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Enhancing Skincare Formulas with Olive Oil-Infused Citrus Peel Extracts: A Comprehensive Study

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Abstract: The increasing global demand for natural and organic ingredients in cosmetics has spurred a surge in research exploring innovative formulations. This study delves into the utilization of citrus fruit peels and seeds macerated in olive oil as a foundation for novel ointments. Through the analysis of extracts from a variety of citrus sources, including orange, lemon, tangerine, grapefruit, and citron peels, as well as lemon and tangerine seeds, their physicochemical properties and antioxidant activity were meticulously examined using thin-layer chromatography (TLC). A groundbreaking aspect of this research is the revelation of the *in vitro* antioxidant potential of DMSO extracts obtained from these citrus-infused olive oils. Chemical assays unequivocally confirmed the presence of phenols and flavonoids, renowned for their robust antioxidant properties, across all extracts. These significant findings not only reinforce the well-documented benefits of citrus fruits in combating premature aging and diseases but also underscore the untapped potential of citrus by-products as valuable natural cosmetic ingredients. This preliminary investigation serves as a beacon illuminating the promising prospects of integrating citrus fruit remnants into cosmetic formulations. The imperative for further exploration in this realm is evident, aiming to refine formulations and advocate for the sustainable exploitation of citrus resources within the cosmetics sector. By embarking on deeper research endeavors, an inclusive comprehension of leveraging the inherent potency of citrus fruits for the creation of cutting-edge and efficacious natural cosmetic products can be achieved.

Keywords: Citrus by-products, Infused olive oil, Cosmetic formulations, Antioxidant, Skincare.

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1. INTRODUCTION

Olive oil plays a crucial role in the Mediterranean diet (1,2), with extensive research conducted to showcase its numerous health benefits. In particular, olive oil contains more than 30 different types of phenolic compounds, which are powerful antioxidants that help protect the body from free radicals (3). Extra Virgin Olive Oil (EVOO), renowned for being the highest quality among all categories of olive oils, is one of the most extensively studied food sources of antioxidants. This is attributed to the presence of various antioxidant compounds in EVOO (4). Their potent bioactive components exhibit robust cardio-protective attributes, aiding in the reduction of blood pressure and the prevention of atherosclerosis (5,6).

In recent times, there has been a growing trend to incorporate aromatic herbs, spices, and vegetables into extra virgin olive oil (EVOO) to create flavored oils. This practice aims to improve the sensory qualities, nutritional value, health benefits, and shelf life of the oil (7,8).

Among the natural flavors used with olive oil is *Citrus*, which is a genus belonging to the Rutaceae family of trees and shrubs, including oranges, lemons, grapefruits, tangerines, and limes (9). The citrus fruits were the second most produced fruits worldwide in 2021, accounting for 161.8 million tons produced in more than 10.2 million hectares (10). Therefore, Citrus by-products are immense and can be classified into peels (flavedo and albedo), seeds, and pulp residues, where the flavedo is the outer colored part of the bark that contains the oil sacs. In

contrast, the albedo is the white inner part of the bark, which is rich in pectin (9). In addition, peels are also high in sugars and have a high concentration of D-limonene, a potent antimicrobial compound (11-13). The residual pulp consists of the membranes and partitions of the segments that once contained the juice. As for the seeds, they are mainly composed of non-nitrogenous extracts, fats, crude proteins, and fibers (14).

Citrus peels, typically discarded as waste, are abundant in beneficial compounds such as Vitamin C, fiber, phenolics, and flavonoids, providing antioxidant, anticarcinogenic, and anti-inflammatory properties. Citrus fruits, thanks to their content of ascorbic acid, antiseptics, and antioxidants, are an effective natural weapon against acne. They help regulate sebum production, eliminate bacteria, and protect the skin from free radical damage (15,16). These criteria make it the best choice to be incorporated in organic and natural ointments, which are semi-solid and greasy preparations for external application to the skin, rectum, or nasal mucosa (17). The use of synthetic ointments encompasses the risk of skin irritation and allergic reactions triggered by specific cosmetic chemicals, such as formaldehyde-releasing preservatives, which can lead to skin problems. Additionally, these ointments may contain harmful components like petrolatum, propylene glycol, and synthetic colors, which have the potential to induce various health problems. Nowadays, the use of organic ointments possesses a considerable advantage thanks to the progressive discovery of the applications of plant oils in health care and in areas of economic interest, and they are also cheaper and without adverse effects. Recently, homemade medicines such as ointments have gained popularity without having been tested for compliance with standards. Therefore, the objective of this study is to develop natural citrus-based ointments incorporated in extra virgin olive oil (EVOO) and to evaluate the quality attributes of natural citrus ointment on the basis of various physicochemical characteristics.

2. MATERIAL AND METHODS

2.1. Chemicals and Reagents

1,1-diphenyl-2-picrylhydrazyl radical (DPPH), quercetin, catechine, and rutin were purchased from Sigma Aldrich; all other chemicals and solvents used were of analytical grade investigations.

2.2. Materials

Citrus fruits were purchased directly from the market; citron was obtained from a farm in Laghouat Province, South of Algeria. After air-drying in shaded areas at room temperature for a week, citrus peels and seeds were coarsely powdered using a dry grinder. Subsequently, the resulting powder was stored in airtight bags under dark conditions until further utilization.

For the maceration procedure, EVOO was purchased from a local certified producer. Beeswax, one of the natural raw materials employed in the ointment formulations, was purchased from a local beekeeper.

2.3. Preparation of Olive Oil-Infused Citrus Peels and Seeds

We have outlined the protocol for preparing Evoo-infused citrus peels and seeds, detailed in Figure 1.

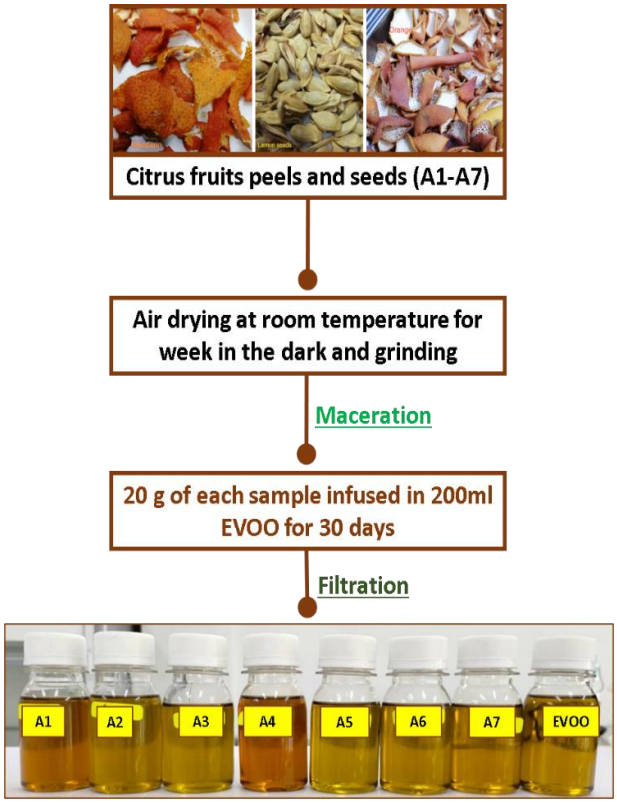


Figure 1: Description of the process for extracting citrus peels and seeds with EVOO through maceration. **A1:** Orange peels, **A2:** Lemon Peels, **A3:** Lemon Seeds, **A4:** Mandarine Peels, **A5:** Mandarine Seeds, **A6:** Grapefruits Peels, **A7:** Citron Peels, **EVOO**: Extra Virgin Olive Oil

The EVOO infused with citrus peels and seeds, displaying varying colors and fragrances, has been carefully preserved in bottles within a dark environment at room temperature until ready for use.

2.3.1 Physical analysis of the oil samples (Refractive Index)
Measurement of the refractive index of the infused samples was done by means of a refractometer according to the method of AOAC (2000) (17).

2.4. Phytochemical Tests
2.4.1. Extraction with DMSO
An extract solution was prepared using the methodology outlined by Erdal Eroglu et al. (2021) (18), with some modifications. Initially, a precise volume of DMSO, combined with double the volume of citrus peels and seeds-infused EVOO, was mixed in a beaker. This mixture was then subjected to gentle stirring on a hot plate at 37°C for 2 hours. Subsequently, the amalgam was allowed to rest undisturbed in darkness for 1 hour until a clear phase separation between the DMSO and the citrus-infused EVOO occurred. Following phase separation, the DMSO fraction was carefully transferred into a glass tube. Simultaneously, a control test tube containing solely EVOO was subjected to the same extraction protocol for comparative purposes. All DMSO-dissolved extracts were promptly stored in a refrigerated environment for subsequent utilization. The major families of secondary metabolites were elucidated through tube-colored reactions employing conventional characterization methods (19).

2.4.2. Phenolic compounds test
The total phenolic contents of all DMSO extracts were quantified utilizing the Folin-Ciocalteu test (20), as per preliminary assessments. Following the established protocol, 100µL of extract samples were mixed with 250 µL of Folin-Ciocalteu reagent in a test tube and allowed to incubate for 2 minutes at room temperature. Subsequently, 1000 µL of sodium carbonate (Na_2CO_3) was added to the same tube, and the samples were further incubated for 30 min at room temperature in darkness. The emergence of a blue coloration signified the presence of total phenolic compounds.

2.4.3. Flavonoids test
The flavonoid contents of DMSO extracts were determined by the $AlCl_3$ test (21). 500 µL of an $AlCl_3$ solution (10%) is taken with 500 µL of each sample in test tubes. The whole was incubated for 15 min at room temperature. The appearance of the yellow color indicates the presence of flavonoids.

2.4.4. Thin-layer chromatography analysis (TLC)
TLC was performed on silica gel 60 f 254, 20X10 cm HPTLC plates (Merck, Darmstadt, Germany) with ethyl acetate : methanol : water (10:1.35:1) (v/v/v) as a mobile phase (22).

Standards, solutions such as quercetin, catechin, and Rutin (2.5 mg/mL each) were applied with DMSO extracts to the plates in the form of strips. After development, the plates were dried with a flow of cold air using a hair dryer; the plates were documented under ultraviolet (UV) light 254 and 366 nm lamp by a camera device. Then, the plate was immersed in iodine (I_2) as a developer. The areas of the molecules appeared as brown spots, so another plate that was prepared with the same protocol was sprayed in the DPPH solution at one mM and dried quickly with a dryer. Next, antioxidant activity was visualized on the basis of trapping the stable artificial radical molecule (DPPH). The zones with an antioxidant effect appeared as yellow spots on the dark purple background and were photographed by a professional camera in visible light after the plates were dried (23,24). All retention factor values were calculated as described in different literature sources (23,24).

2.5. Ointments Preparation
To formulate the citrus-infused EVOO ointments, the following process was followed:
- 2 g of beeswax were melted over low heat until the temperature reached 60-70°C.
- 10 mL of the EVOO infused with the various citrus fruit peels was then added to the melted beeswax.
- The mixture was simmered until a homogeneous oily solution was obtained.
- The mixture was allowed to cool, resulting in the formation of an ointment with a desirable consistency.
- The prepared ointments were then transferred into storage containers for future use.

This straightforward yet effective method enabled the successful incorporation of the citrus-infused EVOO into a stable and well-textured ointment formulation. The controlled heating and cooling process ensured the homogeneity and appropriate consistency of the final ointment products.

2.5.1. Testing of Physical Properties of Ointments
Based on the search results, the key points regarding the evaluation of the physical properties of the citrus-based semi-solid ointments are organoleptic properties, homogeneity, pH, water resistance, skin absorption, and skin sensitivity. By thoroughly evaluating these physical properties, we will be able to assess the quality, stability, and suitability of the citrus-based semi-solid ointment formulations for topical application.

2.5.2. Organoleptic properties
The ointments were assessed for their appearance, color, odor, and texture to ensure they met the desired sensory characteristics.

2.5.3. Homogeneity test
The ointments were evaluated for their homogeneity, ensuring the active ingredients and excipients were uniformly distributed throughout the formulation. A 0.5 g of formulated ointments was placed on the skin and then leveled and observed based on color uniformity and the absence of lumps and granules.

2.5.4. pH test
The pH of the ointments was measured to confirm they were within the acceptable range for topical application, typically close to the skin's natural pH.

Using the pH paper, a thin layer of the ointment was applied, and it was then given some time to stand. The pH level was then determined by comparing it to a color chart.

2.5.5. Water resistance test

The ointments were tested for their ability to resist water and maintain their integrity when exposed to moisture, an important property for topical products. On glass plates, a thin layer of ointment was spread, then a drop of water was added. If they are non-miscible, this means that ointments are resistant to water.

2.5.6. Skin absorption

The ointments were evaluated for their ability to be absorbed into the skin, which is crucial for delivering the active ingredients to the target site. A small amount of ointment was applied to a specific area of the hand; we made circular movements, and then the time taken to absorb the ointment was recorded. Each type of ointment was repeated three times.

2.5.7. Topical Sensitivity Test

The ointments were tested for any potential skin irritation or sensitization reactions to ensure they were well-tolerated when applied topically. All ointments were tested for their skin sensitivity tests by applying them to the skin for a week and observing the side effects, if any, as a set of parameters like skin inflammation, skin irritation, reddening of the skin (allergic reactions) …etc.

3. RESULTS AND DISCUSSION

3.1. DMSO Extraction of EVOO-Infused Citrus Peels and Seeds

The analysis of phytochemicals in plant-based products, particularly in oil seed plant extracts with complex compositions, continues to be a major challenge. Previous research has commonly used methanol as the preferred solvent to extract bioactive compounds from plants infused oil to perform phytochemical characterization (25,26).

This study used DMSO for the first time to extract bioactive compounds from EVOO—citrus peels and seeds. The extraction of bioactive components of infused EVOO—citrus peels was successfully obtained. The transparent color of DMSO has become different according to the color of each sample. This study specifically selected EVOO for the preparation, adhering to a traditional medicine recipe.

Extractive conditions such as incubation time, darkness, and temperature decrease the rate of oxidation, which alters the organoleptic properties of the oil compared to other studies (18).

3.2. Qualitative Phytochemical Screening

Preliminary phytochemical screening of DMSO extracts revealed the presence of various bioactive components, including phenolics and flavonoids (Figure 2). The current study found that DMSO-citrus peel olive oil extracts show a positive test (+++++) for phenols and flavonoids with the exception of A1 and A3 (++), which could be attributed to the improved solubility of the active compounds in organic solvents.

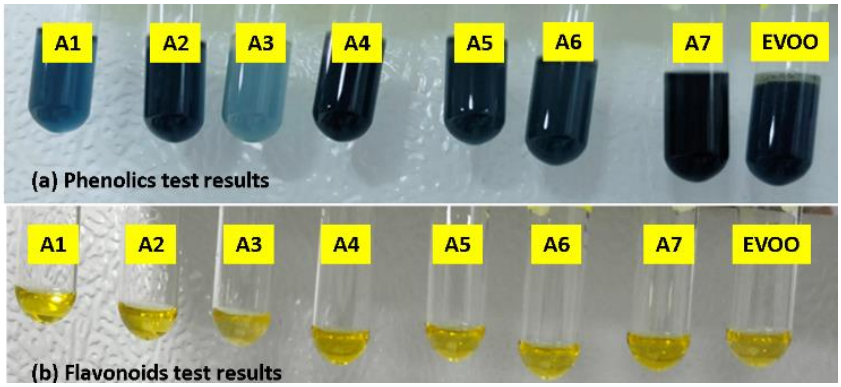


Figure 2: Phytochemical Screening of phenolics and flavonoids.

3.3. Thin-Layer Chromatography Analysis

To avoid false positive results when using TLC-DPPH to analyze antioxidant compounds, it is recommended to dry the chromatograms and shoot them directly under white light after spraying with the DPPH solution. This procedure helps to ensure the stability of the resulting coloring.

The UV detection of the spots showing the antioxidant effect can also be significant since they are visible at 366 nm but not at 254 nm (Figures 3A and 3B).

The iodine developer plate has areas of the molecules in the form of brown spots (Figure 3C). From this plate, Rf is calculated for quercetin, catechine, and rutin (Rf values of 0.86, 0.78, and 0.24, respectively) where quantified in all the TLC plates (Table 1). Among the compounds available as standards, quercetin, and catechine were present in all DMSO-citrus peels and seeds-EVOO extracts. Visualization of the plate in UV light over time wavelength (366 nm) indicates the presence of multiple spots with varying degrees of Polarization and coloration in all extracts. The iodine developer plate (Figure 3C) revealed the presence of various phenolic compounds with differing polarities. These compounds appeared as multiple spots with Rf values ranging from 0.28 to 0.95, which were not readily apparent under short-wavelength UV light (254 nm),

as shown in Figure 3A. In iodine plat, all DMSO- citrus - EVOO extracts revealed four spots except the DMSO- Grap fruits Peels - EVOO (A6) and DMSO-Citron Peels - EVOO (A7) extracts have five spots at R_f =0.94, 0.88, 0.8, 0.74, 0.34 of A6 and R_f =1, 0.86, 0.857, 0.73, 0.34 of A7.

Monitoring the transformation of the purple DPPH solution to a yellow color identified the presence of positive antioxidant activity. The TLC–DPPH analysis of the DMSO-Citrus—EVOO and DMSO-EVOO extracts revealed several characteristic antioxidant zones. DMSO-Orange peels—EVOO (A1), DMSO-Lemon Seeds—EVOO (A3), and DMSO-Grapefruit Peels—EVOO (A6) extracts displayed weak antioxidant activity.

Previous works assessed the DPPH radical scavenging ability of different citrus species and indicated that the antioxidant activity and phenolic content are strongly affected by the species and extraction solvent (27,28). However, it should be noted that no published work has been done on thin-layer chromatography analysis of DPPH in Citrus peels infused olive oil.

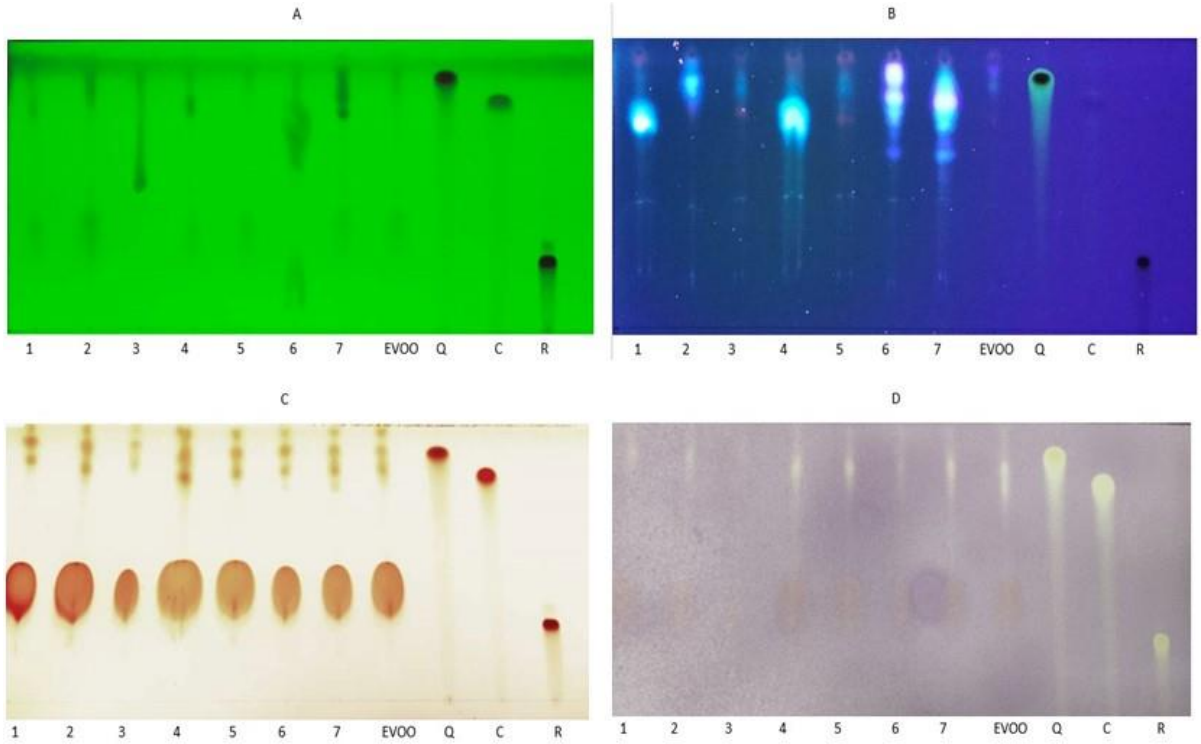


Figure 3: TLC chromatograms of DMSO-Citrus-EVOO, DMSO-EVOO extracts, and standards at 254 nm (A), at 366 nm (B), iodine(C), and at white light after DPPH assay (D).
A1: Orange peels, **A2:** Lemon Peels, **A3:** Lemon Seeds, **A4:** Mandarine Peels, **A5:** Mandarine Seeds, **A6:** Grapefruits Peels, **A7:** Citron Peels, **EVOO**: Extra Virgin Olive Oil, **Q:** Quercetin, **C:** catechin, **R:** rutin

Table 1: R_f values of DMSO-Citrus-EVOO, DMSO -EVOO and standards extracts.

Compounds	R_f
A1	0.95 /0.86 /0.79 /0.3
A2	0.94 /0.88 /0.82 /0.32
A3	0.93 /0.86 /0.8 /0.28
A4	0.94 /0.89 /0.82 /0.32
A5	0.93 /0.86 /0.79 /0.34
A6	0.94 /0.88 /0.8 /0.73 /0.34
A7	0.93 /0.86 /0.8 /0.73 /0.34
EVOO	0.94 /0.87 /0.81 /0.33
Quercetin	0.86
Catechin	0.78
Rutin	0.24

3.4. Physical Analysis of the Infused-EVOO Samples

Table 2 shows the refractive index of EVOO-infused citrus peels and seeds. The values of all oil samples ranged from 1.4659 to 1.4669. The results are within the limits set by Codex Alimentarius (2017) standards for olive oils (29).

Table 2: Refractive index of EVOO-infused citrus peels and seeds.

The oil samples	Refractive index at 23 C°
A1	1.4662
A2	1.4663
A3	1.4665
A4	1.466
A5	1.4665
A6	1.4669
A7	1.4662
EVOO	1.4659

3.5. Testing of Physical Properties of Ointment

3.5.1. Organoleptic test

Organoleptic characteristics refer to the sensory properties of a product, including its appearance, smell, taste, and texture. For EVOO-infused Citrus Peel-derived ointments, the organoleptic characteristics are primarily influenced by the type of citrus fruit used and the quality of the olive oil. The color and appearance of the formulated ointment vary depending on the kind of citrus peels and seeds used. Lemon peel-infused olive oil ointment has a lighter color compared to orange and tangerine peel-infused olive oil ointment (Figure 4). The aroma is typically a pleasant combination of the citrus fruit and the olive oil, with the citrus notes being more prominent. The taste is a balance of the citrus and olive oil flavors, with the citrus peel adding a slightly sweet and tangy taste. The texture is smooth and creamy, with the ointment being easily spreadable.

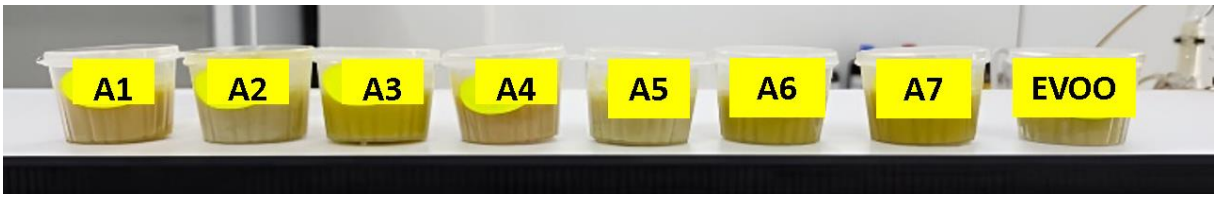
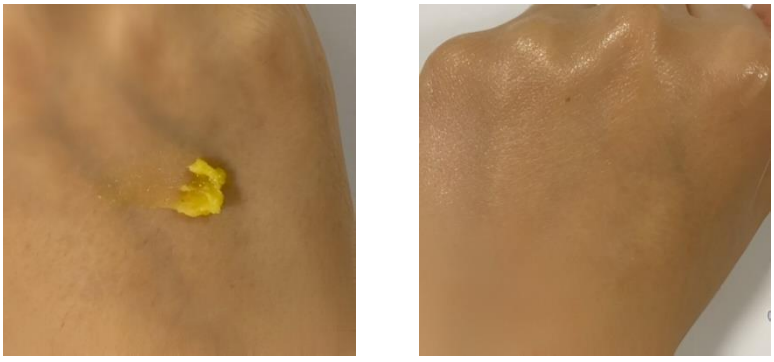


Figure 4: Formulated ointments.
A1: Orange peels, **A2:** Lemon Peels, **A3:** Lemon Seeds, **A4:** Mandarine Peels
A5: Mandarine Seeds, **A6:** Grapefruits Peels, **A7:** Citron Peels, **HO:** EVOO

3.5.2. Homogeneity test

A homogeneity test is performed to analyze whether the basic materials have been mixed homogeneously during the ointment's synthesis process. The ointment's dosage is visually observed based on color uniformity and the absence of lumps and granules. The results of this test showed that all formulated ointments from EVOO-infused citrus peels and seeds present homogeneity before and after application to the skin (Figure 5).



Before application After application

Figure 5: Homogeneity test.

3.5.3. pH test

Monitoring the pH value of ointments is essential for ensuring their stability, efficacy, and safety. It is an important aspect of quality control and regulatory compliance. The pH value of the skin is typically between 5 and 6 (30), and maintaining this pH level is crucial for the skin's barrier function, which helps prevent the entry of harmful microbes and other substances. Changes in the skin's pH level, particularly if it becomes more alkaline, can lead to impaired barrier function, dryness, and irritation. The pH value of ointments can also affect their physical properties, such as their viscosity and spreadability. Changes in pH can lead to changes in the ointment's consistency, which can affect its application and effectiveness. The pH values of all formulated ointments fall within the range of 5 to 6 (Figure 6). This aligns well with the average skin surface pH. Therefore, our results can be considered acceptable, minimizing the risk of irritation upon application.

3.5.4. Water resistance

All formulated ointments are anhydrous in nature and composed of water-insoluble components. They

exhibit a non-miscible and non-absorbent relationship with water, presenting an almost spherical shape (Figure 7). They tend to be more water-resistant compared to water-based emulsions. Therefore, they remain on the skin surface for a long time without drying out. When applied topically, they form a barrier on the skin's surface, which helps restrict the evaporation of water naturally present in the skin, ultimately increasing its hydration levels.

3.5.5. Skin Absorption

The absorption of ointments by the skin is an essential factor in the effectiveness of topical medications. Ointments are a common formulation for dermatological drugs due to their ability to enhance drug penetration and absorption into the skin. The factors affecting the absorption of ointments by the skin include the cornified layer, medication particle size, degree of skin hydration, contact time, skin temperature, and epidermal damage. The cornified layer, the top layer of the epidermis, is crucial in determining the absorption of ointments (31). Moistening the skin can enhance the absorption of medication by softening the cornified layer, allowing the ointment to penetrate more effectively. The size of the medication particles is also an essential factor; solutions such as olive oil or lanolin base have greater absorption (32). Table 3 demonstrates that all ointment formulations exhibited a short absorption duration. This trend suggests a direct relationship between the active ingredient percentage and the absorption duration. All formulations contain a considerable proportion of oils and are greasier, thereby facilitating faster absorption rates into the skin.

Figure 6: pH of formulated ointments.

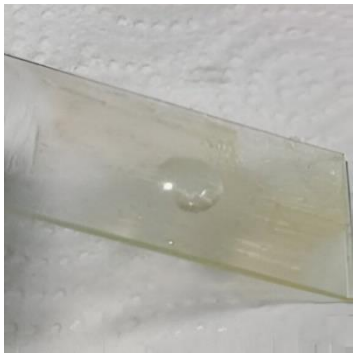


Figure 7: Water resistance test of ointments.

Table 3: Duration of absorption by the skin.

Sample	Duration of absorption by skin (s) per 1 cm
A1	33.93 ±2.04
A2	33.04±2.42
A3	30.06±0.55
A4	29.68±1.39
A5	30.16±0.97
A6	34.1±2.27
A7	34.65±3.86
EVOO	30.94±0. 67

3.5.6. Topical sensitivity test

No side effects were observed, such as skin inflammation, irritation, or reddening of the skin (allergic reaction); i.e., these formulations did not produce any skin irritation for about a week when applied to the skin.

4. CONCLUSION

The study conclusively demonstrates the significant antioxidant properties of citrus extracts infused in extra virgin olive oil (EVOO), highlighting their ability to trap DPPH radicals and reduce oxidative potency effectively. This innovative approach underscores the potential of utilizing citrus fruit peels and seeds, often considered waste, as valuable resources in combating oxidative stress-related diseases, enhancing food products, and formulating natural cosmetic products. By integrating these citrus-infused oils into ointments, the research not only showcases their antioxidant benefits but also emphasizes their favorable physicochemical properties, such as optimal pH, water resistance, and efficient skin absorption, making them suitable for topical applications. The detailed documentation of the methodologies, including the novel use of DMSO for bioactive compound extraction, ensures reproducibility and serves as a solid foundation for future research. This study paves the way for further exploration into optimizing formulations, understanding the individual roles of bioactive compounds, and expanding the use of other natural oils for infusion, thereby contributing to the development of effective, sustainable, and environmentally friendly natural products.

5. ACKNOWLEDGMENT

Declared none.

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