UNIVERSITÀ DEGLI STUDI DI PADOVA
Dipartimento di Biomedicina comparata e alimentazione
Department of Comparative Biomedicine and Food Science

Corso di laurea magistrale/Second Cycle Degree (MSc)
in Biotechnologies for food science

Estrazione e isolamento di composti bioattivi da *Carlina acaulis*

Relatore/Supervisor
Prof. Fabio Vianello
Co-supervisor
Prof. Stefano Dall'acqua

Laureanda/o
/Submitted by
Maryam Monazzah
Matricola n./Student n.
2039463

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Table of Contents

Abstract in English

Carlina acaulis L. is a perennial herbaceous plant in the Asteraceae family commonly known as dwarf carline thistle and stemless carline thistle, this plant is renowned for its traditional medicinal uses. The aim of this study is extraction and isolation of bioactive fractions and compounds from *Carlina acaulis*, and evaluating their cytotoxic effects. Using a combination of Soxhlet extraction, column chromatography with silica gel, and preparative thin-layer chromatography (TLC), some fractions were separated from *C. acaulis* roots. One fraction was purified and the isolated compound was analyzed by NMR spectroscopy, which confirmed the identity of carlina oxide as main constituent. For cytotoxicity assessment, the effect of *Carlina acaulis* extract (dissolved in hexane, methanol, ethyl acetate, acetone, and dichloromethane) and Carlina oxide (purified compound) on the viability of two cell lines, A431 (susceptible to cisplatin) and A431-PT (resistant to cisplatin), was examined at different concentrations. The Crystal Violet Staining (CVS) was employed to conduct the assay. Cells were cultured, seeded, and treated with varying concentrations of Carlina extract and carlina oxide. Following a 24-hour incubation period, cells were fixed, stained, and their optical density was measured. The percentage of viability and IC_{50} were calculated. Results indicated that *Carlina acaulis* extract has potential as anti-cancer agent, and its effects are more pronounced at higher concentrations. Further research is required to understand the mechanisms underlying its cytotoxicity and finding its mode of action. In our study, the purified compound Carlina oxide had the lowest IC_{50} values, suggesting that it is the most potent extract. The least potent extract was the ethyl acetate extract, which has the highest IC_{50} values, which highlighting the potential for cytotoxic effects depending on the specific extract or compound used. Also, the data showed that two cell lines has varying sensitivity to different extracts and the purified compound.

Keywords: Bioactive compounds, *Calina acualis*, Carlina oxide, Cytotoxicity

Abstract in Italian

Carlina acaulis L. è una pianta erbacea perenne della famiglia delle Asteraceae, comunemente conosciuta come cardo carlino nano o cardo carlino senza fusto. Lo scopo di questo studio è l'isolamento e l'estrazione di composti bioattivi da *Carlina acaulis*, una pianta nota per i suoi usi medicinali tradizionali, e la valutazione dei loro effetti citotossici. Utilizzando una combinazione di estrazione Soxhlet, cromatografia a colonna con gel di silice e cromatografia su strato sottile preparativa (TLC), sono stati isolati composti dalle radici di *C. acaulis*. I materiali estratti sono stati analizzati mediante spettroscopia NMR, che ha confermato l'identità dell'ossido di carlina come componente predominante. Per la valutazione della citotossicità, è stato esaminato l'effetto dell'estratto di *Carlina acaulis* (dissolto in esano, metanolo, acetato di etile, acetone e diclorometano) e dell'ossido di carlina (composto purificato) sulla vitalità di due linee cellulari, A431 (sensibile al cisplatino) e A431-PT (resistente al cisplatino), a diverse concentrazioni. È stato impiegato il saggio di colorazione con violetto di cristallo (CVS). Le cellule sono state coltivate, seminate e trattate con diverse concentrazioni di estratto di Carlina e ossido di carlina. Dopo un'incubazione di 24 ore, le cellule sono state fissate, colorate e la loro densità ottica è stata misurata. Sono state calcolate la percentuale di vitalità e l'IC_{50}. I risultati hanno indicato che l'estratto di *Carlina acaulis* ha potenziale come agente antitumorale, con effetti più pronunciati a concentrazioni più elevate. Ulteriori ricerche sono necessarie per comprendere i meccanismi alla base della sua citotossicità e per individuare il suo meccanismo d'azione. Nel nostro studio, il composto purificato ossido di carlina ha mostrato i valori di IC_{50} più bassi, suggerendo che sia l'estratto più potente. L'estratto meno potente è stato l'acetato di etile, che ha presentato i valori di IC_{50} più alti, evidenziando il potenziale di effetti citotossici a seconda dell'estratto o del composto specifico utilizzato. Inoltre, i dati hanno mostrato che le due linee cellulari hanno una sensibilità variabile ai diversi estratti e al composto purificato.

Parole chiave: Composti bioattivi, *Carlina acaulis*, Ossido di Carlina, Citotossicità

Chapter 1

1. Introduction

1.1. Carlina acaulis

Carlina acaulis L. is a perennial herbaceous plant in the Asteraceae family commonly known as dwarf carline thistle and stemless carline thistle. The plant is known by the genus name "Carlina" because of the mythology that it was employed by Charlemagne (Carolus Magnus) to cure his soldiers during a plague because he thought it had medicinal capabilities. The specific name 'acaulis,' which translates to "without a stem," describes how the flowerhead rests directly on a basal rosette of leaves. Usually, from August to October, *Carlina acaulis* blossoms in late summer or early fall. Plant enthusiasts and botanists will find it to be an intriguing subject and a great addition to gardens due to its unique look and rich history (Spinozzi et al., 2023).

Carlina acaulis holds significant value in European traditional medicine, particularly in Poland. Traditionally, the plant's components have been utilized in traditional medicine. It was thought that the root in particular had a number of therapeutic benefits, such as diuretic and antibacterial qualities. Dried flower heads are used as a decorative element in traditional cuisines in several cultures.

It is extracted with wine or vodka in Polish folk medicine and used to stimulate the mental and digestive systems, and as a diuretic, anthelmintic, laxative, and emetic. It is also recommended for dermatological conditions such as eczema and mycoses (Strzemski et al., 2019). In Italy, it is used as a diuretic, cholagogue, and antibacterial agent (Menale et al., 2006). In Bosnia and Herzegovina, and Montenegro, the aerial parts are used for gastritis, while in central Serbia and Bosnia, the roots treat skin diseases, acne, eczema, and ulcers (Rexhepi et al., 2013; Šarić-Kundalić et al., 2010). Historically, the plant was consumed as food during famines; its inflorescences and roots are edible (Pavela et al., 2020). The receptacle can substitute artichoke and is used in alcoholic drinks and snacks (Pieroni and Giusti, 2009). The root's sweet smell has also made it popular in dessert preparation in southern Italy (Guarino et al., 2008).

1.1.1. Morphological and anatomical features

Carlina acaulis is renowned for having a unique and attractive look. *Carlina acaulis* features a basal rosette of elliptical-oblong, spiky, pinnatilobate leaves, approximately 20 cm in diameter, and a large flowerhead that can reach up to 10 cm. The flowerhead consists of silvery white ray flowers surrounding a yellow-brown central disc (Erzsebet et al., 2009). The plant has a substantial, vertically-oriented rhizome, and the stem rarely exceeds 15 cm, typically appearing stemless. Capitula measure 25 to 50 mm in diameter and are encircled by silver/white bracts (Trejgell et al., 2009). The flowerhead closes in moist conditions to protect pollen, a traditional indicator of impending rain. The roots are grey-brown to dark-brown with a large longitudinal fold. The oblong fruits, known as cypselae, are 4–6 mm long and about 1 mm wide, brown, and covered with silvery hairs, having an elliptical cross-section (Strzemski et al., 2020).

Anatomically, the leaves are dorsiventral and amphistomatic with anomocytic stomata and a smooth cuticle. The main leaf nerve contains a conducting bundle and sporadic non-glandular hairs. The root has numerous secretory cavities containing essential oil (EO), located in the cortex and woody parenchyma (Đordevic et al., 2004).

1.1.2. Habitat and distribution

Carlina acaulis is native to northern Europe, typically found in pastures, rocky regions, and dry meadows at altitudes from 0 to 2800 meters. It prefers xerothermic and calcareous grasslands in mountainous areas and is rare in lowland regions (Pavela et al., 2020). The plant is resilient and able to survive the severe conditions seen in alpine settings. It likes soil that drains well and full light. In Europe, it is mainly distributed in southern Poland, including the Carpathian and Sudeten Mountains, Lubelska Upland, and the Warmia and Mazury Lake District (Zając and Zając, 2001). It is also present in southern European countries such as Italy, France, Austria, and Romania. It is possible to multiply *Carlina acaulis* from seeds. Cultivation from seeds occurs between April and May in very sunny, rocky, and well-drained calcareous soils (Strzemski et al., 2019). After sowing, the seeds need to be maintained wet until germination in a well-drained medium. Once established, it takes little care, which makes it an excellent choice for rock gardens and wildflower meadows (Spinozzi et al., 2023; Strzemski et al., 2019).

It is not officially classified as endangered, although changes in land use and agricultural activities may result in habitat loss in some locations. The main goals of conservation efforts are to protect its natural ecosystems and advance sustainable land management techniques (Pruchniewicz et al., 2023).

1.2. Chemical Compounds

A variety of chemical substances found in *Carlina acaulis* which have been investigated for possible medical benefits. Triterpenes, polyphenols, volatile chemicals, and polyacetylenes are the most prominent secondary metabolites found in *Carlina acaulis*.

1.2.1. Triterpenes: Triterpenes are abundant and diverse compounds in plants, making up a significant portion of the secondary metabolites in *Carlina acaulis*. These compounds contribute to the medicinal properties of *C. acaulis*, supporting its use in treating skin disorders, and functioning as an antidiuretic, anti-inflammatory, anthelmintic agent, and in the treatment of dental and gastrointestinal issues (Mioc et al., 2022; Strzemski et al., 2019). The primary triterpenes in *Carlina acaulis* are pentacyclic triterpenes, mainly oleanolic and ursolic acid (Strzemski et al., 2021; Strzemski et al., 2019). The levels of these metabolites vary with cultivation methods, being higher in field and hydroponic crops compared to natural environments (Strzemski et al., 2020). In contrast, in vitro micropropagation techniques seem to reduce their production. Additionally, α-amyrin, β-amyrin, β-amyrin acetate, lupeol, lupeol acetate, and betulinic acid have also been identified (Strzemski et al., 2016).

1.2.2. Polyphenols: Phenolic acids, which have antioxidant properties, such as chlorogenic acid and caffeic acid. (Strzemski et al., 2019). *Carlina acaulis* contains valuable polyphenols responsible for its antioxidant, radical scavenging, anti-inflammatory, and antimicrobial activities (Strzemski et al., 2019). The major phenolic acids in *C. acaulis* are chlorogenic acid and 3,5-di-caffeoylquinic acid, which are beneficial to human health (Jaiswal et al., 2011). Chlorogenic acid is primarily found in the leaves, while 3,5-di-caffeoylquinic acid is more abundant in the roots (Strzemski et al., 2019). The cypsela of *C. acaulis* is rich in chlorogenic acid, containing about 22 g/kg, higher than other Asteraceae plants (Strzemski et al., 2020). Neochlorogenic acid is also present in cypsela part. Jaiswal et al. (Jaiswal et al., 2011) identified various chlorogenic acids in the leaves, including several regioisomeric forms. Additionally, the herb contains flavonoids such as homoorientin, isoschaftoside, orientin, vitexin, apigenin 7-O-glucoside, luteolin and apigenin. flavonoids are well-known for their anti-oxidant properties (Đorđević et al., 2012).

1.2.3. Volatile compounds: Volatile compounds, produced by plants in response to their environment, include terpenoids, phenylpropanoids, benzenoids, fatty acids, and amino acid derivatives. Over 1700 such compounds have been identified from more than 90 plant families (Knudsen and Gershenzon, 2020). *Carlina acaulis* roots contain 1–2% essential oil (EO), primarily composed of the polyacetylene Carlina oxide, which constitutes 80-99% of the EO (Benelli et al., 2021; Herrmann et al., 2011; Kavallieratos et al., 2022a). This is one of the main bioactive substances in the essential oil that is taken from the *Carlina acaulis* roots. It is well renowned for having antimicrobial properties. Other minor components include benzaldehyde, ar-curcumene, (E,Z)-α-farnesene, β-sesquiphellandrene, and 1,8-cineole (Chalchat et al., 1996). Carlina oxide serves as a chemotaxonomic marker for the genus, being predominant in *C. acaulis* and other Carlina species like *C. acanthifolia*, *C. diae, C. vulgaris*, and *C. hispanica*. It is also present in other Asteraceae family plants, such as *Atractylis gummifera* and *Carthamus caeruleus* (Mejdoub et al., 2020).

1.2.4. Polyacetylenes: Polyacetylenes are biologically active compounds with multiple carbon-carbon triple bonds, found in plants, lichens, mosses, fungi, sponges, and marine algae. They are known for their antifungal and antibacterial activities. Notably, over 1100 of the 2000 characterized acetylenes are found in Asteraceae plants. Polyacetylenes are synthesized from saturated fatty acids, which are formed by adding malonyl units to an acyl chain. These fatty acids are then functionalized by enzymes, with triple bonds being formed through the oxidation of double bonds(Christensen, 1992). The primary biosynthesis products, crepenynic acid, stearolic acid, and tariric acid, undergo various reactions to produce a wide range of polyacetylenes (Minto and Blacklock, 2008). These compounds exhibit significant chemical diversity and have been structurally classified by Christensen and Brandt (2006): acyclic C_8-C_{13} acetylenes, acyclic C_{14}-C_{18} acetylenes, acetylenes with the allene structure, aromatic and heterocyclic acetylenes. Subgroups of polyacetylenes, such as thioesters, thiophenes, sulfones, sulfoxides, furans, and pyrans, are defined based on specific functional groups (Christensen and Brandt, 2006).

The main polyacetylene in *Carlina acaulis* is carlina oxide, first isolated in 1889 (Semmler, 1906). This aromatic polyacetylene, featuring benzyl and furan moieties connected by a triple bond, is primarily found in the roots' essential oil. Carlina oxide is extracted using hydrodistillation and organic solvents like methanol and ethanol (Đorđević et al., 2012; Strzemski et al., 2019). Methods for its extraction include ultrasonic bath, Soxhlet apparatus, and reflux condenser with solvents such as hexane and dichloromethane (Herrmann et al., 2011; Link et al., 2016). Purification typically employs silica gel column chromatography with n-hexane as the mobile phase (Pavela et al., 2021; Rosato et al., 2021). Characterization techniques for carlina oxide include NMR, IR, GC-MS, GC-FID, HPLC-PDA, and Raman spectroscopy (Pavela et al., 2021; Strzemski et al., 2019).

1.2.5. Inulin: this is a kind of polysaccharide that functions as a store carbohydrate. It is used in nutritional supplements and has prebiotic qualities (Strzemski et al., 2019).

1.3. Biological Activities

Carlina acaulis has long been used in a variety of folk remedies. It has been used historically as a diuretic, helping to promote the excretion of excess fluids and salts from the body, and current scientific research is starting to identify the biological processes that underlie its medicinal applications.

1.3.1. Insecticidal and acaricidal activity

Research has been done on the insecticidal and acaricidal properties of *C. acaulis* essential oil (EO) and its main constituent, carlina oxide, for a variety of species with medicinal, urban, and agricultural relevance. Formulations based on *Carlina acaulis* have been tried on a number of economically significant insect species, such as mite (*Acarus siro*), moths (*Lobesia botrana*), tephritid flies (*Ceratitis capitate*), and stored-product beetles (Benelli et al., 2019; Kavallieratos et al., 2022b; Rizzo et al., 2021). By encapsulation of EOs in stable nanoformulations, including nanoemulsions (NEs), nanotechnology can effectively support the usage of EOs by enhancing their stability and efficacy (Benelli et al., 2020).
The formulation proved to be extremely toxic to medflies *Ceratitis capitata* and, caused a reduction in aggressive behavior and duration (Benelli et al., 2022). Carlina oxide exhibited a high degree of insecticidal action against aphid *Metopolophium dirhodum*, with an efficacy that was comparable with the tested commercial pesticide (Novák et al., 2024). Regarding beetles, *C. acaulis* EO showed a reliable control of *Prostephanus truncatus* and *Trogoderma granarium* (Kavallieratos et al., 2020). *C. acaulis* EO and carlina oxide showed larvicidal activity against *Culex quinquefasciatus,* a mosquito which is a significant threat to human and animal health as the main vector of West Nile virus (WNV) (Benelli et al., 2019). The effect

of *C. acaulis* root EO has been also investigated against *Musca domestica* L., an insect that can carry over a hundred diseases. The findings demonstrated that the F1 individuals' vitality, lifespan, and numbers were lower than those of their untreated ones (Pavela et al., 2020).
The insecticidal action of *Carlina acaulis* essential oil (EO) is primarily linked to Carlina oxide; with acetylcholinesterase (AChE) inhibition being the only confirmed mechanism (Benelli et al., 2019). Other potential mechanisms include modulation of GABAA receptors, inhibition of octopamine receptors, and P450 cytochromes (Benelli et al., 2019; Czyzewska et al., 2014). Carlina oxide, a phytoalexin, is sensitive to UV radiation and can undergo photoactivation, producing radicals and causing phototoxicity, leading to oxidative damage in insect tissues (Konovalov, 2014). The triple bond of its propynyl chain may be key to this radical production, suggesting Carlina oxide could act as a photosensitizer, enhancing its effects under UV light (Benelli et al., 2022; Pavela et al., 2020).

1.3.2. Antimicrobial activity

The antibacterial and antifungal activity of *Carlina acaulis* is one of its most well-known biological traits, and it is mainly due to the presence of carlina oxide in the essential oil that is derived from the plant's roots (Rosato et al., 2021). Significant effectiveness has been shown by carlina oxide against a range of bacterial such as *Staphylococcus pyogenes* and *Escherichia coli* and fungal infections such as *Candida albicans* and *Candida parapsilosis* (Herrmann et al., 2011; Rosato et al., 2021). According to research, this substance causes microbial cellular integrity to be disrupted, which either inhibits or destroys the bacteria. Given the rising worry about antibiotic resistance in this day and age, *Carlina acaulis* is a promising option for the development of natural antimicrobial drugs due to its antibacterial properties (Rosato et al., 2021).
Herrmann et al. (2011) reported that *Carlina acaulis* hexane extract and carlina oxide exhibit antitrypanosomal activity against Trypanosoma brucei brucei, potentially targeting the active center of trypanothione reductase by forming irreversible linkages with SH groups. Significant antibacterial activity was also shown by the methanolic extract of *Carlina acualis*, especially against methicillin-resistant *Staphylococcus aureus* and *Bacillus cereus* (Wnorowska et al., 2024). Chloroform and ethanol extract of *C. acaulis* showed anti-mycobacterial activity against Nontuberculous mycobacteria (Puk et al., 2023). *Carlina acaulis* also showed antiviral activities against SARS-CoV-2. Wnorowska et al. (2022) demonstrated that carlina oxide

inhibits the interaction between the SARS-CoV-2 spike protein's receptor binding domain (RBD) and the human ACE2 receptor.

1.3.3. Antioxidants activity

Carlina acaulis is also well known for its antioxidant qualities due to the presence of flavonoids and phenolic acids including luteolin, apigenin, caffeic acid, and chlorogenic acid. These substances have the ability to effectively eliminate free radicals, which are unstable chemicals that can lead to oxidative stress and cell damage. *Carlina acaulis* root dichloromethane extract and carlina oxide demonstrated antioxidant effects in the model nematode C. elegans by activating DAF-16, a transcription factor for anti-stress genes (Link et al., 2016). Additionally, the herb extract demonstrated radical scavenging properties and concentration-dependent antioxidant activity in DPPH tests. Furthermore, in an ethanol-induced stress gastric ulcer test in rats, extracts from the herb and roots showed gastroprotective properties and decreased carrageenan-induced rat paw edema in a dose-dependent manner (Đorđević et al., 2012).
Carlina acaulis antioxidants help protect the body against a range of chronic illnesses, such as cancer, heart disease, and neurological conditions, by scavenging free radicals. The plant's anti-aging properties are further enhanced by its antioxidant activity, which is why the cosmetics industry finds value in it.

1.3.4. Anticancer activity

A panel of human skin-derived cell lines, comprising BJ normal fibroblasts, UACC-903, UACC-647, and C32 melanoma cells, were used to evaluate the in vitro cytotoxicity of Carlina oxide. Carlina oxide was shown to have a harmful impact in vitro by inducing necrosis and apoptosis in both normal and UACC-647 melanoma cells. The melanoma cell line showed decreased levels of extracellular signal-regulated kinase 1/2 (ERK1/2) and AKT kinase. Additionally, it was noted that in the examined cell lines, Carlina oxide changed the expression of programmed cell death-ligand 1 (PD-L1) (Wnorowski et al., 2020).
Carlina oxide was tested for its cytotoxic effects on breast adenocarcinoma (MCT116 and MDA-MB 231) and human dermal fibroblasts (HuDe) (Benelli et al., 2019). In accordance with the results of Wnorowski (Wnorowski et al., 2020), the compound did not show selectivity between tumor and non-tumor cells. Moreover, Pavela et al. (2021) demonstrated that *C. acaulis* essential oil (EO) exhibited moderate toxicity towards human fibroblasts, with an IC_{50}

value of 115.92 ± 6.1 μg/mL. In contrast to these findings, Wnorowska et al. (2024) reported that the methanolic extract of *Carlina acaulis* exhibited no significant cytotoxic effects on normal human BJ foreskin fibroblasts, enterocytes derived from CaCo2 cells, or zebrafish embryos.

However, it is worthwhile to conduct more research to propose Carlina oxide as an anticancer drug due to its demonstrated cytotoxicity on human tumor cell lines. It is possible that tumor cells are more susceptible to Carlina oxide toxicity than non-tumor cells. Furthermore, research has shown that encapsulating the EO into a microemulsion reduced significantly its toxicity on human fibroblasts (IC_{50} of 5392.8 ± 315 μg mL^{-1}) (Pavela et al., 2021). This suggests that encapsulating Carlina oxide into nanocarriers such as nanoemulsions, nanoparticles, or liposomes may enable targeted delivery, reducing its cytotoxic effects on non-tumor cells while enhancing its therapeutic efficacy. Further research is essential to fully uncover the potential benefits of this approach.

1.3.5. Anti-inflammatory Activity

Another important component of *Carlina acaulis'* biological actions is its anti-inflammatory qualities. The plant contains a number of triterpenes and flavonoids that have potent anti-inflammatory properties. These substances lessen inflammation and its associated symptoms by blocking the synthesis of pro-inflammatory cytokines and enzymes. Because of this, *Carlina acaulis* is very helpful in the treatment of inflammatory diseases such inflammatory bowel disease, asthma, and arthritis. The anti-inflammatory activity also complements its antioxidant properties, offering a comprehensive strategy for lowering oxidative stress and chronic inflammation (Achiri et al., 2021; Đorđević et al., 2012).

1.3.6. Herbicidal and nematicide activity

C. acaulis EO has shown great promise as a natural product with strong phytotoxic effects on weeds. This EO, which included 98% of the polyacetylene compound Carlina oxide, showed significant phytotoxic effects. These included causing necrosis on leaves, reducing relative water content and total leaf area, and raising the ratio of dry to fresh weight, indicating a change in the water status of the plant. The EO also produced osmotic stress and interfered with photosynthetic activities, further highlighting its potential as an effective herbicidal agent (Álvarez-Rodríguez et al., 2023).

In addition, *C. acaulis* EO has exhibited significant activity against *Meloidogyne incognita*, both in vivo and in vitro (Ntalli et al., 2023).

Aim of this study:

The aim of this project is to extract and isolate bioactive compounds from *Carlina acaulis* and evaluate the bioactivities of the fractions.

Chapter 2

2. Material and methods

2.1. Plant material and extraction

The commercial batch of *C. acaulis* (code MP0136, no C-250518310518) roots were sourced from the A. Minardi & Figli Srl (Bagnacavallo, RA, Italy). They were obtained from an Albanian accession harvested in autumn 2017. The roots were ground into a fine powder.

The powdered plant material was weighed and extracted using a Soxhlet extractor with 300 mL of each of solvents of differing polarities: hexane, diethyl ether (non-polar), ethyl acetate, dichloromethane, acetone (moderately polar), methanol (polar) (Merck, Italy). The obtained extracts were evaporated to dryness under reduced pressure at 50°C using a rotary evaporator. By using the following formula, the extraction yield was calculated:

$$\text{Yield (\%)} = (\text{Weight of Dry Extract (g)} / \text{Weight of Dried Plant Material (g)}) \times 100$$

2.2. Thin layer chromatography

TLC plates (Sigma-Aldrich) were cut to the desired size, a baseline was drawn with a pencil 1 cm from the bottom edge of the plate. The dried extracts were re-dissolved in a small volume of the respective solvents (hexane, ethyl acetate, methanol). Using capillary tubes, small spots of each extract were applied onto the baseline of the TLC plate, ensuring spots were not too large to prevent streaking. The spots were allowed to dry completely before development.

The mobile phase was prepared using an appropriate solvent mixture, typically a combination of hexane, ethyl acetate, and methanol in varying ratios, depending on the polarity of the compounds to be separated. In this study, for hexane and ethyl acetate crude extracts, mixture of hexane:ethyl acetate (99:1) and hexane:ethyl acetate (95:5) was used as the mobile phase. The developing chamber was filled with the mobile phase to a depth of 0.5 cm. The TLC plate was placed in the chamber, ensuring the baseline was above the solvent level. The chamber was sealed with a lid to maintain a saturated atmosphere. The solvent was allowed to ascend the plate until it was 1 cm from the top edge, then the plate was removed, and the solvent front was marked immediately.

The developed TLC plates were dried completely. Plates were observed under a UV lamp to detect fluorescent spots. For non-fluorescent compounds, the plates were sprayed with a mixture of methanol and sulfuric acid (50:2) and then placed in an oven at 120°C.

2.3. Separation and purification in column chromatography

Silica column chromatography was employed to separate and purify bioactive compounds from the plant extract.

2.3.1. Column chromatography for hexane extracts

To prepare the column, the plastic column was filled with dry silica gel (Merck, Italy). The plant extracted by hexane replicate 1 was dissolved in a minimal amount of the initial solvent (hexane) and carefully layered onto the top of the silica gel column. Elution was initiated with 100% hexane to remove non-polar compounds. This was followed by a series of hexane and ethyl acetate mixtures, gradually increasing the ratio of ethyl acetate (95:5 and 70:30).

The eluent was collected in separate tubes, each containing an equal volume (20 mL per tube), and each fraction collection tube was sequentially labeled to keep track of the elution order. Thirty-five fractions were collected. Samples from each fraction were spotted onto TLC plates to analyze their composition. The TLC plates were developed using an appropriate solvent system -hexane:ethyl acetate (95:5, 90:10, and 70:30)- to obtain sharp and distinct spots, which were then visualized under UV light. Fractions containing similar compounds, as determined by TLC analysis, were pooled together, resulting in six pooled fractions. The solvents from the pooled fractions were evaporated under reduced pressure at 33°C using a rotary evaporator. The pooled fractions were spotted onto TLC plates again to analyze their composition.

The first fraction, containing a single spot, was sent for analysis by NMR. The purified compounds were then stored in labeled vials at -20°C for further analysis and bioactivity testing.

Fraction CH12, which had three spots, was re-subjected to column chromatography on silica gel and eluted with 100% hexane and hexane:ethyl acetate (95:5) to obtain 13 sub-fractions which were pooled into 3 fractions. The TLC plates were developed using hexane:ethyl acetate (95:5) as mobile phase.

The procedure was repeated for the plant extract using hexane replicate 2. Thirty-nine fractions were collected. Fractions containing similar compounds, as determined by TLC analysis, were combined, resulting in nine pooled fractions.

2.3.2. Column chromatography for ethyl acetate extracts

The procedure was repeated for ethyl acetate extraction. The silica column was eluted with hexane, hexane:ethyl acetate (95:5, 70:30, 50:50), ethyl acetate, and methanol, yielding 74 fractions which were pooled into 6 fractions. For the TLC plates, a mixture of hexane:ethyl acetate (70:30) and dichloromethane:methanol:H_2O:toluene (20:5:1:15) was used as the mobile phase, and the spots were then visualized under UV light.

Fraction CE16 was re-subjected to column chromatography on silica gel and eluted with dichloromethane:methanol 80:20, 13 sub-fractions were collected which were pooled into 2 fractions. The TLC plates were developed using dichloromethane:methanol:H_2O:toluene (20:5:1:15) as mobile phase.

2.4. Preparative TLC

Preparative Thin Layer Chromatography (TLC) is a method of choice when it comes to purifying and isolating chemicals from small to moderate quantities of material.

First, the plant extract was dissolved in a small amount of a suitable solvent (hexane, methanol, or ethyl acetate). The CAMAG® Linomat 5 semi-automatic sample dispenser was used to carefully spray samples (100 µl) onto TLC plates. To make sure that enough sample was sprayed on the plates, this process was repeated three or four times.

The TLC plate was then developed in a chamber containing the solvent, enabling the solvent to move up the plate until it is about two centimeters from the top. For hexane extracts (CH12), hexane:ethyl acetate (95:5) was used as a mobile phase; for ethyl acetate extracts (CE16), a combination of dichloromethane:methanol:H_2O:toluene (20:5:1:15) was employed. The plate was gently taken out from the chamber and let to air dry.

In order to identify the separated compounds, the developed plate was visualized under a UV light. Any visible bands were marked with a pencil, then the appropriate silica gel pieces that contain the compounds was scraped out using a scraper. The scraped silica gel was moved to a small flask and methanol was added to elute it. By using a filter paper, silica gel particles were removed. The solvent was evaporated at 33°C and reduced pressure using a rotary evaporator in order to concentrate the separated compound. NMR was used to characterize the separated compounds.

2.5. Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR were acquired using a Bruker Avance III spectrometer operating at 400 MHz for 1H and 100 MHz for 13C. Standard Bruker sequences were used for the acquisition of 1H, HSQC, HMBC and COSY experiments.

2.6. Cytotoxicity assay

The effect of *Carlina acaulis* extract (hexane, ethyl acetate, methanol, dichloromethane, acetone, and diethyl ether) and the purified compound Carlina oxide on the viability of two cell lines at various concentrations was studied.

Cervix squamous cell carcinoma cell line was used to evaluate the cytotoxic effect of Carlina extract, carcinoma A431 cells was susceptible to the chemotherapeutic drug cisplatin and A431 pt showed resistance. The Crystal Violet Staining (CVS) was employed to conduct the assay.

A stock solution of the compounds was prepared through a 1:1 dilution of DMSO in the culture medium, ensuring that the final DMSO concentration did not surpass 0.5% at the maximum working concentration. The control group was comprised of cells only treated with 0.5% DMSO in the growth media.

The cell lines were first cultured in DMEM medium containing antibiotics and incubated at 37 °C. The cells were trypsinized and then seeded into 96-well plates at a density of 3,500 cells per well. The plates were then incubated for 24 hours at 37°C to promote cell attachment. After the incubation period, the cells were treated with various doses of Carlina extracts, ranging from 10 µg/ml to 150 µg/ml, and with the purified compound Carlina oxide, ranging from 5 µg/ml to 75 µg/ml. For the methanol extract, treatment doses ranged from 50 µg/ml to 500 µg/ml. The treatment period lasted for 24 hours.

After the treatment period, the media was aspirated and the cells were carefully washed with phosphate-buffered saline (PBS). The cells were fixed by adding 100 µL of 4% paraformaldehyde in each well and incubating them for 15 minutes at room temperature. The fixing agent was removed, and the cells were stained for 20 minutes using a 0.5% crystal violet solution. The remaining stain was removed by washing with distilled water, and then the plates were allowed to air dry. To dissolve the bound dye, 200 µL of a 1% acetic acid solution was then added to each well. At 570 nm, the optical density (OD) was measured with a microplate reader (Titertek Multiscan microElisa) and the IC_{50} value was calculated as a cytotoxic parameter. For every experiment, five replicates were carried out.

By comparing the optical density (OD) values of the treated cells with those of the control group, the cytotoxicity of the Carlina extract was evaluated. The results were displayed as a percentage of the control, which was set at 100%. Then, the percentage of cell viability was plotted against different concentrations of Carlina extract. To determine the percentage viability of the cells, the mean absorbance of the treated group is divided by the mean absorbance of the control group. This ratio is then multiplied by 100 to obtain the final percentage viability:

Cell Viability (%) = (Absorbance of treated cells/Absorbance of control cells)×100

The relationship equation between the logarithm of the concentration and the percentage of cell viability yields the IC_{50} cytotoxic test parameter. By substituting 50 for y in this equation, the corresponding x value was found. To determine the concentration that inhibits 50% of cell viability, this x value was then converted back using its antilog (IC_{50}).

2.7. Statistical analysis

The statistical analysis was performed using Student's t-test to compare the viability of treated samples with control samples. The purpose of this test is to determine whether the means of the two groups differ significantly from one another. The results are expressed as mean ± standard deviation. A p-value of less than 0.05 was considered statistically significant. All analyses were conducted using SPSS software.

Chapter 3

3. Results

Yields of extracts were: 2.36% for hexane extraction, 1.53% for ethyl acetate extraction, 5.19% for methanol extraction. The result indicates the percentage of the original dried plant material that was successfully converted into the final dry extract.

3.1. Hexane extract

For the plant extract using hexane replicate 1, thirty-five fractions were collected. Fractions containing similar compounds, as determined by TLC analysis, were pooled together, resulting in six pooled fractions (Table 1; Fig. 1).

In preparative TLC, three visible bands (A, B, and C) were identified for CH12 and CH12-S2, and two visible bands (A and B) were identified for CH12-S3 (Fig. 2). Due to the insufficient quantity of fractions, the NMR results for CH12 (PB and PC) were not sufficient to establish a main constituent, fraction mostly contains fatty acid derivatives and some polyacetylene. The remaining fractions CH12-S2 and CH12-S3 (PA-PB-PC) need to be further analyzed using NMR.

Table 1. Number and name of fractions for hexane extract replicate 1

Extraction by hexane- replicate 1 (CH1)						
Pooled fractions	**CX2-10**	**CX11-13**	**CX14-17**	**CX21-28**	**CX29-30**	**CX31-35**
Code	CH11	CH12	CH13	CH14	CH15	CH16
		Preparative TLC				
		CH12-PA CH12-PB CH12-PC				
		Sub-fractions				
		CH12-S1 (CX4-7) CH12-S2 (CX8-10) CH12-S3 (CX11-13)				
		Preparative TLC for CH12-S2				
		CH12-S2-PA CH12-S2-PB CH12-S2-PC				
		Preparative TLC for CH12-S3				
		CH12-S3-PA CH12-S3 -PB				

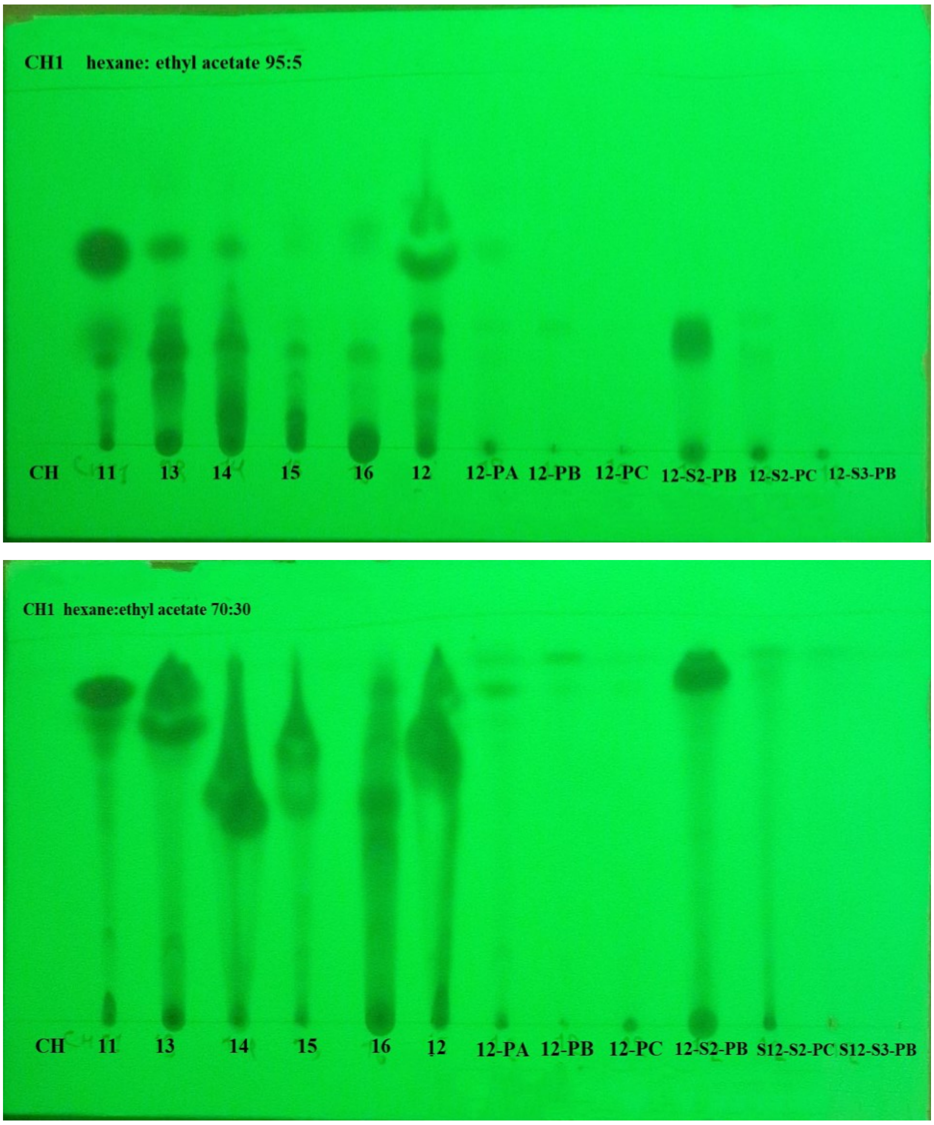


Fig. 1. TLC plates for plant roots extracted with hexane replicate 1. The mobile phase used was hexane:ethyl acetate (95:5 and 70:30). The names of the samples are provided in Table 1.

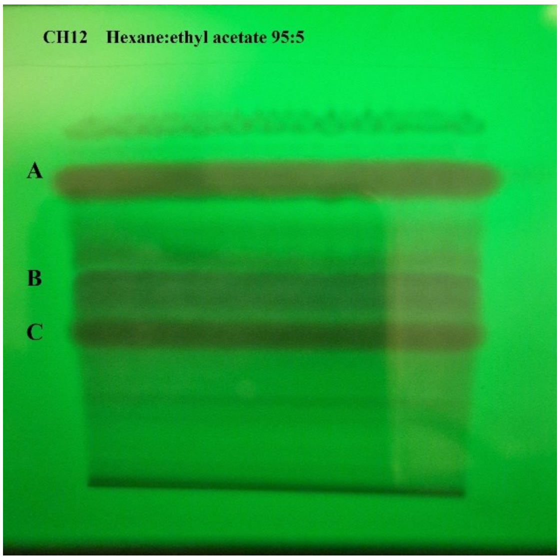


Fig. 2. Preparative TLC for CH12. The mobile phase was hexane:ethyl acetate (95:5).

For the plant extract using hexane replicate 2, thirty-nine fractions were collected. Fractions containing similar compounds, as determined by TLC analysis, were combined, resulting in nine pooled fractions (Table 2; Fig. 3).

Table 2. Number and name of fractions for hexane extract replicate 2

Extraction by hexane- replicate 2 (CH2)									
Pooled fractions	**CX3-6**	**CX7-8**	**CX9-12**	**CX13-18**	**CX19-21**	**CX22-23**	**CX24-28**	**CX29-32**	**CX33-39**
Code	CH21	CH22	CH23	CH24	CH25	CH26	CH27	CH28	CH29

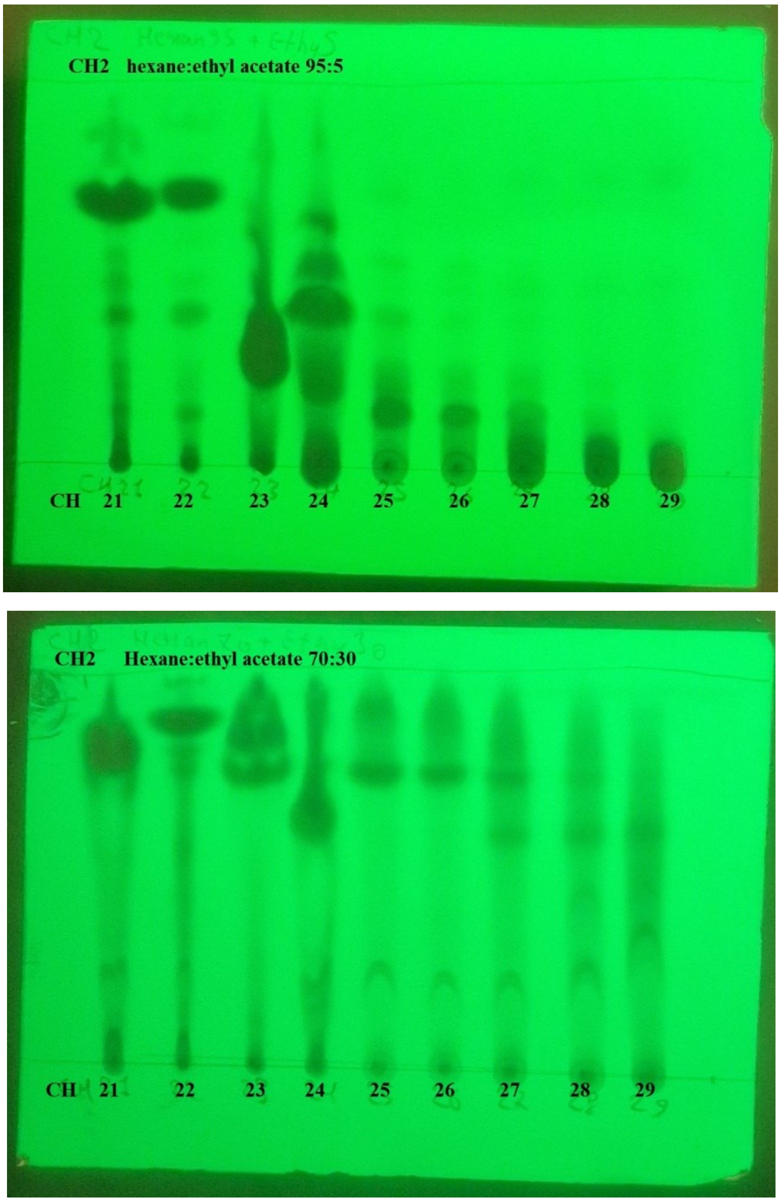


Fig. 3. TLC plates for plant roots extracted with hexane replicate 2. The mobile phase used was hexane:ethyl acetate (95:5 and 70:30). The names of the samples are provided in Table 2.

3.2. NMR analysis of the *Carlina acaulis* essential oil

The hexane extract of *C. acaulis* (CH11 and CH12-PA) presented a main spot in TLC suggesting that they are mainly composed by a single compound. For this reason, the fractions were pooled and analyzed using NMR experiments, which led to the identification of Carlina oxide as the main constituent (Fig. 4).

The spectra of the isolated compound are reported in the following figure. The H-NMR spectrum of the Carlina oxide is characterized by the presence of the signals ascribable to the aromatic benzene ring that appear as multiple signals in the spectrum region δ 7.4 while two group of signals, one doublet at δ 6.6 and two overlapped doublets at δ 6.4 are ascribable to the proton signals of the furane ring.

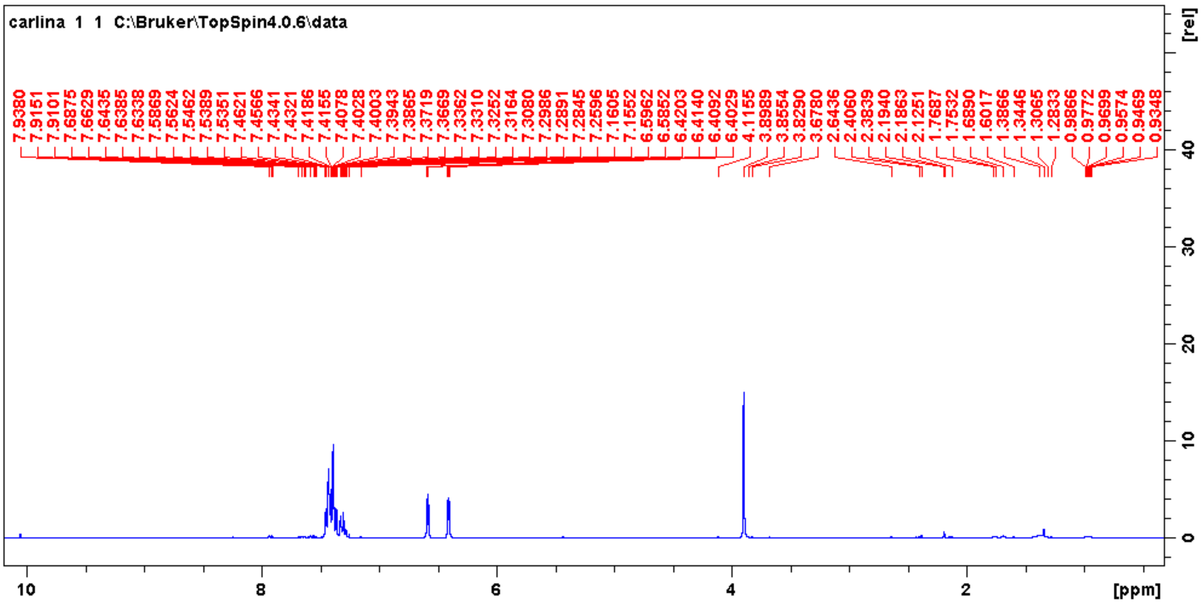
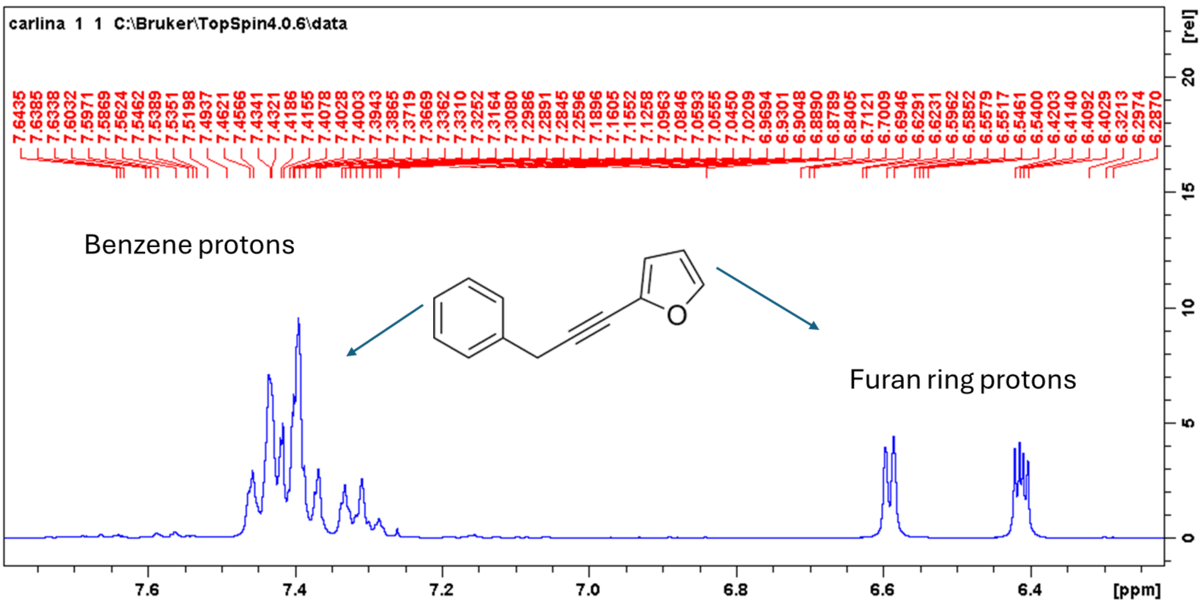


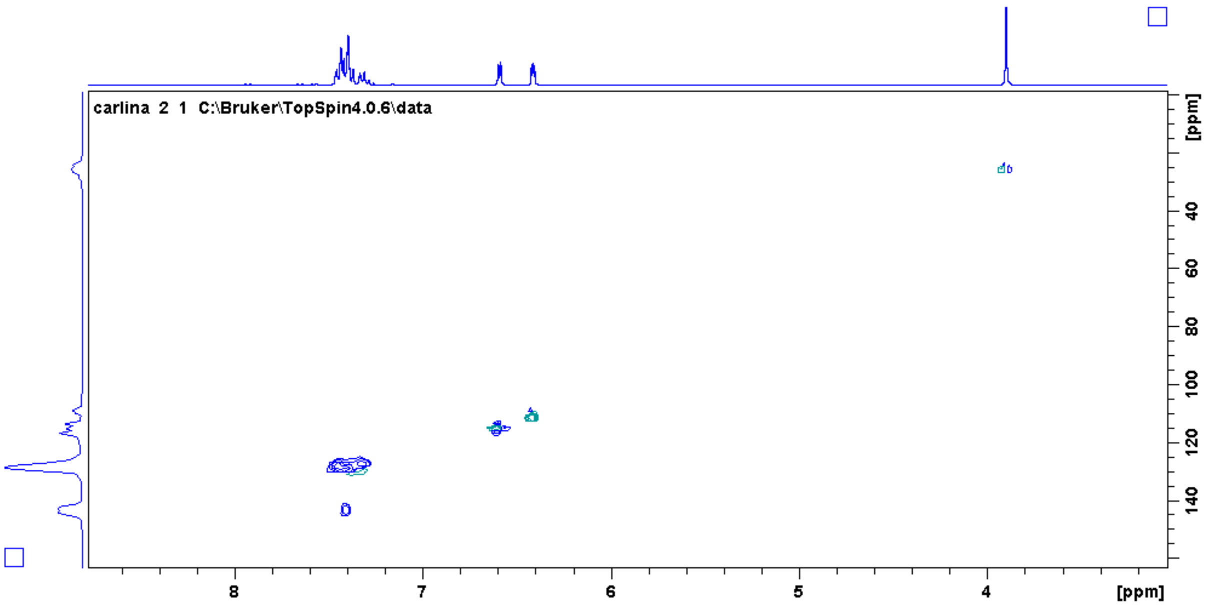
Fig. 4. ^{1}H NMR spectrum of the *Carlina acaulis* root.

A detail of this part of the spectrum with the structure of Carlina oxide is reported in the figure below.

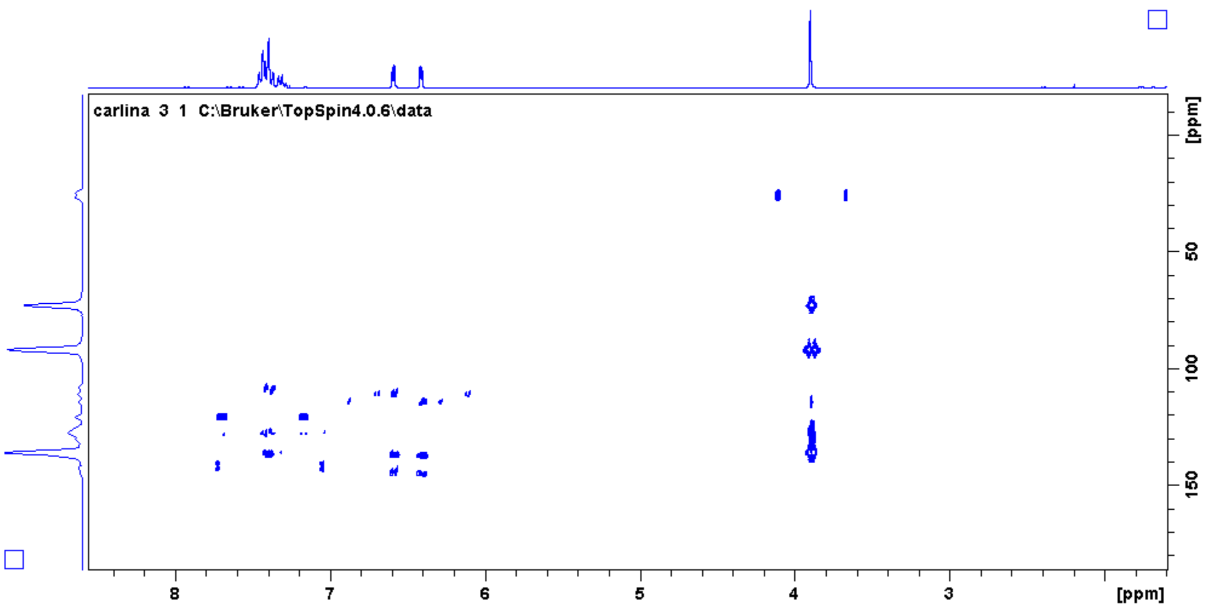


Further diagnostic signal is the singlet at δ 3.89 that represent the benzyl CH2.

2D spectra were also acquired allowing the confirmation of the structure and here we report the HSQC-DEPT and HMBC:



HSQC spectrum (Heteronuclear Single Quantum Coherence)



HMBC (Heteronuclear Multiple Bond Correlation)

The Spectra allowed the complete characterization of Carlina oxide that was also used for the bioassays.

3.3. Ethyl acetate extract

For the plant extract using ethyl acetate, 74 fractions were collected. Fractions containing similar compounds, as determined by TLC analysis, were pooled together, resulting in six pooled fractions (Table 3; Fig. 5). In preparative TLC, two visible bands (A and B) were identified for CE16, which need to be further analyzed using NMR.

Table 3. Number and name of fractions for ethyl acetate extract

Extraction by ethyl acetate (CE1)						
Pooled fractions	**CX3-16**	**CX24-25**	**CX26-30**	**CX34-41**	**CX42-54**	**CX55-74**
Code	CE11	CE12	CE13	CE14	CE15	CE16
						Sub-fractions
						CE16-S1 (CX 1-5) CE16- S2 (CX 6-9)
						Preparative TLC for CE16-S1
						CE16-S1-PA CE16-S1-PB

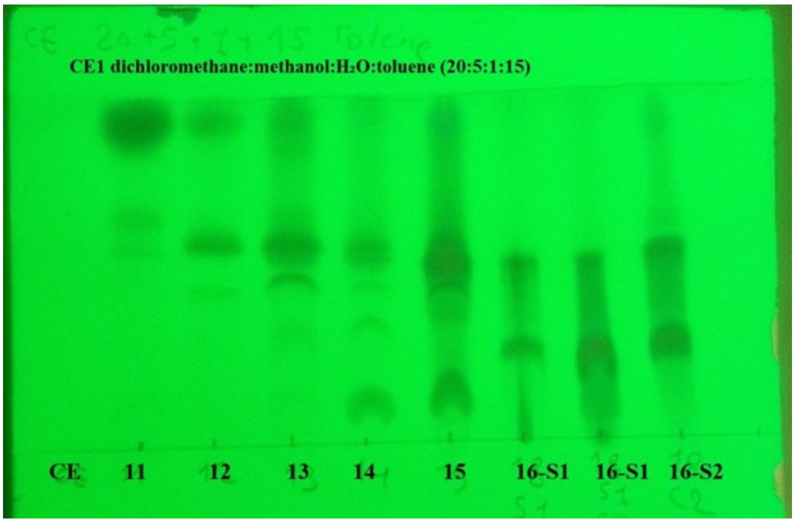


Fig. 5. TLC plates for plant roots extracted with ethyl acetate. The mobile phase used was dichloromethane:methanol:H_2O:toluene (20:5:1:15). The names of the samples are provided in Table 3.

All fractions and sub-fractions obtained in this study were dried under nitrogen and stored for future bioactivity assays.

3.4. Cytotoxicity assay

The effect of *Carlina acaulis* extract (dissolved in hexane, methanol, ethyl acetate, acetone, and dichloromethane, diethyl ether and purified compounds carlina oxide) on the viability of two cell lines, A431 (susceptible to cisplatin) and A431-PT (resistant to cisplatin), was examined at different concentrations.

3.3.1. Hexane extract

Both cell lines (A431 and A431-PT) exhibit 100% viability in the control treatment. The *Carlina acaulis* hexane extract reduces cell viability in both A431 and A431-PT cell lines in a dose-dependent manner, with A431-PT cells being more sensitive to the extract at various concentrations.

A significant decrease in the viability of A431 cells is observed as the concentration of the extract increases ($p < 0.001$, $p < 0.0001$). At 100 µg/ml, the viability of the cell lines is around 50%, and it decreases to approximately 23% as the concentration of the extract increases to 150 µg/ml.

The A431-PT cells also show a decrease in viability with increasing concentrations, but the decline in cell viability begins at a lower concentration, 50 µg/ml. The viability of the cell line decreases fivefold as the concentration of the extract doubles. At 100 and 125 µg/ml, the viability reduction is approximately 15%, and it continues to decrease significantly at higher concentrations of 150 µg/ml.

There is a statistically significant difference in cell viability between A431 and A431-PT at 100 µg/ml concertation ($p < 0.05$), suggesting that A431-PT cells are more sensitive to the extract than A431 cells at this concentration (Fig 6A).

3.3.2. Ethyl acetate extract

Both cell lines (A431 and A431-PT) exhibit 100% viability in the control samples. The *Carlina acaulis* ethyl acetate extract reduces cell viability in both A431 and A431-PT cell lines in a dose-dependent manner. The A431-PT cell line appears to be more sensitive to the extract, especially at higher concentrations.

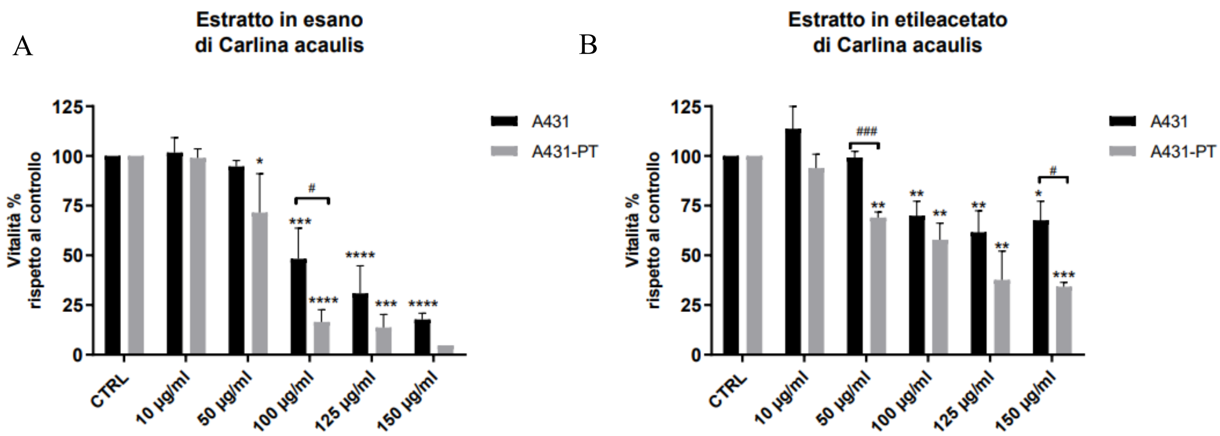
The viability of A431 cells decreases at 100 µg/ml, 125 µg/ml, and 150 µg/ml compared to the control; however, there are no significant differences in cell viability among these concentrations.

The A431-PT cells show a decrease in viability with increasing concentrations of the extract. This decrease starts at a lower concentration of 50 µg/ml. At 100 µg/ml, cell viability is around 60% and drops to approximately 40% as the concentration increases to 125 µg/ml and 150 µg/ml.

At 50 µg/ml, a significant difference in viability between A431 and A431-PT cells is observed ($p < 0.01$), indicating a difference in sensitivity to the extract at this concentration. Similarly, at 150 µg/ml, there is a significant difference between the cell lines ($p < 0.05$), with A431-PT cells being more sensitive to the extract than A431 cells (Fig 6B).

3.3.3. Methanol extract

Both cell lines (A431 and A431-PT) exhibit 100% viability in the absence of the extract. The methanol extract does not significantly affect the viability of either A431 or A431-PT cell lines at the tested concentrations. The viability of both cell lines maintains nearly 100% across all tested concentrations (50 µg/ml to 500 µg/ml), and no significant reduction is observed even at the highest concentration of 500 µg/ml (Fig 6C).



C

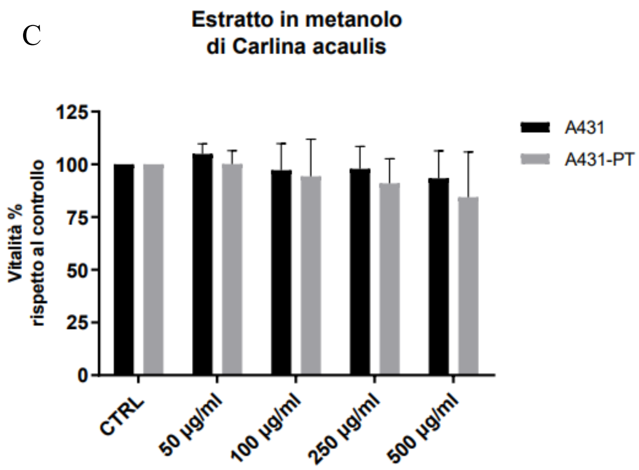


Fig. 6. Effect of hexane extract of *Carlina acaulis* (A), ethyl acetate extract of *Carlina acaulis* (B), methanol extract of *Carlina acaulis* (C) on cell viability of A431 (susceptible to cisplatin) and A431-PT (ressitant to cisplatin) cell lines after 24h of treatment. Data expressed as % viability compared to control, resulting from the average of n= 2-5 experiments ± dev. Standard.

* p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, treated on the respective control

# p<0.05, ## p<0.01, ### p<0.001, #### p<0.0001, A431-PT vs A431

3.3.4. Dichloromethane extract

Both A431 and A431-PT cell lines have 100% viability in the control samples. The viability of both A431 and A431-PT cell lines at 10 μg/ml are around 100%, indicating that 10 μg/ml of the extract does not significantly affect cell viability.

A dose-dependent decrease in cell viability can be observed after treatment with dichloromethane extract. At 50 μg/ml concentration viability decreases to approximately 75% for A431 and A431-PT. A significant reduction in viability is observed at 75 μg/ml concentration, with A431-PT around 60% and A431 around 50%. At 100 μg/ml concentration, viability further decreases to around 50% for A431-PT and 40% for A431. The extract has a stronger inhibitory effect at 75 μg/ml concentration compared to 50 μg/ml. The lowest viability is observed at 150 μg/ml, with A431 and A431-PT around 20%. This suggests that the extract has a strong cytotoxic impact at high doses.

However, there is no significant differences in cell viability between two cell lines at any concentrations (Fig. 7A).

3.3.5. Acetone extract

Both A431 and A431-PT cell lines have 100% viability in the absence of the extract. The extract shows a dose-dependent decrease in cell viability.

Viability remains close to 100% for A431 at 10 µg/ml and, but there is a significant effect at this concentration for A431-PT ($p<0,05$). There is a significant reduction in viability for both cell lines at 50 µg/ml. At 50 µg/ml, viability decreases to approximately 50% for A431 and A431-PT. It continues to decrease significantly at higher concentrations,75 µg/ml, with A431 around 40% and A431-PT around 30%. At 100 µg/ml, viability drops even further to around 30% for A431, suggesting a more potent inhibitory action at this dose. The lowest viability is observed at 150 µg/ml, with A431 and A431-PT around 10%. This implies a potent cytotoxic effect of the extract at high concentrations.

However, no significant differences were observed in cell viability between two cell lines at any concentrations (Fig. 7B).

3.3.6. Diethyl ether extract

Both A431 and A431-PT cell lines have 100% viability in control treatments. The extract shows a dose-dependent decrease in cell viability.

No significant differences were observed between viability of treated cell lines and controls at 10 µg/ml. This is close to 100% for both A431 and A431-PT cell lines. At 25 µg/ml, a slight decrease in viability is observed for A431, but it is not significant. A431-PT also remains close to the control level. Viability decreases significantly at 50 µg/ml to approximately 40% for A431 and 50% for A431-PT. The viability of the cell line decreases two times as the concentration of the extract doubles. This suggests a significant reduction in cell viability for both cell lines, with A431 being more affected. A stronger cytotoxic effect of the extract is observed at higher concentrations. A more pronounced reduction in viability is observed at 75 µg/ml and 100 µg/ml, with A431 and A431-PT around 25%.

A statistical difference in viability is observed between A431 and A431-PT cells at a concentration of 25 µg/ml. However, decrease in viability at this concentration is not significant. No significant differences in cell viability between the two cell lines are observed at other concentrations (Fig. 7C).

3.3.7. Carlina oxide

Both A431 and A431-PT cell lines exhibit 100% viability in the absence of the extract. No significant differences in viability were observed between the treated cell lines and controls at 5 and 10 μg/ml concentrations, with viability close to 100% for A431-PT and 90% for A431. However, the viability percentages of the treated cell lines differ significantly from the controls at 25 μg/ml ($p < 0.01$), and at 50 and 75 μg/ml ($p < 0.001$ and $p < 0.0001$). At 25 μg/ml, the viability of A431 cells decreases significantly to approximately 50%, while A431-PT cells decrease to around 60%.

The viability continues to decrease significantly at higher concentrations, reaching around 25% at 50 μg/ml. At 75 μg/ml, viability drops further to approximately 15% for A431 and 10% for A431-PT, indicating a more potent inhibitory effect at this concentration.

No significant differences in cell viability were observed between the two cell lines at any concentration (Fig. 7D).

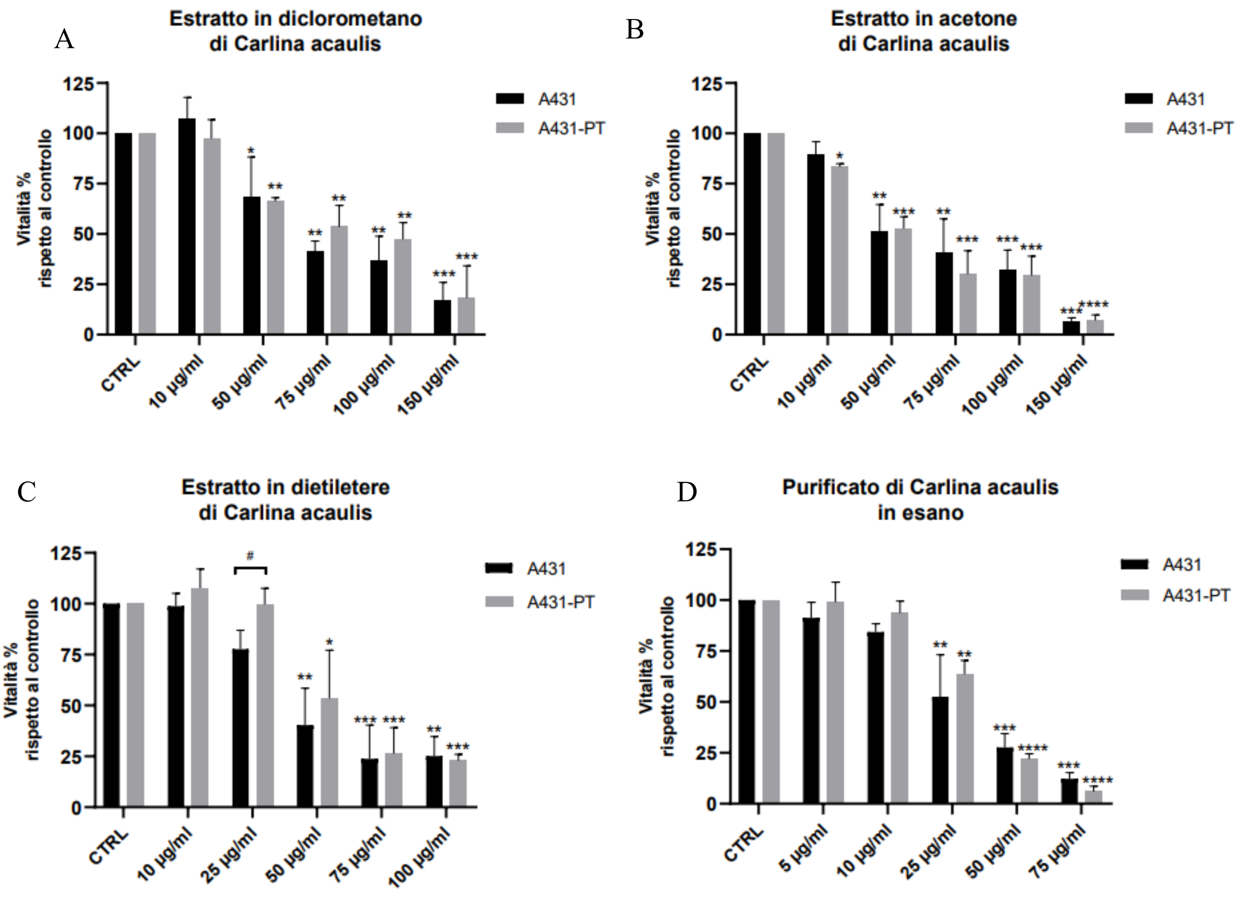


Fig. 7. Effect of *Carlina acaulis* dichloromethane extract (A), *Carlina acaulis* acetone extract (B), *Carlina acaulis* diethyl ether extract (C) and *Carlina acaulis* purified in hexane (D) on cell viability of A431 (susceptible to cisplatin) and A431-PT (resistant to cisplatin) cell lines after 24h of treatment. Data expressed as % viability compared to control, resulting from the average of n= 2-5 experiments ± dev. Standard.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, treated on the respective control

# $p<0.05$, ## $p<0.01$, ### $p<0.001$, #### $p<0.0001$ A431-PT vs A431

Carlina oxide had the highest cytotoxicity on both cell lines at 50 µg/ml. Among the various crude extracts tested, diethyl ether and acetone demonstrated the highest cytotoxicity on A431, resulting in the lowest cell viability at concentrations of 50 and 100 µg/ml. Specifically, at a concentration of 150 µg/ml, the acetone extract showed the most significant reduction in cell viability of A431 compared to all other extracts.

For the A431-PT cell line, at a concentration of 50 µg/ml, both diethyl ether and acetone extract also showed significant cytotoxic effects on cells. At the concentration of 100 µg/ml, the hexane extract exhibited the highest cytotoxicity against the A431-PT, resulting in the lowest cell viability. Notably, at a concentration of 150 µg/ml, the hexane extract led to the most substantial reduction in cell viability of A431-PT cells compared to all other extracts tested. These data are preliminary and allow us to do some consideration but need more replicate to be statistically significant.

It can be concluded that the most active fractions are Carlina oxide, diethyl ether, acetone and hexane.

3.3.8. IC_{50} values for Carlina acaulis extracts and purified compound

The IC_{50} value is the concentration at which the drug inhibits cell viability by 50%. Lower IC_{50} values imply stronger potency of substance.

Ethyl Acetate Extract: The ethyl acetate extract shows moderate cytotoxicity, with IC_{50} values of 131.50 ± 0.43 µg/mL for A431 cells and 94.73 ± 13.97 µg/mL for A431-PT cells. This indicates that the extract is slightly more effective against the cisplatin-resistant A431-PT cells (Table 4).

Hexane Extract: The hexane extract has a stronger cytotoxic effect than the ethyl acetate extract. At 102.79 ± 1.58 µg/mL, the hexane extract inhibits 50% of the A431 cell's viability and at 63.21 ± 5.99 µg/mL, it inhibits 50% of the A431-PT cell's viability. The cisplatin-resistant A431-PT cells is more sensitive to the hexane extract than the A431 cell line, as indicated by the lower IC_{50} value (Table 4), suggesting greater effectiveness against this cell line.

Dichloromethane Extract: The dichloromethane extract shows comparable cytotoxic effects on both cell lines. At a concentration of 75.90 ± 10.16 µg/mL, the dichloromethane extract reduces the viability of A431 cells by 50%. For the A431-PT cells, which are resistant to cisplatin, a slightly higher concentration of 93.63 ± 18.55 µg/mL is required to achieve the same 50% reduction in cell viability. The higher concentration needed to achieve 50% inhibition in A431-PT cells indicates that these cells are somewhat less sensitive to the dichloromethane extract (Table 4).

Acetone Extract: The acetone extract exhibits relatively strong cytotoxic effects, with IC_{50} values of 72.94 ± 14.31 µg/mL for A431 cells and 59.71 ± 7.41 µg/mL for A431-PT cells, showing a slightly higher potency against cisplatin-resistant cells (Table 4).

Diethyl Ether Extract: The diethyl ether extract shows potent cytotoxicity with IC_{50} values of 53.25 ± 9.84 µg/mL for A431 cells and 54.06 ± 8.52 µg/mL for A431-PT cells, indicating comparable effectiveness against both cell lines (Table 4).

Purified Compound (Carlina Oxide): Carlina oxide shows the highest cytotoxicity among all extracts, with the lowest IC_{50} values of 27.91 ± 3.84 µg/mL for A431 cells and 26.54 ± 4.13 µg/mL for A431-PT cells. This suggests that Carlina oxide is the most potent component against both cisplatin-sensitive and cisplatin-resistant cell lines (Table 4).

Overall, the purified compound Carlina oxide exhibits the strongest cytotoxic effects against both cell lines, followed by the diethyl ether and acetone extracts, making these the most promising candidates for further study as potential anticancer agents. The cisplatin-resistant A431-PT cells appears to be more sensitive to the ethyl acetate, hexane and acetone extracts than the cisplatin-sensitive A431 cell line.

Table 4. IC_{50} (µg/ml) of *Carlina acaulis* extracts

***Carlina acaulis* extracts**	**A431**	**A431-PT**
Methanol extract	/	/
Ethyl acetate extract	131,50 ± 0,43	94,73 ± 13,97
Hexane extract	102,79 ± 1,58	63,21 ± 5,99
Dichloromethane extract	75,90 ± 10,16	93,63 ± 18,55
Acetone extract	72,94 ± 14,31	59,71 ± 7,41
Diethyl ether extract	53,25 ± 9,84	54,06 ± 8,52
Purified compound (Carlina Oxide)	27,91 ± 3,84	26,54 ± 4,13

4. Discussion

Plants are a rich source of potential therapeutic compounds. By studying their properties, we can uncover new molecules with beneficial effects on human health. Despite being mentioned in ethnomedicine, *Carlina acualis* remains largely unexplored, offering an exciting opportunity for discovery (Puk et al., 2023).

In this study, the *Carlina acaulis* extract shows a dose-dependent decrease in cell viability for both A431 and A431-PT cell lines and the most potent fractions identified were diethyl ether, and acetone. Isolation of bioactive compounds from fractions, particularly those from diethyl ether and acetone, should be pursued and continued in future studies. The least potent extract was the ethyl acetate extract, which had the highest IC_{50} values (Table 4).

For methanol extracts, no cytotoxicity was observed in either cell line. These findings highlight that the potential for cytotoxic effects associated with the specific extract or compound used. For instance, human cervical cancer (HeLa) cells showed significant resistance to Carlina oxide (IC_{50} 446.0 µg/mL), hexane and dichloromethane extracts (1722.4 µg/mL and 1995.0 µg/ml, respectively) (Hermann et al., 2011). In contrast, the ethyl acetate fraction significantly reduced HeLa cell viability (Sowa et al., 2023).

Similarly, the methanolic extract of *Carlina acaulis* did not show cytotoxicity on normal human BJ foreskin fibroblasts (Wnorowska et al., 2024). The methanolic extract can be considered a good candidate for use as an antimicrobial or insecticidal agent, as it did not exhibit cytotoxicity towards human cell lines.

In the hexane, ethyl acetate, and acetone extracts, the A431-PT cell line shows a significant decrease in viability at lower concentrations, particularly at 10 and 50 µg/ml. While, the A431 cell line is slightly more resistant at lower concentrations but exhibits a significant reduction in viability at higher concentrations (100 µg/ml and above). The data of IC_{50} also confirmed that A431-PT cells are more sensitive to these extracts than A431 cells (Table 4). This data emphasizes the varying sensitivities of the two cell lines to different extracts and the purified compound.

This suggests that *Carlina acaulis* extract has potential as an anti-cancer agent, with A431-PT being slightly more sensitive, and its effects are more pronounced at higher concentrations. Further investigation is imperative to elucidate the mechanisms underlying its cytotoxicity and to uncover its mode of action.

Previous results showed that the main components of Carlina is Carlina oxide (Benelli et al., 2019; Spinozzi et al., 2023). There are several reports on the cytotoxicity of Carlina extracts and carlina oxide against various cell lines. Wnorowski et al. (2020) demonstrated that Carlina oxide exhibits selective cytotoxicity towards human skin cell lines. Specifically, BJ fibroblasts and UACC-647 melanoma cells were sensitive to Carlina oxide, whereas UACC-903 and C32 melanoma cells showed significant resistance, with minimal or no reduction in cell viability observed. Herrmann et al. (2011) investigated the cytotoxic effects of Carlina oxide and hexane and dichloromethane extracts on the HeLa cervical cancer cell line. Their findings revealed that HeLa cells exhibited significant resistance to Carlina oxide and crude extracts (Herrmann et al., 2011). Sowa et al. (2023) reported that the ethyl acetate fraction of *Carlina acaulis*, abundant in polyphenolic compounds, possesses remarkable anti-cancer properties. This fraction significantly decreased the viability of human colorectal adenocarcinoma (HT29) and human cervical cancer (HeLa) cells, while causing minimal impact on normal cells (Sowa et al., 2023). This selective toxicity raises the possibility that the toxic effects of Carlina oxide depend on the existence of a specific receptor or metabolizing enzyme.

In our study, the purified compound Carlina oxide had the lowest IC_{50} values, followed by the diethyl ether extract, in cervix squamous cell carcinoma (both cisplatin-resistant and sensitive cell lines), suggesting that Carlina oxide is the most potent and active compound.

One of the most effective and often used chemotherapeutic agents for the treatment of various solid tumors is cisplatin (Cocetta et al., 2020). Nevertheless, its efficacy is hindered by drug resistance and potential toxicity to multiple organs. Several factors contribute to this resistance, such as reduced drug uptake (due to the knockout of the copper transport protein CTR1) and efflux of the drug, overexpression of metallothionein, enhanced DNA damage repair system (repair of DNA intrastrand cross-links formed by cisplatin-DNA adducts), nuclear respiratory factor 2 (Nrf2) signaling, epithelial-mesenchymal transition, overexpression and activity of enzymes involved in pentose phosphate pathway, higher levels of PD-1 and PD-L1 and autophagy (Dasari et al., 2022; Giacomini et al., 2020; Xiao et al., 2021; Yan et al., 2016).

The results of this study highlight the extensive therapeutic potential of Carlina extract. Carlina extract demonstrates promising efficacy in treating cancer cells that have developed resistance to traditional chemotherapy drugs like cisplatin, showing high therapeutic potential and broad applicability. The effectiveness of Carlina extract on both cell lines suggests a unique mode of action that could potentially overcome well-known resistance mechanisms associated with cisplatin, such as enhanced drug efflux, apoptosis evasion, and DNA repair, which opening new avenues for further research in the future.

The mode of action of Carlina oxide in the studied cell lines (A431 and A431-PT) likely involves suppressing the expression of protein kinase B (AKT) and extracellular regulated protein kinases 1/2 (ERK1/2), as demonstrated by Wnorowski et al. (2020) and Strzemski et al. (Strzemski et al., 2017) in the human melanoma line. These signaling nodes are crucial for cell proliferation and survival. AKT and ERK1/2 signaling is overactive in many cancers including cervix squamous cell carcinoma cell because of mutations or overexpression of AKT and MAPK pathway components (Bao et al., 2023; Chen et al., 2007; Ghafouri-Fard et al., 2022; Glaviano et al., 2023).

AKT, also known as protein kinase B (PKB), is a serine/threonine kinase, plays a crucial role in promoting cell proliferation and survival in various complex ways through phosphorylation, making it a promising target for cancer treatment (Franke, 2008). AKT promotes cell proliferation by activating mTOR (mechanistic target of rapamycin), leading to enhanced protein synthesis and cell growth (Ersahin et al., 2015). Furthermore, AKT contribute to cell survival by inhibiting pro-apoptotic proteins such as BAD (Bcl-2-associated death promoter), phosphorylating and inhibiting caspase family of proteases and by activating anti-apoptotic proteins (Mitsiades et al., 2002; Sangawa et al., 2014; Shimamura et al., 2003). AKT also affects metabolic pathways by regulating glucose metabolism and glycolysis (Elstrom et al., 2004).

In both animal models and human settings, targeted therapies against this pathway have been shown to be successful in reducing the squamous cell carcinomas burden (Ghafouri-Fard et al., 2022). Carlina oxide's suppression of AKT expression can lead to decreases the activation of downstream pathways such as mTOR and removing the inhibitory effects on pro-apoptotic proteins like BAD. It results in reduced protein synthesis and cell growth, which eventually inhibits cell proliferation, triggers the apoptotic cascade and leads to programmed cell death. Additionally, AKT inhibition may also result in reduced glucose uptake and glycolysis, effectively starving the cancer cells of the energy needed for rapid growth and division.

ERK1/2, as components of the mitogen-activated protein kinase (MAPK) pathway, play a crucial role in promoting cell cycle progression, cell proliferation, and survival. Upon activation, ERK1/2 translocate to the nucleus, leading to the phosphorylation of various transcription factors. This process triggers changes in gene expression that support cell function (Lefloch et al., 2009). Similar to AKT, ERK1/2 signaling, enhances cell survival by increasing anti-apoptotic proteins (e.g., Bcl-2) and reducing pro-apoptotic proteins (e.g., Bim), while also influencing proteins involved in cell cycle checkpoints (Balmanno and Cook, 2009).

By suppressing ERK1/2, carlina oxide can disrupt the phosphorylation and activation of downstream transcription factors that promote cell proliferation. This disruption leads to a decrease in the expression of genes necessary for cell cycle progression, ultimately inhibiting cell growth and division. Additionally, cells are more prone to undergo programmed cell death in the absence of activated ERK1/2 which can upregulate anti-apoptotic proteins and downregulate pro-apoptotic proteins.

Overexpression of AKT and ERK1/2 has been consistently associated with the development of chemotherapy resistance (Kaboli et al., 2020; West et al., 2002). Research has highlighted the significant contribution of ERK1/2 and AKT pathways to cisplatin resistance in human small cell lung cancer cells, suggesting that targeting these pathways could greatly enhance the efficacy of cisplatin as an anticancer treatment (Wang et al., 2013).

Therefore, Carlina oxide is a potentially promising compound for treating cancer cells that depend on ERK1/2 and AKT signaling for survival, such as cisplatin-resistant cell lines. It may also help in overcoming resistance mechanisms that reduce the effectiveness of cisplatin as an anticancer treatment. This suggests it could be used in combination with cisplatin to overcome drug resistance. With inactive AKT and ERK1/2, the signals for cell survival diminish, making cells more susceptible to apoptotic triggers, including those induced by chemotherapeutic agents. Moreover, the suppression of AKT and ERK1/2 by Carlina oxide can be synergistic with other therapies. For instance, combining Carlina oxide with inhibitors of the mTOR or MAPK pathways may result in more efficient death of cancer cells by attacking multiple nodes within the same pathway.

The PD-1 (Programmed Death-1) receptor is located on the surface of T cells, which are critical components of the immune system responsible for recognizing and eliminating cancer cells. PD-L1 is a protein expressed on the surface of cancer cells and other cells within the tumor microenvironment. Tumors with high levels of PD-L1 expression can effectively suppress T cells, which hinders the immune system from attacking the cancer cells. The cancer cells are able to hide from the immune system and evade destruction because of this interaction (Blank and Mackensen, 2007). However, immune checkpoint inhibitors or immunotherapies, which block the PD-1/PD-L1 interaction, can reverse this process. By preventing PD-L1 from binding to PD-1, these drugs can reactivate the T cells, enabling them to effectively attack and eliminate the cancer cells (Makuku et al., 2021).

Recent studies have demonstrated that carlina oxide induces the expression of PD-L1 (Wnorowski et al., 2020). This immune evasion strategy enables cancer cells to persist and maybe proliferate even in the presence of lethal chemicals such as Carlina oxide. However, elevated levels of PD-L1 in a tumor indicate abundant targets for checkpoint inhibitors to target. By using checkpoint inhibitors to block these interactions in tumors with high PD-L1 expression, we can significantly reverse immune suppression and boost T cell activity (Akinleye and Rasool, 2019; Doroshow et al., 2021). Research has also demonstrated that patients with tumors expressing elevated levels of PD-L1 are more likely to respond to PD-1/PD-L1 inhibitors such as Atezolizumab. This is attributed to the greater extent of immune suppression that can be reversed, resulting in a more robust anti-tumor immune response (Rosenberg et al., 2016; Wnorowski et al., 2020).

In our study we found that Carlina oxide has the ability to kill directly cancer cells, including those that are resistant to cisplatin. Beside direct toxicity, Carlina oxide maybe improve the effectiveness of therapy on resistant cancer cells by modulating immune responses inside the tumor microenvironment. By triggering the induction of PD-L1, Carlina oxide can boost the effectiveness of immunotherapies, especially by making resistant cancer cells more susceptible to immune-mediated destruction, as tumors with increased PD-L1 expression tend to respond better to treatments that block the PD-1/PD-L1 pathway.

5. Conclusion

This study evaluated the cytotoxic effects of *C. acualis* on cervix squamous cell carcinoma cell lines, leading to the isolation of Carlina oxide with potent cytotoxic activities. The results showed that the bioactive compounds in Carlina not only trigger apoptosis in tumor cell lines but also hold promise in overcoming resistance to conventional therapies. This indicates their high potential in enhancing the overall effectiveness of cancer treatment strategies.

There is limited knowledge about the mode of action of Carlina oxide. Further research is imperative to unravel the modes of action of these components. Additionally, investigating the cytotoxicity of Carlina extracts on a broader range of cancer and non-tumor cell lines, as well as exploring their potential to enhance chemotherapy efficacy in combination with cisplatin, could provide valuable insights.

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