In Vivo Wound-Healing Activity of Oleanolic Acid Derived from the Acid Hydrolysis of *Anredera diffusa*

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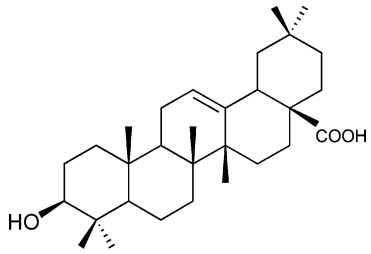
Anredera diffusa is used as a wound-healing agent in traditional Peruvian medicine. Acid hydrolysis of the bioactive ethanolic extract, followed by in vivo activity-guided fractionation, yielded oleanolic acid, with a wound-healing activity equivalent to 42.9% ($p < 0.01$) above the control. The highest cicatrizant activity in mice was obtained by applying 40 μg of oleanolic acid per gram of body weight.

Impaired wound healing may cause severe health-related complications, such as infections and tissue necrosis. These ailments have spurred the search for wound-healing (cicatrizant) agents derived from ethnomedicinal sources.[1] Because there are several stages in the cicatrization process—each of which is not fully understood—the use of animal models is necessary for full scientific assessment.[2] In vivo assays require a large amount of resources and materials. Hence, it is not surprising to find that the vast majority of published papers using in vivo models have focused on crude or partially purified plant extracts.[3] Although the merits of in vivo versus in vitro assays of wound-healing plants have been recently questioned,[4] to the best of our knowledge, there are no commercial applications that use active principles identified through in vitro assays. However, taspine, the active principle in *Croton lechleri*, which we discovered through an in vivo guided fractionation,[5] is a constituent already present in two patented applications.[6]

The emphasis of our international and multidisciplinary research group is to seek, insofar as possible, wound-healing compounds whose structures are either readily available, amenable to scale-up, or easily derivatized. This effort was typified in our earlier work with *Peperomia galioides*,[7] where we found that (+)-anymol was the wound-healing principle. After screening other terpenoids that were structurally similar to (+)-anymol, we discovered that α-terpineol and α-bisabolol also exhibited wound-healing activity, but unlike (+)-anymol, they are readily available and inexpensive. As part of our ongoing search on traditional Peruvian wound-healing plants, we became interested in *Anredera diffusa* (Bassellaceae), commonly known as "Lloto". An infusion is used traditionally to wash external wounds, and the wet leaves are used as wound dressing. In a preliminary screening, we found that an ethanolic extract of the fresh leaves and stems of *A. diffusa* exhibited significant cicatrizant activity in mice and was found nontoxic in an acute toxicity assay.[8] A phytochemical inspection signaled the possible presence of saponins. This led us to speculate that if the cicatrizant activity was associated with a saponin, then perhaps its corresponding aglycon might retain the activity, which would in the end facilitate the isolation process and later quantitative structure−activity relationship studies.

The ethanolic extract of *A. diffusa* was then hydrolyzed in a mixture of dilute sulfuric acid and toluene without loss of wound-healing activity (see hydrolysate under fraction heading in Table 1). The ensuing chromatography of the toluene layer, without the presence of glycosides, was straightforward, yielding oleanolic acid (**1**), with a cicatrizant activity equivalent to 42.9% ($p < 0.01$) above the control at a concentration of 12.5 mg/mL (Table 1).



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As oleanolic acid (**1**) is commercially available (Sigma-Aldrich), we purchased a sample to carry out a preliminary dose−response study of its cicatrizant activity (Table 2). The highest activity was obtained by applying 40 μg of oleanolic acid per gram of body weight in mice. For the purpose of comparison, the ED_{50} values for taspine hydrochloride[5] and (+)-anymol[7] are 15 and 155 μg per gram of body weight in mice, respectively. However, the cytotoxicity of taspine is much higher.

In summary, acid hydrolysis of an ethanolic extract from the fresh leaves and stems of *A. diffusa*, followed by in vivo assay-guided fractionation, yielded oleanolic acid (**1**) as the major wound-healing constituent. This outcome, based on an ethnobotanical observation, resulted in the discovery of a secondary metabolite with wound-healing activity of potential significance in an animal model. The fact that oleanolic acid (**1**) is widely available should facilitate future structure−activity relationship studies on wound-healing potential.

Experimental Section

General Experimental Procedures. NMR spectra were obtained on a Bruker AC 300 spectrometer. Analytical TLC was performed using Macherey-Nagel Polygram Sil G/UV254-precoated plates. Packing employed for column chromatography was silica gel 40−63 μm (Lagand Chemicals, Inc.). Radial chromatography was performed on 1 or 2 mm silica gel-coated circular glass plates using a Chromatotron apparatus. HPLC was performed on a Rainin SD-200 instrument equipped with a multiwavelength detector using a reversed-phase C-18, 5 μm analytical, or semipreparative column.

Plant Material. *Anredera diffusa* (R. et P.) Soukup (Basellaceae) is a liana that grows in Lima and in some Andean valleys of Perú. The first plant collection took place in the valley of Ollantaytambo, Department of Cuzco, at 2800 m of altitude. Lic. Irma Fernández identified the plant and deposited a voucher specimen (IFV416) at the

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Table 1. Cicatrizant Activity Corresponding to the Fractionation of *Anredera diffusa*

fraction	concentration applied to mice (mg/mL)	fraction WBS ± SD[a] (g)	control WBS ± SD[a] (g)	cicatrizant activity (%)
ethanolic extract	25	42.5 ± 2.87	30.83 ± 4.56	37.9[b]
hydrolysate[c]	4	42.22 ± 3.77	33.68 ± 5.61	25.3[b]
oleanolic acid (**1**)	12.5	49.33 ± 7.03	34.5 ± 2.07	42.9[d]
(+)-anymol[e]	50	35.92 ± 4.66	30.56 ± 2.42	17.6[f]

[a] Wound-breaking strength ± standard deviation. [b] Significantly different from the control ($p < 0.05$). [c] Ethanolic extract was heated in a mixture of toluene and 1 M sulfuric acid for 42 h at 80 °C; the toluene layer was then separated and concentrated until dryness. [d] $p < 0.01$. [e] Positive control.[7] [f] $p < 0.05$.

Table 2. Relationship between Dose and Cicatrizant Activity of Commercial Oleanolic Acid (Sigma-Aldrich)

treatment[a] μg/g mouse	solution conc[b] (mg/mL)	WBS ± SD[c] (g)	cicatrizant activity (%)	significance[d]
0	control	31.92 ± 2.08		
30	7.5	40.02 ± 5.00	25.4	$p < 0.05$
40	10	44.46 ± 6.03	39.3	$p < 0.001$
50	12.5	41.78 ± 3.70	30.9	$p < 0.01$
60	15	40.28 ± 1.9	26.2	$p < 0.05$

[a] Total amount of oleanolic acid received by mouse after 48 h treatment. [b] Concentration of oleanolic acid solution applied to mouse every 12 h for 48 h in 25 μL dose. [c] Wound-breaking strength ± standard deviation. [d] Significantly different from the control.

Museo de Historia Natural Javier Prado de la Universidad Nacional Mayor de San Marcos in Lima, Perú. The plant is cultivated for ornamental purposes in residential areas of Lima. Later re-collections took place in Lima between March 1998 and April 2000.

Extraction and Isolation. Fresh leaves and stems of *A. diffusa* (269 g) were finely chopped and crushed in a Waring blender and macerated with 90% ethanol at room temperature. The concentrated extract (40 g) was hydrolyzed by refluxing in a mixture of 1 M H_2SO_4 (350 mL) and toluene (350 mL) for 36 h. The toluene layer was concentrated, and the residue (17.1 g) was extracted successively with petroleum ether (250 mL), ethyl acetate (250 mL), and methanol (250 mL), for 6 h each, using a Soxhlet apparatus. The methanolic phase was concentrated, and the resulting dark green oil (10.64 g) was subjected to flash chromatography (hexane/ethyl acetate gradient) followed by a secondary purification (cyclohexane/ethyl acetate gradient). Major fractions were evaluated for their wound-healing activity. An aliquot (100 mg) corresponding to the active fraction (695 mg) was fractionated using a Chromatotron apparatus (silica gel, cyclohexane/ethyl acetate gradient). The subfraction (32 mg) with R_f between 0.3 and 0.5 (hexane/ethyl acetate, 70:30) was further purified using HPLC (acetonitrile/water gradient), furnishing oleanolic acid (**1**, 10 mg), which was identified by comparing its spectroscopic data with those reported in the literature[9] and with an authentic sample purchased from Sigma-Aldrich.

Bioassay. The procedure described below is a modified version of our initial method to determine tensile strength.[5,8] Male mice (strain A) 2−3 months old, weighing approximately 25 g, were maintained in a room at 20−25 °C and received food and water ad libitum. Before making the wounds, the backs of the mice were shaved at the level of the scapular waist and then depilated with Opilca (Hoescht). After 48 h, the mice were weighed and grouped randomly. Each mouse was placed in a separate cage. A minimum of six mice was used for each fraction or compound tested. Next, the mice were anesthetized with diethyl ether vapor, and a 1 cm incision was made perpendicular to the axis of symmetry of the animal and the two borders of the wound were stitched together at its center. Each substance to be tested was dissolved in minimum amounts of DMSO or ethanol to achieve a maximum concentration of 50 mg/mL (stock solution). These stock solutions were further diluted with appropriate volumes of water to yield final concentrations of 4 to 25 mg/mL. Treatment was started immediately by applying the solution to be tested (25 μL) directly to the wounded area. This treatment was repeated every 12 h. The controls received only the solvent mixture in which the compound was diluted. After 48 h, the mouse was sacrificed with an ether overdose, and the wound-breaking strength (WBS) was quantified by fixing one of the borders of the wound (after cutting the stitch), while applying a measurable force to the other one.

The data were analyzed using the Student's *t*-test. Values are significant when $p < 0.05$.

The percentage of activity was calculated according to the following formula:

$$\% \text{ activity} = \frac{\text{WBSt} - \text{WBSc}}{\text{WBSc}} \times 100$$

WBSt = average of the force necessary to open the wound of a treated mouse and WBSc = average of the force necessary to open the wound of an untreated mouse (control).

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