Mixture of edible flowers as a food alternative with bioactive properties

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List of Abbreviation

AA: absorbance of the sample

A-DPPH: absorbance of the control

ANOVA: Analysis of Variance

anti-HIV: anti Human Immunodeficiency Virus

b.c.e: before Common Era

C100%: *Calendula officinalis* L. 100%

CFU: Colony Forming Unit

CO_2: Carbon dioxide

DAD: Diode Array Detector

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DPPH: 2-diphenyl-1-picrylhydrazyl

ESI: Electrospray Ionization

HAE: Heat Assisted Extraction

HBSS: Hank's Balanced Salt Solution

HPLC: High Performance Liquid Chromatography

INT: Iodo-Nitro-Tetrazolium

MA: Malt Agar

MBC: Minimum Bactericidal Concentration

MFC: Minimum Fungicidal Concentration

MIC: Minimum Inhibitory Concentration

MS: Mass Spectrometry

n.d: not detected

R100%: *Rosa Canina* L. 100%

R50%C50%: *Rosa Canina* L.50% + *Calendula officinalis* L. 50%

R25%C75%: *Rosa Canina* L.25% + *Calendula officinalis* L. 75%

R75%C25%: *Rosa Canina* L.75% + *Calendula officinalis* L. 25%

SRB: Sulfurrodamine B

TSB: Triptych Soy Broth

UFLC: Ultra Fast Liquid Chromatography

Abstract

Edible flowers are increasingly recognized as a functional food alternative due to their rich nutritional and bioactive properties. The incorporation of edible flowers in the diet, either directly or through infusions, has garnered significant interest, particularly in the context of the revival of healthy lifestyles, such as the Mediterranean diet. Numerous studies have delved into the health benefits, nutritional value, and bioactive properties of edible flowers, positioning them as a driving force in the food industry and appealing to consumers seeking healthier and more attractive food products to enhance their diet.

The scientific community aims to investigate the bioactive potential of edible flower mixtures and their suitability as a food alternative with noteworthy health advantages. *Calendula officinalis* L. and *Rosa Canina* L. are known to be sources of various bioactive compounds, such as phenolic acids, flavonoids, and other phenolic compounds, which have demonstrated antioxidant, and antimicrobial properties. These bioactive substances are believed to contribute to the health benefits associated with the consumption of edible flowers.

In the present study, we intend to define the phenolic profile and investigate the bioactive properties of different mixtures of *Calendula officinalis* L. and *Rosa Canina* L. using maceration extraction. The phenolic compounds will be determined by high-performance liquid chromatography coupled with a diode array detector and a mass spectrometry detector (HPLC-DAD-ESI/MS). We will analyze various bioactivities, including antioxidant, antimicrobial, and cytotoxicity activities.

This study investigates the bioactive potential of various formulations of Rosa canina and Calendula officinalis, focusing on their major compounds and overall effectiveness. The principal bioactive compounds identified include digalloylhexoside, galloyl quinic acid, isorhamnetin-*O*-rhamnosyl hexosiderhamnoside, and isorhamnetin-*O*-rutinoside. Among the mixtures tested C25% R75% (Calendula officinalis L. 25% + Rosa Canina L. 75%) and C75% R25% (*Calendula officinalis* L.75% + *Rosa Canina* L. 25%) demonstrated the most promising results. Notably, C75%R25% exhibited superior antioxidant activity, while C25%R75% showed enhanced antibacterial activity.

Our obtained results underscore the significant health benefits and functional potential of these edible flower mixtures, paving the way for their application in the food industry for the formulation of innovative functional food products.

Keywords: Bioactive compounds, *Calendula officinalis*L., edible flowers, functional food, health benefits and *Rosa Canina* L.

Resumo

As flores comestíveis são cada vez mais reconhecidas como uma alternativa alimentar funcional devido às suas ricas propriedades nutricionais e bioativas. A incorporação de flores comestíveis na dieta alimentar, quer diretamente, quer através de infusões, tem suscitado um interesse significativo, nomeadamente no contexto do renascimento de estilos de vida saudáveis, como a dieta mediterrânica. Numerosos estudos investigaram os benefícios para a saúde, o valor nutricional e as propriedades bioativas das flores comestíveis, posicionando-as como uma força motriz na indústria alimentar e apelando aos consumidores que procuram produtos alimentares mais saudáveis e atrativos para melhorar a sua dieta.

A comunidade científica visa investigar o potencial bioativo das misturas de flores comestíveis e a sua adequação como alternativa alimentar com notáveis vantagens para a saúde. A *Calendula officinalis* L. e a *Rosa Canina* L. são conhecidas por serem fontes de vários compostos bioativos, como ácidos fenólicos, flavonóides e outros compostos fenólicos, que demonstraram propriedades antioxidantes e antimicrobianas. Acredita-se que estas substâncias bioativas contribuem para os benefícios para a saúde associados ao consumo de flores comestíveis.

No presente estudo pretendemos definir o perfil fenólico e investigar as propriedades bioativas de diferentes misturas de *Calendula officinalis* L. e *Rosa Canina* L. utilizando a extração por maceração. Os compostos fenólicos serão determinados por cromatografia líquida de alta eficiência acoplada a um detetor de arranjo de díodos e a um detetor de espectrometria de massas (HPLC-DAD-ESI/MS). Analisaremos várias bioatividades, incluindo atividades antioxidantes, antimicrobianas e de citotoxicidade.

Este estudo investiga o potencial bioativo de diversas formulações de *Rosa canina* e *Calendula officinalis*, com foco nos seus principais compostos e eficácia global. Os principais compostos bioativos identificados incluem o digaloilhexosídeo, o ácido galoilquínico, a isorhamnetina-*O*-ramnosil hexosídeoramnosídeo e a isorhamnetina-*O*-rutinosídeo. Entre as misturas testadas, C25%R75% (*Calendula officinalis* L25% +*Rosa Canina* L 75%) e C75%R25% (*Calendula officinalis* L75% + *Rosa Canina* L 25%) demonstraram os resultados mais promissores. Notavelmente, C75%R25% exibiram uma atividade antioxidante superior, enquanto C25%R75% mostraram uma atividade antibacteriana aumentada.

Os resultados apresentados sublinham os benefícios significativos para a saúde e o potencial funcional destas misturas de flores comestíveis, abrindo caminho para a sua

aplicação na indústria alimentar para a formulação de produtos alimentares funcionais inovadores.

Palavras-chave: Alimentos funcionais, benefícios para a saúde, *Calendula officinalis* L., compostos bioativos, flores comestíveis; *Rosa Canina* L.

1. Introduction

Food and nutrition are ever-evolving fields, with a growing focus on the exploration of healthier and sustainable food alternatives. In this context, edible flowers are emerging as a source of nutrient-rich bioactive that deserve special attention(Takahashi et al., 2020). Often used for aesthetic and ornamental purposes, edible flowers also present an untapped potential as functional food ingredient. Their diversity in terms of taste, aroma, and color, combined with their unique nutritional profile, makes them promising candidates for diversifying our diets and enhancing our well-being (Zhao et al., 2019).

Bioactives are natural compounds found in foods that have a positive impact on health, ranging from disease prevention to immune system enhancement (Akhtar et al., 2019).

The food industry is constantly searching for new ingredients that meet consumer expectations for healthier and more attractive food products. Flowers have been traditionally used for medicinal purposes, and recent studies have identified and quantified the chemical profile of bioactive compounds in edible flowers, linking their presence to various health benefits (Takahashi et al., 2020). The incorporation of different edible flowers in the diet, either directly or through infusions, has been gaining more and more interest, particularly in the context of the revival of healthy lifestyles related to specific regions of the world, such as the Mediterranean diet (Amrouche et al., 2022). Moreover, the high number of studies that explore the health benefits, the nutritional value, and the bioactive properties of edible flowers are the key drivers for the food industry and the consumers seeking healthier and more attractive food products that can improve their diet(Pires et al., 2021). Therefore, edible flowers have the potential to be used as a food alternative with bioactive properties, contributing to the expanding field of functional foods and natural food additives.

In this sense, the present study aims to explore an innovative perspective on nutrition by showcasing edible flowers and their potential as a food alternative with bioactive properties.

2. State of art

2.1. Edible flowers

Edible flowers are flowers from various plants that are safe and suitable for human consumption. Edible flowers, including nasturtium, rose, lavender, chamomile, and others, have a rich culinary history, adding color, flavor, and visual appeal to various dishes, as garnishes, ingredients in salads, desserts, or even in teas and beverages (Rivas-García et al., 2021). It's important to note that flowers are edible, and it is essential to be certain of the flower's identity and origin before consuming it, as some flowers can be toxic (Takahashi et al., 2020).

Edible flowers have a rich historical context, with their culinary use dating back to ancient cultures. The Romans, for instance, utilized violets and roses in various culinary applications, while the South American Aztecs and Mayans frequently incorporated edible flowers into their cooking (Guiné et al., 2020). Additionally, the Chinese employed edible flowers for their medicinal qualities, recognizing improve health. However, despite their historical significance, edible flowers were largely overlooked and underutilized in the 20th century, primarily due to challenges in commercializing them for industrial production(Rodrigues & Spence, 2023).

In recent years, there has been a resurgence of interest in edible flowers, particularly with the rise of social media and the visual appeal they lend to dishes (Takahashi et al., 2020). The food industry's intense search for new ingredients that consumer expectations has led to a renewed focus on the potential of edible flowers (Amrouche et al., 2022). These flowers are not only visually appealing but also offer a rich composition of vitamins, antioxidants, and other bioactive compounds that contribute to health and well-being. They are sources of a wide variety of bioactive compounds, including phenolic compounds, carotenoids, betalains, and alkaloids, which have been associated with several health benefits (Pires et al., 2021).

Despite their potential nutritional value, there are current challenges in integrating edible flowers into diets, such as limited awareness or accessibility. This has led to a gap in existing research or literature regarding the comprehensive exploration of edible flowers as a food alternative. There is a need for a deeper understanding of their bioactive properties and culinary potential, as well as their potential use in food development (Pensamiento-Niño et al., 2023). Therefore, further research is warranted to establish the safety, efficacy, and sensory properties of edible flowers as a food ingredient, as well as to explore their potential

applications in the food industry, such as in the development of functional foods, nutraceuticals, and natural food colorants.

2.1.1. History and health benefits

Several civilizations, from ancient Greece and Rome to medieval France and Victorian England, have embraced the use of edible flowers to enhance the flavors of many dishes(Jadhav et al., 2023). Many ancient texts refer to edible flowers, for example, the Bible cites dandelions as one of the "bitter herbs" eaten as salads, while in the Song of Solomon saffron, the stamens of *Crocus sativus*, is mentioned. In Italy, evidence on the use of flowers can be found in some refined preparations, such as, for example, in vino violatum (violet wine) or in rosatum (rose petal wine), safflower flower sauce, and marjoram flower meatballs, whose recipes are reported in *Apicius De Re Coquinaria*, a famous cookbook from imperial Rome (Motti et al., 2022). Flowers have been employed in culinary dishes from Ancient Greece, Egypt, and Rome, with the oldest reference dating back to 140 b.c. (Santos & Reis, 2021).

Around the globe, there are primarily three different kinds of edible flowers namely fruit flowers, vegetable lowers, and aromatic medicinal flowers (Zhao et al., 2019).The differentiation between them is based on the source of the flower and its intended use (Pires et al., 2021). Fruit flowers come from plants that produce edible fruits, such as apple, pear, and strawberry. Vegetable flowers are those that come from plants that are typically grown for their edible leaves, stems, or roots, such as broccoli, cauliflower, and artichokes. Finally, aromatic medicinal flowers are those that are used for their medicinal properties, such as chamomile, lavender, and calendula (Selli et al., 2021).

Edible flowers have a wide range of applications, including their use as a flavoring agent and garnish in gourmet cuisine, as well as in the development of food and beverage products, herbal remedies, and weight loss supplements (Rivas-García et al., 2021). They are also used in garnishing cakes, cookies, and chocolates. The global edible flowers market is expected to experience significant growth, with emerging economies and the booming nutritional food industry driving demand (Faisal et al., 2022).

This natural matrices have long been utilized in indigenous medicine to treat ailments, current research has confirmed these conventional health advantages by demonstrating their high bioactive component concentration, which has been linked to functional qualities(Rao

&Poonia, 2023). **Table 1** summarizing some examples of edible flowers which highlight the diverse uses across different cultures and regions, showcasing their potential contributions to the nutritional value and culinary appeal of various dishes.

Table1: Use of edible flowers across the world.

Edible flower	Popular name	Use	Geographical location	Reference
Calendula officinalis L.	Pot marigold	The petals of flowers are sun-dried and are used for edible decorative purposes and also as an alternative for saffron.	India	(Panda et al., 2019)
Tulbaghiaviolacea Har.	Wild garlic	It is used for culinary purposes.	South Africa	(Trinh et al., 2018)
Oxalis pescaprae L.	Suring	It is consumed fresh or cooked	South Africa	
Viola tricolor L.	Heartsease	It is used in preparation of soups, salads, drinks and as a colorant.	Europe	(Navarro-González et al., 2014)
Camellia sinensis L.	Chiya	Used as a vegetable in the meal.	Nepal	(Rajbanshi & Thapa, 2019)
Centaurea cyanus L.	Corn flower	It is used as a colorant, to garish dishes, and infusions.	Europe	
Rosa canina L.	Dog rose	The fruit is used to make syrup, tea, and preserves. The flowers can be eaten in salads or preserved in vinegar and honey.	Europe & North Africa	(Jadhav et al., 2023)

2.1.2. *Calendula officinalis* L.

Calendula officinalis L. (**Figure1**), commonly known as pot marigold, is an edible flower from the Asteraceae family, characterized by vibrant orange or yellow petals and a mild, peppery flavour (Chitrakar et al., 2019).Native to Europe but now widely cultivated, this member of the Asteraceae family typically grows 12 to 18 inches tall and is prized for its ease of cultivation and self-seeding nature (Jakubczyk et al., 2022).

Figure 1: *Calendula officinalis* L.(Patil et al., 2022)

Its importance as an edible flower stems from its nutritional value, culinary versatility, and potential health benefits. *Calendula* is rich in dietary fiber, protein, and bioactive compounds, primarily polyphenols, which exhibit various biological activities (Jakubczyk et al., 2022).

It is used in culinary applications to enhance visual appeal and flavor in dishes like salads, soups, and as a saffron substitute. In herbal medicine, *Calendula officinalis* L. is valued for its potential anti-inflammatory, antimicrobial, and gastroprotective properties (Arora et al., 2013). The flower's composition includes dietary fiber, protein, bioactive compounds, vitamins, antioxidants, essential oils, flavonol glycosides, triterpene oligoglycosides, and saponins (Goni & Hervert-Hernandez, 2011). These attributes make *calendula* an attractive ingredient for functional foods and herbal remedies, aligning with current trends favoring natural and healthy food options.

2.1.3. *Rosa canina* L.

Rosa canina L. (**Figure 2**), is a perennial shrub that belongs to the Rosacea family. The plant is commonly known as Dog Rose, and its specific epithet, "canina," comes from the Latin word "caninus," meaning "of the dog," due to the ancient belief that the plant's root could cure dog bites (Moldovan et al., 2021). The plant is native to a vast region in the temperate zones of the Old World, including Africa, Asia, and Europe, and it has been naturalized in other parts of the world, such as America and Australia (Rodrigues & Spence, 2023).

R. canina has been used for several medicinal purposes, including the treatment of vitamin C deficiency, diarrhea, gastrointestinal tract disorders, and problems related to kidneys, liver, and bladder (Ayati et al., 2019).

Traditionally, *R. canina* is used in drink, food, and medicine form in many countries (Ahmad et al., 2016).The fruit of *Rosa Canina* L. is rich in vitamins, particularly vitamin C, which is essential for immune function and collagen production (Selahvarzian et al., 2018).

Figure 2: Plant and flower of *Rosa canina*L.(https://www.alamyimages.fr/photos-images/fruits-d'%C3%A9glantier.html?sortBy=relevant)

R. canina contains great amounts of pharmacologically active compositions: flavonols, carotenoids, tannins, and organic acids. So, *R. canina* has been utilized to cure disorders of the kidneys urethra and viral infections, anxiety, osteoarthritis, hypertension, immunomodulatory and antidiabetic, antimicrobial, etc. When clinical studies, toxicity, and side effects about *R. canina* were investigated as pharmaceutical preparations, further clinical

trials are needed to confirm the reported promising experimental effects in clinical use (Arslan et al., 2020).

Having explored the rich tapestry of edible flowers, we now turn our attention to their chemical aspects and bioactive properties.

2.2. Edible flowers as source of bioactive compounds

Table 2, provide valuable insights into the bioactive potential of edible flowers. They demonstrate that edible flowers can serve as a source of phenolic compounds with bioactive potential, which can be applied in the food sector (Pires et al., 2018).

Table2: Studies on bioactive compounds in edible flowers.

Edible Flowers	Bioactive compounds	References
Tagetes erecta L., *Butea monosperma* Lam. and *Calendula officinalis* L.	Total of phenolic compounds, carotenoids, betalains, alkaloids.	Pires et al., 2021
Viola × wittrockiana (Pansy)	Carotenoids, xanthophylls, flavonoids, anthocyanins.	(Kozicka & Hallmann, 2023)
Borago officinalis L., *Calendula officinalis*L.,*Tagetespatula* L. and *Tropaeolum majus* L.	Vitamin C, phytochemicals with bioactive properties.	(Demasi et al., 2021)
*Osmanthus fragrans*Lour, and *FlosSophorae*	Carotenoids, dietary fiber, vitamins, minerals.	(Zhao et al., 2019)

These findings collectively demonstrate the diverse and abundant bioactive compounds present in edible flowers, supporting their potential health benefits and nutritional value.

2.2.1. Chemical aspects and bioactive properties

Edible flowers can be divided into their different parts such as: pollen, petals, nectar, stigma and other parts (Palaric et al., 2023). The pollen is considered as rich source of proteins, carbohydrates, amino acids, flavonoids and carotenoids but is least recommended to have in diet because of the various allergic reactions associated with it (Gardana et al., 2018). The different parts of edible flowers, including petals, stamens, and ovaries, have unique chemical compositions and bioactive properties that contribute to their potential health benefits. These properties make edible flowers a valuable addition to the human diet, particularly in the context of functional foods and dietary supplements (Chen et al., 2020).

Edible flowers contain various therapeutic and pharmaceutic components in them, such as natural antioxidants in the form of vitamin E (tocopherol), which acts as a natural antioxidant protecting cells from oxidative stress and supporting the immune system (Skrajda-Brdak et al., 2020), natural pigments, glycosides, flavonoids, phenolic acids, which have been shown to exhibit antioxidant properties and may have potential anti-cancer properties (Skrajda-Brdak et al., 2020), homogentisic acid, tannins that have been shown to possess antioxidant properties and may reduce the risk of chronic diseases (Yasar et al., 2022), anthocyanidins and proanthocyanins (condensed tannins) (Purohit et al., 2021).

Many researchers analyzed the phenolic profiles and bioactive properties of four edible flower species: *Dahlia mignon*, *Rosa damascena* and *R. gallica* grafts, *Calendula officinalis* L., and *Centaurea cyanus* L. (**Figure 3**) The study examined both hydromethanolic extracts and infusions, evaluating their antioxidant, antiproliferative, and antibacterial capacities (Pires et al., 2018).

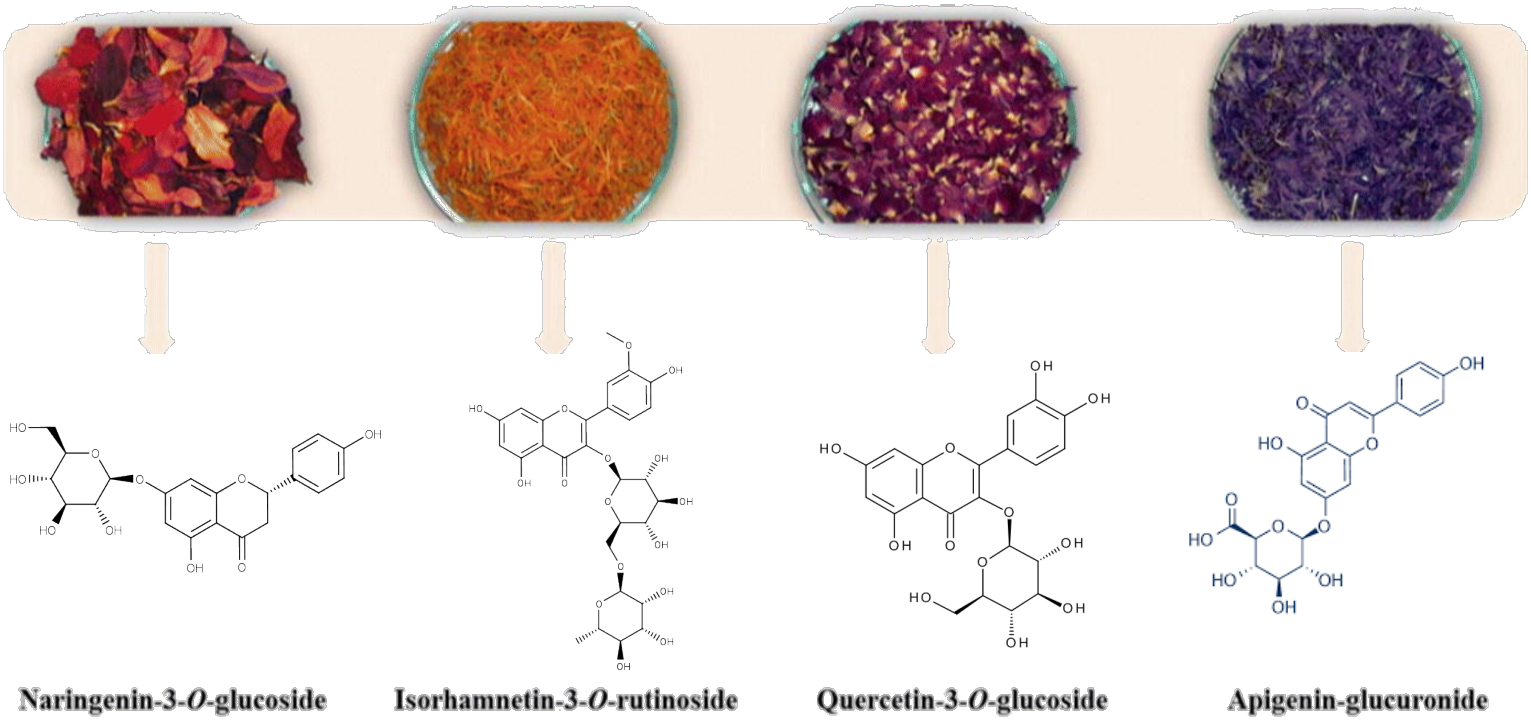

Figure 3: Edible flowers as source of phenolic compounds with bioactive potential (Pires et al., 2018).

- **Phenolic compounds** (**Figure 4**) are found in edible flowers and have been the subject of various studies. Research has shown that edible flowers, such as *Calendula officinalis*, *Centaurea cyanus*, and *dahlia mignon*, contain phenolic compounds with bioactive potential health (Lucarini et al., 2020). Furthermore, studies have aimed to determine the phenolic profile of edible flowers using chromatographic methods (Thorvaldsson et al., 2022).

- **Phenolic acids** commonly found in edible flowers include compounds such as chlorogenic acid, gallic acid, and caffeic acid (Pires et al., 2021).These compounds contribute to the antioxidant activity of edible flowers, which may offer various health benefits, including anti-inflammatory, antimicrobial, and anticancer properties (Prabawati et al., 2021).

- **Flavonoids** are a diverse group of natural compounds and are among the most important phenolic compounds found in edible flowers (Gonçalves et al., 2020). Research has shown that various edible flowers, such as red carnation, Mexican marigold, and pink rose, are rich sources of flavonoids, with significant antioxidant capacity (Pires et al., 2018).

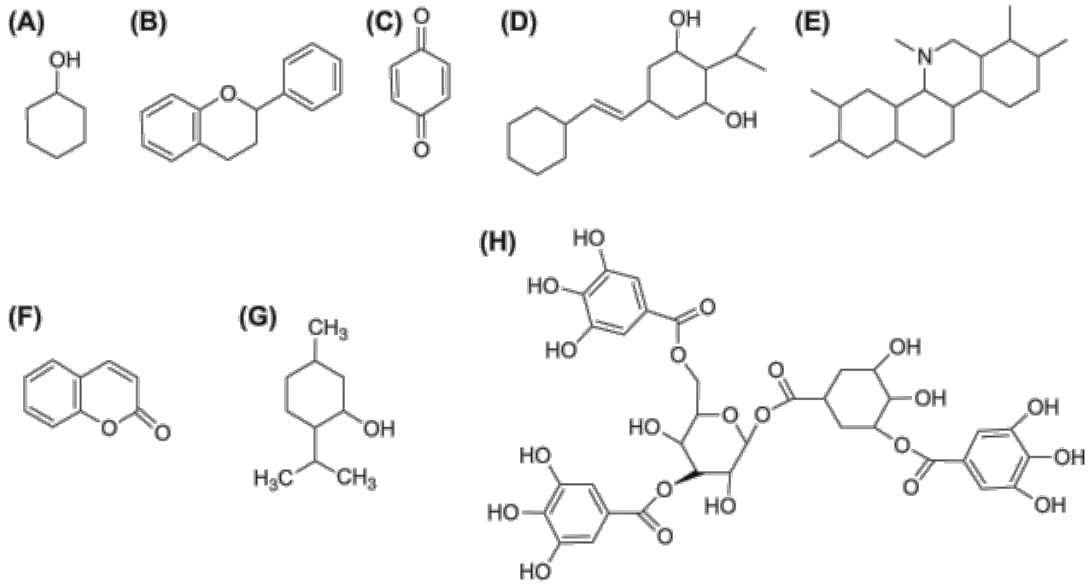

Figure 4: Structure of some bioactive compounds from plants (Egbuna et al., 2020):(A) phenol, (B) flavonoid, (C) quinone, (D) stilbenoid, (E) alkaloid, (F) coumarin, (G) terpenoid, (H) tannin.

With a comprehensive understanding of edible flowers and their benefits, let's delve deeper into the extraction techniques that reveal their potential.

2.2.2. Extraction technique

Extraction is the process of transferring compounds from a solid or liquid into a different solvent or phase, commonly used in analytical chemistry, natural product extraction, and pharmaceutical processing to isolate and purify specific compounds or substances (Zhang et al., 2018). There are two main types of extraction techniques: conventional and emerging techniques. Conventional methods include solvent extraction, which uses organic solvents to extract specific compounds, and liquid-liquid extraction, involving the exchange of compounds between two immiscible solvents (Molinari et al., 1996). Emerging techniques, such as supercritical fluid extraction, pressurized liquid extraction, and microwave-assisted extraction, offer advantages such as reduced solvent consumption, shorter extraction times, and higher extraction yields (Zhang et al., 2018). The choice of extraction technique depends on the nature of the compounds to be extracted, the matrix, and the desired purity of the final product. Each matrix has different needs for extraction variables, which should be considered to ensure efficient extraction and minimal loss of target compounds (Fotsing Yannick Stéphane et al., 2022).

Choosing the most suitable extraction method is crucial, considering the flower species and relevant tissues. This ensures both the yield and quality of the extract.

- ✓ **Maceration extraction:**

Maceration or heat-assisted (HAE) extraction is a conventional solid-liquid extraction technique that has been widely used for the extraction of bioactive compounds from various plant materials, including edible flowers (Gori et al., 2021). The technique involves soaking the plant material in a solvent, such as water or ethanol, at room temperature or under mild heating conditions to facilitate the extraction of the target compounds (Linz &Lunte, 2013). The use of heat can enhance the extraction efficiency by increasing the solubility of the target compounds in the solvent. However, the excessive consumption of time and energy is a major disadvantage of heat-assisted extraction. The optimization of the extraction conditions, such as the choice of solvent, temperature, and extraction time, is crucial for achieving high extraction yields and preserving the bioactive properties of the target compounds (Chuo et al., 2022).

After discussing the significance of edible flowers, we're ready to uncover their role as a source of bioactive compounds.

2.3. Bioactive compounds with potential use in the food industry

2.3.1. Natural ingredients vs artificial ingredients

Consumer preferences for "clean labels" are driving the substitution of synthetic compounds with natural ones. This trend is motivating the scientific community to explore new sources of natural alternatives, with a particular focus on utilizing agro-food by-products. Among the most prominently reported sources are edible flowers because they are rich in diverse compounds that can serve various purposes (Akhtar et al., 2019).

The different types of natural bioactive compounds found in the plants are as follows:

- ✓ **Alkaloids:** Defined as the group of naturally occurring plant product that contains mostly basic nitrogen atoms. Alkaloids are most diverse, effective, and medicinally important plant substances, bitter in taste, optically active, colorless, and crystalline or liquid at room temperature (Bhambhani et al., 2021). It was found that 20% of plant species contain alkaloids, but the major source of alkaloid is flowering plants (Saboon et al., 2019).

✓ **Polyphenols:** are one of the largest classes of phytochemicals and more than 8000 polyphenolic compounds have been identified in various plant species. They are only found in plants, and over 500 polyphenols have been identified in edible plants (Zagoskina et al., 2023).In food, polyphenols contribute toward the quality of food by giving oxidative stability, astringency, color, odor, flavor, bitterness, and nutrition (Saboon et al., 2019).

✓ **Carotenoids:** are a diverse group of pigments found in many fruits and vegetables, contributing to their yellow, orange, and red colors. Key carotenoids include beta-carotene, lutein, and zeaxanthin, which play essential roles in photosynthesis by capturing light energy and protecting chlorophyll from photodamage (Hashimoto et al., 2015). In addition to their function in plants, carotenoids are vital for human health, serving as antioxidants that help neutralize free radicals and reduce oxidative stress (Schiller et al., 2004).

✓ **Anthocyanins:**have garnered significant attention due to their potent antioxidant properties and numerous health benefits, including anti-inflammatory, anti-proliferative, and neuroprotective effects (Manzoor et al., 2022). These compounds play crucial roles in plant biology, such as attracting pollinators, protecting against UV radiation, and modulating reactive oxygen species signaling (Massa et al., 2022).

While both natural and artificial ingredients have their place in the food industry, there is a growing trend towards using natural ingredients with bioactive compounds due to their perceived health benefits and alignment with consumer preferences for clean, minimally processed foods. However, formulators often face challenges in achieving desired functionalities solely from natural sources, leading to a balance between both types of ingredients in some products (Rivas-García et al., 2021).

Natural bioactive compounds, such as alkaloids, polyphenols, carotenoids, and anthocyanins, are widely distributed in various plant matrices, including flowers, fruits, vegetables, and herbs (Zhao et al., 2019). These compounds are found in different parts of the plants, such as the petals, stamens, ovaries, and other floral components, as well as in the fruits, leaves, and roots of different plant species (Antolak&Kregiel, 2017). The specific composition and distribution of these bioactive compounds can vary significantly depending on factors such as the plant species, growth conditions, and environmental factors.

For example, phenolic compounds, which are widely distributed and an important group of bioactive compounds in plants, can be found in various plant matrices and are known for their antioxidant and other bioactive properties (Yasar et al., 2022). Similarly, alkaloids, polyphenols, carotenoids, and anthocyanins are present in different plant matrices and are

known for their diverse biological effects and potential health benefits (Sytar&Smetanska, 2022). The presence of these bioactive compounds in a wide range of plant matrices makes edible flowers, fruits, and vegetables valuable sources of natural bioactive compounds with potential health-promoting properties (Yasar et al., 2022).

As we progress through the exploration of edible flowers, the next section will uncover the intricate chemical properties that make them invaluable in various applications.

2.3.2. Application of bioactive compounds obtained from edible flowers as ingredients in industry

Current global market trends and government regulations are driving a shift towards industrial alternatives that prioritize sustainable and efficient production processes. Consequently, recent studies have concentrated on identifying and extracting bioactive compounds from various sources, aiming to incorporate them as natural ingredients into diverse food matrices (Dos Santos et al., 2018).In response to this, natural polymers, bioactive materials, and biocomposite substances are being proposed as alternative packaging technologies (Hosseini et al., 2022). These innovations not only aim to preserve the quality, safety, and sensory properties of food but also contribute to environmental preservation through their biodegradability and renewability (Takahashi et al., 2020).

Those bioactive compounds must follow the legal requirements and evaluations to assess the risks for human health and their toxicity must be considered before being launched into the market (Vettorazzi et al., 2020). To overcome the potential health risk while increasing the biological activity, stability and biodistribution of the supplements technological alternatives have been studied such as encapsulation of bioactive compounds into micro or nanoparticles or nano-emulsions. This will allow enhancing the stability and release along the gastrointestinal tract in a controlled manner into the specific tissues (Vilas-Boas et al., 2021).

The development of functional foods harnesses the potential of bioactive compounds, such as alkaloids, polyphenols, carotenoids, and anthocyanins, to enhance the nutritional value and provide health benefits in food products. Functional foods, which extend beyond basic nutrition, are designed to reduce the risk of specific chronic diseases and improve overall well-being (Vlaicu et al., 2023). By incorporating bioactive compounds, these foods offer health-promoting properties, including antioxidant, anti-inflammatory, and anti-cancer

effects. The utilization of bioactive compounds in functional foods is a subject of ongoing research, with a focus on identifying and incorporating these compounds from various sources, such as plants, fruits, vegetables, and animals (Martirosyan et al., 2022). The potential of bioactive compounds in functional foods lies in their ability to contribute to health promotion and disease risk reduction, making them valuable components in the quest for healthier food options. Additionally, the incorporation of bioactive compounds in functional foods aligns with the growing consumer demand for products that offer both nutritional benefits and positive health outcomes (Fernandes et al., 2019). This approach supports the development of innovative food products that can positively impact public health and well-being.

For example, the addition of polyphenols from fruits and vegetables to dairy products can enhance their antioxidant and anti-inflammatory properties, while the incorporation of carotenoids from fruits and vegetables into functional foods can improve their pro-vitamin A activity and antioxidant capacity(Nath et al., 2023).

The use of bioactive compounds from edible flowers offers opportunities for innovation and market differentiation in various industries, such as food and beverage production, pharmaceuticals, and cosmetics. By incorporating these compounds, companies can develop unique products that stand out in the market due to their health benefits and functional properties (Zhang et al., 2018).The utilization of bioactive compounds in pharmaceutical formulations offers potential in drug development, whether as active ingredients, supplements, or in formulations aimed at improving health outcomes (Skrajda-Brdak et al., 2020). Some edible flowers have been studied for their potential pharmaceutical applications, such as wound healing, hepatoprotective, and antinociceptive properties. These properties suggest that bioactive compounds from edible flowers could be used as active ingredients, supplements, or in formulations aimed at improving health outcomes (Yasar et al., 2022).

For instance, the industrial application of edible flowers has focused on the production of flower tea, jelly, salads, herbal infused drinks, candied flowers, and juice production, showcasing the incorporation of bioactive compounds into a variety of food and beverage products (Zhang et al., 2018).Additionally, edible flowers have been recognized as sources of valuable phytonutrients, including phenolic compounds, carotenoids, and tocols, which are biologically active low molecular phytochemicals. This recognition has led to the utilization of these compounds in the development of innovative and attractive food products that can improve the diet and provide health benefits (Pires et al., 2021).

Table 3: List of edible flowers related to beneficial properties and food use (Demasi, Mellano, et al., 2021).

Species (Common Name)	Flower Properties	Eaten in/as	References
Borago officinalis L. (borage)	Purifying, emollient, antitussive, diuretic, sudorific, anti-inflammatory	Salads, soups, desserts, syrups and drinks. Cucumber taste.	(Fernandes et al., 2019)
Rosa Canina L. (dog rose)	Anticancer, diuretic, laxative, anti-rheumatic, anti-inflammatory.	Salads, jellies, syrups, teas.	(Lattanzio et al., 2011)
Tropaeolum majus L. (nasturtium)	Disinfectant, antimicrobial, expectorant, diuretic, anti-inflammatory.	Salads, flavoring of soups, meat, pasta, cheese, vinegar. Peppery flavor.	(Garzón & Wrolstad, 2009)
Salviapratensis L. (meadow sage)	Anti-inflammatory, antibacterial, antiseptic, eupeptic.	Flavoring of butter, vinegar, oil, salads and creams, soups. Essential oil to flavor food.	(Guarrera & Savo, 2016)
*Robiniapseudoacacia*L. (black locust)	Antispasmodic, antiviral, diuretic, emollient, febrifuge, laxative, purgative, tonic.	Flavoring liquors, jams, honey, pancakes.	(Loizzo et al., 2016)
Lavandula angustifolia Mill. (lavender)	Antispasmodic, antiseptic, sedative, carminative, cicatrizing.	Flavoring and decoration of cakes, soups, salads, jellies. Essential oil to flavor food.	(Rashed et al., 2017)

Having examined the current landscape of research on flower compounds, we now turn our attention to the specific methods employed in our investigation. The following section outlines the experimental procedures used to evaluate the efficacy of flower extracts.

3. Objectives

With a focus on *Calendula officinalis* L. and *Rosa canina* L. the main objective of this thesis is to investigate the bioactive potential of edible flower mixtures and their suitability as a food alternative with noteworthy health advantages (**Figure 5**).

Thus, the work presents the following specific objectives:

i) Characterization of the phenolic profile of the flower mixtures.

ii) Evaluation of the bioactivities (antioxidant, antibacterial and antifungal of the flower mixtures.

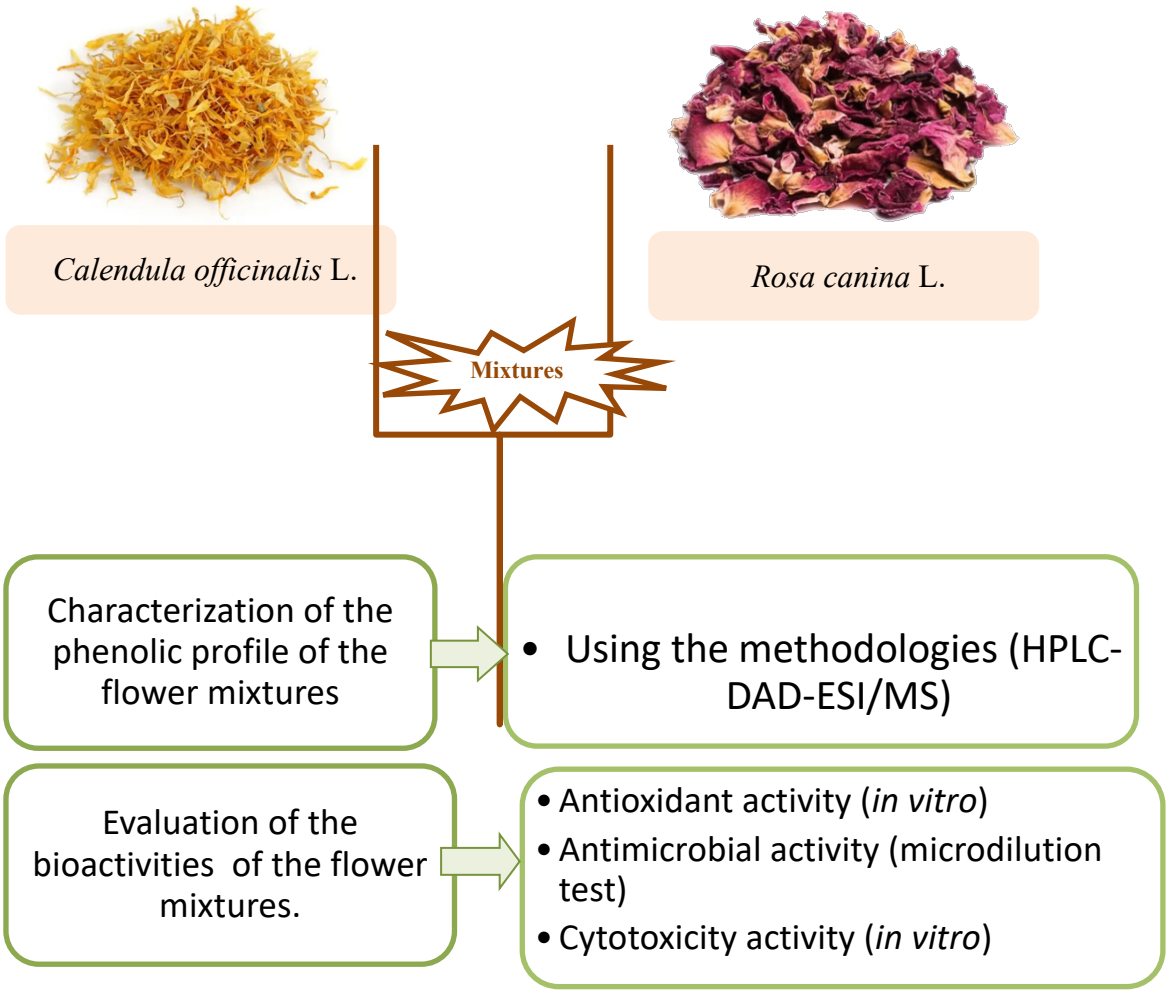

Figure 5: Diagram of the main objectives and procedures of this study.

4. Methodology

4.1 Sample collection and preparation

The samples were obtained from a local store in Bragança (Portugal), in a dry form as shown in **Figure 5**, where (A) indicates *Rosa Canina* L. and (B) indicates *Calendula officinalis* L.

Figure 6: The samples of (A) and (B) (Source: from the author)

For this study, a total of five samples were prepared to investigate the properties of (A) and (B), as illustrated in **Figure 6**. The samples were formulated based on their respective weights as follows:

R100%: This sample contained 50 mg of (A).

C100%: This sample contained 50 mg of (B).

R50%C50%: This sample contained equal parts of (A) and (B) with each contributing 25 mg (totaling 50 mg).

R25%C75%: This sample contained 12.5 mg of (A) and 37.5 mg of (B).

R75%C25%: This sample contained 37.5 mg of (A)and 12.5 mg of (B).

Figure 7: Sample weights of (A) and (B) Mixtures (Source: from the author)

All samples were accurately weighed using an analytical balance to ensure precision (**Figure 7**) and were then subjected to maceration extraction (**Figure 8**) for subsequent assays. The process involved several key steps: first, an appropriate solvent mixture of 80% ethanol and 20% water was chosen to optimize extraction. The plant material was immersed in the solvent for one hour, allowing for the release of bioactive compounds, followed by an additional hour of immersion. After maceration, the mixture was filtered through a 0.22 µm filter to remove solid residues. The alcoholic fraction was then evaporated using a rotary evaporator to eliminate ethanol, while the aqueous fraction was lyophilized at 47°C to preserve heat-sensitive compounds and ensure the final extracts were stable for analysis.

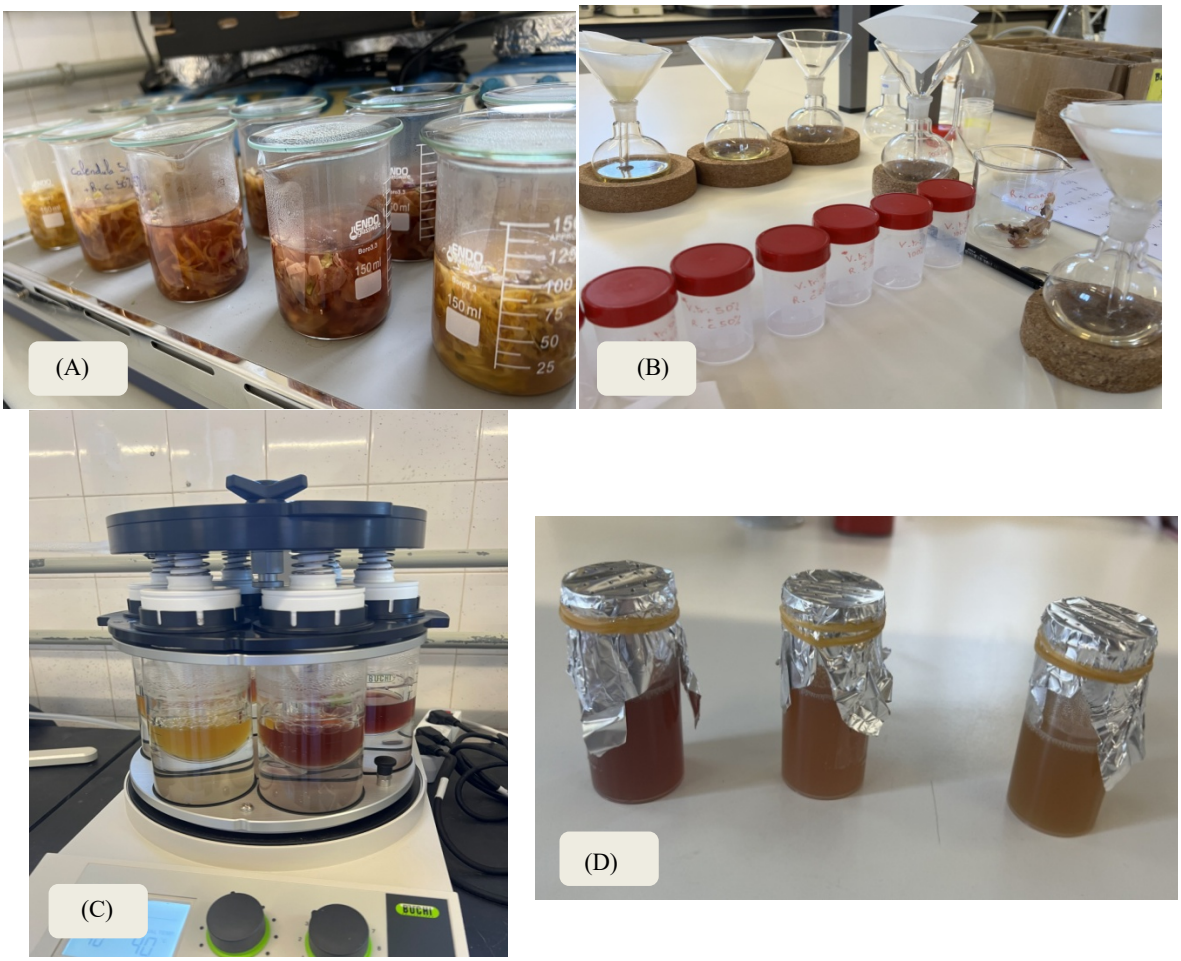

Figure 8: Procedures of the maceration extraction: **(**A): 50 mg of the extraction by maceration was dissolved in 2 mL of a 20:80 (v/v) ethanol:water on a plate at room temperature; (B): The solution filtered through 0.22 µm LC disposable discs; (C):Evaporation of the alcoholic fraction of the extract using the rotary evaporator; (D):Lyophilization of the aqueous fraction (47°C). (Source: from the author)

4.2 Determination of the individual phenolic profile of flower mixtures

The procedure of identification and quantification of the phenolic was performed according to Bessada et al. (2016).

For HPLC analysis, 10 mg of the dry extract was re-dissolved in 2 mL of a 20:80 (*v/v*) ethanol:water solution and then filtered through 0.22 µm LC disposable discs.

The UFLC system utilized was equipped with a quaternary pump, an automatic injector (set at 5°C), a degasser, and a column compartment with an automated thermostat. To detect phenolic compounds, a diode array detector (DAD) was employed, operating at wavelengths of 280, 330, and 370 nm, in conjunction with a mass spectrometry detector (MS).

The reverse-phase column used was a Waters Spherisorb, functioning at 35°C, with the mobile phase consisting of formic acid/water (A, 0.1%) and acetonitrile (B). The elution gradient was isocratic and followed this sequence: 10-15% B for 5 minutes, 15-20% B for 5 minutes, 20-25% B for 10 minutes, 25-35% B for 10 minutes, and 35-50% B for 10 minutes, followed by a 10-minute column rebalancing. The flow rate was maintained at 0.5 mL/min, and the HPLC system was connected to a mass spectrometer (MS).

For compound detection in the MS, a Trap Linear LTQ XL mass spectrometer was used, equipped with an electrospray ionization source (ESI). Nitrogen was used as the carrier gas at 50 psi. The initial temperature was set at 325°C, with a capillary voltage of -20 V, and the tube lens offset voltage maintained at -66 V. Spectra were recorded in negative ion mode across a range of 100 to 1500 *m/z*, with a collision energy of 35 arbitrary units. Data collection and analysis were conducted using the Xcalibur® software.

Compound identification was achieved by comparing the acquired data with literature values and, where possible, with standards. To create calibration curves for quantitative analysis, standard solutions with known concentrations were injected. Results were expressed in mg of compound per g of extract; if standards for specific compounds were unavailable, quantification was performed using calibration curves for compounds within the same phenolic group.

4.3 Determination of bioactive potential profile of flower mixtures

4.3.1 Evaluation of antioxidant activity of flower mixtures

The evaluation of antioxidant activity of the flower mixtures was conducted using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, following the protocol established by Barros et al. (2013).

Initially, the flower mixtures were prepared based on defined compositions (0.6, 0.3, 0.15, 0.07, 0.03, 0.015, 0.0075 and 0.0038 mg/ml). A DPPH solution was then prepared by dissolving a specific amount of DPPH in methanol to achieve a concentration of 0.1 mM.

In the subsequent step, a predetermined volume of the flower extract was added to the DPPH solution in test tubes, maintaining a consistent extract-to-DPPH ratio across all samples. The reaction mixture was incubated in the dark at room temperature for

approximately 30 minutes to facilitate the interaction between the DPPH radicals and the antioxidants present in the flower extracts.

After incubation, the absorbance of the resulting solution was measured at 517 nm using a spectrophotometer, with a decrease in absorbance indicating the scavenging ability of the antioxidants. The antioxidant activity was calculated using this equation:

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

Which A_{DPPH} corresponds to the absorbance of the control and A_A to the absorbance of the sample.

4.3.2. Evaluation of antimicrobial activity of flower mixtures

4.3.2.1. Antibacterial activity

The antimicrobial activity was evaluated using the microdilution method by applying INT dye (*p*-iodonitrotetrazolium chloride) (Dias et al., 2016).

For clinical bacteria, the bacterial strains were clinical isolates obtained from patients hospitalized in various departments at the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal). The tested Gram-negative bacteria included *Escherichia coli* (VRU12881), *Proteus mirabilis* (VRU17684), *Klebsiella pneumoniae* (VRI17214), *Pseudomonas aeruginosa* (VRU14123) and *Morganella morganii* (VRU14272) and three Gram-positive bacteria: *Enterococcus faecalis* (VRU14041), *Listeria monocytogenes* (VRU17684), and methicillin-resistant *Staphylococcus aureus* (MRSA)(VRI17654).

Regarding food contaminants, the extracts were tested against five Gram-negative bacteria, namely *Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica subsp* (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610) and three Gram-positive bacteria, namely *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111) and *Staphylococcus aureus* (ATCC 25923). All these microorganisms are purchase at Frilabo, Porto, Portugal. The bacteria's were incubated at 37 ºC an appropriate fresh medium, for 24 h before analysis to maintain the exponential growth phase.

The microdilution method was used to determine the minimum Inhibitory Concentration (MIC) for all the analyzed extracts. Bacterial cultures were standardized to a concentration of 1×10^5 CFU/mL in each well using a densitometer. For the preparation of

extracts 50 mg of the extract were dissolved in 5% (*v/v*) Dimethyl sulfoxide (DMSO) with autoclaved distilled water to a final volume of 2.5 mL, obtaining an initial concentration of 20 mg/mL.In96-well plates, 100 µL of the prepared solution was pipetted in duplicate into the first well containing 90 µL of tryptic soy broth (TSB), as well as the remaining wells. Successive dilutions of the solution were then performed, resulting in different concentrations. Finally, 10 µL of inoculum was added to all wells and the microplates were incubated under the previously described grow conditions.

After the incubation period, 40 µL of iodonitrotetrazolium chloride (INT) (0.2 mg/mL) were added to all wells, and the microplates were incubated at 37°C for 30 minutes. The results were evaluated considering the change in color to pink.

Concerning the MBC (Minimum Bactericidal Concentration), it was determined by sub-cultures in series, which consists of adding 10 µL from wells that did not change color in microplates containing 100 µL of TSB. The MBC was defined as the lowest concentrations that did not show growth once this subculture is achieved. For the clinical bacteria, the positive controls were ampicillin, imipenem and vancomycin whereas for food bacteria streptomycin, methicilin and ampicillin were used as controls. The MIC and MBC results were expressed in mg/mL.

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

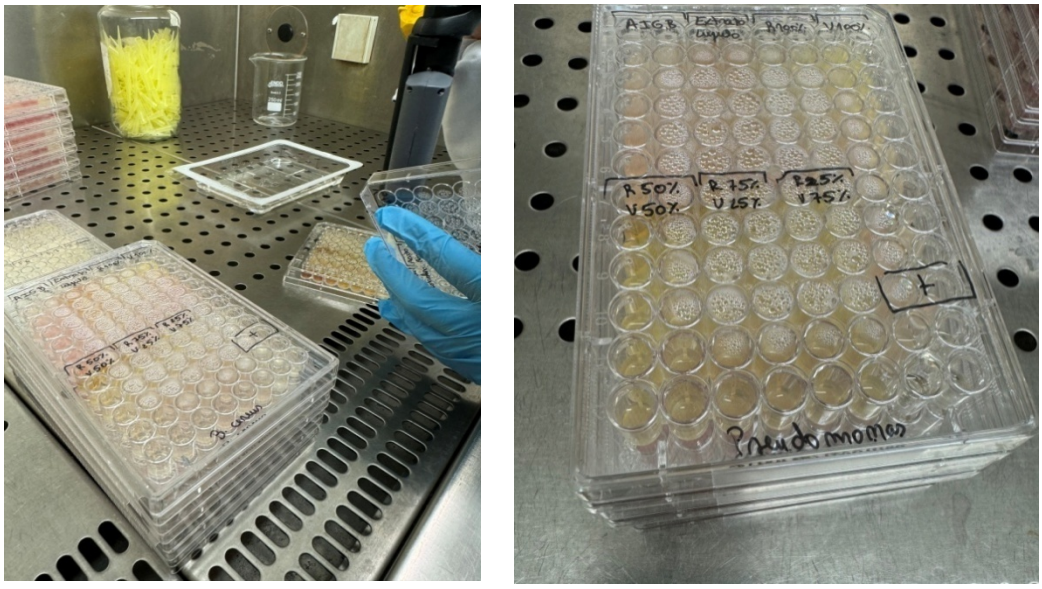

Figure 9: Procedures for the evaluation of antibacterial activity (Source: from the author)

4.3.2.2. Antifungal activity

For the evaluation of antifungal activity, two micromycetes were used: *Aspergillus brasiliensis* (ATCC16404)and *Aspergillus fumigatus*(ATCC 204305).These micromycetes were cultured on malt agar (MA) and stored at 4°C, with monthly sub-culturing to maintain their viability. Spores were collected from the agar plates using a 0.85% sterile saline solution containing 0.1% (*v/v*) Tween 80. The spore suspension was adjusted to approximately 1.0×10^5 CFU/mL in a final volume of 100 µL per well. Inocula were kept at 4°C for later use. To ensure the absence of contamination and the validity of the inoculum, dilutions were grown on solid MA. The minimum inhibitory concentration (MIC) was determined using a serial dilution method in 96-well microplates.

The sample solution was added to the malt medium with the fungal Inoculum, and the microplates were incubated at 28°C for 72 hours. The lowest concentration that showed no visible growth (assessed with a binocular microscope) was recorded as the MIC. Minimum fungicidal concentrations (MFCs) were determined by sub-culturing 2 µL into microplates containing 100 µL of malt broth, followed by incubation at 28°C for 72 hours. The lowest concentration with no visible growth was designated as the MFC, indicating a 99.5% reduction of the original inoculum. Results for MIC and MFC were expressed in mg/mL.

4.3.3 Cytotoxicity

For hepatotoxicity evaluation, the extracts were re-dissolved in water to obtain stock solutions of 4 mg/mL and then submitted to further dilutions., a porcine liver cells primary culture (PLP2) was prepared from a freshly harvested porcine liver obtained from a local slaughterhouse, according to a procedure established by the authors (Abreu et al., 2011). Tissues were washed in Hank's saline solution containing 100 U/mL penicillin and 100 µg/mL streptomycin and divided into 1x1 mm^3 explants. The explants were placed in culture boxes with DMEM medium supplemented with FBS (Fetal Bovine Serum; 10%), 2 mM non-essential amino acids, 100 U/mL penicillin and 100 µg/mL streptomycin and placed in the incubator. The culture medium was changed every 2 days, monitoring using an inverted microscope (Icon Eclipse Ts 100). Cells were transferred to a 96-well plate at a density of $1x10^4$ cells/well, and cultured in DMEM medium with 10% FBS, 100 U/mL penicillin, and

100 μg/mL streptomycin. The cells were treated with different concentrations of sample and the SRB (Sulforhodamine B) assay was performed as described in detail by the authors. Ellipticine was used as the standard. The results were expressed in GI_{50} values (concentration responsible for 50% inhibition of cell growth).

4.4. Statistical analysis

The quantitative results are reported as mean ± standard deviation, except for the antibacterial activity and cytotoxicity results. We conducted statistical analyses to identify significant differences among the various mixtures, and was carried out through a one-way ANOVA analysis of variance, taking into account the different types of comparisons. Each table specifies the statistical test used. For this purpose, the RStudio program, version 4.1.1 was used with the multcomp package.

With the methodology outlined, we will now turn our attention to the results and discussion.

5. Results and Discussion

5.1. Evaluation of individual phenolic profile

The **Table 4** presents a comprehensive analysis of individual phenolic compounds across five different samples, labeled as C100%, R100%, C50% R50%, C25% R75%, and C75% R25%, likely representing varying ratios of plant extracts. The result reveals a diverse profile of 35 phenolic compounds, including flavonoids, phenolic acids, and their derivatives, with concentrations varying widely from trace amounts to as high as 19.9 ± 0.1mg/mL.

The analysis of total phenolic acids, total flavonoids, and total phenolic compounds across different formulations of *R. canina* and *C. officinalis* reveals significant variations in phytochemical profiles. The R100% sample of *R. canina* exhibits the highest total phenolic acids at 55.6 ± 0.5 mg/mL, significantly surpassing other samples. This finding aligns with research by Veneziano (2004), which reported that *R. canina* is a rich source of phenolic compounds, known for their antioxidant and antimicrobial properties. In contrast, the C100% sample of *C. officinalis* shows a much lower total phenolic acids value of 1.58 ± 0.04 mg/mL, suggesting less phytochemical richness compared to *R. canina*.

In terms of total flavonoids, the R100% formulation again ranks lowest at 27.3 ± 0.3 mg/mL, while the C100% formulation demonstrates a robust level of 46.4 ± 0.2 mg/mL. This finding supports studies like those by Garcia-Amezquita et al. (2018), which highlight the superior flavonoid content in *C. officinalis* compared to *R. canina*.

Mixed formulations, particularly C25%R75% and C75%R25%, show moderate total flavonoid levels (20.6 ± 0.3 mg/mL and30.4 ± 0.1 mg/mL, respectively), suggesting that combining these extracts can yield beneficial phytochemical properties.

Moreover, total phenolic compounds further emphasize *Rosa canina*'s potency, with R100% yielding 83.2 ± 0.6 mg/mL compared to 47.9 ± 0.2 mg/mL in C100%. This is consistent with findings from Zhang and Yao (2011), which indicated that *R. canina* extracts contain higher concentrations of beneficial compounds compared to those from *C. officinalis*.

The results indicate that targeted combinations of *R. canina* and *C. officinalis* not only preserve the individual benefits of each plant but may also boost their overall

effectiveness, positioning them as promising candidates for the development of natural antimicrobial agents.

Additionally, our findings demonstrate that maceration is an efficient technique for extracting phenolic compounds from edible flowers, achieving concentrations comparable to or greater than those found in existing literature. This underscores maceration's potential as a practical extraction method for exploring the health-promoting properties of edible flowers.

Table 4: Quantification of individual phenolic compounds (mg/g) in different formulation of edible flowers

Peak	RT(min)	λmax(nm)	M-H	MS^2(*m/z*)	Tentative identification	C100%	R 100%	C50%R50%	C25%R75%	C7
1	4.21	273	483	331, 313, 169	Digalloylhexoside	n.d	13.43 ±0.3[a]	9.92 ± 0.2[b]	n.d	6.
2	4.21	274	343	191, 169	Galloylquinicacid	n.d	13.43 ± 0.3[a]	n.d	13.55 ± 0.1[a]	
3	4.24	273	191	111, 173	Quinicacid	t.r	n.d	9.92 ± 0.2[a]	8.44 ± 0.1[b]	6.
4	4.3	273	627	465, 447, 489, 303, 285	Taxifolin-*O*-hexoside-*O*-hexoside	n.d	8.91 ± 0.2	n.d	n.d	
5	4.3	273	633	301, 615, 481, 331, 249, 275	HHDP galloyl glucose	n.d	13.43 ±0.3	n.d	n.d	
6	5.75	270	785	301, 483, 633, 419, 331, 275, 249	Digallolyl HHDP glucose	n.d	1.8 ± 0.1[a]	n.d	0.8 ± 0.1[b]	
7	5.76	326	353	191, 179, 173, 135	5-Caffeoylquinic acid	0.210 ± 0.001[a]	n.d	0.1600 ± 0.0003[b]	n.d	0.2
8	6.74	330	341	179	Caffeicacidhexoside	0.28 ± 0.01	n.d	n.d	n.d	
9	8.26	271	953	909	Unidentifiedellagitannin	n.d	1.80 ± 0.03[a]	n.d	0.8 ± 0.1[b]	
10	8.39	325	353	191, 173, 179, 135	Chlorogenicacid	0.73 ± 0.04[b]	n.d	0.45 ± 0.01[c]	0.24 ± 0.02[d]	0.8
11	10.41	321	179		Caffeicacid	0.28 ± 0.01[a]	n.d	0.064 ± 0.001[c]	0.06 ± 0.01[d]	0.12
12	14.46	256	755	301, 300, 609, 489, 591	Quercetin-O-rhamnosylhesoxide-rhamnoside	1.08 ± 0.01[a]	0.41 ± 0.01[e]	0.490 ± 0.003[c]	0.43 ± 0.03[d]	0.77
13	15.15	310	313	153, 181	Maloylsyringicacid	0.080 ± 0.001[a]	n.d	0.007 ± 0.001[c]	n.d	0.02
14	16.03	256	609	300, 489	Quercetin-O-rhamnosylhexoside	0.590 ± 0.004[a]	0.29 ± 0.01[c]	0.25 ± 0.01[d]	0.21 ± 0.01[e]	0.3
15	16.41	355	739	284, 285, 575, 593	Kaempferol-O-rhamnosylhexoside rhamnoside	0.170 ± 0.003[a]	0.10 ± 0.01[b]	0.090 ± 0.001[c]	n.d	0.10
16	16.83	254	769	314, 315	Isorhamnetin-O-rhamnosylhexoside rhamnoside	16.82 ± 0.04[a]	n.d	6.32 ± 0.05[c]	3.14 ± 0.04[d]	9.3
17	17.71	255	609	301	Rutin	0.38 ± 0.01[d]	1.320 ± 0.001[a]	0.63 ± 0.01[c]	1.29 ± 0.02[b]	0.6
18	18.91	350	623	314, 315	Isorhamnetin-O-rhamnosylhexoside	2.11 ± 0.01[b]	n.d	1.62 ± 0.02[c]	n.d	2.7
19	18.96	255	463	301	Quercetin-3-O-glucoside	n.d	6.69 ± 0.02[a]	1.62 ± 0.02[d]	5.6 ± 0.1[b]	2.7
20	20.3	254	609	315, 459, 477	Isorhamnetin-O-hexosylpentoside	1.23 ± 0.01[a]	n.d	0.360 ± 0.004[c]	n.d	0.6
21	20.32	256	615	315, 463	Quercetingalloylhexoside	n.d	0.4 ± 0.1[b]	n.d	0.540 ± 0.004[a]	
22	20.55	256	433	301	Quercetin-O-pentoside	n.d	0.7 ± 0.1[a]	n.d	0.39 ± 0.02[b]	
23	20.92	270	435	211, 271, 313, 253	Gallicacidderivative	n.d	1.39 ± 0.02[a]	0.68 ± 0.01[c]	1.33 ± 0.01[b]	0.28
24	20.94	355	593	282	Kaempferol-O-rutinoside	0.18 ± 0.01[a]	n.d	0.070 ± 0.001[b]	n.d	
25	21.88	354	623	315	Isorhamnetin-O-rutinoside	19.9 ± 0.1[a]	n.d	5.13 ± 0.02[c]	3.9 ± 0.1[d]	9.5
26	22.50	350	447	301	Quercetin-O-rhamnoside	n.d	5.9 ± 0.1[a]	0.41 ± 0.02[d]	3.51 ± 0.03[b]	0.5
27	23.04	360	709	665	Isorhamnetin malonyl rhamnosylhexoside	0.44 ± 0.02[a]	n.d	0.160 ± 0.001[c]	n.d	0.270
28	23.34	255	477	314, 315	Isorhamnetin-*O*-hexoside	0.84 ± 0.04[a]	n.d	0.14 ± 0.01[d]	0.17 ± 0.01[c]	0.
29	24.23	360	599	285, 313	Kaempferol-O-galloylhexoside	n.d	0.39 ± 0.01	n.d	n.d	
30	27.12	360	431	285	Kaempferol-*O*-rhamnoside	n.d	0.48 ± 0.02[a]	0.070 ± 0.001[c]	0.310 ± 0.001[b]	0.06
31	27.35	258	651	609, 300, 301	Quercetinacetylrutinoside	n.d	1.1 ± 0.2[a]	0.070 ± 0.001[c]	0.29 ± 0.03[b]	0.05
32	27.73	355	563	519	Isorhamnetinmalonylhexoside	2.5 ± 0.01[a]	n.d	0.8 ± 1.2[c]	0.47 ± 0.01[d]	1.5
33	29.12	360	519	314, 315, 459	Isorhamnetinacetylhexoside	0.100 ± 0.004	n.d	n.d	n.d	
34	30.74	350	635	285, 593, 575	Luteolinacetylrutinoside	n.d	0.220 ± 0.003[a]	n.d	0.140 ± 0.002[b]	
35	32.16	360	301	151, 179	Quercetin	n.d	n.d	n.d	n.d	0.24
					Total Phenolicacids	1.58 ± 0.04[e]	55.9 ± 0.5[a]	21.2 ± 0.3[c]	26.8 ± 0.2[b]	13
					Total Flavonoids	46.4 ± 0.2[a]	27.3 ± 0.3[c]	18.4 ± 1.2[e]	20.6 ± 0.3[d]	30
					Total Phenolic compounds	47.9 ± 0.2[b]	83 ± 1[a]	39.6 ± 1.2[d]	47.4 ± 0.4[b]	44

Retention time (Rt), wavelengths of maximum absorption in the UV-visible region (λmax), mass spectral data (MS), tentative identification and quantification (mg/g of extract) of phenolic compounds present in the extracts of the different mixtures of flowers (C100%, R100%, C50%R50%, C25%R75% and C75%R25%), n.d.: not detected.

In each row different letters mean significant differences between samples ($p< 0.05$), where "a" and "e" correspond to the highest and lowest values, respectively.

5.2.Evaluation of bioactive properties of flower mixtures

5.2.1. Antioxidant Activity

The **Table 5** presents the antioxidant activity of various flower mixtures, measured by the DPPH assay. The DPPH antioxidant activity results demonstrate that the antioxidant capacity of the samples varies significantly, with lower values indicating stronger activity. The sample C75% R25% shows the most potent antioxidant activity, with a DPPH value of 0.0072 ± 0.0002 mg/ml, suggesting a highly effective ability to scavenge free radicals. Following this, the sample C25%R75% demonstrates considerable antioxidant potential with a value of 0.030 ± 0.001 mg/ml.

In contrast, the C100% sample has a much higher DPPH value of 0.40 ± 0.01 mg/ml, indicating weaker antioxidant activity. Overall, the results clearly indicate that the combinations of *Rosa Canina* and *Calendula* samples at varying percentages influence antioxidant activity, with the most effective combinations exhibiting significantly lower DPPH values.

Table 5: Antioxidant activity of flower mixture

Samples	C100%	R100%	C50% R50%	C25% R75%	C75% R25%
DPPH (mg/ml)	0.40 ± 0.01^{a}	0.078 ± 0.002^{c}	0.131 ± 0.004^{b}	0.03 ± 0.001^{d}	0.0072 ± 0.0002^{e}

Different letters mean significant differences between samples ($p< 0.05$), where "a" and "e" correspond to the highest and lowest values, respectively.

A study by Ulu (2004) demonstrated that extracts of *Rosa canina* exhibited a DPPH value of approximately 0.02 mg/ml, reflecting its potent antioxidant capacity. This is comparable to our most effective sample, C75% R25%, which also showed strong scavenging activity. Additionally, *Rosa canina* is known for its high vitamin C content, which contributes to its antioxidant properties, as highlighted by Stuper-Szablewska et al. (2022), who reported significant DPPH scavenging effects in various extracts of *Rosa canina*.

Moreover, the study conducted by Ghasemzadeh et al. (2016)indicated that different extraction methods significantly influence the antioxidant activity of *Calendula*, with some methods yielding DPPH values as low as 0.03 mg/ml, which emphasizes the potential for optimizing extraction techniques to enhance antioxidant capacity.

Furthermore, a comparative study by Moayyedkazemi et al. (2021) analyzed the antioxidant activities of several medicinal plants, including both *Calendula officinalis* and *Rosa canina*, and found that extracts from these flowers were among the most effective in scavenging DPPH radicals, highlighting their importance in natural antioxidant sources. This suggests that not only do these flowers individually possess strong antioxidant properties, but their potential synergy when combined could further enhance their effectiveness.

5.2.2. Antibacterial and antifungal activity

The results of antibacterial activity from clinical isolates are presented in table 6 and reveal significant variability in the efficacy of various treatment formulations against both Gram-negative and Gram-positive bacteria, as measured by minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC), considering that the maximum tested concentration was 10 mg/mL.

The antibacterial activity results of R100% sample, representing *Rosa canina*, displayed a notable MIC of 2.5 mg/mL against *Enterococcus faecalis*, which aligns with previous findings that highlight the antimicrobial properties of this plant. For instance, (Ulu, 2004)reported MIC values for *R. canina* extracts ranging from 5 to 10 mg/mL against similar Gram-positive bacteria, indicating that our R100% sample demonstrates enhanced potency.

In contrast, the R50% C50% sample, which combines 50% *R. canina* and 50% *C. officinalis*, showed moderate effectiveness against several bacterial strains, including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Listeria monocytogenes*, with MIC values of 5 mg/mL. This level of antibacterial activity suggests that the combination of these two plants can enhance their antimicrobial effects compared to when they are used individually. Research by Guilhon-Simplicio et al. (2017) has demonstrated that *C. officinalis* exhibits significant antibacterial activity against a range of Gram-positive and Gram-negative bacteria, further supporting the effectiveness of this combination.

The synergistic effects observed in our R50% C50% sample may be attributed to the phytochemical profiles of both plants, which contain bioactive compounds known for their antibacterial properties. Research has shown that combining extracts can enhance antimicrobial efficacy; for example, studies by Soares and Sato (2000) demonstrated that synergistic combinations of plant extracts often yield lower MIC values compared to single extracts alone.

This reinforces the notion that formulations leveraging the strengths of both *R. canina* and *C. officinalis* could be particularly effective, especially against a range of pathogens. Overall, our findings contribute to the growing body of literature suggesting that specific combinations of natural extracts offer a viable alternative to traditional antibiotics.

Table6: Antibacterial activity (MIC and MBC, mg/mL) of the clinical bacteria

											Positive Control					
	R100%		C100%		R50% C50%		R75% C25%		R25% C75%		Ampicillin (10mg/mL)		Imipenem (1mg/mL)		Vancomycin (1mg/mL)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria																
Escherichia coli	10	>10	>10	>10	5	>10	10	>10	10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	10	>10	>10	>10	5	>10	10	>10	10	>10	10	>10	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	5	>10	>10	>10	10	>10	10	>10	10	>10	>10	>10	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	10	>10	>10	>10	10	>10	10	>10	10	>10	<015	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	10	>10	>10	>10	5	>10	10	>10	>10	>10	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria																
Enterococcus faecalis	2.5	>10	>10	>10	10	>10	10	>10	>10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Listeria monocytogenes	5	>10	10	>10	5	>10	10	>10	10	>10	<0.15	<0.15	n.t.	n.t.	0.25	0.5
MRSA	10	>10	>10	>10	10	>10	10	>10	10	>10	<0.15	<0.15	n.t.	n.t.	0.25	0.5

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; n.t. Not Tested.

The **Table 7** summarizes the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of various formulations against different bacterial strains, including both Gram-negative and Gram-positive bacteria.

Notably, the R100% sample of *R. canina* exhibited the lowest MIC values against Gram-positive bacteria, specifically *Bacillus cereus* and *Listeria monocytogenes*, at 2.5 mg/mL, indicating strong antimicrobial potential. This finding aligns with studies by Stuper-Szablewska et al. (2022), which reported similar low MIC values for *R. canina* extracts against Gram-positive strains, emphasizing the species effectiveness in targeting these pathogens. Additionally, a study by Mothana et al. (2011) also supports this, finding that *R. canina* extracts significantly inhibited the growth of various Gram-positive bacteria, further confirming its potential as a natural antimicrobial agent.

In contrast, the C100% sample of *C. officinalis* showed higher MIC values of 10 mg/mL against all tested bacterial strains, indicating less potency compared to *Rosa canina*. This lower efficacy aligns with findings from Ghasemzadeh et al. (2016), who reported that while *C. officinalis* extracts do possess antibacterial properties, they typically require higher concentrations to effectively inhibit bacterial growth. This may be due to the differing phytochemical compositions between the two plants, as *R. canina* is known to have a higher concentration of phenolic compounds, which are potent antibacterial agents.

However, the combination samples, particularly R50% C50% and R75% C25%, maintained moderate effectiveness across various strains, with MIC values ranging from 5 to 10 mg/mL. This mixed approach is supported by research from Ghasemzadeh et al. (2016), which highlighted the enhanced antibacterial activity of mixed plant extracts. Their study demonstrated that combining extracts from different plants can lead to synergistic effects, resulting in lower MIC values compared to individual extracts alone. This is particularly relevant in our findings, where the mixtures of *R. canina* and *C. officinalis* show improved antimicrobial activity, suggesting that such combinations could be explored further for their potential in treating infections.

Additionally, Shukla et al. (2021) highlighted the influence of different extraction methods on the antibacterial activity of plant extracts. They found that certain methods resulted in enhanced activity against resistant strains, suggesting that optimization of extraction parameters is crucial for maximizing efficacy. This aligns with our findings that treatment conditions significantly impact the antibacterial properties of the tested formulations.

Collectively, these studies underscore the potential of natural compounds as antimicrobial agents and align closely with our findings, emphasizing the importance of further research into the antibacterial properties of plant extracts to develop effective treatments against bacterial infections.

Table7: Antibacterial activity (MIC and MBC, mg/mL) of food contaminants bacteria

											Positive Control					
	R100%		C100%		R50%C50%		R75%C25%		R25%C75%		Streptomicin 1mg/mL		Methicilin 1mg/mL		Ampicillin 10mg/mL	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negativebacteria																
EnterobacterCloacae	5	>10	10	>10	5	>10	5	>10	5	>10	0.007	0.007	n.t.	n.t	n.t.	n.t.
Escherichia coli	10	>10	10	>10	10	>10	10	>10	10	>10	0.01	0.01	n.t.	n.t.	n.t.	n.t.
Pseudomonas aeruginosa	5	>10	10	>10	5	>10	10	>10	10	>10	0.06	0.06	n.t.	n.t.	n.t.	n.t.
Salmonella enterica	5	>10	10	>10	5	>10	5	>10	5	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Yersiniaenterocolitica	5	>10	10	>10	10	>10	5	>10	10	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Gram-positivebacteria																
Bacillus cereus	2.5	>10	10	>10	5	>10	5	>10	10	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Listeriamonocytogenes	2.5	>10	10	>10	5	>10	5	>10	5	>10	0.007	0.007	n.t.	n.t.	0.25	0.5
Staphylococcus aureus	10	>10	10	>10	5	>10	5	>10	10	>10	0.007	0.007	0.007	0.007	0.25	0.5

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

The antifungal activity of the tested mixtures against *Aspergillus brasiliensis* and *Aspergillus fumigatus* was assessed by measuring the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC), as shown in **Table 8.** The maximum concentration tested was 10 mg/mL for all formulations.

Notably, both *Aspergillus brasiliensis* and *Aspergillus fumigatus* displayed a MIC of 10 mg/mL for the C100%, C50%/R50% and C25%R75% formulations, indicating a lack of efficacy at these concentrations, as evidenced by the MFC values exceeding 10 mg/mL. Conversely, the R100% and C75%/R25% formulations exhibited a MIC of 5 mg/mL against *Aspergillus brasiliensis*, suggesting a moderate antifungal effect.

However, for *Aspergillus fumigatus*, all tested formulations resulted in a MIC of 10 mg/mL, showing that this strain was resistant to the antifungal effects of the formulations under the tested conditions. These findings underscore the varying susceptibility of fungal strains to antifungal agents and highlight the importance of optimizing formulations for enhanced efficacy against specific pathogens.

Table8: Antifungal activity (MIC/MFC, mg/mL)

	Aspergillus brasiliensis	***Aspergillus fumigatus***
	MIC/MFC	**MIC/MFC**
C100%	10/>10	10/>10
R100%	5/>10	10/>10
C50%/R50%	10/>10	10/>10
C75%/R25%	5/>10	10/>10
C25%/R75%	10/>10	10/>10

In comparison to previous studies, the antifungal activity observed in our formulations aligns with findings from various researchers, demonstrating the potential of plant-derived compounds in combating fungal infections. A comprehensive study by Rayanakorn et al. (2020) reported that formulations derived from *R. canina* and *C. officinalis* exhibited MIC values ranging from 5 to 15 mg/mL against *Aspergillus* species. This finding is particularly relevant, as our R100% formulation of *R. canina* achieved a MIC of 5 mg/mL against *A.brasiliensis*, demonstrating comparable efficacy.

Further supporting these findings, Khoshnoudi-Nia and Moosavi-Nasab (2019) demonstrated that certain essential oils could inhibit *A. fumigatus* with MIC values between 10 and 20 mg/mL. This reinforces the notion that resistance in this fungal strain is common and requires further exploration of alternative extraction methods and formulations. Our results, showing varying degrees of effectiveness across different formulations, align with this observation and highlight the importance of optimizing extraction and formulation techniques.

The significance of extraction parameters was emphasized by Das et al. (2021), who noted that optimized conditions can significantly enhance the antifungal properties of plant extracts. This finding resonates with our results, suggesting variations in activity among different formulations and underscoring the need for careful consideration of extraction methodologies in developing effective antifungal agents.

Adding to this body of knowledge, Iqdiam et al. (2021) investigated the synergistic effects of combining different plant extracts, reporting enhanced antifungal activity against *Candida albicans* when certain compounds were used in combination. Their work achieved MIC values as low as 2.5 mg/mL for some synergistic formulations, suggesting potential avenues for improving the efficacy of our extracts through strategic combinations.

Lastly, Jankowska and Łozowicka (2022) conducted a comparative study of antifungal activities across different plant families, finding that extracts from certain families consistently outperformed others against a range of fungal pathogens. Their work provides a broader context for our findings, suggesting that the source of our plant extract may play a crucial role in its antifungal potency.

5.3.Cytotoxicity activity

In cytotoxicity evaluation, the results showed that none of the mixtures demonstrated any toxic effects in non-tumoral cells, highlighting their potential for application in the food industry as safe natural ingredients. This finding is particularly significant given the growing consumer demand for natural additives and the increasing scrutiny on synthetic food preservatives.

Previous studies have documented low cytotoxicity levels for *R. canina* extracts. For instance, Zhang and Yao (2011) reported that the methanol extracts of *Rosa canina* exhibited minimal cytotoxic effects against various human cell lines, suggesting their safety

for consumption and potential use in food products. This aligns with our findings that indicate the absence of toxicity in our mixtures.

Similarly, research on *C. officinalis* reinforces its reputation as a safe natural ingredient. Garcia-Amezquita et al. (2018) found that *Calendula* extracts not only possess antimicrobial properties but also demonstrate low toxicity, making them suitable for incorporation into food formulations. The study highlighted that *Calendula* is often used as a natural coloring and flavoring agent in the food industry due to its benign profile.

Moreover, a study by García-Segovia et al. (2020) also supports the safety of these plants, indicating that their bioactive compounds can provide health benefits without compromising safety.

These insights collectively underscore that our mixtures, which combine *R. canina* and *C. officinalis*, can be confidently regarded as safe, effective, and beneficial natural ingredients for the food industry.

6. Conclusion

In conclusion, this study demonstrates the efficacy of maceration extraction in obtaining bioactive compounds from edible flower mixtures. The comprehensive analysis of individual phenolic profiles and bioactive potential, encompassing antimicrobial, antibacterial, and antifungal activities, provides compelling evidence for the potential of these flower mixtures as functional food ingredients.

Our findings consistently highlight the superior performance of two mixtures: R50%C50% and R75%C25%. These balanced mixtures of edible flowers exhibited the most promising results across multiple parameters among the five samples tested. Both formulations showcased diverse and rich phenolic profiles, indicating a high concentration of bioactive compounds. Furthermore, R50%C50% and R75%C25% demonstrated exceptional antimicrobial properties, effectively inhibiting the growth of various pathogenic microorganisms. The observed antibacterial and antifungal activities further underscore the potential of these formulations as natural preservatives and functional food additives.

Importantly, cytotoxicity assays revealed that both R50%C50% and R75%C25% mixtures maintain favorable safety profiles, indicating their potential for incorporation into food products without adverse effects on human health.

The promising results obtained from the R50%C50% and R75%C25% edible flower mixtures pave the way for exciting future applications in the food industry. These formulations, with their rich bioactive profiles and favorable safety characteristics, have the potential to enhance both the nutritional value and functional properties of various food products.

7. Future perspectives

Future research should focus on incorporating these mixtures into a wide range of foods, such as functional beverages, dairy products, baked goods, and culinary innovations. Additionally, their strong antimicrobial properties suggest potential use as natural food preservatives. To fully realize these applications, further studies are needed to optimize extraction and processing methods for large-scale production, conduct sensory evaluations, perform stability studies, and carry out *in vivo* research to confirm the health benefits observed *in vitro*.

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