“BABEȘ-BOLYAI” UNIVERSITY FROM CLUJ-NAPOCA
Faculty of Biology and Geology
Doctoral School of Integrative Biology

Antioxidant, Antimicrobial, and Antitumor Properties of *Allium* Ursinum (Wild Garlic) Leaf Extracts: A Comparison Between Conventional Maceration and Ultrasound-Assisted Extraction Techniques.

-SUMMARY-

PhD student:
Oravetz (căs. Moldovan) Kinga

Supervisor:
Prof. dr. Rakosy-Tican Elena

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CHAPTER I – From plants to therapies: phytocompounds in the fight against cancer and resistant bacteria

1. Phytocompounds: definition, classification, and role

Plants have been used for therapeutic purposes since ancient times, when various extracts were used to treat different diseases. The path to using plants in therapy has been marked by numerous trials and errors, and gradually, humans transformed this practice into one of the oldest sciences—phytotherapy (Jamshidi-Kia et al., 2017). The knowledge transmitted over the years, supported today by scientific evidence, demonstrates that plants are a valuable source of phytochemicals with both preventive and therapeutic roles, contributing to the reduction of major chronic disease risks (Liu, 2004).

Phytochemicals are classified into six major groups, including phenolic compounds, alkaloids, nitrogen-containing compounds, organosulfur compounds, phytosterols, and carotenoids, with phenolic compounds being the most extensively studied (Liu, 2004, 2013).

Phenolic compounds exhibit numerous therapeutic roles and have been extensively investigated. Caffeic acid and chlorogenic acid are recognized for their antioxidant and cardioprotective properties, reducing blood pressure (Agunloye et al., 2019). Gallic acid has been shown to protect cells from oxidative (Dludla et al., 2019). Vanillic acid exerts antitumor effects by inhibiting hypoxia-inducible factor-1 (HIF-1), a key factor in cancer cell adaptation to low oxygen environments (Gong et al., 2019). Among flavonoids, naringenin and hesperidin have demonstrated cardioprotective, antiviral, and antitumor effects against breast, prostate, lung, and colon cancer (Motallebi et al., 2022; Pyrzynska, 2022; Stabrauskiene et al., 2022; Syahputra et al., 2022). Flavones and isoflavones exhibit anti-inflammatory, antimicrobial, and anticancer properties (D. Catarino et al., 2015; Jiang et al., 2016). Quercetin has notable antioxidant effects, neutralizing free radicals such as hydrogen peroxide, superoxide, and hydroxyl radicals (Deepika & Maurya, 2022). Furthermore, quercetin has demonstrated antitumor activity against breast (H. Zhang et al., 2014), ovarian (Gao et al., 2012), colorectal (Darband et al., 2018), and liver cancer (Fernández-Palanca et al., 2019), as well as significant antibacterial activity against various antibiotic-resistant bacterial species (Nguyen & Bhattacharya, 2022).

Thus, the consumption of a diverse range of plant-based products provides an array of nutrients and bioactive compounds with multiple therapeutic properties, contributing to the reduction of various chronic diseases (Liu, 2004).

2. Epidemiology of colorectal and breast cancer: cancers with increased incidence

Colorectal cancer (CRC) and breast cancer are two of the most common types of cancer worldwideCommon Cancer Sites - Cancer Stat Facts).

2.1. Incidence of breast and colorectal cancer

According to data provided by GLOBOCAN in 2022, breast cancer is the second most common type of cancer globally, following lung cancer, with approximately 2.3 million new cases diagnosed that year. This accounts for 11.5% of all new cancer cases worldwide across both sexes. Breast cancer is the leading cause of cancer-related mortality among women, representing 15.4% of total cancer deaths and causing 666,103 deaths globally (Global Cancer Observatory, n.d.).

Colorectal cancer (CRC) is the third most frequently diagnosed cancer worldwide, following breast and lung cancer, and is the third leading cause of cancer-related deaths. CRC accounts for approximately 9.8% of all cancer cases, with 1.85 million new cases reported annually. This type of cancer affects both men and women, with a slightly higher incidence in men (Marcellinaro et al., 2023).

The average age at diagnosis is 68 years for men and 72 years for women. The high CRC incidence in developed countries is associated with factors such as longer life expectancy and an aging population, which contribute to an increased burden of colorectal cancer (Goodarzi et al., 2019).

2.2. Risk factors

The occurrence of breast cancer and colorectal cancer (CRC) is influenced by a variety of non-modifiable and modifiable risk factors, reflecting similarities between the two conditions. Among the common non-modifiable factors are age, sex, genetic predisposition, family history, and medical history. Modifiable risk factors are largely influenced by lifestyle, diet, physical activity, alcohol consumption, and smoking, which are common modifiable factors for both types of cancer (Haggar & Boushey, 2009; Majeed et al., 2014).

2.3. Current prevention and treatment strategies for colorectal and breast cancer

Ranked third in terms of incidence, CRC has become a financial burden and a global challenge; therefore, prevention strategies are crucial for reducing CRC risk and the number of new cases (Xi & Xu, 2021). Unlike other types of cancer, CRC is a disease that can be prevented, and it is estimated that approximately 75% of CRC cases could be avoided by adopting a healthy lifestyle (Giovannucci, 2001).

2.3.1. Primary prevention

Certain studies have highlighted that genetic defects are responsible for only 5%-10% of all cancer cases, with the remaining 90%-95% being attributed to environmental and lifestyle factors. Thus, the best strategy for combating cancer is prevention (Anand et al., 2008). Primary prevention aims to reduce disease incidence by promoting a healthy lifestyle and proactive approaches to eliminate risk factors. It targets healthy individuals before the disease manifests and

includes a combination of interventions focused on lifestyle modifications, diet, pharmacological interventions, and, in some cases, surgical interventions.

2.3.2. Secondary prevention

Secondary prevention focuses on early cancer detection, before symptoms become visible, to enable early intervention and reduce disease progression. It includes screening methods that help identify individuals at high risk of cancer, aiming to reduce mortality through early detection (Kisling & Das, 2025).

2.3.3. Tertiary prevention

Tertiary prevention aims to reduce the impact of the disease after its onset by preventing complications, recurrences, and metastases, thereby improving patients' quality of life (Kisling & Das, 2025). In the case of cancer, tertiary prevention may include a combination of surgical interventions, radiotherapy, and chemotherapy to remove the tumor from the body and prevent its recurrence (Feinleib, 2001).

3. Antibiotic resistance: the impact of phytocompounds on multidrug-resistant bacteria

Managing pathogens that cause numerous infectious diseases has always been a global priority. Moreover, due to cross-contamination and antibiotic overuse, resistant microbial strains have emerged, making this issue far more complex and urgent (Kraemer et al., 2019).

Antimicrobial resistance (AMR) is a globally recognized problem, estimated to be directly responsible for 1.27 million deaths worldwide in 2019 and contributing to 4.95 million deaths. The World Health Organization (WHO) emphasizes the importance of prudent antibiotic use and the urgent development of new antibiotic classes, as in the past 40 years, only two new classes of antibiotics have been developed (World Health Organisation, 2015).

Furthermore, despite the wide range of antibiotics available on the global pharmaceutical market, there is a critical need to identify new potential antimicrobial agents that utilize novel antibacterial mechanisms to prevent cross-resistance and provide effective therapeutic options against infections caused by antibiotic-resistant bacteria. Given the resistance of biofilm structures to current antibacterial treatments, the development of effective antibiofilm agents is also urgently needed (Ivanov et al., 2022).

Thus, exploring plant-based antimicrobials to address the AMR crisis represents a new research direction, offering an opportunity to discover new therapeutic solutions that have the potential to provide safe and effective alternatives to conventional antibiotics, to which many bacteria have developed resistance (Ranjbar & Alam, 2023).

CHAPTER II. Phytocompounds from the *Allium* genus with medicinal properties

Plants from the *Allium* genus have been cultivated since antiquity, being valued for their culinary and medicinal properties. The most well-known representatives of this genus—*Allium cepa* (onion), *Allium sativum* (garlic), *Allium porrum* (leek), and *Allium ascalonicum* (shallot)—are distinguished by their numerous therapeutic properties, including hypoglycemic, hypolipidemic, antimicrobial, anti-inflammatory, antitumor, and platelet aggregation inhibitory effects, which have been recognized and utilized in traditional medicine (Rabinowitch & Goldstein, 2020).

The therapeutic properties of these plants are attributed to active compounds that can be extracted or sometimes isolated in their pure form, providing a valuable basis for their medicinal applications (Stănescu et al., 2002). Through the processing of these plants, extracts have been obtained that, depending on the solvent and plant organs used for extraction, differ qualitatively and quantitatively in terms of chemical composition and therapeutic action. This diversity contributes to their versatility in both traditional and modern medicine (Rodrigues & Delerue-Matos, 2022).

1. Compounds with medicinal properties from the *Allium* genus

Species within the *Allium* genus generally share a similar chemical composition due to their phylogenetic relationships, but variations can occur in concentration and proportion of these compounds, which may influence the biological activity of these species. The most common phytochemicals include organosulfur compounds, polyphenols, saponins, vitamins, volatile oils, and prostaglandins (Beretta et al., 2017). These bioactive compounds provide numerous health benefits. Variations may arise due to genetic differences, environmental factors, plant developmental stages, interactions with abiotic factors, and post-harvest treatments (Shahid et al., 2022; Vuković et al., 2023).

For example, garlic is known for its high allicin content, a sulfur compound formed through the action of the enzyme alliinase on alliin. The characteristic taste and smell of these plants are mainly attributed to thiosulfinates and alliin (S-allyl cysteine sulfoxide). In addition to allicin, degradation products of alliin include diallyl disulfides, ajoene, and vinyl dithiins (Jabbes et al., 2012). The breakdown of thiosulfinates produces sulfides such as diallyl disulfide, allyl methyl sulfide, and allyl methyl disulfide (Lanzotti et al., 2014).

Beyond their aromatic properties, these sulfur compounds provide a range of therapeutic benefits, being responsible for their anticancer (Khanum et al., 2004), antidiabetic (Eidi et al., 2006), anti-inflammatory (Lee et al., 2012), antimicrobial (Harris et al., 2001; Noman et al., 2023), antioxidant (Capasso, 2013; Chen et al., 2013), cardioprotective (Higuchi et al., 2003), and immunomodulatory properties (Schafer & H. Kaschula, 2014). Additionally, garlic is one of the

richest sources of polyphenolic compounds, making it an important dietary source of polyphenols (Martins et al., 2016).

In addition to allicin and polyphenols, garlic also contains lectins, prostaglandins, fructan, pectin, lipids, adenosine, vitamins B1, B2, B6, C, and E, biotin, nicotinic acid, fatty acids, glycolipids, phospholipids, minerals, and essential amino acids (Fenwick & Hanley, 1985). Certain saponins, such as β-chlorogenin, have antifungal, antibacterial, antitumor, anti-inflammatory, and hypocholesterolemic effects (Lanzotti, 2006; Rekowska & Skupień, 2009). Among these medicinal phytochemicals, sulfur compounds, flavonoids, and phenolic compounds are the most extensively studied (Lanzotti et al., 2014).

2. Phytocompounds from the *Allium* genus with antitumor potential in colorectal cancer

Since Weisberger and Pensky (1958) demonstrated *in vitro* and *in vivo* that garlic extract inhibits tumor cell growth, research on garlic has significantly increased (Weisberger & Pensky, 1958). Alongside garlic, other *Allium* species have also been studied to determine their chemopreventive effects, particularly regarding tumor growth inhibition and cell proliferation suppression. Studies show that garlic and onion reduce the risk of sarcomas and carcinomas in various organs, including the stomach, colon, esophagus, prostate, liver, lungs, skin, and brain (Corzo-Martínez et al., 2007).

Garlic (*Allium sativum*), a bulbous plant from the *Amaryllidaceae* family, is one of the most extensively studied species within the *Allium* genus, with a vast scientific literature demonstrating its antitumor effects. As mentioned, garlic contains a complex mix of metabolites, including phenolic compounds, flavonoids, carotenoids, alkaloids, and organosulfur compounds (OSCs) (Ansary et al., 2020). Among these, the bioactive components that appear to prevent CRC include organosulfur compounds such as alliin, allicin, ajoene, diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), S-allylcysteine (SAC), and S-allylmercaptocysteine (SAMC) (Mitra et al., 2022; Nagini, 2008; Omar & Al-Wabel, 2010).

Thus, garlic can be considered an excellent model for illustrating the antitumor potential of the entire *Allium* genus, due to its high content of bioactive compounds and its important role in human nutrition, making it one of the most extensively studied plants for its beneficial effects, particularly in colorectal cancer prevention.

Since other *Allium* species contain similar active compounds that contribute to their antitumor effects, they will not be detailed in this chapter. Therefore, this chapter reviews the latest updates on the antitumor activity of *Allium sativum*, as the main representative of the *Allium* genus, both *in vitro* and *in vivo*, along with its phytochemical constituents responsible for such biological activities, focusing on the cellular and molecular mechanisms of action.

2.1. *In vitro* antitumor mechanisms of action of phytocompounds from the *Allium* genus in colorectal cancer

By studying the phytochemical mechanisms of garlic and designing effective treatments, scientists have used well-characterized colorectal cancer (CRC) cell lines as biological models. To support *in vitro* studies, 10 CRC cell lines, such as Caco-2, DLD-1, HCT-15, HCT116, HT-29, COLO 205, SW480, and SW620, have been used over the years.

Numerous *in vitro* studies have demonstrated the antiproliferative (cytostatic), apoptosis/necroptosis-inducing (cytotoxic), anti-migration, anti-invasion, and immunomodulatory activities of garlic and its constituents against colorectal cancer.

2.2. *In vivo* antitumor mechanisms of action of phytocompounds from the *Allium* genus in colorectal cancer

The *in vivo* activity of several bioactive compounds from *Allium sativum* has been investigated to determine their ability to inhibit CRC carcinogenesis and/or tumor progression in various animal models. The antitumor activity of DAS, DADS, and DATS was studied in thirty-six-week-old male BALB/c nude mice previously injected with COLO 205 cells. Two of these compounds, DADS and DATS, but not DAS, significantly suppressed tumor xenografts (both weight and size) in this animal model, with DATS being the most cytotoxic among all tested compounds (Lai et al., 2012). DATS was also found to be effective in mice injected with CT-26 cells, where a reduction in both tumor volume and weight was observed (Wu et al., 2011). However, both DATS and DADS were shown to promote the expression of multiple multidrug resistance genes *in vivo*, including *MDR1*, *MRP1*, *MRP4*, and *MRP6*, a factor that should be considered in future studies (Lai et al., 2012).

3. Phytocompounds from the *Allium* genus with antitumor potential in breast cancer

3.1. *In vitro* antitumor mechanisms of action of phytocompounds from the *Allium* genus in breast cancer

Although epidemiological studies have not shown a significant correlation between garlic consumption and a reduced risk of breast cancer, *in vivo* and *in vitro* studies have demonstrated that various garlic compounds and whole garlic extracts significantly reduce breast cancer risk (Tsubura et al., 2011). These studies suggest that garlic and its compounds may interfere through multiple molecular mechanisms, including the inhibition of intracellular signaling pathways that contribute to tumor cell development, survival, and proliferation, promotion of cell death, and reduction of metastases. These effects are associated with changes in the expression of key proteins involved in cell cycle regulation and cellular migration and invasion processes (Pandey et al., 2023). Their mechanisms of action include apoptosis induction through specific signaling

pathways, inhibition of cell proliferation, and blocking metastasis processes by modulating key proteins such as Bcl-2, Bax, and E-cadherin.

3.2. *In vivo* antitumor mechanisms of action of phytocompounds from the genus *Allium* in breast cancer

Numerous researchers have also investigated the in vivo activity of garlic extracts and their compounds in Swiss albino mice, BALB/c mice, Sprague-Dawley rats, and severe combined immunodeficiency (SCID) mice. Garlic extracts and their compounds exhibit chemopreventive, antitumor, and anti-metastatic activity. Their mechanisms of action include apoptosis stimulation through the regulation of Bax and Bcl-2 protein expression, blockade of signaling pathways involved in metastasis, and downregulation of metalloproteinases MMP-2 and MMP-9, as well as key angiogenesis factors such as VEGF-A and HIF-1α. Furthermore, the inhibition of ALDH1 suggests that these compounds may contribute to the depletion of cancer stem cells, which are responsible for relapse and metastasis (Oravetz et al., 2023).

4. Phytocompounds from the *Allium* genus with antibacterial action

Numerous studies on the antibacterial effects of raw garlic extracts have investigated their activity against both commensal and pathogenic bacterial species, (*including Escherichia coli, Staphylococcus aureus, Salmonella typhi, Listeria monocytogenes,* multidrug-resistant (MDR) STEC *(*Shiga toxin-producing *Escherichia coli), Campylobacter jejuni, Vibrio parahaemolyticus, Mycobacterium spp., MRSA (*methicillin-resistant *Staphylococcus aureus), Bacillus subtilis, Streptococcus mutans, Clostridium difficile, Clostridium perfringens, Bacteroides* spp.*, and Lactobacillus casei* (Daka, 2011; Jain et al., 2015; Kumar & Berwal, 1998; Roshan et al., 2017; Viswanathan et al., 2014; Vuddhakul et al., 2007).

The synergistic effect between garlic extracts and antibiotics has been demonstrated in several studies, where the combination of fresh garlic extract with gentamicin enhanced antimicrobial efficacy against resistant strains such as MRSA and ESBL-producing Klebsiella pneumoniae (Magryś et al., 2021).

5. The species *Allium ursinum*: general characteristics and biological activities

5.1. General characteristics of the plant

Ramsons (*Allium ursinum*) is a monocotyledonous plant belonging to the *Amaryllidaceae* family and is commonly known by various names such as gypsy or forest garlic (Lachowicz et al., 2017). It is a wild plant widely distributed in the forests of Europe and Northern Asia (Schmitt et al., 2005). It grows at altitudes of up to 1900 meters and no further than 64° north latitude, extending from the Mediterranean region to Scandinavia. It prefers humid, shaded areas but can adapt to a variety of climatic and soil conditions (Oborny et al., 2011). The plant emerges in spring,

usually between March and June, before deciduous trees such as beech and oak fully leaf out, which is where it is commonly found (Jandl et al., 1997).

Ramsons is a perennial vernal geophyte, reaching up to 50 cm in height and propagating through seeds (Sobolewska et al., 2015). Contractile roots begin developing around the third year and function to pull the bulb deeper into the soil, securing it and facilitating nutrient uptake. The bulb is narrow and elongated, measuring approximately 1.5–6 cm in length, and is surrounded by layers of transparent tunics (Clapham et al., 1990). The leaves are elliptical, bright green on the upper surface, and matte on the underside. The leaf blade (lamina) is entire, elliptical-lanceolate in shape, measuring up to 25 cm in length and 7 cm in width, while the petiole can reach up to 20 cm (Clapham et al., 1990). A comparative study of specimens collected from different regions of Poland demonstrated variations in leaf width, petiole and stem length, and the number of flowers per inflorescence. These differences are likely due to adaptations to local environmental factors, including soil type, light availability, and climate (Błażewicz-Woźniak & Michowska, 2011). The umbel-like inflorescences consist of 3-30 star-shaped white flowers, surrounded by 2-3 bracts. Each flower has six tepals measuring 16–20 mm, a superior trilocular ovary, a simple stigma, and stamens shorter than the perianth (Błażewicz-Woźniak & Michowska, 2011). Flowering typically begins in April and ends in the first half of May.

5. 2. Biological activities of *Allium ursinum* species

Literature data have shown that *Allium ursinum* exhibits anti-inflammatory, antioxidant, antitumor, antimicrobial, and cardioprotective activities.

Numerous studies analyzing extracts from different parts of *A. ursinum* have demonstrated that all of them exhibit antioxidant activity (Putnoky et al., 2013; Štajner et al., 2006). Additionally, Pavlović et al. (2017) reported that *A. ursinum* extracts have superior antioxidant capacity compared to other wild *Allium* species (Pavlović et al., 2017). This superiority was also confirmed in a comparative study analyzing extracts from garlic, ramsons, chives, red onion, yellow onion, and white onion, ranking *A. ursinum* second after garlic in terms of antioxidant activity

Allium ursinum exhibits antimicrobial activity, with its potential as a natural antibacterial agent supported by a large number of studies highlighting the effectiveness of *A. ursinum* extracts against numerous bacterial strains, both Gram-positive and Gram-negative (Ivanova et al., 2009; Lupoae et al., 2013; Putnoky et al., 2013; Rankovic et al., 2021; Tomšik et al., 2019).

The available literature data regarding the antitumor effects of *A. ursinum* extracts are very limited. One study reported low to moderate cytotoxicity of methanolic extracts from *A. ursinum* leaves against murine B16 melanoma and XC sarcoma cell lines (Sobolewska et al., 2006). Additionally, cytotoxic effects have been demonstrated against HeLa (human cervical

adenocarcinoma) and LS174 (human colon adenocarcinoma) cell lines (Stanisavljević et al., 2020) as well as against AGS and MKN74 (gastric cancer cells) (Singleton et al., 1999; Xu et al., 2013).

Allium ursinum extracts have been characterized by strong cardioprotective activity demonstrated through platelet aggregation inhibition. Ethanolic extract exhibited inhibitory activity against aggregation induced by adenosine diphosphate (ADP), with a mechanism of action similar to that of clopidogrel, a class of antiplatelet drugs (Hiyasat et al., 2009).

Aim and objectives of the study

Aim of the study

Optimization of the extraction of phytochemicals from Allium ursinum to preserve their biological properties

Objectives of the study

Objective 1. Preparation of plant extracts from *Allium ursinum* leaves using conventional maceration (CM) and ultrasound-assisted extraction (UAE) for comparative analysis.

Objective 2 Biochemical characterization of methanolic plant extracts obtained from *A. ursinum* leaves using ultrasound-assisted extraction (UAE) and conventional maceration (CM), in terms of polyphenol and sulfoxide content, through spectrophotometric methods and LC/ESI+-MS.

Objective 3. Determination of the biological activity of *A. ursinum* extracts in terms of antioxidant activity using the CUPRAC method and hydrogen peroxide scavenging assay, antibacterial activity using the diffusion method on five pathogenic strains, and antitumor activity on two breast cancer and two colorectal cancer cell lines, using the MTT method.

Objective 4. Comparative analysis of the efficiency of ultrasound-assisted extraction (UAE) versus conventional maceration (CM) in terms of extraction yield and exerted biological activity.

CHAPTER III. Materials and methods

1. Plant material and preparation of plant extracts

The plant material consisted of *Allium ursinum* leaves. These were collected in April 2019 from the wild flora of the Cărbunari Forest, Dumbrăvița Commune, Maramureș County, Romania (47°34'28.4"N 23°40'13.9"E).

After collection, the leaves were immediately prepared for the drying process, being left to air-dry naturally in a controlled environment at room temperature, protected from direct light exposure, for a period of four weeks.

Allium ursinum leaves were extracted using 70% methanol (12.5 mL/g) via conventional maceration (CM) and ultrasound-assisted extraction (UAE).

To remove the solvent, methanol was evaporated at 40°C under reduced pressure using a rotary evaporator. The dried residues of both extracts were weighed and subsequently used for the preparation of stock solutions at a concentration of 100 mg/mL. Methanol 70% was chosen as the solvent for biochemical analysis, while dimethyl sulfoxide (DMSO) was used for cytotoxicity experiments, with extracts in both solvents being used for antibacterial activity screening. Before performing the analytical procedures, the extracts were further filtered through a filter with a pore size of 0.22 μm.

2. Qualitative and quantitative chemical analysis of *Allium ursinum* extracts

To emphasize the spectrum of compounds in the two extracts and to quantify the bioactive principles, advanced analytical methods were used, such as high-performance liquid chromatography (HPLC) and spectrophotometric analysis.

2.1. Determination of the total polyphenol and flavonoid content using spectrophotometric methods

The determination of the total polyphenol content characteristic of each extract was performed using the Folin-Ciocâlteu colorimetric method (Singleton et al., 1999) with adaptations.

The total flavonoid content (TFC) of each extracts was determined using a spectrophotometric method based on the formation of a flavonoid-aluminum complex, following the methodology described by Zhishen and collaborators (Zhishen et al., 1999).

2.2. Identification and quantification of phenolic compounds and sulfoxides by LC/ESI$^+$-MS

In this study, phenolic compounds and sulfoxides were identified and quantified using liquid chromatography (LC) combined with mass spectrometry (MS), employing electrospray ionization in positive mode (ESI$^+$) (LC/ESI$^+$-MS). This method allowed the efficient separation of compounds, followed by their detailed analysis based on mass spectra.

To identify the compounds and assign the corresponding peaks on the chromatograms, the retention time (Rt), UV-VIS absorption spectrum, and mass spectrum of each phytoconstituent were compared with the peaks of commercial standards under the same chromatographic conditions. The standards included chlorogenic acid, caffeic acid, quercetin-rutinoside, quercetin-glucoside, ellagic acid, and myricetin.

For the quantification of phenolic compounds, standard calibration curves were first determined for gallic acid and rutin. Specifically, the aliin content was determined using a calibration curve based on gallic acid (R^2 = 0.9978), within a concentration range of 10–100 µg/mL, while the flavonol content was evaluated using a calibration curve based on rutin (R^2 = 0.9981) with the same concentration range.

3. Determination of the biological activity of the extracts

3.1. Determination of the antioxidant activity of the extracts

The antioxidant activity of the extracts was determined using two methods: the cupric reducing antioxidant capacity (CUPRAC) assay and the hydrogen peroxide scavenging method (HPS).

The results were expressed as Trolox equivalents in micromoles per liter of sample (TE µmol/L), based on a standard curve previously determined using different concentrations of Trolox (Fig. 1).

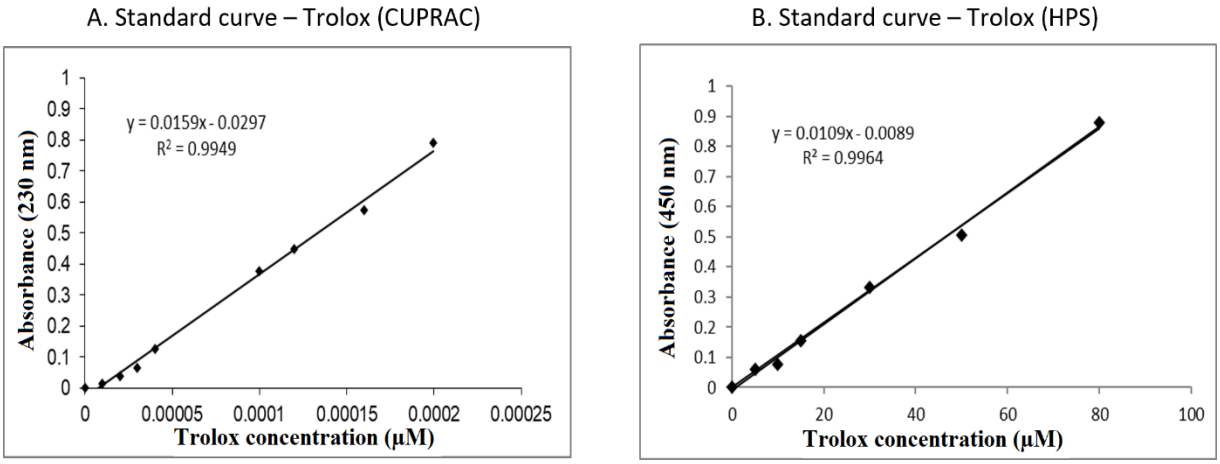

Fig. 1. Calibration curves for Trolox standards in **A.** the CUPRAC method and **B**. the hydrogen peroxide scavenging (HPS) method, used for determining the antioxidant activity of the two plant extracts of *A. ursinum.*

3.2. *In vitro* evaluation of the antimicrobial activity of methanolic extracts of *A. ursinum*

The antimicrobial activity of the methanolic extracts of *Allium ursinum* was assessed using the diffusion method, a widely recognized technique for determining bacterial susceptibility or resistance to various antimicrobial substances (Bălan, 2022).

For the antibacterial testing of wild garlic extracts, five reference bacterial strains were used: *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Streptococcus mutans* ATCC 25175, *Porphyromonas gingivalis* ATCC 33277, and *Escherichia coli* ATCC 25922.

The results were recorded by measuring the diameter of the inhibition zones using a ruler, with larger diameters indicating higher bacterial sensitivity to the tested extract (Carpa et al., 2014).

3.3. *In vitro* evaluation of the antitumoral activity of methanolic extracts of *A. ursinum*

3.3.1. Cell lines and culture conditions

The antitumor activity of *Allium ursinum* extracts was tested on two breast cancer cell lines (T47D and MDA-MB-231) and two colorectal carcinoma cell lines (DLD-1 and HT29).

All the cell lines used in this study were purchased from the European Collection of Authenticated Cell Cultures (ECACC) and cultured in RPMI-1640 medium.

Cells were maintained in an incubator at 37°C in a humidified atmosphere (90% humidity) with 5% CO_2.

3.3.2. Determination of the antitumor activity of the extracts using the MTT assay

The presence of viable cells in cultures incubated with extracts was assessed using the MTT assay.

In essence, cells were seeded in 96-well plates at an initial density of 2×10^4 cells/well, with 200 µL of culture medium per well. After 24 hours, the culture medium in each well was replaced with 180 µL of fresh medium, to which the plant extracts were added in nine progressively increasing concentrations: 50, 100, 150, 200, 250, 350, 500, 750, and 1000 µg/mL, with each concentration tested in six technical replicates. The treatment, administered in a volume of 20 µL per well, consisted of a mixture of the stock extract solution (100 mg/mL concentration) and sterile physiological saline.

After an incubation period of 48 hours at 37°C, in a 5% CO_2 atmosphere, the supernatant was removed, and 100 µL of MTT solution was added to each well. After one hour of incubation, the MTT solution was removed, and 150 µL of 100% DMSO (Carl Roth GmbH) was added to each well to solubilize the formazan formed. Absorbance was measured spectrophotometrically at a wavelength of 570 nm.

To exclude the possibility that the solvent used in the final extracts, DMSO, influenced cell viability, separate experiments were conducted. DMSO (≤1%) showed no cytotoxicity on breast (T47D, MDA-MB-231) and colorectal (DLD-1, HT29) cancer cell lines.

CHAPTER IV – Results

1. Extraction yields using conventional maceration and ultrasound-assisted extraction techniques

The extracts from *A. ursinum* leaves were obtained using 70% methanol, starting from 4 g of dried plant material, employing two alternative extraction techniques: conventional maceration (CM) and ultrasound-assisted extraction followed by maceration (UAE+CM).

It was observed that the extraction yield for the conventional maceration (CM) method was 41.325%. On the other hand, the combined UAE+CM method exhibited greater efficiency, yielding an extraction rate of 45.825% (Table 1).

Table 1. Extraction yield for the two plant extracts obtained through conventional maceration (CM) and the ultrasonication technique followed by maceration (UAE+CM), starting from 4 g of dried plant.

Extract	Dried plant material [DW (g)]	Extracted powder [DW (g)]	Yield (%)
CM	4 g	1.653 g	41.325 %
UAE+CM	4 g	1.833 g	45.825 %

2. Qualitative and quantitative chemical analysis of *Allium ursinum* extracts

The phytochemical profile of the two *A. ursinum* extracts was assessed using spectrophotometric methods to identify the major constituents with potential antibacterial and antitumor properties. Thus, the polyphenols and sulpfur-containing compunds were evaluated in both extracts

The total polyphenol and flavonoid contents of the extracts were determined using spectrophotometric techniques, while the identification of phenolic compounds was performed using high-performance liquid chromatography with photodiode array detection (HPLC-PDA). The quantification of these compounds was carried out through electrospray ionization mass spectrometry (ESI-MS).

2.1 Determination of total polyphenol and flavonoid content using spectrophotometric techniques

The results showed that the extract obtained through conventional maceration (CM) was characterized by a total polyphenol content (TPC) of 47.63 mg GAE (gallic acid equivalent)/100 mL, indicating the presence of significant amounts of phenolic compounds.

In comparison, the extract obtained through ultrasonic-assisted maceration (UAE+CM) exhibited a polyphenol content of 48.14 mg GAE/100 mL, representing a slight increase of 3.23% compared to the CM extract. Thus, both extracts presented similar values, with this relatively small difference suggesting that both extraction methods are effective in obtaining a significant polyphenol content from *Allium ursinum* leaves.

Regarding the total flavonoid content, the analysis revealed that the two *A. ursinum* extracts obtained through conventional maceration (CM) and ultrasound-assisted maceration (UAE+CM) exhibit similar values. The extract obtained through conventional maceration has a content of 4.68 mg QE (quercetin equivalents)/mL, while the one obtained through UAE+CM is 1.92% higher, with a value of 4.59 mg QE/mL (Fig. 2).

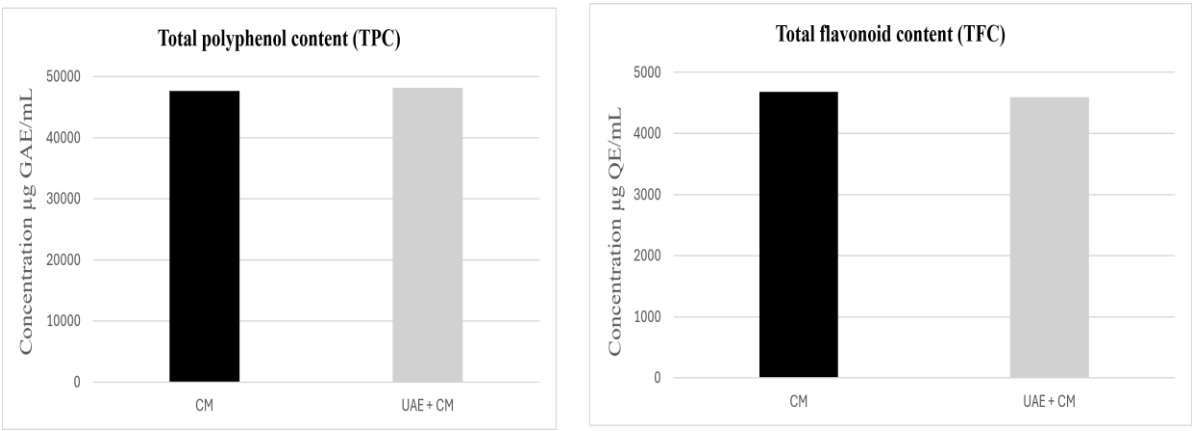

Fig. 2. Total polyphenols and total flavonoids in extracts obtained from *A. ursinum* leaves, according to spectrophotometric methods. Results are expressed in µg gallic acid equivalents (GAE)/mL extract and µg quercetin equivalents (QE)/mL extract, respectively.

2.2. Identification and quantification of phenolic compounds and sulfoxides by LC/ ESI^{+}-MS

The chromatographic analysis revealed the presence of seven major peaks in the obtained spectra, six of which were attributed to flavonoids, and one peak corresponds to the sulfoxide alliin (peak 1), a bioactive compound specific to the *Allium* genus (Table 2).

This study found that both extracts, obtained by conventional maceration (CM) and ultrasound-assisted maceration (UAE+CM), contain notable amounts of alliin, which was the major compound identified in the sulfoxide category. Furthermore, the concentration of alliin was slightly higher in the extracts obtained using the UAE+CM method compared to the traditional method.

The six flavonols present in the extracts were identified as kaempferol-glucosyl-glucosyl-rhamnoside (peak 2), kaempferol-diglucoside (peak 3), kaempferol-galactosyl-rhamnoside (peak 4), kaempferol-rutinoside (peak 5), quercetin-rutinoside (peak 6), and kaempferol-glucoside (peak

7). Notably, the quercetin derivative, rutin, was found in significant amounts in both analyzed extracts.

The total quantity of phenolic compounds determined in the extract obtained through conventional maceration (CM) was 12.539 mg/mL. Kaempferol-glucosyl-glucosyl-rhamnoside was the major component, accounting for 29.49% of the total identified compounds, followed by kaempferol-rutinoside, which represented 17.75%. Additionally, the sulfoxide compound alliin was identified as a significant constituent, comprising 26.05% of the total compounds present in the CM extract.

For the UAE extract results showed a similar composition regarding both diversity and the proportion of individual compounds. However, the use of UAE as an additional step in the extraction process resulted in an overall increase of approximately 20% in the concentrations of both main categories, flavonols and alliin. This increase was observed consistently across all analyzed compound categories.

Table 2. Identification and quantification of phenolic compounds in methanolic extracts obtained from *Allium* ursinum leaves. Rt – retention time; UV λmax – wavelength of maximum absorbance; [M+H]+ – protonated molecular ions. Quantity expressed in mg/mL.

Peak No.	**R_t (min)**	**UV λ_{max} (nm)**	**$[M+H]^+$ (m/z)**	**Phenolic Compound**	**Subclass**	**Ramson CM**	**Ramson UAE**
1	2.91	230	178	Aliin	Sulfoxide	4.641	5.575
2	11.38	260, 350	757, 611, 449, 287	Kaempferol-glucozil-glucozil-ramnozid	Flavonol	5.068	5.967
3	12.65	260, 351	611, 449, 287	Kaempferol-diglucozid	Flavonol	1.759	2.259
4	14.65	261, 350	595, 287	Kaempferol-galactzil-raamnozid	Flavonol	0.925	1.120
5	15.56	261, 350	595, 287	Kaempferol-rutinozid	Flavonol	3.050	3.450
6	16.05	250, 350	611, 303	Quercetin-rutinozid	Flavonol	1.248	1.572

7	17.15	260, 351	449, 287	Kaempferol-glucozid	Flavonol	0.489	0.620
				Total compounds		**17.181**	**20.563**

3. Antioxidant activity of the extracts

Using the CUPRAC method, the UAE extract showed an antioxidant activity of 87.07 µM Trolox/mL. However, the difference compared to the extract obtained by conventional maceration (CM), which had a value of 85.68 µM Trolox/mL, was not considered significant (Fig. 3).

On the other hand, the use of the HPS method to determine hydrogen peroxide scavenging capacity revealed more pronounced differences between the two types of extracts. The UAE extract exhibited an antioxidant capacity of 37.71 µM Trolox/mL, significantly higher than the 22.82 µM Trolox/mL recorded for the extract obtained by CM (Fig. 3).

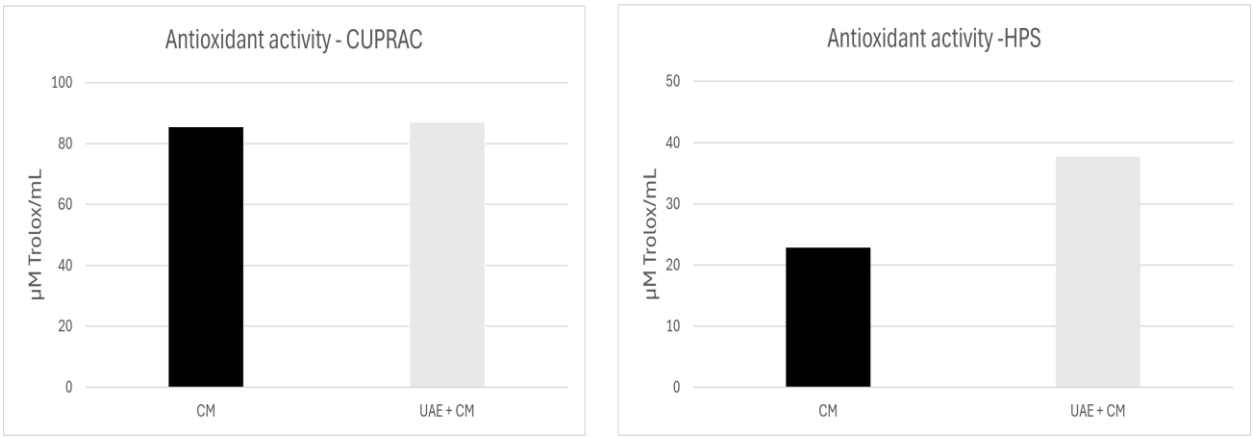

Fig. 3. Antioxidant activity of extracts obtained from *A. ursinum* leaves, according to: **A.** CUPRAC method and **B.** the HPS method.

According to the available information, this is the first time the antioxidant capacity of methanolic *A. ursinum* extracts has been measured using the hydrogen peroxide scavenging method.

4. Antibacterial activity of plant extracts

The antimicrobial activity of *A. ursinum* extracts was evaluated against five clinically relevant bacterial strains: *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Escherichia coli*. The evaluation was conducted using the disc diffusion method.

The results showed that both types of *A. ursinum* extracts demonstrated significant antibacterial activity against all bacterial strains tested, in comparison to the vehicle solvents,

which showed no antimicrobial effect. Detailed values of the inhibition zones for each extract and microorganism combination are summarized in Table 3.

Table 3. Antimicrobial activity of *Allium* ursinum leaf extracts obtained by conventional maceration (CM) and ultrasound-assisted extraction (UAE) against Gram-positive and Gram-negative pathogenic microorganisms

Antimicrobial activity	Extracts								Controls					
	MET CM (1)		DMSO CM (2)		MET UAE (3)		DMSO UAE (4)		MET (M)		DMSO (D)		Gentamicin	
	DIZ (mm)	PI	DIZ (mm)	PI	DIZ (mm)	PI	DIZ (mm)	PI	DIZ (mm)	PI	DIZ (mm)	PI	DIZ (mm)	PI
Gram-positive														
Staphylococcus aureus	10.0	+ ±	10.0	+ ±	11.9	+ ±	12.0	+ ±	0	− −	0	− −	20	++
Enterococcus faecalis	8.5	+ −	11.0	+ ±	8.5	+ −	12.1	+ ±	0	− −	0	− −	17.8	++
Streptococcus mutans	11.9	+ ±	12.0	+ ±	11.5	+ ±	12.5	+ ±	0	− −	0	− −	18.2	++
Gram-negative														
Porphyromonas gingivalis	12.9	+ ±	12.0	+ ±	15.1	++	10.5	+ ±	0	− −	0	− −	14.5	+ ±
Escherichia coli	8.0	+ −	17.1	++	8.5	+ −	15.0	++	0	− −	0	− −	18.5	++

MET—70% methanol (solvent); DMSO—100 %=% dimethyl sulfoxide (solvent); DIZ—diameter of inhibition zone; PI—inhibition potential.

An important aspect of our study is that, to the best of our knowledge, this represents the first evaluation of the antibacterial activity of *A. ursinum* extracts against the *S. mutans* and *P. gingivalis* strains, bacterial organisms often involved in oral and gingival infections.

In conclusion, the results obtained in this study emphasize that the antimicrobial activity of *A. ursinum* extracts was strongest against Gram-negative bacteria, particularly *Escherichia coli* and *Porphyromonas gingivalis*, for which significant inhibition zones (17.1 mm and 15.1 mm, respectively) were obtained. These values indicate an efficiency comparable to that of gentamicin, a reference agent in positive control tests. The importance of the solvent and the extraction method proved essential in achieving optimal antimicrobial activity. Thus, the use of DMSO as a solvent or the application of the UAE method, which demonstrated a higher capacity for extracting bioactive compounds from the plant, led to a significant increase in antimicrobial efficiency, especially against *E. coli*. The observations were similar for Gram-positive bacterial strains, where both DMSO and the UAE method were key factors in improving the antimicrobial activity of the extracts. Therefore, the extract obtained through UAE using DMSO exhibited the strongest antimicrobial activity against all tested bacterial strains, highlighting the potential of this combination in developing effective antimicrobial treatments based on medicinal plants (Fig. 4).

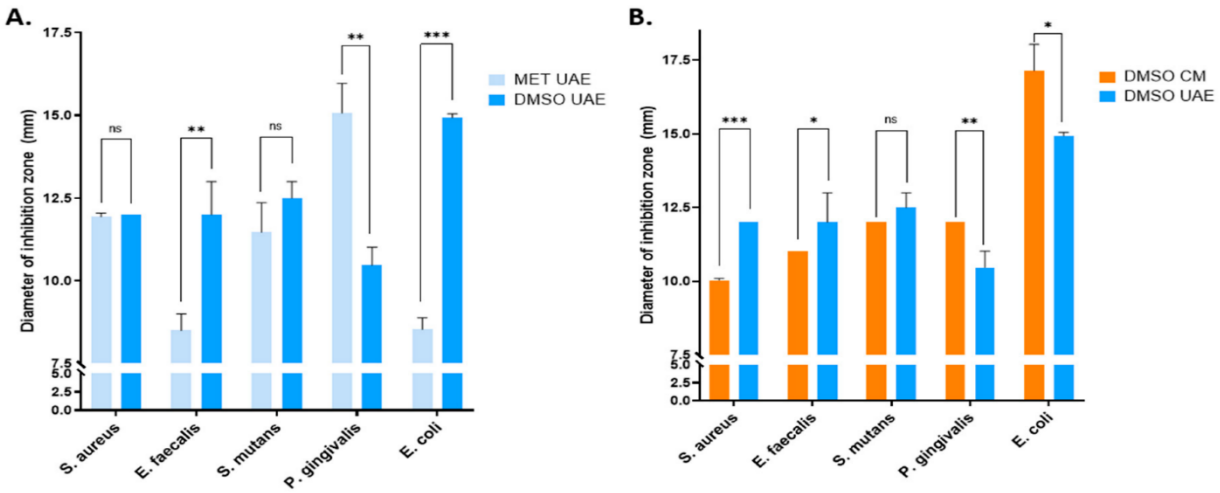

Fig 4. Antimicrobial activity of *Allium ursinum* leaf extracts obtained by conventional maceration (CM) and ultrasound-assisted extraction (UAE) against Gram-positive and Gram-negative pathogenic microorganisms: **A.** Comparing UAE extracts in 70% methanol and DMSO solvents based on inhibition zone diameter (DIZ) for all bacterial strains. **B.** Comparing UAE extract with CM extract in DMSO solvent based on inhibition zone diameter (DIZ) for all bacterial strains ($0.05 > p^* \geq 0.01$ significant; ns, no significant).

5. Antitumor activity of plant extracts

The present study aimed to evaluate the *in vitro* antitumor activity of *Allium ursinum* leaf extracts against four tumor cell lines: two breast cancer cell lines (T47D and MDA-MB-231) and two colorectal cancer cell lines (DLD-1 and HT29). According to available literature, this study represents the first documented investigation testing *Allium ursinum* extracts on breast cancer cell lines and the colorectal cancer cell lines DLD-1 and HT29.

In the experiment, both types of extracts tested demonstrated dose-dependent antitumor activity, indicating a direct proportional relationship between extract concentration and cytotoxic effect. A progressive reduction in cell viability was observed for all four tumor cell lines as the concentration of administered extracts increased. However, the observed cytotoxicity can be considered moderate, in line with general pharmacological standards, as it did not reach high toxicity levels.

The lowest concentrations required to inhibit 50% of cell viability (IC50), 658.1 µg/mL and 755.8 µg/mL, were obtained for the CM extract against HT29 and DLD-1 cells, respectively. For the other cell lines, IC_{50} values exceeded the 1 mg/mL threshold (Table 4). Although the CM extracts showed more promising activity against colorectal cancer cell lines (HT29 and DLD-1), the UAE extract proved to be more effective against breast cancer cell lines (T47D and MDA-MB-231). However, the differences in cytotoxic effectiveness between the two extracts were not statistically significant.

Table 4. IC50 (µg/mL) values of *A. ursinum* extracts on T47D, MDA-MB-231, DLD-1, and HT29 cell lines

IC_{50}[a]	**CM Extract**	**UAE Extract**
T47D	1,693 µg/mL	1,361 µg/mL
MDA-MB-231	0,9948 µg/mL	0,9454 µg/mL
DLD-1	0,7558 µg/mL	9,444 µg/mL
HT29	0,6581 µg/mL	1,092 µg/mL
[a] Concentrations of plant extract at which only half of the treated cells remain viable.		

CHAPTER V – Discussion

1. Extract Analysis: Yield and Phytochemical Characterization

1.1. Extraction Yield of Wild Garlic Extracts

The results showed a significant difference between the two extraction techniques. The extraction yield obtained for CM was 41.32%, whereas the UAE+CM method resulted in a higher extraction rate of 45.82%. This increase in yield using the UAE+CM method aligns with previous studies demonstrating that the incorporation of ultrasound in extraction processes enhances efficiency and yield in extracting bioactive compounds compared to traditional methods (Chemat et al., 2017). Ultrasound affects the plant cell wall, facilitating the release of intracellular compounds into the solvent, thereby maximizing the extraction of bioactive substances.

The extraction yield obtained in this study using 70% methanol exceeds both the maximum value of 33.50% reported in a previous study for the same solvent and the 43.06% yield obtained with distilled water, considered the highest in the study conducted by (Pavlović et al., 2017).

In addition, smaller particle sizes, such as those obtained by grinding the plant material into a fine powder, increase the surface area available for interaction with the solvent, facilitating the diffusion of compounds from the plant material into the solvent, which can lead to higher extraction yields (Meng & Cheng, 2019). Thus, grinding *Allium ursinum* leaves to obtain a fine powder is a step that can significantly contribute to increased yield (Meng & Cheng, 2019).

In conclusion, our study demonstrates that using an advanced extraction technique, such as UAE together with an efficient solvent like 70% methanol, significantly contributes to increasing the extraction yields of *Allium ursinum* leaves. This method allowed for an extraction yield of 45.82%, superior to traditional methods or those reported in other similar studies. Furthermore, optimizing particle size through grinding plays an important role in maximizing extraction efficiency.

1.2. Phytochemical profile

The sulfoxide and polyphenol profiles of *Allium ursinum* vary based on factors like plant organ, harvest time, and processing. Thus, flower extracts contain more allicin (1.946 mg allicin/ml) than leaf extracts (0.028 mg allicin/ml) (Pârvu, 2011) and the highest amount of alliin was observed in leaf extracts that were harvested between March and April (Schmitt et al., 2005). Also, dried wild garlic leaves contain 0.7–1.7% alliin, whereas fresh wild garlic leaves contain lower amounts ranging between 0.25 and 1.15% (Bagiu et al., 2012). In accordance with this data, in this study, significant amounts of alliin were observed in both types of analyzed extracts, obtained from *A. ursinum* leaves harvested in April and air-dried.

Also, *A. ursinum* leaves have a significantly higher total polyphenol content compared to other plant organs, such as flowers or bulbs (Kovarovič, 2019). Kaempferol derivatives were predominant in the leaves compared to seeds and stems. Leaves collected in May exhibited the highest concentration of phenolic compounds, reaching 128 mg/100 g FW (Mahmutovic et al., 2009). A study showed that in the ethanolic extract from wild garlic leaves obtained by maceration at room temperature for 12 hours, the amount of polyphenols expressed in gallic acid equivalents was higher than that obtained by ultrasound-assisted extraction. In contrast, in another study, ultrasound-assisted extraction was found to be more efficient in maximizing polyphenol extraction from *A. ursinum* (Gitin et al., 2012; Tomšik et al., 2016).

In the present study, the total polyphenol content (TPC) and flavonoid content (TFC) were similar for both types of extracts investigated. In our study, the differences between the two extraction methods are minor and could indicate similar efficiency in polyphenol extraction (Gitin et al., 2012). However, it is important to consider that these quantification methods are still quite rudimentary.

In the present study, the use of 70% methanol resulted in a total polyphenol content (TPC) comparable to the values reported in the literature, which may indicate that a lower methanol content does not significantly compromise the overall extraction of polyphenols. The differences between the values obtained in this study and those reported previously could be attributed to several factors, such as the extraction method used, the origin of the plant material, the harvesting period, or environmental conditions that can influence the phytochemical composition of wild garlic.

The tentative identification of the six main flavonols confirmed the presence of kaempferol and quercetin derivatives, in agreement with the specientific literature. Furthermore, the phytochemical analysis revealed that the most important phenolic compounds detected in our extracts are kaempferol glycosides, which support the results of previous studies (Burton et al., 2023; Furdak et al., 2023; Oszmiański et al., 2013).

2. Extracts of *A. ursinum* and their therapeutic potential

2.1. Antioxidant activity of the Extracts

The determination of the antioxidant capacity of *A. ursinum* extracts is of particular importance and has been the subject of numerous studies highlighting its potential health benefits and therapeutic applications (Stefan et al., 2023). The highest antioxidant activity has been observed in leaves, while the lowest was recorded in bulbs, with values of 83.7 µmol Trolox/g for leaves and 33.9 µmol Trolox/g for bulbs (Lachowicz et al., 2018). These findings are supported by the research of Voća et al. (2022), who reported that the antioxidant activity of wild garlic is up to 28% higher before flowering than during flowering, suggesting higher levels of bioactive compounds before this stage (Voća et al., 2022). Petkova et al. (2021) demonstrated that the UAE method applied to *A. ursinum* flowers resulted in high antioxidant activity according to the CUPRAC test, demonstrating the significant influence of extraction techniques on the yield of bioactive compounds.

The present study showed a higher polyphenol content in the extract obtained by UAE compared to that obtained by conventional maceration (CM), suggesting that ultrasound-assisted extraction can enhance the extraction of compounds with antioxidant potential. The determinations performed using the CUPRAC and HPS methods indicated a higher antioxidant potential for the extracts obtained by UAE, further confirming the literature data that suggest extraction methods can significantly influence the antioxidant activity of *A. ursinum* extracts.

2.2. Antimicrobial activity of extracts

Numerous studies have demonstrated the antimicrobial activity of *Allium ursinum* extracts against various pathogens; however, their efficacy varies significantly across studies, depending on the solvent and extraction method used, the plant organs selected, and the characteristics of the pathogens included in the experiments.

The methanolic extract demonstrated greater efficacy, inhibiting the growth of bacteria such as *Staphylococcus aureus, Escherichia coli, Proteus mirabilis,* and *Salmonella enteritidis,* as well as fungi including *Candida lipolytica* and *Aspergillus niger* (Sobolewska et al., 2015).

Ethanolic extracts from *A. ursinum* leaves and flowers exhibited high efficacy against fungi, such as *Aspergillus niger, Botrytis cinerea, Botrytis paeoniae, Fusarium oxysporum, Penicillium gladioli,* and *Sclerotinia sclerotiorum*. The results indicated that flower extracts exhibited greater antifungal activity, likely due to their higher allicin content (Pârvu, 2011).

In this study, the strongest antimicrobial effects were observed against Gram-negative bacteria, particularly *E. coli* and *P. gingivalis*, with the extracts exhibiting antimicrobial activity comparable to that of gentamicin, which was used as a positive control.

Our results appear to contradict previous reports, which suggest that *A. ursinum* extracts are more effective against Gram-positive bacteria than against Gram-negative ones, and that *E. coli* strains are less sensitive than *E. faecalis* (Stupar et al., 2022) or *S. aureus* (Barbu et al., 2023; Ivanova et al., 2009; Krivokapic et al., 2022). It is important to note that none of these studies employed ultrasound-assisted extraction (UAE) or used DMSO as the final solvent, both of which could strongly influence the results.

When 70% methanol was used as the solvent in conventional maceration, our results indicated that the *A. ursinum* extract exhibited the lowest antimicrobial activity against *E. coli* (DIZ = 8.0) compared to all Gram-positive bacterial strains, as expected. However, the use of DMSO, known for its excellent solvent properties for bio-organic compounds at high concentrations (Modrzyński et al., 2019) or the application of the UAE method, which was shown in this study to extract higher amounts of bioactive compounds, significantly enhanced the antimicrobial activity of the extract against *E. coli*.

A similar trend was observed for all Gram-positive bacterial strains used in this study: both DMSO and UAE extraction significantly increased the antimicrobial activity of the extracts (Figure 2). Consequently, for all bacterial strains tested, the extract obtained through UAE using DMSO exhibited the strongest antimicrobial activity.

The biochemical composition of the extracts explains their recorded antimicrobial activity, as various thiosulfinates and polyphenolic compounds are well known for their ability to confer antimicrobial activity in *Allium* species (El-Saber Batiha et al., 2020; Stupar et al., 2022).

2.3. Antitumoral activity of extracts

Allium ursinum extracts have shown notable *in vitro* anticancer effects on several cell lines, including murine melanoma (B16), murine sarcoma (XC), and human gastric cancer (AGS).

Both breast and colorectal cancers used in this study are significantly influenced by dietary factors, suggesting that specific foods may play an important role in their prevention (Buja et al., 2020; Hou et al., 2013). Furthermore, plant-derived compounds have been proposed as valuable complementary therapies in the treatment of these cancers (Rejhová et al., 2018; Y. Zhang et al., 2020). However, the high heterogeneity of tumors, both among different cancer types and within the same type, leads to considerable variations in cancer cell sensitivity to natural compounds. In this context, the present study represents the first investigation testing *A. ursinum* extracts on breast cancer cell lines, as well as on colorectal cancer cell lines DLD-1 and HT29.

Although a dose-dependent decrease in cell viability was observed for both extract types, CM and UAE+CM, across all four cancer cell lines, the cytotoxicity of these extracts remains relatively moderate when analyzed from the perspective of general pharmacological standards.

Conclusions and Research Perspectives

The two methanolic extracts obtained from the leaves of *A. ursinum* by conventional maceration and ultrasound-assisted extraction were rich in polyphenols and sulfoxides.

Ultrasound-assisted extraction (UAE) was more efficient than conventional maceration, yielding 20% more bioactive compounds. LC/ESI+-MS analysis identified six flavonoids and one sulfoxide, with the main constituents being kaempferol derivatives, rutin, and alliin—compounds known for their biologically active properties. The study's findings support existing literature data, confirming that *A. ursinum* leaves are an important source of bioactive compounds.

Regarding the therapeutic potential of *A. ursinum*, the extracts exhibit strong antioxidant and antibacterial activity but moderate antitumor activity. Both extracts demonstrated antimicrobial activity against Gram-positive bacterial strains *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Streptococcus mutans* ATCC 25175, and Gram-negative strains *Porphyromonas gingivalis* ATCC 33277 and *Escherichia coli* ATCC 25922. The antimicrobial activity, particularly notable against Gram-negative bacteria, underscores the importance of this plant in managing bacterial infections. The results indicated moderate, dose-dependent cytotoxicity for both extract types analyzed (CM and UAE). Although there were no statistically significant differences between the two types of extracts, a trend was observed where the CM extract appeared more effective against colorectal cancer, whereas the UAE extract exhibited stronger cytotoxic activity against breast cancer. This variability in cellular response highlights the influence of tumor heterogeneity on the efficacy of natural compounds.

The originality of this study lies in the first-time investigation of the antioxidant capacity of *A. ursinum* extracts using hydrogen peroxide (H_2O_2) scavenging activity. Furthermore, this is the first study evaluating *A. ursinum* extracts against *Streptococcus mutans* and *Porphyromonas gingivalis* bacterial strains, as well as testing them on breast cancer cell lines and colorectal cancer cell lines DLD-1 and HT29.

Future studies should explore the effects of thermal processing on its biochemical profile, investigate the molecular and antimicrobial mechanisms involved, and assess the potential for synergism with antibiotics. Additionally, research should aim to develop nanoparticle-based formulations to improve bioavailability and to evaluate the selective cytotoxicity of *A. ursinum* extracts on tumor versus normal cells.

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