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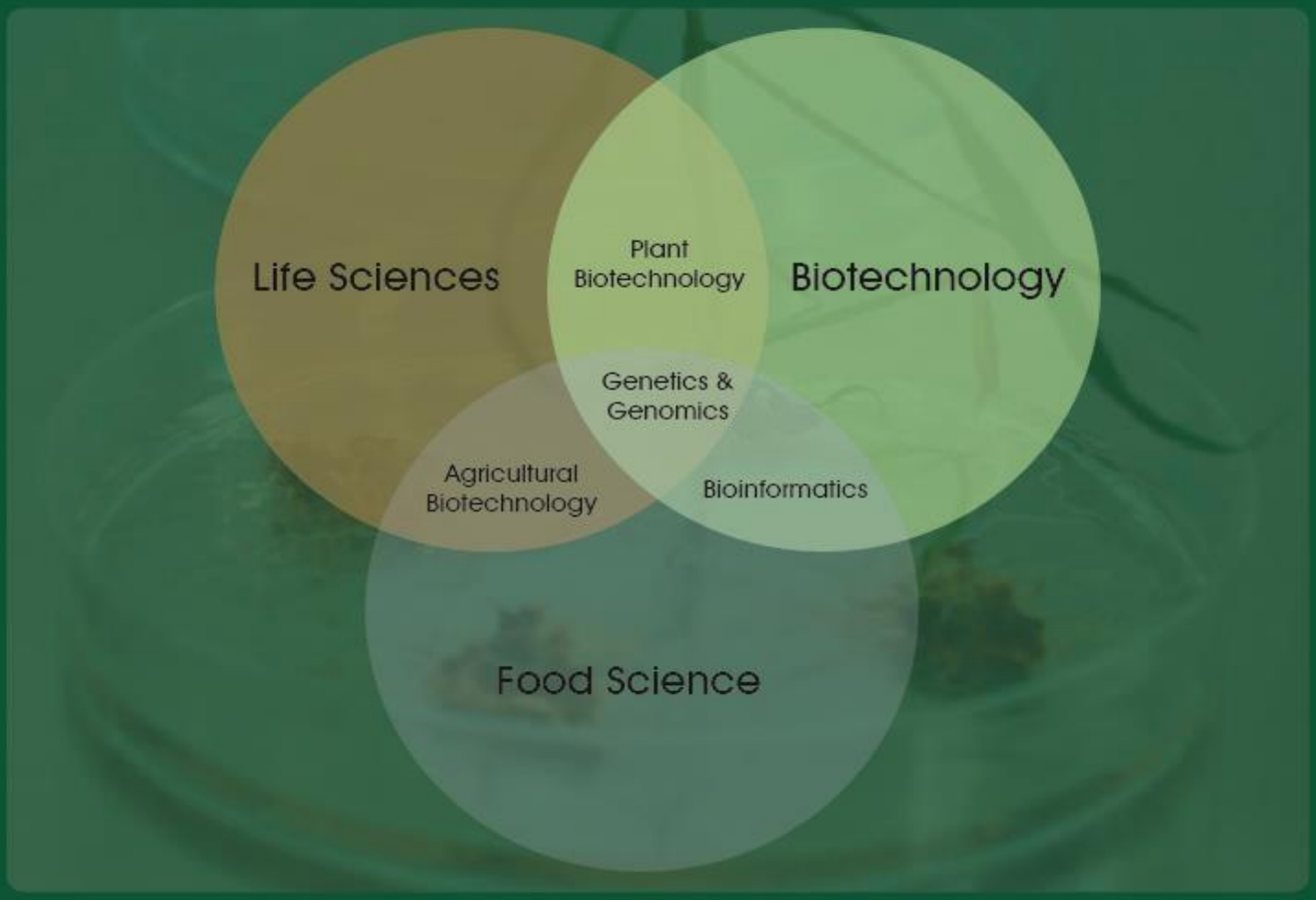

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Yildiz Technical University, Faculty of Chemical and Metallurgical Engineering.
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From The Editor;

Dear Readers and Authors,

As “International Journal of Life Sciences and Biotechnology”, we are pleased and honored to present the 21th issue of the journal. "International Journal of Life Sciences and Biotechnology" is an international double peer-reviewed open access academic journal published on the basis of research- development and code of practice.

The aims of this journal are to contribute in theoretical and practical applications in relevant researchers of Life Sciences, Biology, Biotechnology, Bioengineering, Agricultural Sciences, Food Biotechnology and Genetics institutions and organizations in Turkey, and to publish solution based papers depending on the principle of impartiality and scientific ethics principles, focusing on innovative and added value work, discussing the current and future.

With these thoughts, We are especially thankful to academicians honoring with the articles, valuable scientists involved in editorial boards and reviewers for their contributions to the evaluation processes with through their opinions/ideas/contributions/criticisms in the first issue of 2025 "International Journal of Life Sciences and Biotechnology". Hope to see you in the next issue…

15. 04. 2025
Editor in Chief
Prof. Dr. Ali Aslan

Editörden;

Değerli okurlar ve yazarlar,

“International Journal of Life Sciences and Biotechnology” olarak dergimizin yirminci birinci sayısını yayın hayatına sunmaktan mutluluk ve onur duyuyoruz. “İnternational Journal of Life Sciences and Biotechnology” dergisi araştırma- geliştirme ve uygulama ilkeleri baz alınarak yayınlanan uluslararası hakemli açık erişimli akademik bir elektronik dergidir.

“İnternational Journal of Life Sciences and Biotechnology” dergisi Yaşam Bilimleri, Biyoloji, Biyoteknoloji, Biyomühendislik, Ziraat Bilimleri, Gıda Biyoteknolojisi ve Genetik alanlarındaki ilgili araştırmacılara, kurum ve kuruluşlara teorik ve pratik uygulamalarda katkı sağlamayı, tarafsızlık ve bilim etiği ilkelerine bağlı kalarak çözüm temelli, yenilikçi ve katma değeri olan çalışmalara odaklanan, günceli ve geleceği tartışan çalışmaların yayınlanmasını hedeflemektedir.

Bu düşüncelerle 2025 yılı birinci sayısını yayınladığımız “Internatıonal Journal of Life Sciences and Biotechnology” dergisini, makaleleri ile onurlandıran akademisyenlere, Fikir / Görüş / Öneri / Katkı ve Eleştirileri ile değerlendirme süreçlerine katkılarından dolayı hakem ve yayın kurullarında yer alan kıymetli bilim insanlarına yürekten teşekkür ediyoruz. Bir sonraki sayıda görüşmek ümidiyle…

15.04. 2025
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Prof. Dr. Ali Aslan

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Research article

Challenges and Opportunities for Women Farmers in Afghanistan

Asooda Afshar[1], Mina Hakimi[1], Farzaneh Razmju[1], Bahram Yaqooby[1], Mir Abdullatif Yahya[5]

ABSTRACT
Women play a crucial role in the country's development, particularly in Afghanistan's agricultural industry, where they face numerous challenges and opportunities in Afghanistan. However, this topic has not been extensively investigated in the literature, leaving a gap in understanding the unique experiences of women in this sector. This study examines the challenges and opportunities faced by women farmers in Afghanistan, drawing on survey data from 81 participants across 16 provinces. Results indicate significant barriers to women's participation in the agriculture industry, including restricted access to land (31), financial constraints (87%), limited training opportunities (57%), and insufficient institutional support (83%). Cultural and social norms further impede these concerns by limiting women's roles and decision-making abilities. Despite these challenges, the study conceives anew of potential avenues for empowerment through community-based solutions, evidenced by 69% of respondents interested in joining women's agricultural activities. Other potential avenues could be microfinance programs, financial literacy courses, mobile or community-based learning solutions, and region-specific programs. Our findings highlight a pressing need for targeted and multi-sectoral efforts to overcome systemic barriers, promote gender equality, and release the potentials of women in agriculture. These initiatives could greatly enhance the lives of women and contribute towards Afghanistan's larger economic growth. This study concludes that while women in Afghanistan's agricultural sector face significant challenges, there are substantial opportunities to empower them through targeted interventions and community-based initiatives.

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Agricultural development, gender equity, women and agriculture, women farmers Afghanistan

Introduction

The economic landscape of Afghanistan is significantly shaped by the contributions of women, who make up 50% of the population. Their roles, both visible and behind the scenes, are crucial to the country's development. Notably, their influence within domestic spheres has a substantial impact on economic growth. Agriculture, which is the backbone of Afghanistan's economy, supports 80% of the population living in rural areas. When addressing women farmer's challenges, one must consider how the NGO (none-profit organization) interventions have had or could have positive and negative effects on the wider Afghanistan population, as explored by [1] in the case of ABCO's efforts to alleviate poverty in the region.
Despite women's extensive involvement in various agricultural and animal husbandry tasks, the contributions of rural Afghan women are often overlooked. They engage in essential traditional practices that, while crucial, may not immediately result in financial gain. According to [2] statistics, there has been a steady decline over the years, with female participation in Agricultural activities dropping from around 75% in 2011 to under 50% by 2022 (fig 1). While there were brief periods of stabilization, such as from 2014 to 2017, the trend is predominantly downward, indicating a significant reduction in women's employment in the agricultural sector. This decline may reflect broader socio-economic, cultural, or policy-related challenges affecting women's labor force participation in Afghanistan. From 2011 to 2022, female employment in Afghanistan's agriculture industry decreased as a result of restrictive regulations, economic uncertainty, and cultural limitations. The

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International Labor Organization estimated a 25% decrease in female employment by late 2022 compared to mid-2021, attributing this loss to limitations on women's engagement in the labor market [19].

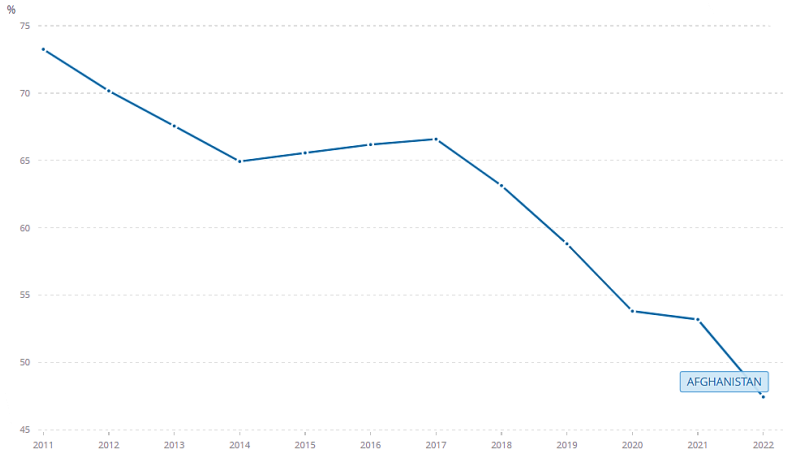

Fig 1 Female Employment in Agriculture in Afghanistan (2011–2022) [2]

Moreover, economic difficulties have resulted in substantial income declines, with 82% of Afghan households seeing salary decreases since August 2021 [20]. Cultural conventions also restrict women's responsibilities in agriculture, exacerbating this decline. Reference [3] highlights the significant roles these women play in family-run agricultural businesses, where they enhance the value of crops like wheat through activities such as storage, cleaning, seed selection, planting, and harvesting. However, these critical contributions frequently go unrecognized in agricultural research and development initiatives. The work of these women is vital to the operation of small-scale dairy farms, with responsibilities including feed collection, animal care, and health management [4]. Comprising 33% of the agricultural labor force, rural Afghan women are integral to various farming activities. They participate in dairy and poultry farming, sheep rearing, mushroom cultivation, and the production of high-value vegetables and other plants in small greenhouses and home gardens, underscoring their indispensable role in agriculture [4]. Research by [5] emphasizes the vital role women play in agriculture and calls on the Afghan authorities to address the challenges rural women face in livestock management and to organize training workshops to enhance their participation.

The aim of the current study was to explore the challenges and opportunities faced by women involved in Afghanistan's agricultural sector. Specifically, it sought to identify the systemic barriers that hinder women’s participation and progress in agriculture. Additionally, the study aimed to highlight potential pathways for empowering women farmers, with a focus on leveraging community-based initiatives, improving access to training and infrastructure, and fostering gender equity in agricultural practices.

Literature Review

In the present study, given the dearth of research on the role of women in Afghan agriculture, analogous examples from other countries were employed for the literature review. For instance, reference [6] identified various institutional barriers, both governmental and non-governmental, that rural women face when creating small businesses in the Lower Dehestan of Rokh Rural District, Torbat-e Heydarieh County, Iran. These barriers included the lengthy process of obtaining business permits, lack of support in marketing women’s products, difficulty in accessing raw materials, insufficient organization of educational and skill-enhancing courses, and inadequate efforts to eliminate middlemen. Economically, the challenges were insufficient financial resources, limited access to raw materials, lack of loans and guarantors, and inadequate space and capital. Socially and culturally, significant barriers included gender discrimination, lack of community trust in women’s work, non-participation in rural affairs, male dominance over women, and unsafe rural environments. Individual and family barriers, although rated the lowest by women, included household chores, lack of time, spousal opposition, objections from children and parents (especially for unmarried daughters), limited skills, low literacy and knowledge levels, lack of self-confidence, and aversion to risk-taking.

reference [7] analyzed the role of rural women in economic activities and the factors influencing it in Jahrom County showed that the results indicate a negligible share and role of women in conducting and developing

economic activities in general, although this situation varies with different types of economic activities. Among the influencing factors, two variable environmental factors and household income status—had a significant and positive impact on the level of women's economic activities. This impact was not statistically significant concerning other independent variables related to institutional factors, supportive policies, education, motivations, and individual factors.

The participation of rural women in the workforce of third-world countries is higher than that of men, and higher than that of women and men in developed countries, due to the traditional nature of production structures and labor relations [8]. According to the findings of [9] in four rural communities in Greece, the attitude of young rural women towards agricultural employment was negative, considering it a necessity only while waiting for better job and income opportunities. Reference [10] conducted documentary research on the problems of developing women's entrepreneurship in rural India, highlighted the lack of a specific life plan, financial freedom, direct ownership of assets, awareness of their capacity, and low risk-taking as the most prominent of these problems.

A study conducted in the eastern and northern districts of Bonab in East Azerbaijan revealed that women play a more active role in agricultural activities, particularly during the harvest phase. However, various individual, familial, and organizational factors have been identified as barriers to their full participation in agriculture. Despite women constituting 49% of the rural population, they are responsible for 65% of the agricultural activities in these areas [11]. The United Nations has reported that achieving sustainable development is not possible without the active participation of women in all spheres, including family, economic, and social domains [12].

Reference [13] emphasized that entrepreneurship has increasingly attracted the attention of planners and policymakers in various countries. Many nations have realized that entrepreneurship is crucial for economic success on both national and international levels, serving as the main agent of change and the primary driver of development, leading to continuous creativity and innovation. Challenges such as personal, character, academic, familial, economic, socio-cultural, and environmental factors are identified as the most significant obstacles for women in agricultural entrepreneurship.

Table 1 Summary of key findings on challenges and roles of women in agriculture and rural development

Reference	Key Findings
Nasibeh G. et al. (2017) [6]	- Institutional barriers: lengthy business permits, lack of marketing support, difficulty accessing raw materials, insufficient educational courses, and inadequate efforts to eliminate middlemen. - Economic barriers: insufficient financial resources, limited access to loans, and inadequate space and capital. - Social and cultural barriers: gender discrimination, lack of community trust, male dominance, and unsafe rural environments. - Individual and family barriers: household chores, lack of time, spousal opposition, and low literacy levels.
Dadvar-Khani (2006) [15]	- Economic, geographical, and cultural factors (e.g., household income, social class, literacy, and isolation) diminish women's role in rural development. - Gender-blind rural development policies offer no bright prospects for improving women's status, especially young girls.
Amini & Taheri (2016) [7]	- Women's role in economic activities is negligible, but environmental factors and household income positively influence their participation. - Institutional factors, supportive policies, education, and motivations have no significant impact.
Kumbhar (2013) [10]	- Women in rural India face challenges such as lack of financial freedom, asset ownership, awareness, and low risk-taking.
Olawoye (1985) [11]	- Women play a more active role in agricultural activities during harvest but face individual, familial, and organizational barriers. - Women constitute 49% of the rural population but are responsible for 65% of agricultural activities.
Dadoukolaei & Alikhani (2015) [13]	- Entrepreneurship is crucial for economic success, but women face challenges such as individual, personality, scientific, family, economic, socio-cultural, and environmental factors in agricultural entrepreneurship.
World Bank (2005) [14]	- Women constitute 50% of the global agricultural workforce and are responsible for preparing food for two-thirds of the world's population.

According to the world bank statistics [14] women constitute 50% of the global agricultural workforce and are responsible for preparing food for two-thirds of the world's population. This highlights the critical role of

women in the agricultural sector and the need for their increased involvement to promote sustainable development and economic growth.
Table 1 provides an overview of the key barriers and challenges faced by women in rural development and agriculture that have been identified in various studies.

The obstacles reported (table 1) to hinder women include institutional barriers such as no access to loans, education, and technology; economic obstacles in the form of insufficient resources and a constrained market space; and socio-cultural hurdles such as gender discrimination, male chauvinism, and social violence. These studies have highlighted the absence of gender-specific focus in development policies, which has contributed to the barriers of multifaceted exclusion of women. Despite their superior contributions to agriculture, more so than those made by men, innate and systemic factors such as low literacy levels, a lack of financial self-confidence, and cultural norms have impeded women's participation in decision-making and entrepreneurial activities.

Material and Methods

Respondent-driven sampling (RDS) and snowball sampling (SBS) are two different methods used to investigate groups that are difficult to access. Reference [16] provides a detailed explanation of the differences between these techniques, including the use of different estimators. Snowball sampling depends on the social networks of initial participants [17] enables a more natural and gradual growth of the sample, which has the potential to encompass a wider variety of viewpoints from the intended population [18]. By utilizing pre-existing social relationships, this approach motivates participants to offer comprehensive views and experiences, so enhancing the depth of understanding of the study subject.
This study used purposive sampling to select a wide range of demographics, including women participating in all kinds of agricultural activities in the various locations around Afghanistan. Primary data was gathered through Google Forms, semi structured interviews, and focus group discussions, providing participants with a platform to express their viewpoints, experiences, and challenges.
Qualitative data undergo thematic analysis, where patterns, themes, and categories are discerned from interview transcripts and focus group discussions. This method entails coding, categorizing, and interpreting qualitative data to extract meaningful insights and themes concerning their challenges and opportunities in the agriculture industry. The study adhered rigorously to ethical rules and principles, obtaining informed consent from all participants thoroughly ensures their voluntary participation and data confidentiality. Moreover, the study places paramount importance on upholding, promoting, and guaranteeing fairness in all interactions with participants and stakeholders.

Results

Snowball sampling resulted in a total of 81 individuals participating in the survey who were directly or indirectly involved in the agriculture industry. This approach facilitated the inclusion of participants from diverse backgrounds and regions across Afghanistan, including Jowzjan (9.9%, *n*=8), Badakhshan (16%, *n*=13), Balkh (12.2%, *n*=10), Kabul (19.6%, *n*=16), Herat (7.4%, *n*=6), Ghazni (4.9%, *n*=4), Bamiyan (3.7%, *n*=3), Daykundi (3.7%, *n*=3), Farah (2.5%, *n*=2), Nimruz (2.5%, *n*=2), Parwan (1.2%, *n*=1), Ghor (1.2%, *n*=1), Baghlan (1.2%, *n*=1), Kunduz (2.5%, *n*=2), Kapisa (1.2%, *n*=1), and Faryab (1.2%, *n*=1). The presence of women in the agricultural sector was observed to vary significantly, with some engaging in small-scale farming, cattle rearing, or laboring on larger farms in Afghanistan. However, the data indicated a dearth of women's involvement in agricultural initiatives. This finding underscores the pressing need for enhanced support and opportunities to integrate women into agricultural activities, as it suggests a considerable degree of uncertainty and minimal participation among most women in this context. This unfulfilled engagement is intricately linked to significant barriers in accessing land. The data indicates that 42% of respondents had complete access to land, 37% had partial access, and 31% reported zero access. These restrictions on women's capacity to expand their agricultural activities also impede their attainment of financial independence, thereby underscoring the necessity to address constraints on land tenure in rural areas.
Financial challenges further exacerbate these issues, as an overwhelming 87% of participants reported significant difficulties in securing funds for agricultural investments, while 18% faced such challenges occasionally, and only 5% experienced no financial constraints. This financial insecurity severely limits women farmers' capacity to expand their businesses or adopt modern agricultural techniques, thereby perpetuating the cycle of limited productivity. Cultural norms and societal expectations that restrict women's roles in agriculture further compound these difficulties. The survey results indicate that more than half of the

respondents (56%) reported facing significant cultural barriers, 34% experienced these barriers occasionally, and only 20% did not face such issues.

Access to training was identified as a significant impediment, with 57% of respondents encountering challenges in participating in training programs, compared to 32% who reported no barriers and 20% who occasionally experienced such difficulties. This dearth of access to training has been shown to impede women's capacity to adopt contemporary farming practices, thus constraining their productivity and efficiency. Furthermore, the awareness of policies designed to support women in the agricultural sector remains limited. Forty-eight percent of respondents expressed uncertainty regarding the existence of such policies, while 30% indicated that no such policies were in place. Only 32% expressed confidence in the existence of these policies. This discrepancy indicates a deficiency not only in the implementation of policy but also in the dissemination of information regarding existing support mechanisms.

Institutional support in the form of agricultural services is notably inadequate. A significant 83% of respondents reported having no access to extension services or agricultural programs, while 19% occasionally received such support, and only 8% had regular access. This lack of institutional engagement severely hampers women's ability to improve their agricultural practices or benefit from technological advancements. Similarly, infrastructure and technology remain significant barriers, with 80% of participants citing difficulties in accessing essential agricultural infrastructure, 27% facing occasional challenges, and only 3% reporting no such issues. These barriers limit women's ability to modernize their operations and scale up their activities effectively.

Given these challenges, many women turn to non-agricultural income-generating activities to diversify their income sources, with 64% of respondents indicating participation in such activities, while 36% did not. This reliance on additional income highlights the inadequacy of agriculture alone in providing economic stability for women farmers. However, there is substantial potential for community-based solutions, as 69% of respondents expressed interest in joining women's agricultural groups, even though only 11% were currently members. This suggests significant opportunities for fostering collaboration, resource-sharing, and advocacy through cooperatives and similar initiatives. All together, these findings (table 2) paint a complex picture of the challenges and opportunities faced by women farmers in Afghanistan, emphasizing the need for targeted interventions to address systemic barriers and unlock their potential in the agricultural sector.

Discussion

This study provides a detailed view on the complex challenges and opportunities confronting Afghan women in agriculture. Our findings suggest a set of formidable barriers that are hindering women participation in and productivity of agriculture. These have to do with their restricted access to land, finance-capital-inefficient training programs, and lack of institutional support, while cultural and traditional norms handicap women's roles and decision-making capacities in farming. Under poor technology and infrastructure, Afghan women have limited ability to scale up and modernize their operations. Access to land remains a critical issue, with nearly one-third of respondents reporting no access and a significant portion indicating only partial access. This lack of access not only restricts women's agricultural output but also impedes their financial independence. Similarly, the financial challenges, with 87% of participants citing challenges in securing funds, underscores the urgent need for financial inclusion initiatives. These aspects emphasize the critical role of policies and programs that address systemic financial barriers and promote equitable access to land ownership.

Cultural and societal norms present another significant challenge, with more than half of the respondents encountering severe cultural barriers that restrict their involvement in agriculture and related decision-making processes. Training and education are further hindered by these norms, leaving women ill-equipped to adopt modern farming practices. The lack of awareness regarding supportive policies and the inadequacy of extension services exacerbates these issues, leaving many Afghan women unable to benefit from institutional support or technological advancements. Despite these challenges, our findings also reveal significant opportunities for empowering women in agriculture. The strong interest in joining women's agricultural groups, expressed by 69% of respondents, indicates the potential for collective action through cooperative and community-based solutions. Such initiatives could foster resource-sharing, advocacy, and skill development while addressing cultural barriers through collaborative efforts. Additionally, there is considerable potential for financial and educational interventions. Microfinance programs, financial literacy training, and access to grants or subsidies could enable women to overcome financial constraints, while mobile or community-based training initiatives could address logistical and cultural challenges to skill development. Tailored interventions for specific regions, particularly those affected by harsh climatic conditions or limited infrastructure, could further enhance the effectiveness of such programs.

Table 2 Survey Findings Summary: Challenges and Opportunities for Women Farmers in Afghanistan

Category	Challenges	Opportunities
Cultural and Social Barriers	- Societal expectations and gender roles limit participation. - Cultural norms restrict decision-making power. - Limited access to training due to cultural restrictions.	- Interest in joining women's agricultural groups. - Potential for collective action and empowerment through cooperatives.
Financial Challenges	- Lack of capital for farming investments (seeds, tools, equipment). - Limited access to formal financial institutions. - Difficulty in accessing markets.	- Potential for microfinance programs. - Opportunities for financial literacy training. - Access to grants or subsidies for women farmers.
Education and Training	- Limited access to agricultural training programs. - Logistical challenges in attending workshops. - Cultural barriers to education.	- Strong interest in training programs. - Potential for mobile or community-based training initiatives. - Collaboration with NGOs for skill development.
Land Access and Ownership	- Limited access to agricultural land. - Legal and cultural barriers to land ownership. - Dependence on family-owned land.	- Advocacy for women's land rights. - Potential for land-sharing programs. - Government initiatives to promote land access for women.
Infrastructure and Technology	- Lack of access to irrigation systems and modern farming equipment. - Poor transportation facilities. - Limited use of improved seeds and tools.	- Investment in rural infrastructure. - Introduction of affordable farming technologies. - Training on modern farming techniques.
Diversification of Income	- Balancing multiple income-generating activities is challenging. - Limited resources for diversification.	- Diversification into livestock, handicrafts, and small businesses. - Potential for value-added agricultural products. - Access to markets for diversified products.
Regional Variations	- Urbanization challenges in Kabul. - Harsh climatic conditions in Badakhshan and Balkh. - Limited infrastructure in rural areas.	- Tailored interventions for specific regions. - Leveraging local knowledge and traditional practices. - Focus on climate-resilient farming in vulnerable areas.
Policy and Support	- Lack of awareness of existing policies. - Limited access to government or NGO support programs.	- Increased awareness of supportive policies. - Expansion of agricultural extension services. - Collaboration with international organizations for funding and support.

Cultural Barriers

Cultural norms and societal expectations play a significant role in shaping the participation of women in Afghanistan's agricultural sector. As highlighted in the study, more than half of the respondents (56%) reported facing significant cultural barriers, which restrict their involvement in agriculture and related decision-making processes. These findings align with the work of [6], who identified gender discrimination, lack of community trust, and male dominance as key social and cultural barriers for rural women in Iran. Similarly, [15] emphasized that gender-blind rural development policies often fail to address the cultural constraints that limit women's roles in agriculture. In Afghanistan, cultural norms often confine women to domestic roles, limiting their ability to engage in agricultural activities outside the home. This is further exacerbated by the lack of access to education and training, as cultural restrictions often prevent women from attending workshops or

participating in skill development programs. The findings of this study are consistent with those of [10], who noted that women in rural India face similar challenges, including low literacy levels and a lack of financial self-confidence, which are deeply rooted in cultural norms.

Economic Barriers

Economic challenges are another major barrier to women's participation in agriculture. The study found that 87% of respondents faced significant difficulties in securing funds for agricultural investments, which severely limits their ability to expand their businesses or adopt modern farming techniques. This is consistent with the findings of [7], who highlighted the economic barriers faced by rural women in Jahrom County, Iran, including limited access to financial resources and markets. Similarly, [6] identified insufficient financial resources, limited access to loans, and inadequate space and capital as key economic barriers for rural women in Iran. In Afghanistan, the lack of access to formal financial institutions and markets further exacerbates these challenges, leaving women farmers reliant on informal and often exploitative lending practices. The World Bank has also emphasized the critical role of financial inclusion in empowering women in agriculture, noting that women constitute 50% of the global agricultural workforce but often lack access to the financial resources needed to improve their productivity [14].

Opportunities to Enhance Women's Involvement in Agricultural Endeavors

Despite the significant challenges, there are substantial opportunities to enhance women's involvement in agricultural endeavors. The study found that 69% of respondents expressed interest in joining women's agricultural groups, indicating a strong potential for collective action through cooperatives and community-based solutions. This aligns with the findings of [11], who noted that women in rural Nigeria play a more active role in agricultural activities during the harvest phase, and that collective action through cooperatives can enhance their participation. Similarly, [13] emphasized the importance of entrepreneurship in empowering women in agriculture, noting that targeted interventions such as microfinance programs and financial literacy training can help women overcome economic barriers. In Afghanistan, there is considerable potential for financial and educational interventions, including mobile or community-based training initiatives, which can address logistical and cultural challenges to skill development. Additionally, tailored interventions for specific regions, particularly those affected by harsh climatic conditions or limited infrastructure, could further enhance the effectiveness of such programs.

The findings of this study are consistent with previous research on the challenges faced by women in agriculture, particularly in developing countries. For instance, reference [6] identified similar institutional, economic, and cultural barriers for rural women in Iran, while [10] highlighted the lack of financial freedom and asset ownership as key challenges for women in rural India. The study also corroborates the findings of the reference [14], which emphasized the critical role of women in agriculture and the need for their increased involvement to promote sustainable development and economic growth. However, this study adds to the existing literature by providing a detailed analysis of the challenges and opportunities faced by women farmers in Afghanistan, a context that has been under-researched in the literature. The findings underscore the need for targeted, multi-dimensional interventions to address the systemic barriers faced by women farmers in Afghanistan, including improving access to land, financial resources, training programs, and infrastructure.

Future Perspectives

Looking ahead, there is a pressing need for more research on the role of women in agriculture, particularly in conflict-affected regions like Afghanistan. Future studies could explore the impact of specific interventions, such as microfinance programs or community-based training initiatives, on women's participation in agriculture. Additionally, there is a need for more longitudinal studies to assess the long-term impact of these interventions on women's livelihoods and the overall economic development of rural areas. Policymakers and development organizations should also prioritize gender-sensitive policies and programs that address the unique challenges faced by women in agriculture, including cultural barriers, economic constraints, and limited access to education and training.

Conclusion

In conclusion, this study highlights the significant challenges faced by women farmers in Afghanistan, including cultural and economic barriers, limited access to land, and insufficient institutional support. These challenges are deeply rooted in societal norms and systemic inequalities, which restrict women's participation in agriculture and hinder their ability to achieve financial independence. However, the study also identifies substantial opportunities for empowering women through targeted interventions, such as community-based solutions, microfinance programs, and financial literacy training. By fostering collaboration through women's agricultural groups and cooperatives, it is possible to unlock the untapped potential of women farmers and promote gender equality in the agricultural sector. The findings of this study underscore the need for a holistic approach to addressing the challenges faced by women in agriculture, one that combines policy reforms, community-based initiatives, and partnerships with NGOs and international organizations. Increased awareness and implementation of supportive policies, alongside investments in rural infrastructure and technology, can further catalyze progress in creating a more inclusive and productive agricultural sector. Addressing these challenges will not only enhance women's livelihoods but also contribute to the overall economic and social development of Afghanistan. Future research should continue to explore the impact of specific interventions on women's participation in agriculture, with a focus on long-term outcomes and the broader implications for rural development.

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Data Availability statement
The authors confirm that the data supporting this study are cited in the article.

Conflict of interest
The authors declare no conflict of interest.

Authors' contributions
The authors confirm contribution to the paper as follows: Study Conception and Design: Yahya, M. A., Yaqooby, B., data collection: Afshar, A., Hakimi, M., Analysis and Interpretation of Results: Yahya, M. A., Draft Manuscript Preparation: Razmju, F., Yaqooby, B. All authors reviewed the results and approved the final version of the manuscript.

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Research article

Analysis of SATB1 And SATB2 Expression in The Mouse Model of Chemically Induced Skin Carcinogenesis

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ABSTRACT
Special AT-rich sequence binding proteins (SATB) 1 and the closely related SATB2 have been proposed to act as genome organizers that regulate chromatin structure and gene expression by recruiting chromatin remodeling/modifying enzymes and transcription factors to genomic DNA. Despite the fact that the changes in the expression levels of SATB1 and SATB2 were shown to be associated with tumor growth and metastasis development in various cancer cells such as lymphoma, colorectal and breast cancer cells, the potential role of SATB1 and SATB2 gene activity in tumors of the skin is still unknown. In the present study, SATB1 and SATB2 expression levels were investigated in mouse skin at early and middle stages of chemically induced carcinogenesis by quantitative RT-PCR analysis. Here, it was found that both SATB1 and SATB2 were down-regulated during the middle stage (papillomas) of skin carcinogenesis. Furthermore, the comparison of the relative expression levels of SATB1 to SATB2 has shown that SATB2 has a greater down-regulation in the middle stage of skin carcinogenesis. These data provide a fundamental knowledge and insight about SATB1 and SATB2 association with the skin carcinogenesis by determining and comparing their relative gene expression levels.

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Introduction

Mammalian skin is composed of at least three differentiating epithelial compartments: the epidermis, the hair follicle and the associated glands (e.g. sebaceous glands) [1]. The growth of epidermis and sebaceous glands is continuous, while hair growth is cyclic [1]. In all continually renewing tissues, a stem cell population is present to provide a source of differentiating cells [2]. In mouse skin, an epithelial stem cell population is thought to localize to the bulge region of the hair follicle at the arrector pili muscle attachment site, the segment that does not undergo regression during the hair cycle [3]. It has been shown that the hair follicles play an important role in mouse models of chemically induced skin carcinogenesis [4] and topical application of a carcinogen at specific phases of the hair cycle results in striking differences in tumor formation [4].

Tissue development and homeostasis are controlled by establishing specific gene expression programs in defined populations of stem cell progeny. Epidermal stem cells have been classically characterized as slow-cycling, long-lived cells that reside in discrete niches in the skin [1]. Stem cells (SCs) in the epidermis have a crucial role in maintaining tissue homeostasis by providing new cells to replace those that are constantly lost during tissue turnover or following injury [5]. Different resident skin stem cell pools contribute to the maintenance and repair of the various epidermal tissues of the skin, including inter-follicular epidermis, hair follicles and sebaceous glands. Interestingly, the basic mechanisms and signaling pathways that orchestrate epithelial morphogenesis in the skin are reused during adult life to regulate skin homeostasis [5]. Adult SCs may be the initial target cells, as they self-renew for extended periods of time, providing increased opportunity to accumulate the mutations required for cancer formation [6].

Interestingly, cancer stem cells derived from epidermal tumors exist independent of the classic skin stem cell niche, yet also have stem cell properties [7]. Epidermal cancer stem cells are thought to be rapid-cycling cells and exist in the absence of normal niche signals, yet it has been postulated they still retain the capacity for multi-lineage differentiation [7]. New evidence suggests that in the mouse, cancer stem cells are reliant on expression of the cell surface antigen, CD34, and intact Wnt/β-catenin signaling [7]. Evidence suggests that

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the p63 and c-Myc networks are likely to be involved [7]. Human cancer stem cells are thought to express high levels of the cell surface marker, CD44, but have reduced levels of Lrig1 and MAP4, two proteins that putatively regulate quiescence in normal human epidermal stem cells [7].

Special AT-rich sequence-binding protein 1 (SATB1) is a nuclear protein that functions as a genome organizer essential for proper T-cell development and expression of the Th-2 cytokine locus [8]. SATB1 is the most well-characterized matrix attachment region (MAR) binding protein (MBP) that participates in the maintenance and compaction of chromatin architecture by organizing it into distinct loops via periodic anchoring of MARs to the nuclear matrix [9]. SATB1 constitutes a functional nuclear architecture that has a 'cage-like' protein distribution surrounding heterochromatin. This architecture is referred to as 'the SATB1 regulatory network', as SATB1 regulates gene expression by recruiting chromatin remodeling or modifying enzymes and transcription factors to genomic DNA, which it tethers via base-unpairing regions (BURs) [10]. SATB1 also acts as a 'docking site' for several chromatin modifiers including ACF, ISWI, and HDAC1 [10] and these chromatin modifiers were suggested to suppress gene expression through histone deacetylation and nucleosome remodeling at SATB1-bound MARs [10].

Special AT-rich sequence-binding protein 2 (SATB2) has high structural similarity to SATB1 and can also bind to AT-rich DNA sequences [11] and interact with chromatin remodeling complexes [12]. Mouse studies have shown that SATB2 has a key role in B-cell differentiation [13], in osteoblast differentiation [14], in the establishment of neural corticocortical connections across the corpus callosum [15], in the developing jaw and incisors [16] and in developmental regulation of neuronal differentiation [11]. Recently, a loss-of-function study in the mouse has demonstrated that SATB2 is essential for proper facial patterning of the embryo and for normal bone development [11, 14] and also the Inactivation of the SATB2 gene by homologous recombination leads to perinatal lethality because of the multiple cranio-facial abnormalities [17].

The nuclear matrix (NM) is an elusive structure according to many studies and its existence and structure has long been questioned [18]. It has been described as a dynamic sponge with open compartments for free diffusion in the nucleoplasm [19] and also it has been argued that the NM structure is an artefact generated by the sample preparation [20]. Perhaps the NM can best be described as a structural component inside the nucleus, to which chromatin binds via matrix (or scaffold) attachment regions of the DNA forming looped chromatin structures [21]. These specialized genomic sequences possessing high affinity for the nuclear matrix and also for various chromatin architectural proteins are termed matrix attachment regions (MARs) and are utilized in a selective and dynamic manner to tether chromatin loops in vivo [22]. MARs have been implicated in the regulation of transcription by altering the organization of eukaryotic chromosomes and augmenting the potential of enhancers to act over large distances [22]. They are frequently located at the boundaries of transcription units where they are likely to delimit the ends of the active chromatin domains in terms of transcription as well as replication [21]. The MAR sequences commonly contain regions where base pairs tend to break under an unwinding stress (base-unpairing region (BUR)), which is important in binding to the nuclear matrix [23]. Chromatin architecture plays an important role in the regulation of nuclear function [24]. Since the chromatin structure and dynamics are crucial in gene regulation, miss-regulation of gene expression caused by the defects in chromatin remodeling leads to numerous diseases including cancer [24].

SATB1 is not expressed in all cells and it seems particularly important in cells that must change their function – as many progenitor cells do, including the thymocytes that turn into T cells, and as cancerous cells must do to turn into metastatic cells [25]. In most recent studies, it was shown that SATB1 is over-expressed in breast cancer cells and it coordinates expression of a large number of genes to induce metastasis [26]. SATB1 was also found to be highly expressed in multidrug-resistant breast carcinoma cell lines and tissues with P-glycoprotein (Pgp) over-expression [27]. Removal of SATB1 from aggressive breast cancer cells not only reverse metastatic phenotypes but also inhibit tumor growth, indicating its key role in breast cancer progression [26]. SATB1 is overexpressed in many cancer cells, such as colorectal cancer cells where it is associated with tumor progression, invasion, and metastasis [27]. In lymphomas, SATB1 plays a role in T-cell development and differentiation, particularly in aggressive subtypes like T-cell lymphomas. Its expression is linked to increased proliferation and survival of malignant lymphocytes [28]. SATB1 also correlates with high expression of tumor necrosis factor (TNF)-a, which mediates the inflammatory processes in tumor invasion, angiogenesis, and metastasis [27]. On the other hand, SATB1 influences the expression of anti-inflammatory interleukin (IL)-4 and IL-10, which in turn are known to lead to escape from cancer immune surveillance [28]. SATB1 also suppresses the apoptosis rate, which is known to allow cells to escape from chemotherapy [27].

It has been also found that low expression of SATB2 is correlated with tumor progression and poor prognosis in patients with colorectal cancer (CRC), suggesting that SATB2 is a novel potential prognostic marker for CRC [29]. In osteosarcoma, SATB2 promotes osteogenic differentiation, and its expression is linked to tumor progression and aggressiveness by enhancing cell proliferation and invasion [30]. Similarly, in prostate cancer, SATB2 is implicated in tumor progression and resistance to therapy possibly through regulating genes involved in cell survival and metastasis [31]. Additionally, in Gastric and Esophageal Cancers, SATB2 downregulation is associated with poor prognosis and increased tumor invasiveness [32].
While high SATB1 expression is linked to tumor growth and metastasis in breast cancer, colorectal cancer, and lymphoma, its role in other tissues, including skin, remains unknown. Similarly, the role of SATB2 in most tumors is also still remains unclear [26]. Therefore, the main aim of this study is the analysis of SATB1 mRNA expression and SATB2 mRNA expression at different stages of tumor development in the mouse model of chemically induced skin carcinogenesis using qRT-PCR technique.
Mouse skin has provided an excellent paradigm for studies of multistage chemical carcinogenesis in epithelial cells [33]. The most common chemical carcinogenesis regimen is two stage induction, which involves the administration of a single dose of the polycyclic aromatic hydrocarbon 7, 12 - dimethylbenz[a]anthracene (DMBA), followed by weekly applications of the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) [33]. This treatment results in the development of numerous benign papillomas, some of which progress to malignant squamous cell carcinomas in 20 to 40 weeks after the first exposure to carcinogens [33]. This common chemical carcinogenesis regime (two-stage induction) closely mimics the multistep process of human skin carcinogenesis, including initiation, promotion, and progression, making it a well-established model for studying tumor development and molecular changes in the skin. By inducing carcinogenesis in mouse skin, SATB1 and SATB2 expressions during the different stages of the tumor formation can be investigated to reveal their possible roles in skin cancer.

Material and Methods

Mouse skin sample collection, RNA extraction and Reverse transcription

All animal works were performed under the license of the University of Bradford (Bradford, UK) and the Institutional Animal Care and Use Committee protocol of Boston University (Boston, MA, USA). Skin samples were collected from neonatal C57BL/6 mice at postnatal days 12–23 (P12–P23), as well as from 8- to 10-wk-old adult mice. Skin was frozen in liquid nitrogen and embedded, as described elsewhere [34]. Chemically induced skin carcinogenesis mouse model has been previously described [34]. Mouse skin tissue collection at different stages of chemically induced skin carcinogenesis, total RNA extraction and nucleic acid quantity and quality analysis were performed as previously described by the manufacturers. Briefly, tissues were homogenized in a lysis buffer, and RNA was isolated using silica column-based purification, followed by on-column DNase treatment to remove contaminating genomic DNA. The quality and quantity of the RNA were assessed using a spectrophotometer and agarose gel electrophoresis. The cDNAs were synthesized using the same technique, as described below for the reference cDNA.

Reference cDNA Template Preparation (Reverse Transcription)

Reference cDNA template preparation was performed by using the two-step method. Total mouse embryonic RNA at E 19.5 was reverse transcribed to cDNA using the following cycling conditions in the thermal cycler:

Table 1 Cyclic conditions for cDNA synthesis

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25ºC	37ºC	85ºC	4ºC
Time	10 minutes	120 minutes	5 minutes	hold

High-Capacity cDNA reverse transcription kit 200 reactions without RNAse inhibitors and with random primers by Applied Biosystems was used to make the master mix. The master mix was prepared for 5 reactions and each reaction had 10µl of the 2X reverse transcription master mix and 10µl of the RNA sample (2µg/10µl). All preparations were done on ice and all solutions were centrifuged briefly during the experiment.
After the reverse transcription, the synthesized cDNA was amplified by regular PCR. Eppendorf licensed and authorized PCR machine was used and the amplification was performed by following the cyclic conditions:

Table 2 Cyclic conditions for cDNA amplification

Steps	Temperatures	Times	Number of cycles
Initial Denaturation	94ºC	3 minutes	1 cycle
Denaturation	94ºC	30 seconds	35 cycles
Annealing	58ºC	10 seconds	
Extension	72ºC	30 seconds	
Final Extension	72ºC	3 minutes	1 cycle
Soak	4ºC	indefinite	1 cycle

Reagents (Promega) with GAPDH reverse and forward primers were used to make master mix for 10 reactions. Taq polymerase was added last to the master mix, each component was mixed and centrifuged briefly and all preparations were done on ice. Each reaction had 24.5µl of the master mix and 0.5µl of the template. 3 different templates were used for 3 different reactions; water control, reference RNA control (1µg/µl) and the reference cDNA.

Annealing Temperature Optimization for the Real Time PCR

Annealing temperature optimization reaction was performed in BIO-RAD, MyiQ, Single Color Real Time PCR machine. Perfecta SYBR Green Supermix for IQ (VWR) was used as a Pre-formulated real-time PCR master mix. Commercially available software program Beacon Designer was used to perform both primer design and amplicon selection for SATB1 and SATB2 (Appendix I). Two different master mixes were prepared for SATB1 and SATB2 primer pairs with adding water and the reference cDNAs with the SYBR Green supermix. Each PCR plate well had 10µl of this master mix. After the loading, the plate was sealed with the BIO-RAD microseal® 'B' Film PCR sealer and centrifuged for 2 minutes at 4000rpm. qPCR reaction was run by using the iCycler iQ® system on the qPCR instrument and the temperatures B (65.9), C (63.8), D (60.8), E (56.5), and F (53.4) were used to find the annealing temperature.

Melting-curve analysis along with the Agarose gel electrophoresis was used to check the specificity of this real time PCR amplification reaction and to identify any additional non-specific products and Primer-dimers. 26 PCR cycles were chosen for melting-curve analysis because they are sufficient to amplify the target DNA to detectable levels without over-amplifying, which could lead to non-specific products or primer-dimers. This cycle number typically ensures that the reaction is in the exponential phase of amplification, where each cycle produces a measurable increase in target DNA. Therefore, 26 is a common choice for balancing amplification efficiency and specificity for melting-curve analysis. Each well on the agarose gel had 8µl of the samples from the PCR plate and 2µl of the loading buffer. 5µl of the molecular weight marker was used to identify the sizes of the DNA fragments in the gel.

Assay Performance Evaluation Using Dilution Series

2-Fold dilution series from the reference cDNA tube, which had 6µl of the cDNA and 54µl of water, were prepared to make the dilution factors of 1, ½, ¼, 1/8 and 1/16. Two different master mixes were prepared for SATB1 and SATB2 with the SYBR Green supermix, forward and reverse primers and water, similarly to the description above for annealing temperature optimization. Each master mixes were prepared for 12 reactions. Each well on the PCR plate had 2µl of the cDNA dilution and 8µl of the master mix. Duplicate samples were used in order to make a statistical analysis of the experiment and to perform a performance evaluation of the assay. A Control reaction also included to the PCR plate with adding water to the master mix. The determined annealing temperature from the optimization reaction above was used and the real time PCR was performed by following the cyclic conditions:

Table 3 Cyclic conditions for Real Time PCR

Steps	Temperatures	Times	Number of cycles
Initial Denaturation	95ºC	3 minutes	1 cycle
Denaturation	95ºC	10 seconds	40 cycles
Annealing	56.5ºC	30 seconds	
Extension	72ºC	15 seconds	

Melting curve analysis	65ºC - 95ºC	30 seconds	26 cycles

The standard curves were constructed by plotting the dilution factor against the threshold cycle (CT) value obtained during amplification of each dilution. Then, the linear regression lines were constructed by plotting the log2 of the dilution factors against the threshold cycle (CT) value obtained during amplification of each dilution. The equation of the linear regression line along with the coefficient of determination and the amplification efficiency equation were then used to evaluate whether the qPCR assay was optimized.

Gene Expression Analysis with the qRT-PCR

After the optimization of qPCR conditions by using the reference cDNA, and after the assay performance evaluation, the relative expression levels of SATB1 and SATB2 at different stages of the mouse skin carcinogenesis were measured by qRT-PCR. The experiment was performed twice in duplicates in order to determine if the data was reproducible. In the first experiment, three different conditions (three different cDNAs) were used: 12 days after the depilation (late anagen skin) as a control, early carcinogenesis (15 weeks after the initiation of carcinogenesis) and middle carcinogenesis (25 weeks after). In the second experiment, the 3 days cDNA after the depilation (early anagen skin) was also added as a second control to the above conditions. In this study, the GAPDH gene was used as housekeeping gene for normalization. SATB1, SATB2 and GAPDH primer pairs were used in each experiment and the master mixes were prepared for these each pair of primers as described previously. For experiments 1 and 2, the master mixes were prepared for 18 and 24 reactions (addition of the early anagen control) respectively. Each well had 8µl of the master mix and 2µl of the cDNA template. The same cyclic conditions of the dilution series experiment above were used. Depending on both practical and scientific considerations, the gene expression analysis was performed as a singleplex assay and DNA-binding dye SYBR Green 1 (BIO-RAD) was used.

Agarose Gel Electrophoresis

1.5% Agarose gel was prepared with TAE buffer and the products of the regular PCR amplification were run at 100volts for 40 minutes to observe the synthesized and amplified reference cDNAs. 6µl of the Invitrogen 1Kb plus DNA Ladder (50ng/1µl) was used as a molecular weight marker. Each well had 20µl of the sample and after 40 minutes, the gel was stained with Ethidium bromide (0.5µg/ml). The gel documentation system (Ingenius Syngene Bioimaging) was used to document and analyze the agarose gel image.

Data Analysis

The relative quantification was performed by using the reference gene GAPDH. After determining the CT values of both the target and the reference genes, the relative expression levels of the target genes in the test samples (in each condition) were determined by using the 2–ΔΔCT (Livak) Method.

All statistical analyses were performed using SPSS software program version 17.0 (PASW Statistics). Data are presented as mean and standard deviation. The relative expression levels between the conditions were statistically analyzed using an independent two-sample t-test that does not assume equal variances (with a significance level at the $p \leq 0.05$ level).

Results

Reference cDNA synthesis and amplification

First, the reference cDNA was synthesized from total mouse embryonic RNA at E 19.5 to optimize the qRT –PCR assay conditions for SATB1 and SATB2. The quality of cDNA was checked by regular PCR using GAPDH specific primers. The gel electrophoresis analysis demonstrated the presence of one specific product of expected size (128 bp) confirming the good quality of the cDNA. The clear single bands can be observed in lanes B to E on the gel image below (Figure 1). These results indicate that the reference cDNA samples can be used for the annealing temperature optimization of SATB1 and SATB2 and also for the dilution curve experiment.

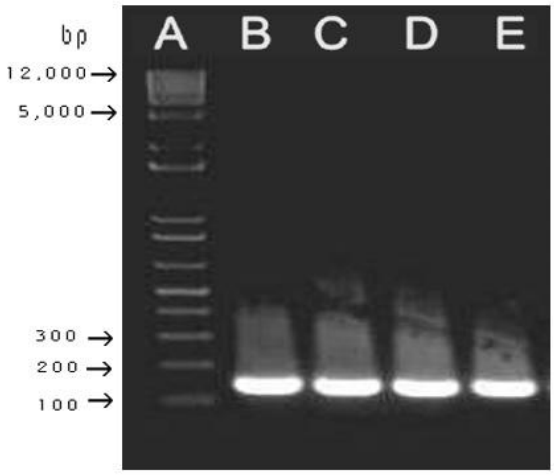


Fig 1 Reference cDNA synthesis and amplification. Lane A: Invitrogen 1Kb plus DNA Ladder, Lanes B to E: PCR products after the amplification of reference cDNA with GAPDH primers

Annealing Temperature Optimization for SATB1 and SATB2 qRT–PCR Assay

Annealing temperature was chosen as the major parameter for PCR optimization. Amplification with SATB1 and SATB2 primers was tested at five different annealing temperatures (0C): B (65.9), C (63.8), D (60.8), E (56.5), F (53.4). Table 1 below shows the Threshold Cycle (CT) values for the SATB1 and SATB2 primers at each temperature.

Table 4 CT values of SATB1 and SATB2 at all temperatures during the amplification of the optimization

Temperature (^{0}C)	Identifier	Threshold Cycle (Ct)	Set Point
B	SATB1	25.43	65.87
B	SATB2	N/A	65.87
C	SATB1	24.63	63.849
C	SATB2	36.56	63.849
D	SATB1	24.33	60.758
D	SATB2	23.56	60.758
E	SATB1	23.90	56.462
E	SATB2	22.86	56.462
F	SATB1	24.10	53.395
F	SATB2	22.92	53.395

These results indicate that the optimum annealing temperature is 56.50C (E) for both SATB1 and SATB2 primers. At the temperature 56.50C, SATB1 and SATB2 primers showed the lowest CT values indicating the highest amplification efficiency at this temperature. Appendix II contains the amplification graphs of the SATB1 and SATB2 at the temperature 56.5 and at all tested temperatures during the optimization process.

Melting-curve analysis along with the agarose gel electrophoresis was used to check the specificity of this real time PCR amplification reaction during the annealing temperature optimization.

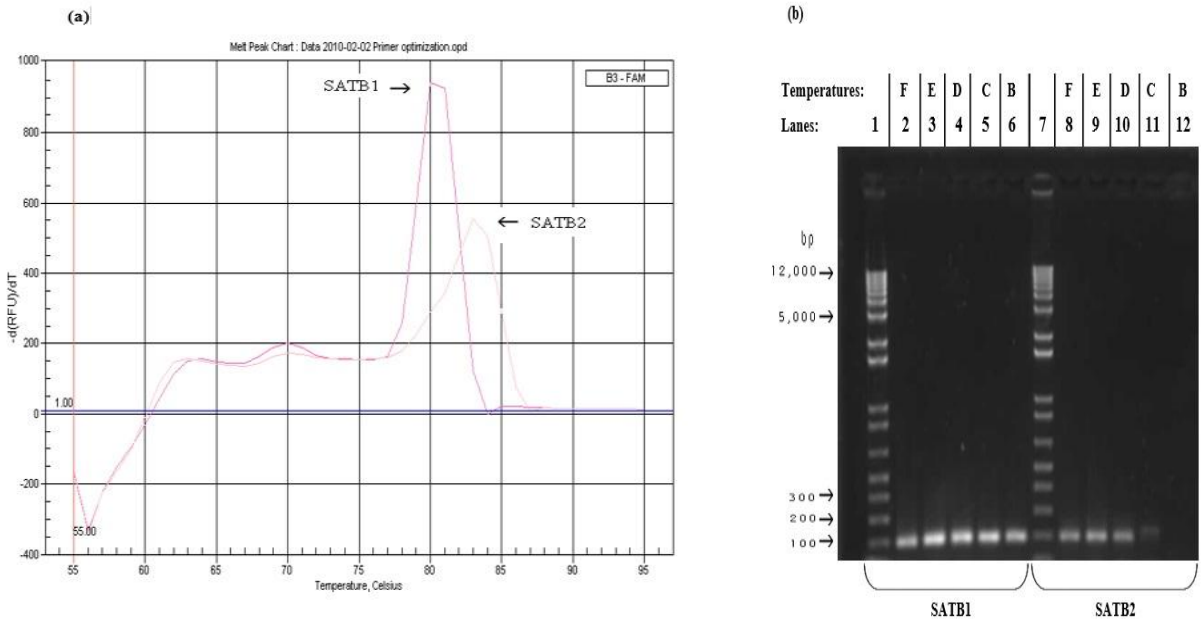


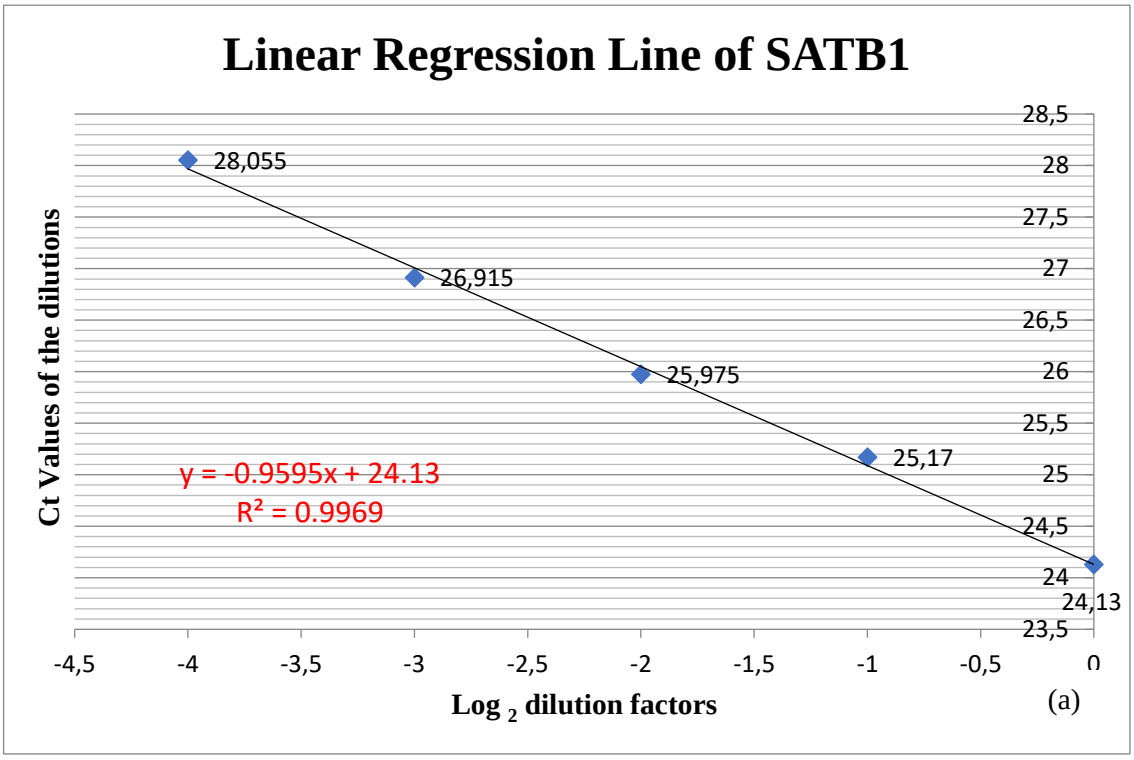
Fig 2 (a) Melting-curve analysis chart of SATB1 and SATB2 at 56.50C (Temperature E). (b) The Agarose Gel Electrophoresis of SATB1 and SATB2 PCR products at all temperatures with the molecular marker (Lane 1 and 7) Invitrogen 1Kb plus DNA Ladder (5µl/lane).

The melting-curve analysis of the SATB1 and SATB2 at the annealing temperature 56.60C has shown only one peak for each primer pairs demonstrating the high specify of the amplification. The agarose gel electrophoresis also confirmed the presence of the single specific product for each primer pair, of expected sizes (184 bp for SATB1 and 188 bp for SATB2). These results demonstrated that the melting curve analysis was consistent with the agarose gel electrophoresis and there were no contamination or non-specific products during the PCR amplification. The annealing temperature of 56.60C was chosen for SATB1 and SATB2 primer pairs based on maximal amplification efficiency and specificity.

Assay Performance Evaluation

2-Fold dilution series of the reference cDNA were made to prepare the mixtures with the dilution factors 1 (undiluted), 1/2, 1/4, 1/8 and 1/16. The amplification CT values were obtained in each dilution factor for SATB1 and SATB2 at the end of the real time PCR (Appendix III). The mean value of the duplicate samples at each dilution were calculated and the standard curves were constructed for SATB1 and SATB2 by plotting the dilution factor against the threshold cycle (CT) value obtained during amplification of each dilution (Appendix IV).

By using these standard curves, the linear regression lines were constructed, by plotting the log2 of the dilution factors against the threshold cycle (CT) value obtained during amplification of each dilution (Figure 3). After plotting the linear regression lines, the coefficient of determinations (R2), Slopes and amplification efficiencies were calculated (Appendix V). For SATB1 assay, the R2 was 0.9969 and the amplification efficiency was 94.6%. For SATB2 assay, R2 was 0.9984 and the amplification efficiency was 104.6%. The parameters of the dilution curves are in the range considered suitable for the SATB1 and SATB2 assays. These results indicate that the optimized assay conditions can be used to determine the SATB1 and SATB2 mRNA expression levels in the experimental samples.



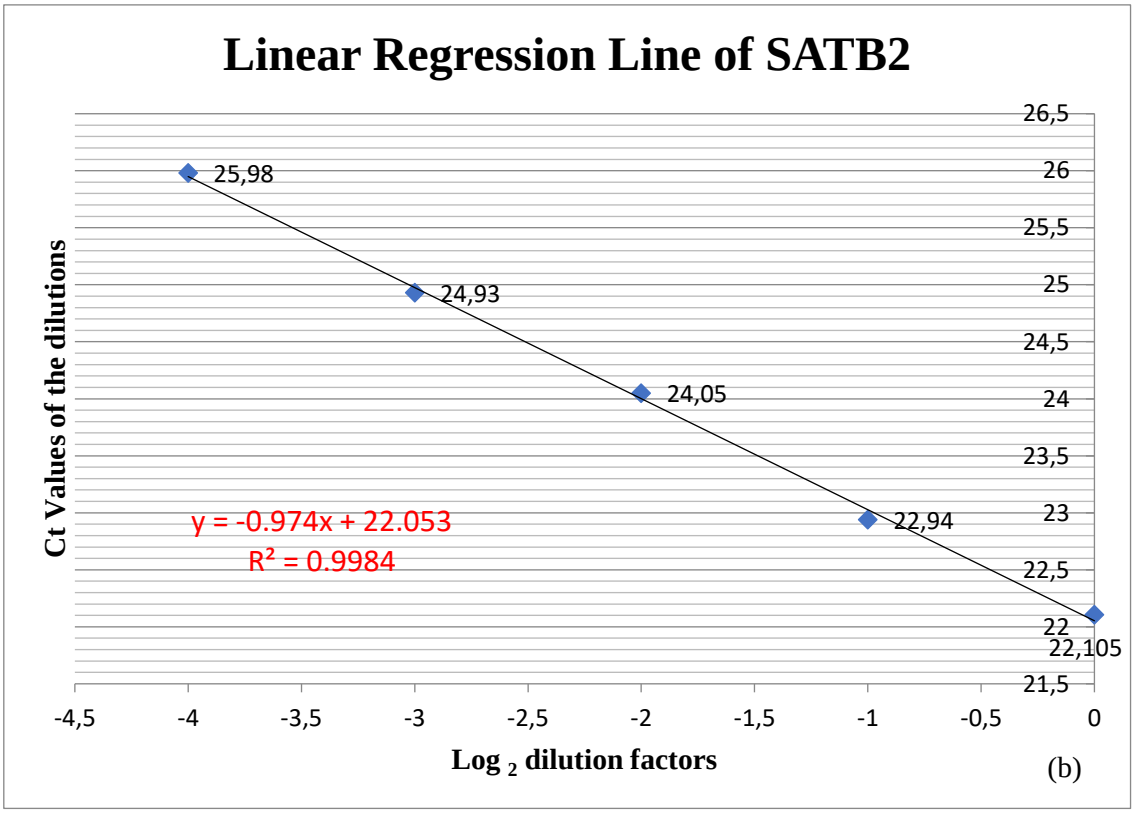


Fig 3 (a) Linear Regression Line, formula and R2 value of SATB1 (b) Linear Regression Line, formula and R2 value of SATB2.

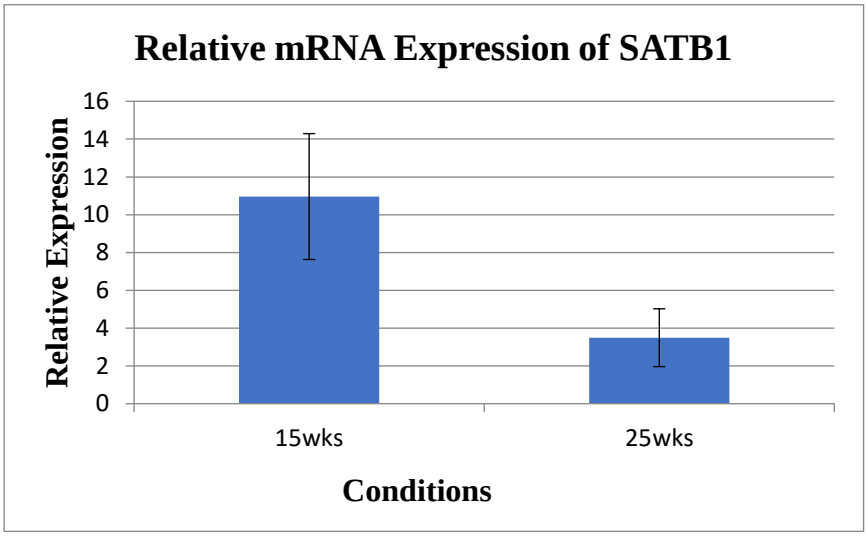
SATB1 and SATB2 Expression Analysis in Mouse Skin at Early and Middle Stages of Chemically Induced Carcinogenesis

The expression levels of SATB1 and SATB2 mRNAs were determined by quantitative RT-PCR. The experiment was performed twice in duplicates with GAPDH as the housekeeping gene and the amplification CT values were obtained from both experiments (Appendix VI). These CT values demonstrated that the cDNAs for the control conditions had poor quality. Therefore, the comparison between SATB1 and SATB2 expression levels at early (hyperproliferative epidermis) and middle (papillomas) stages of skin carcinogenesis was performed.

Figure 4 below was constructed by combining the results from both experiments. It shows the relative expression levels of the SATB1 and SATB2 mRNAs at early and middle stages of the skin carcinogenesis. The bars in Figure 4 represent the mean of duplicate samples from both experiments and the error bars were constructed by calculating the standard deviation of these samples.

These results demonstrated that in comparison to the early stage of skin carcinogenesis, both SATB1 and SATB2 mRNA expressions are lower in the middle stage of skin carcinogenesis. Comparing to SATB1, a stronger down-regulation of the SATB2 in the middle stage of skin carcinogenesis was also observed.

(a)



(b)

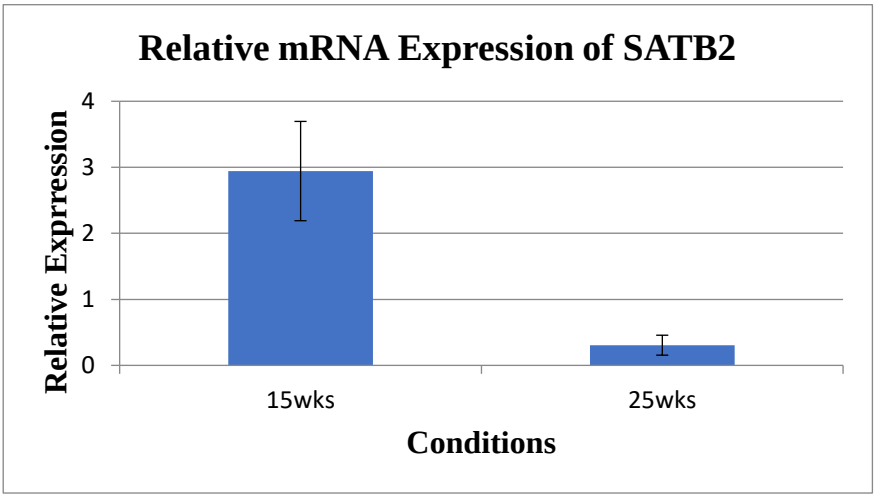


Fig 4 (a) The relative expression levels of SATB1 at early (15 wks) and middle (25 wks) stages of skin carcinogenesis. (b) The relative expression levels of SATB2 at early (15 wks) and middle (25 wks) stages of skin carcinogenesis.

The two-sample F-test for variances was used to test the equality of variances (Appendix VII). It was determined that the variances of the two groups in SATB1 expressions and in SATB2 expressions were significantly different, p=0.0288 and p=0.0002 respectively. Therefore, the relative expression levels between the experimental conditions were statistically analyzed using the independent two-sample t-test that does not assume equal variances.

The statistical analysis confirmed the significant downregulation of SATB1 ($p = 0.0001$) and SATB2 ($p = 1.05 \times 10^{-5}$) expression in papilloma-containing skin compared to hyperproliferative skin in the mouse model of chemically induced carcinogenesis (Appendix VIII).

These results show a significant downregulation of SATB1 and SATB2 mRNA expression in the middle stage of skin carcinogenesis compared to the early stage.

Conclusion and Discussion

The findings of the present study demonstrate the SATB1 and SATB2 expression levels in mouse skin at early and middle stages of chemically induced carcinogenesis. It was determined that there was a down-regulation of SATB1 and SATB2 during the middle stage of (papillomas) skin carcinogenesis. Results of the statistical analysis demonstrate a significant difference in the expression levels of SATB1 and SATB2 between the stages of early and middle skin carcinogenesis. The comparison of SATB1 to SATB2 in the expression levels has shown that SATB2 has a greater down-regulation in the middle stage of skin carcinogenesis.

In contrast to the present study, a previous report suggested high-expression levels of SATB1 in metastatic breast cancer cells and in multidrug-resistant breast carcinoma cell lines [26]. However, the findings of the present study are consistent with the previous report that indicates normal SATB1 expression levels in other many cancer cells [27]. In the present study, results obtained from measurements of SATB2 expression are consistent with the previous report that suggests a relationship between low-expression levels of SATB2 and tumor progression in colorectal cancer [17]. However, significantly higher levels of SATB2 mRNA expression were determined in breast cancer specimens and expression was associated with increase tumor grade [35].

Since the potential role of SATB1 and SATB2 gene activity in tumors of the skin is unknown, this study provides a fundamental knowledge about their association with skin cancer by measuring the expression levels during the different stages of the skin carcinogenesis. However, this study only demonstrates the expression levels of SATB1 and SATB2 at early and middle stages of skin carcinogenesis. Since the data for the expression levels of SATB1 and SATB2 in the normal skin cells is lacking, a confident comparison of SATB1 and SATB2 expression levels in mouse skin carcinogenesis to expression levels in normal mouse skin cells can not be stated. Although this result was not achieved, it can be speculated that both SATB1 and SATB2 were not found to be over-expressed during this study.

Due to the time and resource limitations, the late stage of skin carcinogenesis (carcinomas) wasn't added to the testing conditions. Future research should include appropriate controls such as early and late anagen skin samples along with a telogen skin sample and also the late stage of skin carcinogenesis and metastatic tumors conditions should be included to measure the expression levels at all different stages and make a more reliable conclusion and comparison. Also, the SATB1 and SATB2 gene expression analysis was performed using only the qRT-PCR method with SYBR Green Dye. Therefore, the analysis of the gene expression could be also performed with qRT-PCR with TaqMan probes to increase the specificity of the detection during the expression analysis. Other methods such as Northerring blotting or Tag based serial analysis of gene expression (SAGE) along with the qRT-PCR method could be also used for the gene expression analysis.

The future aspect of this study would be the extension in variety of the techniques used during the experiments of the present study. These techniques can be used to reveal the roles of SATB1 and SATB2 in normal and cancer mouse skin cells by studying their functions, structures, locations in the nucleus and their interactions with the transcriptional factors and chromatin modifying molecules. Such studies would provide a comprehensive knowledge about SATB1 and SATB2 genome-wide targets and about the various factors recruited by SATB1 and SATB2. This progress would further enhance our understanding of the intricate relationship between chromatin architecture and genome function [36].

There is growing evidence that the transcriptional activity of genes might be influenced by nuclear organization [37] and as mentioned before SATB1 has a structure that provides the ability to bind to AT-rich DNA sequences and interact with chromatin remodeling complexes. It functions as a genome organizer and

regulates gene expression by recruiting chromatin remodeling/modifying enzymes and transcription factors to genomic DNA, which it tethers via BURs. It has been shown that a variety of genes involved in many aspects of tumorigenesis are regulated by SATB1, indicating that a large group of SATB1-targeted genes collectively induce tumor growth and metastasis [26]. According to these findings, high expression levels of SATB1 can be associated with carcinogenesis by causing the accumulation and binding of these transcriptional factors and chromatin modifying enzymes to the targeted DNA sites.

Few studies have investigated the role of the SATB2 gene in skin carcinogenesis. The present study raises the intriguing possibility that the observed downregulation of SATB2 during the middle stage of skin carcinogenesis may contribute to tumor development, similar to its role in colorectal cancer (CRC), where its reduced expression correlates with tumor progression and poor prognosis [17]. In CRC, SATB2 downregulation has been linked to increased tumor invasion, lymph node metastasis, distant metastasis, and advanced Dukes' classification [17]. The stronger downregulation of SATB2 in skin carcinogenesis suggests that its loss may play a critical role in disrupting normal epidermal differentiation and promoting a more aggressive tumor phenotype. SATB2 is known to function as a chromatin organizer, regulating gene expression programs essential for cell identity and differentiation. A significant decrease in SATB2 expression may lead to widespread epigenetic changes that favor tumor progression by suppressing differentiation-associated genes while upregulating pathways involved in proliferation, invasion, and survival.

Moreover, given that chromatin remodeling is increasingly recognized as a key regulatory mechanism in cellular differentiation [38], the downregulation of both SATB1 and SATB2 in this study may reflect a broader disruption of differentiation pathways in skin carcinogenesis. A previous study suggested that SATB2 can inhibit SATB1's effects, indicating potential regulatory crosstalk between these two chromatin remodelers [17]. This raises the possibility that the loss of SATB2 may enhance SATB1-driven oncogenic programs or lead to an imbalance in gene expression networks critical for maintaining normal epithelial homeostasis.

Furthermore, SATB1 and SATB2 play crucial roles in regulating gene expression in embryonic stem cells, where their relative levels help balance self-renewal and differentiation [35]. Thus, the significant downregulation of SATB2 observed in this study may contribute to a shift toward a less differentiated, more tumorigenic state in skin carcinogenesis. Further investigations are needed to determine the precise molecular mechanisms through which SATB2 loss influences tumor progression and whether restoring its expression could have therapeutic potential in skin cancer.

Genetic studies in mice have identified multiple signaling pathways and transcriptional regulators that are essential for proper epidermal stratification, acquisition of the skin barrier function [6], maintenance, differentiation and lineage commitment of the epidermal stem cells (SCs) [7]. These pathways involve Notch, Wnt/β-catenin, Hedgehog, c-Myc, mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB) and the transcriptional regulators p63 (which is related to p53), the AP2 family, the CCAAT/enhancer-binding protein (C/EBP) transcriptional regulators, interferon regulatory factor 6 (IRF6), grainy head-like 3 (GRHL3) and Kruppel-like factor 4 (KLF4) [39]. These signaling pathways regulate the functions of skin epithelial SCs, and deregulation of these signaling pathways leads to the development of cancer in various tissues [6]. An important question for the future is whether adult skin SCs are the only cells that sufficiently accumulate mutations to cause cancer and whether the SATB1 and SATB2 play an important role in this process. This hypothesis is attractive because adult SCs exist and proliferate for a long time. Also, they do not protect their genome by asymmetric chromosome segregation [40], providing further opportunities to accumulate oncogenic mutations and potentially induce cancer formation.

SATB1 and SATB2 have been extensively studied in various cancers, highlighting their roles in tumor progression and differentiation. As mentioned before, SATB1 is frequently overexpressed in breast and colorectal cancers, where it promotes metastasis, epithelial-mesenchymal transition (EMT), and tumor cell survival [41]. In lymphomas, SATB1 influences T-cell differentiation and proliferation [41]. Conversely, SATB2 often acts as a tumor suppressor in colorectal cancer but has oncogenic roles in osteosarcoma and certain breast cancer subtypes [42]. These findings suggest that SATB1 and SATB2 may contribute to skin carcinogenesis through similar mechanisms, such as chromatin remodeling, transcriptional regulation of differentiation pathways, and modulation of the tumor microenvironment. Further studies are needed to elucidate their precise roles in skin cancer progression and therapeutic resistance.

In the present study, the common chemical carcinogenesis regime (two-stage induction) was used to promote the skin carcinogenesis in the mouse model. Current information suggests that skin tumor promoters are not mutagenic but bring about a number of important epigenetic changes, such as epidermal hyperplasia and increase in polyamines, prostaglandins and dark basal keratinocytes [43]. The generality of the two-stage

system of inducing tumors has been shown to exist in a number of experimental carcinogenesis systems, such as liver, lung, colon, stomach, bladder and pancreas. However, it is not presently known if other experimental carcinogenesis systems and the induction of human cancer involve a series of stages similar to that in the mouse skin [43].

One subject that remains to be explored is how SATB1 and SATB2 expression levels are associated with the tumor growth and metastasis in various tissues including the skin. Despite the recent interests to SATB1 and SATB2 gene activity, the structural and functional properties of these special AT-rich sequence-binding proteins and their possible roles in various cancer cells are still not fully understood. Since the association of these proteins with cancer is a relatively new field of study, further studies of the biological functions of SATB1 and SATB2 are needed to understand the mechanisms of occurrence and development of various cancers including skin cancer.

Abbreviations

SATB1: Special AT-rich sequence-binding protein 1, SATB2: Special AT-rich sequence-binding protein 2

RT-PCR: Reverse Transcription Polymerase Chain Reaction, SCs: Stem Cells, SP: Side-population, NM: Nuclear Matrix, MARs: Matrix Attachment Regions, BURs: Base-unpairing Regions, CRC: Colorectal Cancer, qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction, CT: Threshold Cycle

DMBA: 7,12-dimethylbenz[a]anthracene, TPA: 12-O-tetradecanoylphorbol-13-acetate, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase, SPSS: Statistical Package for the Social Sciences

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Data Availability statement

The author confirms that the data supporting this study are cited in the article.

Compliance with ethical standards

Conflict of interest

The author declares no conflict of interest.

Ethical standards

The study is proper with ethical standards.

Authors' contributions

During the study, VY conducted the research and wrote the article.

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Impact of Green Synthesis of Silver Nanoparticles on Antioxidant Activity in Drought-Sensitive and Drought-Tolerant *Pimpinella anisum* L.

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ABSTRACT
The rapid advancement of nanotechnology has raised concerns about nanoparticles (NPs) potentially entering the environment, particularly their effects on plants, which are vital to ecosystems. This study explores the effects of green-synthesized silver nanoparticles (AgNPs) on three important antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), and guaiacol peroxidase (GPOx). We examined two varieties of anise (*Pimpinella anisum* L.), specifically the drought-sensitive (DS) and drought-tolerant (DT) varieties. The objective was to assess how these two types of anise respond to stress caused by silver nanoparticles. Anise plants were exposed to silver nanoparticles (AgNP) at 1, 5, and 10 mg/L concentrations for 21 and 28 days. The activity of catalase (CAT) did not show significant changes across all concentrations and remained inactive. Only a significant increase in CAT activity was observed in the plants treated with 5 mg/L AgNPs after 28 days. Superoxide dismutase (SOD) activity significantly increased in the treated plants exposed to 5 mg/L AgNPs after 21 days. For guaiacol peroxidase (GPOx), the treated plants showed significant increases at both 1 mg/L and 5 mg/L AgNPs after 21 days, while other results varied and lacked statistical significance. Overall, the increased enzyme activity in DT anise at low AgNP concentrations suggests a low level of toxicity and indicates that these plants are more resilient to AgNP stress. These findings highlight the complexity of AgNP effects on antioxidant activity in anise and underline the need for further research on the long-term impacts of AgNP exposure, particularly concerning the preservation of drought-tolerant anise in the face of global warming

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Introduction

Nanoparticles have a long history, with Michael Faraday describing the optical properties of metallic nanoparticles in 1857 [1]. While the global nanotechnology market had a market share of 79.14 billion US dollars in 2023, the market is expected to be worth 91.18 billion US dollars in 2024 [2]. More than 1,000 commercial products on the market currently contain nanoparticles (NPs). Common NPs used in household, industrial, and healthcare products include Au (gold), Ag (silver), ZnO (zinc oxide), CuO (copper oxide), TiO2 (titanium dioxide), Fe3O4/Fe2O3 (iron oxides), and CeO2 (cerium oxide)[3]. In addition, incorporating Ag, ZnO, TiO, and SiO (silicon dioxide) NPs into agrochemicals has great potential in nanotechnology-based smart agriculture [4]. With the expansion of nanotechnology applications in various sectors, the possibility of NPs entering the environment as waste-containing nanomaterials increases, necessitating research on plant responses to NPs [5]. Silver nanoparticles have gained attention for their unique properties and diverse applications, including medicine and environmental therapy [6]. These nanoparticles exhibit antimicrobial properties, which makes them effective against microorganisms and have potential in drug delivery systems [7]. The exploration and understanding of metallic and natural nanoparticles have contributed to various fields, including nanotechnology and the use of plants such as *Pimpinella anisum* for their valuable properties [8]. *Pimpinella anisum* L.(anise), a member of the Apiaceae family, is a versatile aromatic plant with numerous historical uses. Its dried fruits, known as anise seeds, are popular as flavouring agents in confectionery and liqueurs [9]. Anise has been traditionally used to treat digestive disorders and possesses potential antimicrobial, antioxidant, and anti-inflammatory effects [10]. It

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exhibits analgesic, antispasmodic, anticonvulsant, and potential antidepressant-like activities [11]. Chemical analysis reveals bioactive compounds like anethole with antimicrobial and antioxidant activity [12]. Anise seed extract has been shown to have cytotoxic effects on cancer cells, like the chemotherapy drug cisplatin, and may have potential applications in nanotechnology. Despite their essential applications, nanoparticles are still being debated regarding their beneficial effects on plants on the one hand and their harmful and toxic effects on the other [13]. AgNPs inhibited Ribulose-1,5-bisphosphate carboxylase/oxygenase activity (Rubisco) and the protective ability of PSII in the higher aquatic plant model *Spirodela polyrhiza* L. [14]. In addition to lower photosynthetic rates, growth inhibition induced by NPs has also been associated with increased oxidative stress [15] However, whether the arrest of photosynthesis or the induction of oxidative stress is the dominant effect of NPs is a matter of debate, as they go hand in hand Although the accumulation of NPs in chloroplasts and damage to the photosynthetic apparatus supports the former [16], the fact that to reach chloroplasts, they must cross the plasma membrane, where they can catalyze reactive oxygen species (ROS) via NADPH oxidases [17]. ROS production, membrane structure and function damage, and antioxidant enzymatic activity fluctuations across plant species have been documented as typical responses to NPs [18]. However, some studies also showed that the treatment plants and photosynthetic microorganisms with NPs increased phenols' production, which may function as antioxidants to suppress ROS [19]. Because of the toxicity associated with nanoparticles remains a concern in terms of their effect on cellular enzymes such as superoxide dismutase, catalase, and Guaiacol peroxidase, which is critical [20] because these enzymes are essential for cellular defense against oxidative stress [21]. They act as antioxidants, preventing the formation of harmful free radicals and reactive species in cells [22]. Several toxicological studies have been performed to measure their levels [22]. Examining the functions and mechanisms for analyzing the effects of AgNPs (Silver Nanoparticles) on the enzymes CAT (Catalase), SOD (Superoxide Dismutase), and GPOx (Glycol Peroxidase) [23]. Overall, CAT, SOD, and GPOx are vital enzymes in the plant's antioxidant defense system [24]. When studying the effects of AgNPs on these enzymes, it is essential to assess how their activity and expression may be altered, as any disruptions to their functions could have significant implications for the plant's overall health and stress response [25]. Detoxification mechanisms to eliminate the toxic effects of AgNPs may differ from plant to plant. In this case, it is not easy to about conclude how different detoxification pathways are activated in response to different AgNPs conditions in different plant species [26]. These different results vary depending on the size, shape, exposure concentration, and amount of aggregation of AgNPs. Therefore, the physical and chemical properties of AgNPs and their effects on plant morphology, physiology, and biochemistry vary greatly depending on the plant type or variety [27]. Considering that the most important factors affecting the penetration of NPs into cells in the literature review are Np type, amount, and plant type, studying the effect of NP is essential for scientific studies [28]. In addition to all this information, the nanoparticle synthesis method is crucial in scientific studies [29]. Chemical methods used for silver nanoparticle synthesis can often lead to toxic solvents, high energy consumption, and the production of harmful by-products. It is important to use environmentally friendly methods for synthesizing silver nanoparticles (AgNPs) [30]. In our study, we synthesized AgNPs that are expected to have a circular shape and dimensions ranging from 10 to 100 nm, utilizing an environmentally friendly green synthesis method [31]. In the synthesis, we used *S. sclaera* leaves [32]. Whose optimization we know well. Anise is a highly valuable medical ingredient plant, and silver nanoparticles have shown promising potential in many applications. The study's results can advance our understanding of the effects of AgNPs on plant growth and their potential applications in agriculture and medicine. Our objective is to synthesize silver nanoparticles (AgNPs) and apply the synthesized product to calluses, followed by an evaluation of the antioxidant activity of these calluses. This research will investigate, for the first time, the effect of AgNPs synthesized from *Salvia sclarea* on the toxicity of calluses in *P. anisum* L. The summary of history is illustrated in Figure 1.

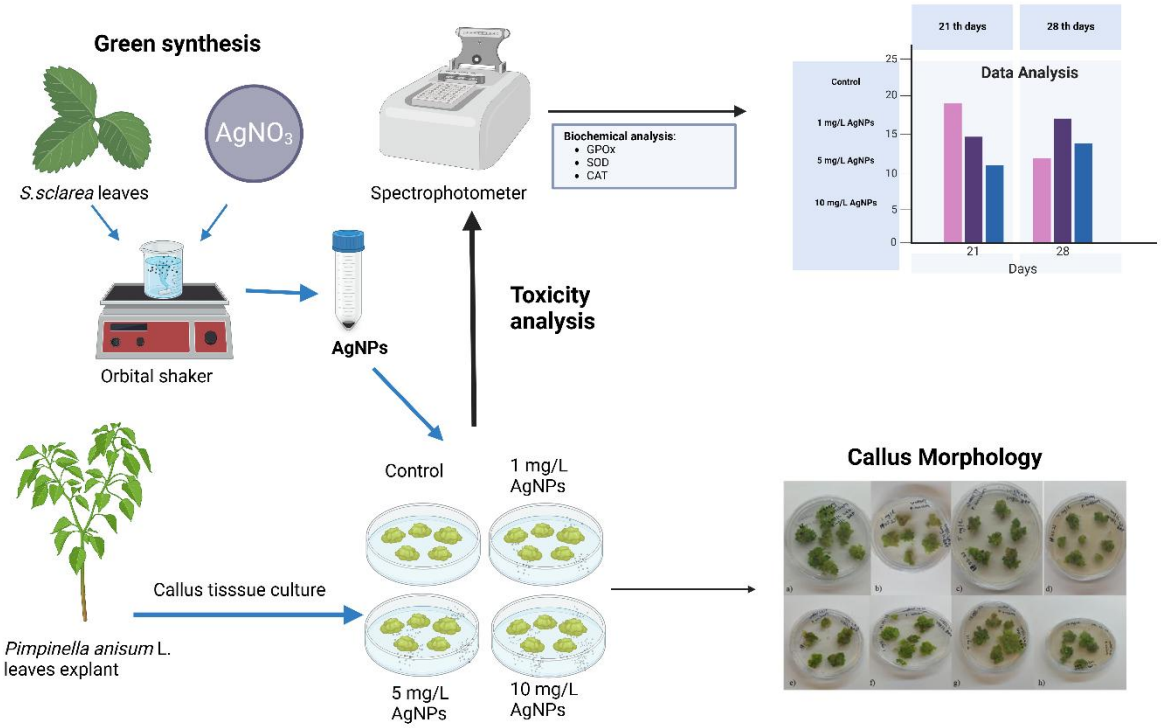

Fig.1 The flowchart summarizing the production of AgNPs through green synthesis, callus acquisition, and analysis is presented above. Created in BioRender. Ulusoy, E. (2025) https://BioRender.com/q83l859.

Material and Methods

Synthesis and Characterization of AgNPs.

Green Synthesis of AgNPs. Synthesis of Silver Nanoparticles (AgNPs) Using *Salvia* Leaf Extract: *Salvia* leaf extract was prepared by boiling 10 g of leaves in distilled water. (1 g:10ml) for 30 min and then filtering the mixture. The filtered extract was then added to 1M AgNO3 solution at a ratio of 1:9. The mixture was heated to 50˚ in a water bath and stirred continuously overnight for incubation. The solution was covered to prevent evaporation. The formation of AgNPs was indicated by the yellow to deep black of the solution. The obtained nanoparticles were precipitated by centrifugation at 10,000 rcf for 1 h. The preparation is illustrated in Figure 2.

Characterization of nanoparticles using scanning electron microscopy (SEM) and zeta potential measurements. The absorption spectra of the AgNP solution were recorded using a ThermoScientific-Genesys 180 UV-Vis spectrophotometer over a range of 300 to 600 nm, along with Zeta size measurements. Beginning with SEM, we deposited a colloidal silver nanoparticle solution onto a gold-coated substrate, allowing us to visualize particle morphology, size distribution, and surface features in detail (Malvern Zetasizer-NanoS, Cambridge, England). Concurrently, Zeta measurements and information on particle surface charge and stability were obtained by consulting the literature [33].

Pimpinella anisum Callus Production:

Seed Sterilization. The anise seeds used in this study were collected in 2022 from plants cultivated in the natural habitats near Kozagac village (37°05’84.0"N, 29°65’97.0” E, 15902 Cavdır, Burdur, Turkey). The seeds were provided by Durmuş EFE, a local breeder, who supplied two varieties: drought-sensitive (DS) and drought-tolerant (DT), the latter of which requires minimal irrigation for growth. Both DS and DT seeds were thoughtfully chosen to compare and understand a range of potential responses. *P. anisum* seeds were sterilized using a 25% bleach solution. A sterilization solution was prepared by mixing 50 ml of commercial bleach with 150 ml of distilled water. The seeds were subsequently submerged in the prepared bleach solution within the confines of a controlled laminar flow hood environment. Following this exposure, the seeds underwent a meticulous sterilization process through a sequence of three consecutive rinses utilizing sterile distilled water. Each rinse cycle was extended to a duration of 10 minutes, making sure that this rigorous rinsing protocol was executed meticulously [34].

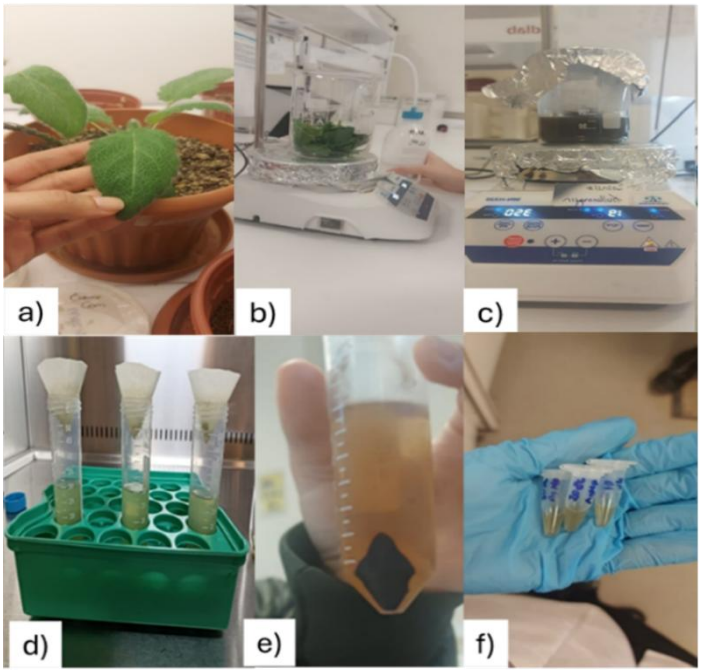

Fig. 2 a) *S. sclarea* grown in the Tissue Culture Lab at Yıldız Technical University (YTU). b) Preparation of *S. sclarea* leaves for extraction. c) AgNPs UV 400-467 nm d) Filtration of *S. sclarea* leaf extract. e) AgNPs after centrifugation. F) Eppendorf tube containing AgNPs.

***Callus formation*.** Fostering Natural Germination of *Anisum* Seeds. A growth hormone-free medium was prepared by dissolving 4.4 g of Murashige and Skoog (MS), 30 g of sucrose, and 7 g of agar in half the final volume of distilled water [35]. The pH of the solution was adjusted to 5,7. Following pH adjustment, the agar was added. The medium was autoclaved. The medium was poured into sterile Petri dishes within a laminar flow hood and allowed to cool until the point of seed placement—this hormone-free medium was composed without the addition of growth hormones. Seeds germinated in darkness were incubated in a medium at a constant temperature of 25±1°C, following a photoperiod of 16 hours of light and 8 hours of darkness.

***Leaf explant preparation and callus formation*.** Ripe anise leaves can be expected approximately 4 to 6 weeks after seed germination. In this study, ten leaf explants were taken, with the medium tested in three repetitions for each experimental group. A systematic approach was employed using a modified MS medium enriched with growth hormones to promote vigorous leaf growth in mature anise plants. For callus formation, leaf segments from aseptic anise seedlings that were 4 to 6 weeks old were utilized as explants. Explants measuring 1 cm square were placed in an MS medium containing 2 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) hormone [36].

Application of AgNPs to *P. anisum L.* Callus Cultures

According to Jebor (2016), we transferred to different plant growth medium the callus formed over a period of 4 to 6 weeks, cutting it into pieces weighing up to 250 mg. These pieces were placed into a plant growth medium, specifically prepared with control and silver nanoparticles (AgNPs) groups at concentrations of 0, 1, 5, and 10 mg in MS medium containing 2 mg/L of BAP (Benzyl aminopurine) and 2 mg/L of 2,4-D. Our study will evaluate the zero-concentration group as the control. The calli will be incubated in a climate cabinet (SANYO MLR-351H) at a temperature of 25 ± 2°C for 16 hours under a photoperiod provided by white fluorescent light at an intensity of 50 µmol/m²/s. Reactive oxygen species (ROS) analyses of the calli will be conducted 21 and 28 days after the initiation of the culture.

Antioxidant enzyme analyses

SOD, GPOx, and CAT analyses, for which the methods are detailed below, were performed on the 21st and 28 th days and examined whether they caused oxidative stress in Calli. In this process, 1 g of callus tissue is homogenized at +4°C using 2 ml of extraction solution containing 0.1 mM

EDTA, 1% PVP, 0.5% Triton X-, and 100-mM PBS at pH 7.8. After homogenization, the mixture was centrifuged at +4°C at 18,000 g for 20 min, and the supernatant was collected. This supernatant will be used to measure antioxidant enzyme activity [37].

Catalase (CAT) activity. A 1.5 ml measurement buffer of 200 mM PBS at pH 7 and 71 mM H_2O_2 was incubated at 30˚ for 3 minutes. After the incubation, the measurement buffer was transferred to a quartz cuvette, and 37.5 µl of supernatant was added. Spectrophotometric measurements were conducted kinetically at 240 nm for 2 minutes. The catalase activity was calculated using the formula Δ Abs (240 nm) / (minute x mg protein) and expressed in units per mg of protein (U/mg protein) [38].

Superoxide dismutase (SOD) activity. Two µl of supernatant and twenty µl of riboflavin (0.2 mM) were added to 2 ml of buffer (100 mM PBS pH7, 2 M Na_2CO_3, 0.5 M EDTA, 300 mM L-Methionine, 7.5 mM NBT and the samples are exposed to 15 W white, fluorescent light until a visible colour change occurs. Following the colour change, the absorbance of the samples at 560 nm was measured spectrophotometrically. SOD enzyme activity was determined using a formula that considers the change in absorbance over time and protein content and expressed as U/mg protein [39].

Guaiacol peroxidase (GPOx) activity. A measurement buffer of 100 mM PBS at pH 5.8, 5 mM H_2O_2, and 15 mM Guaiacol was prepared. Then, ten µl of supernatant was introduced into this buffer and was placed in 1.5 ml quartz tubes. The spectrophotometric analysis (IMPLEN Nanophotometer-P330, Germany) is conducted kinetically at 470 nm for 120 seconds. GPOx activity is quantified using the formula Δ Abs (470 nm) / (minutes x mg protein) and expressed as U/mg protein [40].

Results

Characterization of AgNPs

AgNPs synthesized from *S. sclaera* leaf extract were measured by zeta particle size, potential, and multiple distribution index analysis. The particle size dispersion is shown in Figure 3. The size distribution of the synthesized NPs varied between 13,54-342 nm, with an average size of ~95 nm. The zeta potential was measured as (mV): -10,7. Additionally, UV-Vis measurements indicated wavelength peaks in the range of 400 nm on average.

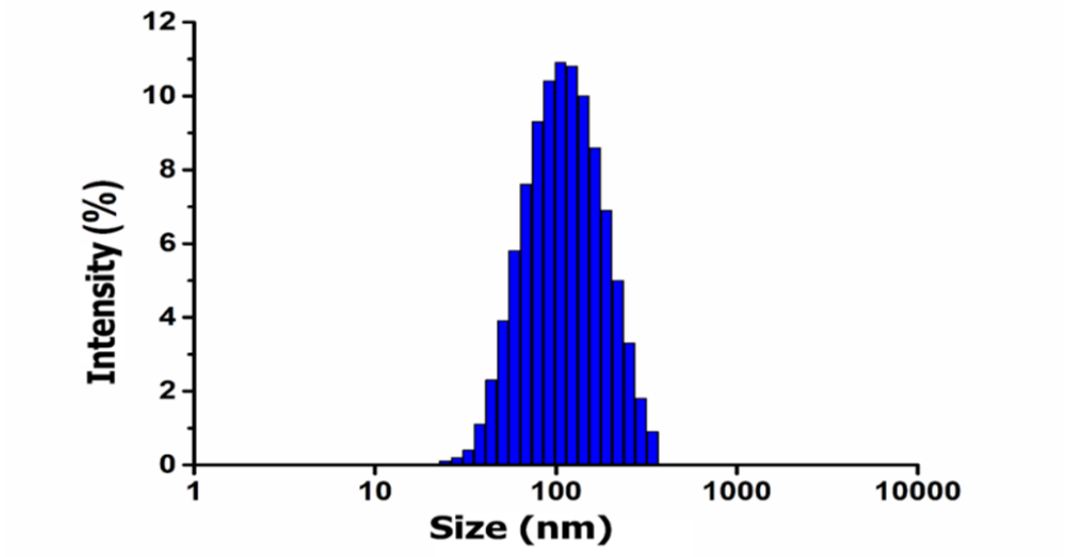

Fig.3 Intensity-based size was measured by Zeta Sizer; here is the distribution of the synthesized silver nanoparticles.

SEM images Figure 4 indicated that the NPs displayed a spherical morphology. The SEM findings aligned with the size measurements obtained using dynamic light scattering, confirming that the particles exhibited a uniform size distribution.

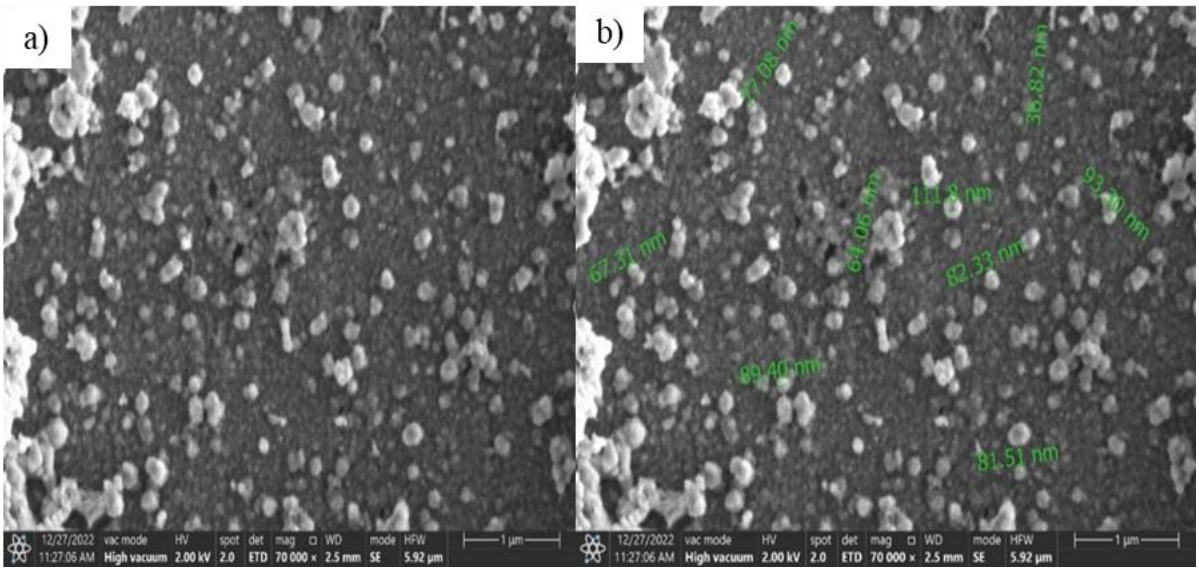

Fig.4 High-resolution SEM image showing spherical structure with a detailed focus on size, shape, and surface features for further analysis. a) High-resolution SEM image (70000x) acquired under high vacuum (2 kV). This focus on size, shape, and surface features allows for further analysis. b) This complements the high-resolution view of Figure 4a and captures the broader distribution and potential cluster formation within the synthesized AgNPs.

Application of AgNPs to *P. anisum L.* Callus Cultures

All morphological and physiological characteristics were observed to be affected by AgNPs stress, with a more pronounced effect on the transformation of callus color from green to yellowish as the amount of AgNPs increased.

Changes in AgNPs concentration had a noticeable impact on the morphological attributes. As the concentration of AgNPs increased, a more prominent effect was observed, particularly in the DT variety, which is much bigger and greener in colour than DS. DS and DT comparison of different concentrations of AgNPs in 21st days. Figure 5 indicated the callus formations.

Antioxidant enzyme activation

In our experiment, we studied the toxicity of AgNPs at three different concentrations (1 mg/L, 5 mg/L, and 10 mg/L) on Anis plants. Next, we evaluated the activation of antioxidant enzymes, including CAT, SOD, and GPOx, 21 and 28 days after AgNPs were applied to the plant. It is worth noting that the results showed distinct trends in enzyme activity.

***CAT Activation results*.** The CAT enzyme activity results in both DS and DT conditions after 21 and 28 days displayed inconsistencies, lacking coherent trends or statistical significance across all concentrations, as shown in Figure 6. However, the 5 mg/L AgNPs concentration after 28 days in DS conditions showed a substantial increase, reflecting a noteworthy **p, suggesting a statistically significant change at that concentration and time point (Table 1&2).

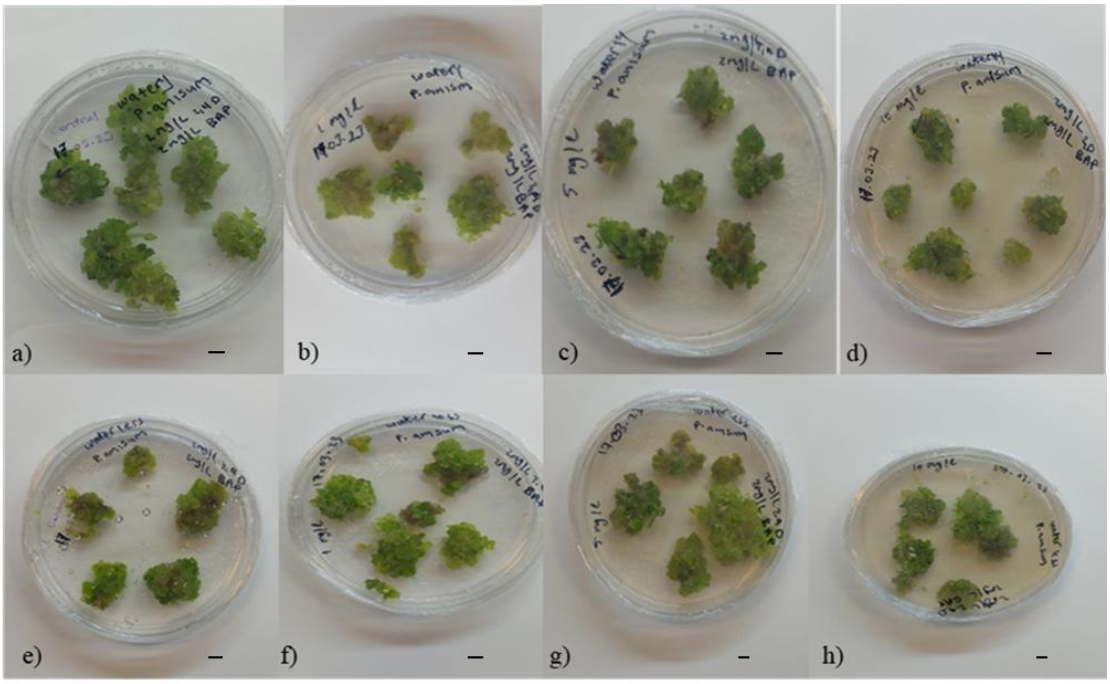

Fig.5 The images represent the progressive transformation of callus color from green to yellowish, reductions in size, and textural changes. 21st day, The DS (top row) and DT (bottom row) are shown for comparison. (a) DS control (b) DS 1mg/L AgNPs (c) DS 5mg/L AgNPs (d) DS 10mg/L AgNPs (e) DT control. (f) DT1mg/L AgNPs (g) DT 5mg/L AgNPs (h)DT 10 mg/L AgNPs. The scale bar was calculated and added as 1 cm based on the size of each image.

Table 1 Compared to the control group, Catalase (CAT) activities in response to AgNPs treatment in **DS** *P. anisum* plants. Change and significance levels in the distribution of AgNPs according to days **P <0.01 and ns (no significant difference).

	21 days		**28 days**	
1 mg/L AgNPs	51.98 %	ns	22.77 %	ns
5 mg/L AgNPs	33.36 %	ns	83.76 %	**
10 mg/L AgNPs	28.32 %	ns	40.71 %	ns

Table 2 Catalase (CAT) activities in response to AgNPs treatment in **DT** *P. anisum* plants in comparison to the control group. Change and significance levels in the distribution of AgNPs are determined by the days. ns (indicates no significant difference).

	21 days		**28 days**	
1 mg/L AgNPs	158.37%	ns	74.36%	ns
5 mg/L AgNPs	50.00 %	ns	57.00 %	ns
10 mg/L AgNPs	49.00 %	ns	40.51 %	ns

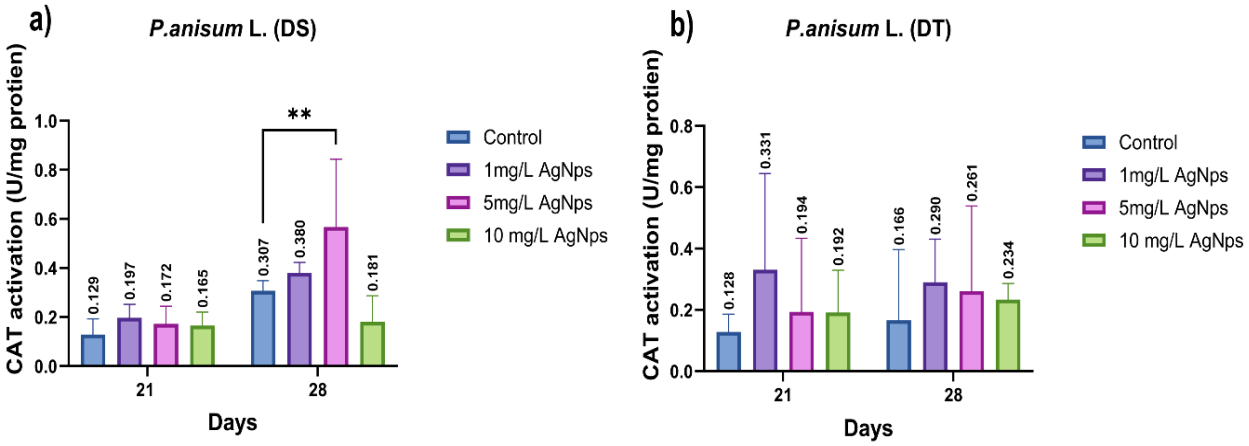

Fig.6 a) CAT enzyme activity graph on the 21st and 28th day applied to control and 1, 5 and 10 mg/L AgNPs Drought-sensitive (DS) anise. ** indicates P < 0.01. b) In DT conditions, the CAT enzyme activity did not produce statistically significant findings.

SOD Activation results. SOD activity in DS after 21 days indicated a significant increase with a 48.42% increase in 1 mg/L AgNPs and an increase in 5 mg/L AgNPs, although 10 mg/L AgNPs did not statistically significant results, as shown in Figure 7. However, after 28 days, all concentrations showed a decrease in SOD activity in DS. On the contrary, the SOD activity in DT showed a decrease, particularly evident with a decrease of 1 mg/L AgNPs and a significant decrease of -57.60% in 10 mg/L AgNPs. However, it showed an increase of 42.26% in the condition of 5 mg/L AgNPs after 21 days. After 28 days, a similar decrease pattern emerged within 1 mg/L AgNPs and 10 mg/L AgNPs, with a significant increase of 322.88% in 5 mg/L AgNPs. At both 21 and 28 days, SOD enzyme activity in DS conditions exhibited compelling results. These results highlight the robust impact of AgNPs on SOD activity over time in DS conditions. In contrast, the 10 mg/L AgNPs concentration demonstrated a p, suggesting a lack of statistical significance in this case (Table 3). In DT conditions, SOD enzyme activity displayed highly noteworthy results both after 21 and 28 days of exposure to AgNPs. For all three concentrations (1 mg/L, 5 mg/L, and 10 mg/L AgNPs), the ****P were consistently found to be, at both time points. This indicates a strong and consistent level of statistical significance, emphasizing the substantial impact of AgNPs on SOD activity under the conditions (Table 3&4).

Table 3. Superoxide dismutase (SOD) activities were examined in response to AgNPs treatment in (DS) *P. anisum* plants compared to the control group. The change and significance levels in the distribution of AgNPs were analyzed over several days. The statistical significance was indicated as follows: ****P < 0.0001, ***P < 0.001, and ns (no significant difference).

	21 days		**28 days**	
1 mg/L AgNPs	48.42 %	**** ↑	- 37.96 %	**** ↓
5 mg/L AgNPs	21.01 %	**** ↑	- 81.13 %	**** ↓
10 mg/L AgNPs	-0.78 %	ns ↓	- 78.49 %	**** ↓

Table 4. Superoxide Dismutase (SOD) activities significantly increased in DT *P. anisum* plants treated with AgNPs compared to the control group, with a high level of statistical significance (****P <0.0001) observed after both 21 and 28 days of treatment.

	21 days		**28 days**	
1 mg/L AgNPs	- 38.14 %	****	- 42.42 %	****
5 mg/L AgNPs	42.26 %	****	322.88 %	****
10 mg/L AgNPs	- 57.60 %	****	- 63.58 %	****

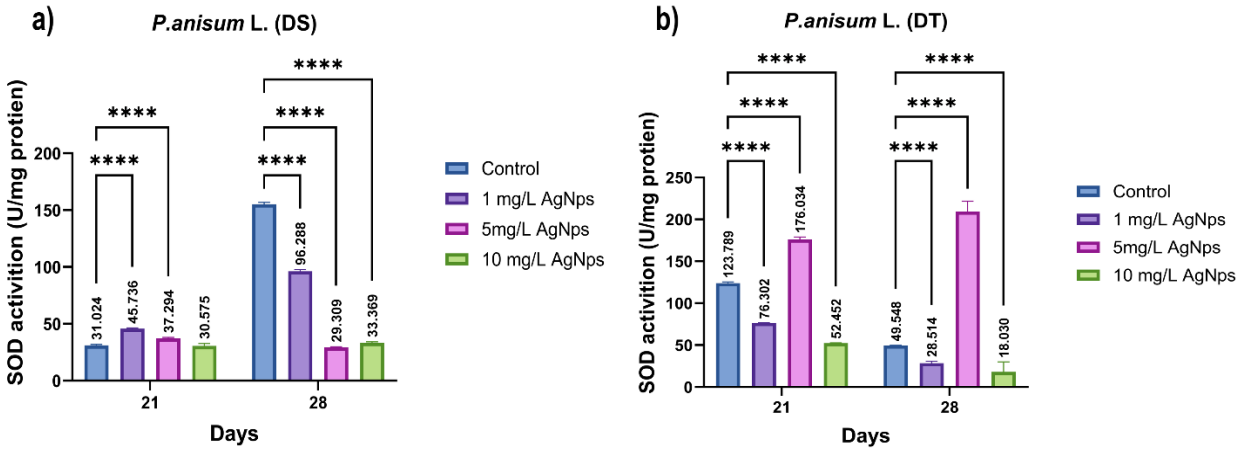

Fig.7 a) SOD enzyme activity graph on 21 and 28 days applied to control and 1, 5, and 10 mg/L AgNPs DS anise. **** indicates P <0.0001. b), SOD enzyme activity graph on 21 and 28 days applied to control and 1, 5, and 10 mg/L AgNPs DT anise. **** indicates P <0.0001.

GPOx Activation results. Turning to GPOx activity in DS condition after 21 days, it showed an impressive increase of 5 mg/L AgNPs, although the results were less interpretable for 1 mg/L and 10 mg/L AgNPs. After 28 days, a significant increase of was recorded for 1 mg/L AgNPs, but the results remained elusive for 5 mg/L and 10 mg/L AgNPs. On the contrary, in DT conditions, after 21 days, the enzyme response appeared inconsistent, with no sense in the case of 10 mg/L AgNPs and a striking increase of 1 mg/L AgNPs, an increase in 5 mg/L. AgNPs. After 28 days, a significant increase was seen in the case of 5 mg/L AgNPs, with an increase of 160.00%, and 10 mg/L AgNPs, which showed an increase of 150.43%, while 1 mg/L AgNPs continued to give results without statistical significance.

In DS conditions, GPOx enzyme activity exhibited varying results after 21 and 28 days of AgNPs exposure. After 21 days, for the 1 mg/L AgNPs concentration, the p indicated no statistical significance, while the 5 mg/L AgNPs concentration displayed a p suggesting a significant impact. However, the 10 mg/L AgNPs concentration had a p of indicating no statistical significance. After 28 days, the 1 mg/L AgNP concentration showed a p of indicating a significant impact, while the 5 mg/L and 10 mg/L AgNP concentrations had ps of signifying no statistical significance, as shown in Figure 8. In DT conditions, GPOx enzyme activity exhibited diverse outcomes following 21 and 28 days of AgNPs exposures. After 21 days, the 1 mg/L AgNPs concentration displayed a p, indicating a highly significant impact, while the 5 mg/L AgNPs concentration showed a p, denoting a significant effect. In contrast, the 10 mg/L AgNPs concentration yielded a p marked as 'ns' (non-significant). After 28 days, the 1 mg/L AgNPs concentration had a p labelled as 'ns,' implying no statistical significance, whereas the 5 mg/L AgNPs concentration presented a p a significant effect. Similarly, the 10 mg/L AgNPs concentration had a p representing a significant impact (Table 5&6).

Table 5. Guaiacol peroxidase (GPOx) activities in DS *P. anisum* plants in response to AgNPs treatment compared to the control group, showing changes and significance levels over time. Significance levels are denoted as **P < 0.01** for significant differences, and 'ns' indicates no significant difference.

	21 days		**28 days**	
1 mg/L AgNPs	22.55 %	ns ↑	93.01 %	** ↑
5 mg/L AgNPs	98.12 %	** ↑	26.00 %	ns ↑
10 mg/L AgNPs	35.5 %	ns ↓	25.57 %	ns ↑

Table 6. Guaiacol peroxidase (GPOx) activities in response to AgNPs treatment in DT *P. anisum* plants compared to the control group. The table presents changes in GPOx activity and significance levels of AgNPs distribution over different time points. Significance levels are indicated as P < 0.01 for significant differences and 'ns' for no significant difference.

	21 days		**28 days**	
1 mg/L AgNPs	186.24%	*** ↑	11.65 %	ns ↑
5 mg/L AgNPs	109.53%	* ↑	160.00 %	** ↑
10 mg/L AgNPs	-37.40 %	ns ↓	150.43 %	* ↑

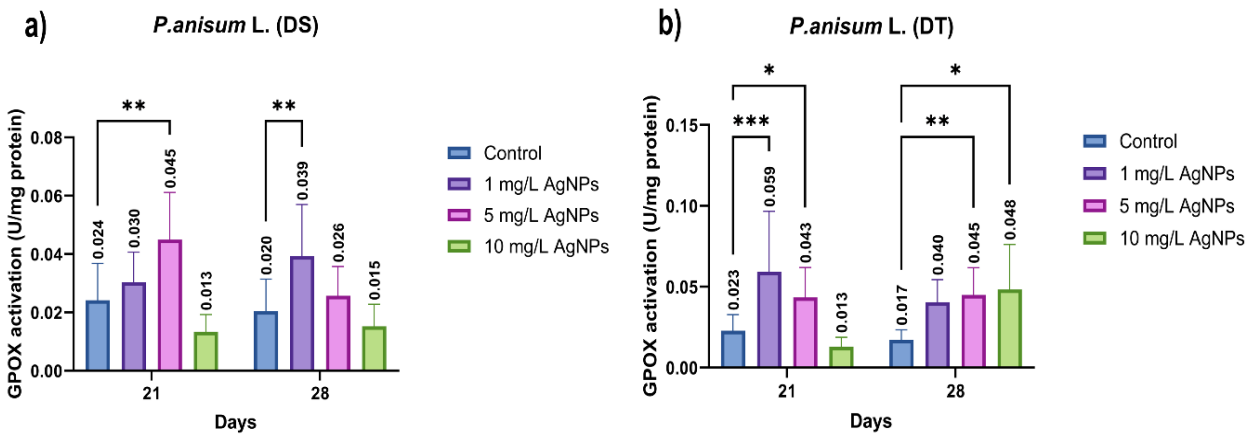

Fig.8 a), GPOx enzyme activity graph on 21 and 28 days applied to control and 1, 5 and 10 mg/L AgNPs DS anise. **** indicates ** indicates P < 0.01. b), GPOx enzyme activity graph on 21 and 28 days applied to control and 1, 5 and 10 mg/L AgNPs DT anise. *** indicates P < 0.001, ** indicates P < 0.01 and * indicates P < 0.05.

Discussion and Conclusion

The goal of this study is to analyse the antioxidant activity of different enzymes, such as CAT, SOD, and GPOx when exposed to AgNPs in two anise types: DT and DS. In 2018, [41] found that the CAT activity was consistent across all the tested AgNPs concentrations in the *Lemna minor*. Also, the study of [42] found

that the effects of AgNPs on CAT activity were not significant in *Allium cepa* roots. Another study by [43]noted that AgNPs had a lesser impact on *Brassica* seedlings as they reduced the accumulation of AgNPs and improved the activities of CAT. However, other studies like [44]. They recorded that AgNPs at 10 mg/L significantly increased the maximum level of CAT activity in plantlets. They also noted that the activity increased. Our results support these findings, indicating that the treatment of AgNPs at varying concentrations did not significantly alter the levels of the CAT enzyme. CAT enzyme activity results after 21 and 28 days, in both DS and DT conditions, displayed inconsistencies, lacking coherent trends or statistical significance across all concentrations.

Continuing with the SOD result, in a study conducted by [44], researchers observed that plants treated with 20 mg/L AgNPs experienced a 17.6% reduction in their SOD activity. Optimal SOD values were found at 2 mg/L for both Ag ion and Ag NP treatments. On the other hand, the highest SOD levels were observed in the treated Ag ions at 10 mg/L. The researchers observed that concentrations of antioxidant enzymes at this level significantly increased SOD enzyme activity. The decrease in SOD activity exhibited at higher concentrations was also notable. In a study conducted by [45], researchers observed a significant increase in SOD activity levels upon exposure to 10 mg/L AgNP. Also, a study conducted by researchers [46] revealed that SOD activity levels were higher in all low AgNPs treatments. This is consistent with the results of our study; we observed that enzyme activity significantly increased in the DT after 21 at 5 mg/L AgNPs days of AgNPs exposure. This is in line with the results of another study [47], which showed that SOD activity levels were higher in tomato plants when exposed to 5 mg/L AgNPs. Also, a study [48] confirmed that the SOD activity was higher when compared to the activity of the control group when the AgNPs concentrations were 2 and 5 mg/dm3. But when AgNPs concentrations were 10, 20, and 50 mg/dm3, the SOD activity decreased. The results of these two studies highlight the complex relationship between plant enzymes and AgNPs. These results support our hypothesis that the increase in SOD activity is due to exposure to different AgNPs concentrations and environmental conditions. The most striking increase in activity, a 322.88% increase, was observed in the DT SOD enzyme after 21 days at 5 mg/L AgNPs, indicating higher enzyme performance under these conditions. Conversely, the most significant decrease, a −63.58% reduction, in DT SOD enzyme was observed after 21 days at 10 mg/L AgNPs, indicating a significant reduction in enzyme functionality at this concentration. These results underscore the complex interaction between AgNPs and plant enzymes, highlighting their potential impact on plant antioxidant defence mechanisms. Similarly, in the investigation of Guaiacol Peroxidase (GPOx) activity in response to silver nanoparticles (AgNPs), multiple studies have contributed to our understanding of the concentration-dependent effects of AgNPs on this antioxidant enzyme. The study of [49] observed a reduction in GPOx activity with AgNPs treatment at 2 mM, indicating an inhibitory effect at this concentration. As the concentration increased to 6 mM, GPOx activity showed a gradual decrease, suggesting a dose-dependent response. This concentration-dependent modulation implies that higher doses of silver nanoparticles may down-regulate the enzyme's functionality, potentially impacting the plant's antioxidant defence mechanism. Moreover, it explored the impact of different AgNPs concentrations on GPOx activity, showing altered GPOx activity in radish plants treated with 50 mg/L and 100 mg/L of AgNPs [50]. The results demonstrated that varying concentrations of AgNPs could influence GPOx activity, highlighting a nuanced response to different nanoparticle concentrations. Another study contributed to this understanding by reporting continuous increases in GPOx activity in *B. juncea* seedlings with increasing concentrations of Ag-NPs from 25 ppm to 400 ppm. This observation aligns with the notion that GPOx activity can be positively influenced by higher concentrations of silver nanoparticles [51]. In our specific investigation, we explored the concentration-dependent effects of AgNPs on GPOx activity in both DS and DT conditions. In the DS condition after the 21st day, GPOx activity displayed a notable increase at 5 mg/L AgNPs, while results for 1 mg/L and 10 mg/L AgNPs were less interpretable. After the 28th day, a significant increase was recorded for 1 mg/L AgNPs, but the results remained elusive for 5 mg/L and 10 mg/L AgNPs. In DT conditions after the 21st day, the enzyme response appeared inconsistent, with no clear trend for 10 mg/L AgNPs, a significant increase for 1 mg/L AgNPs, and an increase for 5 mg/L AgNPs. After the 28th day, a significant increase was observed for 5 mg/L AgNPs (160.00%), and 10 mg/L AgNPs showed an increase of 150.43%, while 1 mg/L AgNPs continued to yield results without statistical significance. These detailed findings underscore the complexity of the relationship between AgNPs concentrations and GPOx activity, providing valuable insights into the nuanced responses of antioxidant enzymes under different exposure conditions.

In conclusion, under DT conditions, there was an increase in the working capacity of plant enzyme activities, with a significant rise at 10 mg/L on the 28th day. These results collectively emphasize the concentration-dependent and complex impact of AgNPs on GPOx enzyme activity in plant systems. According to the results obtained, antioxidant enzyme activity works actively in 1 and 5 mg/L AgNPs concentrations in both

groups, but at the highest dose of 10 mg/L AgNPs concentration reduced the working capacity of the plant's defense mechanism. As a result, it was observed that plant tolerance of 1 mg/L AgNPs dose was high in both groups. However, despite the increase in toxic values at 1 and 5 mg/L, it was observed that the tolerance capacity increased more in the DT condition compared to the DS after the 21st day. While toxic values were evaluated to be relatively high at 10 mg/L, it was observed that it seriously limited the ROS enzyme working capacity of the plant.

Abbreviations

NPs: Nanoparticles; *P. anisum: Pimpinella anisum*; *S. sclarea*: *Salvia sclarea*; AgNPs: silver nanoparticles; ROS: Reactive Oxygen Species; CAT: Catalase enzyme; SOD: Superoxide Dismutase enzyme; GPOx: Guaiacol Peroxidase enzyme; DS: drought-sensitive; DT: drought-tolerant type.

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Data Availability statement

The author confirms that the data supporting this study are cited in the article.

Compliance with ethical standards

Conflict of interest / Çıkar çatışması

The author declare no conflict of interest.

Ethical standards

The study is proper with ethical standards.

Authors' contributions

During the study Esma Ulusoy (E.U.) and Esraa Gaber (E.G.) designed the project, experiment, and acquired data. E. U. supervised the work, while both authors performed the experiments and statistical analysis.

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Research article

Exosome isolation by ultracentrifugation and precipitation methods from human adipose-derived mesenchymal stem cells and the effects on human colon cancer cell line

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ABSTRACT
Exosomes, one of the extracellular vesicles, mediate molecular information and intercellular communication between cells by containing various proteins, lipids, mRNA, metabolic enzymes and numerous essential molecules such as miRNAs. The therapeutic potential of paracrine factors of exosomes released from human adipose mesenchymal stem cells is still under investigation. In our study, we isolated exosomes released from human adipose mesenchymal stem cells by two different methods. To evaluate the cytotoxic effect of the obtained exosomes, we performed MTT assay in SW-480 cell line at 24, 48 and 72 hours. In the results obtained, no statistically significant difference was observed at 24 and 72 hours, while statistically significant differences were determined between the concentration groups at 48 hours.

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Introduction

Many cells release exosomes, tiny membrane vesicles with an endocytic origin. Additional groups reported that cultured reticulocytes secreted vesicles of endocytic origin, whereas exosomes were first identified as microparticles with 5'-nucleotidase activity produced from neoplastic cell lines [1]. Exosomes are endosomal-derived and the smallest extra vesicles (EV), measuring between 30 and 150 nm in diameter [2]. The unifying features of EVs and exosomes are that they operate as carriers of molecular information in cell-cell communication and convey cargo molecules important in both physiological and pathological processes to recipient cells [3]. In addition, the prevalence of exosomes in all bodily fluids indicates their stability in extracellular contexts and explains their potential for endocrine communication. Recent clinical interest in these vesicles has arisen due to their potential use in diagnostic applications and as part of innovative treatment techniques [4]. Secreted from almost all cell types, exosomes have become an area of extensive research, extending beyond immunology to neurobiology, stem cell and tumor biology, and their use as biomarkers or therapeutic tools in clinical applications [5]. Under the right circumstances, human mesenchymal stem cells (hMSCs) can develop into mesenchymal tissue lineages, including muscle, bone, cartilage, and adipose. They have also been extensively researched as a potential reserve cellular fraction for tissue maintenance and repair [6]. Recently, human adipose mesenchymal stem cells (hAMSCs) are become a potential source for stem cell banks and an ideal source of cell cultures for tissue engineering. Studies have shown that hAMSCs can be easily obtained from adipose tissue without ethical concerns or transplantation issues. These cells have high proliferation rates and multilineage differentiation capacity for cultivation in vitro [7]. In research about MSCs, bioactive compounds released by MSCs are thought to be the primary therapeutic approach with cell adhesion and differentiation; they have varying therapeutic benefits in conditions such as liver damage and arthritis. [8]. Abundant data have shown that human adipose mesenchymal stem cells derived exosomes (hAMSCs-exo) are involved in angiogenesis, immunomodulation, reduction of ischemia-reperfusion-induced injury, and other processes. It is then explained by the latest research studies such as myocardial repair, neuroprotection and neurotrophic effects, hepatic repair, renal repair, skin repair, and regeneration [9]. Exosomes are small extracellular vesicles that contain functional molecules from host cells and participate in intercellular communication. Among all the bioactive substances contained in these vesicles, microRNAs and nucleic acids are widely investigated. However, exosomal proteins play an equally important role in signal transduction and regulation [10]. The

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target cells of their microenvironment can absorb exosomes released into the extracellular environment, and biological fluids like blood, urine, breast milk, and cerebrospinal fluid can carry them to distant locations. When disease or damage disrupts the microenvironment, MSC-derived exosomes are essential for maintaining tissue homeostasis and allowing the tissue to respond to external stimuli. Therefore, it might provide insight into the paracrine activity regulation mechanism of MSCs that underlies their tissue-specific regeneration capabilities [11]. Considering all these properties of MSCs, their role in tumor development has also been intensively studied. Studies have reported that the effects of MSCs on the initiation and progression of tumor development are both pro-tumorigenic and anti-tumorigenic. Currently, this paradox is poorly understood due to the paucity of mechanisms governing the interactions between MSCs and tumor cells. These mechanisms suggest that MSCs may act on tumor cells through direct cell-cell interactions or by secreting various diffusible factors such as growth factors and cytokines [12]. MSC-derived exosomes (MSC-exo) have been proven to be promising therapeutic tools because they include anti-tumor mediators of MSCs on cancer cells, which can decrease tumor growth. Nevertheless, MSC-exo, which are divided into three categories—using MSC-exo as therapeutic carriers, using tumor suppressor MSC-exo as therapeutic vehicles, and suppressing tumorigenic MSC-exo as therapeutic targets offer a practical delivery vector that can prevent the degradation of therapeutic molecules due to their lipid bilayer membranes [13].

Exosome isolation is one of the most important steps for accurately detecting exosomal contents. Many methodologies are used to isolate exosomes, including ultracentrifugation, size exclusion chromatography, ultrafiltration, immunoaffinity isolation, microfluidic techniques, and polymeric precipitation method [14]. The research has led to the lack of a standardized technique for isolating exosomes. To assess the quantity and effectiveness of exosomes and to provide insight into previous researches, we employed ultra-centrifugation and the Total Exosome Isolation Kit (TEI) in our investigation. Determining the exosomes lethal impact on colon cancer cells was another goal.

Material and Methods

Growing cells

Human adipose-derived mesenchymal stem cells (Merck, USA, catalog number: SCC038)) were purchased commercially. Human Mesenchymal-LS Growth Medium (SCM023, Merck) and Dulbecco's Modified Eagles Medium (DMEM) low glucose culture medium (Catalog number: 11885084, Gibco) were used for cell growth. For SW-480 cells, DMEM high glucose culture medium, 10% Fetal bovine serum (FBS) (ECS0182L, Euroclone) was used. Under a microscope, the growing cell morphology was examined (Figure 1. A, B).

Exosome isolation from cell culture medium

The hAMSCs grown in cell culture were incubated in a serum-free medium for 72 hours for exosome isolation. Cells kept in a serum-free medium were checked for density and viability under a microscope. Then, at the end of the period, the medium was collected and made ready for isolation using both ultracentrifugation and TEI kit methods.

Exosome isolation by ultracentrifugation

Exosome isolation using the ultracentrifugation method was performed at Kocaeli University Stem Cell and Gene Therapy Research and Application Center (KOGEM). Here, the amount of media collected for exosome isolation and the total number of cells were determined. Using 120 ml of cell culture medium, the steps of exosome isolation by ultracentrifugation method are briefly given (Figure 1. C). Accordingly, the collected medium was centrifuged at 300 xg for 10 min, 15.000 xg for 10 min and the supernatant was passed through a 0.22 μm sterile filter. Then 120.000 xg was centrifuged for 70 min and the supernatant was removed. After washing with PBS and centrifugation at 120.000 xg for 70 min, the exosome pellet was obtained and dissolved with PBS.

Exosome isolation by Total Exosome Isolation Kit method

The amount of medium required for exosome isolation and the total number of cells were determined. TEI reagent (Catalog number: 4478359, Thermo Fisher Scientific) exosome isolation kit was used. Isolation steps were then followed according to the kit instructions (Figure 1.D.). According to the amount of medium collected, 500 μl of TEI reagent was added to 1ml of cell culture media and left to incubate at 4 ºC overnight. The next day, centrifugation was performed at 10.000 xg for one hour at 4 ºC. The supernatant was then removed and the exosome pellet obtained was dissolved in phosphate-buffered saline (PBS). Isolated exosomes were kept at 2 to 8 ºC for short-term storage and at -80 ºC for long-term storage.

Protein quantification by Bradford method

A popular technique for animal proteins is the Bradford protein test [15], which is based on the Coomassie brilliant blue G-250 dye binding to a protein complex that produces absorbance at 595 nm. Here, the protein

content of exosomes isolated by ultra-centrifugation and kit method was evaluated by plotting the standard bovine serum albumin (BSA) graph to determine the stability of the analysis. For Bradford analysis, the soluble protein was extracted from the isolated exosome samples using RIPA buffer. Bradford analysis and protein extraction using RIPA buffer were carried out in accordance with Yıldırım et al. 2023 [16]. Since no separate method was used for exosome quantification and exosome treatment at different concentrations, the amount of protein determined by the Bradford assay was used.

MTT Assay

3-[4, 5- dimethylthiazol- 2- yl]- 2, 5 diphenyltetrazolium bromide (MTT), one of the cytotoxicity tests, is one of the tests that precisely measures cell viability and proliferation in vitro by measuring metabolic activity in cells [17,18]. Briefly, SW-480 cells were incubated overnight in 96-well tissue culture plates at 10,000 cells/well. Then, 24, 12, 6, 3, 1.5, 0.750, 0.375, 0.187 ng/µl hAMSC-derived exosomes were applied and incubated at 37 ºC with 5% CO_2 for 24, 48, and 72 hours. At the end of the time, MTT was applied with a final concentration of 0.5 mg/ml and absorbance at 550 nm was taken at the end of 4 hours**.**

Statistical analysis

The software program Graph Pad Prism version 8.0.1 (GraphPad Software Inc., La Jolla, CA) was used to graph all of the results. The Graph Pad Prism One Way ANOVA test was used for all statistical analyses. The Shapiro-Wilk test, a numerical technique for assessing data normality, was used to verify that the results from the experimental investigations had a normal distribution. To look for significant differences between groups, Tukey's post hoc test was used in conjunction with a one-way analysis of variance for all normally distributed data. When $P < 0.05$, the results were deemed significant. The computer program Graph Pad Prism 9 was used to graph the results.

Results and Discussion

As mediators of chemical exchange and intercellular communication, exosomes are involved in both physiological and pathological processes. Exosomes can simultaneously transport a range of bioactive compounds and quickly deactivate or readily degradable components via several nummber of different pathways and locations. They can take part in immune system functions, tumor diagnostics and treatment, and tissue healing by securely delivering their cargo molecules to target cells [19]. In our study, it was aimed to grow hAMSCs healthily and perform exosome isolation studies using ultracentrifugation and kit protocol. In addition, protein quantification of the isolated hAMSCs-derived exosomes was performed. Then, the cytotoxic effects of the quantified exosomes on SW-480 cells were evaluated. Accordingly, the amount of exosomes isolated from 120 ml medium by ultracentrifugation method was determined as 3400 ng/µl by Bradford protein assay. The amount of exosomes isolated from 12 ml of medium with TEI kit was determined as 1193 ng/µl by Bradford protein assay.

Cells readily absorb MTT, which is used to measure cell viability and proliferation. It is then enzymatically converted to formazan, a black substance that builds up in cytoplasmic granules [20]. SW-480 cells, a colon cancer cell line, and hAMSC cells as exosome sources were used for MTT assay. SW-480 cells were seeded in 96-well plates and exosome treatment was performed. Exosomes were applied at 24, 12, 6, 3, 1.5, 0.75, 0.375, and 0.187 ng/µl and cell viability was determined by MTT assay. In the MTT study, cell viability in SW-480 cells after exosome treatment was determined in Figure 1. E, F, G. While no dose-dependent cytotoxic effect was observed in the results obtained, no statistically significant difference was observed when the control and PBS groups were compared at 24, 48 and 72 hours. However, the groups treated with varying quantities of exosomes at 24 and 72 hours did not differ statistically significantly from the control and PBS groups. Exosome treatment caused a statistically significant decrease in cells when the 0.75 ng/µl group, which was exosome treated for 48 hours only, was compared with the control group. When cell viability between exosome-treated groups was analyzed, statistically significant differences were found between 6 ng/µl and 0.75 ng/µl groups and between 0.75 ng/µl and 0.375 ng/µl groups.

Exosomes, which are tiny vesicles that range in size from 30 to 150 nm and are released by MSCs, can facilitate cell-to-cell communication by carrying a variety of biomolecules, including proteins, mRNAs, microRNAs, and long non-coding RNAs. In experimental models of tissue repair injury and aging, these nanoparticles exhibit regenerative benefits [23] Mesenchymal stem cells derived from bone marrow, adipose tissue, and the umbilical cord have been shown in numerous studies to be able to enhance wound healing by producing exosomes. Moreover, mesenchymal stem cell exosomes derived from adipose tissue are thought to promote vascularization, hastening the repair of diabetic skin wounds [24].

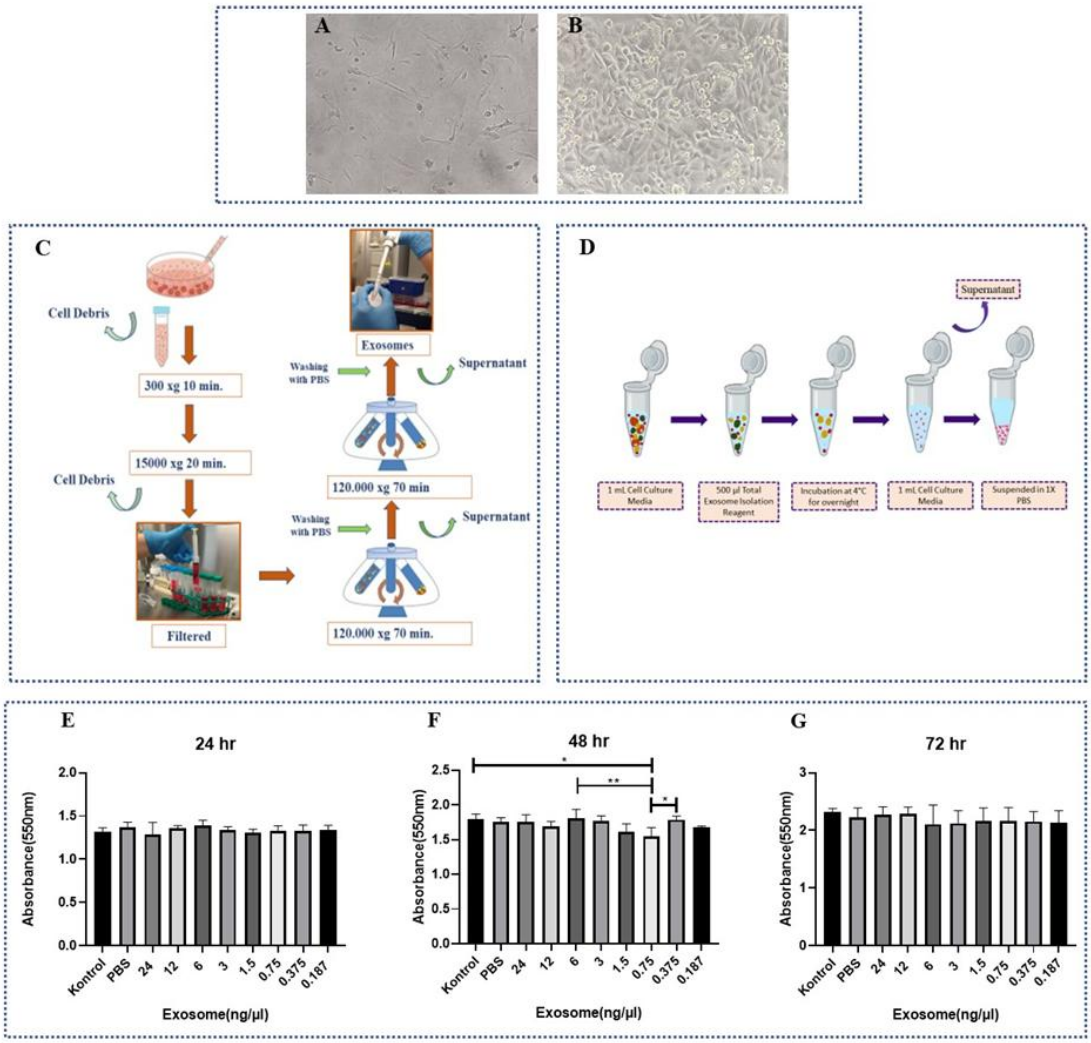

Fig 1 Application steps of two different methods used in exosome isolation with cell lines used in the study. MTT analysis results of 24h, 48h and 72 hours of exosome application. (A) microscopic image of hAMSCs (X10), (B) microscopic image of SW-480 cells, (C) exosome isolation by ultracentrifugation method, (D) exosome isolation by TEI kit method, (E) 24 h MTT analysis results, (F) 48 h MTT analysis results, (G) 72 h MTT analysis results. Some of the images in Figure 1. C are modified from Tang et al. 2017 and Willis et al. 2017 [21, 22].

Exosomes are believed to have antitumor capabilities and to be engaged in restricting the course of cancer, even though they have primarily been found to stimulate the growth of tumors. Exosomes and their contents have been shown in recent research to be a possible source of regulatory information on tumor progression and metastasis, particularly in the diagnosis of cancer. To ascertain the intricacy and functional diversity of exosomes, more research is required. Nevertheless, the typical physiological roles of exosomes in tissue homeostasis in the pathological remodeling of organs are unknown [25].

In our study, hAMSCs-exo were isolated using two different methods. The protein content of the exosomes obtained by ultracentrifugation and kit methods was calculated. There are many different methods in the literature on exosome isolation. In a study by Wang et al. 2015, they used three different methods to purify and analyze exosomes and to provide a better and more economical method for exosome research. These methods were the density gradient ultracentrifugation method, ultracentrifugation and ultrafiltration method and ExoQuick™ Extraction kit method. Exosomes obtained from THP-1 macrophage-like cells were isolated by 3 methods. As a result of Bradford analysis, the amount of protein obtained by the density gradient ultracentrifugation method was 1.09 µg/µl, the amount of protein obtained by the ultracentrifugation method was 1.38 µg/µl and the amount of protein obtained by the ExoQuick™ Extraction kit method was 1.82 µg/µl [26]. It was determined that the protein amounts obtained by ultracentrifugation and the kit method used in

our study were compatible with the studies in the literature and exosome isolation using the kit was more advantageous in terms of the amount of exosomes obtained.

Regarding exosome isolation, Helwa et al. 2017 conducted an exosome isolation study with commercial kits and ultracentrifugation method. Different kits and ultracentrifugation methods were evaluated in the results obtained. As a result of exosome isolation obtained from different amounts of serum samples, it was determined that the ultracentrifugation method had relatively larger particles than the kits. Centrifugation at such high speeds, however, may harm the physical characteristics of exosomes and the sensitivity of proteomic analysis by causing particles in the serum to fuse with impurities and other proteins. Using commercial exosome isolation kits has been linked to decreased exosome purity because more particles from reagents and components based on polyethylene glycol (PEG) may precipitate nanoparticles outside the exosome. Ultracentrifugation isolation is a feasible substitute for kit isolation of exosomes in serum samples isolated at varying volumes, as evidenced by the consistent diameter and concentration of exosome particles in serum samples. The conventional method for isolating exosomes, ultracentrifugation, has been used for a long time, but it requires expertise and an ultracentrifuge. Nonetheless, both kit and ultracentrifugation isolation produced consistent exosome particle diameters and concentrations in serum samples, suggesting that it is a good substitute for exosome isolation in serum samples isolated at various volumes. Despite being the conventional method for isolating exosomes for a long time, ultracentrifugation necessitates both experience and the availability of an ultracentrifuge. It is also technically delicate, time-consuming, and very tedious [27]. Based on this, we preferred the kit method in our study and continued our experimental studies.

The size, concentration, morphology and purity of the exosomes obtained in exosome isolation are of utmost importance [28]. However, the characterization of exosomes is one of the most important steps. Our studies on the characterization of the exosomes isolated within the scope of our study are ongoing. However, within the scope of this article, it was aimed to isolate exosomes with only two different methods and to determine the cytotoxic effect of the isolated exosomes. The study requires an isolation procedure that meets these conditions and a standardized protein analysis. For this purpose, exosome isolation and quantification methods differ in the literature. Kechik et al.2018 isolated exosomes from human saliva in their study. Then, they tried to characterize the obtained exosomes by SEM, NTA, Bradford and western blot analysis. They also evaluated the effect of protease inhibitors on the storage of exosomal protein. They concluded that the analyses largely agreed with one another. They specifically claimed that there was no discernible variation in exosomal protein concentration results across all settings in the protein assay. While clustering of exosomes was observed in SEM and NTA results, especially in NTA analysis, the concentration of isolated exosomes was calculated, but due to the clustering of exosomes, they stated that it is more reliable to use the exosomal protein concentration obtained from the protein assay result for in vitro studies of cells [29]. Based on this, we used Bradford analysis to determine the amount of exosomes isolated in our study and used the amount of protein for different concentrations of exosomes.

MTT assay of the isolated exosomes was performed in SW-480 colon cancer cells. Thus, cell viability and the cytotoxic effect of hAMSC-exo in colon cancer cells were evaluated. In Figure 1. E, F, it was concluded that there was no statistically significant difference between the control and PBS groups in the MTT analysis at 24 and 72 hours, and there was no statistically significant difference between the groups in the exosome-treated groups. In a study conducted by Hosna et al. 2018, antitumour effects were evaluated in different cancer cell lines by isolating exosomes from mesenchymal stromal cells. Accordingly, in the MTT analysis, no statistically significant difference was observed in the BT-474 cell line treated with exosome and the BT-474 cell line treated with exosome and doxorubicin [30]. In MTT analysis, no statistically significant difference was found between the control and PBS groups at 48 hours, but statistically significant differences were found between the control group and 0.75 ng/µl group, 6 ng/µl and 0.75 ng/µl group, 0.75 ng/µl and 0.375 ng/µl groups. Accordingly, there was a significant decrease between the control group and the 0.75 ng/µl group, a significant increase between the 6 ng/µl group and the 0.75 ng/µl group, and a significant decrease between the 0.75 ng/µl group and the 0.375 ng/µl group. In the literature, Kheradjoo et al. (2022) evaluated the cytotoxic effect of MSC-exo on retinoblastoma cells in a study they conducted. In the results obtained, it was stated that exosome application inhibited cell proliferation. At the same time, it was shown that exosome application induced apoptosis in retinoblastoma cells upregulated microRNA-143 expression and decreased Bcl-2 expression [31].

Conclusion

Overall, our study supports the feasibility of using these two isolation techniques to isolate sufficient exosomes. Thus, we conclude that the kit method is relatively more advantageous than the ultracentrifugation method. In summary, although the commercial kit in this study allows the isolation of a

limited amount of exosomes, it is an adequate alternative to the ultracentrifugation method and will encourage the understanding of the function of exosomes and support exosome studies. hAMSCs-exosomes have been shown to reduce proliferation in SW-480 cells. As a result of this study, characterization studies of exosomes are ongoing. In addition, the effect of hAMSCs-exosomes on SW-480 cells is being studied in more detail and transcriptome analysis studies are being carried out.

Abbreviations

EV:Extravesicles; hMSCs: Human mesenchymal stem cells; hAMSCs :Human adipose mesenchymal stem cells; hAMSCs-exo:human adipose mesenchymal stem cells derived exosomes; MSC-exo: MSC-derived exosomes; TEI:Total Exosome Isolation Kit; DMEM: Dulbecco's Modified Eagles Medium; FBS: Fetal bovine serum; PBS: Phosphate-buffered saline; BSA: Bovine serum albümin; MTT: 3-[4, 5- dimethylthiazol- 2- yl]- 2, 5 diphenyltetrazolium bromide.

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Availability of data and material

Please contact the corresponding author for any data request.

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Research article

Assessing of antioxidant activities of, L-carnitine, Coenzyme Q10 and Beta Carotene dietary supplements

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ABSTRACT
In this study, common tests such as 2,2-diphenyl-l-picrylhydrazyl (DPPH) assay, hydrogen peroxide (H_2O_2) and superoxide anion ($O^{\cdot-}_2$) radical scavenging activity were exerted to measure the antioxidant activities of L-carnitine, Coenzyme Q10 (Co-Q10) and β-carotene dietary supplements. Antiradical and antioxidant capacities were evaluated comparatively with these tests. The highest free radical scavenging activity was observed in β-carotene (H_2O_2 73%; $O^{\cdot-}_2$ 69%), while the lowest activity was recorded in L-carnitine (H_2O_2 59%; $O^{\cdot-}_2$ 67%). The radical scavenging activity of these dietary supplements was discovered to be significantly different from each other ($P < 0,05$). The antiradical activities of these dietary supplements were measured colorimetrically based on their DPPH radical scavenging ability. At the end of the incubation period (30 minutes), the effective concentration (EC50) values of β-carotene, Co-Q10, L-carnitine and methanol solutions were measured as 10.060, 28.990 and 146.682 µg/mL, respectively. These results demonstrate the potent antioxidant and antiradical activities of all three dietary supplements compared to ascorbic acid (standard). It can only be said that the antiradical activity of L-carnitine is lower than the others, suggesting that this may be due to kinetic behavior.

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Introduction

Free radicals can occur through the environment (external sources) or through natural physiological processes. It can be produced externally as a result of stress, diet, smoking, inflammation, ozone, exercise, drugs, alcohol air pollutants, industrial chemicals, and exposure to X-rays and sunlight. Free radicals, of which there are many types. However, the most common in aerobic (oxygen breathing) organisms are Reactive Oxygen Species (ROS), which are oxygen free radicals. Examples include hydroxyl, superoxide anion, singlet oxygen and hydrogen peroxide. Superoxide radical is taken into account of a primary ROS because it is generated in mitochondria by electron escape from the ETS (electron transport chain) to molecular oxygen in oxidative phosphorylation [1]. The addition of an electron to dioxygen forms the superoxide anion radical ($O^{\cdot-}_2$). Superoxide, usually known radical in biological systems, is produced largely in the mitochondria organelle of the cell. Mitochondria perform the task of energy production and cellular respiration through the "electron transport chain" mechanism. Electrons produce chemical energy by transferring from one molecule to another in the ETS. Oxygen, which is ranked last in the ETS. Some of the electrons leaking from the ETS react with oxygen to compose superoxide radicals [2-3]. Approximately 3% of the oxygen in the mitochondria is transformed to superoxide [4-5]. Mitochondrial DNA (mtDNA) is the basic place of radical oxygen injury from superoxide. Although cells can repair most nuclear DNA damage, they cannot easily repair mtDNA damage, and over time mtDNA injury gatheres in cells and closes mitochondria. This causes cells to die and the organism to age [1]. However, superoxide radical is also generated by phagocytes to kill pathogens during infection. Superoxide radicals can be produced by both enzymatic and non-enzymatic pathways.
Superoxide radicals, which are radicals, are relatively unreactive with biological molecules such as lipids, proteins and DNA. Under physiological situation, the superoxide radical usually exists largely as the radical anion ($O^{\cdot-}_2$) and in a very small proportion (0.6%) as the hydroperoxyl radical ($HOO\cdot$) [6]. Superoxide radicals do not interact straight with DNA Radical anion can be occured in biological systems as a result of thiol mediated mending of radicals by thiols, outcomes in the occurring of thiyl radicals [7]. Hydrogen peroxide (H_2O_2) is a non-free radical, oxidizing agent generated by the two electron reduction of oxygen (O_2). Hydrogen

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peroxide can produce OH (hydroxyl) through the fenton reaction in existence molecular oxygen (O_2) and transition metal ions. Superoxide and hydrogen peroxide are the two main ROS, which form other radicals such as the hydroxyl radical and peroxynitrite. DPPH is a stable free radical discovered by Blois method [8]. In the DPPH method, the antioxidant capacity is evaluated by measuring the DPPH radical scavenging capacity of antioxidants spectrophotometrically. Supplements are widely used today to protect against various diseases, to strengthen the immune system and for supportive and complementary treatment. The most notable of these supplements are Coenzyme Q10 (Co-Q10), β-carotene and L-carnitine. Each of these has its own specific purpose. Co-Q10, also known as ubiquinone, is a lipid-soluble (lipophilic) intracellular antioxidant that delays aging, improves human immunity, increases body resistance and vitality, reduces oxidative stress in coronary artery disease, and increases antioxidant enzyme activity [9-10]. Co-Q10 is synthesized internally in the human and acting an important role in the formation of cellular respiration in the mitochondria for the production of ATP (adenosine triphosphate). It protects proteins (mitochondrial), cholesterol (low-density lipoprotein) and phospholipids (membrane) from oxidative cell damage caused by free radicals [11]. Co-Q10 behaves as an intracellular antioxidant by behaving as the main quenching of free radicals and reactive oxygen species. β-carotene is one of more than 600 naturally occurring compounds called carotenoids. Carotenoids are pigments that assist in photosynthesis in plants. The antioxidant effect of β-carotene is based on its capability to quenching peroxyl radicals and quench singlet oxygen [12-13]. It protects lipids from free radical oxidation by binding to aggressive peroxyl free radicals. They can terminate oxidation chain reactions [14-15]. L-carnitine has significant physiological duty ATP manufacturing in peripheral tissues and in carrying long chain fatty acids over the internal mitochondrial membrane for β-oxidation [16-17]. L-carnitine, obtained from both dietary and internally biosynthesis, is an significant co-factor of peroxisomal oxidation of long-chain fatty acids and has significant duties in the regulation and intermediary metabolism of neural functions in humans [18].The objective of this study is to comparatively appraise the antioxidant capacities of antioxidant supplements such as Co-Q10, l-carnitine and β-carotene, which are used by consumers to protect their general health and support their immunity.

Material and Methods

Material

Ministry of Agriculture and Forestry approved L-carnitine (500 mg per tablet), Coenzyme Q10 (30 mg per tablet), Beta Caroten capsules (7 mg per tablet) were commercially obtained from Solgar .

Sample Preparation

L-carnitine, Co-Q10 (ubiquinone) and β-carotene tablets were homogenized by mortar and pestle. Each 100 mg of homogenized sample was dissolved by incubating in methanol (1:100 ml ratio) for DPPH analysis and in ultrapure water (UPW) for superoxide anion radical and hydrogen peroxide scavenging activity for 24 hours in a dark medium. At the end of this period, these specimens were centrifuged (1800 rpm, 20 min) and the supernatant was collected for analysis.

DPPH (2,2-diphenyl-l-picrylhydrazyl) free radical scavenging activity

Free radical scavenging activities of Co-Q10, L-carnitine and β-carotene were measured against DPPH (2,2-diphenyl-1-picrylhydrazyl) [19]. DPPH is a stable free radical that absorbs at 517 nm, and its absorption decreases when reduced with antioxidants. Briefly, 2 mL of methanol solutions of supplements prepared at different concentrations (5 μg/mL, 10 μg/mL, 20 μg/mL, 40 μg/mL, 80 μg/mL, 160 μg/mL, 320 μg/mL, 640 μg/mL and 1280 μg/mL) were combined with DPPH (3 mL) also prepared in methanol. For the reaction, the solutions were incubated in the dark for 30 minutes. Then, absorbance values of the solutions were taken at 517 nm using a spectrophotometer against the DPPH only solution (blank solution). % inhibition activity was calculated according to the equation [(%)=[(Acontrol−Asample/Acontrol)×100]. Then, % inhibition was drawn against concentration and EC_{50} was reckoned from this graph. EC_{50} values express the concentration at which 50% of radicals are scavenged by antioxidants, with lower EC_{50} values indicating higher antioxidant capacity. In this study, L-ascorbic acid was employed as the standard antioxidant.

Hydrogen peroxide scavenging activity

H_2O_2 scavenging capability of Co-Q10, L-carnitine and β-carotene was identified by the method of Ruch et al. [20]. For this purpose, 40 mM H_2O_2 solution was made in pH 7.4 PBS (phosphate buffer). 0.6 mL of H_2O_2 was added to Co-Q10, L-carnitine and β-carotene at a concentration of 80 μg/mL. The spectrophotometer absorbance of the reaction mixture was taken at 230 nm after 10 min. PBS was employed as a blank and L-ascorbic acid was employed as a standard. The H_2O_2 clearance percentage of supplements and the standard was calculated according to the equation [(%Cleaned H_2O)=[(Acontrol−Assample/Acontrol)×100].

Superoxide anion radical scavenging activity

$O^{\cdot-}_2$ scavenging activity of Co-Q10, L-carnitine and β-carotene was measured according to the procedure specified by Nishikimi et al. [21]. Superoxide radicals are formed as a result of the oxidation of NADH and the analysis is based on the reduction of nitroblue tetrazolium (NBT). Briefly, superoxide radicals were produced by mixing 1 ml of NBT solution that was prepared with 156 µM NBT in 100 mM PBS at pH 8.0, 1 ml of NADH solution that was prepared with 468 µM NADH in 100 mM PBS at pH 8.0, and 0.1 ml of supplements solution. To initiate the reaction, 100 µl of PMS solution, which was prepared with 60 µM PMS in 100 mM PBS at pH 8.0, was added to the mixture. These reaction mixture was kept at 24°C for 7 min and the absorbance at 560 nm was read against blank. L-Ascorbic acid was employed as control. The reducing absorbance of the reaction mixture stated rising superoxide anion scavenging activity. Percent inhibition of superoxide anion formation by supplements and standards was calculated according to the equation [(% SOSA)=[(Acontrol−Asample/Acontrol)×100]. Blank, in which NBT + NADH + PMS solution without supplement was used.

Statistical Analysis

The results obtained were given as mean. Significant differences were defined by one-way analysis of variance (ANOVA) employing the SPSS 26.0 statistical package program. Differences were accepted as significant at the 0.05 level ($P < 0.05$). All experiments were repeated at least three times.

Results and Discussion

Scanning electron analysis results

The $O^{\cdot-}_2$ (superoxide anion radical) is a poor oxidant. However, it is quite toxic (dangerous) and causes oxidative stress by causing the manufacture of singlet oxygen and hydroxyl radicals, which are stronger [22]. Enzymatic reactions in the xanthine oxidase system can produce superoxide anion radical. Hydrogen peroxide (H_2O_2) radical can be produced in organisms by reactions catalyzed by superoxide dismutase (SOD) and many oxidizing enzymes (glucose oxidase, xanthine oxidase and other oxidases). Since the H_2O_2 formed is uncharged, it can pass through biological membranes and oxidize many biomolecules. However, this oxidation is very slow because the reaction kinetics are slow, which makes it possible for them to accumulate in large quantities [23]. The radical quenching abilities of antioxidant compounds are generally related to their potential to generate stable radicals. In this study, the ability of Co-Q10, L-carnitine and β-carotene to scavenge hydrogen peroxide and superoxide anion is demonstrated in Figure 1. The ability of these supplements to scavenge H_2O_2 and $O^{\cdot-}_2$ was measured in comparison to the standard antioxidant ascorbic acid. At the same concentrations (80 µg/mL), the $O^{\cdot-}_2$ and H_2O_2 scavenging activities (%) of L-carnitine, Co-Q10, and β-carotene and ascorbic acid were 67, 69, 69, 81 and 59, 71, 73, 84, respectively. These results showed that these three supplements have effective superoxide anion radical and hydrogen peroxide scavenging activity and have statistically significant different scavenging effects from each other ($P < 0.05$). At concentrations of 80 µg/mL, the $O^{\cdot-}_2$ scavenging effect of the supplements and the standard declined in the order of ascorbic acid > Co-Q10 and β-carotene > L-carnitine. H_2O_2 scavenging effect declined in the order of ascorbic acid >β-carotene > Co-Q10 > L-carnitine.

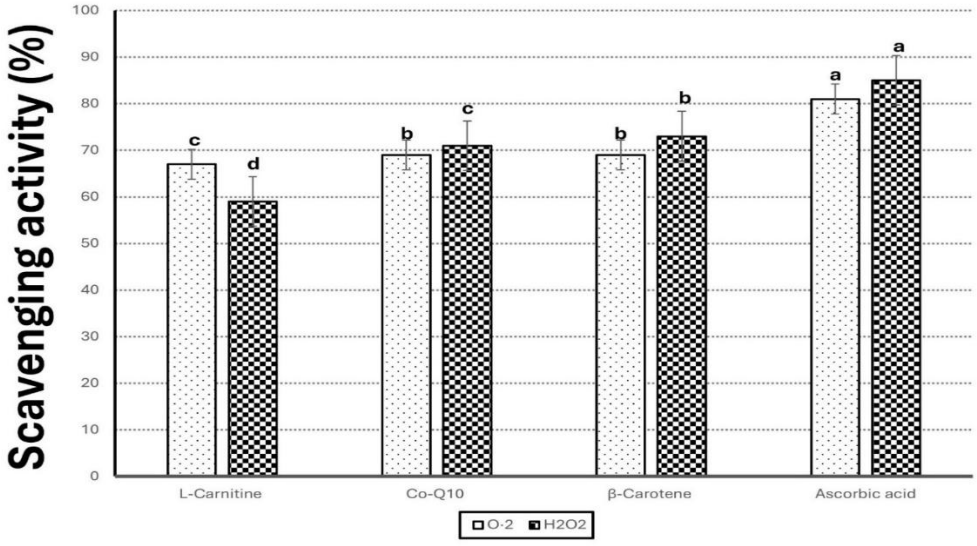

Fig 1 Comparison of $O^{\cdot-}_2$ and H_2O_2 radical scavenging activity of Co-Q10, L-carnitine, β-carotene and ascorbic acid at 80 µg/mL concentration. Lower case letters (a,b and c) indicated that the concentrations for each supplements are significantly different from each other according to Tukey's HSD test ($P < 0.05$).

Again, the antioxidant activities of supplements such as L-carnitine, Co-Q10, and β-carotene in the DPPH test with different concentrations of standard antioxidant (ascorbic acid) and regression EC_{50} values were calculated. The results of the DPPH analysis are expressed as EC_{50}, the value corresponding to the antioxidant concentration necessary to drop the early concentration of the DPPH radical by 50% [24].

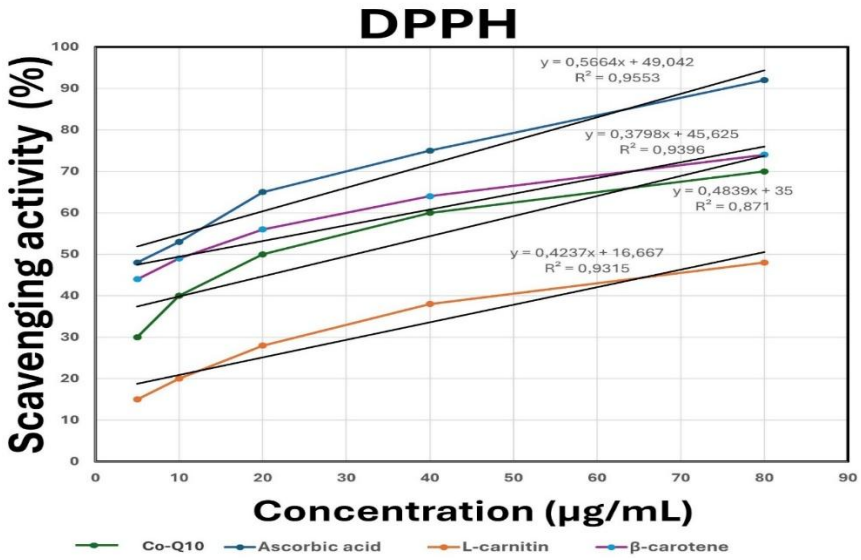

Fig 2 Relationship between Co-Q10, Ascorbic acid, L-carnitine and β-carotene supplement at increasing concentrations and DPPH free radical scavenging activity

In Figure 2, the antioxidant activities of the supplements at changing concentrations (5, 10, 20, 40 and 80 µg/ml) were tested using the DPPH method. In the DPPH assay, antioxidants (i.e., molecules that can donate electrons or hydrogen) colorimetrically drop the purple stable radical DPPH to the yellowish DPPH2 (diphenyl-picrylhydrazine). DPPH gives strong absorption at 517 nm due to unpaired electron. When the radical pairs its unpaired electron in the presence of antioxidant (hydrogen donor), its absorption decreases and its purple color turns into yellow. In other words, the lower the absorption, the higher the antioxidant activity. In our study, it was witnessed that the free radical scavenging activity of Co-Q10, L-carnitine and β-carotene antioxidants changed depending on the concentration change. When the DPPH radical scavenging efficiency of the supplements was tested, β-carotene, Co-Q10 and L-carnitine showed DPPH free radical scavenging efficiency of 74%, 70% and 48% at 80 µg/ml.

Table 1 Co-Q10, L-carnitine, β-carotene and ascorbic acid EC_{50} values

Samples	EC50
Ascorbic acid	5.424 µg/mL
β-carotene	10.060 µg/mL
Co-Q10	28.990 µg/mL
L-carnitine	146.682 µg/mL

In the present study, the antiradical activity effective concentrations (EC_{50}) of Co-Q10, L-carnitine and β-carotene were calculated and expressed in Table 1. The EC_{50} value is a very useful measurement parameter for evaluating antioxidant potential [25]. However, the non-linear relationship between antiradical activity and antioxidant concentration in the calculation of EC_{50} values makes the EC_{50} calculation somewhat difficult. The R^2 values in our study being between 0.9553 that is, very close to 1, indicate that the calibration ranges are

quite linear. In general, the lower the EC_{50} value, the less antioxidant is needed to scavenge DPPH radicals, indicating the radical scavenging effect of the antioxidant. Of these supplements, β-carotene and Co-Q10 appear to have relatively high antiradical activity compared to L-carnitine. In addition, although the R^2 (0.9315) value of L-carnitine is high, the linear link between its the percentage of inhibition and concentration is limited. This is clearly seen when we look at Figure 2. For L-carnitine, the Max. concentration (80 µg/ml) needs to be increased to obtain a better result and a non-linear relationship is likely to occur with increasing concentration. A similar situation has been observed in previous studies. For example, in one study, researchers reported that the maximum concentration should demonstrate 670 percent radical scavenging efficiency for the results to be more satisfactory [26]. In a different study, a linear relationship was demonstrated to link antioxidant concentration and percent inhibition in a limited concentration range [27].

DPPH is on a large scale employed to estimate the free radical scavenging efficiency of diverse antioxidant substances. Some of these studies are as follows; To evaluate the $O^{\cdot-}_2$ radical scavenging activity of *Vaccinium myrtillus* L. (blueberry), natural antioxidants vitamin C, phenolic acids and flavonoids found in *V. myrtillus* were separated by solid phase extraction. These antioxidants have been shown to have very strong free radical quenching capacities as a result of the superoxide anion free radical test [28]. In the study investigating the role of Coenzyme Q10 in improving the harmful effects of oxidative stress caused by high doses of H_2O_2, it was noted that the oxidative stress effect caused by H_2O_2 could be reduced by the application of Coenzyme Q10 [29]. The antioxidant properties of L-carnitine was researched *in vitro* using the DPPH test, H_2O_2 and $O^{\cdot-}_2$ scavenging activities. As a result, it was found that L-carnitine has strong H2O2 and O·2 scavenging activities. Its EC50 for DPPH is very high compared to acarbic acid. Therefore, it cannot be said that L-carnitine has strong DPPH scavenging activity. [30]. In the study researching the link between plasma L-carnitine amount and antioxidant effects, it was noted that L-carnitine increased the capacities of total antioxidant and antioxidant enzymes capacity in healthy individuals. As a result, researchers have suggested that it can be used as a complementary treatment in chronic diseases that cause oxidative stress [31]. In another study, L-carnitine and its effects on antioxidant status and lipid peroxidation were examined in the liver, kidneys, and blood of aged and young rats. L-carnitine has been shown to improve over time the excessively elevated lipid peroxides and excessively decreased antioxidants in elderly individuals [32]. Researchers have highlighted that carnitine is highly effective in normalizing age-related antioxidant status and lipid peroxidation and in regressing age-related disorders of free radicals [32]. In a study evaluating the potential protective effect of L-carnitine, it was shown to be effective in reducing some biochemical and hematological changes and chromosomal damage induced by free radicals caused by radiation in mice [33].

Conclusion

According to the results of the study, commercially available Co-Q10, L-carnitine and β-carotene supplements were found to be effective antioxidants in decreasing power, DPPH radical and H_2O_2 and $O^{\cdot-}_2$ scavenging activity assays when encounter with standard antioxidant compounds such as ascorbic acid.

Abbreviations

DDPH: 2,2-diphenyl-l-picrylhydrazyl; ETS: electron transport chain; $O^{\cdot-}_2$: Superoxide Anion Radical Canadian; ROS: Reactive Oxygen Species; Co-Q10: Coenzyme Q10; mtDNA: Mitochondrial DNA; HOO·: Hydroperoxyl Radical; H_2O_2: Hydrogen Peroxide; SOD: Superoxide Dismutase; ANOVA: one-way analysis of variance; UPW : Ultrapure Water; PBS: Phosphate Buffer; SD: Standard Deviation

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Availability of data and material

Please contact the corresponding author for any data request.

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Oviposition preferences of Black Soldier Fly (*Hermetia illucens* (L., 1758) (Diptera: Stratiomyidae)) on different manures

Gökhan Aydın [1], Ergin Turantepe[2]

ABSTRACT
Hermetia illucens L. (Diptera: Stratiomyidae) called as black soldier fly (BSF), is known as an efficient bio-converter of organic waste are mostly rearing for use as fish and farming feed. Studies on BSF cultivation are generally related to the discovery of the optimum organic substrates of the larval stage. The 'preference-performance principle' suggests that female adults prefer to oviposit in substrates that maximise offspring fitness. Therefore, the substrate needs to be also attractive to females for oviposition. To reveal the best substrates in which is attractive for the female to lay eggs is also extremely important for the better larval development. In this study, five different poultry manures, such as goat, peafowl, pheasant, and chicken, quail manure and a non-manure environment (control) were used in the experimental setup for BSF females to lay eggs in the study. Goat manure was least preferred by BSF adult females for laying eggs during the experiments. The most preferred manures for laying eggs by BSF were found peafowl, pheasant, and chicken, respectively. In the percentage similarity analysis, it was calculated that the number of eggs obtained from goat manure was the least and was different from all other manure tested. According to percentage similarity results the most similar group were found peafowl manure and chicken manure with 58.83%. The study also provides detailed information on the BSF breeding.

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Introduction

Due to its geographical location, Türkiye hosts three different Hotspots (protection priority areas), namely Irano-Anatolian, Caucasus, and Mediterranean [1-3]. In parallel with the plant species richness and high endemism rate (almost 33%), insect biodiversity is also quite rich in the country [4-6]. While new insect species are being discovered every day due to the richness of plants and the favourable location of countries, some species are unfortunately included in the "critically endangered (CR)" or "extinct (EX)" category due to the negative effects of some agricultural policies [7-11]. The populations of insect species living in their natural habitats are significantly decreasing due to some negative anthropogenic and/or ecological factors, some tropical insect species, such as *Hermetia illucens* L. (Diptera: Stratiomyidae) called black soldier fly (BSF), is known as an efficient bio-converter of organic waste, are rearing for use as fish and farming feed in Türkiye [12-17].
Although not yet widely used, it is possible to use *H. illucens* for waste management other than farming feed [18-19]. While there are numerous studies on usage of BSF as farming feed, there are limited numbers of research that provide insight into the production procedures of it.
Insects play an important role in many areas around the world, including food security, environmental health, biotechnology, agriculture and medicine [20]. The production process (optimum temperature, humidity, food preferences, oviposition preference, etc.) of beneficial insects used as food, such as the *H. illucens*, needs to be improved and standardized. Assume that the Black Soldier Fly (BSF), designated for use as aquaculture feed, is subjected to testing with "Food A" as part of the production process. The larvae, which are nourished with "Food A," are subsequently desiccated and processed into fish meal, and the resultant impact on the development of Rainbow Trout is assessed. If, conversely, the BSF larvae were to be fed "Food B" instead of "Food A," it is likely that the effects on the development of Rainbow Trout would differ. Thus, the anticipated response would be "most probably NOT." It is worth noting that numerous studies have examined the implications of BSF as fish feed and/or poultry feed on the development of aquatic species or livestock, yet

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they often fail to disclose pertinent information regarding the production procedures employed [21-24]. Although the primary objective of our investigation is not to detail the production procedures for *H. illucens*, such information is included in the Materials and Methods section to assist researchers intending to conduct analogous studies in the future.

The aim of this study was to investigate the egg laying preferences of the BSF during the oviposition period, which is the most important of the production stages, using different types of manure.

Material and Methods

Rearing of *Hermetia illucens*

Before the experiments began, the *H. illucens* was allowed to produce one generation under laboratory conditions. To this end, eggs were began to be reared in a nutrient medium prepared by moistening with a 50:50 (%) mixture of goat manure and wheat bran, with the addition of water (50-60%) in a 5.5-liter production container, maintained at a constant temperature of 27 °C and 60% humidity. The production container was covered with a tulle large enough for the larvae to pass through, preventing the eggs from contacting water. The nutrient medium was kept moist by spraying a certain amount of water each day. After the first-stage larvae hatched from the eggs and passed through the tulle and reached the nutrient medium, organic waste supplement (homemade meals, fruit peels, etc.) was added at a rate of one-tenth of the bottom of the production container. When the larvae reached an average size of 6-7 mm, they were transferred to larger production containers and continued to be fed with organic waste at 2-day intervals depending on the number of larvae. As the larvae reached maturity, the number and size of the production containers were also increased in accordance with the growth of the larvae. The supplementation of organic waste was terminated upon the alteration of the larvae's color from white-yellowish to brown. At the onset of the prepupa stage, the larvae were separated from one another through a process of sieving, whereby they were passed through a series of progressively finer sieves in order to remove residual organic waste. Larvae in the prepupa period were placed in a different climate cabin in production containers with a wide base area and low height. The pupal period individuals were placed on shelves with a volume of 3 m^3 surrounded by tulle at 4 corners, and kept at 85% humidity and 29 ℃ until they emerged as adults. Artificial flowers with broad leaves were placed to resemble the natural environment for the adults emerging from the pupa. The environment where the adults were kept was sprayed 3 times a day. Mated females were taken to the experimental setup to lay eggs. Each time, 10 mated females were taken into the experimental setup prepared to lay eggs. This application was repeated a total of 10 times to determine the number of replications.

Preparation of the Experimental Setup

The experimental setup was designed by us to hypothesize adult female *Hermetia illucens* individuals would prefer which manure to lay eggs (Fig. 1). Five different poultry manure, goat manure and a non-manure environment (as control) were used in the experimental setup.

In the mechanism, the "release box" where adult females were placed was designed in the area where six different pipes meet. Six devices were designated at the ends of the pipes with lids where different moistened manure would be placed were designed. Goat manure, quail manure, chicken manure, pheasant manure and peafowl manure were placed in these devices. One device was left empty as a control group. A vacuum pump was used to ensure that the manure odors could reach the release box where the adult females were located. The areas where these connected to the pipes were designed to be funnel-shaped towards the inside of the chamber and are designed to be large enough for adult female individuals to pass through (Fig. 2). In addition, 1 mm diameter holes were drilled on the lidded boxes to reduce the vacuum created by the vacuum pump connected to the top cover of the release box and to allow the poultry manure odors in the lidded chambers to reach the release box.

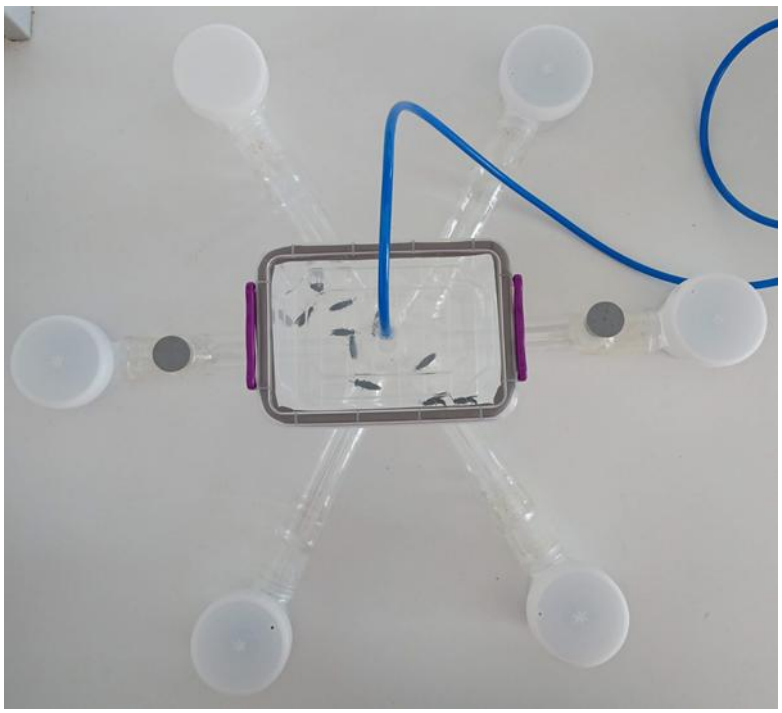

Fig 1 Experimental setup used to test which manure *Hermetia illucens* adult females would prefer to lay eggs

The location of the experimental setup was kept fixed and the lidded boxes and the experimental setup were cleaned in each new trial. In the experiment, the manures were placed in lidded boxes moistened by 95%. The vacuum pump's suction pressure was adjusted to 0.132 atm (100 mm Hg) (Preliminary studies conducted prior to the experiment concluded that this pressure was the most appropriate). After the experimental setup was completed, the captured females were transferred to the box called the release box connected to the system and the study was started. A total of 10 replications were conducted with 10 females in each experiment.

Fig 2 Lidded plastic container designed to be funnel-shaped for storing manures (left), the vacuum pump (middle), and vacuum pump's suction pressure (right)

Statistical Analysis

The means of two samples with equal variance obtained from manure X and manure Y were analysed by t - test to understand whether there was a significant difference in the effect of manure on the oviposition behaviour preferences of female adults of *H. illucens* in the experiments ($P<0.01$). LSD test was performed in order to difference between eggs laid of female adults of BSF on different manures.

The Multi Variate Statistical Package (MVSP) 3.11c program was used to classify the manures were taken into the experiment [25]. The classification method was chosen as the arithmetic group averages (Unweighted Pair Group Method with Arithmetic Mean - UPGMA) in the evaluation of the obtained data. Percentage similarity was used to calculate the similarities of different manures used in the experiments.

Results and Discussion

Mean number of *Hermetia illucens* eggs on manures of chicken and goat, pheasant and goat, peafowl and goat, peafowl and quail, peafowl and release box, peafowl and empty, peafowl and chicken were found statistically different by t - test while the rest combination manures in terms of average of eggs were found not significant ($P<0.01$) (Table 1).

Boafo et al. (2023) have been used six organic substrates for *H. illucens* production on their study and it has been evaluated for their suitability as oviposition attractants and larval development. In the oviposition tests, millet porridge mash has been found the most preferred substrate for egg laid, whereas from the other substrates (chicken manure, pig manure, fruit waste, pito mash, and waste from roots and tubes) have been recovered only a few eggs [26]. *H. illucens* larva has been found strongly preferred pig manure over the mass-rearing diet [27]. In another study, chicken, pig and cow manures have been used for evaluated to larval development of *H. illucens*. Larva on pig and cow manure have been recorded relatively greater abundance [28]. Agricultural waste, rice bran, vegetable waste, fruits waste, and household waste, have been mixed with goat manure, have been used to determine the preference to eggs laid by *H. illucens*. According to study; the highest number of eggs of BSF have been declared in household waste then vegetable waste, rice bran, and fruits waste while the lowest eggs have been found on agricultural waste [29].

Table 1 Number of eggs (Mean±SD) laid by *Hermetia illucens* adult females on different manures.

Manure	n	Mean±SD	Goat	Quail	Release Box	Empty	Chicken	Pheasant	Peafowl
Goat	10	0.2±0.17 c*	1.00000						
Quail	10	0.7±1.12 bc	0.18246	1.00000					
Release Box	10	0.9±0.54 bc	0.01792	0.63012	1.00000				
Empty	10	1.1±2.54 bc	0.10166	0.51725	0.72314	1.00000			
Chicken	10	1.6±1.82 b	**0.00578**	0.11452	0.16734	0.45905	1.00000		
Pheasant	10	2±3.33 ab	**0.00708**	0.06724	0.09427	0.25573	0.58433	1.00000	
Peafowl	10	3.5±2.27 a	**0.00000**	**0.00014**	**0.00012**	**0.00282**	**0.00825**	0.06053	1.00000

* The same letters in the column containing the mean and Standard Deviation (SD) values indicate that the means are not statistically different from each other according to LSD test (P<0.01). The P values indicated in bold were found to be statistically significant according to the t - test (P < 0.01; t = 2,10092204).

The most preferred substrate for oviposition by BSF for laid eggs were calculated peafowl manure, pheasant manure and chicken manure, respectively. Goat and quail manures proved be the least attractive manures for BSF to lay eggs. The number of eggs counted in these manures was found even lower than the non-manure environment used as control.

Percentage similarity results showed that the most similar group were found peafowl and chicken manure with 58.83%. The similarity rate of the group consisting of peafowl and chicken to pheasant was calculated as 25.27%. According to the similarity analysis results, it was noticed that the most different manure was found as goat manure and its similarity rate to all other groups was found only 11.10% (Fig 3).

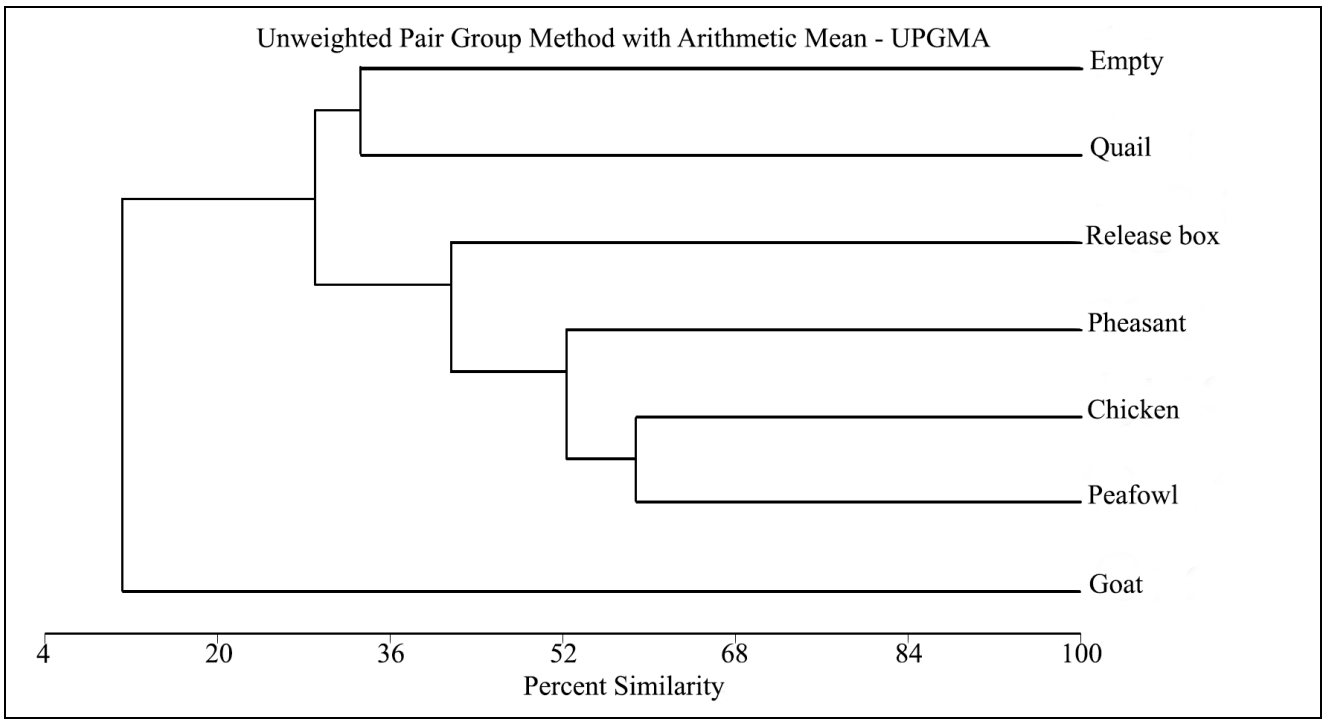

Fig 3 Percentage similarity rates of the manures used in the experiments where *Hermetia illucens* adult females laid eggs

Conclusion

Many previous studies have focused on which substrate is the most nutritious for larval development. The substrate must also be attractive to females for oviposition. The 'preference-performance principle' suggests that adult females prefer to oviposit in substrates that maximize offspring fitness [26]. To reveal the best substrates that are attractive for the females for oviposition is also extremely important for the better larval development.

According to our results; goat manure, which was found to be statistically different from the other substrates tested, was found to be the least preferred manure for BSF when compared with poultry manure. Therefore, it is recommended that goat manure should not be used as an egg-laying substrate for BSF. Adult females of BSF favoured to oviposit to peafowl, pheasant and chicken and no statistically significant difference was found between these poultry manure. Peafowl, pheasant and chicken or a mixture of these manures in certain proportions can be recommended for future studies. However, it is not easy to find peafowl and pheasant manures in Turkey. Chicken manure, which has no statistical difference between peafowl and pheasant, can be easily found.

In the calculated percentage similarity analysis showed that chicken and peafowl manures were found the most similar group with 58.82%. Therefore, chicken manure can be recommended as a suitable substrate for both the insect's egg laid preference and larval development.

Abbreviations

Standard Deviation (SD); critically endangered: CR; Extinct: EX; *H. illucens*: *Hermetia illucens*;
BSF: Black Soldier Fly; LSD: MVSP: The Multi Variate Statistical Package; UPGMA: Unweighted Pair Group Method with Arithmetic Mean; LSD: Least Significant Difference; °C: Degree (Celsius); atm: atmospheric pressure/A standard atmosphere.

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Data Availability statement

The author confirms that the data supporting this study are cited in the article.

Compliance with ethical standards
Conflict of interest / Çıkar çatışması
The author declare no conflict of interest.
Ethical standards
The study is proper with ethical standards.
Authors' contributions
During the study, Ergin TURANTEPE conducted field and lab. research, Gökhan AYDIN wrote the article.

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Review article

Advancements in 3D *in vitro* Cell Culture Systems: Enhancing Drug Pharmacokinetics and Toxicity Assessment in Pharmaceutical Development

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ABSTRACT
The development and screening of pharmaceuticals encounter significant predictive inaccuracies when transitioning from animal models to human trials, primarily due to interspecies differences in drug metabolism and effects. Traditional 2D and animal models, although fundamental in early drug development stages, often do not accurately reflect human physiological responses, leading to high attrition rates in clinical phases. This review highlights the emerging role of three dimensional (3D) *in vitro* models, including organoids and tissue chips, as more predictive and ethically favorable alternatives. These models mimic human physiological and pathophysiological conditions more closely, providing an enhanced platform for drug pharmacokinetics and toxicity assessment. Although there are some disadvantages, innovations in scaffold-based and scaffold-free 3D cultures, bioprinting techniques, and organ-on-chip technologies not only address the limitations of traditional models but also offer profound insights into complex tissue dynamics and drug behaviors. This paper discusses the significant advances in 3D *in vitro* technologies that promise to refine predictive accuracy, reduce reliance on animal testing, and streamline the pharmaceutical development pipeline.

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Introduction

Pharmaceutical companies face a challenge in the development process, which requires significant investments of time and resources [1]. Despite considerable efforts, over 90% of drug candidates face rejection after phase I clinical trials [2], [3]. A key factor contributing to this rejection rate is the lack of alignment between the results obtained from preclinical animal studies and those observed in phase I clinical studies involving human subjects. The difference between the results obtained in preclinical animal studies and those observed in human trials is due to inherent variations in species-specific factors such as physiology and drug sensitivity [4]. Moreover, ethical concerns regarding animal studies have gained increasing attention. Therefore, the development of 3D *in vitro* models, such as organoids and tissue chips, is not only a promising approach to address the limited predictive accuracy associated with animal studies, but also to address ethical issues related to animal testing.

Pharmacokinetics studies the interaction between a pharmaceutical compound and the human body. This is achieved by investigating four main parameters (ADME); the entry into the body (absorption), the moving through the body (distribution), the changes undergoing in the body (metabolism), and the leaving of the body (excretion) of the drug compound [5], [6]. The outcomes of ADME investigations play a pivotal role in determining the effectiveness, safety and potential off-target effects of the pharmaceutical compound. The ADME parameters are crucial in the early drug development process.

In early drug development, traditional 2D *in vitro* and animal models remain crucial for assessing a drug's ADME, safety and effectiveness prior to clinical trials. These models aim to simulate human diseases and predict how humans will respond to the drug candidate. However, both traditional approaches showcase limitations. Traditional

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2D *in vitro* models exhibit limitations due to unnatural growth kinetics, cell attachment characteristics, and cell polarity. These methods fail to accurately represent essential cell-cell and cell-extracellular matrix (ECM) interactions, significantly impacting the drug development process [7]. While animal models offer improved reliability for evaluating human safety and efficacy compared to traditional 2D *in vitro* models, their predictive accuracy is continuously questioned. Species-specific differences often hinder their ability to fully replicate the biology and mechanisms of human diseases [8]. To address these shortcomings, *in vitro* 3D models have emerged as a promising alternative [9]. These models exhibit a remarkable capacity to recapitulate the complexities of solid tumors, including features like reduced oxygen availability leading to the formation of hypoxic regions within spheroids or organoids. Additionally, they effectively mimic the nutrient gradients and elevated glucose metabolism characteristic of tumors, offering significant advantages over traditional 2D culture models [10]. Furthermore, 3D *in vitro* models are demonstrating promising results in simulating drug disposition and pharmacokinetics, crucial factors for predicting drug safety and efficacy during early-stage drug development [11]. However, despite these promising advancements, further research is necessary to address remaining uncertainties associated with 3D *in vitro* models.

Three-Dimensional (3D) *in vitro* Models

Scaffold-based 3D models

Scaffold-based 3D models involve the strategic seeding of cells onto or within a prefabricated, 3D structure known as a scaffold. These scaffolds, typically composed of biocompatible biopolymers, are designed to mimic the natural extracellular matrix (ECM) which contains secretion of growth factors, cytokines or angiogenic factors that play a critical role in supporting various cellular functions [12]. By providing essential physical cues and facilitating cell attachment, migration, proliferation, and differentiation, scaffolds ensure optimal access to nutrients and oxygen while enabling efficient waste removal [13]. The pioneering work of Del Buono et al. established the foundation for scaffold-based 3D models by demonstrating the capability of colorectal adenocarcinoma cells to exhibit morphological differentiation within a collagen gel scaffold, marking a significant advancement from traditional models utilizing basement membrane extracts or normal rat mesenchymal cells [14]. Furthermore, the growth of prostate cancer cells was extensively higher in 3D forms because the upregulation of CXCR4 and CXCR7 signaling pathway which has cross-talk with tumor microenvironment [15]. Since this initial breakthrough, the field has witnessed continuous evolution driven by emerging technologies, leading to the development of increasingly intricate systems incorporating diverse scaffold types. Three primary categories of scaffolds are employed in this technique: membranes, matrices, and hydrogels. Moreover, scaffold-based 3D cell culture models can be classified as polymer-based, hydrogel, decellularized tissue scaffolds and microfluidics. Cell seeding strategies within scaffolds can also vary, with two prevalent approaches: seeding cells onto pre-fabricated, cell-free scaffolds or incorporating cells directly into the scaffold during its fabrication process with the usage of cell-laden bioinks [10].

3D scaffold-based models offer numerous advantages that make them invaluable tools in drug discovery. One of the primary benefits is their ability to closely mimic the *in vivo* cellular organization, providing a more accurate representation of human tissues compared to traditional 2D cultures. This 3D scaffold-based model supports cell growth, differentiation, and function in a way that closely resembles native conditions. These models also allow for the incorporation of co-culture systems, enabling the study of complex cell-cell and cell-matrix interactions. This is particularly useful for creating more physiologically relevant models, such as human-on-chip systems, which can simulate the interactions between different tissues and organs. Additionally, scaffold-based models offer a solution to one of the biggest problems in drug development assays with traditional 2D cultures, which is the control of the extracellular matrix (ECM) compounds. Earlier studies have found that ECM compounds and cell-ECM interactions play a crucial role in cellular response [7]. To overcome this limitation and mimic the biological environment and structure, the use of biologically derived (ECM-based) bioinks in the production of 3D printed ECM-mimicking scaffolds has shown great potential [23], [24]. The versatility of scaffold-based models is another significant advantage. They can be constructed from a wide range of materials, including natural and synthetic polymers, which can be tailored to possess specific mechanical, chemical, and biological properties. This customization allows for the creation of cell-, patient-, and disease-specific models, enhancing the relevance of experimental findings. Scaffold-based models are also more resilient to external factors, providing a stable environment for long-term studies [25]. Additionally, the ability to create complex tissue constructs supports the study of organ-level responses, offering insights into tissue-specific drug effects and toxicities. These models

facilitate advanced tissue engineering applications, enabling the development of functional tissues and organs. This capability is particularly important for regenerative medicine and the creation of organotypic models that can be used for disease modeling and drug testing. Furthermore, scaffold-based models are highly adaptable and can be integrated into high-throughput screening platforms. This integration allows for the simultaneous testing of multiple drug candidates, significantly accelerating the drug discovery process. Their ability to provide more predictive toxicology data also enhances drug safety assessments, reducing reliance on animal models. In the context of personalized medicine, scaffold-based models hold immense potential [26]. They can be derived from patient-specific cells, providing tailored disease models and treatment strategies. This personalization improves the accuracy of drug efficacy and toxicity predictions, leading to more effective and individualized therapies.

Table 1 The advantages and disadvantages of the scaffold types

Scaffold type	Characterization	Advantages	Disadvantages	References
Polymer-based	Mimics the ECM with sources of natural polymers such as collagen, gelatin, chitosan, silk fibroin, alginate.	Cells can easily adapt to the ECM-like environment, proliferate and differentiate. High biocompatibility and facilitate contribution to tissue regeneration during the transplantation phase.	Since they are natural ECM materials, they may vary depending on the time they are obtained. Mechanically sensitive and may cause an immune response during transplantation.	[16]–[18]
Hydrogel	Natural and synthetic molecules with extensively higher water contents. Poly(ethylene glycol) (PEG), oly(2-hydroxy ethyl methacrylate) are such samples of synthetic hydrogels.	Mimic the nature of most soft tissues. Facilitate the transition of nutrients and oxygen or organic waste with its elasticity feature. Biodegradable.	Low mechanical strength, lack of long-term stability.	[19], [20]
Decellularized tissue	Cellular components are removed and remained the native tissue structure with ECM and bioactive compounds.	Decellularized scaffolds are biologically identifiable which is advantageous for cell adhesion, proliferation, and survival. They have low immunogenicity.	Quantity of the decellularized tissues variable and hard to obtain. Because the residual DNA contents, the immune response can be triggered. Decellularization process consists of many steps which may disadvantage for preparation of successful material.	[21], [22]

Despite the significant advancements in 3D scaffold-based models, several areas require further development to maximize their potential in drug discovery. One major limitation is the complexity and technical expertise required to fabricate and utilize these models [27]. Simplifying protocols and integrating automated systems could make scaffold-based models more user-friendly, reducing the reliance on highly specialized skills. Reproducibility remains a challenge, as variability in scaffold production and cell culture can lead to inconsistent results. Standardizing procedures and improving manufacturing processes are essential steps toward achieving reliable and reproducible outcomes across different laboratories. Another critical aspect is the compatibility of scaffold-based models with a wide range of cell lines and diseases. Developing versatile scaffold materials that can be

customized to replicate various tissue microenvironments would enhance their applicability [28]. This customization is particularly important for creating disease-specific models that accurately mimic pathological conditions. Cost-effectiveness is also a concern, as the high cost of materials and production can limit the widespread adoption of scaffold-based models. Identifying more affordable materials and scalable production methods could reduce costs and make these models more accessible to the research community. Improving the clinical relevance of scaffold-based models involves enhancing their physiological accuracy and stability for long-term studies. Advanced characterization and monitoring techniques are needed to better understand and optimize scaffold performance. Integrating sensors and developing high-resolution imaging and omics technologies can provide detailed insights into cellular responses and scaffold interactions in real-time. Finally, fostering interdisciplinary collaboration between cell biologists, engineers, material scientists, and clinicians is crucial for overcoming the multifaceted challenges associated with scaffold-based models [29]. By addressing these limitations, future advancements can make 3D scaffold-based models more user-friendly, reproducible, versatile, cost-effective, and clinically relevant, thereby enhancing their impact on drug discovery.

Bioprinting-based scaffolds

Advancements in medical imaging and structure design software have empowered the development of 3D bioprinting, a novel technology that enables the fabrication of complex tissue constructs. This technique utilizes bioinks, a composite material composed of hydrogels, cells, and biological molecules, to replicate the intricate geometry of various tissues as visualized through imaging techniques [30]. Notably, the first successful 3D printing using stereolithography was achieved by Charles W. Hull in 1986, laying the foundation for this transformative technology [31].

In the realm of tissue engineering, the development of bioinks that are robust and biocompatible is essential for successful 3D bioprinting. GelMA is a gelatin-based hydrogel modified with methacrylate groups to enhance its cross-linking capabilities and mechanical properties. This material is particularly noted for its ability to be finely tuned through photopolymerization, allowing for precise control over its physicochemical properties which is crucial for the integrity and functionality of bioprinted constructs. GelMA-based bioinks can incorporate various cell types and have been successfully used to bioprint complex tissue constructs such as skin and cartilage. These constructs exhibit significant intercellular communication and maintain specific tissue functions, which are vital for their integration and functionality post-implantation. Moreover, the versatility of GelMA bioinks is further demonstrated through the incorporation of different cell types within a single bioprinted construct, leading to the fabrication of tissues with distinct zones of extracellular matrix composition, mimicking the natural tissue heterogeneity. This adaptability highlights the potential of GelMA not only as a standalone bioink but also in combination with other materials, such as synthetic polymers and bioceramics, to enhance the mechanical strength and biological performance of the final constructs. Such developments are pivotal for advancing 3D bioprinting technologies towards more realistic, functional, and sustainable tissue-engineering solutions.

The tumor microenvironment plays a crucial role in drug behavior, highlighting the need for *in vitro* models that accurately mimic its characteristics. Naturally derived bioinks, possessing superior biocompatibility and ECM-like properties, offer significant advantages in this regard. These bioinks closely resemble the native extracellular matrix, facilitating the creation of tumor-specific architectures and fostering improved cell-material interactions. Bioinks encompass a diverse range, including natural materials sourced from living organisms, synthetic materials like engineered protein polymers, and hybrid combinations incorporating both elements [10]. Selection of the appropriate bioink type hinges on the specific research application and desired properties. Printability, characterized by optimal viscosity and structural integrity, remains a critical factor throughout the process, ensuring precise control and successful construct formation.

3D bioprinting can be performed through various techniques, each offering distinct advantages and limitations [10], [30]. Light-based or laser-assisted bioprinting employs focused light energy to precisely deposit bioink droplets, enabling high-resolution patterning. Extrusion-based bioprinting utilizes continuous extrusion of bioink filaments through a nozzle, offering efficient fabrication of larger structures. Inkjet bioprinting, also known as droplet-based bioprinting, leverages piezoelectric or thermal actuation to dispense bioink droplets in a controlled manner, facilitating the creation of complex cell arrangements.

In their innovative approach Kim et al. addressed the limitations of lung disease modeling and drug efficacy testing by developing a 3D Inkjet-Bioprinted Lung-on-a-Chip model [32]. This innovative approach utilizes a culture insert containing a micron-thick, three-layered human alveolar barrier model fabricated by drop-on-demand

piezoelectric inkjet bioprinting. These inserts are placed on a custom-made biochip that facilitates nutrient flow throughout the cultures. The three layers mimic the human lung structure, incorporating human lung microvascular

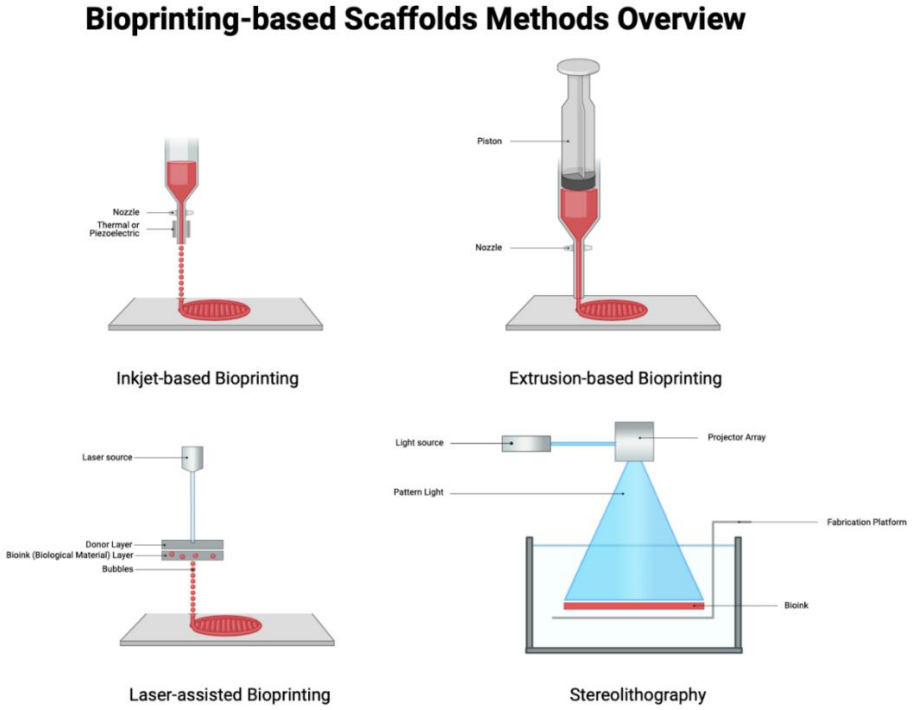

Fig 1 Schematic diagram of different bioprinting techniques, providing a comprehensive overview of the four main bioprinting methods used to fabricate scaffolds for tissue engineering.

endothelial cells, a collagen solution with lung fibroblasts, and both type I and type II alveolar cells. After incubation and mounting on a lung-on-a-chip device, the tissues were analyzed for morphology, structure, and function. The results demonstrated that this model successfully recapitulates the key features of human lung tissue within a microfluidic device. The authors propose that this approach, combining inkjet bioprinting with microfluidics, holds promise for high-throughput analysis in drug discovery.

Bioprinting-based scaffolds offer numerous advantages that enhance their utility in drug discovery. They enable the creation of complex tissue constructs with precise architectural and functional properties through layer-by-layer deposition of cell-laden or acellular bioinks [30]. This precision allows for reproducible and detailed structures, reducing variability and improving research reliability. Bioprinting supports the incorporation of multiple cell types within a single construct, facilitating co-culture systems that better mimic *in vivo* tissues. The customizable properties of bioinks, including mechanical strength and bioactivity, allow for the development of models that closely replicate the extracellular matrix and support desired cellular behaviors [33]. A significant advantage of bioprinting is the ability to include vascularization within constructs, which is essential for nutrient delivery, waste removal, and long-term cell viability [27]. Bioprinting also facilitates the creation of patient-specific models using cells derived from individual patients, promoting personalized medicine by developing tailored therapies.

Bioprinting-based scaffold models, despite their advantages, face several limitations. One major challenge is the complexity and technical expertise required for their fabrication and use. Developing and optimizing bioinks and bioprinting protocols can be time-consuming and require specialized skills and equipment. Reproducibility is another issue, as the precision of bioprinting can lead to variability in the produced constructs, which may affect experimental consistency [33]. Ensuring uniformity across different bioprinted samples remains a significant hurdle. Cost is also a concern, with high expenses associated with the materials, equipment, and skilled labor needed for bioprinting. This limits the accessibility and scalability of the technology for many research laboratories.

Organoid models

Organoids are described as *in vitro* 3D structures grown from stem cells, that with the usage of organ-specific growth factors form a self-organized organ-like structure [34]. Organoid models are groups of cells, which can be derived from genetically modified stem cells or isolated cells from patient-derived tissues [9]. Organoids show physiological similarity to that of *in vivo* tissues and organs [35]. The first organoid was developed by Sato et al. in 2009 while studying the self-renewing epithelium of the small intestine. The renewal process is carried out by Lgr5+ stem cells in the crypt. Sato aimed to cultivate these stem cells in a 3D matrigel culture to observe their proliferation. Rather than observing mere proliferation, the stem cells unexpectedly formed a structural development that replicated the cell types and functions of the gut, creating a miniature version [36]. These engineered tissues mimic the structure and functionality of their natural counterparts, and they can be either transplanted to a specific location or maintained in culture for an extended duration [37]. Currently, multiple types of organoids have been successfully adapted to 3D culture systems.

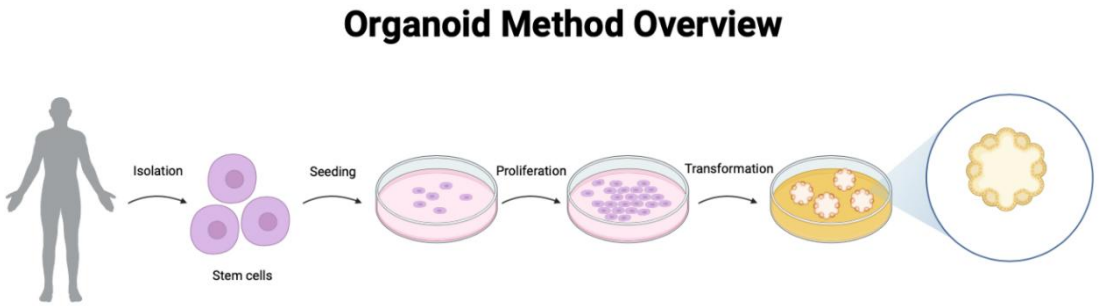

Fig 2 Organoid method overview. The organoid methodology begins with the isolation of human-derived stem cells, sourced either from commercially available cryopreserved human stem cells or obtained from biopsy-derived human stem cells. Following isolation, these stem cells are cultured *in vitro*, undergoing controlled proliferation. Through the induction of organ-specific growth factors, the stem cells are directed to undergo organoid formation.

In their study Okada et al. investigated the passage of orally administered drugs through the gastrointestinal tract, a crucial step for drugs to reach the bloodstream [38]. To address this challenge, they explored an innovative strategy utilizing human intestinal organoids (HIOs). These organoids, derived from LGR5+ stem cells residing in the intestinal crypts, exhibit a remarkable capability for long-term preservation. Notably, HIOs express various pharmacokinetic-related enzymes and transporters, including key players like cytochrome P450 3A4 (CYP3A4), carboxylesterase 2 (CES2), P-glycoprotein (P-gp), and breast cancer resistance protein (BCRP). This elevated expression makes HIOs a promising resource for advancing pharmacokinetic investigations.

Organoid models offer significant advantages in drug discovery by closely mimicking human organ structure and function. These 3D models provide a more accurate representation of tissue-specific processes, enhancing experimental accuracy. Patient-derived cancer organoids show promising results for personalized medicine by reflecting individual tumor biology and predicting patient responses to therapy, facilitating the development of tailored treatments [39]. Organoids retain the gene expression and mutation profiles of the original tumors, making them robust and clinically relevant disease models. They address the limitations of traditional 2D cultures and animal studies, which often fail to closely replicate human disease conditions, offering better translatability of research findings [40]. Additionally, organoids are versatile, supporting applications in drug screening, toxicology, and regenerative medicine, and they maintain stability for long-term studies, making them invaluable tools in advancing medical research and therapeutic development.

Despite their advantages, organoid models face several limitations. One major challenge is assay reproducibility, which is critical for reliable and standardized drug screening [39]. Variability in organoid formation and response can lead to inconsistent results, although strategies like using reference organoids as internal controls can help mitigate this issue. Another significant limitation is the lack of vascularization in organoids, which restricts their growth, differentiation, and functional complexity [40]. This deficiency impacts their ability to accurately model drug delivery and metabolism. Cost and accessibility are also concerns, as the creation and maintenance of organoids can be expensive and require specialized equipment and expertise.

Microfluidic 3D models

In the 1990s, the advent of microfluidic devices revolutionized 3D cell culture systems. By controlling the flow of fluids through microchannels, these devices enable the creation of more intricate and flexible 3D environments. This advancement has been particularly influential in drug development and personalized medicine, where the precision and adaptability of microfluidic 3D models are invaluable. Their ability to mimic complex biological systems makes them a critical tool in these cutting-edge fields of biology [41]. Microfluidics technology extends its applications beyond cell culture models, finding significant utility in high-throughput drug screening, single-molecule analysis, and the development of advanced therapeutics [42].

Organ-on-chip (OoC) models

Organ-on-chip (OoC) models represent an interdisciplinary advancement at the intersection of biology and microtechnology. These scientific and technological systems serve as *in vitro* models designed to replicate the complexity of human physiology [43]. The chips used in these models are microfluidic devices constructed with microchannels, allowing for the precise control of solution volumes ranging from picolitres to millimeters per minute. OoC models provide a platform for recreating, *in vitro*, the complex array of mechanical, fluidic, spatial, and chemical stimuli that tissues experience *in vivo* [44]. OoC models can form intricated cellular microenvironments, which is key in drug assays to recreating *in vivo* conditions and results [42].

Evaluating ADME properties involves a complex process, starting from the drug's entry into the human body, passing in the bloodstream and its distribution to various organs. To replicate this intricate process in OoC models, researchers have developed multi organ human-on-chip (HoC) or body-on-chip (BoC), using various OoC models, which mimic the complexity of the human body's multi-organ systems [45], [46].

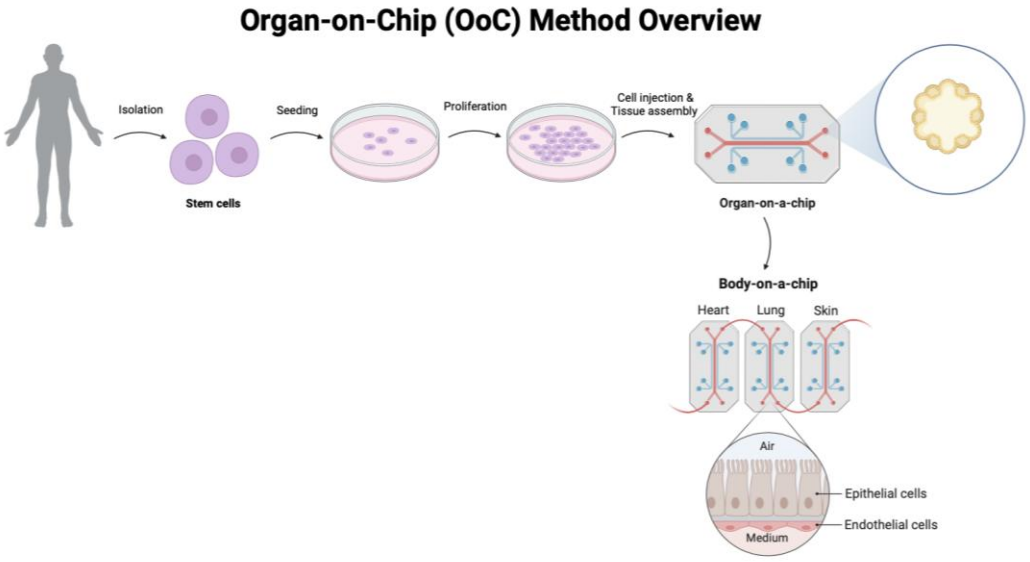

Fig 3 Organ-on-chip (OoC) and body-on-chip (BoC) method overview. Both methodologies begin with the isolation of human-derived stem cells, sourced either from commercially available cryopreserved human stem cells or obtained from biopsy-derived human stem cells. Following isolation, these stem cells are cultured *in vitro*, undergoing controlled proliferation. Through the induction of organ-specific growth factors, the stem cells are directed to undergo organoid formation. Nutrients are brought to cells through microchannels. After having formed OoC's these can be brought together in a BoC to perform ADME and toxicity assessments.

In a study to analyze the mechanisms underlying drug transport, Tsamandouras et al. pioneered the development of the first multi-organ *in vitro* systems by combining gut and liver on a chip model [44]. This innovative approach enabled conducting complex pharmacokinetic assays like bioavailability, drug clearance, and interactions, which are challenging in traditional *in vitro* systems. However, limitations exist, such as the Caco-2/HT29-MTX cells not fully replicating *in vivo* metabolic functions, suggesting patient-derived cells as a potential solution. Additionally, the liver component tends to under-predict hepatic clearance, posing challenges for accurate drug screening.

Lee et al. developed a novel co-culture model using bovine lung-on-chip to evaluate the transport and efficacy of the antibiotic drug danofloxacin [47]. This model incorporated primary bovine bronchial epithelial cells and bovine pulmonary arterial endothelial cells. While the model successfully replicated *in vivo* plasma pharmacokinetics in the endothelial channel, it did not detect consistent levels of danofloxacin in the epithelial channels, likely due to a dilution effect during media sampling. This research represents a significant advancement in creating species-appropriate *in vitro* models, suggesting the potential of the bovine lung-on-chip as a viable alternative for *in vivo* studies in drug and pathogen research.

Microfluidic 3D models, offer several advantages in biomedical research and drug discovery. These models provide precise control over the cellular microenvironment, allowing for the replication of complex tissue and organ functions [43]. By integrating multiple cell types and tissues within a single device (HOC), they can mimic the interactions between different organs, providing a close resemblance to human physiology. One of the key strengths of microfluidic 3D models is their ability to simulate physiological fluid dynamics, including shear stress and nutrient gradients, which are crucial for maintaining cell function and viability [41], [48]. This feature enhances the relevance of these models for studying drug delivery, pharmacokinetics, and disease mechanisms. Microfluidic models also support real-time monitoring and high-resolution imaging, enabling detailed analysis of cellular responses and tissue development. Their compatibility with advanced sensing technologies allows for continuous observation of various parameters, such as pH, oxygen levels, and metabolic activity, improving the accuracy and depth of experimental data [49].

Despite their advantages, microfluidic 3D models, face several limitations. One major challenge is the complexity and technical expertise required for their design and fabrication. Developing and optimizing these systems can be time-consuming and require specialized skills and equipment, limiting their accessibility [27]. Reproducibility is another issue, as slight variations in the fabrication process or cell handling can lead to inconsistent results. Ensuring uniformity and standardization across different devices and experiments remains a significant hurdle. Cost is also a concern, with the high expenses associated with materials, fabrication, and maintenance. This limits the widespread adoption of microfluidic models, especially in resource-limited settings. Microfluidic models often struggle to fully replicate the complexity of living tissues and organs. While they can simulate certain physiological conditions, they may not capture all the intricate interactions and environmental factors present *in vivo*. Integration of multiple organ systems in a single chip, while promising, remains technically difficult and can lead to issues with scaling and inter-system communication [50]. This complexity can also complicate data interpretation and analysis.

Scaffold-free 3D models

In scaffold-free 3D models, unlike those incorporating structural scaffolds, cells are introduced into systems leveraging various forces to facilitate aggregation and spheroid formation. The concept of spheroids was initially introduced by Sutherland and colleagues in the 1970s [51]. Since then, diverse techniques have been developed to cultivate spheroid cultures. Spheroids arise through spontaneous aggregation driven by differentiated forces, where subsequent cell surface integrin interactions with the extracellular matrix (ECM) initiate the process. Following the initial cell-cell attachments, elevated E-cadherin expression enhances the establishment of robust intercellular bonds. The formation of spheroids is influenced by essential factors such as nutrient availability, oxygen levels, growth factors, and paracrine signals.

Scaffold-free 3D models provide several distinct advantages that significantly enhance their utility in drug discovery. One of the primary benefits is their ability to form more natural cell-cell interactions without the influence of an artificial scaffold [27]. This closely mimics *in vivo* conditions, providing a more physiologically relevant environment for studying cellular behaviors and responses. These models are particularly useful for creating uniform and reproducible spheroids, which can be easily scaled up for high-throughput screening applications. The simplicity of scaffold-free methods, such as forced floating, hanging drop, and magnetic levitation, makes them accessible and cost-effective, requiring less specialized equipment compared to scaffold-based techniques [52]. Scaffold-free models are also versatile, accommodating a wide range of cell types, including primary cells, stem cells, and cancer cells. This versatility allows for the development of various disease models and the study of different biological processes, such as tumor growth, drug resistance, and tissue regeneration. Another significant advantage is the ability to rapidly generate spheroids, which can be used to assess drug efficacy and toxicity in a timely manner. This speed is crucial for accelerating the drug discovery process and evaluating potential therapeutics more efficiently. Additionally, scaffold-free models support the formation of more homogenous cell aggregates, which is important for consistency in experimental outcomes [53]. They also

facilitate long-term culture, enabling chronic exposure studies and providing insights into long-term drug effects and cellular adaptation mechanisms.

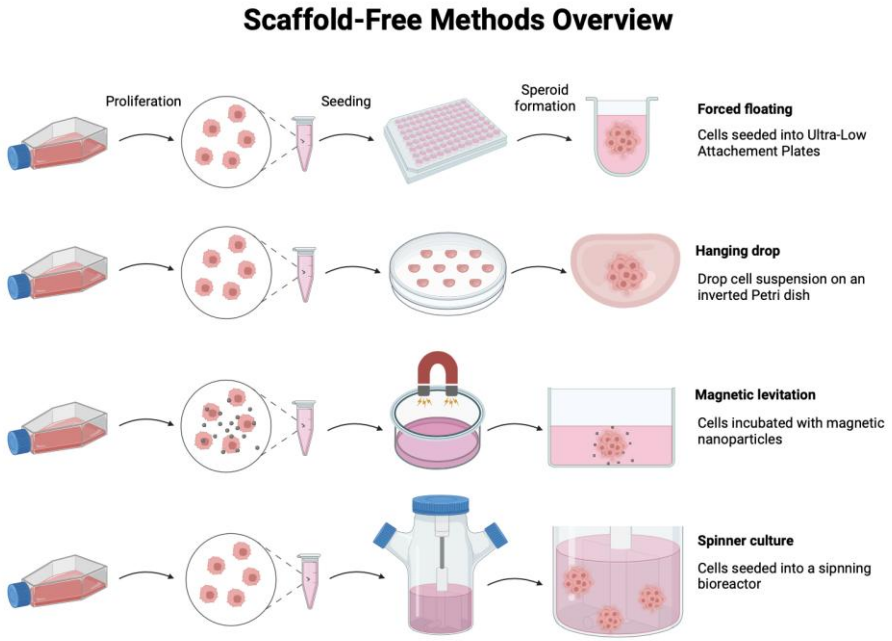

Fig 4 Scaffold-Free Methods Overview. All spheroid cultures are formed from primary sources or cell lines and proliferation. After they are being seeded in various techniques, with the usage of different types of forces, cells start to aggerate and form spheroids.

Scaffold-free 3D models, while advantageous, have several limitations that need to be addressed. One major challenge is the difficulty in controlling the size and shape of spheroids, which can lead to variability in experimental results. Ensuring uniformity and consistency across different samples remains a significant hurdle. Another limitation is the lack of structural support, which can result in less stable and less robust tissue models. Without a scaffold, it can be challenging to maintain the integrity of larger or more complex tissue constructs over time, limiting their use in certain applications. Scaffold-free models also struggle with nutrient and oxygen diffusion, particularly in larger spheroids. The absence of a vascular network can lead to the development of necrotic cores, reducing the viability and functionality of the tissue model. This limitation affects the ability to accurately model physiological conditions and drug responses. Furthermore, the scalability of scaffold-free models can be problematic. While they are suitable for high-throughput screening, the production of large-scale or more complex tissues for therapeutic applications remains challenging. Developing methods to scale up these models without compromising their integrity and functionality is an ongoing area of research.

Forced Floating

The Forced Floating technique involves culturing cells in ultra-low attachment plates to promote spheroid formation. By preventing cell adhesion to surfaces with surface coating, cells are held in a suspended culture causing cells to form cell-cell attachment, which leads to aggregation and spheroid formation [54].

Bell et al. investigated whether primary human hepatocyte (PHH) spheroids could serve as a viable model for studying drug-induced liver injury (DILI) [55]. Their research primarily focused on assessing the functional and metabolic activity of PHH spheroids, which is crucial for DILI drug screening. The study involved monitoring major cytochrome P450 enzymes (CYPs), particularly five major CYPs, and the formation of drug metabolites. CYPs are responsible for the oxidative transformation of a wide variety of drugs and endogenous compounds [56]. Notably, the research demonstrated stable hepatic function in PHH spheroids by sustained ATP levels, albumin secretion and preserved CYP enzyme activity. These findings suggest the potential suitability of PHH spheroids for chronic drug toxicity assays.

The forced floating technique for 3D cell culture offers advantages such as promoting natural cell-cell interactions, simplicity, cost-effectiveness, versatility, and scalability. However, it also faces limitations including difficulty in controlling spheroid size and shape, lack of structural support, issues with nutrient and oxygen diffusion, scalability

challenges for complex tissues, and reproducibility concerns. Addressing these limitations will enhance the utility of the forced floating technique in drug discovery.

Hanging Drop

The Hanging Drop technique involves cellular cultivation within droplets suspended on the interior of culture dish lids. Droplets hang on the surface with surface tension utilizing gravitational forces to instigate spontaneous cellular aggregation within suspended droplets of culture medium. Among scaffold-free techniques, Hanging Drop is distinct in its ability to control the size of the resulting spheroids.

Michael et al. developed innovative approach to enhance the hanging drop method as the Paper Hanging Drop Chip (PHDC), facilitating the stable culture of 3D spheroids for over ten days [57]. A key feature of PHDC is its ability to allow uninterrupted and periodic medium exchange, enabling efficient nutrient and oxygen transport through its porous substrate. This was demonstrated to yield results consistent with conventional hanging drop cultures in drug screening assays. Further advancement led to the creation of Networked PHDCs (N-PHDCs), combining multiple units. This advancement is significant in mimicking complex *in vivo* microphysiology, thereby offering a viable tool for multispheroid-based studies that closely emulate human *in vivo* systems.

Limitations associated with media exchange in the hanging drop technique have prompted the development of alternative approaches for 3D spheroid formation. Liu et al. introduced the SimpleDrop Chip, a novel microfluidic device constructed from polydimethylsiloxane (PDMS) with integrated micro-chambers [32]. These chambers facilitate the introduction of cell suspensions, culture medium, and even drugs for anti-cancer drug assays. The SimpleDrop Chip offers potential advantages for drug testing, particularly in fluorescent dye-based (immunofluorescence) analyses, due to its simplified handling compared to the hanging drop technique.

The hanging drop method for 3D cell culture offers advantages such as high reproducibility, minimal equipment requirements, natural cell-cell interactions, versatility, simplicity and ease of observation. However, it also faces limitations including difficulties with media exchange, scalability issues, size limitations of spheroids, uncontrolled spheroid size and formations, handling and stability challenges, and the need for specialized plates for optimization [32]. Addressing these limitations will enhance the utility of the hanging drop method in drug discovery.

Magnetic Levitation

The Magnetic Levitation method, utilizing magnetic nanoparticles (MNP), is a pivotal technique for regulating nutrient distribution within cell cultures, subsequently influencing cell aggregation patterns. This process magnetizes cells through electrostatic forces and non-specific binding to the cell membrane [58]. The controlled manipulation of MNPs via magnets facilitates cell levitation, enhancing cell-to-cell interactions and promoting spheroid formation. There are two distinct approaches to this method: Positive magnetophoresis / Paramagnetic manipulation and Negative magnetophoresis. Positive magnetophoresis involves integrating MNPs into cells, while Negative magnetophoresis levitates cells without MNP labeling [59], [60]. Souza et al. were pioneers in applying this method, aiming to develop a 3D cell culture system that employs magnetic levitation in conjunction with phage-based hydrogels containing nanoparticles [61] This system is designed to closely mimic *in vivo* protein expression and is conducive to long-term multicellular studies.

Roth et al. developed the Spatially Patterned Organoid Transfer (SPOT) platform, a novel approach leveraging magnetic levitation for optimized organoid cytoarchitecture conservation [62]. This platform utilizes iron-oxide nanoparticle-laden hydrogel and magnetized 3D printing, enabling controlled assembly of organoids. Consequently, SPOT facilitates the creation of well-organized assembloids that replicate key aspects of developmental processes and disease etiologies.

The magnetic levitation technique for 3D cell culture offers advantages such as rapid formation of spheroids, natural cell-cell interactions, minimal physical handling, versatility, cost-effectiveness, and enhanced diffusion. However, it also faces limitations including challenges with uniformity and reproducibility, potential interference from magnetic particles, limited size of constructs, scalability issues, handling and optimization complexities, and the need for specialized equipment. Addressing these limitations will enhance the utility of the magnetic levitation technique in drug discovery.

Spinner Culture

The spinner culture technique involves culturing cells in bioreactors with constant stirring, promoting cells to form cell-cell adhesion and aggerate. These spinner cultures are mostly used in mass productions of spheroids. Magnetic spinners facilitate stirring, ensuring even distribution of oxygen and nutrients. Critical parameters in this technique

include the type and size of the paddle and impeller, as well as the stirring rate, which are essential for maintaining cell-cell attachment and minimizing stress on the cells [63], [64].

As a result, it is more advantageous to study 3D cell culture forms in order to increase the reliability of the unreliable and unrealistic results produced by 2D *in vitro* cell forms in pharmacokinetic studies. 3D culture forms can be used to evaluate both the impact of the tumor microenvironment and the possible effects of pharmacokinetics on tumor forms.

The spinner culture technique for 3D cell culture offers advantages such as enhanced nutrient and oxygen supply, scalability, cost-effectiveness, rapid formation of spheroids, versatility, and suitability for long-term culture. However, it also faces limitations including shear stress, control over spheroid size, complexity of optimization, handling and sampling challenges, limited structural complexity, and scalability issues for complex tissues. Addressing these limitations will enhance the utility of the spinner culture technique in drug discovery.

Discussion

While 3D cell cultures offer numerous advantages over their 2D counterparts, certain drawbacks currently limit their widespread adoption in drug discovery. Developing 3D models can be more expensive, time-consuming, and intricate, with variability observed across cell lines that can hinder reproducibility [27]. Despite these challenges, 3D models are demonstrating promising results in the drug development pipeline, leading to their emergence as a preferred method. Furthermore, the application of 3D models is expanding into the fields of organ transplantation (regenerative medicine) and personalized medicine, where patient-derived tumor models hold immense potential [65]. Future advancements aim to refine 3D models, making them more user-friendly, cost-effective, and ultimately more clinically relevant.

Scaffold-based models are gaining traction in drug discovery due to their advantages, including high reproducibility, the ability to incorporate co-cultures, and the potential to mimic *in vivo* cellular organization. However, achieving cell-specificity and reproducibility with these models necessitates extensive research and development [50]. Different cell types require unique combinations of growth factors and ECM components, making scaffold development a complex process that demands significant time, resources, and advanced technologies. 3D bioprinting is a cutting-edge technology with immense potential for creating complex tissue models. It utilizes a layer-by-layer deposition of cell-laden or non-laden hydrogels to create complex 3D structures that replicate the architecture and function of natural tissues and organs. The tumor microenvironment (TME) plays a critical role in drug efficacy, necessitating the development of *in vitro* models that faithfully recapitulate its characteristics. Innovative 3D bioprinting technology offers a promising avenue for precisely controlling and mimicking the TME, encompassing all its components, including both mechanical and fluidic forces [10]. However, a major challenge lies in constructing and utilizing intricate, composite tissue structures that resemble solid organs. This challenge arises from the interplay of numerous factors, including the selection of appropriate cells, bioink composition, the type of bioprinter and the bioprinting technique employed [66].

Gelatin methacryloyl (GelMA) is a widely used biomaterial in 3D bioprinting due to its tunable properties. The concentration of GelMA plays a critical role in mimicking *in vivo* tissue structure and cellular behavior. Arya et al. demonstrated that 10% GelMA hydrogels exhibited morphological features similar to decellularized human breast tumor tissue, as confirmed by compression testing and microscopic evaluations [67]. Interestingly, a decrease in GelMA concentration resulted in enhanced cell proliferation, with the highest rates observed in 5% GelMA hydrogels. However, the superior mechanical properties of 10% GelMA hydrogels make them more suitable for long-term cell culture, making them a favorable matrix for mimicking the *in vitro* breast microenvironment. This study also highlights the importance of 3D models in drug discovery. When compared to traditional 2D models, 3D spheroid models derived from breast cancer cells exhibited decreased sensitivity (higher IC50) to paclitaxel, a first-line chemotherapeutic drug for advanced breast cancer. This finding suggests the development of drug resistance within the 3D microenvironment, potentially due to the role of cell-cell and cell-ECM interactions in spheroid formation. Cell adhesion-mediated drug resistance (CAM-DR) is currently being investigated through the lens of integrin-mediated adhesion [33]. Integrins are cell adhesion molecules that can influence cell survival and potentially inhibit apoptosis. While studies suggest a link between integrin signaling and drug resistance, further investigation is necessary to fully elucidate the underlying mechanisms.

3D organoid cultures offer significant advantages over traditional 2D models in drug development. They effectively mimic *ex vivo* organ development and function, making them valuable tools for disease modeling. Additionally, organoids exhibit well-differentiated behaviors, enabling predictions of therapeutic response and

pharmacokinetic (PK) studies. These features facilitate rapid and multiplexed assays, streamlining the drug development process [46]. However, despite these advantages, recent research has highlighted a critical factor: the composition of the culture medium. Studies have shown that growth factors and molecular inhibitors within the medium can significantly influence organoid drug response [68], [69]. This finding underscores the importance of optimizing culture media to achieve reliable and informative results in drug development using organoid models.

Microfluidic devices offer a valuable approach in drug discovery while holding current limitations. The fabrication process typically involves specialized equipment and materials, requiring significant technical expertise. This complexity translates to higher costs compared to traditional 2D cultures. Maintaining consistent results in 3D cell cultures presents a significant challenge due to the inherent complexity of these systems [29]. The sensitivity of 3D cultures to even minor perturbations extends from the biological variability of primary cells to subtle variations in culture conditions. The intricate nature and specialized equipment requirements inherent to scaffold-based 3D cultures present significant barriers to their large-scale adoption in industrial and clinical applications.

Microfluidic devices offer a distinct advantage for 3D cell culture by employing oxygen and growth factor-permeable materials, a feature that addresses hypoxia, a major challenge encountered in scaffold-free models [41], [48]. OoCs and HoCs represent a significant advancement in mimicking human physiology, aiming to replicate organ size, interactions, and functions. However, achieving accurate relative size scaling across individual OoCs within these multi-organ systems remains a challenge, as highlighted by Wikswo et al. [70]. Allometric and functional scaling approaches are crucial for overcoming this limitation and enabling OoCs/HoCs to effectively replicate human drug response and pharmacokinetics.

Another critical limitation of OOC chips lies in the control of essential factors for cell function. Due to the inherent complexity and heterogeneity within a chip, meticulously regulating parameters like pH, temperature, organ-specific growth factors, oxygen levels, and fluid flow is crucial for optimal cell proliferation and 3D structure formation [71]. To address this challenge, researchers are exploring the integration of sensors within OOC models. These sensors would enable real-time monitoring and precise control of these critical parameters without disrupting the cultured tissues, cells, or organoids. Integration of sensors within OoC models is another key advantage, allowing for continuous monitoring and control of crucial parameters without disrupting the cultured tissues/cells/organoids [49]. Notably, nutrient/fluid flow rate is a critical parameter. Excessive flow rates can lead to cell disintegration, hindering organoid/tissue formation and potentially generating misleading results in pharmacokinetic studies. OoCs offer several advantages over 2D models. Their more accurate flow control translates to efficient nutrient utilization and enables rapid, multiplexed analyses.

Scaffold-free spheroid models hold promise for 3D cell culture, but a critical limitation remains: the risk of spheroid disintegration [58]. Magnetic levitation emerges as a solution, minimizing this disintegration risk while effectively producing homogeneous and compact cell aggregates. Compared to other 3D culture techniques, magnetic levitation offers significant advantages. It requires minimal specialized equipment, facilitating its implementation across diverse cell lines and enabling easier large-scale production. Furthermore, this versatile technique demonstrates efficacy in various applications within biotechnology, pharmaceutical development, stem cell research, and personalized medicine. However, a limitation associated with paramagnetic manipulation, a method used in magnetic levitation, is the potentially time-consuming and labor-intensive process of magnetically labeling cells [60].

Spinner cultures offer a valuable tool for large-scale production of spheroids due to their automation capabilities, facilitating rapid and efficient aggregation. This automation is particularly advantageous for companies managing numerous drug development projects. However, this rapid process can potentially lead to variability in spheroid size and shape. Additionally, prolonged stirring within the culture can have detrimental effects on cell physiology, potentially causing spheroid disintegration. This size variation can pose significant challenges during drug screening assays [41], [63]. While spinner cultures offer automated control of pH and dissolved oxygen levels compared to static plate systems such as forced floating, the automated stirring itself can have both advantageous and limitations. One of the limitations is that high stirring rates can disrupt spheroid integrity, while low rates can lead to cell sedimentation, impeding spheroid formation [64]. Another limitation of spinner cultures is the requirement for a larger volume of culture medium compared to other 3D culture techniques. This increased media volume translates to higher operational costs [41].

The hanging drop method has traditionally been employed for spheroid formation due to its high reproducibility and ability to generate single spheroids. However, limitations such as difficulties in media exchange using standard culture dishes, the high cost and inflexibility of specialized hanging drop plates, and the extensive time required

for optimization have hindered its widespread use [57]. Furthermore, the hanging drop method is limited by the restricted size of the spheroids that can be formed due to the nature of the technique. Additionally, media exchange can be challenging due to the setup, making it less suitable for drug assays where frequent addition or removal of compounds is necessary [32].

The Paper Hanging Drop Chip (PHDC) presents another option, offering advantages in simplicity and cost-effectiveness [57]. Additionally, PHDC enables spheroid culture for extended periods, up to 10 days. However, a limitation of PHDC is its white paper color, which hinders the optical imaging of non-fluorescent cells.

The SimpleDrop Chip is an innovative approach to 3D cell culture. It eliminates shear forces typically encountered in other techniques, making it a potentially gentler and more biocompatible approach for cell cultures [32]. Additionally, the polydimethylsiloxane (PDMS) material contributes to the environmental friendliness of this method compared to some traditional methods.

Ultra-Low Attachment (ULA) plates, also known as forced floating, have emerged as a more favorable alternative due to their ability to address these drawbacks. ULA plates offer a simpler and more cost-effective approach, facilitating media exchange and streamlining the process [72].

While the hanging drop method remains favored for certain applications due to its high reproducibility, ULA plates represent a more practical and adaptable choice for many researchers.

Conclusion

Three-dimensional (3D) models offer significant advantages over traditional 2D and animal models, making them highly favorable in drug development. By successfully replicating *in vivo*-like structures with accurate cell-cell and cell-ECM interactions, 3D models have shown promising results in pharmacokinetic and toxicology assessments [9]. The advantages of 3D models are evident in their ability to replicate key aspects of human tissue dynamics and drug behavior, reducing the ethical and practical limitations associated with animal testing and thereby addressing the high attrition rates observed in clinical trials.

3D models can be produced in three main forms: scaffold-free models, scaffold-based models, and organoid models. Scaffold-free models were the first to be developed and offer advantages such as lower complexity, cost-effectiveness, reproducibility, and accessibility. Despite these benefits, they face challenges like lack of size controllability, which hinders uniformity and consistency across samples, and structural fragility, increasing the risk of spheroid disintegration. Scaffold-based models address some of these limitations by using scaffolds to control size and provide structural support, making them less fragile [12]. However, these models are more complex, requiring substantial resources and specialized knowledge [27]. The high cost of materials and the need for interdisciplinary collaboration among engineers, biologists, and chemists add to the challenges associated with scaffold-based models, hindering their large-scale adoption in industrial and clinical applications. Organoid models, particularly OoC and HoC systems, have shown promising results in drug assessments. While these models offer significant potential for mimicking human physiology and studying drug responses, achieving relative size scaling across individual OoCs remains a major challenge [42]. The use of allometric and functional scaling approaches is necessary for accurate results in drug assays, necessitating further research to optimize their use and address existing limitations.

In conclusion, while 3D models provide a promising alternative to traditional methods, continued advancements and interdisciplinary efforts are essential to overcome current challenges and enhance their application in drug development.

Future studies

Future research should focus on overcoming the current limitations of 3D cell culture models to maximize their impact on drug development. The biggest problems 3D models currently face include lack of reproducibility, scalability, biological and disease relevance, high costs, and the interdisciplinary knowledge required for further development. Key areas for future studies include technological advancements, reproducibility and scalability, enhanced predictive models, interdisciplinary collaboration, and regulatory and ethical considerations.

Technological advancements are essential for improving the mechanical and biological properties of scaffolds and bioinks used in 3D bioprinting [27]. Previous studies have shown that the extracellular matrix (ECM) significantly affects drug responses [7]. Therefore, further research into the ECM and its compounds is needed to create more accurate models [23], [24]. Additionally, research should prioritize developing more sophisticated microfluidic devices that better mimic the dynamic interactions within human tissues. One of the main issues with current microfluidic devices is the lack of flow control, which leads to inaccurate drug responses. Integrating biosensors

offers a promising future for system optimization, which is essential for drug discovery [49]. These innovations will contribute to creating more accurate and reliable 3D models.

Reproducibility and scalability are crucial for the widespread adoption of 3D culture techniques. Reproducibility is a concern for their use in high-throughput screening (HTS) and high-content screening (HCS), which are important steps in drug discovery [28]. To address this, there is a need for the development of a versatile 3D culture system capable of seamless integration into mainstream drug discovery pipelines while also permitting precise modulation to replicate the tissue-specific attributes of an *in vivo*-like microenvironment. Additionally, making these technologies scalable and more accessible is vital for their extensive use in research and industry [70]. Improving scalability requires the development of universally adapted scales based on allometric and functional scaling.

Enhancing the predictive accuracy of these models for human drug responses, particularly in personalized medicine, is another important area for future research. Using patient-derived cells in organoid and other 3D models can tailor drug development and testing more closely to individual patient profiles, improving the relevance and effectiveness of therapeutic strategies [65].

Interdisciplinary collaboration is essential to address the multifaceted challenges associated with 3D cell culture models. Collaboration between biologists, engineers, material scientists, and clinicians can further our understanding of the complex interactions within these models and their applications in various fields of biomedical research [29]. This collaborative approach will drive innovation and overcome technical and methodological barriers.

Addressing regulatory and ethical aspects is also important for the adoption of 3D cell culture models in drug development. These models have the potential to meet regulatory standards and reduce ethical concerns associated with animal testing, paving the way for their acceptance and implementation in the pharmaceutical industry. By addressing these areas, future advancements can make 3D cell culture models more user-friendly, reproducible, versatile, cost-effective, and clinically relevant, ultimately enhancing their role in the drug development pipeline and beyond.

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Review article

Fish Immunity Against Nervous Necrosis Virus (NNV) Infection: A Review

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ABSTRACT
Fish are unique organisms fitted with a certain degree of immune system that are similar to mammals; consists of innate and adaptive immunity. Innate immunity comprised of non-specific cellular and the nonspecific humoral components while adaptive immunity consists of specialized systemic cells and mechanisms that are categorized into two main groups: the humoral and cellular components. In response to nervous necrosis virus (NNV) infection, both innate and adaptive immunity mechanisms play crucial roles. NNV invasion in fish triggers phagocytosis by antigen-presenting cells (APCs). These cells process viral proteins into peptide fragments, presenting them on major histocompatibility complex (MHC) class II molecules, a crucial protein component that initiate specific adaptive immune response against NNV infection. This will eventually activate adaptive immunity pathway to stimulate the proliferation and maturation of the B cells. As B cells matured, antibodies will be produced to suppress the pathogen through series of process known as opsonization and neutralization before phagocytosis takes place. Following secondary exposure with the same antigen, B cells will rapidly proliferate, releasing significant number of antibodies to fight the antigen. Overall, this review elucidates the complex interplay between innate and adaptive immune responses in fish, highlighting their crucial roles in combating NNV infection through mechanisms such as phagocytosis, antigen presentation, and antibody production.

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Introduction

Aquaculture in Malaysia

Malaysian aquaculture production for freshwater species were dominated by red tilapia (*Oreochromis* spp.), freshwater catfish (*Clarias gariepienus*) and striped catfish (*Pangasionodon hypophthalmus*) with recorded production at 30,022 tonnes, 31,987 tonnes and 21,144 tonnes and wholesale value of RM 322 million, RM 159 million and RM 165 million respectively [1]. While for marine species, the most cultured species were seabass (*Lates calcarifer*), snapper (*Lutjanus* spp.) and grouper (*Epinephelus* spp.) whose production stood at 34,186 tonnes, 5417 tonnes and 2584 tonnes with wholesale value worth RM 480 million, RM 277 million and RM 96 million respectively 1AFS., Annual Fisheries Statistic. Department of Fisheries, Ministry of Agriculture & Agro-Based Industry, Malaysia. However, expansion of aquaculture industry in Malaysia was hampered by the amount of good quality fish seeds [2], leading to the fish fries and fingerlings importation from neighboring Asian countries such as Thailand, Taiwan, Indonesia and Vietnam. Nevertheless, fish importation without improper biosecurity practices and measures may lead to the introduction of diseases causing agents [3]. Hastein (2001) stressed out that importation of live aquatic animals possessed higher risks of pathogen transfer which has led to the transmission of aquatic animals' diseases worldwide. This has been documented [5] where infectious diseases caused by bacteria, virus, fungi and parasites were found to be introduced at imported countries within 1978 to 2005. In 1996, importation of common carp (*Cyprinus carpio*) as ornamental pet trade has led to the outbreak of contagious viral disease, Koi herpesvirus disease (KHD) with major outbreaks observed in farms culturing common and Koi carp in Israel next two years [6].

In aquaculture, diseases are caused by a diverse array of pathogenic agents, including parasites, bacteria, fungi, protozoa, and viruses [7]. These infectious organisms can significantly impact fish health, growth, and survival

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rates, leading to substantial economic losses in the aquaculture industry. The severity and prevalence of these diseases can vary depending on environmental factors, host susceptibility, and the virulence of the pathogen [8]. Among these pathogens, viral diseases have emerged as a particularly concerning issue over the past two decades. Outbreaks of viral infections have been frequently reported, with consequences that cannot be overlooked. These viral pathogens often result in high mortality rates, sometimes exceeding 90% in susceptible populations, and can lead to significant economic losses due to reduced production, increased treatment costs, and trade restrictions [8,9].

Fish Immune System

Immune system can be characterized as a set of humoral and cellular component to barricade the body against foreign substances including pathogens and toxins following the presence of endogenous and exogenous substances that initiate this system [10].

Although fish immune system is physiologically comparable to higher vertebrates including mammals, there are few peculiarities between the fish immune system and the immune system of other vertebrates [11]. From birth, fish have a well-developed and robust innate immunity mechanisms, with the presence of crucial innate immune cells to recognize and eliminate pathogen including phagocytes, macrophages and natural killer cells. However, the adaptive immune system, that crucial for the secretion of specific antibodies, takes time to develop and mature. In contrary, innate immune system mechanisms of mammals is not well developed at birth, but becomes more effective over time. For adaptive immunity, both fish and mammals include B and T cells to provoke immune responses. Unlike mammals, fish lack discrete lymph nodes. Instead, their immune system relies on diffuse lymphoid tissues distributed throughout their body. These tissues, which include the thymus, kidney, and spleen, serve analogous functions to mammalian lymphoid organs, supporting immune cell development and facilitating immune responses. This diffuse organization of lymphoid tissues in fish is an evolutionary adaptation to their aquatic environment and provides effective immune surveillance across their body systems.

When immune responses take place, mammals produce higher antibody compared to fish [12]. While mammals produce five main classes of antibodies (IgM, IgG, IgA, IgE, and IgD), fish have a more limited antibody class. Fish primarily produce three classes of immunoglobulins: IgM, which is the predominant systemic antibody; IgD, whose function is not fully understood but is believed to play a role in antigen recognition; and IgT (or IgZ in some species), which is specialized for mucosal immunity. This difference in antibody diversity reflects the evolutionary divergence between fish and mammals and the unique adaptations of the fish immune system to aquatic environments. The presence of IgT in fish and its absence in mammals is a result of evolutionary divergence and adaptation to different environments. IgT, discovered early 20s, is a teleost-specific immunoglobulin that plays a crucial role in mucosal immunity, particularly in the gut and gills of fish [12]. This specialization reflects the unique challenges faced by aquatic organisms in protecting their mucosal surfaces from pathogens. Mammals, evolving in terrestrial environments, developed different strategies for mucosal immunity, primarily relying on IgA [12]. The absence of IgT in mammals and its presence in fish highlight the distinct evolutionary paths of their immune systems, shaped by their respective environmental pressures and physiological needs. While both mammals and fish are equipped with mucosal immunity responses, mucosal immunity in fishes are more noticeable compared to mammals. Mucosal surfaces in fishes include skin, gills and digestive tract incorporated with specialized immune cells that produce antibodies to provide protection against pathogen. On the other hand, although the role of classical fish major histocompatibility complex (MHC) in fish appear to be similar to those in mammals [13]. The fish MHC are clear, precise and easier to distinguish compared to mammals. Researchers defined that fishes typically have less highly polymorphic MHC genes as well as less complex MHC system compared to mammals.

As fishes are classified as ectothermic or cold-blooded animals where its body temperature follows surrounding temperature [14], immune system plays an essential role in maintaining its internal homeostasis. This complex system made up of specific organs to detect and identify any mobilizing pathogen that attack the hosts in the bloodstream. Despite the diversification and variations, fishes' immune system was comparable across the vertebrate linage, comprise of two important mechanisms, innate and adaptive immunity [15]. Across species and environment, freshwater and marine fishes are said to generally have immune system mechanisms that are alike and comparable [16]. In general, freshwater and marine fishes possess similar innate immune mechanisms, including physical barriers like skin and mucous, antimicrobial peptides, phagocytic cells (such as macrophages and neutrophils) and complement proteins. These components initially offer primary protection and help to provokes the immune response against pathogen [15]. Besides, IgM were found to be expressed in both freshwater and marine species [17]. Previous study [18] also reported that IgT or IgZ antibody classes were identified in both marine and freshwater fishes. Hence, those clearly supports that both freshwater and marine species fishes possessed similar adaptive immunity mechanisms. Overall, while

adaptations and modifications might be observed on specific circumstances, freshwater and marine fish species commonly possess similar immune system mechanisms in order to protect themselves from pathogens.

Viral Nervous Necrosis

Viral nervous necrosis (VNN) or also known as Viral encephalopathy and retinopathy (VER) is one of the most devastating viral diseases that leads to significant mortality rate, particularly in larval stages [19,20]. The causative agent for VNN/VER is nervous necrosis virus (NNV), a betanodavirus class of virus that belongs to the *Nodaviridae* family [21]. Betanodavirus are non-enveloped and icosahedral in structure. This small virus (25-20 nm in diameter) was made up of two positive-sense ssRNA molecules labelled as RNA1 and RNA2 (Figure 1). The RNA1 (3.1kb) consists of RNA dependent RNA polymerase (RdRps) that are subjected to viral replication, whilst RNA2 (1.4 kb) encodes the viral capsid protein (42 kDa) [21]. Previously, it was reported that additional segment, designated as RNA3, was sub-genomically synthesized from RNA1 that encodes polypeptide B2 [23]. However, this non-structural protein that inhibits the cell's RNA silencing mechanisms only exists in infected cells as it was not captivated into the viral particles [21].

The common entry of NNV infection is through the eyes and brain organ, [24] before spreading through the blood circulation and rapidly attack the host central nervous system via the peripheral nervous system, causing death to the fish [26]. Following the infection, virus will proliferate, leading to the blockage of the blood circulation and increased capillary pressure [27]. Accumulation of fluid will ultimately increase the force between the capillary membrane and interstitial space, caused edema and ruptured the fish's inner eye lining. This will eventually lead to the dysfunction of the eye's organ from its normal physiological state [25]. Similarly, Yuwanita and Yanuhar (2013) also find out that signs of bleeding (hemorrhage) in eye's organ were common during the time of NNV infection in humpback grouper as a result of burst blood vessels in the eye's lining. In addition, NNV infected fish will usually possess weaker nervous system, hence, fish will lose its nerve control, develop lethargy motion [28] and cause death up to 100% especially in the juvenile stages [29].

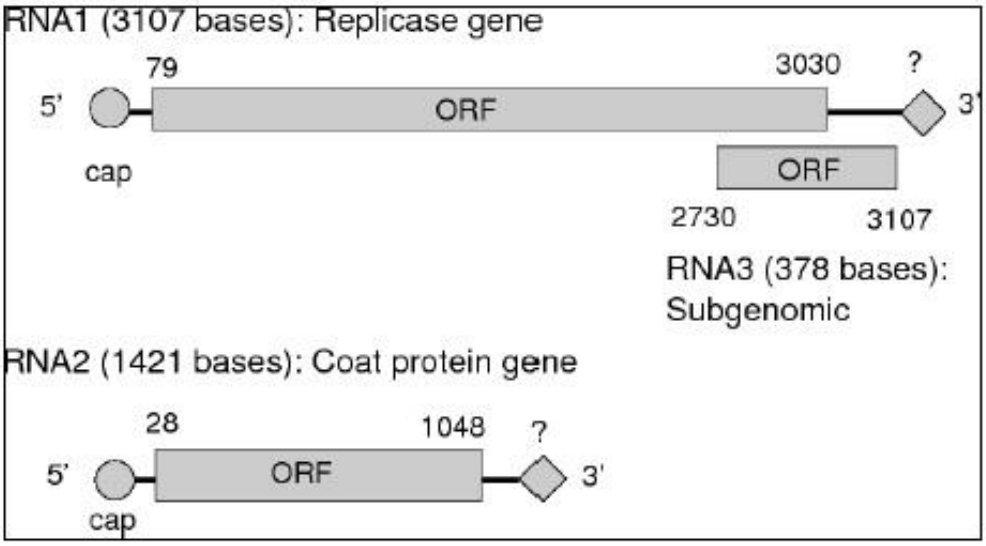

Fig 1 Betanodavirus genome organization. The genome of betanodaviruses consists of two RNA segments: RNA1 and RNA [35]. These two segments encode different viral proteins and play distinct roles in the viral life cycle.

To date, betanodavirus has been reported in more than 120 cultured and wild species [30], becoming a major threat affecting the global aquaculture production. Betanodavirus infections have been reported globally particularly in regions with extensive aquaculture activities [31] including south and east Asia (Japan, Korea, Taiwan, China, Phillipines, Thailand, Singapore, Indonesia, Vietnam, Brunei and Malaysia), Mediterranean (Bosnia, Greece, Portugal, Italy, France and Spain) and Oceania (Australia and Tahiti). Scientists reported through several studies [33] that both marine and freshwater species are vulnerable to betanodavirus infection. Infections in marine species has been reported in several species including Asian seabass (*Lates calcarifer*), European seabass (*Dicentrarchus labrax*), Japanese parrotfish (*Oplegnathus fasciatus*), turbot (*Scophthalmus maximus*), Redspotted grouper (*Epinephelus akaara*), Striped jack (*Pseudocaranx dentex*), Giant grouper (*Epinephelus lanceolatus*), Sevenband grouper (*Epinephelus septemfasciatus*), Atlantic cod (*Gadus morhua*), Golden pompano (*Trachinotus blochii*) and Red snapper (*Lutjanus campechanus*) [34]. Most recently, betanodavirus has also been isolated in freshwater species such as Chinese catfish (*Silurus asotus*), Australia catfish (*Tandanus tandanus),* medaka (*Oryzias latipes*), freshwater tilapia (*Oreochromis* spp.), guppy (*Poicelia reticulata*) and zebrafish (*Danio rerio*) [32,33,37,38,39]. Although researchers [32,33,34] proved that

betanodavirus infections are susceptible for both marine and freshwater species, few experiments suggested that Asian seabass at juveniles' stages are more prone to betanodavirus as early as 10 days post hatching (DPH) onwards with high mortalities rate recorded at 80% 19. Apart from small size fishes that are more vulnerable to viral infections than bigger size fishes, [19] stressed out that this could also be associated with several factors including cannibalistic nature of seabass and stress factors such as overcrowding in the tanks or cages.

Innate Immunity

Innate immunity, or commonly referred as natural immunity is the first line of defense that protect the hosts against any foreign agent [15]. Innate immunity is immensely crucial for several reasons. Firstly, this defense mechanism is non-specific, hence, do not rely on individual molecular structure for detection of the foreign pathogen. Therefore, the protection provided is relatively quick, limiting pathogen ability to survive. Thirdly, innate immunity works regardless surrounding temperature fluctuation, serves as useful tool for ectothermic vertebrates since adaptive immunity takes relatively longer time and rely on the surrounding temperature to mount a response. Before specific response take place, innate immunity will react against invading pathogen, initiating several immunological responses. Verrier et al., (2012) stressed that innate immune response played a crucial part to counter virus infection as quick induction of this non-specific mechanisms is essential to prevent viral replication. Although without any immunological memory [41], innate immune response serves as an early signal that will eventually initiate adaptive immune system to establish its response [42].

Innate Response Against NNV Infection

Interferons (IFNs)

Following infection, fishes will establish a quick antiviral response through their innate immune system [43] before complex response in form of antibody and memory cells secretion takes place [44].

Following NNV infection, quick induction of innate immune response by teleost fishes lead to the production of Interferons (IFNs). IFN can be characterized as a pH resistant cytokine, with a low molecular weight (20-23- KDa), produced prior to the viral infection [45]. Deonarain et al., (2004) stated that IFN acts as an indicator that determine the stage and development of the viral infection, hence, serves as a crucial element in the innate immune system. Although the reasons for persistence of VNN in adult stage is still in doubt, the awful effect during larval stage infection comes out with a solid justification that innate immune system have a huge role to play in persistent infection [41]. Generally, IFN works by binding to receptors located at the cell membrane, initiating several processes that lead to the stimulation of interferon-stimulated genes (ISGs) [47] .Then, IFN produced will makes its way out of the cell, and directly generate the production of protein including 2',5'-oligoadenylate synthetase, protein kinase P1 and Mx proteins that inhibit viral replication as well as degenerating viral RNA [48]. Normally, production of IFN happened rapidly as early as two days post infection as reported by [49] where IFN were presence in virus infected teleost fish 48 hours post-injection.

In teleost fish weighing less than 200 grams, the production of interferons (IFNs) plays a pivotal role in the innate immune response. These small yet potent signalling molecules serve as a critical first line of defense, offering robust protection to the host during early developmental stages when the adaptive immune system is not yet fully matured. IFNs are rapidly produced in response to viral infections and other pathogenic stimuli, initiating a cascade of antiviral responses within the fish's body. This was justified by [48] as organs in NNV infected groupers showed relatively higher percentage value of IFN in brain (0.55%), eyes (17.73%) and kidney (16.08%) compared to healthy fishes whereby the IFN percentage recorded in healthy fishes are 0.21%, 14.05% and 8.54% in brain, eyes and kidney respectively. Other than that, previous study by [43] through in-vivo and in vitro test suggested that IFN-like cytokines and Mx gene have a major role in assisting the persistence infection in Seabass and Grouper. Indeed, for seabass, within six to twelve hours post infection, published data displayed that infected seabass exhibit intense IFN and Mx responses in kidney and brain organs [50,51]. This may indicate that the capsid protein of nodaviruses is capable enough to mount innate immune genes transcription ahead of NNV infection [52].

Complement system

The involvement of complement system in combating viral infection has been reported previously [53]. The complement can be activated or initiated via a combination of three pathways: the alternative, the lectin and the classic pathways. In vertebrates including fishes, complement system, composed of 35 soluble proteins mostly reported to play its part in innate immunity by directly bind to the viral surfaces [54]. Whilst most researchers are focusing on classical complement pathway, alternative and lectin complement pathway has also been reported to engage against viral infection [55]. Fish complement system has been reviewed by [56] and was shown to be involved in opsonization, phagocytosis and inflammation to combat viral infection.

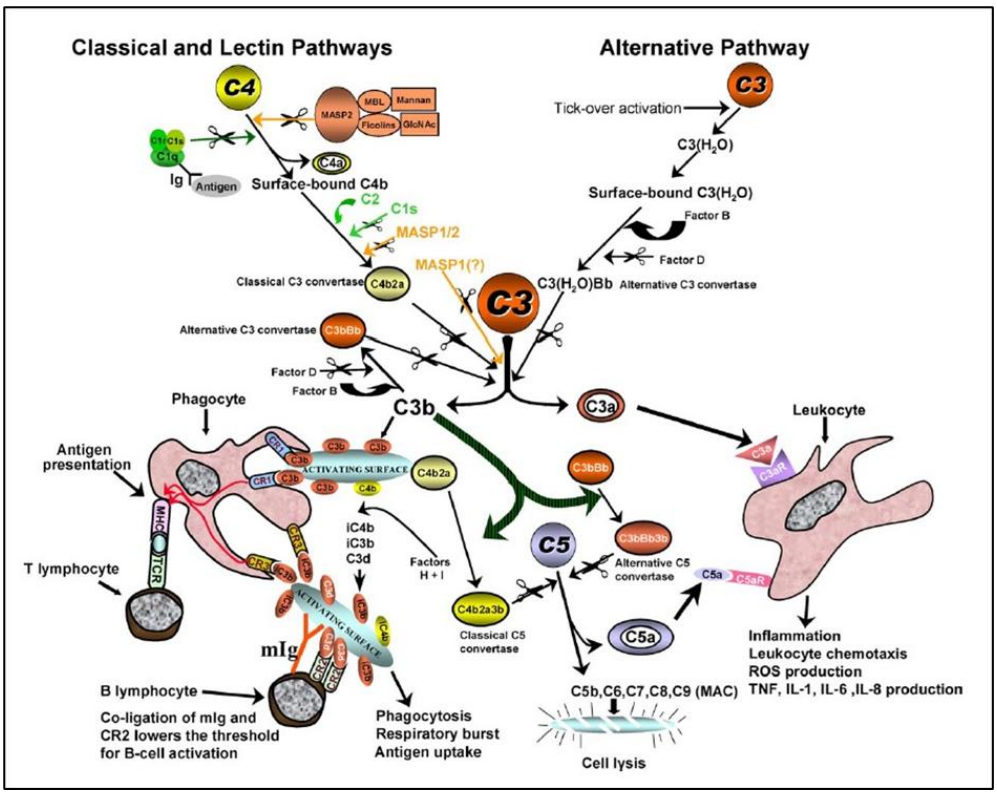

Fig 2 Complement pathway mechanism in teleost fish. Complement system in teleost fish plays a crucial role in their immune defense, contributing their ability to combat infections in aquatic environments [56].

The classical pathway, predominantly studied in mammals, is initiated by the interaction between antigen-antibody complexes and the C1q component of the C1 complex. Specifically, the Fc portion of IgG or IgM binds to C1q, triggering the activation of C1r and C1s proteases. The alternative pathway, more prevalent in fish serum compared to mammals, is characterized by spontaneous activation or "tick-over" mechanism. This pathway is initiated by the hydrolysis of C3 to C3(H_2O), which can then interact with factor B and factor D to form the C3 convertase (C3bBb). This convertase can cleave more C3, amplifying the response. The lectin pathway is activated when pattern recognition molecules, such as mannose-binding lectin (MBL) or ficolins, recognize carbohydrate moieties on microbial surfaces. This recognition leads to the activation of MBL-associated serine proteases (MASPs), which then cleave C4 and C2 to form the C3 convertase (C4b2a), similar to the classical pathway. All three pathways converge at the formation of C3 convertases, leading to the generation of C5 convertases and ultimately the assembly of the MAC. This system provides rapid and effective defense against a wide range of pathogens, including viruses playing a critical role in fish immunity against NNV infection.

Overall, activation of complement pathway assists the innate immunity especially during viral infection through phagocytosis and cytolysis of pathogens, solubilization of immune complexes, and inflammation [57] In fact, scientists reported that apart from its crucial role in activating innate immune response, complement helps to enhance and intensify humoral immunity [58]. Specifically, it has been demonstrated that bounding of C3 and C4 molecules to antigen during the activation process boost the intake and production of antigen presenting cells (APCs), hence, in turn, induce and results to more effective primary and secondary immune responses during viral infection [59].

Natural killer cells (NC) and non-specific cytotoxic cells (NCC)

Natural killer cells (NKC) are crucial element that ensure innate immunity works against viral infection [41]. NKC carry out lysis and apoptosis, as well as secreting cytokines (group of signaling proteins produce to regulate humoral responses) to destroy impaired cells attacked by virus during antiviral response [60]. NKC are proved to have positive link with macrophages as NKC are responsible to initiate macrophages by triggering them to produce type I IFN. In fact, those induced cytokines acted as a key bridge between innate and adaptive immunity.

Similar to mammals, teleost fishes have non-specific cytotoxic cells (NCC) that are engaged to trigger immune response during viral infections [61]. Basically, NCC will be able to recognize specific size protein (40 KD) and in turn directly produce cytotoxic cells when NCC are in contact with susceptible target cell membrane [62]. As in mammals, perforin also have its own role to barricade viral infections in teleost fishes. Perforin is

the most common signaling proteins associated with natural killer cells (NKC) and NCC during viral infections [63]. They act by forming pores, allowing cytotoxic molecules such as granzymes to enter the target cells and directly initiate programmed cell death (apoptosis) to remove the infected cells [62] (Figure 3).

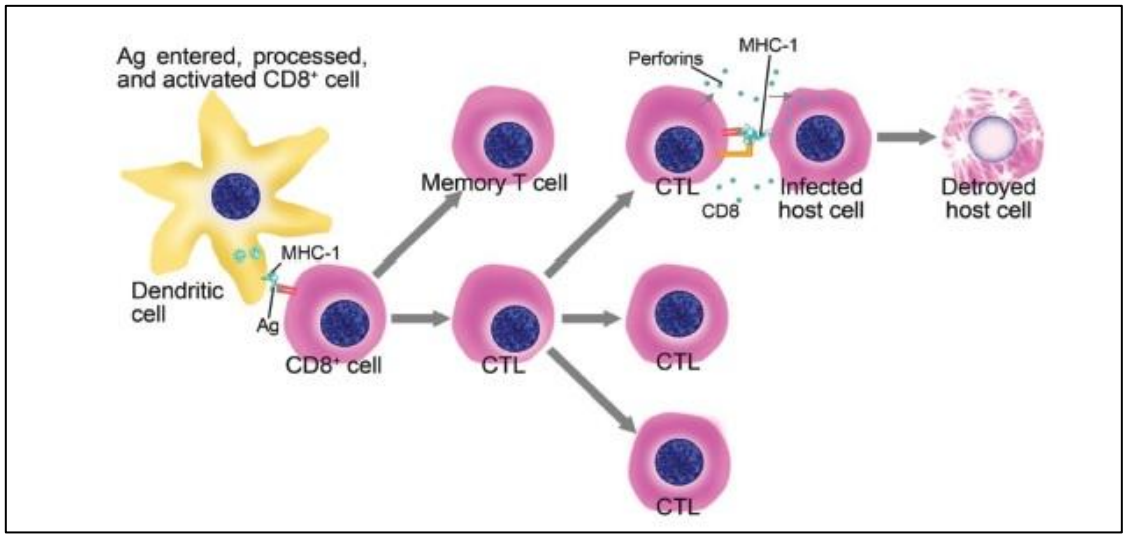

Fig 3 Destruction of infected cells during viral infection. Perforin is found to possess a role regarding the immune defense against virus infections among teleosts fishes [14].

Pathogen recognition receptors (PRRs)

Koyama et al., (2008) mentioned that recognition of viral pathogen is the first crucial early step that serves as a connector between innate and adaptive immunity. Pathogen Recognition Receptors (PRRs) families include the C-type lectins, complement receptors, cytosolic nucleotide-binding domain, LRR proteins and the most commonly identified in fish, Toll-like Receptors (TLRs) [65] . Although TLRs molecules in mammals was reported to be more specific and complex, teleost fish are well conserved with TLRs molecules that are able to at least detect the amino acid sequence level of viral molecules. In fact, TLR3 gene which are responsible for the recognition of dsRNA molecule have been isolated in rainbow trout [65] from leukocytes and tissues following viral infection. Recently, [66] also reported the presence of TLR7 and TLR8 genes specifically to detect ssRNA virus. TLRs operates by detecting the unique conserved molecules of the microbes, labeled as pathogen-associated molecular patterns (PAMPs) before stimulates inflammatory reaction that triggers the innate immunity response [67]. Lee and Kim (2007) explained that PRRs mount the innate immune response through several process including complement pathway, apoptosis, leukocyte activation and cytokine secretion. To date, a total of 13 TLR have been identified in teleost fishes based on research conducted on zebra fish as immunological model [68].

Adaptive Immunity

Compare to innate immunity, adaptive immunity is made up of complex system consist of highly specific systemic cells and processes that interrupt and impede pathogenic growth following an infection. In general, adaptive means the distinction between specific and non-specific and reshaping the immune response against particular foreign pathogen. Rubio-Godoy (2010) mentioned that adaptive immunity was established following early response by non-specific immunity. Although it took longer time to mount a response during the first infection, adaptive immunity is crucial due to its specificity for the particular agent [11]. In fact, subsequent exposure commonly happened in shorter time and higher immensity than the earlier response.

Adaptive immunity mechanisms are incorporated with two major components which are antibodies and lymphocytes [11]. B cells, T cells and lymphocytes are the cells that makes up the adaptive immunity response. B cells, originated from the bone marrow, responsible for the antibody production whilst T cells, found and mature in thymus, differentiate into cells that eventually involved in lymphocyte maturation or in discarding virus-infected cells. To be precise, the secretion of memory cells by B cells is the key feature of adaptive immunity from innate immunity [14].

Adaptive Immune Response Against NNV Infection

B Cells

B cells are necessary type of lymphocyte that established the anti-viral response against infection majorly through humoral immunity [44]. Upon activation, B cells will produce plasma cells and memory cells. In return, plasma cells will generate antibodies that provokes response for the destruction of antigen as well as acting as antigen presenting cells (APCs) [14,45]. In teleost fishes, B cells originated in the head kidney, hence,

labelled as the primary lymphoid tissue [70]. Furthermore, B cells are also found in the spleen of teleost fishes, making spleen as the secondary lymphoid tissues. Bromage (2004) stated that B cells activation takes place in the spleen before differentiated into plasma cells and migrate to the head kidney, hence, justified the presence of antibody secreting cells in the spleen compared to head kidney. Danilova et al., (2005) stressed out that antibodies generate in fin fishes following viral infections are the major key to achieve the long-term adaptive immunity. In addition, immunoglobulin isotypes such as IgD, IgM and IgT were presence in teleost fishes, indicates that IgM expressing B cells responds to antigen stimulation including viral infection [73].

The role of B cells to induce antiviral immunity is undoubted since B cells are responsible to secrete antibodies, handing over protection against viral diseases [74]. In response to viral infection, teleost fishes have the capability to produce IgMs serum that able to identify viral antigens and this been demonstrated in wide range of fish species. For instance, in the course of viral infection, the presence of fully functional serum IgMs that are able to neutralize virus were recognized in rainbow trout. Although IgM is the common isotypes studied in teleost fishes, several studies have also highlighted the production of IgD and IgT following viral infections. As scientists stated that teleost fishes have three different classes of immunoglobulin (IgM, IgD and IgT), those classes of antibodies that are crucial to identify and counteract pathogen during viral infections can be distinguished through several characteristics [18]. IgM in general was the primary antibody classes that can be found in most species particularly in the earlier stages of the immune response and is the first class of antibodies to be produce upon infection. In fact, IgM was observed to be involved in both systemic and mucosal immunity mechanism in fishes [75]. IgD on the other hand, was observed only in certain species. For instance, IgD transcription was expressed during viral infections as [76] reported that IgD mRNA levels in freshwater carp (*Catla catla*) and rohu (*Labeo rohita*) gradually increase following infection against inactivated rabies virus via intramuscular injection. Several studies reported that IgD is usually observed in low concentrations, hence, its role in fish immune response still remain elusive, but it may have a role in initiating innate immune response [77]. Next, IgT or also labbeled as IgZ, is another class of unique antibody found in teleost fishes such as zebrafish and rainbow trout. Junirahma et al., (2021) found out that grouper (*Epinephelus coioides*) generates specific levels of IgT in specific organs, following vaccination with nodavirus inactivated vaccine. Previous study [18] reported that IgT is important for mucosal immunity, triggering immune response at specific sites as it was primarily exposed at mucosal surfaces including gut and, skin and gills.

T Cells

T cells are another crucial component in adaptive immune mechanisms, provoke its reaction through cell-mediated immunity response **[**78]. Similar as B cells, T cells are also type of lymphocytes that originated and matured in thymus, hence, labelled as T cells or also known as thymocytes. T cells are able to be differentiated from other lymphocytes by the presence of T-cell receptor or also known as antigen-specific receptor on its surface.

T cells families include T helper cells (Th cell), cytotoxic T cells, memory T cells, regulatory T cells and gamma delta T cells. Exposure to NNV infection will lead to the proliferation and differentiation of dendritic cells into Th1 cells and Th2 cells, generating cellular immune responses against the viral infection [25]. In fact, the proliferation of immune cells expressed on the surface of Th cells, known as CD4 plays a vital role in coordinating the adaptive immune response as T cells receptors only distinguish antigen represented by MHC class II molecules [28]. Saito et al., (2010) explained that Th cells expressing CD4, labelled as $CD4^+$ Th cells play a part to provoke the immune response as effector cells or memory cells. Naive $CD4^+$ T cells are able to differentiate into five different effector cells (Th1, Th2, Th17, Th9 and Th22), three subclass of regulatory T cells (Treg, Th3 and Tr-1) and memory T cells. Hence, unique abilities of this immune cells give grounds for their crucial role in synchronizing the immune system, immune pathogenesis and host defense mechanism [80]. In fact, [61] described $CD4^+$ T cells as flexible mainly due to their multiformity as recent finding figured out that T cells related genes such as TCR, CD4 and CD8 as well as MHC class I and II genes are detected in several teleost fish species. Study by [28] demonstrated that proliferation of CD4 and CD8 cells immune cells are detected in Humpback groupers (*Cromileptes altivelis*), following NNV infection. The presence of CD4 and CD8 cells in tissues and organ of groupers explained that the exposure to foreign antigen is able to mount cellular immune response, produced by the differentiation of cytotoxic T cells [81].

CD4 and CD8 cells were formed as a result of the proliferation and differentiation process, initiate from the entry of the antigen into the cells and tissues of the infected fishes. During NNV infection, the virus, acting as an intracellular antigen, undergoes proteolytic degradation within infected cells. The resulting peptide fragments are then transported to the endoplasmic reticulum by the transporter associated with antigen processing (TAP). This process is crucial for the subsequent presentation of viral antigens on MHC class I molecules, facilitating recognition by cytotoxic T lymphocytes and initiating a specific immune response against NNV-infected cells. Following that, the peptide will bound with MHC class I molecules, before being

presented on the surface by T cells $CD8^+$ (Cytotoxic T cells (Tc)). Then, cell destruction process (cytolysis), will be carried out by Tc cells with two different mechanism which are exocytic pathway and interaction between Fas ligand (FasL) and Fas expressed on target cells. In addition, Fas ligand is a type-II transmembrane protein that belongs to the tumor necrosis factor (TNF) family and interaction with its receptor induces apoptosis. After the binding of Tc cells with target cells, perforin and granzyme will be secreted in the cytosol of the host cells. Next, perforin spread through the cells and form pores, allowing granzyme to enter the host cells. Granzyme will eventually induce the process of apoptosis, hence, ending the programmed cell death. According to [82], perforin/granzyme-induced apoptosis is the main pathway used by cytotoxic lymphocytes to kill virus-infected and transformed cells.

Overall Mechanisms of Fish Immune System Against NNV Infection

Fish viruses most likely have the same capacity with mammalian viruses, where they developed few approaches to withstand the immune system [41]. Previously, [83] described that IFN suppression is the most common strategy for innate immune evasion following viral infection. Several studies have reported the occurrence of IFN related gene suppression following viral infection in salmon and rainbow trout fishes [84]. Other than IFN suppressions, restriction of apoptosis is also one of the viruses' mechanisms to escape the innate immunity response. By inhibiting apoptosis, infected cells are alive until all the particles were released [85]. Hence, innate immune evasion apparently justified the importance of both innate and adaptive immunities to work in complement [14].

In attempt to mount antiviral response against an infection, both innate and adaptive immunity mechanisms play their own parts. Following NNV infection, innate immunity directly works through TLRs. Once NNV particles successfully pass the physical barrier, invasion starts and this initiate innate cellular and humoral immunities, as well as the specific adaptive immunity. Innate immunity discards the invading pathogen through phagocytosis and involved several components including granulocytes, phagocytes and non-specific cytotoxic cells. During the same course of infection, humoral immunity mechanisms consist of protein and glycoproteins, works together with cellular innate immunity to suppress the growth of microorganisms [86]. Following phagocytosis, small protein fragments were presented by macrophages on MHC class II molecules (Figure 4). Upon activation of adaptive immunity, antigen-presenting cells (APCs) such as macrophages present processed antigens on MHC class II molecules. T helper (Th) cells recognize these antigens via their T cell receptors (TCRs), forming an APC-Th cell complex. This interaction triggers the secretion of cytokines by activated Th cells. These cytokines, including interleukins and interferons, stimulate B cell proliferation and differentiation. As B cells mature, they differentiate into two distinct cell types: plasma cells, which secrete antibodies, and memory B cells, which provide long-term immunological memory. This process is crucial for mounting an effective and specific immune response against pathogens like NNV [87]. Antibodies produced by the plasma cells are responsible to suppress the pathogen through opsonization and neutralization before phagocytosis takes place to destroy and remove the disabled pathogen [14,87].

Intracellular antigen including virus undergo another functional mechanism before being eliminated from the fish body. In case of NNV infection, NNV pathogen undergo phagocytosis and being displayed on MHC Class I by APC. This attracts CD8+ cells to bind with the MHC I of APC through CD8+ receptors. Then, activated CD8+ cells will start to proliferate and produce memory T cells and cytotoxic T Lymphocytes (CTL) that discard the infected cells via cell apoptosis. Following subsequent exposure with the same antigen, as memory cells meet the specific antigen again, b cells will rapidly proliferate and differentiate into plasma cells. This will result in plasma cells releasing significant number of antibodies to clear the antigen.

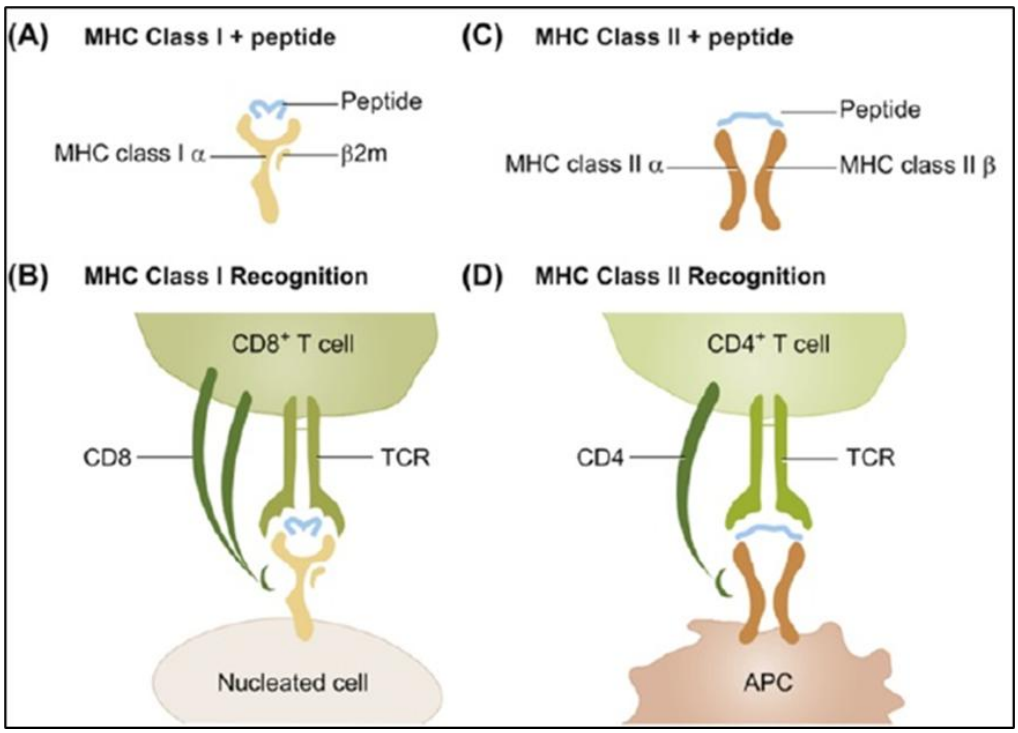

Fig 4 Major Histocompatibility Complex (MHC) Class I and Class II [88]. MHC system is crucial for the adaptive immune response in fish, enabling them to recognize and combat a wide array of pathogens.

Conclusion

Viral nervous necrosis is a serious disease, leading to significant economic loss in aquaculture. Better understanding on key factors including fish immune system mechanisms, the pathogens itself and its entry mechanisms can leave a significant impact to the farming industry. While vaccination seems to be the most effective strategy to prevent the emergence of various diseases in the future, findings on how the fish's immune system works serves as a strong basis as well as lay the foundations for further research, particularly on vaccination. Therefore, the necessary knowledge is crucial and helps in efforts to boost the health and disease protection of fish.

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Examples

Table 1 Possible effects of genetically modified organisms

Table 2 Methods of tissue culture in tomato plants

Fig 1 Amount of GMO production in the world

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Aksoy, H.M., Y. Kaya, and T.H. Tengku Abdul Hamid, Expression of the dspA/E gene of Erwinia amylovora in non-host plant Arabidopsis thaliana. Biotechnology & Biotechnological Equipment, 2017. 31(1): p. 85-90.Doi:xxxxxxxxxxxxxxxxxxxxxxxxx

Examples of articles with four or more authors;
Kaya, Y., et al., Evaluation of transgenic Nicotiana tabacum with dehE gene using transposon based IRAP markers. American Journal of Plant Sciences, 2013. 4(08): p. 41. Doi:xxxxxxxxxxxxxxxxxxxxxxxxx

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Kocalishkan, I., Allelopathy. 2006, Ankara, Turkey: Our Office Press-In Turkish. ISBN:xxxxxxxxxxxxxx

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Kaya, Y., F.Z. Huyop, and M.F. Edbeib, Genetic Diversity in Plants, Advances in Biosciences, F.Z. Huyop and S. Mohammed, Editors. 2019, Penerbit UTM Press. Malaysia: Malaysia. p. 04-24. Doi:xxxxxxxxxxxxxxxxxxxxxxxxxx or ISBN:xxxxxxxxxxx

Thesis
Kaya, Y., Regeneratıon of Dalapon Tolerant Nicotıana Tabacum By Expressıng Dehalogenase E Gene Through Agrobacterıum Tumefaciens – Medıated Transformatıon. 2013, Universiti Teknologi Malaysia: Malaysia.

Example for congress/conferences paper;

Arvas, Y.E., et al., Possible Effects of Genetically Modified Plants on Biodiversity, in I. International Organic Agriculture and Biodiversity Symposium. 2017: Berlin- Germany. p. 65.

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DECLARATION:

This work is part of Gülfidan KUYUMCU's MA thesis. Articles on 27-29 September 2017

held in Bayburt in Turkey was presented as a Oral Presentation I. International Organic Agriculture and

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Genetic Analysis Related To Organized Genetic Changes in Potato And Processed Potatoes

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