ا لمجهروية الجزائرية اديلمقريطاة اليبعشة

People's Democratic Republic of Algeria

وزارة العتليم العلاي والثحب العلمي

Ministry of Higher Education and Scientific Research

Ibn Khaldoun University, Tiaret
Faculty of Natural and Life Sciences
Department of Biology

Dissertation

Submitted in partial fulfilment of the
requirements for the degree of

Master of Biological Sciences

Field: **Natural and Life Sciences**
Branch: **Biological Sciences**
Speciality: **Cell and Molecular Biology**

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Title

Study of cytogenotoxicity of Atlas Cedar *Cedrus atlantica* (Endl.) Manetti ex Carriere.

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July 2024

الجمهورية الجزائرية الديمقراطية الشعبية
République Algérienne Démocratique et Populaire
وزارة التعليم العالي والبحث العلمي
Ministère de l'Enseignement Supérieur et de la Recherche Scientifique

Université Ibn Khaldoun, Tiaret
Faculté des Sciences de la Nature et de la Vie
Département de biologie

Mémoire

Présenté en vue de l'obtention du diplôme de

Master académique

en

Domaine :	**Sciences de la Nature et de la Vie.**
Filière :	**Sciences Biologiques.**
Spécialité :	**Biologie Moléculaire et Cellulaire.**

Présenté par :

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Intitulé

Étude de la cytogénotoxicité du Cèdre de l'Atlas *Cedrus atlantica* (Endl.) Manetti ex Carriere

Soutenu publiquement le :

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Année universitaire 2023-2024

الملخص

تعد (*Cedrus atlantica*) نوع من أشجار الصنوبر المهددة بالانقراض وموطنه الأصلي الجزائر و المغرب. وهو معروف بخصائصه الطبية ويظهر مجموعة واسعة من الأنشطة البيولوجية، بما في ذلك القدرات المضادة للأكسدة والمضادة للالتهابات والمسكنات والمضادة للبكتيريا.

تهدف الدراسة الحالية إلى استكشاف التأثيرات السمية الخلوية للمستخلصات المائية والإيثانولية لأوراق شجرة الأرز والتي تم تحليلها باستخدام اختبارالبصل، تم أيضًا تقييم المركبات الكيميائية النباتية الرئيسية ونشاط مضادات الأكسدة.

يحتوي المستخلص الإيثانولي على نسبة أعلى من المركبات الكيميائية النباتية مقارنة بالمستخلص المائي. أظهر تحليل الخصائص السامة للخلايا تأثيرات تعتمد على الجرعة في كل من مؤشر الانقسام الفتيلي ونمو الجذر. لم يكن للمستخلصات أي تغيرات في نسبة الطور الخلوي عند زيادة التراكيز.

بناءً على مقايسة الكروموسومات، وجد أن مستخلصات شجرة الأرز لها أيضًا إمكانات سمية جينية ولكنها تظل أقل من تلك الخاصة بالشاهد. في كل من المستخلصات المائية والإيثانولية، أظهرت المعالجة بـ 0.1 ملغم/مل أكبر عدد من الحالات السامة بينما تم الحصول على أقل عدد تحت العلاج بـ 1 ملغم/مل.

تشير هذه النتائج إلى أن النبات المدروس له تأثيرات سامة للخلايا على أطوار الخلية التي توقف الطور الاستوائي. وفي الوقت نفسه كانت آثارها السمية الجينية منخفضة مقارنة بالشاهد.

الكلمات الدالة

السمية الخلوية؛ السمية الجينية؛ الأرز الأطلسي؛ البصل؛ المركبات الكيميائية النباتية؛ النشاط المضاد للأكسدة؛ الجزائر.

Abstract

Cedrus atlantica is an endangered endemic pine tree species native to north west Africa. It is known for its medicinal characteristics and exhibits a wide range of biological activities, including antioxidant, anti-inflammatory, analgesic, and antibacterial capabilities.

The current study aimed to explore the cyto-genotoxic effects of the needles aqueous and ethanolic extracts, which were analyzed using *Allium cepa* test. The main phytochemical compounds and antioxidant activity (DPPH) were also evaluated.

The ethanolic extract has a higher content of phytochemical compounds than the aqueous extract. Analysis of cytotoxic properties demonstrated dose-dependent influences in both the mitotic index (MI) and root growth. The extracts had no significant changes in the prophase percentage when increasing concentrations. On the contrary, the metaphase percentage experienced a noticeable drop; it was around 8,24±3,22% for the aqueous extract and 3,81±3% for the ethanolic extract under the treatment with 0,1 mg/mL. These values decreased to 1,92±2,17% and 0,96±0,83% respectively for aqueous and ethanolic extracts under the treatment with 0,75 and 1 mg/mL.

Based on *Allium cepa* chromosomal aberration assay, it was found that the extracts of *C. atlantica* have also genotoxic potential but remains below that of the negative control. In both aqueous and ethanolic extracts, treatment with 0,1 mg/mL displayed the highest number of anomalies while the lowest number was obtained under treatment with 1 mg/mL.

These findings suggest that the studied plant has cytotoxic effects on the cell phases arresting metaphase. Meanwhile its genotoxic effects were low compared to negative control.

Keywords

Cytotoxicity; Genotoxicity; *Cedrus atlantica*; *Allium cepa*; Phytochemical compounds; Antioxidant activity; Algeria.

Résumé

Cedrus atlantica est une espèce en voie de disparition, endémique du Maroc et l'Algérie. Il est connu pour ses caractéristiques médicinales et présente un large éventail d'activités biologiques, notamment des capacités antioxydantes, anti-inflammatoires, analgésiques et antibactériennes.

La présente étude vise à explorer les effets cytogénotoxiques des extraits aqueux et éthanoliques d'aiguilles du cèdre, par le test *Allium cepa*. Les principaux composés phytochimiques et l'activité antioxydante (DPPH) ont également été évalués.

L'extrait éthanolique a une teneur plus élevée en composés phytochimiques que l'extrait aqueux. L'analyse des propriétés cytotoxiques a démontré des influences dose-dépendantes sur l'indice mitotique (IM) et la croissance des racines. Les extraits n'ont présenté aucun changement significatif dans le pourcentage de prophase lors de l'augmentation des concentrations. Au contraire, le pourcentage de métaphase a connu une baisse notable ; elle était d'environ 8,24 ± 3,22% pour l'extrait aqueux et 3,81 ± 3% pour l'extrait éthanolique sous traitement avec 0,1 mg/mL. Ces valeurs ont diminué respectivement à 1,92 ± 2,17 % et 0,96 ± 0,83 % pour les extraits aqueux et éthanoliques sous le traitement avec 0,75 et 1 mg/mL.

Sur la base du test d'aberration chromosomique d'*Allium cepa*, il a été constaté que les extraits de *C. atlantica* ont également un potentiel génotoxique mais restent inférieur à celui du contrôle négatif. Dans les extraits aqueux et éthanoliques, le traitement avec 0,1 mg/mL a présenté le plus grand nombre d'anomalies tandis que le nombre le plus faible a été obtenu avec le traitement avec 1 mg/mL.

Ces résultats suggèrent que la plante étudiée a des effets cytotoxiques sur les phases cellulaires arrêtant la métaphase. Pendant ce temps, ses effets génotoxiques étaient faibles par rapport au contrôle négatif.

Mots clés

Cytotoxicité ; Génotoxicité ; *Cedrus atlantica*; *Allium cepa*; Composés phytochimiques ; Activité antioxydante; Algérie.

Acknowledgement

First of all, we would like to thank Allah, the only one to whom we owe all our obedience and Who allowed us to complete this work.

All the expressions of esteem and gratitude in the world are insufficient to express our thanks to our parents who accompanied us throughout our study.

We would like to express our deepest and warmest thanks to our supervisor, Prof. Khaled TAIBI, for his valuable advice, his availability, and his close supervision throughout the completion of this work.

Our thanks go to all the members of the jury Prof. Mohamed BOUSSAID and Dr. Mohamed ACHIR who agreed to examine our work.

I am indebted to Prof. Mohamed BOUSSAID and Mme. Khadija MEKNASSI for their valuable advice and help throughout this work. My sincere thanks are due to Mme. Fatima Zohra SAMMAR for her untiring efforts to help in the laboratory.

Our thanks also go to all the teachers, especially Prof. Leila AIT ABDERRAHIM Leila, Dr SOUANA, and Dr. TADJ

Finally, I thank all my friends and anyone who has contributed directly or indirectly to the development of this work.

Dedications

To my beloved parents, who continually provided their love and support both emotionally and financially.

To my brother and sisters, who have been a source of inspiration.

To my dearest friend Yasmine, for keeping me motivated and for always believing in me.

To the friends and classmates, I met along the way, who in a w ay or another had an impact on my life.

Lastly to me, for the long nights of research, the moments of self-doubt, and the sheer perseverance required to reach this milestone. This achievement is a reflection of your unwavering commitment to growth.

Karima

"Life can be heavy, especially if you try to carry it all at once."

_Taylor Swift

Dedication

This study is wholeheartedly dedicated to my beloved parents, who have been my source of inspiration, strength and confidence when I thought of giving up, they continually provide their moral, spiritual, emotional, and financial support.

To my sisters Yamna and Aicha, my brother Mohamed, relatives, mentors, friends, and classmates who shared their words advice and encouragement to finish this study

Lastly, I dedicate this work to myself. For the countless hours of hard work, perseverance, and determination, I acknowledge the effort and resilience that have brought me to this moment. This is a testament to my dedication and passion for knowledge.

Djihane

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ALCL3:	Aluminum chloride
AP:	Advanced placement
BER:	Base excision repair
CAs:	Chromosomal aberrations
CAt:	Cedrus atlantica extract
DNA	Deoxyribonucleic acid
DPPH:	2,2-diphenyl-1-picrylhydrazyl
DSB:	Double strand break
DSBR:	Defense science board report
GAE/mL:	Gallic acid equivalent per milliliter
GBM:	Glioblastoma
GG- NER:	Global genome nucleotide excision repair
HCC:	Hepatocellular Carcinoma
HCL:	Hydrochloric acid
HR:	Homologous recombination
MI:	Mitotic index
MMR:	Mismatch Repair
MMS:	Methyl methanesulfonate
NER:	Nucleotide excision repair
NHEJ:	Non-homologous end-joining
NOR:	The nucleolus organizer region
PI:	Phase Index
QE/mL:	Quercetin equivalent per milliliter
RNA:	Ribonucleic acid
SD:	Standard deviation
TAE/mL:	Tannic acid equivalent per milliliter
TC-NER:	Transcription coupled nucleotide excision repair
UV:	Ultraviolet

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State of the Art

With the rise of side effects of drugs nowadays, people tend to return to the traditional ways by using medicinal plants to boost their well-being. Cedrus atlantica is one of the plants used in traditional medicine. It is an endemic plant in Algeria and Morocco with numerous biological activities such as anticancer, antibacterial and antioxidant activity.

However, medicinal plants can be toxic due to the non-definite dose. For this reason, this study aimed to investigate the toxic and mutagenic effects of *Cedrus atlantica* by assessing its cytotoxic and genotoxic properties using the *Allium cepa* assay.

1. Cytotoxicity

The term "cytotoxic" refers to a substance or process that causes cell damage or death. In which "cyto" denotes a cell while "toxic" translates into a poison (Eldridge 2022). The strict meaning of the word cytotoxicity refers to the extent to which a substance can harm a cell. A toxicant's effects are typically species- and dose-specific. Examples of cell toxicants include chemical agents, environmental pollutants, extracts from natural plants, and prescription drugs (Zhang 2018). Toxic agents typically harm molecular targets that are crucial for a cell's ability to reproduce and survive, such as metabolic sites, signaling proteins, or DNA (Moghadam et al 2020).

The International Organization for Standardization 109993-5 listed three different types of cytotoxicity tests: extract, direct contact, and indirect contact tests (Li et al 2015).

(i) Direct cytotoxicity is the most sensitive of the three methods and can detect even the smallest cytotoxic effects. It includes exposing a medical device or material sample directly to a monolayer of cultured cells, making it suitable for detecting cytotoxicity caused by direct contact between the device/material and the cells (Li et al 2015).

(ii) Indirect cytotoxicity concerns the extraction into a fluid medium and subsequent contact with cultured cell(s) via the agar overlay or filter diffusion method in the biological evaluation of medical devices or materials. This provides a mediated evaluation of cytotoxic or other effects from the device or material via putative soluble substances (Li et al 2015).

(iii) Extract method is particularly important in the toxicological evaluation of soluble extracts released by medical devices. Cultured cells are exposed to extracts collected from the device or material so that researchers can determine the impact of these leachable substances on cell viability and function (Li et al 2015).

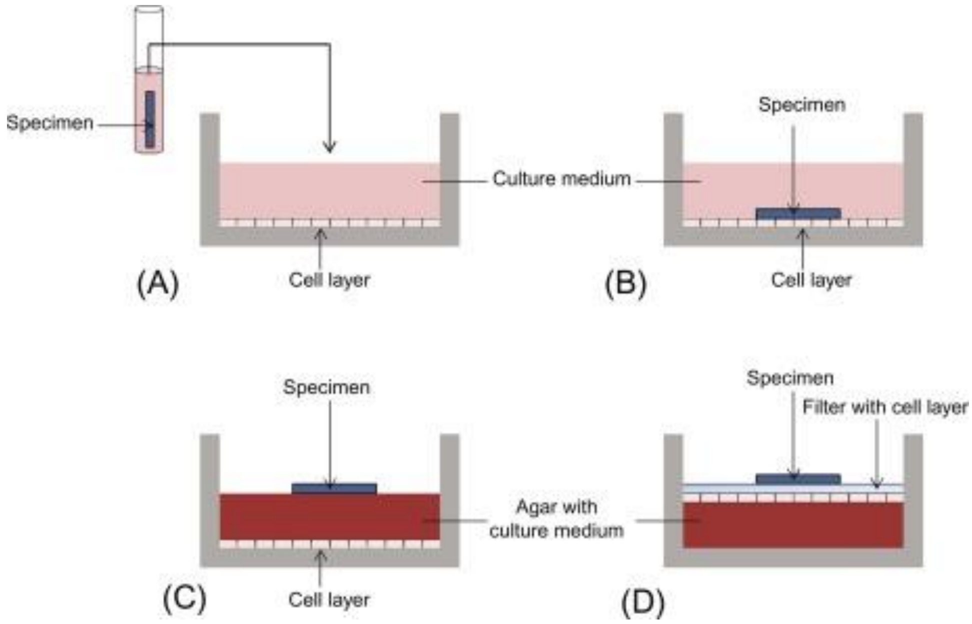

Fig. 1. Cell-culture based tests for cytotoxicity assessment of biomaterial specimen (Gola 2019). (A) Extract test; (B) direct contact test; (C) indirect contact test: agar diffusion assay; (D) indirect contact test: filter diffusion assay.

The list of ways chemicals might affect the health and survival of cells is long and so therefore are the strategies used to detect cytotoxicity. Typical approaches include:

Membrane impermeable dyes or primarily DNA stains, which are frequently used in cell viability assays to stain cells with damaged cell membranes (Chiaraviglio & Kirby 2014).

Metabolic activity which is the assessment of the activity of cellular enzymes involved in metabolic pathways; a reduction in metabolic activity indicates cytotoxicity (Tolosa et al 2014).

Cell proliferation includes assays for measuring DNA content or synthesis in replicating cells providing important insights into the viability and potential for cell proliferation. Such assays are required to assess the health and proliferative capacity of cells (Sapkota 2022).

Cytotoxicity assays are an effective first step when assessing the possible toxicity of a test substance, such as plant extracts or biologically active compounds that have been extracted from plants (Çelik 2018). The type of signal measured (absorbance, fluorescence, luminescence, radioactivity, etc.), the reagents used, the conditions of the reaction, the analytical and automation equipment, and the statistical models used for the data analysis all affect how well an assay works (Riss et al 2019). It is considered one of the most crucial indicators of the biological evaluation system *in vitro* for observing the effects of chemicals on cell growth, reproduction, and morphology. As modern cell biology advances, more and more experimental techniques for assessing cytotoxicity are being created and refined (Çelik 2018).

There are three potential outcomes for the cells that can result from cytotoxicity;

(i) Necrosis is a term used to describe irreversible cell injury and eventual cell death resulting from pathological processes. It is an uncontrollable cell death that causes swelling of the cell organelles, rupture of the plasma membrane and sequent cell lysis, and spillage of intracellular contents into the surrounding tissue causing tissue damage (Khalid & Azimpouran 2024).

(ii) Apoptosis, also known as apoptotic cell death, is an active cellular process that needs specific apoptotic pathways to be activated. It is physiologically necessary for the formation of tissues or organs during development or lifecycle, naturally occurring apoptosis is evolved to have advantageous effects on the organism by getting rid of unwanted cells (Hu and Liu 2017).

(iii) Autophagic cell death which is an activation of the autophagy pathway results in the cellular process known as autophagy, which involves sequestering cytoplasmic components, such as organelles and macromolecules, in double-membrane autophagosomes and targeting them for lysosomal degradation (Park et al 2023). Breakdown products including lipids, amino-acids, and carbohydrates are either metabolized to provide energy or used to synthesize new proteins and organelles (Yonekawa and Thorburn 2013).

2. Genotoxicity

Genotoxicity refers to when some chemical agents exert the toxicity to the DNA and/or RNA molecules of a cell. As a bystander, it causes mutations that can eventually give rise to cancer (Nagarathna et al 2013), severe genetic disorders, and various types of degenerative diseases (Fortin et al 2023). The agents that cause genotoxicity are called genotoxins, they are mutagens, they can cause mutations. Genotoxins include both radiation and chemical genotoxins (Savale 2018). Their effects can be of the following class; (i) carcinogens or cancer-causing agents, (ii) mutagens or mutation-causing agents, and (iii) teratogens or congenital disability causing agents (Radhika et Jyothi 2019).

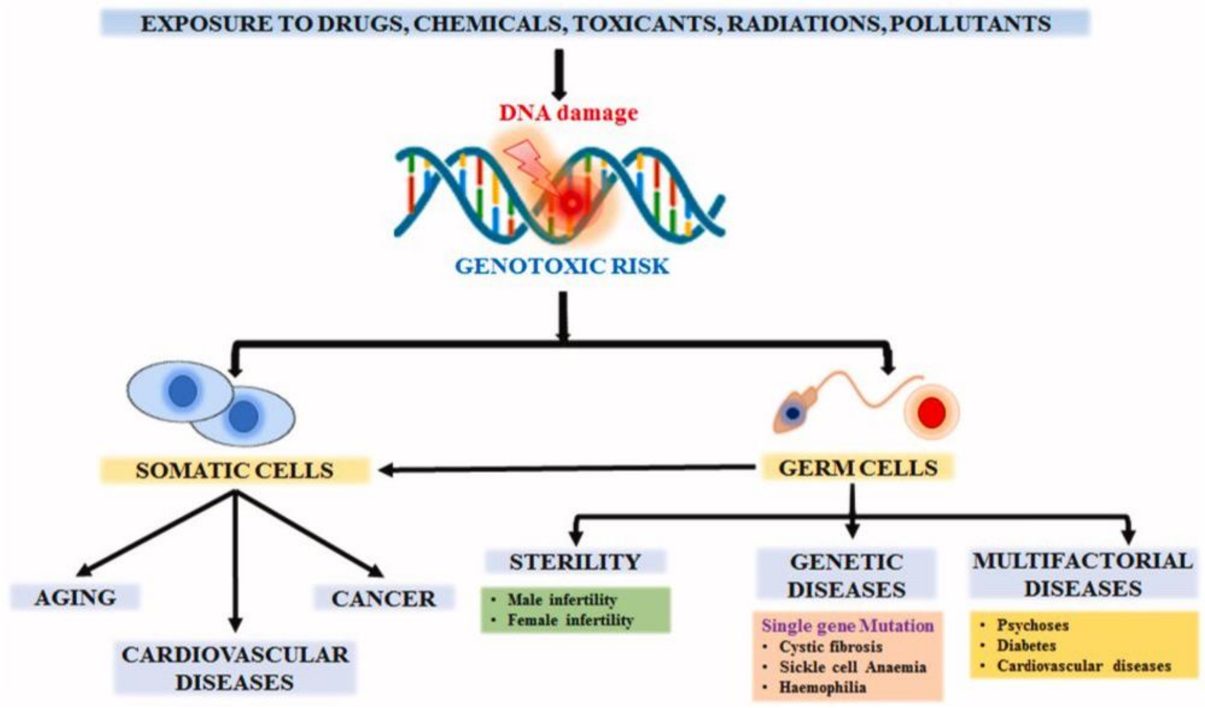

Fig. 2. Major effects on human health on exposure to genotoxins (Choudhuri et al 2021).

The interactions of the genotoxic substance with the DNA structure and sequence lead to damage to the genetic material, this damage is caused by various genotoxic substances that interact with a specific location or base sequence of the DNA structure, causing lesions, breakage, fusion, deletion, mids-segregation, or non-reactive oxygen species (Saks et al 2023).

Cells have the ability to protect themselves from potentially lethal or mutagenic, genotoxic impacts by many repair processes, so not all genotoxic events result in mutations. Yet, the ability to damage the genome is a sign of potential mutagenicity. Therefore, some methods used to measure genotoxicity may not provide direct evidence of heritable mutation. Testing for genotoxicity and mutagenicity is crucial for regulatory purposes when assessing the hazards of chemicals (Savale 2018).

Numerous factors must be involved in order to effectively respond to DNA damage. To prevent cellular damage or the formation of tumors, the genome's integrity must be maintained, and any potentially harmful mutations must be avoided. Efficient repair can be facilitated by establishing a background context that signals the presence of DNA lesions. Genotoxic chemicals have been used for many years to cause DNA damage, as DNA repair mechanisms can also be used as anti-cancer treatments in medical practice. Multiple mechanisms, including base excision repair (BER), nucleotide excision repair (NER), mismatching repair (MMR), and double strand break repair (DSBR), can be used for DNA repair (Chen et al 2024).

Mismatch Repair (MMR) is a DNA repair system that is effective in maintaining genomic stability. It is responsible for repairing mismatched normal bases after DNA replication, which contributes to the remarkable fidelity of DNA replication. Cells that lack MMR exhibit a mutation phenotype, where the rate of spontaneous mutations is significantly higher, and defects in MMR are linked to a greater risk of various types of cancer. Moreover, the MMR system plays a vital role in killing cells in response to alkylating agents, and cells that are deficient in MMR are roughly 100 times more resistant to the cytotoxic effects of such agents (Hartwig et al 2020).

Base excision repair (BER) is a mechanism that repairs damaged DNA in cells during the cell cycle. This cellular mechanism helps to protect cells from the harmful effects of endogenous DNA damage caused by various metabolic processes, such as hydrolysis and reactive oxygen species. It also plays a crucial role in removing many lesions that are caused by ionizing radiation and strong alkylating agents. The two main enzymes involved in BER are DNA glycosylases and AP endonucleases. DNA glycosylases are responsible for removing the damaged base, while the remaining a-basic site is further processed by AP endonucleases. Short-patch repair and long-patch repair are the two types of BER. In short-patch repair, a single nucleotide is replaced, while in long-patch repair, 2-10 nucleotides are replaced (Nagarathna et al 2013).

Nucleotide excision repair (NER) or nucleotide excision repair, is a crucial process that helps to protect cells from various kinds of DNA damage (Petruseva et al 2014). This process is responsible for repairing bulky DNA lesions that are caused by exposure to UV radiation, environmental mutagens, and certain types of chemotherapy drugs (Schärer 2013). NER can be divided into two categories: global genome repair (GG-NER) and transcription-coupled nucleotide excision repair (TC-NER), which specifically targets bulky DNA lesions that cause transcriptional blockage. In mammalian cells, at least 30 different proteins and enzymes are required to carry out this process (Hartwig et al 2020).

Double-strand DNA (DSB) breaks are usual in eukaryotic cells. Two major pathways are common for repairing them: homologous recombination and nonhomologous DNA end-joining (NHEJ) (Lieber 2010). DSB is essential for preserving genomic stability (Scully et al 2019). DNA double-strand breaks can be initiated by both external and internal factors, such as DNA crosslinking agents, topoisomerase inhibitors, and endogenous processes. There are two major methods of repairing DSBs, namely, non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ can be employed without the need for sequence homology, while HR utilizes sister chromatids as homologous templates to repair the damaged chromatid. NHEJ occurs during the entire cell cycle, while HR is limited to the S- and G2 phases. Both DNA repair pathways have distinct effects on genomic stability. The NHEJ pathway protects against cytotoxicity but is prone to errors, making it a pro-mutagenic process. On the other hand, the HR pathway is almost error-free, unlike NHEJ (Hartwig et al 2020).

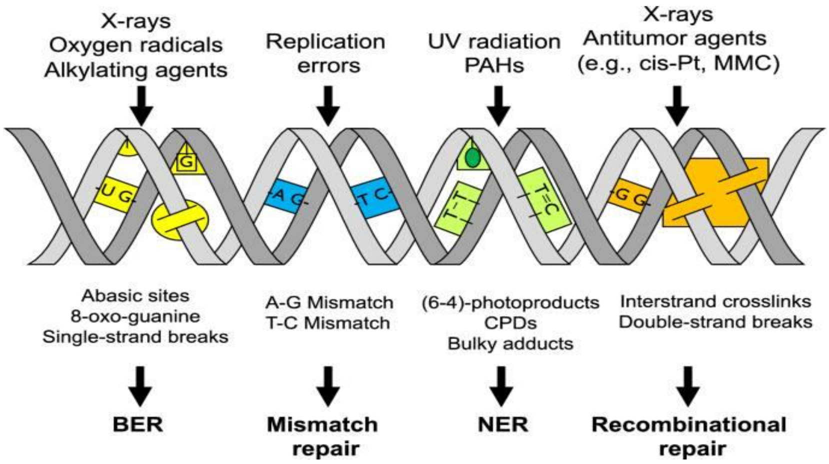

Fig. 3. Major cause of DNA damage and DNA repair pathways (Hartwig et al 2020).

Genotoxicity testing is critical in determining the safety of chemicals for industrial and regulatory applications. Although many *in vitro* tests are widely recognized and approved by regulatory bodies, the accuracy of human predictions of mutagenic/genotoxic potency is frequently questioned. Managing real and functional genetic toxicology issues requires a profound understanding of DNA damage mechanisms at the molecular, subcellular, cellular, tissue, organ system, and organism level (Choudhuri et al 2021). To assess genotoxicity and/or mutagenicity, different endpoints must be taken into consideration. A compound can induce changes in chromosomal number or structure, such as polyploidy or aneuploidy, breaks, deletions, and rearrangements. However, aneuploidy can arise as a result of both genotoxic and non-genotoxic events. Due to the diversity of endpoints, the potential genotoxicity or mutagenicity of a compound cannot be assessed by a single assay system (Savale 2018).

Chromosomal anomalies are the visible part of a large number of DNA changes (Obe et al 2002) which are a result of DNA Double Strand Breaks. It is known to be induced by non-carcinogenic agents at toxic levels (Hilliard et al 1998).

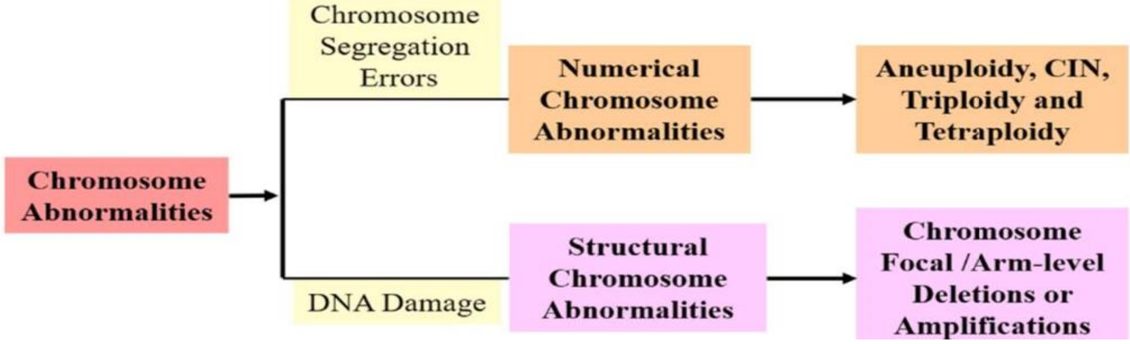

Fig. 4. Classification of Chromosome Abnormalities (Kou et al 2020).

Generally, they are important biological consequences of human and other organisms' exposure to ionizing radiation and other genotoxic agents. In general, chromosomal abnormality is considered numerically, for example, euploidy: monoploidy, polyploidy, aneuploidy: monosomy, trisomy (Holečková et al 2021) lead by chromosome segregation errors in mitosis, while structural chromosome abnormalities occur due to DNA damage and gain or loss of comprise focal/arm-level chromosome (Kou et al 2020).

3. Atlas Cedar

The Atlas cedar (*Cedrus atlantica* (Endl.) Manetti ex Carriere), also called Arz El Atlas in Arabic, Beguenoun or Idil in Berber (Sbabdji et al 2009), is an endemic species belonging to the *Pinaceae* family, a near-related species of tall, monoecious, coniferous, evergreen trees (Pijut 2000). It is believed to be the oldest tree after Pinus genus (Annemer et al 2023), holding a tremendous value for its technological, ecological, and biogeographical contributions. Its various applications, including sawing and charcoal production, make it a coveted resource in high demand (Ez-Zriouli et al 2023). Renowned for its superior wood utilized in both furniture crafting and construction endeavors (Moustaid et al 2023).

Fig. 5. Trees of *Cedrus atlantica* from the national parc of Chrea, Algeria (© 2024).

It is a native of North Africa (Morocco and Algeria) that grows at high altitudes on mountain slopes. It prefers moist and dry soil and can grow in both full and partial sunlight. However, because of human exploitation for wood and timber, this plant is endangered (Saini 2023). In Algeria, where the species naturally occupies 33,000 hectares spread among several massifs (Rabhi et al 2018) including Aures, Belezma, Hodna, Djbel Babor, Djurdjura, Blida and

Ouarsenis (Linares et al 2011). However, it grows on about 133,000 hectares in Morocco's Middle Atlas (Moustaid et al 2023).

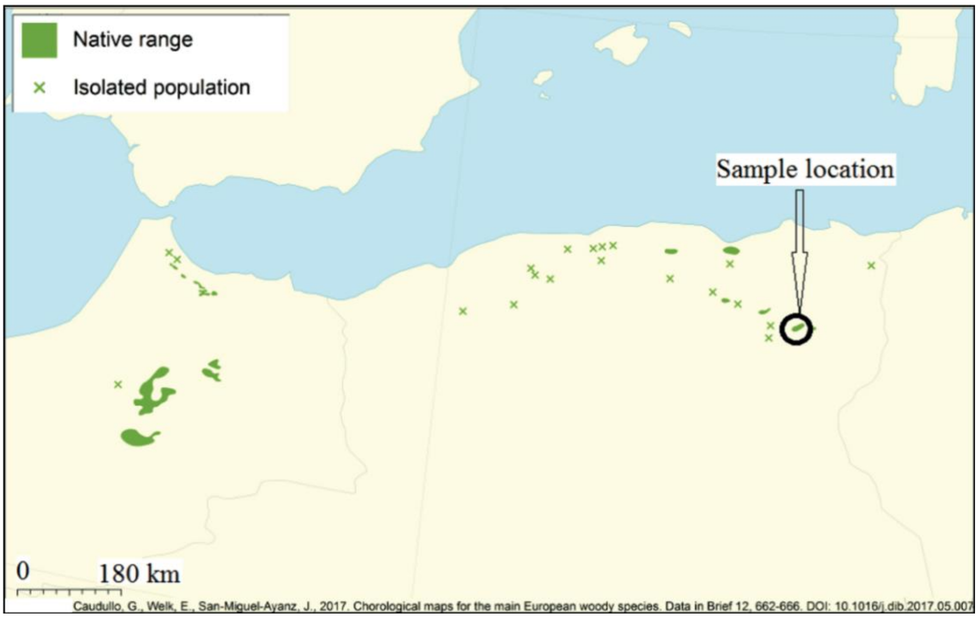

Fig. 6. The location of the study site (Batna) and distribution of *Cedrus atlantica* (Benhizia et al 2023).

Cedrus atlantica has a long history of use in traditional medicine, it is used for managing depression, stress-related disorders, anxiety, certain cognitive disorders, insomnia, postoperative pain (Martins et al 2015) and hair loss issues (Ezekwe et al 2020). It is renowned for its therapeutic properties and manifests a spectrum of biological activities, such as antioxidant, anti-inflammatory, analgesic and antimicrobial activities. It holds also other activities, such as anti-tumor and antiseptic activities (Belkacem 2021). A phytochemical study uncovered that it contains a range of bioactive compounds, including sesquiterpene hydrocarbons, monoterpene hydrocarbons, and oxygenated monoterpenes (Maya et al 2017).

4. *Allium cepa* assay

Higher plants are widely employed in monitoring toxicity studies because they are acknowledged as superior genetic models for identifying environmental mutagens (Leme and Marin-Morales 2009). *Allium cepa* (Onion) for instant is a tool used to identify and quantify the extent of changes in an organism's system caused by carcinogens, mutagens, or chemicals that can cause harm. By examining chromosomal aberrations, this method allows researchers to describe the effects of these damages and better understand the impact of these substances on the system (Cabuga et al 2017). The species is commonly used to assess the cytotoxic, genotoxic, and mutagenic effects of various substances and environmental samples. One key indicator of this is the mitotic index (MI), which can be used as a reliable measure to evaluate the cytotoxicity of different agents and substances (Özkara et al 2014). Its root meristem cells are extremely vulnerable to chemical-induced genetic damage (Firbas and Amon 2014). Under a microscope, *A. cepa*'s monocentric chromosomes are simple to view and examine due to their large size and small number (Nagaki et al 2012).

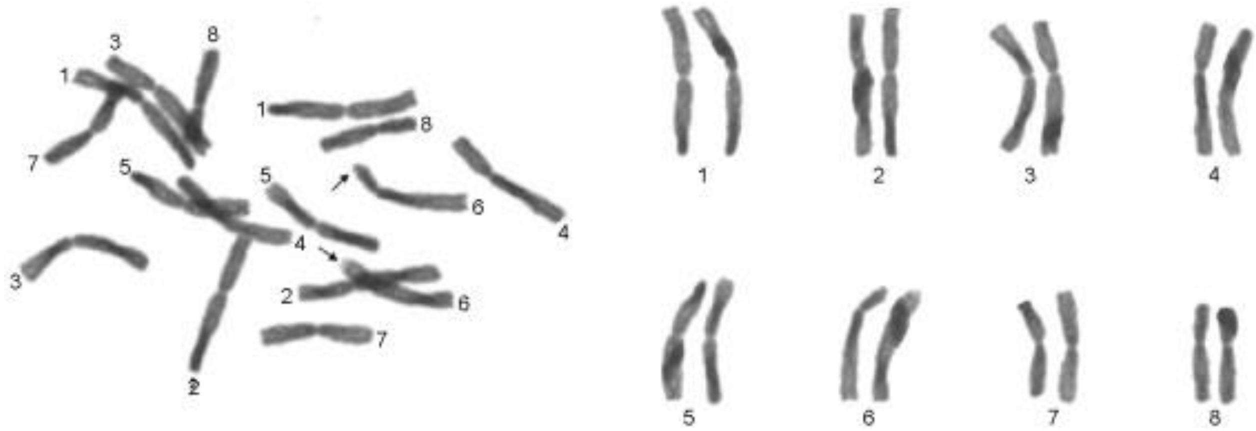

Fig. 7. *A. cepa* karyotype ($2n = 16$). Arrow indicating the nucleolus organizer region (NOR) located in the terminal portion of the short arm of pair 6 (Leme and Marin-Morales 2009).

Methodology

1. Preparation of plant extracts

Needles of *Cedrus atlantica*, collected from the national parc of Belezma (Btana, Algeria), were cleaned of any impurities then air dried in the dark at room temperature. After that, needles were crushed in an electric blinder to generate a somewhat soft powder that was stored in a well-covered jar and kept out of light.

The aqueous extract was prepared through macerating 50 g of plant powder and 500 mL of distilled water in a glass flask. The mixture was left with stirring at room temperature in the dark for 24 hours. The extract was then filtered using Whatman paper, and the obtained filtrate was dehydrated in an incubator at 37 °C to obtain a dry residue, which was then stored in a clean box.

The ethanolic extract was prepared by putting 33.5 g of powder in a glass flask containing 335 mL ethanol 70 %. The mixture was agitated for 3 hours and then macerated for 24 hours. The resulting extract was filtered using Whatman paper, and the filtrate was dehydrated in an incubator at 37 °C to obtain a dry residue, which was then stored in a clean container.

Extraction yield is the proportion of the amount of extracted analyte to the quantity of plant sample:
Yield (%) = Weight of the dry extract x 100/ Initial weight of the dry plant.

2. Phytochemical compounds

2.1. Polyphenols

Using a micropipette, a volume of 200 µL of each extract (aqueous and ethanolic) with the same concentrations as before (0.1, 0.5, 0.75, 1 mg/mL), was added to Eppendorf tubes along with a ten-fold diluted 1 ml of Folin-Ciocalteu reagent. The tubes are shielded from light and incubated for 5 min at room temperature. Next, each tube is filled with 800 µl of a 7.5% sodium carbonate (Na2CO3) solution. After shaking, the tubes are held for 30 min. The absorbance is measured at 765 nm. The total polyphenol content is derived from the calibration curve, and the findings are expressed as mg gallic acid equivalent (GAE)/mL of the extract. Each experiment was replicated a minimum of three times.

2.2. Flavonoids

In an Eppendorf tube, 1 mL of each extract was mixed with 1 ml of Aluminum chloride ($AlCl_3$) with 2% methanol. The solution is forcefully stirred using a vortex before being incubated in the dark for 15 minutes. The absorbance is instantaneously measured at 430 nm against a blank (distilled water). The total flavonoid content is estimated by utilizing a calibration curve, and the findings are presented as mg quercetin equivalent (QE)/mL extract. Each experiment was repeated a minimum of three times.

2.3. Tannins

Each extract was added in a volume of 50 µL to 1500 µL of the 49% vanillin/methanol solution and then mixed with force. Afterward, 750 µl of concentrated hydrochloric acid (HCl) was added. For 20 min, the resulting mixture is set to react at room temperature. Absorbance is measured at 550 nm to a blank. The overall tannin content is assessed using a calibration curve, and the results are indicated as mg quercetin equivalent (TAE/mL) extract. Each experiment was replicated at least three times.

3. Antioxidant activity using DPPH radicals scavenging assay

The radical scavenging activity of the extract was assessed utilizing 1,1-diphenyl-2-picrylhydrazine (DPPH). 0,2 ml of plant extract was put in Eppendorf tubes, then 1 ml of DPPH solution (2mg in 100 ml of methanol) was added. The tubes were incubated in the dark for 30 minutes. After the incubation, the absorbance measurements for each sample were set to 517 nm.

DPPH Scavenged (%) = [(Acontrol – Asample)/ Acontrol] × 100

4. Mitotic index analysis

Onion plant, *Allium cepa* (2n = 16), is a member of the Alliaceae family. Onion bulbs were purchased from a local market in Tiaret (Algeria) according to their size and were cleaned in the distilled water, after removing the roots and first peel. Prior to initiating the *Allium* anaphase-telophase test, outer scales of the bulbs and the dry bottom plate were removed without destroying the root primordia. After 2 days of contact with the distilled water in the darkness at 22 ± 4 °C, bulbs which roots have a length from 1 to 2 cm were used for this study. This consists on placing each of the germinated bulbs on a test tube containing distilled water so that only roots are immersed.

After that, the best growing four bulbs, were exposed for 48 h to different aqueous and ethanolic concentrations of *Cedrus atlantica* solutions (0.1, 0.5, 0.75 and 1 mg/mL respectively). The concentrations used in this study are chosen to match those found in our previous ethnopharmacological studies regarding the traditional medicinal use of *Cedrus atlantica* in Algeria (Taïbi et al. 2021). Distilled water serves as negative control while methyl methane sulfonate (MMS, 10 ppm) was employed as positive control.

After 48 h of exposure in the darkness, the last 2 cm of root was taken and fixed in Carnoy's solution at 4 °C for at least one night and then stored for long term in ethanol 70 % at 4 °C. These roots were then hydrolyzed in a solution 1 N HCl during 8 min and stained with Feulgen dye shielded from the light (Silva et al. 2011).

The mitotic index (MI), poly-nucleus, and the frequencies of chromosomal aberrations (Cas) were carried out according to Saxena et al. (2005). For each sample, five roots were prepared by crushing the root ends with 45 % acetic acid (1 root tip/slide); 5000–6000 cells were counted for determining the different stages of mitosis and were expressed as percentage. For the chromosome aberration test, 500–600 cells in anaphase or telophase were examined. Types of aberrations scored include disturbed anaphase-telophase, chromosome laggards, stickiness and anaphase bridges.

Results

1. Evaluation of the yield of extraction

The ethanolic extract yield of Cedrus atlantica was higher than the aqueous extract yield. The respective recorded values are 15,01% and 13,8%.

Table 1. Yield of the plant extracts.

Extract	Yield %
Aqueous extract	13,8
Ethanolic extract	15,01

2. Evaluation of the phytochemical compounds

2.1. Total phenolics content

The examination of the data reveals a notable difference in the total phenolic content among aqueous and ethanolic plant extracts in the various studied concentrations (Fig. 9).

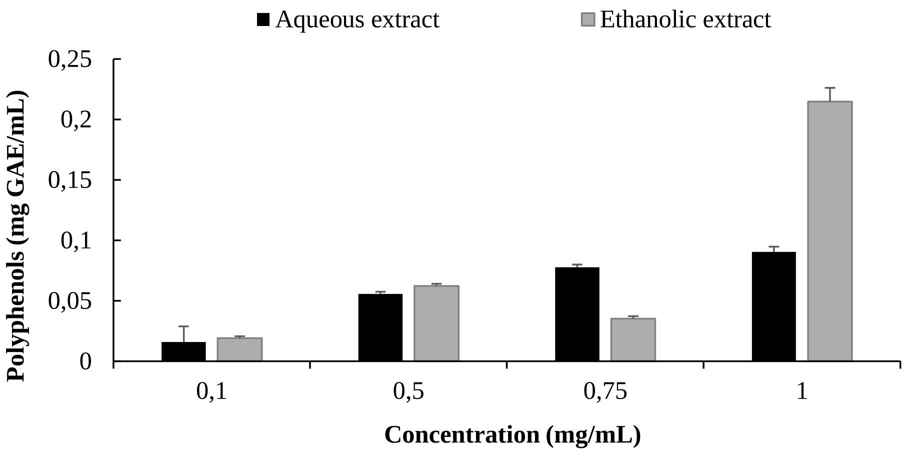

Fig. 9. Variation of total phenolic contents in the aqueous and ethanolic extracts of *C. atlantica*.

In general, ethanolic extract marked the higher content of total phenolic content than aqueous extract, except in the third concentration of 0,75 mg/mL. The content of phenolic compounds ranged between a higher value of 0,21±0,01 mg GAE/mL, in the treatment with 1 mg/mL of ethanolic extract, and a lower value of 0,016±0,01 mg GAE/mL, in the treatment with 0,1 mg/mL of the aqueous extract.

2.2. Total flavonoids content

The analysis of the data reveals a notable difference in the flavonoids content among aqueous and ethanolic plant extracts (Fig. 10).

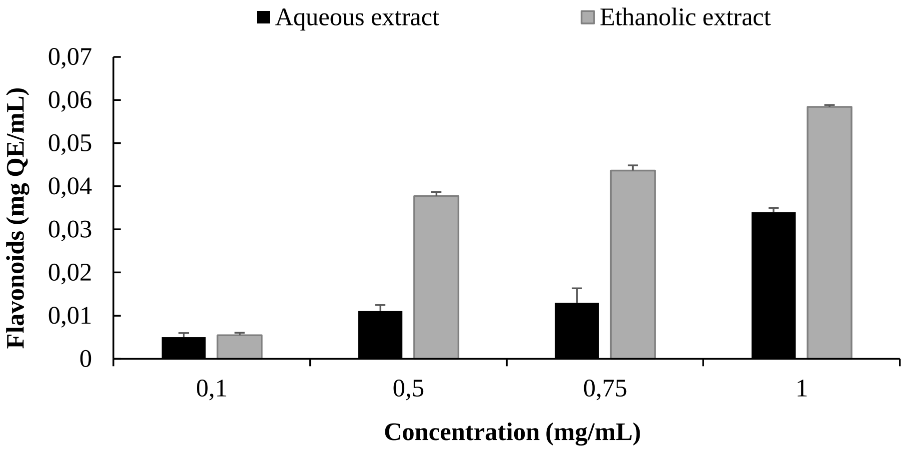

Fig. 10. Variation of flavonoids contents in the aqueous and ethanolic extracts of *C. atlantica*.

The ethanolic extract exhibited a higher content of total flavonoids than the aqueous extract. The flavonoids content ranged between 0,005±0,001 mg QE/mL in the treatment with 0,1 mg/mL and 0,03±0,001 mg QE/mL in the treatment with 1 mg/mL of the aqueous extract while it ranged between 0,005±0,001 mg QE/mL and 0,06±0,001 mg QE/mL respectively in the treatment with ethanolic extract.

2.3. Total tannins content

By the same, the content of tannins differs among aqueous and ethanolic plant extracts (Fig. 11). The aqueous extract exhibited higher tannins content under treatments bellow 0,75 mg/mL while the opposite is observed in the treatment with 1 mg/mL.

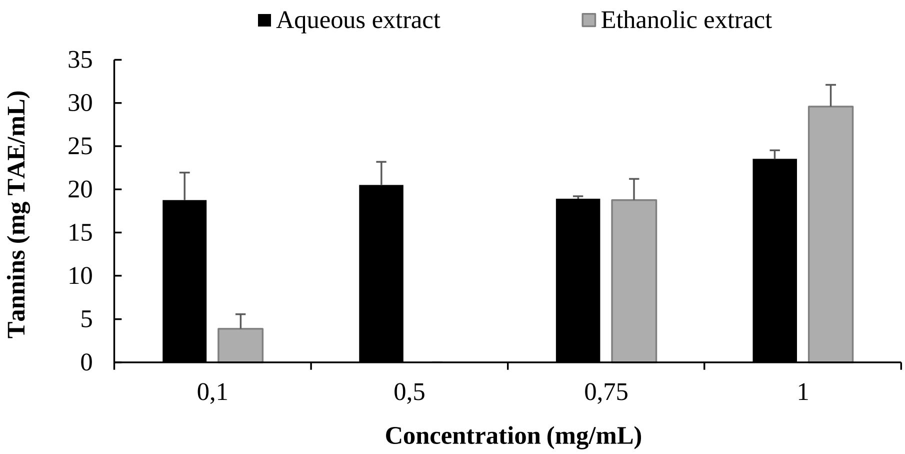

Fig. 11. Variation of tannins contents in the aqueous and ethanolic extracts of *C. atlantica*.

The content of tannins varied between a higher value of 23,5±0.99 mg TAE/mL in the treatment with 1 mg/mL aqueous extract and a lower value of 18,8±3,2 mg TAE/mL in the treatment with 0,1 mg/mL aqueous extract. Nonetheless, it ranged between 29,6±2,5 mg TAE/mL and 3,85±1,7 mg TAE/mL respectively in the ethanolic extract.

3. Evaluation of the antioxidant activity using DPPH assay

The antioxidant activity was measured by the DPPH scavenging assay. The antioxidant activity was higher in the ethanolic extract in comparison with the aqueous extract (Fig. 12).

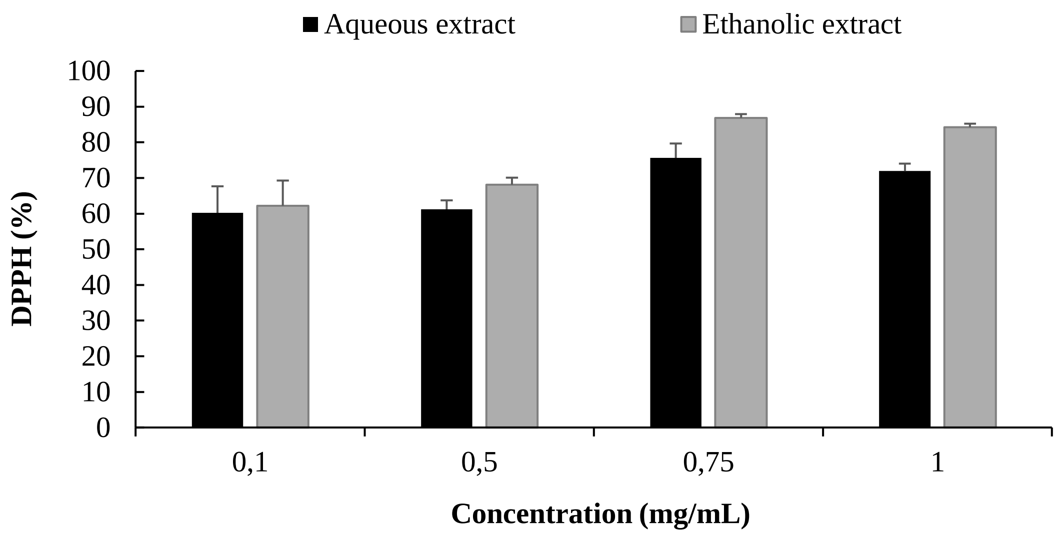

Fig. 12. Variation of antioxidant activity of the aqueous and ethanolic extracts of *C. atlantica*.

The highest antioxidant activity was observed in the treatment of 0.75 mg/mL for both extracts. The scavenging activity ranged between 60.24 and 71.99 % for the aqueous extract and between 62.18 and 84.2 % in the ethanolic extract.

4. Evaluation of the cytotoxicity

4.1. Root morphometry

- Roots number

Roots number of *Allium cepa* has been measured in each treatment before and after addition of plant extracts to the growth medium.

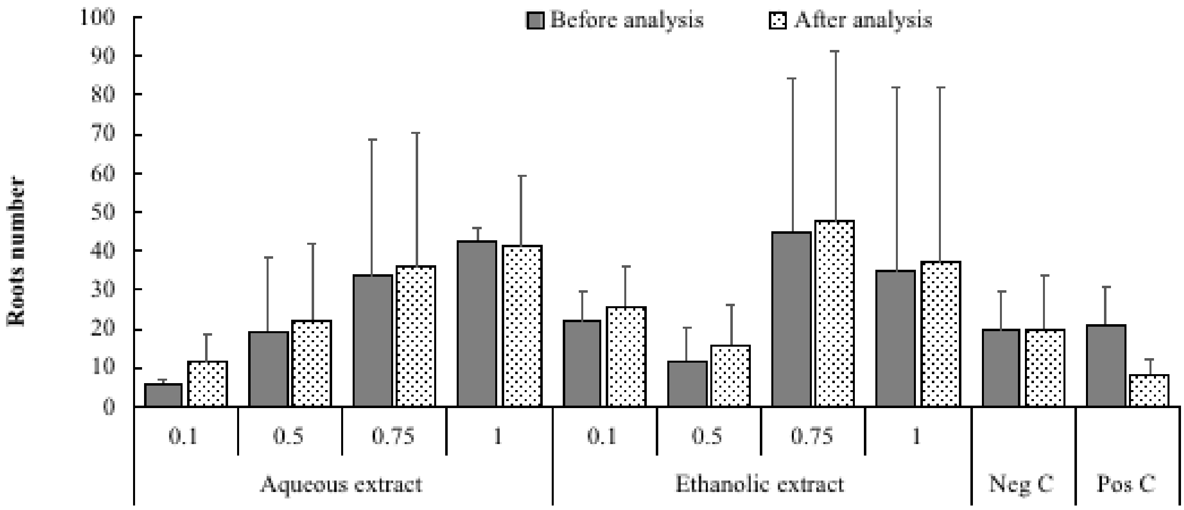

Fig. 13. Roots number before and after treatment with plant extracts.

There was a significant decrease in the number of roots in the positive control after the addition of MMS solution (from 21 to 10). However, roots number remains unchanged in the negative control with distilled water (20). As for the aqueous and ethanolic extracts, there was significant increase in roots number correlated positively with the concentration of the extract in the medium. Under each concentration of plant extract, the difference among roots number remains nonsignificant.

- Root length

Similarly, roots length has been measured before and after the addition of plant extracts. Growth of roots length was arrested after the addition of MMS solution as a positive control. However, it triplicated in length in the negative control with distilled water (Fig. 14).

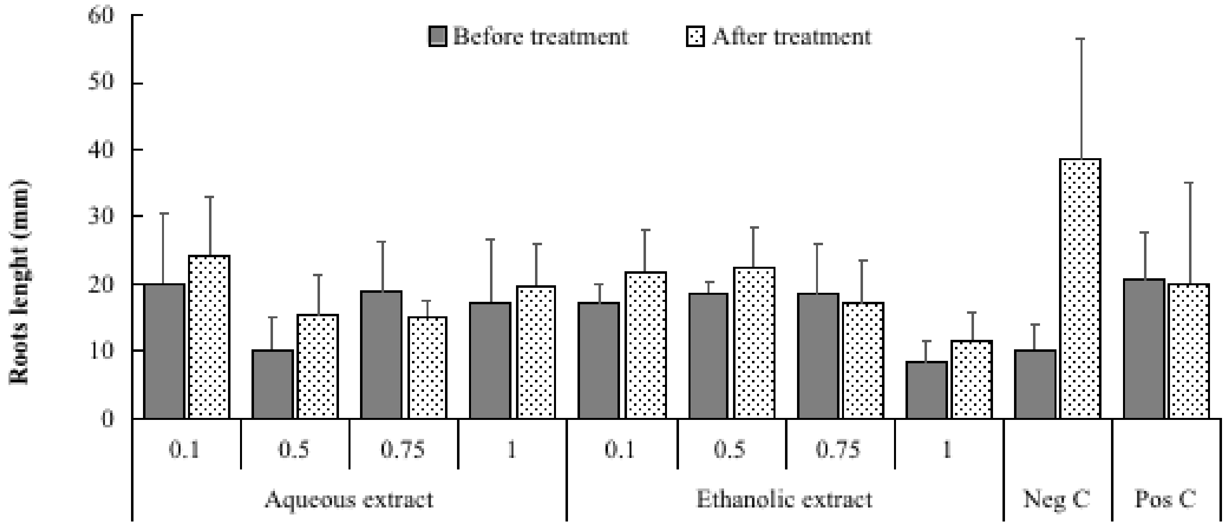

Fig. 14. Roots length before and after treatment with plant extracts.

Both ethanolic and aqueous extracts have exhibited roots length bellow that observed in the negative control. However, a slight increase was noted within each treatment when comparing before and after addition of plant extracts.

- Root diameters

Roots diameter was also evaluated before and after plant extracts addition (Fig. 15). There was a notable decrease of roots diameter in positive and negative controls. However, it significantly increased in treatments with ethanolic extract. The highest diameter was recorded in the treatment with 0,5 mg/mL ethanolic extract with 1,2 mm.

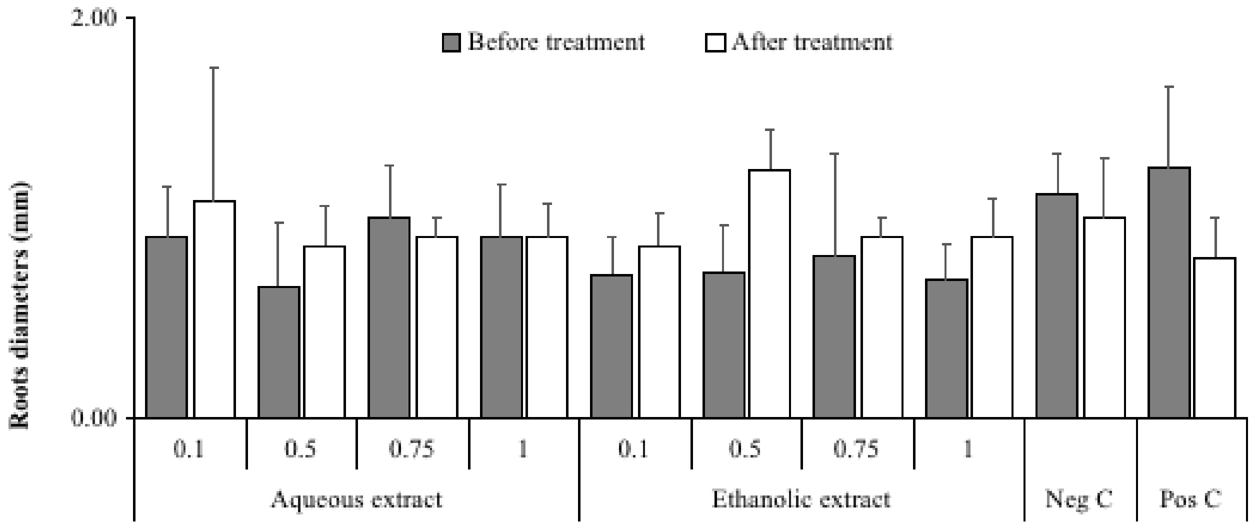

Fig. 15. Roots diameters before and after treatment with plant extracts.

The roots diameter increased also after addition of aqueous extract at 0,1 and 0,5 mg/mL while it remained unchanged above these concentrations. The highest value was observed in the treatment at 0,1 mg/mL with 1,1 mm.

4.2. Cell phases

- Mitotic index

Analysis of data demonstrated that *C. atlantica* extracts affects significantly the mitotic index (MI, %) and mitotic phases of *A. cepa* meristematic root cells. The rate of mitotic index decreased when the concentrations of the extracts increased (Fig. 16).

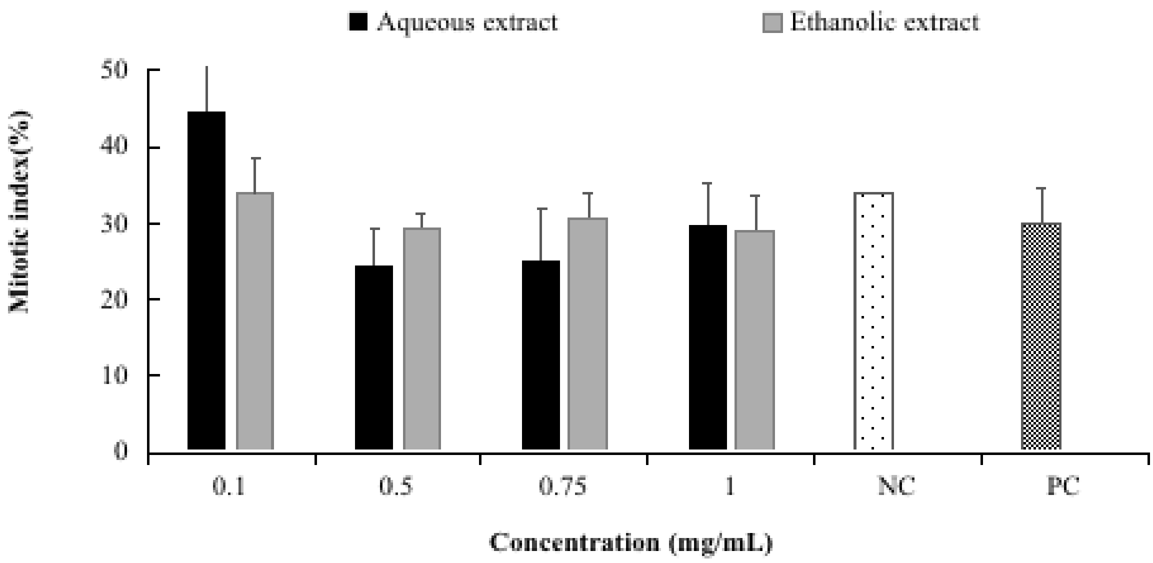

Fig. 16. The effect of *Cedrus atlantica* extracts on MI and mitotic phases in the root cells of *A. cepa*.

The highest MI percentages are obtained under treatments with aqueous extract at 0,1 mg/mL (44,08±11,54%). However, the lowest percentage was observed under treatment with 0,5 mg/mL (28.,14±5,44%). As for the ethanolic extract, the highest percentage of MI was recorded under treatment with 0,1 mg/mL (33,65±5,2%), while the lowest percentage was observed at 0,1 mg/mL (29,30±2,21%).

- Prophase

Analysis of data showed that the prophase cells number was significantly higher among all treatments. It appears that *C. atlantica* extracts did not affect the prophase cells number (Fig.17).

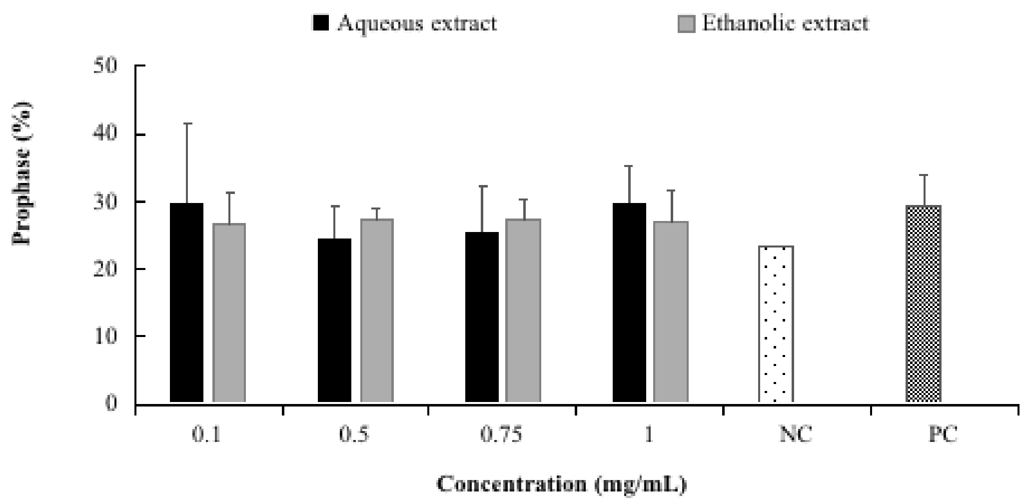

Fig. 17. The effect of *Cedrus atlantica* extracts on prophase in the root cells of *A. cepa.*

The highest prophase cells number was marked in aqueous extract under the treatment of 1 mg/mL (29,5±5,23%). While the lowest cells number was obtained under the treatment with 0,5 mg/mL (24,44±11,52%). On the other hand, the highest prophase cells number was observed in the treatment with 0,75 mg/mL (27,3±3,09%) of the ethanolic extract while the lowest cells number was observed at 0,1 mg/mL (26,6±4,32%).

- Metaphase

The data analysis showed that the *C. atlantica* extract had a significant impact on the metaphase stages of *A. cepa* meristematic root cells. As the concentration of the extract increased, there was a notable decrease in the metaphase cells number (Fig. 18).

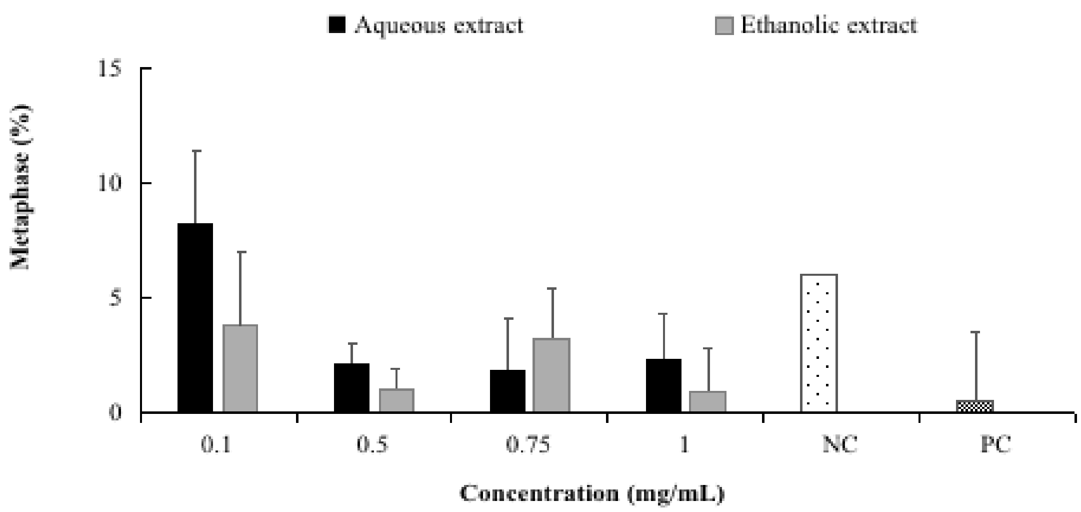

Fig. 18. The effect of *Cedrus atlantica* extracts on metaphase in the root cells of *A. cepa.*

The metaphase cells number was higher under the treatment with 0,1 mg/mL (8,24±3,22%) aqueous extract. However, the lowest cells number was observed under the treatment of 0,75 mg/mL (1,92±2,17%) aqueous extract. Nevertheless, the number of cells in metaphase ranged between 3,81±3 % and 0,96±0,83% in ethanolic extract treatments.

- Anaphase

C. atlantica extract affected significantly the anaphase phase of *A. cepa* meristematic root cells. As the concentration of the extract increases, the number of cells in the anaphase decreases (Fig. 19).

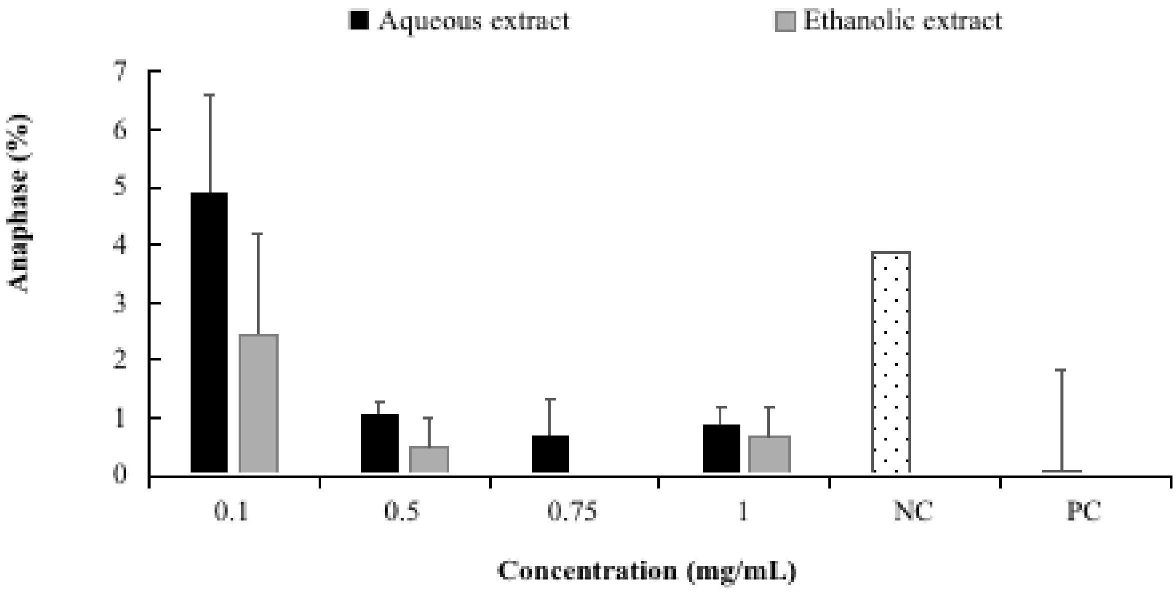

Fig. 19. The effect of *Cedrus atlantica* extracts on anaphase in the root cells of *A. cepa.*

The highest cells number in anaphase was recorded in the treatments with 0,1 mg/mL aqueous extract (4,49±1,7%). However, the lowest cells number was observed in the treatment with 0,75 mg/mL (0,71±0,64%). In contrast, the treatment with 0,1 mg/mL ethanolic extract demonstrated the highest cells number (2,44±1,76%) while the lowest cells number was observed in the treatment with 0,75 mg/mL (0%).

- Telophase

Analysis of data revealed that *C. atlantica* extract had a significant impact on the telophase phase of *A. cepa* meristematic root cells. The increase of the extract concentration is correlated with a decrease in telophase cells number (Fig. 20).

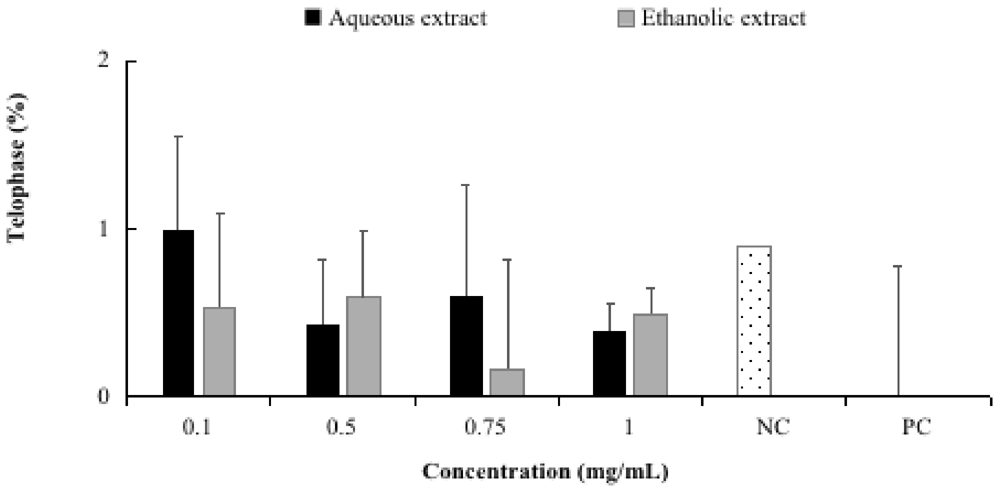

Fig. 20. The effect of *Cedrus atlantica* extracts on telophase in the root cells of *A. cepa.*

In aqueous extract, the highest telophase cells number was observed under treatment with 0,1 mg/mL (0,99±0,56%) while the lowest cells number was observed under treatment with 1 mg/mL (0,39±0,16%). Whereas, the highest cells number in ethanolic extract was observed in treatment with 0,5 mg/mL (0,59±0,56%). However, the lowest telophase cells number was observed at 0,75 mg/mL (0,16±0,12%).

5. Evaluation of the genotoxicity

The overall chromosomal abnormalities in *A. cepa* root tips were observed microscopically after treatment with *C. atlantica* extracts. The data analysis revealed that both water and ethanolic extracts had substantial effects on the chromosomes of *A. cepa* root tips. The ethanolic extract displayed more aberrations than the aqueous extract, with 395 and 327 anomalies, respectively. For both extracts, the rates of anaphase-telophase aberrations (disturbed anaphase-telophase, chromosomal laggard(s), adherence, and chromosomic bridge) were lower than the total rates of other anomalies (c-metaphase, binuclear cell, polyploidy, micronucleus, metaphase adherence).

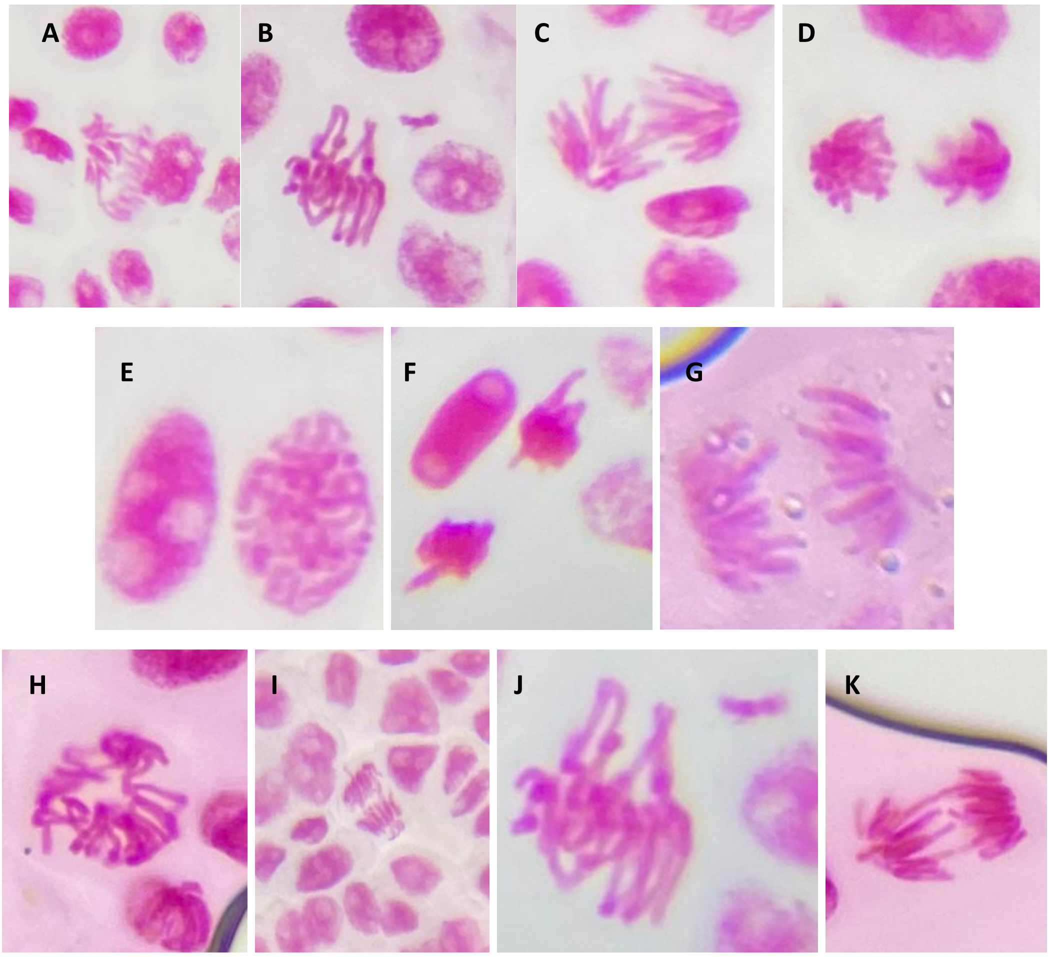

Fig. 21. Chromosomal aberrations induced by C. atlantica extracts.

A; Chromosome laggards, B; Polar slip and chromosomal bridge on anaphase, C; Telophase, D; Late chromosome in telophase, E; Late chromosome in anaphase, F; C-metaphase, I; Polyploid cell in prophase

The increase in total anomalies is also influenced by dose. The higher the concentration, the fewer anomalies are detected. This is could be due to the arrest of cells cycle in metaphase. Polyploidy was the most frequent chromosomal abnormality with a percentage of 47,18% out of the total amount of anomalies, followed by chromosomic bridge, disturbed anaphase, and micronucleus with 15,49%, 13,17%, and 7,74% respectively. The lowest rate of anomalies was observed in metaphase adherence with 0,4% while telophase adherence did not occur in all treatments.

Disturbed anaphase displayed a higher perturbation with 19 anomalies at 0,5 mg/mL in the aqueous extract. In addition, chromosome laggards highest rank was 13 anomalies at 0,1 mg/mL and none at 0,75 mg/mL, both in the aqueous extract.

Chromosomic bridges were frequent by 37 distributions at 0,1 mg/mL and none at 0,75 mg/mL of the ethanolic extract. C-metaphase highest rate was noticeable with 14 anomalies at 1 mg/mL of ethanolic extract and none at 0,1 mg/mL of the aqueous extract.

Binuclear cells displayed a higher rate with 26 anomalies at 1 mg/mL of aqueous extract, while the rest concentration had below 5 anomalies. Polyploid had a higher rate with 130 anomalies at 0,1 mg/mL and a lower rate with 11 anomalies at 1 mg/mL of aqueous extract. Micronucleus ranked higher anomalies at 1 mg/mL with 14 of ethanolic extract, and lowest at the same concentration of aqueous extract. Metaphase adherence was displayed only at 0,75 mg/mL aqueous extract with 4 anomalies.

In both aqueous and ethanolic extracts, treatment with 0,1 mg/mL displayed the highest rates of abnormalities while the lowest rates were obtained from treatment with at 1 mg/mL.

Discussion

Discussion

Aromatic plants have an ancient history in traditional medicine of numerous nations. Currently, there is a rise of interest in researching active biological substances of aromatic plant extract due to their variety and availability (Fitsiou and Pappa 2019). However, they are not free of side effects or toxicity. The level of toxicity can depend on the chemical composition of the plant extract itself, it can emerge from acute or chronic exposure even when using extracts with low cytotoxicity (Anywar et al. 2021). Aromatic plants could be cytotoxic, genotoxic and mutagenic (Bnouham et al. 2006).

The dosage can be a problem during the usage of medicinal plants in a traditional medicine due to the non-specification of the prescribed dosage (Ramulondi et al 2019). On that account, the objective of this study is to assess the cytotoxic and genotoxic effects of an endemic species that is used in Algerian traditional medicine, namely *C. atlantica*, using *Allium cepa* tests. The phytochemical compounds and the antioxidant activity were also evaluated.

Aqueous extracts have high polarity and are capable of dissolving a broad spectrum of compounds. Moreover, they are cost-effective, non-toxic, and non-flammable (Abubakar and Haque 2020). Many natural products are poorly soluble in water, making it nearly impossible to properly solubilize or extract them using only water (Lajoie et al. 2022). Unfortunately, there are some cons to consider as well. Water can promote bacteria and mold growth, as well as produce specific chemical changes or hydrolysis (Abubakar and Haque 2020). However, a combination of water and alcohol (ethanol) may be a more effective solvent mixture than a single solvent (Plaskova and Mlcek 2023).

Alcohols are polar molecules that fall somewhere between those that are lipophilic, and hydrophilic (Wade 2024). Unlike water, alcohol cures itself in strengths above 20%, which means it does not have to be maintained as much (Abubakar and Haque 2020).

The results of yields vary between extracts; the ethanolic extract exhibited the highest yield with 15,01% making it a suitable solvent for phytochemical extraction due to its ability to effectively extract a wide range of compounds (Stanciauskaite et al. 2021). Belkacem et al. (2021) reported that the type of solvent used in the extraction process affected the various yields of the branch extracts.

The phenolic content increased according to the rise of the extract concentrations. Studies conducted by Belkacem et al. (2021) and Ameggouz et al. (2024) demonstrate that *C. atlantica* is rich in phenolic compounds nearly about 143.5±12.72 mg EqC/g extract and 237.23±1.61 mg

GAE/g in ethanolic extract respectively. Total phenolic content of our study was about 0.2±1 mg GAE/mL, different result when compared with the other published studies. It could be due to the variation of region, plant parts, harvesting period, soil composition, and storage process. It can be also because of the different concentrations and the amount of the used powder.

The flavonoid content increased in response to the rise of the extract concentrations. It was proved by Ameggouz et al. (2024) and Deffa and Daikh (2024) that macerated *C. atlantica* extract had a higher flavonoid content of 81.53±1.13 mg QE/g extract. Tannins content rose in tandem with the increase in extracts concentrations. The tannin content of the aqueous extract compared to the ethanolic one was higher when treated below 0,75 mg/mL, while it was lower when treated at 1 mg/mL. Bouyahia et al. (2022) reported that high amounts of tannins were found in the acetone, methanol, and ethanol extracts of *C. atlantica*, with 189,4 to 237,23 mg catechin equivalents per gram of extract.

Antioxidant assays are essential for the high-throughput, cost-effective evaluation of antioxidant capabilities in natural goods, such as food samples and medicinal plants (Sadeer et al 2020). In this study, the DPPH test was used to determine the antioxidant activity of the plant extracts under study. The process of DPPH testing involves removing DPPH, which is a free radical that has stabilized. When the free radical DPPH interacts with an odd electron, it produces a strong absorbance at 517 nm (Baliyan et al. 2022). according to Ameggouz et al. (2024), it was revealed that ethanolic extracts had stronger antioxidant activities. Bouyahia et al. (2022) demonstrated that the high concentration of flavonoids and phenolic compounds in the *Cedrus atlantica* extracts was thought to be the cause of their strong antioxidant activity. It is well known that polyphenolic substances have antioxidant qualities (Kačániová et al. 2022).

The cytotoxic impacts of both aqueous and ethanolic extracts as indicated by *Allium cepa* assay show a growth inhibition in all the treatments. The ethanolic extract has the highest cytotoxic effect compared to the aqueous extract. The concentration that arrests the phases is the fourth concentration at 1 mg/mL. As for genotoxicity, ethanolic extract caused more chromosomal aberration when the concentration increased. Therefore, the fourth concentration 1 mg/mL caused the most chromosomal damage.

Several *in vivo* and *in vitro* studies have been conducted to assess the cytotoxic and genotoxic effects of *C. atlantica* extracts. For instance, Huang et al. (2020) revealed the anti-liver cancer potential of *C. atlantica* extract *in vitro* and *in vivo* by using HCC cells treated with concentration of 25 µg/mL and 50 µg/mL. *In vivo* experiments on mouse with HepG2 liver cancer treated with *C. atlantica* extract (200 mg/kg) demonstrated that *C. atlantica* extract has anti-tumor activity and improve survival rate.

Chang et al. (2021) evaluated the anti-Glioblastoma (GBM) activity of *C. atlantica* extract with a concentration of 40 µg/mL using human GBM cells (DBTRG-05MG) and rat GBM cells (RG2), *in vitro* and *in vivo*. *In vivo* study based on F344 mice were injected with *C. atlantica* extract at a dose of 200 mg/kg every 2 days via subcutaneous administration for 20 days. The results indicated that *C. atlantica* extract significantly inhibited the growth of GBM cells both in the laboratory and in living organisms and improved survival rates in animal models with subcutaneous and orthotopic tumors. Additionally, *C. atlantica* extract elevated the levels of reactive oxygen species (ROS), leading to DNA damage, which resulted in the arrest of the cell cycle at the G0/G1 phase and induced cell apoptosis.

López-Romero et al. (2018) and Sevastre et al. (2022) reported that phytochemicals like flavonoids (flavanols, catechins, flavones, anthocyanidin), tannins and phenolics (hydroxy-benzoic acid and hydroxy-cinnamic acids) can induce genotoxic and cytotoxic abnormalities. They can also disrupt cell signaling pathways, such as cell cycle, survival, and even death. Madani et al. (2021) have shown that metabolites such as alkaloids and phenols can cause polyploidy. Alexa-Stratulat et al. (2017) showed that vinca alkaloids affect cell-cycle in the metaphase. They have antitumor activity by attaching to microtubular proteins and inhibiting tubulin polymerization. This disrupts the assembly of microtubule during cell division and causes the cell to stop in metaphase. Hervás et al. (1974) showed that Colchicine inhibits the fibrous arrangement of the material forming the spindle and causes the disintegration of these fibers after they have formed.

Exposure of *A. cepa* root tips to the studied plant extracts led to various chromosomal abnormalities, including disrupted anaphase-telophase, chromosomal laggard(s), adhesion, and chromosomic bridge, as well as c-metaphase, binuclear cells, polyploidy, micronucleus, and others. The genotoxic effects of plant extracts are caused by their richness of specific chemicals (Bardoloi and Soren 2022). As a result, the findings indicate that the effects of *C. atlantica* are concentration-specific (Oney-Birol and Balkan 2019). Anaphase-telophase aberration was found to have a lower overall rate than other abnormalities (c-metaphase, binuclear cell, polyploidy, micronucleus, and metaphase adherence). Possibly because *C. atlantica* extracts primarily inhibit or halt the metaphase which explain why the number of cells in cycle phases decreased along with the number of anomalies at higher concentrations. Mercykutty et Stephen (1980) demonstrated that the C-metaphase observed in this study seems to result from inhibiting protein synthesis during mitosis. Hence, the presence of binucleate or multinucleate cells in treated materials may result from disruptions that prevent cytokinesis. Numerous chemicals are recognized to possess this capability. Khallef et al. (2013) stated that chromosome laggards and chromosome bridge could occur by the effect of bromoform and chloroform on microtubule formations that may arise due to inhibition of tubulin polymerization. Chromosome laggards at anaphase may be due to the failure of the chromosomes or acentric chromosome fragments to move to either of the pole and may result in micronucleate cells.

The mitotic index indicates the percentage of cells undergoing mitosis at a given time, serves as a gauge of cells' ability to divide and the pace of cell division. Its purpose is to identify areas of growth within a tissue and to classify the cell types engaged in division (Campbell 1983). The mitotic index serves as a measurement for detecting cytotoxic substances. A notable decrease in mitotic activity is an indication of genotoxic potential. Decreases in the mitotic index are usually linked to arrest of DNA synthesis or a stop in the G2 phase. Conversely, an elevation in the mitotic index can be the result of a shortened period required for DNA repair (Graña 2018).

Conclusion

Conclusion

Several plant species are used to treat a wide range of ailments. For instance, the Atlas cedar *Cedrus atlantica*, endemic to North Africa, is highly valued for its hard, fragrant timber and the essential oil that has been used traditionally to treat inflammation. However, as any other plant, *C. atlantica* has its own side effects.

The present study demonstrates for the first time the cytotoxic and genotoxic effects of *C. atlantica* aqueous and ethanolic extracts using *Allium cepa* test. A significant toxicity effect was observed in the ethanolic extract compared to the aqueous extract at concentrations above of 1 mg/mL.

Precautions must be taken due to toxicity concerns. Certainly, more studies are required to investigate the toxicological potential of this plant species.

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