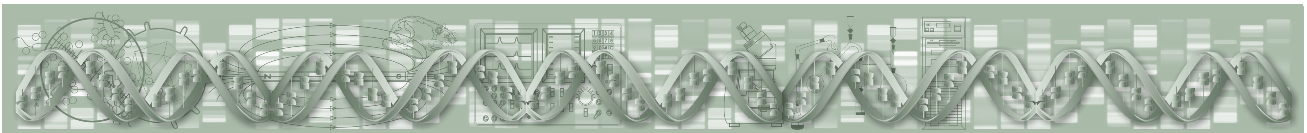

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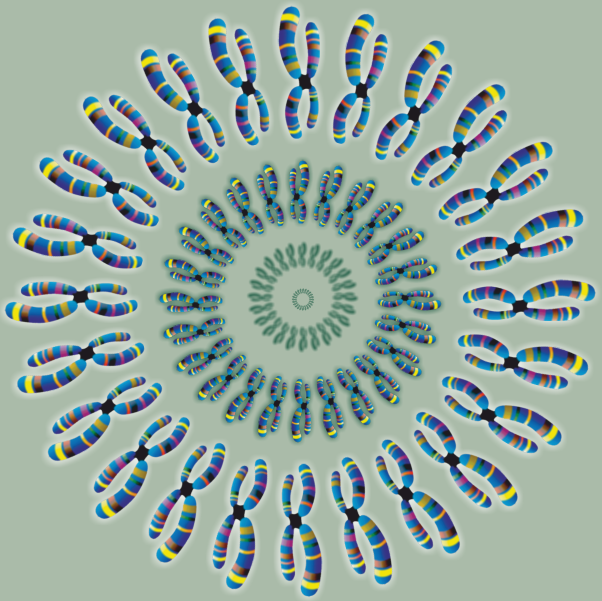

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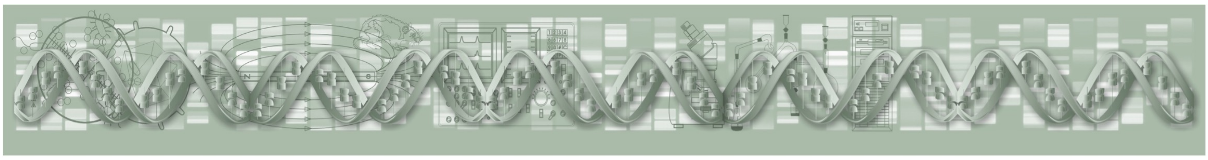

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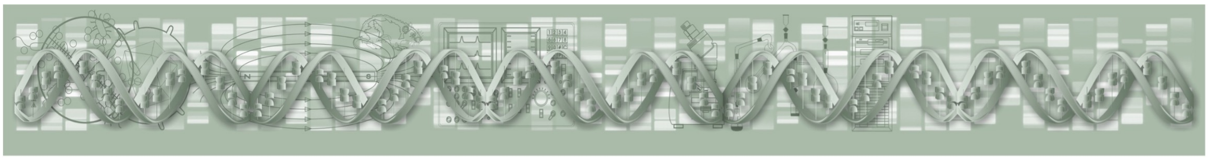

FROM ERLENMEYER FLASK TO MICROPLATES – LESSONS LEARNED FROM MEASURING *ESCHERICHIA COLI* GROWTH

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Abstract

Growth curve measurements are one of the fundamental operations in microbiology and molecular biology. Modern microplate readers have the advantage of high-throughput and ease of automation. This allows for real-time monitoring of many small bacterial cultures and a significant decrease labor and materials costs. Using these devices, tens to hundreds of absorbance readings can be collected simultaneously for hundreds of samples. The small volume and the geometry of a well from a microplate implies that calibration and comparative studies must be performed before switching from one cultivation and growth monitoring system to another. Here, *Escherichia coli XL1* Blue was used as a reference strain in order to test the feasibility of using a common, filter-based microplate reader for recording growth curves when performing antibiotic and toxicity testing. Two different cultivation conditions (flask cultivation and microplate cultivation) and three inoculum/media ratios were tested. The recorded growth curves indicated that the growth of the bacteria is much slower, the turbidity levels are lower, and reading are more variable in the microplate compared with the Erlenmeyer flask. Several major technical difficulties must be carefully dealt with when measuring bacterial growth curves in microplates: efficiency of oxygenation that leads to slow growth rates and low densities, condensation on the microplate lead that leads to interference and evaporation of the growth media.

Keywords: *E. coli*, growth curve, microplate

Introduction

Growth curve measurements are one of the fundamental operations in microbiology and molecular biology, many studies starting with a culture of bacterial cells and the assessment of the bacterial cells proliferation (Kurokawa and Ying 2017). Hence, most of knowledge on bacterial life cycle is derived from monitoring cell growth over time in liquid media. Analytical data on bacteria proliferation can be obtained by timed sampling of a bacterial culture followed by determination of the cells number by either measuring optical turbidity or performing colony-forming unit (CFU) assays (Kurokawa and Ying 2017). Due to its simplicity, the most routinely used method is measuring optical turbidity or optical density (OD). Applications of OD measurements range from studying cellular metabolism or physiology, preparing competent cells for cloning, heterologous overexpression of proteins, monitoring biomass accumulation during fermentation processes or determining growth rate for antibiotic resistance studies (Stevenson et al. 2016).

Modern microplate readers have the advantage of high-throughput and ease of automation (Zimmermann et al. 2004). This allows for real-time monitoring of many small bacterial cultures and a significant decrease labor and materials costs. Using these devices, tens to

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hundreds of absorbance readings can be collected simultaneously for hundreds of samples and the data can be processed with freely available software packages that allow modeling and interpretation (Hall et al. 2014, Sprouffske and Wagner 2016).

Commonly, bacterial cultures in liquid media are performed in Erlenmeyer flasks (baffled or not) or Falcon tubes with a ratio of media volume: flask volume anywhere between 1/10 to 1/4 depending on the size of the culture (Kram and Finkel 2014). The most common practice is to use a ratio of 1/5 for 100 ml to 1L media. This, combined with proper agitation ensures the formation of a thin film of culture media which is essential for optimum aeration and fast growth rates (Somerville and Proctor 2013). The small volume and the geometry of a well from a standard 96 well microplate is far from achieving the ideal conditions for maximal growth. Hence, correct calibration and comparative studies must be performed before switching from one cultivation and growth monitoring system to another. This is especially important when microplate readers are to be incorporated into routinely used experimental designs and data comparability is essential.

Here, *Escherichia coli* XL1 Blue was used as a reference strain in order to test the feasibility of using a common, filter-based microplate reader for recording growth curves when performing antibiotic and toxicity testing. Two different cultivation conditions (flask cultivation and microplate cultivation) and three inoculum/media ratios were tested. The recorded growth curves were compared in terms of lag phase length, growth rate and cultivation time required to reach stationary phase. The direct comparation of the growth curves was preferred instead of a CFU assays as these assays have been reported as being unsuitable for calibrating OD readings when antibiotics are tested (Stevenson et al. 2016).

Material and Methods

Strains, media and growth conditions. *Escherichia coli* XL1 Blue (Stratagene, endA1 gyrA96(nalR) thi^{-1} recA1 relA1 lac glnV44 F'[::Tn10 proAB^{+} lacI^{q} Δ(lacZ)M15] hsdR17(r$_{K}^{-}$ m$_{K}^{+}$) were kept as glycerol stocks at -80^{0}C and revived by plating on LB plates supplemented with 10 microg/ml tetracycline (Sigma Aldrich, Germany). Plates were incubated overnight at 37^{o}C and one colony was used to inoculate 10 ml LB broth, 10 microg/ml tetracycline in a 50 ml Falcon tube. After 16 hours at 37^{o}C, 190 rpm in a GFL-3031 (GFL, Germany) orbital shaker, the preculture was used to inoculate the main cultures. Flask cultivation was performed in a 500 ml not baffled Erlenmeyer flask containing 200 ml LB broth, 10 microg/ml tetracycline. Microplate cultivation was done in standard 96 well microplates, each well containing 200 microL LB broth, 10 microg/ml tetracycline. Each culture was started by adding the inoculum in various ratios and incubated at 37^{o}C, 190 rpm. For shaking the microplates, an microplates adapter was printed using a Creality3D Ender 5 printer (Figure 1). A model of the adapter and instructions are available on NIH 3D Print Exchange server (Coakley et al. 2014), Model ID 3DPX-015527. LB medium (Sambrook et al. 1989) was prepared as following: 10 g NaCl (Chemical Company, Romania), 5 g yeast extract (Merck, Germany), 10 g Peptone from casein (Carl Roth, Germany) were dissolved in 1 L distilled water and autoclaved at 115^{0}C for 20 min using a AES-50 (Raypa, Spain) autoclave. For solid medium, 16 g/L of microbiology grade agar-agar (Roth, Germany) was added prior to sterilization.

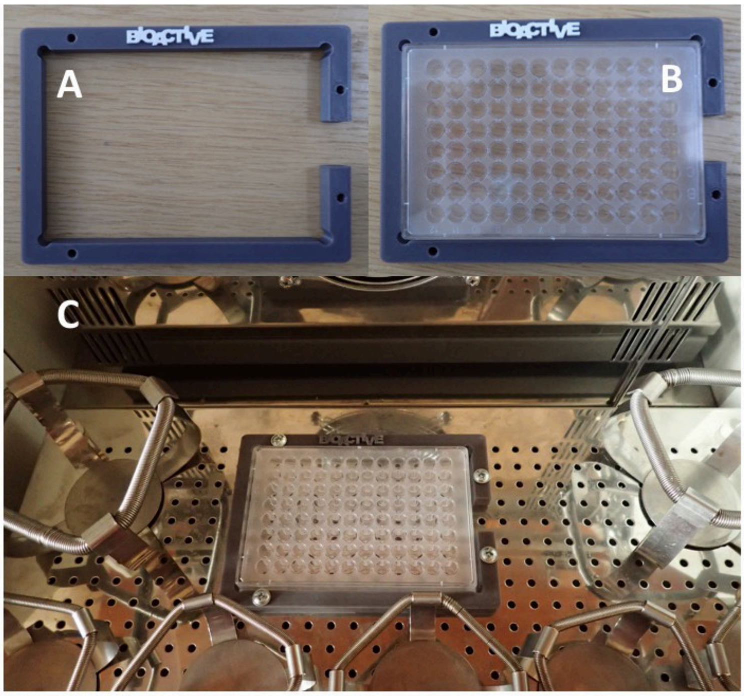

Figure 1. Adapter for secured fixation of the microplates to the GFL-3031 shaking platform fabricated using 3D printing (**A** and **B** adapter ready to be mounted; **C** adapter attached to the shaker platform)

OD measurements. 0.8 ml samples were taken every hour from the Erlenmeyer flasks and OD at 590 nm were recorded against a blank with LB medium using a DU 730 UV/VIS Spectrophotometer (Beckman Coulter). Plates were removed from the shaker and OD were read using a Tristar^2 Multimode Reader LB942 (Berthold Technologies). The reading program consisted of 2 s shaking (orbital motion, normal speed) fallowed by the endpoint measurement (counting time 0.2 s) with a “by well” operation mode. A microplate adapter was fabricated to allow the reading of the microplates with the leads on. The adapter was printed using a Creality3D Ender 5 printer. A model of the adapter and printing instructions are available on NIH 3D Print Exchange server (Coakley et al. 2014), Model ID 3DPX-015703.

Data processing. Flask cultures were performed in triplicate, while microplates cultures were repeated at least 12 times (one row of wells in the microplate). OD readings from the spectrophotometer and multiplate reader were imported into Microsoft Excel spreadsheet program. Means and standard error were calculated using the Data Analysis function from the Analysis ToolPak and plotted using the same program.

Results and Discussions

General aspect of the growth curves. *Escherichia coli* XL1 Blue strain was grown in both classic shaken Erlenmeyer flask and a 96 well microplate using the same batch of LB media. For each cultivation system, three ratios of the same inoculum have been used: 0,5%, 1% and 2%. The growth of the cultures was recorded at 590 nm using either a spectrophotometer or multiplate reader and the data is plotted in figure 2.

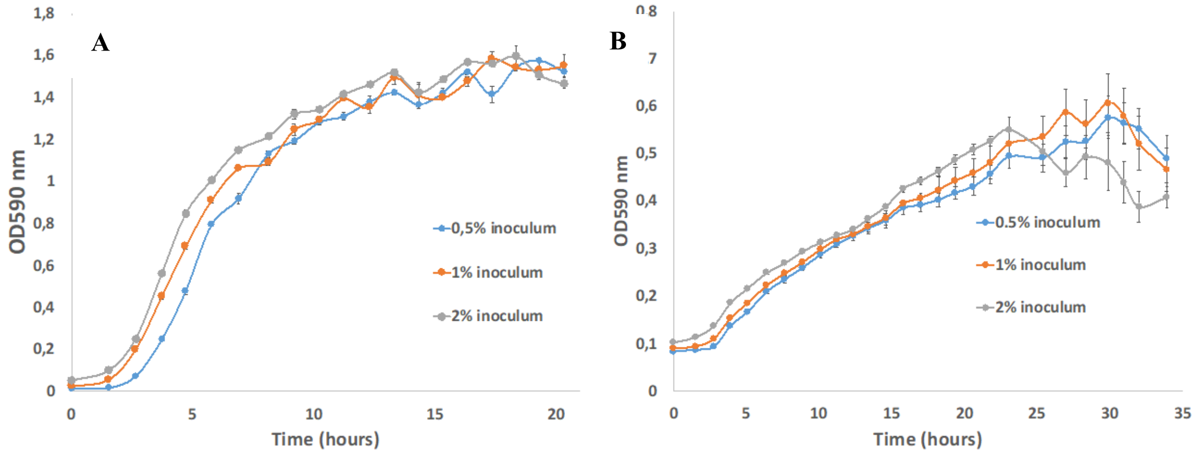

Figure 2. Growth curves of *Escherichia coli* XL1 Blue when cultivated in (**A**) Erlenmeyer flask and (**B**) 96 well microplate, both incubated at 37°C and 190 rpm. Each point is a mean of 3 measurements for the Erlenmeyer flask cultivation and at least 12 measurements for the microplate. Vertical error bars represent standard error.

A striking difference between the two cultivation systems can be easily observed. Disregarding the amount of inoculum used, the growth of the bacteria is much slower in the microplate compared with the Erlenmayer flask. This is also clearly demonstrated by the calculated slope of the exponential phase. In the case of the microplate culture, the calculated slopes are 0,0169 OD units/hour (R^2=0.947) for 0.5% inoculum, 0,0175 OD units/hour (R^2=0.9899) for 1% inoculum and 0,0187 OD units/hour (R^2=0.9963) for 2% inoculum, while for the Erlenmeyer flask culture the calculated slopes are 0,2197 OD units/hour (R^2=0.970) for 0.5% inoculum, 0,1934 OD units/hour (R^2=0.987) for 1% inoculum and 0,1808 OD units/hour (R^2=0.960) for 2% inoculum.

Basically, the flask cultures grow at least 10 times faster than the multiplate cultures, this difference in growth rate translating into a delayed stationary phase in the case of the multiplate culture.

Another striking difference between the two cultivation and growth monitoring system is that the recorded turbidity in the stationary phase is less than half in the multiplate compared with the flask cultures. As the relative amounts of nutrients are basically the same in both cultivation systems, these differences must be related to shaking differences and hence oxygen availability. Both the Erlenmeyer flasks and the multiplate were incubated in the same time on the same shaker, so the shape of the multiplate well does not allow for good aeration in our experimental conditions. Reports in the literature agree with our conclusions and recommend a 8-shaped shaking motion as a method to improve aeration in microplates (Kurokawa and Ying 2017) or the usage of a 48-well microplates that provide a better small-scale fermentation systems for microbial cultivation (Zimmermann et al. 2004).

Another particularity of the microplates cultures is a great variability of the OD reads in the stationary phase. A close visual inspection of the microplates indicated the presence of particulate matter in the center of the wells (Figure 3) that would interfere with the path of light in the microplate. This is caused by either improper or low frequency shaking that causes the cells to stack in the center at a high density (Kurokawa and Ying 2017).

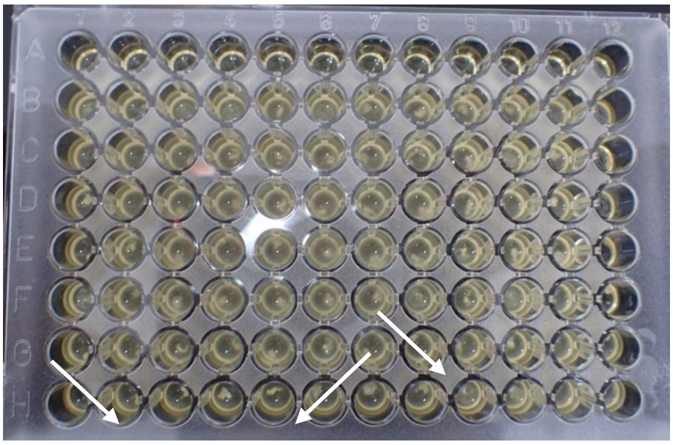

Figure 3. At high densities, bacterial cells tend to stack in the center of the well due to improper shaking causing unreliable OD readings in the microplate reader. Arrows indicate particulate matter in the wells due to cells stacking.

Technical difficulties encountered. During the course of the experiments, several technical difficulties were encountered. First, the wells located on the edges of the microplate suffer from evaporation of the medium that has a great impact on the reads due to the small culture volume (i.e., 200 microL). Hence, it is advisable that these wells should only contain blank medium and that cultivation time should be less then 48 h (Kurokawa and Ying 2017).

Secondly, as the plate was incubated at 37ºC and the multiplate reader was at room temperature, the temperature differences caused extreme condensation on the lead that interfered with the readings. Hence, a lead coating procedure must be used, as the one based on Triton X-100 described by Krishnamurthi et al. 2021 (Krishnamurthi et al. 2021).

Third, repeated loading and unloading of the plate in the microplate reader caused scratches on the lead that interfered with the OD readings, most probably due to an incompatibility between the plates used and the adapter provided with the multiplate reader. As a solution, another adapter was fabricated using 3D printing that placed the plate 2 mm lower, hence removing the scratching and allowing the reads to be performed with the lead on the microplates. A model of the adapter and printing instructions are available on NIH 3D Print Exchange server, Model ID 3DPX-015703.

In conclusion, common filter-based microplate readers can be used for recording growth curves for antibiotic and toxicity testing, but careful calibration and comparative studies must be taken into account before incorporating such devices into routine experimental designs. Several major technical difficulties must be carefully dealt with when measuring bacterial growth curves in microplates: efficiency of oxygenation that leads to slow growth rates and low densities, condensation on the microplate lead that leads to interference and evaporation of the growth media.

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Declarations

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Code availability (software application or custom code): Not applicable
Authors' contributions: Abalașei Marinela and Pioară Marinela performed the experiments, acquired data, performed initial data analysis, corrected the manuscript; Mihășan Marius - acquired funding, devised the experiments, provided lab space and materials, performed data analysis and interpretation, wrote the manuscript. All authors read and approved the final manuscript.
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YEW EXTRACTS - A POSSIBLE SOURCE OF BIOACTIVE COMPOUNDS WITH POTENTIAL ALLELOPATHIC PROPERTIES

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Abstract

Representative species of the *Taxus* genus, known since antiquity for their toxicity, have been an essential plant resource for the production of plant extracts with medicinal properties (anti-inflammatory, antifungal, and antibacterial) and, more recently, for the study of their allelopathic properties with significant implications in organic agriculture (bioherbicidal potential). With a view to the development of this new direction of research, the present work aims: to present a qualitative evaluation of two types of plant extracts (aqueous and alcoholic) of different concentrations (1% and 5%) obtained from various organs (bark, leaves, arils, and seeds) belonging to three *Taxus* taxa: a spontaneous taxa - *Taxus baccata* L. (**T1**) and two cultivated taxa - *Taxus baccata* (**T2**) and *Taxus baccata 'Robusta'* (**T3**) at different times of the phenological cycle by determining their absorption spectra (of both types of extracts), total amounts of polyphenols and flavonoids, and by evaluating their antioxidant capacity (alcoholic extracts); to investigate, under experimental conditions of cultivation the possible allelopathic effects induced by the aqueous extracts on the germination, growth, and development of the seedlings in two test plant species: *Amaranthus retroflexus* L. (ruderal species) and *Lycopersicon esculentum* Mill, variety Silvia (crop species). The data indicates the presence of phenolic and compounds, alkaloids, and carotenoid pigments in the alcoholic extracts prepared from the different organs of the studied yew taxa, showing higher amounts of polyphenols and flavonoids in the extracts obtained from the leaves of taxon **T2** compared to taxon **T1.** The effect of 1% and 5% aqueous extracts obtained from the bark of the three investigated yew taxa, as well as 1% aqueous extracts prepared from the leaves of taxon **T3** on seed germination, seedling growth, and development was more pronounced in the crop species *Lycopersicon esculentum* Mill, variety Silvia, compared to the ruderal broadleaf weed *Amaranthus retroflexus* L., both species having rapid germination stimulated by ambient light. Aqueous extracts of 5% concentration obtained from the arils of plants belonging to the three yew taxa stimulated the elongation of the seedlings' hypocotyls of both test species.

Keywords: *Taxus*, plant extracts, seed germination

Introduction

Germination and plant growth are highly complex phenomena that result in the development of new plant individuals. These processes can be influenced by a relatively large number of both abiotic and biotic factors. The former includes humidity temperature, the intensity of light radiation present in the plant's growing environment, etc. At the same time, plant development can be affected by interactions with other organisms of microbial, plant, or animal origin. Interactions between plants and biotic and abiotic factors have led to the synthesis of large numbers of secondary metabolites, which are products with specific biological properties.

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These properties are of great interest to the pharmaceutical and agricultural industries, currently the subject of intensely local, national, and international research.

The effort put into weed control has created a strong dependence on the synthesis and application of a wide range of herbicides, which has led to significant changes in weed flora and the selection of herbicide-resistant crop plant biotypes (Kudsk and Streibig 2003). In Romania, the areas in which herbicides were applied in 2007-2020 increased by 31.1%, compared to the previous period, with herbicides becoming the main tool for weed control in most areas of the country. Today, the reliance on herbicides continues to grow internationally as the global population migrates from rural areas to cities and the agricultural labor force declines. Consequently, the use of these synthetic chemicals has risen significantly, increasing the selection pressure induced by their intensive application on plants of agricultural interest, which has invariably led to an increase in multiple weed resistance in crops. Today's efforts to research new weed control technologies and integrated weed management systems aim to counteract the effects of their herbicide resistance (Peterson et al. 2018).

In this context, allelopathy has emerged as a pragmatic approach to solving problems in modern agriculture. Multiple crop approaches such as crop rotation, cover crops, intercropping, mulching, incorporation of crop residues, and application of aqueous extracts are some of the research directions developed with allelopathic themes to manage agricultural pests, mitigate abiotic and biotic stresses on plants of interest and improve and increase crop production (Farooq et al. 2013). Allelopathy is a naturally occurring ecological phenomenon in which different organisms affect the functioning of other crops. Allelopathy is a natural ecological phenomenon in which different organisms affect the functioning of other organisms in their vicinity, in a negative or positive way (Rice 1984), by releasing secondary metabolites (Farooq et al. 2011a). At the level of plant-plant interactions, the links between partner organisms are generated by the chemicals they produce and release into the environment through various pathways (volatilization, exudation, dissolution, etc.) (Wier et al. 2004).

In a plant’s metabolism, the synthesized compounds have various roles: informational, structural, energetic, synthetic, and signaling, some of them with defensive or competitive roles (Soltys et al. 2013). There are many categories of these compounds, such as alkaloids, flavonoids, tannins, organic acids, or volatile compounds, that can induce stimulation or inhibition of seed germination and growth of surrounding plants (Li et al. 2010). Thus, phenolic compounds and terpenoids act differently in different organisms. For example, in plant organisms, they can inhibit lipid and protein synthesis, alter photosystem I (Hirata et al. 2003) and photosystem II (Einhellig 1993, Dayan and Duke 2006), inhibit nutrient processing or seed germination, or alter transpiration and respiration rates (Rimando et al. 1998, Abrahim et al. 2003, Dayan and Duke 2006).

The biological properties of secondary metabolites have long attracted the attention of specialists and have been the basis for the development of psychostimulants or phytoinhibitors. The elucidation of the mechanisms that determine the biological properties of secondary metabolites biosynthesized by plants under certain growth conditions requires complex investigations, starting from observations in nature and continuing by deciphering specific molecular aspects. In this sequence, testing the effects of these compounds under various experimental conditions and quantifying them through interdisciplinary analyses using test organisms is an important step. Of particular relevance among these test methods is the testing of aqueous extracts produced by plants, as this method can more accurately reflect the response of test plants to favorable or unfavorable factors in their natural environment (Farooq et al. 2010).

In the present work, various yew taxa were chosen as potential sources of biocontrol compounds (Reigosa et al. 1999), being rich in compounds with phytotoxic activity (phenols, flavonoids, alkaloids) (Das et al. 1998, Parmar et al. 1998). Yew is widely recognized as a source of paclitaxel, a compound initially known as taxol, patented by Bristol-Myers Squibb under the generic name paclitaxel, used as an antineoplastic agent in the treatment of common cancers, such as ovarian and breast cancer (Onrubia et al. 2010). It also has other biological properties, including anti-inflammatory (Küpeli et al. 2003) and antifungal activity against *Cladosporium oxysporum* Berk. Curt, *Fusarium culmorum* W. G. Smith (Sacc.), *Alternaria alternata* (Fr.) Kiessler) (Baranowska and Wiwart 2003), anti-ulcerogenic (Gurbuz et al. 2004), antioxidant and antibacterial (Prakash et al. 2018), or insecticidal against *Tribolium confusum*, *Trogoderma granarium*, *Sitophilus granarius* (Daniewski et al. 1988).

The present research aims, to characterize the biochemistry of two types of plant extracts (aqueous and alcoholic) of different concentrations (1% and 5%) obtained from different organs (bark, leaves, arils, and seeds) belonging to three yew taxa (one spontaneous and two cultivated), at different times of their phenological cycle, by determining their absorption spectra (for both types of extracts) and the total amount of polyphenols and flavonoids, and their antioxidant capacity (for alcoholic extracts), and to evaluate the possible allelopathic (bioherbicidal) potential of 1% and 5% aqueous extracts obtained from various organs of the respective taxa, interpreting their effects on the germination, growth, and seedling development of two test plant species under experimental laboratory conditions: *Amaranthus retroflexus* L. (broad-leaved ruderal species) and *Lycopersicon esculentum* Mill, variety Silvia (crop species).

Materials and Methods

Study area, plant sample collection, and authentication

The biological material is represented by different organs (bark, leaves, arils, and seeds) collected from female yew individuals belonging to the spontaneous species *Taxus baccata* L. (**T1**), from the Yew Reserve - Tudora Forest, Botoșani County (lat. 47.524909° N, long. 26.691887° E, alt. 444 m) (access to the Reserve was ensured based on ANANP agreement no. 8147/26.07.2019 issued by the National Agency for Protected Natural Areas) and from cultivated taxa: *Taxus baccata* (**T2**) and *Taxus baccata 'Robusta'* (**T3**), procured from the nursery S.C. Doropad S.R.L. Suceava, based in Dorohoi and cultivated in Vorniceni commune, Botoșani county (lat. 47.986328° N, long. 26.663299° E, alt. 185 m).

Biological sampling was carried out in April (IV), June (VI), and September (IX) of the year 2021. According to the literature (Robakowski et al. 2018), the selected harvesting times correspond to the specific seasons of the phenological cycle of the analyzed species: strobili production in spring, intense vegetative growth in summer, and biomass allocation to roots in autumn. No plant material was collected for the winter period, as the weather conditions recorded during that period did not allow field access to the Yew Reserve in Tudora Forest. Vouchers of the taxa taken and used in the study were deposited, after identification (by Dr. Irina Irimia) at the Herbarium of the Faculty of Biology of "Alexandru Ioan Cuza" University of Iasi, with the corresponding identification numbers: 186539 - taxon **T1**, 186537 - taxon **T2**, 186538 - taxon **T3**. The processing of the plant material was carried out in the research facilities of the Faculty of Biology of "Alexandru Ioan Cuza" University of Iași, using the equipment from the Plant Biology Laboratory and the Integrated Centre for Environmental Science Studies for the North-East Development Region (CERNESIM), organized with funds obtained through grant No. 257/28.09.2010, SMIS/CNR 13984/901.

Experimental design

The biological material collected for analysis (bark, leaves, arils, and seeds) was subjected to heat treatment at 60°C in a ventilated oven for 60 minutes for enzyme inactivation. Subsequently, the material was dried at room temperature in adequately ventilated areas away from direct sunlight. The dried material was ground using an electric grinder and used for alcoholic and aqueous extracts preparation. In parallel, the determination of the water and dry matter content of the samples was carried out to report the results of the laboratory analyses (Boldor et al. 1983). Alcoholic extracts of 1% and 5% concentration, respectively, were prepared from the harvested plant material for a qualitative evaluation, and aqueous extracts of the same concentrations were prepared and used to investigate, under experimental laboratory conditions, their potential allelopathic effects on the germination, growth, and development processes of the seedlings, using seeds belonging to the test species *Amaranthus retroflexus* L. - (ruderal broad-leaved weed) and *Lycopersicon esculentum* Mill. Silvia variety - (crop plants), species with rapid germination stimulated by ambient light (Boldor et al. 1983, Asaad et al. 2017).

Preparation of alcoholic extracts

Alcoholic extracts were prepared using 70% ethyl alcohol (Chemical Company S.A., Iasi, Romania) as an extractant. According to the literature, extracts prepared using solvents of various polarities (water, ethyl alcohol, methyl alcohol, acetone, chloroform) allow the extraction of the compounds of interest from the plant material (Naczk 2004). Extraction was carried out by maceration for 48 h in closed plastic Falcon tubes, stored in a dark place to avoid photodegradation of the constituent compounds. Thus, 5 ml of 70% ethyl alcohol was added to 1 g of powder (rhytidome, leaves, seeds), and the tubes were shaken on a mechanical shaker at 300 RPM for 24 hours. Afterward, the extracts were centrifuged for 15 minutes at 4000 RPM and the supernatant was collected in separate tubes. Over the residue 5ml of 70% ethyl alcohol were added, repeating the extraction procedure for another 24 hours. The final (combined) extracts were centrifuged for 15 minutes at 4000 RPM with the supernatant used for further analysis. Aril extracts were obtained according to the method used by Tabaszewska et al. (2021), with 10 g of fresh material extracted in 150 ml of 80% ethyl alcohol. Alcoholic extracts of 5% concentration were obtained at the following dilutions: 1:4 for qualitative spectrophotometric evaluation and 1:9 for quantitative biochemical analysis.

Preparation of aqueous extracts

Aqueous extracts of bark, leaves, arils, and seeds of 1% and 5% concentrations were prepared using 0.5 and 2.5 grams of powdered plant material suspended in 49.5 and 47.5 ml of distilled water, respectively. The Erlenmeyer flasks with the obtained mixture (plant material and solvent) were kept for 150 minutes in a water bath at 50°C to facilitate the extraction of the bioactive compounds and to avoid their degradation due to the high temperature. Subsequently, the extracts were filtered through filter paper and stored at 4°C away from any light source to prevent the decomposition of biologically active components until use (Lobiuc et al. 2016).

Qualitative evaluation of absorption spectra of aqueous and alcoholic extracts

For the qualitative evaluation of the chemical composition of the extracts, their UV-vis absorption spectra were determined (Baciu et al. 2013) using a Beckman DU - 730 spectrophotometer in the 190 - 700 nm range. Before evaluating the absorption spectra, it was necessary to dilute the aqueous and alcoholic extracts of 1% and 5% concentration (25µl extract + 675 µl solvent, respectively).

Determination of total polyphenol content in alcoholic extracts

A 1:9 dilution was made from the initial 5% alcoholic extracts using the Folin-Ciocalteu method (Herald et al. 2012). Absorbance readings were performed at $\lambda = 760$ nm against distilled water on a Shimadzu UV - mini spectrophotometer. The calibration curve was generated using gallic acid of 0-400 µg/ml concentrations. Polyphenol concentrations in extracts were calculated using a calibration curve with $R^2=0.9977$ and expressed as mg gallic acid/g dry matter.

Determination of flavonoid content in alcoholic extracts

Determination of flavonoid compound content was carried out on 1:9 diluted alcoholic extracts using the method of Jia et al. (1999) and Herald et al. (2012). The analysis was performed at λ = 510 nm against distilled water. Standard solutions were prepared for the calibration curve using quercetin in 0-500 µg/ml concentrations. The flavonoid content in the extracts was calculated from the calibration curve with R^2=0.9879 and expressed as mg quercetin/g dry matter.

Determination of the antioxidant activity of alcoholic extracts

The antioxidant activity of alcoholic extracts was determined according to the method of Thaipong et al. (2006) and Herald et al. (2012). A 60 µM DPPH alcohol solution was prepared. The samples were obtained by mixing 0.1 ml diluted plant extract (1:9) with 2.9 ml DPPH solution. The resulting mixture was incubated in the dark at room temperature for 3 hours. The analysis was performed at λ= 515 nm against ethanol. Standard solutions were prepared to obtain the calibration curve, using ascorbic acid concentrations of 0-200 µg/ml. Antioxidant activity was calculated using the formula obtained from the calibration curve (R^2=0.9936) and expressed as mg ascorbic acid equivalent per gram of dry matter.

Experimental conditions for testing aqueous extracts

Seeds of *Lycopersicon esculentum* Mill., variety Silvia - tomato, crop species - purchased from Agrosel, and of redroot pigweed - *Amaranthus retroflexus* L., a broad-leaved ruderal weed, were used to test the effects of aqueous extracts on seed germination. Seed sterilization was carried out in two stages: a first stage with 2.5% sodium hypochlorite for 3 minutes, followed by three successive rinses with sterile distilled water, and a second stage with 3% hydrogen peroxide for 3 minutes, followed by three rinses with distilled water. Two sets of experimental variants were set up for each species; each variant consisted of 6 tubes with equal volumes of 5 ml aqueous extracts of 1% and 5% concentrations of bark, leaves, arils, and seeds, respectively, and one tube with distilled water (control variant). The aqueous extracts prepared from the plant material corresponded to the specific times of harvesting (IV-April, VI-June, IX-September for leaves and bark, respectively, and IX-September for arils and seeds). After sterilization, test plants seeds (15 *Amaranthus* and 10 *Lycopersicon* seeds for each experimental variant, respectively) were subjected to imbibition for 24 hours. Afterwards, the seeds were placed on filter paper in Petri dishes (previously sterilized in an autoclave sterilizer at 180°C for 2 hours to avoid microbial contamination); the handling of the biological material was carried out in a vertical 700 laminar flow hood. This resulted in 98 experimental variants (48 treatments x 2 test species + 1 control x 2 test species), each of the 98 variants being set up in replicates of three plates. The filter paper in the Petri dishes was moistened with 2 ml of sterile distilled water at the start of the experiment, and the filter paper was moistened with 1 ml of distilled water as needed several times a day during the duration of the study. Petri dishes with seeds were initially maintained for 24 hours in the dark in a thermostat at 24°C, and then for 13 days in the laboratory at room temperature (26±2°C). The experiment lasted 14 days (336 hours), including the 24 hours of imbibition.

Germination index analysis

The effects of aqueous extracts on the germination process in the test species were evaluated by recording the total number of germinated seeds in each variant at 24-hour intervals throughout the experiment. The values were used to calculate four germination indices (Table 1) according to the formulas described by Boldor et al., (1983) and Mominul Islam and Kato-Noguchi, (2014), with the results being calculated as the average of the values obtained in triplicate for each experimental variant.

Table 1. The equations used to calculate different germination indices

Germination parameters	**Equations**	**References**
Germination percentage (**GP**)	$\mathbf{GP} = \left[\frac{\text{Number of germinated seeds at final count}}{\text{Total number of seeds sets for bioassay}}\right] \text{x } 100$	Mominul Islam and Kato-Noguchi 2014.
Speed of emergence (**SE**)	$\mathbf{SE} = \left(\frac{\text{Number of germinated seeds at the starting day for germination}}{\text{Number of germinated seeds at the final days of measuremnet}}\right)$	Mominul Islam and Kto-Noguchi 2014
Germination energy (**GE**)	$\mathbf{GE} = \left(\frac{\text{Number of germinated seeds at the 120 h}}{\text{Total number of seeds sets for bioassay}}\right) \text{x}$	Boldor et al. 1983, Mominul Islam and Kato-Noguchi 2014
Seedling vigour index (**SVI**)	$\mathbf{SVI} = \left(\frac{\text{Seedling length (mm)x Germination percent}}{100}\right)$	Mominul Islam and Kato-Noguchi 2014

Biometric Determinations of newly formed seedlings

To assess the influence of aqueous extracts on the initial growth of newly formed seedlings, the following seedling parameters were measured: root length (mm), hypocotyl length (mm), and seedling mass (g) (Dayan and Duke 2006). Measurements were made 336 hours after the experiment was set up on 15 seedlings of the shoot and up to 10 seedlings of the tomato, or the maximum number of seedlings available for each variant, using ImageJ software.

Statistical analysis

The data reported for all parameters represent the mean value ± standard error (SEM). Statistically significant differences between variables were assessed using one-way ANOVA and Tukey's multiple comparisons tests using GraphPad Prism 9.2.0. Significant differences between variants were considered at $p \leq 0.05$ and are marked on the graphs as follows: **** = $p<0.0001$, *** = $p<0.001$, ** = $p<0.01$, * = $p<0.05$.

Results and discussions

Summary qualitative evaluation of aqueous and alcoholic extracts

The obtained values indicate the presence of several types of compounds, with variations according to a specific taxon, the season/time of investigation, type of extract, organ, and extract concentration (Plates I and II). According to the literature, the presence of alkaloids was indicated by the existence of maxima in the 220 nm region and in the 280 nm region of the analysis spectrum, maxima that correspond to groups with nitrogen atoms (Porto 2016), being known that in the case of alkaloids synthesized by *Taxus* species, the absorption maxima are for taxine A - 220 and 255 nm, for 2-deacetyltaxine A - 224 and 264 nm, for taxine B - 210 and 277 nm, and for isotaxine B - 282 nm (Wilson and Hooser in Gupta 2018).

From our investigations, it can be observed that during the period of the formation of the strobiles (April), both aqueous and alcoholic extracts of 1% and 5% concentration of bark and leaf show the same absorption maxima in the 220 and 250 nm region for all taxa. Differences appear in June (intense growth stage) when it is observed, for example, that the alcoholic extracts of bark and leaf of 1% concentration in the case of spontaneous taxon **T1** show the lowest absorbances, compared to the other two cultivated taxa, in the 220-250 nm region, and that the highest values were recorded for the bark of taxon **T2** and the leaves of taxon **T3**. In aqueous extracts of the same concentration, only the bark of the three investigated taxa shows compounds in the range mentioned above, where once a new spontaneous taxon **T1** records lower values, and taxon **T2,** the highest value. In September, when the allocation of newly formed biomass to the roots of yew plants takes place, it is observed that in the 220-250 nm range of the spectrum, all alcoholic extracts of 1% concentration from bark, leaves, and seeds show approximately the same absorbance, which leads us to consider that all these organs contain substantially similar amounts of compounds. The higher concentrations of compounds, by the intensity of absorbance, were recorded in extracts of 5% concentration belonging to the same variants. Generally, aqueous extracts, as opposed to alcoholic extracts, have higher amounts of compounds, with the highest concentrations observed in seed extracts, which show the highest absorbance for all taxa.

According to data presented in the literature (Kajdžanoska et al. 2010, Baciu et al. 2013, Bunghez et al. 2013, Butnariu 2014), the main classes of compounds identified with absorption maxima in the 260-280 nm region are phenolic compounds. Consistent with this information, from our analyses, we can consider that during June, alcoholic extracts are higher in phenolic compounds compared to aqueous extracts; thus, for 1% leaf extracts, cultivated taxa show higher concentrations compared to the spontaneous species, and at 5% concentration, alcoholic extracts display approximately the same absorbance for all organs and taxa. Differences between taxa occur for aqueous extracts from bark and leaf at 5% concentration, where the spontaneous taxon **T1** shows the lowest absorbances. The 1% and 5% alcoholic extracts obtained during June and September show approximately the same absorbance/concentration of phenolic compounds, with slight differences between taxa at 1% concentration. Aqueous extracts show the highest absorbances for leaf extracts, where **T2** and **T3** taxa are richer in phenolic compounds. The maxima recorded in the 440 nm region of the absorption spectrum, according to the literature, indicates the presence of carotenoid pigments (Horváth et al. 2010, Zăvoi et al. 2011), which have appeared in the experiments carried out on yew in aqueous extracts from arils of 5% concentration in taxa **T1** and **T3**. The results obtained after the experiments are in agreement with the literature, which mentions the presence of phenolic compounds in different yew organs (Das et al. 1993, Das et al. 1998), as well as alkaloids (Parmar et Jha 1998), the synthesis of the latter in plants of this taxonomic group and their pharmacological activity (Malik et al. 2011) being the reason why yew specimens have been intensively exploited over time (Dhar et al. 2013).

The total polyphenol content of alcoholic extracts

Phenolic compounds are the major groups of allelochemical compounds present in plants. These are a group of organic compounds that have in their structure an aromatic nucleus onto which one or more hydroxyl (-OH) groups are grafted. Phenolic compounds with allelopathic effect include (Inderjit 1996) simple aromatic phenols, hydroxylated and substituted benzoic acids, aldehydes, hydroxylated cinnamic acids, as well as coumarins, tannins, and some flavonoids (Liu et al. 2008) in agreement with John et Sarada, (2012). Phenols are known to be produced by plants in response to stress caused by pollutants or pathogens, functioning as a defense mechanism in their metabolism (Smolders et al., 2000). Higher values of this parameter may indicate a non-enzymatic antioxidant response of plants, and lower values at lower concentrations may suggest that the stress level exceeds the metabolic capacity of the synthesis of these compounds by those organisms. The practical results obtained during the present experiment on the total polyphenol content of the alcoholic extracts showed variations between taxa, organs, and time of analysis (Figure 1).

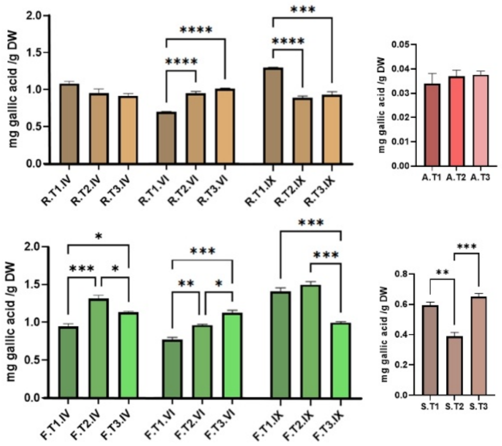

Figure 1. Total polyphenol content

R = bark; **F** = leaf; **A** = aril; **S** = seed; **T1** = *Taxus baccata* – spontaneous; **T2** = *Taxus baccata* – cultivated; **T3** = *Taxus baccata 'Robusta'* – cultivated; **IV** – April (strobili production); **VI** – June (intense vegetative growth); **IX** – September (biomass allocation to roots)

Leaf extracts had the highest content of polyphenols; this biochemical parameter showed statistically significant differences between taxa and analysis times, with spontaneous taxon **T1** and cultivated taxon **T2** showing the highest levels of these compounds during September. The extracts obtained from bark show the highest level of polyphenols at the time of biomass allocation to roots (September) in spontaneous taxon **T1,** and the lowest during June, when cultivated taxa **T2** and **T3** show statistically significant higher levels of phenolic compounds, compared to taxon **T1**. In the case of extracts prepared from seeds, the highest level of polyphenols is recorded in taxon **T3**, followed by taxa **T1** and **T2**. The literature data indicates that shaded *Taxus* individuals have a higher total content of phenolic compounds than those growing in the light. While the activity of phenylpropanoid enzymes (phenylalanine, ammonia-lyase, and chalcone synthase) is light-dependent, the accumulation of polyphenolic compounds is probably due to specific functional adaptations of these trees to shaded conditions. Thus, a high level of secondary metabolites in yew specimens and, in addition, a higher level of specific biochemical compounds in their young leaves represent important metabolic properties of these trees; similar tendencies were also observed in Scot's pine (Giertych 2001) in agreement with Brzezińska et Kozłowska (2008).

The data obtained in our research confirm the information presented in the literature for the specimens belonging to the analyzed spontaneous species (taxon **T1**), which has the highest concentration of polyphenols, given the specific vegetation conditions, namely the deciduous forest in the area of the Yew Reserve in Tudora, with a rich canopy, which does not easily allow sunlight to reach the yew trees. In addition, physiological research carried out in the field and the laboratory during the same study period revealed that specimens of the spontaneous taxon **T1** in that reserve, which does not benefit from optimal illumination, recorded the lowest photosynthetic and transpiration rate values under conditions of maximum leaf assimilatory pigment content (Hageneder 2013, Perrin et Mitchell 2013, Zarek 2016).

Total flavonoid content of alcoholic extracts

Flavonoids are an important group of compounds, widely studied due to their possible beneficial effects on human health (Luo et al. 2014, Karak et al. 2019). These biologically active natural compounds are currently an essential phytochemical substrate for a wide variety of applications aimed at obtaining medicinal, pharmaceutical, and cosmetic preparations (Panche et al. 2016, Karak et al. 2019), due to their anti-inflammatory, antioxidant, anticarcinogenic, and antimutagenic properties, along with their ability to modulate their cellular key enzyme function (Panche et al. 2016, Alseekh et al. 2020). Flavonoids are diverse bioactive compounds that can be classified into different classes as flavones, flavonols, flavan-3-ols, flavanonols, isoflavones, and bioflavonoids (Pietta et al. 2000, Andrade et al. 2018) and can be extracted from a diverse range of sources, from microorganisms to higher plants (Verma et al. 2020) according to Bekhouche et al. (2022).

The practical results obtained during our research (Figure 2) show that the bark and leaves of *Taxus* are the organs richest in such compounds. Significant amounts of such compounds are found in the plant material analyzed during autumn (IX - September) for the spontaneous taxon **T1** and the cultivated taxon **T2**. At the same time, the seeds of the cultivated taxon **T3** show significantly higher amounts of flavonoids compared to the other two taxa studied, and the arils of the cultivated taxa **T2** and **T3** show significantly higher contents compared to the spontaneous taxon **T1**.

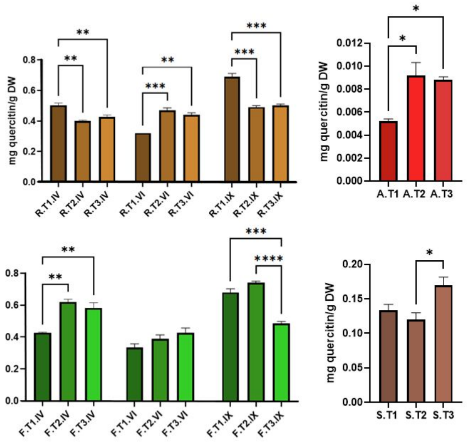

Figure 2. Total flavonoid content

R = bark; **F** = leaf; **A** = aril; **S** = seed; **T1** = *Taxus baccata* – spontaneous; **T2** = *Taxus baccata* – cultivated; **T3** = *Taxus baccata 'Robusta'* – cultivated; **IV** – April (strobili production); **VI** – June (intense vegetative growth); **IX** – September (biomass allocation to roots)

Little information is known on the chemical composition and biological activity of extracts prepared from various organs belonging to the species *Taxus baccata*. For example, Milutinović et al. (2015) reported that the total flavonoid content in a methanolic extract prepared from leaves and seeds of *Taxus baccata* growing in Serbia was, at the time of conducting experiments, 161.98 ± 1.02 mg rutin equivalent/g dry extract, while Senol et al. (2015) reported a total flavonoid content of 48.89 ± 0.76 mg quercetin equivalent/g in an ethanolic extract from leaves and shoots of *Taxus baccata* growing in Turkey. According to the literature, the differences in total flavonoid content can be attributed to genetic variations, geographical origin, climatic growing conditions, and yew tree populations investigated by Bekhouche et al. (2022).

Antioxidant activity of alcoholic extracts

The antioxidant activity and its intensity in plants depend on the existence of different compounds in the plant species. The antioxidant and radical scavenging activities of flavonoid compounds in plant organisms are well studied and presented in the literature (Das et al. 1992). Some of the phenolic compounds isolated from plants (anthocyanidin, catechins, flavones, flavonols, and isoflavones), tannins (ellagic acid, gallic acid), phenyl isopropanol (caffeic acid, coumaric acids, ferulic acid), lignans, catechol, and many others are compounds with significant antioxidant properties (Rice-Evans et al. 1996 according to Emami et al. 2007). There is very little information in the literature on the antioxidant activity of extracts prepared from different organs of *Taxus baccata*; among these, most data discuss antioxidant activities, along with other biological activities of individual components derived from this taxon (Erdemoglu et al. 2004, Kucukboyaci et al. 2010 cf. Milutinović et al. 2015.) For example, taxifolin present in several plant species, including *T. baccata*, is thought to scavenge DPPH free radicals (Topal et al. 2016), and other flavonoid compounds, such as proanthocyanidins, amentoflavone, kaempferol, myricetin, isorhamnetin, and apigenin, have been reported to scavenge free radicals (Bekhouche et al. 2022).

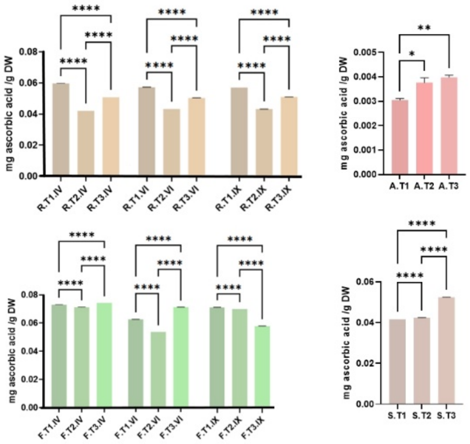

Figure 3. Antioxidant activity of alcoholic extracts

R = bark; **F** = leaf; **A** = aril; **S** = seed; **T1** = *Taxus baccata* – spontaneous; **T2** = *Taxus baccata* – cultivated; **T3** = *Taxus baccata 'Robusta'* – cultivated; **IV** – April (strobili production); **VI** – June (intense vegetative growth); **IX** – September (biomass allocation to roots)

The data obtained in our research using alcoholic extracts from various organs of the studied *Taxus* taxa (Figure 3) show the highest values of antioxidant activity for extracts obtained from leaves, followed by those from bark, seeds, and arils. Statistically significant differences appear between all taxa at all time points of analysis (IV, VI, IX) as spontaneous species (**T1**) show the highest antioxidant activity in ethanolic extracts from the bark. Extracts obtained from leaves, arils, and seeds, taken from the cultivated taxon **T3** show high antioxidant activity at the timing of strobila formation (IV-April) and intense vegetative growth (VI-June).

Germination indexes

The aqueous extracts obtained from the organs of the studied *Taxus* taxa had different effects on the germination indices (germination percentage, germination rate, germination energy, and vigor index) in the two test species. The effects were more pronounced in the case of seeds of tomato - *Lycopersicon esculentum* Mill., variety Silvia (crop plant), compared to those of *Amaranthus retroflexus* L. (ruderal weed). For both test species analyzed, seed germination was monitored over 336 hours (14 days), following the working protocol for this type of determination presented in the literature (Yarnia et al. 2009, Singh et al. 2012). The application of 5% aqueous extracts obtained from the bark of the cultivated taxon **T3** induced significantly reduced germination percentage values in *Amaranthus* seeds during the period of biomass allocation to roots (IX), (Plate III, A); the other types of extracts (prepared from leaves, arils, and seeds of all studied taxa) resulted in slightly lower values of this germination index, close to those obtained in the control variant (distilled water treatment). In the case of the second germination index - germination speed, it was found that following the application of the 5% aqueous leaf extract treatment, taxon **T3** showed statistically significant differences compared to the control variant at all times of analysis (IV, VI, IX); this treatment is followed by the treatments with 5% and 1% arils extracts, respectively, in all taxa tested, as well as the 1% extracts from the seeds of the spontaneous taxon **T1** and the cultivated taxon **T3** (Plate III, B). Germination energy showed significantly lower values, compared to the control variant, following treatments with 5% aqueous extract obtained from the bark of cultivated taxon **T3** at the time of biomass allocation to the roots (IX). At the same time, the 5% aqueous extract prepared from the arils of spontaneous taxon **T1** resulted in significantly lower mean values of this germination index compared to the mean values recorded with the control variant (Plate III, C). Significantly reduced vigour index values compared to the control variant were recorded following treatments with 5% aqueous extracts obtained from the bark of spontaneous taxon **T1** (VI) and cultivated taxon **T3** (IX). In the case of the application of treatments with 5% extracts obtained from bark (the cultivated taxon **T2)** and leaves (all three taxa) in April (strobili formation), it can be observed that this time, the extracts have a stimulating effect on the respective physiological parameter (Plate III, D), due to the significantly increased values obtained.

The cumulative analysis of the obtained data shows that the most obvious effects of aqueous extracts prepared from bark, leaves, arils, and seeds from specimens belonging to the three *Taxus* taxa on the germination process in *Amaranthus retroflexus* were produced by extracts of 5% concentration from bark and leaves of cultivated yew taxa specimens, especially taxon **T3**. Among the analyzed germination indices, the emergence rate recorded the lowest values in response to the application of the treatments, compared to the control variant, being the least influenced parameter of the germination percentage.

The germination percentage for the crop species *Lycopersicon esculentum* Mill., variety Silvia showed significantly reduced values following the application of aqueous extracts treatments obtained from the 1% concentration bark at the times of intense vegetative growth (VI) and biomass allocation to roots (IX), but also when applying extracts from the 5% concentration bark in June (time of intense vegetative growth). The other aqueous extracts from leaves, arils, and seeds applied to *Lycopersicon* seeds resulted in slightly reduced values of this germination index compared to the control, but statistically insignificant (Plate III, a). As a result of treatments with 1% aqueous extracts prepared from the bark of cultivated taxa **T2** and **T3**, the germination rate shows significantly reduced values compared to the control variant in April, when strobili formation occurs. In the period of vegetative growth (June), treatments applied with 5% aqueous extracts obtained from the leaves of the investigated taxa show significantly lower values when compared to the control sample (Plate III, b). The highest effects on germination energy were recorded for this test species following treatments with aqueous bark extracts of 5% concentration (VI - intense vegetative growth stage) and 1% (IX - stage of biomass allocation to roots), respectively, in which case significantly lower values are observed, compared to the control, for all three investigated taxa. The other aqueous extracts (leaf, arils, and seed preparations) applied to *Lycopersicon* seeds resulted in slightly reduced values of this germination parameter compared to the control, but not significant (Plate III, c). The vigour index is the germination parameter which, in the case of this test species, for each time of analysis (IV - April, VI - June, IX - September), recorded statistically significantly lower values than the control variant, in the case of treatments with aqueous extracts of concentration 1 and 5%, respectively, obtained from the rhytidome. Significantly lower values were also recorded when applying treatments with aqueous extracts of 1% concentration obtained from the leaves of taxa **T1** - spontaneous taxon and **T3** - cultivated taxa during the formation of strobila (Plate III, d). Overall, in the case of the test species *Lycopersicon esculentum*, variety Silvia the germination indexes considered showed different values, allowing their ranking, in descending order, in the following sequence: vigour index, germination energy, emergence speed, germination percentage; the treatments applied with aqueous extracts of 1 and 5% concentration obtained from the bark of the three *Taxus* taxa induced the most pronounced effects on the seeds of the test species plants during the germination process under experimental conditions.

Biometric determinations

In the experiments with the test species of the ruderal redroot pigweed - *Amaranthus retroflexus* L. - some of the aqueous extracts of *Taxus* tested generally inhibited the analyzed biometric parameters. In this species, stronger influences were recorded when treatments with the extracts obtained from the bark and leaves of all three yew taxa were applied. The mass of newly formed seedlings by germination was generally reduced, but statistically insignificant. In the case of the application of 5% aqueous extracts obtained from leaves of individuals belonging to all three yew taxa under consideration in the period of strobila formation (IV), a stimulation of mass accumulation in newly formed seedlings was observed (Plate IV, E). The mean root length of newly formed seedlings was statistically significantly reduced following treatments with 5% aqueous extracts obtained from the bark of taxa **T1** harvested in June (stage of intense vegetative growth) and **T3** harvested in September (biomass allocation to roots stage). The application of treatments with aqueous extracts obtained from leaves belonging to the cultivated taxon **T3** also had a slightly stimulatory effect on this parameter, in which case statistically significantly higher values of the mean root length of newly formed seedlings were recorded compared to the control variant (Plate IV, F). The same slightly stimulatory effect was observed for the mean hypocotyl length of newly formed seedlings when applying 5% aqueous extracts obtained from the bark of taxa **T2** and **T3**, as well as from the leaves of all three yew taxa studied (**T1**, **T2**, **T3**) - extracts prepared from material collected in April when the strobiles are formed. Similar results, but with significantly higher values than the control sample, were also obtained in June (stage of intense vegetative growth), following treatment with the aqueous extract obtained from the leaves of the cultivated taxon **T2** (Plate IV, G).

In the case of the crop species *Lycopersicon esculentum* Mill, variety Silvia, the comparative analysis of the biometric parameters characterizing the newly formed seedlings in the germination process following treatments with aqueous extracts of rhytidome, leaves, arils, and seeds obtained from the three yew taxa allows us to consider that they were, in general, significantly reduced compared to the control sample. The mass of fresh newly formed seedlings was significantly statistically reduced following treatments with 1% aqueous extracts obtained from the bark of all three yew taxa studied (VI - June) (Plate IV, e). The other aqueous extracts from leaves, arils, and seeds applied to *Lycopersicon* seeds showed slightly reduced or increased values of this parameter compared to the control, but statistically insignificant. The mean root length of newly formed seedlings generally showed significantly reduced values after treatments with aqueous extracts obtained from the three yew taxa studied. The lowest values were recorded for seedlings from seeds treated with aqueous extracts of 1 and 5% concentration obtained from the rhytidome of the three yew taxa investigated, obtained at two moments of their phenological cycle, namely intense vegetative growth (VI) and biomass allocation to roots (IX). For the time of strobila formation (IV), the 1% aqueous extracts prepared from the leaves of taxa **T1** (spontaneous taxon) and **T3** (cultivated taxon) showed statistically significantly lower values of this parameter compared to the control (Plate IV, f). The most significantly reduced effects in the mean hypocotyl length of newly formed seedlings (Plate IV, g) were recorded following the application of 1 and 5% aqueous bark extracts obtained from the three yew taxa in June (stage of intense vegetative growth) and September (biomass allocation to roots stage). Also, the 5% aqueous leaf extract obtained from the cultivated taxon **T3** affected the hypocotyl elongation of newly formed seedlings during September (biomass allocation to roots stage), with significantly lower values than the control. For the aqueous extracts obtained from the arils of the three yew taxa investigated, it can be observed that the elongation of the hypocotyl of the seedlings is significantly higher after the treatments compared to the control.

The inhibitory effects of aqueous extracts of yew plants on seed germination and growth parameters of newly formed seedlings of the test species may be due to specific allelochemicals (including phenolic compounds) in their composition. Furthermore, the toxicity of aqueous extracts might be due to the interactions of the allelochemicals present in them rather than the effects of a single allelochemical (Dadkhah 2012). The toxicity of *Taxus baccata* species has been known since antiquity, and yew leaf extracts have been used for both homicides and suicides. Intoxication with parts of the yew plant (seeds, bark, leaves) is well described in the literature, but cases of suicide by yew ingestion are rarely reported. *Taxus baccata* species contains a complex mixture of compounds, including phenolic constituents (for example, 3,5-dimethoxyphenol), non-alkaloid diterpenoids (for instance, 10-deacteylbaccatin III), alkaloid diterpenoids (for example, paclitaxel, taxin B) or flavonoids (ex. myricetin) and bioflavonoids (bilobetin) (Gupta 2005), compounds that could be responsible for such a severe toxic effect.

Germination is now known to be inhibited by phenolic compounds (Li et al. 2014) or alkaloids (Lovett and Hoult 1998), which can reduce mitotic activity in roots and hypocotyls, suppress hormonal activity, reduce nutrient uptake rates, inhibit photosynthesis and respiration as well as enzymatic action, reduce cell membrane permeability (Rice 1984, Dadkhah 2012), elongate plant roots, cell division, alter cell ultrastructure and subsequently interfere with the growth and normal development of the whole plant (John and Sarada 2012).

Comparative interpretation of the data from the present experiments allows us to consider that the inhibition of germination of the test plants was most strongly manifested in the case of application to their seeds of aqueous extracts of 1% and 5% concentration, respectively, prepared in descending order of their effectiveness from the bark, leaves, seeds, and arils of the three yew taxa. The literature discusses the allelopathic effect of extracts obtained from the integument and endosperm of Chinese yew (*Taxus chinensis* (Rehder & E.H.Wilson) Rehder var. mairei) seeds which, according to the authors, inhibited the germination of cabbage (*Brassica oleracea* L.) seeds and the growth of newly formed seedlings (Zhang et al. 2010). Although there is information in the literature that several gymnosperm genera have demonstrated allelopathic effects on other plants in either *in vitro* tests or *in situ* community studies, studies using such plant material, with the exception perhaps of some members of the genus *Pinus*, are still in their infancy, leaving room for much future research. The possible application of plant extracts or chemicals extracted from these plant species as possible bioherbicides are research topics of genuine interest for the future, with the idea of founding technologies to obtain from this group of plants biologically active compounds of broad interest for organic agriculture (Teixeira da Silva et al. 2015).

Conclusions

Following the investigations carried out on the *Taxus* taxa under study, at three defining moments of the phenological cycle, the chemical characterization of the plant extracts obtained and the testing of their effects on the germination, growth, and development of the seedlings of the test species chosen for analysis, we can conclude the following: in the composition of alcoholic and aqueous extracts of the investigated taxa, the presence of phenolic, alkaloid and carotenoid compounds was revealed; according to the literature, the analysis and discussion of the absorption spectra of these extracts constitutes a novelty for science; the amount of polyphenolic and flavonoids, and the antioxidant activity of alcoholic extracts obtained from rhytidome, leaves, arils and seeds in the analyzed yew taxa showed slight quantitative variations between taxa and times of analysis; in this respect, the cultivated taxon **T2** shows higher amounts of polyphenolic and flavonoid compounds than the spontaneous taxon **T1**; aqueous extracts, especially those obtained from bark, exert inhibitory effects on the analyzed germination parameters (germination percentage, germination speed, germination energy, vigour index); the application of 5% aqueous extracts obtained from the rhytidome of spontaneous taxon **T1** and cultivated taxon **T3** significantly inhibits root elongation of newly formed seedlings of *Amaranthus retroflexus* L. ; in the case of the test species *Lycopersicon* *esculentum* Mill, variety Silvia, the application of aqueous extracts of 1 and 5% concentration obtained from the bark of the three yew taxa and extracts of 1% concentration prepared from the leaves of the cultivated taxon **T3** inhibited root elongation of newly formed seedlings; hypocotyl elongation of newly formed seedlings in both test plant species was stimulated by the application of 5% aqueous extracts obtained from arils harvested from all three yew taxa; seeds of *Lycopersicon esculentum* Mill. , variety Silvia showed higher sensitivity to the applied aqueous extracts compared to seeds of *Amaranthus retroflexus* L. The allelopathic effects produced by the aqueous extracts obtained from the three yew taxa on the test plants can be continued and completed by increasing the number and diversifying the physiological and biochemical analyses on a wider range of target plants of agricultural interest, as an important scientific research step in the work of identifying, isolating and testing compounds with implications in organic agriculture (with bioherbicidal potential).

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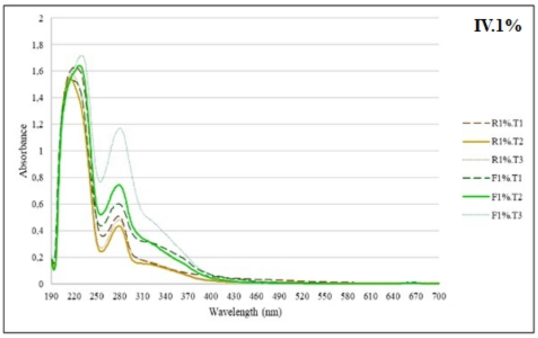
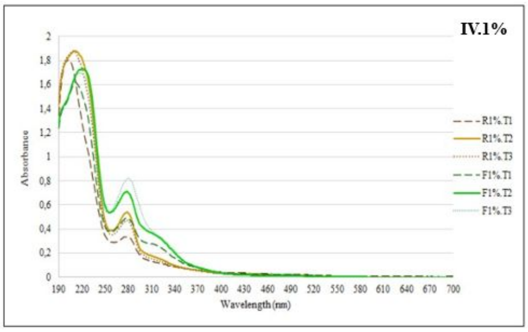
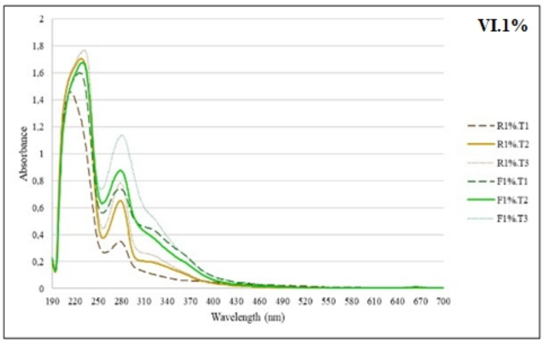
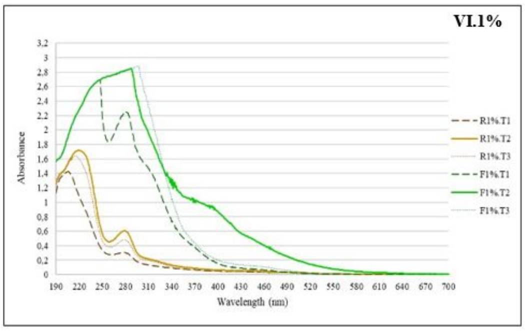
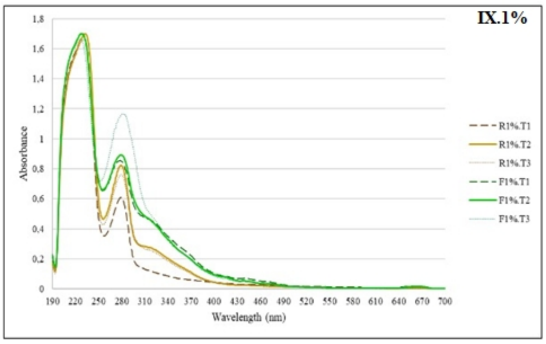
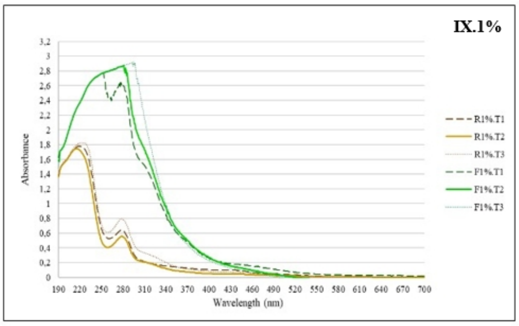
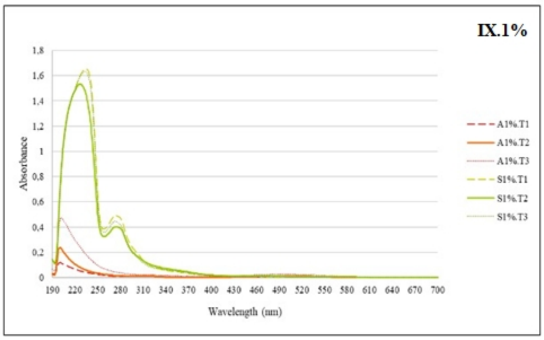
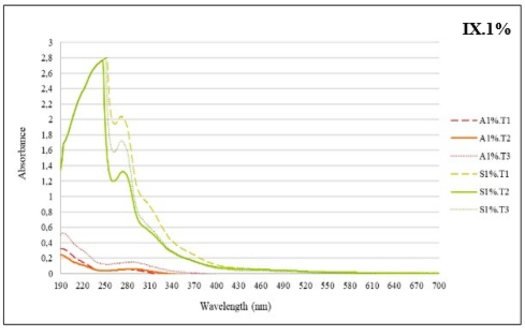
PLATE I

Absorption spectra of alcoholic and aqueous extracts of 1% concentration from *Taxus* bark, leaf, arils, and seeds

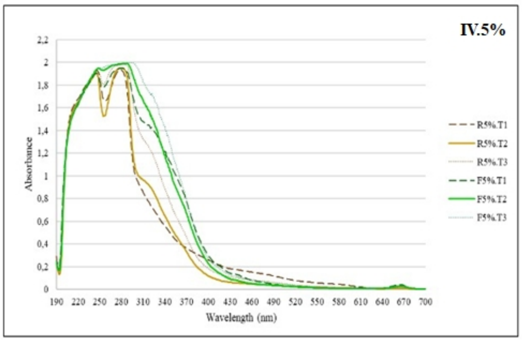
Alcohol extracts	Aqueous extracts
	
	
	
	

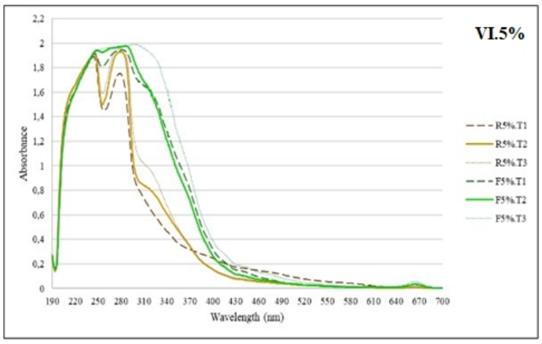
Legend: **IV** – April (strobili production); **VI** – June (intense vegetative growth); **IX** – September (biomass allocation to roots); **R** = bark; **F** = leaf; **A** = aril; **S** = seed; **T1**= *Taxus baccata* – spontaneous; **T2**= *Taxus baccata* – cultivated; **T3**= *Taxus baccata 'Robusta'* – cultivated

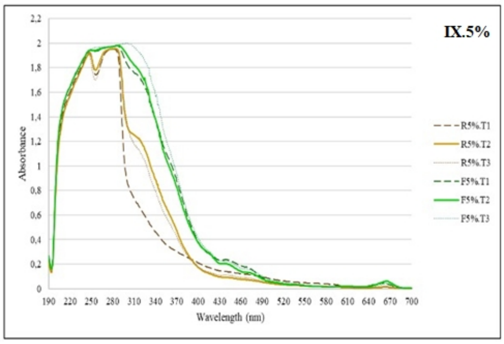
PLATE II

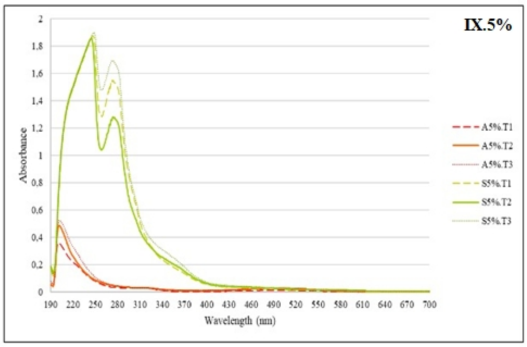
Absorption spectra of alcoholic and aqueous extracts of 5% concentration from *Taxus* bark, leaf, arils, and seeds

Alcohol extracts

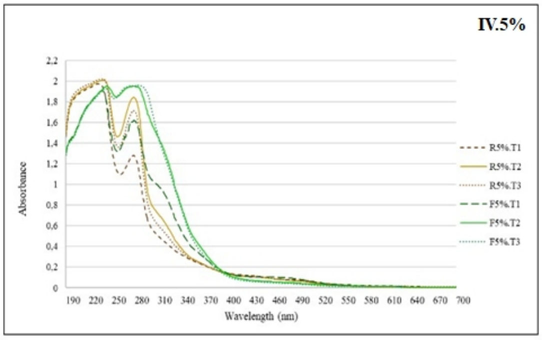


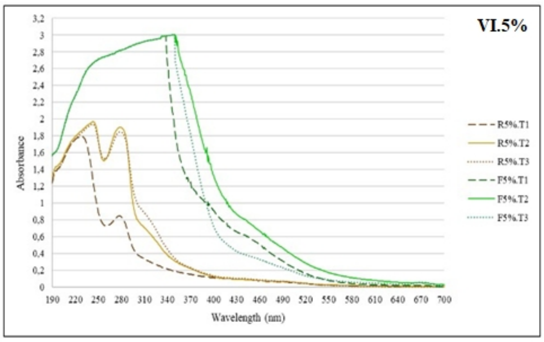


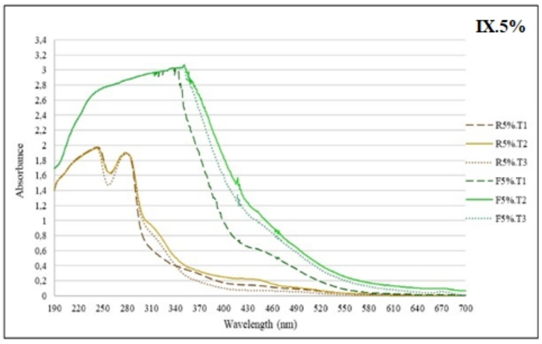


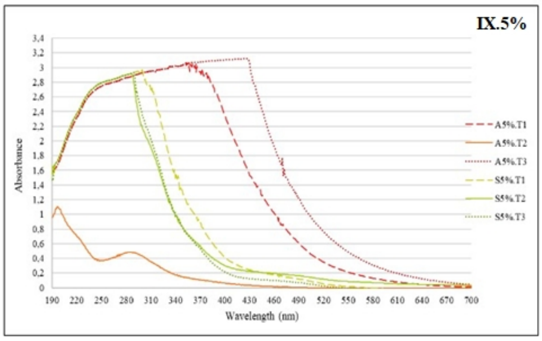


Aqueous extracts







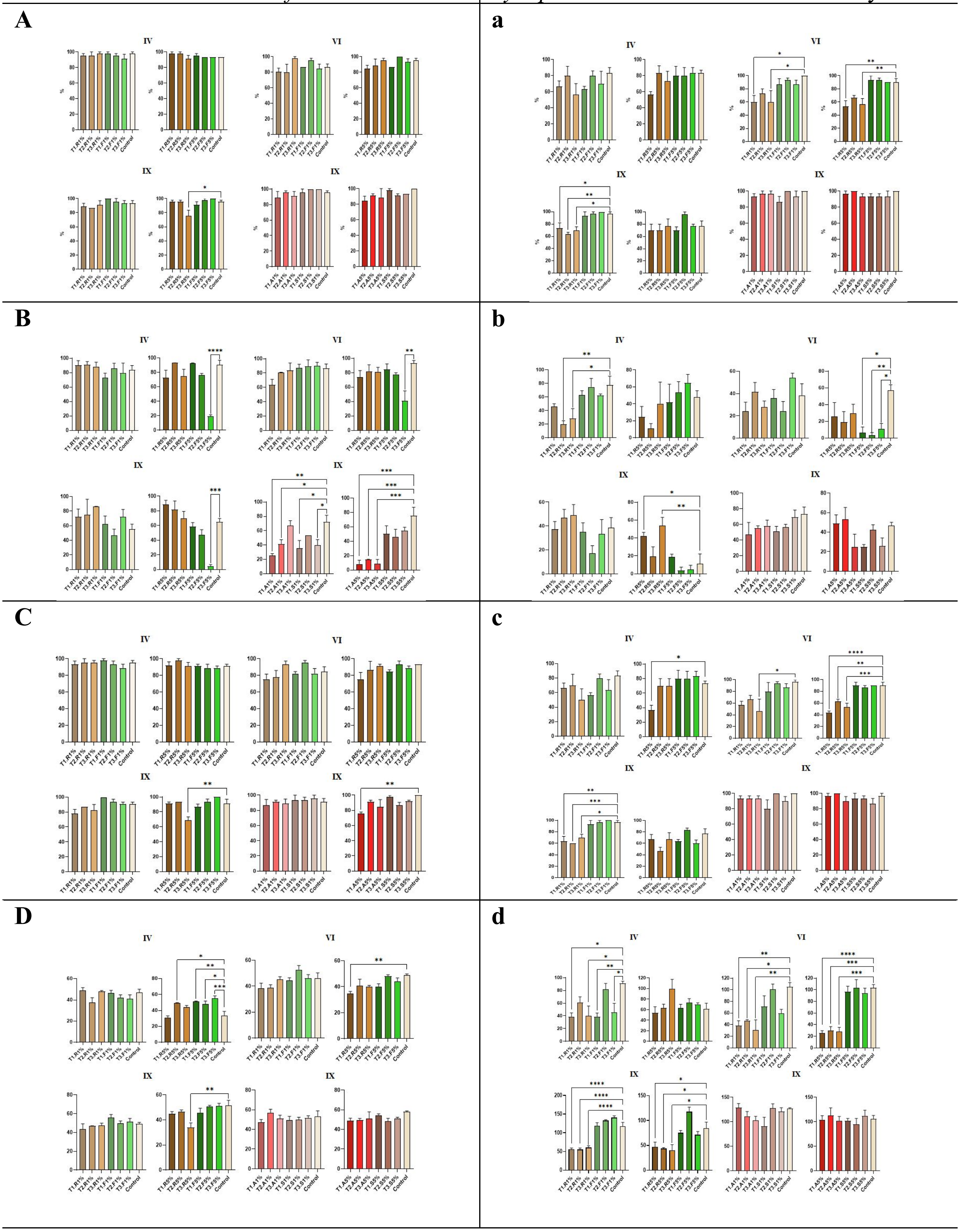


Legend: **IV** – April (strobili production); **VI** – June (intense vegetative growth); **IX** – September (biomass allocation to roots); **R** = bark; **F** = leaf; **A** = aril; **S** = seed; **T1**= *Taxus baccata* – spontaneous; **T2**= *Taxus baccata* – cultivated; **T3**= *Taxus baccata 'Robusta'* – cultivated

PLATE III

Effects of *Taxus* extracts on germination indices in test plants

Amaranthus retroflexus L. | *Lycopersicon esculentum* Mill. variety Silvia



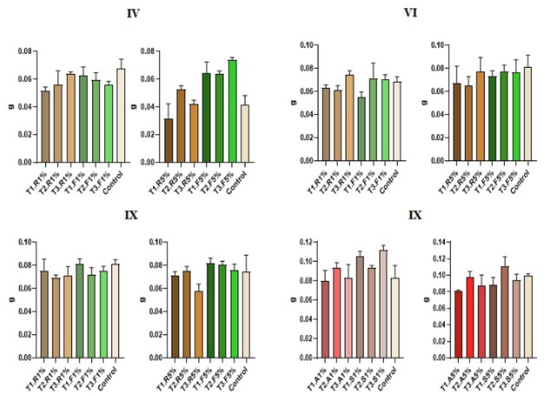
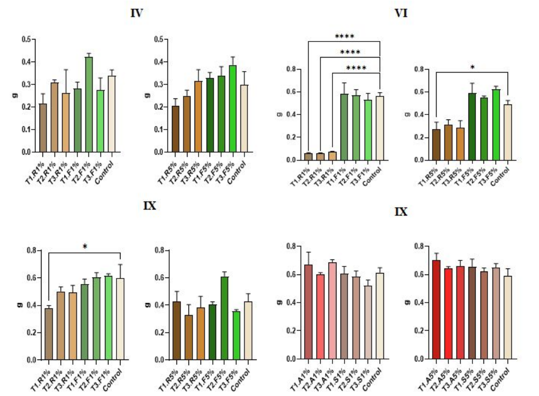
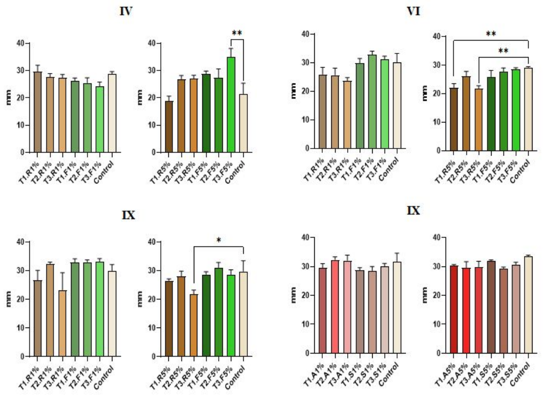
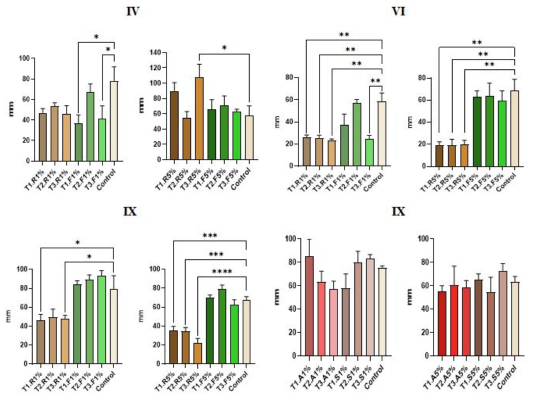
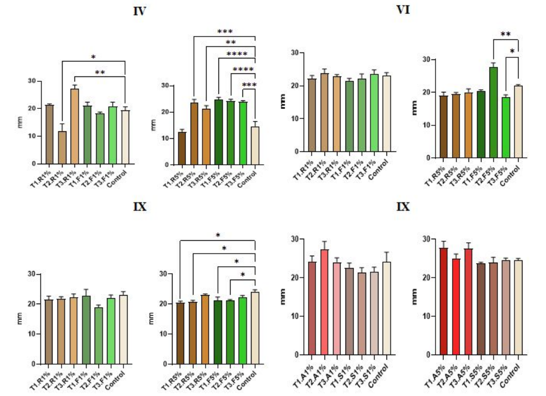
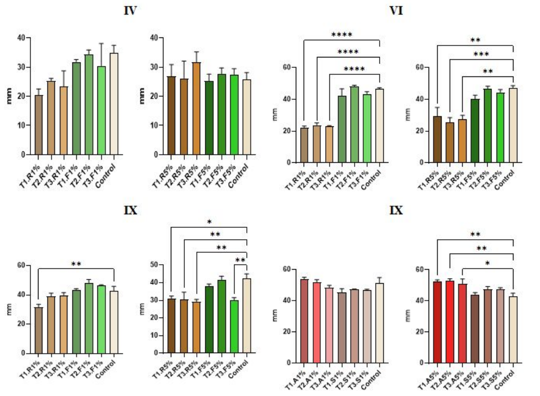
Legend:

A, a - Germination percentage; **B, b** - Speed of emergence; **C, c** - Germination energy; **D,d** - Seedling vigour index. **IV**-April (strobili production); **VI-**June (intense vegetative growth); **IX-**September (biomass allocation to roots); **T1** = *T. baccata* – spontaneous; **T2** = *T. baccata* –

cultivated; **T3** = *T. baccata 'Robusta'* – cultivated; **R** = bark; **F** = leaf; **A** = aril; **S** = seed; 1%, 5% = extracts of concentration; **** =$p<0.0001$; *** = $p<0.001$; ** = $p<0.01$; * = $p<0.05$

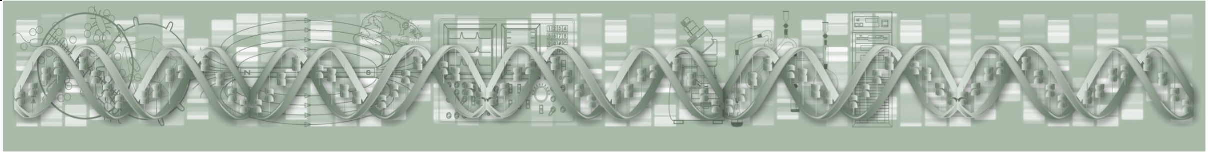
PLATE IV

Biometric determinations of new seedlings from test plants

Amaranthus retroflexus L.	*Lycopersicon esculentum* Mill. variety Silvia
E 	**e** 
F 	**f** 
G 	**g** 

Legend:

E, e - Fresh seedling mass; **F, f** - Mean root length of seedlings; **G, g** - Mean hypocotyl length of seedlings; **IV**-April (strobili production); **VI**-June (intense vegetative growth); **IX**-September (biomass allocation to roots); **T1** = *T. baccata* – spontaneous; **T2** = *T. baccata* – cultivated; **T3** = *T. baccata 'Robusta'* – cultivated; **R** = bark; **F** = leaf; **A** = aril; **S** = seed; 1%, 5% = extracts of concentration; **** =$p<0.0001$; *** = $p<0.001$; ** = $p<0.01$; * = $p<0.05$



COLD ATMOSPHERIC PLASMA TREATMENT MODULATES THE EXPRESSION OF *cdk1*, *tnf α* AND *tp53* GENES IN HUMAN OSTEOSARCOMA CELLS

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Abstract

Osteosarcoma (OS), a malignancy primarily affecting children and adolescents, is the most frequently encountered malignant, non-hematologic, bone tumor. Despite the gradual improvement of survival rates, the management of this disease remains problematic due to challenges such metastasis development, its heterogeneous characteristics, and resistance to cytostatic drugs. Cold atmospheric plasma (CAP), a partially ionized gas operating at near room temperature, which is comprised of free carriers, excited or neutral molecules, and active radicals capable of initiating diverse physical phenomena and chemical reactions represent a new and innovative potential solution in cancer therapy. The aim of this study was to evaluate the capacity of CAP produced by our custom-build plasma source to induce cytotoxic effects to HOS cells and to analyze post-treatment modulations of *cdk1*, *tnf α* and *tp53* genes expression. Direct and indirect CAP treatments effectiveness on HOS cells were evaluated by MTT assay and the regulation of interest genes expression were carried out by RT-qPCR analysis. Cell viability analysis revealed a strong cytotoxic effect of direct CAP treatment on HOS cells, while the indirect treatment resulted in a slight decrease of cells viability. Direct CAP treatment modulates the expression of all analyzed genes, both at 2- and 24-hours post-treatment. In conclusion, direct CAP treatment produced by our custom-build plasma source have cytotoxic effects on HOS cells up to 24 hours post-treatment. Furthermore, direct CAP treatment induces cell cycle arrest of HOS cells, and the CAP-induced cell death is independent of *tp53* gene.

Keywords: cold atmospheric plasma, osteosarcoma, *cdk1*, *tnf α*, *tp53*.

Introduction

Osteosarcoma (OS) is a primary bone cancer that predominantly affects children and young adults. This type of cancer has mesenchymal origins and usually develops around the metaphysis of long bones, producing immature bone tissue (Lindsey et al. 2016, Eaton et al. 2021). Although the survival rate for osteosarcoma has increased from below 30% to approximately 70% over time, the treatment is still challenging due to metastasis, its

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heterogeneous nature, and resistance to cytostatic drugs (Misaghi et al. 2018, Yang et al. 2020; Sheng et al. 2021). The current treatment consists of surgical intervention combined with neoadjuvant and post-operative chemotherapy, using mainly cisplatin, doxorubicin, methotrexate and isophosphamide (Lindsey et al. 2016). Due to the risk of local recurrence, tumor resections must be performed with negative margins, otherwise the affected limb may be amputated (Eaton et al. 2021). Although this radical intervention can result in a significant increase in the survival prognosis, a decrease in the patient's quality of life in terms of social relationships has been reported (Eaton et al. 2021). The recurrence rate, as well as the poorer prognosis that accompanies it, are mainly defined by the microlesions around the tumor and the microresidual tumors that cannot be completely removed by surgery. Therefore, in order to overcome these challenges, as well as better manage the metastatic nature of osteosarcoma and its chemoresistance, new alternative or complementary treatment methods are needed in addition to the current ones (Gümbel et al. 2017c, Xu et al. 2020). Cold atmospheric plasma (CAP), a partially ionized gas consisting in ions, free electrons, molecules, photons, free radicals, and a large specter of reactive species, represent a promising new alternative in cancer therapy (Yan et al. 2017, Baik et al. 2023, Cui et al. 2023). An increasing number of studies demonstrate the potential of CAP treatment in cancer therapy. Thus, by studying various cancer types, diverse advantageous outcomes of CAP treatment have been identified, including selective cytotoxicity, modifications in the tumor microenvironment, sensitization of tumor cells, enhancement of nanoparticles effects, improvements in the antitumor effectiveness of cytostatics and the overcoming of drug resistance (Zhu et al. 2016, Dai et al. 2020, Yan et al. 2021, Mateu-Sanz et al. 2022, Min et al. 2022, Murillo et al. 2023). In biomedical studies, two main methods of CAP treatment are widely used: direct treatment, where the target to be treated is subjected to CAP discharges, and indirect treatment, where a solution is first exposed to CAP and then transferred to the target (Malyavko et al. 2020). Although Saadati et al. asserted that the location of the tumor is an important factor in deciding the best treatment approach and direct exposure may not be feasible for tumors located inside the body (Saadati et al. 2018), Malyavko et al. proposed direct treatment as a substitute for surgical intervention or a complementary method to it, and indirect treatment as a pharmacological procedure (Malyavko et al. 2020). Until now, the best treatment method between the two has not been clearly identified. Depending on the type of cancer, the type of study, the cell culture media used, the molecular mechanisms or the reactive species monitored, these two treatment methods alternate in terms of efficiency and effectiveness. *In situ* and *in vivo* experiments conducted by Zhou et al. demonstrated that, although similar efficiencies were recorded in terms of reducing cancer cell proliferation and migration, recovering of white blood cell indexes, and regulating renal dysfunction, higher survival rates of treated mice, along with reduced costs and improved safety, promote direct treatment as the better method (Zhou et al. 2020). Analyzing the influence of cancer type and cell culture media on the effects of direct and indirect treatments, Biscop et al. demonstrated that variation in these parameters strongly influence the indirect treatment compared to the direct one (Biscop et al. 2019). Malyavko et al. demonstrated that although reactive oxygen and nitrogen species (RONS) underlie the antitumor effects of both indirect and direct treatment, the ability of the latter to induce CAP-treated cells into an activated state makes them more vulnerable to RONS, thereby making the direct treatment method superior to the indirect one (Malyavko et al. 2020). In a study conducted by Saadati et al., even though the direct treatment demonstrated a more pronounced cytotoxic effect on tumor cells than the indirect treatment, the latter presented less toxic side effects. Additionally, by combining the indirect treatment with chemotherapy, the most effective method of antitumor treatment was obtained (Saadati et al. 2018). Similarly, in a study comparing the effects of both types of CAP treatments on normal bone marrow mesenchymal stem cells (BMMSCs), Hajizadeh et al. demonstrated the superiority of the indirect method due to the reduced cellular damage it caused (Hajizadeh et al. 2019). Thus, it can be observed that there is still an uncertainty regarding the selection of one of the methods as being superior to another. While direct treatment is the most commonly used method in studies exploring the effects of CAP in cancer therapy, there has been a continuous increase in the number of studies using indirect treatment (Dubuc et al. 2018). Regarding the evaluation of the effects of CAP treatment on osteosarcoma, it appears that the field of interest is still in its early stages. In 2018, Dubuc et al. presented in a systematic review that out of a total of 190 articles, only 6 focused on osteosarcoma (Dubuc et al. 2018). By analyzing articles on the Web of Science core collection, it can be observed that there are only 34 articles specifically dedicated to the study of CAP treatment for osteosarcoma. Furthermore, although there is constant progress in this field, the mechanisms of action as well as the signaling pathways activated by CAP treatment are not fully known or understood and require further studies to lead the use of CAP as a therapeutic method against cancer (Haralambiev et al. 2019, Hasse et al. 2019, Malyavko et al. 2020, Murillo et al. 2023). Considering the aspects that have been mentioned earlier, the aim of this study is to assess the ability of CAP produced by our custom-made source to induce cytotoxic effects on osteosarcoma cells, using both direct and indirect treatment methods, and to investigate the impact of CAP treatment on the expression levels of specific genes involved in cell cycle regulation and cell death.

Materials and Methods

Cell culture. Human osteosarcoma cells (HOS) (CRL-1543, American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 medium (Sigma Aldrich, MO, USA) supplemented with fetal bovine serum 10% (Sigma Aldrich, MO, USA) and penicillin-streptomycin antibiotics 1% (Biological Industries, Kibbutz Beit-Haemek, Israel). Cells were maintained and expanded at 37°C, 5% CO_2 and 95% humidity in a Certomat CO_2 Incubator (Sartorius Stedim Biotech GmbH, Göttingen, Germany).

CAP treatment. In order to produce CAP, a custom-made dielectric barrier discharge without flow system was used. CAP discharges took place in a polypropylene 24-well cell culture plate (TPP Techno Plastic Products AG, Trasadingen, Switzerland), between a high voltage electrode made of 4 cylindrical stainless-steel subunits and a ground electrode represented by a copper plate placed under the culture plate. Between the bottom of the culture plate and the ground electrode was positioned a 0.1 mm dielectric layer. The system was powered up by an AC power supply with maximum voltage of 15 kV and a sinusoidal waveform. Due to the high-voltage electrode configuration, CAP exposures were carried out one after the other, exposing one column of the culture plate at a time. Thus, the discharge parameters were identical for all 4 wells of the column. In this study, in order to evaluate the biological effects of CAP treatment on HOS cells, three exposure times were used: 30 seconds, 60 seconds and 120 seconds. This represents the periods of time in which the source was turned on and the CAP discharges occurred.

Direct CAP treatment. Direct treatments were performed by exposing 5 x 10^5 HOS cells/well to CAP discharges, for previously presented periods of time. Depending on these exposure times, we will address samples directly treated as CAP 30s, CAP 60s and CAP 120s. Cells were seeded in the culture plate 1-day prior exposures, in 500 µl complete RPMI 1640 medium. Before treatments, culture medium was replaced with 150 µl Dulbecco's phosphate-buffered saline (PBS) (Biological Industries, Kibbutz Beit-Haemek, Israel). After treatment, PBS was removed and 500 µl complete RPMI 1640 medium per well was added.

Indirect CAP treatment. One day before treatment, 5 x 10^5 HOS cells were seeded in a 24-well cell culture plate, in 500 µl complete RPMI 1640 medium. For indirect treatment, 150 µl PBS was dispensed into each well of the discharge vessel and exposed to CAP for previously mentioned exposure times, thus obtaining plasma-activated medium (PAM). In this study, depending on the exposure time used, we will refer to the CAP exposed PBS for the indirect treatment as PAM-PBS 30s, PAM-PBS 60s and PAM-PBS 120s. After PAM generation, the medium from the seeded cells was discarded and, after a washing step with PBS, 150 µl of PAM/well were added. For each exposure time, four wells/plate were treated with PAM for three minutes. After treatment, PAM was replaced with 500 µl complete RPMI 1640 medium per well.

Cell viability assay. In order to evaluate the cytotoxic effects of CAP treatment, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed at 2- and 24-hours post-treatments. After this period of incubation, cell culture medium was discarded and the cells were washed with PBS. Subsequently, 550 µl of MTT working solution consisting of 500 µl RPMI 1640 and 50 µl MTT 5mg/ml were added in each well. After a two hours incubation step at 37°C, 5% CO_2 and 95% humidity, 450 µl of MTT working solution was discarded followed by the addition of 350 µl DMSO. Cells were incubated for 10 minutes, afterwards the absorbance was measured by using the multimodal plate reader FilterMax F5 (Molecular Devices LLC, CA, USA) at 570 nm wavelength.

Gene expression analysis. Total ARN was extracted at 2- and 24-hours post treatment using SV Total RNA Isolation System (Promega, WI, USA) following manufacturer's instructions and spectrophotometrically quantified by measuring its absorbance at 260 nm by NanoDrop 2000 Spectrophotometer (ThermoFisher, Waltham, MA, USA). The reverstranscription and real-time PCR amplification of *cdk1*, *cdk2*, *tnf α* and *tp53* genes was performed in a single step reaction by GoTaq® 1-Step RT-qPCR System (Promega, Wi, USA) on a Rotor Gene 6000 Q 5-plex HRM thermocycler (Corbett Research, QIAGEN Corporation, Germany). The primer sequences were CDK1 F 5'- GAATCCGGGGCCCTTTAGCG -3', CDK1 R 5'- ACAACAACCGCGTCGCTCTCC -3', TNF α F 5'- ATGAGCACTGAAAGCATGATCC -3', TNF α R 5'- GAGGGCTGATTAGAGAGAGGTC -3', TP53 F 5'- CAGCACATGACGGAGGTTGT -3', TP53 R 5'- TCATCCAAATACTCCACACGC -3'. In order to amplify and quantify the expression level of each targeted gene, the following thermal cycling parameters were used: 40°C for 15 minutes and 95°C for 10 minutes followed up by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Data acquisition was performed with Rotor-Gene Q - Pure Detection Software v. 2.2.3. (QIAGEN Corporation, Germany). The gene expression modifications were evaluated by comparing the gene expression levels in CAP treated samples with the gene expression levels in untreated control samples and reported as a fold change. Each measurement was performed in triplicate.

Signaling pathways identification for simultaneous regulation of the analyzed genes expression. The evaluation of cellular signaling pathways was conducted using the GeneCards database – the human gene database (Stelzer et al. 2016), and the analysis platform The SIGnaling Network Open Resource (SIGNOR 3.0) (Lo Surdo et al. 2023). The analysis aimed to identify direct or indirect signaling pathways between the explored genes in this study, which exhibited a similar modulation of their expression levels. It consisted of the following steps: a. Investigation of direct signaling pathways. b. Investigation of indirect signaling pathways. c. Identification of regulators whose activity involves transcriptional regulation of the target genes. d. Identification of signaling pathways connecting regulators identified in the previous step (referred in this study as interrelating regulators).

Results

Cell viability. In order to evaluate the response of osteosarcoma cells to treatment with CAP, in this study the viability of HOS cells treated with CAP was checked both by the direct method and by the indirect method. For this, three CAP exposure times were evaluated: 30 seconds, 60 seconds and 120 seconds. Control groups were used for each individual treatment method, the cells within these groups being manipulated identically to those treated with CAP, only that they were not subjected to the treatments. The results are expressed in percentages and represent the changes identified in relation to the values of the samples from the control groups. Two hours after the direct treatment, the cells showed a decrease in viability of down to 27.4% for the 30s exposure, 9.9% for the 60s exposure and 2.1% for the 120s exposure. According to Dunnett's post hoc test, all results are statistically significant. In the case of the indirect treatment, two hours post-treatment the viability of cells treated with PAM-PBS 30s was 103.3%, of those treated with PAM-PBS 60s was 91%, and of those treated with PAM-PBS 120s was 85.3%. The Dunnett post-hoc test reveals that only in the case of treatment with PAM-PBS 120s the change in viability is statistically significant (Figure 1).

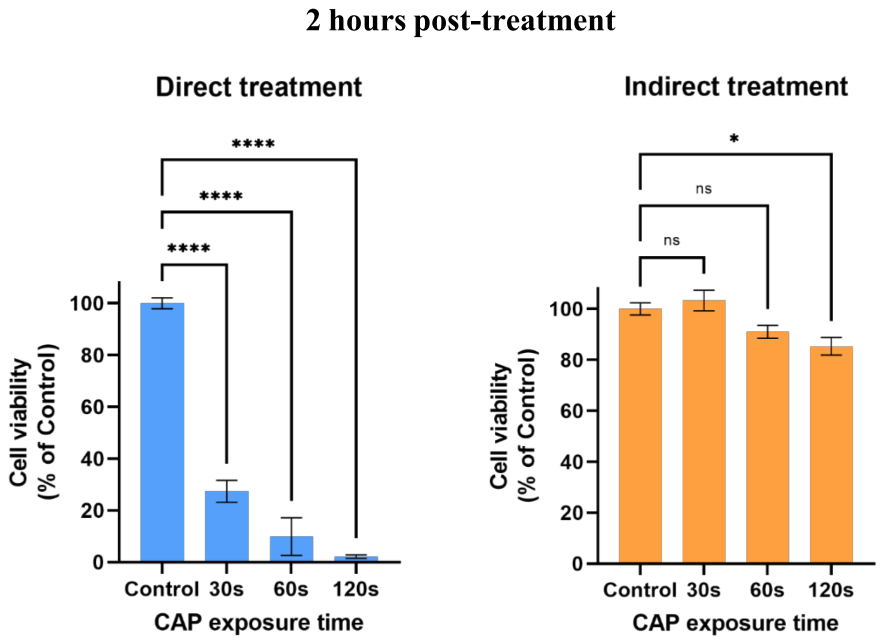

Figure 1. Viability of human osteosarcoma cells (HOS) treated with CAP by direct method (left) and indirect method (right) at 2 hours post-treatment. Values are means ±SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – nonsignificant, * $p<0.05$, **** $p<0.0001$).

Twenty-four hours after the treatment, the cells showed relatively the same trend of change in cell viability as after the evaluation two hours post-treatment. In the case of direct treatment, the viability of cells treated with CAP decreased to 51.2% for the 30s exposure, 6.7% for the 60s exposure and 13.4% for the 120s exposure. The indirect treatment had the effect of changing cell viability, as follows: 99.6% for PAM-PBS 30s, 96.3 for PAM-PBS 60s and 83% for PAM-PBS 120s. According to the Dunnett post-hoc test, in the case of direct treatment all changes in cell viability were statistically significant, and in the case of indirect treatment only the observed effects of the treatment with PAM-PBS 120s were significant (Figure 2). Comparing the effects of the two treatment methods for each individual exposure time, according to the Šídák post-hoc test, there are statistically significant differences for all the experimental variants analyzed in this study, both at 2- and 24-hours post-exposure (Figure S1 and Figure S2).

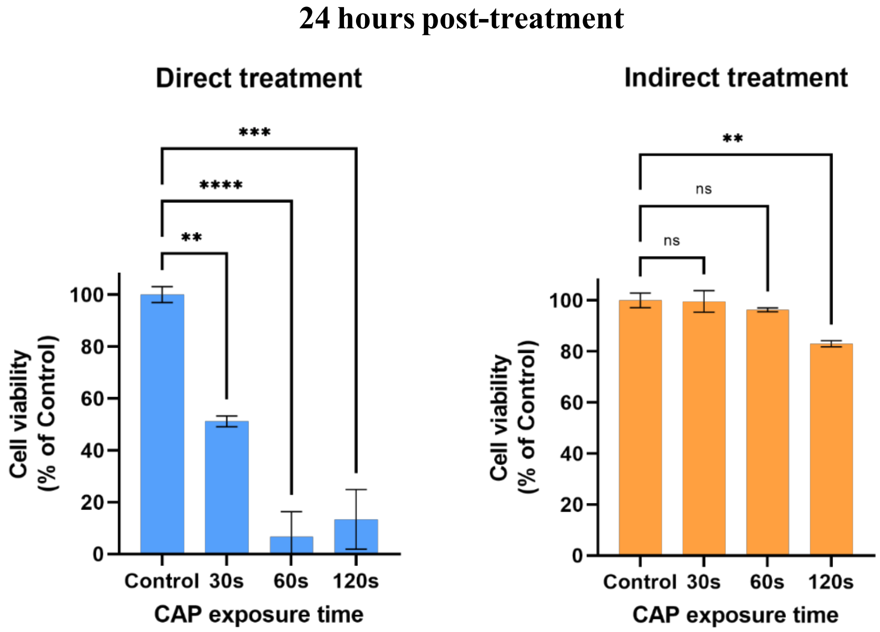

Figure 2. Viability of human osteosarcoma cells (HOS) treated with CAP by direct method (left) and indirect method (right) at 24 hours post-treatment. Values are means ±SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – nonsignificant, ** p<0.01, *** p<0.005, **** p<0.0001).

Gene expression.

CDK1. Direct treatment with CAP on osteosarcoma cells resulted in a decrease in *cdk1* gene expression levels at two hours post-treatment. When compared to control samples, exposure to CAP for 30s, 60s, and 120s downregulated *cdk1* gene by 0.52-fold, by 0.77-fold, and by 0.29-fold, respectively. However, after 24 hours, all experimental variants showed an upregulation of the *cdk1* gene by 1.24-fold, by 2.61-fold, and by 3.18-fold for the 30s, 60s, and 120s exposures, respectively. According to the Dunnett post-hoc test, except for the change in the expression level of the *cdk1* gene evaluated 24 hours after treatment in cells exposed to CAP 30s, all other identified results were statistically significant (Figure 3).

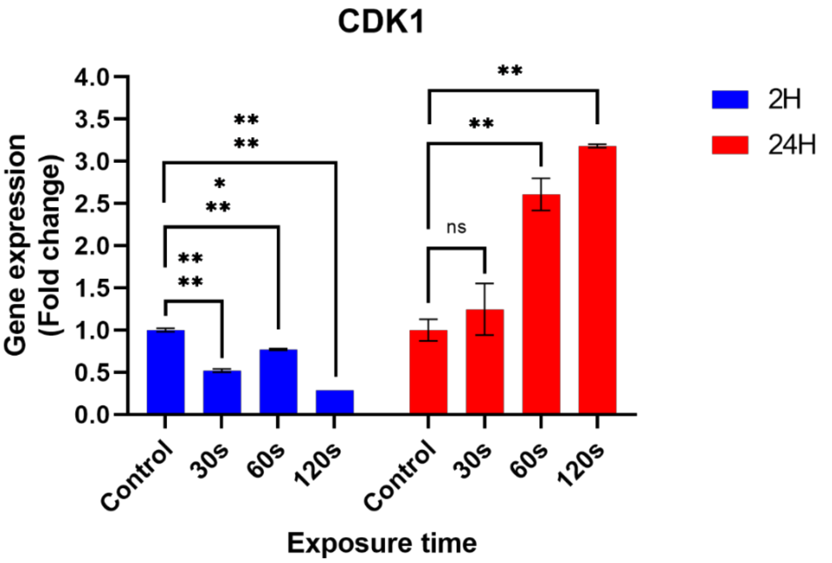

Figure 3. Analysis of *cdk1* gene expression in CAP-treated HOS cells at 2- and 24-hours post-treatment. Values are mean ± SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – nonsignificant, ** p<0.01, *** p<0.005, **** p<0.0001).

TNF α. After treatment with CAP, the expression level of the *tnf α* gene in osteosarcoma cells was analyzed. Results showed that two hours after treatment, the expression level of *tnf α* decreased in all experimental variants compared to control samples (by 0.73-fold for CAP 30s, by 0.59-fold for CAP 60s, and by 0.68-fold for CAP 120s). However, twenty-four hours after treatment, the samples treated with CAP 30s presented a similar gene expression level to the control samples, while CAP 60s and CAP 120s treatments upregulated *tnf α* gene by 1.52-fold and by 1.32-fold, respectively. Despite that, according to the Dunnett post-hoc test, no change in the level of *tnf α* gene expression showed statistical significance (Figure 4).

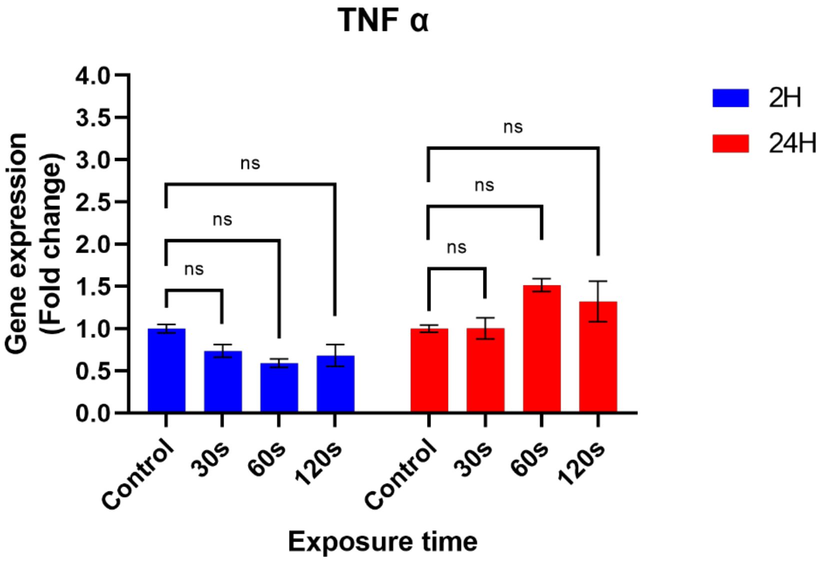

Figure 4. Analysis of *tnf α* gene expression in CAP-treated HOS cells at 2- and 24-hours post-treatment. Values are mean ± SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – nonsignificant).

TP53. Two hours after CAP 30s treatment, *tp53* gene was upregulated by 1.47-fold. In contrast, the 60s and 120s treatments resulted in its downregulation by 0.91-fold and by 0.1-fold, respectively). The evaluation of the *tp53* gene expression twenty-four hours after the treatment revealed its downregulation for all experimental variants, as follows: by 0.78-fold for CAP 30s, by 0.16-fold for CAP 60s, and by 0.08-fold for CAP 120s. The Dunnett post-hoc test shows that all identified changes in the expression levels of the *tp53* gene are statistically significant (Figure 5).

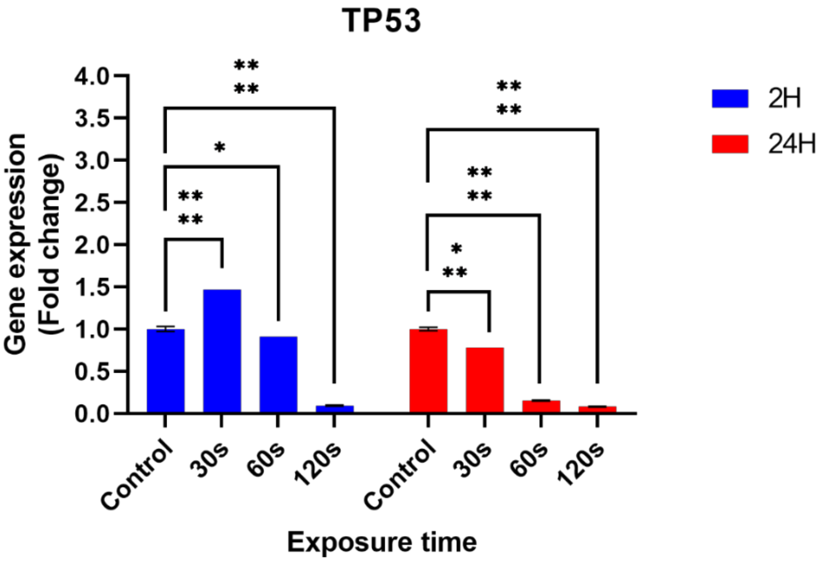

Figure 5. Analysis of *tp53* gene expression in CAP-treated HOS cells at 2- and 24-hours post-treatment. Values are mean ± SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – nonsignificant, * p<0.05, *** p<0.005, **** p<0.0001).

Identification of the signaling pathways linkage. At 2 hours post-treatment both *cdk1* and *tnf α* genes were downregulated. According to SIGNOR tool the *cdk1* gene is downregulated by KDM2B and Noncanonical PRC1, and *tnf α* gene is downregulated by MCR1, KLF4, MFGE8 and FUBP1. Evaluating the signaling linkage between the identified regulators of both genes, we have identified two interrelating regulators, MCR1 and KLF4 (Figure 6).

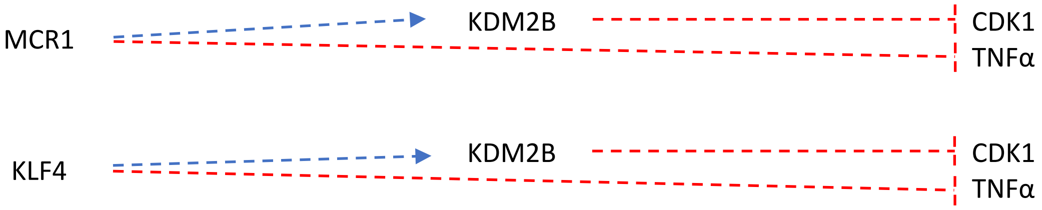

Figure 6. Evaluation of signaling pathways linkage between *cdk1* and *tnf α* resulting in downregulation of both genes (- - -▸ indirect upregulation, - - -┤ indirect downregulation).

At 24 hours post-treatment, *cdk1* and *tnf α* genes were upregulated, while *tp53* was downregulated. Using the SIGNOR tool, we have evaluated the possible signaling pathways which modulates the expression of our interest genes as previously presented.

The *cdk1* gene is upregulated by E2F1 and TFDP1, *tnf α* gene is upregulated by A9/b1 integrin, NfKb-p65/p50, RBM10, HBA1, IRF5, ANXA1, HBB and IL1A, and *tp53* gene is downregulated by KLF4, DRAM2, CBFbeta-MYH11 and U2AF1. Firstly, in order to evaluate common signaling pathways whose activity have as result modulation of *cdk1*, *tnf α* and *tp53* genes in the same manner as we observe following RT-qPCR post-CAP treatment analysis, we evaluated the interrelating regulators between each gene.

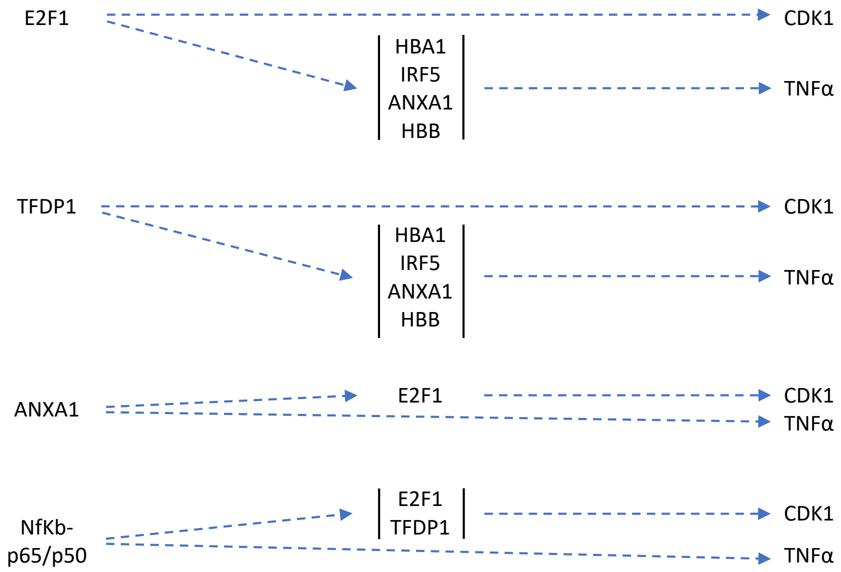

Figure 7. Evaluation of signaling pathways linkage between *cdk1* and *tnf α* resulting in upregulation of both genes (- - -▶ indirect upregulation).

As result, we identified four interrelating regulators of *cdk1* and *tnf α* (E2F1, TFDP1, ANXA1 and NfKb-p65/p50) (Figure 7), four interrelating regulators of *cdk1* and *tp53* (E2F1, TFDP1, KLF4 and CBFbeta-MYH11) (Figure 8) and five interrelating regulators of *tnf α* and *tp53* (KLF4, CBFbeta-MYH11, ANXA1, IL1A and NfKb-p65/p50) (Figure 9).

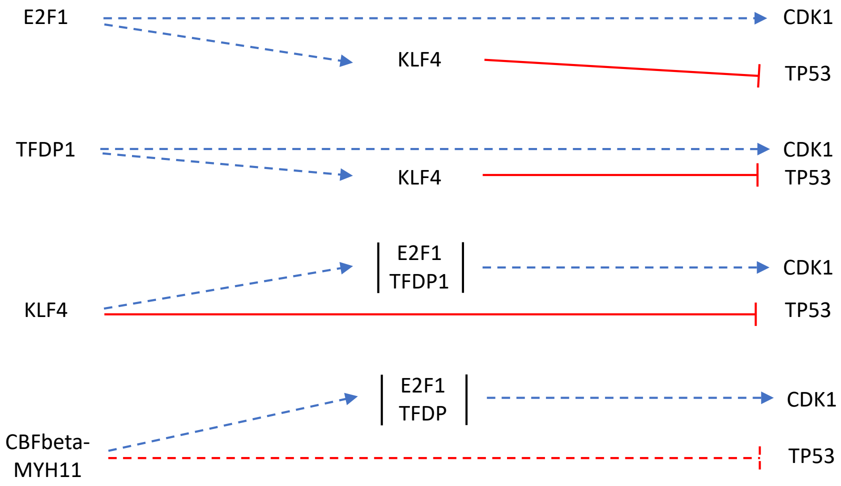

Figure 8. Evaluation of signaling pathways linkage between *cdk1* and *tp53* resulting in upregulation of *cdk1* gene and downregulation of *tp53* gene (- - -▶ indirect upregulation, —┤ direct downregulation, - - ┤ indirect downregulation).

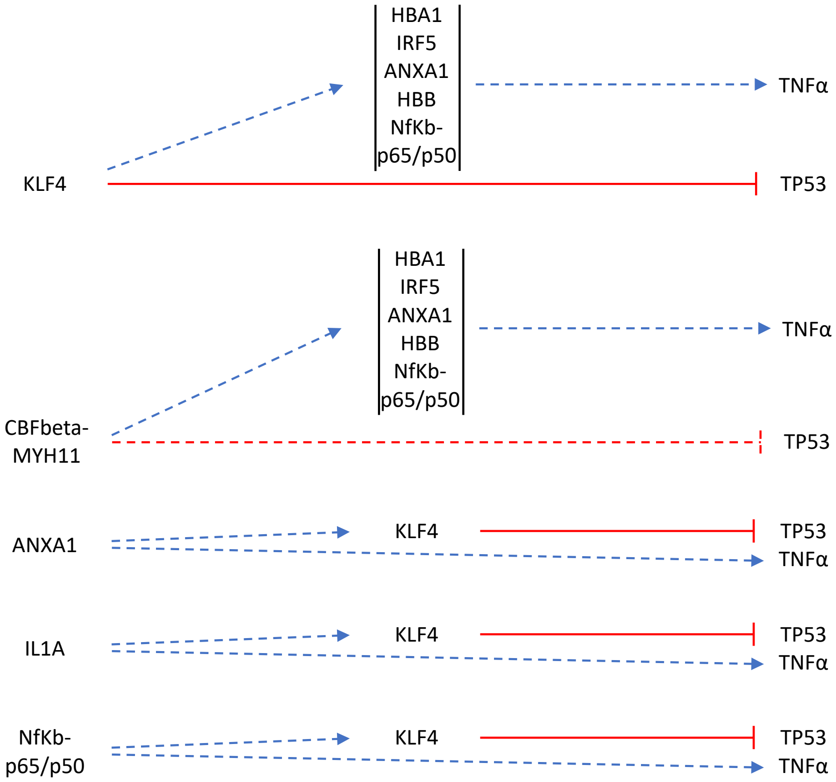

Figure 9. Evaluation of signaling pathways linkage between *tp53* and *tnf α* resulting in upregulation of *tnf α* gene and downregulation of *tp53* gene (- - -▸ indirect upregulation, —| direct downregulation, - - -¦ indirect downregulation).

Analyzing the interrelating regulators of all 3 genes, we observed a common signaling pathway composed of TFDP1, E2F1 and KLF4, whose activity can simultaneously upregulate *cdk1* and *tnf α*, and downregulate *tp53* (Figure 10).

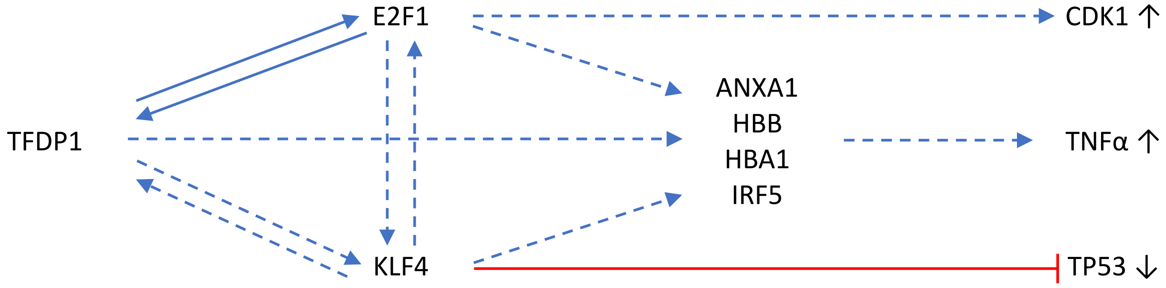

Figure 10. Evaluation of common signaling pathways linkage between *cdk1*, *tnf α* and *tp53* resulting in upregulation of *cdk1* gene, upregulation of *tnfα* and downregulation of *tp53* gene (⟶ direct upregulation, - - -▸ indirect upregulation, —| direct downregulation).

Discussions

Different methods of producing and administering CAP to tumor cells, yielding diverse and variable outcomes in terms of cytotoxic effects, proliferation rates, and modulation of various cellular signaling pathways have been evaluated in biomedical researches. These studies have also highlighted differences in treatment methods used and the effectiveness of CAP treatment (Volotskova et al. 2012, Keidar et al. 2013, Siu et al. 2015, Malyavko et al. 2020, Min et al. 2022). Although progress has been made in recent years regarding the mechanisms underlying the anti-tumor effects of CAP, they are still not fully understood, and further studies are needed to identify which cellular signaling pathways are modulated by CAP treatment (Turrini et al. 2017, Semmler et al. 2020, Tan et al. 2020, Motaln et al. 2021, Murillo et al. 2023). Therefore, in this study, we aimed to identify the cytotoxic effects of direct and indirect treatment with CAP generated from our custom-built source on human osteosarcoma cells. In addition, our objective was to evaluate the modifications that the treatment induces on the expression level of three representative genes involved in regulating the cell cycle and cell death. The results obtained in this study revealed that each experimental variant of the direct treatment with CAP had a drastic effect on reducing the viability of osteosarcoma cells, both at 2 hours and 24 hours after exposure, while the indirect treatment induced minor changes. In both treatment methods, the exposure time of 120s showed the most pronounced cytotoxic effect. Similar results of cytotoxicity from direct treatment with CAP have been observed in previous studies on various bone cancer cell lines (Gümbel et al. 2016, Gümbel et al. 2017b, Saadati et al. 2018, Malyavko et al. 2020). However, regarding indirect treatment, unlike the results obtained in this study, previous studies show effects of reduced viability of bone tumor cells through the use of much longer treatment times (Canal et al. 2017, Saadati et al. 2018, Tornin et al. 2019, Mateu-Sanz et al. 2020, Xu et al. 2020, Hamouda et al. 2021). Interestingly, under certain treatment conditions, Biscop et al. obtained better cytotoxic results with indirect treatment compared to direct treatment (Biscop et al. 2019). Due to its superior efficiency demonstrated in this study, direct method was selected for CAP-treating HOS cells in order to evaluate changes in the expression levels of genes of interest. *cdk1* is a member of the cyclin-dependent kinase family and plays a crucial role in promoting the progression of the cell cycle through the G2/M phase in eukaryotic cells (Liao et al. 2017, Lemonnier et al. 2020). Our study results revealed that at 2 hours after direct CAP treatment, HOS cells exhibited a downregulation of *cdk1* gene which was not found to be correlated with the exposure time. Thus, we assume that HOS cells may undergo G2/M arrest due to the *cdk1* downregulation by CAP treatment (Badie et al. 2000, Yang et al. 2018). Surprisingly, at 24 hours post-treatment, upregulation of the *cdk1* gene was identified, this time in a dose-dependent manner, thus promoting the proliferation of the cell population that survived the CAP treatment (Zhang et al. 2018, Liu et al. 2022). Similar to our results obtained 2 hours post-exposure, Yan et al. detected a downregulation of *cdk1* gene in HepG2 cells 48 hours after direct treatment with CAP (Yan et al. 2010). While Nakai et al. showed increased levels of phosphorylated CDK1 protein and Lin et al. observed an elevated level of phosphorylated CDK1 protein and no effect on CDK1 protein levels after CAP treatment (indicating cell cycle arrest in G2/M phase due to the regulation of cyclin B1/CDK1 complex), Han et al. reported increased levels of CDK1 proteins in Human uterosacral ligament fibroblasts treated with CAP (Nakai et al. 2014, Lin et al. 2018, Han et al. 2021). To the best of our knowledge there are no studies regarding the evaluation of *cdk1* gene expression in CAP-treated osteosarcoma cells, apart from assessments considering the protein level reduction as a result of overexpression of the *p53*, *p21* and *bax* genes (Gümbel et al. 2017a). Tumor necrosis factor alpha (TNF α) is a cytokine that exerts pleiotropic effects on various cell types. It is initially synthesized as a precursor form called transmembrane TNF α, which can function as both a ligand and a receptor. TNF α plays a role in the development of inflammatory and autoimmune diseases and is involved in regulating cytotoxic activity against normal and tumoral cells, being part of the extrinsic apoptosis pathway (Cavalcante et al. 2019a, Mercogliano et al. 2020, Jang et al. 2021). Although according to the Dunnett post hoc test, the differences observed in the levels of *tnf α* gene expression within the three experimental variants compared to the control sample were not statistically significant, following CAP treatment, a modulation of *tnf α* gene expression similar to that of *cdk1* was observed. Specifically, downregulation of *tnf α* was observed at 24 hours post-treatment, followed by its upregulation at 24 hours post-treatment. Previous studies have reported overexpression of *tnf α* following CAP treatment on tumor cells (Hou et al. 2015, Xia et al. 2019, Murthy et al. 2022), while studies regarding the ability of CAP to heal wounds have shown both increases and decreases in the expression level of the *tnf α* gene (Arndt et al. 2018, He et al. 2020). Due to the fact that TNF α has an ambivalent effect, either promoting cell death or cell survival (Wang et al. 2005, Kim et al. 2018, Kokolakis et al. 2021) it cannot be determined exactly whether the decrease in viability of HOS cells in this study can be correlated with the identified *tnf α* gene expression modulation. The tumor suppressor gene, *tp53*, plays crucial roles in various cellular processes such as apoptosis, cell cycle control, genomic stability, regulation of cell proliferation, metabolism, and immune response. *tp53* gene modulate the expression of more than 900 genes and has the ability to induce apoptosis through both intrinsic and extrinsic pathways (Gümbel et al. 2017a, Cavalcante et al. 2019b, Marvalim et al. 2023, Wang et al. 2023). The CAP treatment of HOS cells induced downregulation of the *tp53* gene at both 2 and 24 hours after treatment, except for HOS cells exposed to CAP for 30 seconds and evaluated at 2 hours post-treatment, where upregulation of *tp53* gene was recorded. Similar results were obtained by Shi et al., Eggers et al., and Adil et al., who identified low levels of *tp53* mRNA in various types of CAP-treated cells (Shi et al. 2017, Adil et al. 2020, Eggers et al. 2021). In contrast, other studies have reported increases in the *tp53* gene expression level in various tumor cells types as a result of CAP treatment (Turrini et al. 2017, Yadav et al. 2020, Sadoughi et al. 2021). According to the obtained results, we can state that treatment with CAP induces a decrease in the viability of HOS cells, but the mechanism of action appears to be independent of *tp53*. By comparatively evaluating the results obtained from assessing the expression of *cdk1*, *tnf α*, and *tp53* genes in CAP-treated HOS cells, it can be presumed that there might be a connection between them determined by a direct or indirect regulatory mechanism of expression among these three genes or a common regulator whose activity modulates their level of expression. Additionally, we must also consider the possibility that, following CAP treatment, the expressions of the three genes are individually modulated through distinct signaling pathways. Analyzing the signaling pathways, we have observed that there is no direct or indirect connection between the three genes, meaning that the overexpression or underexpression of one of them does not influence the expression levels of the others. However, we have identified signaling pathways that include interrelating regulators, whose activation can lead to the regulation of *cdk1*, *tnf α*, and *tp53* genes expression. Therefore, we can assume that the effects of CAP treatment on the expression of *cdk1*, *tnf α*, and *tp53* genes in HOS cells could be based on the activation of one, two or all of the identified interrelating regulators. Further studies are necessary to evaluate the level of the proteins encoded by the analysed genes, and to confirm the activation of these interrelating regulators by CAP treatment. Moreover, new signaling pathways which promotes the proliferation or death of the cells should be evaluated in order to elucidate the underlying mechanism of CAP action on HOS cells.

Conclusions

CAP generated by our custom-build source greatly reduces HOS cells viability via direct treatment, whereas the indirect CAP treatment with PAM-PBS has negligible effects. In addition to its cytotoxic effects, direct treatment modulates *cdk1* gene expression, leading to cell cycle arrest of HOS cells at two hours after the treatment. However, an opposite effect is observed at twenty-four hours following treatment. According to the Dunnett post-hoc test the expression level of the *tnf α* showed no statistical significance. Our results revealed that the molecular mechanism of cells death in HOS treated cells does not involve the expression of *tp53* gene, on the contrary, direct CAP treatment downregulate *tp53* gene expression. Furthermore, we emphasize the need to further evaluate caspases signaling pathways to establish the molecular mechanism modulated by direct CAP treatment, produced with our custom-build plasma source, which induces cell death in HOS cells. The analysis of interrelating regulators for the 3 interest genes showed that the *cdk1* and *tnf α* can be simultaneously upregulated, while *tp53* will be downregulated.

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Supplementary files

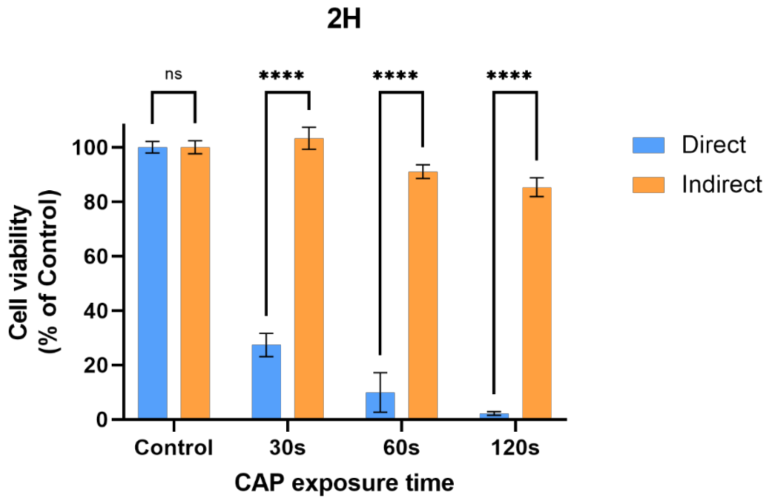


Figure S1. Comparison between the viability of HOS cells directly and indirectly treated with CAP, 2 hours post-treatment. Values are means ±SEM (n=3). Statistical significance was determined by two-way ANOVA followed by Šídák's post hoc multiple comparison test (ns – nonsignificant, **** p<0.0001).

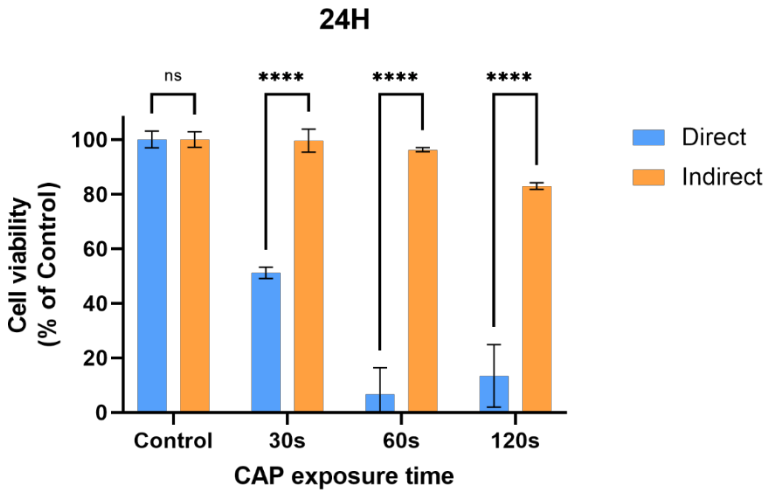
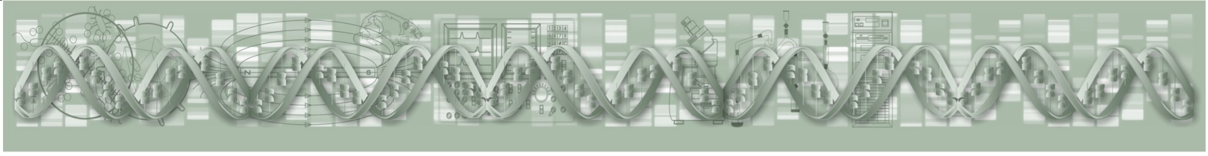


Figure S2. Comparison between the viability of HOS cells directly and indirectly treated with CAP, 24 hours post-treatment. Values are means ±SEM (n=3). Statistical significance was determined by two-way ANOVA followed by Šídák's post hoc multiple comparison test (ns – nonsignificant, **** p<0.0001).



APPLICATION OF GENETIC MARKER FOR DIVERSITY ASSESSMENT AND CONSERVING OF PLANT GENETIC RESOURCE

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Abstract

Several molecular markers have been used for various purposes since the beginning of contemporary molecular technology: genetic resource characterization, core genetic resource collection, mapping, marker-assisted selection and marker-assisted backcrossing etc. The variety of all plant species, their genetic make-up and the environments in which they dwell are considered to be components of biological diversity. Plant classification and identification can be done most quickly and simply using morphological examination. It has been established that molecular markers are effective instruments for evaluating genetic diversity in groups and individuals as well as germplasm resources. To preserve biodiversity more than just genetic testing and DNA polymorphism detection are needed. It examines significant issues regarding managing plant germplasm both ex-situ and in-situ in order to support decision-making. Significant progress has been made in recent years in mapping, tagging and isolating many important genes for agriculture using molecular markers like (Restriction Fragment Length Polymorphisms, Random Amplified Polymorphic DNAs, Amplified Fragment Length Polymorphisms, Simple Sequence Repeats and Single Nucleotide Polymorphism). Each marking technique has unique benefits, drawbacks and applications. If one was aware of the presence of relevant traits, genes and alleles one might make judgments on the extension of accessions and the preservation of seed stocks to meet an anticipated rise in demand for materials. By including genotypes with well-known and useful genes and alleles in the core collections and then breeders can use them. Utilizing crop plant genetic resources will be simpler as a result increasing their potential.

Keywords: DNA, Germplasm Conservation, Genome editing, Marker assisted selection, Quantitative Trait Loci, Polymerase Chain Reaction

Introduction

The diversity of all plant and animal species, their genetic makeup and the ecosystems in which they cohabit are all seen to be instances of biological diversity. The three basic types of diversity are genetic, theoretical and ecological. The assortment of genotype and gene variations is referred to as genetic variety (Species communities and the ecosystems they inhabit). Many would argue that diversity is vital for the feasibility and sustainability of many human efforts,and in recent years the worth of biodiversity to humans has received significant acknowledgement. (Shiva, 1994). Morphological analysis is the quickest and least technical way for classifying and identifying plants. The strategy involves writing down and keeping track of features that are simple to see such form and structure. Prior to the development of biotechnology, distinct cultivars were characterized by their morphological and physical characteristics. Numerous studies have evaluated the genetic diversity of various crops based on differences in morphological and agronomic features or ancestry data. (Sneller et al. 1997).

It has been demonstrated that molecular markers are useful tools for assessing germplasm resources and assessing genetic variation both among and within populations. The area of plant genomics that has really made the most strides in the use of DNA marker technology is population genetics. Nevertheless, markers produced from the polymerase chain reaction (PCR) and restriction length polymorphism (RFLP) have also been extensively used in the mapping of Mendelian genes and QTLs in plants. It is crucial to use molecular markers for genetic resource management and research so that breeders can add critical genetic and behavioral data to core collections. Recent advances in the techniques have led to the mapping, tagging and isolation of a number of agriculturally significant genes. Microsatellites, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and PCR-based DNA markers like sequence-characterized amplifiable regions (SCARs) or sequence-tagged sites are among the methods that are particularly promising (STS). These methods make use of F2 and backcross populations near isogenic lines, doubling haploids and recombinant inbred lines to aid in the direct selection of numerous desired traits simultaneously (FAO 2002).

Managing biodiversity entails more than just genetic characterization via DNA polymorphism detection; it also calls for data that may be used to address important challenges in the management of both ex situ and in situ plant germplasm and to aid in decision-making. Molecular technologies may help with sampling, management, the creation of "core" collections and the use of genetic diversity for in situ agricultural germplasm maintenance. The best management and utilization strategies for the in situ and "on farm" preservation strategies of genetic resources may be determined using molecular markers. These markers can also help identify the most representative populations within a landrace's "gene pool" as well as the best management and usage tactics (Lanteri and Barcaccia 2005).Therefore,, the objective of these review article is to describe molecular marker approaches and discuss how to use molecular markers to assess and protect plant genetic resources.

Methods of Molecular Markers and Their Application

A DNA sequence on an area in an organism's genome where the DNA genomic sequence changes between members of a population are referred to as a molecular marker. The way that molecular markers function is by exposing variations in DNA sequences (polymorphism) among various members of the population. Insertions, deletions, point mutations and translocations are a few of these alterations. A desirable biomarker has the following qualities: high levels of polymorphism that are evenly distributed throughout the genome, the absence of a requirement for prior knowledge of an organism's genome, the ability to generate multiple, distinct and reliable markers that provide an adequate level of resolution, the need for little startup material, simplicity, speed and low cost and the lack of pleiotropic or epistatic links to distant phenotypes.

Restriction Fragment Length Polymorphism (RFLP)

Refers to Variations in the size of DNA fragments generated from the genomic DNA of two or more individuals of a species by a particular restriction endonuclease (Kahl 2001). An infinite number of RFLPs can be produced by digesting entire DNA with particular restriction enzymes. RFLPs are co-dominant in nature and are comparatively tiny in size. The restriction enzyme will only cut the DNA of one person if there is even a little nucleotide difference between the two in the restriction site. Thus, restriction fragments of various lengths are produced. It is bands that identify DNA fragments that have been produced as a result of the digestion of genomic DNA by restriction enzymes. These pieces of DNA are typically between 2 and 10 kb in size.

Southern blot hybridization to a tagged DNA probe is used to detect DNA fragments after they have been separated using agarose gel electrophoresis. Digoxigenin or fluorescein are examples of non-radioactive stains that can be used to mark the probe instead of a radioactive isotope. The homologous sequence of a certain chromosomal region makes up the locus-specific RFLP probes. The creation of genomic or complementary DNA (cDNA) libraries yields the creation of probes, which can be made of either a specific sequence of unknown identity (genomic DNA) or a segment of the sequence of a functional gene (Exons only, cDNA). In suitable bacterial vectors, RFLP probes are kept as clones, making it simple to isolate the DNA fragments they contain.

Techniques applied: - To separate the fragments of genomic DNA based on size, electrophoresis is performed through an agarose gel after one or more restriction enzymes have broken down the DNA. The DNA is subsequently moved from the gel to a nylon membrane following in situ denaturation. The positioning of the DNA fragments relative to one another is not altered during passage to the filter. Following the hybridization of the DNA with radioactive or (in this case) non-radioactively labelled DNA probes, autoradiography or alternative enzyme-linked detection methods can be used to determine the locations of the probe's complementary bands.

Random Amplified Polymorphic DNA (RAPD)

Any DNA fragment amplified using the polymerase chain reaction with short oligodeoxy nucleotide primers of any nucleotide sequence (amplifiers) (Kahl, 2001). RAPDs are DNA fragments generated by PCR using short, synthetic primers with random sequences (often 10 bp). These oligonucleotides often have the capacity to amplify fragments from 1–10 genomic locations simultaneously and function as forward and reverse primers. Agarose gel electrophoresis is used to separate amplified fragments, which are typically in the 0.5–5 kb size range. Ethidium bromide staining and the presence or absence of bands of particular diameters are used to identify polymorphisms. These polymorphisms can occur for a variety of reasons, including variations in the primer annealing sites and length differences in the amplified sequence between the primer annealing sites.

Amplified Fragment Length Polymorphism (AFLP)

AFLPs are DNA fragments (80–500 bp) produced by restriction enzyme digestion, oligonucleotide adapter ligation to the digestion products, and targeted PCR amplification. Therefore, RFLP and PCR are both used in AFLPs. The restriction enzyme-specific sequence, 1–5 selected nucleotides and a core sequence (a component of the adaptor) make up the PCR primers. Variations in the restriction sites or the surrounding area lead to changes in the AFLP banding profiles. The AFLP method produces fragments from numerous genomic locations concurrently (about 50–100 fragments per reaction), which are then sorted by polyacrylamide gel electrophoresis. Selective Fragment Length Amplification (SFLA) and Selective Restriction Fragment Amplification (SRFA) are synonyms sometimes used to refer to AFLPs. A variation of the AFLP technique is known as Selectively Amplified Microsatellite Polymorphic Locus (SAMPL). This technology amplifies microsatellite loci by using a single AFLP primer in combination with a primer complementary to compound microsatellite sequences, which do not require prior cloning and characterization.

Simple Sequence Repeats SSR (Microsatellite)

Any of a number of DNA sequences that are scattered throughout the genomes of fungi, plants, animals and humans that are very short (2-10 bp), largely repetitive, tandemly organized and highly variable (hyper variable) (Kahl, 2001). A kind of repeated DNA components are called microsatellites or simple sequence repeats (SSR) (Tautz and Rentz 1984, Tautz 1989). The 5- to 50-copy tandem arrays of di-, tri-, or tetra-nucleotide repeats such as $(AT)_{29}$, $(CAC)_{16}$, or

(GACA)32, are used to organize the repeats. Plants have many SSRs, with one on average per 6-7 kb. In order to PCR-amplify the DNA portion containing the SSR, forward and reverse primers can be created using the conserved nucleotide sequences that flank these repeat motifs. By using gel electrophoresis, SSR alleles, which are amplified products with varied lengths may be distinguished and seen by silver-staining, autoradiography (if primers are radioactively labelled) orvia automation (if primers are fluorescently labelled). SSR analysis is amenable to automation and multiplexing), and allows genotyping to be performed on large numbers of lines and multiple loci to be analyzed simultaneously. SSRs can be identified by searching among DNA databases (e.g. EMBL and Gene bank) or alternatively small insert (200-600bp) genomic DNA libraries can be produced and enriched for particular repeats (Powell et al. 1996). From the sequence data primer pairs.

Single nucleotide polymorphism (SNP)

Any variation between two genomes that is caused by a minor deletion, insertion or exchange of a single nucleotide (Kahl 2001). The novel marker technology known as small nucleotide polymorphism (SNP) was first created in nonhuman primates. SNPs, which have two to three severe polymorphic sites per site, are the most prevalent polymorphism markers (Cooper et al. 1985). SNPs were initially found in humans but they are currently used to genotype plants. Sequence information is a key component of SNP technology.

Table 1. Widely used molecular markers in conservation of plant genetic resource

Feature and description	RFLP	RAPD	AFLP	SSR	SNP
Genomic abundance	High	High	High	Moderate to high	Very high
Expression/inheritance Co-dominant	Co-dominant	Dominant	Dominant/co-dominant	Co-dominant	Co-dominant
Number of loci	Small (< 1000)	Small (< 1000)	Moderate (1000s)	High (1000s–10,000s)	Very high (> 100,000)
Level of polymorphism	Moderate	High	High	High	High
Type of polymorphism	Single base change, indel	Single base change, Indel	Single base change, indel	Changes in length repeat	Single base change, indel
Cloning and/or sequencing	Yes	No	No	Yes	Yes
Type of probes/primer	Low-copy DNA or cDNA clones	10 bs random	Nucleotides Specific	sequence Specific	sequence Allele-specific PCR primer
PCR-based	Usually no	Yes	Yes	Yes	Yes
Radioactive detection	Usually yes	No	Yes or NO	Usually no	No
Reproducibility/reliability	High	Low	High	High	High
Amount of DNA required	Large (5–50 μg)	Small (0.01–0.1 μg)	Moderate (0.5–1.0 μg)	Small (0.05–0.12 μg)	Small (> 0.05 μg)
Genotyping throughput	Low	Low	High	High	High

Cost	Moderate to high	Low	Moderate	Moderate to high	High
Marker index Moderate	Low	Moderate	Moderate	Moderate to high	Moderate
Time demanding	High	Low	Moderate	Low	Low
Number of polymorphic per loci	1-3	1.5-5	20-100	1-3	1
Primary application	Genetic	Diversity	Diversity and genetic	All purpose	All purpose

Due to the timeline of technological advancement, molecular markers are divided into three main categories or generations: (i) hybridization-based markers, such as restriction fragment length polymorphism (RFLP), (ii) PCR-based markers, such as random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellites or simple sequence repeat (SSR) and (iii) sequence-based markers, such as single nucleotide (SNP). In various areas of plant science study, including genetic and phylogenetic investigations and ecological, evolutionary and taxonomic studies, DNA-based molecular markers are frequently used.

Table 2. Strength, weakness and application of molecular markers

Markers type	**Strength**	**Weakness**	**Application**
Restriction Fragment Length Polymorphism (RFLP)	Moderately polymorphic. Show co-dominant alleles and having high reproducibility	Requirement of laborious and technically demanding methodological procedures Not amenable to automation and collaboration among research teams requires distribution of probes.	Applied in diversity and phylogenetic studies Widely used in gene mapping studies They also have been used to investigate relationships of closely related taxa
Random Amplified Polymorphic DNA (RAPD)	They are quick and easy to assay. No sequence data for primer construction are needed. Have a very high genomic abundance and are randomly distributed throughout the genome.	Low reproducibility highly standardized experimental procedures are needed Markers are not locus-specific, band profiles cannot be interpreted in terms of loci and alleles (dominance of markers), and similar sized fragments may not be homologous.	Studies at the individual level. Applied in gene mapping studies. Include Arbitrarily Primed Polymerase Chain Reaction (AP-PCR)
Amplified Fragment Length Polymorphism (AFLP)	High genomic abundance Considerable reproducibility	The need for purified, high molecular weight DNA The dominance of alleles, and the possible non-	Applied in studies involving genetic identity, parentage and identification of clones and cultivars, and

	No sequence data for primer construction are required.	homology of co-migrating fragments belonging to different loci.	phylogenetic studies of closely related species. Widely valued technology for gene mapping studies Considered more applicable to intraspecific than to interspecific studies
Simple Sequence Repeats SSR (Microsatellite*)*	Fast, highly polymorphic Required only very small DNA Does not require radioactivity.	High developmental and startup cost Species-specific Sometimes difficult interpretation	Fingerprinting, Varietal/line identification Genetic maps, Diversity studies, Marker-assisted selection
Single nucleotide polymorphism (SNP)	Robust in usage Polymorphism are identifiable Different detection methods available Suitable for high throughput Can be automated	Very high development costs Requires sequence information Can be technically challenging	Genetic maps, Diversity studies, Marker-assisted selection

Role of Molecular Marker for Plant Genetic Resource Conservation

Characterization of plant genetic resources refers to the procedure and equipment used to assess, classify and identify accessions. This identification, in broad terms, can apply to any morphological descriptor or molecular polymorphism of an accession. Characterization refers to the description of qualitative or quantitative features that are highly heritable, detectable by the eye, and equally expressed in all situations. It is a word commonly used in the management of germplasm collections and gene banks (Van and M.C. 2005). Strong genetic characterization makes it possible for decisions to be made regarding conservation measures to be backed by this data which leads to better management of the germplasm. Plant genetic resources are enhanced by experimental work related to them, as well as by morpho-phenological and molecular analyses of germplasm.

Customary efforts to directly employ plant accessions kept in germplasm banks in breeding programs have generally been focused on locating the origins of interesting genes, including resistance to plant diseases or pests, and their transfer to cultivated materials. Because better material from sophisticated breeding programs is significantly more alluring than any germplasm resource with unknown genetic origin and phenotypic adaptability or performance, linkage drag has frequently prevented breeders from taking the initiative to employ accessions from germplasm banks (Ferreira 2006).

Molecular Markers' Function in Characterization

The use of genomic DNA-based marker assays has modernized and changed the way we can characterize genetic diversity and explain genetic selection (Lanteri and Barcaccia 2006). For the characterization of genome architectures and the analysis of gene polymorphisms in agricultural plants, molecular markers are recognized as being particularly efficient and trustworthy methods. The plant DNA polymorphism assays are effective instruments for assessing and examining germplasm resources and genetic relatedness, in addition to linkage mapping, gene targeting and aided breeding. The most popular methods for characterizing crop plant genetic resources are SSR and AFLP markers, which both provide an almost infinite variety of molecular traits for distinct fingerprinting and genotyping of plant materials, both with and without prior knowledge of the target DNA sequences.

In order to detect polymorphism, molecular markers typically assess a portion of the total amount of DNA sequence variation in a genome. The polymorphisms that the RFLP assay finds are a reflection of the range of restriction fragment sites. The causes of PCR-based polymorphisms are changes in DNA sequence at primer binding sites as well as variations in DNA length between primer binding sites. In the SSR experiment, which employs pairs of primers flanking each simple sequence repeat, polymorphisms depend on the number of repeating di-, tri-, or tetra nucleotide units that are present at one locus. To find polymorphisms at various loci, the AFLP assay combines primers specific for two separate four-base and six-base long restriction sites that flank the target sequence unit.

The selective potency of RFLP markers has generated significant debate, despite the fact that they have assisted in quantifying the genetic diversity and relatedness of crop plants. More probe-enzyme combinations may increase the number of loci that RFLP markers can identify, but PCR-based markers can still detect a higher level of polymorphism. SSR markers often detect multiple alleles at a specific locus because of their own genetic make-up, in contrast to AFLP tests, which typically detect single alleles at various randomly distributed loci in the genome. SNP markers, more contemporary methods, directly sequence the DNA of target gene regions to detect single-nucleotide polymorphisms. In actuality, AFLP markers have so far provided the widest the largest.

Population-Level Characterization of Germplasm

Determining the genetic make-up of societies is greatly aided by measures of genetic diversity and similarity. The genetic composition of a crop plant species natural populations is significantly influenced by both an individual's reproductive system and the kinds of unions that occur within populations. Plant reproductive barriers and mating systems determine the breeding methods that can be used and the types that can be produced. Normally dominated by a small number of genotypes with advantageous adaptations for genotype separation, natural populations of animals that reproduce vegetatively or via apomixes are polyclonal, consisting of multiple genetically distinct clones (Spooner 2005).

Land races of self-pollinated plants, such beans, lentils, wheat, and barley, are made up of pure lines that are reproductively separate from one another genetically. Genetic and phenotypic variation is most obvious within lines because natural populations frequently have fixed genotypes, primarily homozygous for different alleles. Yet, depending on the species, environmental factors and germplasm availability, some spontaneous hybridization is conceivable. The cultivated forms of selfing species are often pure lines made through the repeated self-pollination of a large number of hybrid individuals descended from two parental lines chosen for complementary morphological and commercial features. One of the most significant cross-pollinated plants for trade is maize (Spooner 2005).

Molecular Markers for Plant Genetic Resource Conservation

Knowing the genetic make-up of gene bank accessions facilitates decision-making for conservation operations, which range from acquiring and managing genetic resources to choosing genes that provide value for breeding purposes. The adoption of educated sampling methods for germplasm material intended for ex-situ conservation and the choice of prioritized in-situ conservation sites are essential for the success of conservation initiatives. Hence, developing solutions requires knowledge about the location, distribution and degree of genetic diversity. Molecular marker-based characterization provides reliable information on a variety of factors, including the degree of genetic diversity/similarity, the structure of genetic variation in samples and populations rates of genetic differentiation among populations and the distribution of biodiversity in populations from different geographical locations.

Molecular characterization is useful for understanding the reproduction of species, the reproduction and adaptation of individuals and the existence of gene flow across individuals, i.e., the movement of alleles within and among populations of the same or related species (Papa and Gepts 2003). Molecular data, which also provide the essential knowledge required to understand taxonomy, domestication and evolution increase or even enable the understanding of phylogeny. Moreover, molecular marker data sets provide a baseline for monitoring changes in the genetic makeup of accessions that occur naturally as well as those brought on by human intervention help prevent issues later (De Vicente et al. 2006). Many molecular genetics-related methods, such as Mendelian gene tagging and QTL mapping, have been helpful in characterizing. This research, where cutting-edge techniques have found beneficial variation that may aid in varietal development, has highlighted the significance of wild relatives.

Current Advancement of Genetic Marker

The inter simple sequence repeat (ISSR)-PCR technique uses microsatellite sequences as primers in a polymerase chain reaction to produce multi-locus markers. The bulk of the advantages of microsatellites (SSRs) and amplified fragment length polymorphism (AFLP) are combined in a quick and simple technology. This technology combines the universality of random amplified polymorphic DNA with these advantages (RAPD). Given that they have a high degree of polymorphism, ISSR markers are useful in studies of genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology (Reddy 2002). Sequences amplified by ISSR-PCR can be used to identify DNA. Sequence diversity is larger than in actual gene sequences but lower than in SSR-PCR. This method is better suited for phylogeographical research or perhaps taxonomic delimitation rather than human identification because an ISSR may be a conserved or non-conserved area. In addition, microsatellite sequencing and ISSR sequencing are advantageous to one another since each produces primers for the other (Pradeep et al. 2002) (Uddin and Cheng 2015).

Applications of ISSR

For the first time in spiders, the Inter Simple Sequence Repeat (ISSR) method was used to assess the genetic diversity of Brachypelma vagans populations in Mexico. Seven ISSR primers were tested and six populations on the Yucatan peninsula were sampled non-lethally. Four of these primers generated fragments (bands) that were sufficiently distinct and reproducible to generate a binary matrix and determine the parameters of genetic variability. There was the highest level of polymorphism (P 5 98.7%) ever seen in tarantula spiders. The results show that the ISSR-PCR strategy is promising for tarantula spider intraspecific variation (Uddin and Cheng 2015).

Application of Marker Assisted Selection

Local damages in the genome were caused by targeting the majority of crop plants can benefit from the non-transgenic reverse genetics technique known as targeted induced local lesions in the genome (TILLING). In an effort to comprehend how two genes in Arabidopsis plants function, McCallum created TILLING in 1990. (McCallum et al. 2000). When using TILLING procedures, the mutagenic population is initially established by treating seeds with a common chemical mutagen like methyl methanesulfonate (MMS) or ethyl methanesulfonate (EMS). Using the most crucial methods; including mass spectroscopy, liquid chromatography, array-based technologies and enzymatic mismatch cleavage. It is possible to determine the differences in target nucleotide sequences of mutant people in the population (Kurowska et al. 2011). Eventually more significant bioinformatics methods are used to analyze mutations brought on by certain mutagens, such as project aligned relevant sequences and assessed SNPs

(PARSESNP). While TILLING is applicable to all plant species, ploidy level and genome size shouldn't have an impact on it. The discovery of a higher rate of gene mutations is the technique's most significant benefit. This method can be utilized in molecular genetics during plant breeding projects since it allows for the rapid and accurate identification of novel alleles at a cheaper cost and in less time (Hasan et al. 2021)

Genome editing (CRISPR)

The use of the CRISPR genome editing method has enhanced many crop plants (Feng et al. 2013). Because to its many benefits, including ease of use, the capacity to cleave the methylation loci and the versatility of genome editing, the new emerging technique of Cas9 technology is quickly replacing other methods (Hsu et al. 2013, Lozano-Juste and Cutler 2014). The two most crucial components of the CRISPR method are CRISPR RNAs and the Cas protein. Two short-length RNA molecules, trans-encoded CRISPR RNA (tracrRNA) and CRISPR RNA (crRNA), can cleave a specific target site with the aid of Cas9 endonuclease (the most explored Cas protein). Single guide RNA, or sgRNA, is the hybrid formed when tracrRNA and crRNA are artificially combined (Qi LS, Larson et al. 2013). The sgRNA and Cas proteins come together to produce the RNA-guided endonuclease, which facilitates the cleave of a specific sequence in the genome (Niewoehner et al. 2013). The CRISPR-Cas system is divided into three types—I, II, and III—based on this Cas protein. Two distinct proteins called Cas1 and Cas2 are frequently found in all three types. Type III is most frequently found in archaea but can also be found in some bacteria, whereas type I is present in both archaea and bacteria and type II is only present in bacteria (Makarova et al. 2011). Genome editing have been performed fruitfully in model plants like *Nicotiana tabacum* (Shan et al. 2013), *Arabidopsis* (Ali et al. 2018) and some economically important crops like maize (Svitashev et al. 2018) and wheat (Hasan et al. 2021)

Investigations of genome-wide associations in plants in order to fairly precisely identify the genomic areas causing symptoms genome-wide association studies (GWAS) take advantage of an ancient recombination event. After a decade of intense research, the GWAS approach was well established in the field of human genetics. GWAS are already developing as a potent tool for discovering natural variation underlying complex traits in cops due to the rapid advancement of sequencing technologies and computational methodologies (Zargar et al. 2015). GWAS in crops typically utilizes a permanent resource—a population of various (and preferably homozygous) varieties that can be rephenotyped for multiple traits and only need to be genotyped once—and one can then produce specific mapping populations for a particular trait or QTLs in crops (Atwell et al. 2010). GWAS have now been successfully conducted in a variety of crops, including sorghum, foxtail millet, rice and maize (Zhao et al. 2016). Rice and maize are the two main crop GWAS models based on the volume of resources already created and published, and both include panels of tens of thousands of genotyped inbred and multiple environment experiments undertaken for numerous characteristics. Low genome coverage sequencing was done on 446 wild rice accessions (*Oryza rufipogon*) and 1083 cultivated *O. sativa* spp. *indica* and *O. sativa* spp. *japonica* varieties of rice (Huang et al. 2012). Data imputation was used to create a high-density haplotype map of the rice genome, and a GWAS was then carried out utilizing the extensive data set of 1.3 million SNPs to describe the allele linked to 10 grain-related variables and blooming time. A GWAS was also carried out in 446 O. *sativa* for the traits leaf sheath color and tiller angle since these traits would have better mapping power because the wild species exhibit a greater level of genetic diversity Rufipogon accessions. In addition, the GWAS was carried out using a genotyping method based on microarrays. The genotyping of 413 different *O. sativa* accessions at 44,100 SNP variations and the phenotyping of 34 phenotypes revealed the intricate genetic architecture of rice attributes. By combining linkage mapping and GWAS in the NAM panel, the genetic architecture of maize's flowering time, leaf angle, leaf size and disease resistance features was analyzed, and numerous associated candidate genes were found (Juliana et al. 2018). The GWAS results showed that several QTLs with minor effects dominate the genetic architecture of these traits. A GWAS in maize was recently conducted to determine the composition of maize kernel oil, which is a significant food and energy source (Li 2013). A total of 368 maize lines were examined using a genome-wide SNP analysis and 74 loci were discovered to be connected to the quantity and makeup of maize kernel oil. These results demonstrate that the GWAS method in crops is a reliable and practical approach that complements traditional biparental cross mapping and has the capacity to simultaneously genetically map many variables. In order to better research the genetic basis of plant shape, yield, and physiology in more grasses as well as close wild family of cultivated crops, GWAS results are anticipated to be used. It is significant to emphasize that uncommon alleles, which account for a considerable fraction of natural variation, have a low power in GWAS 44% of the SNPs in rice have low frequency. The use of a large sample size or method for high-throughput virus-induced gene silencing is recommended when studying uncommon alleles (VIGS). VIGS is performed by cloning a short stretch of sequence from a candidate gene or random cDNAs into a virus genome under the control of promoter within a binary vector (Hasan et al. 2021).

Conclusions

Molecular markers give better conservation tactics a strong base. Some molecular methods can be used to accurately and cheaply identify the genotype profiles and gene haplotypes of accessions generated by DNA sequencing and fingerprinting. These methods can also detect contaminants, especially in the case of mixtures, contaminating genes from commercial or other accessions, as well as the existence of redundant materials or duplicated accessions. In terms of the use of molecular marker technologies, applied breeding programs are probably the area of agricultural plant genomics that will experience the most expansion. Genetic characterization serves an increasingly important function of identification. Using cutting-edge molecular markers, it is now possible to conserve desirable treat at the DNA level. The most recent developments in molecular plant breeding technology include marker aided selection, marker assisted back crossing and marker assisted plant breeding. On the other hand, genome editing or CRISPR technology is currently being used to improve several crop plants. Although each of the aforementioned markers has its own benefit and negative, they are all utilized to conserve and make use of plant genetic resources.

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Hasan et al. 2021. Journal of Genetic Engineering and Biotechnology (2021) 19:128 Pp 15-26 the construction of multiple biparental cross population (e.g. NAM or MAGIC) may be helpful.

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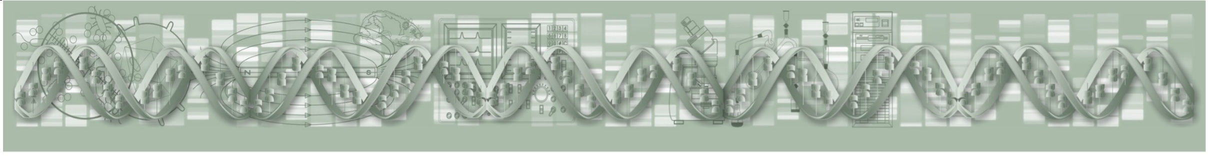

Remember PhD Prof. Dumitru Cojocaru

(1951-2023)

By the mid-1980s, I crossed paths with a young biochemist named Dumitru Cojocaru, who would become my friend a decade later. He worked alongside professor (at that time lecturer) Vlad Artenie, whom I often visited when I went to Iași, in a work space assigned to this discipline in the old building (Body A) of "Alexandru Ioan Cuza" University. I discovered that we were actually colleagues, researchers within the same institution – the Centre for Biological Research (CCB) in Iași: while he was affiliated at Iași Section, I was at the one in Piatra Neamț. Most of the colleagues from Iași were then working in the premises of the Faculty of Biology (Body B). After 1990, at the initiative of Prof. Artenie, the Faculty established a Department (specialisation) of Biochemistry in the building where it also operated, by vacating some spaces belonging to other disciplines to create laboratories and offices for this new Department. The new faculty specialisation entailed new study subjects and academic positions, which enabled some specialists from CCB Iași to compete for and hold some of the teaching positions that were created. Among them was the recent doctor in biochemistry Dumitru Cojocaru, who became a lecturer. In fact, it was in this position that I would get to know him personally, and, gradually, through our professional obligations in the faculty, we would collaborate and establish a friendship that, over the years, became closer.

Dumitru Cojocaru was born on May 15, 1951 in Săveni village (which became a town in 1968) from Botoşani county, in a family of farmers with many children, being the youngest of the brothers and the only one who was supported by his family to learn and to finally reach the highest level in education, that of university professor. He began his schooling in 1958 at the General School in Petricani (Săveni), then attended (between 1966-1970) the courses of the Theoretical High School (Department of Sciences) in Săveni town; in 1970, he took the entrance examination at the Faculty of Chemistry, Department of Chemistry - Physics of "Alexandru Ioan Cuza" University in Iași. After finishing the second year of college, he was selected and sent to study in Ukraine, where (between 1972-1976) he attended the courses of the Faculty of Biology, Department of Biochemistry, of "Maxim Gorki" University (currently "V. N. Kazarin") in Kharkiv. After completing his university studies, he worked as a biochemist at the Biochemistry Laboratory of Botoșani County Hospital. In 1981, he moved to Iași where he continued his professional activity as a biochemist in the Industrial Microbiology Laboratory of the Biological Research Centre. During the 9 years of working in this laboratory, he participated in contract-based research projects of his group, attended professional scientific meetings, published a series of articles in the field of biochemistry, etc.; consequently, the results of his hard work helped him to promote to the positions of Scientific Researcher (SR)

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and SR, qualification level III. In 1986, he enrolled on the doctoral specialisation, first with Prof. Dr. Napoleon Topală as his scientific supervisor (who passed away) and then with Prof. Vlad Artenie. His doctoral thesis entitled "Research on the immobilization of enzymes and microbial cells with applications in biotechnology" was publicly defended in 1990.

The same year, in autumn, he entered the competition for a lecturer position, which he obtained at the Faculty of Biology of "Alexandru Ioan Cuza" University - Iaşi, Department of Genetics, Biochemistry, Microbiology. In 1994 and 1995, Prof. Dr. Dumitru Cojocaru benefited from two specialisation internships (of two months each), within the "TEMPUS" program, at the University of Lille I (France), where he had as scientific advisors Dr. Rita Barot (from CNRS Paris) and Professor Jean Montreuil from the University of Lille (honorary member of the Romanian Academy). Between 1995-2004 he participated in the training of those interested in the field of "Glycosciences and Glycotechnologies", courses and activities organised within the French-Romanian Summer School, with Professor Emeritus Jean Montreuil as the director of this school. At the last five editions of this school, Prof. Dumitru Cojocaru was selected among the specialists who gave conferences to the participants, the chosen topics being: "Fundamental enzymology" and "Competitive and non-competitive enzyme inhibitors".

The main fields of activity in which Professor Cojocaru was reputed were: general biochemistry, applied enzymology, biochemistry of vitamins and hormones, metabolism of proteins and nucleic acids, enzyme biotechnologies (extraction and purification of enzymes, immobilization of enzymes and microbial cells on solid supports, use of enzymes in industry and medicine). He taught biochemistry, protein and nucleic acid metabolism, enzyme biotechnologies, biochemistry of informational molecules to undergraduate and master's students. The scientific and didactic activity supported in the laboratory and in the department allowed him to promote swiftly from the position of lecturer in 1993 to that of university professor in 1997. Moreover, in 2008 he requested and was approved by the faculty and university management the quality of doctoral supervisor, which was subsequently confirmed by the CNATDCU Profile Commission, within the Ministry of Education. Professor Dumitru Cojocaru's scientific activity was conducted in high-value research projects and contracts, financed by economic enterprises or from the National Research and Development Plan. This research concerned the testing of macromolecular acrylic supports in the purification and immobilisation of enzymes; the immobilisation by physical methods and by covalent binding of some microbial enzymes; the characterisation of active plant and fungal principles with cytostatic, immunomodulatory and neurotropic action; the use of cereals and cereal waste to obtain bio-ethanol; the ecological reconstruction through micromediation processes of soils degraded by mining activities, etc.

His scientific activity resulted in more than 100 articles published in ISI or BDI indexed journals, in the development and publication of 9 manuals and specialised books, in the preparation and support of scientific communications at professional meetings, but also of conferences intended for the students of the previously mentioned Summer School, organised by the Department of Biochemistry of "Alexandru Ioan Cuza" University. The experience acquired as a researcher and teacher, as well as the prestige he enjoyed among his colleagues, entitled him to hold highest positions in committees/commissions at the national level: he was a member of the Biology Commission of CNATDCU for the confirmation of the titles of professor, associate professor, respectively scientific researcher II and I; he was a permanent expert evaluator in the "Exact Sciences and Natural Sciences" Commission within ARACIS; about a decade ago, he was proposed and elected an associate member of the Romanian Academy of Scientists, Biology Section.

The professional achievements of researcher and professor Dumitru Cojocaru were undoubtedly remarkable, but I was very impressed by his human qualities, his manner of being and treating his peers, the respect he showed towards values of any kind. He was a reliable person, who was supportive for better or worse, who did not cause problems around him because he did not like tensions between people. I had the opportunity to get to know him better during our official visits to some universities in the country for the evaluation and accreditation of specialisations and master's degrees in Biology and Ecology. Being a well-educated, honest and balanced person, he did not assume the role of bossy "inspector" who would put his hosts in difficulty; on the contrary, he behaved as a well-meaning, fair and constructive colleague of theirs. We got along perfectly in all these working visits and I think no one had anything to comment or reproach us about the way we did our duty and how we treated the colleagues who were undergoing evaluations. These trips were also opportunities to talk more, to learn about each other, about our concerns, our values, our passions, our way of thinking and acting. A relationship of great trust and sincere friendship has been established between us. We often talked on the phone in a relaxed manner and in our long discussions we covered all kinds of topics, from family and professional ones to history, politics, music, etc.

Compared with his career path, which was smooth, obstacle-free and always ascending, Dumitru Cojocaru's personal life did not take a similar course. He married in 1981 Doina Irina Anisie, a chemist whose parents were teachers in Botoșani. They were a happy family, free from material worries – having an apartment and good jobs. God had given them a child, Sabina-Ioana, who promised to follow in their footsteps. Everything seemed to be on the upswing in their lives, until one day – when his wife contracted a disease that still claims many lives among her fellows and ended her days at the turn of the millennium, at the age of just 44. That was an extremely difficult moment for the whole family, but especially for Professor Cojocaru, left alone at a young age, going to take care of both his 17-year-old daughter and his in-laws (who, meanwhile, had moved to Iași), who were elderly people with health problems. As a firm believer, he just called on divine help, gritted his teeth and moved on, he did not complain to anyone, but managed on his own. To his credit, he did not think of rebuilding his life with another woman, which is a sign of great gentleness. He understood that he had an even greater responsibility, that he had to take care of his daughter, to whom he was not only a loving father, but a friend and even a colleague, making sure that she did not lack anything and that she developed her full potential.

After the death of his parents-in-law, Sabina inherited the apartment in which they had lived, located in the city centre and proposed to her father to sell it and buy a house (at an advanced stage of construction) in Aroneanu commune, a locality located at approximately 5 km from Iasi. They were both delighted that their initiative was successful. Reaching the threshold of retirement, the teacher had at his disposal the dream place where he could spend a large part of his time, create a household to his taste, which would remind him of his childhood years spent in Săveni. As the works on the house progressed, the two began to set up spaces on the land next to it for a small orchard (cultivated with various fruit trees and shrubs) and a vegetable garden, which would provide them with organic produce. Professor Cojocaru was very proud of the living conditions he had created at his home in the country, of his work as a "farmer" in the garden and orchard and of its fruits. When some agricultural work or landscaping was beyond him, he called on his brothers and nephews to help him. I was deeply impressed hearing him tell me how well he felt among his people in Săveni, how much care and respect they had for him. Rarely have I heard of such families among us! The coming of the pandemic was no longer a nightmare for my friend, as it was for most townspeople. Father and daughter now realised, once more, how inspired their idea was to acquire this home, where they had freedom of movement and action, a wonderful place to relax and spend time usefully.

Because of the pandemic, I have not been able to meet Professor Cojocaru physically in the last three years. In our phone conversations, we covered a wide variety of topics. They gave me a good mood, I could feel how at peace he was with himself and how happy he felt with the new occupation added to the old ones. He confessed to me that the activity of "farmer", in addition to some material satisfaction offered, had helped him to get rid of the extra kilos he had and to normalise all his medical tests. Even though he did not lack anything and had good medical test results, I am convinced that it was not easy for him alone between four walls with enough professional and personal worries on his head! I think loneliness was his biggest enemy, which affected his health without realising it. More than 10 years ago he had to undergo some surgery to have a coronary stent implanted. Although he became cautious from that moment on, careful about his diet, alcohol consumption, physical exertion, etc., I think, made him forget about his blood pressure, which was probably not always within normal limits. The fact that he had visibly lost weight, that he had adopted a balanced diet and lifestyle, that he seemed very satisfied with his life lately, made me believe that he would live and enjoy for many years all that he had achieved together with his daughter. Unfortunately, fate decided otherwise and on February 4, 2023, he suffered a severe stroke. Its consequences, as well as a series of complications in the respiratory system, could not be controlled and improved medically, which sent him to the world of shadows on the night of March 18 to 19. A heavy loss not only for his loving family, colleagues and friends, but also for Iasi biology!

To me, the news that my friend Dumitru Cojocaru suffered a stroke was deeply distressing, it seemed so unbelievable because it came like a storm out of nowhere, while in our phone conversations I felt him optimistic and happy with the life he had he lived. His timely passing away affected me even more strongly, as I was parting forever with a friend who was somewhat unique. You rarely meet people like him, discreet, honest, God-loving, content with what he has, free from temptations, restrained, devoted to his family. Many friends have left me for some time now and their disappearance has deeply affected me, but when Professor Dumitru Cojocaru left us, I experienced a very painful feeling, I could not banish the thought of his merciless fate, the fact that life had left him precisely when he was happier, when he had created for his soul a real corner of heaven in Aroneanu. The memory of my friend Dumitru Cojocaru will remain forever alive in me, and I comfort myself with the thought that our Lord took him from us because he needed him very much up there!

Gogu Ghiorghiță

In memoriam Prof. univ. dr. Dumitru Cojocaru
(1951-2023)

Pe la jumătatea anilor 1980, întâlneam în cale pentru prima oară un tânăr biochimist, pe numele său Dumitru Cojocaru, care avea să-mi devină un deceniu mai târziu prieten. Lucra alături de profesorul (pe atunci conferențiar) Vlad Artenie, pe care îl vizitam adesea când mergeam la Iași, într-un spațiu de lucru alocat acestei discipline în clădirea veche (Corpul A) a Universității ”Alexandru Ioan Cuza”. Am aflat atunci că eram de fapt colegi, cercetători la aceeași instituție - Centrul de cercetări biologice (CCB) din Iași, el la Secția din Iași a acestuia, eu la cea de la Piatra Neamț. Majoritatea colegilor ieșeni ai CCB Iași își desfășurau pe atunci activitatea în spațiile Facultății de Biologie a universității (Corpul B). După 1990, la inițiativa prof. Artenie, Facultatea a înființat în corpul de clădire în care funcționa și o Secție (specializare) de Biochimie, eliberând unele spații aparținând altor discipline pentru a crea laboratoare și cabinete de lucru acestei noi Secții. Noua specializare a facultății însemna noi discipline de studiu și norme didactice, astfel că unii specialiști ai CCB Iași au dat concurs și au ocupat unele din posturile didactice care au fost create. Printre aceștia s-a numărat și proaspătul doctor în biochimie Dumitru Cojocaru, devenit Șef de lucrări. De fapt, în această nouă calitate, aveam să-l cunosc personal și treptat, prin obligațiile noastre profesionale în cadrul facultății, să colaborăm și să stabilim o relație de prietenie care, cu trecerea anilor, a devenit tot mai strânsă.

Dumitru Cojocaru s-a născut la 15 mai 1951 în comuna Săveni, jud. Botoșani (devenită oraș în 1968), într-o familie de gospodari cu mulți copii, fiind cel mai mic dintre frați și singurul care a fost sprijinit de ai lui să învețe și să ajungă în final pe cea mai înaltă treaptă în învățământ, cea de profesor universitar. Și- a început instruirea în 1958 la Școala generală din Petricani (Săveni), a urmat apoi (între 1966-1970) cursurile Liceului teoretic (Secția Reală) din orașul Săveni, în 1970 a dat examen de admitere la Facultatea de Chimie, Secția Chimie-fizică a Universității ”Alexandru Ioan Cuza” din Iași. După terminarea anului II de facultate a fost selecționat și trimis la studii în Ucraina, unde (între 1972-1976) urmează cursurile Facultății de Biologie, Secția de Biochimie, a Universității ”Maxim Gorki” (în prezent ”V. N. Kazarin”) din Harkov. După finalizarea studiilor universitare s-a angajat ca biochimist la Laboratorul de Biochimie al Spitalului Județean din Botoșani. În 1981 s-a mutat cu activitatea profesională la Iași, unde a ocupat prin concurs un post de biochimist în cadrul Laboratorului de Microbiologie industrială a Centrului de Cercetări Biologice. În cei 9 ani cât a slujit acest laborator a participat la realizarea proiectelor de cercetare pe bază de contract ale grupului în care lucra, a fost prezent la reuniuni științifice de profil, a publicat o serie de articole în domeniul biochimiei etc., rezultatele activității sale ajutându-l să promoveze în funcțiile de cercetător științific (CS) și de CS gradul III. În 1986 s-a înscris la specializarea prin doctorat, având mai întâi ca îndrumător științific pe prof. dr. Napoleon Topală și apoi (din cauza decesului acestuia), pe prof. dr. Vlad

Artenie, iar ca subiect de studiu ”*Cercetări privind imobilizarea enzimelor şi celulelor microbiene cu aplicaţii în biotehnologie*”, teza de doctorat fiind susținută public în 1990.

În toamna aceluiași an, cum am arătat deja, a dat concurs și a devenit Șef de lucrări la Facultatea de Biologie a Universității ”Alexandru Ioan Cuza” - Iași, Departamentul de Genetică, Biochimie, Microbiologie. În 1994 și 1995, conf. dr. Dumitru Cojocaru a beneficiat de două stagii de specializare (de câte două luni), în cadrul programului ”TEMPUS”, la Universitatea din Lille I (Franța), unde i-a avut ca îndrumători științifici pe dr. Rita Barot (de la CNRS Paris) și pe profesorul Jean Montreuil de la Universitatea din Lille (membru de onoare al Academiei Române). Între 1995-2004 a participat la perfecționarea celor interesați în domeniul ”Glicoştiinţe şi Glicotehnologii”, cursuri și activități organizate în cadrul Şcolii de vară franco-române, avându-l ca director al acestei școli pe prof. emerit Jean Montreuil. La ultimele cinci ediții ale acestei școli, prof. Dumitru Cojocaru a fost selectat printre specialiștii care au ținut conferințe participanților, temele alese fiind: ”Enzimologii fundamentale” și ”Inhibitori enzimatici competitivi și necompetitivi”.

Principalele domenii de lucru în care s-a făcut cunoscut profesorul Cojocaru au fost: biochimie generală, enzimologie aplicată, biochimia vitaminelor și hormonilor, metabolismul proteinelor și acizilor nucleici, biotehnologii enzimatice (extracția și purificarea enzimelor, imobilizarea enzimelor și celulelor microbiene pe suporturi solide, utilizarea enzimelor în industrie și medicină). A predat studenților din ciclul licență și master cursuri de biochimie, metabolismul proteinelor și acizilor nucleici, biotehnologii enzimatice, biochimia moleculelor informaționale. Activitatea științifică și didactică susținută în laborator și la catedră i-au permis să promoveze rapid, astfel că în 1993 devine conferențiar, iar în 1997 profesor universitar. Mai mult, în 2008 solicită și primește din partea conducerii facultății și universității calitatea de conducător de doctorate, confirmată apoi de către Comisia de profil a CNATDCU, din cadrul Ministerului Învățământului. Activitatea științifică a profesorului Dumitru Cojocaru s-a desfășurat în cadrul unor proiecte și contracte de cercetare de mare valoare, finanțate de unități economice sau din Planul Național de Cercetare Dezvoltare. Acestea au vizat: testarea unor suporturi acrilice macromoleculare în purificarea și imobilizarea enzimelor; cercetări privind imobilizarea prin metode fizice și prin legare covalentă a unor enzime microbiene; caracterizarea unor principii active vegetale și fungice cu acțiune citostatică, imunomodulatoare și neurotropă; folosirea cerealelor și deșeurilor cerealiere la obținerea bio-etanolului; reconstrucția ecologică prin procedee de micromediere a solurilor degradate de activități miniere etc.

Rezultatele activității sale științifice s-au materializat într-un număr de peste 100 articole publicate în reviste ISI sau indexate BDI, în elaborarea și publicarea a 9 manuale și cărți de specialitate, în pregătirea și susținerea de comunicări științifice la reuniuni de profil, dar și a unor conferințe destinate cursanților Școlii de Vară amintită anterior, organizată de către Departamentul de Biochimie al Universității ”Alexandru Ioan Cuza”. Experiența dobândită ca cercetător și profesor, prestigiul de care se bucura în rândul colegilor de breaslă, l-au recomandat în funcții de răspundere la nivel național: a fost membru în Comisia de Biologie a CNATDCU pentru confirmarea titlurilor de profesor și conferențiar universitar, respectiv cercetător științific II și I; a fost cooptat ca expert evaluator permanent în Comisia de ”Științe exacte și Științe ale naturii” din cadrul ARACIS; în urmă cu cca un deceniu a fost propus și ales membru asociat al Academiei Oamenilor de Știință din România, Secția de Biologie.

Desigur, realizările profesionale ale cercetătorului și profesorului Dumitru Cojocaru au fost remarcabile, dar pe mine m-au impresionat mult calitățile sale umane, maniera de a fi și a trata semenii, respectul pe care îl manifesta față de valorile de orice fel. Un tip serios, de cuvânt, pe care puteai conta la bine și la rău, echilibrat, care nu crea probleme în jurul său pentru că nu agrea tensiunile între oameni. Am avut ocazia să-l cunosc mai bine în deplasările noastre la unele universități din țară pentru evaluarea și acreditarea specializărilor și masteratelor în

Biologie și Ecologie. Fiind un tip cu educație aleasă, onest și așezat, nu încerca să facă pe șeful aflat în ”inspecție”, să-și pună gazdele în dificultate, ci dimpotrivă se comporta ca un coleg al acestora bine intenționat, corect și constructiv. Ne-am înțeles perfect în toate aceste vizite de lucru și cred că nimeni nu a avut ce să comenteze sau să ne reproșeze în privința modului cum ne-am făcut datoria și cum i-am tratat pe colegii supuși evaluărilor. Aceste deplasări au fost și prilejuri de a sta mai mult de vorbă, de a afla unul despre celălalt, preocupările, valorile pe care punem preț, pasiunile, modul de a gândi și de a acționa. Între noi s-a stabilit o relație de mare încredere și de prietenie sinceră. Vorbeam adesea la telefon relaxați, abordam în discuțiile noastre îndelungi tot felul de subiecte, de la cele legate de familie și profesionale, până la cele de istorie, politică, muzică ș. a.

Dacă în plan profesional traseul urmat de Dumitru Cojocaru a fost lipsit de obstacole, lin și mereu ascendent, în viața personală lucrurile nu au avut același curs. S-a căsătorit în 1981 cu Doina Irina Anisie, chimist, fiică a unor profesori din Botoșani. Erau o familie fericită, lipsită de griji materiale - având un apartament la bloc și slujbe bune. Dumnezeu le dăruise un copil, Sabina-Ioana, ce promitea să le calce pe urme. Totul părea să aibă un curs ascendent în viața lor, până într-o zi - când soția sa a contractat o boală care seceră încă multe vieți printre semeni și care i-a pus capăt zilelor la trecerea între milenii, la vârsta de doar 44 de ani. Moment extrem de greu pentru întreaga familie, dar mai cu seamă pentru profesorul Cojocaru, rămas singur la o vârstă tânără, urmând a purta doar el de grijă atât fiicei sale de 17 ani, cât și socrilor (care, între timp, se mutaseră în Iași), oameni în vârstă și cu probleme de sănătate. Credincios fiind - a invocat doar ajutorul divin, a strâns din dinți și a mers mai departe, nu s-a plâns nimănui, s-a descurcat cum numai el a știut. Spre cinstea sa, nu s-a gândit să-și refacă viața lângă o altă femeie, dovadă de mare gentilețe sufletească. A înțeles că are o responsabilitate și mai mare, că trebuie să poarte de grijă fiicei sale, căreia i-a fost nu doar tată iubitor, ci prieten și chiar coleg, îngrijindu-se să nu-i lipsească nimic și să evolueze potrivit potențialului de care dispunea.

După decesul socrilor, Sabina a moștenit apartamentul în care locuiseră aceștia, situat în centrul orașului, care după o vreme i-a propus tatălui să vândă acest apartament și să cumpere o casă (aflată în stadiu avansat de construcție) în comuna Aroneanu, localitate aflată la cca 5 km de Iași. Ambii erau încântați că această inițiativă a lor a reușit. Ajuns în prag de pensie, profesorul avea acum la dispoziție locul mult visat, unde să-și petreacă o mare parte din timp, să realizeze o gospodărie pe gustul său, care să-i amintească de anii copilăriei petrecuți la Săveni. Pe măsură ce lucrările de finalizare a casei avansau, cei doi au început să amenajeze pe terenul de lângă ea spații destinate unei mici livezi (cultivată cu diverși pomi și arbuști fructiferi) și grădinii de legume, care să le furnizeze produse ecologice. Tare mândru era profesorul Cojocaru de condițiile de viață pe care și le crease la locuința de la țară, de munca de ”fermier” în grădină și livadă și de roadele ei. Atunci când unele lucrări agricole sau amenajări îl depășeau, apela la frații și nepoții săi să-l ajute. Eram profund impresionat auzindu-l povestind cât de bine se simțea printre ai săi la Săveni, câtă grijă și respect îi purtau aceștia. Mai rar mi-a fost dat să aflu că există printre noi asemenea familii! Venirea pandemiei nu a mai fost pentru prietenul meu un coșmar, așa cum a fost cazul majorității orășenilor. Tată și fiică realizau acum, o dată în plus, ce inspirată a fost ideea lor de a procura această locuință, unde aveau libertate de mișcare și acțiune, un loc minunat de relaxare și de petrecere cu folos a timpului.

Din cauza pandemiei, nu am reușit să-l mai întâlnesc pe profesorul Cojocaru în ultimii trei ani. În discuțiile telefonice cu el abordam subiecte dintre cele mai diverse. Ele îmi dădeau o stare de bine, îl simțeam cât de împăcat cu sine era și cât de fericit se simțea cu noua ocupație adăugată la cele vechi. Îmi mărturisea că activitatea de ”fermier”, pe lângă unele satisfacții materiale oferite, îl ajutase să se elibereze de kilogramele în plus pe care le avea și să-și normalizeze toate analizele medicale. Chiar dacă nu-i lipsea nimic și avea analize bune, sunt convins că nu i-a fost ușor singur între patru pereți cu destule griji de ordin profesional și personal pe cap! Singurătatea i-a fost cred cel mai mare duşman, care i-a afectat sănătatea fără să bage de seamă. Cu mai bine de 10 ani în urmă fusese nevoit să se supună unei intervenții chirurgicale pentru implantarea unui stent coronarian. Deși a devenit prudent din acel moment, atent la alimentație, consumul de alcool, la efortul fizic etc., analizele medicale bune, l-au făcut cred să uite de tensiunea arterială, care probabil nu era întotdeauna în limite normale. Faptul că scăzuse vizibil în greutate, că adoptase un regim de viață şi alimentar echilibrat, că părea foarte mulțumit cu viața lui din ultima vreme, mă făcea să cred că va trăi și se va bucura ani mulți de tot ceea ce agonisise şi realizase împreună cu fiica sa. Din nefericire, soarta a decis altfel și, în ziua de 4 februarie 2023, a fost afectat de un accident vascular cerebral (AVC) grav. Consecințele acestuia, precum și o serie de complicații la nivelul sistemului respirator, nu au putut fi controlate și ameliorate medical, ceea ce l-a trimis în lumea umbrelor în noaptea de 18 spre 19 martie. O pierdere grea nu doar pentru familia lui iubitoare, colegi și prieteni, ci și pentru biologia ieșeană!

Pentru mine, vestea că prietenul Dumitru Cojocaru a suferit un AVC m-a răvășit, mi s-a părut de necrezut, pentru că a venit precum o furtună de nicăieri, în timp ce în discuțiile noastre telefonice îl simțeam tonic, optimist, bucuros de viața pe care o trăia. Dispariția sa m-a marcat și mai tare, întrucât mă despărțeam pentru totdeauna de un prieten, într-un anume fel, unic. Rar întâlnești oameni pe tiparul lui, discret, onest, iubitor de Dumnezeu, mulțumit cu ce are, lipsit de tentații, reținut, dedicat întru-totul familiei. M-au părăsit de o vreme încoace mulți prieteni, a căror dispariție m-a afectat profund, dar la plecarea profesorului Dumitru Cojocaru dintre noi, am trăit un sentiment foarte dureros, nu mi-am putut alunga gândul sorții sale neîndurătoare, faptul că viața l-a părăsit tocmai atunci când era mai fericit, când își croise pentru sufletul său un adevărat colț de rai la Aroneanu. Amintirea prietenului Dumitru Cojocaru îmi va rămâne veșnic vie și mă mângâi cu gândul că Bunul Dumnezeu l-a luat dintre noi pentru că avea mare nevoie de el acolo Sus!

Gogu Ghiorghiță