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Effect of hydrolat from *Melaleuca alternifolia* (Maiden & Betche) Cheel leaves in rats skin wound healing

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ABSTRACT: *Melaleuca alternifolia* (Maiden & Betche) Cheel was introduced in Brazil from Australia and is commonly known as the tea tree. The trees reach 5m in height, growing in sandy soil with low water retention capacity. The objectives of the present study were to identify the species cultivated, analyze tea tree oil and hydrosol, and evaluate the effect of hydrosol from its leaves on skin wound healing in rats. *Melaleuca* samples were obtained from Sitio Melaleuca, Ibiuna, São Paulo, and the oil was obtained using steam drag distillation technique, with the posterior collection of hydrosols containing 3 to 5% oil. A gel with 10% hydrosol was prepared. Thirty rats (Ethics Committee UNINOVE AN 37/2014) were separated into three groups: one group was treated with 1mL of hydrosol gel, another group with 1mL of distilled water/gel (negative control), and the third group with a thin layer of fibrinase® (positive control). Daily application was performed on a 4cm^2 square wound in the animal dorsal region and observed for 14 days. Results indicated a statistical difference in the wound areas of animals treated with hydrosol from *Melaleuca* gel and the control animals. At the end of 14 days, the wounds of animals treated with hydrosol gel were fully healed, corresponding to 91.3% better response when compared to the control group. Animals treated with fibrinase® achieved only 63.9% more than the control animals. Conclusion: Tea tree (*Melaleuca alternifolia* (Maiden & Betche) Cheel) hydrosol can help with skin healing and can be used as an alternative treatment for healing wounds in humans at a low cost.

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KEYWORDS: hydrosol, *Melaleuca alternifolia*, healing wounds, gel

1. INTRODUCTION

Melaleuca alternifolia (Maiden & Betche) Cheel was introduced in Brazil from Australia and is known as "tea tree". The trees reach 5m in height, growing in Sandy soil with low water retention capacity (Craven and Lepschi, 1999).

Inflorescences are terminal or axillary, growing on branches extremities (Lee et al., 2002), with tree bark presenting a paper-like shedding. Leaves submitted to extraction produce volatile oil and hydrosol (Craven, 1999). Identification of the tree species is performed using identification keys described by the Australian government as "ANH – Australian National Herbarium" Australia (ANH, 2012)", and the volatile oil composition is regulated by standards established by the International Organization for Standardization (ISO 1996, 2004).

Essential oils (volatile) are a complex mixture of natural compounds of an extremely volatile nature with hydrophobic characteristics, characterized by strong odor and possessing a diverse range of therapeutic properties. The characteristic aroma originates due to the high variability of chemical compounds (Costa, 2008, Cunha et al., 2009). Tea tree oil has been commonly extracted by drag distillation of *M. alternifolia* leaves vapor. The oil is liquid, viscous, yellowish, and has the characteristic smell of a mixture composed of monoterpene, terpineol, cineol, and other compounds (Southwell and Russel, 2002). Tea tree oil exhibits a broad-spectrum antimicrobial activity, that may be attributed mainly to terpinen-4-ol (Carson et al, 2006; Russell and Southwell, 2003, Lam et al, 2020; Yang et al, 2020).

According to ISO 4730:2004 "Oil of *Melaleuca*", the main constituents present in the oil are terpinen-4-ol (30% minimum), 1,8-cineole, γ-terpineol, and α-terpinene. However, there can still be isolated: α-pinene, sabinene, *p*-cimene, terpinolene, α-terpineole, aromadendrene, viridiflorene, δ-cadinene, globulol and viridiflorol (Hammer et al., 2012; ISO, 1996).

The oil presents antimicrobial activity against both gram-positive and gram-negative bacteria (Carson et al., 2006; Hammer et al., 2003; Lam et al., 2018; LAM et al, 2020). These antimicrobial activities have allowed tea tree oil to be introduced in cosmetics aiming to treat acneic skin, with, or without, associated inflammation (Enshaieh *et al.*, 2007; Sharifi-Rad et al, 2017). The oil has been demonstrated as effective against some multi-resistant strains of bacteria, therefore acting as an alternative treatment in cases of grave infections (Furneri et al., 2006; Warnke *et al.*, 2013). The volatile tea tree oil should not be used orally, with reports of toxicity after consumption of the compound (Hammer et al, 2006)

It is known that the compound 1,8-cineole causes skin rash in concentrations over 10%, developing an allergic dermatitis on contact. Due to this property, the concentration commonly employed in cosmetics is 7% or lower (Groot and Schmid, 2016; Hammer et al., 2006; Southwell et al, 1997). The oil has been used in Australia, however in the present study will be used a melaleuca hydrosol, containing between 3-5% of oil extracted from Brazil-grown trees.

Thus, the objective of the present study was to identify the variety of plants being farmed, analyze the oil extracted from given plants and evaluate melaleuca tree leaves hydrosol, using gel as pharmaceutical presentation, and assess the effects of the hydrosol-gel over skin wound healing in rats through macroscopic evaluation of the skin healing process.

2. MATERIAL AND METHODS

Identification of Melaleuca species and oil extraction

The species was identified by key taxa from the Australian National Herbarium and confirmed by photos "ALA - Atlas of Living Australia (ALA, 2012)" and by Chah (Chah, 2010). Key taxa identify the species by characteristics of leaves, flowers, fruit, inflorescences, and bark. All observations led to identifying the species as *Melaleuca alternifolia* (Maiden & Betche) Cheel.

Melaleuca alternifolia plants of 1 year of age were used for the experiment, and fresh leaves were harvested, and immediately submitted to a volatile oil extraction process by steam drag and separating the oil from the hydrosol. All procedures were performed at Sitio das Melaleucas, Ibiuna-SP, which farms the species used in the present study. Hydrosol-gel was prepared to contain 10% of hydrosol, and this hydrosol contained from 3 to 5% oil.

Chromatography Conditions- GC/MS

The GC (gas-chromatography) analysis for identification of constituents present in the essential oil was carried out with Agilent 7820A with Colum: HP-5 30m x0.25um (Agilent Technologies, Saint Clare, USA). Injector: 200ºC split: 1/50. Detector FID:220ºC, injection volume: 1uL (conc 1% in chloroform). Helium gas was used as the carrier gas at a constant flow rate of 0.5mL.min^{-1}.

Animals

The experiment was approved by UNINOVE´s Ethics Committee with protocol number 37/2014. A total of 30 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 animal care unit and were kept in cages, grouped in groups of 5 animals/cage, and kept under a controlled environment, with an 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 began. Animals were separated in individual cages and submitted to the following treatment: a) a syringe containing 1mL of gel-water (negative control); b) a syringe containing 1mL of gel + 10% hydrosol; c) a thin layer of gel-free fibrinase® (SG) (positive control). Skin healing results were observed on days 2, 5, 7 and 14. Wounds were photographed, and the total wound area was evaluated by ImageJ software (NIH, USA).

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. RESULTS AND DISCUSSION

Identification of species from Melaleuca

Melaleuca genus belongs to the Myrtaceae family, and almost all 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 for approximately 8 meters of height, compared to 5 meters when farmed (Colton and Murtagh, 1999).

For taxonomic identification, the "ANH - Australian National Herbarium (ANH, 2012)" is available online, and can be used to compare photos from all species in "ALA - Atlas of living Australia (ALA, 2012)". Chah (Chah, 2010) proceeded a new revision from all species.

The identification was based on morphological analysis of plants structures: Leaves: that is usually larger than 5mm long, petiolate, or sessile, flat or concave on the lower surface. Flowers: white, staminal claw longer than 5mm long. Inflorescences: terminal or axillary, borne at the ends of branchlets. Bark: brown with a paper-shedding aspect. All morphological characteristics indicated that the species was *Melaleuca alternifolia*.

In conclusion the plant cultivated in Ibiuna was identified as *Melaleuca alternifolia* (Maiden & Betche) Cheel. (Chah, 2010; Cowley et al., 1990) (Figure 1).

Figure 1. Flower from *Melaleuca alternifolia* (Ibiuna, SP). Photo by Erna Bach (September 2022).

Extraction

Plant harvest was made in the early morning, avoiding intense sunlight that may lead to a lower oil yield (Simões et al., 2003). After extraction, hydrosol was separated from the oil. Hydrosols contained from 3-5% oil concentration. The hydrosols were added to a gel and submitted to gas-chromatography analysis. Results demonstrated a peak of terpinen-4-ol (Table 1), a compound present in *Melaleuca alternifolia* according to the International Organization from Australia (Altman, 1989; AS, 1989; ISO, 1996, 2004).

Table 1: Chemical constituents [%] of the essential oil from leaves of *Melaleuca alternifolia* (Maiden & Betche) Cheel.

N°	IK	Compound	% relative
1	977	alfa-thujene	1.1
2	980	alfa-pinene	2.8
3	1000	sabinene	0.9
4	1002	Beta-pinene	0.9
5	1012	mircene	0.9
6	1019	Alfa-felandrene	0.4
7	1027	Alfa-erpinene	11.0
8	1033	p-cimene	2.2
9	1035	limonene	1.8
10	1037	1,8-cineole	2.4
11	1059	gama-terpinene	22.6
12	1081	terpinolene	4.0
13	1166	Terpinen-4-ol	32.1
14	1179	alfa-terpineol	2.5
15	1424	Beta-gurjunene	2.1
16	1484	viridiflorine	2.8
17	1516	cis-calamene	2.1
		others	7.5

Animals

Animals from groups treated with gel with hydrosol (gel-hydrosol) started to present a skin wound healing process on par with the observed in animals of groups treated with fibrinase® and control. On the 14th day, the group treated with gel + hydrosol presented a total healed area of 91.3% when compared to the control group. Fibrinase® acted as a positive control, thus promoting skin healing, however when compared to the control group, only a 63.9% healing was observed. These results demonstrated that gel + hydrosol was more effective than fibrinase in the skin healing process.

Rats submitted to treatment with pharmaceutical presentation of gel + hydrosol, besides decreasing the lesioned area during skin healing, did not present any evidence of allergic reaction, and skin growth was observed in these animals.

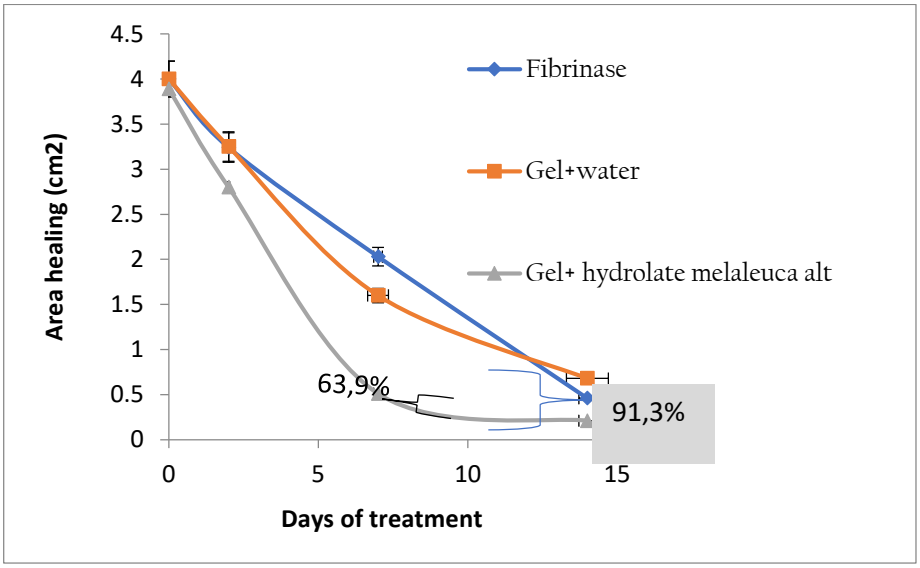

Figure 2: Graphic displaying time and area of skin healing in rats treated with gel + hydrosol; gel + water; fibrinase. Points based on the average value of n = 5 rats, with standard deviation bars displayed.

Gel is an easily appliable pharmaceutical presentation, and hydrosol is a byproduct of the melaleuca oil extraction process, being commonly discarded by the manufacturer, due to being the resultant water from the distillation process. However, it still contains the same volatile oil in its composition, but in lower concentrations, therefore the hydrosol could still be used as an adjuvant for skin healing processes, keeping the lesion humid, thus creating a better environment for skin healing, a hypothesis that was verified in the present study.

The present study demonstrated that it is possible to use melaleuca hydrolat efficiently to help in skin healing processes, and in future works, clinical trials with this gel in venous lesions are to be evaluated, due to the low cost and easy applicability of the product.

4. CONCLUSION

Melaleuca alternifolia (Maiden & Betche) Cheel hydrosol could be used to help in the skin healing process and be an alternative for a low-cost skin wound treatment in human medicine.

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