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In vitro and *in vivo* assessment of the effect of *Laurus novocanariensis* oil and essential oil in human skin

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Synopsis

Laurus novocanariensis is an endemic plant from the Madeira Island forest that derives a fatty oil, with a strong spicy odour, from its berries that has been used for centuries in traditional medicine to treat skin ailments. This work aimed to investigate the effect of the application of both the oil and its essential oil on normal skin, to assess their safety and potential benefits. Diffusion studies with Franz cells using human epidermal membranes were conducted. The steady-state fluxes of two model molecules through untreated skin were compared with those obtained after a 2-h pre-treatment with either the oil or the essential oil. Additionally, eleven volunteers participated in the *in vivo* study that was conducted on the forearm and involved daily application of the oil for 5 days. Measurements were performed every day in the treated site with bioengineering methods that measure erythema, irritation and loss of barrier function. Slightly higher steady-state fluxes were observed for both the lipophilic and the hydrophilic molecule when the epidermal membranes were pre-treated. Nevertheless, such differences had no statistical significance, which seems to confirm that neither the oil nor the essential oil impaired the epidermal barrier. Results collected with the Chromameter, the Laser Doppler Flowmeter and the visual scoring are in agreement with those established in the *in vitro* study. They indicate that the repeated application of the oil did not cause erythema, because the results observed in the first day of the study were maintained throughout the week. Application of the oil did not affect the skin barrier function, because the transepidermal water loss remained constant throughout the study. The stratum corneum hydration was slightly reduced on days 4 and 5. This work shows that both the oil and the essential oil were well tolerated by the skin and did not cause significant barrier impairment or irritation.

Résumé

Laurus novocanariensis est une plante endémique de la forêt l'île de Madère qui contient une huile grasse de forte odeur épicée dans ses baies qui a été utilisée pendant des siècles en médecine traditionnelle pour traiter des maladies de la peau. Ce travail visait à étudier l'effet de l'application de l'huile et de son huile essentielle sur la peau normale, afin d'évaluer leur innocuité et les avantages potentiels. Des études de diffusion ont été menées avec des membranes épidermiques humaines en utilisant les cellules de Franz. Le flux à l'équilibre de deux molécules modèles à travers la peau non traitée a été comparé à celui obtenu après quelques 2 heures de prétraitement avec soit de l'huile ou de l'huile essentielle. En outre, onze volontaires ont participé à l'étude *in vivo*, qui a été menée sur l'avant-bras et a impliqué l'application quotidienne de l'huile pendant 5 jours. Des mesures ont été effectuées chaque jour sur le site traité avec des méthodes de bio-ingénierie qui mesurent l'érythème, l'irritation et la perte de la fonction de barrière. Des flux à l'équilibre légèrement plus élevés ont été observés à la fois pour la molécule lipophile et la molécule hydrophile, lorsque les membranes épidermiques ont été prétraitées. Néanmoins, ces différences n'étaient pas statistiquement significatives, ce qui semble confirmer que ni l'huile ni l'huile essentielle porté atteinte à la barrière épidermique. Les résultats recueillis avec le Chromamètre, le débitmètre Laser Doppler et par la notation visuelle sont en accord avec ceux établis dans l'étude *in vitro*. Ils indiquent que l'application répétée de l'huile ne cause pas d'érythème, puisque les résultats observés lors du premier jour de l'étude ont été maintenus tout au long de la semaine. L'application de l'huile n'a pas d'incidence sur la fonction barrière de la peau, puisque la perte insensible en eau est restée constante tout au long de l'étude. L'hydratation du *stratum corneum* était légèrement réduite aux jours 4 et 5. Ce travail montre que l'huile et l'huile essentielle ont été bien tolérées par la peau et n'ont pas causé de détérioration de la barrière significative ou d'irritation.

Introduction

Plant extracts and essential oils are classic ingredients in both drugs and cosmetics; however, in recent years, a considerable effort has been made by the industry to discover, or even rediscover in most cases, natural resources with effective bioactivities [1]. 'Naturals' are usually considered safer than synthetic materials by consumers; nevertheless, physicians often establish a link between the use of such products and the occurrence of adverse effects, as well as interactions with prescribed drugs [2, 3].

Laurus novocanariensis (the *Laurus* subspecies found in the Madeira archipelago, Portugal) produces ripe berries from which an oil can be expressed that has been used for centuries in traditional medicine. The Mediterranean species, the common *Laurus nobilis*, produces a solid fatty material that is used to make soaps.

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This fat has a melting point of 40–60°C with *essential oil* content of 2.5–3.5% and is also commercialized for veterinarian uses.

Madeira Laurel oil has a melting point > 5°C, and it is mostly constituted by triglycerides (oleic acid (30.1%), linoleic acid (20.5%), palmitic acid (22.4%), lauric acid (14.0%)) and lactones (5%) and exhibits an unusually high content in essential oil (7–10%) [4]. The main terpene present in its essential oil is (cis + trans) β-ocimeno (40%) [4].

Although its clinical properties remain scientifically unproven, ingestion of the oil is recommended to treat gastritis, constipation and respiratory ailments, but it is also applied topically to treat infections or to promote skin healing [5]. Madeira laurel oil can be mechanically extracted and percolated at room temperature, but a higher yield is obtained with the method practiced by local producers, where the berries are boiled in water before crushing and pressing [4].

The literature contains few references to the bioactivity of laurel oil, and its claimed medicinal properties have not yet been validated. To date, only anti-mycobacterial activity was established, linked to two sesquiterpenic lactones – costunolide and dehydrocostuslactone [5, 6]. Because sesquiterpene lactones are referenced as source of contact dermatitis [7], this study aimed to assess the safety of both the oil and essential oil, as well as any benefits of the application of these ingredients on normal skin.

Fatty acids, essential oils and terpenes have been extensively reported as skin penetration enhancers [8–10]. The first part of the work investigated the influence of pre-treating epidermal membranes with the two materials in the percutaneous penetration of a hydrophilic and a lipophilic model molecule. *In vitro* diffusion studies with Franz cells were conducted, and the steady-state fluxes of caffeine and ibuprofen obtained through treated and untreated skin were compared. The second part of the investigation addressed the *in vivo* effects on the skin through bioengineering methods that measure erythema, irritation and loss of barrier function.

Materials and methods

Laurus novocanariensis oil (vegetative cycle 2010/2011) was purchased from local producers at Ponta do Pargo (Madeira, Portugal); *Laurus novocanariensis* essential oil was obtained from its oils by hydrodistillation in a Clevenger type apparatus for 4 h. The isolated oil was dried with anhydrous sodium sulphate and stored at 10°C in the dark prior to analyses; caffeine was purchased from Fragon Ibérica (Barcelona, Spain); ibuprofen from Sigma-Aldrich GmbH (Steinheim, Germany); propylene glycol (PG) from José M. Vaz Pereira SA (Lisbon, Portugal). Solvents and other reagents were of analytical grade (Sigma-Aldrich, Steinheim, Germany).

Donor solutions

Caffeine solution: ethyl alcohol absolute 60%, propylene glycol 35%, distilled water 4%, caffeine 1%; Ibuprofen solution: Ethyl alcohol absolute 60%, propylene glycol 31%, distilled water 4%, ibuprofen 5%.

In vitro permeation studies

Human abdominal skin tissue from a female donor, obtained following informed consent, was used to produce epidermal membranes. Ethical approval was provided by the Ethics Committee of the Faculty of Health Sciences of the Lusófona University. After removal of the adipose tissue by blunt dissection, the epidermis was separated by immersing the skin in water at 60°C for 1 min [11]. It was then pinned on a corkboard, the epidermis was carefully peeled away from the dermis and mounted on filter paper, after which it was stored in a freezer at −20°C until required. Prior to the diffusion experiment, the epidermis was defrosted and cut to appropriate size.

Permeation experiments (n = 5) with epidermal membranes were conducted on glass Franz type diffusion cells with a receptor volume of ~4 mL and a diffusional area of 0.95 cm^2. The continuously stirred receptor medium was isotonic phosphate-buffered saline (PBS, pH = 7.4). The receptor compartment was thermostated at 37°C.

For each model drug, diffusion experiments were conducted with 3 series of 5 replicates each. In the first series, 95 μL of laurel oil was placed in the donor compartment and left for 2 h, after which the oil was removed with a Pasteur pipette, and the surface was washed 3 times with water. The same procedure was conducted in the second series, but this time, 150 μL of the essential oil was applied. The third series was used as an untreated control. After this pre-treatment, 500 μL of the donor solution containing the model drug (caffeine or ibuprofen) was applied to each cell of the three series.

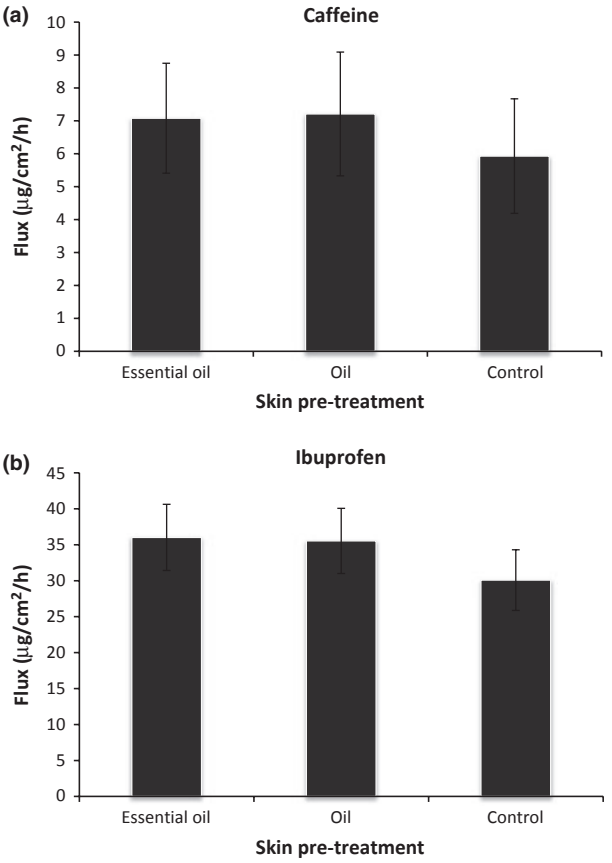

Figure 1 Steady-state fluxes of caffeine (a) and ibuprofen (b) after different skin pre-treatments (mean ± SD, n = 5).

The diffusion experiments were performed under occluded conditions by sealing the donor compartment with microscope coverslips. At designated time intervals, the receiver solution was withdrawn completely from the receptor compartment and immediately replaced with fresh and pre-thermostated PBS. Quantitative analysis of the drugs was performed on a UV-Vis spectrophotometer (Evolution 600, Thermo Scientific, U.K.) at 273 nm for caffeine and at 221 nm for ibuprofen [12].

Flux values for each individual diffusion experiment were calculated by monitoring the cumulative amount of drug diffused and measuring the slope of the graph once steady-state diffusion was reached.

In vivo studies

Skin tolerance studies using the 'open-test' methodology [13] were conducted in 11 male and female volunteers, mean age 41 ± 18 years old, who were informed of the study and all procedures involved. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration.

Ten μL of *Laurus novocanariensis* oil was applied once daily in the volar forearm of each volunteer for 5 consecutive days in an area of 9 cm^2. Measurements were performed with a Corneometer CM825 (CK Electronics, GmBh, Köln, Germany), a Tewameter TM300 (CK Electronics) and a Laser Doppler Flowmeter LDF (Periflux PF5010, Perimed, Järfälla, Sweden), following published guidelines [14, 15]. The erythema was also quantified by Colorimetry using a Minolta Chroma Meter CR-300 (Minolta Camera Co. Ltd., Osaka, Japan). All measurements were performed in triplicate and using the CIE Lab system [16]. The parameter a* reflects the red chromaticity and was therefore used to quantify the increase in blood perfusion.

The basal values were determined in the first day of the study, and further measurements were made at 24, 48, 72 and 96 h. To minimize the effect of inter-individual variability, the results were analysed as the ratio between the values obtained after applying the oil and the basal values.

Statistical analysis

One-way ANOVA was used in this study (SPSS Statistics 17.0, IBM Corporation, Somers, NY, U.S.A.). A 0.05 significance level was adopted.

Results

In vitro diffusion studies were conducted to determine the impact of pre-treatment with the oil and the essential oil in the steady-state fluxes of a hydrophilic (caffeine) and lipophilic (ibuprofen) model

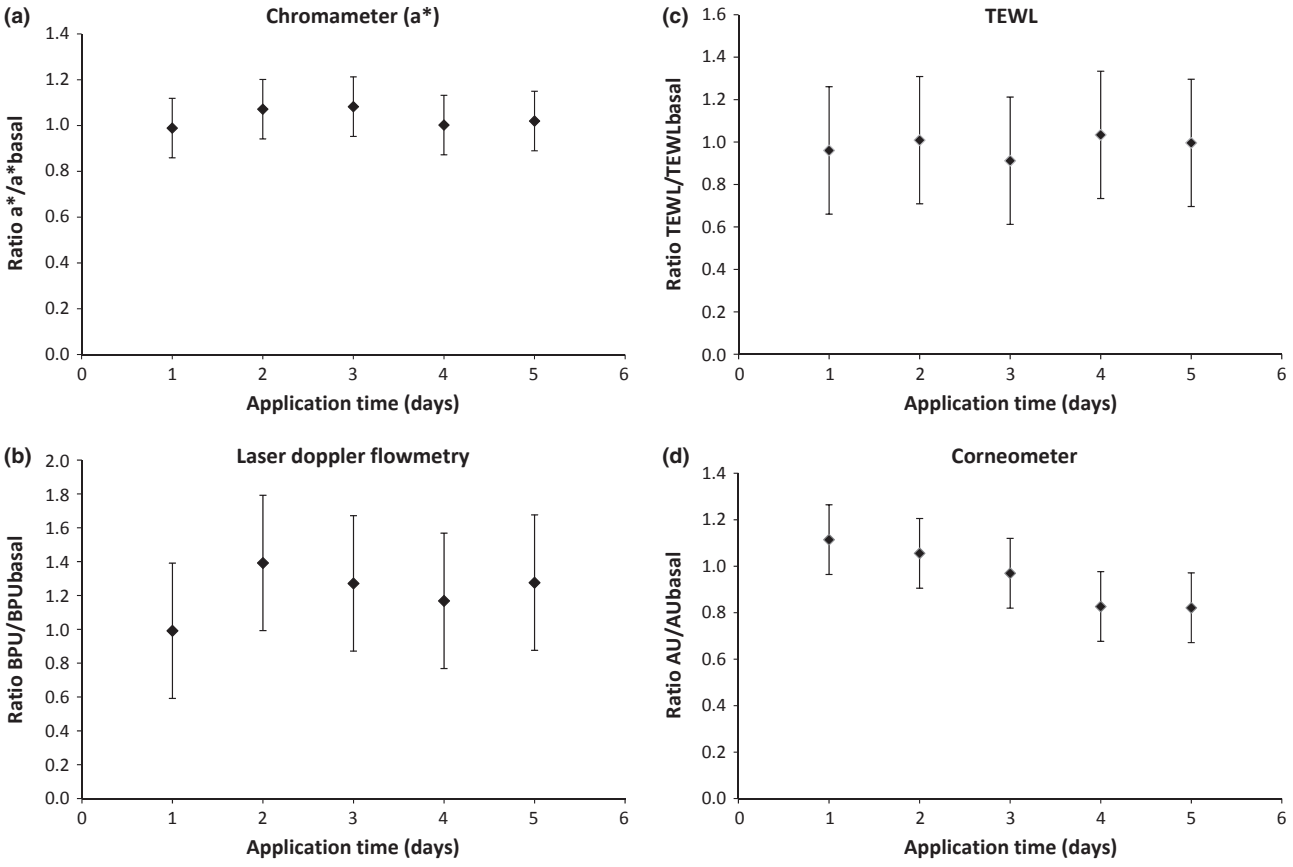

Figure 2 Variation in different skin properties at each application day (mean + SD, $n = 11$) (a) a* obtained with a Chromameter, (b) blood flow, (c) transepidermal water loss, (d) stratum corneum hydration.

permeants. For caffeine (Fig.1a), slightly higher fluxes were observed when the epidermis was pre-treated with laurel oil, whereas in the case of ibuprofen (Fig. 1b) the essential oil provided higher values. Nevertheless, no statistical differences were established between the fluxes obtained in the pre-treated epidermal membranes and the untreated controls (caffeine $P = 0.27$ and ibuprofen $P = 0.12$).

Several bioengineering methodologies were employed in the *in vivo* study to assess the impact of the application of laurel oil for 5 consecutive days. To decrease the impact of the inter-individual variability, results were analysed as the ratio between the values obtained in each day of the study and the basal values. Measurements with the Chromameter did not detect any increase in the skin redness ($P = 0.47$), and no significant variations in the blood flow were detected with the Laser Doppler Flowmeter ($P = 0.21$). This indicates that the repeated application of the oil did not cause erythema, because the results observed in the first day of the study were maintained throughout the week (Fig. 2a,b). The transepidermal water loss remained constant during the study ($P = 0.73$), which indicates that the oil did not affect the skin barrier function (Fig. 2c). The stratum corneum hydration was slightly reduced on days 4 and 5 (Fig. 2d) and statistical analysis confirmed that the differences were significant ($P = 0.01$).

Discussion

Madeira laurel oil has a high content in fatty acids and an unusually high content of essential oils [4]. The first part of this investigation aimed to ascertain *in vitro* its capacity to alter the skin barrier by pre-treatment of epidermal membranes with either the oil or its isolated essential oil for 2 h. All cells were dosed with the same caffeine or ibuprofen solutions, and the putative permeation enhancers were completely removed after pre-treatment. Therefore, thermodynamic activity and donor concentration were the same in each replicate, and differences would be attributable only to impact in barrier function by the oil and essential oil. Nevertheless, despite the presence of substances with the potential to be penetration enhancers, none of the two materials had the capacity to significantly affect the *in vitro* permeation of the lipophilic or the hydrophilic model molecules.

Most of the fatty acids present in the triglycerides of laurel oil have percutaneous penetration enhancement effects described in the literature. Oleic acid has been shown to be effective for both lipophilic and hydrophilic molecules and is thought to cause perturbation of the lamellar stratum corneum lipids [17–19]. Linoleic and lauric acid were able to promote piroxicam flux when the skin was pre-treated with 5% solutions of the fatty acids in propylene glycol [20].

The main chemical components present in laurel essential oil are monoterpene hydrocarbons (trans-β-ocimene: 32.37%, cis-β-ocimene: 8.09%, α-pinene: 8.44%, β-pinene: 4, 21%) and sesquiterpene hydrocarbons (germacrene D: 16.87%, β-elemene: 5.68%) [21]. Terpenes present in essential oils are usually good candidates to promote the percutaneous penetration of both lipophilic and hydrophilic molecules [10, 22, 23]. Cornwell and Barry reported that pre-treatment of epidermal membranes with sesquiterpene oils, or solid sesquiterpenes saturated in dimethyl isosorbide, increased the rate of absorption of a model hydrophilic permeant, 5-fluorouracil [24]. However, it was observed in this study that pure hydrocarbons were less potent enhancers than those with polar functional groups, which is the case of laurel essential oil, that has only vestigial amounts of the oxygenated compounds.

The lack of activity of laurel essential oil could be attributed to an insufficient contact time between the material and the epidermal membranes. Nevertheless, a similar protocol was followed by Ballam *et al.* investigating the potential of *Aloe vera* juice as a penetration enhancer [25]. Even though no significant differences in the transdermal permeation of ketoprofen were found for the *Aloe vera* pre-treatments, 1 h of pre-treatment with the positive control (tea tree oil) originated higher fluxes.

The second part of this study addressed the *in vivo* effects of the repeated application of laurel oil on the skin through bioengineering methodologies. Overall results reflect the innocuous nature of the material and are in agreement with those established in the *in vitro* study. Measurements performed with Chromameter and the Laser Doppler Flowmeter indicate that the repeated application of the oil did not cause erythema, because the basal values observed in the first day of the study were maintained throughout the week. Application of the oil did not impair skin barrier function, because the transepidermal water loss remained constant throughout the study. Nevertheless, there was a slight reduction in stratum corneum hydration on days 4 and 5.

This work shows that both the oil and the essential oil were well tolerated by the skin and did not cause significant perturbation of the barrier or irritation. The wound-healing properties of these products will be addressed in further studies.

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