CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

Effect of Artificial Polyploidization on the Biological Activities of Medicinal and Ornamental Plants

Doctoral dissertation thesis

Author: Neha Gupta, M.Sc.

Supervisor: prof. Ing. Eloy Fernández Cusimamani, Ph.D.

Praha, 2025

Abstract

Medicinal and ornamental plants show emerging phytomedicinal potential which boost their economic and therapeutic value in pharmaceutical, nutraceutical, and cosmetic industries. Plant extracts and essential oils from these plants are rich in phytochemicals, offering a plethora of pharmacological properties such as antimicrobial, antiviral, anti-inflammatory, and antioxidant activities. Yet, a knowledge gap remains in understanding the molecular interactions of phytochemicals and their biological activities. Additionally, the low phytochemical yield and bioactivity of these plants, combined with high market demand, highlight the need for solutions.

To circumvent this, artificial polyploidization was employed to enhance the metabolite biosynthesis, stress tolerance, and biological activities via chromosomal duplication. Integrating findings from three peer-reviewed publications, this thesis evaluates the impact of oryzalin-induced polyploidization on *Thymus vulgaris* L., a medicinal aromatic plant, and *Callisia fragrans* (Lindl.) Woodson, an ornamental species. In *T. vulgaris*, polyploidization significantly increased essential oil yield, enriched phytochemical composition, and enhanced *in vitro* antimicrobial, antioxidant, and anti-inflammatory activities. Furthermore, it also addresses the molecular interactions of phytochemicals through *in silico* molecular docking analysis of major compounds of *T. vulgaris*. This analysis demonstrates high binding affinity to essential biological proteins, supporting the correlation between phytochemical content and biological activities. Similarly, polyploid *C. fragrans* exhibited enhanced volatile and non-volatile phytochemical content, correlating with superior *in vitro* antioxidant, antimicrobial, and anti-inflammatory activities, alongside with pronounced cell viability effects. Additionally, this thesis incorporates a comprehensive mini-review highlighting the potential of artificial polyploidization in medicinal and aromatic plants to optimize metabolites biosynthesis and enhance biological activity, with a focus on insights for upregulating natural metabolites responsible for higher biological activities.

By integrating plant biotechnology, enhanced phytochemical profiling, bioactivity assays, and computational biology, this doctoral thesis presents a robust framework for enhancing pharmaceutical and economic value of these plants through artificial polyploidization to meet growing global demand.

Keywords: artificial polyploidization, biological activities, *in vitro*, medicinal plants, metabolites

Declaration

I hereby affirm that the content of this thesis, titled "**Effect of Artificial Polyploidization on the Biological Activities of Medicinal and Ornamental Plants**", submitted in partial fulfillment of the requirements for the Ph.D. degree at the Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague, is my original work, except where explicitly acknowledged in the references or acknowledgments sections. Furthermore, I confirm that no part of this work has been submitted for any other academic degree at this or any other institution.

Place and Date: Prague, 2025 **……**

Neha Gupta

Acknowledgement

With immense gratitude and profound humility, I wish to express my heartfelt appreciation to my supervisor, Professor Eloy Fernández Cusimamani. His unwavering belief in my potential, insightful guidance, and the academic freedom he granted me have been the bedrock of this research. His mentorship has not only shaped my scholarly journey but has also inspired me to strive for excellence with unwavering dedication.

I am beyond grateful to my partner, Mr. Soham Bhattacharya, from the Department of Agroecology and Crop Production, whose unwavering support has been my anchor through the highs and lows of this journey. His wisdom, patience, love and endless encouragement have been a source of strength, and his unwavering presence through every challenge has been nothing short of a blessing.

I owe an immeasurable debt of gratitude to my grandfather, Lt. Shiba Prasad Gupta; my father, Mr. Shashi Kant Gupta; my mother, Mrs. Krishna Gupta; my brother, Mr. Vineet Gupta; my grandmother, Smt. Jyotsna Gupta; and every cherished member of my family. Their unconditional love, sacrifices, and unwavering faith in me have been my guiding light, reminding me that no distance is too great when hearts remain connected. Without them, this journey would have been incomplete.

To my incredible collaborators, your generosity, efficiency, and steadfast support have ensured that I had everything I needed to pursue my research. I deeply appreciate your commitment and contributions, which have been instrumental in shaping this work.

I am endlessly grateful to my dear friends and colleagues whose camaraderie turned challenges into cherished memories. A special mention to Mr. Adrish Dutta, whose kindness, encouragement, and shared experiences have enriched this journey beyond measure.

Lastly, with the deepest reverence, I bow in gratitude to lord Shiva, whose grace, strength, and divine guidance have carried me through every moment of doubt and perseverance. This dissertation is a testament to His unwavering presence in my life.

List of abbreviations

ABTS: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

AMA: Antimitotic Agent

AP: Artificial polyploidization

APPI: Antimitotic Agent-induced Polyploidy Induction

COX-1: Cyclooxygenase-1

COX-2: Cyclooxygenase-2

DNA: Deoxyribonucleic Acid

DPPH: 2,2-Diphenyl-1-picrylhydrazyl

EO: Essential Oil

GMO: Genetically Modified Organism

MAP: Medicinal and Aromatic Plant

MD: Molecular docking

MOP: Medicinal and Ornamental Plant

ORAC: Oxygen Radical Absorbance Capacity

PE: Plant Extract

PGR: Plant Growth Regulator

RT-PCR: Reverse Transcription Polymerase Chain Reaction

SM: Secondary metabolite

SP: Synthetic Polyploidization

TFC: Total Flavonoid Content

THC: Tetrahydrocannabinol

TPC: Total Phenolic Content

List of tables

List of figures

Table of Contents

1. Introduction

Medicinal and ornamental plants (MOPs) have been used for millennia in both conventional and complementary medical systems. They are also employed in the food and cosmetics industries for flavoring and fragrance (Ghorbanpour et al., 2017). Additionally, MOPs hold significance from conceptual, landscape architecture, and commercial viewpoints. Plant extracts (PEs) from MOPs contain various phytochemicals, including alkaloids, flavonoids, terpenoids, and phenolic compounds, responsible for biological activities such as antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, and anticancer properties (Sharma et al., 2024; Gupta et al., 2023; Gupta et al., 2024). Essential oils (EOs) from plants like *Lippia alba*, *Melissa officinalis*, *Thymus vulgaris*, *Sideritis cypria*, *Origanum dubium*, *Mentha piperita*, *Thymus capitatus*, and *Salvia fruticosa* exhibit notable antioxidant, antimicrobial, and anti-inflammatory activities (Velásquez et al., 2023; Chrysargyris et al., 2024).

With growing recognition of the health benefits of MOPs, there has been a shift towards plant-based products in cosmetics, pharmaceuticals, food packaging, aquaculture, and fodder industries. This trend is driven by concerns over the toxicity of synthetic alternatives and environmental degradation, leading to the global rise of organic, natural, and green consumerism (Kuebutornye et al., 2024; Malik et al., 2023; Priya et al., 2024). However, this growing demand creates a gap between supply and market needs, exacerbated by low PEs yields and high labor requirements. Over-harvesting and gene depletion further complicate the situation (Jaouadi et al., 2023). Additionally, the chemical composition and biological activity of PEs are influenced by various factors such as geography, environment, agroclimatic conditions, and genetics (Mehalaine et al., 2021), making sustainable production increasingly challenging. Thus, improvised breeding approaches are needed to balance cultivar production with biodiversity preservation. To meet significant market demand, improving MOP quality by enhancing their phytochemical yield and biological activities is crucial. These traits are often inherently low, but various breeding approaches, such as backcross breeding, mass selection, pure-line selection, precursor feeding, *in vitro* culture, genome editing, and metabolic engineering, can reinforce them (Ahmar et al., 2020). However, traditional breeding methods are time- and capital-intensive, typically requiring 8 to 10 years or more to develop new cultivars (Begna 2022).

Conversely, artificial polyploidization (AP) or synthetic polyploidization (SP) has emerged as a promising breeding strategy due to its efficiency and applicability in both *in vitro* and *in vivo*

conditions. AP involves chromosome doubling, typically using antimitotic agents (AMAs) like colchicine or oryzalin, which disrupt normal cell division (Soltis et al., 2015). In MOPs, polyploidization can enhance physiological, morphological, anatomical, and biochemical traits (Mohammadi et al., 2023; Bharati et al., 2023). However, its effects are sometimes unpredictable, and gene duplication may negatively impact certain traits (Xie et al., 2012; Carusa et al., 2013). Selecting the right genotypes and traits is essential for maximizing the benefits of this technique (Lamlom et al., 2024). Studies suggest that AP can enhance the production of primary and secondary metabolites (SMs), influencing the biological activities of polyploid plants (Gupta et al., 2024; Herawati et al., 2024). Despite these findings, research on the bioactivity of SMs in polyploid plants is still in its emerging stages.

This thesis focuses on the comprehensive evaluation of oryzalin-induced polyploidization in *Thymus vulgaris* L. and *Callisia fragrans* (Lindl.) Woodson, with the aim of enhancing phytochemical composition and biological activities. In the first study, the effects of polyploidization on *T. vulgaris* demonstrated a significant increase in EO yield, an enriched phytochemical profile, and a marked improvement in its *in vitro* antimicrobial, antioxidant, and anti-inflammatory activities. Further investigations through *in silico* molecular docking (MD) analysis, examining major phytochemicals from *T. vulgaris* was conducted. The analysis revealed that these compounds exhibit high binding affinity to critical biological proteins, reinforcing the correlation between enhanced phytochemical content and improved biological activity observed in the polyploid plants. This set the foundation for further investigations into how polyploidization can impact other MOPs. In the second study, *C. fragrans*, upon undergoing polyploidization, showed a substantial increase in phytochemical content, which was directly correlated with enhanced *in vitro* antimicrobial, antioxidant, and anti-inflammatory properties, alongside elevated cell viability effects. Building on these findings, the final and the third study delves into the potential of AP across medicinal and aromatic plants (MAPs) more broadly, emphasizing its ability to optimize SM biosynthesis.

Succinctly, this thesis integrates plant biotechnology, phytochemical analytics, bioactivity assays, and computational biology to leverage AP for optimizing phytochemical richness, biological efficacy, stress resilience, and economic potential in MOPs. Such interdisciplinary approaches are vital for addressing challenges in climate-resilient medicinal plant breeding, sustainable phytopharmaceutical production, and functional food development.

2. Literature review

2.1 Polyploidization

Polyploidization, characterized by the presence of more than two sets of chromosomes in a cell, is a significant evolutionary mechanism contributing to plant species' genetic diversity and adaptability. This phenomenon has played a crucial role in the evolutionary dynamics of plants, enabling them to thrive in diverse and challenging environments. It is estimated that approximately 50-70% of angiosperms have undergone polyploidy during their evolutionary history (Renny-Byfield et al., 2010). Advances in genomics and the increasing availability of genomic data have revealed that plants have experienced multiple genome duplication events throughout their evolutionary trajectory (Panchy et al., 2016).

Polyploidy can arise through processes such as endomitosis and endoreduplication. In endomitosis, mitosis occurs without the breakdown of the nuclear membrane, leading to chromosome doubling within the nucleus. Endoreduplication involves DNA replication without chromatid segregation, resulting in 2n chromatids without altering the chromosome count. The nucleus undergoing endoreduplication can continue DNA replication without entering mitosis, producing nuclei with ploidy levels of 4n, 8n, 16n, and beyond (Jafarkhani-Kermani & Emadpour, 2019). Polyploidy can also arise through somatic doubling and the formation of unreduced (2n) gametes (Ramsey & Schemske, 1998). Polyploidy is traditionally categorized into two primary types: (i) allopolyploidy, which is more common in nature and results from the hybridization of two distinct species, with heterozygosity varying based on the divergence of parental genomes, and (ii) autopolyploidy, which involves the doubling of homologous genomes within a single species (Ramsey & Schemske, 1998; Hegarty et al., 2013) (Figure 1). An example of autopolyploidy is the AAA genomic group, a key component of commercially used *Musa acuminata* (A-genome) cultivars. Interspecific crosses between *M. acuminata* and *Musa balbisiana* give rise to various allopolyploids, including AB, AAB, ABB, and ABBB (Subbaraya et al., 2011).

The duplication of entire chromosome sets increases the copy number of existing genes and induces additional changes, such as epigenetic modifications and altered gene expression, influencing a wide range of phenotypic traits. Consequently, agricultural and horticultural crops with increased ploidy levels often exhibit anatomical and morphological changes (known as the Gigas effect), enhanced biomass, yield, vigor, stress tolerance, and adaptive advantages contributing to their commercial success. Polyploidy can also promote self-incompatibility and

may lead to dwarfism (Parida & Misra, 2015; Hannweg et al., 2016; He et al., 2016). Over recent decades, new and improved cultivars of economically essential species exhibiting these enhanced traits have been developed through the induction of AP using mutagenic agents such as colchicine, trifluralin, and oryzalin (Eng & Ho, 2019; Dhooghe et al., 2011). The discovery of polyploid genomes in various plant species has garnered significant interest from plant breeders for applying AP in crop improvement.

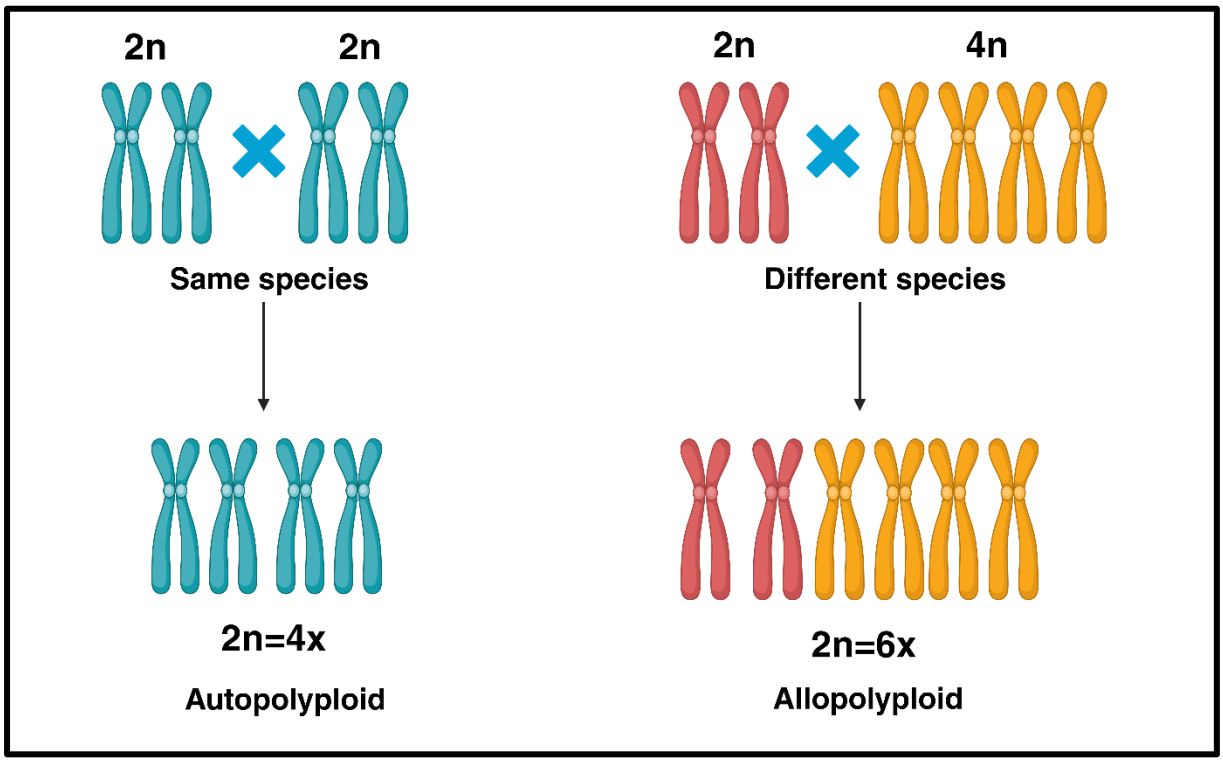

Figure 1. Simplified illustration of the mechanisms involved in autopolyploid and allopolyploid formation. Image source: Author

2.2 Natural polyploidization

Natural polyploidization, a process where plants acquire additional chromosome sets without human intervention, is a pivotal phenomenon that has profoundly influenced plant evolution and biodiversity. This genetic variation, particularly in angiosperms, also occurs in some amphibians and fish (Woodhouse et al., 2009). Polyploidy plays a critical role in plant adaptability, speciation, and diversity, arising through mechanisms such as whole-genome duplication, errors in cell division, and hybridization events. Advances in genomic technologies have deepened our understanding of the mechanisms and evolutionary significance of natural polyploidization. Several prominent plant lineages have undergone natural polyploidization, contributing to their genetic complexity and ecological success. For example, wheat (*Triticum*

aestivum), a major staple crop, is an allohexaploid with six chromosome sets originating from three distinct ancestral species. This intricate polyploidization event has provided wheat with extensive genetic diversity, enhancing its adaptability and agricultural value (Dubcovsky & Dvorak, 2007). Similarly, the genus *Brassica*, which includes critical crops such as cabbage, broccoli, and mustard, features many polyploid species. These species have evolved through natural hybridization and chromosome-doubling events, significantly contributing to their diversification and ecological adaptation (Pradhan et al., 2010).

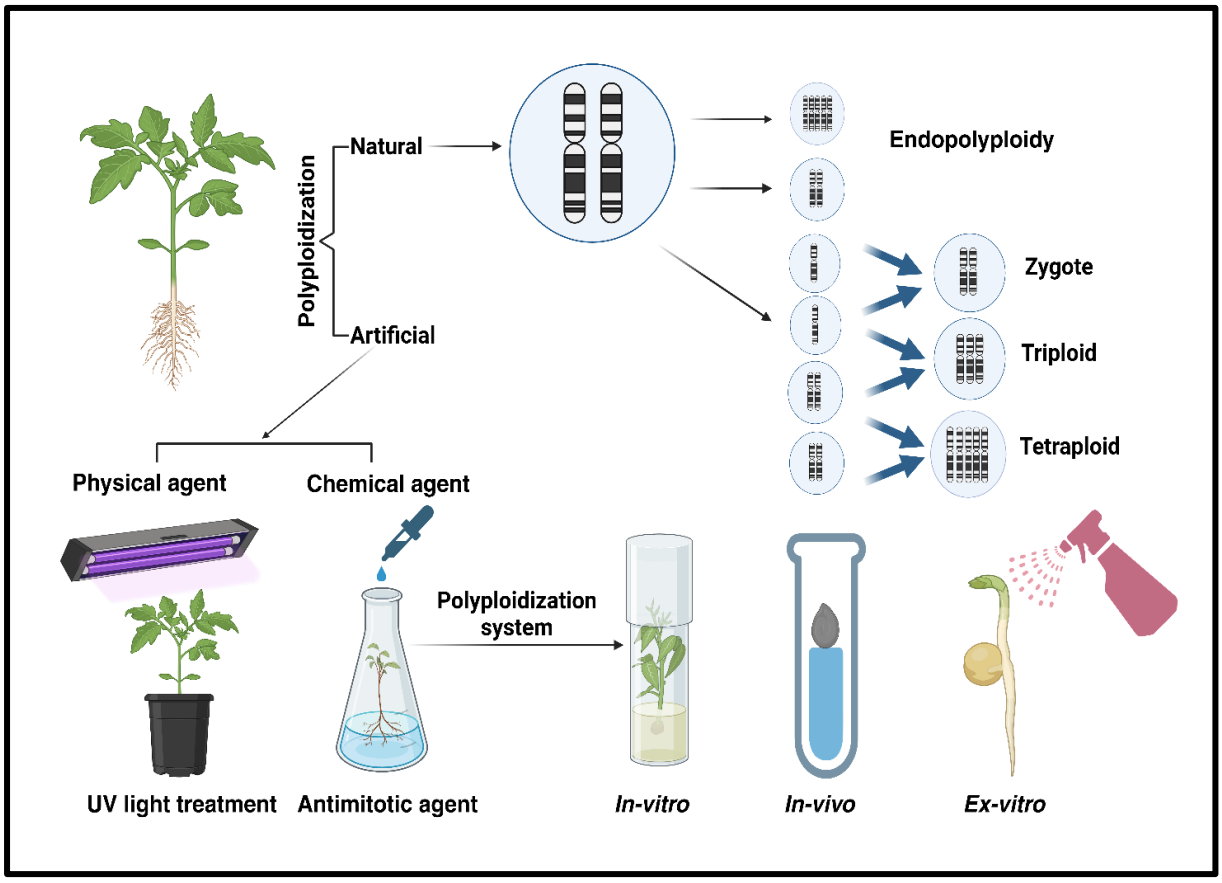

Figure 2 Schematic representation of different polyploidization systems. Image source: Author

2.3 Artificial polyploidization

Artificial polyploidization (AP) is a technique used to induce polyploidy in plants that possess more than two sets of chromosomes through chemical treatments, physical interventions, or hybridization. This approach is increasingly recognized as a critical strategy in plant mutation breeding, mainly because it involves genomic mutation, which typically results in more substantial phenotypic variation than gene mutations. Such variation can lead to enhanced traits, particularly those advantageous for medicinal applications. AP varieties can emerge spontaneously or through deliberate induction using chemical or physical agents (Figure 2).

2.3.1 Physical agents

Physical mutagens, such as electromagnetic radiation (including X-rays and UV light) and particle radiation (such as fast and thermal neutrons, as well as β and α particles), are commonly used to induce mutations (Maluszynski et al., 2000) (Figure 2). These irradiations cause various disruptions in DNA molecules, including hydrogen bond breakage, single- and double-strand breaks, base loss, base changes, formation of pyrimidine dimers, DNA cross-linking, and DNA-protein cross-linking. These effects result from the intense external energy applied to DNA, disrupting its thermodynamically stabilized state (Gupta et al., 2019). Despite these efforts, the efficiency and success rate of polyploidy induction in plants remain low due to undesirable chromosomal alterations, the formation of chimeric plants, poor root development, or even plant death.

2.3.2 Chemical agents

The assortment of an antimitotic agent (AMA) is one of the critical factors while conducting polyploidization (Figure 2). One should consider the agent type, minimum effective concentration, exposure duration, and application method during polyploidization. These AMAs act as spindle inhibitors. Colchicine is a well-known mitotic arrest alkaloid that ceases microtubule formation (Chaikam et al., 2019). However, it is considered toxic due to its lower affinity for plant cell tubulins and a higher affinity for tubulins in animal cells (Sivakumar et al., 2017). Alternatively, less toxic AMAs, such as oryzalin, trifluralin, flufenacet amiprophos-methyl combined with pronamide and dimethyl sulfoxide, and N_2O gas, can be a promising candidate as a substitute for colchicine (Chaikam et al., 2019). Oryzalin is safer than colchicine as it does not bind to animal tubulin sites, has a higher specificity for plant tubulins, and requires lower dosages than colchicine (Grosso et al., 2018; Eng & Ho, 2019). For example, in the *in vitro* polyploidy induction of *Trachyspermum ammi* L., the maximum tetraploidy

induction was depicted using 0.05% colchicine for 24 hours (Noori et al., 2017). In contrast, *Gerbera jamesonii* gave the highest numbers of tetraploids on treatment, with 0.2% colchicine applied for 2 hours (Khalili et al., 2020). The AMA concentration is inversely proportional to the duration of the treatment, which often leads to desirable results, achievable either by using high concentrations of AMA for short durations or low concentrations for more extended periods (Ye et al., 2010; He et al., 2016). Several environmental factors, such as the composition of the medium and culture temperature, are crucial for establishing an efficient *in vitro* AP (Xu et al., 2018). Qi-Qing et al. (2018) reported the highest polyploid production in *Andrographis paniculata* when treated with colchicine at 4°C for 40 minutes, followed by exposure to fresh colchicine solution at 40°C for 20 minutes.

2.4 Polyploid induction application systems

2.4.1 *In vitro* polyploidization

In vitro polyploidization is conducted under sterile, controlled conditions using plant tissue culture techniques. AMAs are introduced directly into the culture medium, allowing localized, precise application to explants such as shoot apices, nodal segments, seeds, and embryogenic suspension cultures (Touchell et al., 2020; Acanda et al., 2015). This environment facilitates efficient polyploid induction, enhanced by early screening tools such as flow cytometry and chromosome counting. This method typically exhibits higher mutation rates, lower mortality, and reduced mixoploidy compared to *ex vitro* or *in vivo* approaches (Fu et al., 2019). The effectiveness of *in vitro* protocols is influenced by several factors, including plant genotype, explant age, culture medium composition, plant growth regulator (PGR) ratios, exposure duration, and temperature (Niazian, 2019; Niazian & Shariatpanahi, 2020). For example, in *Allium hirtifolium*, colchicine (0.5% w/v for 36 h) and oryzalin (0.001% w/v for 8 h) induced polyploidy rates of 35.0% and 45.5%, respectively, with significant improvements in morphological traits, antioxidant activity, and enzyme expression (Farhadi et al., 2023). In *Pennisetum* × *advena*, colchicine-induced hexaploids displayed desirable dwarfism and delayed flowering, aligning with ornamental market preferences (Yue et al., 2020). Likewise, oryzalin-induced tetraploids of *Rhododendron fortunei* exhibited thicker, curled leaves and darker green coloration, enhancing aesthetic appeal (Lan et al., 2020). Moreover, hormonal balance during *in vitro* culture plays a critical role. For instance, the auxin-to-cytokinin ratio significantly influenced polyploid regeneration in both *Trachyspermum ammi* and *Paeonia sect. Moutan* (Sadat-Noori & Norouzi, 2017; Du et al., 2020).

2.4.2 *Ex vitro* polyploidization

Ex vitro polyploidization involves the application of AMAs to plant materials grown outside of tissue culture, typically in greenhouse or field settings. This method is suitable for large-scale operations, allowing treatment of numerous seedlings, plantlets, or mature plants. Application methods include foliar sprays and cotton-plugging, which are operationally simple and less reliant on laboratory infrastructure. Although effective in some contexts, *ex vitro* treatments often suffer from low induction efficiency, high mixoploid rates, and chemical wastage due to evaporation (Salma et al., 2017). For instance, in *Citrullus lanatus*, colchicine applied at 0.1% and 0.2% to shoot apices and inverted hypocotyls resulted in a 29.5% increase in tetraploid induction (Noh et al., 2012). Similarly, a 14.5% increase in polyploid count was reported in *Dendranthema* following 24-hour seed soaking in 0.1% colchicine (He et al., 2016). *Sesamum indicum* treated with 0.6% colchicine for 4 hours exhibited delayed maturity (Anbarasan et al., 2014). Despite these successes, *ex vitro* polyploidization remains less efficient than *in vitro* methods and is more prone to producing chimeric plants (Miri & Roughani, 2018).

2.4.3 *In vivo* polyploidization

In vivo polyploidization bridges the gap between *ex vitro* and *in vitro* methods. It involves direct treatment of plants at early developmental stages typically through immersion**,** seed soaking, or droplet application of AMAs (Figure 2). This method is often employed due to its technical simplicity and cost-effectiveness, requiring minimal laboratory resources (Castillo et al., 2009). In *Gerbera hybrida*, a prominent ornamental species and a model for SMs studies, seedlings treated with 0.2% colchicine successfully developed into pure tetraploids. These exhibited thicker leaves and scapes and reduced stomatal density, contributing to enhanced abiotic stress resistance (Bhattarai et al., 2021). However, high mortality, inconsistent outcomes, and frequent chimerism remain persistent limitations at low AMA concentrations.

While both methods involve treatment of whole plants outside sterile culture, *ex vitro* typically refers to the treatment of tissue culture-derived plantlets during acclimatization, often under semi-controlled conditions. In contrast, *in vivo* polyploidization is applied directly to naturally grown plants in non-controlled, open environments. This differentiation is well-established (Castillo et al., 2009; Miri & Roughani, 2018) and reflects important methodological and biological differences, particularly regarding treatment precision, plant developmental stage, and outcomes such as mixoploidy rates and mortality.

2.5 Polyploidization in medicinal and ornamental plants

Polyploidization in MOPs often leads to distinct characteristics, such as altered phytochemical profiles, increased concentrations of pharmaceutically valuable compounds, and alterations in morphological traits, including flower color, size, fragrance, and shelf life. These enhanced traits offer significant economic potential in both medicinal and ornamental plant breeding. By employing both *in vitro* and *in vivo* chromosome doubling techniques, researchers can develop custom-designed plants with increased market appeal.

2.5.1 Polyploidization in medicinal plants

Medicinal plants are valuable due to their bioactive components, which possess therapeutic properties and have bio-prospecting potential (Pradhan et al., 2018). Finding effective drug candidates with minimal side effects remains a challenge in the pharmaceutical industry. Plant-based medicines are in great demand due to their lower side effects compared to synthetic drugs (Niazian et al., 2019). For instance, plant-based anti-obesity drugs are preferred over synthetic alternatives like Orlistat and Sibutramine due to their lower cost and reduced side effects (Mopuri & Islam, 2017). Global demand for medicinal plants has risen due to their health benefits, economic value, and industrial applications. To circumvent this demand gap, sustainable production systems are required to prevent the extinction of these plants (Niazian et al., 2017; Niazian et al., 2018).

One effective breeding method for medicinal plants is antimitotic agent-induced polyploidy induction (APPI) (Niazian, 2019). For instance, oryzalin-induced tetraploids in *T. vulgaris* L. (Figure 3) exhibited increased levels of EO (1.19%) with enhanced thymol (18.01%), and carvacrol (0.49%) content compared to diploids (Shmeit et al., 2020). Similar effects were observed in other medicinal plants, such as *L. alba* and *Tetradenia riparia*, where the EO composition was influenced (Julião et al., 2020; Hannweg et al., 2016). Since polyploidization produces varieties of autopolyploid lines, proper screening must be conducted to select the superior genotypes in terms of the desired phytochemical profiles. Certain reports suggested that polyploid-induced gene silencing processes lead to transcription repression. One such example is of *Citrus limon* (L.), where reverse transcriptase polymerase chain reaction (RT-PCR) analysis revealed upregulation of *limonene synthase*, *phenylalanine ammonia-lyase*, and *chalcone synthase* genes in colchicine-induced tetraploids, leading to alterations in EO composition, which resulted in the loss of iso-carveol, geranial, and trans α-bergamotene components and the emergence of a new component of β-bisabolene in colchicine-induced

tetraploids (Bhuvaneshwari et al., 2020). Another example is *T. riparia,* which reported the occurrence of α-humulene, α-terpineol, and viridiflorol in the polyploids, which was absolutely absent in their diploid counterpart (Hannweg et al., 2016).

Polyploidy can also enhance tolerance to biotic and abiotic stresses (Tossi et al., 2022; Parsons et al., 2019; Šedivá et al., 2019). Alternatively, increased levels of SMs have been observed in various medicinal plants when subjected to abiotic stress conditions (Yeshi et al., 2022; Niazian et al., 2019). Therefore, cultivation of induced polyploid medicinal plants under stressful environments has the double benefit of improving their SM content. Another advantage of polyploidization is enhanced tolerance to biotic and abiotic stresses (Van de Peer et al., 2021; Parsons et al., 2019). Abiotic stress conditions can further increase the production of SMs in medicinal plants (Mansinhos et al., 2024), making the cultivation of polyploid medicinal plants in stressful environments a beneficial strategy.

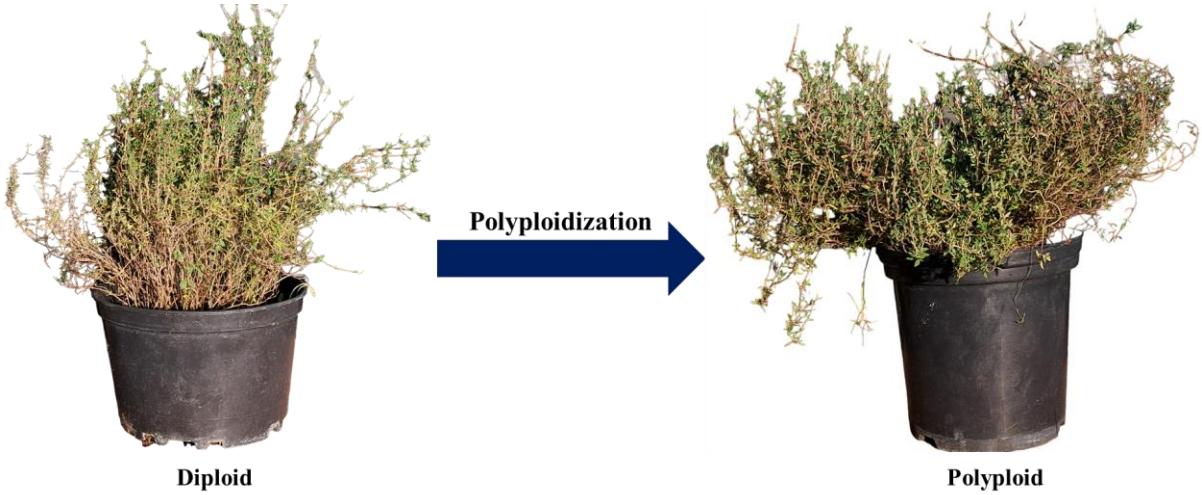

Figure 3 The plant on the left represents the diploid *T. vulgaris*, while the plant on the right represents the polyploid *T. vulgaris.* Image source: Author

2.5.2 Polyploidization in ornamental plants

There is a continuous demand for novel varieties with new traits, such as enhanced flower color, fragrance, and shape, which is why genetic diversity is critical for advancing in ornamental plant industries (Katoch et al., 2024). Artificial *in vitro* polyploidy induction has been widely used in breeding programs to create new ornamental varieties with desirable characteristics (Eeckhaut et al., 2018). Phenotypic alterations in ornamental plants caused by polyploidy are attributed to factors such as allelic diversity (heterozygosity), gene silencing, gene dosage effects, and epigenetic and genetic interactions (Manzoor et al., 2019). Polyploid

induction, especially triploids and tetraploids, is valuable for improving traits such as fragrance, plant structure, flower color, and extended flowering periods (Kumar et al., 2024). The most common AMA used in ornamental plant APPI is colchicine and oryzalin, which successfully produced larger flowers in various species, including *Gerbera jamesonii, Chrysanthemum boreale*, *Callicarpa bodinieri,* and *C.fragrans*, respectively (Figure 4) (Khalili et al., 2020; Hoang et al., 2020; Beranová et al., 2022). For instance, colchicine-induced triploids of lavandin (*Lavandula* × *intermedia*) have shown potential for oil production (Urwin, 2014). Similar methods have been used in species like *Leptospermum scoparium* and *Populus canescens*, leading to improvements in flower diameter, growth traits, and stress resistance (Bicknell et al., 2019; Zhou et al., 2020).

However, the relationship between chromosome duplication and desired traits is not always linear, as seen in *Hyoscyamus muticus* L., where polyploidy led to reduced organ size (Shahriari-Ahmadi et al., 2008). Flower color is a critical trait in ornamental breeding, and generating novel colors has become increasingly important. Studies have demonstrated that polyploidy can generate novel flower colors, such as in *Impatiens walleriana,* where oryzalin treatment leads to increased anthocyanin content in the polyploid (Ghanbari et al., 2019). Studies indicate that key factors influencing ornamental polyploidization include plant species, explant type, culture medium, AMA type and concentration, and inoculation time (Fomicheva et al., 2024; Ghanbari et al., 2019; Subramanian et al., 2024; Fatima et al., 2024; Miler et al., 2023). For instance, Esmaeili et al. (2020) studied the effects of colchicine, oryzalin, and trifluralin on chromosome doubling in *Calendula officinalis* cultivars which depicted that colchicine was most effective in the Nova (200 ppm) and Orange Beauty (400 ppm) cultivars, while oryzalin (20 ppm) worked best for WUR 1553-7. Meanwhile, colchicine-treated polyploid *Petunia* plants with distinct morphological traits possess compact growth (Regalado et al., 2017). This benefits the ornamental plant breeders as it reduces reliance on hormones and fertilizers while achieving higher yields (Eng & Ho 2019).

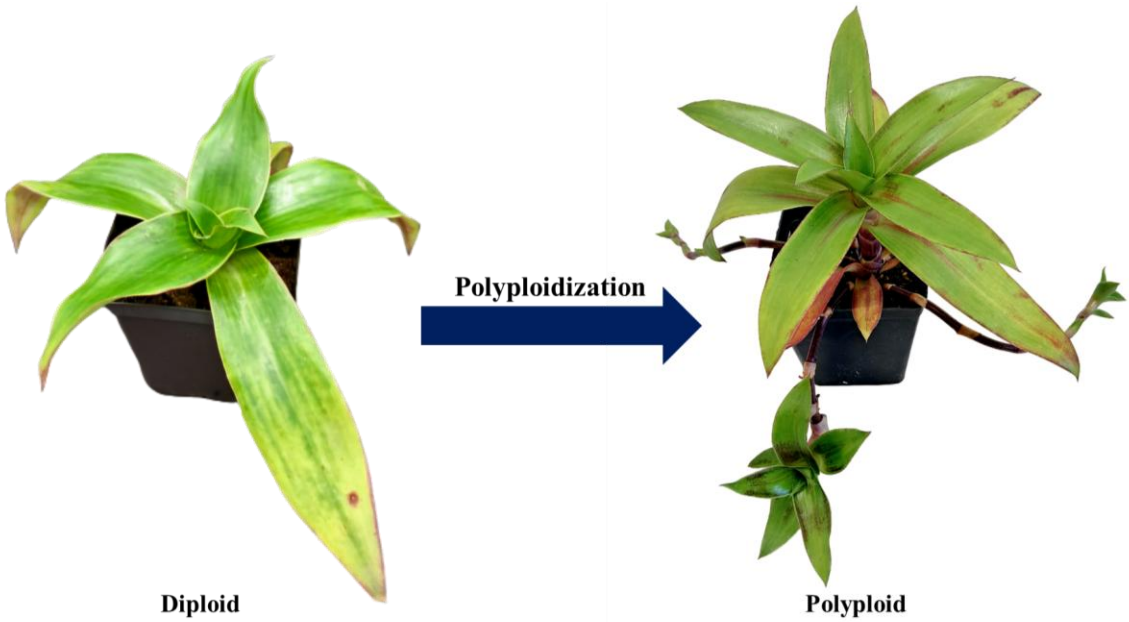

Figure 4 The plant on the left represents the diploid C. *fragrans*, while the plant on the right represents the polyploid *C. fragrans.* Image source: Author

In summary, AP is an effective breeding strategy for developing ornamental plants with enhanced commercial traits, such as larger flowers, increased fragrance, and improved stress tolerance. Additionally, it holds promise in medicinal plant breeding by improving phytochemical profiles and stress resistance.

2.6 The botany of medicinal and ornamental plants used in this study

2.6.1 *Thymus vulgaris* L. (Garden thyme)

T. vulgaris L., commonly referred to as garden thyme or common thyme, is a perennial, bushy, woody-based aromatic herb of the Lamiaceae family, typically reaching heights between 10 and 30 cm. It is native to the Mediterranean region, and is also found in parts of Asia, Southern Europe, and North Africa (Hossain et al., 2022). The genus *Thymus* includes approximately 400 species, traditionally used in the treatment of various ailments, such as cough, bronchitis, sore throat, arthritis, and rheumatism (Horváth & Ács, 2015; Kowalczyk et al., 2020; Hosseinzadeh et al., 2015), in addition to its culinary applications for flavor enhancement and preservation of food (Neito, 2020). *T. vulgaris* is an evergreen subshrub characterized by an upright, woody stem (Figure 3). Its leaves are arranged in whorls around the stem and are evergreen, simple, ovate, and fine-textured, emitting a pleasant fragrance. The leaves are primarily the edible part of the plant. The flowers are cymose, bisexual, and two-lipped, with

a purple and white color, and possess a hairy glandular calyx, which also contributes to the plant's aromatic properties. Blooming typically occurs in spring and summer (Kosakowska et al., 2020). *T. vulgaris* exhibits a moderate growth rate and, upon maturity, can reach heights of 0.5 to 1 m, with a similar spread, although it may extend up to 15 m when acting as ground cover. It takes approximately 2 to 5 years to achieve its full height (Talhouk et al., 2015). The species is tolerant of both frost and drought and thrives in poor, salty environments. It grows well in loamy and sandy soils with neutral to alkaline pH. The plant has minimal water requirements but requires full sun exposure. It has a life span of up to 25 years. The plant can be propagated through seeds, cuttings, or layering (Hammoudi et al., 2022).

Traditionally, *T. vulgaris* has been used to treat various health issues, particularly those affecting the respiratory and digestive systems, as well as for its antimicrobial properties (Patil et al., 2021). More recent ethnomedicinal studies highlight its continued relevance. For instance, breast cancer patients in Algeria have used infusions of *T. vulgaris* aerial parts (Taïbi et al., 2020), while in southeastern Serbia, thyme tea is traditionally used to treat stomach disorders (Jarić et al., 2015), and in Spain, it is consumed as a medicinal infusion (Rivera et al., 2019). In Europe, it is recognized for its medicinal applications, particularly as a cough remedy and for treating urinary and genital disorders (Iftikhar et al., 2023). The European Medicines Agency classifies therapeutic effects of thyme under 'traditional use,' supported by its safe application over at least 30 years, including a minimum of 15 years within the European union (Bodalska et al., 2021).

In the natural environment, EOs play a crucial role in plant defense, exhibiting antiviral, antibacterial, antifungal, insecticidal, and herbivore-deterrent activities (Hammoudi et al., 2022). *T. vulgaris* EO is particularly rich in thymol, along with other compounds such as carvacrol, geraniol, α-terpineol, 4-thujanol, linalool, 1,8-cineole, myrcene, γ-terpinene, and *p*-cymene (Najar et al., 2021). These constituents are responsible for a range of therapeutic effects, including antitussive, antibronchial, antispasmodic, anticancer, and other medicinal properties (Silvi et al., 2021; Salehi et al., 2019).

2.6.2 *Callisia fragrans* (Lindl.) Woodson (Basket plants or Golden moustache)

C. fragrans, commonly referred to as the Basket Plant or False Bromeliad is a diminutive herb belonging to Commelinaceae family is known for its rosette growth form and waxy leaves. It is native to Mexico and has been cultivated as an ornamental houseplant for over a century, primarily in subtropical and tropical regions. Recently, interest in its medicinal properties has

grown, particularly in Eastern Europe, where its leaves are utilized for the treatment of skin conditions, burns, and joint disorders. Their leaves are rich in biologically active compounds, including flavonoids, neutral glycolipids, and phospholipids, along with their associated fatty acid profiles (Yarmolinsky et al., 2010; Le et al., 2022). Their leaves measure 6 to 10 inches in length, with a glossy green color and a subtle purplish tint on the underside. These leaves often overlap tightly at the base and may develop a more pronounced purple hue when exposed to sufficient light (Figure 4). It produces clusters of small, fragrant white flowers that emerge on long, erect stems, adding to its unique ornamental appeal (Beranová et al., 2022). Indigenous to Mexico and parts of South America, this plant thrives in temperatures between 30-32°F (3-5°C) and can be cultivated as a potted or basket plant, or used as ground cover. In Eastern Europe, it has been traditionally used for its medicinal properties, particularly for treating skin diseases, burns, and joint pain, where it is believed to alleviate inflammation and promote healing (Yarmolinsky et al., 2010; Huynh et al., 2023). Additionally, the plant has been employed as remedies to treat cardiovascular conditions, bronchial asthma, and mucosal infections (El Sohafy et al., 2021). Beyond traditional uses, the Basket Plant has garnered attention for its potential in the pharmaceutical industry, thanks to its abundance of phytochemicals, antioxidants, minerals, and vitamins. The plant's chemical constituents include compounds such as aloe-emodin, umbelliferone, scopoletin, ethyl acetate, quercetin, gallic acid, caffeic acid, and chicoric acid, which contribute to its medicinal properties (Le et al., 2015; Le et al., 2022; Olennikov et al., 2008).

In addition to its therapeutic potential, *C. fragrans* is appreciated as an ornamental plant, often admired for its visual resemblance to bromeliads, with trailing stems that enhance its aesthetic appeal (Beranová et al., 2022). Its distinct appearance, coupled with its medicinal benefits, makes it a valuable plant for both horticultural and pharmaceutical applications.

2.7 Significance of polyploidization in enhancing the phytochemical profile of plant extracts

The rising demand for valuable plant SMs across various industries has made AP a promising breeding approach for enhancing the phytochemical efficacy of MOPs. This method is particularly advantageous as it aligns with consumer preferences for natural genetic profiles, bypassing concerns associated with genetically modified organisms (GMOs). A research study by Navratilova et al. (2022) reported that oryzalin-induced tetraploid genotypes of *Ajuga reptans* exhibited elevated levels of key compounds, including trans-teupolioside, trans-verbascoside, and 20-hydroxyecdysone, compared to diploid genotypes. Similarly, Priya and

Pillai (2023) found a 160-fold increase in andrographolide production in colchiploid calluses of *Andrographis paniculata*, a compound known for its pharmacological activities such as anticancer, antimicrobial, antiparasitic, and anti-inflammatory properties.

Another example includes *Artemisia annua*, where artemisinin, an effective antimalarial compound, increased from 39% to 56% in tetraploid plants induced by colchicine treatment (Lin et al., 2011). Research by Tavan et al. (2021) also demonstrated that polyploidization in *Salvia officinalis* influenced phytochemical traits, including alterations in rosmarinic acid content. In a separate study, Shmeit et al. (2020) observed increased levels of key compounds such as carvacrol, thymol, trans-caryophyllene, γ-terpinene, and 4-cymene in polyploid plants. Pansuksan et al. (2014) identified 40 unique bioactive compounds in tetraploid *Mitracarpus hirtus* that were absent in its diploid progenitor. Bharati et al. (2023) reported successful SP of *Mentha spicata* using oryzalin, which resulted in increased levels of significant compounds like carvone and limonene. Additionally, oryzalin-induced polyploidization in *M. officinalis* led to elevated levels of geranial and neral (Bharati et al., 2023). Several other plants, including *Anoectochilus formosanus, Cichorium intybus, Datura stramonium, Papaver bracteatum, Salvia miltiorrhiza, Stachys byzantina, and T. ammi*, have also shown enhanced SM production following polyploidization (Huang et al., 2022; Ghotbi Ravandi et al., 2014; Al-Taweel et al., 2019; Madani et al., 2019; Ma et al., 2023; Hamarashid et al., 2021; Sadat Noori et al., 2017). A study by Keshtkar et al. (2019) demonstrated that colchicine treatment of *Trigonella foenumgraecum* resulted in an enhanced phytochemical profile, with increased concentrations of alkaloids (trigonelline, choline, carpaine), flavonoids (saponaretin, isovitexin, isoorientin, quercetin, luteolin), and saponins (fenugrin, foenugracin, yamogenin, trigonoesides). In another case, oryzalin-induced polyploidization in *Cannabis sativa* led to larger plant morphology and higher levels of valuable phytochemicals, including α-humulene, guaiol, tetrahydrocannabinol (THC), cannabidiol (CBD), and α-bisabolol, when compared to their diploid counterparts (Parsons et al., 2019). Lastly, research by Zhang et al. (2018) highlighted an increase in total alkaloid content in colchicine-treated *Dioscorea zingiberensis*, showcasing the potential of polyploidization in boosting phytochemical production.

2.8 Polyploidization influences the biological activities of medicinal and ornamental plants

AP is often proposed to enhance both primary and SM production by inducing chromosome doubling, which subsequently affects the biological activities of polyploid plants. While research on the bioactivity of polyploid medicinal plants is still in its early stages, several studies have characterized induced polyploid plants, focusing on metabolite enhancement and biological effects. For instance, Gupta et al. (2024) demonstrated that oryzalin-induced polyploid *T. vulgaris* EO exhibited stronger antibacterial, antioxidant, and anti-inflammatory activities, along with higher concentrations of thymol and *γ*-terpinene compared to the diploid counterpart. The study highlighted that the higher thymol content in the tetraploid genotype was primarily responsible for the enhanced biological activity. Similarly, another study on induced tetraploid *T. vulgaris* revealed greater insecticidal activity, along with elevated levels of bioactive compounds such as carvacrol, thymol, trans-caryophyllene, *γ*-terpinene, and 4-cymene compared to the diploid genotype (Navrátilová et al., 2021). Further examples include studies by Bhuvaneswari et al. (2019) and Mei et al. (2020), which demonstrated higher antioxidant activities in colchicine-induced *C. limon* and *E. purpurea*, respectively, alongside increased production of SMs. In the case of *Acorus calamus*, tetraploid plants were found to contain over 95% *β*-asarone in their EO, contributing to enhanced anticancer activity, a compound typically absent in diploid plants (Song et al., 2015). Polyploid *T. riparia* EO also showed increased antifungal activity against *Geotrichum candidum* (Hannweg et al., 2016). Additionally, Pansuksan et al. (2014) reported that tetraploid *M. hirtus* exhibited higher antibacterial activity and contained 40 unique bioactive compounds not present in its diploid progenitor. However, it is essential to note that elevated concentrations of specific bioactive components in SMs do not necessarily guarantee improved biological activity. The efficacy of bioactivity depends on multiple factors, including compound synergism, metabolic fluxes, target specificity, and bioavailability. Therefore, comprehensive phytochemical profiling combined with mechanistic biological assays is essential to confirm and rationalize functional enhancements in polyploid plants.

2.9 Methods for evaluating biological activities and phytochemical characterization of polyploid plant extracts

The imperative to evaluate qualitative attributes of synthetically induced polyploid genotypes is paramount, given the nascent state of research into the bioactivity of SMs from such novel polyploids. The comparative evaluation of phytochemical profile and biological activities between different genotypes is classified into four different categories: Ploidy level estimation, extraction methods, analytical methods, and *in vitro* bioassays (Figure 5).

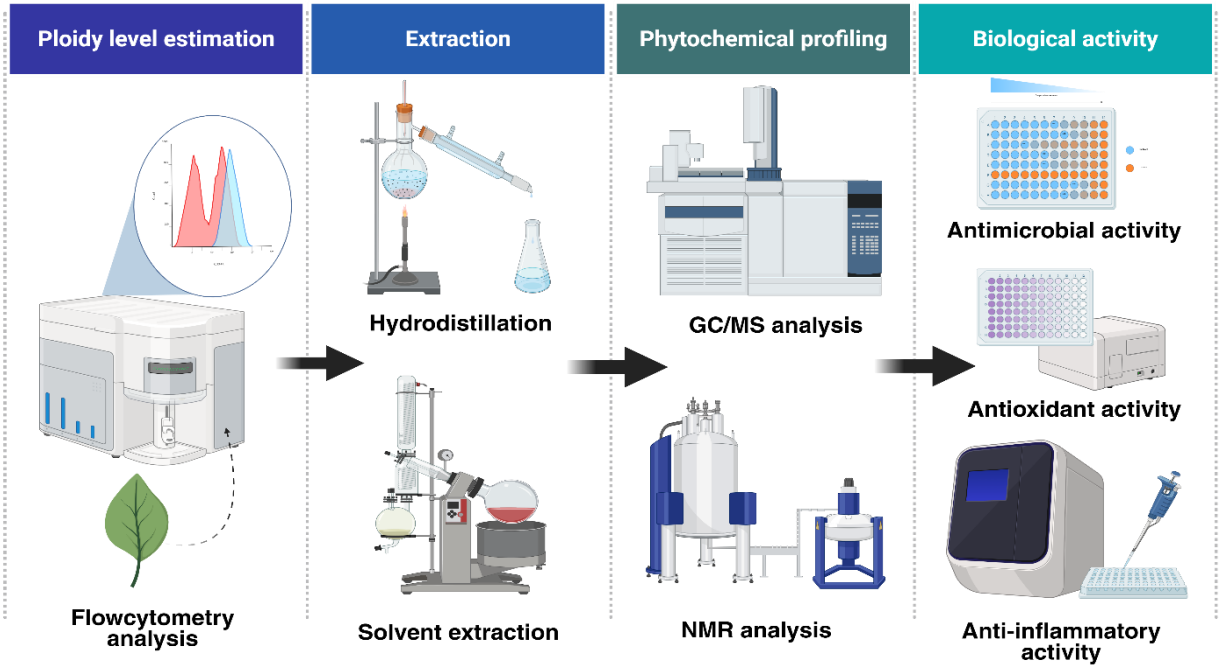

Figure 5 Schematic representation for evaluating biological activities and phytochemical characterization of polyploid plants. Image source: Author

For the confirmation of genetic stability in polyploid plants, flow cytometry offers rapid and accurate ploidy assessment by measuring nuclear DNA content (Bharati et al., 2023). This is often complemented by cytogenetic techniques such as chromosome counting, molecular marker profiling using Simple Sequence Repeat (SSR) and Amplified Fragment Length Polymorphism (AFLP), and Fluorescence In Situ Hybridization (FISH), which collectively provide deeper insights into chromosomal integrity, structural variation, and genome stability across generations (Dhooghe et al., 2011). Once genetic stability is confirmed, polyploid plants become valuable candidates for further phytochemical and biological evaluation due to their enhanced SM production resulting from gene duplication.

PEs such as EOs and phenolic compounds are typically extracted using traditional techniques like cold expression, hydrodistillation, and steam distillation, though heat-sensitive compounds may degrade during these processes. To overcome such limitations, modern green extraction technologies like supercritical fluid extraction (SFE) with CO_2 (Nahar & Sarker, 2012), ultrasound-assisted extraction (Rodrigues & Fernandes, 2009), and microwave-assisted extraction (Lee et al., 2017) can be employed to improve selectivity, efficiency, and thermal protection. However, for the extraction of non-volatile PEs conventional solvent extraction using polar solvents like methanol or ethanol is widely adopted due to their strong ability to dissolve a broad range of polar phytochemicals such as phenolics, tannins, and flavonoids (Azmir et al., 2013).

High-throughput analytical techniques such as Gas Chromatography–Mass Spectrometry (GC-MS) for volatiles, Nuclear Magnetic Resonance (NMR) spectroscopy, Liquid Chromatography–Mass Spectrometry (LC-MS), Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS), Fourier-Transform Infrared Spectroscopy (FTIR), and Ultraviolet–Visible Spectrophotometry (UV-Vis) are essential tools for analyzing phytochemical composition between polyploid and diploid counterparts (Munjal et al., 2022). These methods provide a precise understanding of chemical composition differences between the two genotypes. Therefore, these chromatographic and spectrometric methods are currently the most effective tools for investigating metabolic insights across different ploidy levels.

The metabolite diversity in polyploids directly correlates with their bioactivity; therefore, it is necessary to understand the specific biological activities of polyploid plants and compare them with their parental genotypes. Several *in vitro* bioassays can be performed in this regard. However, the selection of appropriate biological activity assessments should be based on previously reported bioactivities to understand the current status post-polyploidization.

To assess antimicrobial activity, the broth microdilution volatilization (BMV) method to measure minimum inhibitory concentrations (MICs) is a good approach (Gupta et al., 2024). Antioxidant capacity of plant extracts can be evaluated via 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Oxygen Radical Absorbance Capacity (ORAC), Ferric Reducing Antioxidant Power (FRAP) assays. Additionally, analyzing total phenolic (TPC) and flavonoid content (TFC) can help clarify the underlying mechanisms supporting antioxidant activity (Anand et al., 2018).

Several plant extracts also exhibit anti-inflammatory activity, making its assessment a valuable approach. This can be evaluated by various methods, with one of the most popular involving the inhibition of cyclooxygenase (COX) enzymes, specifically COX-1 and COX-2 (Ahmed et al., 2025). Moreover, many studies have reported other bioactivities to explore differences across ploidy levels, including insecticidal, anticancer, and antiproliferative effects (Navrátilová et al., 2021; Madani et al., 2021). However, research on elucidating these biological activities in relation to ploidy variation remains in its early stages. Thus, further experimental studies are essential to establish the efficacy of induced polyploid varieties for improved product development.

Equally important is the assessment of potential toxicity of these PEs, especially for their future applications. Cytotoxicity testing is therefore a crucial part of this research. In this regard, the MTT colorimetric assay is a widely used and reliable method to measure cell viability and cytotoxic effects (Mosmann 1983).

Beyond these, polyploid extracts can also be screened for antidiabetic activity *via* α-glucosidase and α-amylase inhibition (Kumar et al., 2011), anticancer activity through cytotoxicity against cancer cells and apoptosis assays (Mosmann 1983), wound-healing potential using *in vitro* scratch tests (Liang et al., 2007), and neuroprotective effects via acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition (Orhan et al., 2007). Together, these interconnected analyses validate the enhanced therapeutic potential of genetically stable polyploid MOPs.

3. Hypotheses and Objectives

3.1 Hypotheses

- Artificial polyploidization in selected medicinal and ornamental plants significantly increases the yield of plant extracts compared to their diploid progenitors
- Induced polyploidy enhances the biosynthesis and accumulation of secondary metabolites, leading to greater phytochemical diversity and elevated concentrations of bioactive compounds
- Polyploid derivatives exhibit significantly improved biological activities namely antioxidant, antimicrobial, and anti-inflammatory properties, relative to their diploid counterparts

3.2 Objectives

The main objective of this doctoral thesis is to characterize the effectiveness of artificial polyploidization on medicinal and ornamental plants, focusing on the phytochemical profile and biological activity.

The specific objectives of this doctoral thesis are:

- To verify the relative DNA content and ploidy stability of induced polyploid in selected medicinal and ornamental plant species using flow cytometry
- To extract, quantify, and compare the yield of plant extracts between diploid and polyploid genotypes
- To identify and characterize the phytochemical profiles (volatile and non-volatile constituents) of diploid and polyploid extracts using chromatographic and spectrometric techniques
- To assess and compare the *in vitro* biological activities (antioxidant, antimicrobial, and anti-inflammatory) of extracts from diploid and polyploid plants

4. Synthetic polyploidization induces enhanced phytochemical profile and biological activities in *Thymus vulgaris* L. essential oil

Adopted from: **Gupta, N.,** Bhattacharya, S., Dutta, A., Tauchen, J., Landa, P., Urbanová, K., Houdková, M., Fernández-Cusimamani, E., & Leuner, O. (2024). Synthetic polyploidization induces enhanced phytochemical profile and biological activities in *Thymus vulgaris* L. essential oil. *Scientific Reports, 14*(1), 5608.

This chapter explores the effects of synthetic polyploidization on T. vulgaris, resulting in a pronounced enhancement of its phytochemical profile, correlate with enhanced antimicrobial, anti-inflammatory and antioxidant activities.

Author contributions: Conceptualization, Investigation, Methodology, Writing original draft, Writing review & editing, Formal analysis.

DOI: https://doi.org/10.1038/s41598-024-56378-7

Abstract

Essential oil from *Thymus vulgaris* L. has valuable therapeutic potential that is highly desired in pharmaceutical, food, and cosmetic industries. Considering these advantages and the rising market demand, induced polyploids were obtained using oryzalin to enhance essential oil yield. However, their therapeutic values were unexplored. So, this study aims to assess the phytochemical content, and antimicrobial, antioxidant, and anti-inflammatory activities of tetraploid and diploid thyme essential oils. Induced tetraploids had 41.11% higher essential oil yield with enhanced thymol and γ-terpinene content than diploid. Tetraploids exhibited higher antibacterial activity against all tested microorganisms. Similarly, in DPPH radical scavenging assay tetraploid essential oil was more potent with half-maximal inhibitory doses (IC_{50}) of 180.03 µg/mL (40.05 µg TE/mg) than diploid with IC_{50} > 512 µg/mL (12.68 µg TE/mg). Tetraploids exhibited more effective inhibition of *in vitro* catalytic activity of pro-inflammatory enzyme cyclooxygenase-2 (COX-2) than diploids at 50 µg/mL concentration. Furthermore, molecular docking revealed higher binding affinity of thymol and γ-terpinene towards tested protein receptors, which explained enhanced bioactivity of tetraploid essential oil. In conclusion, these results suggest that synthetic polyploidization using oryzalin could effectively enhance the quality and quantity of secondary metabolites and can develop more efficient essential oil-based commercial products using this induced genotype.

4.1 Introduction

Thymus vulgaris L., popularly known as garden thyme or common thyme, is a perennial bushy and wood-based aromatic herb belonging to the Lamiaceae family predominantly found in the Mediterranean regions, Asia, Southern Europe, and North Africa [1]. The genus *Thymus* comprises approximately 400 species, widely used in traditional for treating ailments such as cough, bronchitis, sore throat, arthritis, and rheumatism [2, 3, 4, 5] in addition to its culinary use for taste enhancement and preventing food spoilage [6]. Thymol is found in abundance in *T. vulgaris* followed by carvacrol, geraniol, α-terpineol, 4-thujanol, linalool, 1,8-cineole, myrcene, γ-terpinene, and *p*-cymene solely responsible for antitussive and antibroncholitic [7], antispasmodic anti-cancer, and several medicinal properties [8, 9, 10]. The bioactivities of *T. vulgaris* essential oil mostly depend on its terpene and terpenoid contents [11, 12]. There has been a plethora of reports validating the *in vitro* antibacterial activity of thyme essential oil on some respiratory disease-causing pathogens including *Staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus influenza,* and *Streptococcus pneumonia* [13, 14]. Thyme essential oil is used commercially as a natural preservative in food industries to prevent spoilage as well as for food packaging systems [15, 16, 17].

Essential oil-based products promote organic, natural, and green consumerism throughout the global marketplace leading to escalating rivalry to create high-quality cultivars and to reduce production costs. The gap between the supplier and the market demand created due to the lesser yield of essential oils and heavy use of labor has led to over-harvesting from the wild causing gene-pool deficiency [18]. Several pieces of literature highlighted the fact that geographical, environmental, agroclimatic, and various genetic factors can influence the quantity of essential oil production in the plant along with chemical composition and its biological activity [19, 20, 21].

Keeping in mind that consumers prefer natural products over genetically modified plants due to safety issues [22] and other conventional plant breeding techniques are quite expensive and time-consuming, synthetic polyploidization is considered one of the safest and ideal contemporary breeding approaches [23, 24]. Synthetic polyploidization is chromosome duplication of the whole genomic constitution of an organism creating genetic uniqueness using chemical AMAs like oryzalin, colchicine, trifluralin, etc. [25], may result in superior or inferior genotypes with enhanced or reduced morphological, physiological, and biochemical properties as the result of gene duplication is unknown [26]. It is reported that oryzalin has

fewer side effects compared to colchicine and possesses a higher affinity towards plant tubulin [27].

Several studies reported and it is often hypothesized that synthetic polyploidization enhanced primary and secondary metabolite production due to chromosome doubling that influenced the biological activities of the polyploid plants [28, 29, 30, 31, 32]. However, one of such reports has proven it as a myth [33]. Thus, it is important to assess the quality of these induced genotypes. However, the bio-activity analysis of polyploid plants' secondary metabolites is still in its budding stage, and as of now, there has been no research article reporting the biological activities of induced tetraploid *T. vulgaris* essential oil by broadening the concern on antimicrobial, anti-inflammatory, and antioxidant activities. Therefore, the objective of this study was to extract essential oil from induced tetraploid thyme plants and characterize the effectiveness of polyploidization on *T. vulgaris* essential oil on its chemical composition and biological activities such as antimicrobial, anti-inflammatory, and antioxidant activities compared to essential oil extracted from diploid thyme. The current findings may elucidate the increase of secondary metabolite production in the polyploid genotype through synthetic polyploidization that positively influences the biological activities of plants that are of great economic importance to the pharmaceutical, cosmetic, and food industries.

4.2 Materials and methods

4.2.1 Plant material acquisition

Artificially induced autopolyploid plants of *T. vulgaris* (2n=4x=60) and *T. vulgaris* control plants (2n=2x=30) were obtained from the previous study by Homaidan Shmeit et al., 2020 [34] and maintained in the field condition at the botanical garden of the Faculty of Tropical Agrisciences (FTA), Czech University of Life Sciences Prague (CZU). The control *T. vulgaris* plants were obtained from the botanical garden (Index seminum number-343, year: 2019) identified by Marie Hlaváčová (Botanist and Curator) of botanical garden FTA, CZU. The plant materials were not deposited in the herbarium repository as they were obtained from seeds and through plant tissue culture for experimental purposes and later maintained in the field of the botanical garden. For experimental purposes, the plant materials were collected from the parental plants maintained in field conditions at the botanical garden of FTA, CZU (50.131115 N, 14.370528 E) with permission and relevant institutional guidelines. The flow cytometric analysis was conducted as a confirmatory test using a Partec PAS flow cytometer (Partec

GmbH, Munster Germany) equipped with a high-pressure mercury arc as described by Bharati et al. 2023 [35] and the results can be found in Supplementary (Fig. S1a-b).

4.2.2 Essential oil extraction

Fresh aerial parts of *T. vulgaris* were obtained from tetraploid and diploid control plants and dried at 30°C. Dried samples were then ground and homogenized using a Grindomix apparatus (GM 100 Retsch, Haan, Germany). The residual moisture content was evaluated gravimetrically in triplicate by Scaltec SMO 01 Analyzer (Scaltec Instruments, Gottingen, Germany) at 130°C for 1 h and expressed as arithmetic averages. Ground samples were then hydro-distilled using a Clevenger-type apparatus. The extracted essential oils were collected in air-tight glass vials and stored at 4°C until further use.

4.2.3 Chemical analysis of essential oils

Chemical characterization of essential oils has been done using the Agilent GC-7890B system (Agilent Technologies, Santa Clara, CA, USA) equipped with autosampler Agilent 7693, non-polar HP-5MS column (30 m × 0.25 mm, film thickness 0.25 µm, Agilent 19091s-433), and a flame ionization detector (FID) coupled with single quadrupole mass selective detector Agilent MSD-5977B. Samples were diluted in n-hexane for GC-MS analysis at a concentration of 20 µl/mL. 1 µl of the solution was injected in splitless mode. The injector temperature was 250°C. The initial temperature of the oven was 60°C for 1 minute and then increased to 240°C at a rate of 3°C/min. The transfer line temperature was kept at 250°C. We used helium as a carrier gas and the flow rate was 1 ml/min. The FID was programmed with a heating temperature of 250°C, an H_2 flow rate of 40 ml/min, an airflow rate of 400 ml/min, and a make-up flow rate of 30 ml/min. The MS analysis was carried out with the following conditions: ionization energy 70 eV, ion source temperature 230 °C, and mass range 30–550 m/z. The identification of chemical components was based on the comparison of their retention indices (RIs), retention times (RT), spectra with the National Institute of Standards and Technology Library (NIST 2.0.f), and the available literature [36]. The RI of the separated compounds was calculated using the retention times of the n-alkanes series ranging from C8 to C40 (Sigma-Aldrich, Prague, Czech Republic). The relative percentage content of chemical components was determined from FID.

4.2.4 Bacterial strain and culture media

For the antimicrobial assay, American Type Culture Collection (ATCC) that includes *Haemophilus influenzae* ATCC 49247, *Staphylococcus aureus* ATCC 29213, *Streptococcus pneumoniae* ATCC 49619, and *Streptococcus pyogenes* ATCC 19615 was used. The cultivation and assay media (broth/agar) were Mueller Hinton (MH), complemented with Haemophilus Test Medium and defibrinated horse blood for *H. influenzae*, MH only for *S. aureus,* and Brain Heart Infusion for both *S. pneumoniae* and *S. pyogenes*. The pH of the broth was adjusted to a final value of 7.6 using Trizma base (Sigma-Aldrich, Prague, Czech Republic). All microbial strains, growth media, and other supplements were purchased from Oxoid (Basingstoke, Hampshire, UK).

Stock cultures of bacterial strains were cultivated in appropriate media at 37°C for 24h before testing. The turbidity of the bacterial strains was adjusted to 0.5 McFarland standard using Densi-La-Meter II (Lachema, Brno, Czech Republic) to reach the final concentration of 10^7 CFU/mL. Ampicillin and amoxicillin were purchased from Sigma-Aldrich (Prague, Czech Republic) and assayed as positive antibiotic controls for all the strains used (CLSI).

4.2.5 Antimicrobial assay

In vitro growth-inhibitory effect of essential oils was assessed using the Broth Microdilution Volatilization (BMV) method that allows the assessment of the antibacterial activity of essential oils at different concentrations in both liquid and vapor phases as described by Hudokova et al. (2017) & (2018) [37, 38]. A standard 96-well microtiter plate (well volume = 400µL) with tight-fitting lids and flanges was used for this experiment. Each essential oil sample was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Prague, Czech Republic) at a maximum concentration of 1% and diluted in appropriate broth medium, and seven two-fold serial dilutions of samples of all essential oils starting from 1024 µg/mL were prepared with 100 µL as the final volume of each well. The plates were then inoculated with bacterial suspension using a 96-pin multi-blot replicator (National Institute of Public Health, Prague, Czech Republic) and incubated at 37°C for 24h. The wells containing inoculated and non-inoculated broth were simultaneously prepared for growth and purity controls. The Minimum Inhibitory Concentrations (MIC) were evaluated by visual assessment of bacterial growth after the coloring of metabolically active bacterial colonies with thiazolyl blue tetrazolium bromide dye (MTT) at a concentration of 600µg/mL (Sigma-Aldrich, Prague, Czech Republic). The MIC values were determined as the lowest concentrations that inhibited

the bacterial growth compared to compound-free control and expressed in µg/mL (1024, 512, 256, 128, 64, 32, and 16 µg/mL, respectively). In the case of vapor phase, these concentrations can be expressed as weight of volatile agent per unit volume of a well and the MIC values would be expressed in µg/cm^3 (256, 128, 64, 32, 16, 8, and 4 µg/cm^3, respectively). All the experiments were performed in triplicate with three independent measurements and the results were expressed as median/modal MIC values.

4.2.6 Antioxidant Activity

The radical scavenging assay using DPPH (2,2-diphenyl-1-picrylhydrazyl) was tested to determine the ability of samples to scavenge the DPPH radicals using the method described by Stastny et al., 2022 [39]. Samples were diluted in analytical-grade methanol to obtain the initial concentration of 1024 µg/mL. Subsequently, the serial dilution of each sample was prepared in methanol (100 µL) in 96-well microtiter plates. The radical scavenging reaction was started after the addition of 100 µL of freshly prepared 0.25 mM DPPH in methanol to each well along with samples, thus creating a range of 512 to 0.5 µg/mL. Trolox was used as a standard reference material and pure methanol as a blank control. The absorbance was measured at 517 nm using Synergy H1 multi-mode reader (BioTek, Winooski, Winooski, VT, USA). The results were expressed as half-maximal inhibitory concentrations (IC_{50} in µg/mL) and Trolox equivalents (mg TE/g extract).

4.2.7 *In vitro* anti-inflammatory activity

For evaluating anti-inflammatory activity, the inhibitory activity against cyclooxygenases was determined using the previously described method by Langhansova et al., 2017 [40] with slight modifications. COX-2 (0.125 units/reaction) was added to 180 µL of incubation mixture consisting of 100 mM Tris buffer (pH 8.0), 5 µM hematin porcine, 50 µM Na_2EDTA, and 18 mM L-epinephrine. The essential oil samples were dissolved in DMSO and 10 µL was added to incubation mixture in the 96-well microplate with 5 µL of COX enzyme. After adding 10 µM arachidonic acid the reaction was initiated. After 20 mins of incubation at 37^0 C, the reaction was ceased by adding 10 µL of 10% formic acid. The PGE_2 concentration was determined by PGE_2 ELISA kit according to the manufacturer's instructions and the final solutions were diluted at 1:15 in assay buffer. The absorbance was measured with a microplate reader (Tecan Infinite M200) at 405 nm and the inhibitory activity was calculated as the percentage inhibition of PGE_2 production compared to blank. (S)-(+)-ibuprofen was used as a

reference inhibitor and DMSO as the blank. The experiment was repeated at least two times with at least two technical replicates in each experiment.

4.2.8 Molecular docking study

Molecular docking was done to understand the interaction of major compounds identified from essential oils with bacterial protein receptors along with confirming their antioxidant and anti-inflammatory properties. The docking study was conducted according to the previously described method by Gupta et al., 2023 [41]. The crystal structure of seven universal bacterial proteins such as isoleucyl-tRNA synthetase (PDB ID: 1JZQ), DNA gyrase (PDB ID: 1KZN), dihydropteroate synthase (PDB ID: 2VEG), D-alanine: D-alanine ligase (PDB ID: 2ZDQ), topoisomerase IV (PDB ID: 3RAE), dihydrofolate reductase (PDB ID: 3SRW), penicillin-binding protein 1a (PDB ID: 3UDI), and also protein human cyclin-dependent kinase 2 complex (PDB ID: 1HCK), and cyclooxygenase-2 (PDB: 1CX2) were obtained from Protein Data Bank (https://www.rcsb.org/, accessed on 12 November 2023). Ascorbic acid was used in antioxidant activity as a positive reference as described by Mendes-da-Silva et al., 2014 [42]. Each center and size were submitted to AutoDock Tools (https://autodock.scripps.edu/download-autodock4/) for docking using the interface of the command prompt and the interaction and visualization were performed for the best-docked complexes using LigPlot + ver. 2.2 (https://www.ebi.ac.uk/thornton-srv/software/LigPlus/download.html).

4.2.9 Statistical analysis

The data obtained from the antioxidant and anti-inflammatory activity of control diploid and induced polyploid genotypes were presented as means ± SD. The IC_{50} of antioxidant activity (half-maximal inhibitory concentration) was calculated by plotting the values for % inhibition ($absorbance_{blank} - absorbance_{sample}/absorbance_{blank} \times 100$) to the particular concentration of the sample/positive control. The IC_{50} was expressed as the concentration (in µg/mL) corresponding to the 50% inhibition of the DPPH radical with the use of the Gen5 microplate and imager software ver 3.04 (BioTek, Winooski, USA) (https://www.agilent.com/en/product/cell-analysis/cell-imaging-microscopy/cell-imaging-microscopy-software/biotek-gen5-software-for-imaging-microscopy-1623226). All obtained values from essential oil yield, antioxidant, and anti-inflammatory activity were analyzed and compared based on Tukey's post hoc analysis (5% significance level) in the Microsoft Excel 2021 software package (https://softwarekeep.eu/microsoft-office-2021-home-and-student-pc.html).

Ethical statement

All experiments conducted in this study, including essential oil extraction (according to European Pharmacopoeia) [43], antimicrobial activity (according to CLSI) [44], and the collection of plant material, comply with relevant institutional, national, and international guidelines and legislation.

4.3 Results

4.3.1 Essential oil yield and chemical composition

T. vulgaris essential oils from tetraploid and diploid genotypes were extracted by hydro-distillation method with an average moisture content of 12.77% and 13.45%, respectively. The essential oil yield values were 1.2% and 0.85% in tetraploid and diploid plants, respectively. The polyploid plants yielded higher amount of essential oil than the control diploid (Figure 6). All essential oils presented a strong fragrance with a palish-yellow color. GC-MS analysis resulted in the identification of 16 compounds in total in both genotypes representing 99.86% (diploid) and 99.88% (polyploid) of their corresponding total constituents (Table 1) as well as Supplementary (Figure S2 a-b). Monoterpenoid represented by thymol was the most abundant compound found in tetraploid and diploid (53.5% and 50.65%, respectively), followed by monoterpenes comprising of γ-terpinene and *p*-cymene constituting 21.81% and 7.85% in tetraploid and 5.55% and 20.40% in diploid, respectively. The major compounds considered were based on the significant amount of the total composition present in both diploid and polyploid essential oils which is above 5%. These three major compounds together constituted 83.16% and 76.67% of the total composition of tetraploid and diploid *T. vulgaris* essential oil, respectively. To a lesser extent, other compounds were identified such as caryophyllene (5.8% and 2.13%), caryophyllene oxide (0.67% and 1.71%), borneol (1.34% and 3.78%), d-camphor (0.21% and 4.21%), eucalyptol (1.01% and 3.74%) which had significant differences between tetraploid and diploid genotypes, respectively. The differences in the content of other components did not exceed 1%.

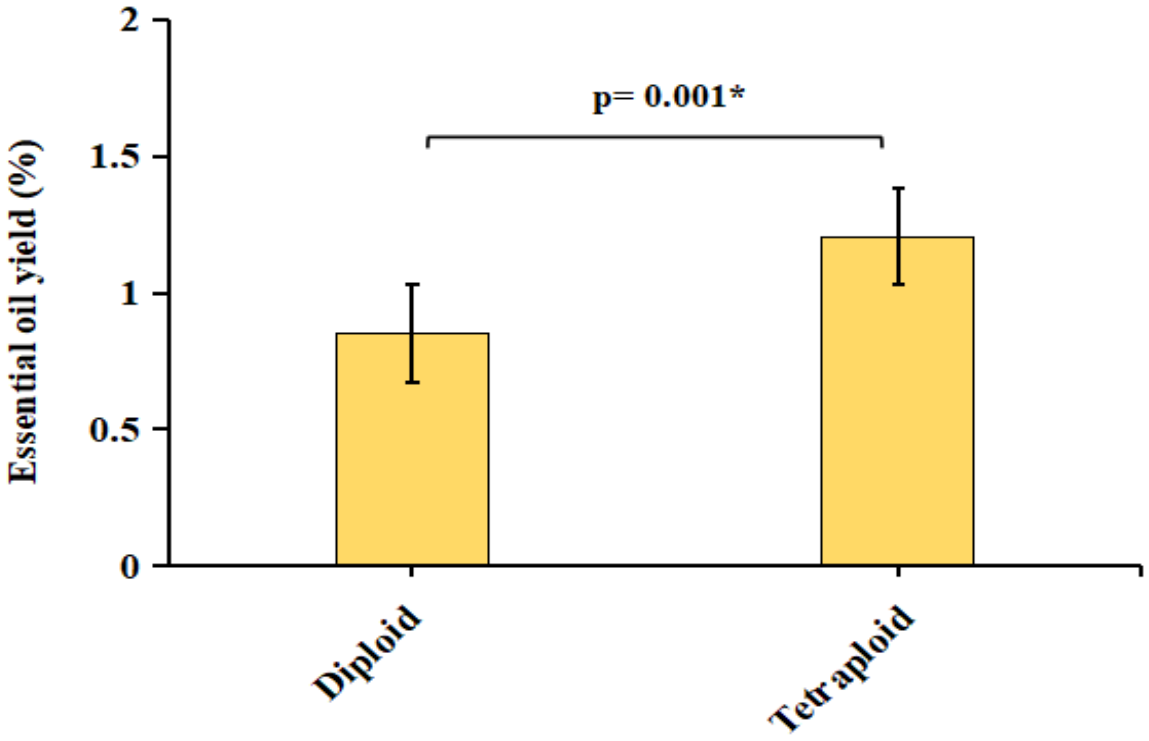

Figure 6 Essential oil yield in control diploid and induced polyploid of *T. vulgaris*. "*" expressed a significant difference (Tukey HSD Test, $p < 0.05$).

Table 1 Essential oil constituents of diploid (Control) and polyploid genotype of *T. vulgaris*.

Compound name	RI[a]		Content [%][c]	
	Observed	Literature[b]	Diploid	Tetraploid
3-Thujene	926	931	0.26	0.30
α-Pinene	933	937	0.54	0.24
Camphene	948	953	0.87	0.16
Myrcene	991	991	0.68	0.76
4-Carene	1017	1009	0.70	1.5
p-Cymene	1026	1026	**20.40**	**7.85**
Eucalyptol	1031	1033	3.74	1.01
γ-Terpinene	1060	1062	**5.55**	**21.81**
Linalool	1103	1098	2.82	2.79
d-Camphor	1146	1143	4.21	0.21
Borneol	1171	1165	3.78	1.34
4-Terpineneol	1181	1175	1.70	1.29

Thymol	1307	1290	**50.65**	**53.5**
Caryophyllene	1423	1418	2.13	5.8
D-Germacrene	1485	1480	0.12	0.65
Caryophyllene oxide	1589	1581	1.71	0.67
Total			**99.86**	**99.88**

a: Kovats' retention indices measured on HP-5MS column; b: retention indices from literature; c: relative percentage content based on the total area of all peaks [45].

4.3.2 Antimicrobial activity

Samples of essential oil from diploid and tetraploid *T. vulgaris* were tested against four standard bacterial strains related to respiratory infections (Table 2). All essential oils offered a certain degree of antibacterial efficacy ranging from 128 to 1024 µg/mL in both liquid and vapor phases. The tetraploid essential oil was the most active with the lowest MIC value of 128 µg/mL in liquid and 1024 µg/mL in vapor phase whereas, diploid essential oil showed the lowest MIC value of 256 µg/mL in liquid and 1024 µg/mL in vapor phase. *H. influenzae* growth was most sensitive to *T. vulgaris* essential oil where tetraploid presented a MIC value of 128 µg/mL and diploid presented a MIC value of 256 µg/mL in the liquid phase. However, in the vapor phase both essential oils presented a MIC value of more than 1024 µg/mL. Mild activity against *S. pyogenes* and *S. aureus* (512 µg/mL) for tetraploid and weak activity (1024 µg/mL) for diploid in the liquid phase was recorded. For vapor phase, MIC value of 1024 µg/mL was observed for both essential oils against *S. pyogenes,* however, a concentration greater than 1024 µg/mL was needed for diploid essential oil against *S. aureus.* Against *S. pneumoniae* both essential oils showed the same activity (512 µg/mL and 1024 µg/mL) for liquid and vapor phases, respectively. It was observed that both essential oils were more effective in the liquid phase against all tested microorganisms than in the vapor phase.

Table 2 *In vitro* growth-inhibitory effect of *T. vulgaris* L. essential oils (control and tetraploid) in liquid and vapor phases against respiratory infection bacteria.

Bacterium/Growth Medium/Minimum Inhibitory Concentration								
Essential Oil	***Haemophilus influenzae***		***Staphylococcus aureus***		***Streptococcus pneumoniae***		***Streptococcus pyogenes***	
	Agar*	Broth	Agar	Broth	Agar	Broth	Agar	Broth
	(µg/mL)							
Diploid	>1024	256	>1024	1024	1024	512	1024	1024
Tetraploid	>1024	128	1024	512	1024	512	1024	512
Positive antibiotic control								
Amoxicillin	NT	NT	NT	NT	>2	>2	NT	NT
Ampicillin	>2	0.25	>2	2	NT	NT	>2	2

NT: Not tested; *: If the distribution of volatiles is uniform in liquid and gaseous phase, the concentrations can be expressed as weight of volatile agent per volume unit of a well, whereas their real values will be 256, 128, 64, 32, 16, 8, 4 and 2 µg/cm^3 for 1024, 512, 256, 128, 64, 32, 16, and 8 µg/mL, respectively.

4.3.3 Antioxidant activity

The DPPH radical scavenging assay was used for the screening of the antioxidant activity between *T. vulgaris* diploid and polyploid essential oils. The antioxidant activity results are summarized as IC_{50} and TE (Trolox equivalent) in Table 3. It was observed from the results that the tetraploid essential oil was more potent in inhibiting the DPPH radical. The antioxidant activity of essential oil from tetraploid thyme was stronger with half-maximal inhibitory concentrations (IC_{50}) of 180.03 ± 51.50 µg/mL (40.05 ± 14.01 µg TE/mg), while the diploid essential oil was found to be an IC_{50} value of more than 512 µg/mL (less than 12.68 µg TE/mg). The antioxidant activities were found highly significant ($p < 0.05$) in the tetraploid compared to the diploid. It was also observed that none of the tested essential oils had significantly higher activity as compared to trolox (IC_{50} 6.49 ± 1.01 µg/mL). The tetraploid genotype possessed the highest DPPH radical scavenging activity and therefore, has the ability to prevent oxidative stress better than the diploid genotype.

Table 3 Antioxidant activity of *T. vulgaris* diploid and polyploid essential oils.

Plant sample	DPPH	
	IC_{50} ±SD[1](µg/mL)	**µg TE/mg ± SD**
Diploid	> 512	>12.68
Tetraploid	180.03 ± 51.50*	40.05 ± 14.01*
Positive control Trolox	6.49 ± 1.01	-

[1]IC_{50} ± SD: half maximal inhibitory concentration ± standard deviation; TE = Trolox equivalent; *: shows a significant difference between the diploid and tetraploid genotype based on Tukey's test for post hoc analysis at 5% significance level.

4.3.4 Anti-inflammatory activity

In vitro anti-inflammatory activity of *T. vulgaris* diploid and polyploid essential oils was tested as inhibition of COX-2 catalytic activity. In comparison to untreated control, the PGE_2 production was significantly reduced in the presence of 500 and 50 µg/mL of essential oils whereas they were inactive at 5 µg/mL concentration. The activity of both essential oils was comparable (Table 4). The anti-inflammatory activity between tetraploid and diploid essential oil was found to be significant ($p < 0.05$) at 50 µg/mL concentration with an inhibition value of 83.74 ± 5.8 and 70.53 ± 11.86, respectively.

Table 4 *In vitro* anti-inflammatory activity of *T. vulgaris* diploid and polyploid essential oils determined by inhibition of COX-2 enzyme.

Samples	Concentration (µg/mL)	Inhibition %
Diploid	500	80.96 ± 7.2
	50	70.53 ± 11.86
	5	2.02 ± 17.13
Tetraploid	500	85.57 ± 7.5
	50	83.74 ± 5.8*
	5	6.74 ± 23.48
Ibuprofen	5	74.52 ± 10.2

The results are expressed as means ± SD for two independent experiments measured in duplicate. The results were compared by Tukey's test for post hoc analysis at 5% significance level. *: shows a significant difference between the diploid and tetraploid genotypes.

4.3.5 Molecular docking

The molecular interactions between major volatile compounds like *p*-cymene, γ –terpinene, and thymol of *T. vulgaris* essential oil and the vital enzymes involved in biosynthesis and repair of cell walls, nucleic acids, and proteins in bacteria along with protein human cyclin-dependent kinase 2 complex and cyclooxygenase-2 are summarized in Table 5. All the abundant compounds were found to be most actively binding with D-alanine: D-alanine ligase (PDB ID: 2ZDQ) enzyme with the highest binding affinity of *p*-cymene (-7.9 kcal/mol) followed by γ –terpinene (-7.8 kcal/mol) and thymol (-7.7 kcal/mol). It was found that the DNA gyrase (PDB ID: 1KZN) depicted highest binding affinity towards thymol (-6.3 kcal/mol) with a hydrogen bond of bond length 3.01 Å between hydroxyl group of thymol and ASP A:73 of 1KZN and also a hydrophobic interaction involving A chains of ALA47, ASN46, GLU50, ILE78, THR165, VAL43, VAL71, and VAL167, followed by *p*-cymene (-5.8 kcal/mol) and γ –terpinene (-4.6 kcal/mol). The difference in binding affinity of the three major compounds was very similar for the other bacterial protein receptors. A good binding affinity of cyclooxygenase-2 (PDB ID: 1CX2) enzyme protein with all abundant compounds was observed, where the binding affinity of γ-terpinene (-6.3 kcal/mol) and *p*-cymene (-6.3 kcal/mol) were the same, but thymol (-6.5 kcal/mol) showed slightly higher affinity due to presence of hydrogen bond of bond length 2.81 Å between hydroxyl group of thymol and ARG A:376 of 1CX2. In addition, there were hydrophobic interactions involving A chains of ALA151, ALA378, ARG150, ASP125, ASN375, ILE124, PHE529, and THR149. The binding affinity of thymol towards the protein human cyclin-dependent kinase 2 complex (PDB ID: 1HCK) with a value of -6.4 kcal/mol was considered better than ascorbic acid (-5.0 kcal/mol). There were two hydrogen bonds each of bond length 2.95 Å and 3.32 Å present between the hydroxyl group of thymol with GLU A:81 and LEU A:83, respectively. There were also hydrophobic interactions involving A chains of ALA31, ALA144, ILE10, LEU134, PHE82, VAL18, VAL64, and PHE80. Since, thymol depicted the best docking scores for 1KZN (-6.3 kcal/mol), 1CX2 (-6.5 kcal/mol), and 1HCK (-6.4 kcal/mol), binding analysis was conducted to reveal the interactions between ligands and protein-binding sites (Figure 7 & 8).

Table 5 Binding free-energy values of major volatile compounds of *T. vulgaris* essential oil.

Ligand	Binding Free Energy ΔG (kcal/mol)								
	1JZQ*	1KZN	2VEG	2ZDQ	3RAE	3SRW	3UDI	1CX2	1HCK
γ -terpinene	-5.7	-4.6	-4.5	-7.8	-5.3	-5.6	-5.0	-6.3	-5.2
***p*-cymene**	-5.8	-5.8	-4.6	-7.9	-5.8	-5.6	-5.1	-6.3	-4.5
Thymol	-5.4	-6.3	-4.7	-7.7	-5.6	-5.7	-5.2	-6.5	-6.4
Ascorbic acid**	-	-	-	-	-	-	-	-	-5.0

*Protein PDB ID:1JZQ- isoleucyl-tRNA synthetase, 1KZN- DNA gyrase, 2VEG-dihydropteroate synthase, 2ZDQ-D-alanine:D-alanine ligase, 3RAE-topoisomerase 4, 3SRW-dihydrofolate reductase, 3UDI-penicillin-binding protein 1a, 1CX2- cyclooxygenase-2, and 1HCK- protein human cyclin-dependent kinase 2 complex. **Ascorbic acid: Used as a reference for antioxidant activity.

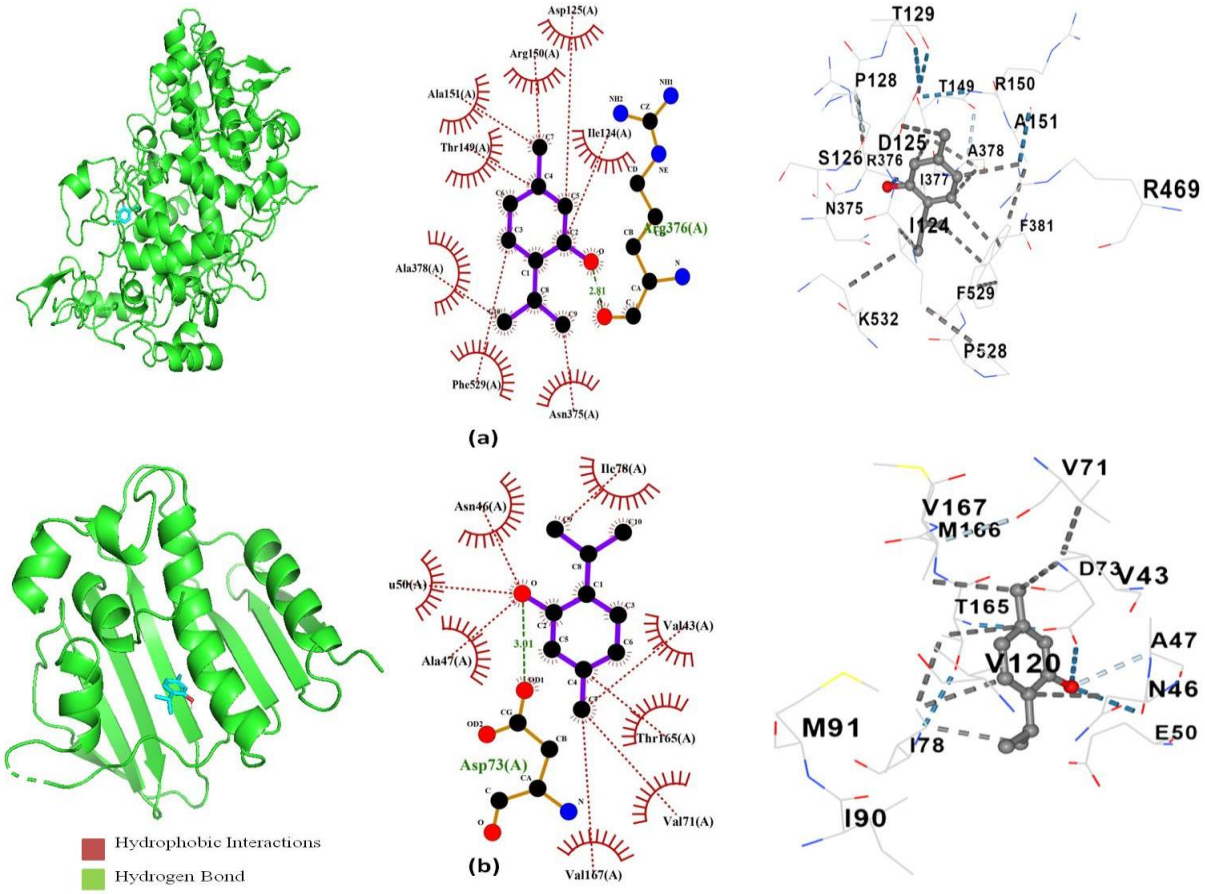

Figure 7 Interactions and docked 3D structures of (**a**) thymol with cyclooxygenase-2 enzyme 1CX2, (**b**) thymol with DNA gyrase 1KZN.

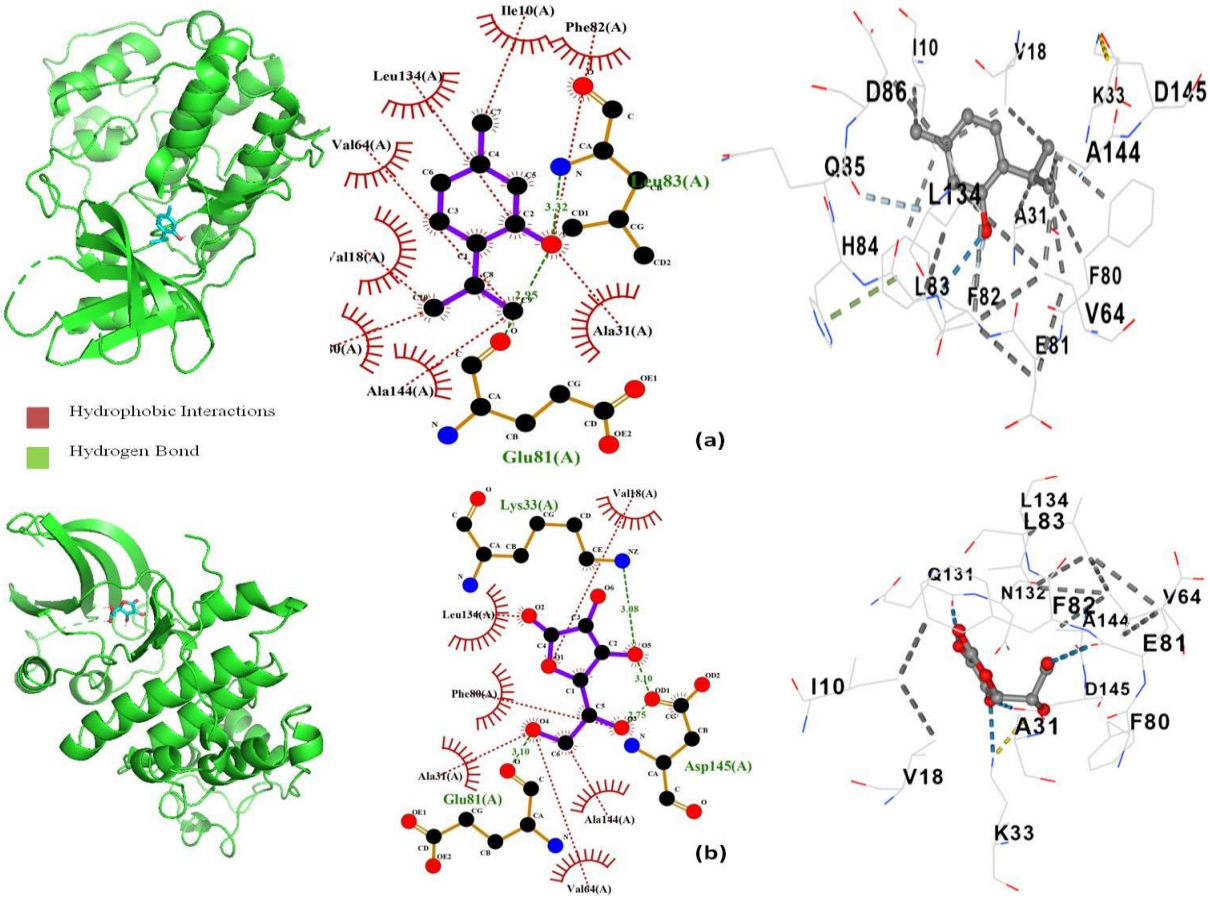

Figure 8 Interactions and docked 3D structures of (**a**) thymol with protein human cyclin-dependent kinase 2 complex 1HCK and (**b**) ascorbic acid with protein human cyclin-dependent kinase 2 complex 1HCK as control.

4.4 Discussion

In vitro polyploidization using synthetic AMAs can be an effective method to generate polyploid plants with enhanced biological traits. Still, it is not applicable every time as the result of gene duplication is unknown. [46, 47]. In the Lamiaceae family, polyploidization has drawn attention to crop improvement because of its potential to achieve higher secondary metabolites as well as increased essential oil content [34, 35]. Therefore, it is important to analyze the effect of polyploidization on the biological activity of essential oils.

Previously, it has been reported that the average essential oil yield of *T. vulgaris* ranges between 0.3 to 1.2% [7]. In our study, we found that the essential oil yield of the diploid control was 0.85% whereas the polyploid genotype exhibited an increased amount of essential oil content (1.2%) which is an increase of 41.11% compared to the control diploid genotype. Similarly, the increased essential oil content in the induced polyploids of *T. vulgaris* has been reported

previously [34, 48]. The enhanced essential oil yield in polyploid plants has also been observed in other Lamiaceae family species such as *Mentha spicata* [35] and *Tetradenia riparia* [49]. However, significantly lower essential oil content was observed in polyploid *Humulus lupulus* than in diploid as an effect of synthetic polyploidization [50]. Although, there is not always an increase in essential oil quantity, however, our results indicated the potential to enhance essential oil quantity through synthetic polyploidization that can be used as an important tool for crop breeding.

Essential oil yield along with its phytochemical constituents can be affected by polyploidization [34]. GC-MS analysis revealed that the essential oil of both *T. vulgaris* genotypes consisted of three major components thymol, γ-terpinene, and *p*-cymene. When compared with the diploid control, thymol and γ-terpinene contents increased in tetraploid essential oil whereas *p*-cymene was found in higher amounts in the diploid control. These major compounds were reported to have antimicrobial, antioxidant, and anti-inflammatory activities [12, 51, 32, 52] and are also widely used in pharmaceutical and food industries. Similarly, Homaidan Shmeit et al., 2020 [34] and Navratilova et al., 2021 [48] have reported increased thymol and γ-terpinene contents in the polyploid *T. vulgaris* essential oil and decreased *p*-cymene content. However, a decrease in the amount of major compounds has been reported in some polyploid plants [53]. It can be assumed that the increased amount of these secondary metabolites in *T. vulgaris* polyploid essential oil is majorly responsible for its enhanced biological activities compared to the control diploid.

In this study, the antimicrobial activity on respiratory pathogens such as *H. influenzae*, *S. aureus*, *S. pneumoniae*, and *S. pyogenes* revealed that the induced tetraploid *T. vulgaris* essential oil has higher antibacterial activity in comparison to diploid control, although, both genotypes exhibited the best results in the liquid phase. Several works on antibacterial activity have been previously reported for diploid *T. vulgaris* essential oil that showed similar results to our findings [7, 14] but this is the first report on the antibacterial activity of induced tetraploid *T. vulgaris* essential oil. The antimicrobial activity of tetraploid essential oil against these tested microbes revealed that higher concentrations of abundant compounds may be majorly responsible for the increased antimicrobial activity in the tetraploid line as they have previously been well established for their antimicrobial activity [51,52]. The molecular interaction study revealed that thymol has a higher binding affinity towards DNA gyrase than other major compounds which is an essential target for antibacterial agents as It regulates DNA structure during transcription and replication by introducing breaks in both DNA strands, which is

crucial for bacterial survival. Therefore, the higher amount of thymol content in polyploid essential oil probably contributes to its higher antibacterial activity. A similar increased antibacterial activity has been reported for tetraploid-induced *Mitracarpus hirtus* where the tetraploid line exhibited higher antibacterial activity against *S. aureus and B. subtilis* [54].

The tetraploid essential oil showed an increased amount of DPPH radical scavenging activity which means it is a better hydrogen provider compared to the diploid control genotype. The compounds present in *T. vulgaris* essential oils contain conjugated carbon double bonds and hydroxyl groups that readily inhibit free radicals that lead to antioxidant effects [32]. Several works described significant results for the antioxidant activity of *T. vulgaris* essential oil [55, 56, 57]. However, this is the first reported study demonstrating the antioxidant activity of induced polyploid *T. vulgaris* essential oil. It can be assumed that chromosome doubling genetically influenced the secondary metabolite production which resulted in increased antioxidant activity in the tetraploid genotype. Previously, it was reported that the effect of colchicine-induced tetraploid *Citrus limon* exhibited higher antioxidant activity than the diploid genotype [58]. Another study reported that the radical scavenging activity of *Geranium macrorrhizum* was related to the plant ploidy level [59]. Also, the molecular docking study revealed that thymol has a high binding affinity towards the protein human cyclin-dependent kinase 2 complex more than the known antioxidant agent ascorbic acid. It can be expected that the effectiveness of polyploid *T. vulgaris* essential oil in scavenging the DPPH radical is probably due to the increased substantial content of monoterpenoids and monoterpenes that were previously identified as potential antioxidants [51, 32].

COX-1 and COX-2 are two cyclooxygenase isoforms. COX-2 is an inducible form that catalyzes the biosynthesis of pro-inflammatory prostanoids (actually, the role of both COX forms is much more complex). COX inhibitors are used to relieve acute and chronic pain and inflammation [60]. We observed slightly higher activity of essential oil isolated from tetraploid plants. The major compound of thyme essential oil thymol is known as a potent COX inhibitor [61]. Also, the docking study showed a higher binding efficacy of thymol with the cyclooxygenase-2 protein. Therefore, a slightly higher amount of thymol found in essential oil from tetraploid can contribute to its higher activity. However, it is possible that other compounds contained in essential oils could also influence the overall activity of essential oils. Polyploidization can result in the opposite effect as reported for *Gynostemma pentaphyllum* leaf extracts where diploid showed the strongest inhibitory effects on the expression of TNF-

α, IL-6, and COX-2 mRNA [33]. However, our results indicate that polyploidization could be an effective strategy for obtaining plant products with enhanced bioactivity.

4.5 Conclusion

In the current study, the characterization of valuable biological activities of oryzalin-induced polyploid *T. vulgaris* essential oil has been acquired for the first time. These findings also indicate the effectiveness of synthetic polyploidization in *T. vulgaris.* The induced genotype exhibited a significant increase in essential oil yield with simultaneously higher concentrations of biologically active compounds such as thymol and γ-terpinene. The polyploid genotype exhibited enhanced antibacterial, antioxidant, and anti-inflammatory activities compared to the diploid genotype. Additionally, this study suggests that the induced genotype of *T. vulgaris* has improved traits that can be embraced for commercial use to obtain economic advantage, especially in the pharmaceutical and food industries due to the enhanced quantity and quality of essential oil. Synthetic polyploidization may perform a crucial role in the breeding of plants with high biological activities. However, further *in vivo* studies should be assessed to confirm their practical application in the above-mentioned industries.

References

[1] Ghorab, H., Kabouche, A. & Kabouche, Z. Comparative compositions of essential oils of Thymus growing in various soils and climates of North Africa. (2014).

[2] Horváth, G. & Ács, K. Essential oils in the treatment of respiratory tract diseases highlighting their role in bacterial infections and their anti-inflammatory action: a review. Flavour Fragr. J. 30, 331–341 (2015).

[3] Kowalczyk, A., Przychodna, M., Sopata, S., Bodalska, A. & Fecka, I. Thymol and Thyme Essential Oil—New Insights into Selected Therapeutic Applications. Molecules 25, 4125 (2020).

[4] TSAI, M.-L., LIN, C.-C., LIN, W.-C. & YANG, C.-H. Antimicrobial, Antioxidant, and Anti-Inflammatory Activities of Essential Oils from Five Selected Herbs. Biosci. Biotechnol. Biochem. 75, 1977–1983 (2011).

[5] Van Vuuren, S. F., Suliman, S. & Viljoen, A. M. The antimicrobial activity of four commercial essential oils in combination with conventional antimicrobials. Lett. Appl. Microbiol. 48, 440–446 (2009).

[6] Senatore, F. Influence of Harvesting Time on Yield and Composition of the Essential Oil of a Thyme (*Thymus pulegioides* L.) Growing Wild in Campania (Southern Italy). J. Agric. Food Chem. 44, 1327–1332 (1996).

[7] Borugă, O. et al. *Thymus vulgaris* essential oil: chemical composition and antimicrobial activity. J. Med. Life 7, 56–60 (2014).

[8] Tohidi, B., Rahimmalek, M., Arzani, A. & Sabzalian, M. R. Thymol, carvacrol, and antioxidant accumulation in *Thymus* species in response to different light spectra emitted by light-emitting diodes. Food Chem. 307, 125521 (2020).

[9] Salehi, B. et al. *Thymus* spp. plants - Food applications and phytopharmacy properties. Trends Food Sci. Technol. 85, 287–306 (2019).

[10] Fachini-Queiroz, F. C. et al. Effects of Thymol and Carvacrol, Constituents of *Thymus vulgaris* L. Essential Oil, on the Inflammatory Response. Evid. Based Complement. Alternat. Med. 2012, e657026 (2012).

[11] Marchese, A. et al. Antibacterial and antifungal activities of thymol: A brief review of the literature. Food Chem. 210, 402–414 (2016).

[12] Kazemi, M. Chemical Composition, Antimicrobial, Antioxidant and Anti-inflammatory Activity of *Carum copticum* L. Essential Oil. J. Essent. Oil Bear. Plants 17, 1040–1045 (2014).

[13] Reyes-Jurado, F., Cervantes-Rincón, T., Bach, H., López-Malo, A. & Palou, E. Antimicrobial activity of Mexican oregano (*Lippia berlandieri*), thyme (*Thymus vulgaris*), and mustard (*Brassica nigra*) essential oils in gaseous phase. Ind. Crops Prod. 131, 90–95 (2019).

[14] Antih, J., Houdkova, M., Urbanova, K. & Kokoska, L. Antibacterial Activity of *Thymus vulgaris* L. Essential Oil Vapours and Their GC/MS Analysis Using Solid-Phase Microextraction and Syringe Headspace Sampling Techniques. Molecules 26, 6553 (2021).

[15] Quesada, J., Sendra, E., Navarro, C. & Sayas-Barberá, E. Antimicrobial Active Packaging including Chitosan Films with *Thymus vulgaris* L. Essential Oil for Ready-to-Eat Meat. Foods 5, 57 (2016).

[16] Orhan-Yanıkan, E., Gülseren, G. & Ayhan, K. Antimicrobial characteristics of *Thymus vulgaris* and *Rosa damascena* oils against some milk-borne bacteria. Microchem. J. 183, 108069 (2022).

[17] Mahmoodi, M. et al. Beneficial effects of *Thymus vulgaris* extract in experimental autoimmune encephalomyelitis: Clinical, histological and cytokine alterations. Biomed. Pharmacother. 109, 2100–2108 (2019).

[18] Jaouadi, R., Boussaid, M. & Zaouali, Y. Variation in essential oil composition within and among Tunisian *Thymus algeriensis* Boiss et Reut. (Lamiaceae) populations: Effect of ecological factors and incidence on antiacetylcholinesterase and antioxidant activities. Biochem. Syst. Ecol. 106, 104543 (2023).

[19] Patra, B., Schluttenhofer, C., Wu, Y., Pattanaik, S. & Yuan, L. Transcriptional regulation of secondary metabolite biosynthesis in plants. Biochim. Biophys. Acta BBA - Gene Regul. Mech. 1829, 1236–1247 (2013).

[20] Guimarães, A. F., Vinhas, A. C. A., Gomes, A. F., Souza, L. H. & Krepsky, P. B. essential oil of *Curcuma longa* L. Rhizomes chemical composition, yield variation and stability. Quím. Nova 43, 909–913 (2020).

[21] Gomes, A. F. et al. Simultaneous determination of iridoids, phenylpropanoids and flavonoids in *Lippia alba* extracts by micellar electrokinetic capillary chromatography. Microchem. J. 138, 494–500 (2018).

[22] Zhang, C., Wohlhueter, R. & Zhang, H. Genetically modified foods: A critical review of their promise and problems. Food Sci. Hum. Wellness 5, 116–123 (2016).

[23] Niazian, M. & Nalousi, A. M. Artificial polyploidy induction for improvement of ornamental and medicinal plants. Plant Cell Tissue Organ Cult. PCTOC 142, 447–469 (2020).

[24] Baiton, A. Novel Strategies for Sustainable Rapid Breeding of *Cannabis sativa* L. (University of Guelph, 2024).

[25] Soltis, P. S., Marchant, D. B., Van de Peer, Y. & Soltis, D. E. Polyploidy and genome evolution in plants. Curr. Opin. Genet. Dev. 35, 119–125 (2015).

[26] Salma, U., Kundu, S. & Mandal, N. Artificial polyploidy in medicinal plants: Advancement in the last two decades and impending prospects. J. Crop Sci. Biotechnol. 20, 9–19 (2017).

[27] Beranová, K. et al. Morphological, Cytological, and Molecular Comparison between Diploid and Induced Autotetraploids of *Callisia fragrans* (Lindl.) Woodson. Agronomy 12, 2520 (2022).

[28] Jadaun, J. S., Yadav, R., Yadav, N., Bansal, S. & Sangwan, N. S. Influence of Genetics on the Secondary Metabolites of Plants. in Natural Secondary Metabolites: From Nature, Through Science, to Industry (eds. Carocho, M., Heleno, S. A. & Barros, L.) 403–433 (Springer International Publishing, 2023).

[29] Jmii, G., Gharsallaoui, S., Mars, M. & Haouala, R. Polyploidization of Trigonella foenum-graecum L. enhances its phytotoxic activity against *Cyperus rotundus* L. South Afr. J. Bot. 153, 336–345 (2023).

[30] Farhadi, N. & Moghaddam, M. Application of Recent Advanced Technologies for the Improvement of Medicinal and Aromatic Plants. in Biosynthesis of Bioactive Compounds in Medicinal and Aromatic Plants: Manipulation by Conventional and Biotechnological Approaches (eds. Kumar, N. & S. Singh, R.) 235–255 (Springer Nature Switzerland, 2023).

[31] Saleem, A. et al. HPLC, FTIR and GC-MS Analyses of *Thymus vulgaris* Phytochemicals Executing *In Vitro* and *In Vivo* Biological Activities and Effects on COX-1, COX-2 and Gastric Cancer Genes Computationally. Molecules 27, 8512 (2022).

[32] Diniz do Nascimento, L. et al. Bioactive Natural Compounds and Antioxidant Activity of Essential Oils from Spice Plants: New Findings and Potential Applications. Biomolecules 10, 988 (2020).

[33] Xie, Z. et al. Chemical composition and anti-proliferative and anti-inflammatory effects of the leaf and whole-plant samples of diploid and tetraploid *Gynostemma pentaphyllum* (Thunb.) Makino. Food Chem. 132, 125–133 (2012).

[34] Homaidan Shmeit, Y. et al. Autopolyploidy effect on morphological variation and essential oil content in *Thymus vulgaris* L. Sci. Hortic. 263, 109095 (2020).

[35] Bharati, R. et al. Oryzalin induces polyploids with superior morphology and increased levels of essential oil production in *Mentha spicata* L. Ind. Crops Prod. 198, 116683 (2023).

[36] Adams, R. P. Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy. Identif. Essent. Oil Compon. Gas Chromatogr. Mass Spectrosc. (2001).

[37] Houdkova, M., Rondevaldova, J., Doskocil, I. & Kokoska, L. Evaluation of antibacterial potential and toxicity of plant volatile compounds using new broth microdilution volatilization method and modified MTT assay. Fitoterapia 118, 56–62 (2017).

[38] Houdkova, M. et al. *In vitro* growth-inhibitory effect of Cambodian essential oils against pneumonia causing bacteria in liquid and vapour phase and their toxicity to lung fibroblasts. South Afr. J. Bot. 118, 85–97 (2018).

[39] Stastny, J. et al. Antioxidant and Anti-Inflammatory Activity of Five Medicinal Mushrooms of the Genus *Pleurotus*. Antioxidants 11, 1569 (2022).

[40] Langhansova, L. et al. Myrica rubra leaves as a potential source of a dual 5-LOX/COX inhibitor. Food Agric. Immunol. 28, 343–353 (2017).

[41] Gupta, N. et al. Systematic analysis of antimicrobial activity, phytochemistry, and *in silico* molecular interaction of selected essential oils and their formulations from different Indian spices against foodborne bacteria. Heliyon 9, e22480 (2023).

[42] Mendes-da-Silva, R. F. et al. Prooxidant versus antioxidant brain action of ascorbic acid in well-nourished and malnourished rats as a function of dose: A cortical spreading depression and malondialdehyde analysis. Neuropharmacology 86, 155–160 (2014).

[43] Essential oils: revised monograph and new general chapter in the Ph. Eur. European Directorate for the Quality of Medicines & HealthCare https://www.edqm.eu/en/-/essential-oils-revised-monograph-and-new-general-chapter-in-the-ph.-eur

[44] Clinical & Laboratory Standards Institute: CLSI Guidelines. Clinical & Laboratory Standards Institute https://clsi.org/.

[45] Aggio, R. B., Mayor, A., Reade, S., Probert, C. S. & Ruggiero, K. Identifying and quantifying metabolites by scoring peaks of GC-MS data. BMC Bioinformatics 15, 374 (2014).

[46] Tavan, M., Mirjalili, M. H. & Karimzadeh, G. *In vitro* polyploidy induction: changes in morphological, anatomical and phytochemical characteristics of *Thymus persicus* (Lamiaceae). Plant Cell Tissue Organ Cult. PCTOC 122, 573–583 (2015).

[47] Sattler, M. C., Carvalho, C. R. & Clarindo, W. R. The polyploidy and its key role in plant breeding. Planta 243, 281–296 (2016).

[48] Navrátilová, B., Švécarová, M., Bednář, J. & Ondřej, V. *In Vitro* Polyploidization of *Thymus vulgaris* L. and Its Effect on Composition of Essential Oils. Agronomy 11, 596 (2021).

[49] Hannweg, K., Visser, G., de Jager, K. & Bertling, I. *In vitro*-induced polyploidy and its effect on horticultural characteristics, essential oil composition and bioactivity of *Tetradenia riparia*. South Afr. J. Bot. 106, 186–191 (2016).

[50] Trojak-Goluch, A. & Skomra, U. Artificially induced polyploidization in *Humulus lupulus* L. and its effect on morphological and chemical traits. Breed. Sci. 63, 393–399 (2013).

[51] Kazemi, M. Phytochemical Composition, Antioxidant, Anti-inflammatory and Antimicrobial Activity of *Nigella sativa* L. Essential Oil. J. Essent. Oil Bear. Plants 17, 1002–1011 (2014).

[52] Nikolić, M. et al. Chemical composition, antimicrobial, antioxidant and antitumor activity of *Thymus serpyllum* L., *Thymus algeriensis* Boiss. and Reut and *Thymus vulgaris* L. essential oils. Ind. Crops Prod. 52, 183–190 (2014).

[53] Tan, F.-Q. et al. Polyploidy remodels fruit metabolism by modifying carbon source utilization and metabolic flux in Ponkan mandarin (*Citrus reticulata* Blanco). Plant Sci. 289, 110276 (2019).

[54] Pansuksan, K., Sangthong, R., Nakamura, I., Mii, M. & Supaibulwatana, K. Tetraploid induction of *Mitracarpus hirtus* L. by colchicine and its characterization including antibacterial activity. Plant Cell Tissue Organ Cult. PCTOC 117, 381–391 (2014).

[55] Mancini, E. et al. Studies on Chemical Composition, Antimicrobial and Antioxidant Activities of Five *Thymus vulgaris* L. Essential Oils. Molecules 20, 12016–12028 (2015).

[56] Dash, K. T. et al. Chemical Composition of Carvacrol Rich Leaf Essential Oil of *Thymus vulgaris* from India: Assessment of Antimicrobial, Antioxidant and Cytotoxic Potential. J. Essent. Oil Bear. Plants 24, 1134–1145 (2021).

[57] Galovičová, L. et al. *Thymus vulgaris* Essential Oil and Its Biological Activity. Plants 10, 1959 (2021).

[58] Bhuvaneswari, G., Thirugnanasampandan, R. & Gogulramnath, M. Effect of colchicine induced tetraploidy on morphology, cytology, essential oil composition, gene expression and antioxidant activity of *Citrus limon* (L.) Osbeck. Physiol. Mol. Biol. Plants 26, 271–279 (2020).

[59] Ćavar Zeljković, S., Siljak-Yakovlev, S., Tan, K. & Maksimović, M. Chemical composition and antioxidant activity of *Geranium macrorrhizum* in relation to ploidy level and environmental conditions. Plant Syst. Evol. 306, 18 (2020).

[60] Stiller, C.-O. & Hjemdahl, P. Lessons from 20 years with COX-2 inhibitors: Importance of dose–response considerations and fair play in comparative trials. J. Intern. Med. 292, 557–574 (2022).

[61] Marsik, P. et al. *In vitro* Inhibitory Effects of Thymol and Quinones of *Nigella sativa* Seeds on Cyclooxygenase-1- and -2-Catalyzed Prostaglandin E2 Biosyntheses. Planta Med. 71, 739–742 (2005).

5. Oryzalin-Induced Polyploidy Enhances Natural Metabolite Accumulation and Functional Bioactivities in Novel *Callisia fragrans* Genotype

Adopted from: **Gupta, N**., Bhattacharya, S., Žiarovská, J., Dutta, A., Fialková, V., Farkasová, S., Bergo, A. M., Havlik, J., Tauchen, J., Novy, P., Urbanová, K., Milella, L., & Fernández-Cusimamani, E. (2025). Oryzalin-induced polyploidy enhances natural metabolite accumulation and functional bioactivities in novel *Callisia fragrans* genotype. *Scientific Reports, 15*, 25652.

This chapter explores the effects of synthetic polyploidization on C. fragrans, resulting in a pronounced enhancement of its phytochemical profile, correlate with enhanced antimicrobial, cell viability, anti-inflammatory and antioxidant activities.

Author contributions: Investigation, Methodology, Writing original draft, Writing review & editing, Formal analysis.

DOI: https://doi.org/10.1038/s41598-025-10588-9

Abstract

The reorientation of *Callisia fragrans* (Lindl.) Woodson, from therapeutic to ornamental use, exemplifies a broader domestication trend, favoring aesthetics over medicinal properties. Renewed phytomedicinal interest and the rise of plant extract markets drive demand for organic, sustainable consumer products, heightening the competition for superior cultivars. Synthetic polyploidization using oryzalin was conducted to obtain high-quality cultivars of *C. fragrans*, facilitating enhanced phenotypic and biological traits without genetic modification. This study aimed to explore the metabolic spectrum and biological activities of oryzalin-induced polyploid *C. fragrans* for its advanced medicinal application. Flow cytometric analysis confirmed the ploidy level of the plants. Consequently, GC-FID and ^{1}H NMR analyses revealed distinct metabolite profiles, with increased ethyl stearate, malic acid, gallic acid, fumaric acid, and unique compounds like (*Z*)-11-eicosenoic acid and dodecan-1-ol in polyploids. Polyploid extracts demonstrated exceptional antioxidant capacity, with DPPH, ORAC, and ABTS assays showing higher radical scavenging and oxygen absorbance abilities than diploid extracts. The polyploid extract showed enhanced antimicrobial activity against skin pathogens, including Methicillin-resistant *Staphylococcus aureus* (MRSA). *Callisia* extracts, meticulously at a low concentration of 25 µg/mL, showed cytoprotective effects on HT-29 cells, mitigating H_2O_2-induced oxidative stress. Furthermore, treatment with polyploid extract was associated with the downregulation of the expression of pro-inflammatory enzymes COX-1 and COX-2, suggesting a potentially greater anti-inflammatory effect compared to the diploid extract. These findings depict enhanced metabolite accumulation and biological activities in polyploid compared than diploid progenitor, highlighting the potential of the novel polyploid *C. fragrans* variety for future therapeutic applications, particularly in pharmaceutical and cosmetic industries.

Keywords

Antioxidant activity, Anti-inflammatory activity, GC-FID, NMR, Polyploidization, Skin infection

Abbreviations

AAPH, ((2,2′-azobis(2-amidinopropane) dihydrochloride); ABTS, (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); ATCC, American Type Culture Collection; BBFO, broadband fluorine observation; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GAE, gallic acid

equivalent; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC-FID, gas chromatography-flame ionization detector; HT-29, human colorectal adenocarcinoma cell line; MIC, minimum inhibitory concentrations; MRSA, methicillin-resistant *Staphylococcus aureus*; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NMR, Nuclear Magnetic Resonance; ORAC, Oxygen radical absorbance capacity; QE, quercetin equivalent; RIs, retention indices; RTs, retention times; TE, trolox equivalent; TFC, total flavonoid content; TPC, total phenolic content.

5.1 Introduction

The anthropogenic transition of *C. fragrans* from medicinal to ornamental use exemplifies a broader paradigm in plant domestication, where aesthetic attributes often supersede traditional therapeutic potential. However, a resurgence of interest in phytomedicinal research has positioned *C. fragrans* as a potential subject for advanced pharmacological investigations. *C. fragrans* or basket plant, a perennial ornamental plant of the Commelinaceae family, native to Mexico but globally cultivated, has traditionally been used for treating various skin diseases, burns, oncological diseases, tuberculosis, asthma, wounds, and joint disorders [1]. It exhibits significant anti-oxidant, anti-inflammatory, anti-cancer, and antimicrobial activities, etc. [2-5], which attributes to its rich phytochemical profile, including phenolic acids, flavonoids, including luteolin derivatives, and coumarins [6].

The global marketplace has witnessed a proliferation of plant extract-based products, catalyzing a shift towards organic and sustainable consumerism. This trend has intensified competition in developing high-quality cultivars and optimizing production methodologies [7]. While standardization, stability, and quality control of these preparations are possible, they present significant challenges due to the inherent variability of plant-based products. Besides these, geographical location, cultivation practices, and harvesting methods also influence the phytochemical composition. Additionally, rigorous evaluations through well-controlled, double-blind clinical trials and toxicological studies to establish their efficacy and safety are relatively scarce [8]. Given consumer preferences for non-genetically modified products and the constraints of conventional breeding techniques, synthetic polyploidization has emerged as a promising approach [9]. Synthetic polyploidization involves artificial duplication of an organism's genomic constitution using antimitotic agents like oryzalin, colchicine, and trifluralin. This genetic reconfiguration may produce superior or inferior phenotypes with altered morphological, physiological, and biochemical characteristics [10]. Oryzalin, notably, exhibits reduced cytotoxicity compared to colchicine and demonstrates a higher affinity for plant tubulin [11].

Multiple studies hypothesize that synthetic polyploidization augments primary and secondary metabolite production due to chromosomal doubling, potentially modulating the biological activities of polyploid plants [12,13]. However, contradictory findings underscore the need for rigorous assessment of these artificially induced genotypes [14]. The imperative to evaluate qualitative attributes of synthetically induced polyploid genotypes is paramount, given the nascent state of research into the bioactivity of secondary metabolites from such novel

polyploids. Considering the known biological activities of *C. fragrans*, this study aims to conduct a comprehensive analysis of the antimicrobial, anti-inflammatory, and antioxidant properties of extracts derived from induced tetraploid *C. fragrans* (Figure 9). Additionally, this research characterizes the bioactive compounds in the tetraploid variety, comparing their chemical composition and biological efficacy to those of their diploid counterparts. The findings elucidate the impact of genomic duplication on phytochemical profiles and concomitant bioactivities in *C. fragrans*. Such insights could catalyze renewed interest in this species within pharmaceutical and cosmetic industries, transcending its current role as an ornamental plant. This research represents a pivotal step towards realizing the full biotechnological potential of *C. fragrans* through advanced breeding techniques, potentially revolutionizing its utilization in pharmaceutical and cosmetic industries.

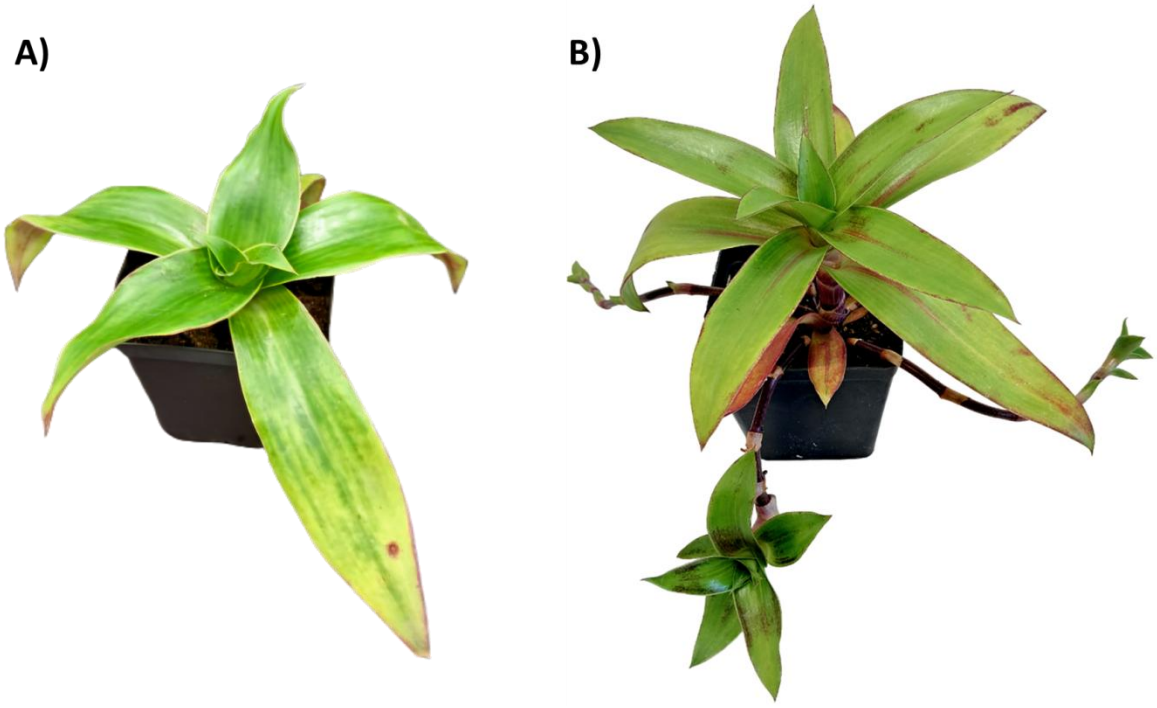

Figure 9 *C. fragrans* plant A) control diploid B) polyploid.

5.2 Materials and methods

5.2.1 Plant material acquisition

Autopolyploid *C. fragrans* plants (2n=4x=24) were obtained from the previous study of our group by Beranová et al. (2022) [10] and maintained with their diploid counterparts (2n=2x=12) in the field condition at the botanical garden of the Faculty of Tropical Agrisciences (FTA), Czech University of Life Sciences Prague (CZU). The plant materials were not archived in a herbarium repository, as they originated from seeds and were propagated

through plant tissue culture for experimental use. Following propagation, they were cultivated and sustained in the field of botanical garden of FTA, CZU (coordinates: 50.131211 N, 14.370589 E). The ploidy level stability of all maintained plants was confirmed through Flow cytometry analysis, using a Partec PAS flow cytometer (Partec GmbH, Munster, Germany) equipped with a high-pressure mercury arc lamp, as detailed by Bharati et al. (2023) [15]. After the ploidy confirmation, leaf samples were collected from the diploid and polyploid parental plants for further experiments, following institutional protocols and necessary permissions.

5.2.2 Preparation of methanolic plant extract

The harvested samples were air-dried for five days, finely ground using a Grindomix GM100 homogenizer (Retsch, Germany), and stored in dark conditions until further use. Approx 2 g of samples were subjected to extraction at room temperature in 80% methanol using an orbital shaker at 150 rpm for 24 hours. The extract was then centrifuged at 9000 rpm for 10 minutes at 4 °C. The supernatant was transferred into an evaporation flask and evaporated using a Büchi R-200 rotary evaporator (Büchi, Switzerland) under vacuum at 40 °C. After the evaporation, the dry extracts were weighed and dissolved in 100% dimethyl sulfoxide (DMSO) to yield a stock solution with a 51.2 mg/mL concentration for further antibacterial, antioxidant, and anti-inflammatory studies. Aqueous methanol was chosen for its broad-spectrum extraction of bioactive metabolites, its traditional use in plant-based medicines [16], and its superior biological activity for *C. fragrans* in prior studies.

5.2.3 Chemical analysis

5.2.3.1 Quantitative ^{1}H Nuclear Magnetic Resonance (NMR) for the determination of secondary metabolites present in *Callisia* extract

The sample preparation for ^{1}H NMR analysis was carried out according to Kim et al. (2010) [17]. All chemicals and reagents used were of analytical grade. Young and old leaves from the 5th leaf stage of polyploid and diploid *Callisia* plants (5 plants per genotype) were pooled, snap-frozen, and then freeze-dried for three days. The dried material was ground into a fine powder using a Mixer Mill MM 200 (Retsch) at 25 Hz for 40 seconds. Approximately 40 ± 1 mg of the powder was weighed and extracted by adding 500 µL of MeOD and 500 µL of KH_2PO_4 buffer in D_2O containing 0.01% 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt (TSP) (*w/v*). The mixture was vortexed for 1 minute at room temperature, ultrasonicated for 15 minutes, and centrifuged at 24,400× g for 10 minutes. A 600 µL aliquot of the supernatant was then transferred to a 5 mm tube. The phosphate buffer was prepared with 90 mM KH_2PO_4 and 0.01% TSP, and the pH was adjusted to 6.0 using 1.0 M NaOD [17]. The ^{1}H-NMR parameters for

spectral acquisition followed the guidelines set by Mascellani et al. (2021) [18]. Spectra were acquired at 298 K (25 °C) using a Bruker Avance III HD spectrometer with a broadband fluorine observation (BBFO) SmartProbe™ (Bruker BioSpin GmbH, Rheinstetten, Germany), operating at a ^{1}H-NMR frequency of 500.18 MHz. Metabolites were identified and quantified using Chenomx NMR Suite 8.5 (Chenomx Inc., Canada) with an in-house database, while unannotated peaks were analysed as bins. For this, the spectra were imported into MATLAB® R2022a (MathWorks, Natick, MA, USA) and underwent baseline correction via an in-house script for multipoint baseline correction within user-defined segments. Spectra were divided into bins ranging from δH 0.5 to 9.0 ppm (excluding the water region at δH 4.70–5.10 ppm), with each bin ideally representing a pure, distinct, and quantitative spin system. Bin intervals are presented in Table S1. Each bin was scaled to the unitary integral of the TSP signal for statistical analysis.

5.2.3.2 GC-FID analysis

GC-FID analysis was conducted following the method by Gupta et al. (2024) [11]. Young and mature 5th-stage leaves from polyploid and diploid *Callisia* plants were pooled, air-dried, and extracted in n-hexane using an Automatic Solvent Extractor (SER 158, Usmate, Italy). The Agilent GC-7890B system (Santa Clara, CA, USA), equipped with an Agilent 7693 autosampler and non-polar HP-5MS column (30 m × 0.25 mm, 0.25 µm film), was used. 1 µL sample was injected in splitless mode with helium as the carrier gas at 1 mL/min. The oven was programmed with an initial temperature of 60 °C for 1 min and then increased to 240 ◦C at a rate of 3 °C /min, and it was kept on hold for 20 mins. The transfer line temperature was kept at 250 °C. Extracts were diluted in n-hexane for GC-FID analysis at 20 µL/mL concentration. The FID was set at 250 °C with 40 mL/min H_2, 400 mL/min air, and 30 mL/min make-up flow. Chemical components were identified by comparing retention indices (RIs), retention times (RTs), and spectra against the NIST 2.0.f library and existing literature [19]. RIs were calculated using RTs of *n*-alkane series (C_8–C_{40}, Sigma-Aldrich, Prague, Czech Republic). Relative percentages of components were derived from FID data.

5.2.4 Estimation of antioxidant activity, phenolic and flavonoid content

5.2.4.1 DPPH radical-scavenging assay

A modified method from Sharma and Bhat (2009) [20] was employed for this experiment. Two-fold serial dilutions of each sample were prepared in analytical grade methanol in 96-well plates, followed by the addition of 100 µL of 0.25 mM DPPH in methanol, yielding final concentrations of 512 to 2 µg/mL in a 200 µL reaction volume. The mixture was incubated in

the dark at room temperature for 30 minutes, and absorbance was measured at 517 nm using a multi-mode reader (Synergy H1, BioTek, Winooski, Winooski, VT, USA). Trolox was used as a positive control, along with methanol as blank. The results were expressed as Trolox equivalents (mg TE/g extract).

5.2.4.2 Oxygen radical absorbance capacity (ORAC) assay

A modified version of the Ou and Hampsch-Woodill (2001) [21] method was used to assess the sample's capacity to retard the AAPH-induced oxidant decay of fluorescein (FL). To enhance thermal stability, the outer wells of black 96-well microtiter plates were filled with 200 µL of distilled water. Stock solutions of AAPH ((2,2′-azobis(2-amidinopropane) dihydrochloride)) and FL in 75 mM phosphate buffer (pH 7.0) were prepared. Each sample (25 µL) was diluted in 150 µL of FL (54 nM) and pre-incubated at 37 °C for 10 minutes. The reaction was initiated by adding 25 µL of AAPH (153 mM), giving a final volume of 200 µL per well. Extracts were tested at a concentration of 32 µg/mL. Trolox was used as a positive standard at a range of 8, 4, 2, 1, and 0.5 µg/mL. Fluorescence readings were recorded at 1-minute intervals for 120 minutes, using an excitation wavelength of 487 nm and an emission wavelength of 528 nm. The ORAC values were quantified by calculating the area under the calibration curve, following the method proposed by Ou and Hampsch-Woodill (2001) [21], and expressed as Trolox equivalents (µg TE/mg extract).

5.2.4.3 ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) assay

ABTS radical cation decolorization method was conducted according to the modified version of Re et al. (1999) [22]. ABTS radical cations were generated by reacting 5.05 µL of ABTS (7 mM) with 500 µL of ammonium persulfate (245 mM) and incubating overnight in the dark. Before the assay, the ABTS solution was diluted with PBS to achieve an absorbance of ~0.700 at 734 nm. In a 96-well plate, 10 µL of each sample was mixed with 190 µL of ABTS solution and incubated for 5 minutes. Absorbance was measured at 734 nm. The extracts were tested at a final concentration of 128 µg/mL. The Trolox calibration curve was prepared with concentrations of 0.156, 0.313, 0.625, 1.25, 2.5, 5, and 10 µg/mL, and the results were calculated as the area under the curve and expressed in Trolox equivalents (µg TE/mg extract).

5.2.4.4 Total phenolic content (TPC)

The total phenolic content was determined using a modified version of the method described by Singleton et al. (1998) [23]. 100 µL of each sample (128 µg/mL) was added to a 96-well plate, followed by 25 µL of Folin–Ciocalteu reagent. After shaking at 40 rpm for 10 minutes,

75 µL of 12% sodium carbonate (Na_2CO_3) solution was added. The mixture was incubated at 37 °C in the dark for 2 hours, and absorbance was measured at 760 nm. Gallic acid was used for preparing a standard calibration curve (0.012–50 µg/mL), and the results were expressed as gallic acid equivalents (µg GAE/mg extract).

5.2.4.5 Total flavonoid content (TFC)

The total flavonoid content was determined using a modified aluminium chloride ($AlCl_3$) method [24]. In a 96-well plate, 100 µL of sample (512 µg/mL) was mixed with 100 µL of 10% $AlCl_3$ and incubated in the dark at room temperature for 60 minutes. Absorbance was measured at 420 nm. Quercetin served as the standard, and a calibration curve was prepared using seven concentrations (1.563, 3.125, 6.25, 12.5, 25, and 50 µg/mL). Flavonoid content was expressed as quercetin equivalents (µg QE/mg dry weight, DW).

5.2.5 Antimicrobial assay

5.2.5.1 Bacterial strain and culture media

For the antimicrobial assay, the skin disease-causing bacteria and fungus were obtained from the American Type Culture Collection (ATCC) that includes *Staphylococcus aureus* (ATCC 29213), Methicillin-resistant *S. aureus* (MRSA) (ATCC 43300), *Staphylococcus epidermidis* (ATCC 12228), *Streptococcus pyogenes* (ATCC 19615), and *Candida albicans* (CRM-10231). The cultivation and assay media used were Mueller-Hinton broth for *S. aureus*, *S. epidermidis*, and MRSA, Brain Heart Infusion broth for *S. pyogenes*, and Sabouraud dextrose broth for *C. albicans*. The broth pH was adjusted using Trizma base (Sigma-Aldrich, Prague, Czech Republic). All microbial strains, media, and supplements were sourced from Oxoid (Basingstoke, Hampshire, UK). Bacterial stock cultures were grown in the appropriate media at 37°C for 24 hours before testing. For the antifungal assay, fungal inoculum suspensions were prepared from fresh 5-day-old cultures grown on Sabouraud dextrose agar (pH 5.6) at 37°C as described by Petrikkou et al. (2001) [25]. Bacterial suspensions were adjusted to 0.5 McFarland, and fungal suspensions to 0.8 McFarland, using a Densi-La-Meter II (Lachema, Brno, Czech Republic) to achieve a final concentration of 10^7 CFU/mL. Ampicillin, tetracycline, oxacillin, and amphotericin B (Sigma-Aldrich, Prague, Czech Republic) were used as positive controls, following CLSI guidelines.

5.2.5.2 Antimicrobial activity via Broth microdilution method

The *in vitro* antimicrobial activity was evaluated using the broth microdilution method in 96-well microtiter plates, following the protocol outlined by Kudera et al. (2021) [26]. Extracts

were prepared by two-fold serial dilution in the appropriate growth media, starting from an initial 512 µg/mL concentration. Standardised cultures were inoculated into 96-well plates and incubated following the same protocols as initial cultivation. Growth and purity controls were prepared with inoculated and non-inoculated broth. Minimum inhibitory concentrations (MICs) were determined visually after staining active bacterial cells with MTT (thiazolyl blue tetrazolium bromide) dye (600 µg/mL). MIC values, defined as the lowest concentrations inhibiting growth compared to the control, were expressed in µg/mL. All experiments were performed in triplicate, and results were reported as median or modal MIC values.

5.2.6 Cell viability assay and protection against hydrogen peroxide-induced oxidative stress

5.2.6.1 Cell culture and treatment

The human colorectal adenocarcinoma cell line (HT-29) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultivated in RPMI 1640 medium with 10% heat-inactivated FBS, 2 mM L-glutamine, and 1% antibiotics (penicillin, streptomycin, and amphotericin B). The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. HT-29 cells were grown in 75 cm² flasks to 90% confluence and used for experiments between passages P10 and P31. Cell density was measured using an automated cell counter (Bio-Rad), and cells were regularly harvested with 0.25% trypsin-EDTA and used for subsequent experiments.

5.2.6.2 Cell viability assay

The cell viability assay was determined by a metabolic activity (MTT) assay. The HT-29 cells were seeded into 96-well plates (Falcon, Corning, Poland) at the seeding density of $1x10^4$ cells/well and cultured for 24 hours at 37 °C in a humidified atmosphere with 5% CO_2. The cells were then treated with the appropriate concentrations of *C. fragrans* polyploid and control extract (25; 50; 100; 200; 300 µg/mL) and H_2O_2 (3,125; 6,25; 10; 12,5; 25; 50; 100 and 200 µM). Subsequently, the treated cells were incubated with 1 mg/mL of MTT (Sigma-Aldrich, St. Louis, MO, USA) for 3 hours under dark conditions (37ºC and 5% CO_2). The formazan crystals were dissolved in 150 µL of DMSO (Sigma-Aldrich, St. Louis, MO, USA). The absorbance was determined spectrophotometrically at a measuring wavelength of 570 nm against 620 nm as a reference by a microplate reader (Multiskan FC, ThermoFisher Scientific, Finland). The data were presented as a percentage relative to the control group. DMSO alone was used as a vehicle control. The concentration of DMSO was < 0.5% throughout all the treatments.

5.2.6.3 Anti-inflammatory activity via gene expression analysis

The HT-29 cells were seeded in 6-well plates ($5x10^5$ cells/well) and treated with nontoxic concentrations of *C. fragrans* polyploid and diploid extract (25 and 200 µg/mL) in combination with H_2O_2 (25 µM). The cells were incubated for 24 hours to assess gene expression. Total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, USA), and RNA quantity and purity were assessed with an Implen NanoPhotometer (München, Germany). Complementary DNA was synthesized using the RT^2 First Strand cDNA Synthesis Kit (Qiagen, USA) as per the manufacturer's instructions. Relative mRNA expressions of *COX-2* (Prostaglandin-Endoperoxide Synthase 2) and *COX-1* (Prostaglandin-Endoperoxide Synthase 1) were analyzed using the Stratagene Mx3005P Real-Time PCR System (Agilent Technologies, USA) under the following cycling conditions: 95°C for 2 min, followed by 45 cycles of 95°C for 5s and 60-65°C for 25s. *GAPDH* was used as the reference gene. All the primers were designed (Table S2) using Primer-BLAST software based on publicly available sequences. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and presented as fold changes compared to the vehicle control group. PCR for each sample was conducted in triplicate from at least three independent experiments.

5.3 Statistical analysis

The antioxidant, cell viability, and GC-FID results for both control diploid and induced polyploid genotypes were expressed as means ± standard deviation (SD), while anti-inflammatory results were reported as mean ± standard error of the mean (SEM). Antimicrobial results were expressed as median/modal MIC values. All values from antioxidant, cell viability, anti-inflammatory activity, and GC-FID results were analyzed using Tukey's post hoc analysis (5% significance level) in Microsoft Excel 2021. Normalised bins and concentrations of NMR data were analyzed using MetaboAnalyst version 6.0, followed by an independent T-test and PCA analysis.

Ethical statement

This article does not contain any studies with human or animal subjects. All experiments conducted in this study along with the collection of plant material, comply with relevant institutional, national, and international guidelines and legislation.

5.4 Results

5.4.1 Ploidy level estimation

Flow cytometric analysis was performed on all maintained *C. fragrans* plants to assess their ploidy levels. The resulting histograms effectively differentiated induced polyploids from diploids, showing distinct differences in DNA content across genotypes (Figure 10A and B). The analysis confirmed that the relative DNA content of the polyploid genotype was doubled compared to control diploids, verifying significant ploidy variation amongst the tested genotypes.

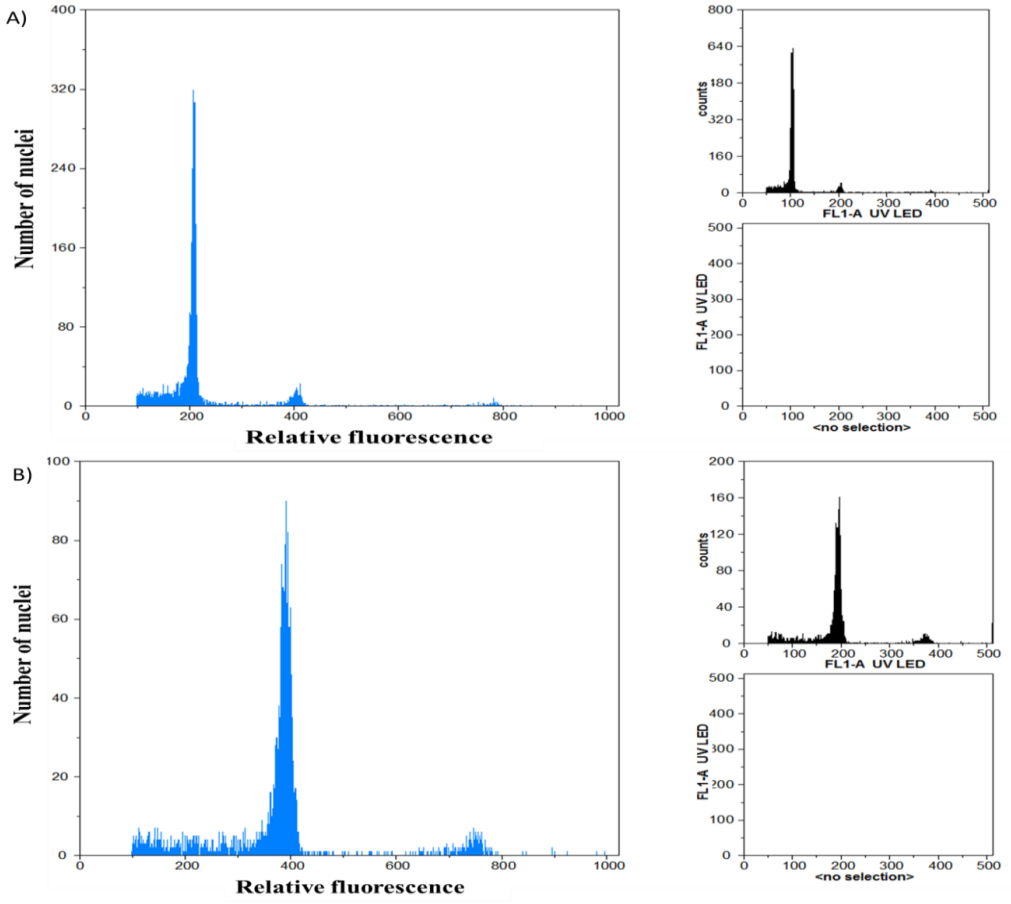

Figure 10 Flow cytometric analysis of *C. fragrans* **(A)** histogram of relative DNA content of control diploid and **(B)** tetraploid plant.

5.4.2 Extraction Yield of *C. fragrans*

Figure 11 illustrates the percentage yield of extracts from diploid and tetraploid *C. fragrans*, revealing a notable enhancement in the yield of plant extract with increased ploidy. Specifically, the percentage yield of extracts of 80% methanolic extract was 12.88% in tetraploid plants, whereas it was 8.50% in diploid plants. These results demonstrate a

significant difference in extraction yields between the diploid and polyploid samples using 80% methanol as the extraction solvent.

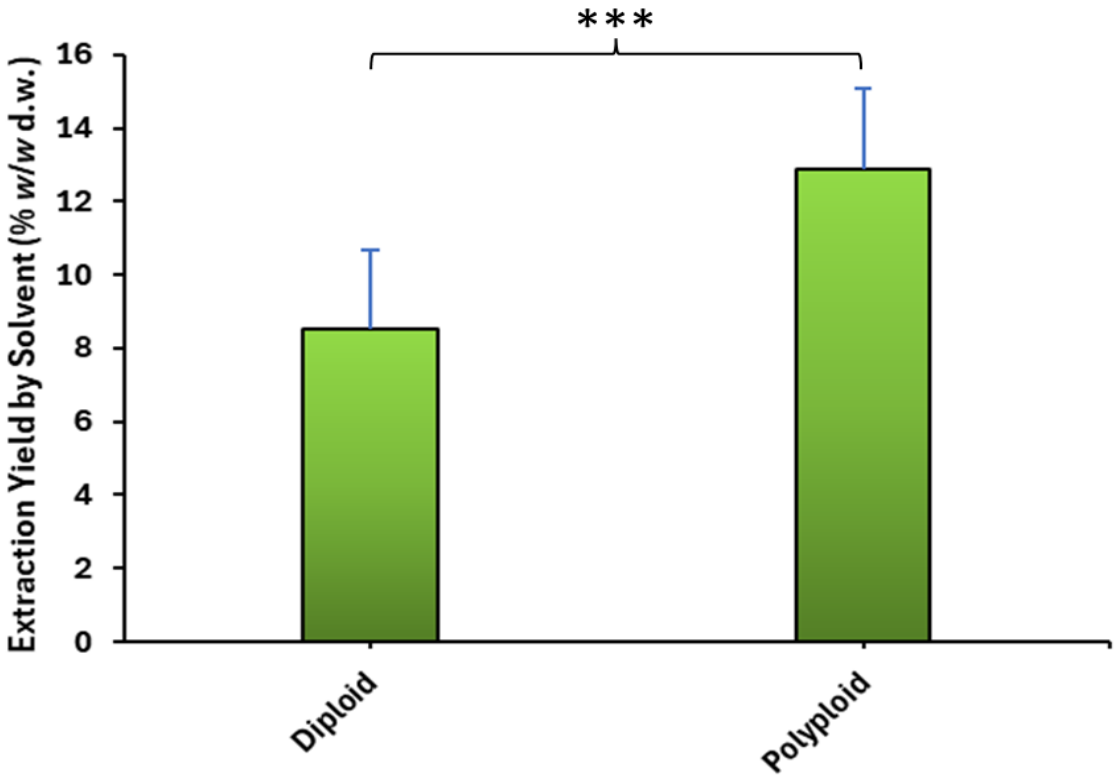

Figure 11 Extraction yield (80% MeOH) in control diploid and induced polyploid of *C. fragrans*. "***" expressed a significant difference (Tukey HSD Test, $p < 0.001$).

5.4.3 Chemical composition of *C. fragrans* extracts

5.4.3.1 GC-FID analysis

GC-FID analysis identified 22 compounds from the polyploid extract and 21 compounds from the diploid extract, representing 98.44% and 98.06%, respectively, of their corresponding total constituents (Table 6 and Fig. S1). Fatty acid ester, represented by ethyl stearate, was the most abundant compound found in both extracts, showing a significantly higher concentration in the polyploid (79.74%) than in the diploid (76.52%). In contrast, mandenol was significantly more concentrated in the diploid extract (8.06%) than in the polyploid (2.84%). Additionally, the polyploid extract uniquely contained two compounds, (*Z*)-11-eicosenoic acid (1.08%) and dodecan-1-ol (0.26%), which were not present in the diploid. Conversely, nonadecanoic acid (0.52%) was identified only in the diploid extract. An enhanced concentration of neophytadiene was also found in polyploid extract compared to diploid. To a lesser extent, other identified compounds such as hexadecen-1-ol (1.50% in polyploid *vs.* 0.42% in diploid), phytol (2.12%

in polyploid *vs.* 1.18% in diploid), and eicosane (0.57% in polyploid *vs.* 1.25% in diploid) exhibited notable differences between the genotypes. Variations in the concentrations of other components between genotypes were minimal, with differences of less than 1%.

Table 6 Chemical constituents of diploid (Control) and polyploid extract of *C. fragrans* through GC-FID analysis.

RI obs[a]	RI lit[b]	Compound name	(%) content[c] ± SD	
			Diploid	Polyploid
1104	1105	Nonanal	0.29 ± 0.01	0.32 ± 0.01 *
1480	1480	Dodecan-1-ol	n.d.	0.26 ± 0.01
1489	1490	*β*-Ionone	0.26 ± 0.01	0.32 ± 0.04 ns
1515	1517	Butylated hydroxytoluene	0.27 ± 0.04	0.48 ± 0.02 *
1532	1533	Dihydroactinidiolide	0.11 ± 0.01	0.45 ± 0.03 ***
1809	1811	2-Ethylhexyl salicylate	0.63 ± 0.02 *	0.31 ± 0.07
1838	1840	Neophytadiene	1.66 ± 0.34	3.84 ± 0.63 *
1845	1847	Hexahydrofarnesyl acetone	0.32 ± 0.01	0.45 ± 0.03 *
1885	1883	Hexadecen-1-ol	0.42 ± 0.08	1.50 ± 0.15 **
1893	1893	Corymbolone	0.27 ± 0.01	0.77 ± 0.02 ***
1897	1899	1,2-Epoxyoctadecane	0.58 ± 0.06 *	0.47 ± 0.02
1926	1927	Methyl palmitate	0.22 ± 0.03	0.32 ± 0.04 *
1969	1971	Sandaracopimaradiene	0.69 ± 0.18	0.93 ± 0.09 ns
1994	1995	Ethyl palmitate	3.89 ± 0.24 ***	0.30 ± 0.05
1997	1999	Eicosane	1.25 ± 0.02 ***	0.57 ± 0.05
2025	2026	Isopropyl palmitate	0.20 ± 0.01	0.37 ± 0.03 *
2102	2104	Methyl petroselinate	0.59 ± 0.01 ***	0.30 ± 0.01
2120	2121	Phytol	1.18 ± 0.03	2.12 ± 0.26 *

2163	2164	Mandenol	8.06 ± 0.22 **	2.84 ± 0.61
2195	2196	**Ethyl stearate**	**76.52 ± 1.05**	**79.74 ± 0.74 ***
2236	2236	Nonadecanoic acid	0.52 ± 0.03	n.d.
2363	2364	(*Z*)-11-Eicosenoic acid	n.d.	1.08 ± 0.07
2389	2392	n-Butyl stearate	0.13 ± 0.01	0.70 ± 0.02 ***
		Total	**98.06**	**98.44**

a: Kovats' retention indices measured on HP-5MS column; b: retention indices from literature; c: relative percentage content based on the total area of all peaks expressed in mean ± SD. Major compounds are in bold. Asterisks indicate statistical significance. (Tukey HSD Test, *p < 0.05; **p < 0.001; *** p < 0.0001; ns: non-significant).

5.4.3.2 NMR Analysis

The analysis through ^{1}H NMR revealed quantification of 13 metabolites (Table 7). Spectrum from polyploid sample leaves of *C. fragrans* is shown in Fig. S2. Significant metabolic changes were reflected between the diploid and polyploid leaves extract (Fig. S3), showing a significantly higher sucrose concentration in the polyploid variety (440.45 µg/mL). A significantly increased concentration of organic acids such as fumaric acid (5.56 µg/mL), gallic acid (2.97 µg/mL), and malic acid (673.74 µg/mL) was also observed in the polyploid variety. However, citric acid was observed in a significantly higher concentration in the diploid variety (307.64 µg/mL) (Table 7). The PCA score plot (Figure 12A) reveals a mild separation between diploid and polyploid within the first two principal components, accounting for 46.1% and 21.8% of the variation, respectively. Within the variance captured by component 2, polyploid leaves exhibit significantly greater composition variability than diploid leaves. Diploid samples are characterised by elevated levels of citric acid and unknown signals (unknown 1, unknown 2, unknown 4, and unknown 10) (Figure 12B). In contrast, polyploid samples are distinguished by contributions from malic acid, gallic acid, fumaric acid, sucrose, and multiple unknown signals. The levels of some metabolites, such as sucrose, fumaric acid, and gallic acid, vary significantly between individual polyploid plants (Figure 12B).

Table 7 Metabolite concentrations of *C. fragrans* leaves extracted in MeOD-D_20 (1:1, *v/v*).

Compound	Diploid	Polyploid
	Concentration (µg/mL)	
Alanine	5.66 ± 3.51	13.00 ± 7.17
Chlorogenic acid derivative-1	67.48 ± 23.83	42.19 ± 25.84
Chlorogenic acid derivative-2	4.16 ± 0.06	13.13 ± 10.98
Citric acid	307.64 ± 67.64	110.65 ± 50.41*
Ethanolamine	4.95 ± 0.61	4.20 ± 2.78
Fructose	403.36 ± 39.97	359.65 ± 138.98
Fumaric acid	1.46 ± 1.19	5.56 ± 2.57*
Gallic acid	1.76 ± 0.64	2.97 ± 1.01*
Glucose	3072.05 ± 649.11	2559.56 ± 121.96
Malic acid	341.67 ± 214.09	673.74 ± 105.40*
Putrescine	3.11 ± 0.86	5.89 ± 1.31
Sucrose	141.68 ± 54.71	440.45 ± 114.56*
Valine	0.65 ± 0.20	2.98 ± 2.56

The results are expressed as means ± SD. *Shows a significant difference between the diploid and tetraploid genotypes ($p < 0.05$).

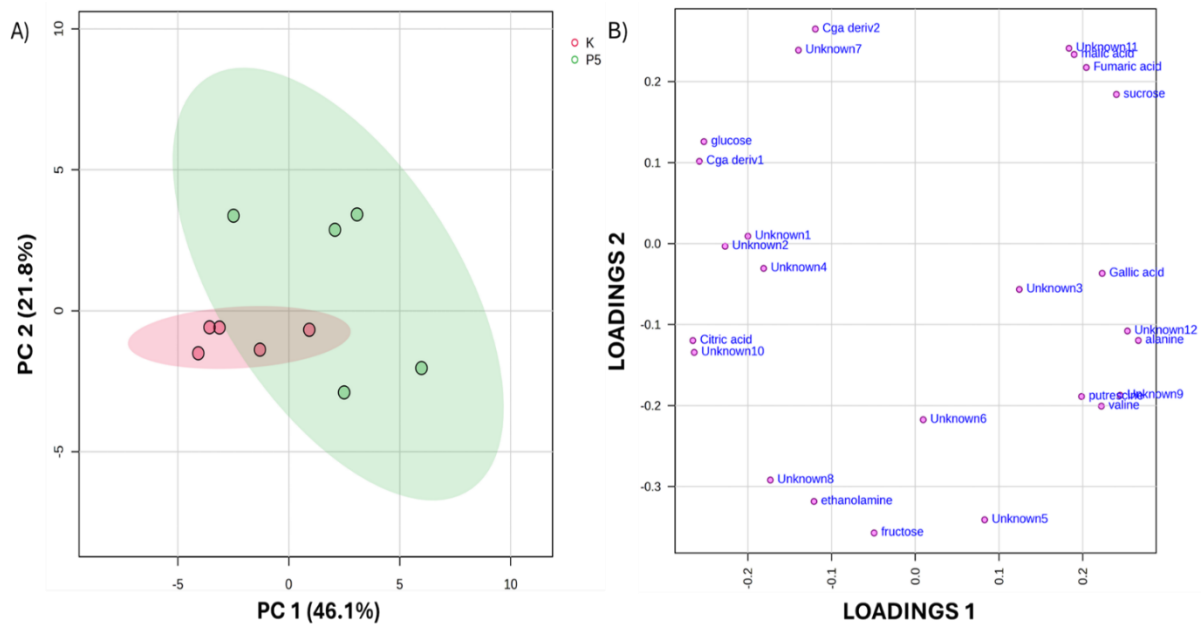

Figure 12 PCA score plot **(A)** and loadings plot **(B)** derived from metabolites detected via ^{1}H NMR of diploid and polyploid *C. fragrans*. K- control diploid, P5- polyploid variety, Cga deriv1- Chlorogenic acid derivative-1, and Cga deriv2- Chlorogenic acid derivative-2.

5.4.4 Antioxidant activity, total phenolics, and flavonoids content

5.4.4.1 DPPH radical scavenging assay

The DPPH radical scavenging assay was conducted to compare the antioxidant activities of diploid and polyploid *C. fragrans* extracts, as summarized in Table 8. Results showed that the polyploid extract exhibited significantly higher DPPH radical scavenging activity, measuring 13.01 µg TE/mg extract, while the diploid extract demonstrated a lower activity of 5.10 µg TE/mg (Table 8). This substantial increase in antioxidant capacity in the tetraploid genotype suggests a significantly greater efficacy in mitigating oxidative stress, highlighting its enhanced bioactive potential relative to the diploid genotype.

5.4.4.2 ORAC assay

The ORAC assay was utilized to evaluate the inhibitory effects of *C. fragrans* phenolic compounds on peroxy-radical-induced oxidation, focusing on hydrogen atom transfer reactions. Results indicated that the polyploid extract exhibited a significantly higher antioxidant activity at 126.53 µg TE/mg compared to the diploid extract's 89.75 µg TE/mg (Table 8). This significant difference underscores the enhanced antioxidant capacity of the polyploid genotype relative to the diploid.

5.4.4.3 ABTS assay

The ABTS assay assesses the antioxidant capacity of *C. fragrans* by measuring its ability to reduce the ABTS green-blue cation radical via an electron transfer mechanism, which is observed as a decolourization. Our results showed that the polyploid extract has significantly higher ABTS activity compared to the diploid. The polyploid showed an ABTS value of 61.48 µg TE/mg, while the diploid extract showed a value of 37.83 µg TE/mg (Table 8).

5.4.4.4 Total phenolics and flavonoids content

The total phenolic content (TPC) and total flavonoid content (TFC) of both diploid and polyploid genotypes were summarized in Table 8. Notably, the polyploid genotype exhibited a significantly elevated TPC of 30.22 µg GAE/mg, nearly double that of the diploid genotype at 13.20 µg GAE/mg, highlighting a marked enhancement in phenolic accumulation. In contrast, TFC values were comparable between genotypes, with the polyploid and diploid genotypes showing 9.03 and 8.80 µg QE/mg, respectively, indicating no significant variation in flavonoid content.

Table 8 Antioxidant activity of control diploid and polyploid *C. fragrans* extract

Sample	DPPH	ORAC	ABTS	TPC	TFC
	µg TE/mg extract	µg TE/mg extract	µg TE/mg extract	µg GAE/mg extract	µg QE/mg extract
Diploid	5.10 ± 1.28	89.75 ±25.27	37.83 ± 9.35	13.20 ± 1.13	8.80 ± 0.48
Tetraploid	13.01 ± 2.59**	126.53 ± 12.84**	61.48 ± 9.46***	30.22 ± 1.49***	9.03 ± 1.60ns

DPPH, ORAC, ABTS, TPC, and TFC results are expressed as means ± SD for three independent experiments measured in triplicate. TE- Trolox equivalent, GAE- Gallic acid equivalent, QE- Quercetin equivalent. Asterisks indicate statistical significance. (Tukey HSD Test, *$p < 0.05$; **$p < 0.01$; *** $p< 0.001$; ns, non-significant).

5.4.5 Antimicrobial activity

The diploid and polyploid *C. fragrans* extracts were evaluated for antimicrobial activity against four standard bacterial strains and one fungal strain associated with skin infections, as summarized in Table 9. Both diploid and polyploid *C. fragrans* extracts demonstrated antimicrobial efficacy against the tested microbial strains, with MIC values ranging from 16 to

512 µg/mL. Across all strains tested, the polyploid extract demonstrated greater efficacy. *S. epidermidis* was the most susceptible among all the tested microbes, with the polyploid extract achieving a significantly lower MIC of 16 µg/mL compared to the diploid's 64 µg/mL. Notably, *S. pyogenes*, the diploid extract demonstrated no inhibitory activity across the tested concentration range, while the polyploid extract exhibited a mild effect, with a MIC value of 512 µg/mL. Similarly, the diploid extract exhibited an inhibitory effect against both *S. aureus* and the fungal strain *C. albicans*, with a MIC value of 512 µg/mL. In contrast, the polyploid extract demonstrated greater efficacy against these microbes, achieving inhibition at a lower MIC value of 256 µg/mL. Additionally, the polyploid extract demonstrated significant effectiveness against the antibiotic-resistant MRSA strain, with a MIC of 256 µg/mL, compared to the diploid extract, which required a concentration of over the tested concentration range for minimum inhibition. This highlights the polyploid extract's enhanced potency against resistant pathogens like MRSA. These findings highlight the polyploid extract's superior antimicrobial activity compared to its diploid counterpart, indicating its potential as an effective remedy for pathogens responsible for skin infections.

Table 9 *In vitro* growth-inhibitory effect of *C. fragrans* extract (control diploid and tetraploid) against skin infection-causing bacteria.

Sample	**Minimum inhibitory concentrations (µg/mL)**				
	Candida albicans	**MRSA**	***Staphylococcus aureus***	***Staphylococcus epidermidis***	***Streptococcus pyogenes***
Diploid	512	>512	512	64	>512
Polyploid	256	256	256	16	512
Positive antibiotic control					
Amphotericin B	0.125	N.T.	N.T.	N.T.	N.T.
Ampicillin	N.T.	N.T.	N.T.	N.T.	0.5
Oxacillin	N.T.	64	0.125	0.125	N.T.
Tetracycline	N.T.	1	N.T.	N.T.	N.T.

NT: Not tested; results were expressed as median/modal MIC values.

5.4.6 Effect of *C. fragrans* extract on cell viability and oxidative stress-induced cell death assay

The human colorectal adenocarcinoma cell line (HT-29) was used as a cell model to evaluate the cytotoxic effect of *C. fragrant* extract. Cell viability was assessed by MTT assay after the cells were treated with different concentrations of diploid and polyploid *C. fragrans* extracts (25-300 µg/mL). The extracts demonstrated no cytotoxic effects on HT-29 cells at any tested concentrations. Interestingly, *C. fragrans* extracts slightly enhanced cell viability, with the most pronounced effect at 25 µg/mL. At higher concentrations (50 and 100 µg/mL), the polyploid extract exhibited significantly greater activity than the diploid extract (Figure 13A).

To evaluate the extract's potential to prevent oxidative stress-induced cell death, an initial test was conducted to determine the cytotoxic concentration range of H_2O_2. HT-29 cells were exposed to H_2O_2 (3.125–200 µM), and cell viability was assessed via MTT assay. Significant cytotoxicity was observed at 25 µM H_2O_2 ($p < 0.01$) compared to controls (Figure 13C). Thus, this concentration was selected for further testing to examine the extracts' protective effects against oxidative stress.

To assess the preventive effect, HT-29 cells were co-treated with extracts during oxidative stress exposure. Both extracts effectively mitigated H_2O_2-induced viability loss in a dose-dependent manner (Figure 13B), with the highest activity at 25 µg/mL, which significantly enhanced cell viability compared to cells under oxidative stress alone ($p < 0.0001$). Notably, both diploid and polyploid extracts exhibited similar protective effects against oxidative stress.

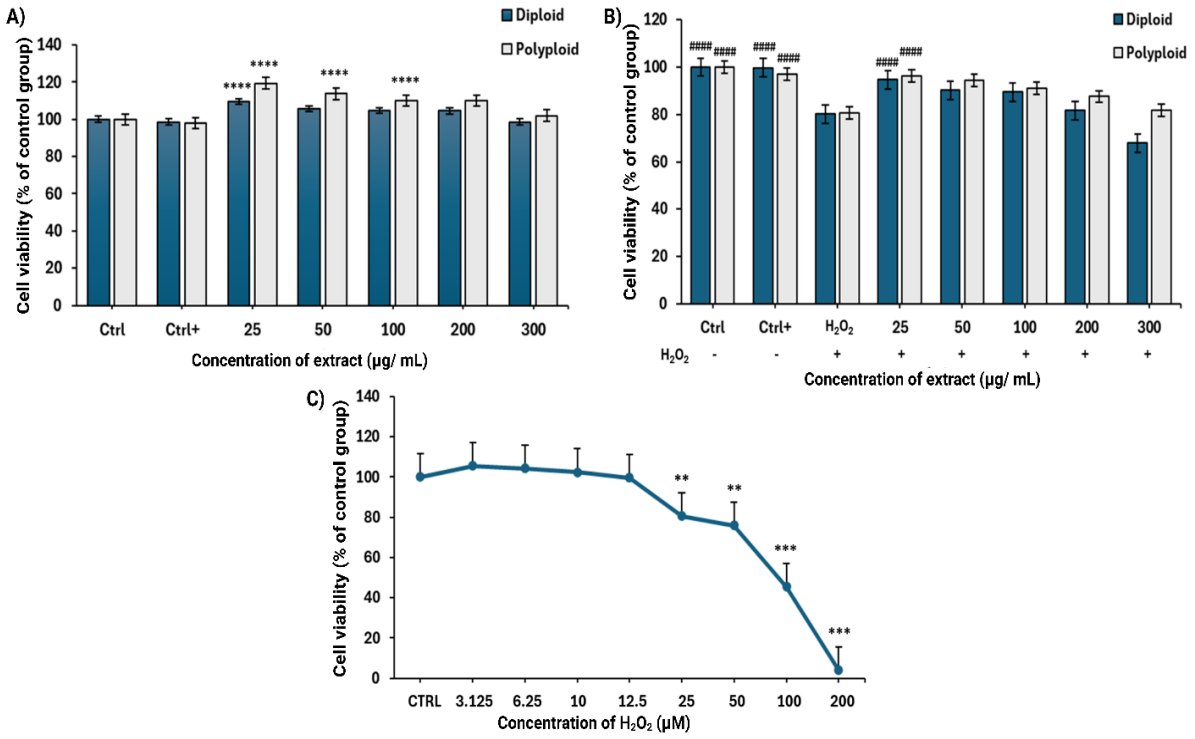

Figure 13 Cell viability of HT-29. **(A)** The effects of *C. fragrans* extracts on the cell viability of HT-29. The cells were treated with different concentrations of *C. fragrans* (25, 50, 100, 200, and 300 µg/mL) for 24 hours. **(B)** The effects of *C. fragrans* extracts and H_2O_2 on the cell viability of HT-29. The cells were treated with different concentrations of *C. fragrans* (25, 50, 100, 200, and 300 µg/mL) in combination with H_2O_2 (25 µM) for 24 hours. **(C)** The effects of H_2O_2 on the cell viability of HT-29. The cells were treated with different concentrations of H_2O_2 (3.125, 6.25, 10, 12.5, 25, 50, 100, and 200 µM) for 24 hours. The treated groups are compared to the control (Ctrl) and H_2O_2. The data are presented as mean ± SD from at least three independent experiments. Ctrl = untreated control group. Ctrl+ = vehicle control corresponding to the final concentration of DMSO ≤ 0.6 % (v/v) in cell culture media. **** ($p< 0.0001$) vs. Ctrl. #### ($p< 0.0001$) *vs*. H_2O_2.

5.4.7 Anti-inflammatory activity

To evaluate the anti-inflammatory potential of *C. fragrans* extracts on HT-29 cells, their effects on cyclooxygenase isoforms COX-1 and COX-2 were investigated under oxidative stress induced by H_2O_2 exposure (Figure 14A and B). Quantitative RT-PCR was utilized to determine the expression levels of *COX-1* and *COX-2* upon treatment with diploid and polyploid extract (25 and 200 µg/mL). Our findings reveal that H_2O_2 exposure markedly increased *COX-1* and *COX-2* expression in HT-29 cells. Treatment with *C. fragrans* extracts effectively counteracted

this effect, reducing the expression of both cyclooxygenase isoforms. Notably, the polyploid extract displayed a more potent anti-inflammatory effect than the diploid extract. Specifically, co-treatment with H_2O_2 and the diploid extract significantly elevated *COX-1* expression at both tested concentrations (25 and 200 µg/mL) compared to untreated controls. In contrast, the polyploid extract significantly suppressed *COX-1* expression in H_2O_2-treated cells at both concentrations, achieving the strongest reduction at 200 µg/mL. Similarly, co-treatment with H_2O_2 and the diploid extract led to an increase in *COX-2* expression at 25 µg/mL concentration, though a significant reduction in *COX-2* expression was observed at 200 µg/mL. Additionally, exposure of HT-29 cells to the polyploid extract of *C. fragrans* at both tested concentrations resulted in a marked decrease in *COX-2* expression compared to cells treated solely with H_2O_2, demonstrating active result at 25 µg/mL. These findings further highlight the polyploid extract's superior efficacy in downregulating inflammatory markers under oxidative stress conditions.

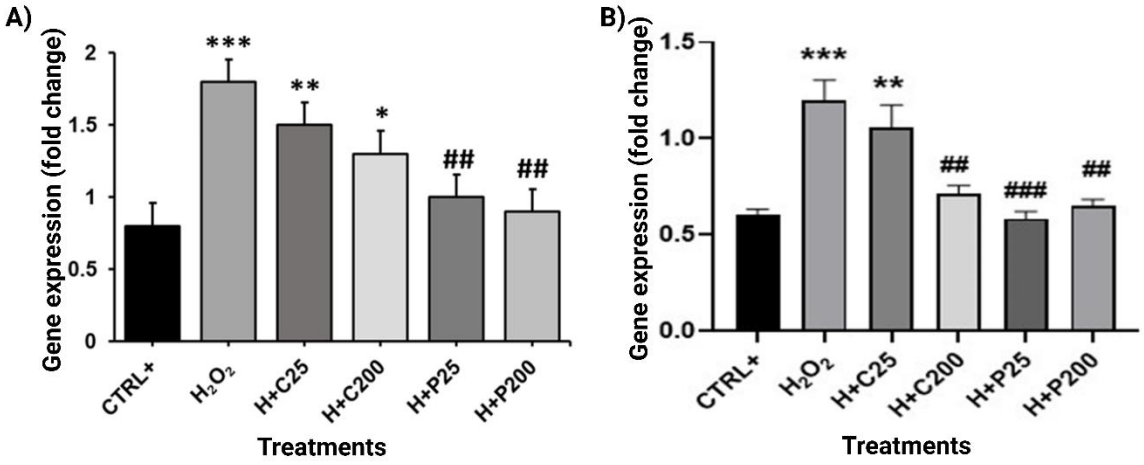

Figure 14 Relative gene expression of **(A)** COX-1 and **(B)** COX-2. The cells were treated with different concentrations of *C. fragrans* extracts (25 and 200 µg/mL of control and polyploid extract) with H_2O_2 (25 µM) for 24 hours. The treated groups are compared to the control (CTRL+) and H_2O_2. The data are presented as mean ± SEM from at least three independent experiments. H+C25/200 = H_2O_2 + *C. fragrans* control diploid extract. H+P25/200 = H_2O_2 + *C. fragrans* polyploid extract. *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$) vs. CTRL+. ### ($P < 0.001$), ## ($P < 0.01$) and # ($P < 0.05$) vs. H_2O_2. The picture of gel is presented in Supplementary Figure S3

5.5 Discussion

Polyploidization has emerged as a promising approach in crop enhancement, particularly for increasing secondary metabolite concentrations that boost therapeutic efficacy for medicinal and ornamental plants. However, it is not always the same as the result of gene duplication is unknown [27]. In our previous study, oryzalin proved to be an effective anti-mitotic agent, successfully inducing polyploidy in *C. fragrans*. The resulting polyploid plants exhibited both enhanced morphological traits and distinct genetic profiles compared to their diploid counterparts, underscoring oryzalin's potential for polyploid induction in this species [10]. Given the traditional medicinal significance of *C. fragrans*, a detailed analysis of how polyploidization influences its metabolic profile is crucial for optimizing its potential in advanced pharmaceutical applications. As far as we know, this study is the first to document the phytochemical profile and therapeutic effects of induced somatic polyploid in *C. fragrans* or any *Callisia* species.

In this experiment, methanol was used as a solvent, and a significant difference in the extraction yield was found between the diploid (8.50%) and polyploid (12.88%) varieties. This difference may be due to polyploid plants containing more polar and non-polar compounds, as methanol effectively extracts a wide range of these substances [28].

However, plant metabolomic profile can be affected by polyploidization [15], therefore, determining the chemical profile in the newly developed genotype holds important significance. GC-FID analysis identified ethyl stearate as the predominant compound in both diploid and polyploid plant extracts, with significantly higher concentrations in polyploid plants. Ethyl stearate, a major fatty acid ester found in medicinal plants such as *Neolamarckia cadamba* (Roxb.) Bosser [29] and *Coreopsis* cultivars [30] are well-documented for their antimicrobial and antioxidant properties [31]. The increased concentration of this compound in polyploid plants suggests an enhancement of these beneficial properties, likely contributing to the broader bioactivity observed in polyploid variants. Additionally, polyploid extracts uniquely contained (*Z*)-11-eicosenoic acid and dodecan-1-ol, which are absent in diploids. This unique metabolic profile in polyploid plants may introduce new therapeutic potentials, as these compounds could contribute to the enhanced antimicrobial advantages seen in polyploidy [32,33]. In polyploid plants, further insights from ^{1}H NMR analysis indicated significantly elevated levels of key bioactive compounds, including fumaric acid, gallic acid, and malic acid. These compounds are widely valued for antimicrobial, antioxidant, and anti-inflammatory activities and are widely utilized in pharmaceutical and food industries [34-40]. The increased

abundance of these phytochemicals underscores the enhanced bioactive potential of polyploid plants. Additionally, a noteworthy increase in sucrose concentration in polyploid plants was also observed, potentially stimulating metabolic pathways critical for growth and vitality [41]. These cumulative enhancements in bioactive compounds are boosting the therapeutic values in polyploid plants. Similar effects of oryzalin-induced polyploidization on increased bioactivity have been observed in *Thymus vulgaris* L. [10], *Ajuga reptans* L. [42], *Mentha spicata* L. [15], *Melissa officinalis* L. [43], and *Cannabis sativa* L. [12], supporting the role of polyploidy in improving medicinal qualities.

Likewise, antioxidant, antimicrobial, and anti-inflammatory activities are used as a key approach to evaluate the impact of polyploidization on the pharmaceutical value of plants. Free radicals and oxidative stress are linked to numerous health issues. Recent studies show that natural compounds like phenolics and vitamins can help reduce oxidative stress, offering a safe, nonmedicinal approach that patients may find appealing [44]. Our results revealed that the polyploid sample exhibited a twofold higher phenolic content compared to the diploid extracts, consistent with our NMR findings, which indicated elevated levels of gallic acid in the polyploid sample. However, given the relatively low concentration of gallic acid in the extract, it is insufficient to attribute the enhanced antioxidant activity solely to this compound. This suggests the potential involvement of other bioactive constituents present in the extract, as indicated by its chemical composition profile, some of which may not have been detected in the current study. However, both extracts showed a similar TFC level. Research indicates that synthetic polyploidization often raises phenolic content in plants but can have mixed effects on flavonoid levels. For instance, polyploidization increased phenolics but decreased flavonoids in *Ponkan mandarin* [45], while in *Lonicera japonica* Thunb., certain growth stages in diploids showed higher levels of specific flavonoids like luteoloside [46]. Thus, despite higher phenolic content overall, polyploid plants may have lower flavonoid levels than diploids, depending on the species and growth stage. Although phenolic compounds are responsible for bioactivities, including antioxidant activity, the antioxidant activity should be evaluated through multiple *in vitro* tests. The scavenging of the stable DPPH radical is widely used to evaluate the antioxidant activity of bioactive compounds extracted from plants. Our results revealed a significantly higher DPPH radical scavenging activity (13.01 µg TE/mg) than the diploid (5.10 µg TE/mg). A previous study by Tan et al. (2014) [47] also reported a lower DPPH activity of *C. fragrans* (262.5 mg AA/100g extract) than our polyploid variety. Additionally, the polyploid extracts showed a significantly higher ability to scavenge ABTS radicals as well as increased activity

of subsequent reduction in reactive oxygen species (ROS) compared to the diploid one. Several works described significant results for the antioxidant activity of *C. fragrans* extracts [3,5,47]. However, this polyploid variety showed better antioxidant activity compared with the previous reports. Similar enhanced antioxidant properties have also been reported in induced tetraploid *T. vulgaris* [15] and tetraploid *Citrus limon* [48] compared to their diploid counterpart. So, it can be inferred that the increased production of bioactive metabolites, particularly phenols and fatty acid esters, due to the genetic influence of chromosome doubling likely boosts antioxidant activity in the tetraploid genotype [35,37,49,50].

The antimicrobial activity of *C. fragrans* extracts on skin infection-causing pathogens revealed that the polyploid extracts showed a higher antimicrobial efficiency than the diploid extract, specifically on *S. epidermidis*, *S. aureus*, *C. albicans*, and MRSA. It is more interesting that the polyploid extract showed good efficacy against the resistant strain, making it a potent candidate for advanced medical applications. While several studies have explored the antibacterial and antifungal properties of *C. fragrans* extract [47,51], this is the first report on the antimicrobial activity of induced polyploid plants of this species. Notably, the polyploid extract demonstrated greater efficacy than previously reported results. Similar enhanced antibacterial activity has been observed in tetraploid *Mitracarpus hirtus* against *S. aureus*, *Bacillus subtilis* [52], and *T. vulgaris* against respiratory pathogens [10]. The enhanced antimicrobial activity in polyploids may be attributed to the presence of higher levels of compounds like ethyl stearate, fumaric acid, gallic acid, and malic acid, which are also recognized for their antimicrobial properties [31,34,38,53,54].

HT-29, a human colorectal adenocarcinoma cell line characterized by its epithelial morphology, was used as an *in vitro* model for our study. Our result demonstrated a significantly higher protective activity in polyploid extract compared to diploid. Upon treatment with curative intent after oxidative stress induced by H_2O_2, both extracts demonstrated comparable cytoprotective effects on the HT-29 cell line at a low concentration of 25 µg/mL. This study represents the first investigation into the cytoprotective activity of *C. fragrans* extract against oxidative stress-induced cell death in HT-29 cells. The observed protective effect is likely attributed to the enhanced concentration of bioactive compounds, which may play a crucial role in mitigating H_2O_2-induced cell death [55].

COX-1 and COX-2 are cyclooxygenase isoforms with distinct functions. COX-1 is consistently expressed and essential for normal physiological processes, including gastric protection and

platelet support. In contrast, COX-2 is an inducible enzyme that increases in response to inflammation, producing pro-inflammatory prostanoids. Despite their complex interactions, COX inhibitors are commonly used to alleviate acute and chronic pain and reduce inflammation by blocking these enzymes [56]. Our gene expression study revealed that the polyploid extract showed a downregulation of *COX* gene expression, showing the best results at 25 µg/mL compared to the diploid extract, which showed a similar COX expression as oxidative stress induced by H_2O_2 in cells. So, the polyploid extract established its superior anti-inflammatory activity than the diploid genotype. Therefore, a significantly higher amount of fumaric acid, gallic acid, and malic acid can contribute to this higher anti-inflammatory activity as they are potential anti-inflammatory agents [36,40,57,58]. Similarly, Gupta et al. (2024) [15] showed that the higher thymol content in polyploid *T. vulgaris* essential oil exhibited enhanced COX-2 inhibition efficacy. Given that the polyploid extracts significantly downregulated *COX* gene expression in treated cells, this suggests a potential COX-inhibitory mechanism and indicates their ability to modulate inflammatory pathways at the molecular level. Considering the central role of COX enzymes in the biosynthesis of pro-inflammatory prostaglandins, such inhibition is consistent with established anti-inflammatory pharmacodynamics and may contribute to the extracts' potential in alleviating inflammation by targeting this key enzyme. Furthermore, results from the cytotoxicity assay demonstrated that the extracts exhibited no significant toxicity up to a concentration of 100 µg/mL, thereby defining a clear therapeutic safety margin. Notably, the most potent anti-inflammatory activity was observed at a concentration of 25 µg/mL, which is well below the cytotoxic threshold. This suggests that the active dose for anti-inflammatory efficacy falls well within the established safety range and may be suitable for the treatment of skin infections associated with inflammatory responses. Overall, our results indicate that synthetic polyploidization induced by oryzalin enhanced the natural metabolites as well as biological activities in this novel *C. fragrans* variety. Although this study demonstrates the enhanced potential of *C. fragrans* polyploid extracts as potent antibacterial, antioxidant, and anti-inflammatory agents, the findings are based solely on preliminary *in vitro* analyses. To fully establish their pharmacological efficacy, further investigations are necessary, including comprehensive preclinical studies and *in vivo* evaluations using appropriate animal models. Such studies will be essential to validate the therapeutic potential and ensure translational relevance for future clinical applications.

5.6 Conclusion

This investigation marks the first instance of elucidating the significant biological activities related to oryzalin-induced polyploidy in *C. fragrans*. These results further substantiate the efficacy of synthetic polyploidization in this species. The polyploid genotype demonstrated a remarkable enhancement in the extract yield, concurrently exhibiting elevated concentrations of biologically active metabolites, including ethyl stearate, gallic acid, fumaric acid, and malic acid. Furthermore, novel compounds such as dodecane-1-ol and (*Z*)-11-eicosenoic acid have also been identified as contributors to the augmented biological activities observed in polyploid specimens. The polyploid variant illustrated enhanced antimicrobial efficacy against skin infection-causing pathogens, including the resistant *S. aureus* variant, and showed superior antioxidant, cytoprotective, and anti-inflammatory attributes when contrasted with its diploid counterpart. Furthermore, this study asserts that the induced genotype of *C. fragrans*, beyond its aesthetic applications, exhibits superior characteristics that can be harnessed for commercial purposes, particularly within the pharmaceutical and cosmetic industries, due to the enhanced bioactivity of plant extracts. The process of synthetic polyploidization may play a pivotal role in the cultivation of plant varieties exhibiting heightened biological activities. Nevertheless, additional clinical and toxicological investigations are warranted to validate their practical applicability within the aforementioned industries.

References

[1] Chernenko, T. V., Ul'chenko, N. T., Glushenkova, A. I. & Redzhepov, D. Chemical investigation of *Callisia fragrans*. *Chem. Nat. Compd.* **43**, 253–255 (2007). https://doi.org/10.1007/s10600-007-0110-5.

[2] El Sohafy, S. M., Nassra, R. A., D'Urso, G., Piacente, S. & Sallam, S. M. Chemical profiling and biological screening with potential anti-inflammatory activity of *Callisia fragrans* grown in Egypt. *Nat. Prod. Res.* **35**, 5521–5524 (2021). https://doi.org/10.1080/14786419.2020.1847669.

[3] Phan, L. T. M. *et al.* Supercritical fluid extraction of polyphenols from Vietnamese *Callisia fragrans* leaves and antioxidant activity of the extract. *J. Chem.* **2020**, 9548401 (2020). https://doi.org/10.1155/2020/9548401.

[4] Nguyen, L. A. T. *et al.* Green synthesis of silver nanoparticles using *Callisia fragrans* leaf extract and its anticancer activity against MCF-7, HepG2, KB, LU-1, and MKN-7 cell lines. *Green Process. Synth.* **12**, 20230024 (2023). https://doi.org/10.1515/gps-2023-0024.

[5] Le, T. *et al.* Changes in total phenol, flavonoid contents and anti-*Lactobacillus* activity of *Callisia fragrans* due to extraction solvent. *AIP Conf. Proc.* **1954**, 1 (2018). https://doi.org/10.1063/1.5033315.

[6] Olennikov, D. N., Zilfikarov, I. N., Toropova, A. A. & Ibragimov, T. A. Chemical composition of *Callisia fragrans* wood juice and its antioxidative activity (*in vitro*). *Chem. Plant Raw Mater.* **4**, 95–100 (2008).

[7] Mykhailenko, O., Ivanauskas, L., Bezruk, I., Petrikaitė, V. & Georgiyants, V. Application of quality by design approach to the pharmaceutical development of anticancer crude extracts of *Crocus sativus* Perianth. *Sci. Pharm.* **90**, 19 (2022). https://doi.org/10.3390/scipharm90010019.

[8] Kunle, O. F., Egharevba, H. O. & Ahmadu, P. O. Standardization of herbal medicines—A review. *Int. J. Biodivers. Conserv.* **4**, 101–112 (2012). https://doi.org/10.5897/IJBC11.163.

[9] Zhang, C., Wohlhueter, R. & Zhang, H. Genetically modified foods: A critical review of their promise and problems. *Food Sci. Hum. Wellness* **5**, 116–123 (2016). https://doi.org/10.1016/j.fshw.2016.04.002.

[10] Beranová, K. *et al.* Morphological, cytological, and molecular comparison between diploid and induced autotetraploids of *Callisia fragrans* (*Lindl.*) Woodson. *Agronomy* **12**, 2520 (2022). https://doi.org/10.3390/agronomy12102520.

[11] Gupta, N. *et al.* Synthetic polyploidization induces enhanced phytochemical profile and biological activities in *Thymus vulgaris L.* essential oil. *Sci. Rep.* **14**, 5608 (2024). https://doi.org/10.1038/s41598-024-56378-7.

[12] Parsons, J. L. *et al.* Polyploidization for the genetic improvement of *Cannabis sativa*. *Front. Plant Sci.* **10**, (2019). https://doi.org/10.3389/fpls.2019.00476.

[13] Shmeit, Y. H. *et al.* Autopolyploidy effect on morphological variation and essential oil content in *Thymus vulgaris L. Sci. Hortic.* **263**, 109095 (2020). https://doi.org/10.1016/j.scienta.2019.109095.

[14] Xie, Z. Chemical composition and anti-proliferative and anti-inflammatory effects of the leaf and whole-plant samples of diploid and tetraploid *Gynostemma pentaphyllum* (*Thunb.*) Makino. *Food Chem.* **132**, 125–133.

[15] Bharati, R. *et al.* Oryzalin induces polyploids with superior morphology and increased levels of essential oil production in *Mentha spicata L. Ind. Crops Prod.* **198**, 116683 (2023). https://doi.org/10.1016/j.indcrop.2022.116683.

[16] Huie, C. W. A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Anal. Bioanal. Chem.* **373**, 23–30 (2002). https://doi.org/10.1007/s00216-002-1265-3.

[17] Kim, H. K., Choi, Y. H. & Verpoorte, R. NMR-based metabolomic analysis of plants. *Nat. Protoc.* **5**, 536–549 (2010). https://doi.org/10.1038/nprot.2009.237.

[18] Mascellani, A. *et al.* Moderate salinity stress affects expression of main sugar metabolism and transport genes and soluble carbohydrate content in ripe fig fruits (*Ficus carica L.* cv. Dottato). *Plants* **10**, 1861 (2021). https://doi.org/10.3390/plants10091861.

[19] Adams, R. P. *Identification of essential oil components by gas chromatography/mass spectrometry* (Allured Publishing Corporation, 2007).

[20] Sharma, O. P. & Bhat, T. K. DPPH antioxidant assay revisited. *Food Chem.* **113**, 1202–1205 (2009). https://doi.org/10.1016/j.foodchem.2008.08.008.

[21] Ou, B., Hampsch-Woodill, M. & Prior, R.L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **49**, 4619–4626 (2001). https://doi.org/10.1021/jf010586o

[22] Re, R. *et al.* Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* **26**, 1231–1237 (1999). https://doi.org/10.1016/S0891-5849(98)00315-3

[23] Singleton, V.L., Orthofer, R. & Lamuela-Raventós, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Meth. Enzymol.* **299**, 152–178 (1999). https://doi.org/10.1016/S0076-6879(99)99017-1

[24] Christ, B. & Müller, K.H. Zur serienmäßigen Bestimmung des Gehaltes an Flavonol-Derivaten in Drogen. *Arch. Pharm.* **293**, 1033–1042 (1960). https://doi.org/10.1002/ardp.19602931210

[25] Petrikkou, E. *et al.* Inoculum standardization for antifungal susceptibility testing of filamentous fungi pathogenic for humans. *J. Clin. Microbiol.* **39**, 1345–1347 (2001). https://doi.org/10.1128/JCM.39.4.1345-1347.2001

[26] Kudera, T. *et al. In vitro* selective antibacterial and antiproliferative effects of ethanolic extracts from Cambodian and Philippine plants used in folk medicine for diarrhea treatment. *Front. Pharmacol.* **12**, 746808 (2021). https://doi.org/10.3389/fphar.2021.746808

[27] Sattler, M.C., Carvalho, C.R. & Clarindo, W.R. The polyploidy and its key role in plant breeding. *Planta* **243**, 281–296 (2016). https://doi.org/10.1007/s00425-015-2450-x

[28] Santos-Buelga, C., Gonzalez-Manzano, S., Dueñas, M. & Gonzalez-Paramas, A.M. Extraction and isolation of phenolic compounds. In *Natural Products Isolation* 427–464 (2012). https://doi.org/10.1007/978-1-61779-624-1_16

[29] Zayed, M., Ahmad, F., Ho, W.-S. & Pang, S. GC-MS analysis of phytochemical constituents in leaf extracts of *Neolamarckia cadamba* (Rubiaceae) from Malaysia. *Int. J. Pharm. Pharm. Sci.* **6**, 123–126 (2014).

[30] Kim, B.-R. *et al.* Composition and antioxidant activities of volatile organic compounds in radiation-bred *Coreopsis* cultivars. *Plants* **9**, 717 (2020). https://doi.org/10.3390/plants9060717

[31] Lazarevic, J. *et al.* Chemical composition and screening of the antimicrobial and antioxidative activity of extracts of *Stachys* species. *J. Serb. Chem. Soc.* **75**, 1347–1359 (2010). https://doi.org/10.2298/JSC100601117L

[32] Togashi, N. *et al.* Antibacterial activity of long-chain fatty alcohols against *Staphylococcus aureus*. *Molecules* **12**, 139–148 (2007). https://doi.org/10.3390/12020139

[33] Al-Rajhi, A.M.H. & Abdel Ghany, T.M. Nanoemulsions of some edible oils and their antimicrobial, antioxidant, and anti-hemolytic activities. *BioResources* **18**, 1465–1481 (2023). https://doi.org/10.15376/biores.18.1.1465-1481

[34] Unver, T. A preliminary study of fumaric acid, called allomaleic acid, as a pharmaceutical antimicrobial compound. *Int. Med. J.* **13**, 383–387 (2024). https://doi.org/10.5455/medscience.2024.04.031

[35] Kokkaew, H., Srithanyarat, N. & Pitirit, T. Optimization of ingredients using response surface methodology and effects of organic acids on phytochemicals and antioxidant activities in extruded purple corn noodle (2020).

[36] Kaur, G., Shivanandappa, T.B., Kumar, M. & Kushwah, A.S. Fumaric acid protects cadmium-induced hepatotoxicity in rats: Owing to its antioxidant, anti-inflammatory action and aid in recast the liver function. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **393**, 1911–1920 (2020). https://doi.org/10.1007/s00210-020-01900-7

[37] Kweon, D.K., Choi, J.K., Kim, E.K. & Lim, S.T. Adsorption of divalent metal ions by succinylated and oxidized corn starches. *Carbohydr. Polym.* **46**, 171–177 (2001). https://doi.org/10.1016/S0144-8617(00)00310-4

[38] Bajko, E. *et al.* 5-O-Caffeoylquinic acid: A spectroscopic study and biological screening for antimicrobial activity. *LWT* **65**, 471–479 (2016). https://doi.org/10.1016/j.lwt.2015.08.024

[39] Karpiński, T.M. & Ożarowski, M. Plant organic acids as natural inhibitors of foodborne pathogens. *Appl. Sci.* **14**, 6340 (2024). https://doi.org/10.3390/app14146340

[40] Cai, L. *et al.* Gallic acid mitigates LPS-induced inflammatory response via suppressing NF-κB signalling pathway in IPEC-J2 cells. *J. Anim. Physiol. Anim. Nutr.* **106**, 1000–1008 (2022). https://doi.org/10.1111/jpn.13698.

[41] Zheng, Z. *et al.* The protein kinase SnRK2.6 mediates the regulation of sucrose metabolism and plant growth in *Arabidopsis*. *Plant Physiol.* **153**, 99–113 (2010). https://doi.org/10.1104/pp.109.150789.

[42] Navrátilová, B. *et al.* Impact of artificial polyploidization in *Ajuga reptans* on content of selected biologically active glycosides and phytoecdysone. *Horticulturae* **8**, 581 (2022). https://doi.org/10.3390/horticulturae8070581.

[43] Bharati, R. *et al.* Synthetic polyploid induction influences morphological, physiological, and photosynthetic characteristics in *Melissa officinalis* L. *Front. Plant Sci.* **14**, 1332428 (2023). https://doi.org/10.3389/fpls.2023.1332428.

[44] Lee, K. W., Kim, Y. J., Kim, D.-O., Lee, H. J. & Lee, C. Y. Major phenolics in apple and their contribution to the total antioxidant capacity. *J. Agric. Food Chem.* **51**, 6516–6520 (2003). https://doi.org/10.1021/jf034475w.

[45] Tan, F.-Q. *et al.* Polyploidy remodels fruit metabolism by modifying carbon source utilization and metabolic flux in Ponkan mandarin (*Citrus reticulata* Blanco). *Plant Sci.* **289**, 110276 (2019). https://doi.org/10.1016/j.plantsci.2019.110276.

[46] Wang, H. *et al.* Comparative transcriptomic analyses of chlorogenic acid and luteolosides biosynthesis pathways at different flowering stages of diploid and tetraploid *Lonicera japonica*. *PeerJ* **8**, e8690 (2020). https://doi.org/10.7717/peerj.8690.

[47] Tan, J. B. L., Yap, W. J., Tan, S. Y., Lim, Y. Y. & Lee, S. M. Antioxidant content, antioxidant activity, and antibacterial activity of five plants from the Commelinaceae family. *Antioxidants* **3**, 758–769 (2014). https://doi.org/10.3390/antiox3040758.

[48] Bhuvaneswari, G., Thirugnanasampandan, R. & Gogulramnath, M. Effect of colchicine-induced tetraploidy on morphology, cytology, essential oil composition, gene expression and antioxidant activity of *Citrus limon* (L.) Osbeck. *Physiol. Mol. Biol. Plants* **26**, 271–279 (2020). https://doi.org/10.1007/s12298-020-00771-8.

[49] Cvetković, B. *et al.* Assessing antioxidant properties, phenolic compound profiles, organic acids, and sugars in conventional apple cultivars (*Malus domestica*): A chemometric approach. *Foods* **13**, 142692 (2024). https://doi.org/10.3390/foods13142692.

[50] Badhani, B., Sharma, N. & Kakkar, R. Gallic acid: A versatile antioxidant with promising therapeutic and industrial applications. *RSC Adv.* **5**, 27540–27557 (2015). https://doi.org/10.1039/C5RA01911G.

[51] Gurbanov, N. H., Yusifova, M. R., Omarova, E. M. & Maharramova, M. H. Physicochemical parameters, antibacterial and antifungal characteristics of preparations from the ground parts of the golden mustache (*Callisia fragrans* wood) grown in the conditions of Azerbaijan.

[52] Pansuksan, K., Sangthong, R., Nakamura, I., Mii, M. & Supaibulwatana, K. Tetraploid induction of *Mitracarpus hirtus* L. by colchicine and its characterization including antibacterial activity. *Plant Cell Tiss. Organ Cult.* **117**, 381–391 (2014). https://doi.org/10.1007/s11240-014-0437-6.

[53] Zhang, R. *et al.* Antibacterial activity of ethanol extract from Australian finger lime. *Foods* **13**, 2465 (2024). https://doi.org/10.3390/foods13152465.

[54] Flores-Maldonado, O., Dávila-Aviña, J., González, G. M., Becerril-García, M. A. & Ríos-López, A. L. Antibacterial activity of gallic acid and methyl gallate against emerging non-fermenting Bacilli. *Folia Microbiol.* (2024). https://doi.org/10.1007/s12223-024-00948-2.

[55] Benedetto, N. *et al. Malus pumila* Mill. cv Annurca apple extract might be therapeutically useful against oxidative stress and patterned hair loss. *FEBS Open Bio*. (2024). https://doi.org/10.1002/2211-5463.13397.

[56] Stiller, C.-O. & Hjemdahl, P. Lessons from 20 years with COX-2 inhibitors: Importance of dose–response considerations and fair play in comparative trials. *J. Intern. Med.* **292**, 557–574 (2022). https://doi.org/10.1111/joim.13458.

[57] Vijeesh, V., Vysakh, A., Jisha, N. & Latha, M. S. Malic acid attenuates potassium oxonate-induced gouty inflammation in Wistar rat. *Biointerface Res. Appl. Chem.* **12**, 1682–1691 (2022). https://doi.org/10.33263/BRIAC122.16821691.

[58] Lee, S. *et al.* Anti-inflammatory function of arctiin by inhibiting COX-2 expression via NF-κB pathways. *J. Inflamm.* **8**, 1–9 (2011). https://doi.org/10.1186/1476-9255-8-1.

6. *In Vitro* Synthetic Polyploidization in Medicinal and Aromatic Plants for Enhanced Phytochemical Efficacy—A Mini-Review

Adopted from: **Gupta, N**., Bhattacharya, S., Dutta, A., Cusimamani, E. F., Milella, L., & Leuner, O. (2024). *In vitro* synthetic polyploidization in medicinal and aromatic plants for enhanced phytochemical efficacy—A mini-review. *Agronomy, 14*(8), 1830.

This chapter highlights the various mechanisms involved in synthetic polyploidization to enhance phytochemical profiles in medicinal and aromatic plants, focusing on improved secondary metabolite production and bioactivity.

Author contributions: Investigation, Methodology, Writing original draft, Writing review & editing, Formal analysis.

DOI: https://doi.org/10.3390/agronomy14081830.

Abstract

Medicinal and aromatic plants (MAPs) are well known for their valuable secondary metabolites and diverse phytochemicals responsible for a plethora of medicinal properties such as antimicrobial, antioxidant, anti-inflammatory, anticancerous, and analgesic activities, making them essential for various industries. Therefore, this significant market demand has led to the need to improve the quality and quantity of secondary metabolites and thus develop high-quality commercial products. In this context, polyploidization is considered a sound contemporary approach that produces new genotypes, leading to the overexpression of genes involved in biosynthesizing crucial metabolites. Enhanced natural metabolite production increases the biological activities of plant extracts along with enhanced tolerance against abiotic and biotic stresses to achieve homogeneity. This improvisation in the quality and quantity of plant secondary metabolites can maximize the medicinal value of the plants. Therefore, this mini-review aims to explore the importance of enhancing biological activity in medicinal plants, summarize the progress of synthetic polyploidization as a breeding tool in MAP species, and elucidate how this technique plays an important role in improving medicinal values. This breeding strategy could significantly advance future research and industrial applications by inducing superior genotypes with enhanced genomic complexity and improving traits like increased biomass, stress tolerance, and novel biochemical pathways. So, it can be concluded that *in vitro* synthetic polyploidization can be an effective tool for promoting the production of more distinctive genotypes with immense medicinal properties for a variety of commercial and pharmaceutical purposes.

Keywords: biological activity; epigenetic regulation; MAP species; polyploidization; secondary metabolites

6.1 Introduction

Medicinal and aromatic plants (MAPs) have been extensively utilized throughout millennia to alleviate a wide range of diseases in both conventional and complementary medical systems, as well as in the food and cosmetic sectors for flavouring and fragrance [1]. The extracts and the essential oils from MAPs contain an array of phytochemicals such as alkaloids, flavonoids, terpenoids, and phenolic compounds, which are responsible for performing a plethora of biological activities such as antimicrobial, anti-viral, antioxidant, anti-inflammatory, and anticancer activities [2,3]. For instance, essential oils from *Melissa officinalis* L., *Sideritis cypria* Post, *Origanum dubium* Boiss., *Mentha piperita* L., *Thymus capitatus* (L.) Cav., and *Salvia fruticose* Mill. were found to exhibit antioxidant, antimicrobial, and cytotoxic properties [4]. By 2050, the value of the world's medicinal plant trade is estimated to exceed USD 5 trillion [5]. Industrialists are switching to plant-based products in cosmetics, pharmaceuticals, food packaging, aquaculture, fodder industries, etc. [6,7,8], as synthetic products lead to the emergence of multidrug resistance microbial strains, high toxicity, and environmental degradation [9,10]. Due to huge market and economic demand, there is an utmost need to improve the quality of plants by enhancing their phytochemical yield, which is naturally low, and their biological activities [11].

To enhance their yield and biological activities, various breeding approaches, such as backcross breeding, mass selection, pure-line selection, precursor feeding, *in vitro* culture, genome editing, and metabolic engineering, were introduced [12,13]. Then again, these techniques are quite time-consuming, labour-intensive, and expensive. Also, current methods often struggle with limited genetic diversity and stability, whereas synthetic polyploidization overcomes these limitations by expanding the genetic toolkit to create organisms with expanded genomic complexity and greater trait stability [13,14]. Synthetic polyploidization, however, is one of the safest and most ideal contemporary breeding approaches conducted under *in vitro* and *in vivo* conditions to induce polyploidy in organisms. This technique involves inducing chromosome doubling in cells or tissues, typically through treatment with antimitotic agents such as colchicine or oryzalin, which disrupt normal cell division. Alternatively, synthetic polyploidization can also be achieved through the fusion of cells from different species or individuals with varying chromosome numbers that generate hybrid cells with an increased number of chromosome sets [14]. Polyploidization in MAPs enhances their physiological, morphological, anatomical, and biochemical traits, etc. [15,16,17,18]. However, this is not

always the case because gene duplication results are uncertain and can adversely influence plant traits [19]. Hence, selecting the appropriate genotypes and traits (which will be positively influenced) is necessary to overcome this problem [20].

Several studies have been conducted on the morphological, biochemical, and anatomical features of polyploids in MAP species [16,21,22]; however, studies on their enhanced biological activities and elucidating insights are scarce. Hence, it is important to understand the mechanism of polyploidization involved in increasing the production of major plant metabolites and biological activities. This study aims to highlight and explore the immense potential and current status of synthetic polyploidization in MAP species for enhancing plant secondary metabolites and their biological activities, emphasizing their insights for upregulating natural metabolites solely responsible for higher biological activities. Furthermore, it would illustrate the importance of the improvisation of MAP species and endorse breeders to accept synthetic polyploidization with an integrated omics-based selection approach as one of the sound breeding techniques facilitating economically important MAP species in the current as well as future pharmaceutical, food, and cosmetic industries.

6.2 *In Vitro* Synthetic Polyploidization in MAP Species Improvement: Current Status Focusing on Enhancement of Natural Metabolite Production and Biological Activity

Given the high demand for valuable plant secondary metabolites across industries, synthetic polyploidization emerges as a promising breeding method to boost the phytochemical efficacy of MAPs [23], while aligning with consumer preferences for natural genetic profiles and avoiding concerns about genetically modified organisms [24]. New polyploid lineages in plants emerge through chromosomal doubling in somatic cells and the reunion of unreduced gametes [25]. This can be achieved via *in vitro*, *ex vitro*, and *in vivo* systems. *In vitro* polyploidization is the most common and preferred method in research and commercial breeding due to its controlled environment, enabling precise application of growth regulators, leading to high chromosome doubling efficiency, low mortality, and minimal mixoploidy [26,27]. However, *in vivo* polyploidization applies antimitotic agents to intact plants, often resulting in variable outcomes due to less precise control, lower induction rates, and higher mixoploidy. In contrast, *ex vitro* polyploidization, using methods like foliar spraying, is even less efficient due to chemical evaporation, reduced absorption, and further increased mixoploidy [28,29]. In specific contexts where high consistency and uniformity are critical, *in vitro* polyploidization is favoured for its superior efficiency in producing stable polyploids with precise, desired

phenotypic traits. For instance, a study by Navratilova et al. (2022) [30] reported that the tetraploid genotype of *Ajuga reptans* induced by oryzalin treatment showed increased levels of trans-teupolioside, trans-verbascoside, and 20-hydroxecdysone content than the diploid genotype. These results indicated that synthetic polyploidization can induce better genotypes to enhance substances with potential pharmaceutical and economic applications. Similarly, another study by Priya and Pillai (2023) [21] reported a 160-fold increase in the production of major compound andrographolide in colchiploid calluses than the diploid, which has high economic values due to its pharmacological properties including anticancer, antimicrobial, antiparasitic, choleretic, hypocholesterolemia, anti-inflammatory, antidiabetic, hepatoprotective, immunomodulatory, and cardiovascular activity [31]. Artemisinin, a valuable plant secondary metabolite used as the most effective antimalarial drug, was reported to increase from 39% to 56% in induced tetraploid *Artemisia annua* plants using colchicine. This study also reported the upregulation of FPS, HMGR, and artemisinin metabolite-specific Aldh1 genes related to the artemisinin biosynthetic pathway. These results suggested that synthetic polyploidization positively influenced the key enzymes for the biosynthesis of artemisinin, which resulted in the increased production of this valuable metabolite. The successful induction of polyploid medicinal plants and enhancing plant secondary metabolites through colchicine has been reported in several plants such as *Anoectochilus formosanus* [32], *Cichorium intybus* [33], *Datura stramonium* [34], *Papaver bracteatum* [35], *Salvia miltiorrhiza* [36], *Stachys byzantine* [37], and *Trachyspermum ammi* [38]. Similarly, Bharati et al. (2023) [16] reported the successful induction of polyploid *Mentha spicata* by synthetic polyploidization using oryzalin as an anti-mitotic agent with an increased amount of valuable major compounds such as carvone and limonene compared to their diploid control. Another study by Bharati also reported an increased amount of geranial and neral in oryzalin-induced tetraploid *Melissa officinalis* [17].

Even though bioactivity analysis of polyploid medicinal plants is still in the nascent stage, some studies have characterized induced polyploid medicinal plants focusing on metabolite enhancement and biological activity. A study by Gupta et al. (2024) [3] elucidated that oryzalin-induced *Thymus vulgaris* essential oil showed higher antibacterial, antioxidant, and anti-inflammatory activities along with higher concentrations of thymol and γ-terpinene than diploid essential oil. This study also indicated that a high concentration of thymol content in the tetraploid genotype is mainly responsible for its enhanced biological activity. Similarly, another study on induced tetraploid *Thymus vulgaris* exhibited higher insecticidal activity

along with an increased amount of bio-active compounds such as carvacrol, thymol, trans-caryophyllene, γ-terpinene, and 4-cymene than the diploid genotype [39]. Bhuvaneswari et al. (2019) [40] and Mei et al. (2020) [41] also exhibited higher antioxidant activity in colchicine-induced *Citrus limon* and *Echinacea purpurea* with increased secondary metabolites. Additionally, Pansuksan et al. (2014) [42] reported higher antibacterial activity along with 40 unique bio-active compounds in tetraploid *Mitracarpus hirtus* compared to its diploid progenitor. However, elevated concentrations of specific bioactive components in secondary metabolites do not automatically translate to improved biological activity. The effectiveness is primarily driven by the synergistic interactions among these components.

In vitro polyploidization facilitated by synthetic antimitotic agents presents a potentially effective approach for generating polyploid plants with augmented biological traits. However, it is not applicable every time as the outcomes of gene duplication remain skeptical. For that, a genome selection-based predictive accuracy model can be employed to accomplish desirable genotypes through synthetic polyploidization. However, the overall findings suggest that synthetic polyploidization could be a sustainable approach for improving MAP species by focusing on the enhancement of metabolite production and biological activity. Table 10 summarizes the major attempts of *in vitro* synthetic polyploidization conducted in MAP species focusing on the enhancement of their secondary metabolites and biological activity.

Table 10 List of major attempts of *in vitro* synthetic polyploidization in MAP species focusing on enhanced secondary metabolite production and biological activity

Species Name	Family name	Antimitotic agent	Short Description	References
Ajuga reptans	Lamiaceae	Oryzalin	Tetraploid variants showed increased content of 20-hydroxyecdysone along with an increased amount of trans-verbascoside, and trans-teupolioside compared to diploids.	[30]
Allium sativum L.	Alliaceae	Colchicine	A 30.7% increase in the pharmaceutically active metabolite allium, which exhibits anti-bacterial, anti-fungal, and anti-atherosclerotic activities, was observed in the polyploids	[43]
Andrographis paniculata (Burm.f.) Wall. ex. Nees.	Acanthaceae	Colchicine	Andrographolide was observed to be increased by 160-fold compared to diploids.	[21]
Anoectochilus formosanus Hayata	Orchidaceae	Colchicine	A higher content of total flavonoid and gastrodin was observed in the leaves, stem, and whole plant of the polyploid when compared to diploid.	[32]
Artemisia annua	Asteraceae	Colchicine	Tetraploids produced an increase in levels of artemisinin content from 39% to 56% compared to diploids.	[44]
Bacopa monnieri	Plantaginaceae	Colchicine	Tetraploids exhibited a 2.3-fold increase in total bacoside content compared to diploids.	[45]

Cannabis sativa L.	Cannabaceae	Oryzalin	An increase in terpene content was observed in tetraploid by 71.5% along with other compounds like α-humulene which doubled and cis-nerolidol. The content of cannabidiol increased by 8.9%, guaiol by 60%, and 15.2% in cannabidivarinic acid in tetraploids. α-bisabolol was also found in teraploid leaves which was absent in diploid.	[46]
Cichorium intybus L.	Asteraceae	Colchicine	Tetraploids showed an increase of 1.9-fold in total phenolics content and a 10-fold increase in chlorogenic acid in the leaves.	[33]
Citrus limon (L.) Osbeck	Rutaceae	Colchicine	Limonene and lanceol increased drastically in tetraploids than in diploids and showed the presence of β-bisabolene that was absent in diploids. The increase in antioxidant activity of tetraploids was also observed due to increased limonene and lanceol content.	[40]
Datura stramonium L.	Solanaceae	Colchicine	An increase in total alkaloid content by 7.25 % was observed. For instance, Hyoscyamine and scopolamine raised to 2.6 and 3.0 times respectively, in contrast to diploid control.	[34]
Dracocephalum kotschyi Boiss	Lamiaceae	Colchicine	Tetraploid genotypes represented an increased production of flavonoids by 19.37% compared to diploids.	[47]
Echinacea purpurea (L.) Moench	Asteraceae	Colchicine	Increased production of cichoric acid, caffeic acid derivatives and alkamides in polyploids	[48]

Echinacea purpurea (L.) Moench	Asteraceae	Colchicine	Higher contents of cichoric acid, caffeic acid, chlorogenic acid, caftaric acid, and 1,5-dicaffeoyl quinic acid were observed in tetraploids compared to diploids. An increase in antioxidant activity and total phenolic content was also observed in tetraploids.	[41]
Melissa officinalis L.	Lamiaceae	Oryzalin	An increase in essential oil content by 75 % was reported, along with an increase in geranial and neral by 11.06% and 9.49%, respectively, in tetraploid compared to diploid.	[17]
Mentha spicata L.	Lamiaceae	Oryzalin	Higher essential oil yield is observed in polyploids, along with a higher concentration of carvone and limonene	[16]
Mitracarpus hirtus L.	Rubiaceae	Colchicine	Increased antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* with the detection of 9-Octadecyne (2); Stigmast-5-en-3-ol, oleate; 9,12,15-Octadecatrienoic acid, methyl ester, (Z, Z, Z) that were absent in diploids.	[42]
Nigella sativa L.	Ranunculaceae	Colchicine	An increase of 46.3% of thymoquinone was observed in the polyploid variants.	[49]
Papaver bracteatum Lindl	Papaveraceae	Colchicine	Tetraploids exhibited increased benzylisoquinoline alkaloid content. Increased production of noscapine (30.55-fold increase) and thebaine (5.86-fold increase) with increased antioxidant activity in polyploid.	[35]
Salvia miltiorrhiza Bunge	Lamiaceae	Colchicine	Enhanced production of dihydrotanshinone I and total tanshinones in polyploid compared to diploid.	[36]

Stachys byzantina L.	Lamiaceae	Colchicine	An increase in linalool, α-cadinol, cubenol, α-terpineol, and menthone content was reported in polyploid.	[37]
Stevia rebaudiana (Bertoni)	Asteraceae	Colchicine	An approximate 2.4% increase in stevioside production was observed in the polyploid variants.	[50]
Taxus baccata L.	Taxaceae	Colchicine	Tetraploid variety exhibited a 10.8-fold higher taxane content compared to diploids, indicating increased production of the chemotherapeutic drug.	[51]
Tetradenia riparia (Hochst.) Codd	Lamiaceae	Colchicine	An increase in the percentage of fenchone from 25.42% in diploids to 48.4% in tetraploids was observed. Additionally, major bioactive compounds with antifungal properties, such as γ-terpineol (10.16%) and viridiflorol (5.58%), were found exclusively in tetraploids and not in diploids.	[52]
Thymus persicus L.	Lamiaceae	Colchicine	Increased production of betulinic acid (69.73%), oleanolic acid (42.76%), and ursolic acid (140.6%) in their polyploids	[53]
Thymus vulgaris L.	Lamiaceae	Oryzalin	With an increase of 41.11% in essential oil yield, enhanced production of thymol and *γ*-terpinene was observed in polyploid. Similarly, an increase in antibacterial, antioxidant, and anti-inflammatory activities was observed in the polyploid compared to the diploid.	[3]

Thymus vulgaris L.	Lamiaceae	Oryzalin	An increase in the contents of carvacrol, thymol, trans-caryophyllene, *γ*-terpinene, and 4-cymene was observed in the polyploid. Increased insecticidal activity was observed in polyploids rather than diploids.	[39]
Trachyspermum ammi L.	Apiaceae	Colchicine	Thymol was reported to increase from 49.67% in diploids to 69.2% in tetraploids. The essential oil yield was 2.5 times more in tetraploids than in diploids.	[38]

6.3 Unveiling the Mechanisms: Insights into *In Vitro* Artificially Induced Polyploid Plants for Enhanced Phytochemicals and Biological Activities

Often, it is postulated that synthetic polyploidization augments both primary and secondary metabolite production by inducing chromosome doubling, thereby influencing the biological activities of polyploid plants [54]. However, one such report contradicted this notion, revealing that the diploid plants had higher flavonoid and phenolic content, including increased rutin and quercetin levels, which enhanced their anti-proliferative and anti-inflammatory effects compared to tetraploids [19]. Polyploidization impacts genetic composition and gene expression, facilitating the emergence of new regulatory pathways [55]. This leads to enhanced adaptability, expanded geographical niches, and altered community structures in various plant species [56]. Molecular mechanisms involved include transcriptome changes [57], microRNAs [58], alternative splicing [59], histone modifications [60], chromatin remodelling, RNA-binding proteins [61], DNA methylation [62], and N6-methyladenosine RNA methylation [63] contributing to the evolutionary process of polyploids by which polyploidy reshapes gene expression, expands proteome diversity, and alters epigenetic landscapes, leading to the differential regulation of duplicated genes. These modifications enhance genetic and epigenetic plasticity, driving the adaptation, stability, and evolutionary diversification of polyploids. Antimitotic agents involved in synthetic polyploidization like colchicine, oryzalin, trifluralin, and amiprophosmethyl disrupt spindle formation during cell division by binding with tubulin dimers, preventing microtubule formation and chromatid migration leading to chromosome doubling and conversion to higher ploidy levels such as triploids, tetraploids, hexaploids, and octaploids [64].

Gene duplication leads to DNA amplification, increasing the copy number of individual genes. This amplification enhances mRNA expression, leading to the overproduction of key biosynthetic enzymes involved in secondary metabolite synthesis. As a result, enzyme activity is elevated, and metabolic pathways are upregulated, thereby influencing the quantity, composition, and proportions of secondary metabolites [65,66] (Figure 15). According to Lavania (2005) [67], genome duplication causes a decrease in the ratio of the nuclear membrane to chromatins so that more chromatins come into contact with the nuclear membrane, which enhances the genetic activity of the cell and influences the production of secondary metabolites. The duplicated gene copy leads to the production of more RNA molecules and was found to be dominant in the whole genome duplication, which is retained

in the polyploids [68]. Comai (2005) [69] also stated that an increase in gene number increased the overall gene expression. Gene duplication leading to increased protein expression, which could ultimately increase the production of target enzymes or metabolites, has also been reported by Dar and Rehman (2017) [70].

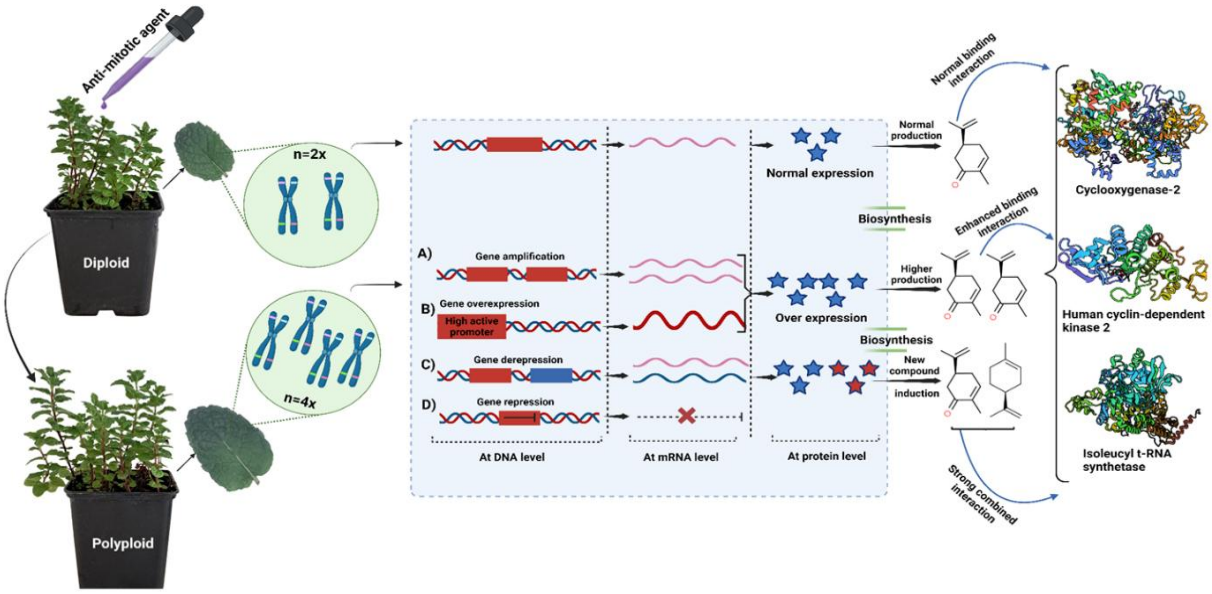

Figure 15 A schematic diagram showing the mechanism of synthetic polyploidization for enhancing major phytochemicals and bio-activity of MAPs. The treatment with antimitotic agents such as oryzalin or colchicine leads to chromosome doubling that results in amplification, overexpression, depression, or repression of genes encoding key biosynthetic enzymes of the plant's major metabolites due to the epigenetic and transcriptional regulation. A. Gene duplication leads to DNA amplification, boosting expression by replicating the coding region of the target gene, resulting in additional copies within the genome. This results in enhanced expression at the mRNA level and contributes to the overexpression of key biosynthetic enzymes. B. Overexpression of the coding region of the desired gene resulting in the over-production of the key enzyme. C. Derepression of previously silenced or weakly expressed gene that encodes another key enzyme for new metabolites. D. Repression or inhibition of gene encoding key biosynthetic enzyme for metabolites that are expressed in the diploid genotype. This over-production of newly expressed key biosynthetic enzymes enhances the production of major metabolites or induces new metabolites in the phytochemical composition of plants that show strong binding or combined interactions with targeted proteins, and it enhances biological activities such as anti-inflammatory, antioxidant, and antibacterial activities.

Simultaneously, polyploidy can influence different mechanisms that can increase, decrease, or even silence the gene expression that influences plants' physiological and biochemical traits [15]. A study by Hassanzadeh et al. (2020) [71] described that physiological functions or gene expressions were greatly influenced by polyploid induction. Javadian et al. (2017) [72] reported that the expression levels of some key genes encoding specific enzymes involved in the podophyllotoxin biosynthetic pathway were enhanced by increasing the plant ploidy, including phenylalanine ammonia-lyase (PAL), cinnamoyl-CoA reductase (CCR), cinnamyl-alcohol dehydrogenase (CAD), and pinoresinol-lariciresinol reductase (PLR).

As the chromosome number increased, DNA content and enzyme activity per cell were also increased [73]. Talebi et al. (2017) [74] reported increased enzyme activity (CAT and POD) and protein content in the tetraploid *Agastache foeniculum* plants. Enhancement in enzyme activity with increased ploidy level has also been confirmed by some other researchers [15,75]. The emergence of novel compounds in the polyploid genotype is attributed to the derepression of previously silenced or weakly expressed genes, while the absence of specific compounds results from the epigenetic modification of gene expression, leading to the inhibition of expression of previously active genes (Figure 15). A study by Parsons et al. (2019) [46] reported the presence of *α*-bisabolol in oryzalin-induced *Cannabis sativa*, which was absent in the diploid genotype.

In medicinal plants, the production of the per unit biomass of secondary metabolites is of immense economic importance [76]. Synthetic polyploidization involves alterations of cellular dynamics that are positively influenced by increased cell size, organelle size and numbers, transcriptome products, net photosynthetic rate, and upraised metabolic pathways [77]. The accumulation of favourable alleles in one organism, along with the induced doubling of chromosome number, further adds to the pharma-chemical productivity, which promotes partitioning of cell energy resources for secondary metabolism and cuts down lengthy pathways via improvised enzyme kinetics [66]. The polyploidization of *Catharanthus roseus* enhances the expression of genes related to alkaloid biosynthesis, boosting vindoline, catharanthine, and vinblastine content in leaves by 130.9%, 188.6%, and 122.6%, respectively, compared to the diploid genotype. These alkaloids, particularly vinblastine and vincristine, are prized for their potent anticancer properties, making *C. roseus* a crucial species for the pharmaceutical industry [78]. On the other hand, in hop tetraploids, the composition of the major chemical compounds used in beer production had an insignificant change with genome

doubling compared to diploids where the essential oil content was lower, but the proportion of beneficial components such as humulene, limonene, caryophyllene, and farnesene was higher [79]. Some other studies also reported enhanced metabolite induction in plants with increased ploidy levels, such as a 56% increase in artemisinin content in *Artemisia annua* [44], high triterpenoid levels in *Centella asiatica* [80], and a 50% increase in morphine in *Papaver somniferum* [81].

Metabolites interact with proteins via binding, allosteric regulation, or post-translational modifications, influencing protein function, stability, localization, and cellular processes like signal transduction, gene expression, and metabolism. The specificity and affinity of these interactions determine their impact on protein function and cellular responses. As the concentration of bioactive secondary metabolites increases, their affinity for binding with targeted proteins also rises. This enhanced interaction can significantly boost the biological activity of plant extracts. The overall effect largely depends on the synergistic interplay among these bioactive metabolites [82,83] (Figure 15). Many studies reported that an enhanced phytochemical profile in the polyploid genotype resulted in enhanced biological activities such as antibacterial, antioxidant, or anti-inflammatory activities [3,40,41].

In conclusion, synthetic polyploidization boosts gene copy numbers for key enzymes in biosynthetic pathways, elevating protein expression. This regulation enhances secondary metabolite production and augments biological activities in polyploid plants (Figure 15).

6.4 Summary and Outlook: Current Challenges and a Way Forward

In this mini-review, we attempted to highlight the potential of synthetic polyploidization as one of the powerful breeding approaches for improving MAP species. In this study, we have already proven its success in different MAP species for enhancing secondary metabolites as well as the biological activity of plants (Table 10). However, induced polyploids may show a reduced level of metabolite production due to unpredictable gene duplication and increased concentrations of certain secondary metabolites do not guarantee enhanced biological activity, as component synergy is crucial [53,84]. Polyploidization in MAPs enhances both agronomic performance and biochemical activities. For example, nearly one-quarter of global chamomile varieties are now colchicine-induced tetraploids, highlighting the effectiveness of this method [85]. In addition, synthetic polyploidization offers substantial benefits for MAP species by enhancing agronomic traits, improving stress tolerance, and increasing genetic diversity, which facilitates the development of novel traits. Simultaneously, it boosts secondary metabolite production,

thereby augmenting medicinal and aromatic properties, and promotes hybrid vigour. Furthermore, it bridges reproductive barriers, enabling the creation of new hybrids, fosters novel phenotypes, and extends growth cycles, resulting in crop varieties with specialized and optimized characteristics tailored to specific agricultural or industrial requirements. In summary, the industrial implementation of polyploidization supports sustainable production practices and meets the growing demand for natural bioactive compounds in diverse industries.

Despite so many advantages, this uncertainty and selection of the desired genotype is one of the major challenges for plant breeding. However, the genomic selection process for polyploid screening and integration of multi-omics strategies can overcome this issue. Utilizing multi-omics data, including genomics, transcriptomics, proteomics, and metabolomics, can offer not only valuable insights but also novel approaches to estimating the genomic correlation in polyploids and helping in precision screening for desired traits. However, multi-omics applications for genomic selection can themselves be a challenge because of their complex data output, which makes it difficult to manage and interpret, and also due to the limited data availability of MAP species, specifically transcriptome, genome, reference genome, as well as their integrated approach.

Numerous studies have highlighted the influence of geographical, environmental, agroclimatic, and genetic factors in shaping both the quantity and quality of secondary metabolite production in plants [86]. Synthetic polyploids demonstrate superior adaptability across diverse environments compared to diploids, which can influence the screening of superior polyploid genotypes [87]. So, analyzing the interaction between genotypes and various environmental factors is crucial. Additionally, synthetic polyploidization can affect the selection of desired genotypes through epigenetic instability, disrupted genomic imprinting, and unwanted epistasis. Epigenetic instability can cause variable gene expression, complicating trait selection. Disrupted genomic imprinting may lead to inconsistent trait outcomes, while unwanted epistasis can obscure genotype-phenotype relationships, making it challenging to stabilize and select beneficial traits. These complexities need to be managed to effectively achieve the desired polyploid genotypes. Another potential challenge is that substantial non-additive effects in MAP species during vegetative propagation can influence biochemical traits and stress responses. Hence, understanding additive effects is essential for effective genomic selection. For that, disruptive technologies like CoPhMoRe (corona phase molecular recognition) nanosensors can measure targeting molecules in real time, offering high sensitivity, and non-destructive analysis of plant signalling pathways. This is achieved by

creating various corona phases based on the structure of amphiphilic polymers. When combined with machine learning models like random forest, support vector machine, and deep neural network, these tools can accurately capture complex marker–trait relationships and improve marker selection, which can be highly useful for agricultural precision [88]. Despite having several challenges, synthetic polyploidization with genomic selection offers significant potential for enhancing the medicinal properties of plants. However, further investigation is required to optimize ideal polyploidization protocols, elucidate genomic and epigenomic alterations, and perform the comprehensive phenotypic and metabolite profiling of synthetic polyploids. Additionally, research should focus on assessing fertility, reproductive viability, and the environmental and economic implications of polyploidization. Also, integrating the polyploid approach with advanced biotechnological tools, such as CRISPR/Cas9 and marker-assisted selection, is essential. A deeper understanding of these mechanisms could facilitate the development of superior MAP cultivars with enhanced medicinal properties.

References

[1] Barata, A.M.; Rocha, F.; Lopes, V.; Carvalho, A.M. Conservation and Sustainable Uses of Medicinal and Aromatic Plants Genetic Resources on the Worldwide for Human Welfare. Ind. Crops Prod. 2016, 88, 8–11. [Google Scholar] [CrossRef]

[2] Sethi, S.; Prakash, O.; Kumar, R.; Dubey, S.K.; Arya, M.; Pant, A.K. Phytochemical Analysis, Antioxidant and Antifungal Activity of Essential Oil and Extracts of *Alpinia malaccensis* (Burm.f.) Roscoe Flowers. Braz. J. Pharm. Sci. 2023, 58, e201209. [Google Scholar] [CrossRef]

[3] Gupta, N.; Bhattacharya, S.; Dutta, A.; Tauchen, J.; Landa, P.; Urbanová, K.; Houdková, M.; Fernández-Cusimamani, E.; Leuner, O. Synthetic Polyploidization Induces Enhanced Phytochemical Profile and Biological Activities in *Thymus vulgaris* L. Essential Oil. Sci. Rep. 2024, 14, 5608. [Google Scholar] [CrossRef]

[4] Chrysargyris, A.; Petrovic, J.D.; Tomou, E.-M.; Kyriakou, K.; Xylia, P.; Kotsoni, A.; Gkretsi, V.; Miltiadous, P.; Skaltsa, H.; Soković, M.D.; et al. Phytochemical Profiles and Biological Activities of Plant Extracts from Aromatic Plants Cultivated in Cyprus. Biology 2024, 13, 45. [Google Scholar] [CrossRef]

[5] World Health Organization. The Selection and Use of Essential Medicines: Report of the WHO Expert Committee on Selection and Use of Essential Medicines, 2021 (Including the

22nd WHO Model List of Essential Medicines and the 8th WHO Model List of Essential Medicines for Children); World Health Organization: Geneva, Switzerland, 2021; ISBN 978-92-4-004113-4.

[6] Prakash, S.; Radha; Kumar, M.; Kumari, N.; Thakur, M.; Rathour, S.; Pundir, A.; Sharma, A.K.; Bangar, S.P.; Dhumal, S.; et al. Plant-Based Antioxidant Extracts and Compounds in the Management of Oral Cancer. Antioxidants 2021, 10, 1358. [Google Scholar] [CrossRef] [PubMed]

[7] Haikal, A.; El-Neketi, M.; Awadin, W.F.; Hassan, M.A.; Gohar, A.A. Essential Oils from Wild *Mentha longifolia* Subspecies typhoides and Subspecies schimperi: Burn Wound Healing and Antimicrobial Candidates. J. King Saud. Univ. Sci. 2022, 34, 102356. [Google Scholar] [CrossRef]

[8] Dawood, M.A.O.; Gewaily, M.S.; Sewilam, H. The Growth Performance, Antioxidative Capacity, and Histological Features of Intestines, Gills, and Livers of Nile Tilapia Reared in Different Water Salinities and Fed Menthol Essential Oil. Aquaculture 2022, 554, 738122. [Google Scholar] [CrossRef]

[9] Catalano, A.; Iacopetta, D.; Ceramella, J.; Scumaci, D.; Giuzio, F.; Saturnino, C.; Aquaro, S.; Rosano, C.; Sinicropi, M.S. Multidrug Resistance (MDR): A Widespread Phenomenon in Pharmacological Therapies. Molecules 2022, 27, 616. [Google Scholar] [CrossRef]

[10] Ali, B.M.; Ang, F.; van der Fels-Klerx, H.J. Consumer Willingness to Pay for Plant-Based Foods Produced Using Microbial Applications to Replace Synthetic Chemical Inputs. PLoS ONE 2021, 16, e0260488. [Google Scholar] [CrossRef] [PubMed]

[11] Favela-González, K.M.; Hernández-Almanza, A.Y.; De la Fuente-Salcido, N.M. The Value of Bioactive Compounds of Cruciferous Vegetables (*Brassica*) as Antimicrobials and Antioxidants: A Review. J. Food Biochem. 2020, 44, e13414. [Google Scholar] [CrossRef]

[12] Anand, A.; Subramanian, M.; Kar, D. Breeding Techniques to Dispense Higher Genetic Gains. Front. Plant Sci. 2023, 13, 1076094. [Google Scholar] [CrossRef] [PubMed]

[13] Acquaah, G. Conventional Plant Breeding Principles and Techniques. In Advances in Plant Breeding Strategies: Breeding, Biotechnology and Molecular Tools; Al-Khayri, J.M., Jain, S.M., Johnson, D.V., Eds.; Springer International Publishing: Cham, Switzerland, 2015; pp. 115–158. ISBN 978-3-319-22521-0. [Google Scholar]

[14] Miri, S.M. Artificial Polyploidy in the Improvement of Horticultural Crops. J. Plant Physiol. Breed. 2021, 10, 1–28. [Google Scholar] [CrossRef]

[15] Mohammadi, V.; Talebi, S.; Ahmadnasab, M.; Mollahassanzadeh, H. The Effect of Induced Polyploidy on Phytochemistry, Cellular Organelles and the Expression of Genes Involved in Thymol and Carvacrol Biosynthetic Pathway in Thyme (*Thymus vulgaris*). Front. Plant Sci. 2023, 14, 1228844. [Google Scholar] [CrossRef]

[16] Bharati, R.; Fernández-Cusimamani, E.; Gupta, A.; Novy, P.; Moses, O.; Severová, L.; Svoboda, R.; Šrédl, K. Oryzalin Induces Polyploids with Superior Morphology and Increased Levels of Essential Oil Production in *Mentha spicata* L. Ind. Crops Prod. 2023, 198, 116683. [Google Scholar] [CrossRef]

[17] Bharati, R.; Gupta, A.; Novy, P.; Severová, L.; Šrédl, K.; Žiarovská, J.; Fernández-Cusimamani, E. Synthetic Polyploid Induction Influences Morphological, Physiological, and Photosynthetic Characteristics in *Melissa officinalis* L. Front. Plant Sci. 2023, 14, 1332428. [Google Scholar] [CrossRef]

[18] Homaidan Shmeit, Y.; Fernandez, E.; Novy, P.; Kloucek, P.; Orosz, M.; Kokoska, L. Autopolyploidy Effect on Morphological Variation and Essential Oil Content in *Thymus vulgaris* L. Sci. Hortic. 2020, 263, 109095. [Google Scholar] [CrossRef]

[19] Xie, Z.; Huang, H.; Zhao, Y.; Shi, H.; Wang, S.; Wang, T.T.Y.; Chen, P.; Yu, L. (Lucy) Chemical Composition and Anti-Proliferative and Anti-Inflammatory Effects of the Leaf and Whole-Plant Samples of Diploid and Tetraploid *Gynostemma pentaphyllum* (Thunb.) Makino. Food Chem. 2012, 132, 125–133. [Google Scholar] [CrossRef]

[20] Lamlom, S.F.; Yehia, W.M.B.; Kotb, H.M.K.; Abdelghany, A.M.; Shah, A.N.; Salama, E.A.A.; Abdelhamid, M.M.A.; Abdelsalam, N.R. Genetic Improvement of Egyptian Cotton (*Gossypium barbadense* L.) for High Yield and Fiber Quality Properties under Semi Arid Conditions. Sci. Rep. 2024, 14, 7723. [Google Scholar] [CrossRef] [PubMed]

[21] Priya, L.; Pillai, P.R.U. *In Vitro* Effect of Colchiploidy on Andrographolide Enhancement in *Andrographis paniculata* (Burm.f.) Wall. Ex. Nees. S. Afr. J. Bot. 2023, 163, 786–793. [Google Scholar] [CrossRef]

[22] Liu, Y.; Duan, S.-D.; Jia, Y.; Hao, L.-H.; Xiang, D.-Y.; Chen, D.-F.; Niu, S.-C. Polyploid Induction and Karyotype Analysis of *Dendrobium officinale*. Horticulturae 2023, 9, 329. [Google Scholar] [CrossRef]

[23] Niazian, M.; Nalousi, A.M. Artificial Polyploidy Induction for Improvement of Ornamental and Medicinal Plants. Plant Cell Tissue Organ. Cult. 2020, 142, 447–469. [Google Scholar] [CrossRef]

[24] Zhang, C.; Wohlhueter, R.; Zhang, H. Genetically Modified Foods: A Critical Review of Their Promise and Problems. Food Sci. Hum. Wellness 2016, 5, 116–123. [Google Scholar] [CrossRef]

[25] Tayalé, A.; Parisod, C. Natural Pathways to Polyploidy in Plants and Consequences for Genome Reorganization. Cytogenet. Genome Res. 2013, 140, 79–96. [Google Scholar] [CrossRef]

[26] Eng, W.-H.; Ho, W.-S. Polyploidization Using Colchicine in Horticultural Plants: A Review. Sci. Hortic. 2019, 246, 604–617. [Google Scholar] [CrossRef]

[27] Fu, L.; Zhu, Y.; Li, M.; Wang, C.; Sun, H. Autopolyploid Induction via Somatic Embryogenesis in *Lilium distichum* Nakai and *Lilium cernuum* Komar. Plant Cell Tissue Organ. Cult. 2019, 139, 237–248. [Google Scholar] [CrossRef]

[28] Salma, U.; Kundu, S.; Mandal, N. Artificial Polyploidy in Medicinal Plants: Advancement in the Last Two Decades and Impending Prospects. J. Crop Sci. Biotechnol. 2017, 20, 9–19. [Google Scholar] [CrossRef]

[29] Roughani, A.; Miri, S.M. Polyploidy Induction in Ornamental Plants. In Proceedings of the 2nd International and 3rd National Congress on Flower and Ornamental Plants, Mahallat, Iran, 23–25 October 2018. [Google Scholar]

[30] Navrátilová, B.; Ondřej, V.; Vrchotová, N.; Tříska, J.; Horník, Š.; Pavela, R. Impact of Artificial Polyploidization in *Ajuga reptans* on Content of Selected Biologically Active Glycosides and Phytoecdysone. Horticulturae 2022, 8, 581. [Google Scholar] [CrossRef]

[31] Zeng, B.; Wei, A.; Zhou, Q.; Yuan, M.; Lei, K.; Liu, Y.; Song, J.; Guo, L.; Ye, Q. Andrographolide: A Review of Its Pharmacology, Pharmacokinetics, Toxicity and Clinical Trials and Pharmaceutical Researches. Phytother. Res. 2022, 36, 336–364. [Google Scholar] [CrossRef]

[32] Chung, H.-H.; Shi, S.-K.; Huang, B.; Chen, J.-T. Enhanced Agronomic Traits and Medicinal Constituents of Autotetraploids in *Anoectochilus formosanus* Hayata, a Top-Grade Medicinal Orchid. Molecules 2017, 22, 1907. [Google Scholar] [CrossRef]

[33] Ghotbi Ravandi, E.; Rezanejad, F.; Zolala, J.; Dehghan, E. The Effects of Chromosome-Doubling on Selected Morphological and Phytochemical Characteristics *of Cichorium intybus* L. J. Hortic. Sci. Biotechnol. 2013, 88, 701–709. [Google Scholar] [CrossRef]

[34] Al-Taweel, S.; Al Amrani, H.; Alrawi, T. Induction and Flow Cytometry, Gc-Ms Identification of Tetraploids through Colchicine Treatments in *Datura stramonium* L. Medical Plant Breeding View Project Improving of Broad Bean Production View Project Induction and Flow Cytometry, Gc-Ms Identification of Tetraploids through Colchicine Treatments in *Datura stramonium* L. Plant Arch. 2019, 19, 972–5210. [Google Scholar]

[35] Madani, H.; Hosseini, B.; Karimzadeh, G.; Rahimi, A. Enhanced Thebaine and Noscapine Production and Modulated Gene Expression of Tyrosine/Dopa Decarboxylase and Salutaridinol 7-O-Acetyltransferase Genes in Induced Autotetraploid Seedlings of *Papaver bracteatum* Lindl. Acta Physiol. Plant. 2019, 41, 194. [Google Scholar] [CrossRef]

[36] Chen, E.G.; Tsai, K.-L.; Chung, H.-H.; Chen, J.-T. Chromosome Doubling-Enhanced Biomass and Dihydrotanshinone I Production in *Salvia miltiorrhiza*, A Traditional Chinese Medicinal Plant. Molecules 2018, 23, 3106. [Google Scholar] [CrossRef] [PubMed]

[37] Hamarashid, S.H.; Khaledian, Y.; Soleimani, F. *In Vitro* Polyploidy-Mediated Enhancement of Secondary Metabolites Content in *Stachys byzantina* L. Genet. Resour. Crop Evol. 2022, 69, 719–728. [Google Scholar] [CrossRef]

[38] Sadat Noori, S.A.; Norouzi, M.; Karimzadeh, G.; Shirkool, K.; Niazian, M. Effect of Colchicine-Induced Polyploidy on Morphological Characteristics and Essential Oil Composition of Ajowan (*Trachyspermum ammi* L.). Plant Cell Tissue Organ. Cult. 2017, 130, 543–551. [Google Scholar] [CrossRef]

[39] Navrátilová, B.; Švécarová, M.; Bednář, J.; Ondřej, V. *In Vitro* Polyploidization of *Thymus vulgaris* L. and Its Effect on Composition of Essential Oils. Agronomy 2021, 11, 596. [Google Scholar] [CrossRef]

[40] Bhuvaneswari, G.; Thirugnanasampandan, R.; Gogulramnath, M. Effect of Colchicine Induced Tetraploidy on Morphology, Cytology, Essential Oil Composition, Gene Expression and Antioxidant Activity of *Citrus limon* (L.) Osbeck. Physiol. Mol. Biol. Plants 2020, 26, 271–279. [Google Scholar] [CrossRef]

[41] Mei, B.; Xie, H.; Xing, H.; Kong, D.; Pan, X.; Li, Y.; Wu, H. Changes of Phenolic Acids and Antioxidant Activities in Diploid and Tetraploid *Echinacea purpurea* at Different Growth Stages. Rev. Bras. Farmacogn. 2020, 30, 510–518. [Google Scholar] [CrossRef]

[42] Pansuksan, K.; Sangthong, R.; Nakamura, I.; Mii, M.; Supaibulwatana, K. Tetraploid Induction of *Mitracarpus hirtus* L. by Colchicine and Its Characterization Including Antibacterial Activity. Plant Cell Tissue Organ. Cult. 2014, 117, 381–391. [Google Scholar] [CrossRef]

[43] Dixit, V.; Chaudhary, B.R. Colchicine-Induced Tetraploidy in Garlic (*Allium sativum* L.) and Its Effect on Allicin Concentration. J. Hortic. Sci. Biotechnol. 2014, 89, 585–591. [Google Scholar] [CrossRef]

[44] Lin, X.; Zhou, Y.; Zhang, J.; Lu, X.; Zhang, F.; Shen, Q.; Wu, S.; Chen, Y.; Wang, T.; Tang, K. Enhancement of Artemisinin Content in Tetraploid *Artemisia annua* Plants by Modulating the Expression of Genes in Artemisinin Biosynthetic Pathway. Biotechnol. Appl. Biochem. 2011, 58, 50–57. [Google Scholar] [CrossRef] [PubMed]

[45] Inthima, P.; Sujipuli, K. Improvement of Growth and Bacoside Production in *Bacopa monnieri* through Induced Autotetraploidy with Colchicine. PeerJ 2019, 7, e7966. [Google Scholar] [CrossRef] [PubMed]

[46] Parsons, J.L.; Martin, S.L.; James, T.; Golenia, G.; Boudko, E.A.; Hepworth, S.R. Polyploidization for the Genetic Improvement of *Cannabis sativa*. Front. Plant Sci. 2019, 10, 476. [Google Scholar] [CrossRef] [PubMed]

[47] Zahedi, A.A.; Hosseini, B.; Fattahi, M.; Dehghan, E.; Parastar, H.; Madani, H. Overproduction of Valuable Methoxylated Flavones in Induced Tetraploid Plants of *Dracocephalum kotschyi* Boiss. Bot. Stud. 2014, 55, 22. [Google Scholar] [CrossRef] [PubMed]

[48] Xu, C.; Tang, T.; Chen, R.; Liang, C.; Liu, X.; Wu, C.; Yang, Y.; Yang, D.; Wu, H. A Comparative Study of Bioactive Secondary Metabolite Production in Diploid and

Tetraploid *Echinacea purpurea* (L.) Moench. Plant Cell 2014, 116, 323–332. [Google Scholar] [CrossRef]

[49] Dixit, V.; Verma, S.; Chaudhary, B.R. Changes in Ploidy and Its Effect on Thymoquinone Concentrations in *Nigella sativa* L. Seeds. J. Hortic. Sci. Biotechnol. 2015, 90, 537–542. [Google Scholar] [CrossRef]

[50] Yadav, A.; Singh, S.; Yadav, S.; Dhyani, D.; Bhardwaj, G.; Sharma, A.; Singh, B. Induction and Morpho-Chemical Characterization of *Stevia rebaudiana* Colchiploids. Indian. J. Agric. Sci. 2013, 83, 159–169. [Google Scholar]

[51] Escrich, A.; Hidalgo, D.; Bonfill, M.; Palazon, J.; Sanchez-Muñoz, R.; Moyano, E. Polyploidy as a Strategy to Increase Taxane Production in Yew Cell Cultures: Obtaining and Characterizing a Taxus baccata Tetraploid Cell Line. Plant Sci. 2023, 334, 111776. [Google Scholar] [CrossRef]

[52] Hannweg, K.; Visser, G.; de Jager, K.; Bertling, I. *In Vitro*-Induced Polyploidy and Its Effect on Horticultural Characteristics, Essential Oil Composition and Bioactivity of *Tetradenia riparia*. S. Afr. J. Bot. 2016, 106, 186–191. [Google Scholar] [CrossRef]

[53] Tavan, M.; Mirjalili, M.H.; Karimzadeh, G. *In Vitro* Polyploidy Induction: Changes in Morphological, Anatomical and Phytochemical Characteristics of *Thymus persicus* (Lamiaceae). Plant Cell Tissue Organ. Cult. 2015, 122, 573–583. [Google Scholar] [CrossRef]

[54] Madani, H.; Escrich, A.; Hosseini, B.; Sanchez-Muñoz, R.; Khojasteh, A.; Palazon, J. Effect of Polyploidy Induction on Natural Metabolite Production in Medicinal Plants. Biomolecules 2021, 11, 899. [Google Scholar] [CrossRef]

[55] Scarrow, M.; Wang, Y.; Sun, G. Molecular Regulatory Mechanisms Underlying the Adaptability of Polyploid Plants. Biol. Rev. 2021, 96, 394–407. [Google Scholar] [CrossRef]

[56] Segraves, K.A. The Effects of Genome Duplications in a Community Context. New Phytol. 2017, 215, 57–69. [Google Scholar] [CrossRef]

[57] Zhang, Z.; Fu, T.; Liu, Z.; Wang, X.; Xun, H.; Li, G.; Ding, B.; Dong, Y.; Lin, X.; Sanguinet, K.A.; et al. Extensive Changes in Gene Expression and Alternative Splicing Due

to Homoeologous Exchange in Rice Segmental Allopolyploids. Theor. Appl. Genet. 2019, 132, 2295–2308. [Google Scholar] [CrossRef]

[58] Dong, B.; Wang, H.; Song, A.; Liu, T.; Chen, Y.; Fang, W.; Chen, S.; Chen, F.; Guan, Z.; Jiang, J. miRNAs Are Involved in Determining the Improved Vigor of Autotetrapoid *Chrysanthemum nankingense*. Front. Plant Sci. 2016, 7, 1412. [Google Scholar] [CrossRef]

[59] Wang, R.; Liu, H.; Liu, Z.; Zou, J.; Meng, J.; Wang, J. Genome-Wide Analysis of Alternative Splicing Divergences between *Brassica* Hexaploid and Its Parents. Planta 2019, 250, 603–628. [Google Scholar] [CrossRef]

[60] Song, Q.; Chen, Z.J. Epigenetic and Developmental Regulation in Plant Polyploids. Curr. Opin. Plant Biol. 2015, 24, 101–109. [Google Scholar] [CrossRef]

[61] Peal, L.; Jambunathan, N.; Mahalingam, R. Phylogenetic and Expression Analysis of RNA-Binding Proteins with Triple RNA Recognition Motifs in Plants. Mol. Cells 2011, 31, 55–64. [Google Scholar] [CrossRef]

[62] Jackson, S.A. Epigenomics: Dissecting Hybridization and Polyploidization. Genome Biol. 2017, 18, 117. [Google Scholar] [CrossRef]

[63] Liang, Z.; Geng, Y.; Gu, X. Adenine Methylation: New Epigenetic Marker of DNA and mRNA. Mol. Plant 2018, 11, 1219–1221. [Google Scholar] [CrossRef]

[64] Koefoed Petersen, K.; Hagberg, P.; Kristiansen, K. Colchicine and Oryzalin Mediated Chromosome Doubling in Different Genotypes of *Miscanthus sinensis*. Plant Cell Tissue Organ. Cult. 2003, 73, 137–146. [Google Scholar] [CrossRef]

[65] Álvarez-Lugo, A.; Becerra, A. The Role of Gene Duplication in the Divergence of Enzyme Function: A Comparative Approach. Front. Genet. 2021, 12, 641817. [Google Scholar] [CrossRef]

[66] Lavania, U.C.; Srivastava, S.; Lavania, S.; Basu, S.; Misra, N.K.; Mukai, Y. Autopolyploidy Differentially Influences Body Size in Plants, but Facilitates Enhanced Accumulation of Secondary Metabolites, Causing Increased Cytosine Methylation. Plant J. 2012, 71, 539–549. [Google Scholar] [CrossRef]

[67] Lavania, U. Genomic and Ploidy Manipulation for Enhanced Production of Phyto-Pharmaceuticals. Plant Genet. Resour. 2005, 3, 170–177. [Google Scholar] [CrossRef]

[68] Woodhouse, M.R.; Cheng, F.; Pires, J.C.; Lisch, D.; Freeling, M.; Wang, X. Origin, Inheritance, and Gene Regulatory Consequences of Genome Dominance in Polyploids. Proc. Natl. Acad. Sci. USA 2014, 111, 5283–5288. [Google Scholar] [CrossRef]

[69] Comai, L. The Advantages and Disadvantages of Being Polyploid. Nat. Rev. Genet. 2005, 6, 836–846. [Google Scholar] [CrossRef]

[70] Dar, T.-U.-H.; Rehman, R.-U. Polyploidy in Changing Environment. In Polyploidy: Recent Trends and Future Perspectives; Dar, T.-U.-H., Rehman, R.-U., Eds.; Springer: New Delhi, India, 2017; pp. 89–99. ISBN 978-81-322-3772-3. [Google Scholar]

[71] Hassanzadeh, F.; Asghari-Zakaria, R.; Hosseinpour Azad, N. Polyploidy Induction in *Salvia officinalis* L. and Its Effects on Some Morphological and Physiological Characteristics. Cytologia 2020, 85, 157–162. [Google Scholar] [CrossRef]

[72] Javadian, N.; Karimzadeh, G.; Sharifi, M.; Moieni, A.; Behmanesh, M. *In Vitro* Polyploidy Induction: Changes in Morphology, Podophyllotoxin Biosynthesis, and Expression of the Related Genes in *Linum album* (Linaceae). Planta 2017, 245, 1165–1178. [Google Scholar] [CrossRef]

[73] Molin, W.T.; Meyers, S.P.; Baer, G.R.; Schrader, L.E. Ploidy Effects in Isogenic Populations of Alfalfa 1: II. Photosynthesis, Chloroplast Number, Ribulose-1,5-Bisphosphate Carboxylase, Chlorophyll, and DNA in Protoplasts. Plant Physiol. 1982, 70, 1710–1714. [Google Scholar] [CrossRef]

[74] Talebi, S.F.; Saharkhiz, M.J.; Kermani, M.J.; Sharafi, Y.; Raouf Fard, F. Effect of Different Antimitotic Agents on Polyploid Induction of Anise Hyssop (*Agastache foeniculum* L.). Caryologia 2017, 70, 184–193. [Google Scholar] [CrossRef]

[75] Yildiz, M. Plant Responses at Different Ploidy Levels. In Current Progress in Biological Research; IntechOpen: Rijeka, Croatia, 2013; ISBN 978-953-51-1097-2. [Google Scholar]

[76] Selwal, N.; Rahayu, F.; Herwati, A.; Latifah, E.; Supriyono, S.; Suhara, C.; Suastika, I.; Makarti, W.; Wani, A. Enhancing Secondary Metabolite Production in Plants: Exploring Traditional and Modern Strategies. J. Agric. Food Res. 2023, 14, 100702. [Google Scholar] [CrossRef]

[77] Gantait, S.; Mukherjee, E. Induced Autopolyploidy—A Promising Approach for Enhanced Biosynthesis of Plant Secondary Metabolites: An Insight. J. Genet. Eng. Biotechnol. 2021, 19, 4. [Google Scholar] [CrossRef]

[78] Xing, S.-H.; Guo, X.-B.; Wang, Q.; Pan, Q.-F.; Tian, Y.-S.; Liu, P.; Zhao, J.-Y.; Wang, G.-F.; Sun, X.-F.; Tang, K.-X. Induction and Flow Cytometry Identification of Tetraploids from Seed-Derived Explants through Colchicine Treatments in *Catharanthus roseus* (L.) G. Don. J. Biomed. Biotechnol. 2011, 2011, 793198. [Google Scholar] [CrossRef]

[79] Rutnik, K.; Ocvirk, M.; Košir, I.J. Changes in Hop (*Humulus lupulus* L.) Oil Content and Composition during Long-Term Storage under Different Conditions. Foods 2022, 11, 3089. [Google Scholar] [CrossRef] [PubMed]

[80] Kaensaksiri, T.; Soontornchainaksaeng, P.; Soonthornchareonnon, N.; Prathanturarug, S. *In Vitro* Induction of Polyploidy in *Centella asiatica* (L.) Urban. Plant Cell Tissue Organ Cult. (PCTOC) 2011, 2, 187–194. [Google Scholar] [CrossRef]

[81] Mishra, B.K.; Pathak, S.; Sharma, A.; Trivedi, P.K.; Shukla, S. Modulated Gene Expression in Newly Synthesized Auto-Tetraploid of *Papaver somniferum* L. S. Afr. J. Bot. 2010, 76, 447–452. [Google Scholar] [CrossRef]

[82] Venegas-Molina, J.; Molina-Hidalgo, F.J.; Clicque, E.; Goossens, A. Why and How to Dig into Plant Metabolite–Protein Interactions. Trends Plant Sci. 2021, 26, 472–483. [Google Scholar] [CrossRef] [PubMed]

[83] Matsuda, R.; Bi, C.; Anguizola, J.; Sobansky, M.; Rodriguez, E.; Vargas Badilla, J.; Zheng, X.; Hage, B.; Hage, D.S. Studies of Metabolite–Protein Interactions: A Review. J. Chromatogr. B 2014, 966, 48–58. [Google Scholar] [CrossRef]

[84] Sattler, M.C.; Carvalho, C.R.; Clarindo, W.R. The Polyploidy and Its Key Role in Plant Breeding. Planta 2016, 243, 281–296. [Google Scholar] [CrossRef]

[85] Tsuro, M.; Kondo, N.; Noda, M.; Ota, K.; Nakao, Y.; Asada, S. *In Vitro* Induction of Autotetraploid of Roman Chamomile (*Chamaemelum nobile* L.) by Colchicine Treatment and Essential Oil Productivity of Its Capitulum. Vitr. Cell. Dev. Biol.-Plant 2016, 52, 479–483. [Google Scholar] [CrossRef]

[86] Gomes, A.F.; Ganzera, M.; Schwaiger, S.; Stuppner, H.; Halabalaki, M.; Almeida, M.P.; Leite, M.F.; Amaral, J.G.; David, J.M. Simultaneous Determination of Iridoids,

Phenylpropanoids and Flavonoids in *Lippia alba* Extracts by Micellar Electrokinetic Capillary Chromatography. Microchem. J. 2018, 138, 494–500. [Google Scholar] [CrossRef]

[87] Ruiz, M.; Oustric, J.; Santini, J.; Morillon, R. Synthetic Polyploidy in Grafted Crops. Front. Plant Sci. 2020, 11, 540894. [Google Scholar] [CrossRef] [PubMed]

[88] Bharati, R.; Sen, M.K.; Severová, L.; Svoboda, R.; Fernández-Cusimamani, E. Polyploidization and Genomic Selection Integration for Grapevine Breeding: A Perspective. Front. Plant Sci. 2023, 14, 1248978. [Google Scholar] [CrossRef] [PubMed]

7. Discussion

MOPs serve as vital reservoirs of bioactive compounds with well-established relevance in pharmaceutical, nutraceutical, and cosmetic applications. However, a critical knowledge gap remains in understanding the molecular mechanisms governing phytochemical–biotarget interactions and their associated biological activities. Additionally, many of them exhibit inherently low phytochemical yield and limited bioactivity, which, when coupled with escalating global demand, highlights the urgent need for innovative approaches. AP emerges as a promising strategy to enhance the phytochemical richness and biological efficacy of MOPs, thereby addressing key constraints in metabolite production and therapeutic performance (Gupta et al., 2024). Inducing polyploidy in plants through synthetic antimitotic agents can enhance desirable traits, particularly by boosting SM levels and EO yields in MOPs. Despite this potential, outcomes are not always predictable, as gene duplication can lead to variable or even reduced SM production in polyploid genotypes compared to their diploid counterparts (Xie et al., 2012). Therefore, a deeper understanding of how polyploidy affects the phytochemical composition and biological activity of plant extracts is crucial (Gupta et al., 2024; Gupta et al., 2025). Therefore, this doctoral thesis characterizes the effectiveness of AP specially on two MOP species such as *T. vulgaris* and *C. fragrans*, focusing on their phytochemical profile and biological activities. The thesis comprises three distinct, peer-reviewed scientific studies, each contributing to a comprehensive understanding of the main objectives of this study.

In the first study, Gupta et al. (2024) demonstrated that AP substantially improved both yield and *in vitro* biological efficacy of *T. vulgaris* EO, with the tetraploid genotype producing 1.2% EO, thereby, representing a 41.11% increase over the diploid control (0.85%), which is consistent with earlier observations in *T. vulgaris* (Shmeit et al., 2020; Navrátilová et al., 2021) and other Lamiaceae species (Bharati et al., 2023; Hannweg et al., 2016). However, contrasting reports in *H. lupulus* (Trojak-Goluch et al., 2013) reflect species-specific metabolic responses to genome duplication. GC-MS profiling revealed elevated levels of thymol and γ-terpinene and reduced *p*-cymene in the tetraploid, a pattern also documented by Shmeit et al. (2020) and Navratilova et al. (2022), with these compositional shifts directly correlating to enhanced *in vitro* antimicrobial, antioxidant, and anti-inflammatory activities due to the well-established bio-efficacy of thymol and *γ*-terpinene (Kazemi et al., 2014; Kazemi et al., 2014; Diniz do Nascimento et al., 2020; Nikolić et al., 2014).

Functionally, the tetraploid EO outperformed the diploid in antimicrobial assays against *H. influenzae, S. aureus, S. pneumoniae,* and *S. pyogenes*, particularly in the liquid phase. This marks the first such report for induced polyploid *T. vulgaris*, and molecular docking confirmed thymol's superior binding to DNA gyrase, thereby validating its mechanistic role. Similarly, the tetraploid EO exhibited significantly higher DPPH radical scavenging activity, which can be attributed to the increased monoterpenoid content, primarily thymol, as it readily donates hydrogen atoms and neutralizes free radicals. This finding is further supported by docking data showing stronger thymol binding to human CDK2 than ascorbic acid. Anti-inflammatory efficacy, though moderately improved, also reflected the phytochemical enrichment, particularly in thymol, which is a known COX inhibitor (Marsik et al., 2005). Its docking affinity for COX-2 supports its role in attenuating prostaglandin biosynthesis. However, the complexity of EO synergy and contrary findings in species such as *G. pentaphyllum*, where diploids showed stronger anti-inflammatory gene suppression (Xie et al., 2012), underscore the genotype-dependent nature of polyploid responses, as we also described earlier. Overall, the interrelated enhancements in EO yield, phytochemical composition, and multi-target bioactivity in the tetraploid *T. vulgaris* affirm AP as a robust biotechnological strategy for maximizing the therapeutic and commercial potential of aromatic medicinal plants.

Similarly, in the second study conducted on *C. fragrans* by Gupta et al. (2025), oryzalin-induced polyploidy resulted in significantly enhanced SM yield and *in vitro* bioactivity compared to diploids, corroborating the improvements observed in our first study on *T. vulgaris* (Gupta et al., 2024). Methanolic extraction yielded 12.88% in polyploids versus 8.50% in diploids, reflecting an expanded metabolite profile encompassing both polar and non-polar compounds (Santos-Buelga et al., 2012). GC-FID identified ethyl stearate as the predominant fatty acid ester, markedly elevated in polyploids, consistent with its known antimicrobial and antioxidant properties (Zayed et al., 2014; Kim et al., 2020). The presence of (*Z*)-11-eicosenoic acid and dodecan-1-ol exclusively in polyploids suggests the emergence of novel bioactive constituents unique to the polyploid genotype (Togashi et al., 2007; Al-Rajhi et al., 2023). NMR analysis revealed increased levels of fumaric, gallic, and malic acids, which are known for their antimicrobial, antioxidant, and anti-inflammatory properties (Unver et al., 2024; Kakkaew et al., 2020; Kaur et al., 2020; Kweon et al., 2001; Bajko et al., 2016; Karpiński & Ożarowski, 2024; Cai et al., 2022), along with higher sucrose content, indicating enhanced metabolic activity. While total phenolic content doubled in polyploids, total flavonoid levels remained comparable, reflecting a complex modulation of phytochemical classes. In line with

these findings, the study reported higher antioxidant and antimicrobial activities in the polyploid extract, particularly against bacteria responsible for skin infections. This enhanced bioactivity is directly linked to the elevated secondary metabolite levels identified in the phytochemical analysis.

Furthermore, gene expression analysis showed that the polyploid extract significantly downregulated *COX* gene expression compared to the diploid extract, indicating stronger anti-inflammatory potential. The increased levels of fumaric acid, gallic acid, and malic acid in the polyploid extract likely contribute to this effect, as these compounds are well-known for their anti-inflammatory properties (Vijeesh et al., 2022; Lee et al., 2011; Kaur et al., 2020; Cai et al., 2022). These findings suggest that the polyploid extract may exert its anti-inflammatory effects via COX inhibition, modulating inflammatory responses at the molecular level. Finally, cytotoxicity assays defined the toxic concentration range of the extracts; notably, the biologically active dose identified in this study fell well below the toxicity threshold. This indicates a clear therapeutic window and supports the potential application of the polyploid extract for treating inflammation-related skin infections. Collectively, these results demonstrate that SP enhances the phytochemical complexity and pharmacological potential of *C. fragrans*, making it a promising candidate for advanced pharmaceutical development.

Beyond empirical data, a comprehensive mini-review by Gupta et al. (2024) reinforced the potential of *in vitro* synthetic polyploidization to enhance secondary metabolite production and biological activity in MAPs. This review highlighted plethora of studies based on how antimitotic agents like colchicine and oryzalin resulted in increased yields of thymol, artemisinin, andrographolide, carvone, and geranial on *T. vulgaris*, *A. annua*, *A. paniculata*, *M. spicata*, and *M. officinalis*, respectively. These chemical enhancements were often accompanied by elevated antibacterial, antioxidant, and anti-inflammatory activity (Gupta et al., 2024). Mechanistically, gene duplication, transcriptomic reprogramming, and epigenetic modifications (e.g., DNA methylation, chromatin remodeling) were implicated in upregulating biosynthetic pathways, was also depicted via this review. Furthermore, this review also suggest combining polyploidy with genomic selection and multi-omics tools to improve predictability and stability of desired traits. Overall, Enhanced phytochemical content and biological activity leads to better therapeutic profiles.

Collectively, this thesis advances the frontiers of plant biotechnology by demonstrating the dual impact of AP and PE bioactivity studies. The findings contribute to ongoing efforts in metabolic phytochemical enhancement, providing insights for optimizing the pharmaceutical and therapeutic potential of SP-induced MOPs. Through increased SM production, improved bioactivity, and greater phytochemical diversity, polyploidization emerges as a transformative approach with scalable applications across the pharmaceutical, nutraceutical, and agricultural sectors. However, future research should focus on validating the *in vivo* efficacy and safety of polyploid-derived extracts, and assessing the commercial viability of large-scale cultivation.

8. Conclusion and future perspectives

This dissertation establishes how enhanced SM biosynthesis, phytochemical diversity, and biological activities in MOPs result from AP. Through three interrelated studies, this research not only provides compelling evidence that genome duplication enhances bioactive compound production, thereby improving pharmacological efficacy and industrial applicability, but also elucidates the relationship between phytochemicals and essential biological target proteins.

Study 1 demonstrated that AP in *T. vulgaris* significantly increased EO yield, with elevated phytochemical levels correlating with enhanced *in vitro* antimicrobial, anti-inflammatory, and antioxidant activities. Furthermore, molecular interaction analyses *via in silico* molecular docking and MD simulations confirmed strong binding affinities between these phytochemicals and biological target proteins, explaining their improved bioactivity. **Study 2** extended these insights to *C. fragrans*, where AP led to substantial improvements in polyphenolic and fatty acid compositions. These metabolic shifts translated into superior *in vitro* antimicrobial, antioxidant, anti-inflammatory, and cell viability effects, reinforcing the pharmaceutical and nutraceutical potential of polyploid PEs. **Study 3** provided a comprehensive review on the various mechanisms involved in AP to enhance phytochemical profiles in MAPs, focusing on improved SM production and enhanced bioactivity.

In conclusion, this doctoral thesis successfully addressed all the proposed hypotheses and achieved its designated objectives. Beyond its scientific contributions, AP holds far-reaching implications across multiple industries. In the pharmaceutical sector, AP-induced MOPs offer a sustainable source for synthesizing enhanced bioactive compounds, particularly for antimicrobial, anti-inflammatory, and antioxidant therapies. In the nutraceutical industry, polyploid PEs can be formulated into functional foods and dietary supplements with superior health benefits. Within the food industry, improved PE profiles can serve as natural flavoring agents and preservatives, reducing dependence on synthetic additives while enhancing product stability and shelf life. Despite these promising applications, future research must prioritize the large-scale validation of polyploid-derived bioactive components. Although our *in vitro* results demonstrate significant antibacterial, antioxidant, and anti-inflammatory effects, further investigations especially preclinical and *in vivo* studies, broader taxonomic assessments, and large-scale industrial validations are necessary to confirm these findings and to evaluate their potential for clinical application. Besides, the economic feasibility and regulatory considerations surrounding artificial polyploidization warrant thorough investigation in future

applied research to facilitate its practical implementation across pharmaceutical, nutraceutical, and agricultural industries

9. References and Bibliography

1. Acanda, Y., Martínez, Ó., Prado, M. J., González, M. V., & Rey, M. (2020). Changes in abscisic acid metabolism in relation to the maturation of grapevine (*Vitis vinifera* L., cv. Mencía) somatic embryos. BMC Plant Biology, 20, 1-16.
2. Ahmar, S., Gill, R. A., Jung, K. H., Faheem, A., Qasim, M. U., Mubeen, M., & Zhou, W. (2020). Conventional and molecular techniques from simple breeding to speed breeding in crop plants: recent advances and future outlook. International journal of molecular sciences, 21(7), 2590.
3. Ahmed, A. K. M., Beshr, E. A., Salem, I. M., Ahmed, O. A., Ibrahim, T. S., Abouzed, D. E. E., ... & Mohamed, M. F. (2025). Novel chalcone candidates as potential *in vitro* and *in vivo* anti-inflammatory agents: Synthesis, in-silico docking, multitarget bio evaluation and molecular dynamic simulation. *Bioorganic Chemistry*, 108540.
4. Al-Rajhi, A.M.H. & Abdel Ghany, T.M. Nanoemulsions of some edible oils and their antimicrobial, antioxidant, and anti-hemolytic activities. *BioResources* **18**, 1465–1481 (2023). https://doi.org/10.15376/biores.18.1.1465-1481
5. Al-Taweel, S. K., Al-Amrani, H. A., & Al-Rawi, T. K. (2019). Induction and flow cytometry, GC-MS identification of tetraploids through colchicine treatments in Datura stramonium L. *Plant Arch*, *19*(1), 241-250.
6. Anand, S., Pang, E., Livanos, G., & Mantri, N. (2018). Characterization of physico-chemical properties and antioxidant capacities of bioactive honey produced from Australian grown *Agastache rugosa* and its correlation with colour and poly-phenol content. *Molecules*, *23*(1), 108.
7. Anbarasan, K., Rajendran, R., Sivalingam, D., & Chidambaram, A. C. (2014). Studies on the effect of EMS and colchicine in M1 generation of sesame (*Sesamum indicum* L.) var. TMV3. International Letters of Natural Sciences, 11(2).
8. Azmir, J., Zaidul, I. S. M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., ... & Omar, A. K. (2013). Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of food engineering*, *117*(4), 426-436.
9. Bajko, E. *et al.* 5-O-Caffeoylquinic acid: A spectroscopic study and biological screening for antimicrobial activity. *LWT* **65**, 471–479 (2016). https://doi.org/10.1016/j.lwt.2015.08.024
10. Begna, T. (2022). Speed breeding to accelerate crop improvement. International Journal of Agricultural Science and Food Technology, 8(2), 178-186.
11. Beranová, K., Bharati, R., Žiarovská, J., Bilčíková, J., Hamouzová, K., Klíma, M., & Fernández-Cusimamani, E. (2022). Morphological, cytological, and molecular comparison

between diploid and induced autotetraploids of *Callisia fragrans* (Lindl.) woodson. *Agronomy*, *12*(10), 2520.

12. Bharati, R., Fernández-Cusimamani, E., Gupta, A., Novy, P., Moses, O., Severová, L., ... & Šrédl, K. (2023). Oryzalin induces polyploids with superior morphology and increased levels of essential oil production in *Mentha spicata* L. *Industrial Crops and Products*, *198*, 116683.
13. Bharati, R., Gupta, A., Novy, P., Severová, L., Šrédl, K., Žiarovská, J., & Fernández-Cusimamani, E. (2023). Synthetic polyploid induction influences morphological, physiological, and photosynthetic characteristics in *Melissa officinalis* L. *Frontiers in Plant Science*, *14*, 1332428.
14. Bhattarai, K., Kareem, A., & Deng, Z. (2021). *In vivo* induction and characterization of polyploids in gerbera daisy. Scientia Horticulturae, 282, 110054.
15. Bhuvaneswari, G., Thirugnanasampandan, R., & Gogulramnath, M. (2020). Effect of colchicine induced tetraploidy on morphology, cytology, essential oil composition, gene expression and antioxidant activity of *Citrus limon* (L.) Osbeck. Physiology and Molecular Biology of Plants, 26, 271-279.
16. Bicknell, R., Boase, M., & Morgan, E. (2019). Developing polyploid genotypes of *Leptospermum scoparium*. *New Zealand Journal of Crop and Horticultural Science*, *47*(4), 273-281.
17. Bodalska, A., Kowalczyk, A., & Fecka, I. (2021). Quality of herbal medicinal products based on sage and thyme preparations. *Acta Poloniae Pharm.-Drug Res*, *78*, 539-561.
18. Boo, H. O., Kim, Y. S., Kim, H. H., Kwon, S. J., & Woo, S. H. (2015). Evaluation of cytotoxicity, antimicrobial and antioxidant enzyme activity of diploid and tetraploid *Platycodon grandiflorum*. *Korean Journal of Crop Science*, *60*(2), 239-247.
19. Cai, L. *et al.* Gallic acid mitigates LPS-induced inflammatory response via suppressing NF-κB signalling pathway in IPEC-J2 cells. *J. Anim. Physiol. Anim. Nutr.* **106**, 1000–1008 (2022). https://doi.org/10.1111/jpn.13698
20. Castillo, A. M., Cistué, L., Vallés, M. P., & Soriano, M. (2009). Chromosome doubling in monocots. Advances in haploid production in higher plants, 329-338.
21. Chaikam, V., Gowda, M., Nair, S. K., Melchinger, A. E., & Boddupalli, P. M. (2019). Genome-wide association study to identify genomic regions influencing spontaneous fertility in maize haploids. Euphytica, 215(8), 138.
22. Chrysargyris, A., Petrovic, J. D., Tomou, E. M., Kyriakou, K., Xylia, P., Kotsoni, A., ... & Tzortzakis, N. (2024). Phytochemical Profiles and Biological Activities of Plant Extracts from Aromatic Plants Cultivated in Cyprus. Biology, 13(1), 45.

23. Dhooghe, E., Van Laere, K., Eeckhaut, T., Leus, L., & Van Huylenbroeck, J. (2011). Mitotic chromosome doubling of plant tissues in vitro. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *104*(3), 359-373.
24. Diniz do Nascimento, L., Barbosa de Moraes, A. A., Santana da Costa, K., Pereira Galúcio, J. M., Taube, P. S., Leal Costa, C. M., ... & Guerreiro de Faria, L. J. (2020). Bioactive natural compounds and antioxidant activity of essential oils from spice plants: New findings and potential applications. *Biomolecules*, *10*(7), 988.
25. Du, Y., Cheng, F., & Zhong, Y. (2020). Induction of direct somatic embryogenesis and shoot organogenesis and histological study in tree peony (*Paeonia* sect. Moutan). Plant Cell, Tissue and Organ Culture (PCTOC), 141, 557-570.
26. Dubcovsky, J., & Dvorak, J. (2007). Genome plasticity a key factor in the success of polyploid wheat under domestication. Science, 316(5833), 1862-1866.
27. Eeckhaut, T., Van der Veken, J., Dhooghe, E., Leus, L., Van Laere, K., & Van Huylenbroeck, J. (2018). Ploidy breeding in ornamentals. Ornamental Crops, 145-173.
28. El Sohafy, S. M., Nassra, R. A., D'Urso, G., Piacente, S., & Sallam, S. M. (2021). Chemical profiling and biological screening with potential anti-inflammatory activity of *Callisia fragrans* grown in Egypt. *Natural Product Research*, *35*(23), 5521-5524.
29. Eng, W. H., & Ho, W. S. (2019). Polyploidization using colchicine in horticultural plants: A review. Scientia horticulturae, 246, 604-617.
30. Esmaeili, G., Van Laere, K., Muylle, H., & Leus, L. (2020). Artificial chromosome doubling in allotetraploid *Calendula officinalis*. *Frontiers in Plant Science*, *11*, 622.
31. Farhadi, N., Panahandeh, J., Motallebi-Azar, A., & Mokhtarzadeh, S. (2023). Production of autotetraploid plants by *in vitro* chromosome engineering in *Allium hirtifolium*. Horticultural Plant Journal, 9(5), 986-998.
32. Fatima, T., Mujib, A., Bansal, Y., Dewir, Y. H., & Mendler-Drienyovszki, N. (2024). Indirect Organogenesis of *Calendula officinalis* L. and Comparative Phytochemical Studies of Field-Grown and *In Vitro*-Regenerated Tissues. *Agronomy*, *14*(8), 1743.
33. Favela-González, K. M., Hernández-Almanza, A. Y., & De la Fuente-Salcido, N. M. (2020). The value of bioactive compounds of cruciferous vegetables (Brassica) as antimicrobials and antioxidants: A review. Journal of Food Biochemistry, 44(10), e13414.
34. Fomicheva, M., Kulakov, Y., Alyokhina, K., & Domblides, E. (2024). Spontaneous and Chemically Induced Genome Doubling and Polyploidization in Vegetable Crops. *Horticulturae*, *10*(6), 551.

35. Fu, L., Zhu, Y., Li, M., Wang, C., & Sun, H. (2019). Autopolyploid induction via somatic embryogenesis in *Lilium distichum* Nakai and *Lilium cernuum* Komar. Plant Cell, Tissue and Organ Culture (PCTOC), 139, 237-248.
36. Ghanbari, M. A., Jowkar, A., Salehi, H., & Zarei, M. (2019). Effects of polyploidization on petal characteristics and optical properties of *Impatiens walleriana* (Hook.). *Plant Cell, Tissue and Organ Culture (PCTOC)*, *138*, 299-310.
37. Ghorbanpour, M., Hadian, J., Nikabadi, S., & Varma, A. (2017). Importance of medicinal and aromatic plants in human life. *Medicinal plants and environmental challenges*, 1-23.
38. Ghotbi Ravandi, E., Rezanejad, F., & Dehghan, E. (2014). *In vitro* regeneration ability of diploid and autotetraploid plants of *Cichorium intybus* L. *Cytology and genetics*, *48*, 166-170.
39. Grosso, V., Farina, A., Giorgi, D., Nardi, L., Diretto, G., & Lucretti, S. (2018). A high-throughput flow cytometry system for early screening of *in vitro* made polyploids in Dendrobium hybrids. Plant Cell, Tissue and Organ Culture (PCTOC), 132, 57-70.
40. Gupta, N., Bhattacharya, S., Dutta, A., Cusimamani, E. F., Milella, L., & Leuner, O. (2024). *In Vitro* Synthetic Polyploidization in Medicinal and Aromatic Plants for Enhanced Phytochemical Efficacy—A Mini-Review. *Agronomy*, *14*(8).
41. Gupta, N., Bhattacharya, S., Dutta, A., Tauchen, J., Landa, P., Urbanová, K., ... & Leuner, O. (2024). Synthetic polyploidization induces enhanced phytochemical profile and biological activities in *Thymus vulgaris* L. essential oil. *Scientific Reports*, *14*(1), 5608.
42. Gupta, N., Bhattacharya, S., Urbanová, K., Dutta, A., Hazra, A. K., Fernández-Cusimamani, E., & Leuner, O. (2023). Systematic analysis of antimicrobial activity, phytochemistry, and in silico molecular interaction of selected essential oils and their formulations from different Indian spices against foodborne bacteria. *Heliyon*, *9*(12).
43. Gupta, N., Bhattacharya, S., Žiarovská, J., Dutta, A., Fialková, V., Farkasová, S., Bergo, A. M., Havlik, J., Tauchen, J., Novy, P., Urbanová, K., Milella, L., & Fernández-Cusimamani, E. (2025). Oryzalin-induced polyploidy enhances natural metabolite accumulation and functional bioactivities in novel *Callisia fragrans* genotype. *Scientific Reports, 15*, 25652. https://doi.org/10.1038/s41598-025-10588-9
44. Gupta, S., Tiwari, N., & Munde, M. (2019). A comprehensive biophysical analysis of the effect of DNA binding drugs on protamine-induced DNA condensation. Scientific reports, 9(1), 5891.
45. Hamarashid, S. H., Khaledian, Y., & Soleimani, F. (2021). *In vitro* polyploidy-mediated enhancement of secondary metabolites content in *Stachys byzantina* L. *Genetic Resources and Crop Evolution*, 1-10.

46. Hammoudi Halat, D., Krayem, M., Khaled, S., & Younes, S. (2022). A focused insight into thyme: Biological, chemical, and therapeutic properties of an indigenous Mediterranean herb. *Nutrients*, *14*(10), 2104.
47. Hannweg, K., Visser, G., De Jager, K., & Bertling, I. (2016). *In vitro*-induced polyploidy and its effect on horticultural characteristics, essential oil composition and bioactivity of *Tetradenia riparia*. South African Journal of Botany, 106, 186-191.
48. He, M., Gao, W., Gao, Y., Liu, Y., Yang, X., Jiao, H., & Zhou, Y. (2016). Polyploidy induced by colchicine in *Dendranthema indicum* var. aromaticum, a scented chrysanthemum. Eur. J. Hortic. Sci, 81(4), 219-226.
49. Hegarty, K., O'Doherty, L., Taft, A., Chondros, P., Brown, S., Valpied, J., ... & Gunn, J. (2013). Screening and counselling in the primary care setting for women who have experienced intimate partner violence (WEAVE): a cluster randomised controlled trial. The Lancet, 382(9888), 249-258.
50. Herawati, M. M., Wibisono, A. P., Setiawan, A. W., & Pudjihartati, E. (2024). The increase of steviol glycosides in stevia (*Stevia rebaudiana* Bertoni) through artificial polyploidy. In BIO Web of Conferences (Vol. 101, p. 01004). EDP Sciences.
51. Horváth, G., & Ács, K. (2015). Essential oils in the treatment of respiratory tract diseases highlighting their role in bacterial infections and their anti-inflammatory action: a review. *Flavour and Fragrance Journal*, *30*(5), 331-341.
52. Hossain, M. A., Alrashdi, Y. B. A., & Al Touby, S. (2022). A review on essential oil analyses and biological activities of the traditionally used medicinal plant *Thymus vulgaris* L. *International Journal of Secondary Metabolite*, *9*(1), 103-111.
53. Hosseinzadeh, S., Jafarikukhdan, A., Hosseini, A., & Armand, R. (2015). The application of medicinal plants in traditional and modern medicine: a review of *Thymus vulgaris*. *International Journal of Clinical Medicine*, *6*(9), 635-642.
54. Huang, X., Ouyang, K., Luo, Y., Xie, G., Yang, Y., & Zhang, J. (2022). A comparative study of characteristics in diploid and tetraploid *Anoectochilus roxburghii*. *Frontiers in nutrition*, *9*, 1034751.
55. Huynh, D. T. M., Le, M. N. T., De Tran, V., Tran, V. H., & Pham, D. T. (2023). Native Medicinal Plants (*Moringa oleifera* Lam, *Brucea javanica* (L.) Merr., *Eclipta prostrata* (L.), *Callisia fragrans* (Lindl.) Woodson, and *Zingiber zerumbet* (L.) Smith) in An Giang, Vietnam: A Preliminary Investigation for Rhabdomyosarcoma Treatments using *in vitro* RD cell cytotoxicity test. *Jordan Journal of Pharmaceutical Sciences*, *16*(4), 830-841.

56. Iftikhar, T., Majeed, H., Zahra, S. S., Waheed, M., Niaz, M., & Bano, N. (2023). Thyme. In *Essentials of Medicinal and Aromatic Crops* (pp. 399-429). Cham: Springer International Publishing.
57. Jafarkhani Kermani, M., & Emadpour, M. (2019). Application of polyploidization in breeding of ornamental plants. Flower and Ornamental Plants, 3(2), 77-89.
58. Jaouadi, R., Boussaid, M. & Zaouali, Y. Variation in essential oil composition within and among Tunisian *Thymus algeriensis* Boiss et Reut (Lamiaceae) populations: Effect of ecological factors and incidence on antiacetylcholinesterase and antioxidant activities. Biochem. Syst. Ecol. 106, 104543 (2023).
59. Jarić, S., Mačukanović-Jocić, M., Djurdjević, L., Mitrović, M., Kostić, O., Karadžić, B., & Pavlović, P. (2015). An ethnobotanical survey of traditionally used plants on Suva planina mountain (south-eastern Serbia). *Journal of ethnopharmacology*, *175*, 93-108.
60. Julião, S. A., Lopes, J. M., Zorzatto, C., Matos, E. M., & Viccini, L. F. (2020). Genome Status of *Lippia alba* Polyploid Complex Long-term *in vitro* Cultivated. *European Journal of Medicinal Plants*, *30*(4), 1-14.
61. Karpiński, T.M. & Ożarowski, M. Plant organic acids as natural inhibitors of foodborne pathogens. *Appl. Sci.* **14**, 6340 (2024). https://doi.org/10.3390/app14146340
62. Katoch, M., Verma, K., Sharma, D., Priti, & Bhargava, B. (2024). Ornamental Plant Breeding for Improved Floral Attributes: Entering a New Era. In Ornamental Horticulture: Latest Cultivation Practices and Breeding Technologies (pp. 45-62). Singapore: Springer Nature Singapore.
63. Kaur, G., Shivanandappa, T.B., Kumar, M. & Kushwah, A.S. Fumaric acid protects cadmium-induced hepatotoxicity in rats: Owing to its antioxidant, anti-inflammatory action and aid in recast the liver function. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **393**, 1911–1920 (2020). https://doi.org/10.1007/s00210-020-01900-7
64. Kazemi, M. (2014). Chemical composition, antimicrobial, antioxidant and anti-inflammatory activity of *Carum copticum* L. essential oil. *Journal of Essential Oil Bearing Plants*, *17*(5), 1040-1045.
65. Kazemi, M. (2014). Phytochemical composition, antioxidant, anti-inflammatory and antimicrobial activity of *Nigella sativa* L. essential oil. *Journal of Essential Oil Bearing Plants*, *17*(5), 1002-1011.
66. Keshtkar, A. H., Fallahi, N., Abdollahi, M. R., Sarikhani, H., Safari, H., & Mohseni Araghi, Z. (2019). Effect of colchicine on polyploidy induction and its effects on morphophysiological and biochemical properties of fenugreek (*Trigonella foenum-graecum*). *Journal Of Horticultural Science*, *33*(1), 127-139.

67. Khalili, S., Niazian, M., Arab, M., & Norouzi, M. (2020). *In vitro* chromosome doubling of African daisy, *Gerbera jamesonii* Bolus cv. Mini Red. The Nucleus, 63, 59-65.
68. Kim, B. R., Kim, H. M., Jin, C. H., Kang, S. Y., Kim, J. B., Jeon, Y. G., ... & Han, A. R. (2020). Composition and antioxidant activities of volatile organic compounds in radiation-bred Coreopsis cultivars. *Plants*, *9*(6), 717.
69. Kosakowska, O., Bączek, K., Przybył, J. L., Pawełczak, A., Rolewska, K., & Węglarz, Z. (2020). Morphological and chemical traits as quality determinants of common thyme (*Thymus vulgaris* L.), on the example of 'Standard Winter' cultivar. *Agronomy*, *10*(6), 909.
70. Kowalczyk, A., Przychodna, M., Sopata, S., Bodalska, A., & Fecka, I. (2020). Thymol and thyme essential oil—new insights into selected therapeutic applications. *Molecules*, *25*(18), 4125.
71. Kuebutornye, F. K. A., Roy, K., Folorunso, E. A., & Mraz, J. (2024). Plant-based feed additives in *Cyprinus carpio* aquaculture. Reviews in Aquaculture, 16(1), 309-336.
72. Kumar, M., Sirohi, U., Yadav, M. K., & Chaudhary, V. (2024). *In vitro* culture technology and advanced biotechnology tools toward improvement in gladiolus (Gladiolus species): Present scenario and future prospects. Molecular Biotechnology, 66(8), 1806-1835.
73. Kumar, S., Narwal, S., Kumar, V., & Prakash, O. (2011). α-glucosidase inhibitors from plants: A natural approach to treat diabetes. *Pharmacognosy reviews*, *5*(9), 19.
74. Kweon, D.K., Choi, J.K., Kim, E.K. & Lim, S.T. Adsorption of divalent metal ions by succinylated and oxidized corn starches. *Carbohydr. Polym.* **46**, 171–177 (2001). https://doi.org/10.1016/S0144-8617(00)00310-4
75. Lamlom, S. F., Yehia, W. M. B., Kotb, H. M. K., Abdelghany, A. M., Shah, A. N., Salama, E. A., ... & Abdelsalam, N. R. (2024). Genetic improvement of Egyptian cotton (*Gossypium barbadense* L.) for high yield and fiber quality properties under semi-arid conditions. Scientific Reports, 14(1), 7723.
76. Lan, M. O., Chen, J. H., Fei, C. H. E. N., Xu, Q. W., Tong, Z. K., Huang, H. H., ... & Lin, E. P. (2020). Induction and characterization of polyploids from seeds of *Rhododendron fortunei* Lindl. Journal of integrative agriculture, 19(8), 2016-2026.
77. Le, T., Cao, D. K., Pham, T. V., Huynh, T. D., Ta, N. T. A., Nguyen, N. T. L., ... & Truong, D. H. (2018, April). Changes in total phenol, flavonoid contents and anti-Lactobacillus activity of *Callisia fragrans* due to extraction solvent. In *AIP Conference Proceedings* (Vol. 1954, No. 1). AIP Publishing.
78. Le, X. T., Thi Nguyen, L. T., Nguyen, P. T., Nguyen, T. V., Nguyen, H. V., Nguyet Pham, H. T., ... & Matsumoto, K. (2022). Anti-hypertensive effects of *Callisia fragrans* extract on Reno-vascular hypertensive rats. *Clinical and Experimental Hypertension*, *44*(5), 411-418.

79. Lee, S. *et al.* Anti-inflammatory function of arctiin by inhibiting COX-2 expression via NF-κB pathways. *J. Inflamm.* **8**, 1–9 (2011). https://doi.org/10.1186/1476-9255-8-1.
80. Lee, Y. Y., Jeong, M. C., & Jang, H. W. (2017). Determination of volatile compounds by headspace-solid phase microextraction-gas chromatography/mass spectrometry: Quality evaluation of Fuji apple: Quality evaluation of Fuji apple. *분석과학*, *30*(2), 68-74.
81. Liang, C. C., Park, A. Y., & Guan, J. L. (2007). *In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature protocols*, *2*(2), 329-333.
82. Lin, X., Zhou, Y., Zhang, J., Lu, X., Zhang, F., Shen, Q., ... & Tang, K. (2011). Enhancement of artemisinin content in tetraploid *Artemisia annua* plants by modulating the expression of genes in artemisinin biosynthetic pathway. Biotechnology and applied biochemistry, 58(1), 50-57.
83. Ma, S., Sun, C., Su, W., Zhang, L., Zheng, J., Su, S., ... & Kong, L. (2023). Induced Autotetraploids in *Salvia miltiorrhiza* and Their Agronomic Traits. *Cytologia*, *88*(2), 127-136.
84. Madani, H., Escrich, A., Hosseini, B., Sanchez-Muñoz, R., Khojasteh, A., & Palazon, J. (2021). Effect of polyploidy induction on natural metabolite production in medicinal plants. *Biomolecules*, *11*(6), 899.
85. Madani, S. H., Hosseini, B., Karimzadeh, G., & Rahimi, A. (2019). Effects of polyploidy induction on antioxidant capacity and some phytochemical and morphological characteristics of Iranian poppy (*Papaver bracteatum* Lindl.). *Iranian Journal of Medicinal and Aromatic Plants Research*, *35*(2), 170-181.
86. Malik, S., Brudzyńska, P., Khan, M. R., Sytar, O., Makhzoum, A., & Sionkowska, A. (2023). Natural plant-derived compounds in food and cosmetics: A paradigm of shikonin and its derivatives. Materials, 16(12), 4377.
87. Maluszynski, M., Nichterlein, K., Van Zanten, L., & Ahloowalia, B. S. (2000). Officially released mutant varieties-the FAO/IAEA Database.
88. Mansinhos, I., Gonçalves, S., & Romano, A. (2024). Impact of abiotic stresses on *in vitro* production of secondary metabolites. *In Vitro* Propagation and Secondary Metabolite Production from Medicinal Plants: Current Trends (Part 1), 62-90.
89. Manzoor, A., Ahmad, T., Bashir, M. A., Hafiz, I. A., & Silvestri, C. (2019). Studies on colchicine induced chromosome doubling for enhancement of quality traits in ornamental plants. Plants, 8(7), 194.
90. Marsik, P. et al. *In vitro* Inhibitory Effects of Thymol and Quinones of *Nigella sativa* Seeds on Cyclooxygenase-1- and -2-Catalyzed Prostaglandin E2 Biosyntheses. Planta Med. 71, 739–742 (2005).

91. Mehalaine, S., & Chenchouni, H. (2021). Quantifying how climatic factors influence essential oil yield in wild-growing plants. *Arabian Journal of Geosciences*, *14*(13), 1257.
92. Mei, B., Xie, H., Xing, H., Kong, D., Pan, X., Li, Y., & Wu, H. (2020). Changes of phenolic acids and antioxidant activities in diploid and tetraploid *Echinacea purpurea* at different growth stages. *Revista Brasileira de Farmacognosia*, *30*, 510-518.
93. Miler, N., Tymoszuk, A., Rewers, M., & Kulus, D. (2023). *In Vitro* Regeneration of Chrysanthemum from Ovaries and Ovules Treated with Thermal and Chemical Stimuli: Morphogenic and Cytogenetic Effects. *Agriculture*, *13*(11), 2069.
94. Miri, S. M., & Roughani, A. (2018, October). *Allium* species growing in Iran: Chemical compositions and pharmacological activity. In Proceedings of the First National Congress and International Fair of Medicinal Plants and Strategies for Persian Medicine that Affect Diabetes, October (pp. 9-11).
95. Mohammadi, V., Talebi, S., Ahmadnasab, M., & Mollahassanzadeh, H. (2023). The effect of induced polyploidy on phytochemistry, cellular organelles and the expression of genes involved in thymol and carvacrol biosynthetic pathway in thyme (*Thymus vulgaris*). Frontiers in Plant Science, 14, 1228844.
96. Mopuri, R., & Islam, M. S. (2017). Medicinal plants and phytochemicals with anti-obesogenic potentials: A review. Biomedicine & Pharmacotherapy, 89, 1442-1452.
97. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, *65*(1-2), 55-63.
98. Munjal, Y., Tonk, R. K., & Sharma, R. (2022). Analytical techniques used in metabolomics: A review. *Systematic Reviews in Pharmacy*, *13*(5), 515-521.
99. Nahar, L., & Sarker, S. D. (2012). Supercritical fluid extraction in natural products analyses. In *Natural products isolation* (pp. 43-74). Totowa, NJ: Humana Press.
100. Najar, B., Pistelli, L., Ferri, B., Angelini, L. G., & Tavarini, S. (2021). Crop yield and essential oil composition of two *Thymus vulgaris* chemotypes along three years of organic cultivation in a hilly area of central Italy. *Molecules*, *26*(16), 5109.sale
101. Navrátilová, B., Ondřej, V., Vrchotová, N., Tříska, J., Horník, Š., & Pavela, R. (2022). Impact of Artificial Polyploidization in *Ajuga reptans* on Content of Selected Biologically Active Glycosides and Phytoecdysone. *Horticulturae*, *8*(7), 581.
102. Navrátilová, B., Švécarová, M., Bednář, J., & Ondřej, V. (2021). *In vitro* polyploidization of *Thymus vulgaris* L. and its effect on composition of essential oils. *Agronomy*, *11*(3), 596.
103. Niazian, M. & Nalousi, A. M. Artificial polyploidy induction for improvement of ornamental and medicinal plants. Plant Cell Tissue Organ. Cult. PCTOC 142, 447–469 (2020).

104. Niazian, M. (2019). Application of genetics and biotechnology for improving medicinal plants. Planta, 249, 953-973.

105. Niazian, M., & Shariatpanahi, M. E. (2020). *In vitro*-based doubled haploid production: recent improvements. Euphytica, 216(5), 69.

106. Niazian, M., Sadat Noori, S. A., Tohidfar, M., & Mortazavian, S. M. M. (2017). Essential oil yield and agro-morphological traits in some Iranian ecotypes of ajowan (*Carum copticum* L.). Journal of Essential Oil Bearing Plants, 20(4), 1151-1156.

107. Niazian, M., Sadat-Noori, S. A., Abdipour, M., Tohidfar, M., & Mortazavian, S. M. M. (2018). Image processing and artificial neural network-based models to measure and predict physical properties of embryogenic callus and number of somatic embryos in ajowan (*Trachyspermum ammi* (L.) Sprague). *In Vitro* Cellular & Developmental Biology-Plant, 54, 54-68.

108. Nikolić, M., Glamočlija, J., Ferreira, I. C., Calhelha, R. C., Fernandes, Â., Marković, T., ... & Soković, M. (2014). Chemical composition, antimicrobial, antioxidant and antitumor activity of *Thymus serpyllum* L., *Thymus algeriensis* Boiss. and Reut and *Thymus vulgaris* L. essential oils. *Industrial crops and products*, *52*, 183-190.

109. Noh, J., Sheikh, S., Chon, H. G., Seong, M. H., Lim, J. H., Lee, S. G., ... & Huh, Y. C. (2012). Screening different methods of tetraploid induction in watermelon [*Citrullus lanatus* (thunb.) Manst. and Nakai]. Horticulture, Environment, and Biotechnology, 53, 521-529.

110. Olennikov, D. N., Ibragimov, T. A., Zilfikarov, I. N., & Chelombit′ ko, V. A. (2008). Chemical composition of *Callisia fragrans* juice 1. Phenolic compounds. *Chemistry of natural compounds*, *44*, 776-777.

111. Orhan, I., Kartal, M., Naz, Q., Ejaz, A., Yilmaz, G., Kan, Y., ... & Choudhary, M. I. (2007). Antioxidant and anticholinesterase evaluation of selected Turkish Salvia species. *Food Chemistry*, *103*(4), 1247-1254.

112. Panchy, N., Lehti-Shiu, M., & Shiu, S. H. (2016). Evolution of gene duplication in plants. Plant physiology, 171(4), 2294-2316.

113. Pansuksan, K., Mii, M., & Supaibulwatana, K. (2014). Phytochemical alteration and new occurring compounds in hairy root cultures of *Mitracarpus hirtus* L. induced by phenylurea cytokinin (CPPU). *Plant Cell, Tissue and Organ Culture (PCTOC)*, *119*, 523-532.

114. Parida, B. P., & Misra, B. B. (2015). Is a plant's ploidy status reflected in its metabolome. Journal of Postdoctoral Research, 1(11).

115. Parsons, J. L., Martin, S. L., James, T., Golenia, G., Boudko, E. A., & Hepworth, S. R. (2019). Polyploidization for the genetic improvement of *Cannabis sativa*. *Frontiers in plant science*, *10*, 449166.

116. Patil, S. M., Ramu, R., Shirahatti, P. S., Shivamallu, C., & Amachawadi, R. G. (2021). A systematic review on ethnopharmacology, phytochemistry and pharmacological aspects of *Thymus vulgaris* Linn. *Heliyon*, *7*(5).

117. Pradhan, A., Plummer, J. A., Nelson, M. N., Cowling, W. A., & Yan, G. (2010). Successful induction of trigenomic hexaploid *Brassica* from a triploid hybrid of *B. napus* L. and *B. nigra* (L.) Koch. Euphytica, 176, 87-98.

118. Pradhan, S. K., Gupta, R. C., & Goel, R. K. (2018). Differential content of secondary metabolites in diploid and tetraploid cytotypes of *Siegesbeckia orientalis* L. Natural product research, 32(20), 2476-2482.

119. Priya, A. K., Muruganandam, M., Kumar, A., Senthilkumar, N., Shkir, M., Pandit, B., ... & Ubaidullah, M. (2024). Recent advances in microbial-assisted degradation and remediation of xenobiotic contaminants; challenges and future prospects. Journal of Water Process Engineering, 60, 105106.

120. Priya, L., & Pillai, P. U. (2023). *In vitro* effect of Colchiploidy on andrographolide enhancement in *Andrographis paniculata* (Burm. f.) Wall. ex. Nees. *South African Journal of Botany*, *163*, 786-793.

121. Qi-Qing, L. I., Zhang, J., Ji-Hua, L. I. U., & Bo-Yang, Y. U. (2018). Morphological and chemical studies of artificial *Andrographis paniculata* polyploids. Chinese journal of natural medicines, 16(2), 81-89.

122. Ramsey, J., & Schemske, D. W. (1998). Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annual review of ecology and systematics, 29(1), 467-501.

123. Regalado, J. J., Carmona-Martín, E., Querol, V., Veléz, C. G., Encina, C. L., & Pitta-Alvarez, S. I. (2017). Production of compact petunias through polyploidization. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *129*, 61-71.

124. Renny-Byfield, S., Ainouche, M., Leitch, I. J., Lim, K. Y., Le Comber, S. C., & Leitch, A. R. (2010). Flow cytometry and GISH reveal mixed ploidy populations and Spartina nonaploids with genomes of *S. alterniflora* and *S. maritima* origin. Annals of botany, 105(4), 527-533.

125. Rivera, D., Verde, A., Fajardo, J., Obón, C., Consuegra, V., García-Botía, J., ... & Laguna, E. (2019). Ethnopharmacology in the Upper Guadiana River area (Castile-La Mancha, Spain). *Journal of ethnopharmacology*, *241*, 111968.

126. Rodrigues, S., & Fernandes, F. A. (2009). Ultrasound-assisted extraction. *Stewart Postharvest Review*, *5*(5), 1-11.

127. Sadat Noori, S. A., Norouzi, M., Karimzadeh, G., Shirkool, K., & Niazian, M. (2017). Effect of colchicine-induced polyploidy on morphological characteristics and essential oil

composition of ajowan (*Trachyspermum ammi* L.). Plant cell, tissue and organ culture (pctoc), 130, 543-551.

128. Salehi, B., Abu-Darwish, M. S., Tarawneh, A. H., Cabral, C., Gadetskaya, A. V., Salgueiro, L., ... & del Mar Contreras, M. (2019). *Thymus* spp. plants-Food applications and phytopharmacy properties. *Trends in Food Science & Technology*, *85*, 287-306.

129. Salma, U., Kundu, S., & Mandal, N. (2017). Artificial polyploidy in medicinal plants: advancement in the last two decades and impending prospects. Journal of crop science and biotechnology, 20, 9-19.

130. Santos-Buelga, C., Gonzalez-Manzano, S., Dueñas, M. & Gonzalez-Paramas, A.M. Extraction and isolation of phenolic compounds. In *Natural Products Isolation* 427–464 (2012). https://doi.org/10.1007/978-1-61779-624-1_16

131. Šedivá, J., Mrázková, M., Zahumenická, P., Cusimamani, E. F., & Zahradník, D. (2019). Identification of Phytophthora tolerance in the *Anemone sylvestris* tetraploid. Scientia Horticulturae, 256, 108579.

132. Shahriari-Ahmadi, F., Dehghan, E., Farsi, M., & Azizi, M. (2008). Tetraploid induction of *Hyoscyamus muticus* L. using colchicine treatment. Pakistan Journal of Biological Sciences, 11(24), 2653.

133. SHARMA, A. D., Inderjeet, K. A. U. R., Jasprret, K. A. U. R., & CHAUHAN, A. (2024). Chemical profiling and *in-vitro* anti-oxidant, anti-diabetic, anti-inflammatory, anti-bacterial and anti-fungal activities of essential oil from *Rosmarinus officinalis* L. Notulae Scientia Biologicae, 16(1), 11756-11756.

134. Sharma, N., Yeasmen, N., Dube, L., & Orsat, V. (2024). Rise of Plant-Based Beverages: A Consumer-Driven Perspective. Food Reviews International, 1-27.

135. Shmeit, Y. H., Fernandez, E., Novy, P., Kloucek, P., Orosz, M., & Kokoska, L. (2020). Autopolyploidy effect on morphological variation and essential oil content in *Thymus vulgaris* L. Scientia Horticulturae, 263, 109095.

136. Sivakumar, R., Nandhitha, G., & Nithila, S. (2017). Impact of drought on chlorophyll, soluble protein, abscisic acid, yield and quality characters of contrasting genotypes of tomato (*Solanum lycopersicum*). British Journal of Applied Science & Technology, 21(5), 1-10.

137. Soltis, P. S., Marchant, D. B., Van de Peer, Y. & Soltis, D. E. Polyploidy and genome evolution in plants. Curr. Opin. Genet. Dev. 35, 119–125 (2015).

138. Song, B. H., Varshney, V. K., Mittal, N., & Ginwal, H. S. (2015). High levels of diversity in the phytochemistry, ploidy and genetics of the medicinal plant *Acorus calamus* L. *Med. Aromatic Plants*, *1*, 002.

139. Subbaraya, U., Saraswathi, M. S., & Pillay, M. (2011). 2 Evolution and Genetic. Banana breeding: Progress and challenges, 21.

140. Subramanian, M., Koh, K. S., Gantait, S., & Sinniah, U. R. (2024). Advances on *in vitro* regeneration and microrhizome production in Zingiberaceae family. *In Vitro Cellular & Developmental Biology-Plant*, 1-19.

141. Taïbi, K., Abderrahim, L. A., Ferhat, K., Betta, S., Taïbi, F., Bouraada, F., & Boussaid, M. (2020). Ethnopharmacological study of natural products used for traditional cancer therapy in Algeria. *Saudi Pharmaceutical Journal*, *28*(11), 1451-1465.

142. Talhouk, S. N., Fabian, M., & Dagher, R. (2015). Landscape plant database. Department of Landscape Design & Ecosystem Management, American University of Beirut. Available online: https://landscapeplants. aub. edu. lb/Plants/PlantProfile, 20(20), 20.

143. Tavan, M., Sarikhani, H., Mirjalili, M. H., Rigano, M. M., & Azizi, A. (2021). Triterpenic and phenolic acids production changed in *Salvia officinalis* via *in vitro* and *in vivo* polyploidization: A consequence of altered genes expression. *Phytochemistry*, *189*, 112803.

144. Togashi, N. *et al.* Antibacterial activity of long-chain fatty alcohols against *Staphylococcus aureus*. *Molecules* **12**, 139–148 (2007). https://doi.org/10.3390/12020139

145. Tossi, V. E., Martínez Tosar, L. J., Laino, L. E., Iannicelli, J., Regalado, J. J., Escandón, A. S., ... & Pitta-Álvarez, S. I. (2022). Impact of polyploidy on plant tolerance to abiotic and biotic stresses. Frontiers in Plant Science, 13, 869423.

146. Touchell, D. H., Palmer, I. E., & Ranney, T. G. (2020). *In vitro* ploidy manipulation for crop improvement. Frontiers in Plant Science, 11, 722.

147. Trojak-Goluch, A., & Skomra, U. (2013). Artificially induced polyploidization in *Humulus lupulus* L. and its effect on morphological and chemical traits. *Breeding Science*, *63*(4), 393-399.

148. Unver, T. A preliminary study of fumaric acid, called allomaleic acid, as a pharmaceutical antimicrobial compound. *Int. Med. J.* **13**, 383–387 (2024). https://doi.org/10.5455/medscience.2024.04.031

149. Urwin, N. A. (2014). Generation and characterisation of colchicine-induced polyploid Lavandula× intermedia. Euphytica, 197, 331-339.

150. Van de Peer, Y., Ashman, T. L., Soltis, P. S., & Soltis, D. E. (2021). Polyploidy: an evolutionary and ecological force in stressful times. The Plant Cell, 33(1), 11-26.

151. Velásquez, M. M., Lattig, M. C., Chitiva, L. C., Costa, G. M., Sutachan, J. J., & Albarracin, S. L. (2023). Dendritogenic Potential of the Ethanol Extract from *Lippia alba* Leaves in Rat Cortical Neurons. *Molecules*, *28*(18), 6666.

152. Vijeesh, V., Vysakh, A., Jisha, N. & Latha, M. S. Malic acid attenuates potassium oxonate-induced gouty inflammation in Wistar rat. *Biointerface Res. Appl. Chem.* **12**, 1682–1691 (2022). https://doi.org/10.33263/BRIAC122.16821691.

153. Woodhouse, M., Burkart-Waco, D., & Comai, L. (2009). Polyploidy. Nature Education, 2(1), 1.

154. Xie, Z., Huang, H., Zhao, Y., Shi, H., Wang, S., Wang, T. T., ... & Yu, L. L. (2012). Chemical composition and anti-proliferative and anti-inflammatory effects of the leaf and whole-plant samples of diploid and tetraploid *Gynostemma pentaphyllum* (Thunb.) Makino. *Food chemistry*, *132*(1), 125-133.

155. Xu, C., Zhang, Y., Huang, Z., Yao, P., Li, Y., & Kang, X. (2018). Impact of the leaf cut callus development stages of *Populus* on the tetraploid production rate by colchicine treatment. Journal of Plant Growth Regulation, 37, 635-644.

156. Yarmolinsky, L., Zaccai, M., Ben-Shabat, S., & Huleihel, M. (2010). Anti-herpetic activity of *Callissia fragrans* and *Simmondsia chinensis* leaf extracts *in vitro*. *The open virology journal*, *4*, 57.

157. Ye, Y. M., Tong, J., Shi, X. P., Yuan, W., & Li, G. R. (2010). Morphological and cytological studies of diploid and colchicine-induced tetraploid lines of crape myrtle (*Lagerstroemia indica* L.). Scientia Horticulturae, 124(1), 95-101.

158. Yeshi, K., Crayn, D., Ritmejerytė, E., & Wangchuk, P. (2022). Plant secondary metabolites produced in response to abiotic stresses has potential application in pharmaceutical product development. *Molecules*, 27(1), 313.

159. Yue, Y., Fan, X., Hu, Y., Han, C., Li, H., Teng, W., ... & Wu, J. (2020). *In vitro* induction and characterization of hexaploid *Pennisetum* × *advena*, an ornamental grass. *Plant Cell, Tissue and Organ Culture* (PCTOC), 142, 221-228.

160. Zayed, M., Ahmad, F., Ho, W.-S. & Pang, S. GC-MS analysis of phytochemical constituents in leaf extracts of *Neolamarckia cadamba* (Rubiaceae) from Malaysia. Int. J. Pharm. Pharm. Sci. 6, 123–126 (2014).

161. Zhang, X., Jin, M., Tadesse, N., Dang, J., Zhou, T., Zhang, H., ... & Ito, Y. (2018). *Dioscorea zingiberensis* CH Wright: An overview on its traditional use, phytochemistry, pharmacology, clinical applications, quality control, and toxicity. *Journal of Ethnopharmacology*, *220*, 283-293.

Neha Gupta

Phone: (+420)731215680
Email Address: guptan@ftz.czu.cz ; nehagupta18041997@gmail.com
Date of Birth: 18th April, 1997
Nationality: Indian
Orcid ID: 0000 -0002 -1725 -7908
Google Scholar: https://scholar.google.com/citations?user=yqdd_CkAAAAJ&hl=en

RESEARCH INTEREST

My research focuses on the discovery, isolation, and structural elucidation of secondary metabolites from medicinal and ornamental plants for herbal medicine development. I use various analytical methods GC/MS, NMR, MALDI-TOF, and HPLC for comprehensive phytochemical profiling and identify bioactive compounds through antioxidant, antimicrobial, and anti-inflammatory assays *in vitro* and in cell culture. I also explore polyploidization to enhance phytochemical yields, integrating natural products chemistry and plant biotechnology to advance novel herbal therapeutics and nutraceutical applications.

EDUCATION

1. **Ph.D. in Tropical Agrobiology and Bioresource Management,** Czech University of LifeSciences, Prague, Czechia (September 2022 – September 2025)
   Thesis: Effect of Artificial Polyploidization on the Biological Activities of Medicinal and Ornamental Plants.
   Supervisor: Prof. Dr. Eloy Fernandez Cusimamani

2. **M.Sc. in Agricultural Biotechnology**, Ramakrishna Mission Vivekananda Educational and Research Institute (RKMVERI), India (2018 - 2020)
   Thesis: Studies on comparative assessment of phytochemistry and antimicrobial activity of essential oils from different Indian spices and their formulations
   Supervisor: Dr. Alok Kumar Hazra

3. **B.Sc. in Biotechnology**, Maulana Abul Kalam Azad University of Technology (MAKAUT), India (2015 - 2018)

RESEARCH EXPERIENCE

Research Assistant

Laboratory of Plant Tissue Cultures, Czech University of Life Sciences Prague, Czechia

Roles: Media preparation, glassware and equipment sterilization, surface sterilization, micropropagation, and subculturing, inventory management, helping, guiding bachelor and master students, and maintaining safety measures

Research Associate

Tissue culture and seed technology department, Debgiri AgroProducts Pvt. Ltd, India (2020 - 2022)

Roles: Development and optimization of advanced plant tissue culture techniques and seed production technologies. Conducting research on the G-9 variety of bananas, maintaining aseptic culture conditions, developing propagation protocols, and collaborating with multidisciplinary teams to enhance the quality and yield of agricultural products.

TECHNICAL SKILLS

1. ***In vitro* bioassays**: Proficient in antioxidant (DPPH, ABTS, FRAP, ORAC, TFC, TPC), cytotoxicity (MTT, SRB), anti-inflammatory (NO inhibition, ELISA), and enzyme activity assays.
2. **Polyploidization & micropropagation**: Expertise in *in vitro* polyploidization and largescale propagation of medicinal and ornamental plants
3. **Molecular biology**: Skilled in PCR/qRT-PCR for gene expression analysis and molecular characterization of plant genotypes
4. **Analytical chemistry**: Experienced in GC/MS, LC/MS, NMR, MALDI-TOF, and HPLC for phytochemical profiling and metabolomics
5. **Computational biology**: Proficient in *in silico* molecular docking and SAR studies for bioactive compound analysis
6. **Antimicrobial assays**: Competent in disk diffusion, broth microdilution, and time-kill kinetics for bioactivity screening
7. **Cell culture**: Expertise in mammalian and plant cell culture for pharmacological and toxicological studies

8. **Microbial identification**: Skilled in biochemical, molecular, and MALDI-TOF-based bacterial characterization
9. **Flow cytometry**: Specialized in ploidy estimation and genome size determination for plant breeding

PROJECTS

1. Internal Grant Agency 2022/2023 (Co-investigator)
2. Internal Grant Agency 2023/2024 (Co-investigator)
3. Internal Grant Agency 2024/2025 (Co-investigator)

TEACHING EXPERIENCES

Undergraduate Lecturer, Faculty of Tropical AgriSciences, Czech University of Life Sciences, Prague, Czechia

Subject: Tropical Agricultural Systems (IRI007E) (2023 - Current)

Mentored three students: 3 Bachelor's and 4 Master's students, providing guidance in their experiments and research projects in Plant tissue culture and biotechnology.

Opponent reviewer for Bachelor's and Master's student thesis, Faculty of Tropical AgriSciences, Czech University of Life Sciences, Prague, Czechia

INTERNSHIPS

1. **University of Basilicata**, Potenza, Italy: Highly advanced enzyme kinetics and tyrosinase activities conducted on different phytochemicals
2. **Slovak University of Agriculture**, Nitra, Slovak Republic: Conducted MALDI-TOF and qRT-PCR on different extracts of polyploid medicinal and ornamental plants
3. **Garden of Medicinal Plants And Facilitation Centre**, Ramakrishna Mission Ashrama, Narendrapur, Kolkata, India: Collected samples of Indian medicinal plant samples that have not yet been explored
4. **Council for Scientific and Industrial Research (CSIR),** Kolkata, India: Flowcytometric analysis of various cancer cells, T-lymphoma cells, sarcoma cells, myeloma cells, etc.

5. **Liebig's Agrochemical Private Limited,** Kolkata, India: Micropropagation of *Musa acuminate* (G-9 banana) and comparative studies amongst various organic fungicides and larvicides of different concentrations
6. **SHRM Private Limited, Kolkata,** India**:** Isolation of genomic DNA from bacterial and plant cells, PCR amplification, Gel electrophoresis, SDS PAGE

LIST OF PUBLICATIONS

1. **Gupta, N**., Bhattacharya, S., Žiarovská, J., Dutta, A., Fialková, V., Farkasová, S., Bergo, A. M., Havlik, J., Tauchen, J., Novy, P., Urbanová, K., Milella, L., & Fernández-Cusimamani, E. (2025). Oryzalin-induced polyploidy enhances natural metabolite accumulation and functional bioactivities in novel *Callisia fragrans* genotype. *Scientific Reports, 15*, 25652.
2. **Gupta, N**., Bhattacharya, S., Dutta, A., Tauchen, J., Landa, P., Urbanová, K., ... & Leuner, O. (2024). Synthetic polyploidization induces enhanced phytochemical profile and biological activities in *Thymus vulgaris* L. essential oil. *Scientific Reports*, *14*(1), 5608
3. **Gupta, N**., Bhattacharya, S., Urbanová, K., Dutta, A., Hazra, A. K., Fernández-Cusimamani, E., & Leuner, O. (2023). Systematic analysis of antimicrobial activity, phytochemistry, and *in silico* molecular interaction of selected essential oils and their formulations from different Indian spices against foodborne bacteria. *Heliyon*, *9*(12)
4. **Gupta, N**., Bhattacharya, S., Dutta, A., Cusimamani, E. F., Milella, L., & Leuner, O. (2024). *In Vitro* Synthetic Polyploidization in Medicinal and Aromatic Plants for Enhanced Phytochemical Efficacy—A Mini-Review. *Agronomy*, *14*(8)
5. Bhattacharya, S., **Gupta, N**., Flekalová, A., Gordillo-Alarcón, S., Espinel-Jara, V., & Fernández-Cusimamani, E. (2024). Exploring Folklore Ecuadorian Medicinal Plants and Their Bioactive Components Focusing on Antidiabetic Potential: An Overview. *Plants*, *13*(11), 1436 **(*Joint 1st author)**
6. Bhattacharya, S., **Gupta, N.**, Dutta, A., Khanra, P. K., Dutta, R., Žiarovská, J., ... & Fernández-Cusimamani, E. (2025). Repurposing major metabolites of lamiaceae family as potential inhibitors of α-synuclein aggregation to alleviate neurodegenerative diseases: an *in silico* approach. *Frontiers in Pharmacology*, *16*, 1519145
7. Bhattacharya, S., Dutta, A., Khanra, P. K., **Gupta, N**., Dutta, R., Tzvetkov, N. T., ... & Ponticelli, M. (2024). *In silico* exploration of 4 (*α*-l-rhamnosyloxy)-benzyl

isothiocyanate: A promising phytochemical-based drug discovery approach for combating multi-drug resistant *Staphylococcus aureus*. *Computers in Biology and Medicine*, *179*, 108907

8. Bhattacharya, S., Khanra, P. K., Dutta, A., **Gupta, N.**, Aliakbar Tehrani, Z., Severová, L., ... & Fernández-Cusimamani, E. (2024). Computational screening of T-muurolol for an alternative antibacterial solution against *Staphylococcus aureus* infections: an in silico approach for phytochemical-based drug discovery. *International Journal of Molecular Sciences*, *25*(17), 9650

UPCOMING PUBLICATIONS

1. Mitigating ROS Signalling Pathway-Mediated Defence Mechanism: A Novel Approach to Counteract Bacterial Resistance Using Natural Antioxidant-Based Antibiotics (Phytochemistry Reviews)
2. Precision Warfare Against Multidrug-Resistant *Staphylococcus aureus*: Advanced Phytochemical-Based Nanotechnology Interventions (Journal of Antimicrobial Chemotherapy)

REVIEW ACTIVITY

Participated as an academic reviewer for the following prestigious journals:

1. Scientific Reports
2. Planta
3. Next research
4. Scientia Agriculturae Bohemica

CONFERENCES

1. **Tropentag 2024** (BOKU, Vienna, Austria)

   Topic: Synthetic Polyploidization a Promising Tool in Crop Management: Induces Enhanced Phytochemical Profile and Biological Activities in *Thymus vulgaris* L.

2. **Agrinatura 2023** (Faculty of Tropical AgriSciences, CZU, Prague, Czechia)

   Topic: Comparative *in vitro* analysis of antimicrobial activity against food-borne pathogens and phytochemistry of essential oils from various Indian spices and their formulations

REFERENCES

Prof. Eloy Fernandez Cusimamani, PhD.

Professor at Faculty of Tropical AgriSciences, Head of Laboratory of Plant Tissue Cultures, Department of Crop Science and Agroforestry at Czech University of LifeSciences Prague, Czech Republic. Email: eloy@ftz.czu.cz

Prof. Luigi Millela, PhD.

Pharmacy Programme Coordinator**,** Associate Professor in Pharmaceutical Biology, Department of Science University of Basilicata in Potenza, Italy. Email: luigi.milella@unibas.it

Prof. Jana Žiarovská, PhD.

Vice-dean for Science, Faculty of Agrobiology and Food Resources, Slovak University of Agriculture in Nitra, Slovak Republic. Email: jana.ziarovska@uniag.sk