 5: Percentage wound contraction on thermal burn injuries in rats.

Treatments	Percentage (%) wound closure		
	5th day	10th day	15th day
Group I: Control (simple ointment)	10.05±1.01	26.33± 0.77	61.09±1.22
Group II: Silver sulfadiazine (1% w/w)	32.33±0.81**	71.23±0.55**	96.03±0.88**
Group III: EEMTAP (5% w/w)	12.03±0.33	39.11±1.22*	71.63±2.08*
Group IV: EEMTAP (10% w/w)	26.22±0.33*	68.01±0.83**	92.33±1.08*

Values are expressed as mean ± S.E. (n = 6). All columns are significant using ANOVA.
* P<0.05, ** P<0.01 when compared to control; Dunnet's t-test.

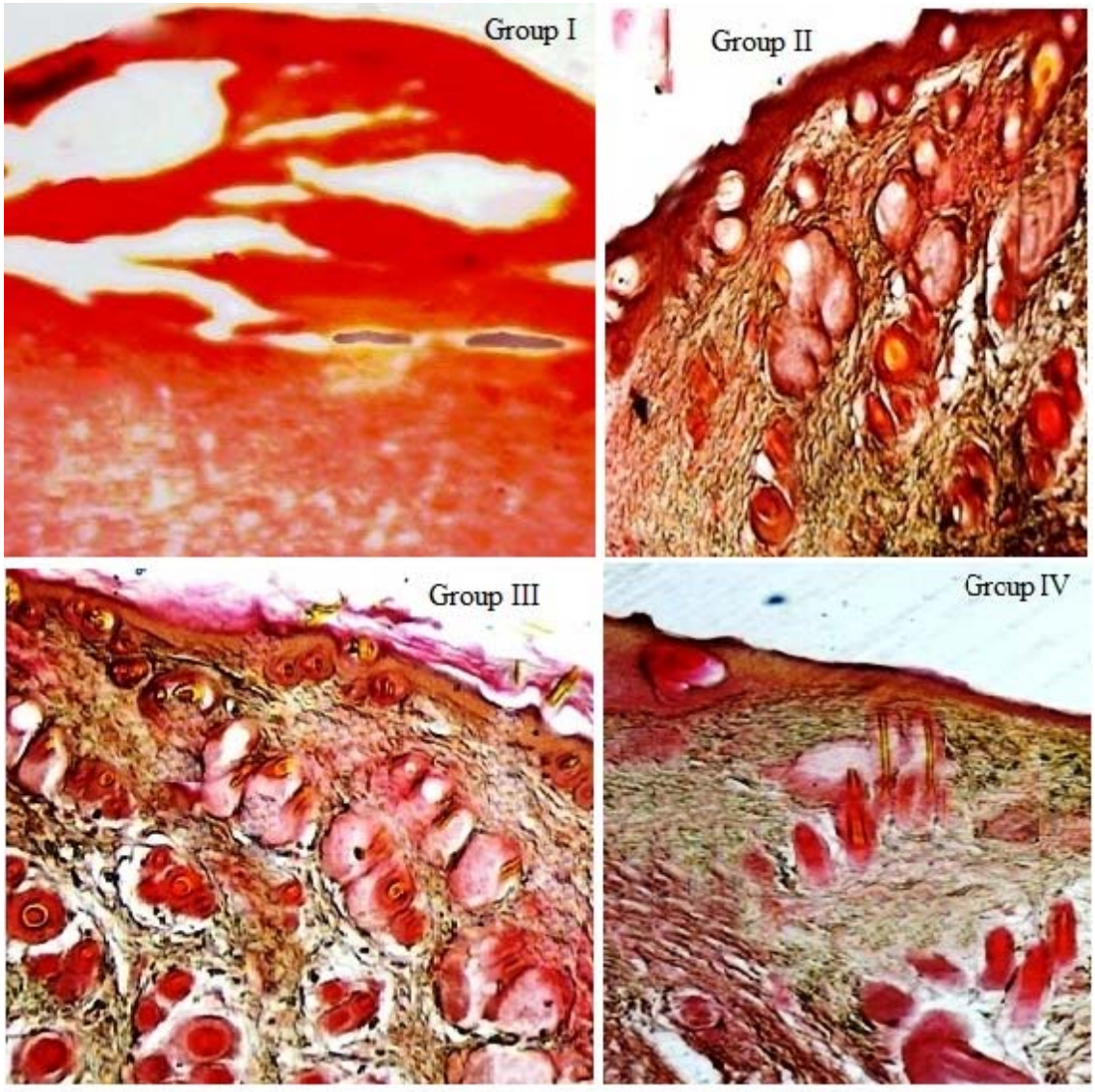

Figure. 1: Histopathology of wound tissue

Group I: (control-treated with simple ointment) showing with less collagen and more macrophages with evidence of chronic inflammation.
Group II: (standard-treated with povidone-iodine) Granulation tissue formation, showing significant collagenation, lesser fibroblasts and capillaries, and re-epithelialization, with reduced pus cells.
Group III: (treated with 5% w/w EEMTAP) Granulation tissue formation, animal showing with moderate dispersed pus cells, increased collagenation, moderate macrophages and re-epithelialization.
Group IV: (treated with 10% w/w EEMTAP) Showing significant increased collagenation, few macrophages and capillaries. Appreciable angiogenesis and granulation tissue formation. Evidence of hair follicle and re-epithelialization.

Discussion

Wound healing is fundamental response to tissue injury, which consists of different phases such as hemostasis, inflammation, proliferative and remodeling or maturation. This is due to the synthesis of the connective tissue matrix [19]. In excision wound model the ethanol extract from *M. torresiana* aerial parts showed significant increase in percentage wound closure and increased rate of epithelization. This enhanced may be due to the enhanced collagen synthesis, because in incision wound we found that the extract treated groups increase in skin breaking strength and tissue breaking strength and in dead space wound model respectively indicated enhanced collagen maturation. Increase in the granulation tissue dry weight and hydroxyproline content indicated the high collagen turnover which may be due to the activity of some phytoconstituents like flavonoids which are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving vascularity [20]. The extent of the collagen formation was also depicted in the breaking strength determination conducted on the healed wound tissues in which 10%w/w the ethanol extract from *M. torresiana* demonstrated a highly significant breaking strength with reference to the untreated wounds. Through breaking strength and tensile strength of a wound can be used to describe the healing rate particularly for incision wounds. The breaking strength gives an indication of collagen accumulation and cross-linking in the healed wound tissues because collagen is a major protein and is the component that ultimately contributes to wound strength [19,21].

Similarly, treatment of burn wounds has always been one of the most challenging clinical problems. In the present study, ethanol extract from *M. torresiana* was applied to the experimental burns induced by chemically and direct heat to test its efficacy on repairing of the burned tissues and wound contraction. A burn always produces an alternation in the skin; a lesion in the corneal strata is sufficient to cause the skin to lose its capacity to act as a barrier [20,22]. The goal of a burn treatment is to produce epithelization as soon as possible. These results showed that the burn wounds of untreated groups were contaminated, as they were not repaired, while those of treated groups by silver sulfadiazine and EEMTAP restored the normal architecture more rapidly and the percentage wound closure time was significantly reduced.

Thus, healing property of the ethanol extract from *M. torresiana* aerial parts may be attributed to the phytoconstituents they contain like, flavonoids, saponins or tannins present in it. This may be either due to their individual or additive effect that fastens the process of both burn and normal wounds healing. The wound treated with vehicle (simple ointment only) exhibited poor wound healing with persistent inflammation. This indicates that the vehicle had no interference with healing effects of the extract and the effects are soley as a result of biological activities of the extract, but at this stage it is difficult to say which component(s) of extract is responsible for wound healing. However, further phytochemical studies are needed to isolate the active compound(s) responsible for these pharmacological activities.

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