https://doi.org/10.31925/farmacia.2025.2.11 **ORIGINAL ARTICLE**

ETHANOLIC EXTRACT OF *JUNIPERUS COMMUNIS* EXHIBITS ANTIMICROBIAL ACTIVITY AND SELECTIVE CYTOTOXICITY IN PANC-1 CANCER CELLS COMPARED TO HEPARG HEPATOCYTES

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Abstract

Pancreatic cancer remains a significant public health burden due to the challenges of detection in the early stages, which contribute to its low rate of survival. Furthermore, treatment options are limited and often accompanied by increased toxicity. For this reason, the need for safer alternatives is in continuous research. *Juniperus communis* is a medicinal plant rich in bioactive compounds that have been traditionally used for their therapeutic effects. However, its anticancer potential is still being explored. This study investigated the antimicrobial and cytotoxic potential of a JCo ethanolic extract, focusing on its selective cytotoxic effects on pancreatic cancer (PANC-1) and healthy human primary hepatocytes (HepaRG) cell lines. LC-MS/MS analysis was also used to identify key phytochemicals in the plant – isoquercitrin, hyperoside, quercitrin, and rutin, which are known for their multiple therapeutic properties. The antimicrobial potential was also evaluated using disk diffusion and MIC methods, revealing significant activity against *Streptococcus pyogenes*. The extract showed cytotoxic effects against the PANC-1 cell line in a dose-dependent manner at the intermediate and the highest concentration tested. In contrast, on HepaRG, it presented a stimulatory effect at all concentrations. Morphological and nuclear staining analyses confirmed apoptotic-like changes, especially at higher doses. The HET-CAM assay confirmed its non-irritant nature, supporting its safety for potential use in several therapeutic applications.

Rezumat

Cancerul pancreatic rămâne o povară semnificativă pentru sănătatea publică din cauza dificultăților de detectare în stadii incipiente, care contribuie la rata scăzută de supraviețuire. În plus, opțiunile de tratament sunt limitate și adesea însoțite de toxicitate crescută. Din acest motiv, nevoia de alternative mai sigure este în continuă cercetare. *Juniperus communis* (JCo) este o plantă medicinală bogată în compuși bioactivi care a fost utilizată în mod tradițional pentru efectele sale terapeutice, cu toate acestea, potențialul său anticancerigen este încă explorat. Acest studiu a investigat potențialul antimicrobian și citotoxic al unui extract etanolic de JCo, concentrându-se pe efectele sale citotoxice selective asupra liniilor celulare de cancer pancreatic (PANC-1) și hepatocite umane primare sănătoase (HepaRG). Analiza LC-MS/MS a fost, de asemenea, utilizată pentru a identifica principalele substanțe fitochimice din plantă - isoquercitrina, hiperosida, quercitrina și rutina - cunoscute pentru proprietățile lor terapeutice multiple. Potențialul antimicrobian a fost, de asemenea, evaluat prin metode de difuzie pe disc și CMI, relevând o activitate semnificativă împotriva *Streptococcus pyogenes*. Extractul a prezentat efecte citotoxice împotriva liniei celulare PANC-1 într-o manieră dependentă de doză la concentrația intermediară și la cea mai mare concentrație testată, în timp ce pe HepaRG, acesta a prezentat un efect stimulator la toate concentrațiile. Analizele morfologice și de colorare nucleară au confirmat modificări de tip apoptotic, în special la doze mai mari. În plus, testul HET-CAM a confirmat caracterul său neiritant, susținând siguranța sa pentru utilizarea potențială în mai multe aplicații terapeutice.

Keywords: biosafety, pancreatic cancer, cytotoxicity, ethanolic extract, *Juniperus communis*, primary hepatocytes

Introduction

The pancreas is an important organ behind the peritoneum, serving both endocrine and exocrine functions. Pancreatic cancer remains a significant public health burden, mainly due to its low rate of survival, with only a small percentage of patients living beyond 5 years. Additionally, because it is quite challenging to detect it in the early stages, it further complicates treatment and contributes to its high mortality rate [29]. According to GLOBOCAN, in 2020, approximately 496,000 new cases and 460,000 deaths from pancreatic cancer were reported, making it the 12th most diagnosed malignancy and the 7th leading cause of cancer-related deaths globally. The highest incidence and mortality rates were recorded in Europe, with 7.6 and 7.1 *per* 100,000 persons, respectively. In Romania, GLOBOCAN 2022 data ranks pancreatic cancer as the 8th most diagnosed cancer and the 5th leading cause of cancer deaths, with 3,389 new cases (9.6 *per* 100,000) and 3,209 deaths (8.9 *per* 100,000) being recorded, highlighting the aggressive nature and poor prognosis of this disease [19, 20, 33].

Non-modifiable risk factors for developing pancreatic cancer include age (over 65 years), gender (males present a higher risk), ABO blood group (especially A and AB), ethnicity (African-Americans, Native Americans, and Japanese-Americans have a higher incidence), family history and genetic predisposition (individuals with a family history of pancreatic cancer or suffering genetic mutations at *BRCA1/2*, *ATM*, *PALB2*, *CDKN2A*), and diabetes mellitus. Modifiable risk can often be preventable and include factors like smoking (increases 2 - 3 times the risk over time), alcohol consumption (> 3 drinks/day), dietary factors (high-fat diets, red meat and processed foods), obesity, chronic pancreatitis, infections (*Helicobacter pylori*), microbiota imbalance and low socioeconomic status [29].

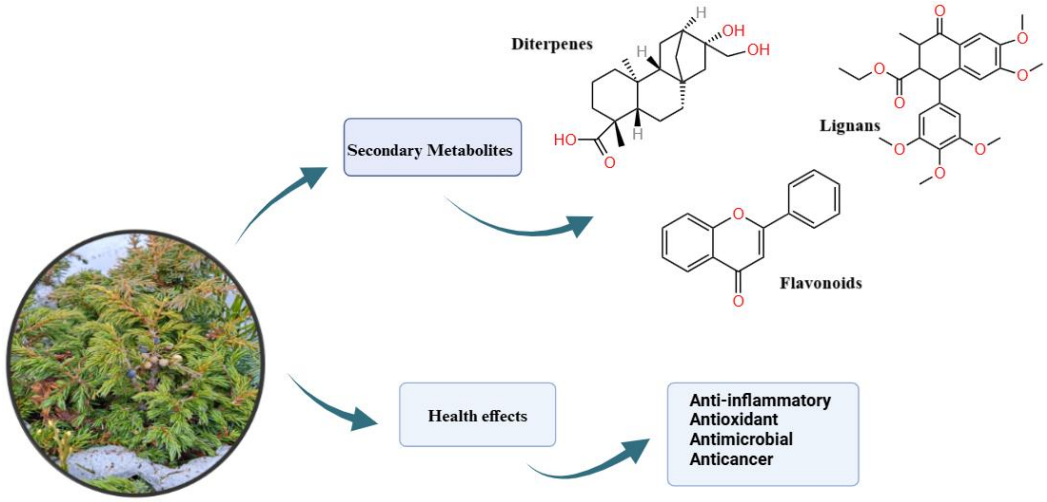

Figure 1.
The representation of *Juniperus communis* L., its secondary metabolites, and health effects
The chemical structures were made with KingDraw, and this image was created using Biorender.com

Surgery remains the only potentially curative option, yet it is feasible for only a small percentage of patients due to late-stage diagnosis. Moreover, surgical resection alone often results in complications and has a high risk of relapse, with a median survival of 15 - 20 months and a 5-year survival rate of only 8 - 15%. For locally advanced and metastatic cases, chemotherapy remains the primary approach to treatment. Standard chemotherapy drugs include the FOLFIRINOX (a combination of 5-fluorouracil, folinic acid, irinotecan and oxaliplatin) and gemcitabine ± nab-paclitaxel, both demonstrating an improved rate of survival. However, these treatments often cause severe side effects, such as cachexia, fatigue and toxicity, affecting the gastrointestinal, haematological and neurological systems, leading to a significant decrease in patients' quality of life [42, 43]. Due to this reason, natural compounds have come into the spotlight for their benefits and low toxicity profile [48]. Among medicinal plants, common juniper (*Juniperus communis* L., zimbro) from the *Cupressaceae* (Figure 1) family has sparked interest due to its rich composition of bioactive compounds, such as terpenes, lignans, flavonoids, proteins and others [38]. Traditionally, this evergreen coniferous shrub has been widely used in folk medicine across Europe and among American tribes for its therapeutic properties (*e.g.*, anti-inflammatory, antioxidant, antimicrobial, anti-septic, as well as for treating digestive problems, migraines, arthritis, gout and diabetes). In Romania, the fruits have traditionally been used as an infusion or tincture to support kidney health and act as a natural antiseptic. Moreover, it was used externally to help with various skin inflammatory conditions

[57]. Although the essential oil extracted from the berries and needles of the plant has been used for centuries for its therapeutic properties, nowadays, attention is directed towards its potential cytotoxic effects against various cancer cell lines [25, 31, 52]. *Juniperus communis* extracts were found to disrupt the cell cycle and induce apoptosis in various types of cancer. It arrests cells in the G2/M and G0/G1 phases by regulating p53, p21, and CDK4/cyclin D1, leading to tumour cell death while exhibiting reduced toxicity toward normal cells. Previous studies have shown that *Juniperus communis* contains various natural compounds with anticancer properties. For instance, deoxypodophyllotoxin induces caspase-dependent apoptosis *via* the mitochondrial (intrinsic) pathway and inhibits cell survival through MAPK/ERK and NFκB pathways in breast cancer cells. Additionally, podophyllotoxin and deoxypodophyllotoxin, found in certain *Juniperus* species, are effective against leukaemia cell lines [44, 57].

For this reason, the focus of the current study is directed toward the antitumoral effect of *Juniperus communis.* Although *Juniperus communis* is generally regarded as a plant with limited adverse effects, further investigation is necessary to confirm its safety in emerging clinical applications [38].

Given this scientific data, the present study aims to explore a *Juniperus communis* ethanolic extract. In this regard, a physicochemical analysis was performed to further correlate its chemical profile with the observed pharmacological effects and to provide a deeper insight into its potential pharmaceutical applications. Also, the extract was evaluated regarding its cytotoxic effects on a pancreatic cancer cell line (PANC-1) while testing its biosafety on a healthy cell line (HepaRG). In addition to *in vitro* assessments, *in ovo* bioassays were employed to investigate the irritant potential of *Juniperus communis* at the vascular level to complete the safety profile of the tested plant.

Materials and Methods

Plant material and preparation

The pseudo-fruits of juniper were purchased from Stef Mar (Râmnicu Vâlcea, Romania, product code: 6422579000788). Key microscopic characteristics were observed to determine the botanical identity using an optical microscope (BH-2, Olympus, Tokyo, Japan) on manually prepared transverse sections [63]. Trace metal control was performed *via* X-ray fluorescence (XRF) with an X-MET8000 series device and a handheld XRF spectrometer from Hitachi (Chiyoda, Japan). The pseudo-fruits were ground into a fine powder using an IKA 11 grinder, with 300 g processed. The resulting powder was extracted with three portions of 1000 mL ethanol (99.5%, Riedel-de Haën, Fisher Scientific, Finland). Each extraction involved 20 min of sonication in an ultrasonic bath (ELMA S120 Elmasonic, Singen, Germany) at room temperature, followed by a filtration using a Whatman Grade 4 filter. The combined extracts were then concentrated using a rotary evaporator (Laborata 4000eco, Heidolph, Schwabach, Germany) at room temperature, yielding 56.69 g of crude extract corresponding to 18.90% w/w relative to the 300 g of processed plant material.

Reagents and bacterial strains

Five microbial strains were used for the antibacterial potential assay (ThermoScientific, USA): *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 19615, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Candida parapsilosis* ATCC 22019. The MTT viability kit, phosphate-buffered saline (PBS), insulin from bovine pancreas, and hydrocortisone 21-hemisuccinate sodium salt were purchased from Sigma-Aldrich, Merck KGaA (Darmstadt, Germany). The cell culture media, William's E Medium (Gibco™; 12551032) was procured from Gibco, Waltham, MA, USA, and Dulbecco's Modified Eagle's Medium (DMEM-ATCC® 30–2002™), foetal bovine serum (FBS-30-2020™), the penicillin/streptomycin mixture and the dimethylsulfoxide (DMSO, 4-X™), trypsin-EDTA solution, and Trypan blue solution 0.4% were purchased from American Type Culture Collection (ATCC) Manassas, VA, USA. Hoechst 33342 dye and MitoTracker™ Red CMXRos were ordered from ThermoFisher Scientific (Waltham, MA, USA). Cytation 5 (plate reader) and Lionheart FX (automated microscope) were supplied by BioTek Instruments Inc. (Winooski, VT, USA), while the SteREO Discovery.V8 stereomicroscope was acquired from ZEISS (Jena, Germany).

Phytochemical analysis of plant extract by LC-MS/MS

The phytochemical profile of the plant extracts was analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) following two previously validated analytical methods [2, 65, 67]. The analysis was conducted using an Agilent Technologies 1100 HPLC Series system (Agilent, Santa Clara, CA, USA), which included an autosampler, column thermostat, binary gradient pump, degasser, and UV detector. This system was interfaced with an Agilent Ion Trap 1100 SL mass spectrometer (LC/MSD Ion Trap VL) for detection [24, 59].

In the first analytical method, chromatographic separation was carried out using a reverse-phase Zorbax SB-C18 analytical column (100 mm × 3.0 mm *i.d.*, 3.5 µm particle size, Agilent Technologies). The mobile phase comprised methanol and 0.1% acetic acid (v/v) in a binary gradient system. The elution process began with a linear gradient, starting at 5% methanol and increasing to 42% over 35 min, followed by an isocratic phase at 42% methanol for 3 min. The system was then re-equilibrated with 5% methanol for an additional 7 min [17]. The column temperature was maintained at 48°C, with a flow rate of 1 mL/min and an injection volume of 5 µL. Detection was

conducted in both UV and MS modes, with the UV detector set at 330 nm for polyphenolic acids for up to 17 min and at 370 nm for flavonoids and their aglycones for up to 38 min. The MS system operated in electrospray ionization (ESI) negative mode, with a capillary voltage of 3000 V, a nebulizer pressure of 60 psi (nitrogen), and a gas flow rate of 12 L/min at 360°C [17, 61].

A second validated LC-MS method was applied to potentially identify six additional polyphenolic compounds, including epicatechin, catechin, syringic acid, gallic acid, protocatechuic acid and vanillic acid. The same analytical column and instrumental settings were utilised, adjusting the mobile phase gradient. The gradient started at 3% methanol, increased to 8% within 3 minutes, reached 20% by 8.5 min, and was maintained until 10 min before decreasing to 3%. Bioactive compounds were detected in MS mode, with ESI conditions matching those used in the first method [61, 67].

Each bioactive compound was identified by comparing MS spectra and traces with library standards, followed by quantification through UV detection using calibration curves of the corresponding analytical standards. Data processing was performed with Agilent's DataAnalysis (v5.3) and ChemStation (vB01.03) software. Quantification results were expressed in micrograms *per* mL of plant extract. The analysis was performed in triplicate for each sample.

In vitro antimicrobial activity

The evaluation was conducted following the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [7] and the Clinical Laboratory and Standards Institute (CLSI) [12] and based on prior studies.

Bacterial strains (*Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa*) were cultured on Columbia agar supplemented with 5% sheep blood. At the same time, *Candida parapsilosis* was grown on Sabouraud agar containing chloramphenicol (Oxoid, Wesel, Germany). Microbial suspensions were prepared in 0.85% NaCl solution, adjusted to a concentration of 0.5 McFarland, corresponding to approximately 1 - 2 × 10^8 colony-forming units (CFU) *per* mL.

Disk diffusion method (DDM)

Mueller Hinton (MH) agar or MH supplemented with blood and β-NAD (MHF) for *S. pyogenes* (Oxoid, Wesel, Germany) was inoculated with microbial suspensions of each tested strain. Once dried, 6 mm blank paper disks (BioMaxima, Poland) were placed on the agar surface and loaded with 5 µL of each tested compound at a 20 mg/mL concentration. Gentamicin or fluconazole disks were positive controls, while disks impregnated with the solvent (DMSO or EtOH/H_2O) were negative controls. The plates were incubated at 35°C for 24 hrs, after which the diameters of the inhibition zones were measured. Strains with inhibition zones ranging from 6 mm to 15 mm were classified as resistant to the tested compounds and were not subjected to further testing. For strains displaying inhibition zones greater than 15 mm, a minimum inhibitory concentration (MIC) test was conducted [14].

Minimum inhibitory concentration (MIC) by dilution method

Serial dilutions of the tested compounds were prepared, ranging in concentration from 10 mg/mL to 1.25 mg/mL. In four test tubes, 100 µL of each dilution was combined with 50 µL of MH/MHF broth (bioMérieux, Marcy-l'Etoile, France) and 50 µL of microbial suspension, adjusted to approximately 2.5 × 10^5 CFU/mL. Following incubation at 35°C for 24 hrs, the minimum inhibitory concentration (MIC) was determined as the lowest concentration at which no visible microbial growth was observed [10, 13, 37].

Preparation of the samples

The test samples for the *in vitro* evaluation of the *Juniperus communis* ethanolic extract (concentrations of 150 µg/mL, 175 µg/mL and 200 µg/mL) were prepared by diluting the stock solution (*Juniperus communis* in DMSO) in the culture medium appropriate for the used cell lines. The DMSO concentration in each test sample was kept below 0.5%. Building on previous studies testing the JCo aqueous extract up to 150 µg/mL, this study expanded the concentration range for the ethanolic extract to 200 µg/mL. Previous studies have tested JCo extract at concentrations below 150 µg/mL. In this study, we extended the concentration range beyond those previously tested for *Juniperus*-based extracts, using doses up to 200 µg/mL [39, 44].

Cell Culture Conditions

The study was conducted using a human pancreatic ductal adenocarcinoma cell line – PANC-1 (passage 10) (PCS-201-018™; ATCC, Manassas, VA, USA) and human immortalised hepatocytes – HepaRG (passage 12) (HPRGC10; ThermoFisher Scientific, Waltham, MA, United States). The cell lines were cultured in their specific medium (DMEM for PANC-1 and William's E Medium enriched with insulin and hydrocortisone 21-hemisuccinate sodium salt at final concentrations of 4 µg/mL and 50 µM, respectively, for HepaRG). Both media contained a mixture of 10% FBS and 1% penicillin (100 U/mL)/streptomycin (100 µg/mL). During the experiments, the cells were kept in an incubator at 37°C and 5% CO_2. The cells presented normal morphology and proliferation during all the experiments.

Cell Viability Evaluation

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) test was used to determine cell viability. The MTT assay was performed following the 24-hour treatment of PANC-1 and HepaRG cells with *Juniperus communis* ethanolic extract at varying concentrations (150 µg/mL, 175 µg/mL and 200

μg/mL). The cells were seeded in flat-bottom 96-well plates at a density of 1 x 10^4 cells/well and treated with the JCo ethanolic. The protocol was conducted in accordance with the method described by Gag *et al.* [22].

Bright-field cellular morphology assessment

The effect of the ethanolic *Juniperus communis* extract on the morphology of PANC-1 and HepaRG cells was examined by capturing representative images of both controls (untreated cells) and treated cells. These cells were cultured in 96-well flat-bottom plates at a density of 1 x 10^4 cells *per* well, and the cell line was maintained at 37°C and 5% CO_2 in a humidified incubator. The cells were then stimulated with *Juniperus communis* extract 150 μg/mL, 175 μg/mL, and 200 μg/mL for 24 hrs. At the end of this period, cell images were captured under brightfield illumination at 20× magnification using the Lionheart FX automated microscope. The acquired images were then analysed and processed with Gen5™ Microplate Data Collection and Analysis Software (Version 3.14), developed by BioTek Instruments Inc. (Winooski, VT, USA).

Hoechst 33342 nuclear staining

To further investigate the effects of *Juniperus communis* extract on cell nuclei, the Hoechst staining method was employed. Cells were seeded in 12-well plates at a density of 1×10^5 and exposed to Jco extract at concentrations of 150 μg/mL, 175 μg/mL, and 200 μg/mL for 24 hrs under standard incubation conditions (37°C and 5% CO_2). The protocol was conducted in accordance with the method described by Manea *et al*. [51].

MitoTracker™ Red CMXRos – Mitochondrial immunofluorescence staining

Through the immunofluorescence staining, the aspect of the mitochondria in PANC-1 cells was analysed through MitoTracker™ Red CMXRos. Briefly, cells were cultured in 12-well plates at a density of 1×10^5 and allowed to adhere until they reached the desired confluence. Then, the cells were stimulated with the test samples at the highest concentration (200 μg/mL) of *Juniperus communis* extract. MitoTracker™ Red CMXRos was first dissolved in DMSO to a final concentration of 1 mM and then further diluted in the specific culture media (Dulbecco's Modified Eagle's Medium) to a concentration of 300 nM. Subsequently, in the prepared solution, the live PANC-1 cells were incubated at standard conditions (37ºC with 5% CO_2) for 30 - 45 min before being washed with PBS. The Lionheart FX automated microscope set at 20x magnification was utilised for imaging, while analysis was performed using the Gen5™ Microplate Data Collection and Analysis Software (version 3.14) from BioTek Instruments Inc. (Winooski, VT, USA).

Fertilised hen eggs preparation for the in ovo experiments

Fertilised hen eggs were used for the chorioallantoic membrane assays. On the first day, the eggs were disinfected with 70% alcohol, labelled with the date, and placed in an incubator for three days. On day four, a small incision was carefully made at the top of each egg, allowing for the extraction of approximately 6 - 7 mL of albumen using a syringe needle. The opening was then sealed with adhesive tape. By day 5, the upper part of the shell was removed to observe the vessels of the chorioallantoic membrane, then each egg was covered with adhesive tape. Afterward, the eggs were returned to the incubator, where temperature (37°C) and humidity (60%) were consistently maintained until the start of the experiments.

Assessment of the irritant potential via HET-CAM method

The HET-CAM assay was conducted in accordance with a protocol adapted from guidelines established by the Interagency Coordinating Committee on the Validation of Alternative Methods [32]. Fertile hen eggs were procured from a rural supplier to ensure viability. The incubation period for the assay spanned 10 days, during which the eggs were maintained at a constant temperature of 37.5°C and a humidity level of 65% in a hatcher. On the 4^{th} day of incubation, approximately 10 mL of egg white was extracted from each egg, followed by the creation of a window on the 5^{th} day. These procedures employed disposable syringes, medical tape, and forceps, with the window subsequently sealed with medical tape to preserve optimal internal conditions throughout the incubation period. On day 10, following sufficient vascular development, the HET-CAM assay was initiated. A total of 600 μL of *Juniperus communis* extract, alongside positive (1% sodium dodecyl sulfate) and negative (distilled water) controls, was applied to the vascularised chorioallantoic membrane of the chick embryo. Observations were conducted using a stereomicroscope (Zeiss SteREO Discovery.V8) connected to a digital camera (Zeiss Axiocam 105) and a computer. The assessment of irritability indicators (namely, bleeding, coagulation, and lysis) was recorded over a 5-minute interval for each sample, allowing for the calculation of an irritability score (IS) based on the observed parameters:

$$\boldsymbol{IS} = 5x\,\frac{301-H}{300} + 7x\,\frac{301-L}{300} + 9x\,\frac{301-C}{300}.$$

Statistical Analysis

All data were statistically analysed using GraphPad Prism software version 10.2.3 – GraphPad Software, San Diego, CA, USA, www.graphpad.com. The statistical methods applied were the one-way ANOVA analysis and Dunnett's multiple-comparison test. The assay was performed in triplicate using a total of 9 eggs.

Results and Discussion

Pancreatic cancer is an intricate malignancy with a high mortality rate and an incidence that has risen

over the past few decades [26, 30]. The management of pancreatic cancer is often complex since it is frequently difficult to detect it in early stages due to the absence of specific symptoms, and treatment options for advanced and metastatic stages remain limited [64]. At the moment, treatment approaches include surgical resection, chemotherapy, radiotherapy, targeted or immunotherapy, and palliative care, which plays a crucial role, especially in advanced stages. Nonetheless, the current treatment options face significant challenges due to limited targeted therapy, drug resistance, substantial toxicity, and lack of survival benefit [17, 54]. Given these limitations, attention has been directed toward natural compounds with potential medicinal benefits. Among them, *Juniperus communis* has been studied for its diverse pharmacological properties. Although, volatile compounds from the juniper plant have been extensively used in traditional phytotherapy for their aromatic and antimicrobial effects, at present, the focus is shifting toward non-volatile compounds, particularly flavonoids, for their anti-inflammatory, antioxidant and anticancer activities [38, 60]. However, despite its therapeutic potential, safety considerations should not be overlooked, as side effects, such as skin irritation, nasal congestion, and blister formation, have been previously reported with the zimbro essential oil [57]. To further explore the presence and concentration of these active principles, an ethanolic extract from juniper cone berries was analysed using the HPLC-MS method. The results presented in Table I highlight that the majority of the compounds of the JCo ethanolic extract are represented by isoquercitrin (0.406 ± 0.036 µg/mL), followed by hyperoside, quercitrin, and rutin, all of which belong to the flavonoid class.

Table I

Identification and quantification of compounds present in the ethanol extracts from juniper cone berries by HPLC-MS

No.	Compound	Concentration in the fraction (µg/mL)
1	Hyperoside	0.254 ± 0.011
2	Isoquercitrin	0.406 ± 0.036
3	Rutin	0.132 ± 0.017
4	Quercitrin	0.183 ± 0.018

The LC-MS analysis is important for phytochemical investigation because it enables the separation, detection and quantification of bioactive compounds in plants. The identified compounds are all well known for their ability to induce apoptosis, inhibit tumour cell proliferation and reduce oxidative stress, thereby exhibiting anti-cancer activities [3, 23]. Our results align with previously reported data, though variations exist in both the quantity and the identity of the compounds. These differences are related to the geographical area where the plant was harvested, the extraction technique, and the solvent used in the extraction process [4].

In addition to their tumour-suppressive properties, these compounds are also recognised for their anti-microbial effects, which are particularly relevant in the context of cancer treatment. Cancer patients, especially those undergoing chemotherapy or immuno-suppressive therapies, are highly vulnerable to the opportunistic infections caused by both fungal and bacterial pathogens [69]. For this reason, the antibacterial activity of the investigated extract was evaluated against 2 Gram-positive bacteria, 2 Gram-negative bacteria and 1 yeast (Table II). The *Juniperus* ethanolic extract revealed significant antimicrobial potential against Gram-positive *Streptococcus pyogenes*, while against the other tested strains, it was not determined. Negative controls (EtOH/H_2O, DMSO) showed no inhibition, confirming the extract's antimicrobial activity. Positive controls (gentamicin, fluconazole) validated the method for comparison.

Table II

In vitro antimicrobial potential of JCo ethanolic extract against different microbial strains

Microbial strains	Disk diffusion method inhibition zone (mm)	MIC (mg/mL)
Staphylococcus aureus	15	ND*
Streptococcus pyogenes	21	5
Escherichia coli	11	ND*
Pseudomonas aeruginosa	10	ND*
Candida parapsilosis	9	ND*

ND* stands for not detected

The incidence of *Streptococcus pyogenes* bacteraemia has increased over the years, with the mortality rate reaching nearly 42% between 2020 and 2024. Due to this alarming rise, the antimicrobial activity of *Juniperus communis* could be particularly valuable in combating group A streptococci (GAS) bacteriaemia, offering a potential natural alternative to antibiotics, which can often cause resistance [1].

Subsequently, the MTT assay was used to determine whether the juniper ethanolic extract exerts a cyto-

toxic activity against malignant and healthy cell lines (Figure 2). For this reason, the current study evaluated the potential of *Juniperus communis* alcoholic extract (JCo) at 150 µg/mL, 175 µg/mL and 200 µg/mL in the treatment of pancreatic cancer in the PANC-1 cell line. A preliminary assessment of the cytotoxic effects was also carried out at the same dosage interval on a non-cancerous cell line, HepaRG, to evaluate the selectivity of the extract. In PANC-1 cells, the extract showed a mild stimulatory effect at the lowest concentration tested (150 µg/mL), but then the cell viability was gradually reduced in a dose-dependent manner. At the intermediate concentration (175 µg/mL), the cell viability slightly decreased, reducing at the highest concentration (200 µg/mL) to approximately 80%. Concerning the treatment of healthy hepatic cell line HepaRG (Figure 2), the JCo ethanolic extract significantly stimulated the cells at 150 µg/mL, with a slight increase observed at 175 µg/mL. However, at 200 µg/mL, cell viability began to decrease, though it remained about 130%.

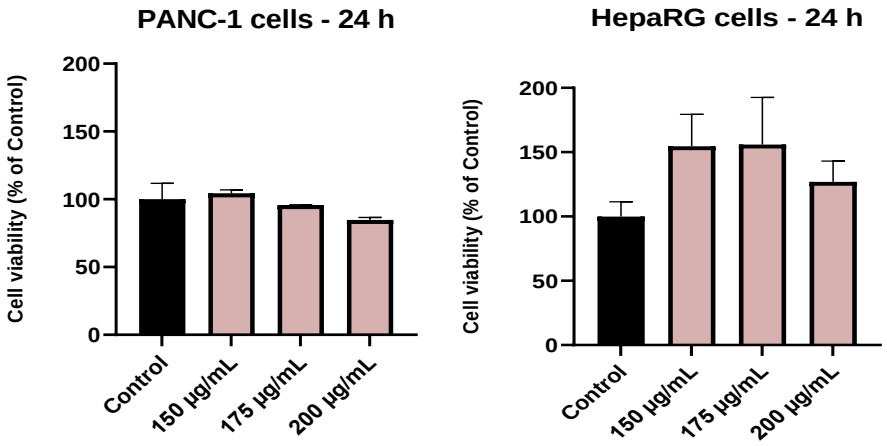

Figure 2.
The evaluation of the *in vitro* cell viability after 24 h of treatment with a JCo ethanolic extract at 150 µg/mL, 175 µg/mL and 200 µg/mL in PANC-1 (pancreas ductal adenocarcinoma cell line) and HepaRG (hepatic immortalised cell line)

After the treatment period (24 h), an MTT colorimetric assay was performed. Results are expressed as viability percentages (%) normalised to control (untreated cells). Presented data are expressed as mean values ± SD of three independent experiments performed in triplicate. A one-way ANOVA test was used to assess the statistical differences between the control and the treated group, and then Dunnett's multiple comparisons post hoc test was performed.

The results of the present study suggested a dose-dependent decrease in the cells' viability induced by JCo. Also, the phytochemicals found in *Juniperus communis* have been increasingly investigated for their potential anticancer properties. Various studies have demonstrated through MTT colorimetric assay that metabolites derived from zimbro, especially from the flavonoid class, such as quercetin, apigenin, and amentoflavone, exhibit potent cytotoxic effects against several pancreatic cancer cell lines (AsPC-1, CD18, MIA PaCa2, S2-013 and PANC-1) [6, 21, 57, 66î. In the present study, the identified compounds derived from *Juniperus communis* – isoquercitrin, hyperoside, quercitrin, and rutin have shown promising results regarding their cytotoxic effect against pancreatic cancer. For instance, isoquercitrin has proven time- and dose-dependent cytotoxic effects on 2 pancreatic cell lines (BxPC-3 and AsPC-1), results obtained after the MTT analysis conducted for 24, 48 and 72 h at 23.19, 46.438, 92.876 and 185.752 µg/mL [11]. In the case of hyperoside, it was shown in a study that, after performing an MTT assay, the compound presented an IC_{50} of approximately 23.22 µg/mL against MIA PaCa-2 cells after 48 h of treatment [9]. In a study conducted by Sarikahya *et al*., it was observed that quercitrin extracted from the leaves of *Pistacia lentiscus*, along with scutellarein, was thought to be responsible for the remarkable cytotoxic effects against the PANC-1 cell line. The quercitrin content varied from about 25.13 µg/mL to a maximum of 631.13 µg/mL [58]. Rutin, another flavonoid found in our ethanolic extract, has been previously tested on the PANC-1 cell line, and it was demonstrated through the MTT assay that it significantly impacts the cell viability at 25 µg/mL, reducing it by under 40% [16].

Pancreatic cancer treatments are primarily metabolised in the liver, which can lead to hepatotoxicity. While gemcitabine rarely causes liver toxicity, its metabolisation *via* the hepatic system is still a factor that should not be neglected [18]. Conversely, folfirinox, another commonly used chemotherapeutic agent, is more prone to causing hepatic toxicity [41].

Since the liver has a central role in drug metabolism and is vulnerable to hepatotoxic effects, it is crucial to evaluate the safety profile of therapeutic compounds. To address this, we used a healthy hepatic cell line (HepaRG), which is a well-established *in vitro* model for toxicity studies [5]. To the best of our knowledge, there is no previous *in vitro* research regarding the toxicological effects of JCo ethanolic extract on the HepaRG cell line. However, other findings have previously stated that the hepatoprotective effect of *Juniperus communis* is also present. In a study, it was demonstrated that ethanolic and aqueous extracts of JCo were able to reduce elevated transaminase (SGPT, SGOT, ALP) and bilirubin levels, which are markers of liver inflammation and damage [8]. The isoquercitrin was shown to significantly reduce the levels of AST, ALT and TGF-β *in vivo* through anti-inflammatory and antioxidant properties, suggesting its hepatoprotective action [40]. In a study conducted by Zhu *et al*., it was reported through MTT assay that isoquercitrin did not affect the cell viability at a much higher concentration (2.5 - 80 μmol/L) compared to the much lower content extracted in our study (0.406 μg/mL) in the context of H_2O_2-induced apoptosis in EA.hy926 cells. Additionally, it was observed that isoquercitrin activated the PI3K/Akt signalling pathway, which plays a crucial role in hepatocyte survival and liver regeneration [34, 70]. Another compound present in our ethanolic juniper extract, hyperoside, has proven hepatoprotective effects in LO2 (human primary hepatocytes) cells, as evidenced by the MTT assay. The study showed that hyperoside significantly enhanced LO2 cell viability in a dose-dependent manner (5, 10 and 20 μM) after 24 h of treatment, counteracting N-acetyl-para-amino-phenol-induced acute hepatic injury. However, its protective effect was abolished when Nrf2 was silenced, indicating its Nrf2-dependent hepato-protective action [36]. Rutin is another interesting phytocompound from the flavonoid class that has proven to have many therapeutic benefits. In a study conducted by Dehelean *et al*., MTT assay results demonstrated that rutoside exhibited a stimulatory effect at concentrations of 1 - 10 μM on the HepaRG cell line, further supporting its cytoprotective role in liver health [15]. Taking these findings into consideration, the hepatoprotective effects of key phytocompounds found in *Juniperus communis* ethanolic extract, such as isoquercitrin, hyperoside, quercitrin and rutin, reinforce the beneficial properties of the plant.

In the next section, the effect of JCo ethanolic extract (150 μg/mL, 175 μg/mL and 200 μg/mL) was evaluated on the morphologic level (Figure 3) after 24 h of treatment. In the case of the pancreatic cell line (PANC-1), the plant extract revealed a slight decrease in confluence (Figure 3A) that was observed in a dose-dependent manner, and at the highest concentration (200 μg/mL), the cells appear to suffer morphological alterations, such as cell shrinkage and traces of cell debris. Furthermore, we further analysed the extract at the same dosages and time intervals on the healthy hepatic cell line (HepaRG) in Figure 3B. The juniper extract noticeably stimulated cell growth, with an apparent increase in cell number at both 150 μg/mL and 175 μg/mL. However, the JCo extract did not exhibit any sign of dysmorphologies in the interval range tested.

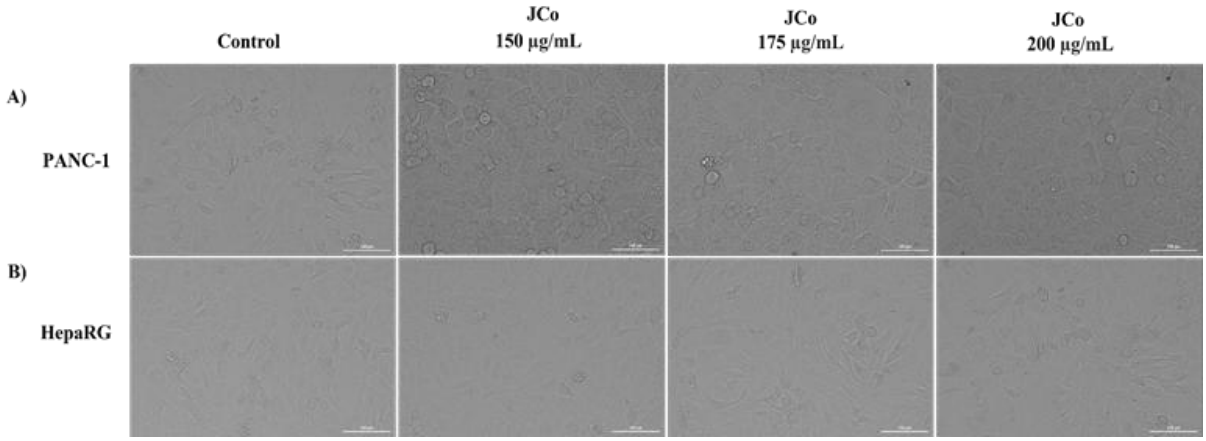

Figure 3.

The presented images portray the morphological aspect 24 h post-stimulation of PANC-1 cells (A) and HepaRG cells (B) with JCo ethanolic extract at 150 μg/mL, 175 μg/mL and 200 μg/mL

The experiment was performed in triplicate

In order to assess cytotoxicity, investigating cell morphology is necessary for detecting potential shape alterations following different treatments. In this manner, the impact of JCo extract and its metabolites on cell morphology in different cell lines has been examined. In a study conducted by Lee. *et al*., JCo (30 μg/mL) determined morphological alterations in the OECM-1 (human oral squamous carcinoma) cell line, in a time-dependent manner (6, 12, 24 and 48 h), causing cell shrinkage, elongation, detachment, and

ultimately cell death [44]. The most abundant metabolite in our ethanolic JCo extract, isoquercitrin, was found to be effective against SK-MEL-2 (malignant melanoma) cells. After 24 hours of treatment, the morphological analysis revealed that isoquercitrin reduced cell growth and confluency in a dose-dependent manner (15, 20 and 25 μM), while also causing cell rounding and shrinkage [68]. Based on morphology analysis, hyperoside caused shrinkage, loose adherence, decreased cell number, and increased apoptosis with the increase in the dosage against SW579 (human thyroid squamous cell carcinoma) cell line at 5 - 20 μg/mL after 24 h of exposure [47]. In addition to evaluating the JCo extract's effect on neoplastic cells, we also investigated how the extract influenced the morphology of healthy hepatic cells. Rutin's activity (a compound found in JCo extract) was assessed on 2 healthy cell lines (HepaRG, HaCaT) *via* morphological analysis and it was determined that after 24 h of exposure at 100 μM (a much higher concentration than the one present in our study), the cells did not suffer any negative changes in their confluency and adherence [15]. Isoquercitrin also proved no toxic effects on normal human keratinocytes (HaCaT) cells at 15 μM, 20 μM and 25 μM after a 24 h period, inducing no observable dysmorphologies in the human keratinocytes [27].

To obtain a more comprehensive picture of the mechanism of action of the JCo ethanolic extract, we further examined the aspect of nuclei on PANC-1 cells through the Hoechst method, commonly used in various fields to observe changes in the nuclear structure (Figure 4). The results obtained after 24 h of treatment indicate that the juniper extract induces non-significant dysmorphologies at 150 μg/mL compared to the control. On the other hand, at 175 μg/mL and 200 μg/mL concentrations, the nuclei appear smaller, and there are also some slight nuclear alterations present, such as chromatin condensation, which marks apoptosis.

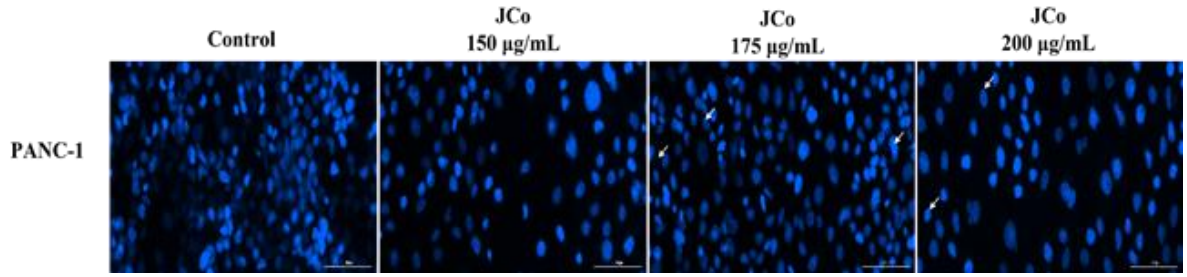

Figure 4.

The presented images portray the morphological changes noticed in nuclei of PANC-1 cells after 24 h of treatment with JCo ethanolic extract at 150 μg/mL, 175 μg/mL and 200 μg/mL. White arrows indicate changes in the aspect of nuclei. The scale bars indicate 100 μm. The experiment was performed in triplicate

Nuclear shrinkage, shape changes, and chromatin condensation all represent key markers of cell death. Hoechst staining is a valuable tool for visualising hallmarks of apoptosis, making it useful for assessing whether a compound of interest exerts toxic effects [51]. In this way, we analysed the obtained results and demonstrated that the JCo ethanolic extract produced cytotoxic effects at the nuclear level against the PANC-1 cell line at higher concentrations (175 and 200 μg/mL) after 24 h of treatment. Similarly, in another study, it was found that JCo essential oil (50 μM) caused chromatin condensation, membrane blebbing and loss of cytoskeletal arrangement in MIA PaCa-2 (pancreatic cancer) cell line 48 h post-treatment, results obtained after Hoechst staining [9]. Through the same method, it has been proven in another study that after 24 h, rutin induced no noticeable alterations in the nuclei of H9c2 (2-1) cardiomyoblasts, HepaRG hepatocytes and HaCaT keratinocytes at the concentration of 100 μM [58]. As rutin is part of our ethanolic extract, it is important to ensure that the extracted compounds exert selective cytotoxicity, as proven in the present study. Marcovici and her associates have shown that a rutin's metabolite, rutin linoleate, triggered nuclear fragmentation at 25 - 50 μM after 24 h of exposure, and at 100 μM, no significant changes were observed within the nuclei of NCI-H23 cells (lung adenocarcinoma). However, at the highest concentration used (125 μM), cell shrinkage was noted, as well as chromatin condensation and cytoplasmic vacuolation [56]. Similar to our results, when the highest concentration of JCo extract was used, the nuclei of the cells appeared smaller, and the chromatin condensation was also present. In another research paper, the nuclear translocation of p53 in *Juniperus communis*-treated cells through DAPI staining (a similar staining method used for nuclear visualisation) was confirmed. This observation aligns with the increase in total p53 levels and PARP cleavage, detected *via* Western blotting, reinforcing juniper's role in triggering p53-dependent apoptotic signalling in non-small lung cancer (A549), 2 prostate cancer (22RV1 and DU145) and hepatocellular carcinoma (HepG2) cell lines. It was previously reported that PARP-1 downregulation enhances p53 expression and apoptosis in PANC-1 cells. Taking these aspects into consideration, future

investigations should explore whether *Juniperus* extract could modulate these pathways in a pancreatic cancer model [28, 62].

As a final step in our *in vitro* exploration of the JCo ethanolic extract's potential cytotoxic effects, an analysis of the alterations in mitochondria (Figure 5) was conducted *via* immunofluorescence staining by using MitoTracker. As depicted in Figure 5, the lowest concentration (150 µg/mL) did not induce any noticeable changes in the mitochondria compared to the control. At the intermediate concentration (175 µg/mL), some slight alterations could be observed at the mitochondrial level, such as mitochondrial condensation. Nevertheless, at the highest dosage (200 µg/mL), JCo ethanolic extract produced massive mitochondrial condensation (changes marked with white arrows) and also reduced their confluency.

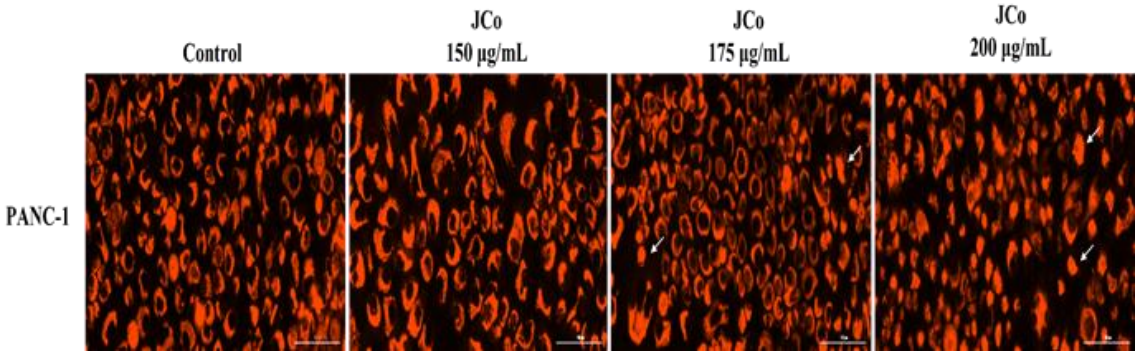

Figure 5.

The presented images portray the morphological changes in mitochondria of PANC-1 cells after 24 h of treatment with JCo ethanolic extract at 150 µg/mL, 175 µg/mL and 200 µg/mL. White arrows indicate changes in mitochondrial morphology. The experiment was performed in triplicate

Mitochondria, often called the "powerhouse of the cell" play an essential role in energy production. Besides their role in energy metabolism, mitochondria synthesise crucial biomolecules, including lipids and amino acids. Moreover, it regulates reactive oxygen species production and is involved in managing stress response. Additionally, it regulates Ca^{2+} intracellular levels and is included in several cellular activities and signalling pathways, including intrinsic pathways of apoptosis [35, 46]. Thereby, by analysing mitochondrial alterations, we can identify crucial signs of cell death, highlighting the connection between metabolism and apoptosis. It was found that a JCo extract (70 µg/mL) induced apoptosis in oesophageal cancer cells (CE81T/VGH) after 24 h of treatment, as observed by fluorescence microscopy. The extract triggered chromatin condensation, anoikis, DNA fragmentation and apoptotic body formation. In the same study, it was discovered that the juniper extract activated both extrinsic (Fas/FasL/caspase-8) and intrinsic (*Bax*/*Bcl-2*/caspase-9) pathways. Additionally, it was noticed that the JCo extract worked synergistically with 5-FU in inhibiting ESCC cells. These findings suggest that *Juniperus communis* could be either used in combinatorial treatment or as an adjuvant therapy for ESCC. Further research may focus on the combination of JCo and 5-FU, as it is a commonly used chemotherapeutic agent in pancreatic cancer [42, 49]. Marković *et al*. demonstrated that JCo berries' essential oil disrupts mitochondrial function in HeLa (cervical cancer) cells at an IC50 of 10.14 ± 2.89 µg/mL and in HCT 116 cells (colon cancer) at an IC_{50} of 29.19 ± 6.12 µg/mL after 48 h. The study confirmed through immunofluorescence techniques that the cytotoxic effects were driven by the intrinsic apoptotic pathway, as indicated by the release of cytochrome C from mitochondria into the cytosol in both cancer cell lines [11]. These findings collectively demonstrate that JCo induces toxicity toward multiple cancer cells by targeting mitochondria. Our investigation supports this by showing that the JCo ethanolic extract produced from the pseudo-fruits induced mitochondrial alterations in PANC-1 cells in a dose-dependent manner, triggering mitochondrial condensation and reduced confluency, indicating disruption of mitochondrial integrity.

To assess the irritant potential of *Juniperus communis* extract utilising the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) method, a concentration of 200 µg/mL was selected as the representative test concentration, which also represents the maximum concentration evaluated *in vitro* (Figure 6). The HET-CAM method involves monitoring alterations in the vascularised chorioallantoic membrane subsequent to the application of test samples. Observations focus on indicators such as bleeding, rupture of blood vessels, clotting, and vascular blockage. The irritability index is calculated based on the timing of these observed processes. Samples are categorised according to their irritation score (IS): an IS of 0 - 0.9 indicates non- irritation, 1 - 4.9 denotes mild irritation, 5 - 8.9 signifies moderate irritation, and 9 - 21 reflects high irritation [53]. For the *Juniperus communis* extract, an IS value of 0.07 was recorded, indicating non-irritating properties. In contrast, the positive control,

sodium dodecyl sulphate (SDS), yielded an IS of 13.043, categorising it as a strong irritant. Distilled water, serving as a negative control, also exhibited an IS of 0.07, confirming its non-irritant status.

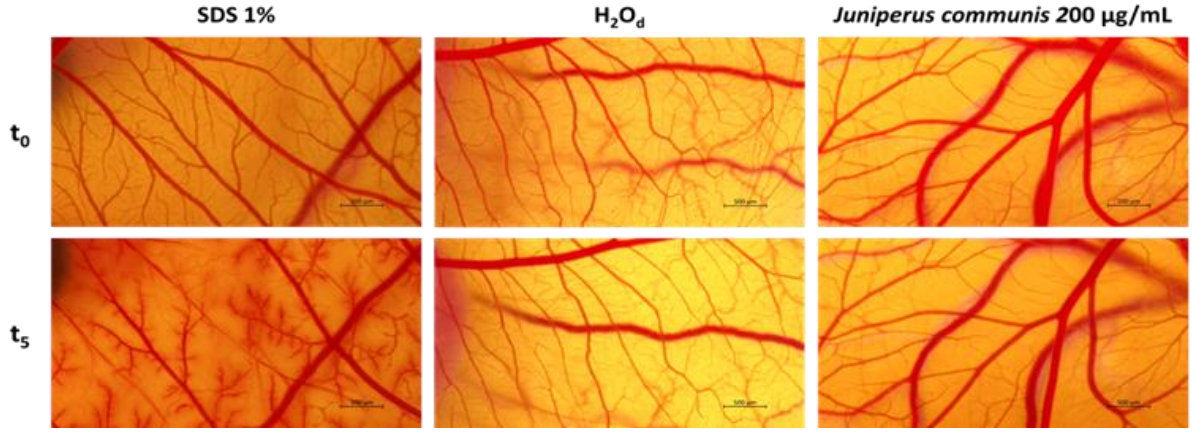

Figure 6.

HET-CAM Assay. Stereomicroscopic images of the chorioallantoic membrane at time the initial time (pre-treatment – t_0) and five minutes post-application of test samples (post-treatment – t_5). Sodium dodecyl sulfate (SDS 1%) served as a positive control, while distilled water (H_2O) was used as a negative control. The extract of interest, *Juniperus communis*, was tested at a concentration of 200 µg/mL. The scale bar in the images indicates a measurement of 500 µm. The experiment was performed in triplicate

The HET-CAM assay is a widely recognised alternative method for assessing the irritancy of plant extracts, particularly in the context of topical applications. This assay uses the chorioallantoic membrane of chick embryos, which is highly supplied with blood vessels and reacts to irritants in a similar manner to human mucosal tissues. The HET-CAM assay has been validated for its sensitivity and specificity in detecting irritants, making it a valuable tool for evaluating the safety of natural compounds [53].

The performed HET-CAM assay demonstrated that the extract of *Juniperus communis* exhibits a favourable biosafety profile at a concentration of 200 µg/mL within the *in ovo* model, as evidenced by the absence of irritation to the chorioallantoic membrane (CAM) vasculature. In stark contrast, the application of sodium lauryl sulphate at a concentration of 1% resulted in a pronounced irritative response, characterised by severe haemorrhaging, blood vessel lysis and coagulation of the vascular structures. Consequently, *Juniperus communis* extract can be regarded as non-irritant, achieving a safety score equivalent to that of distilled water, which served as a negative control and did not induce any alterations in the CAM. This study underscores the potential of *Juniperus communis* extract as being safe for further studies employing its applications for topical formulations.

Conclusions

The findings of this study reveal that *Juniperus communis* can be used as a promising alternative for pancreatic cancer treatment. The juniper extract selectively reduced the viability of the pancreatic cancer (PANC-1) cells while stimulating the healthy hepatic (HepaRG) cells. At higher doses, the JCo ethanolic extract induced noticeable morphological changes in the pancreatic cancer cell line, such as cell elongation and shrinkage, alongside nuclear and mitochondrial alterations. Additionally, it showed antimicrobial activity, particularly against *Streptococcus pyogenes*, highlighting its potential benefits as a natural antibacterial agent. Additionally, it demonstrated antimicrobial activity, particularly against *Streptococcus pyogenes*, highlighting its potential as a natural antibacterial agent. Moreover, the HET-CAM assay confirmed the non-irritant nature of JCo at the tested concentration. These results provide a basis for further investigation into the therapeutic potential of *Juniperus communis*, highlighting the need for additional mechanistic studies, *in vivo* efficacy trials and pharmacodynamic evaluations to fully validate its clinical applicability.

Conflict of interest

The authors declare no conflict of interest.

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