Formulation of a soap enriched with *Zingiber officinale* extract with antibacterial and anti-inflammatory properties

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ABSTRACT

Ginger, derived from the rhizome of the *Zingiber officinale* Roscoe plant, is renowned for its medicinal properties, particularly in alleviating nausea and vomiting. Recent studies have confirmed its efficacy in various medicinal treatments, with antimicrobial, anti-inflammatory, antiseptic, and antioxidant attributes. Ginger is highly valued in dermatology for its antioxidant abilities to combat free radicals that cause skin damage, rich in gingerols and other active compounds, ginger extract demonstrates antimicrobial effects by inhibiting unwanted microorganism growth and aids in reducing inflammation and promoting skin healing.

Consequently, soaps infused with ginger extract may provide a natural and efficient solution for skin hygiene and care. However, it's important to consider limitations such as variations in the effectiveness of ginger extract for wound treatment, which may depend on the concentration of the extract, soap formulation, and severity of the wound. Additionally, the use of plant extracts can trigger allergic reactions in some individuals, which may worsen the skin condition instead of improving it. Therefore, before use, it's crucial to seek guidance from a healthcare professional whenever possible.

In this study, two extraction methods were tested: Microwave-Assisted Extraction and Ultrasound-Assisted Extraction. The optimal points identified were established at 17 minutes, 260 Watts of power, and 93% of hydroethanolic solvent ($EtOH/H_2O$) concentration for the Microwave method. For the Ultrasound-assisted extraction, the ideal parameters were determined as 45 minutes, 20% power, and 80% of hydroethanolic solvent ($EtOH/H_2O$) concentration. The microwave-assisted extraction method achieved a slightly higher yield than the ultrasound method.

Ultrasound extraction showed better antioxidant activity by the DPPH method (0.373 ± 0.005 mg/mL), while microwave extraction demonstrated superior activity by the TBARS method (0.0032 ± 0.0001 mg/mL).

In antibacterial tests, *Cutibacterium acnes*, *Staphylococcus epidermidis*, and Methicillin-resistant *Staphylococcus aureus* (*MRSA)* show comparable efficacies for both microwave and ultrasound extraction methods, indicated by the same minimum inhibitory concentration (MIC) of 5 mg. However, for *Pseudomonas aeruginosa*, a difference is observed, the microwave extraction appears to be less effective (>10 mg) compared to ultrasound extraction (10 mg).

Ginger extracts exhibits anti-inflammatory properties with low cytotoxicity.

Microwave and ultrasound extracts only showed cytotoxic effects at concentrations exceeding 400 μg/mL for HFF1 cell lines. The result of 235.76 EC_{50}, μg/mL indicates the effective concentration of the microwave extract needed to achieve 50% of the desired anti-inflammatory activity in the RAW 267.4 cell line, while ultrasound extract showed 267.9 EC_{50}, μg/mL.

The solid soaps were produced using 25 grams of glycerin, with the addition of 5 and 10 milligrams of each extract that were diluted in 1 milliliter of water, therefore two solid soaps were produced for each extraction method. The soaps were then left at room temperature until solidify, later divided into equal parts for evaluation at 0, 7, and 15 days.

The soap with the most bioactive properties was the one made with ginger extract obtained through the ultrasonication extraction method. The sequence of inhibition zones followed the order from highest to lowest activity: *Pseudomonas aeruginosa* (25 mm), Methicillin-resistant *Staphylococcus aureus* (*MRSA)* (20 mm), *Staphylococcus epidermidis* (20 mm), and *Cutibacterium acnes* (20 mm).

This study aimed to optimize ginger extraction by identifying the optimal extraction points for the two methods tested, aiming to obtain an extract with the best bioactive properties to be incorporated into a soap. The results confirmed that ginger possesses antibacterial, anti-inflammatory, and antioxidant characteristics, crucial for formulating a medicinal soap.

Keywords: Ginger, soap production, antibacterial, anti-inflammatory, antioxidant.

RESUMO

O gengibre, derivado do rizoma da planta *Zingiber officinale* Roscoe, é renomado por suas propriedades medicinais, especialmente na redução de náuseas e vômitos. Estudos recentes têm confirmado sua eficácia em vários tratamentos medicinais, com atributos antimicrobianos, anti-inflamatórios, antissépticos e antioxidantes. O gengibre é altamente valorizado na dermatologia por suas habilidades antioxidantes de combater os radicais livres que causam danos à pele, rico em gingeróis e outros compostos ativos, o extrato de gengibre demonstra efeitos antimicrobianos ao inibir o crescimento indesejado de microrganismos e auxilia na redução da inflamação e na promoção da cicatrização da pele.

Consequentemente, sabonetes enriquecidos com extrato de gengibre podem oferecer uma solução natural e eficaz para a higiene e cuidado da pele. No entanto, é importante considerar limitações, como variações na eficácia do extrato de gengibre para o tratamento de feridas, que podem depender da concentração do extrato, da formulação do sabonete e da gravidade da ferida. Além disso, o uso de extratos vegetais pode desencadear reações alérgicas em algumas pessoas, o que pode piorar a condição da pele em vez de melhorá-la. Portanto, antes de usar, é crucial buscar orientação de um profissional de saúde sempre que possível.

Neste estudo, foram testados dois métodos de extração: Extração Assistida por Micro-ondas e Extração Assistida por Ultrassom. Os pontos ótimos identificados foram estabelecidos em 17 minutos, 260 Watts de potência e 93% de concentração de solvente hidroetanólico ($EtOH/H_2O$) para o método de Micro-ondas. Para a extração assistida por ultrassom, os parâmetros ideais foram determinados como 45 minutos, 20% de potência e 80% de concentração de solvente hidroetanólico ($EtOH/H_2O$). O método de extração assistida por micro-ondas alcançou um rendimento ligeiramente maior do que o método de ultrassom.

A extração por ultrassom apresentou melhor atividade antioxidante pelo método do DPPH (0.373 ± 0.005 mg/mL), enquanto a extração por micro-ondas demonstrou uma atividade superior pelo método TBARS (0.0032 ± 0.0001 mg/mL).

Nos testes antibacterianos, *Cutibacterium acnes*, *Staphylococcus epidermidis* e Methicillin-resistente *Staphylococcus aureus* (*MRSA)* apresentam eficácias comparáveis para ambos os métodos de extração por micro-ondas e ultrassom, indicadas pela mesma concentração inibitória mínima (CIM) de 5 mg. No entanto, para *Pseudomonas aeruginosa*,

observa-se uma diferença, a extração por micro-ondas parece ser menos eficaz (>10 mg) em comparação com a extração por ultrassom (10 mg).

O gengibre apresenta propriedades anti-inflamatórias com baixa citotoxicidade. Os extratos de micro-ondas e ultrassom mostraram efeitos citotóxicos apenas em concentrações acima de 400 μg/mL para as linhas celulares HFF1. O resultado de 235,76 EC_{50}, μg/mL indica a concentração eficaz do extrato de micro-ondas necessária para alcançar 50% da atividade anti-inflamatória desejada na linha celular RAW 267.4, enquanto o extrato de ultrassom mostrou 267.9 EC_{50}, μg/mL.

Os sabonetes sólidos foram produzidos utilizando 25 gramas de glicerina, com a adição de 5 e 10 miligramas de cada extrato diluídos em 1 mililitro de água, portanto, foram produzidos dois sabonetes sólidos para cada método de extração. Os sabonetes foram então deixados em temperatura ambiente até solidificar, posteriormente divididos em partes iguais para avaliação nos dias 0, 7 e 15.

O sabonete com as melhores propriedades bioativas foi aquele feito com extrato de gengibre obtido por meio do método de extração por ultrassom. A sequência das zonas de inibição seguiu a ordem de maior para menor atividade: *Pseudomonas aeruginosa* (25 mm), Methicillin-resistente *Staphylococcus aureus* (*MRSA)* (20 mm), *Staphylococcus epidermidis* (20 mm) e *Cutibacterium acnes* (20 mm).

Este estudo teve como objetivo otimizar a extração de gengibre identificando os pontos ótimos de extração para os dois métodos testados, com o objetivo de obter um extrato com as melhores propriedades bioativas para ser incorporado em um sabonete. Os resultados confirmaram que o gengibre possui características antibacterianas, anti-inflamatórias e antioxidantes, essenciais para a formulação de um sabonete com potencial terapêutico.

PALAVRAS-CHAVE: Gengibre, produção de sabonete, antibacteriana, anti-inflamatória, antioxidante.

TABLE OF CONTENTS

LIST OF EQUATIONS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

Hz - Hertz

% - Percent

°C - Degrees Celsius

rpm - Revolutions per minute

L - Liter

mL - Milliliter

μL - Microliter

kg - Kilogram

g - Gram

mg - Milligram

μg - Microgram

Cm - Centimeter

mm - Millimeter

nm - Nanometer

mM - Millimolar

pH - Potential of Hydrogen

w/v - Weight/Volume

v/v - Volume/Volume

h - Hour

min - Minute

CFU - Colony Forming Unit

INT - 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride

DPPH - 2,2-diphenil-1-picrylhydrazil

TBARS - Thiobarbituric Acid Reactive Substances

MDA - Malondialdehyde

TBA - Thiobarbituric Acid

EtOH - Ethanol

H_2O - Water

$FeSO_4$ - Iron (II) sulfate

CO_2 - Carbon dioxide

NO - Nitric oxide

OS - Oxidative stress

XO - Xanthine oxidase

DNA - Deoxyribonucleic acid

Tris-HCl - Tris(hydroxymethyl)aminomethane hydrochloride

LPS - Lipopolysaccharide

Caco-2 - Colon adenocarcinoma cells (a cell line)

MRSA - Methicillin-resistant *Staphylococcus aureus*

NF-κB - Nuclear Factor kappa B

COX - Cyclooxygenase

COX-2 - Cyclooxygenase-2

LOX - Lipoxygenase

TNF - Tumor Necrosis Factor

TNF-α - Tumor Necrosis Factor alpha

IL-6 - Interleukin-6

IL-1β - Interleukin-1 beta

Nrf2 - Nuclear factor erythroid 2-related factor 2

iNOS - Inducible Nitric Oxide Synthase

ROS - Reactive Oxygen Species

EC_{50} - Concentration that quenches 50% of radicals

IC_{50} - Concentration required to achieve 50% inhibition

GI_{50} - Concentration responsible for causing 50% growth inhibition

LC_{50} - Lethal Concentration 50%

MAE - Microwave-assisted Extraction

UAE - Ultrasound-assisted Extraction

MIC - Minimum Inhibitory Concentration

DMSO - Dimethyl Sulfoxide

TSB - Tryptic Soy Broth

CLSI - Clinical and Laboratory Standards Institute

ECACC - European Collection of Authenticated Cell Cultures

DMEM - Dulbecco's Modified Eagle Medium

NED - N-(1-naphthyl) ethylenediamine hydrochloride

SRB - Sulforhodamine B

ANOVA - Analysis of Variance

HBSS - Hank's Balanced Salt Solution

W - Watts

TPC - Total Phenolic Compounds

GAE - Gallic Acid Equivalents

dw - Dry Weigh

DG - Air-dried Ginger

PDG - Powder Air-dried Ginger

FDG - Gresh Air-dried Ginger

HFF1 - Human Fetal Skin Fibroblasts

cSSSI - Complicated Skin and Skin-structure Infections

BSIs - Bloodstream Infections

VAP - Ventilator-associated Pneumonia

HAIs - Healthcare-associated Infections

1. INTRODUCTION

Ginger is the rhizome of the plant known as *Zingiber officinale* Roscoe, belonging to the family Zingiberaceae. Originally from South Asia, it is currently disseminated in various parts of the world. Since ancient times, ginger has been used to treat ailments such as nausea and vomiting, and recent research has confirmed its efficacy (1).

This herbaceous plant can grow up to 1.5 meters in height, including an articulated stem and a horizontal rhizome that extends laterally, with branches on the same plane. The rhizome (Figure 1) is the commercially used part of the plant and is the source of active compounds like essential oils, gingerols, shogaols, zingiberene, phellandrene, camphene, cineole, borneol, citral, and carbohydrates (2).

Figure 1 - Ginger rhizome

Source (3).

Ginger is widely recognized for its dermatological benefits due to its antimicrobial, anti-inflammatory, antiseptic, and antioxidant properties. These properties play an important role in inhibiting free radicals that contribute to oxidative damage to the skin (4). Additionally, ginger has also been used in the regeneration of skin tissue (5).

However, it is important to emphasize that the direct application of ginger to the skin may be suitable for everyone. In certain individuals, it can provoke adverse reactions, such exacerbating conditions like psoriasis and acne (5).

Ginger extract, rich in gingerols and other bioactive compounds, exhibits antimicrobial properties that inhibit the growth of unwanted microorganisms (6). Its anti-inflammatory properties also help reduce skin inflammation and promoting healing (7).

Soaps containing ginger extract thus offer a natural and effective approach to skin hygiene and care.

Soaps, play a fundamental role in cleaning and in case of certain formulations, disinfecting, with the goal of removing and reducing germs. The antibacterial properties of soaps are particularly important for skin protection, ensuring effective hygiene. They are used in a variety of settings, and selecting an appropriate soap crucial for maintaining skin health and integrity, preventing infections, and promoting proper hygiene (8).

2. OBJECTIVES

2.1 GENERAL OBJECTIVE

Develop a soap containing *Zingiber officinale* Roscoe extract with antibacterial and anti-inflammatory activity for topical application.

2.2 SPECIFIC OBJECTIVE

- Optimization of the extraction methodologies;
- Determination of bioactive properties of the extracts (antimicrobial against *Cutibacterium acnes*, *Staphylococcus epidermidis*, Methicillin- resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* and anti-inflammatory capacity (mouse RAW 264.7 macrophages));
- Evaluation of the cytotoxicity of the extracts (keratinocyte cell line HFF1 (Human Fetal Foreskin Fibroblasts));
- Assessment of the antioxidant activity (DPPH) Radical Scavenging Effect method (2,2-diphenyl-1-picrylhydrazyl) and thiobarbituric acid reactive substances (TBARS);
- Development of the solid soap containing ginger extract;
- Monitorization the bioactivity and the color of the developed soaps.

Based on this, the objective of this study is to produce a soap with anti-inflammatory, antibacterial and antioxidant properties by using a hydroethanolic extract of ginger which inherently contains these bioactive qualities. Two extraction methods, microwave-assisted extraction and ultrasound-assisted extraction will be evaluated which method yields an optimized extract with potent bioactive properties.

The solid soap will be produced using both extraction methods and tested for its antimicrobial capacity immediately, and at intervals of 7 and 15 days, to ascertain the most effective extraction technique.

3. LITERATURE REVIEW

3.1 ETYMOLOGY AND APPLICATIONS

Ginger, scientifically known as *Zingiber officinale*, was first cataloged by the renowned English botanist William Roscoe (1753-1813) in 1807 (3, 9).

Zingiber officinale Roscoe has been cultivated since ancient times, believed to have originated in southern China, Southeast Asia, and India. It was introduced to the Mediterranean in the first century, reached Japan by the third century and arrived in Europe by the fourth century. By the eleventh century, ginger was introduced to both England and America, solidifying its presence in different parts of the world. This historical dissemination highlights the broad reach and cultural significance of ginger throughout the centuries (10). Nowadays, ginger is cultivated in tropical and subtropical countries, with India, China, Nepal, Indonesia, and Nigeria as some of the main producers (11).

It is a monocotyledonous plant belonging to the family Zingiberaceae and the order Zingiberales (6). It belongs to the genus *Zingiber*, subfamily Zingiberoideae, and subgenus *Officinale* (12). Table 1 provides detailed information about ginger.

Table 1 – Characteristics of Ginger (*Zingiber officinale* Roscoe)

Synonyms	Zingiber, *Rhizoma zingiberis*
Family	Zingiberaceae (6).
Biological source	The rhizomes of the plant *Zingiber officinale* Roscoe are used to produce ginger, which is kind of an oleo gum resin.
Geographical source	It is native to Southeast Asia and is grown in India, Jamaica, the Caribbean, Australia, Mauritius, Taiwan and Africa (10).
Colour	Buff or earthy brown
Odour	Characteristic, agreeable and aromatic
Taste	Pungent (3).
Size	Rhizomes range in size from 5 to 20 cm and from 2 to 6 cm (2).
Shape	Rhizomes bear shorts, flat, oval branches that bud at the tip and are laterally compressed (2).

Source: Adapted from (7).

Ginger is widely used in cuisine as a spice and flavoring agent. The distinctive characteristic of its taste and aroma are attributed to the volatile oils present in the plant, zingerone, shogaols, and gingerols identified as the most pungent compounds. Various investigations have been conducted to unravel the extraordinary properties of this plant (7).

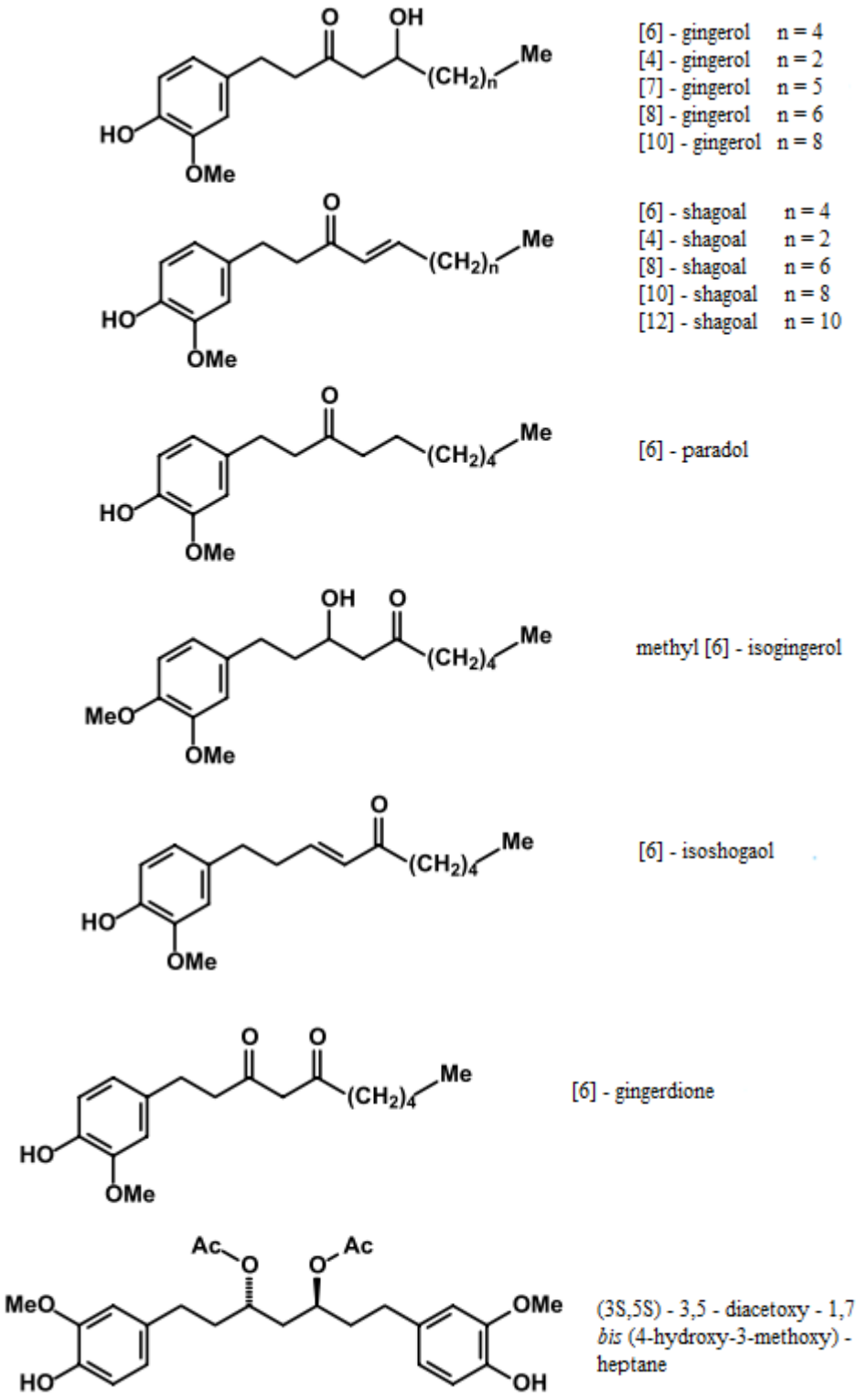
Nowadays, ginger is used to treat several common disease, including headaches, nausea, vomiting, and colds (13). Furthermore, recent research has revealed its diverse biological activities, which include antioxidant (14), antimicrobial (15), anti-inflammatory (16) and anticancer (17) properties.

3.2 CHEMICAL COMPOSITION

The ginger rhizome contains a variety of chemical compounds, that include approximately 3% to 6% of fatty oil, 9% of protein, 60% to 70% of carbohydrates, 3% to 8% of crude fiber, about 8% of ash, 9% to 12% of water, and 2% to 3% of essential oil (10, 18, 19). Ginger is also an important source of sugars, proteins, B-complex vitamins, and vitamin C, as well as minerals, carbohydrates, fats, waxes, extractable oils, resins, and the enzyme zingibain, which work together for the proper functioning of the human body and the diseases prevention (3).

The composition of ginger can vary according to storage conditions, processing, cultivation, and harvesting. These variations can affect the nutritional and therapeutic properties of the plant. Cooking, drying, and steaming are some of the processes that can alter its composition. Additionally, environmental and cultivation factors also play an important role (18). However, the major chemical constituents of ginger are illustrated in the figure 2 below.

Figure 2 - Major chemical constituents of ginger



Source (10).

Research carried out by Singh *et al*., 2004 (20), on acetone oleoresin extracted from fresh ginger rhizome, through gas chromatography-mass spectrometry (GC-MS) identified several chemical compounds, including trans-6-shogaol (26.32%), trans-10-shogaol

(13.0%), α-zingiberene (9.66%), trans-8-shogaol (7.72%), 10-gingerdione (6.80%), cis-6-shogoal (3.31%), β-sesquiphellandrene (2.94%), ar-curcumene (2.76%), 6-gingerdiol diacetate (2.00%), β-bisabolene (1.98%), 6-gingerol (1.87%), (E,E)-α-farnesene (1.63%), 6-paradiol (1.50%), and cis-8-shogaol (1.21%) (21).

Similarly research conducted by Mesomo *el al*., 2013 (22) , on the ether ethylic extract from dried ginger rhizome also identified several compounds using GC-MS, including α-zingiberene (17.5%), β-sesquiphellandrene (13.2%), α-farnesene (6.3%), citral (7.1%), nerol (5.1%), decanal (4.1%), caryophyllene oxide (3.1%), farnesol (2.3%), tetracosane (2.5%), and alloaromadendrene (2.0%) (21).

The most commonly found class of compounds in ginger (*Zingiber officinale*) extracts is gingerols and shogaols. These phenolic compounds are responsible for ginger´s distinct spicy flavor and associated health benefits. Gingerols and shogaols have anti-inflammatory and antioxidant properties, and are known for their ability to alleviate nausea, among other health benefits (20, 21).

Essential oils, or volatile compounds, obtained through steam distillation of the rhizome are responsible for the aromatic characteristics. The non-volatile compounds, responsible for the pungent characteristics, are phenolic compounds (6). The essential oil of ginger contains various compounds, including sesquiterpenes like zingiberene (36% w/w), curcumene (18%), and farnesene (10%). Monoterpenes like cineole (1.3%), linalool (1.3%), borneol (2.2%), geranial (citral a, 1.4%), and neral (citral b, 0.8%) are also present. Non-volatile components of ginger include biologically active compounds such as gingerols, shogaols, paradols, and zingerone (19, 20).

The main constituents of ginger essential oils are terpene components such as β-bisabolene, α-curcumene, zingiberene, α-farnesene, and β-sesquiphellandrene (24). Polysaccharides, organic acids, crude fibers, and lipids are also present in ginger (28, 29).

Terpenoids are compounds with the basic isoprene unit (C_5) and form a broad group of phytochemicals known for their pleasant aroma, spicy flavor, and various pharmacological activities (26). Monoterpenoids have a carbon skeleton formed by two isoprene units (C_{10} skeleton), and may contain cycloalkane units, such as cyclopropane, cyclohexane, and cyclobutene (31, 32). Sesquiterpenoids, characterized by three isoprene units (C_{15} skeleton), are widely found in nature and play a significant role in creating distinct flavors and fragrances (29–31).

Phenolic compounds possess a phenolic hydroxyl group and include polyphenols, which constitute a significant portion of naturally occurring bioactive compounds (20, 36).

In addition to terpenoids, which primarily comprise the volatile oil, the non-volatile compounds responsible for the ginger´s pungent sensation are predominantly gingerols, shogaols, and paradols, all classified as phenolic compounds and their derivatives (33).

Diarylheptanoids are compounds that have two aryl groups linked by a heptane chain and are divided into linear (curcuminoids) and cyclic groups. Curcumin, a primary component of curcuminoids, was initially identified in turmeric (Long turmeric), a member of the Zingiberaceae family (ginger) and is present in significant quantities in ginger (34).

The chemical mixtures found in ginger, such as essential oil and extracts/powders/infusions, exhibit a range of biological effects (35).

The composition of ginger's chemical constituents can vary depending on the extraction methods used. Distillation techniques such as hydrodistillation and steam distillation are typically employed to obtain volatile compounds. In contrast, solvent extraction using substances like acetone, ethyl acetate, ethyl ether, hexane, or pentafluoropropane/heptafluoropropane is used for extracting non-volatile compounds. Methods such as solid-phase microextraction and CO_2 extraction are used to isolate oily or aromatic components (20, 21).

3.3 BIOACTIVE PROPERTIES

Bioactive compounds in plants are substances that have beneficial properties for human or animal health. These compounds can belong to various chemical classes, including polyphenols, terpenoids, alkaloids, flavonoids, carotenoids, steroids, among others. Each class of bioactive compounds has specific properties that contribute to their beneficial health effects. For example, polyphenols are known for their antioxidant, anti-inflammatory, and anticancer properties, while terpenoids have antibacterial and antifungal activities (37). Some of the constituents of ginger and their bioactive properties are listed in Table 2.

Table 2 - Bioactive properties of ginger's constituents

Chemical	Bioactive properties (references)
Monoterpene	
Cineole	Anti-inflammatory (31, 32), antioxidant (27).
Citral	Hepatoprotective, anti-inflammatory (38).

Limonene	Antibacterial (39); antiviral, anticancer, anti-inflammatory (42, 43), antioxidant, antinociceptive, antidiabetic, antihyperalgesic, gastroprotective (40).
α/β-pinenes	Cytogenetic, gastroprotective, anxiolytic, cytoprotective, anticonvulsant, neuroprotective, antioxidant, antipancreatitis, antihyperthermia (41).
Sesquiterpenes	
B-elemene	Anticancer (29–31).
Zerumbone	Antibacterial, fragrance (42).
Phenolics	
Gingerols	Anticancer, anti-inflammatory and antioxidant (37, 46); Anticancer, antimicrobial, antifungal, neuroprotective, antiemetic, antihyperlipidemic (33).
[6]-shagaol	Antioxidant, anti-inflammatory, anticancer, antiemetic (44).
[6]-paradol	Anti-inflammatory, cardioprotective, neuroprotective (45).
Zingerone	Anti-inflammatory, antidiabetic, antidiarrheic, antispasmodic, anticancer, antiemetic, appetite simulant, anxiolytic, antithrombotic, radiation protective, antimicrobial (46).
Diarylheptanoids	
Curcumin	Antioxidant, antiulcer, anticancer, cardioprotective, antidiabetic, antimalarial, antimicrobial, neuroprotective (50, 51).
Other	
Essential oil	Bactericidal, antiviral, fungicidal, antiparasitic, insecticidal, analgesic, sedative, anti-inflammatory, spasmolytic, anesthetic (49); flavor,pungency (35).
Ginger extract/powder	Anticancer (50,51); anti-inflammatory, cardioprotective, neuroprotective (52).

Source: adapted from (23).

Biological effects have been reported for various constituents of ginger, including monoterpenes (such as cineole, citral, limonene, and α/β-pinene), sesquiterpenes (such as β-elemene, farsene and zerumbone), phenolic compounds (such as gingerols, [6]-shogaol, [6]-paradol, and zingerone), and diarylheptanoids (such as curcumin) (23). These compounds have demonstrated antioxidant (14, 23), antibacterial (21,54,55), anti-inflammatory (16, 27) and other biological activities (Table 2).

3.3.1 Antibacterial Activity

Ginger contains active compounds such as sesquiterpenoids (including gingerol and shogaol) and phenolics (like zingerone), that exhibited antimicrobial activity against a range of bacteria and fungi (6–8, 13, 20, 25, 53–55). Sesquiterpenoids which are more abundant in ginger play a significant role in this effect by disrupting bacterial cell membranes,

inhibiting growth, and potentially causing bacterial cell death (6, 7, 13, 55, 56, 60).

Over the years, various researchers have also tested gram-positive and gram-negative bacteria and concluded that both types of bacteria were sensitive to ginger, but gram-positive bacteria showed greater sensitivity. In addition to bacteria, several strains of fungi have also been tested (55, 56).

There are studies indicating that the antibacterial properties of ginger can be superior to some commercial antibiotics, such as chloramphenicol and gentamicin, which have inhibitory effects on microorganisms like *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae* and *Proteus vulgaris*. Ginger has also shown effectiveness antimicrobial activity against Methicillin- resistant *Staphylococcus aureus* (*MRSA*)(6).

The effectiveness of ginger´s antimicrobial activity is influenced by the choice of solvent used for extraction and dissolution. non-volatile compounds with antimicrobial properties tend to be more soluble in organic solvents and research show that extracts in ethanol or methanol generally have significantly higher antimicrobial activity than those in water (58–60).

Antimicrobial activity is also influenced by external factors such as the extract's presentation, temperature, or time. Studies have shown that increases in temperature from 5°C to 15°C, as well as storage time, can negatively impact antimicrobial action (6).

3.3.2 Anti-Inflammatory Activity

Inflammation plays a crucial role in the development and progression of various dermatological diseases, such as acne, dermatitis, and psoriasis (7). The inflammatory process is a complex phenomenon that has multiple response mechanisms, which can act individually or together. Therefore, a compound's ability to block or minimize one or more of these mechanisms gives it anti-inflammatory potential (59).

One of the important inflammatory pathways is the arachidonic acid metabolism, and in this pathway, the arachidonic acid cascade depends on acid oxidation and is divisible into two main pathways: the cyclooxygenase (COX)-dependent pathway, which results in the production of thromboxanes (causing coagulation), prostaglandins (causing pain and fever), and the lipoxygenase (LOX)-dependent pathway, responsible for producing leukotrienes (6).

Studies have shown that ginger has anti-inflammatory potential that can play a

significant role in reducing cutaneous inflammation (7). This inhibition is achieved through the inhibition of multiple genes involved in the inflammatory response, including those responsible for coding cytokines, chemokines, and the enzyme COX-2. Despite blocking genes and inhibiting the synthesis of these compounds, ginger does not interfere with antigen creation (57, 58).

Ginger has demonstrated the ability to suppress the production of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β), by skin cells. Additionally, these compounds have shown inhibitory activity on the activation of nuclear factor-kappa B (NF-κB), a transcription factor essential in the inflammatory response. Furthermore, ginger has been reported as an inhibitor of COX-2, an enzyme involved in inflammation, as well as a modulator of the production of prostaglandins, important inflammatory mediators (7,63).

Compounds such as gingerol, shogaol, and zingerone have demonstrated the ability to inhibit the production of nitric oxide (NO), a pro-inflammatory mediator, by reducing the expression of genes mediated by NF-κB and inhibiting the enzymatic activity of iNOS (inducible nitric oxide synthase), which is the enzyme responsible for NO production. These actions contribute to ginger's anti-inflammatory properties (58).

These anti-inflammatory properties of ginger have the potential to attenuate skin inflammation, alleviate the symptoms of inflammatory skin conditions, and contribute to overall dermatological health improvement (7).

3.3.3 Antioxidant Activity

Ginger has been widely investigated for its antioxidant capabilities. This is attributed to the presence of bioactive compounds in ginger, which possess hydroxyl groups on an aromatic ring, enabling them to neutralize free radicals and reduce oxidative stress (OS) (13). Gingerols, shogaols, flavonoids, and phenolic acids are some of the antioxidant compounds found in ginger that contribute to this property (64).

Oxidative damage has the potential to harm proteins and lipids present in the cell membrane (65). Free radicals can oxidize proteins, compromising their structure and function, as well as affecting the integrity of the cell membrane. Ginger is suggested as a protective agent against oxidative damage, as observed in a study by Romero *et al.*, (2018) (66), in which a ginger extract reduced AKT phosphorylation, protecting cells against damage induced by hydrogen peroxide.

The reactive oxygen species (ROS) and reactive nitrogen species, including nitric oxide (NO), are known to contribute to the onset of diseases, affecting cellular communication and causing damage to DNA. The production of NO is controlled by the inducible nitric oxide synthase enzyme (iNOS), which is increased in response to various stressful stimuli (67).

Oxidative damage to DNA can result in mutations and activation of cellular signaling pathways that may lead to chronic diseases, such as cancer, due to the damage caused by ROS, which are capable of inducing DNA strand breaks and oxidation of nitrogenous bases, resulting in errors during replication and mutation formation (68).

6-gingerol is a substance found in ginger that can inhibit the action of the enzyme xanthine oxidase (XO). This enzyme is important because it is involved in the transformation of hypoxanthine into xanthine and then into uric acid, which are part of the metabolic breakdown of purines. When this breakdown occurs, ROS (reactive oxygen species) are produced. So, when 6-gingerol inhibits the activity of xanthine oxidase, it helps to reduce the production of these ROS, protecting our body against the damage they can cause (68).

4. EXTRACTION METHODS

Int this study two extraction methodologies will be employed: microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE).

In MAE, the magnetron emits electromagnetic microwaves that are absorbed by molecules with a high dielectric constant and dielectric loss. This absorption leads to an increase in temperature, ultimately enhancing the extraction process (69). Microwave-assisted extraction can result in products with higher concentrations of 6-shogaol, which may be linked to enhanced antitumor, anti-inflammatory, and antimicrobial activities (70).

Ultrasound-assisted extraction involves the use of high-frequency pulses that generate cavitation bubbles in the solvent. These bubbles impact cellular structures, leading to the release of extraction compounds when the bubbles collapse on the surface of the solid material. This results in the breakdown of the cell wall and the release of bioactive compounds across the cellular membrane into the solution. In UAE processes, frequency, power, and wavelength are the main physical parameters that affect extraction yields. Furthermore, UAE is economically advantageous due to reduced time requirements and represents an environmentally friendly process (71).

Ultrasound-assisted extraction still offers benefits such as affordability, wide availability, speed, and the absence of the use of harmful solvents. Additionally, the extraction process is carried out at lower temperatures compared to conventional methods, helping to preserve heat-sensitive compounds (72).

5. MATERIAL AND METHODS

5.1 SAMPLE PREPARATION

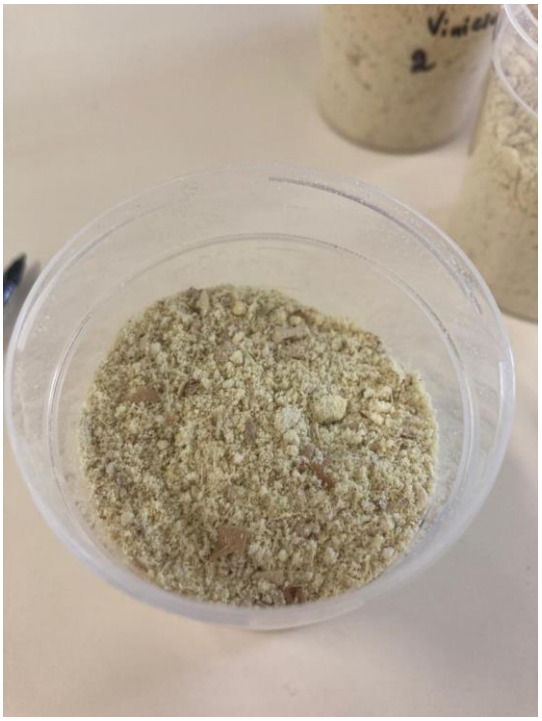
The ginger samples were purchased from a commercial establishment (Pingo Doce) that imported them through the company EUROFRUTAS, SA. The ginger in question was produced in Costa Rica, belonging to lot number 23001288, harvested on 08/04/2023. After acquiring the samples, cleaning and fractionation (Figure 3 and 4) of the ginger were performed to reduce them into smaller pieces to facilitate the freeze-drying process. The lyophilized ginger (Figure 5) was carefully crushed (Figure 6) to be used later in the conducted analyses and extractions.

Figure 3 - Cleaning and selection of the samples

Figure 4 - Fractionation of the samples

Figure 5 - Lyophilized ginger rhizome

Figure 6 - Crushed ginger rhizome post-lyophilization



5.2 OPTIMIZATION OF EXTRACTIONS

For both extraction types, an optimization was carried out using response surface methodology (Design Experto, StatEase, Inc., 1300 Godward Street Northeast, Suite 6400, Minneapolis, MN, USA). This technique allows the variation of three variables, allowing to find the point that optimizes a specific response.

The determination of the optimal point for Microwave-Assisted Extraction (MAE) involved the analysis of 17 extraction points. In this context, different times (5, 17.5, and 30 minutes) were investigated, varying the power between 150, 375, and 600 Watts, and using three concentrations of hydroethanolic solvent (EtOH/H_2O) - 0%, 50%, and 100%. Throughout the experiment, the temperature and rotation were kept constant at 60°C and 1700 rpm, respectively.

The optimization of Ultrasound-Assisted Extraction (UAE) also involved the analysis of 17 extraction points. In this context, different times (15, 37.5, and 60 minutes) were investigated, varying the power between 15, 47.5, and 80%, and using three concentrations of hydroethanolic solvent (EtOH/H2O) - 0%, 50%, and 100%.

The optimization was considered for the antioxidant activity, namely the 2,2-diphenil-1-picrylhydrazil (DPPH) assay. As this assay is expressed in EC_{50} (concentration

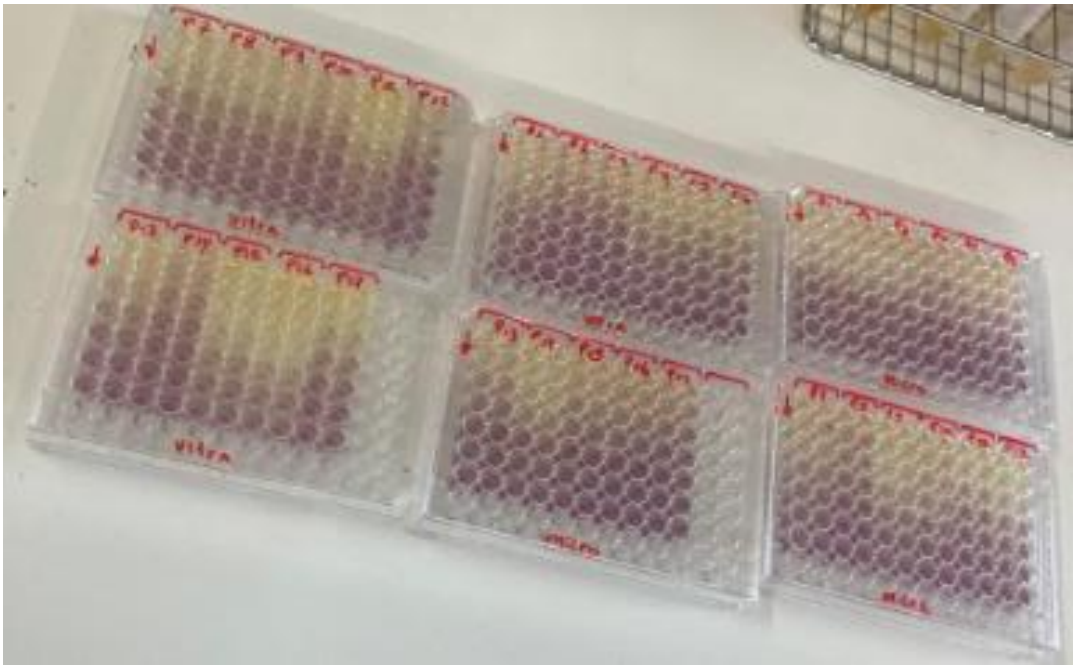
that quenches 50% of radicals), the lower the concentration, the higher the antioxidant capacity, the minimize function was used.

For all 17 extraction points for both techniques, 3 grams of freeze-dried powdered ginger were used along with 90 mL of solvent.

After the extractions, the samples were evaporated using a rotary evaporator to remove all ethanol, followed by freezing and subsequent lyophilization process.

The lyophilized extracts were subjected to antioxidant testing (DPPH) to determine the optimal point. The procedures described in section *5.4.1.1* were followed, but with only ten dilutions (20, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15, 0.075 and 0.0375). After a period of 1 hour at room temperature and protected from light, the plates (Figure 7) were taken for absorbance reading in the spectrophotometer (SPECTROstar Nano) at 515 nm.

Figure 7 – DPPH assay for determination of the optimal point



By opting only for the DPPH test, we aimed to ensure more stable and replicable results, facilitating the identification of the best extraction point for ginger extract with antimicrobial, anti-inflammatory, and antioxidant properties for soap formulation.

5.3 DETERMINATION OF TOTAL PHENOLIC COMPOUNDS

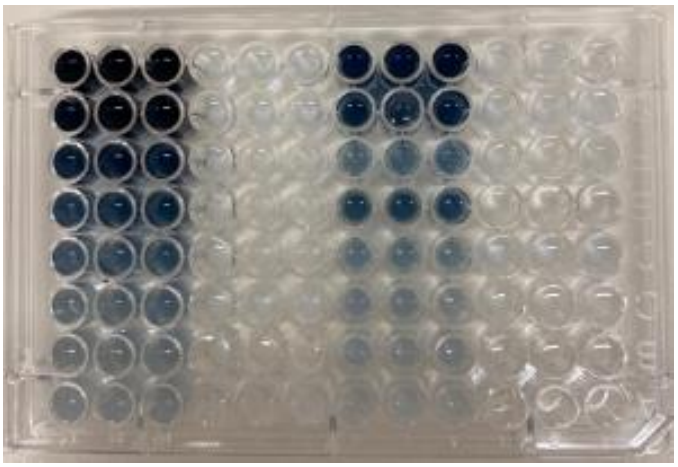
This test was conducted following the procedure described by Pico *et al.*, 2020 (73), with some modifications. The Folin–Ciocalteu reaction method was employed to determine the total extractable phenolic compounds.

In the analysis of the total phenols of the obtained extracts, 30 µL of the extract samples (in triplicate), initially at a concentration of 10 mg/mL, were added to 96-well plates. Then, fourteen dilutions were made, specifically: 5, 2.5, 0.625, 0.3125, 0.1562, 0.0781, 0.0780, 0.0390, 0.0195, 0.0097, 0.0049, and 0.0024 mg/mL. Subsequently, 150 µL of 10% Folin-Ciocalteu reagent and 120 µL of 75 g/L sodium carbonate were added. The resulting solution (figure 8) was left to stand for 2 hours at room temperature and protected from light, and then the absorbance was measured at 760 nm.

A blank analysis was also performed, one with ultrapure water and the other two with the solvents used in the microwave and ultrasound extractions.

To establish the calibration curve for total phenolic analysis, gallic acid was used as the reference standard. The stock solution was prepared by dissolving 0.1 g of gallic acid in 100 ml of deionized water. Various concentrations were prepared using measured volumes of 0.5, 0.6, 0.75, 1, 2, 3, and 4 ml of the stock solution in 25 ml volumetric flasks, which were then topped up with deionized water. The blank was prepared by adding 0.5 ml of deionized water to the reagents (2.5 ml of Folin-Ciocalteu and 2 ml of sodium carbonate). After 2 hours in the dark, the absorbance was measured at 760 nm.

Figure 8 - Total phenolic compounds determination



5.4 DETERMINATION OF BIOACTIVE PROPERTIES

5.4.1 Assessment of Antioxidant Activity

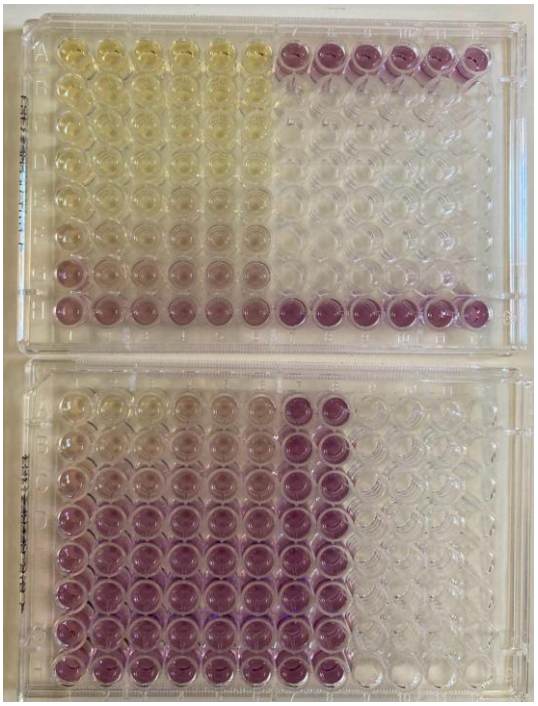
The evaluation of antioxidant activity was conducted using two different methodologies, namely the DPPH method and the TBARS method.

The DPPH Radical Scavenging Effect method (2,2-diphenyl-1-picrylhydrazyl) was performed following the method described by Cardeñosa *et al*. 2015 (74).

The assay was performed in triplicate using 96-well plates. 270 µL of a methanolic solution containing DPPH (6×10^5 mol/L) was added, followed by 30 µL of each extract obtained, as previously detailed, at different concentrations. The hydroethanolic extract was used to prepare a stock solution with a concentration of 20 mg/mL, which was then serially diluted to obtain twelve different concentrations (20, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15, 0.075, 0.0375, 0.01875 and 0.009375 mg/mL).

Following an incubation of one hour at room temperature and protected from light, the plate (Figure 9) was subjected to an absorbance reading using a spectrophotometer (SPECTROstar Nano) at a wavelength of 515 nm. The DPPH molecule presents as an intense violet color, and upon interaction with a substance that has antioxidant properties, it turns into a lighter yellow or violet hue (75).

Figure 9 - Microplate before DPPH reading



The percentage of DPPH inhibition was determined according to Equation 1 presented below. The results were expressed in terms of IC_{50}, representing the extract concentration required to achieve 50% inhibition of DPPH.

$$\%\ of\ inhibition = \frac{A_{DPPH} - A_A}{A_{DPPH}} * 100$$

Equation 1 - Percentage of DPPH inhibition (A_DPPH corresponds to the absorbance of the control and AA to the absorbance of the sample)

The evaluation of the antioxidant potential through the inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS) was carried out according to the methodology described by Barros *et al*. 2013 (76).

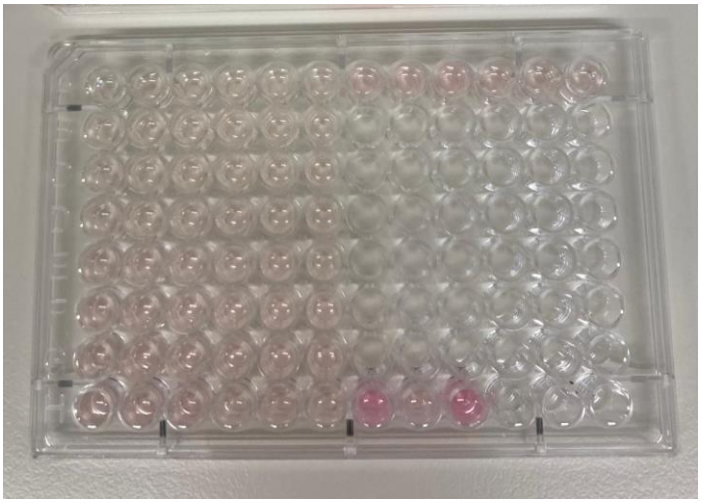
The solutions previously prepared in the DPPH method described in section *5.4.1.1* were used to obtain eight different concentrations of hydroethanolic extracts (0.6, 0.3, 0.15, 0.075, 0.0375, 0.01875, 0.009375 and 0.0046875 mg/mL).

In a Falcon tube, a certain mass of pig brain was weighed and added twice this mass of Tris-HCl buffer (20 mM; pH = 7.4). After agitation, the tube was centrifuged at 3500 rpm for 10 minutes. Subsequently, 100 µL of the supernatant of the pig brain with Tris-HCl buffer was added to 2 mL Eppendorf tubes in triplicate, along with 200 µL of the extract solutions. Additionally, 100 µL of $FeSO_4$ and 100 µL of ascorbic acid were added. After adding all the solutions to the plates, incubation was carried out at 37,5 °C for 1 hour.

After 1 hour, the reaction was stopped by adding 500 µL of trichloroacetic acid (28%, w/v), followed by 380 µL of freshly prepared thiobarbituric acid (2%, w/v). Then, the Eppendorf tubes were heated at 80 °C for 20 min and subsequently centrifuged at 3000 rpm for 5 min to remove the precipitate. 200 µL of the supernatant was added to a 96-well plate for reading in a spectrophotometer.

Malondialdehyde (MDA) reacts with thiobarbituric acid (TBA) to form a pink pigment, called the malondialdehyde (MDA)–TBA complex (Figure 10), whose intensity was measured in the SPECTROstar Nano spectrophotometer at 532nm.

Figure 10 - Microplate before TBARS reading



The calculation of the percentage of inhibition was done according to Equation 2. The results were presented in IC_{50} values, which correspond to the extract concentration that promotes 50% inhibition of lipid peroxidation.

$$\%\ of\ inhibition = \frac{A_{Tris} - A_A}{A_{Tris}} * 100$$

Equation 2 - Percentage of lipid peroxidation inhibition (A_Tris corresponds to the absorbance of the control and AA to the absorbance of the sample)

5.4.2 Assessment of Antibacterial Activity

The antibacterial activity of the extracts was evaluated through microdilution assays using colonizing skin bacteria, following the methodology described by Pires *et al.*, (2018) (77).

The extracts were tested against one Gram-negative bacteria, namely *Pseudomonas aeruginosa* (VRU14123), obtained from patients hospitalized in various departments at the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal) and three Gram-positive bacteria, namely methicillin-resistant *Staphylococcus aureus* (*MRSA*)(VRI17654) also obtained from the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal) and *Cutibacterium acnes* (ATCC 11827), *Staphylococcus Epidermidis* (ATCC 12228) are purchase at Frilabo, Porto, Portugal.

Using the microdilution method, we were able to identify the minimum inhibitory concentration (MIC) for all the analyzed extracts. Bacterial cultures were standardized to a

concentration of 1×10^5 CFU/mL in each well using a densitometer.

MRSA and *P. aeruginosa* were incubated in aerobic conditions at 37°C for 24 hours. While *C. acnes* and *S. epidermidis* were incubated in anaerobic conditions at 37 ºC fresh medium for 24 h to ensure they were in the exponential growth phase.

50 mg of the extract were weighed and dissolved in 5% (v/v) Dimethyl sulfoxide (DMSO) with autoclaved distilled water to a final volume of 2.5 mL, obtaining an initial concentration of 20 mg/mL. Using 96-well plates, 100 µL of the prepared solution was pipetted in duplicate into the first well containing 90 µL of tryptic soy broth (TSB), as well as the remaining wells. Successive dilutions of the solution were then performed, resulting in eight different concentrations (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, and 0.078 mg/mL). Finally, 10 µL of inoculum was added to all wells. The microplates were then incubated following the growth conditions described previously.

After the incubation period, 40 µL of INT (0.2 mg/mL) were added to all wells, and the microplates were incubated in an oven at 37°C for 30 minutes. After this time interval, the results were evaluated considering the change in color to pink (Figures 11, 12, 13, 14 and 15).

All work was carried out with sterile materials manipulated in a laminar flow hood.

Figure 11 - *Staphylococcus epidermidis* assay

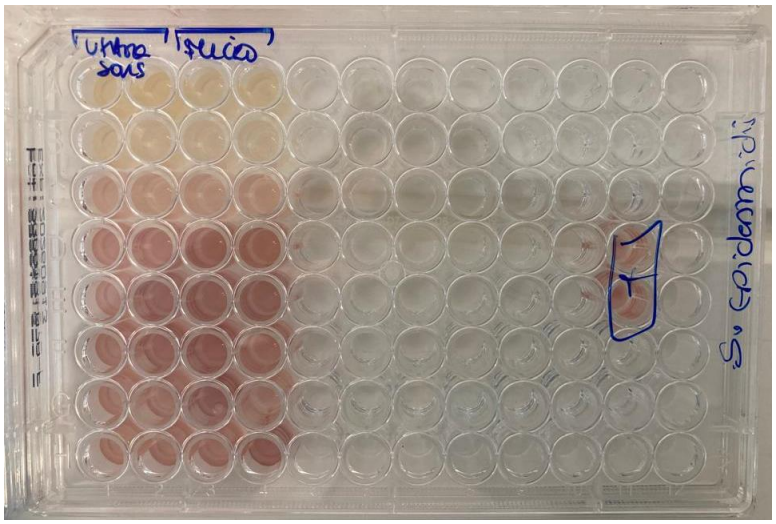


Figure 12 - *Cutibacterium acnes* assay

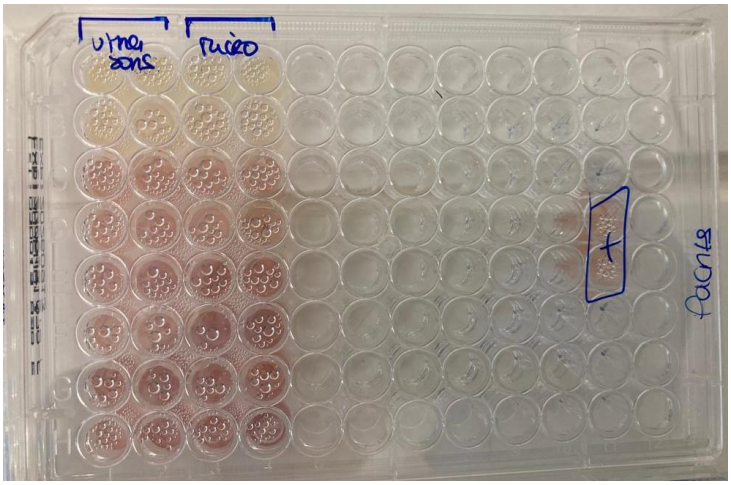


Figure 13 - *Pseudomonas aeruginosa* assay

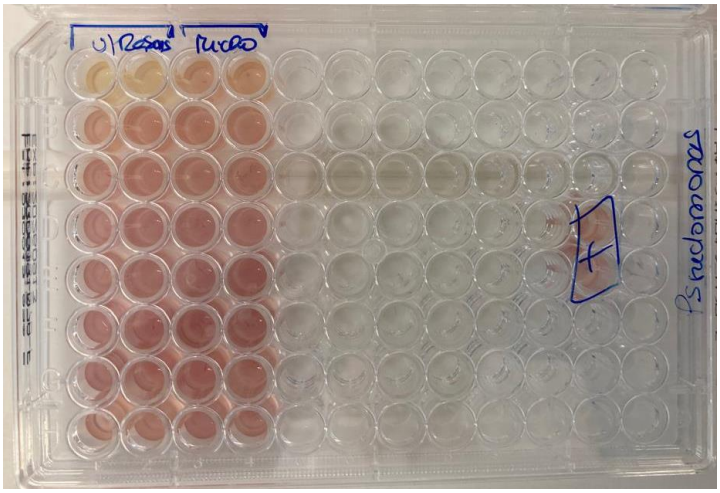


Figure 14 - Methicillin-resistant *Staphylococcus aureus* (*MRSA*) assay

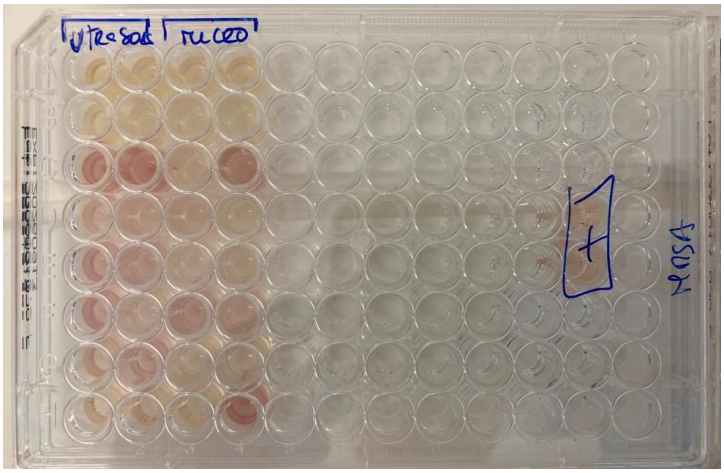
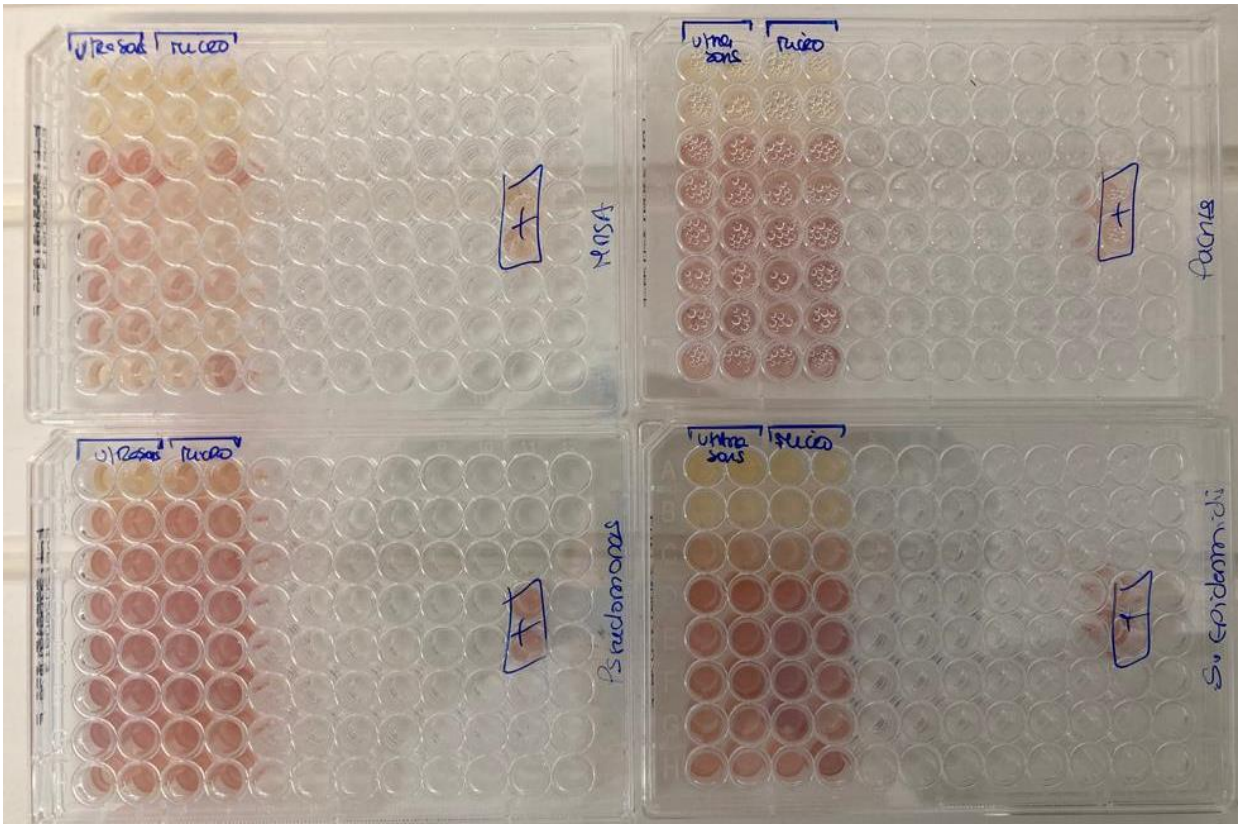


Figure 15 – Antibacterial assay



For the antibacterial tests of solid soaps, the agar diffusion method was used, the established procedure used in numerous clinical microbiology laboratories to conduct routine antimicrobial susceptibility tests. Following the methodology described by the Clinical and Laboratory Standards Institute (CLSI) (78).

The petri dishes, with a diameter of 55 mm, were inoculated on the surface with a standardized concentration of approximately 1-2×10^8CFU/mL of each microorganism. Subsequently, the soaps were divided into squares of similar dimensions and carefully placed in the center of the petri dish. The plates were then incubated for 24 hours at 37°C. After this period, the inhibition zones were analyzed and measured.

The assay was performed in duplicate, testing both extraction methods, with concentrations of 5 and 10 mg of extract, at three different time points (0, 7, and 15 days). Glycerin soap used as the soap base was used as a control.

5.4.3 Assessment of Anti-inflamatory Activity

The evaluation of anti-inflammatory activity was determined according to the

methodology described by Taofiq *et al*. (2015) (79).

Mouse RAW 264.7 macrophages were used to evaluate the anti-inflammatory activity. The cell cultures were obtained from the European Collection of Authenticated Cell Cultures (ECACC) and grown in DMEM medium supplemented with 10% heat-inactivated bovine serum and L-glutamine, maintained at 37°C with humidified air and 5% CO_2. Dexamethasone was the control used.

According to the Trypan Blue exclusion method, cells with successful growth were scraped and balanced to an experimental density of $5x10^5$ cells/mL, with a dead cell ratio of less than 5%. The cells were seeded (300 µL/well) into 96-well microplates and incubated at 37°C and 5% CO_2 for 24 hours to adhere and proliferate.

Afterward, they were incubated for 1 hour with various extract solutions at final concentrations ranging from 400 to 1.56 µg/mL. Then, lipopolysaccharides (LPS, 1 µg/mL, 30 µL) were provided for 18 hours. Negative controls were performed without the addition of LPS to verify if they could induce changes in baseline nitric oxide (NO) levels.

Dexamethasone (50 mM) was used as a positive control. A Griess Reagent kit (Promega) containing sulfanilamide, N-(1-naphthyl) ethylenediamine hydrochloride (NED), and nitrate solutions was employed to detect nitric oxide presence. Subsequently, 100 µL of the cell supernatant solution was transferred to a microplate, along with the sulfanilamide and NED solution, and gently mixed at room temperature for 5 to 10 minutes each.

The amount of nitric oxide released was calculated by comparing the absorbance at 540 nm using an ELX800 Biotek microplate reader with the calibration curve. Finally, the extract concentration required to inhibit the production of NO by 50% (EC_{50}, µg/mL) was determined.

5.4.4 Assessment of Cytotoxicity

Cytotoxic activity was evaluated in vitro through the Sulforhodamine B (SRB) assay as described by Vaz *et al*., (2010) (80).

The keratinocyte cell line HFF1 (Human Fetal Foreskin Fibroblasts) was used for this cytotoxicity assay. The cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC), cultured in Dulbecco's Modified Eagle Medium (DMEM), and maintained in an incubator at 37°C and 5% CO_2. The ginger extracts were dissolved in water and diluted to obtain different concentrations ranging from 400 to 6.25

μg/mL. Ellipticine was the control used.

After verifying cell confluence, they were washed with phosphate-buffered saline (HBSS) and then 2 mL of trypsin were added to detach them from the plate. After approximately 3 minutes, 5 mL of DMEM culture medium was added, and the mixture was centrifuged for 5 minutes at 1200 rpm. A cell suspension solution was prepared with a concentration of 100.000 cells/mL (Countess II FL Automated Cell Counter, InvitrogenTM, Waltham, MA, USA). The cells were cultured in medium containing each concentration of ginger extracts in a 96-well plate for 72 hours under the same conditions mentioned above.

Sulforhodamine is a protein dye capable of binding to protein components of cells fixed on plates, turning them pink to quantify cell proliferation under a specific treatment. For the sulforhodamine B (SRB) assay, trichloroacetic acid (10%, w/v) was added to the wells, and the plates were incubated at 4°C for 1 hour, followed by three washes with water.

Once completely dried, 100 μL of sulforhodamine B solution was added to each well and left for 30 minutes at room temperature. Another wash was performed with acetic acid (1%, v/v), before adding the Tris solution (10 mM, w/v) to solubilize the colored proteins. Absorbance was read at 540 nm using a microplate reader (Synergy H1, Biotek Instruments, Winooski, Vermont, USA).

The results obtained were used to determine the concentration of the extract responsible for causing 50% growth inhibition (GI_{50}).

5.5 DEVELOPMENT THE SOAP CONTAINING GINGER EXTRACT

For the development of solid soaps, a method involving the use of two different concentrations of ginger extract was employed, one at 5 mg/mL and the other at 10 mg/mL. Therefore, two solid soaps were produced for each extraction method.

The soaps were crafted using solid glycerin from the For Bury brand, which composition includes water, glycerin, propylene glycol, stearic acid, sodium laurate, sodium lauryl sulfate, sodium hydroxide, sorbitol, sodium chloride, sodium thiosulfate, and etidronic acid. Glycerin appears solid and opaque at 25°C, with a white color and a water content between 25-30%. Moreover, its pH in a 2% aqueous solution varies between 9.5 and 10.5, and its melting temperature ranges between 60-65°C.

For soap production, the following procedure was adopted: 25 grams of glycerin were weighed, along with 5 and 10 mg of each extract from the optimal points (microwave

and ultrasound). Next, the extracts were diluted in 1 mL of water and incorporated into the previously melted glycerin. The soaps were then left at room temperature until solidify, later divided into equal parts for evaluation at 0, 7, and 15 days.

The soap coloration was analyzed following the procedure described by Pires *et al.*, 2020 (81), using a portable colorimeter CR400 (Konica Minolta, Chiyoda, Tokyo, Japan), employing the C65 illuminant, representing midday light in Europe, and a standard from the International Commission on Illumination (CIE). The CIE L*, a*, and b* color space coordinates were used, where L* represents lightness, a* represents redness (red-green), and b* represents yellowness (yellow-blue), with a 10° observer angle and an 8 mm aperture. The analyses were performed in triplicate and presented as mean followed by standard deviation. The variation in total color difference (ΔE*) between the soap samples of each time was calculated using the following equation 3:

$$\Delta E* = \sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2}$$

Equation 3 - Variation in total color difference

For the antibacterial tests of solid soaps, we applied the agar diffusion method as described in section 5.4.2. Each assay was duplicated, testing both extraction methods. The results are presented as the average of the values obtained.

5.6 STATISTICAL ANALYSIS

The quantitative results were presented as mean ± standard deviation, except for the antibacterial activity and cytotoxicity results. The statistical analysis of the data was conducted to identify significant differences among the various extracts or soaps. We utilized both analysis of variance (ANOVA) and the Student's t-test, considering different types of comparisons. Each table outlines the statistical test applied. To perform these analyses, we employed Excel software.

6. RESULTS AND DISCUSSION

6.1 OPTIMAL POINT DETERMINATION

Regarding the optimization studies for both extraction types, table 3 shows the 17 extractions used to optimize the antioxidant activity through the DPPH assay.

The optimization used the Box-Benken model, as prior knowledge of the intervals of variation were known. Thus, for the microwave assisted extraction, the model was significant while the lack of fit was non-significant, meaning the values were adjusted to the model. The F-value explains the effects of the independent factors on the responses, which was of 71.29. The best factor in terms of effects towards the model was solvent, with an F-value of 124 (result not shown).

Thus, with an F-value of 19.57, there is only 0.04% chance that this F-value could occur due to noise. The R^2 shows how the model explains variability, and thus 99% of variability is explained by the model, which is an excellent precision, while the adjusted R^2 shows the precision in terms of predictors, also showing a very good result of 0.97.

The adequate precision ensures that the experimental design and the resulting regression model adequately capture the variation in the response variable and provide reliable estimates of the effects of the independent variables and should always be higher than 4. In the microwave extraction optimization, it reached 23.

Finally, the coefficient of variance shows the variability of a response variable compared to its mean value and can be useful for assessing the reliability and consistency of experimental results and for comparing the variability of response variables across different experimental settings.

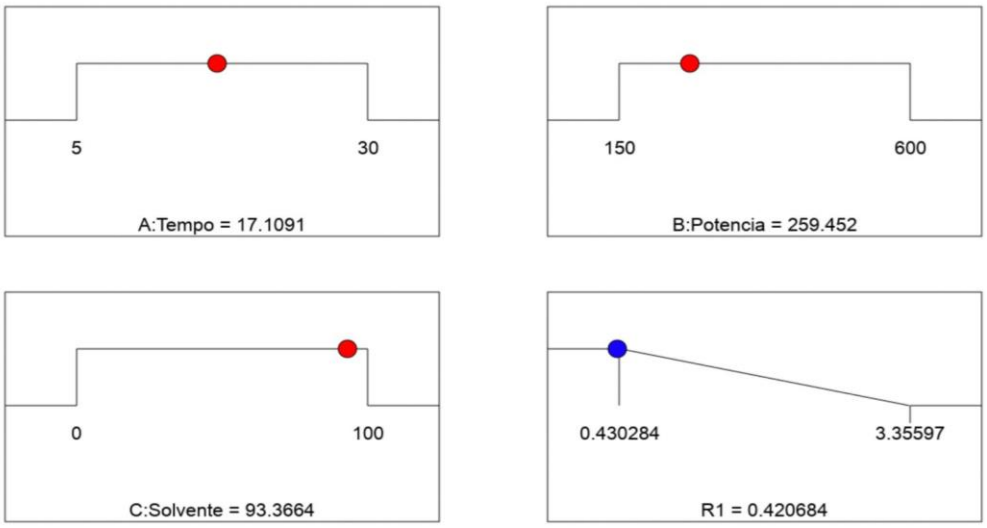
In this case, it reached 19%. The coded equation for the microwave assisted extraction was $R_1^M=0.89+0.25_A+0.05_B-0.91_C+0.11_{AB}-0.40_{AC}+0.02_{BC}+0.31_A^2-0.12_B^2+0.51_C^2$, showing that in terms of the three factors, the one with the most impact on the outcome was factor C (0.91), namely the percentage of ethanol.

Table 3 - Factors (A, B, C) and responses (R_1^M, R_1^U), and statistical data for the optimization of both extractions

Runs	**Microwave Extraction**				**Ultrasound Extraction**			
	A Time (min)	B Power (W)	C Solvent (%)	R_1^M	A Time (min)	B Power (W)	C Solvent (%)	R_1^U
1	17.5	375	50	0.70	15	15	50	0.79
2	17.5	375	50	0.86	60	47.5	0	2.28
3	30	150	50	1.08	15	47.5	0	2.25
4	17.5	600	0	1.95	60	15	50	0.32
5	17.5	375	50	0.99	37.5	47.5	50	0.19
6	5	600	50	0.86	37.5	47.5	50	0.19
7	5	150	50	0.70	37.5	47.5	50	0.23
8	5	375	0	2.16	37.5	47.5	50	0.05
9	17.5	600	100	0.43	37.5	47.5	50	0.25
10	17.5	375	50	1.03	37.5	80	0	~~2.36~~
11	5	375	100	0.87	37.5	80	100	0.53
12	30	375	100	0.46	15	80	50	0.16
13	30	600	50	1.67	60	80	50	0.27
14	17.5	150	100	0.56	37.5	15	0	2.24
15	17.5	375	50	0.86	15	47.5	100	0.78
16	17.5	150	0	2.16	60	47.5	100	0.06
17	30	375	0	3.35	37.5	15	100	0.03
Statistical data								
Model *p*-value				<0.001				0.0004
Model F-value				71.29				19.57
Lack-of-fit				0.0733				0.0537
R^2				0.9907				0.9618
R^2_{adj}				0.9768				0.9126
Adequate precision				23.536				16.7442
Coefficient of variance (%)				18.76				18.98

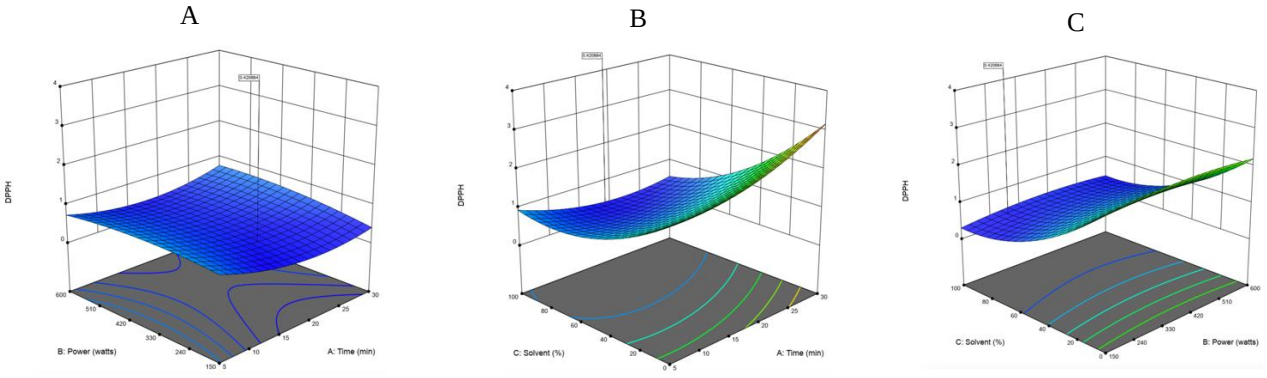
Applying the minimize function to the model, thus finding the conditions that show the lowest point, the model predicted a response of 0.43 mg/mL. For this to be achieved, the conditions of each factor should be 17 minutes, 259W and 93% of ethanol, as shown in Figure 16.

Figure 16 - Optimal point for Microwave-Assisted Extraction



In Figure 17, the 3D plots of the response surface are shown for the microwave assisted extraction, where it is clear that, as stated above, the percentage of solvent shoed the most influence, as in figure 17B, the antioxidant activity decreases with the decrease of ethanol, meaning that this alcohol helps extract antioxidant molecules. The other two factors, times and power seem to be secondary as there are very slight variations in the 3D plots in the axis with these two factors, clearly shown in Figure 17A, although shorter times seem to favor antioxidant activity.

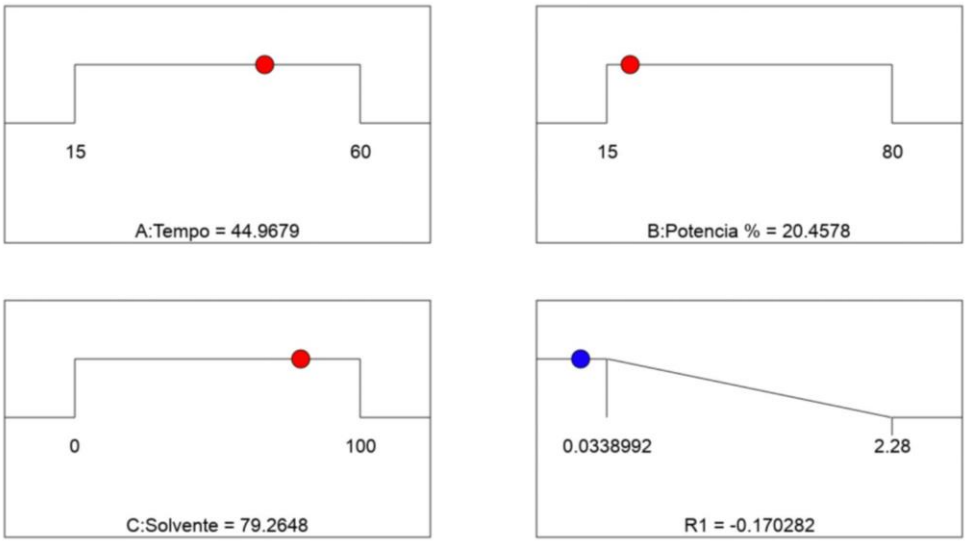
Figure 17 - 3D plots of the response surface for microwave assisted extraction.



Regarding ultrasound assisted extraction, from the values shown in table 3, for this optimization one value was ignored as it constituted an outlier. As for the microwave assisted extraction, once again the model was significative and did not show a significative lack of fit.

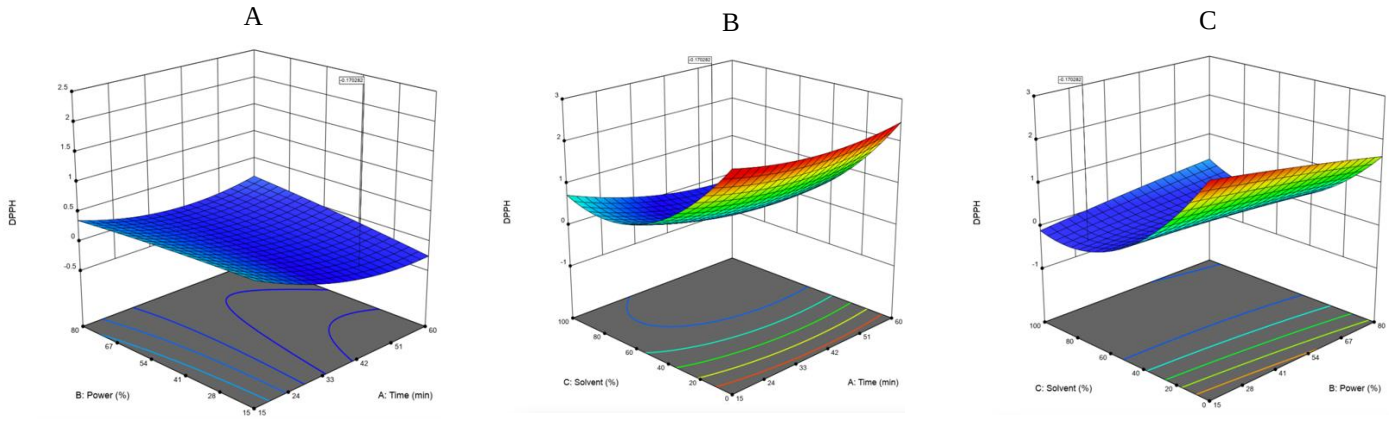
The F-value was set at explains the effects of the independent factors on the responses, which was of 19.57, lower than for the microwave assisted extraction. The R^2 was set at 0.9618 while the adjusted R^2 0.9126. The adequate precision was 16.7442 and the coefficient of variability was 18.98 The coded equation for the ultrasound assisted extraction was $R_1^U=0.18-0.13_A-0.11_B-0.86_C+0.14_{AB}-0.18_{AC}+0.30_{BC}+0.23_A^2-0.02_B^2+0.93_C^2$, showing that in terms of the three factors, the one with the most impact on the outcome were factors A and B, time and power.

Figure 18 - Optimal point for Ultrasound-Assisted Extraction



Using the minimize function to obtain the optimal condition for maximum antioxidant activity the model showed 0.03 mg/mL, showing a value even beyond the experimental conditions, meaning the optimization leads to increased antioxidant activity. Thus, the optimal point was set at 45 minutes, 20 W and 89% of ethanol, as can be seen in Figure 18.

Figure 19 - 3D plots of the response surface for ultrasound assisted extraction.



In Figure 19, showing the 3D plots for the ultrasound assisted extraction, the left figure shows that higher extraction times favor antioxidant activity, while the power does not seem to induce much variation, although these are the two most contributing factors towards the model. In terms of solvent, it is clear in Figure 19B and C, lower quantities of ethanol reduce the antioxidant activity. Overall, ethanol helps increase the antioxidant activity for ultrasound assisted extraction, along with long extraction times and mean power.

6.2 CALCULATION OF YIELD

After extraction at the optimum points and lyophilization of the extracts, the final mass was recorded, and the extraction yield was calculated according to Equation 4.

$$Yield\ (\%) = \frac{Final\ lyophilized\ extract\ (g) * 100}{Initial\ dry\ sample\ (g)}$$

Equation 4 - Equation for calculating extract yield

The extraction results and their respective yields are presented in the following tables 4:

Table 4 - Extraction at the optimum points

Extraction method	Sample (g)	Lyophilized extract (g)	Yield (%)
UAE	3.001 ± 0.02	0.39 ± 0.03	13.15 ± 1.03
MAE	3.002 ± 0.02	0.43 ± 0.01	14.16 ± 0.19

MAE: Microwave-Assisted Extraction. UAE: Ultrasound-Assisted Extraction. The results are presented as mean ± standard deviation.

Regarding the table 4, the microwave-assisted extraction method achieved a slightly higher yield than the ultrasound method. But that doesn't necessarily mean it has the best bioactivities. However, an analysis using the Student's t-test revealed that there were no significant differences in the results, with a p-value > 0.05.

6.3 TOTAL PHENOLIC COMPOUNDS

The Folin–Ciocalteu reaction method was employed to determine the total extractable phenolic compounds. Thee values of total phenolic compounds (TPC) were calculated as gallic acid equivalents (GAE) per gram of dry weigh (mg GAE/g dw).

Based on our results (table 5), we conclude that the ultrasound extraction method was the most effective in extracting phenolic compounds, yielding a result of 0.085 ± 0.003 mg GAE/g dw, while microwave extraction yielded a result of 0.053 ± 0.001 mg GAE/g dw. The Student’s t-test analysis revealed significant differences between these results, with a p-value < 0.05. Therefore, we can assert that the ultrasound extraction method demonstrated superior performance.

Table 5 - Total phenolic compounds of ginger extracts

Extraction	mg GAE/g dw
MAE	0.053 ± 0.001
UAE	0.085 ± 0.003*

MAE: Microwave-Assisted Extraction. UAE: Ultrasound-Assisted Extraction. The results are presented as mean ± standard deviation, as gallic acid equivalents (GAE) per gram of dry weigh (mg GAE/g dw). ‘*’ indicate significant differences ($p < 0.05$), determined by statistical analysis obtained through a t-Student test.

Dessie Ezez and Molla Tefera (2021) (82) found that the amount of total phenolic compounds in ginger samples extracted in an aqueous solution of methanol, acetone, ethanol, and ethyl acetate (1:4, water, solvent, v/v) obtained by maceration was significantly

influenced by the extraction solvent, with a p-value < 0.05. The results ranged from 1.183813 to 0.690152 mg of GAE/g of dry weight for methanol and acetone, respectively. Among the tested solvents, methanol proved to be the most efficient in TPC extraction, followed by ethanol, ethyl acetate, and acetone. This indicates that the TPC extracted from ginger was higher in polar solvents compared to less polar solvents.

Jan *et al.*, (2022) (83) performed an ultrasound-assisted extraction for 15 minutes, with a frequency of 60 Hz, and identified the phenolic and flavonoid compounds present in the ginger extract. The phenolic and flavonoid compounds in ginger were measured using the RP-HPLC method. The ethanol:water (75:25) and ethyl acetate:water (75:25) extracts showed the highest levels of gallic acid, vanillic acid, and ferulic acid, with 41.30 mg/kg, 97.00 mg/kg, and 218.4 mg/kg found in the ethanol:water extract, and 48.31 mg/kg, 94.30 mg/kg, and 190.43 mg/kg in the ethyl acetate:water extract, respectively, while the aqueous extracts showed the lowest levels of these compounds (24.10 mg/kg, 86.43 mg/kg, 94.50 mg/kg).

Similarly, the ethanol:water (75:25) extracts showed the highest levels of flavonoids (quercetin 101.7 mg/kg, kaempferol 37.76 mg/kg, isorhamnetin 247.8 mg/kg, rutin 31.67 mg/kg, catechin 175.1 mg/kg, and caffeic acid 38.23 mg/kg), while the aqueous extracts showed the lowest levels. The results indicated a higher quantity of phenolic and flavonoid compounds in the samples of dried ginger compared to the fresh extracts, with isorhamnetin, ferulic acid, catechin, and quercetin as the main compounds (83).

Therefore, according to a literature review, we found that polar solvents yield higher amounts of total phenols. Also, according to Mohamad et al. (2020) (84), the total phenolic content of dried ginger depends on the drying method used. Overall, the freeze-dried sample shows better results in retaining the total phenolic content among the tested drying methods (sun drying, vacuum oven drying, and freeze drying of ginger).

The study by An et al. (2016) (85) investigated various drying methods to assess their effectiveness in extracting phenolic compounds. The results revealed that freeze-drying was the method that yielded the highest amount of total phenolic compounds, with an average of 13.83 ± 0.31 mg GAE/g dry weight. Fresh ginger root followed with an average of 11.97 ± 0.33 mg GAE/g dry weight, then infrared drying with 11.35 ± 0.66 mg GAE/g dry weight, hot air drying with 9.69 ± 0.54 mg GAE/g dry weight, and finally microwave drying with the lowest yield, averaging 8.41 ± 0.35 mg GAE/g dry weight.

In our study microwave extraction resulted in a lower amount of total phenolics, possibly due to the thermal degradation of these compounds, as mentioned by Dalsasso *et*

al., 2022 (70). On the other hand, according to the same author, certain drying and extraction processes and conditions, such as freeze-drying and cold ultrasound extraction, can preserve sensitive compounds, resulting in extracts richer in total phenolics, gingerols, and antioxidant activity. This observation helps explain why ultrasound extraction performed better in our study.

6.4 GINGER EXTRACT BIOACTIVITIES

6.4.1 Antioxidant Activity

Antioxidant activity was assessed using the TBARS assay (inhibition of lipid peroxidation by thiobarbituric acid-reactive substances) and the DPPH assay (based on the ability to scavenge the colorimetric organic radical 2,2-diphenyl-1-picrylhydrazyl).

The results of antioxidant capacity are expressed in IC_{50} values (extract concentration required to achieve 50% antioxidant activity). Considering that higher antioxidant activity is indicated by a lower concentration required to inhibit 50% oxidation, the lower IC_{50} value reflects greater antioxidant activity (86).

From our results, based on the Student's t-test, we can conclude that there is a significant difference between the DPPH test and the TBARS test, with $p < 0.05$. We can highlight that ultrasound showed better antioxidant activity by the DPPH method, while microwave extraction presented better activity with the TBARS method. In table 6 are presented the results obtained for the antioxidant activity of ginger extracts obtained by ultrasound and microwave.

Table 6 - Antioxidant activity of ginger extracts evaluated by DPPH and TBARS

Method	**DPPH**	**TBARS**
UAE	0.373 ± 0.005* mg/mL	0.0046 ± 0.0002 mg/mL
MAE	0.429 ± 0.008 mg/mL	0.0032 ± 0.0001* mg/mL

MAE: Microwave-Assisted Extraction. UAE: Ultrasound-Assisted Extraction. The results are presented as mean ± standard deviation. '*' indicate significant differences ($p < 0.05$), determined by statistical analysis obtained through a t-student test.

Qadri *et. al.,* (2017) (58) also investigated the antioxidant activity of ginger using the maceration method in different solvents: 80% ethanol, 80% methanol, 80% acetone, and 100% distilled water, employing the DPPH assay. They obtained results of 0.0000325

mg/mL with the aqueous solvent, 0.0000283 mg/mL with acetone, 0.0000246 mg/mL with methanol, and 0.0000178 mg/mL with ethanol.

Similarly, Ivane *et. al.,* (2024) (87) evaluated the antioxidant capacity of ginger peel extract using ethanol extraction process. The DPPH analysis revealed an IC_{50} value for the extract of 0.78 ± 0.012 mg/mL. Jorge-Montalvo *et. al.,* (2023) (88) also measured the antioxidant potential of ginger ethanolic extract, obtaining values between 0.668 to 0.722 mg/mL via the DPPH method, while experimenting with four different extraction techniques: ultrasound, magnetic stirring, maceration, and reflux.

Studies conducted by Jelled *et. al.,* (2015) (89) evaluated the antioxidant activity of different forms of ginger rhizomes (fresh ginger, air-dried ginger (DG), and ginger powder (PDG)) using the thiobarbituric acid reactive substances (TBARS) assay. The fresh ginger underwent a process of washing, cleaning, was cut into small pieces, and air-dried (FDG) until it reached a constant weight. The results obtained were DG 0.12 ± 0.01 mg/mL, PDG 0.23 ± 0.01 mg/mL, and FDG 0.15 ± 0.01 mg/mL. The study also found that drying the whole rhizomes without cutting them prevents adulteration and preserves the internal composition.

Vendruscolo (2017) (90) conducted TBARS tests with lyophilized and fresh ginger samples, finding that the lyophilized samples showed TBARS values of 4.51 ± 0.1 mg of malondialdehyde/kg for the cubed sample and 4.54 ± 0.28 mg of malondialdehyde/kg for the ground sample. It was observed that, with the drying process, there was no significant difference in TBARS levels due to the sample form. Furthermore, compared to the fresh sample (4.30 ± 0.01 mg of malondialdehyde/kg), lyophilization did not result in a significant increase in TBARS levels.

Based on the data found in the literature, we observed that our results (Table 5) showed some similarity since none of the authors optimized the extraction process, and they used different extraction methods before conducting antioxidant analyses.

6.4.2 Antibacterial Activity

Antibacterial activity was assessed through microdilution assays using colonizing skin bacteria (*C. acnes*, *S. epidermidis*, *MRSA* and *P. aeruginosa*). The Table 7 below presents the minimum inhibitory concentrations (MIC) of the extracts and their respective extraction methods, considering that the maximum tested concentration was 10 mg/mL.

The following antibiotics were used as positive controls: Imipenem (1mg/mL),

which has a broad spectrum of activity against a variety of gram-positive and gram-negative bacteria; Ampicillin (10mg/mL), effective against a wide range of gram-positive and some gram-negative bacteria; and Vancomycin (1mg/mL), primarily used in the treatment of infections caused by gram-positive bacteria resistant to other antibiotics, such as methicillin-resistant *Staphylococcus aureus* (*MRSA*).

Table 7 - Antimicrobial activity of ginger extracts (MIC)

Extraction Method	**Bacteria**			
	C. acnes	***S. epidermidis***	***MRSA***	***P. aeruginosa***
MAE	5	5	5	>10
UAE	5	5	5	10
Positive Control				
Imipenem 1mg/mL	n.t.	n.t.	n.t.	0.5
Ampicillin 10mg/mL	0.07	n.t.	<0.15	>10
Vancomycin 1mg/mL	n.t.	0.25	0.25	n.t.

MAE: Microwave-Assisted Extraction. UAE: Ultrasound-Assisted Extraction. *MRSA:* Methicillin-resistant *Staphylococcus aureus*. The results are presented as the minimum inhibitory concentrations (MIC) in mg/mL.

For *C. acnes*, *S. epidermidis*, and *MRSA*, the microwave and ultrasound extraction methods appear to have comparable efficacies, both indicated by the same amount of 5 mg. This may suggest that, for these microorganisms, both methods are equally efficient under the tested conditions.

In the case of *P. aeruginosa*, however, a difference is observed. Microwave extraction appears to be less effective (>10 mg) compared to ultrasound extraction (10 mg), indicating that the ultrasound method is more efficient for this bacterium, ultrasound-assisted extraction also surpassed the positive control of Ampicillin.

Yusuf *et al.* (2018) (91) evaluated the antimicrobial activity of methanolic ginger extracts in vitro against *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, *Salmonella typhi*, and *E. coli*. They found that the methanolic extract exhibited a significant level of inhibition against both gram-positive and gram-negative bacteria, possibly due to the polar nature of

methanol. Additionally, they highlighted that *S. aureus* was the most resistant microorganism among all tested.

Masniari (2011) (92) found that methanolic ginger extracts obtained through the maceration method demonstrated efficacy in inhibiting the growth of *S. epidermidis*. This bacterium was found to be more sensitive to ginger extracts compared to other tested bacteria.

Also, according to Goyal and Kaushik (2011) (93) using the maceration method for extraction, it was found that the methanolic extract exhibited higher antibacterial activity against *S. aureus* (MIC = 0.512 mg/mL) compared to those obtained with ethanol (MIC = 4.096 mg/mL), water (MIC = 2.048 mg/mL), and ethyl acetate (MIC = 4.096 mg/mL).

Karuppiah *et al.*,(2012) (94) Using the maceration method and a ratio of 1:15 (g of sample / mL of solvent), they also investigated the antimicrobial properties of ginger and highlighted that the ethanolic extract of ginger had a high antimicrobial effect against *E. coli* (MIC = 0.0756 mg/mL), *P. aeruginosa* (MIC = 0.0670 mg/mL), *Proteus sp.* (MIC = 0.0702 mg/mL), *S. aureus* (MIC = 0.0685 mg/mL), and *Bacillus sp*. (MIC = 0.0045 mg/mL).

Azhari e Sengaji (2023) (95) They also investigated the Minimum Inhibitory Concentration (MIC) of various bacteria using a ginger extract obtained by maceration with 96% ethanol as the solvent. The results were as follows: *E. coli* (MIC = 0.256 mg/mL), *S. aureus* (MIC = 0.512 mg/mL), *P. aeruginosa* (MIC = 1.024 mg/mL), *C. acnes* (MIC = 0.256 mg/mL) e *Candida albicans* (MIC = 2.048 mg/mL).

Through the studies conducted by Ghasemzadeh, Jaafar, & Rahmat (2016) (96) They found that samples of fresh rhizome extract exhibit significantly higher antibacterial activity compared to stored samples, although they still maintain activity superior to commercial antibiotics.

Additionally, according to Ghasemzadeh, , Jaafar, & Rahmat (2016) (96) The antimicrobial activity of an extract stored for more than 4 months at a temperature of 15°C became null for gram-negative bacteria and greatly reduced for gram-positive bacteria.

Based on all the studies reviewed regarding the antibacterial properties of ginger extracts, including our own study, it can be affirmed that ginger possesses antibacterial capacity. However, this capacity is highly dependent on the extraction method and storage conditions.

6.4.3 Anti-inflammatory activity and cytotoxicity

We assessed the cytotoxic activity of the extracts, HFF1 (human fetal skin fibroblasts) was used. The results are presented in Table 8 and expressed as GI_{50} values, which correspond to the concentration of extract with the ability to inhibit cell growth by 50%.

Table 8 - Cytotoxicity of ginger extracts (GI_{50}, μg/mL)

Method	HFF1
MAE	>400
UAE	>400
Ellipticine	0.9 ± 0.1

MAE: Microwave-Assisted Extraction. UAE: Ultrasound-Assisted Extraction. HFF1: human fetal skin fibroblasts. Ellipticine was the control used. The GI_{50} values correspond to the concentration of extract with the ability to inhibit cell growth by 50%.

To assess the anti-inflammatory activity of ginger extracts by observing their ability to inhibit the production of nitric oxide (NO) in RAW 264.7 mouse macrophages stimulated with lipopolysaccharides (LPS). We used dexamethasone as a positive control. The results (Table 9) are expressed as IC_{50} values, representing the concentration of each extract required to inhibit 50% of nitric oxide production.

Table 9 - Anti-inflammatory activity of ginger extracts (EC_{50}, μg/mL)

Method	RAW 267.4
MAE	235.76 ± 9.04*
UAE	267.9 ± 13.76
Dexamethasone	6 ± 1

MAE: Microwave-Assisted Extraction. UAE: Ultrasound-Assisted Extraction. Dexamethasone was the control used. The results are presented as mean ± standard deviation. The EC_{50} values correspond to the extract concentration required to inhibit the production of NO by 50%. '*' indicate significant differences ($p < 0.05$), determined by statistical analysis obtained through a t-student test.

Based on our results from the anti-inflammatory and cytotoxic analyses, we conclude that our ginger extracts possess anti-inflammatory activity and exhibit low cytotoxicity. However, none of the extracts showed cytotoxic activity at the maximum tested concentration 400 μg/mL for the HFF1 cell lines. Analysis of the anti-inflammatory

activity in the RAW 267.4 cell line resulted in an EC_{50} of 235.76 ± 9.04 µg/mL for the microwave extract, which is the concentration required to achieve 50% of the anti-inflammatory activity. Meanwhile, the ultrasound-extracted sample obtained an EC_{50} of 267.9 ± 13.76 µg/mL.

Kim *et al.,* (2017) (97) In their study, it is suggested that ginger extract, along with its active components such as 6-gingerol and 6-shogaol, help reduce inflammation by lowering levels of inflammatory cytokines. They achieve this by blocking the activity of a protein called NF-κB in Caco-2 cells. Moreover, ginger extract, 6-gingerol, and 6-shogaol were able to significantly decrease the expression of certain inflammation-related genes, such as iNOS and COX-2, while increasing trans-epithelial electrical resistance (electrical resistance across an epithelial cell layer).

Zhou *et al.,* (2022) (98) They found that ginger exhibited moderate inhibitory effects on nitric oxide (NO) production with an IC50 value of 11.8 ± 1.58 µg/mL and on interleukin-6 (IL-6) with an IC_{50} value of 32.91 ± 9.06 µg/mL, while it had no effect on tumor necrosis factor (TNF). Additionally, they found that ginger did not cause significant harm to cells in our body (ginger's lethal concentration 50 (LC_{50}) was 104.3 ± 5.63 µg/mL), even at higher doses, as demonstrated by the absence of cytotoxicity in RAW 264.7 and THP-1 cells when exposed to concentrations of up to 100 µg/mL and 50 µg/mL, respectively. The same was confirmed in our study; ginger extract did not exhibit cytotoxicity to cells. Furthermore, ginger moderately induced the upregulation of the Nrf2 factor by up to 2.04 ± 0.22 times at a concentration of 25 µg/mL (98).

Saanin *et al*., (2020) (99) they employed the maceration technique with 70% ethanol solvent and concluded that the ethanolic extract of ginger possesses anti-inflammatory properties. They observed a reduction in the production of pro-inflammatory mediators such as TNF-α, IL-1β, IL-6, COX-2, and NO. The ethanolic extract of ginger, at a concentration of 50 µg/mL, inhibited the concentration of TNF-α with an inhibitory activity value of 53.09%. Additionally, this extract exhibited the highest inhibitory activity of IL-6 (70.16%) at 50 µg/mL, comparable to the normal cell (negative control). These results suggest promising therapeutic potential for the treatment of inflammation-associated diseases using the ethanolic extract of ginger.

Based on the literature and our results from anti-inflammatory and cytotoxic analyses, we confirm that our ginger extracts possess anti-inflammatory activity and exhibits low cytotoxicity. Our extract reinforces these characteristics.

6.5 DEVELOPMENT THE SOAP CONTAINING GINGER EXTRACT

After the soap manufacturing process with the addition of the extract at each of the concentrations to be tested, the soaps (figure 20 and 21) were then divided into equal parts to be evaluated over three distinct periods: at the time of manufacture (0 days), after one week (7 days), and again after two weeks (15 days). This approach allows for a comprehensive analysis of soap color stability and bioactivity over time.

Figure 20 - Solid soaps Ultrasound-Assisted Extraction method at the time of manufacture (time 0 days)

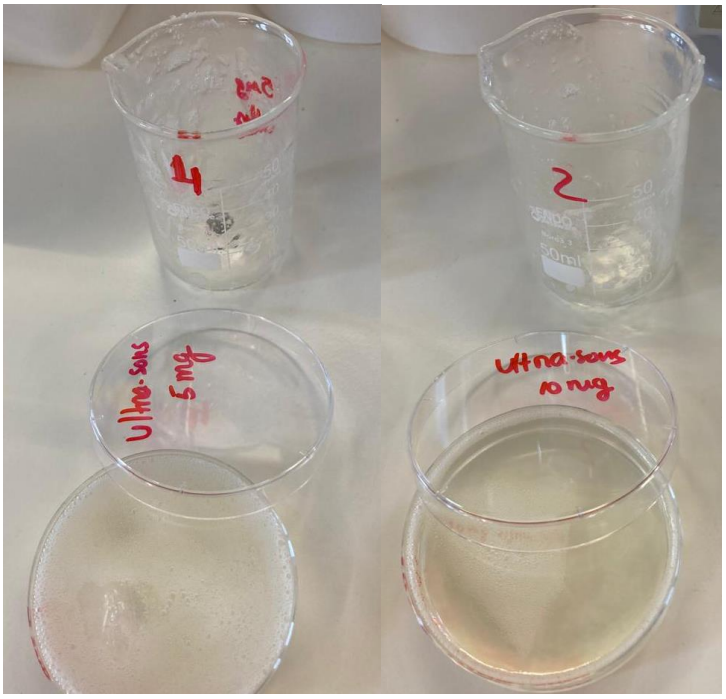
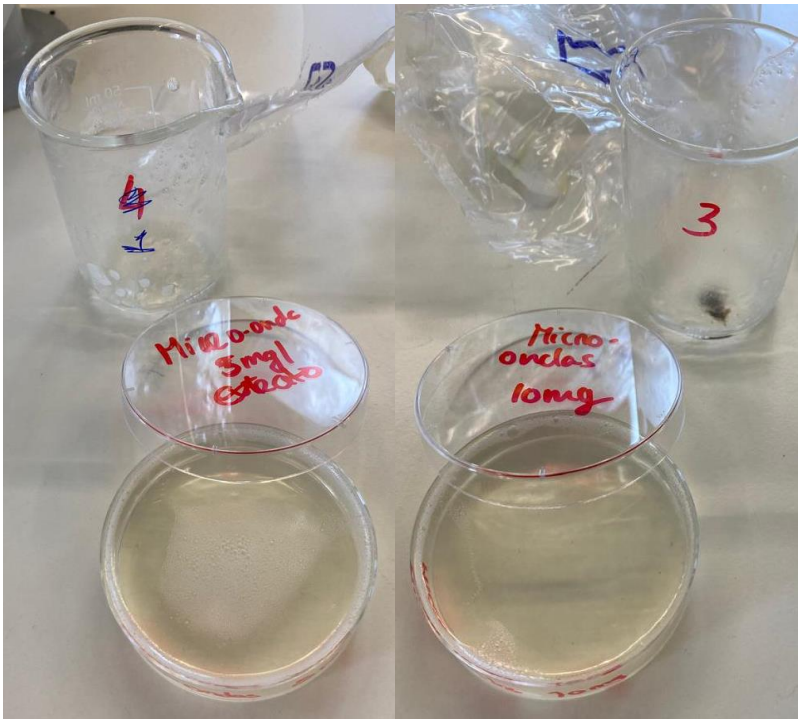


Figure 21 - Solid soaps Microwave-Assisted Extraction method at the time of manufacture (time 0 days)



As depicted in the figures above, no visible differences in the coloration of the solid soaps were observed due to the extract concentration.

As soon as the soaps reached stability, we proceeded to collect the samples (time 0), and each solid soap was divided and stored away from light and moisture at room temperature for further analysis.

After 7 days (figures 22 and 23), soap samples were collected again, assessed for any changes in their physical appearance and color.

Figure 22 - Solid soaps Ultrasound-Assisted Extraction method after 7 days

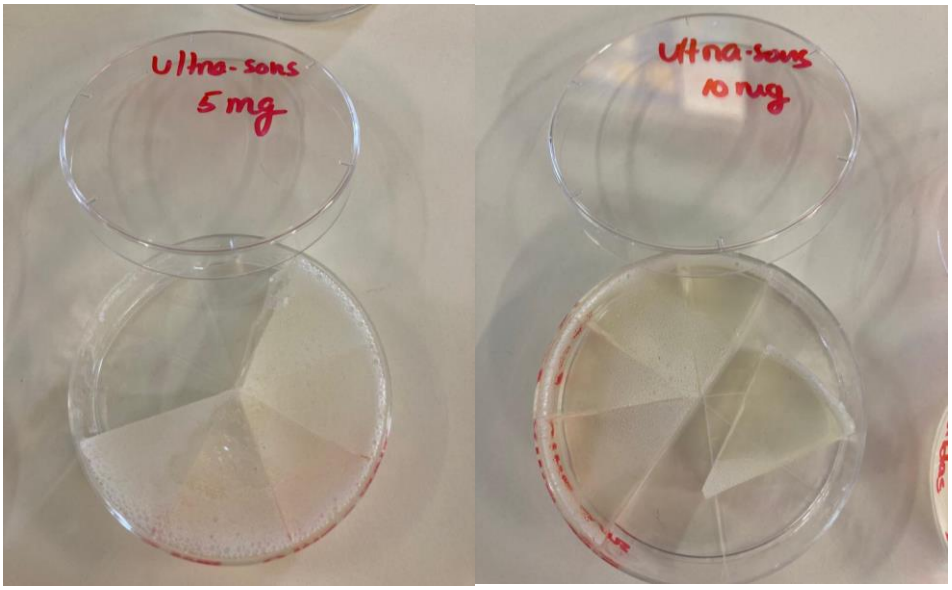
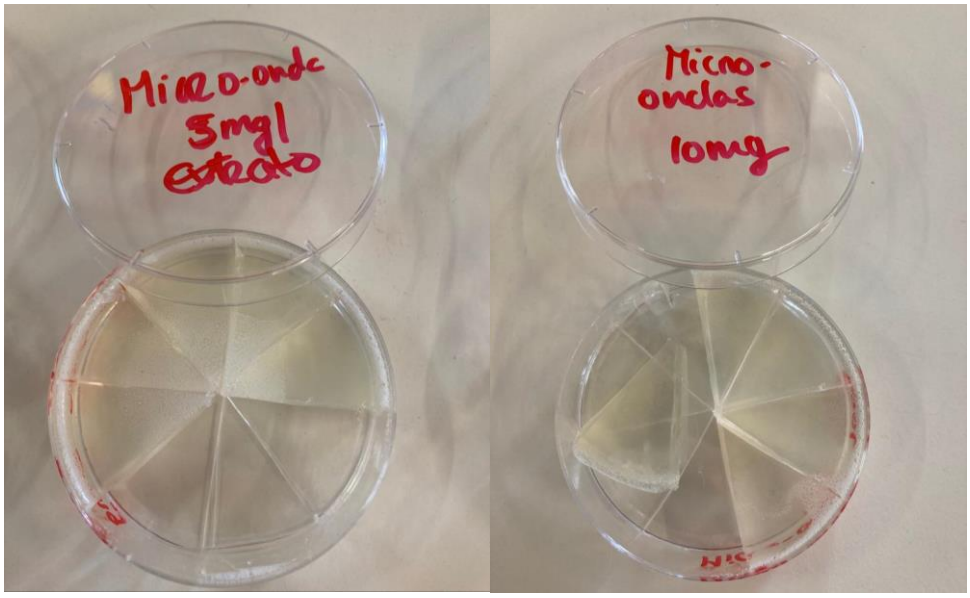


Figure 23 - Solid soaps Microwave-Assisted Extraction method after 7 days



After 15 days (figures 24 and 25), the soaps maintained the same physical appearance, with no apparent visible changes.

Figure 24 - Solid soaps Ultrasound-Assisted Extraction method after 15 days

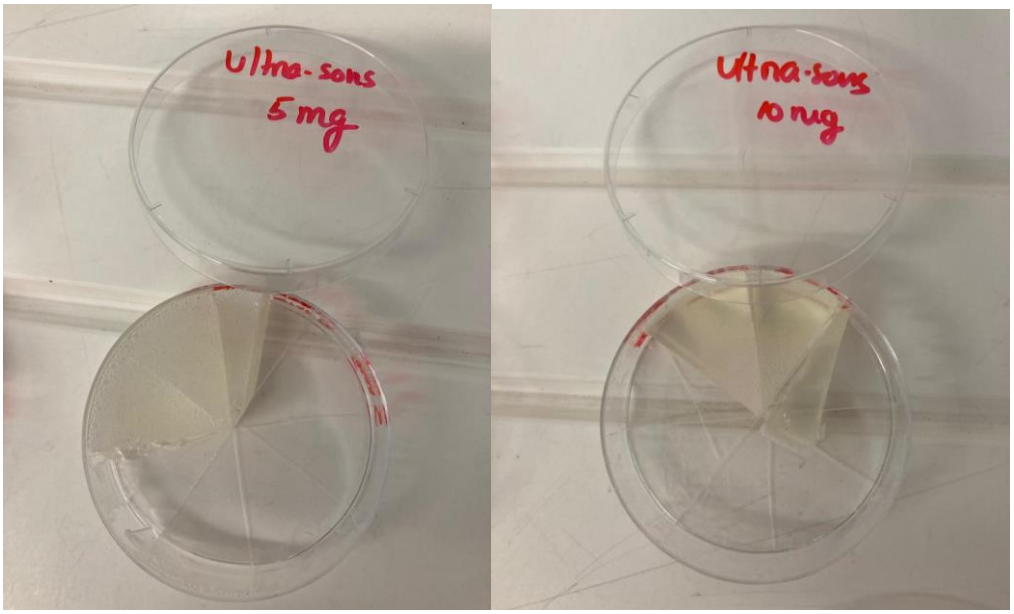
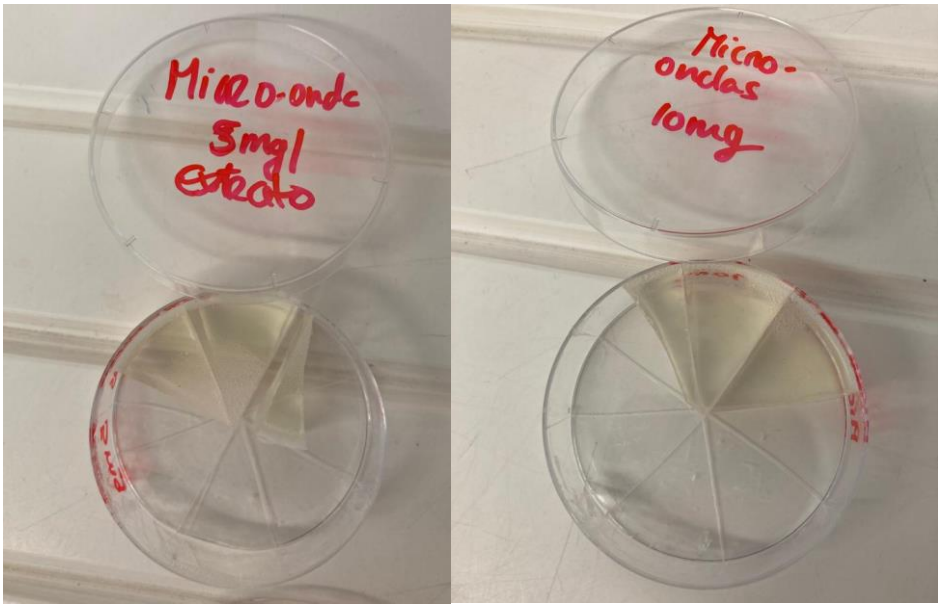


Figure 25 - Solid soaps Microwave-Assisted Extraction method after 15 days



At the end of the 15-day period, antimicrobial tests were conducted on all solid soaps to determine if there were any changes in their bioactive properties. Additionally, the samples were analyzed for color changes using a colorimeter (table 10).

Table 10 - Color of the extracts at 0, 7, and 15 days

SÓLID SOAPS			L	a	b	HEX	RGB	ΔE*
UAE	10 mg	Tempo 0	65.52 ± 0,26	-0.46 ± 5	16.15 = 0.09	A79F82		0
		Tempo 7	56.42 ± 0.60	-0.83 ± 0.02	10.30 ± 0.38	8B8775		10,82
		Tempo 15	56.80 ± 1.03	-0.40 ± 0.06	9.51 ± 1.30	8D8878		0,98
	5 mg	Tempo 0	56.35 ± 1.73	-0.32 ± 0.11	5.31 ± 1.37	8A877E		0
		Tempo 7	50.27 ± 0.85	-0.69 ± 0.04	6.17 ± 0.46	7A786D		6,15
		Tempo 15	52.42 ± 1.78	-0.5 ± 0.01	4.54 ± 0.27	7E7C75		2,71
MAE	10 mg	Tempo 0	62.42 ± 0.45	-0.26 ± 0.05	14.95 ± 0.48	9E967C		0
		Tempo 7	65.93 ± 1.39	0.59 ± 0.05	15.83 ± 0.80	A8A084		3,64
		Tempo 15	64.05 ± 2.44	-1.00 ± 0.07	14.76 ± 0.55	A19B81		2,20
	5 mg	Tempo 0	61.80 ± 1.58	-0.47 ± 0.03	13.99 ± 0.70	9C957C		0,00
		Tempo 7	66.78 ± 0.95	-0.82 ± 0.06	15.70 ± 0.40	A9A286		5,27
		Tempo 15	55.55 ± 0.87	-0.67 ± 0.03	7.54 ± 0.52	888578		13,87

MAE: Microwave-Assisted Extraction. UAE: Ultrasound-Assisted Extraction. ΔE*: variation in total color difference between time 0 and 7 days, and between time 7 and 15 days. The values are presented as mean ± standard deviation.

Based on our analysis of soap coloration using a colorimeter, we can highlight that the ultrasonication extraction method resulted in a soap with a darker hue, while the microwave method resulted in a lighter shade. Additionally, it was observed that both soaps exhibited variations in their coloration over time. We can observe through the total variation in color that the soap with 10 mg made with the extract obtained by ultrasound-assisted extraction showed a greater color variation between days 0 and 7, however, between day 7 and day 15, it exhibited greater stability. On the other hand, the soap with 5 mg, prepared with the extract obtained by microwave-assisted extraction, showed a more significant variation between days 7 and 15.

For the antibacterial activity of solid soaps, the method used was agar diffusion, as described in section 5.4.2, where standardized pieces of the soaps were placed in the center of Petri dishes with the bacteria to be tested. The inhibition zones (Figures 26 and

27) were measured, and the results are expressed in Table 11 below in millimeters of diffusion.

Figure 26 - Inhibition zone in *P. aeruginosa*, soap Microwave-Assisted Extraction method 10mg at time 0 days

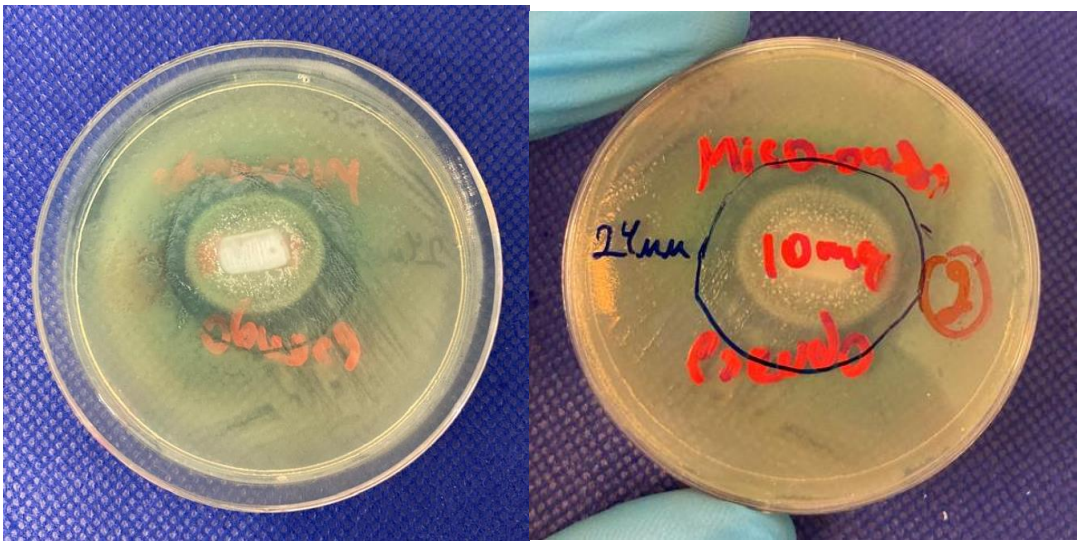


Figure 27 - Inhibition zone in *MRSA*, soap Microwave-Assisted Extraction method 10mg at time 0 days

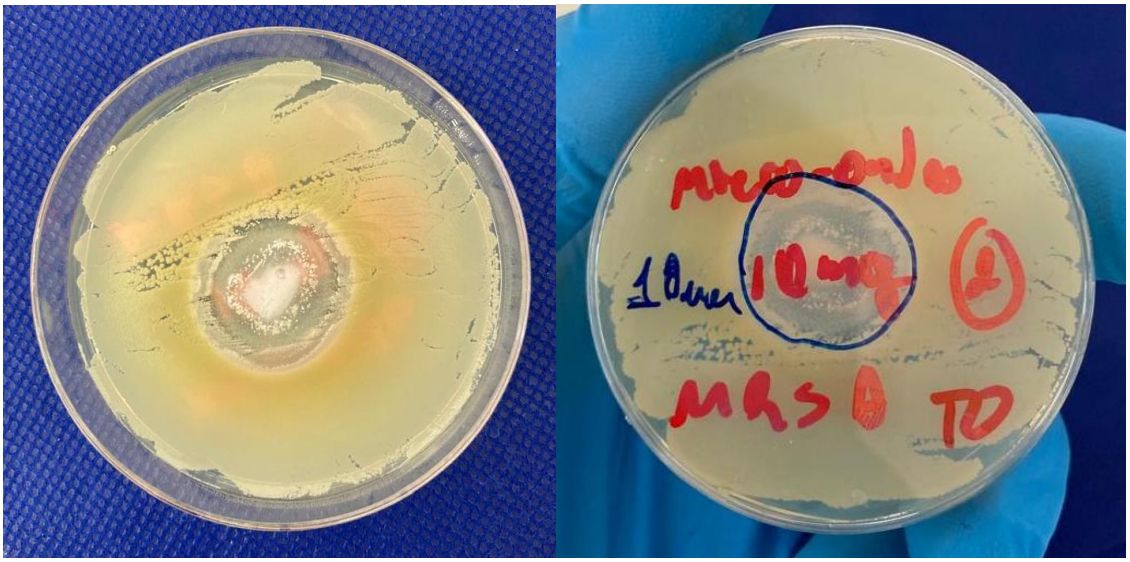


Table 11 - Zones of inhibition of solid soaps in millimeters (mm)

SOLID SOAPS			Bacteria			
			MRSA	*P. aeruginosa*	*S. epidermidis*	*C. acnes*
MAE	10 mg	Time 0	17	24	17.5	15
		Time 7	13.5	18	14	14
		Time 15	13.5	17	13	12
	5 mg	Time 0	15.5	21.5	14	13
		Time 7	14	17	13	13
		Time 15	13.5	11	13	13
UAE	10 mg	Time 0	20*	25	20	20*
		Time 7	20*	20	20	17*
		Time 15	19.5*	15	15	15.5*
	5 mg	Time 0	20	22.5	13.5	20
		Time 7	20	20	N/O	17
		Time 15	15	13	N/O	15
Control			10	12.5	10	9

MAE: Microwave-Assisted Extraction. UAE: Ultrasound-Assisted Extraction. *MRSA:* Methicillin-resistant *Staphylococcus aureus*. Control: Glycerin. The results are presented as mean in millimeters (mm). '*' mean significant differences ($p < 0.05$) using one-way analysis of variance (ANOVA).
The inhibition zone of *S. epidermidis* on the Ultrasound 5 mg soap was not identified for the 7 and 15-day time. Therefore, we marked it as not observed (N/O).

Based on our results (table 11), we found that the soap with the most potent bioactive properties was the one made with ginger extract obtained through the ultrasonication extraction method. The sequence of inhibition zones followed the order from highest to lowest activity: *P. aeruginosa* > *MRSA* > *S. epidermidis* ≥ *C. acnes*. The largest inhibition zone in the concentration of 10 mg was observed for *P. aeruginosa*, at 25 mm, while the smallest was for *C. acnes*, at 15 mm.

We observed that a higher concentration of the extract in the soap positively influenced its antimicrobial activity. Moreover, we observed a decline in bioactivity over time in the soap containing the extract obtained via ultrasonic-assisted extraction. Using the statistical analysis ANOVA, we found that for both the *P. aeruginosa* and *C. acnes* bacteria a significant variance over the time, with a p-value < 0.05.

However, upon statistical analysis using the ANOVA to assess significant differences between the two extraction methods, we found that ultrasound extraction yielded a significant difference against the bacteria *C. acnes* and *MRSA*, with a significance level of $p < 0.05$. Conversely, for the bacteria *P. aeruginosa and S. epidermidis*, there was no significant difference found between the extraction methods, with a p-value > 0.05.

Through their studies Mohammed *et al.,* (2019) (100) obtained an aqueous ginger extract through maceration and analyzed its antimicrobial properties. They found that the inhibition zone increased with concentration (250 and 500 mg/mL were tested), ranging from 12-26 mm for *Klebsiella pneumoniae*, 14 to 25 mm for *Staphylococcus aureus*, and 15 to 19 mm for *E. coli*. Its effects against *Pseudomonas* were also examined, but none of the concentrations showed antibacterial activity.

Upon comparing our results with the bacterial inhibition of the control, we can conclude that our extract had a positive impact when added to the glycerin base. There was an increase in the inhibition zone with the addition of our extract, more significantly for *P. aeruginosa* and less noticeably for *C. acnes*. These same results were observed in the control inhibition. However, there was also a trend of decreasing inhibition zones over time, indicating a reduction in the extract's bioactivity.

Qadri et. al., (2017) (59) They also investigated the antimicrobial activity of ginger extract using the maceration method with different solvents: 80% ethanol, 80% methanol, 80% acetone, and 100% distilled water. The results showed inhibition zones (in millimeters), with higher inhibition against the bacterium *S. aureus* with the ethanolic extract (15.7). The ethanolic extract also proved to be more effective against the bacteria *E. coli*, *P. multocida*, and *B. subtilis*, reaching 11.8, 13.2, and 14.6, respectively.

Our results also surpassed those reported by Sirilak Sanpa & Sirikarn Sanpa (2019) (101), In a study on the antimicrobial activity of ginger extracts obtained by maceration, two extracts were prepared: one aqueous and the other ethanolic, with three different concentrations (100, 200, and 300 mg/mL) tested. Sanpa & Sanpa found that ethanol extraction exhibited superior activity compared to the aqueous extract. Furthermore, they observed that increasing the concentration of the extracts also increased the inhibition zones, achieving an inhibition zone of 13.67 for *S. aureus*, 13.00 for *S. epidermidis*, 10.33 for *P. aeruginosa*, and 15.33 for *C. acnes* at a concentration of 300 mg/mL.

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacillus characterized

by its greenish color, associated with potentially severe forms of dermatoses. This bacterium often colonizes chronic wounds, severe burns, and severe dermatoses, requiring special care due to its high resistance to antibiotics. Additionally, it may be linked to folliculitis, especially in water sports practitioners, and nail disorders. Understanding its ecology, resistance, and biofilm-forming ability is crucial for effective therapy and preventive measures (102).

Also *P. aeruginosa* has one of the largest and most complex bacterial genomes known. Its ability to mutate frequently and to acquire resistance genes through plasmids or transposons explains its capacity to develop resistance to many antibiotics. Additionally, it possesses various factors contributing to its virulence, such as adhesion capability, survival in hostile environments, biofilm formation, and production of enzymes and toxins, including those causing necrosis (103).

P. aeruginosa is common in moist environments such as pools, bathtubs, toilet flushes, showers, and sinks. In hospital settings, it can be found on equipment like humidifiers and respirators, and it can persist in products like soaps, eye drops, ointments, and bandages. Direct or indirect transmission through equipment is crucial in the spread of hospital-acquired infections by *P. aeruginosa* (102).

Staphylococcus aureus is one of the leading causes of morbidity and mortality worldwide due to infections. Treatment of S. aureus infections is complicated by antibiotic resistance, and currently, there is no vaccine available (104).

Methicillin-resistant *Staphylococcus aureus* (*MRSA*) remains one of the leading multidrug-resistant bacterial pathogens causing complicated skin and skin-structure infections (cSSSI) and severe hospital-acquired infections, especially bloodstream infections (BSIs) and ventilator-associated pneumonia (VAP) (105,106). Overall, it is estimated that *MRSA* causes 171,200 healthcare-associated infections (HAIs) in Europe each year, accounting for 44% of all HAIs, as well as causing 5,400 additional attributable deaths and over a million extra days of hospitalization associated with these infections (105).

C. acnes and *S. epidermidis*, two genera of stable Gram-positive bacteria, are essential components of the skin microbiota and are widely distributed throughout the human body according to skin environmental conditions, including temperature (ranging between 31.8 and 36.6 °C) and pH (ranging between 4.2 and 7.9), which are the two main factors influencing their growth (107).

S. epidermidis species are predominantly found in moist regions, congregating

in the axillae, antecubital folds, popliteal folds, and plantar tissues. On the other hand, *C. acnes* species are mainly located in sebaceous areas, such as the face and back (107–109).

C. acnes and *S. epidermidis*, as commensals, establish a symbiotic relationship with the cutaneous system, more specifically, a mutualistic relationship in which both organisms benefit. The skin hosts *S. epidermidis* and *C. acnes*, providing them with nutrients, while, in turn, both bacteria contribute to skin homeostasis, host defense, and innate immunity. They are often viewed as commensal bacteria because they are harmless under healthy conditions and promote skin health when in balance (110).

These bacteria adapt to constantly changing cutaneous microenvironments and can become opportunistic pathogens, forming biofilms and thereby playing a significant role in common cutaneous dysbiosis, such as acne or atopic dermatitis (111).

7. CONCLUSION

In summary, the objectives established in this research were successfully achieved. Determining the optimal extraction point was crucial to ensure an extract with the best bioactivities. The results confirmed that ginger possesses antibacterial, anti-inflammatory, and antioxidant properties, essential for the development of a soap with therapeutic potential. Moreover, the comparison between the ultrasound and microwave extraction methods revealed that the ultrasound method proved more efficient in obtaining the desired bioactive extract. We concluded, therefore, that the soap produced with the extract obtained by the ultrasound method, using 10 mg, exhibited the best bioactivities.

The addition of ginger extract to the glycerin base resulted in a soap with enhanced antimicrobial properties, highlighting the potential of this product as a natural and effective alternative for personal hygiene and skincare.

8. FUTURE PERSPECTIVES

For future studies, we recommend a more comprehensive chemical characterization of ginger to distinguish and identify the compounds influencing each bioactivity. We suggest conducting an in-depth study focusing on the characterization of lyophilized ginger and the extract obtained from this ginger, aiming to enhance its anti-inflammatory, antimicrobial, and antioxidant properties.

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