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Anti-inflammatory and wound healing potential of *Prosthechea michuacana* in rats

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ABSTRACT

The hexane, methanol and chloroform extracts of bulbs of orchid *Prosthechea michuacana* (PMIC) were studied for their wound healing and anti-inflammatory properties. Wound healing effects were studied on incision (skin breaking strength), excision (percent wound contraction). Collagen, hexosamine, total protein and DNA content in the granulation tissues were determined in addition to the rates of wound contraction and period of epithelialization. Topical applications of hexane extract PMIC produced increases in tensile strength, collagen content (hydroxyproline), and is better epithelisation thereby facilitating the healing. The hexane extract also was found possess significant anti-inflammatory activity in both acute (carrageenin-induced edema) as well as subacute (cotton pellet) animals models. Thus it could concluded that PMIC hexane may enhance the process of wound healing by influencing phases such as inflammation, fibroplasias, collagen synthesis and maturation, and wound contraction. Hexane extract significantly inhibited later phase of edema so it seems possible that P. *michuacana* blocks prostaglandins and cyclooxygenase release in later phase of acute inflammation, consequently decrease in granuloma weight indicates the suppression of the proliferative phase, which was effectively inhibited by the PMIC.

Keywords: bulbs, orchid, hexane extract, healing process, inflammation.

INTRODUCTION

Since pre-Hispanic times in Mexico orchids have been given medical application, handmade, edible, narcosis, flavor, poison, glue, foodstuff ceremonial purposes, magic-religious talismans and aphrodisiacs (1). Most orchids are known mainly for its ornamental value in America and Europe but not in Asia where they have a long history in medicine.

Prosthechea michuacana (Lex.) W. E Higgins belong to the family Orchidaceae. Mixtecos and zapotecos of Oaxaca used as food the seudobulbo of *P. michuacana* which chews on the crude to quench thirst or liquefied with a little water to prepare a drink, is also used as an anti-inflammatory kidney, depurative of the circulatory system (diuretic), wound healing and in the treatment of diabetes (2). Also is used in the State of Oaxaca as an ornamental to adorn the traditional "Birth" in the months of November and December.

Some biochemical studies of this orchid found that *P. michuacana* contains 8-C-(6-deoxy-β-D-glucopyranosy) apigenina, l-(3'-hydroxy-5'-methoxyphenyl)-2-(4"-hydroxy-5"-methoxy phenyl) ethane and 2-(4-hydroxybenzyl) malic acid (3). We reported on previous studies the relaxant and antispasmodic effect in isolated guinea pig ileum of *P. michuacana* (4). The current investigation is an attempt to study the antiinflamatory

and wound healing effect of some extract of bulbs of *Prosthechea michuacana* in rats.

MATERIALS AND METHODS

Plant material.

Prosthechea michuacana (Lex.) W. E Higgins belong to the family Orchidaceae, bulbs were collected from El Punto, municipio de Santa Catarina Ixtepeji, distrito de Ixtlán, Oaxaca state, México in may of 2007 and were taxonomically authenticated in the Herbario OAX and The Cassiano Conzatti Botanical Garden, Instituto Politécnico Nacional and a voucher specimen of the plant is stored for reference (No. No.6478).

Animals.

The study was conducted in male Wistar strain albino rats, weighing about 180–225 g. Before and during the experiment, animals were fed with normal laboratory diet and water ad libitium. The animals were acclimatized for a period of 3 days in the new environment before initiation of experiment. The study was approved by the Institutional Animal Ethics Committee.

Preparation of plant extracts.

100 g of the aerial parts and bulbs were dried and powdered in a mechanical grinder. The powdered material was extracted by 500 ml of hexane, chloroform, methanol and water consecutively using soxhlet apparatus. These extracts was filtered and concentrated by rotary vacuum evaporator and kept in a vacuum dessicator for complete removal of solvent. Aqueous suspension of extracts of *P. michuacana* (PMIC) was prepared using 2% (v/v) Tween-80 and simple ointment bases are used for administration.

Wound healing activity

Excision wound model.

An impression was made on the dorsal thoracic region 1 cm away from vertebral column and 5 cm away from ear using a round seal of 2.5 cm diameter on the anaesthetized rat. The skin of impressed area was excised to the full thickness to obtain a wound area of about 500 mm^2 diameter. Contractions, which contributed to wound closure in the first 2 weeks, were studied by tracing the raw wound (5).

Rate of wound contraction and period of epithelialization.

To determine the rate of wound contraction, excision wounds were traced on a transparent paper having a millimeter scale, and the change in wound size was calculated as the percentage of wound area that had healed. The period of epithelialization of the wound was expressed as the number of days taken for complete epithelialization (so that no raw wound was left behind) (6).

Biochemical analyses of excision wounds.

Animals were sacrificed on the 4th, 8th, 12^{th} and 16th days after wound creation, and the entire wound on each animal was cut out and stored at −70°C until analysis.

For estimations of collagen, the weighed granulation tissues were first defatted in chloroform: methanol mixture (2:1 v/v), hydrolysed in 6.0 N HCl for 18 h at 110°C, evaporated to dryness, and then made up with a known volume of water. Collagen content of granulation tissues were determined by the estimation of hydroxyproline, as described by (7). For hexosamine estimations, wound tissues were processed as described above, except that the samples were lyophilized before hydrolysis. The hexosamine content of granulation tissues were then estimated by the method of Elson and Morgan (8).

For estimations of total protein and DNA content, wet granulation tissues were first extracted with TCA by the method of (9). Briefly, the tissue was first homogenized in 5% TCA and centrifuged. The pellet was washed with 100/0 TCA, resuspended in 5% TCA, and kept for 15 min in a water bath maintained at 90°C. The contents were centrifuged and the supernatant was used for the determination of DNA content by the method of Burton (10). The precipitated proteins were suspended in 0.1 M Tris-HC1, pH 7.4, and the protein content estimated by the method of Lowry et al., (11).

Incision wound model.

Rats were anaesthetized and two paravertebral-long incisions were made through the skin and cutaneous muscles at a distance of about 1.5 cm from the midline on each side of the depilated back of the rat. Full aseptic measures were not taken and no local or systemic antimicrobial was used throughout the experiment (12). All the groups were treated in the same manner as mentioned in the case of the excision wound model. No ligature was used for stitching. After the incision was made, the parted skin was kept together and stitched with black silk at 0.5 cm intervals. Surgical thread (No.000) and a curved needle (No.11) were used for stitching. The continuous thread on both wound edges were tightened for good closure of the wounds. The wound was left undressed; ointment, along with water soluble base ointment (control) and nitrofurazone ointment were applied topically twice a day for 7 days. When wounds were thoroughly cured, the

sutures were removed on the 7th day and tensile strength was measured with a tensiometer .

Tensile strength.

The tensile strength of a wound represents the degree of wound healing. Usually wound healing agents promote a gain in tensile strength.

On the 7th day after creating the wound the animals were anaesthetised. Healing tissue along with normal skin at two ends was excised for tensile strength measurement using Tensile Testing Machine TKG-20 (from Fine Testing Machines, Miraz). Strips of 8 mm width and 20 mm length were cut out from the excised tissue in treated and control animals and were loaded between the upper and lower holder of the machine in such a way that the effective load bearing size was 8 × 8 mm with the wound remaining in the centre. The total breaking load is measured in Newtons and the tensile strength was calculated by the following equation:

Tensile strength = Total breaking load/Cross-sectional area

The tensile strength of EMIC ointment treated wounds was compared with control and nitrofurazone ointment as standard. The tensile strength increment indicates better wound healing stimulated by the applied herbal formulation. Further epithelization period and scar area were measured daily for 25 days after determination of tensile strength (13).

Anti-inflammatory activity
Carrageenin-induced rat paw edema.

The anti-inflammatory activity of extract was evaluated by carrageenin-induced rat paw edema method (14).The suspension of the extracts were orally administered to rats (100, 150 and 200mg/kg) (n=6, each dose). After 1 h of administration of extract, 0.1 ml of the carrageenin suspension (0.1% in normal saline) was injected into subplantar region of the right hind paw. The paw edema volume was measured by means of water displacement technique using plethysmometer before and 1 and 3 h after the carrageenin injection. The control rats were received equal volume of the vehicles of the extract and comparable group received indomethacin (10 mg/kg) as a standard anti-inflammatory agent. The results are expressed in terms of mean increase in paw volume at 1 and 3 h and anti-inflammatory activity is expressed in terms of percent inhibition of paw edema at 1 and 3 h.

Cotton pellet-induced granuloma.

After shaving the hairs on its back, rats were anesthetized with light ether and granulomatous lesions were induced by surgically implanting two cotton pellets (10 ± 1 mg) subcutaneously in the dorsal region of the rats, one near each axial as described by Swingle and Shideman (15). Extracts (100, 150 and 200 mg/kg) or vehicle (10 ml/kg body wt.) or diclofenac sodium (10 mg/kg, p.o.) was given orally once daily for 7 days at fixed time of day. On 8th day, the rats were sacrificed by over dose of anesthetic ether, and the pellets covered by granulomatous tissue were dissected and dried to a constant weight at 50°C for 20 hr. The mean weight for different groups were determined and compared to the control group.

Statistical analysis.

The results are expressed as mean ± SEM. Parametric data were assessed by the method of analysis of One-way ANOVA followed by Dunnet's t-test, p-values <0.05 were considered significant.

RESULTS

Topical applications ointment of the bulbs extracts of *P. michuacana* showed effect on the healing process on the rats. In control rats excision type of wound take more than 18 days to heal completely unlike with the hexane extract the wounds heal almost completely around day 16 at doses of 50% (99.2%). The most remarkable effect of cicatrizant activity (with 100%) was observed in the group treated with EMIC in excision wound test at doses of 50%. While the group treated at doses of 20% and 30% showed 79.9% and 83.0% respectively at 18 post-wounding days (Table 1).

Table 2 shows the collagen content (hydroxyproline) in granulation tissues of topically treated rats. A common pattern of change was observed in all the groups. The collagen content of granulation tissues reached maximum levels 8 days after wound creation. Moreover, the steep increase in collagen content observed after day 4 was followed by almost equally steep decreases after day 8 in the treated groups. The levels continued to decrease after day 12, but at a much slower rate. *P. michuacana*-treated wounds also showed an increased rate of wound contraction, leading to quicker healing as confirmed by decreased period of epithelialization when compared to untreated control wounds.

Table 2 shows the hexosamine content in the granulation tissues. It was observed levels reached a high level 8 days after wound creation. In all treated rats, the hexosamine levels were found to decrease gradually and reach near normal levels by day 16.

The protein and DNA contents of the granulation tissues are presented in Table 2, There was a rapid increase in both protein and DNA contents, their values reaching

Table 1. Effect of hexane extract from *P. michuacana* (PMIC) on wound contraction by excision wound method

Post-wounding days	Wound area (mm²) ± S.E.M. % of wound contraction				
		EMIC		Nitrofurazone	
	Control	20%	30%	50%	0.2%
0	520 ± 1.83 (0.0)	517 ± 3.12 (0.0)	523 ± 3.17 (0.0)	519 ± 3.44 (0.0)	509 ± 3.32 (0.0)
2	489 ± 2.41 (5.96)	480 ± 3.34 (7.15)	467 ± 2.78 (10.70)	440 ± 2.90 (15.2)	451 ± 2.37 (11.4)
4	434 ± 2.08 (16.5)	425 ± 2.54* (17.8)	372 ± 2.82 (28.9)	320 ± 3.37* (38.3)	321 ± 2.55* (37)
6	365 ± 1.33 (29.8)	360 ± 3.468* (30.3)	289 ± 2.31* (44.7)	241 ± 3.10** (53.5)	265 ± 2.42** (52.1)
8	339 ± 1.74 (34.8)	329 ± 2.29* (36.3)	246 ± 2.50* (52.9)	178 ± 3.67** (65.7)	187 ± 2.50** (63.3)
10	281 ± 1.15 (45.9)	257 ± 2.71** (50.2)	187 ± 2.08** (64.2)	98 ± 2.81** (81.1)	104 ± 2.16** (79.6)
12	251 ± 1.78 (51.7)	231 ± 1.90** (55.3)	166 ± 1.82** (68.2)	71 ± 1.43** (86.3)	81 ± 2.08** (84.0)
14	178 ± 1.37 (90.8)	155 ± 2.38* (65.7)	125 ± 1.10** (70)	40 ± 4.30** (76.0)	47 ± 1.14* (92.3)
16	151 ± 1.26 (70.96)	143 ± 4.15** (72.3)	112 ± 3.96** (78.58)	4 ± 5.02** (99.2)	9 ± 6.08** (98.2)
18	143 ± 2.56 (72.5)	104 ± 2.48* (79.9)	89 ± 4.12** (83.0)	0.0 (100.0)	0.0 (100.0)

Values are mean ± SEM (n=6); Statistically significant differences with control group;
*p<0.01,
**p<0.05.

Table 2. Collagen (Hydroxyproline), hexosamine, total protein and DNA content in *P. michuacana* (PMIC) treated and untreated wound granulation tissue from rats.

Day	Granuloma dry weight			
	Hydroxyproline (mg/100mg)	Hexosamine (mg/100mg)	Total protein (mg/g)	DNA (mg/g)
Control (4 day)	28.73 ± 1.65	0.81 ± 2.23	31.42 ± 3.12	24.86 ± 1.40
4	35.17 ± 0.98	0.76 ± 3.45*	51.33 ± 4.82*	36.72 ± 2.61**
8	78.69 ± 0.24	0.68 ± 5.21**	98.70 ± 5.21**	68.91 ± 3.53**
12	38.12 ± 1.03**	0.52 ± 4.32**	79.65 ± 6.83**	59.74 ± 4.76*
16	23.18 ± 0.56	0.45 ± 2.98*	61.54 ± 4.80*	51.28 ± 5.12*

Date are given as mean ± SD for six animal in each group. Statistically significant results are indicated as
*p<0.01 and
**p<0.001.

maximum level 8 days after wound creation. Their levels decreased after day 8, but the rate of decrease was much slower. On all days, both the protein and DNA levels were significantly higher in the groups treated with *P. michuacana* when compared to the untreated control wounds.

In the incision wound studies, the administration of ointment of bulbs produced a statistically significant decreased in the epithelization period of 13 days (48%) at doses of 50%, along with a visibly decreased scar area (Table 3). However nitrofurazone ointment (0.2%) produced maximum percentage of decreased of 14 days and were found to be 44%. Also showed a significant increase in tensile strength of the 7-day-old wound due to treatment with PMIC when compared with the control group. Tensile strength of the nitrofurazone and PMIC was almost the same at doses of 0.2 and 50% respectively. The test results are showing in Table 3.

Table 4 showed the aspect and evolution after topical application on rats of the PMIC. The congestion and oedema wounds decreased significantly in the groups treated with doses of 30 at 50 %. The wounds after 7 days treatment exhibited marked dryness of wound edges with regeneration of healing tissue and the wound area was also considerably reduced compared to controls indicating the healing potential of PMIC.

The wounds after 7 days treatment with plant extract exhibited marked dryness of wound edges with regeneration of healing tissue and the wound area was

Table 3. Effect of hexane extract from *P. michuacana* (PMIC) on incision wounds

Topical Treatment	Epithelization period (day)	Scar area (mm^2)	Tensile strength (N/cm^2)
Control	25 ± 0.98	50.16 ± 1.65	6.60 ± 1.52
Hexane extract (PMIC), (20%)	20 ± 1.23 (20)	45.21 ± 4.23* (9.86)	7.79 ± 1.93*
Hexane extract (PMIC), (30%)	16 ± 1.87 (36)	38.63 ± 1.87* (22.9)	10.01 ± 2.30*
Hexane extract (PMIC), (50%)	13 ± 2.01* (48)	29.20 ± 3.54** (41.8)	13.95 ± 5.16**
Nitrofurazone ointment (0.2%)	14 ± 0.65** (44)	30.54 ± 1.78 (39.11)	13.23 ± 5.88 **

The parenthesis indicate the % inhibition. Values are mean ± SEM (n=6); Statistically significant differences with control group;
*p<0.01,
**p<0.05.

Table 4. Aspect and evolution finding of 7 days after topical application of hexane extract from *P. michuacana*

Treatment Extract (%)	Congestion	Oedema
Control	+++	+++
20	+	+
30	-	-
50	-	-
Nitrofurazone (0.2%)	-	-

Slight +, moderate ++, marked +++, extensive++++, absent -

Table 5. Anti-inflammatory effect of hexane extract from *P. michuacana* (PMIC) in carrageenin induced paw edema

Treatment	Dose (mg/kg)	Edema volume 1h	3h
Control	10 ml/kg	0.67 ± 0.24	0.77 ± 0.54
Hexano extract (PMIC)	100	0.55 ± 1.19** (17.95)	0.59 ± 0.34* (23.37)
	150	0.47± 1.15** (29.85)	0.46 ± 0.08** (40.25)
	200	0.40 ± 0.65* (40.29)	0.20 ± 0.71* (74.02)
Indomethacin	10 (58.20)	0.28 ± 0.72* (79.22)	0.16 ± 0.07**

The parenthesis indicate the % inhibition; The results are mean ± SEM from 6 animals;
*p<0.05,
**p<0.01 when compared to vehicle control.

also considerably reduced compared to controls indicating the healing potential of *P. michuacana*. A examination of granulation tissue sections revealed that the tissue regeneration was much faster in the treated group compared to control wounds. The congestion and oedema wounds decreased significantly in the groups treated with hexane extract after 7 days of treatment.

The results of the carrageenan-induced edema test on animal experiment was shown in Table 5. The date indicated that hexane extract with a dose of 200 mg/kg b.w showed maximum anti-inflammatory activity (74.02%) as compared to indomethacin the reference drug, which showed 79.22 % inhibition. Hexane extract with a dose 150 mg/kg b.wt produced 40.25 % of inhibition and is also high as compared to the reference drug. The extract with a dose of 100 mg/kg b. wt produced 23.37% of inhibition. The development of odema in the paw of the rat after the injection of carrageenan is due to release of histamine, serotonin and prostaglandin like substances (16).

In cotton pellet granuloma model, there was statistically significant reduction in the dry weight of granuloma in hexane extract (100, 150 and 200 mg/kg) as well as diclofenac sodium (10 mg/kg, p.o.) treated rats as compared to control group (Table 6). Maximum anti-inflammatory activity on rats when compared with the control group of animals receiving an equal dose of Tween 80 was observed with 200 mg/kg doses of hexane

Table 6. Anti-inflammatory effect of hexane extract from *P. michuacana* (PMIC) on cotton pellet granuloma

Treatment	Dose (mg/kg)	% Increase in granuloma weight (mg/100g)	% Inhibition
Control	10 ml/kg	28.13 ± 5.7	--------
	100	18.21± 4.3**	35.26
Hexano extract (PMIC)	150	9.12 ± 6.1*	67.56
	200	6.83 ± 4.9*	75.71
Diclofenac sodium	10	5.47 ± 4.6**	80.55

The results are mean ± SEM from 6 animals;
*p<0.05,
**p<0.01 when compared to vehicle control.

extract of *P. michuacana* was 75.71%. Diclofenac sodium 10 mg/kg dose produced 80.55% granuloma reduction. However chloroform and methanol extracts to the same doses no produced anti-inflammatory activity.

DISCUSSION

Traditionally, medicinal plants have been used for many years as topical and internal preparations to promote wound repair. Current researches are devoted to validating their efficacy and to uncover the mechanisms responsible for this activity. Medicinal plants have great potentials and have been shown to be very beneficial in wound care, promoting the rate of wound healing with minimal pain, discomfort, and scarring to the patient (17). Some of these plants owe their effects to direct effect on the wound healing processes and some to their anti-inflammatory effects. A combination of these properties is also possible in some of the medicinal plants used in wound care.

Wound healing involves various phases. Initially involves acute inflammatory phase followed by the synthesis of collagen and other extra cellular macromolecules, which are later removed to form a scar (18). Drugs, which influence one phase, may not necessarily influence another. Hence different models have been used in our study to assess the effect of various phases, which run concurrently, but independent of each other. Control group wound showed granulation tissue and fibroblast aggregation in the *P. michuacana* treated group showed extensive growth of granulation also started along its surface as shown subsequently on 14th day. The treated group of wound showed complete healing of wounds with almost normal architecture of the collagen, reticulin, Increase in tensile strength of treated group wound may be due to increase in collagen concentration, extract of *P. michuacana* increase the collagen synthesis.

Further elevated levels of hydroxyproline in regenerated tissue suggests enhanced collagen synthesis, an important constituent of extracellular matrix (19). Collagen not only confers strength and integrity to the tissue matrix but also plays an important role in haemostasis and in epithelisation at later phase of wound healing (20) hence enhanced collagen synthesis by *P. michuacana* may significantly contribute to healing and also provide necessary strength to repaired tissue.

Glycosaminoglycans and proteoglycans are synthesized by fibroblasts in the wound area. These substances form a highly hydrated gel-like ground substance, a provisional matrix. on which collagen fibres are embeded. As collagen accumulates, hexosamine levels decrease (21). Treatment with *P. michuacana* increases the content of ground substance in the granulation tissues. It may be seen that the decrease in hexosamine content was associated with a concomitant increase in collagen content.

The protein and DNA content of granulation tissues indicate the levels of protein synthesis and cellular proliferation. Higher protein and DNA contents (compared to the untreated controls) of the treated wounds suggest that *P. michuacana,* through an as yet unknown mechanism, stimulates cellular proliferation. The collagen/DNA ratio of the granulation tissues also suggest that *P. michuacana,* may increase the synthesis of collagen per cell.

The collagen molecules synthesized are laid down at the wound site and become cross linked to form fibers. Wound strength is acquired from both, remodeling of collagen, and the formation of stable intra- .and inter-molecular cross links.

Since incisional wounds treated with the *P. michuacana,* showed greater tensile strength, it may be inferred that it not only increases collagen synthesis per cell, but also aids in cross linking of the protein. *P. michuacana*-treated wounds also showed an increased rate of wound contraction, leading to quicker healing as confirmed by decreased period of epithelialization when compared to untreated control wounds.

In the present study, the anti-inflammatory activity of the hexane extract of bulbs of *P. michuacana* has been established in acute model i.e., Carrageenan-induced rat paw edema, is a suitable test for evaluating anti-inflammatory drugs which have frequently been used

to assess the anti-edematous effect of natural products (22). The edema formation by subplantar injection of carrageenin is a biphasic response; initial phase (1h) is being due to the release of serotonin and histamine whereas the later phase (over 1h) is attributed to the release of prostaglandins, the cyclooxygenase products and the continuity between two phases is provided by kinins (23). Hexane extract significantly inhibited later phase of edema so it seems possible that EMIC blocks prostaglandins and cyclooxygenase release in later phase of acute inflammation.

In the cotton pellet granuloma model, inflammation and granuloma developed during a period of several days. The dry weight of the pellets correlates with the amount of granulomatous tissue (24). Protein synthesis is necessary for the formation of granuloma. Inflammation involves infiltration of macrophages, neutrophils and proliferation of fibroblasts, which are the basic sources for granuloma formation (25). Consequently decrease in granuloma weight indicates the suppression of the proliferative phase, which was effectively inhibited by the PMIC. Hexane extract (200 mg/kg) appears to be equally effective to that of diclofenac sodium (10 mg/kg) in inhibiting the dry weight of cotton pellets.

The present investigation clearly showed that the topical application of hexane extract of the bulbs of *P. michuacana* produced significant wound healing and anti-inflamatory activities and showed an activity dose-dependently. The wound healing properties and anti-inflammatory activity of the plant validate their uses in traditional medicine for treating injured. The results obtained encourage us to carry out a wider and more profound study of this plant to obtain better knowledge of its therapeutic possibilities.

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