Myrtus communis L.: an unconventional wild berry as a source of bioactive compounds

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Summary

List of abbreviations

BBD: Box-Behnken design

CCD: Central composite design

CO_2: Carbone dioxide

Da: Dalton

DoE: Design of experiment

DPPH: 2,2-diphenyl-1-picrylhydrazy

EAE: Enzyme-assisted extraction

EC_{50}: Half maximal effective concentration

FD: Factorial design

GAE: Gallic acid equivalent

HAE: Heat-assisted extraction

min: Minute

MAE: Microwave-assisted extraction

MBC: Minimum bacterial concentration

MDA: Malondialdehyde

MIC: Minimum inhibitory concentration

MFC: Minimum fungicidal concentration

MS: Mass spectrometry

MRF: Multiple response functions

MRSA: Methicillin-resistant Staphylococcus aureus

ns: Non significant

PF: Pellet fraction

PLE: Pressurized liquid extraction

R^2: Correlation coefficient

R^2 adj: The adjusted determination coefficient model

R_t: Retention time

RSM: Response surface methodology

SD: Standard deviation

SFE: Supercritical fluid extraction

TFC: Total flavonoid content

TPC: Total phenolic content

UAE: Ultrasound-assisted extraction

UV: Ultraviolet

W: Watt

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Abstract

Myrtus communis L., commonly known as myrtle, is a plant found in Mediterranean regions, traditionally used to treat various ailments such as respiratory infections and gastrointestinal disorders due to its anti-inflammatory, antimicrobial, and antioxidant properties. Studies in literature report the chemical composition of its leaves and berries, identifying bioactive compounds responsible for its therapeutic properties. However, few studies have investigated optimized extraction methods to obtain specific bioactive compounds from its berries. In this context, the present study aimed to develop and evaluate extraction methodologies to maximize the yield of bioactive compounds, particularly phenolic compounds, from M. communis berries, highlighting their potential as antioxidant and antimicrobial agents. To this end, two extraction methodologies were tested: heat-assisted extraction (HAE) and microwave-assisted extraction (MAE), evaluating the extraction yield, total phenolic content, and antioxidant activity. Response surface methodology (RSM) was used to optimize extraction parameters, such as time, temperature, and ethanol concentration. The optimized extract was assessed for its antioxidant and antimicrobial capacity, and its phenolic profile was determined by HPLC-LC/MS. As a result, HAE demonstrated superior efficacy in preserving phenolic integrity and antioxidant capacity. The optimal extraction conditions were determined to be 90 minutes at 100°C with 39% ethanol, resulting in a yield of 47.2 ± 0.2%, with the extract containing 50 ± 8 mg GAE/g and capable of scavenging 50% of the DPPH radical at a concentration of 0.041 ± 0.003 mg/mL. The optimized extract also showed the ability to inhibit lipid peroxidation through the thiobarbituric acid reactive substances (TBARS) assay, with an EC_{50} value of 0.045 ± 0.006 mg/mL, and was effective in inhibiting the growth of 16 bacteria, including 8 clinical strains and 8 strains associated with food contamination. In its phenolic composition, four myricetin derivatives and one quercetin derivative were identified, with the most abundant compound being myricetin-*O*-hexoside (3.60 ± 0.04 mg/g extract). In conclusion, *M. communis* berries offer potential applications in food, pharmaceuticals, and cosmetics, providing a sustainable option and an eco-friendly substitute for artificial compounds.

Keywords: *Myrtus communis* L., bioactive compounds, antioxidant activity, phenolic compound, extraction optimization.

Resumo

Myrtus communis L., comumente conhecida como murta, é uma planta encontrada nas regiões mediterrâneas, tradicionalmente utilizada no tratamento de várias doenças, como infecções respiratórias e distúrbios gastrointestinais, devido às suas propriedades anti-inflamatórias, antimicrobianas e antioxidantes. Estudos encontrados na literatura relatam a composição química de suas folhas e bagas, identificando compostos bioativos responsáveis por suas propriedades terapêuticas. No entanto, poucos estudos investigaram métodos otimizados de extração para obter compostos bioativos específicos a partir das suas bagas. Neste contexto, o presente trabalho teve como objetivo desenvolver e avaliar metodologias de extração para maximizar a obtenção de compostos bioativos, nomeadamente compostos fenólicos, das bagas de *M. communis*, destacando seu potencial como agente antioxidante e antimicrobiano. Para isso, foram testadas duas metodologias de extração: a extração assistida por calor (HAE) e a extração assistida por micro-ondas (MAE), avaliando-se o rendimento de extração, o teor de fenóis totais e atividade antioxidante. A metodologia de superfície de resposta (RSM) foi utilizada para otimizar os parâmetros de extração, como tempo, temperatura e concentração de etanol. O extrato otimizado foi avaliado quanto à sua capacidade antioxidante e antimicrobiana, e o seu perfil fenólico foi determinado por HPLC-LC/MS. Como resultado, a HAE demonstrou uma eficácia superior na preservação da integridade fenólica e da capacidade antioxidante. As condições ótimas de extração foram determinadas em 90 minutos a 100°C com 39% de etanol, resultando em um rendimento de 47,2 ± 0,2%, onde o extrato apresentou 50 ± 8 mg GAE/g, sendo capaz de captar 50% do radical livre DPPH numa concentração de 0,041 ± 0,003 mg/mL. O extrato otimizado também demonstrou capacidade de inibir a peroxidação lipídica por meio do ensaio de substâncias reativas ao ácido tiobarbitúrico (TBARS), com um valor de EC_{50} de 0,045 ± 0,006 mg/mL, e capacidade de inibir o crescimento de 16 bactérias, incluindo 8 estirpes clínicas e 8 estirpes associadas à contaminação de alimentos. Em sua composição fenólica, foram identificados 4 derivados de miricetina e um derivado de quercentina, sendo que o composto mais abudante foi a miricetina-*O*-hexosídeo (3,60±0,04 mg/g extrato). Em conclusão, as bagas de *M. communis* apresentam potenciais aplicações em alimentos, medicamentos e cosméticos, oferecendo uma opção sustentável e um substituto ecológico para compostos artificiais.

Palavras-chaves: *Myrtus communis* L., compostos bioactivos, atividade antioxidante, compostos fenólicos, otimização da extração.

1. Introduction

Several industrial sectors (cosmetics, pharmaceuticals, food, food supplements, among others) increasingly incorporate natural bioactive molecules to replace synthetic chemicals. In the case of the food sector, these molecules of natural origin are substantial and are used as food additives, which means they are substances incorporated into food for technological purposes as preservatives or organoleptic during the production of this food (Nieto et al., 2023). Therefore, evaluating these natural substances represents enormous economic potential and products that contain fewer chemicals and are healthier (Garcia-Salas et al., 2010).

Currently, the food industries have shown significant development due to the use of natural products extracted and refined from natural sources through a range of technological methods and processes (Sridhar et al., 2022). Bioactive compounds can be used as natural preservatives due to their antioxidant and antimicrobial activities. In addition, these compounds can also show technology proprieties, such as emulsifying, flavoring, and coloring agents (Nieto et al., 2023). For instance, pomegranate peel extracts have been used to stop the growth of bacteria that cause spoiling, such as *Penicillium digitatum* in oranges and *Botrytis cinerea* in straw berries. By lowering lipid oxidation and microbiological development, grape seed extract can be utilized in coatings to preserve fresh fish fillets (Nieto et al., 2023).

With the aim to explore a new source of bioactive molecules, this work focuses on performing the chemical and bioactive characterization of the berry produced by *M. communis*, a shrub that can reach up to 2 meters in height and can be found in the Mediterranean region. Some studies revealed that this fruit can have several biological properties, such as antioxidant, anti-inflammatory, anticancer, and anti-diabetic activities (Bilawal et al., 2021). *M. communis* berries are considered a rich source of phytochemicals beneficial to humans, including phenolic acids, flavonoids, tannins, and stilbenes (Bilawal et al., 2021).

The main objective of this study was to perform the extraction, identification and quantification of the main bioactive compounds present in *M. communis* berry. In order to obtain an extract with a higher concentration of bioactive molecules and high bioactive potential, the extraction optimization was performed. The results found in this study can contribute to the fields of pharmacology, food, and agriculture by identifying new sources of natural bioactive compounds and will also enhance the knowledge base around this wild berry, which is not typically consumed or commercialized, supporting biodiversity conservation and sustainable use of natural resources.

1.1 Natural products

Natural products occupy a significant place in biomolecular chemistry because they are a rich source of bioactive substances due to their diverse range of biological activities, which can be attractive for their application in pharmaceutical and food fields. These compounds are often divided into two major classes: primary and secondary metabolites. Primary metabolites are organic molecules with an intrinsic function essential to the organism's survival. Some examples of primary compounds are proteins, carbohydrates, and lipids. Secondary metabolites are organic molecules that typically have non-essential functions for the organism; however, they are important to the organism's survival in the environment; they have a fundamental role in defense, competition, and signaling. Extrinsic factors influence their syntheses, and examples of secondary metabolites are alkaloids, polyketides, polyphenols, and terpenoids (Atanasov et al., 2021).

Natural compounds are found in various biological matrices, including microorganisms, fungi, plants, animals, marine species (e.g., algae), among others (Khalifa et al., 2019). Microorganisms produce diverse biomolecules that support their growth, survival, and interactions with their environment and other organisms. These bioactive compounds exhibit important biological properties, including antimicrobial and cytotoxic activities. Well-known examples of natural products from microorganisms are streptomycin, a power antibiotic used to treat tuberculosis, synthesized by *Streptomyces griseus,* and erythromycin, an antibiotic produced by *Saccharopolyspora erythraea* (Peng et al., 2021). An example of the natural compounds derived from the animal source is the venoms obtained from venomous animals. For example, the Brazilian viper venom present a mixture of bioactive peptides and proteins (captopril and a peptide derivative) that are applied in the pharmaceutical field, as an antihypertensive drug (Gurnani et al., 2014). In plants, several classes of secondary metabolites can be produced by different species and be used in various industries for different purposes. In the next section, the main natural products found in plants will be presented in more detail.

1.1.1 Main classes of natural products found in plant tissue

Secondary metabolites produced by plants are extremely rich in bioactive activities that benefit human health. These molecules are extracted, for example, from vegetables, fruits, grains, seeds, and leaves, which may contain different phytochemicals that are a source of therapeutic and preventive agents against diseases (Riaz et al., 2023). The main secondary metabolites in plants are terpenes, alkaloids, polyphenols, and saponins (Staniek et al., 2014). Natural compounds are safer and have a healthier impact on the consumer (Staniek et al., 2014).Therefore, using these secondary metabolites in the food industry can be a great alternative to reducing the application of artificial and synthetical chemical compounds in the foodstuff.

Researchers have shown that natural products recovered from fruits and their by-products can be explored to develop value-added products to be used in the food industry(Ben-Othman et al., 2020). Fruits and vegetables are abundant in phenolic compounds, vitamins, carotenoids, polysaccharides, and various other bioactive substances closely linked to human health. Berries are abundant in anthocyanins, while carrots provide carotenoids, which serve as natural food colorants, imparting color without synthetic additives. Some plant extracts have antimicrobial properties, which can help preserve food and extend shelf-life (Tuberoso et al., 2010). Furthermore, these compounds assist in safeguarding cells from oxidative stress, potentially diminishing the risk of several diseases, including heart disease, cancer, and diabetes (Giampieri et al., 2020).

A huge variety of bioactive compounds with potential bioactivities of interest can be extracted from plant tissue. The following are some examples of the most important biomolecules found in fruits and vegetables.

a) Alkaloids

Alkaloids are nitrogenous bases (usually heterocyclic) found primarily in plants. They are particularly common in certain families of flowering plants such as *leguminosae*, *papaveraceae*, *menispermaceae*, and *loganiaceae* families (Debnath et al., 2018).

Alkaloids are essentially formed from the metabolic processes of plants and normally it is endowed with distinguished physiological and toxicological properties (Kurek 2019; Silva et al., 2020).

The alkaloids effects of alkaloids on cells are multiple; they can be pretty close to neurotransmitters, such as serotonin or dopamine, which will allow them to interact on a large number of receptors and, therefore, to present several activities (Hanapi et al., 2021). Among the effects of alkaloids in the body, them known are their effects on brain activity and on nervous system, which can lead to the use of these molecules as euphoriants, stimulants, hallucinogens, psychedelics, hypnotics, paralytics, analgesics, anesthetics, analgesics, and emetics (Silva et al., 2020). Besides that, these secondary metabolites can affect the cardiovascular system, by helping regulate blood pressure (hypo or hypertension), antiarrhythmics, or arrhythmia (Silva et al., 2020).

Due to their biological proprieties, alkaloids have been used as medicines; for example, morphine, a natural derivative of the opium poppy (*Papaver somniferum*), a powerful painkiller (opiates). It is only used in case of pain when other analgesic treatments are no longer sufficient to soothe the patient. It has even undesirable side effects: sleepiness, vomiting, and nausea. Also, depending on the used dose, it can cause psychological disorders (Heinrich et al., 2021); codeine, another molecule derived from opium, is used for antitussive, analgesic, and narcotic properties (Heinrich et al., 2021); quinine, a natural analgesic and antipyretic alkaloid used to treat malaria (Heinrich et al., 2021); and caffeine, a natural stimulant found mainly in coffee and tea, is used in energy drinks and in the formulation of other medicines (Heinrich et al., 2021).

b) Terpenoids

Terpenoids are natural products present mainly in the essential oils of plants (Masyita et al., 2022). These bioactive molecules have biological activities, including anticancer, antimicrobial, anti-inflammatory, antioxidant, and anti-allergic activities (Masyita et al., 2022). Due to their antimicrobial capabilities, essential oils, rich in terpenoids, have been used against food-borne bacteria. Their use is beneficial in foods as flavoring additives, and they can also be an alternative to standard bactericides and fungicides currently found in the food industry (Perricone et al., 2015)

Terpenoids can be divided according to their number of carbon units into monoterpenes, sesquiterpenes, diterpenes, and triterpenes. Some examples of terpenoids are carvacrol, citronellal, geraniol, linalool, linalyl acetate, piperitone, menthol, and thymol (Wang et al., 2019).

Some terpenoids have been used to treat diseases, such as the taxol (Paclitaxel), extracted from *Taxus brevifolia*, that have been used as an anticancer agent (Sung et al., 2021), and the menthol, obtained from *Mentha* species, used in medicinal and cosmetic products for its cooling properties (Hudz et al., 2023).

c) Peptides and proteins

Proteins are organic molecules composed of amino acids that exist in all living organisms. Peptides are specific protein fragments that benefit human health (Zhang et al., 2023). Peptides and proteins are essential biomolecules in nature. They have vital biological roles in human body such as structural components, enzymes, hormones, and antibodies, catalyzing metabolic reactions, transporting and storing molecules like oxygen, and transmitting nerve impulses. Peptides extracted from natural sources can have interesting bioactivities for these compounds (Dang and Süssmuth, 2017). An example of the application of protein as a pharmaceutical is captopril, derived from a peptide in the venom of the Brazilian pit viper used as an antihypertensive drug (Dang and Süssmuth, 2017).

d) Polysaccharides

Polysaccharides are also known as complex sugars. Depending on the origins, these natural polysaccharides are classified as plant polysaccharides, animal polysaccharides, algal polysaccharides, and microbial polysaccharides (Mozammil Hasnain et al., 2019). The main structural polysaccharides are plant wall cellulose β1 4-homoglucan, the chitin constituting the cuticle of β1 arthropods 4-chitosan, hemicellulose, and pectin (complex heteropolysaccharides) (Gilbert, 2010). Polysaccharides extracted from plants are commonly used for a number of industrial purposes because of their biodegradability, sustainability, lower processing charge, and higher abundance in nature (Mozammil Hasnain et al., 2019). Due to their physicochemical properties, polysaccharides are widely used in the food, pharmaceutical, and cosmetic industries.

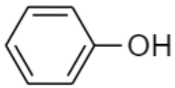
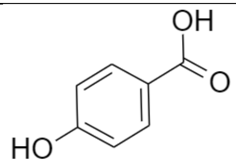
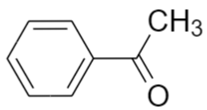
The polysaccharides have various biological properties; they exhibited stronger antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* (Hammami et al., 2018). In addition, extracellular sulfated polysaccharides extracted from eukaryotic algae, such as green algae, red algae, and brown algae, demonstrated antibacterial activity (Dewi et al., 2018). In the long term, antibiotics could be replaced by antibacterial polysaccharides, which would avoid the problems associated with the development of antibiotic resistance but this requires further studies to explore the relationship between the characteristics of plant polysaccharides and their antibacterial activities (Zhou et al., 2022).

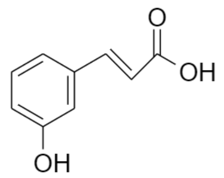
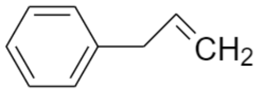
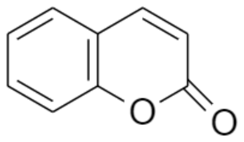
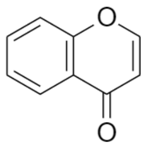
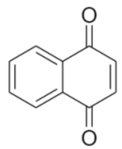
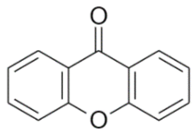
e) Phenolic compounds

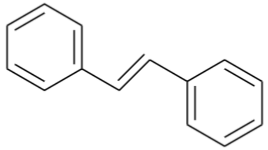
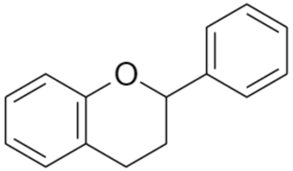
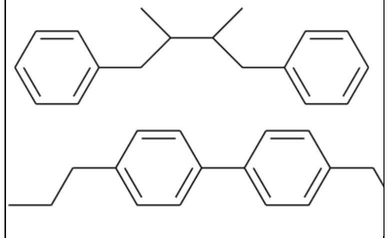
Phenolic compounds represent a significant class of bioactive compounds, exhibiting a variety of biological functions. These are primarily plant secondary metabolites associated with sensory and nutritional quality of plant-based foods. These compounds exhibit significant antioxidant and antimicrobial properties, which may be investigated across various industrial sectors (Chirinos et al., 2009). Research indicates that fruits and vegetables are a significant sources of phenolic compounds; however, certain studies have identified their residues, including rind, peel, and seeds, as having the highest concentrations (Sagar et al., 2018).

Phenolic compounds possess an aromatic ring as their fundamental structure, featuring one or more hydroxyl groups. They encompass about 8,000 distinct compounds, which can be categorized into 10 classes depending on their chemical structure (Garcia-Salas et al., 2010). **Table 1** summarizes the main phenolic compound classes found in natural matrices.

Table 1. Classes, structures, and sources of some phenolic compounds (adapted from Garcia-Salas et al., (2010))

Class	Main activities	Basic structure	Main Sources	Applications	References
Phenols	Antioxidant, anticancer, and antimicrobial		Tomato, olives	Food preservatives, skin care products, drug development and biopesticides	(Kumar and Goel, 2019).
Benzoquinones	Antioxidant, anticancer, and antimicrobial		Plant *Juglans nigra* and *sponges*	Antibiotics, anti-cancer agents, polymers and resins	(Abraham et al., 2011)
Hydroxybenzoic Acid	Anti-inflammatory, cardioprotective, and anti-cancer		Cranberry, cereals	Flavoring agents, and anti-inflammatory supplements	(Ud Din et al., 2023).
Acetophenones	Antioxidant, anti-inflammatory, and antimicrobials		Apple, apricot, banana	Treating infections, skin health, and anti-aging formulations	(Emami and Ghanbarimasir, 2015)
Phenylacetic acid	Antimicrobial and anti-inflammatory		Essential oil of jasmine and some fungi	Fragrance and flavor industry, drug synthesis	(Cook, 2019)

Hydroxycinnamic acid	Antimicrobial, antioxidant, and anti-cancer		Carrot, citrus, tomato, spinach, peaches, cereal, pears, eggplant	Food, nutraceutical, and pharmaceutical industries	(Sova and Saso, 2020).
Phenylpropene	Antimicrobial, antioxidant, and anti-cancer		Spinach, peaches, cereal, pears, eggplant	Food, nutraceutical, and pharmaceutical industries	(Sova and Saso, 2020)
Coumarins	Anticoagulant activity, antioxidant, and anti-inflammatory		Carrot, citrus, parsley	Pharmaceuticalapplications	(Sharifi-Rad et al., 2021)
Chromones	Anti-cancer, anti-inflammatory, and antioxidant		Peaches, cereal, pears, eggplant	Pharmaceutical industries	(Gaspar et al., 2024)
Naphthoquinones	Cytotoxicity and anticancer		Nuts	Pharmaceutical and polypharmacology like chemotherapy	(Durán et al., 2023)
Xanthones	Antioxidant and anti-inflammatory		Mango, Mangosteen	Pharmaceuticalapplications	(Quintana et al., 2021)

Stilbenes	Antiviral, neuroprotective, and antioxidant		Grapes	Anti-aging products	(Duta-Bratu et al., 2023).
Flavonoids	Antioxidant, antidiabetic, and antiviral		Widely distributed	Therapeutic applications and in food industry	(Roy et al., 2022).
Lignans, neolignans	Neuroprotective		Sesame, rye, wheat, flax	Pharmaceutical applications	(Zálešák et al., 2019)
Hydrosoluble tannins	Antioxidant and antimicrobial	Heterogeneous polymer composed of phenolic acids and simple sugars	Pomegranate, raspberry, berries	Food preservatives, food supplements	(Vera et al., 2023)

Phenolic chemicals produced by plants are well known for their antioxidant capabilities. These substances play a critical role in shielding plants against infections, UV rays, and herbivores, among other challenges. Phenolic compounds have gained interest in the field of human health because of their capacity to counteract free radicals and lower oxidative stress, an important element in the progression of numerous diseases, including cardiovascular disease and inflammatory disorders (Lebedev et al., 2022).

Flavonoids are found in plant tissues, and they contribute to the color of berries, flowers, and leaves. This family of biomolecules includes flavanols, flavones, flavanones, isoflavones, and anthocyanins (Pascoalino et al., 2021).

Plants with red, blue, and purple colors are attributed to pigments called anthocyanins, which are subgroups of flavonoids. They have strong antioxidant properties and improve human health by lowering oxidative stress and inflammation. Berries are well recognized for having a high phenolic content, especially in flavonoids such as ellagitannins and anthocyanins. For instance, genotypes of raspberries vary in terms of their antioxidant activity and phenolic content depending on the fruit's developmental stage, color, and genotype (Lebedev et al., 2022). Red raspberries (*Rubus idaeus L.*), for instance, are noted for having high concentrations of phenolic acids, flavonoids, and anthocyanins. Interestingly, the phenolic content of raspberry leaves is noticeably higher than that of the fruits. These phenolic substances facilitate their antioxidant ability, which helps to lower oxidative stress and prevent chronic illnesses (Lebedev et al., 2022).

In conclusion, phenolic compounds in berries enhance their color, flavor, and astringency while providing substantial health advantages, especially due to their antioxidant properties. These chemicals are progressively acknowledged for their potential in preventing chronic diseases and enhancing general health (Djordjević, 2023).

Table 1 proves that some natural compounds, such as lignans and stilbenes, can have a neuroprotective effect (Zálešák et al., 2019; Duta-Bratu et al., 2023). Stilbenes, such as antigen in and cubebin, have been investigated to minimize the Alzheimer's disease, while sesamin can have beneficial effects in the treatment of Parkinson’s disease (Zálešák et al., 2019)

1.1.2 Wild berries as sources of natural products

Many of the health-promoting qualities of berries, including blueberries, raspberries, blackberries, and strawberries, are attributed to their abundance of bioactive molecules, particularly phenolics. The primary bioactivities of wild berries are antioxidant, anti-inflammatory, and anti-proliferative activities. Berries possess strong antioxidant properties that scavenge free radicals and reduce oxidative stress, partly due to their high phenolic content. This is particularly beneficial in preventing chronic diseases like cancer and heart disease (Dróżdż et al., 2017). Some studies have shown that berries' phenolic compounds can reduce inflammation, which may be helpful in the management of inflammatory diseases. Research has linked the antiproliferative qualities of phenolic acids and flavonoids found in berries to inhibiting the growth of cancer cells; these compounds exhibit potential anti-cancer activities by modulating several cellular pathways that regulate cancer progression. These substances, such as resveratrol, gallic acid, and quercetin, have demonstrated efficacy in diminishing cancer cell growth, triggering apoptosis, and decreasing the spreading of cancer cells (Liu et al., 2023).

Berries are a great source of phenolic compounds. Among the noteworthy examples, some wild berries can be highlighted, such as blueberries, which are sources of flavonoids, such as myricetin and quercetin, as well as phenolic acids, like chlorogenic acid. Their potent antioxidant action is attributed to their high total flavonoid content (TFC) and total phenolic content (TPC) (Subbiah et al., 2020); strawberries are rich in pelargonidin and anthocyanin that gives their vivid red color. They also contain large concentrations of phenolic acids, such as ellagic acid, and tannins; blackberries are rich in anthocyanins, especially derivatives of cyanidin, which give them their rich purple color. In addition to having high tannin content, blackberries also contain additional phenolics that strengthen their antioxidant ability, and raspberries can be a source of anthocyanins such as cyanidin and ellagitannins are abundant in raspberries and contribute to their antioxidant qualities (Subbiah et al., 2020).

The high phenolic content of berries is well-known, especially for flavonoids, such as ellagitannins and anthocyanins. Bilberries are rich in anthocyanins, strong antioxidants, whereas black currants are abundant in quercetin and other flavonoids with anti-inflammatory qualities. Lingonberries are abundant in procyanidins, which exhibit potential for cancer prevention. These substances function as antioxidants, aiding in the neutralization of detrimental free radicals and potentially diminishing cancer chances (Tian et al., 2017).

1.1.3 Myrtus communis *L. fruit as a potential natural product source*

M. communis is the only *Myrtacea* species indigenous throughout the Mediterranean region, widely distributed in the coast European, African, and Asian continents. *M. communis* is a shrub one to two meters in height, with a rich history of use in traditional medicine, particularly in Mediterranean cultures (Tuberoso et al., 2010). Its berry is known for its antioxidant, antifungal, and antibacterial activities. Moreover its leaves and flowers are used to obtain essential oils (Özkan and Güray, 2009).

Its flowering begins from May - June and can go until August under the shape of solitary white flowers large 10-15 mm, very fragrant and particular is one of its character traits in the leaf axils (Barboni, 2010; Bouzabata, 2017). It is an edible berry, popularly known as myrtle, and shows a bluish-black color when mature. The full maturity of these fruits is ripe in November and still until February (Medda and Mulas, 2021).

In traditional medicine, this plant has been used as an infusion to treat a wide range of diseases like diarrhea, peptic ulcers, hemorrhoids, inflammation, uterine bleeding, headache, palpitation, leucorrhea, urethritis, epistaxis (nosebleeds), conjunctivitis, excessive perspiration, and pulmonary and skin diseases. The leaves are used for their antiseptic properties and have been employed in treating wounds and disorders of the digestive and urinary systems (Hennia et al., 2018).

The scientific exploration of its chemical composition and antioxidant properties further supports its traditional uses and opens the door for potential modern therapeutic applications. *M. communis* extracts, mainly from leaves and berries, possess significant antioxidant properties. This plant is rich in flavonoids and their derivatives (Hennia et al., 2018). These antioxidants mitigate oxidative stress and safeguard the organism from free radical damage. In addition, leaf extracts have shown anti-inflammatory activity. This effect is attributed to flavonoids, hydrolysable tannins, and nonprenylated acylphloroglucinols such as myrtucommulone (Hennia et al., 2018). *M. communis* has also been used to treat various infections. For instance, its leaves and berries have shown activity against some bacteria, including *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Hennia et al., 2018).

1.2 Extraction methods to obtain natural compounds

An extraction method is a procedure employed to isolate specific natural substances from raw materials, including plants, fungi, or microbes, typically utilizing solvents or mechanical processes. These techniques extract bioactive components such as essential oils, phenolics, and alkaloids for diverse applications in food, medicine, and cosmetics. Some of the common method employed the decoction, hydrodistillation, ultra-sound assisted extraction and micro-wave assisted extraction.

1.2.1 Conventional methods

In order to extract the desired bioactive chemicals from plant materials, such as the phenolic compounds found in plant tissue, conventional extraction procedures employ heat, solvents, and mechanical processes. In the following, some traditional extraction techniques will be described.

Infusion extraction is an accessible method for extracting bioactive compounds from natural products, especially for water-soluble compounds. In this extraction method, the plant powder material is immersed in boiling solvent, particularly water, and left to stand in a covered container for a short time until the bioactive compounds are solubilized in the solvent (Bitwell et al., 2023;Verep et al., 2023). This method is commonly used for preparing teas, herbal infusions, and medicinal extracts. It is characterized as easy to performance low-cost since it does not need complex equipment and materials. On the other hand, it is a method not effective for non-polar compounds, it is not even suitable for compounds that degrade at high temperatures, and it may not extract all bioactive compounds, leading to lower yields compared to other methods (Verep et al., 2023).

Decoction extraction is employed to isolate water-soluble chemicals, including saponins, tannins, and flavonoids, from resilient plant components such as roots, bark, and seeds (Kumar et al., 2023). It relies on prolonged boiling to break down the plant material and release its bioactive compounds into the water (Kumar et al., 2023). The antioxidant activities obtained by decoction and infusion methods are high because they can be ascribed to many parameters concerning the effective extraction of antioxidant components from plant materials by these methods, specifically water-soluble phenolics, flavonoids, and other bioactive chemicals. This explains why using these techniques can result in significant levels of antioxidant activity (Verep et al., 2023). To perform this extraction method, specialized equipment is not required. However, this method is unsuitable for heat-sensitive compounds, which may degrade during boiling. As another negative point, the process can take a long time, and then some volatile compounds may be lost due to prolonged boiling (Verep et al., 2023).

Soxhlet extraction is a commonly used technique for extracting bioactive compounds from natural sources. It is classified as a continuous solvent extraction method and is recognized for its ability to extract bioactive compounds (Yu et al., 2023). In this extraction method, the solvent is recycled through extraction in a closed-loop system, where the liquid solvent successively washes the solid sample, slowly extracting the compounds of interest. To perform this extraction, the solvent is heated to its boiling point. The resulting vapor is then condensed in a cooling tower, typically cooled with water, causing it to return to its liquid state. The liquid solvent then passes through the sample again, carrying the extracted compounds, and returns to the heating vessel for repeated cycles.

As an advantage, this method can lead to efficient extraction of the desired compounds, effectively obtaining high bioactive compound yields (Yu et al., 2023). However, this method is not considered an eco-friendly system and has several disadvantages. The process requires a large volume of solvents, consumes a significant amount of water, involves long extraction times, and the heating needed for solvent evaporation can lead to the degradation of bioactive compounds (Yu et al., 2023).

Another solid-liquid conventional usually used to obtain bioactive molecules is maceration, or heat-assisted extraction HAE, when temperature is applied to improve the extraction. This method is widely used in phytochemistry and herbal medicine to extract bioactive compounds from plant materials. It involves soaking the material in a solvent to dissolve the desired compounds. Heating and stirring can help the extraction and improve its efficiency (Bitwell et al., 2023).

This conventional method can offer several advantages, for example, its simplicity allow its operation without specialization; it does not require a complex and expensive apparatus, it is a method that is easily scale-up; the temperature control and the possibility to operate at room temperature reduces the risk of the degradation of target compounds, and different type of solvent can be used to extract different compounds. These advantages make it is a suitable technique for a wide range of plant materials (Bitwell et al., 2023). However, between the disadvantages of the method, the long-time extraction required, low selectivity, high amount of solvent applied, and low extraction efficiency can be highlighted (Bitwell et al., 2023).

1.2.2 Emergent technologies

Emerging extraction technologies are cutting-edge, innovative techniques created to enhance the sustainability, efficiency, and selectivity of extracting bioactive substances, such as essential oils, flavonoids, and phenolic compounds, from plant materials. These emergent technologies seek to overcome the drawbacks of traditional extraction procedures by using less solvent, completing extractions faster, and maintaining the bioactivity of molecules that are sensitive.

Microwave-assisted extraction is a process that uses microwave energy to heat the sample, which accelerates the breakdown of complex structures within plant material, such as the pectin, cellulose, and hemicellulose, helping the transfer of analytes to solvent (Cui, 2021). It is a powerful and efficient method for extracting a wide range of bioactive compounds, such as phenolic acids, flavonoids, and tannins. Its advantages make it an attractive alternative to conventional extraction techniques in various industries since it can reduce the time of extraction, be more selective, reduce the amount of solvent used, and improve the extraction yield. On the other hand, the equipment used in this method can be very expensive and extraction process can require a high amount of energy (Cui, 2021).

Ultrasound-assisted extraction is a non-thermal process that applies acoustic energy to increase the release and diffusion rates of target materials via solvent cavitation (Misra et al., 2018). This method has gained popularity in various fields, including food, pharmaceutical, and environmental sciences, due to its efficiency, speed, selection, and ability to extract bioactive compounds while preserving their integrity (Cui, 2021). However, the application of high frequency and power can lead to the degradation of some sensitive compounds.

Pressurized-liquid extraction is a solid-liquid extraction method that uses temperatures (50–200°C) and pressures (35 – 200 bars). In this process, the solvents are close to the supercritical region, where higher temperatures produce high rates of solute solubility and diffusion. High pressures increase the force and speed with which liquids penetrate solid matrices, providing high extraction yields and improved reproducibility compared to traditional methods (Costa et al., 2020). PLE is very versatile, applying to a wide range of samples, such as soils, plant materials, foods, pharmaceuticals, and polymers, and can be used with different solvents, allowing the extraction polar and non-polar compounds. Besides that, the specificity of the operating process allows the adjustment of the appropriate conditions for obtaining purer extracts (Verep et al., 2023). In addition, this technology requires a shorter extraction time and a lower amount of solvent than conventional methods (Costa et al., 2020). Nonetheless, some limitations can be obstacles to implementing this method for large-scale extraction, for example, the high costs for instrumentation and energy requirements make the implementation on an industrial scale (Višnjevec et al., 2024).

Supercritical fluid extraction is also an attractive green alternative since non-toxic and non-flammable solvents are used in this process, being that carbon dioxide (CO_2) is the most common solvent used due to its low critical point (temperature at 31ºC and pressure at 73.8 bar). In addition, CO_2 can be easily vaporized from the final product. Supercritical fluid extraction uses a specific solvent at critical pressure and temperature points. Under these conditions, supercritical fluids can show liquid and gas characteristics. Diffusivity and selectivity of the liquids can be controlled through changes in thermodynamic conditions (Gallego et al., 2019). This method is also considered an eco-friendly process, realized as continuous cycles of extraction with minimal emission of extractors and without carbon dioxide CO_2 to the environment (Felix et al., 2019). Despite the high separation efficiency and quality of the end-product, the membrane separation used in this process has a high cost. In addition, it requires expensive investment in robust equipment and safety in the installation and operation, which has hindered its use on a large scale (Felix et al., 2019).

Enzyme-assisted extraction (EAE) is a biological technique that employs specialized enzymes in conjunction with water as a solvent. Enzymes like pectinase, β-glucanase, β-glucosidase, cellulase, and xylanase depolymerize polysaccharides in the cell wall, liberating the associated chemicals (Jeevan Kumar et al., 2017). This approach is primarily employed when phytochemicals are distributed within the cell cytoplasm. It is well used in the food industry to extract flavors, essential oils, antioxidants, and vitamins. This technique can give a higher yield of bioactive compounds compared to conventional methods. On the opposite side, the enzymes are costly (Jeevan Kumar et al., 2017).

1.2.3 Extraction process optimization

Optimization of the extraction process is crucial to maximize the yield and purity of bioactive compounds from natural products. This involves fine-tuning various parameters to achieve the most efficient extraction while minimizing costs, time, and environmental effects. The key factors to consider in optimizing the extraction process include the choice of solvent, temperature, extraction time, solvent-to-material ratio, and particle size of the material. The primary factors optimized are solvent type, substrate concentration, temperature, particle size, and extraction duration. Each variable affects the process's efficiency: solvent type influences solubility and yield, substrate concentration alters diffusion rates, temperature affects the stability and solubility of compounds, and particle size dictates the surface area available for extraction. Moreover, time guarantees the completeness of extraction without deterioration (Sridhar et al., 2022). The techniques typically employed for optimization in extraction procedures encompass Design of Experiment (DoE) methodologies such as Box-Behnken Design (BBD), Central Composite Design (CCD), and Factorial Design (FD). These tools facilitate the simultaneous optimization of various variables by assessing response surface methodology (RSM), contour plots, and desirability indices to identify the optimal compromise among multiple objectives (Boateng, 2023).

1.3 Application of natural products in industry

Natural products have wide-ranging applications in several industries because of their eco-friendliness and bioactive qualities. They provide compounds with antibacterial, anti-inflammatory, and anticancer characteristics, which the pharmaceutical industry uses as a basis for drug development, whilst in the food industry they can be used as natural additives and/or functional ingredients to help prevent some diseases and promote health. In the cosmetic industry, natural extracts are utilized to formulate skincare, hair care, and beauty products because of their nourishing and therapeutic properties. Furthermore, natural products such as bio-pesticides and fertilizers encourage environmentally responsible farming methods by providing sustainable substitutes for artificial chemicals in agriculture (Sorrenti et al., 2023).

1.3.1 Food industry

The food industry is most likely choosing to use non-artificial molecules in the aliment to ensure a healthier and non-harmful product in the long term. The recent advancements in the use of natural products in food applications include the diverse roles that these kinds of compounds can play, from enhancing the shelf-life and sensory qualities of food products to contributing to health benefits for consumers. For this reason, bioactive molecules extracted from natural products are gaining attention and space in the food industry (Lv et al., 2021).

These natural products can exert bioactive proprieties, such as antioxidant and antimicrobial activities, acting, therefore, as food preservatives (Mäkinen et al., 2020). For example, a study on antioxidant sources demonstrated that bilberry and sea buckthorn leaves, extracted using subcritical water, significantly inhibited lipid oxidation in meat products, suggesting a sustainable method for enhancing meat preservation (Mäkinen et al., 2020). Also, essential oils can be used as preservatives in food, as shown in the research on *Satureja macrantha* essential oils, which highlighted the highest antibacterial and antioxidant activities during the flowering stage, making it a potent natural preservative (Aghbash et al., 2020). In another study, red cabbage extracts obtained with chloroform solvent exhibited strong antimicrobial and antioxidant activities, extending the shelf-life of refrigerated beef, showing its potential to be used as a natural preservative (Rubab et al., 2020).

Natural products have also been used for the development of nutraceuticals. Nutraceuticals are food products that provide nutritive food or medical benefits, including the prevention and treatment of disease. The vitamin E supplement can be a good example of a nutraceutical; it is essential for maintaining cellular health due to its antioxidant properties (Nemati et al., 2020). While it shows potential in preventing or slowing the progression of certain diseases, such as cardiovascular diseases, cancer, cataracts, and Alzheimer's, the evidence is mixed, and the benefits may depend on the form of vitamin E and individual health conditions. A study demonstrated that vitamin E supplementation improved goose meat quality and shelf-life, offering a promising strategy for enhancing poultry products (Nemati et al., 2020).

Functional foods are similar to nutraceuticals; however, they are specifically designed to offer additional health benefits beyond essential nutrition (Damián et al., 2022). An example of the production of functional foods is the incorporation of probiotics into food to improve gut health (Damián et al., 2022). Also, omega-3 and other fatty acids are added to food to promote heart health (Damián et al., 2022). The flavonoids found in berries may be enhanced in food to give flavor, provide antioxidant benefits, and also be added as a tannin as a colorant. The numerous health advantages and bioactive characteristics of polyphenols make them highly valued ingredients in functional foods. These substances have potent antioxidant action that helps fight oxidative stress and lowers the risk of chronic illnesses like diabetes and heart diseases. Polyphenol-rich foods, such as berries (e.g., strawberries, blueberries) have been linked to better cardiovascular and cognitive health when included in diets. Furthermore, studies on the anti-obesity and improved metabolic health benefits of green tea, which is high in catechins, have been reported. Moreover, because of their natural preservative qualities that enhance food safety and prolong shelf life, polyphenols are used as natural preservatives in functional foods (Güneş Bayir et al., 2019)

1.3.1 Pharmaceutical industry

Bioactive compounds from natural products significantly contribute to the pharmaceutical industry, providing a basis for many existing therapies and offering potential for new drug discoveries. These compounds, often extracted from plants, marine organisms, fungi, and microorganisms, have diverse biological activities that make them valuable for developing new drugs and therapies. Natural products have been successfully used in developing drugs across various therapeutic areas. These include cancer treatment, infectious diseases, cardiovascular diseases, and neurological conditions fingolimod for multiple sclerosis (Zotchev et al., 2021). Many natural products have evolved to interact with biological targets, making them inherently bioactive. This bioactivity is particularly useful in discovering drugs that target specific proteins or pathways. Natural products are increasingly being integrated with modern drug discovery technologies, including high-throughput screening, omics technologies, and machine learning, to discover and optimize new drug candidates (Zotchev et al., 2021). Examples include quinine, which was collected from the bark of *Cinchona officinalis* and used as a potent painkiller; morphine, which was extracted from *Papaver somniferum* (opium poppy); and paclitaxel, which was taken from *Taxus brevifolia* (Pacific yew) and used to treat diseases such as ovarian and breast cancer. The plant *Artemisia annua* is the source of artemisinin, which is essential for fighting drug-resistant malaria. These natural products demonstrate how the pharmaceutical sector depends on plant-based substances, which provide distinctive chemical variety and provide inspiration for novel therapeutic treatments (Chaachouay and Zidane, 2024).

1.3.3 Cosmetic industry

Natural compounds offer many benefits for the cosmetic industry, enhancing the effectiveness and appeal of skincare, hair care, and personal care products. Several polyphenols can be used in the formulation of cosmetic products. For example, oleuropein extracted from olive leaves exhibits antioxidant properties, reducing skin erythema and water loss. It inhibits reactive nitrogen species and modulates inflammatory pathways (Hsu et al., 2022). Resveratrol, found in grapes and other plants, offers protection from photoaging by reducing UVB-induced skin damage and improving skin firmness and elasticity. It also has estrogen-like effects and regulates tyrosinase activity (Liu, 2022). Niacinamide, also known as vitamin B3, which extracted from a yeast , green vegetables and grains improves skin texture and reduces hyperpigmentation by inhibiting melanosome transfer (Madaan et al., 2021). Retinoids, or vitamin A, derived from plants like carrots and sweet potatoes, can promote cell turnover, reduce wrinkles, and improve skin texture (Sadgrove et al., 2021).

2. Purpose and objectives

The purpose of this work was to explore the potential of natural resources as promising sources of bioactive compounds. Recognizing the growing interest in natural ingredients with diverse bioactivities applicable across various fields, this research seeks to investigate the bioactive properties of *M. communis* berries. Through a comprehensive analysis, this study aims to determine whether these berries can serve as valuable sources of bioactive molecules, contributing to advancements in areas such as health, cosmetics, and nutrition. To achieve this objective, the secondary aims of this study were as follows:

- Identify the extraction method: determine the most effective method for extracting antioxidant compounds from *M. communis* berries.
- Optimize the extraction process: enhance the efficient extraction method to obtain an extract with higher amount of total phenolics and greater antioxidant activity.
- Characterize the phenolic profile: analyze the phenolic profile of the optimized extract.

- Investigate the extract' bioactivities: evaluate the antioxidant activity with TBARS and determine the antimicrobial potential of the optimized extract.

Overall, this research aims to discover new natural products with bioactive properties that can contribute to the development of new therapeutic agents and functional foods. It also promotes biodiversity conservation and sustainable resource use, providing economic and health benefits while reducing dependence on synthetic chemicals.

3. Material and methods

3.1 Plant material

The sample used in the present study was collected in Nabeul, Tunisia (**Figure 1**), at the end of March 2024.

Figure 1. The specific region in Tunisia where berries of *M. communis* were harvested (Green-shaded area circled).

After collecting the *M. communis* fruit, it was washed with distilled water and then traditionally dried under the sun for 2 days, in sequence. To complete the drying, the fruits were put in an oven at 50°C until obtained a constant weight. After that, it is crushed with a grinder and turned into a fine powder (**Figure 2**).

Figure 2. Powdered *M. communis* berries.

3.2 Extraction method

3.2.1 Heat-assisted extraction (HAE)

Maceration extractions were performed using a carousel system (**Figure 3**, CarouselTM 6 plus, Radleys Discovery Technologies, United Kingdom). To evaluate the efficiency of the extraction of the method, three different extraction conditions were tested:

- 1st condition: extraction time of 120 min at room temperature (~20°C).
- 2nd condition: extraction time of 120 min at 50°C.
- 3rdcondition: extraction time of 60 min at 90°C.

The solid/liquid ratio of extraction was kept constant (20 g/L), as well as the ethanol concentration in the solvent (50:50, ethanol:water, *v/v*) and stirring (500 rpm). After the extraction, the ethanol was evaporated at 40°C in a rotary evaporator, and the aqueous phase was frozen at -20°C and, subsequently, freezer-dried until complete dryness.

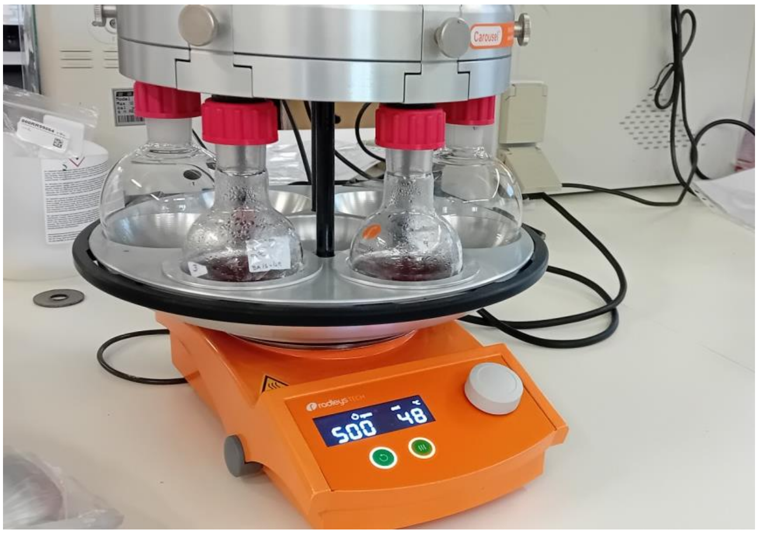


Figure 3. A carousel system used to perform the HAE extraction.

3.2.2 Microwave-assisted extraction (MAE)

The microwave-assisted extraction (MAE) was performed using a microwave extractor (Nµ Tech, NuWav-Uno, Sonilex, West Bengal, India) equipped with a circulating cool-water reflux system, a manual electromagnetic stirrer, and a time controller.

The three different conditions tested were:

- 1st condition: extraction time is 15 min at 70°C and power = 100W.
- 2nd condition: extraction time is 10 min at 50°C and power = 400W.
- 3rd condition: extraction time is 5 min at 50°C and power = 800W.

The solid/liquid ratio of extraction was kept constant (20 g/L), as well as the ethanol concentration in the solvent (50:50, ethanol: water, *v/v*). After the extraction, the ethanol was evaporated at 40°C in a rotary evaporator, and the aqueous phase was frozen at -20°C and, subsequently, freezer-dried until complete dryness.

3.2 Yield determination

The extraction yields (Y_1: %, w/w) were determined gravimetrically and calculated as the ratio of the extract weight to the dry peel weight, according to the Equation (***1***):

$$Yield\ (Y_1, \%) \quad = \frac{Extract\ weight\ (g)}{Dry\ berries\ weight\ (g)} \times 100 \qquad \textbf{(1)}$$

3.3 Total phenolic determination

The total phenol content was measured by spectrophotometric quantification with a modified Folin-Ciocalteau method (Hazrati et al., 2022).In test tubes, the extract (0.5mL) was added to 2.5 mL of Folin-Ciocalteau solution with a concentration of 0.1% and 2 mL sodium carbonate (75g/L); subsequently, the tubes were incubated for 20 min, at 40°C. After that, the extracts were transferred, in triplicate, to 96-well microplates, and the absorbance was read at 765nm. A calibration curve was built using gallic acid at different concentrations (0.6-0.001875 mg/mL). The results were expressed as mg of gallic acid equivalent (GAE)/g of extract (E).

3.3 Evaluation of antioxidant properties by DPPH scavenging activity

For the spectrophotometric analysis using DPPH, the extract was dissolved in ethanol (1:5, *w/v*). A standard solution of DPPH was prepared at a concentration of 6×10^{-5}M. The extract was then diluted in a 96-well plate through eight successive dilutions using 50% ethanol as solvent. The final volume of the extract in each well was 30 µL. Subsequently, 270 µL of the DPPH solution was added to each well. A blank was also prepared in the same condition, composed of 30 µL of 50% ethanol without extract. The plate was incubated at room temperature and protected from the light, for 60 min. After, the absorbance was measured at 515nm (Meng et al., 2016). All determinations were performed in triplicates.

3.4 Optimization of the extraction method

To determine the extraction condition that is able to provide an optimal extract in terms of total phenol content and antioxidant activity, an optimization of the best extraction method was performed. For this, a central composite design (CCD) was applied, and the effect of three extraction variables, namely time (*t*), temperature (*T*), and ethanol concentration (*S*), was assessed. The independent variables were tested in five levels in the following ranges: *t*: 5 min – 90 min, *T*: 20ºC – 100ºC, and *S*: 0% - 100% ethanol. The response surface methodology (RSM) was used to determine the optimal points. The Design-Expert software, Version 11 (Stat-Ease, Inc., Minneapolis, MN, USA) was used to perfume the ANOVA and to build the 3D response surfaces. As dependent (or response) variables, the extraction yield

(Y_1, %, *w/w*), total phenol (Y_2, TAC mg/g extract (E)), and antioxidant activity (Y_3, EC_{50} value for DPPH assay) were evaluated for process optimization.

3.5 Identification and quantification of main phenolic compounds present in the optimal extracts

High-performance liquid chromatography, diode array detection, and tandem mass spectrometry (HPLC-DAD-MS^2) were used to identify and quantify the main phenolic compounds present in the *M. communis* extracts. Then the extracts were filtered with a 0.2 µm filter and injected into the HPLC-DAD-MS^2. The run followed the conditions previously described (Ueda et al., 2023).

The identification and quantification of the phenolic compounds present in the *M. communis* extracts were performed based on a combination of the retention time (R_t), UV-Visible (UV-Vis) spectral characteristics, and mass spectral data, including the deprotonated molecule ($[M-H]^-$), MS^2 ion fragmentation patterns, and the relative abundance of fragment ions. These parameters were compared with reference commercial standards (myricetin-3-*O*-glucoside and quercetin-3-*O*-glucoside) and data from the literature. The results were expressed as mg per g of extract (mg/g E).

3.6 Bioactivities of the optimal extracts

3.6.1 Antioxidant activity

The antioxidant activity of the optimal extracts was determined by DPPH, as described in 3.3, as well as by the thiobarbituric acid reactive substances (TBARS) assay. TBARS was used to determine the potential of the extract to inhibit lipid oxidation (Ghani et al., 2017). To perform this assay, the extract (100 µL) was mixed in an Eppendorf with ascorbic acid (100 µL, 0.1 mM), iron sulfate (100 µL, 10 mM), and porcine brain solution (100 µL). This porcine brain solution was obtained with Tris-HCl buffer (20 mM, pH 7.4) in a ratio of 1:2. Subsequently, the samples were incubated at 37.5°C for one hour. After that, 500µL of trichloroacetic acid (28%, *w/v*) and 380µL of thiobarbituric acid (2%, *w/v*) were added. The reaction to form malondialdehyde (MDA) occurred at 80ºC for 20 min. The Eppendorf tubes were centrifugated at 3800 g for 3 min, and the supernatant was transferred to a 96-well microplate in triplicate. The absorbance was measured at 532 nm. A blank using the solvent extraction was prepared in the same condition. The results were expressed as EC_{50}(mg/mL) values, which mean the extraction concentration that can inhibit 50% of the lipid oxidation (Aguilar Diaz De Leon and Borges, 2020).

3.6.2 Antimicrobial activity

The antibacterial activity of the extract was tested against several bacterial strains, including eight food isolated bacteria (*Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (food isolate), *Listeria monocytogenes* (NCTC 7973), *Escherichia coli* (ATCC 35210), *Salmonella enterica* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), *Pseudomonas aeruginosa* (ATCC 9027), and *Yersinia enterolitica* (ATCC 8610)), and eight clinical isolated bacteria (*E. coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *P. aeruginosa*, *Enterococcus faecalis*, *L. monocytogenes*, and methicillin-resistant *S. aureus* (MRSA)). The antifungal activity was determined on two microfungi (*Aspergillus fumigatus* (ATCC 1022), *Aspergillus versicolor* (ATCC 11730), and *Aspergillus niger* (ATCC 6275)).

The assays were performed following a protocol described by (Corrêa et al., 2015). As a result, the minimum inhibitory concentrations (MIC, mg/mL), as well as the minimum bactericidal or fungicidal concentrations (MBC and MFC, respectively, mg/mL) were determined. Ampicillin, imipenem, and streptomycin were employed as positive controls in the antibacterial assay, while ketoconazole was used in the antifungal assay.

4. Results and Discussion

4.1 Selection of extraction method

HAE and MAE were tested as extraction methods for this optimization due to their ability to recover sensitive phenolic components with less degradation caused by excessive mechanical stress. These techniques entail immersing plant materials in a solvent applying a heating factor and stirring, facilitating bioactive chemical diffusion while preserving their structural integrity. The results found in the tested conditions are presented in **Table 2**.

Table 2. Values of total phenolics, yield, and DPPH of two extraction methods.

Extraction method	Condition	Yield	Total phenolics	DPPH
HAE	1	45±2	32±1	0.80±0.03
	2	43.9±0.5	41±2	0.7±0.1
	3	44.3±0.2	46±2	0.40±0.09
MAE	1	39±6	47±3	1.0±0.3
	2	38±1	33±1	0.60±0.09
	3	41±0.8	35±3	0.50±0.08

4.1.1 Extraction yield

When compared to HAE, the extraction yield from MAE is often lower. Even yet, MAE can extract substances more rapidly and effectively, particularly those that are weakly soluble at room temperature. In the extractions conducted under three distinct settings, it was observed that HAE preserved the integrity of heat-sensitive compounds and somewhat enhanced the yield of particular compounds, such as polyphenols, elucidating the variation in values. The HAE extraction technique yielded a greater quantity of the required component from a specified amount of raw material compared to MAE, with HAE achieving an extraction yield of 44.3%, surpassing MAE's yield of 39.33%.

4.1.2 Total phenolic content

According to **Table 2**, under two of three tested conditions, HAE produced a higher TCP than MAE. The TCP varied depending on the extraction procedure (Ueda et al., 2023).In this study, HAE often yielding the highest concentrations. Generally, heat-assisted extraction is soaking the plant material in a solvent for a long time at room temperature. However, when heat is applied, it can improve the extraction of desired compound; this allows the solvent to penetrate the plant matrix more thoroughly and improves the extraction (Shi et al., 2022). For total phenolics, the first condition of MAE showed a higher result, but for the rest of the condition the HAE was more efficient in the extraction. These results (**Table 2**) prove that phenolic compounds present in the composition of *M. communis* berry are sensitive to microwaves, and the intense, localized heating in MAE can degrade or alter these compounds, leading to lower total phenolics yields. Moreover, larger and more complex phenolic compounds that may be under extracted during the quicker MAE process, in contrast, they extraction can be benefited with the longer extraction time and temperature used in HAE, which can improved solvent penetration and extraction effectiveness (Shi et al., 2022).

4.1.3 Antioxidant activity

The antioxidants naturally present in fruit and vegetables have benefits in human health, through scavenging free radicals from damaging biological organs, tissues and cells. The antioxidant activity is significantly associated with the presence of phenolic compounds (Li et al., 2014). In the DPPH assay, the donation of hydrogen from DPPH forms the stable DPPH molecule, resulting in the scavenging of DPPH radical. Based on their different extraction processes, the DPPH assay results presented in **Table 2** can show differences in antioxidant activity between HAE and MAE extracts. For HAE, the lowest EC_{50}value was achieved by the extract obtained under condition 3, which means that this methodology can enhance the antioxidant activity. However, this does not exclude that under condition 2 and 3 of MAE, also was obtained low EC_{50} values. Nonetheless, fewer than two of three tested conditions, HAE was the most efficient extraction method for obtaining higher antioxidant extracts. Because maceration is a slower procedure, antioxidant integrity is maintained by the gradual and energy-free extraction of phenolic chemicals. On the other hand, MAE uses microwaves causing cavitation effects, which breaks down plant cell walls in order to speed up the extraction process. This can result in enhanced mass transfer, however it also has the potential to degrade sensitive antioxidants (López-Salazar et al., 2023). Due to phenolic compounds are better preserved during the milder extraction process, DPPH results from HAE may have higher antioxidant activity. In contrast, DPPH results from MAE's mechanical disruption may result in a faster release but possibly partial degradation of antioxidant molecules, reducing their DPPH radical scavenging activity.

4.2 Optimization of the extraction method

Considering the results presented in **Table 2**, HAE was selected as the method to optimize for obtaining a phenolic-rich extract from M. communis berries. To achieve this, several critical variables were optimized, including the influence of three factors: ethanol concentration, extraction power, extraction time, and solvent concentration. In order to model these variables and optimize the yield of total phenolic a response methodology was used with specific parameters like the table below (**Table 3**).

Table 3. Natural and coded values (between brackets) of the three independent variables were assessed, and real responses were obtained with the CCD and FD.

	Natural and coded values ()						**HAE**		
Run	*Time* (*A*, min)		*Temperature* (*B*,ºC)		*Ethanol* (*C*, %)		*Yield* (Y_1, %)	*Total phenolics* (Y_2, *mg GAE/g E)*	DPPH (Y_3, EC_{50} (mg/mL)
1	22	(-1)	36	(-1)	20	(-1)	41±3	43.1±0.4	0.23±0.01
2	73	(1)	36	(-1)	20	(-1)	42±2	42±3	0.19±0.01
3	22	(-1)	84	(1)	20	(-1)	49.9±0.4	47±2	0.14±0.01
4	73	(1)	84	(1)	20	(-1)	49±1	51±2	0.13±0.01
5	22	(-1)	36	(-1)	80	(1)	41±3	19±2	0.21±0.01
6	73	(1)	36	(-1)	80	(1)	39±2	19±1	0.19±0.01
7	22	(-1)	84	(1)	80	(1)	46.3±0.1	22±2	0.18±0.005
8	73	(1)	84	(1)	80	(1)	47±1	24±2	0.13±0.01
9	5	(-1.68)	60	(0)	50	(0)	40.8±0.8	47±3	0.11±0.01
10	90	(1.68)	60	(0)	50	(0)	47.0±0.1	52±2	0.13±0.01
11	48	(0)	20	(-1.68)	0	(0)	39±1	46±1	0.14±0.01
12	48	(0)	100	(1.68)	100	(0)	49.9±0.7	55±3	0.11±0.02
13	48	(0)	60	(0)	0	(-1.68)	52±3	34±2	0.46±0.03
14	48	(0)	60	(0)	100	(1.68)	37.7±0.1	20±3	0.51±0.01
15	48	(0)	60	(0)	50	(0)	46±1	50±3	0.17±0.004
16	48	(0)	60	(0)	50	(0)	45.5±0.8	48±5	0.165±0.002
17	48	(0)	60	(0)	50	(0)	43±0.1	59±3	0.106±0.002
18	48	(0)	60	(0)	50	(0)	44.2±0.4	51±3	0.09±0.01
19	48	(0)	60	(0)	50	(0)	46±1	50±3	0.14±0.01
20	5	(-1.68)	100	(-1.68)	0	(-1.68)	57±3	28±2	0.31±0.02
21	90	(1.68)	20	(1.68)	100	(1.68)	32.1±0.8	12±3	0.71±0.06
22	90	(1.68)	100	(1.68)	100	(1.68)	43±3	28.8±0.8	0.156±0.003
23	90	(1.68)	100	(-1.68)	0	(-1.68)	56±1	38±1	0.30±0.02
24	5	(-1.68)	20	(1.68)	100	(1.68)	20±3	17±2	1.15±0.06
25	5	(-1.68)	100	(1.68)	100	(1.68)	43±1	24±2	0.41±0.03
26	5	(-1.68)	20	(-1.68)	0	(-1.68)	52±4	20.4±0.9	0.55±0.02
27	90	(1.68)	20	(-1.68)	0	(-1.68)	49±2	28.6±0.9	0.48±0.01

4.2.1 Experimental results

The goal of this work was to maximize the recovery of bioactive compounds, more particularly the phenolic compounds from plant material by optimizing an extraction technique. The approach attempted to maximize time and solvent savings while optimizing extraction efficiency by varying critical parameters such temperature, solvent-to-solid ratio, extraction time, and solvent concentration. The response surface methodology (RSM) was used to determine the ideal conditions for obtaining the maximum phenolic yield extraction. These results offer important new perspectives on how to enhance and maximize the efficiency of extraction procedures.

In **Table 3** are presented the results obtained with the HAE under 27 runs applied to optimize extraction from *M. communis*. The experimental results showed that for the yield higher percentage was obtained in run 20 (57%) and run 23 (56%), at the highest temperature condition (100 ºC) and the lowest ethanol concentration (0%). The lowest yield was obtained in run 24, at the shortest extraction time (5 min), the lowest temperature (20ºC), and highest ethanol concentration (100%). This difference in yield results obtained can be explained by the solubility and diffusion rate of the compounds present in the composition of *M. communis* berries, which under these circumstances, account for the maximum yield in HAE at the highest temperature and lowest ethanol content. At high temperatures, the physicochemical characteristic of the solvent can change, increasing its kinetic energy and enhancing its ability to penetrate plant cells and dissolve bioactive substances. As a result, the solutes diffuse into the solvent more easily and the cell walls become more porous, increasing the extraction efficiency. Furthermore, as water is a polar solvent that is better able to extract hydrophilic molecule, low ethanol concentration can improve the solubility of polar compounds such as phenolics.

Regarding total phenolics, the highest results was obtained at Run 17 (59 mg GAE/g E), at middle extraction conditions and this proves the literature about the solvent of extraction for *M. communis* should be ethanol 50% to give the most efficient results (Dahmoune et al., 2015). The lowest value obtained was observed at run 21 (21 mg GAE/g E), where the extraction was performed at the highest ethanol (100%) and at the lowest temperature (20°C). These results can be explained by the unique interactions between temperature, solvent polarity, and cell wall permeability. By raising the kinetic energy of molecules, high temperatures improve solvent penetration and improve phenolic chemical solubilization. Additionally, heat encourages the disintegration of plant cell walls, which makes bound phenolics easier to release. Due to the "like dissolves like" concept, lower quantities of ethanol often combined with water provide a more polar solvent environment, improving the extraction of polar phenolic chemicals. Since water is more polar than ethanol, it increases the surface area where the solvent interacts with plant tissues by inducing swelling. Thus, the extraction of polar phenolic compounds is optimized by the combination of high temperature and low ethanol concentration, which also ensures effective solvent penetration and compound dissolution (Meng et al., 2023).

4.2.2 Optimal individual conditions that maximize experimental extraction and verification of predictive models

The analysis of variance (ANOVA) for the experimental results presented in **Table 4** provide the following mathematical models based on coded values, developed to elucidate the effects of the factors and the analyzed responses, based on the experimental data.

$Y1 = 44.91 + 2.85b2 - 3.76b3$ **(2)**

$Y2 = 47.85 + 3.30b2 - 5.37b3 - 8.89b33$ **(3)**

$Y3 = 0.0997 - 0.0493b2 + 0.0250b3 - 0.1277b33$ **(4)**

Table 4. The estimated model coefficients, statistical data, and model-predicted optimal extraction conditions that maximize each response.

a) Estimated model coefficients		Yield (Y_1)	Total phenolics (Y_2)	DPPH (Y_3)
Intercept	b_0	44.91	47.85	0.0997
Linear term	b_1	ns	ns	ns
	b_2	2.85	3.30	-0.0493
	b_3	-3.76	-5.37	0.0250
Quadratic terms	b_{11}	ns	ns	ns
	b_{22}	ns	ns	ns
	b_{33}	ns	-8.89	0.1277
Interaction terms	b_{12}	ns	ns	ns
	b_{13}	ns	ns	ns
	b_{23}	ns	ns	ns
Statistical data	SM	<0.0001	<0.0001	<0.0001
	LF	0.1120	0.1420	0.1112
	R^2	0.8379	0.7681	0.8914
	R^2_{adj}	0.8238	0.7365	0.8759
b) Optimal individual conditions				
Time (A, min)		5	5	73
Temperature (B, ºC)		100	86	84
Ethanol (C, %)		0	43	50
	Optimal predicted response:	56±3	52±8	0.039±0.003
	Optimal real responses	57	48±2	0.038±0.004
c) Optimal global conditions				
Time (A, min)			90	
Temperature (B, ºC)			100	
Ethanol (C, %)			39	
	Optimal predicted responses:	50.0±2.4	52.7±7.2	0.049±0.03
	Optimal actual responses:	47.2±0.2	50±8	0.041±0.003

ns – non significant coefficient; SM - Significance of the model; LF = lack of fit; R^2 = correlation coefficient; R^2adj = the adjusted determination coefficient for the model. The model coefficients were predicted at a 95% confidence level

All mathematical models were significant (p<0.0001), the determination coefficients (R^2) were higher than 0.75, while the adjusted determination coefficient (R^2_{adj}) of the models were higher than 0.73, these values showed a strong correlation between the predicted values and practical values showed in **Table 4**. The R^2 and R^2adj values for the model exhibited minimal variation, as indicated in the **Table 4**, being higher variation observed in model for total phenol response (0.03), which indicate that the addition of non-significant variables at the model, have low impact in the final response (Albuquerque et al., 2023).

For the yield response, the built model showed a highly significant value ($p < 0.0001$). The variation in the yield can depend primly on two significant variables, namely the temperature (b_2 coefficient) and ethanol concentration (b_3 coefficient), being that the temperature has a positive effect, whilst the ethanol concentration has a negative effect on the yield.

Regarding the total phenolics model, three variable effects were significant: the linear effects of temperature (b_2) and solvent concentration (b_3), and the quadratic effect of solvent concentration (b_{33}). This suggests that a higher amount of phenol can be achieved by increasing the temperature and ethanol concentration below 50% (coded value: 0).

In the case of antioxidant activity, higher activity is indicated by a lower EC_{50} value; therefore, negative effects can lead to better responses. Similar to total phenolics, only temperature and ethanol concentration had a significant impact on this response.

These prediction models can facilitate navigation inside the design space. The response surface generated from the models developed for the HAE process is shown in **Figure 4**. The 3D models were constructed to clearly demonstrate the individual and collective impacts of the factors on the desired responses.

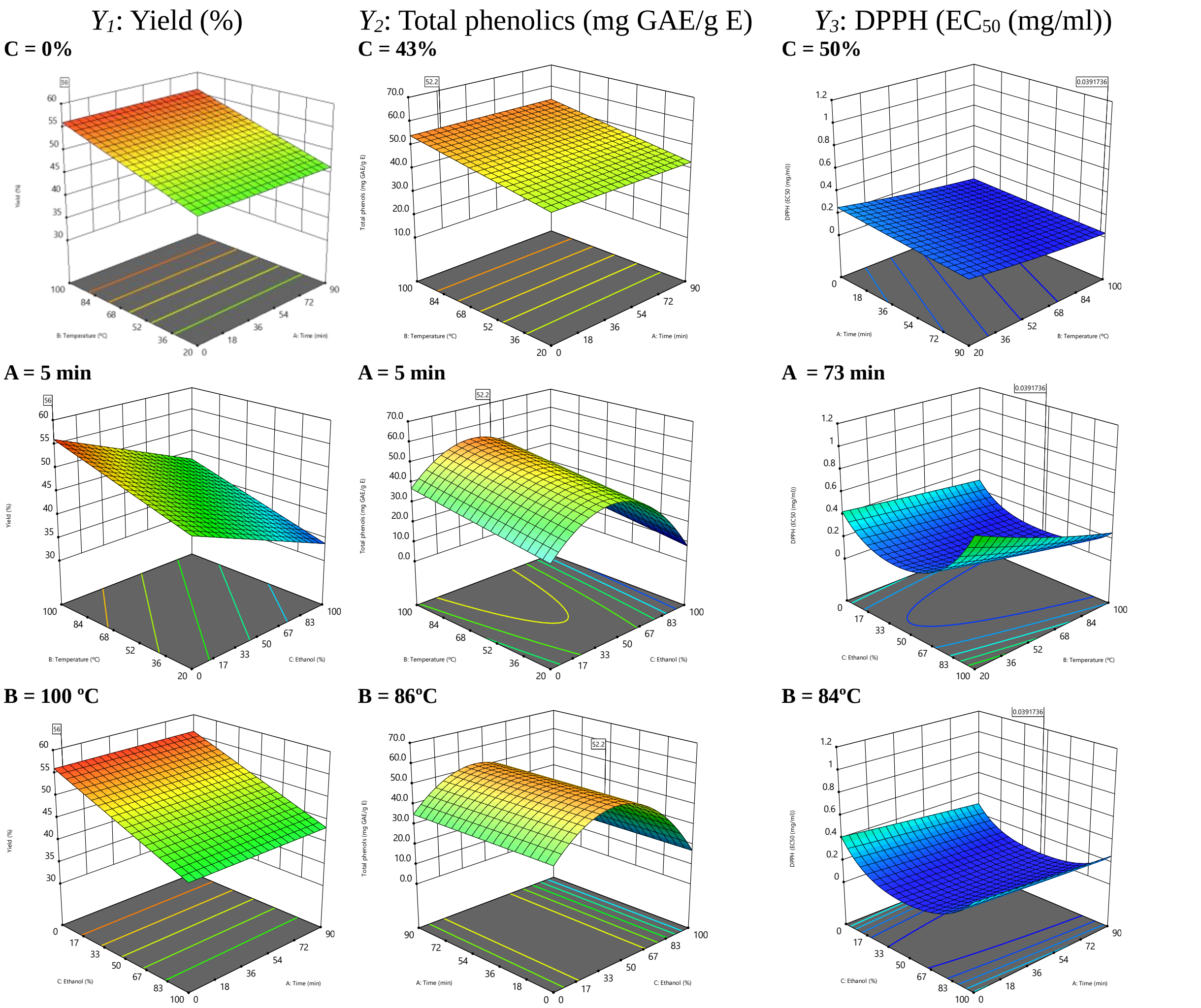


Figure 4. Response surface graphs illustrating the combined effects of the independent variables (*A*: time, *B*: temperature, and *C*: Ethanol (%)) involved in the extraction process on the dependent variables (Y_1: extraction yield, Y_2: total phenolics, and Y_3: antioxidant activity determined by DPPH assay

For the yield response, in the response surfaces built by temperate *x* time and temperature *x* solvent, demonstrate that elevated temperature enhances the extraction yield, with the peak yield (49-57%, w/w) achieved at around 100ºC and ethanol concentration around zero. Under the optimal conditions, described in **Table 4**, the predicted response was 56% and the actual laboratory response achieved was 57%, demonstrating the good adjusting of the model.

Regarding the total phenolics, the 3D models indicate that high temperatures yield better results, while ethanol concentration shows a quadric effect, with a recommended concentration of around 50% to obtain a higher amount of phenolics. When a high temperature is combined with middle ethanol concentration, HAE produced a higher concentration of phenolics (up to 59 mg GAE/mL) owing to the regulated environment that aids in the preservation of sensitive components. The optimal conditions determined for this response were an extraction time of 5 min, temperature of 86ºC, and ethanol concentration of 46%. These conditions were expected to produce an extract with approximately 52 mg GAE/g E. However, when these conditions were tested in the laboratory, the actual result was 48 mg GAE/g E. Although this value is lower than the predicted response, it is within the 95% confidence interval of the response.

Concerning the optimization of the antioxidant activity, the 3D models indicate that the ideal conditions to obtain extracts with a high capacity to scavenge DPPH radicals should involve high temperature, middle ethanol concentration, and long extraction time. In this context, the optimal conditions predicted by the RSM were 73 minutes of extraction at 84ºC, using a solvent with 50% ethanol, which should produce an extract with EC_{50} values of approximately 0.039 mg/mL. This is similar to the actual value of 0.038 mg/mL.

4.2.3 Optimal global conditions to obtain the optimal extract

To obtain an extract with a higher concentration of phenolic compounds, improved antioxidant activity, and better yield, a simultaneous optimization process for the evaluated responses was conducted. In this process, all responses were considered with equal importance. The global optimal condition obtained is presented in **Table 4**. This condition combined a long extraction period, high temperature, and moderate ethanol concentration. The laboratory results showed that the optimal extract contains approximately 50 mg GAE/g E, with an EC_{50} value for oxidation of 0.041 mg/mL, and a yield of around 47%.

4.3 Evaluation of the phenolic profile of the optimal extracts

To a better understanding of the chemical composition of *M. communis* extracts, the identification and quantification of main phenolic compounds found in its composition was performed by a HPLC analyses. The results obtained are presented in **Table 5**. The phenolic profile of *M. communis* berries in the first part (a) of **Table 5** includes the chromatography data, including retention time (R_t), UV-Vis spectra (λ_{max}), molecular ions obtained in the negative model ($[M-H]^-$) and mass fragmentation spectrometry (MS^2), used in the tentative identification of the major phenolic compounds present in the extracts. In the second part (b) of **Table 5** are presented the quantification of each identified compounds, as well as the TPC.

Table 5. Phenolic profile of the optimal extracts obtained under individual and global optimal conditions: a) Tentative identification of major phenolic compounds, and b) Quantification of major phenolic compounds.

a) Tentative identification of the major phenolic compounds in *M. communis* berries

Compounds	**Rt min**	**λmax nm**	**[M-H]$^-$ *m/z***	**MS^2 *m/z***	**Tentative identification**	**References**
1	14.01	363	631	479 (100)	Myricetin galloyl-hexoside	(D'Urso et al., 2019)
2	15.10	357	479	316(100), 317 (73), 205 (8), 245 (5), 369 (4)	Myricetin-*O*-hexoside	(D'Urso et al., 2019)
3	16.99	361	615	463(100)	Myricetin galloyl-rhamnopyranoside	(D'Urso et al., 2019)
4	17.67	357	463	445(15),317 (32), 316 (100), 179(7)	Myricetin-*O*-deoxyhexoside	(Bouaoudia-Madi et al., 2019)
5	22.50	357	447	301 (100)	Quercetin-*O*-deoxyhexoside	(Bouaoudia-Madi et al., 2019)

b) Quantification of the major phenolic compounds in *M. communis* berries. Results are expressed as mg/g of extract (E).

Compounds	**MC1**	**MC2**	**MC3**	**MC4**
1	2.85±0.02	3.272±0.003	2.975±0.003	2.72±0.01
2	2.89±0.02	3.888±0.003	3.81±0.03	3.40±0.06
3	2.87±0.02	3.2017±0.0005	2.867±0.004	2.66±0.01
4	0.537±0.002	3.4817±0.0003	3.22±0.002	2.95±0.03
5	nd	0.7799±0.0002	0.769±0.001	0.69±0.02
TPC	9.15±0.05	14.623±0.001	13.64±0.02	12.84±0.14

MC1 = extract obtained under the optimal individual condition for Y_1; MC2 = extract obtained under the optimal individual condition for Y_2; MC3 = extract obtained under the optimal individual condition for Y_3; MC4 = extract obtained under the optimal global condition; TPC – Total phenolic compounds; nd – not detected.

Five phenolic compounds were detected in *M. communis* berry extracts. Compound 1, with a molecular ion at 631 *m/z*, produced a mass fragment at 479 *m/z*, indicating the loss of a galloyl molecule (-152Da). Based on the literature, this fragmentation behavior is characteristic of myricetin galloyl-hexoside, which was previously identified in *M. communis* berries by D'Urso et al. (2019). Compound 2 showed the same ion fragment found in compound 1. In the MS^2 analyses, five mass fragments were detected, with the major fragment at 316 *m/z*, suggesting a loss of hexose moiety (-162 Da) and the presence of the aglycone myricetin. The chromatography data obtained for this compound was similar to those described for myricetin-*O*-hexoside, as identified by D'Urso et al. (2019). Compound 3, with molecular ion at 615 *m/z*, detached an ion fragment at 463 m/z (-152 Da, galloyl moiety). According to the literature, this molecular ion has been identified as myricetin galloyl-rhamnopyranoside (D'Urso et al., 2019). Compound 4 revealed a pseudomolecular ion at 463 *m/z*and disassociated four mass fragments in MS^2, being again the major fragment at 316 *m/z*. Based on the literature, this compound was tentative identified as myricetin-*O*-deoxyhexoside (Bouaoudia-Madi et al., 2019). Finally, compound 5, with molecular ion at 447 *m/z*, revealed a unique ion fragment at 301 *m/z*, characteristic of aglycone quercetin. This compound has been identified as quercetin-*O*-deoxyhexoside in *M. communis* berries (Bouaoudia-Madi et al., 2019)

Quantification of the phenolic compounds was performed on four distinct extracts which were designated as MC1 – the extract obtained under optimal conditions for yield; MC2 extract obtained under optimal conditions for total phenolics; MC3 – extract obtained under optimal conditions for antioxidant activity; and MC4 – extract obtained under optimal global conditions. Over the four conditions, the concentration of myricetin galloyl-hexoside varied from (2.72- 3.272 mg/g E) with a standard deviation (SD= 0.390). In all extracts, myricetin-*O*-hexoside was the most abundant compound, with concentrations ranging from (2.89- 3.888 mg/g of extract) with (SD= 0.706).

Depending on the extraction conditions, myricetin galloyl-rhamnopyranoside could range from (2.66- 3.2017 mg/g E) (SD= 0.383). The levels of myricetin-*O*-deoxyhexoside ranged from (0.537- 3.4817 mg/g E) (SD= 2.082), indicating that certain conditions may limit the extraction of this compound. The lowest concentration was quantified in MC1, an extract obtained without ethanol in the solvent composition and with a short extraction time. As a deoxyhexoside, by the loss of oxygen, confers a less polar molecule, this may explain its low solubility in pure water (Mansouri et al., 2022).

There was no evidence of quercetin-*O*-deoxyhexoside in MC1, although the concentrations of this compound in the other extracts ranged from 0.69 - 0.7799 mg/g E (SD = 0.064).

The TPC ranged from 9.15 mg/g E in MC1 to 14.623 mg/g in MC2, indicating that the phenolic compounds were better recovery the extraction performed under the optimal condition for total phenolics response (time = 5 min, temperature = 86ºC, and ethanol concentration = 43%). According to the findings of the study, the phenolic composition and concentration in the extracts were significantly impacted by the various extraction conditions. These phenolic compounds, in particular flavonoids such as myricetin and quercetin derivatives, are well-known for the powerful antioxidant properties that they possess (Osaili et al., 2024).

4.4 Evaluation of the bioactivities of the optimal extract

The optimized extract obtained under the global optimal conditions was tested for its antibacterial, antifungal, and antioxidant activities.

4.4.1 Antimicrobial activity

The antibacterial activity was evaluated against 16 bacteria, including 8 clinical strains and 8 foodborne strains. The results obtained against the clinical bacteria are presented in **Table 6**.

Table 6. Antibacterial activity of the optimal extract (MC) against clinical strains.

			Positive Control			
	MC		**Ampicillin (10mg/mL)**		**Imipenem (1mg/mL)**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria						
Escherichia coli	10	>10	<0.15	<0.15	<0.0078	<0.0078
Klebsiella pneumoniae	10	>10	10	>10	<0.0078	<0.0078
Morganella morganii	10	>10	>10	>10	<0.0078	<0.0078
Proteus mirabilis	10	>10	<015	<0.15	<0.0078	<0.0078
Pseudomonas aeruginosa	5	>10	>10	>10	0.5	1
Gram-positive bacteria						
Enterococcus faecalis	10	>10	<0.15	<0.15	<0.0078	<0.0078
Listeria monocytogenes	2.5	>10	<0.15	<0.15	n.t.	n.t.
MRSA	10	>10	<0.15	<0.15	n.t.	n.t.

MIC – minimum inhibitory concentration; MBC – minimum bactericidal concentration; All values are expressed as mg/mL.

On Gram-negative bacteria, MC demonstrated limited efficacy, exhibiting MIC values of 10 mg/mL for *E. coli*, *K. pneumoniae*, *M. morganii*, and *P. mirabilis*, while showing more efficacy to inhibit the proliferation of *Pseudomonas aeruginosa* (MIC = 5 mg/mL), and lacking significant bactericidal activity at the maximum concentration tested (MBC > 10 mg/mL). In the specific case of *K. pneumoniae*, the extract demonstrated inhibitory activity comparable to that of the antibiotic ampicillin. The antibacterial efficacy of this extract is significant; thus, reviewing research on analogous natural extracts is beneficial. For instance, studies on plant-based extracts, such as those from *Thymus vulgaris* (thyme), frequently show broad-spectrum action against both Gram-positive and Gram-negative bacteria. MIC values, which vary greatly depending on the chemical and bacteria examined, typically range from 0.1 to 10 mg/mL. These values, particularly when combined with MBC data, facilitate the evaluation of the potency and efficacy of the examined extract against microorganisms. Comparing this extract with others helps contextualize its efficacy as equivalent, improved, or diminished relative to proven natural antimicrobials (Vassiliou et al., 2023).

In the case of Gram-positive bacteria, MC demonstrated the ability to inhibit the growth of all tested bacteria, with the highest activity against *L. monocytogenes* (MIC = 2.5 mg/mL). However, the extract did not exhibit bactericidal activity at the maximum concentration evaluated.

Table 7 presents the results of the antibacterial activity of *M. communis* berry extract against eight foodborne strains.

Table 7. Antibacterial activity of the optimal extract (MC) against foodborne bacteria.

			Positive Control			
	MC		**Streptomicin 1mg/mL**		**Ampicillin 10mg/mL**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria						
Enterobacter Cloacae	10	>10	0.007	0.007	0.15	0.15
Escherichia coli	5	>10	0.01	0.01	0.15	0.15
Pseudomonas aeruginosa	>10	>10	0.06	0.06	0.63	0.63
Salmonellaenterica	10	>10	0.007	0.007	0.15	0.15
Yersinia enterocolitica	5	>10	0.007	0.007	0.15	0.15
Gram-positive bacteria						
Bacillus cereus	5	>10	0.007	0.007	n.t.	n.t.
Listeria monocytogenes	10	>10	0.007	0.007	0.15	0.15
Staphylococcus aureus	2.5	>10	0.007	0.007	0.15	0.15

MIC – minimum inhibitory concentration; MBC – minimum bactericidal concentration; All values are expressed as mg/mL.

The extract exhibited inhibitory activity against all tested bacteria except *P. aeruginosa*. The highest activity was observed against *S. aureus* (MIC = 2.5 mg/mL), followed by *E. coli*, *Y. enterocolitica*, and B. *cereus*, with a MIC value of 5 mg/mL for each. However, no bactericidal activity was observed at the maximum concentration tested for any of the strains. The literature (Egidio et al., 2024) indicated an inhibitory impact on many bacteria, particularly *S. aureus*, exhibiting a substantial inhibition zone, although revealed minimal or no bactericidal activity at the maximum dosage used. This outcome differs from other extracts that exhibit a wider antibacterial range and efficacy at reduced doses. The results in the literature indicate that several extracts from some plants exhibited differing inhibitory effects, notably the pellet fraction (PF), which demonstrated efficacy at lower concentrations against *S. aureus* and other pathogens such as *E. coli* and *B. cereus* (Egidio et al., 2024). This demonstrates that plant extracts may serve as a standard for comparison in antibacterial investigations of plant-derived extracts.

The antifungal activity of the optimal extract (MC) against *Aspergillus brasiliensis* and *Aspergillus fumigates* is demonstrated in **Table 8**.

Table 8. Antifungal activity of the optimal extract (MC).

	Aspergillus brasiliensis		*Aspergillus fumigatus*	
	MIC	MFC	MIC	MFC
MC	10	>10	10	>10
Ketoconazole	0.06	0.125	0.5	1

MIC – minimum inhibitory concentration; MFC – minimum fungicidal concentration; All values are expressed as mg/mL.

The extract was able to inhibit both fungi at the concentration of 10 mg/ml. However, it did exhibit fungicidal activity for both species at the maximum concentration tested. The results demonstrate that MC has restricted inhibitory and no effective fungicidal activity at the evaluated doses.

4.4.2 Antioxidant activity of the optimal extract

The TBARS assay is commonly employed to assess the antioxidant potential of a sample by measuring its efficacy in inhibiting lipid peroxidation. The EC_{50} value, indicative of the extract concentration necessary to block 50% of TBARS production, serves as a critical measure of antioxidant efficacy. The EC_{50} value for the *M. communis L.* berry extract in this investigation was determined to be 0.045 ± 0.006 mg/mL, signifying robust antioxidant ability. The low EC_{50} value indicates that a little quantity of the extract is sufficient to markedly diminish oxidative damage in lipid membranes, a vital process for averting cellular damage and inflammation. The concentration of phenolic compounds, including myricetin and quercetin derivatives, likely enhances this antioxidant activity, as these compounds are recognized for their effectiveness in neutralizing free radicals and preventing lipid peroxidation (Abreu-Naranjo et al., 2020).

Relative to strawberries, and raspberries reflecting strong antioxidant activity (Günal Köroğlu et al., 2021), this EC_{50} value is notably low, positioning *M. communis* berries among those with a highly efficacious antioxidant profile. The robust antioxidant capacity of these berries renders them significant for use in food preservation, cosmetics, and nutraceuticals, where oxidative stability is crucial.

These result aligns with the growing consumer demand for functional foods that support overall well-being and disease prevention (Mehta et al., 2022). *M. Communis* berries are abundant in phytochemicals, such as flavonoids, which contribute to antioxidant defense and overall well-being. They contain various bioactive molecules containing antioxidant, antimicrobial, flavoring, coloring, nutritional, and therapeutic properties, and they are widely used for preservation in the food industry, which promote the growth of beneficial gut bacteria and support digestive health (Giampieri et al., 2020). It also has a great importance as a food preservative to provide better quality and stability to the food products.

5. Conclusion

This study investigated *M. communis,* an underutilized wild fruit from the Mediterranean region, for its potential as a source of bioactive compounds, especially phenolic compounds with antioxidant and antibacterial activities. Between two extraction methodologies, MAE and HAE, the last one exhibited a higher efficacy, producing superior results in terms of phenol concentration and antioxidant activity.

The study established that *M. communis* is a great source of phenolic chemicals, including myricetin and quercetin derivatives, which demonstrate potent antioxidant and antibacterial characteristics. The study also emphasized the essential importance of optimal extraction conditions, including time, temperature, and solvent concentration, in enhancing phenolic yields and bioactivity. The results indicated that reduced ethanol concentrations along with elevated temperatures were the most efficient in extracting these important compounds. This research has significant implications across multiple sectors. The pharmaceutical sector can utilize the high antioxidant and antibacterial capabilities of *M. communis* for drug development, namely in formulating natural treatments for oxidative stress-related disorders and bacterial infections. In the food sector, the bioactive molecules derived from this berry can work as natural preservatives, extending food shelf-life and aligning with the increasing trend towards functional foods that enhance health. The cosmetic industry may profit from the incorporation of antioxidants derived from *M. communis* in skincare products to safeguard against environmental stressors and premature aging and to increase the shelf-life of products.

This work highlighted the unexploited potential of *M. communis*, thereby promoting the sustainable utilization of natural resources and contributing to the understanding of bioactive compounds derived from wild flora. This facilitates additional research and development in areas such as medicine, food science, and cosmetic formulations, promoting innovation and aiding in biodiversity conservation particularly as a food preservative or dietary supplement for its rich source of phenolic compounds.

When incorporating these bioactive ingredients, it is necessary to consider their impact on flavor and texture of the final product. Experiment with different combinations to achieve a balance between health benefits and sensory appeal. In addition, to ensure the safety use of this extract, future studies should focus on evaluating its cytocompatibility in various cell lines. Subsequently, *in vivo* testing may be necessary to validate the findings.

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