PRODUÇÃO CIENTÍFICA EM CIÊNCIAS BIOLÓGICAS 2

Daniela Reis Joaquim de Freitas
(Organizadora)

Atena Editora
Ano 2022

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Daniela Reis Joaquim de Freitas

SUMÁRIO

CAPÍTULO 7

Melaleuca armillaris (Sol. Ex Gaertn.) HYDROLAT: USE IN RAT SKIN WOUND HEALING AND BLOOD ANALYSIS

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ABSTRACT: *Melaleuca armillaris* (Sol. Ex Gaertn.) Sm was introduced in Brazil from Australia and is commonly known as the honey bracelet. The trees reach 5m in height, growing in sandy soil with low water retention capacity. The objective of the present work was to evaluate the effect of the Melaleuca leaf hydrolate on wound healing in rats, as well as to evaluate the blood of the animals by biochemical tests. The objective of the present work was to evaluate the effect of the Melaleuca leaf hydrolate on wound healing in rats, as well as to evaluate the blood of the animals by biochemical tests. The leaves were collected from trees in Piedade, SP, brought to the laboratory in a Styrofoam refrigerator and the oil was distilled. After oil separation, the hydrolate was removed and the presence of 0.3% of oil was confirmed, containing 91.48% of 1,8 cineol and antioxidant activity equivalent to 494 umol Trolox. A gel with 10% of hydrolate was prepared. After approval by the Ethic Committee, 15 adult male Wistar rats (weight about 200-250g) were used and divided into three groups. The animals in the first group were treated with 1mL of the 10% hydrolate gel, those in the second group with 1mL of distilled water gel, and those in the third group with a thin layer of fibrinase. Daily application was performed on a 4 cm^2 square wound on the dorsal region of each animal. The wound was evaluated macroscopically in the predetermined periods (0, 2, 7, and 14 days). The macroscopic analysis of the evolution of the wound aspect and measurement of wound healing retraction was performed by digital planimetry. The blood biochemical test showed no difference, demonstrating that the hydrolate penetrates into the skin but has no action on the biochemical metabolism. Partial results of the healing indicated a statistical difference in the areas of the wounds treated with melaleuca when compared to the water-gel control. In conclusion, melaleuca hydrolate may come to assist in wound healing and be an alternative in the treatment of wounds.

KEYWORDS: Hidrolate of Melaleuca, rats, wound healing.

RESUMO: *Melaleuca armillaris* (Sol. Ex Gaertn.)

Sm, foi introduzida no Brasil oriunda da Austrália e comumente conhecida como Bracelete de Mel. As árvores chegam a 5m de altura, crescendo em solo arenoso ou com rochas com baixa capacidade de retenção de água. O objetivo do presente trabalho foi avaliar o efeito do hidrolato das folhas de Melaleuca, sobre a cicatrização de feridas em ratos, bem como, avaliar por testes bioquímicos o sangue dos animais. As folhas foram coletadas de árvores em Piedade, SP, trazidas ao laboratório em geladeira de isopor e realizada a destilação do óleo. Após a separação do óleo, foi retirado o hidrolato e confirmada a presença de 0.3% do óleo contendo 91,48% de 1,8 cineol e, atividade antioxidante equivalente a 494 umol Trolox. Gel foi preparado contendo 10% do hidrolato. Após aprovação no Comitê de Ética, foram utilizados 15 ratos da linhagem Wistar, machos, adultos (peso cerca de 200-250g), divididos em três grupos. Os animais do primeiro grupo foram tratados com 1mL do gel a 10% do hidrolato, os do segundo grupo, com 1mL de gel-água destilada e os do terceiro com uma fina camada de fibrinase. Foi realizada aplicação diária sobre ferida quadrada de 4cm^2 na região dorsal de cada animal. A avaliação da ferida foi feita macroscopicamente nos períodos pré-determinados (0, 2, 7, e 14 dias). A análise macroscópica da evolução do aspecto da lesão e medida da retração cicatricial da ferida foi realizada por planimetria digital. O teste bioquímico do sangue não apresentou nenhuma diferença demonstrando que o hidrolato penetra na pele mas não chega a ter ação no metabolismo bioquímico. Resultados parciais da cicatrização indicaram uma diferença estatística nas áreas das feridas tratadas com melaleuca quando comparadas com controle gel-água. Como conclusão o hidrolato de melaleuca poderá a vir auxiliar na cicatrização e ser uma alternativa no tratamento de feridas.
PALAVRAS-CHAVE: Hidrolato de Melaleuca, ratos, cicatrização.

1 | INTRODUCTION

Melaleuca, genus of *Myrtaceae* family, included 100 species that occur in Australia and Islets from Indic Ocean. The plant is known as “Tea Tree”, for *Melaleuca alternifolia* and “honey bracelet” for *Melaleuca armillaris* and produces volatile oil formed by a complex mixture of compounds obtained by steam distillation from leaves. The chemical studies of the volatile oil have reported about 100 constituents such as the actives terpinen-4-ol, α-terpineol and 1,8 cineole (Cox et al., 2001).

In Brazil, many different species from *Melaleuca* have been introduced as fresh tea tree plants, such as: *M. alternifolia* Cheel, *M. leucadendra* (L.). L., *M. armillaris Sm., M. quinquenervia* (Cav.) S.T. Blake, *M. cajuputi* Roxb. subsp*. cajuputi* Powell and *M. cajuputi* Roxb. subsp. *platyphylla* (Lemos et al, 2012; Silva et al., 2010). The species can be identified through key characters described by “ANH - Australian national herbarium (ALA, 2012)” and confirmed by photos of “ALA - Atlas of living Australia (ANH, 2012)” as well as by oil composition. The composition of tea tree oil has been regulated by the International Standard (ISO, 1996, 2004).

For *M. armillaris*, in Australian, a new Plant census was demark with scientific name *Melaleuca armillaris* (Sol. ex Gaertn.) Sm. subsp. *armillaris* (Chah, 2010; Cowley et al., 1990). The seeds from *M. armillaris* came from Australia and grown very well in São Paulo

but don´t have any analysis from the product. *M. armillaris* was cultivated in Bairro Gurgel, Piedade (Prop. Wolfgang Pickert with latitude 23º45`0.5 13``S and longitude 47º19´19.39´´, CEP 18700-000), São Paulo, and grows to about 5 meters high.

The objectives of present study were: identified the specie, analyze the chemical composition of the leaves extracts (hydrolat) and the essential oil and after evaluated the effect on wound healing in rats.

2 I MATERIALS AND METHODS

2.1 Plant Material

Plants of *Melaleuca armillaris* were collected in August 2012, grown in Piedade, São Paulo (Prop. Wolfgang Pickert), from 1-year plantation. Seeds originated from CSIRO-Australia. Voucher specimen has sent to deposited in herbarium of "Prefeitura Municipal de São Paulo (PMSP)". – verificar que no resumo e abstract esta que foi coletada em Ibiúna.

The specie was identified by key taxa from Australian national herbarium and confirmed by photos "ALA - Atlas of living Australia (ALA, 2012)" and by Chah (Chah, 2010). In key taxa must be observed the leaves, flowers, fruit, inflorescences, bark for checked as *Melaleuca armillaris* (Sol. ex Gaertn.) Sm. subsp. *armillaris.*

2.2 Extract from *Melaleuca armillaris* and antioxidants

Leaves were collected from the trees, in a completely randomized way and were transported to UNINOVE (University Nove de Julho, São Paulo) in a Styrofoam cooler. The essential oil was extracted by steam distillation of the leaves and terminal branches of *M. armillaris*. Once condensed, the clear to pale yellow oil is separated from the aqueous distillate, that is Hydrolate.

The technique for determined antioxidants was based on Rufino et al. (2007) and Sousa et al. (2007). The method is based on electron transfer where, by the action of an antioxidant (AH) or a radical species, the DPPH that has purple color is reduced forming diphenyl-picrylhydrazine, yellow, with consequent disappearance of absorption, which can be monitored by the decrease in absorbance. For the DPPH method first the 0.6 mm DPPH stock solution was prepared in methanol and stored at -20 °C and in the dark. Every time it is necessary to use prepare a diluted DPPH solution in methanol with an absorbance of 1.1± 0.02 at 515 nm. Standard solution was evaluated with Trolox at concentrations between 100 to 1000 µM. The reaction involves 975 µL of DPPH reagent at room temperature, adding 25 µL of Trolox and monitoring absorbance at 515nm for 6 minutes. The results were expressed as µmol Trolox.

2.3 Chromatography Conditions- GC/MS

The GC (gas-chromatography) analysis for the identification of constituents present

in the essential oil and aqueous distilled samples were carried out with Hewlett-Packard 5890 Series II apparatus equipped with a FID detector, an Agilent BPX5 cap. column (5%-phenyl)-methylpolysiloxane; 30m x 0.25mm i.d., film thickness 0.5mm), an automatic injector (HP 7673) and an electronic integrator (HP 3396A). The oven temperature was programmed rising from 60 to 320°C at 3ºC.min^{-1} and then held isothermal at 320°C for 9min. The injector and detector temperatures were 280°C. Helium gas was used as the carrier gas at a constant flow rate of 0.5mL.min^{-1}. The calculation of the concentrations of the compounds was based on the areas of the peaks in the chromatogram, following the elution order on the BPX-5 column.

GC/MS analysis: The essential oil was analyzed with a Shimadzu CG-17A apparatus equipped with a MS-QP-5050A mass selective detector operating by electronic impact (70 eV) and a DB-5 cap. column (30 m x 0.25 mm i.d., film thickness 0.25 mm). He (1 mL.min^{-1}) was used as carrier gas. The GC analytical conditions were as described above (see GC Analysis).

Compound identification: The identification of the compounds was based on the comparison of their retention index (RI determined relatively of the t_R of n-alkanes) (Adams, 2001) and mass spectra with those of authentic compounds by means of the program CLASS-5000, version 2.23 (Shimadzu Corporation), equipped with the commercial libraries NIST 21, NIST 107 and Wiley 229.

2.4 Animals

The experiment was approved by UNINOVE´s Ethics Committee with protocol number AN 0037/2013). A total of 15 male Wistar rats were used, with a controlled body mass of 275± 25g, aging from 12 ± 2 weeks old. All animals were obtained from the University vivarium (UNINOVE), and were kept in cages, grouped in groups of 5 animals/cage, and kept under controlled environment, with average temperature of 26°C and 12 hours light/dark cycle, with water *ad libitum* and balanced feed.

Animals were initially weighed, and then anesthetized with xylazine (0.5mg) and ketamine (0.1mg). After 5 minutes of anesthesia started, the dorsal region of each animal was shaved. After local asepsis, a surgical incision of 4cm² was performed at the trichotomized area, and topical treatment begun. Animals were separated in individual cages and submitted to the following treatment: a) syringe containing 1mL of gel-water (negative control); b) syringe containing 1mL of gel + 10% hydrolate; c) a thin layer of gel-free fibrinase (SG) (positive control). Skin healing results were observed at days 2, 4, 7 and 14. Wounds were photographed, and total wound area evaluated by ImageJ software (NIH, USA).

At the end of the experimental period, the animals were anesthetized with a lethal dose of a cocktail containing ketamine (1 mg), xylazine (5 mg), and acepromazine (0.2 mg). Thoracotomy was performed. Blood was collected from the left ventricle and centrifuged.

The plasma was removed and stored at –20º C for no longer than three days before the assay. Total cholesterol, triglycerides, urea and creatinine were measured using test kits (Labtest Diagnostica).

2.5 Statistical analysis

All data was evaluated using average ± standard deviation, and evaluated using One-Way ANOVA variance analysis, using Assistat *software*, with a significant result considered *P <0,05.

3 I RESULTS AND DISCUSSION

3.1 Taxonomic identification and chemical analysis

Melaleuca genus belongs to the *Myrtaceae* family, and almost species are native from Australia (ALA, 2012). When left to grow naturally, the plants demonstrated a difference in height of trees, for example, *M. alternifolia* grows of approximately 8 meters compared to 4-5 meters when is cultivated and chemical compounds presented different concentrations (Colton and Murtagh, 1999). For taxonomic identification, in internet have the "ANH - Australian National Herbarium (ANH, 2012)" that can be compared with the photos from all species in "ALA - Atlas of living Australia (ALA, 2012)". Chah (Chah, 2010) proceeded a new revision from all species.

The first identification was related to leaves: that is usually less than 4mm long, sessile (identify *M. irbyana*) or; more than 5mm long, petiolate or sessile. After the leaves was opposite or scattered. With the flowers that can observed: Flowers are red, staminal bundles more than 20mm long (identify *M. hypericifolia*) or; flowers are mauve, pink, white or yellowish and stamina bundles than 20mm long. When the flowers are white and inflorescences lateral, the specie was *M. acuminata* and, when flower was pink color with the same inflorescences the specie was *M. thymifolia*.

But when the leaves was scattered, alternate; less than 3mm wide; more than 5mm long and fruit also scattered; flowers in spikes with white color; staminal claw more than 5mm long but inflorescences axillary, borne low on the branchlets; bark corky or hard the specie was identified as *Melaleuca armillaris* and Chah (2010) named as *Melaleuca armillaris* (Sol. ex Gaertn.) Sm. subsp. *armillaris*.

When compared with *M. alternifolia* in the set of observations is the same as *M. armillaris*, but the inflorescences were terminal and borne at the ends with bark papery.

In conclusion the plant cultivated in Piedade was identified as *Melaleuca armillaris* (Sol. ex Gaertn.) Sm. subsp. *armillaris* (Chah, 2010; Cowley et al., 1990) (Figure 1).

Table 1 shows the chemical composition of the essential oil from *Melaleuca armillaris* (Sol. ex Gaertn.) Sm. subsp. *armillaris* (extracted August 2012) and the compounds are listed according to their elution order on GC/MS. In total, 15 compounds, representing

100% of the chemical composition of the oil sample, were identified. The major components present in the essential oil were 1,8-cineole (91.48%), myrcene (3.53%) and α-terpineol (3.42%).

Chabir et al. (2011) and Amri et al. (2012) extracted volatile oil from leaves of *Melaleuca armillaris* in Tunisia and observed thirty-two components representing more than 98% of the total composition of the essential oil and identified 85.8% from 1,8-cineole, camphene (5.05%) and α-pinene (1.95%). Farag et al. (2004) analized essential oil from *M. armillaris* leaves in Egypt, and observed 33.9% of 1,8-cineole. When compared results from São Paulo with Tunisia and Egypt, the major component was 1,8-cineole but in different percentual of concentrations. The other compounds were not the same. This difference in the chemical composition of the essential oil could be related to seasonal variations such as: temperature, humidity in environmental, quality, micronutrients (N, P, K) and water availability in soil, and presence of sun (Sayuri et al., 2010; Simões et al., 1999). Other factor that required careful it is in collection from plants that must made in early morning when have no much Sun because can loss compound from oil (Simões et al, 2003).

Figure 1- *Melaleuca armillaris* (Sol. ex Gaertn.) Sm. subsp. *armillaris* from Piedade. Photo by Erna Bach (august 2018).

N°	IK	Compound	% relative
1	991	Myrcene	3.53
2	1031	limonene	0.88
3	1033	1,8-cineole	91.48
4	1062	alfa-terpinene	0.03
5	1118	myrcenol	0.02
6	1177	terpinen-4-ol	0.14
7	1189	1,2 terpineol	3.42
8	1404	(*Z*)-caryophyllene	0.03
9	1418	(*E*)-caryophyllene	0.15
10	1441	alfa-humulene	0.07
11	1454	2-humulene	0.09
12	1524	1-cadinene	0.06
13	1655	alfa-bisabolol B oxide	0.03
14	1683	1-bisabolol	0.04
15	1744	2-bisabolol A oxide	0.03
Total identified			100.0

Table 1: Chemical constituents [%] of the essential oil from leaves of *Melaleuca armillaris* (Sol. ex Gaertn.) Sm. subsp. *armillaris.*

When the aqueous distillate was observed by GC/MS one peak corresponded to 1,8-cineole was present that can be represent from 3 to 5% of oil in hydrolate.

The antioxidant activity based on Trolox showed 494.8 mmol TEAC/g leaf. The antioxidant activity is important in terms of stimulating new skin tissue, collagen production, having anti-inflammatory properties, and decreasing scar formation in chronic wounds.

3.2 Animals

The wound induction consisted of a single dorsal skin fragment, according to the technique described by Prata et al. (1988). The dorsal region was chosen because it is difficult to manipulate by the animals. In this experiment, dressings were not performed, and the wounds remained unprotected, since the use of dressings on the skin surface of rats is difficult to adapt and maintain, besides causing trauma by contact with the wound. It is a complex, dynamic process of reestablishing the cellular structures and, consequently, the layers of epithelial tissue. This process is carried out in order to establish a re-epithelialization closer to its normal state (Kumar et al., 2006).

For the treatments we used a 10% gel, according to the orientation of the Association of Pharmaceuticals-Homeopathy from Brazilian -ABFH- (1995), which in its technical norms for the preparation of medicines, recommends for pharmaceutical forms for external use a 10% preparation, whether for cream, gel or ointment. The option for the gel and not the liquid form of the hydrolat is due to its adherence on the injured site, with longer duration of action.

As for the option for the gel, it is due to the easy acquisition and manipulation of the vehicle, which allows its preparation by hand. This facilitates its use in the human area. To measure the healing areas there are several methods and, in the project were used morphometry system by pachymetry, photo followed by software and computer measurement.

The fibrinase ointment (fibrinolisin, deoxyribonuclease, and chloramphenicol) was used as a positive control because it is indicated for the treatment of infected skin lesions (lesions on the skin with infection) such as burns, ulcers (superficial skin lesions), and wounds. It consists of an association of active enzymes (substances that activate a chemical reaction, in this case accelerating the destruction of cells damaged by inflammation, infection, or cauterization) and a bacteriostatic antibiotic (type of antibiotics that prevent bacteria from reproducing, but do not kill them directly). This combination acts doubly as a debridement agent (removes substances deposited on the skin surface) and a topical antibiotic, keeping the area clean so that healing can occur properly.

Wound areas were measured at all time intervals (2d, 7d, and 14d) and the results were plotted on the graph of the averages of the rats per group (Figure 2). Observing the healed area, the animals treated with gel + melaleuca hydrolate had an increase or acceleration in tissue recovery when compared with control and with the ointment. A macroscopic evaluation of each lesion was also performed, taking the following aspects: presence of edema, hyperemia, exudate, granulation tissue and re-epithelialization.

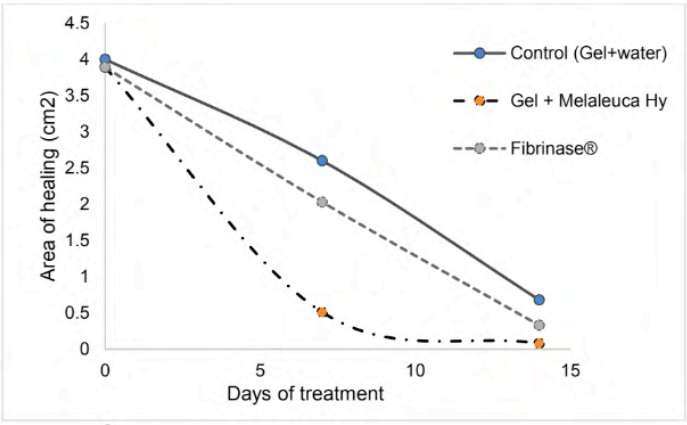

Figure 2: Area in cm^2 of wounds in the healing period in rats using fibrinase, melaleuca in the form of hydrolate (Hy) incorporated with gel and, control (gel-water).

With the macroscopic observation, in relation to the control animals and with fibrinase, these on the second day after surgery, serous-saginolent exudate, hyperemia and edema were observed in the wounds. In the melaleuca group the healing area remained dry with no sero-saginolent sign. On the seventh day in the control group and fibrinase already appeared a thin crust what can be observed in the percentage of healed area where fibrinase

had 49.29% compared to the control with 60% while, with melaleuca, the percentage was 86.89% having a well-defined crust. In 14 days the wounds of the animal with melaleuca practically closed (96.15%) while in fibrinase still had small pieces finishing the healing and reaching 90.75% and the control animal having 73% of healed area. The results were in agreement with the description of Mott et al. (2003), which states that the exponential period occurs between six and fifteen days after the injury, and corresponds to the maximum level of activity of the myofibroblasts that are part of the granulation tissue.

	Time	(days)	
Treatments	**2d**	**7d**	**14d**
Fibrinase®	19.00a*	49.29a	90.75a
Control (Gel+ water)	18.75a	60.00b	73.00b
Gel + melaleuca Hy	28.03b	86.89c	96.15c

*Media from wound area five animals in %.

Different letters in the columns are statistically different from each other ($p<0.05$).
Table 2: Percentage of healing or recovery in the treatment period.

The treatments showed no difference in biochemical tests indicating no metabolic changes when using the melaleuca gel (Table 3) this means that the hydrolat penetrates into the wound but not into the bloodstream and may bring no differences in biochemical metabolism.

Treatments*	Glycemia (mg/dL)	Total Cholesterol (mg/dL)	TAG (triglycerides) (mg/dL)	Urea	Creatinine (mg/dL)
Fibrinase®	91 ± 3	74 ± 10	91 ± 18	32 ± 3	0,55 ± 0,11
Control (Gel+ water)	89 ± 4	72 ± 10	90 ± 17	32 ± 4	0,56 ± 0,10
Gel + melaleuca Hy	90 ± 2	73 ± 10	91 ± 18	34 ± 2	0,57 ± 0,09

No difference statistically in collums (ANOVA + Tukey's test).

Table 3: Biochemical results of plasma from rats submitted to treatments.

In conclusion *Melaleuca armillaris* hydrolate could be used to help in skin healing process and can be an alternative for a low-cost skin wound treatment in humans.

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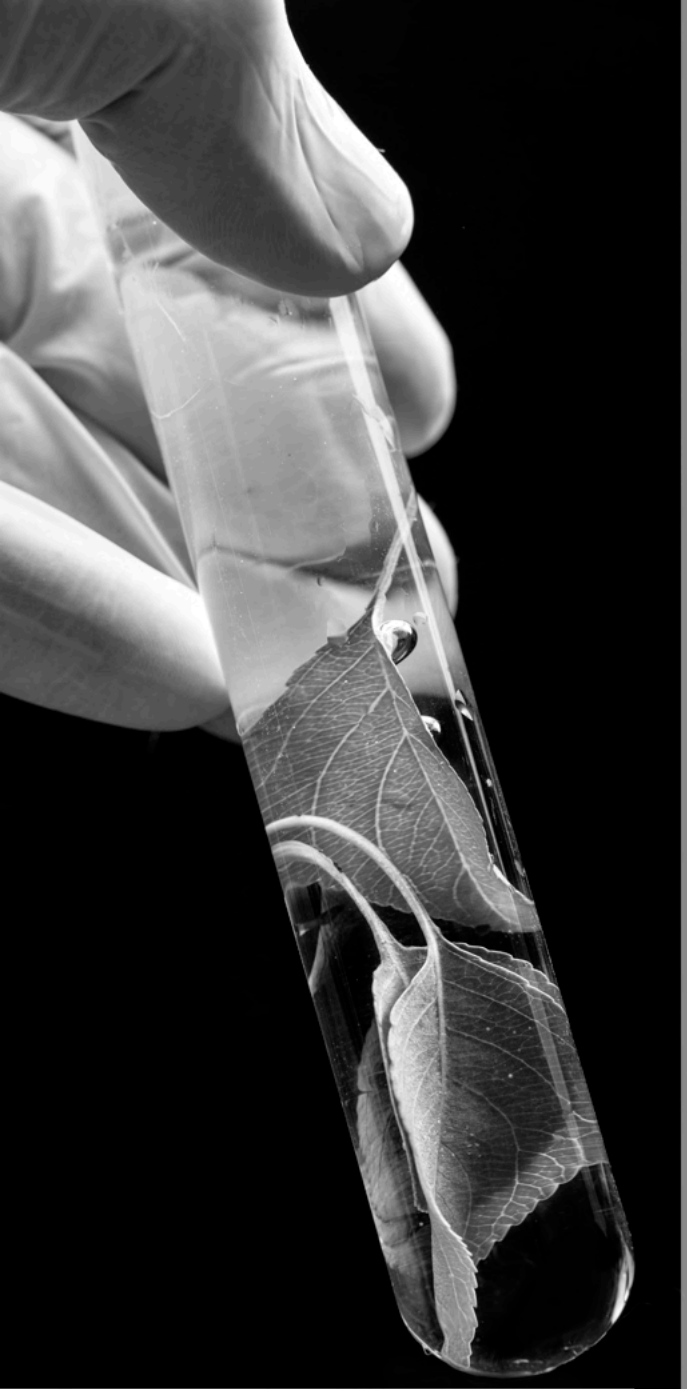

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