**Date:** November 27, 2023

**Title:** Clinical trial in skin conditions of the foot using a cream enriched with *Melaleuca alternifolia* essential oil.

**Description of document:** Materials and Methods (Study Protocol: description of the clinical study, including objective and design. Statistical Analysis Plan (description of the statistical considerations for analyzing the data collected in the study) results of the study (Results obtained in the study) and Supplementary data (Annexes).

## 1. Material and method

### Type of study, permits, and inclusion criteria

A randomized, double-blind, multicenter clinical trial was designed, developed at the facilities of the University Center of Plasencia (University of Extremadura, Spain) and at the Escola Superior de Saúde Dr. Lopes Dias (Polytechnic Institute of Castelo Branco, Portugal), from March to May 2023.

The trial was authorized by the Bioethics Committee of the University of Extremadura (Register No.: 55/2017, Annex 1) and was conducted in accordance with the ethical principles established in the Declaration of Helsinki in accordance with current legislation on biomedical research (Law 14/2007, of 3 June). In addition, all participants joined voluntarily, after signing the informed consent.

The inclusion criteria established were that the participants were free of cosmetic and pharmacological substances in their feet and nails, that they did not have any type of diagnosed allergy to the components of the cream or to the EO of choice and that they maintained correct foot hygiene. Participants who had a condition that compromised their health and had repercussions on the foot or who were under medical treatment incompatible with their participation in the trial were excluded from the study.

### Sample

The initial number of participants was 65, of which 9 were excluded because they did not meet the inclusion criteria or because they did not sign the informed consent form (Figure 1). Of the remaining 56 participants, 7 were excluded due to lack of adherence, as they did not attend the review, so the final number of participants was 49 (Figure 1).

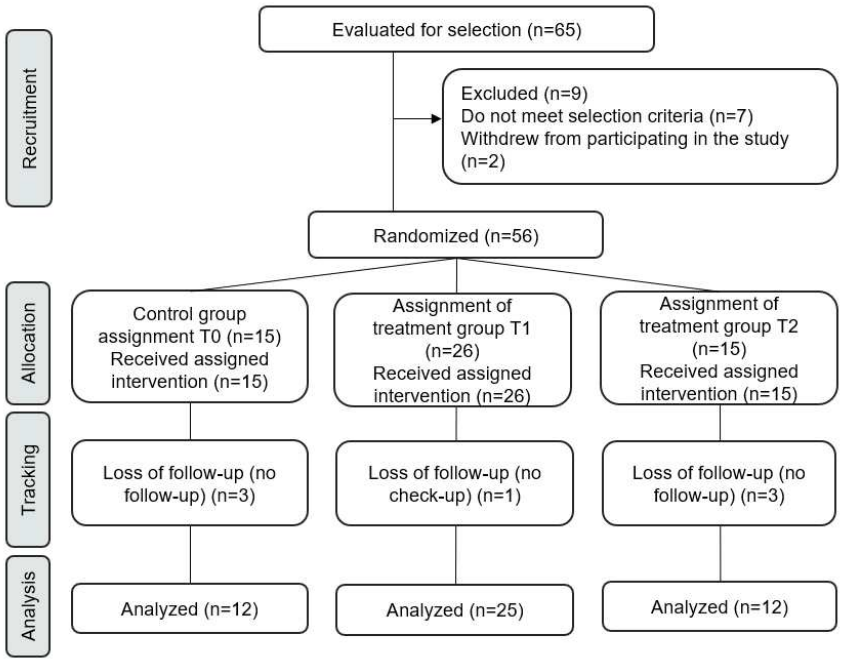



**Figure 1.** Flowchart according to CONSORT standards of the process of inclusion of participants in the study.

The sample consisted of 36 women and 13 men, divided into two age groups: young people between 18 and 30 years old (22,438±3,898) and adults between 31 and 64 years old (50,212±9,633). In terms of origin, 23 were residents of Plasencia (Spain) and 26 in Castelo Branco (Portugal). The descriptive characteristics of the sample are detailed in Table 1.

**Table 1.** Descriptive characteristics of the sample.

| VARIABLES | Total n=49 | Origin | Young Men (mean±SD) | Adult Men (mean±SD) | Young Women (mean±SD) | Adult women (mean±SD) |
|---|---|---|---|---|---|---|
| **AGE (years)** | 41,143± 15,485 | Spain | 20±2,828 | 46.333±13.317 | 24,111±3,723 | 49±9,367 |
| | | Portugal | 26±NP | 46,857±10,495 | 19±2 | 53.50±8.609 |
| **WEIGHT (kg)** | 70,545± 12,705 | Spain | 80±14,142 | 87,333±17,926 | 68,667±13,684 | 64,411±7,193 |
| | | Portugal | 69±NP | 80,714±11,572 | 66,750±8,995 | 66.857±11.016 |
| **HEIGHT (m)** | 1,664±0 ,085 | Spain | 1.82±0.113 | 1.733±0.025 | 1,652±0.074 | 1.618±0.068 |
| | | Portugal | 1.7±NP | 1.757±0.060 | 1.657±0.049 | 1,616±0.057 |
| **IMC ($kg/m^2$)** | 25,404± 3,626 | Spain | 24,026±1,280 | 29,170±6,725 | 25,069±4,334 | 24,611±2,350 |
| | | Portugal | 23,875±np | 26,148±3,488 | 24,372±3,837 | 25,552±3,548 |

n: sample size; SD, standard deviation; NP: Not applicable; kg: kilogram; M: Metro; BMI, body mass index; $^{M2}$: Square meter.

**Randomization, masking, and experimental design**

Participants were randomly assigned to distinct groups using free software Epidat 4.0 (2011, Galicia, Spain). The blinding was double-blind, so that neither the investigators nor the participants knew the group assigned to the participant until the completion of the study and statistical analysis.

The total number of participants was divided into three groups: a control group (T0) (n=12), in which no participants had a skin infection, and two different treatment groups (T1 and T2) (n=37), with diverse levels of EC. The rest of the participants were included in these two treatment groups, including both infected (n=32) and healthy (n=5) participants. Thus, group T1 (n=25) had 23 participants with infection and 2 healthy participants, and group T2 (n=12) had 9 participants with infection and 3 healthy participants.

The clinical evaluation of the participants was carried out before the start of treatment and, subsequently, on days 15, 30, 45 and 60 of treatment, except for cultures, both mycological and bacterial, which were performed at baseline, at 30 days and at 60 days of treatment. All reviews, sampling, and data collected were performed by the same observer.

**Choice of EO, base cream, and preparation of treatments**

After reviewing the available literature, the essential oil (EO) of *M. alternifolia* was chosen for its antibacterial and antifungal characteristics (16–19). There were 3 types of commercial M. *alternifolia* EO from the brands Marnys® (Cartagena, Spain), Naissance® (Neath, United Kingdom) and Esencias Lozano® (Murcia, Spain). However, the choice of the specific commercial EO depended on the results obtained from the analysis of its composition. This analysis was performed using gas chromatography mass spectrometry (CG-MS) and the results obtained were contrasted with those available in the online database SpectraBase (WILEY, John Wiley & Sons).

The cream used as a base for the incorporation of the different concentrations of EO tested was a hand and nail cream (*La Chinata*®, Plasencia, Spain), in which Extra Virgin Olive Oil is used. This cream was chosen after verifying that none of its components were allergens for the skin of the participants according to Regulation 1223/2009(1). In addition, it was the treatment received by patients in the T0 group, in whom no type of EC was added.

To test the possible efficacy of *the chosen M. alternifolia* EO as a treatment against infections, the base cream was enriched with different concentrations of EO. Specifically, 1% for the T1 treatment group

and 0.5% for the T2 treatment group. The emulsion of the EO and the base cream was carried out by means of a magnetic stirrer (Agimatic-N, Barcelona, Spain) under sterile conditions (laminar flow cabinet, Telstar AV-100), adding the necessary volume of EC until the desired two concentrations were achieved.

Treatments were administered to participants in the form of prefilled 15ml syringes under sterile conditions with enough for 15 days of treatment. Along with the treatment, all the necessary information was provided on the hygienic habits to follow (washing the feet properly with soap and water and drying them properly with a towel for exclusive use), the correct way of applying the treatment, the daily volume of treatment for each foot (0.5ml/2 times a day).

**Exploration, sampling, and diagnostic tests**

Sociodemographic data (age and sex), medical and podiatric history, allergies and pharmacological treatments prescribed, as well as hygiene habits, were obtained through the completion of a Google Forms survey (Annex 2).

Scans and measurements were performed in a temperature-controlled room (20-24°C) and participants aired their bare feet for 10 minutes before starting the procedures, to normalize the temperature. In the first session they were weighed and measured (SECA 701, Vogel & Halke Hamburg, Germany), calculating the BMI(2). After normalizing the temperature, the physical examination was carried out, samples were taken, and data were obtained.

The Corneometer CM 825® (Electronic Courage-Khazaka, Cologne, Germany) with a measuring surface of 49 mm, a frequency of 0.9–1.2 MHz and an accuracy of ±3%, was used to assess the superficial hydration of the skin (3). Five reference points were measured, both at the dorsal level and at the plantar level (Figure 2), obtaining three measurements from each point to later calculate the average hydration of each location analyzed.

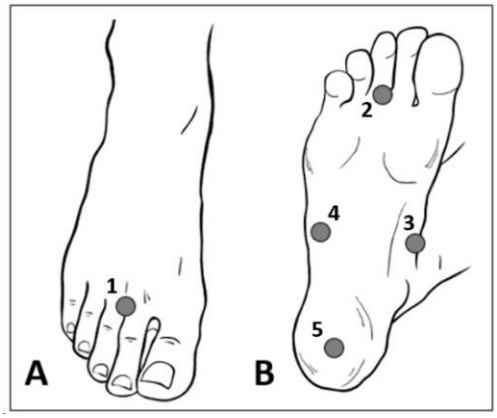
1. 


**Figure 2**. Location of the 5 reference points on the feet for assessment of superficial hydration. (A) Dorsor foot: (1) dorso-distal point, above the central metatarsal heads. (B) Sole of the foot: (2) interdigital-plantar point, between 3rd and 4th digital space, (3) central point of the internal arch, (4) central point of the external arch, and (5) central point of the calcaneus.

Regarding the diagnosis of possible infections present in the feet, the following tests were performed:

- Wood's Light Examination

The feet of the participants were examined with a Wood L1004® lamp (Weelco, Barcelona, Spain) at maximum power (41) and at a distance of 10-15 cm from the surface to be examined. Long-wave ultraviolet radiation allows visual observation of the fluorescence emitted by *Pseudomonas aeruginosa, Microsporum audouinii, Microsporum canis, C. minutissimum, T. schoenleinii,*(4) among

others, whose color varies depending on the pathogen. The areas that fluoresced were recorded along with the color of the fluorescence in each review conducted from the beginning of the study to the day of its completion.

- Potassium hydroxide (KOH) test and mycological culture

A skin sample was taken following the standard procedure described for the diagnosis of mycosis (5). The epithelial surface was disinfected with 70° alcohol and a sample was collected from the affected area by scalpel scraping. Subsequently, the sample was analyzed using two different procedures. The 30% KOH test (35.3g/100ml distilled water) (Labbox, Barcelona, Spain) followed by conventional light microscopy observation to identify the infective fungal agent, and microbiological culture on Sabouraud dextrose agar with chloramphenicol and cycloheximide (Condalab, Torrejón de Ardoz, Madrid), at a constant temperature (between 25°C and 28°C) for 2 to 4 weeks, in order to inhibit molds and bacteria and observe the growth of theinfective fungal agent. Participants with infection were considered to be those who had positive results in both tests, excluding cases in which only one test was positive.

- Bacteriological culture

A skin sample was taken following the standardized procedure for microbiological analysis (6). The skin was disinfected with 70° alcohol and a sample was taken by smear with a sterile swab from the affected surface. Again, the analysis was carried out using two distinct and independent procedures. A Gram stain was performed to identify bacteria and the sample was inoculated with Sabouraud dextrose broth (SDA) (Merck, Germany), at a constant temperature (between 27°C and 31°C) for 1 to 3 days. Participants with infection were considered to be those who had positive results in both tests, excluding cases in which only one test was positive.

**Variables and statistical analysis**

The following variables were analyzed: sociodemographic (sex and age), anthropometric (weight, height, and BMI), hygiene-related habits (frequency of foot washing, use of flip-flops in showers/pools, type of footwear and socks and frequency of sock change), presence or absence and type of infection, degree of hydration and concentration of EC group T0 (0%), group T1 (1%) and group T2 (0.5%). Of these variables, age, weight, height, BMI, and hydration were considered quantitative compared to the rest that were analyzed as qualitative.

Statistical analysis was performed using the statistical analysis program IBM-SPSS Statistics for Windows version 25 (IBM Corp. Armonk, NY, USA). Some figures have been obtained using the Python programming language, using the Seaborn library (Waskom, M. L., (2021). Seaborn: statistical data visualization. Journal of Open-Source Software, 6(60), 3021, https://doi.org/10.21105/joss.03021).

Normality was assessed using the Shapiro-Wilk test. To evaluate the influence of different variables on infection remission, a Cox regression model was used, and survival curves of significant factors were plotted. To compare the efficacy of different EC concentrations, survival curves were compared using the Log-rank test. The Mann-Whitney U test was used to assess hydration averages according to sex. To check if there were improvements in hydration due to the use of the cream with different concentrations of EC (T1 and T2) compared to the control group (T0), hydration levels were compared using Kruskal-Wallis tests, using the Mann-Whitney U test with correction of p-values by the Bonferroni method for multiple comparisons.

In all statistical tests, significance was considered when $p\text{-value}<0.05$ and high significance when $p\text{-value}<0.01$.

## 2. Results

### Analysis of the Elemental Composition of Commercial S. S.

The determination of the specific composition of the 3 types of EO made it possible to quantify the exact concentration of the different components of each of the EO, choosing the EO with the highest concentration of components with antimicrobial activity and the lowest concentration of components susceptible to interaction with other drugs or to produce allergies in the participant, such as terpinolene. alpha-terpinene and sabinene and the aged components of EO(7–10).Specifically, the main component in the 3 EO samples was terpinen-4-ol, with a concentration between 28.47% and 29.15%, followed by gamma-terpinene and alpha-terpinene (Table 2). These three components accounted for approximately 65% of the total EO in the 3 samples analyzed. On the other hand, alpha-terpineol and eucalyptol were found to be the fourth and fifth most concentrated components, with percentages between 3.85%-5.85% and 4.12%-5.81%, respectively. These two, along with m-cymene, alpha-pinene, and terpinolene, made up about 24% of the total EO. The rest of the EO was made up of up to 22 different compounds with minority percentages, considered together as traces. For all the above results, the EO of *M. alternifolia* of the brand Esencias Lozano® was used, since it was the EO that presented the highest percentage of the components of terpinen-4-ol, eucalyptol and alpha-terpineol, whose antimicrobial activity has been described in the bibliography(7,11–14).

**Table 2.** Percentages of the different components according to the EO studied in the CG-MS.

| | **Trademarks of EO** | | |
|---|---|---|---|
| **Components** | **Naissance®** | **Marnys®** | **Esencias Lozano®** |
| Terpinen-4-ol | 28.47% | 29.10% | **29.15%** |
| Gamma-terpinene | 21.92% | 20.42% | 22.86% |
| Alpha-Terpinene | 13.10% | 11.23% | 13.58% |
| Alpha-terpineol | 3.85% | 4.03% | **5.85%** |
| Eucalyptol | 4.12% | 4.82% | **5.81%** |
| M-cymene | 3.40% | 5.28% | 4.96% |
| Alpha-pinene | 3.18% | 3.15% | 5.12% |
| Terpinolene | 4.65% | 4.26% | 3.72% |
| Traces | 17.31% | 17.71% | 8.95% |
| Total | 100% | 100% | 100% |

®: trademark; %: percentage of the component in the sample.

### Test results for microbiological diagnostics

After reading the tests performed for microbiological diagnosis, it was determined that, of the 49 total participants, 32 (65.3%) were positive for skin infections. Of these 32 participants, 4 (12.5%) had fungal infection, 19 (59.4%) showed bacterial infection, and 9 (28.1%) had mixed fungal and bacterial infections simultaneously.

Of the participants who developed skin infections, 23 were women and 9 were men. The prevalence of skin infections by sex is shown in Table 3.

**Table 3.** Prevalence of infections as a function of sex.

| **Sex** | **Infections** | | | |
|---|---|---|---|---|
| | **Fungal** | **Bacterial** | **Mixed** | **Total** |
| **Women** | 3(9.3%) | 13 (40.6%) | 7 (21.8%) | 23 (71.7%) |
| **Men** | 1 (3.1%) | 6 (18.8%) | 2 (6.4%) | 9 (28.3%) |
| **Total** | 4 (12.4%) | 19 (59.4%) | 9 (28.2%) | 32 (100%) |

%: Percentage.

The colorations observed with Wood's light in the interdigital spaces were coral red and opaque blue (Figure 3). The coral-red coloration corresponds to a superficial bacterial infection of the skin caused by C. *minutissimum*, known as erythrasma, and the dull blue coloration corresponds to a fungal infection caused by the dermatophyte Trichophyton *schoenleinii.*

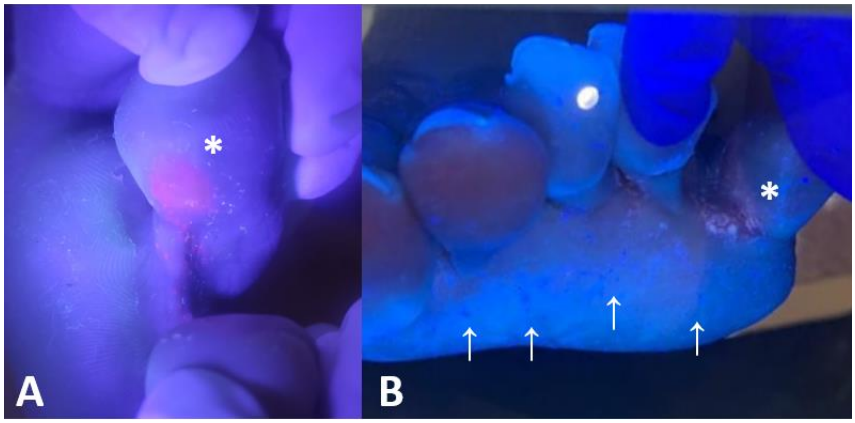



**Figure 3**. Fluorescence of different color emitted by pathogens under Wood's light in interdigital spaces of the feet. (Aand B) erythrasma with coral-red coloration (*) and (B) suspected *Trichophyton schoenleinii* with bluish coloration (↑).

Mycological cultures were used to determine the causative agents of fungal infections. The dermatophytes and yeasts that were identified were: Trichophyton *schoenleinii, Trichophyton verrucosum, Trichophyton tonsurans* and *Candida albicans.* On the other hand, the following bacteria were identified in bacterial cultures: *Corinebacterium minutissimum, staphylococcus haemolyticus, staphylococcus epidermidis, staphylococcus lugdunensis,* micrococcus luteus and *micrococcus sp.*

**Efficacy of treatments**

To test whether the EC-enriched cream was effective against fungal infections, the cumulative survival curves of the two treatments, T1 and T2, were plotted (Figure 4). 100% of fungal infections resolved within 30 days with bothtreatments, although the EC curve at 1% was higher than 0.5%.

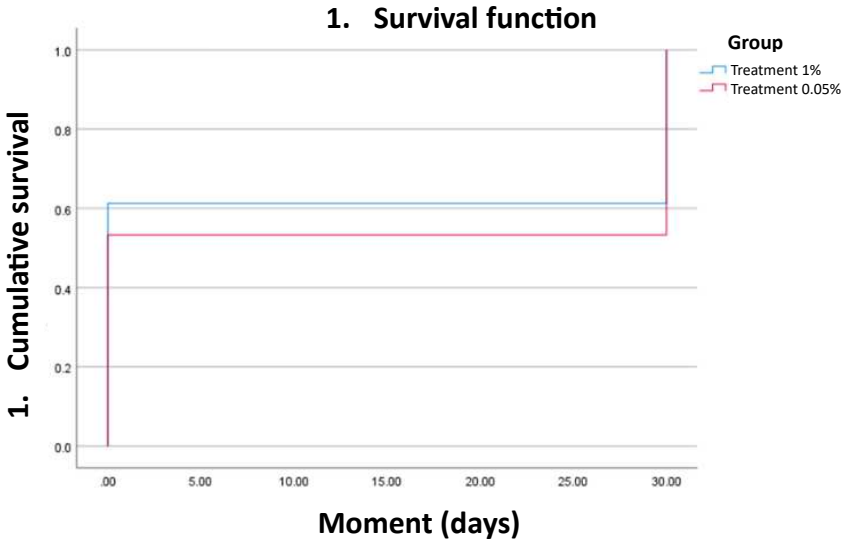



**Figure 4.** Cumulative survival curves as a function of the two treatments for fungal infections.

In fact, the mean time to achieve remission in the T1 group (1%) was 11.61 days, compared to 14.00 days inthe T2 group (Table 4). However, according to the Lo g-ra nk test, the differences between the two groups were not statistically significant (p-value=0.611). Therefore, both concentrations have similar efficacy in terms of the time required to achieve remission of infection.

**Table 4.** Mean and median survival time (in days) of remission of fungal infection for the two EC concentrations.

| Group | Media | | | | Median | | | |
|---|---|---|---|---|---|---|---|---|
| | East. | Err. East | IC 95% | | East. | Err. East | IC 95% | |
| | | | Lim. Inf. | Lim. Sup. | | | Lim. Inf. | Lim. Sup. |
| **Treatment 1%** | 11.61 | 2.68 | 6.36 | 16.86 | - | - | - | - |
| **Treatment 0.5%** | 14.00 | 4.04 | 6.09 | 21.91 | - | - | - | - |

Est: statistical survival test; Err. Est: statistician error; CI, confidence interval; Lim. Inf: lower bound; Lim. Sup: upper limit; "-": data not available.

Similarly, to evaluate whether the EO-enriched cream was effective against bacterial infections, the cumulative survival curves for both treatments were plotted (Figure 5). In this case, the treatment curve at 0.5% concentration showed better results than the 1% treatment curve.

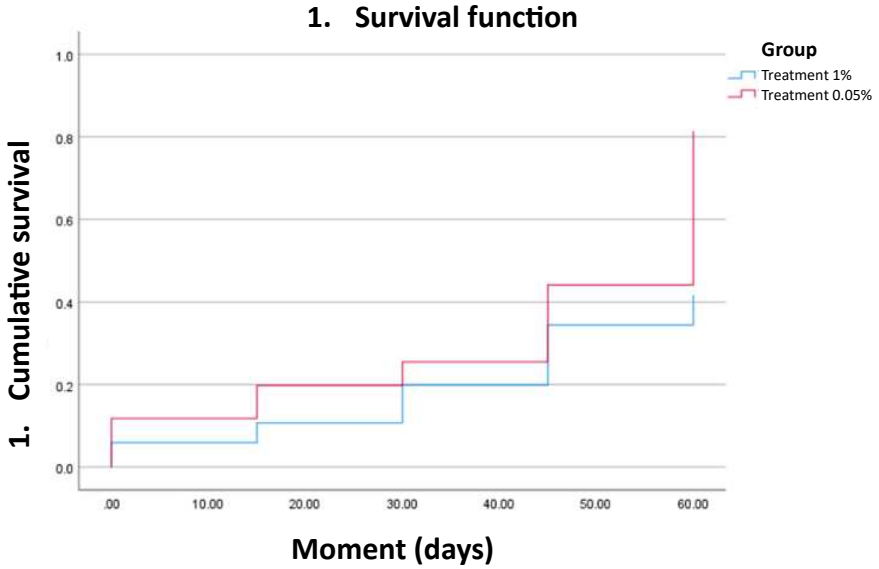



**Figure 5.** Cumulative survival curves as a function of the two treatments for bacterial infections.

Thus, the mean time to achieve remission in the T1 group (1%) was 49.35 days, compared to 44.82 days in the T2 group (0.5%) (Table 5). This indicated that the treatment of the T2 group was better, since the infection remitted 5 days earlier on average, although when comparing both treatments using the Long-rank test, no statistically significant differences were found (p-value=0.130). Median survival was 60.00 days for treatment at 0.5%, so half of the participants in the T2 group achieved remission at the end of the study. This value could not be computed for the 1% treatment, indicating that less than half of the subjects in this group experienced resolution of the infection at the end of the trial (Table 5).

**Table 5.** Mean and mediansurvival time (in days) of remission of bacterial infection for the two EC concentrations.

| Group | Media | | | | Median | | | |
|---|---|---|---|---|---|---|---|---|
| | East. | Err. east. | IC 95% | | East. | Err. east. | IC 95% | |
| | | | Lim. Inf. | Lim. Sup. | | | Lim. Inf. | Lim. Sup. |
| **Treatment 1%** | 49.35 | 2.38 | 44.68 | 54.01 | - | - | - | - |
| **Treatment 0.5%** | 44.82 | 4.38 | 36.2 | 53.40 | 60.0 | 6.37 | 47.51 | 72.49 |

Est: statistical survival test; Err. Est: statistician error; CI, confidence interval; Lim. Inf: lower bound; Lim. Sup: upper limit; "-": data not available.

**Relationship between infections and the rest of the variables analyzed**

In relation to hygiene habits, it was found that most of the participants washed their feet daily, used flip-flops in swimming pools or showers, wore breathable footwear and cotton socks, and changed their socks daily (Table 6), although it is true that these habits did not show a statistically significant relationship with respect to the presence or absence of skin infections (p-value≥0.103) (Table 7).

**Table 6.** Contingency table between hygiene habits and skin infections.

| Category | Fungal infection | | Total | Bacterial infection | | Total |
|---|---|---|---|---|---|---|
| | No | Yes | | No | Yes | |
| **Frequency of foot washing** | | | | | | |
| ***1 time every 2 days*** | 2 (4.88%) | - | 2 (4.08%) | 2 (6.90%) | - | 2 (4.08%) |
| ***Daily*** | 33 (80.49%) | 8 (100.00%) | 41 (83.67%) | 23 (79.31%) | 18 (90.00%) | 41 (83.67%) |
| ***2 times a day*** | 6 (14.63%) | - | 6 (12.24%) | 4 (13.79%) | 2 (10.00%) | 6 (12.24%) |
| ***Total*** | 41 (83.67%) | 8 (16.33%) | 49 | 29 (59.18%) | 20 (40.82%) | 49 |
| **Flip flops in the shower** | | | | | | |
| ***No*** | 7 (17.07%) | 2 (25.00%) | 9 (18.37%) | 8 (27.59%) | 1 (5.00%) | 9 (18.37%) |
| ***Yes*** | 34 (82.93%) | 6 (75.00%) | 40 (81.63%) | 21 (72.41%) | 19 (95.00%) | 40 (81.63%) |
| ***Total*** | 41 (83.67%) | 8 (16.33%) | 49 | 29 (59.18%) | 20 (40.82%) | 49 |
| **Type of footwear** | | | | | | |
| ***Non-breathable*** | 18 (43.90%) | 4 (50.00%) | 22 (44.90%) | 12 (41.38%) | 10 (50.00%) | 22 (44.90%) |
| ***Breathable*** | 23 (56.10%) | 4 (50.00%) | 27 (55.10%) | 17 (58.62%) | 10 (50.00%) | 27 (55.10%) |
| ***Total*** | 41 (83.67%) | 8 (16.33%) | 49 | 29 (59.18%) | 20 (40.82%) | 49 |
| **Sock Type** | | | | | | |
| ***Cotton*** | 21 (51.22%) | 6 (75.00%) | 27 (55.10%) | 17 (58.62%) | 10 (50.00%) | 27 (55.10%) |
| ***Polyester*** | 10 (24.39%) | - | 10 (20.41%) | 6 (20.69%) | 4 (20.00%) | 10 (20.41%) |
| ***I don't know*** | 10 (24.39%) | 2 (25.00%) | 12 (24.49%) | 6 (20.69%) | 6 (30.00%) | 12 (24.49%) |
| ***Total*** | 41 (83.67%) | 8 (16.33%) | 49 | 29 (59.18%) | 20 (40.82%) | 49 |
| **Daily sock change** | | | | | | |
| ***1 time every 2 days*** | 5 (12.20%) | - | 5 (10.20%) | 1 (3.45%) | 4 (20.00%) | 5 (10.20%) |
| ***Daily*** | 32 (78.05%) | 8 (100.00%) | 40 (81.63%) | 26 (89.66%) | 14 (70.00%) | 40 (81.63%) |
| ***2 times a day*** | 4 (9.76%) | - | 4 (8.16%) | 2 (6.90%) | 2 (10.00%) | 4 (8.16%) |
| ***Total*** | 41 (83.67%) | 8 (16.33%) | 49 | 29 (59.18%) | 20 (40.82%) | 49 |

"-": data not available, %: percentage.

**Table 7.** Relationship between hygiene habits and skin infections.

| Variables | Fungal infection | | Bacterial infection | |
|---|---|---|---|---|
| | $X^2$ | p-value | $X^2$ | p-value |
| **Frequency of foot washing** | 1.87 | 0.393 | 1.68 | 0.432 |
| **Flip flops in the shower** | 0.00 | 0.976 | 2.66 | 0.103 |
| **Type of footwear** | 0.00 | 1.000 | 0.09 | 0.761 |
| **Sock Type** | 2.64 | 0.267 | 0.58 | 0.748 |
| **Daily sock change** | 2.15 | 0.542 | 4.08 | 0.252 |

$X^{2:}$ Chi-Square

On the other hand, the possible relationship between anthropometric and sociodemographic variables and treatment efficacy for fungal infections was examined, where no statistically significant association was observed (p-values ≥0.145) (Table 8). Therefore, for this sample, anthropometric and sociodemographic variables did not seem to be critical factors in the efficacy of treatment for fungal infections of the foot.

**Table 8.** Cox model for different anthropometric and sociodemographic variables and fungal infection.

| Variable | B (coef.) | Exp(B) | IC 95% | | p-value |
|---|---|---|---|---|---|
| | | | Lim. Inf. | Lim. Sup | |
| ***Stature*** | -0.02 | 0.98 | 0.73 | 1.30 | 0.874 |
| ***Weight*** | 0.01 | 1.01 | 0.73 | 1.40 | 0.948 |
| ***BMI*** | -0.04 | 0.96 | 0.39 | 2.40 | 0.938 |
| ***Age*** | 0.03 | 1.03 | 0.54 | 1.97 | 0.918 |
| ***Sex*** | -0.64 | 0.52 | 0.20 | 1.35 | 0.181 |

Coef: coefficient; Exp: exponent; B: correlation coefficient; CI, confidence interval; Lim. Inf: lower bound; Lim. Sup: upper limit; *: statistically significant.

Regarding the possible relationship between anthropometric and sociodemographic variables and the efficacy of treatment for bacterial infections, it was observed that only sex showed a statistically significant relationship (p-value=0.028) (Table 9).

**Table 9.** Cox model for different anthropometric and sociodemographic variables and bacterial infection.

| Variable | B (coef.) | Exp(B) | IC 95% | | p-value |
|---|---|---|---|---|---|
| | | | Lim. Inf. | Lim. Sup | |
| ***Stature*** | 0.11 | 1.12 | 0.82 | 1.54 | 0.484 |
| ***Weight*** | -0.08 | 0.93 | 0.64 | 1.34 | 0.684 |
| ***BMI*** | 0.13 | 1.14 | 0.41 | 3.21 | 0.803 |
| ***Age*** | 0.55 | 1.74 | 0.82 | 3.67 | 0.146 |
| ***Sex*** | **1.41*** | 4.11 | 1.16 | 14.50 | **0.028*** |

Coef: coefficient; Exp: exponent; B: correlation coefficient; CI, confidence interval; Lim. Inf: lower bound; Lim. Sup: upper limit; *: statistically significant.

To assess the efficacy of the treatment over time according to sex, a cumulative survival curve was used (Figure 6) and the proportion of individuals in whom the resolution of the infection over time was observed was represented, observing that the healing time was shorter in women than in men and, therefore, a faster resolution of bacterial infection is observed in women.

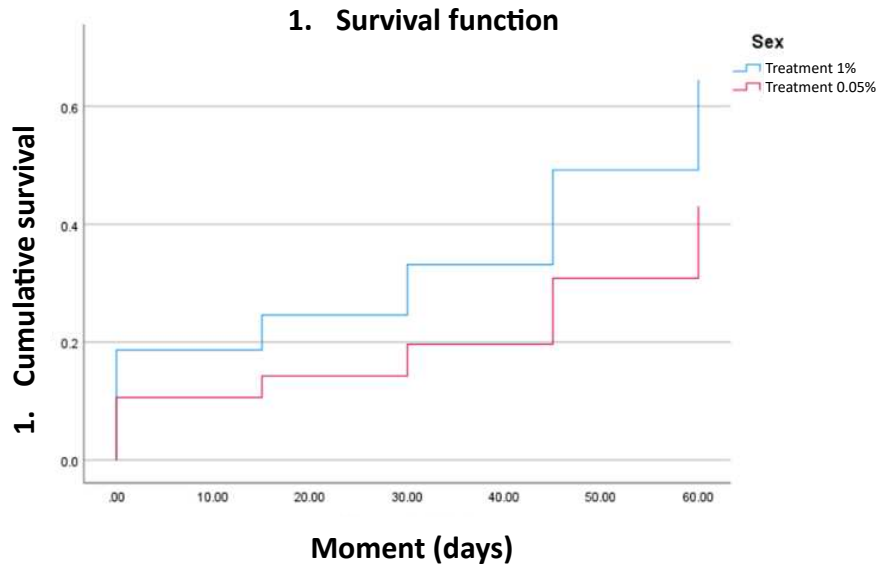



**Figure 6.** Cumulative survival curve as a function of sex.

Finally, in relation to skin hydration, significant differences were obtained according to sex (p-value ≤0.010), with women having higher hydration averages (Table 10).

**Table 10.** Results of the Kruskal-Wallis tests for the analysis of hydration according to sex.

| Landmarks per foot | Sex | Initial | | 15 days | | 30 days | | 45 days | | 60 days | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Media±Sd | p-value | Media±Sd | p-value | Media±Sd | p-value | Media±Sd | p-value | Media±Sd | p-value |
| ***Dorsal R*** | Woman | 34.507±9.834 | 0.146 | 41.904±13.322 | 0.511 | 44.364±13.907 | 0.439 | 42.674±13.989 | 0.408 | 43.871±16.218 | 0.171 |
| | Man | 29.306±9.159 | | 40.966±18.823 | | 44.358±21.589 | | 37.933±10.723 | | 36.837±12.851 | |
| ***Digital Plant R*** | Woman | 29.453±11.772 | 0.115 | 39.738±16.234 | 0.075 | 37.465±14.911 | **0.044*** | 36.737±15.137 | **0.048*** | 45.303±18.070 | **0.015*** |
| | Man | 23.450±10.769 | | 31.391±12.869 | | 27.967±16.632 | | 28.604±10.934 | | 24.685±11.913 | |
| ***Inner Arc R*** | Woman | 28.401±19.944 | 0.221 | 32.539±22.396 | 0.541 | 31.884±20.830 | 0.318 | 29.654±18.746 | 0.318 | 30.272±18.633 | 0.480 |
| | Man | 21.644±15.004 | | 26.911±16.238 | | 24.792±13.615 | | 24.906±15.583 | | 23.601±11.566 | |
| ***External Arc R*** | Woman | 19.661±10.161 | 0.180 | 23.534±14.690 | 0.679 | 24.834±12.267 | 0.147 | 22.125±6.492 | 0.626 | 22.006±12.310 | 0.087 |
| | Man | 15.241±8.282 | | 22.537±15.051 | | 21.120±17.939 | | 21.646±13.670 | | 15.783±6.355 | |
| ***Heel R*** | Woman | 15.655±12.504 | **0.051*** | 22.007±17.183 | 0.087 | 24.039±16.469 | **0.010**** | 20.392±15.543 | **0.044*** | 16.369±9.456 | **0.019*** |
| | Man | 9.405±5.241 | | 13.295±9.737 | | 13.218±11.013 | | 12.085±6.642 | | 10.163±5.772 | |
| ***Dorsal L*** | Woman | 33.660±11.527 | 0.132 | 37.629±10.449 | 0.519 | 41.001±11.869 | 0.075 | 41.194±12.604 | 0.062 | 40.777±13.844 | 0.164 |
| | Man | 28.085±9.337 | | 34.736±10.806 | | 33.799±15.305 | | 33.745±18.116 | | 33.668±12.444 | |
| ***Digital plant L*** | Woman | 27.532±14.373 | **0.028*** | 33.285±15.653 | 0.548 | 32.845±18.542 | 0.180 | 31.167±16.393 | 0.103 | 30.706±15.793 | **0.048*** |
| | Man | 18.307±8.845 | | 31.372±15.814 | | 24.748±11.505 | | 22.863±9.201 | | 20.941±11.391 | |
| ***Inner Arch L*** | Woman | 25.227±18.044 | 0.509 | 25.921±17.105 | 0.497 | 30.068±21.122 | 0.217 | 24.222±14.493 | 0.319 | 27.622±17.542 | 0.111 |
| | Man | 21.662±16.214 | | 23.418±15.999 | | 21.847±11.934 | | 19.917±12.615 | | 20.309±13.074 | |
| ***External Arc L*** | Woman | 18.237±11.858 | **0.022*** | 20.679±12.047 | 0.679 | 22.122±10.429 | **0.045*** | 19.848±13.604 | 0.448 | 19.494±9.876 | **0.012*** |
| | Man | 11.614±4.385 | | 20.008±12.647 | | 16.649±11.251 | | 16.387±8.587 | | 12.575±5.412 | |
| ***Heel L*** | Woman | 16.168±11.812 | **0.012*** | 18.131±13.019 | 0.167 | 24.513±20.395 | 0.062 | 16.580±14.922 | **0.038*** | 14.351±9.984 | **0.010**** |
| | Man | 8.765±4.557 | | 13.095±9.544 | | 12.488±7.218 | | 9.045±5.445 | | 7.762±4.338 | |

R: right; L: Left; Sd: standard deviation; *: significant; **: Highly significant.

On the other hand, in relation to skin hydration, it was obtained that the three groups had similar hydrations at the beginning of the study, although it is true that it was observed that the hydration of the T2 group decreased throughout the treatment, being lower at the end of the treatment in all the measured points except for the left dorsal point, where a marked increase in hydration was observed after a month and a half of treatment (Figure 7).

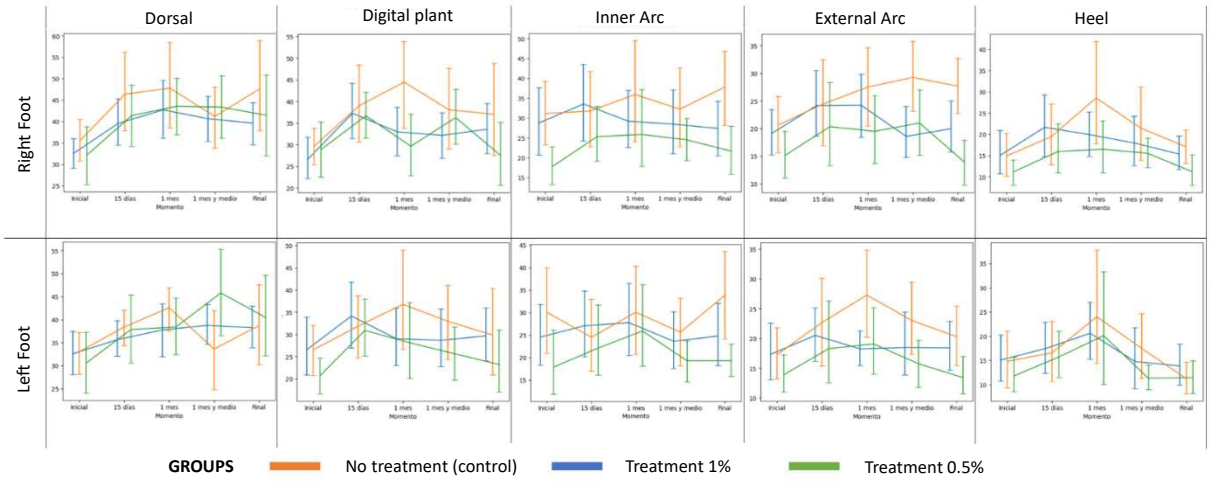



**Figure 7.** Evolution of hydration of the different points of the foot over the study time according to the test group.

Although descriptively variations in skin hydration are observed depending on the treatment applied, only the area of the "Outer Arch" of the right p-ie showed significant differences between groups, both at one and a half months of treatment (p-value=0.028) where the T1 group presented a lower hydration, and at its completion (p-valor=0.002), with the T2 group having the lowest hydration (Table 1-1). After the pairwise comparison, statistically significant differences were obtained between the T0 and T1 groups at one and a half months (p-value=0.016), observing that the control group had greater hydration, and between the T0 and T1 groups (p-value=0.021) and the T0 and T2 groups (p-value=0.003) at the end of treatment where, as in the month and a half, the control group had greater hydration (Table 1-2).

**Table 11.** Results of the Kruskal-Wallis tests for the analysis of hydration as a function of time and treatment applied.

| | | Clinical Evaluation | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Landmarks per foot** | **G** | **Initial** | | **15 days** | | **30 days** | | **45 days** | | **60 days** | |
| | | Media±Sd | **p-value** | Media±Sd | **p-value** | Media±Sd | **p-value** | Media±Sd | **p-value** | Media±Sd | **p-value** |
| ***Dorsal R*** | G. T0 | 35,506±9,364 | 0.600 | 46,356±17,565 | 0.405 | 47,787±18,500 | 0.669 | 41.144±12.744 | 0.775 | 47.612±19.149 | 0.411 |
| | G. T1 | 32,481±9,077 | | 39,537±14,093 | | 42,690±16,888 | | 40,626±13,840 | | 39,587±12,749 | |
| | G. T2 | 32.095±12.123 | | 41,366±13,255 | | 43.522±11.821 | | 43,332±13,482 | | 41.433±17.052 | |
| ***Digital Plant R*** | G. T0 | 29.630±8.215 | 0.592 | 38,992±17,156 | 0.943 | 44.454±18.161 | 0.059 | 38.044±17.682 | 0.474 | 36,998±18,832 | 0.395 |
| | G. T1 | 26.538±13.104 | | 37.230±17.615 | | 32,939±14,430 | | 32.126±14.095 | | 25.531±19.103 | |
| | G. T2 | 28.846±12.171 | | 36,666±10,250 | | 29,616±12,832 | | 36,225±11,886 | | 27,466±13,514 | |
| ***Inner Arc R*** | G. T0 | 31.117±15.186 | 0.135 | 31,721±18,640 | 0.687 | 35,890±23,178 | 0.457 | 32.205±18.994 | 0.633 | 37,761±17,694 | 0.104 |
| | G. T1 | 28,705±22,666 | | 33,491±24,892 | | 29.179±18.094 | | 28.441±20.519 | | 27,355±17,934 | |
| | G. T2 | 17.731±9.006 | | 25.278±12.703 | | 25,831±17,795 | | 24,487±9,525 | | 21.633±11.244 | |
| ***External Arc R*** | G. T0 | 20,622±9,449 | 0.336 | 24.434±14.194 | 0.701 | 27,500±13,395 | 0.317 | 29.243±12.342 | **0.028*** | 27,668±9,032 | **0.002**** |
| | G. T1 | 19.081±10.643 | | 24.125±15.356 | | 24.190±14.910 | | 18,564±12,384 | | 19.955±12.007 | |
| | G. T2 | 15.119±8.104 | | 20.323±14.354 | | 19,487±11,907 | | 20.989±11.010 | | 13,874±7,691 | |
| ***Heel R*** | G. T0 | 14,873±9,495 | 0.654 | 19,435±13,382 | 0.778 | 28,527±20,929 | 0.212 | 21,406±16,765 | 0.898 | 17.062±7.272 | 0.133 |
| | G. T1 | 14,966±14,056 | | 21.629±19.017 | | 19,897±13,953 | | 17.934±15.610 | | 15.314±10.359 | |
| | G. T2 | 11.102±5.294 | | 15,928±10,798 | | 16.457±12.132 | | 15,501±6,685 | | 11,152±6,825 | |
| ***Dorsal L*** | G. T0 | 33.012±8.530 | 0.678 | 38,395±7,423 | 0.586 | 42,575±9,287 | 0.353 | 33.613±15.368 | 0.350 | 38,689±15,840 | 0.964 |
| | G. T1 | 32,565±12,148 | | 35,652±10,539 | | 37,758±15,290 | | 38,750±11,631 | | 38,240±11,576 | |
| | G. T2 | 30,550±12,090 | | 37,848±13,328 | | 38,380±11,603 | | 45,797±17,274 | | 40,447±16,602 | |
| ***Digital plant L*** | G. T0 | 26.513±10.342 | 0.418 | 31,862±12,709 | 0.874 | 36.755±21.018 | 0.378 | 32,947±14,918 | 0.360 | 29.832±17.134 | 0.359 |
| | G. T1 | 26,507±16,982 | | 34.129±18.575 | | 29.028±16.213 | | 28,648±16,907 | | 29,655±15,528 | |
| | G. T2 | 20,692±7,252 | | 30,878±11,501 | | 28.115±15.045 | | 25,639±11,464 | | 23.191±12.778 | |
| ***Inner Arch L*** | G. T0 | 30.085±18.613 | 0.107 | 24,526±14,755 | 0.962 | 30.087±18.498 | 0.663 | 25.663±14.024 | 0.672 | 33,847±17,840 | 0.162 |
| | G. T1 | 24.558±18.120 | | 27.108±18.847 | | 27,772±21,308 | | 23.644±16.181 | | 24.813±18.254 | |
| | G. T2 | 17,902±13,773 | | 22.131±14.279 | | 25.928±17.070 | | 19.322±8.165 | | 19,328±6,947 | |
| ***External Arc L*** | G. T0 | 17.284±8.118 | 0.467 | 22,680±13,574 | 0.767 | 27.284±14.439 | 0.170 | 23.026±11.456 | 0.213 | 20.305±9.256 | 0.114 |
| | G. T1 | 24.558±18.120 | | 20.511±11.508 | | 18,256±7,641 | | 18,480±14,599 | | 18.428±10.461 | |
| | G. T2 | 17,902±13,773 | | 18,300±12,370 | | 19.084±10.512 | | 15,772±7,333 | | 13,408±5,542 | |
| ***Heel L*** | G. T0 | 14.813±11.394 | 0.743 | 16,564±11,596 | 0.963 | 24.016±21.500 | 0.467 | 17.47612.617 | 0.486 | 11.199±5.958 | 0.934 |
| | G. T1 | 15.070±12.280 | | 17.430±14.041 | | 20.608±15.971 | | 14,765±16,442 | | 13,849±11,577 | |
| | G. T2 | 11,789±6,844 | | 15,705±9,684 | | 20.119±21.826 | | 11,302±4,496 | | 11,412±6,345 | |

R: right; L: Left; G: group; G. T0: control group; G T1: T1 treatment group; G T2: T2 treatment group; Sd: standard deviation; *: significant; **: Highly significant.

**Table 12**. Mann-Whitney U test with Bonferroni p-value correction for outer arch hydration data at one and a half months and completion of treatment.

| | | | **p-value** | **Adjusted p-value** |
|---|---|---|---|---|
| **External arch after a month and a half of treatment** | No treatment (T0) | Treatment 1% (T1) | **0.008**** | **0.023*** |
| | No treatment (T0) | Treatment 0.5% (T2) | 0.092 | 0.276 |
| | Treatment 1% (T1) | Treatment 0.5% (T2) | 0.476 | 1.000 |
| **External arch at the end of treatment** | No treatment (T0) | Treatment 1% (T1) | **0.008**** | **0.024*** |
| | No treatment (T0) | Treatment 0.5% (T2) | **0.001*** | **0.002**** |
| | Treatment 1% (T1) | Treatment 0.5% (T2) | 0.211 | 0.632 |

*: meaningful; **: Highly significant.

**Bibliography**


1. Spanish Agency for Medicines and Health Products (AEMPS). Compendium of European standards and guidelines relating to cosmetic products for the application of the regulation 1223/2009 [Internet]. 2019. Available from: https://www.aemps.gob.es
2. Quételet ALJ. Fisica sociale ossia svolgimento delle facolta dell'uomo. 1869.
3. Léveque J, Rigal J. IMPEDANCE METHODS FOR STUDYING SKIN MOISTURIZATION. SOC COSMETIC CHEMISTS. 1983;34(8):419–28.
4. Dyer J, Foy V. Revealing the unseen a review of woodslamps in dermatology. J Clin Aesthet Dermatol. 2022;15(6):25–30.
5. Pérez Pico AM, Mingorance Álvarez E, Pérez Luque C, Mayordomo Acevedo R. Adquisición de competencias para el diagnóstico de onicomicosis mediante entrenamiento práctico podológico preclínico y clínico. Educ Med Super. 2019;33(4):37–49.
6. Burillo A, Moreno A, Salas C. Procedimientos en microbiología clínica. 2006.
7. Carson CF, Hammer KA, Riley T V. Melaleuca alternifolia (tea tree) oil: A review of antimicrobial and other medicinal properties. Clin Microbiol Rev. 2006;19(1):50–62.
8. Lee CJ, Chen LW, Chen LG, Chang TL, Huang CW, Huang MC, et al. Correlations of the components of tea tree oil with its antibacterial effects and skin irritation. J Food Drug Anal [Internet]. 2013;21(2):169–76. Available from: http://dx.doi.org/10.1016/j.jfda.2013.05.007
9. Groot AC De, Schmidt E. Tea tree oil: contact allergy and chemical composition. Contact Dermatitis. 2016; 75:129–43.
10. Rudbäck J, Bergström MA, Börje A, Nilsson U, Karlberg AT. α -Terpinene, an Antioxidant in Tea Tree Oil, Autoxidizes Rapidly to Skin Allergens on Air Exposure. Chem Res Toxicol. 2012;25(3):713–21.
11. Cox SD, Mann CM, Markham JL, Bell HC, Gustafson JE, Warmington JR. The mode of antimicrobial action of the essential oil of Melaleuca alternifolia (tea tree oil). J Chromatogr A. 2000; 1406:170–5.
12. Yang S ah, Jeon S kyung, Lee E jung, Im N kyung, Jung J young, Lee I seon. Bioactivity and Chemical Composition of the Essential oil of Tea Tree (Melaleuca alternifolia). Journal of Life Sciences. 2008;18(12):1644–50.
13. Yu D, Wang J, Shao X, Xu F, Wang H. Antifungal modes of action of tea tree oil and its two characteristic components against Botrytis cinerea. J Appl Microbiol. 2015; 119:1253–62.
14. Winkelman WJ. Aromatherapy, botanicals, and essential oils in acne. Clin Dermatol [Internet]. 2018;36(3):299–305. Available from: https://doi.org/10.1016/j.clindermatol.2018.03.004

UNIVERSIDAD DE EXTREMADURA

**VICERRECTORADO DE INVESTIGACIÓN Y TRANSFERENCIA**

Campus Universitario
Avda de Elvas, s/n
06006 - BADAJOZ

Tel.: 924 28 93 05
Fax: 924 27 29 83

NºRegistro: 195//2022

**D. JOÃO NUNO MEIRELES DA SILVA GONÇALVES RIBEIRO, SECRETARY OF THE BIOETHICS AND BIOSECURITY COMMISSION OF THE UNIVERSITY OF EXTREMADURA**

**REPORTS:**That once analyzed by this Commission, in its session held on 12/19/2022, the request for a Thesis Project entitled "Use of essential oils as natural therapies against dermatophytosis and new diagnostic techniques" , whose Director is Mrs. Raquel Mayordomo Acevedo and whose Principal Investigator is Mrs. Julia Villar Rodríguez has unanimously decided to positively value the aforementioned project considering that it complies with essential ethical standards, complying with current regulations for this purpose.

And so that it is recorded and has the appropriate effects, I sign this report

MEIRELES DA SILVA GONCALVES RIBEIRO JUAN NUNO - 08844642S

Firmado digitalmente por MEIRELES DA SILVA GONCALVES RIBEIRO JUAN NUNO - 08844642S
Fecha: 2022.12.21 18:07:37 +01'00'

DE FRANCISCO MORCILLO JAVIER - 28946338X

Firmado digitalmente por DE FRANCISCO MORCILLO JAVIER - 28946338X
Nombre de reconocimiento (DN): c=ES, serialNumber=IDCES-28946338X, givenName=JAVIER, sn=DE FRANCISCO MORCILLO, cn=DE FRANCISCO MORCILLO JAVIER - 28946338X
Fecha: 2022.12.22 14:29:39 +01'00'

V.º B.º

Signed.: Javier de Francisco Morcillo
President by delegation of the Bioethics and Biosafety Commission

# Anamnesis study creams

You will then be asked a series of questions to find out your previous stage before starting the study. In this study, the possibility of suffering from fungal infections in the feet and its subsequent treatment with natural therapies such as essential oils will be evaluated.

1. Reference

2. Participant's Name

3. Age

4. Contact phone number

5. Dedication?

   *Mark only one oval.*

   - ◯ Student
   - ◯ Unemployed
   - ◯ Worker
   - ◯ Other

6. If you answered student in the previous answer, please tell us that you are studying:

7. If you answered worker in the previous answer, please tell us what you work for:

8. Foot No.

9. If you have any medication/drug allergies please set it to continuation. If you don't have anything, say NO.

10. If you take any medication, please put it below. If you don't take anything put NO.

11. Weight

12. Height

13. Have you had any of these on your feet before?

*Select all that apply.*

- [ ] Discoloration changes
- [ ] Excessive sweating Wounds
- [ ] Cracks between the toes
- [ ] Cracks in another part of the foot
- [ ] Maceration
- [ ] None of the above

14. Do you have any of these on your feet today?

*Select all that apply.*

- [ ] Discoloration changes
- [ ] Excessive sweating Wounds
- [ ] Cracks between the toes
- [ ] Cracks in another part of the foot
- [ ] Maceracion
- [ ] None of the above

15. Have you had any of these symptoms on your feet before?

*Select all that apply.*

- [ ] Itching between the toes
- [ ] Itching on the bottom of the foot
- [ ] Generalized itching of the foot
- [ ] Stinging between the toes
- [ ] Stinging of the sole of the foot
- [ ] Generalized stinging of the foot
- [ ] Burning between the toes
- [ ] Burning of the sole of the foot
- [ ] Widespread burn on the foot
- [ ] None of the above

16. Do you have any of these symptoms on your feet today?

*Select all that apply.*

- [ ] Itching between the toes
- [ ] Itching on the bottom of the foot
- [ ] Generalized itching of the foot
- [ ] Stinging between the toes
- [ ] Stinging of the sole of the foot
- [ ] Generalized stinging of the foot
- [ ] Burning between the toes
- [ ] Burning of the sole of the foot
- [ ] Widespread burn on the foot
- [ ] None of the above

17. Have you been diagnosed with fungal infections on your feet before?

*Mark only one oval.*

- ◯ Yes
- ◯ No

18. Have you used drug treatment to treat fungal infections in your feet?

*Mark only one oval.*

- ◯ Yes
- ◯ No

19. If your previous answers were "YES", could you please tell us what treatment it was?

20. Have you ever been examined by a podiatrist?

*Mark only one oval.*

- ◯ Yes
- ◯ No

21. Have you ever had a mycological foot culture?

*Mark only one oval.*

- Yes
- No

22. If the previous answer was "YES", please indicate why

23. How often do you wash your feet?

*Mark only one oval.*

- Daily
- 2 times a day
- Less

24. How often do you examine your feet?

*Mark only one oval.*

- Daily
- 2 times a day
- Less

25. What kind of sock do you wear?

*Mark only one oval.*

- Cotton
- Polyester
- Don’t know

26. How often do you change your socks?

*Mark only one oval.*

- Daily
- 2 times a day
- Less

27. Do you wear breathable shoes?

*Mark only one oval.*

- Yes
- No

28. What kind of footwear do you usually wear on a daily basis?

*Mark only one oval.*

- Sneakers
- Boots/Trekking Boot/ Work/Protective Boots
- Other Style

29. How many times a day do you change your shoes?

*Mark only one oval.*

- I usually wear the same shoes all day
- I change shoes 2 times a day
- I change my shoes according to the activity
- I go every day with the same shoes

30. Do you play any sports? If yes, tell us which one and if you don't practice sport, put "NO"

31. If you play a sport, do you usually have specific shoes just for it?

*Mark only one oval.*

- Yes
- No

32. Do you wear protection (flip flops) when using showers in gyms, spas, clubs Sports?

*Mark only one oval.*

- Yes
- No