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Jordan Journal of Pharmaceutical Sciences

Aims & Scopes:

***Jordan Journal of Pharmaceutical Sciences* (*JJPS*)** is a bimonthly open-access peer reviewed journal funded by the Scientific Research Fund at Ministry of Higher Education and Research and hosted by the Deanship of Research at the University of Jordan. JJPS is dedicated to various disciplines of pharmaceutical and allied sciences. JJPS publishes manuscripts on original work, either experimental or theoretical in the following areas:

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INTRODUCTION

The Jordan Journal of Pharmaceutical Sciences (**JJPS**) is a peer-reviewed Journal, which publishes original research work that contributes significantly to further the scientific knowledge in pharmaceutical sciences' fields including pharmaceutical/medicinal chemistry, drug design and microbiology, biotechnology and industrial pharmacy, instrumental analysis, phytochemistry, biopharmaceutics and Pharmacokinetics, clinical pharmacy and pharmaceutical care, pharmacogenomics, bioinformatics, and also **JJPS** is welcoming submissions in pharmaceutical business domain such as pharmacoeconomics, pharmaceutical marketing, and management. Intellectual property rights for pharmaceuticals, regulations and legislations are also interesting topics welcomed from our colleagues in Schools of Law.

On a current topic in Pharmaceutical Sciences are also considered for publication by the Journal. **JJPS** is indexed in SCOPUS (Q3). It's a journal that publishes 4 issues per year since 2021 in (**March, June, September, December**). The Editorial Team wishes to thank all colleagues who have submitted their work to JJPS). If you have any comments or constructive criticism, please do not hesitate to contact us at jjps@ju.edu.jo. We hope that your comments will help us to constantly develop **JJPS** as it would be appealing to all our readers.

Prof Ibrahim Alabbadi
Editor-in-Chief
School of Pharmacy- The University of Jordan
Amman 11942- Jordan

Volume 17, 2024

Letter from the Editor-in-Chief

Typically, Food and Drug Administration (FDA) organizations or health authorities in countries worldwide perform all necessary investigations before granting approval for any medication or any type of food suitable for human consumption. But what happens when these authorities do not exist? Can we expect peace in 2024? We all hope every human, irrespective of their geographical location, can live in peace and enjoy good health, with all essential life necessities readily available. The World Health Organization's recent definition of health refers to a state of complete physical, social, and mental well-being, NOT ONLY the absence of disease or infirmity. Health-Related Quality of Life is a fundamental right for any human living on earth. But what if there is no food or medication, or if these essentials are even prohibited?

The Jordan Journal of Pharmaceutical Sciences (JJPS) team includes numerous scholar colleagues from universities in Gaza which have been demolished. These colleagues may still be alive or sadly, they may have already lost their lives. Numerous professors supported the JJPS by acting as peer reviewers and submitting quality scientific articles under incredibly challenging circumstances. We tip our hats to all of them and hope that God, with his immediate power, will grant life, peace, and security as Ramadan commences in March.

The JJPS editorial board has already initiated the second phase of a three-year term, following renewal approval from the Jordanian Ministry of Higher Education, which underscores our tremendous teamwork and significant progress. The JJPS' scores in international scientific databases, such as SCOPUS, continue to improve – our Q3 score is now close to Q2. Additionally, we've seen a continued influx of submissions from increasingly diverse countries, including places in North Africa, Europe, the USA, Canada, Australia, and Southeast Asia. We've also noticed a significant reduction in time from submission through revision to the decision-making process, and with the rise in ambiguity due to AI and ChatGPT programs, the need for similarity report checks has become essential.

Best regards

Prof Ibrahim Alabbadi
Editor-in-Chief

CONTENTS

Jordan Journal of Pharmaceutical Sciences, Volume 17, No. 3, 2024

The Role of Empathy and Relationship Quality in Building Customer Loyalty in Community Pharmacies: Evidence from Jordan

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ABSTRACT

Aim: This study explores how pharmacist empathy influences customer loyalty through the mediating role of relationship quality in community pharmacies.

Methodology: A cross-sectional study utilizing a web-based questionnaire and convenience sampling was conducted in Jordan. Data were analyzed using Structural Equation Modeling (SEM).

Results: A total of 536 responses were gathered and analyzed from all Jordanian regions. The gender distribution was balanced, with 261 males (48.7%) and 275 females (51.3%). The age distribution was concentrated in the 35-44 age group (29.3%), followed by 25-34 (25.6%). The majority had a high level of education, with 323 (60.3%) holding a Bachelor's degree or higher academic qualification (19.0%). Most participants visited pharmacies at least once every six months (50.6%), primarily using independent community pharmacies (52.1%). The study findings highlight a significant positive impact of pharmacist empathy on the three key dimensions of pharmacist-patient relationship quality (trust, satisfaction, and commitment). Furthermore, a significant positive relationship was found between the relationship quality dimensions and both customer loyalty dimensions (attitudinal and behavioral loyalty).

Conclusion: The study findings highlight the central role of pharmacist empathy in establishing a strong and enduring pharmacist-patient relationship quality, which in turn, enhance consumers loyalty, which is essential for thriving and surviving in today's competitive market.

Keywords: Relationship quality; Pharmacist Empathy; Customer Loyalty; Community pharmacies; Jordan

1. INTRODUCTION

In the midst of intense market competition, businesses must establish and sustain strong relationships with their profitable customers over the long term [1]. Building, managing, and maintaining these relationships is not just a business strategy; it is a fundamental approach that contributes to customer loyalty, cost savings, and sustainable competitive advantage in a dynamic market environment [2,3,4,5].

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One substantial indicator of relationship success is relationship quality [6]. Relationship quality goes beyond mere interaction, delving into the depth and strength of the connection between customers and sellers. A high level of relationship quality has the potential to not only strengthen and prolong the bond between customers and sellers but also play a crucial role in enhancing customer loyalty over time, which in turn enhances business sustainability [1,2,3,7,5,8].

Customer loyalty is crucial as it contributes to customer retention, leading to substantial cost savings, including reduced marketing expenses [9]. Acquiring new customers can be up to five times more costly than

maintaining existing ones [10]. Simultaneously, retaining customers has the added benefit of boosting profit rates through heightened purchase frequency and increased referrals [3]. Thus, enhancing customer loyalty is particularly important in sectors where continuous customer involvement and interaction are crucial, as seen in industries like the pharmaceutical sector and businesses such as community pharmacies.

In the pharmaceutical industry, particularly, the community pharmacies, where customers desperately seek solutions to health concerns, pharmacist empathy could be a factor shaping relationship quality. Empathy, defined as the ability to understand and share the feelings of others, forms the building blocks of meaningful interactions [11]. The ability of pharmacists to build an empathetic bond with the patient by actively listen to patients' needs, and understand their concerns could significantly contributes to the quality of the relationship.

The pharmaceutical sector in Jordan is experiencing rapid growth, mirroring global trends. Within the domain of community pharmacies, their numbers have increased by approximately 65.7%, rising from 2,157 in 2012 to 3,576 in 2022 [12,13]. Although chain pharmacies were appeared in Jordan in the last 2 decades with relatively enough investment on the ground, it seems it is unstable and many chains left the market with major financial failures. It's also crucial to emphasize that community pharmacies operate as businesses with limited resources [14]. These aspects emphasize the requirement for sustainability of managerial activities, alongside the primary function of dispensing pharmaceuticals. These activities could include the focus on pharmacist empathy and relationship quality to enhancing community pharmacies sustainability as it enhances customer loyalty. None of the previous studies has explored the impact of empathy and relationship quality, in community pharmacies context, on enhancing customer loyalty. Thus, understanding this relationship is crucial for community pharmacies aiming to grow in an increasingly competitive market.

The current study aims to explore the impact of pharmacist empathy and customer-pharmacist relationship quality on the customer loyalty in the context of community pharmacies in Jordan. Particularly interested in the role of relationship quality, measured through trust, commitment, and satisfaction, as a mediator in this process. By understanding these dimensions, pharmacy owners can develop targeted marketing strategies, benefitting customers through improved service experiences and enhanced healthcare outcomes. Consequently, this leads to an enhancement of loyalty, and contributing significantly to the sustainability and success of community pharmacies.

1.1 Conceptual background and study hypotheses

Customer Loyalty

According to the American Marketing Association, customer loyalty is characterized by a consumer consistently choosing the same product or service from a specific seller over time, rather than making purchases from various sellers within the category [15]. Customer loyalty is a multidimension construct that commonly understood as either an attitude (referred to as attitudinal loyalty) or an intention that prompts specific behaviors (known as behavioral loyalty) [5]. Attitudinal loyalty reflects the customer’s emotional and psychological attachment to a specific offering, leading to positive word-of-mouth and recommending the service to others [16,17,18,19]. While behavioral loyalty involves interpreting repeat purchasing patterns as indicative of customer loyalty [2].

Relationship quality

Relationship quality is core concept in the relationship marketing. Relationship quality is a multi-dimensional concept that refers the comprehensive evaluation of the relationship strength and the extent to which it meets the needs and expectations of the parties based on a history of successful interactions [20]. Relationship quality was initially defined through dimensions like trust and satisfaction [21], the conceptualization was later expanded

to include commitment as an additional dimension to gauge the quality of the relationship [22]. Several previous studies in marketing context adopted this expansion [2,3,5,23]. Our study aligns with this expansion, employing trust, satisfaction, and commitment as dimensions to assess relationship quality.

In the marketing literature, relationship quality consistently emerges as a key factor influencing the attitudinal and behavioral customer loyalty [2,5,24,25,26]. Accordingly, we propose the following hypotheses:

H1: Trust positively affects customer behavioral loyalty to the community pharmacy.

H2: Trust positively affects customer attitudinal loyalty to the community pharmacy.

H3: Satisfaction positively affects customer behavioral loyalty.

H4: Satisfaction positively affects customer attitudinal loyalty.

H5: Commitment positively affects customer behavioral loyalty.

H6: Commitment positively affects customer attitudinal loyalty.

Empathy

Pharmacist empathy is crucial for patient-centered care, plays a crucial role in establishing and maintaining positive patient-pharmacist relationship in community pharmacy settings. Empathy was identified as the ability to comprehend both the spoken and felt aspects of a patient's expression and convey this comprehension verbally [27]. Building on this definition, the concept of empathy was expanded suggesting that it encompasses cognitive attributes involving an understanding of patients' experiences, effective communication of this understanding, and an intention to offer assistance to the patient [28]. Our study explores how empathetic interactions contribute to the three dimensions relationship quality. Accordingly, the following hypotheses has been proposed:

H7: Pharmacist empathy positively affects trust.

H8: Pharmacist empathy positively affects customer satisfaction.

H9: Pharmacist empathy positively affects customer commitment.

1.2 Research model

The current research has adopted the research model presented in Figure 1. This model serves as a comprehensive framework to guide the investigation and analysis conducted in this study.

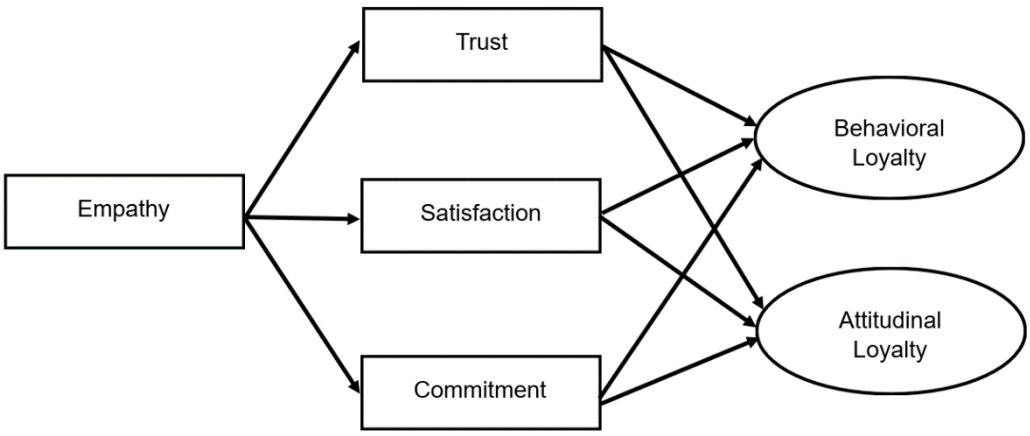

Figure 1. Research Model

2 METHODOLOGY

2.1 The setting

The study was carried out in Jordan. Inclusion criteria were being over 18 years of age and regularly visiting community pharmacies, regardless of whether they were independent community pharmacies or part of larger chain pharmacies.

2.2 Data collection instrument and the sample size

This cross-sectional study utilized a quantitative approach, collecting data through a web-based questionnaire. The questionnaire comprised three sections: the first focused on demographic information, including age, gender, and location of residence. The second part involved inquiries about their preferred community pharmacy and the one where they consistently buy pharmaceutical products including the frequency of visits to pharmacy and the type of pharmacy (independent pharmacy, chain pharmacy, or online pharmacy). The third section consisted of 24 items that used to measure the variables in the research model. A five-point Likert scale was used for all of the questionnaire items.

Table 1 provides details of the measures, their sources (references), and item counts for each variable in this study. References were selected based on their relevance to our focus on empathy, relationship quality (trust, commitment, and satisfaction), and behavioral and attitudinal loyalty. For example, Almomani [2] and Rauyruen et al. [5] investigated the impact of relationship quality dimensions, including trust, satisfaction, and commitment, on attitudinal and behavioral loyalty, aligning closely with our study's focus. Similarly, Alshuriadeh et al. [3] examined the antecedents and consequences of relationship quality in the pharmaceutical industry, using trust, commitment, and satisfaction, which are pertinent to our exploration of relationship quality dimensions. Furthermore, Murray et al. [29] investigated the interaction between empathy and responsiveness in determining consumer perceptions of service quality and loyalty in pharmacy retailing, providing valuable insights into the importance of empathy, a key component of our study's focus on relationship quality. All items utilized from these sources were updated to fit the current study context.

To ensure the instrument's face validity, we conducted a pilot study with 20 participants, including 10 customers, 8 pharmacists, and 2 academic experts in the field. Based on their feedback, we enhanced the questionnaire's clarity by translating it into Arabic and conducting a thorough review to clarify any unclear questions, as well as to correct grammar and spelling errors. These modifications aimed to improve the questionnaire's quality, ensuring accurate and meaningful participant responses.

Regarding the sample size, previous study determined a suitable range of sample size requirements for studies employing Structural Equation Modeling (SEM), ranging from 30 to 460 cases [31]. This study collected a sample size of 536, surpassing the established range. The sample size of 536 was selected to ensure robust statistical power, practical feasibility, and enhanced generalizability, aiming to exceed recommended sample size ranges and ensure more representative and reliable study results.

Table 1. Measures source and count

Variable (Dimensions)	Reference	Items #
Pharmacist Empathy	29, 30	4
Relationship Quality (Trust, satisfaction and commitment)	2, 3, 5	12
Loyalty (Attitudinal and Behavioral)	2, 5, 15, 30	8

2.3 Analysis

The study data underwent analysis using both SPSS and SPSS Amos software. Descriptive statistics using frequencies and percentages were employed for demographic data. To assess the reliability and validity of data collection instrument, Cronbach's Alpha, Exploratory Factor Analysis (EFA), Confirmatory Factor Analysis (CFA), Composite Reliability (CR), Average Variance extracted (AVE) were calculated.

Following the methodology of previous studies [2, 14, 32], the model's fit to the data was evaluated using various fit indices. These indices included the Chi-square of degree of freedom (Chi2 /df), Comparative Fit Index (CFI), normed fit index (NFI), standardized root mean-square residual (SRMR), and Root Mean Square Error of Approximation (RMSEA). For assessing the goodness of measurement model fit using SEM, the evaluation criteria were established as follows: Chi-square ($p < 0.05$); (Chi2/df≤3) goodness-of-fit index (GFI ≥ 0.90); adjusted goodness-of-fit index (AGFI ≥ 0.80); comparative fit index (CFI ≥ 0.90); normed fit index (NFI ≥ 0.90); standardized root mean square residual (SRMR ≤ 0.08); and root mean square error of approximation (RMSEA < 0.10). These indices collectively offer a comprehensive assessment of how well the model aligns with the observed data.

SEM was then applied to test the research model and the hypotheses, with a significance level set at $p < 0.05$ in the analysis.

3 RESULTS

3.1 Participant Demographics

Survey was collected from 536 participants. The sample descriptive analysis (frequencies and percentages) is illustrated in Table 2. There was an approximately equal representation of males and females. Regarding age distribution, the majority of respondents fell within the 35-44 age group (29.3%), followed by the 25-34 age group (25.6%). The majority of participants were relatively highly educated with Bachelor's degree or higher. Moreover, the frequency of visits to the pharmacy varied, with the majority visiting at least once every 6 months (50.6%). Regarding pharmacy type, a slight majority of respondents reported visiting independent community pharmacies (52.1%), followed closely by chain pharmacies (42.9%), with a smaller percentage utilizing online pharmacies (5.0%).

3.2 Instrument reliability and validity

Both types of factor analysis (EFA and CFA) were utilized to assess the construct validity of the data collection instrument. Table 3 demonstrates the appropriateness of using Factor Analysis, supported by a Kaiser-Meyer-Olkin (KMO) value of 0.950 and a statistically significant Bartlett's test for the variables (p-value = 0.000) (Figure 2).

EFA using the SPSS and CFA using the SPSS AMOS were conducted. The results are shown in Table 3. Any item with factor loading less than 0.40 was removed [33].

The instrument's internal consistency was evaluated using Cronbach's alpha coefficient. Table 4 displays the Cronbach's alpha values for each variable before and after factor analysis. An alpha coefficient of 0.6 and above is considered acceptable [34]. Thus, the results from the reliability analysis confirm the internal consistency reliability of all instruments.

3.3 Structural equation model results

The Structural Equation Modeling (SEM) analysis results support for all nine paths (H1-H9) in the research model. The detailed outcomes of hypothesis testing using are provided in Table 5. Notably, pharmacist empathy positively influences the three dimensions of pharmacist-patient relationship quality, trust, satisfaction, and commitment. Additionally, the relationship quality influence both aspects of customer loyalty, the attitudinal and behavioral loyalty.

Table 2. Participants characteristics

Characteristics	Number (Percentage)
Sex	
Male	261 (48.7%)
Female	275 (51.3%)
Age	
18-24	61 (11.4%)
25-34	137 (25.6%)
35-44	157 (29.3%)
45-54	111 (20.7%)
55-64	44 (8.2%)
>=65	22 (4.1%)
Prefer not to say	4 (0.7%)
Education level completed	
No formal education	1 (0.2%)
Secondary school	52 (9.7%)
Diploma	54 (10.1%)
University (Bachelor)	323 (60.3%)
University (Masters or PhD)	102 (19.0%)
Prefer not to say	4 (0.7%)
Frequency of visits to pharmacy rated	
At least once every week	57 (10.6%)
At least once every month	143 (26.7%)
At least once every 6 months	271 (50.6%)
At least once a year	65 (12.1%)
Pharmacy type	
Independent community pharmacy	279 (52.1%)
Chain pharmacy	230 (42.9%)
Online pharmacy	27 (5%)

KMO and Bartlett's Test

Kaiser-Meyer-Olkin Measure of Sampling Adequacy.		.950
Bartlett's Test of Sphericity	Approx. Chi-Square	9360.962
	df	276
	Sig.	.000

Figure 2. KMO and Bartlett's test

Table 3. EFA and CFA Results, Composite Reliability, and Average Variance Extracted.

Constructs and items		EFA results		CFA results		
Pharmacist Empathy		**Factor loadings**	**Eigen Value**	**Factor Loadings**	**Composite reliability**	**Average variance extracted**
Em1	I feel that the pharmacist takes the time to understand my feelings when providing guidance on my health	0.73	5.279	0.81	90.90%	57.17%
Em2	I feel satisfied with the emotional support provided by the pharmacist	0.753		0.88		
Em3	I feel that the pharmacist listens well and shows empathy when discussing my health concerns	0.761		0.85		
Em4	The pharmacist is empathetic when discussing my health condition.	0.748		0.84		
Relationship Quality (Trust)		**Factor loadings**	**Eigen Value**	**Factor Loadings**	**Composite reliability**	**Average variance extracted**
Tr1	I trust the recommendations and advice provided by the pharmacist	0.666	4.176	0.72	86.24%	40.80%
Tr2	I believe that the pharmacist would not provide false information, even if there could be personal gain from doing so	0.667		0.79		
Tr3	I trust that the pharmacist will keep my health-related information confidential	0.637		0.74		
Tr4	I believe that the pharmacist is honest	0.706		0.87		
Relationship Quality (Satisfaction)		**Factor loadings**	**Eigen Value**	**Factor Loadings**	**Composite reliability**	**Average variance extracted**
Sa1	I am satisfied with the overall services provided by the pharmacist	0.654	3.412	0.81	88.11%	52.04%
Sa2	I am satisfied with the speed of service when obtaining medications from the pharmacy	0.572		0.87		
Sa3	I am pleased with the pharmacist	0.728		0.73		
Sa4	I believe that the pharmacist always meets my expectations	0.678		0.81		
Relationship Quality (Commitment)		**Factor loadings**	**Eigen Value**	**Factor Loadings**	**Composite reliability**	**Average variance extracted**
Ct1	I am committed to continuing my relationship with my pharmacist for future healthcare services	0.720	3.089	0.80	86.56%	49.39%
Ct2	I feel a sense of commitment to maintaining a long-term relationship with my pharmacist	0.693		0.83		
Ct3	I am willing to invest time and effort to sustain a lasting relationship with your pharmacist	0.672		0.77		
Ct4	I see my relationship with my pharmacist as similar to that of a family member	0.662		0.74		
Attitudinal Loyalty		**Factor loadings**	**Eigen Value**	**Factor Loadings**	**Composite reliability**	**Average variance extracted**
AL 1	I recommend this pharmacy to anyone who seeks my advice.	0.771	1.226	0.86	85.29%	47.34%
AL 2	I will recommend the pharmacy products to close friends and family	0.773		0.87		
AL3	I would say positive things about the pharmacy to other people.	0.772		0.89		
AL4	I hold a favorable opinion and attitude towards	0.761		0.84		
Behavioral Loyalty		**Factor loadings**	**Eigen Value**	**Factor Loadings**	**Composite reliability**	**Average variance extracted**
BL1	I will buy from this pharmacy in the future	0.695	1.226	0.77	81.40%	35.61%
BL2	I will not switch to another pharmacy even though there are lots of other pharmacy options	0.646		0.74		
BL3	This pharmacy is my first option and I will choose this it for my pharmaceutical needs	0.714		0.80		
BL4	I am willing to travel to my favorite pharmacy to make a purchase, even if there are closer pharmacies to my location.	0.081 (Deleted)		-		

Table 4. Cronbach's Alpha test

Variables	Before factor analysis		After factor analysis	
	Items #	Cronbach's Alpha	Items #	Cronbach's Alpha
Pharmacist Empathy	4	0.908	4	0.908
Trust	4	0.857	4	0.857
Satisfaction	4	0.884	4	0.884
Commitment	4	0.876	4	0.876
Attitudinal Loyalty	4	0.923	4	0.923
Behavioral Loyalty	4	0.692	3	0.761

Table 5. Results of Hypotheses Testing using SEM

Research Proposed Paths	Estimate	Standard Error	t-value	p-value	Result
H1: Empathy → Trust	0.478	.038	12.617	0.000	Confirmed
H2: Empathy → Satisfaction	0.457	.036	12.548	0.000	Confirmed
H3: Empathy → Commitment	0.938	.058	16.184	0.000	Confirmed
H4: Trust → Attitudinal Loyalty	0.274	.078	3.529	0.000	Confirmed
H5: Trust → Behavioral Loyalty	0.121	.062	1.970	.049	Confirmed
H6: Satisfaction → Attitudinal Loyalty	0.291	.071	4.100	0.000	Confirmed
H7: Satisfaction → Behavioral Loyalty	0.273	.057	4.791	0.000	Confirmed
H8: Commitment → Attitudinal Loyalty	0.340	.038	9.001	0.000	Confirmed
H9: Commitment → Behavioral Loyalty	0.326	.032	10.215	0.000	Confirmed

The model fit indices, as shown in Table 6, indicate the appropriateness of the research model in relation to the collected data. The scaled chi-square to degrees of freedom ratio (CMIN/df) stands at 2.938, suggesting a well-fitting model. Further, the Standardized Root Mean Square Residual (SRMR) is reported as 0.0336, denoting a low level of model fit discrepancy. The Goodness of Fit Index (GFI) is estimated at 0.901, while the Adjusted Goodness of Fit Index (AGFI) is 0.867, both indicating favorable fit. The Normed Fit Index (NFI) stands at 0.942, implying a high degree of fit, and the Comparative Fit Index (CFI) registers at 0.965, reinforcing the model's adequacy. Lastly, the Root Mean Square Error of Approximation (RMSEA) is reported as 0.059, confirming a reasonably close fit between the research model and the observed data.

Table 6. Measurement Model Fit Indices

	DF	CMIN/DF	P	SRMR	GFI	AGFI	NFI	CFI	RMSEA
Criteria		≤3	<0.05	≤0.08	≥0.90	≥0.8	≥0.90	≥0.90	<0.1
Study Model	213	2.938	0.00	0.028	0.902	0.873	0.934	0.955	0.060

4 DISCUSSION

The current study highlights the relationship quality as a crucial mediator in the pathway from pharmacist empathy to customer loyalty. This mediation suggests that pharmacist empathy positively influence relationship quality dimensions (trust, satisfaction, and commitment). Trust, serving as the building block of any enduring relationship, emerges as a natural consequence of empathetic interactions. Satisfaction, reflecting the harmony between patient expectations and the care received, is nurtured by the empathetic bond. Commitment, the allegiance to a healthcare provider, finds

its roots in the genuine and empathetic engagements within the pharmacist-patient relationship. Consequently, these dimensions shapes and influences both aspects of customers loyalty (behavioral and attitudinal loyalty) to the community pharmacy.

Enhancing customer behavioral loyalty has the potential to boost overall customer retention, a critical consideration in the competitive field of pharmaceutical care. Retaining customers within the community pharmacy is an economically sound practice. Several studies consistently confirm that acquiring new customers incurs significant costs and challenges compared to retaining existing ones [3,10,35]. Furthermore, long-term customers often contribute to cost savings through valuable, free-of-charge referrals of new customers [36].

The current study provided evidence that empathy and relationship quality affect attitudinal loyalty. This aspect of loyalty emerges as a critical dimension influencing customer behavior within community pharmacies. As customers develop emotional connections to the pharmacist and pharmacy, attitudinal loyalty becomes a motivator and driver force behind their repeat engagements, and customers become advocate powerful advocates through positive word-of-mouth [3,37]. This positive word-of-mouth serves as a substantial factor in attracting and acquiring new customers.

Amid a 65.7% increase in the number of pharmacies in Jordan over the last 10 years, community pharmacies face heightened competition, necessitating a thorough examination of consumer preferences beyond traditional factors. Previous studies explored the traditional determinants of consumer selection of community pharmacies like pharmacy location, medicine availability, satisfaction level of the pharmaceutical services provided as well as the quality of these services, discount prices, pharmacists expertise, and continuous education [38,39,40], all of which remain important. However, in the current competitive market, empathic pharmacist-patient relationships become increasingly crucial in differentiating pharmacies in the marketplace. This finding aligns with broader healthcare studies which emphasize the importance of empathy in the healthcare sector as empathic engagement could lead to better patient compliance and adherence [28,41], reduced anxiety and depression [28], and more accurate prognosis [28,42].

Unlike other sectors, customers visit community pharmacies seeking treatments, advices and guidance for their diseases and illnesses, adding a unique layer of significance to the role of empathy. This uniqueness shed the light on the necessity for pharmacies to strategically invest in training programs focusing on building strong empathetic communication skills, emotional intelligence, active listening to understand customer needs, particularly among pharmacists who serve as primary healthcare interfaces.

In Jordan, the Jordan Food and Drug Administration (JFDA) sets medicine prices, prohibiting pharmacists from offering discounts. Despite this, discounting is common in community pharmacies [43]. While adhering to these strict rules is challenging [44], pharmacies can overcome the negative impact of these rules by focusing on empathy and building strong customer relationships. Our study found that being empathic and building a quality relationship can significantly enhance customer loyalty, even under strict regulations.

Building upon the findings of our study, it's noteworthy to consider the potential consequences of a deficient pharmacist-patient relationship, customer dissatisfaction, and a lack of trust in the pharmacist. Previous studies established a link between these factors and consumers resorting to alternative, potentially unsafe sources for their medications, including online pharmacies and the internet which are a potential source of fake medicines [45,46,47,48]. Interestingly, within our cohort of 536 participants, 27 individuals acknowledged having purchased medicines from online sources. This observation highlights the practical significance of our study, suggesting that a strengthened pharmacist-patient

relationship, cultivated through empathetic communication and enhanced relationship quality, may play a crucial role in preventing consumers from seeking medications from potentially unsafe online sources.

5 Limitations and future research

While this study offers valuable insights into the role of pharmacist empathy in shaping the pharmacist-patient relationship and enhancing customer loyalty, some limitations should be considered. The use of a convenience sampling method and the specific cultural focus may impact the generalizability of our findings. Cultural factors, including norms, values, and communication styles, can influence the perception and expression of empathy in pharmacist-patient interactions. Therefore, the findings may not be directly applicable to other cultural contexts. Future research should consider more diverse samples and explore the different cultural nuances that influence empathy in pharmacist-patient interactions. Moreover, while we concentrated on the patient's perspective, future investigations should also explore the dynamics from the pharmacist's viewpoint. Understanding pharmacists' experiences of empathetic interactions and aligning them with patient experiences can offer a more comprehensive understanding.

6 CONCLUSION

The current study explores the crucial elements of pharmacist-patient interactions in community pharmacies, emphasizing the link between pharmacist empathy, relationship quality, and customer loyalty. Our findings underscore the significant role of pharmacist empathy in fostering strong relationships characterized by trust, satisfaction, and commitment, which in turn enhance customer loyalty. For community pharmacies in highly competitive markets, prioritizing pharmacist empathy is a key strategy to maintain positive relationships and boost customer loyalty—both essential for sustained success. Implementing training programs to enhance pharmacist empathy can be a valuable approach, and future research should delve into the reciprocal nature of empathetic interactions from the pharmacist's perspective.

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Conflict of interest

There are no conflicts of interest to disclose.

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دور التعاطف وجودة العلاقة في بناء ولاء العملاء في الصيدليات المجتمعية: دليل من الأردن

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ملخص

الهدف: يستكشف هذا البحث كيف يؤثر تعاطف الصيدلي على ولاء العملاء من خلال الدور الوسيط لجودة العلاقة في الصيدليات المجتمعية.

المنهجية: تم إجراء دراسة مقطعية باستخدام استبانة عبر الإنترنت وأخذ عينات مريحة في الأردن. تم تحليل البيانات باستخدام نمذجة المعادلات الهيكلية.

النتائج: تم جمع وتحليل ما مجموعه 536 استجابة من جميع مناطق الأردن. كان توزيع الجنس متوازنًا، حيث كان هناك 261 ذكراً (48.7%) و275 أنثى (51.3%). كانت الفئة العمرية الأكثر تركيزاً هي من 35 إلى 44 عامًا (29.3%)، تلتها الفئة من 25 إلى 34 عامًا (25.6%). معظم المشاركين كانوا من ذوي التعليم العالي حيث حصل 323 منهم (60.3%) على درجة البكالوريوس أو أعلى من المؤهلات الأكاديمية (19.0%). الأغلبية يزورون الصيدليات مرة واحدة على الأقل كل ستة أشهر (50.6%)، ويستخدمون بشكل رئيسي الصيدليات المجتمعية المستقلة (52.1%). تبرز نتائج الدراسة تأثيرًا إيجابيًا كبيرًا لتعاطف الصيدلي على الأبعاد الثلاثة الرئيسية لجودة العلاقة بين الصيدلي والمريض (الثقة، والرضا، والالتزام). علاوة على ذلك، تم العثور على علاقة إيجابية كبيرة بين أبعاد جودة العلاقة وأبعاد ولاء العملاء (الولاء السلوكي والولاء التوجهي).

الخلاصة: تسلط نتائجنا الضوء على الدور المركزي لتعاطف الصيدلي في إنشاء علاقة قوية ودائمة بين الصيدلي والمريض، مما يعزز ولاء المستهلكين، وهو أمر ضروري للنجاح والبقاء في السوق التنافسية الحالية.

الكلمات الدالة: جودة العلاقة، تعاطف الصيدلي، ولاء العملاء، صيدليات المجتمع، الأردن.

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Compliance of the Jordanian Population with the Protective Measures during and after the COVID-19 Pandemic; A Nation-Wide Survey

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ABSTRACT

Background: The COVID-19 pandemic has spread globally, with over 695 million confirmed cases and 6.9 million deaths as of September 2023. Compliance with protective measures is considered essential to combat the pandemic.

Objectives: To assess the adherence of the Jordanian population to preventive measures during the COVID-19 pandemic. The survey specifically focused on the habits and practices of Jordanians during the pandemic, as mentioned in the introductory sentence of the Google Forms questionnaire.

Methods: This cross-sectional study was conducted among the general population in Jordan aged 18 and above using an online questionnaire distributed from March to July 2022. The questionnaire was divided into two sections: demographic characteristics and practice-related questions. Data were collected using Google Forms and analyzed using R Statistical Software.

Results: Most of the 409 participants were under 30 years old (65.5%), female (70%), and held a college diploma or higher degree (80%). Around 57% had been infected with COVID-19 at least once, while 60% had a relative, friend, or colleague who died due to COVID-19. More than half of the participants (54%) demonstrated favorable practices. The multivariate analysis revealed that a previous COVID-19 infection significantly increased the odds of having favorable practices (OR=2.44; CI[1.59-3.77]; $p<0.001$).

Conclusion: This study evaluated how Jordanians adhered to COVID-19 preventive measures during the pandemic. It was found that roughly half of the population effectively followed precautions such as using masks and hand sanitizers, although adherence to a balanced diet was less frequent. The likelihood of taking precautions increased after having had COVID-19. The study also reported high vaccine acceptance rates. These findings underscore the importance of public adherence to preventive measures, especially in areas like nutrition, and provide insights for future pandemic responses.

Keywords: COVID-19, preventive measures, Jordanian population, Pandemic, protective practices.

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BACKGROUND

The Coronavirus, initially identified in December 2019 in China, swiftly disseminated worldwide, leading to the World Health Organization (WHO) declaring COVID-19 a global pandemic on March 11, 2020 (1). As of April 12, 2023, WHO records indicate more than 762 million confirmed COVID-19 cases and approximately 6.9 million associated fatalities (1).

Transmission of COVID-19 can occur through intermediary hosts, such as bats, or via direct human-to-human contact through respiratory droplets or physical interaction (2). Key modes of exposure to infected droplets include touching contaminated surfaces and subsequent contact with the face, including the eyes, nose, and mouth (3). Fever, cough, and shortness of breath are the predominant symptoms of COVID-19, typically manifesting 2 to 14 days after exposure (1). In advanced stages of infection, severe acute respiratory distress can ensue, with secondary outcomes potentially encompassing death during the ICU period due to organ dysfunction, hemorrhage, and septic shock (4).

The global ramifications of the pandemic have been profound, affecting public health, economies, and societies, prompting governments and healthcare systems worldwide to grapple with containment efforts and disease-related challenges. Governments have issued specific guidelines and restrictions to mitigate virus transmission (1,4). For example, on March 20, 2020, Jordan's Prime Minister imposed a mandatory curfew from 6 p.m. to 10 a.m., with violations resulting in fines ranging from 100-500 JD or potential arrests. Jordan also implemented stringent measures, including suspending inbound and outbound flights, introducing passenger screening and quarantine protocols, and launching distance learning and medication services on April 13. As of June 6, 2020, Jordan had reported a total of 784 COVID-19 cases, comprising 125 active cases, 9 fatalities, and 571 recoveries (5).

Variations in mortality rates among countries may be attributed to the implementation of various health measures. These health measures included social distancing, mask mandates, work reorganization, closure of commercial establishments, flight cancellations, traffic restrictions, avoidance of physical contact (e.g., handshakes and kissing), minimizing contact with shared surfaces, refraining from direct contact with confirmed cases, avoidance of touching the mouth or eyes, rigorous hand hygiene, abstaining from shared utensils, and seeking COVID-19 testing when symptomatic (6). Consequently, the adherence of the general populace to these protective measures and practices has been pivotal in controlling disease transmission and enhancing individual and community-level outcomes. Thus, this study endeavors to evaluate the compliance of the Jordanian population with protective practices during the COVID-19 pandemic, along with an exploration of factors associated with favorable adherence to these practices.

METHODS

● Study design and population

This was a cross-sectional survey using a self-administered online questionnaire targeting the general Jordanian population over the age of 18. The questionnaire was distributed, and data were collected from March to July 2022. A convenience sample of Jordanian individuals was invited to participate by sending a survey link through social media platforms (Facebook and WhatsApp). The inclusion criteria were adult individuals (≥ 18 years) residing in Jordan. A convenience sampling technique was employed, with a 95% significance level and a 5% margin of error, to determine the minimal required sample size, which was calculated to be 385 participants for a population ranging from 500,000 to infinity (7). The researchers successfully collected 409 completed questionnaires valid for analysis.

● Study tool

The constructs of this study were measured using statements from other studies that examined the knowledge, attitudes, and practices of people during

COVID-19, conducted in Nepal, Nigeria, Venezuela, Syria, multinational (Global), the Middle East, and the North Africa Region, respectively (8–13). We developed an online questionnaire after conducting a thorough literature search based on prior similar studies (8–13). The questionnaire was divided into two sections: demographic information and practice questions:

1- Demographic data included the following variables: age, gender, location, educational level, educational background, monthly income, health status, and history of COVID-19 infection and vaccination.

2- The second section, the practice questions which were in the form of frequency Likert scale from 1 (never) to 5 (always), included questions about (1) using vitamin supplements in general as vitamin D, (2) using supplements specific for the immunity as vitamin C, zinc, and magnesium nutritional supplements, as well as inquiries about (3) using hand sanitizer, medical alcohol, and other sterilizers, (4) sterilizing objects like electronic devices and surfaces, (5) wearing a mask and/or gloves, (6) whether you avoid crowds: less than six people in a confined space, (7) whether you adopted a healthier diet (i.e., did you consume more fruits, vegetables, and protein while consuming less carbohydrates and fats), (8) whether you had a PCR test for the Coronavirus, and finally (9) the question about whether you isolate yourself when you feel the symptoms of Corona or the flu or not.

● Data Collection

The questionnaire was designed using Google Forms and posted on various social media platforms. It was translated into Arabic by Deena Moghrabi, an authorized translator proficient in English, German, and Arabic. The accuracy, clarity, content validity, relevance, and conciseness of the questionnaire items were evaluated by three academics and researchers in the field of pharmacy in Jordan. Suggested amendments were discussed and incorporated before finalizing the questionnaire. Both Arabic and English sentences and answers were included for each question to accommodate Arabic and English speakers.

● Data Analysis

The data were organized in a Microsoft Excel sheet and then imported and analyzed using R Statistical Software (v4.1.3; R Core Team 2022). For baseline demographic characteristics, frequencies and percentages were used to describe categorical variables, while means and standard deviations were used for continuous variables. In the practice section, answers were coded as follows: Never = 1, Rarely = 2, Sometimes = 3, Often = 4, Always = 5. Participants with a total score higher than or equal to the average score were considered to have favorable practices.

The Chi-square test was used to assess the significant association between demographic characteristics and the level of practice. Multivariate regression analysis was performed to assess the association between demographic characteristics and practice level. Results were reported as odds ratios (OR) and 95% confidence intervals (CI). A p-value of ≤ 0.05 was considered significant.

● Ethical considerations

This cross-sectional observational study was conducted in compliance with the Declaration of Helsinki's ethical guidelines. All participants provided informed consent before data collection, and participation was completely voluntary. Confidentiality and anonymity were ensured at every stage of the research, including data gathering, storage, and analysis. Such studies conducted by PhD students are supervised by the University of Debrecen to ensure ethical compliance.

RESULTS:

● Demographic characteristics of the participants

The total number of survey respondents who completed their data was 406 individuals, who were included in the final analyses (Table 1). Most of the participants were under the age of thirty (65.5%), and more than half were female (70%). Of the respondents, 325 individuals (80%) had a bachelor's degree or higher, while only 195 (48%) worked or studied in the health sciences

field. Most (63.8%) of the respondents had a monthly income of $565 USD or less, while only 6.4% had an income greater than $1,963 USD. The majority (84%) had not been diagnosed with any chronic diseases such as diabetes or high blood pressure. The vast majority (96.3%) had received the coronavirus vaccine, while only 238 participants (58.7%) had been infected with the coronavirus. Moreover, 245 individuals (60.3%) reported having a friend or family member who died due to COVID-19.

Table 1- Baseline characteristics of the participants

Label	Frequency (%) (N= 406)
Age, years	
20-29	266 (65.5)
30-39	80 (19.7)
> 40	60 (14.8)
Gender	
Female	287 (70.7)
Male	119 (29.3)
Educational level	
College or above degree e.g. bachelor	325 (80.0)
High school	81 (20.0)
Educational background	
Health-related sciences	195 (48.0)
Non-health-related sciences	211 (52.0)
Monthly income	
565 USD or Less	259 (63.8)
565 USD – 1128 USD	91 (22.4)
1128 - 1693 USD	30 (7.4)
More than 1693 USD	26 (6.4)
Diagnosed with a chronic illness e.g. diabetes or hypertension	
No	341 (84.0)
Yes	65 (16.0)
Received COVID vaccine	
No	15 (3.7)
Yes	391 (96.3)
Infected by COVID	
No	168 (41.3)
Yes	238 (58.7)
A family member, friend, colleague or relative died due to COVID infection	
Not sure	20 (4.9)
Yes	245 (60.3)
No	141 (34.7)

● **Compliance with the protective measures among the participants**

Based on the results of the study, the average total score for participants' practices was 35.8 out of 45, with a standard deviation of 5.5. Only 222 participants (54.7%) had favorable practices (a score equal to or greater than the mean), while the remaining 45.3% had unfavorable practices. The highest average score for an item was for wearing masks and/or gloves (mean = 4.4, SD = 0.8) and for using hand sanitizers, rubbing alcohol, and other antiseptics (mean = 4.4, SD = 0.7). In contrast, the lowest average score was for adopting a healthy diet (containing more fruits, vegetables, and proteins, and fewer fats and carbohydrates) (mean = 3.5, SD = 1.1). The details of the practice section results are summarized in Table 2.

Table 2- Participants' adherence to the preventive measures

Label	Mean (SD)
Consumed vitamin supplements e.g. vitamin D	3.8 (0.9)
Consumed certain supplements with potential to boost immunity (Vitamin C/Zinc/Magnesium)	3.9 (1.0)
Used hand sanitizers, rubbing alcohol and other antiseptics	4.4 (0.7)
Used disinfectants on objects e.g., groceries, electronic devices, surfaces	4.0 (1.0)
Wore face masks and/or gloves	4.4 (0.8)
Avoided crowds of More than 6 people in a closed area	3.8 (1.0)
Chose a healthier diet (more fruits, vegetables and proteins and less fats and carbohydrates)	3.5 (1.1)
Did COVID PCR Diagnostic Test	3.9 (1.2)
Self-isolated when COVID or Flu-like symptoms were experienced	4.1 (1.0)
Total score of practice	35.8 (5.5)
Practice	Frequency (%)
Favorable practice	222 (54.7)
Unfavorable practice	184 (45.3)

Chi-square tests revealed no significant differences between the groups with favorable and unfavorable practices in terms of age, gender, educational background, history of chronic diseases, or COVID-19 vaccination. However, there was a significant difference regarding history of COVID-19 infection (p-value < 0.001) and monthly income (p-value = 0.039). A higher proportion of participants with a history of COVID-19 infection were in the favorable practices group (68.5%) compared to the unfavorable practices group (46.7%). Additionally, more participants with a higher monthly income were in the favorable practices group (Table 3). Regression analysis found that infection with COVID-19 significantly increases the odds of compliance with protective measures or having favorable practices (adjusted OR = 2.44; 95% CI: [1.59-3.77], p-value < 0.001) (Table 4).

Table 3- Distribution of participants according to practice

Label	Favorable practice	Unfavorable practice	p-value
Age, years			0.201
• 20-29	139 (62.6)	127 (69.0)	
• 30-39	44 (19.8)	36 (19.6)	
• > 40	39 (17.6)	21 (11.4)	
Gender			0.332
• Female	152 (68.5)	135 (73.4)	
• Male	70 (31.5)	49 (26.6)	
Educational level			0.844
• College or above degree e.g. bachelor	179 (80.6)	146 (79.3)	
• High school	43 (19.4)	38 (20.7)	
Educational background			0.566
• Health-related sciences	110 (49.5)	85 (46.2)	
• Non-health-related sciences	112 (50.5)	99 (53.8)	
Monthly income			0.039
• 565 USD or Less	131 (59.0)	128 (69.6)	
• 565 USD – 1128 USD	58 (26.1)	33 (17.9)	
• 1128 - 1693 USD	21 (9.5)	9 (4.9)	
• More than 1693 USD	12 (5.4)	14 (7.6)	
Diagnosed with a chronic illness e.g. diabetes or hypertension			0.178
• No	181 (81.5)	160 (87.0)	
• Yes	41 (18.5)	24 (13.0)	
Received COVID vaccine			0.929
• No	8 (3.6)	7 (3.8)	
• Yes	214 (96.4)	177 (96.2)	
Infected by COVID			<0.001
• No	70 (31.5)	98 (53.3)	
• Yes	152 (68.5)	86 (46.7)	
A family member, friend, colleague or relative died due to COVID infection			0.17
• Not sure	9 (4.1)	11 (6.0)	
• Yes	143 (64.4)	102 (55.4)	
• No	70 (31.5)	71 (38.6)	

Table 4- Regression analysis of factors affecting participants' practice

Label	Unfavorable practice	Favorable practice	OR (univariable)	OR (multivariable)
Age, years				
• 20-29	127 (47.7)	139 (52.3)	0.59 (0.32-1.05, p=0.075)	0.65 (0.32-1.33, p=0.243)
• 30-39	36 (45.0)	44 (55.0)	0.66 (0.33-1.31, p=0.234)	0.71 (0.33-1.50, p=0.371)
• > 40	21 (35.0)	39 (65.0)	-	-
Gender				
• Female	135 (47.0)	152 (53.0)	-	-
• Male	49 (41.2)	70 (58.8)	1.27 (0.82-1.96, p=0.281)	1.35 (0.85-2.16, p=0.208)
Educational level				
• College or above degree e.g. bachelor	146 (44.9)	179 (55.1)	-	-
• High school	38 (46.9)	43 (53.1)	0.92 (0.57-1.51, p=0.748)	0.90 (0.52-1.56, p=0.699)
Educational background				
• Health-related sciences	85 (43.6)	110 (56.4)	-	-
• Non-health-related sciences	99 (46.9)	112 (53.1)	0.87 (0.59-1.29, p=0.501)	0.65 (0.40-1.05, p=0.079)
Monthly income				
• 565 USD or Less	128 (49.4)	131 (50.6)	-	-
• 565 USD – 1128 USD	33 (36.3)	58 (63.7)	1.72 (1.06-2.83, p=0.031)	1.40 (0.80-2.47, p=0.241)
• 1128 - 1693 USD	9 (30.0)	21 (70.0)	2.28 (1.04-5.42, p=0.048)	1.99 (0.83-5.05, p=0.132)
• More than 1693 USD	14 (53.8)	12 (46.2)	0.84 (0.37-1.88, p=0.667)	0.61 (0.25-1.47, p=0.273)
Diagnosed with a chronic illness e.g. diabetes or hypertension				
• No	160 (46.9)	181 (53.1)	-	-
• Yes	24 (36.9)	41 (63.1)	1.51 (0.88-2.64, p=0.140)	1.41 (0.74-2.74, p=0.303)
Received COVID vaccine				
• No	7 (46.7)	8 (53.3)	-	-
• Yes	177 (45.3)	214 (54.7)	1.06 (0.36-3.00, p=0.915)	1.04 (0.33-3.15, p=0.951)
Infected by COVID				
• No	98 (58.3)	70 (41.7)	-	-
• Yes	86 (36.1)	152 (63.9)	2.47 (1.65-3.72, p<0.001)	2.44 (1.59-3.77, p<0.001)
A family member, friend, colleague or relative died due to COVID infection				
• Not sure	11 (55.0)	9 (45.0)	-	-
• Yes	102 (41.6)	143 (58.4)	1.71 (0.68-4.40, p=0.250)	1.13 (0.41-3.17, p=0.807)
• No	71 (50.4)	70 (49.6)	1.21 (0.47-3.16, p=0.698)	1.10 (0.40-3.12, p=0.852)

Table 4 presents the results of the regression analysis examining the factors influencing participants' COVID-19 preventive practices. Among the notable findings, age demonstrated a trend towards significance in multivariable analysis, with individuals aged 20-29 and 30-39 years showing a slightly higher likelihood of favorable practices compared to those over 40 years old. Although the p-values for age did not reach conventional significance levels, they suggest a potential age-related influence on preventive practices. Gender did not exhibit a statistically significant association with practice outcomes. While males appeared to have slightly higher odds of favorable

practices, the results did not achieve significance in either univariable or multivariable analysis. Monthly income levels showed interesting patterns. Participants with incomes between $565 and $1,128 per month had significantly higher odds of favorable practices compared to those earning less than $565. This finding suggests a potential economic dimension to preventive behaviors.

The most substantial and statistically significant predictor of favorable practices was a history of COVID-19 infection. Individuals who had been infected with COVID-19 had significantly higher odds of engaging in favorable practices, with an odds ratio (OR) of 2.44 in multivariable analysis. This underscores the impact of personal experience with the virus on adherence to preventive measures.

DISCUSSION

We conducted this study in Jordan to assess adherence to preventive practices during the COVID-19 pandemic among the general population. The study indicated that only half of the population had a favorable level of practice or good adherence to protective measures. A similar result was found in a study in Palestine, where participants had an average score of 50.2% (14). In contrast, studies in the UAE and Iraq reported total scores of 90% and 76%, respectively, for practice questions (15,16). Another study in Palestine showed a generally high level of acceptance of government regulations during the pandemic (17).

The present study indicated that the highest level of adherence was observed in using masks, gloves, hand sanitizers, and/or other antiseptics. This may be attributed to the increased availability of personal protective equipment during the pandemic and the growing culture of proper hand washing (18). These findings support a prior study conducted in Jordan, which summarized safety procedures followed by the majority of neighborhood pharmacies to protect both employees and clients from contracting the virus. The study found that since the pandemic began, about 94% of pharmacists have worn personal protective equipment, while 82% of pharmacies routinely sterilize the premises and the primary door handle, and 82% of pharmacies provide gloves, medical masks, and alcohol at the entrance (19). Additionally, another study in Jordan examining knowledge and practices related to disinfectants and sanitizers during the COVID-19 pandemic found that Jordanian adults increased their use of disinfectants during the outbreak. Although few high-risk activities were identified, research participants generally demonstrated positive practices regarding the use of chemical disinfectants. Interestingly, awareness of the safe and effective use of antimicrobials had little to no impact on the positive behaviors adopted by Jordanian adults (20).

Similarly, other studies in the UAE and Palestine revealed that most of the population agreed on the importance of wearing masks (15,17). The use of hand sanitizers, rubbing alcohol, or other antiseptics was also widely supported in Iraq and Palestine (16,17). Our study showed a good level of practice in using disinfectants among participants, with a mean score of 4.1 out of 5. Another study in Palestine reported that around 68% of participants agreed on the use of disinfectants (14). The current study's results further support a relevant study conducted in Kuwait, which evaluated the parameters associated with beneficial behaviors and compliance with preventive measures among the Kuwaiti community during the COVID-19 pandemic.

In that study, a convenience sample of the Kuwaiti population was surveyed online, and 389 completed questionnaires were evaluated. The analysis revealed that the majority of participants (81.2%) had no congenital or chronic illnesses and were female (59.4%). The COVID-19 infection rate was 54.8%. During the COVID-19 epidemic, more than half of the participants (54.8%) engaged in positive behaviors. Significant differences in medical history and COVID-19 infection history were found between the groups with favorable and unfavorable practices. Approximately 65.3% of those with favorable

practices had been infected with COVID-19 at least once, compared to only 42% of those with unfavorable practices. Similarly, 23.9% of the favorable group and only 12.5% of the unfavorable group had a history of congenital or chronic diseases. The results of the multivariate analysis indicated that prior COVID-19 infection significantly increased the likelihood of having beneficial behaviors. Likewise, having a history of a congenital or chronic illness significantly raised the likelihood of having positive practices (21).

Our analysis revealed that the lowest level of compliance during the COVID-19 pandemic was in following a balanced diet, with a mean score of only 3.5 out of 5. Another study in Palestine showed that 71.7% of participants adhered to a balanced diet (13). Despite the fact that the U.S. National Institutes of Health does not endorse any prophylactic treatments against COVID-19 (21), our study found good adherence among participants to consuming supplements to boost immunity, such as Vitamin C, Zinc, and Magnesium, with a mean score of 3.9 out of 5. A study conducted in Kuwait, which assessed the influence of the COVID-19 pandemic on the purchasing intention of vitamins, revealed that health consciousness positively affected attitudes toward vitamins, thereby increasing purchasing intentions. This could be attributed to heightened health consciousness during the pandemic and more positive attitudes toward supplements and vitamins due to their perceived role in boosting immunity (22). A study aimed at evaluating changes in patient demand for drug and non-drug items in community pharmacies in Jordan during the COVID-19 pandemic found that all participants reported an increase in the demand for minerals and vitamins compared to previous years. This rise in demand can be attributed to the inclusion of these supplements in Jordan's Ministry of Health's treatment protocol for mild COVID-19 cases (23).

Avoiding crowded public spaces was recommended during the COVID-19 era, and our study detected relatively good compliance with this recommendation, with a mean score of 3.8 out of 5. High levels of acceptance and compliance with this measure were also reported in studies conducted in the UAE and Palestine (15,17).

Throughout the epidemic, approximately 57% of participants were infected with COVID-19 at least once. Compliance with precautions was shown to improve with prior exposure to COVID-19. A related study in Palestine (14) found a statistically significant correlation between prior infection and adherence to preventive measures. Notably, a large proportion of our participants had already been vaccinated against COVID-19, with at least one dose.

These results confirm findings from previous research conducted in Jordan. A cross-sectional survey evaluating awareness and attitudes towards COVID-19 vaccination revealed a fairly high level of awareness about the COVID-19 vaccine, with 51.4% of participants perceiving the importance of vaccination. However, only 37.1% agreed that the newly developed vaccine was safe, and 77.4% expressed a preference for natural immunity. Overall, the attitude towards COVID-19 vaccination was cautiously optimistic, with 60.2% of respondents scoring above Bloom's 60.0% cutoff point, despite mixed opinions on vaccine safety and necessity (23).

Many people are reluctant to be vaccinated for various reasons, including misunderstandings and distrust (24), which is a major issue worldwide. Increased vaccine availability and accessibility, public health campaigns, and trust in healthcare institutions (25) may all contribute to higher vaccination uptake rates among the study group.

The purpose of a descriptive questionnaire-based cross-sectional survey among the Jordanian population was twofold: (1) to evaluate the prevalence of COVID-19 in Jordan and (2) to identify socio-demographic and behavioral predictors of infection, with an emphasis on the use of preventive measures (PPMs). Our final statistical analyses included data from 7,746 individuals. The descriptive data revealed that 82.6% of people believed wearing a face mask would protect them from contracting COVID-19. Approximately 69.5% were adamant about

using a face mask, whereas 65% were committed to using hand sanitizer. Female gender (AOR = 1.2; 95% CI: 1.07-1.35; p = 0.002), having a close relative with COVID-19 (AOR = 8.5; 95% CI: 7.51-9.70; p = 0.001), and employment or education in the healthcare field (AOR = 1.2; 95% CI: 1.09-1.38; p = 0.001) were associated with higher adherence to preventive measures. However, the belief that face masks do not protect against COVID-19 was also a factor (AOR = 1.3).

The authors observed that adherence to non-pharmaceutical interventions (NPIs) such as mask use, hand washing, and social isolation fell short of ideal levels among Jordanians. This may be why the COVID-19 infection rate has surged in recent months throughout the nation. A third wave could be devastating, and comparable infectious dangers in the future could be mitigated through more long-term health promotion and stringent policy initiatives (26).

The study's results carry significant implications for the post-COVID-19 period. Firstly, the finding that individuals who have previously been infected with COVID-19 are more likely to exhibit favorable preventive practices underscores the power of personal experience. Moving forward, public health authorities and policymakers should consider tailoring their messages and campaigns to leverage these personal experiences. Stories and testimonials from survivors and those who have battled the virus can be instrumental in emphasizing the importance of preventive measures, potentially resonating more strongly with individuals who have directly experienced the impact of COVID-19.

Furthermore, the study suggests age-related influences on preventive practices, with individuals aged 20-29 and 30-39 showing a slightly higher likelihood of engaging in favorable practices compared to those over 40 years old. Although these differences did not reach conventional significance levels, they hint at a potential generational divide in behavior. In the post-pandemic period, it would be prudent for public health strategies to consider the unique perspectives and needs of different age groups. Tailored educational campaigns and interventions can be designed to address the specific concerns and motivations of these distinct age cohorts, ensuring a more effective and targeted approach to promoting preventive practices.

Additionally, the study's examination of monthly income levels reveals an intriguing economic dimension to preventive behaviors. Participants with higher incomes, particularly those earning between 565 USD and 1128 USD per month, displayed significantly higher odds of favorable practices. This finding highlights the need for equitable access to resources and information in the post-COVID era. Policymakers should focus on addressing economic disparities to ensure that preventive measures remain accessible to all socioeconomic groups. Efforts to provide financial support, affordable healthcare, and resources for preventive tools like masks and sanitizers can contribute to a more equitable approach to pandemic management.

RECOMMENDATIONS:

Our results suggest that extensive and targeted public awareness campaigns should be initiated. The elements of the protective measures should be discussed thoroughly and explained in both Arabic and English. We can leverage individuals with higher education levels and good compliance with these measures to help understand and reach all other population groups. Moreover, governments should work on building trust among the population to facilitate adherence to recommendations and instructions.

Implications of Study Results on the Post COVID-19 period

1- The study emphasizes the need for continued public health messaging and instruction regarding the importance of maintaining preventive measures even after the pandemic's peak. Although vaccination rates have been favorable, there is a risk of complacency as the pandemic recedes. It is imperative to maintain awareness and highlight the ongoing importance of behaviors such as mask-wearing and hand hygiene.

2- The reduced adherence to dietary recommendations suggests that tailored initiatives are needed to promote healthy eating patterns during the post-pandemic phase. Public health initiatives should emphasize the role of nutrition in maintaining overall health and combating infectious diseases.

3- Understanding the factors influencing compliance, such as a history of COVID-19 infection, can inform future outbreak response efforts. Public health officials can use these insights to target specific population groups and enhance preparedness and response strategies.

4- The study's high vaccine uptake rates are encouraging and reflect a level of confidence in immunization campaigns. For the post-pandemic period, it is crucial to sustain and expand this confidence to ensure continued vaccine coverage for COVID-19 and other preventable diseases.

5- These results can be generalized to other nations and regions facing similar issues. The lessons learned from Jordan's experience with COVID-19 compliance can inform global health policies, particularly in areas where vaccine hesitancy remains a challenge.

6- In the post-pandemic phase, healthcare systems can optimize resource allocation by understanding the factors affecting compliance. By identifying high-risk groups and tailoring interventions to meet their specific needs, resource use can be more effectively managed.

CONCLUSIONS

The findings of this study provide valuable insights into the factors influencing individuals' adherence to COVID-19 preventive practices during the pandemic and offer guidance for the post-COVID-19 period. The significant impact of personal experience with COVID-19 infection on the likelihood of engaging in favorable preventive practices underscores the importance of relatable messaging and testimonials in public health campaigns. Leveraging firsthand experiences can be a powerful tool for encouraging continued adherence to preventive measures in the future.

While age-related trends in preventive practices were observed, they did not reach statistical significance. However, this hints at potential generational differences in behavior that merit further exploration and targeted interventions. Public health strategies should consider tailoring their approaches to address the unique concerns and motivations of different age groups, recognizing that one-size-fits-all approaches may not be as effective.

Additionally, the influence of monthly income on preventive practices highlights the need for equitable access to resources and support. In the post-COVID-19 era, policymakers should prioritize measures that reduce economic disparities, ensuring that preventive tools and healthcare remain accessible to all segments of the population.

As we transition into the post-pandemic period, these insights provide a roadmap for public health authorities and policymakers to continue fostering a culture of prevention. By leveraging personal experiences, addressing age-specific needs, and promoting economic equity, we can better navigate the challenges that lie ahead and sustain the progress made in managing ongoing and future health crises.

Declarations:

Ethical considerations

This was an observational study with no interventions or experimental procedures. It was conducted in accordance with the principles outlined in the Declaration of Helsinki. Participation in this survey was voluntary, and participants provided informed consent before completing the questionnaire. Anonymity and confidentiality were maintained throughout the study, including during data collection and analysis.

Consent for publication

Not applicable

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Conflict of Interests

The authors declare no conflict of interest.

Availability of data and materials

Data are available upon reasonable request from the corresponding author.

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امتثال السكان الأردنيين لإجراءات الحماية أثناء وبعد جائحة كورونا-19؛ دراسة على مستوى البلاد

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ملخص

الخلفية: انتشرت جائحة كوفيد-19 على مستوى العالم، حيث بلغ عدد الحالات المؤكدة أكثر من 105 ملايين حالة و2.3 مليون حالة وفاة حتى شباط 2021. ويعتبر الامتثال للتدابير الوقائية ضروريًا لمكافحة الوباء.

الأهداف: تقييم مدى التزام السكان الأردنيين بالإجراءات الوقائية أثناء وبعد جائحة كورونا -19 .

الطُرق: استطلعت هذه الدراسة المقطعية عموم السكان في الأردن الذين تتراوح أعمارهم 18 عامًا فما فوق باستخدام استبيان عبر الإنترنت تم توزيعه في الفترة من اذار إلى تموز 2022. وقد تم تقسيم الاستبيان إلى قسمين: أسئلة ديموغرافية وأخرى عملية. تم جمع البيانات باستخدام نماذج جوجل (Google) وتحليلها باستخدام برنامج R الإحصائي.

النتائج: كان معظم المشاركين البالغ عددهم 409 تحت سن 30 عامًا (65.5%)، والإناث (70%)، وكان لديهم شهادة جامعية أو درجة أعلى (80%). أصيب حوالي 57% بفيروس كورونا مرة واحدة على الأقل بينما توفي 60% لدى منهم قريب أو صديق أو زميل بسبب الإصابة بكورونا -19. وكان لدى أكثر من نصف المشاركين (54%) ممارسات إيجابية. كشف التحليل متعدد المتغيرات أن الإصابة السابقة بفيروس كورونا تزيد بشكل كبير من احتمالات وجود ممارسات إيجابية (0.001>OR=2.44; CI[1.59-3.77]; p).

خاتمة: وسلطت هذه الدراسة الضوء على مدى التزام الأردنيين بالإجراءات الوقائية، حيث كان لدى ما يقرب من النصف ممارسات إيجابية والتي تزداد مع الإصابة السابقة بفيروس كورونا.

الكلمات الدالة: كوفيد-19، وقائي، الأردن.

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Preparation and Evaluation of Nanolipid Carriers of Bedaquiline- *In vitro* Evaluation and *in silico* Prediction

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ABSTRACT

Background: Bedaquiline, a potent antitubercular drug used in the treatment of multidrug-resistant strains, suffers from low oral bioavailability, a slow onset of therapeutic action, and side effects. This investigation proposes the development of nanocarriers for the drug to improve drug release and estimate its effect on oral absorption through an in-silico model. Initially, a custom design was investigated to estimate the effects of composition and process on the entrapment and particle size of the carriers. The nanocarriers were subjected to studies on surface characteristics, surface morphology, thermal properties, drug release, ex vivo permeation, and antimicrobial efficacy. In silico predictions of bioavailability and pharmacokinetic parameters of the optimized formulation were conducted using GastroPlus® software.

Results: The study revealed that bedaquiline entrapped in nano lipid carriers (65.5 nm) of glyceryl behenate and palm oil effectively increased the rate of drug release by more than 80% and led to a 3.5-fold increase in antimicrobial activity against Mycobacterium tuberculosis. Intestinal permeation was enhanced by 3.7 times. Predictions using GastroPlus® software indicated that the nano lipid carrier of bedaquiline could be a promising method for improving the drug's efficacy with better localization in the gastrointestinal compartments and improved pharmacokinetics, achieving 93% bioavailability.

Conclusion: It can be concluded that bedaquiline nanocarriers in a lipid matrix can serve as an effective tool for enhancing the efficacy of bedaquiline in the treatment of tuberculosis.

Keywords: Nano lipid carriers, Bedaquiline, Bioavailability, Permeation, *In Silico* prediction.

1. INTRODUCTION

Tuberculosis (TB), a contagious disease caused by *Mycobacterium tuberculosis*, is one of the leading causes of illness and death worldwide [1]. A major problem in the pharmacotherapy of TB is the occurrence of drug-resistant strains of bacteria [2]. Drug resistance is a man-made phenomenon and occurs due to non-compliance with the long dosage regimen [3]. Intrinsic resistance of the bacterial cell wall to drug penetration and bacterial mutations also contribute to resistance development [4]. The types of resistance include mono, poly, multi, and extensive. Resistance to first-line treatments, such as isoniazid and rifampicin, has become a significant therapeutic challenge in TB treatment, necessitating the development of novel strategies to overcome drug resistance and enhance drug potency against both drug-susceptible and resistant strains.

Bedaquiline is a diarylquinoline derivative indicated for multi-drug-resistant TB, typically used in combination therapy for the treatment of adult and pediatric patients [5]. The oral dose of the drug is 100 mg. It is categorized as a Biopharmaceutical Classification System (BCS) II drug, has a very slow

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onset of action, and is extensively metabolized in the liver [6]. Side effects associated with oral bedaquiline delivery include cardiac arrhythmia, muscle stiffness, and joint pain [7]. According to available reports, the bioavailability of bedaquiline increases twofold in the presence of food [8]. The fatty component of food is a key factor in enhancing the absorption and bioavailability of lipophilic drugs [9].

Therefore, effective and safe development of nanotechnology methods would be beneficial in overcoming the challenges associated with oral drug administration. Nanocarriers can increase drug solubility, enabling lower doses and improving treatment safety and efficacy.

Nano lipid particles appear to be promising systems for the oral delivery of lipophilic drugs among the various types of nanoparticles examined, primarily due to their excellent surface-active characteristics, nontoxicity, stability, and good biocompatibility [8]. Additionally, their small particle size allows them to reach the cellular level, their high surface-to-volume ratio enhances interaction with target cells, and their improved water solubility and ability to penetrate thick bacterial cell walls make them effective carriers for overcoming therapeutic limitations [10].

Nano lipid carriers (NLCs), composed of physiological and biodegradable solid and liquid lipids, belong to second-generation nanoparticles. They are beneficial for drug targeting and improving the oral bioavailability of poorly aqueous soluble drugs [11]. An imperfect or less ordered structure is created in the lipid matrix, which plays a crucial role in enhancing drug entrapment and reducing drug expulsion, thereby providing a continuous release of the drug at a consistent rate. NLCs exhibit a bi-phasic drug release pattern, with an initial burst release from the outer layers followed by a slow release from the solid lipid core [12].

Therefore, the present study focuses on the preparation of nano lipid carriers of the anti-tuberculosis drug bedaquiline to enhance its solubility and bioavailability [6]. Oral drug absorption is a complex process, and predicting it in humans remains a challenge. Pharmaceutical research must translate in vitro data into in vivo data for the safe and effective development of pharmaceuticals. This study includes an in-silico prediction tool to estimate the viability of the proposal. The Advanced Compartmental Absorption Transit (ACAT) model in the GastroPlus® software (Simulations Plus, Lancaster, CA) considers physicochemical, physiological, and formulation factors for predicting oral absorption. It also detects the involvement of transporters and/or enzymes. Hence, the study includes the use of GastroPlus® software to correlate in vitro evaluations with the prediction of in vivo performance of NLCs of bedaquiline.

2. MATERIALS AND METHODS

Bedaquiline was provided as a gift sample from Viatris, Hyderabad, India. Stearic acid, palm oil, oleic acid, vitamin E, and olive oil were purchased from SD Fine Chemicals, Mumbai, India. Glyceryl behenate was gifted by Gattefosse Sas, Mumbai, India.

2.1 Screening of lipids for the preparation of bedaquiline loaded NLC

NLCs represent a binary system with an imperfect lipid core. The drug solubility in this less ordered structure determines its physicochemical properties and the final effectiveness of the formulation. The selection of solid and liquid lipids for preparing bedaquiline-loaded nano lipid carriers was performed using a solubility method [13]. For solid lipids, 10 mg of the drug was added to gradually increasing amounts of molten solid lipids (stearic acid, glyceryl monostearate, and glyceryl behenate). The mixture was heated in a controlled-temperature water bath to obtain a clear molten mass. The minimum amount of molten lipid required to solubilize the drug and form a clear molten state was estimated [14].

Various liquid lipids, including vitamin E, olive oil, palm oil, and oleic acid, were screened. A fixed volume (2 mL) of each liquid lipid was placed in an Eppendorf tube. Each tube, containing an excess of the drug, was shaken in a mechanical shaker for 24 hours at 37°C and 100 rpm. The tubes were then centrifuged at 5000 rpm for 15 minutes to separate the undissolved drug and collect the supernatant [15]. The supernatant was suitably diluted and analyzed chromatographically to estimate drug solubility. Chromatographic analysis was performed using a C18 column, with a mobile phase of acetonitrile and 0.1% trifluoroacetic acid in a 50:50 ratio, at a flow rate of 1 ml/min and ambient temperature. The sample was analyzed at 242 nm [16]. A calibration curve was generated in the dilution range of 50-200 µg/ml with a linearity equation of Y = 2.325X + 21664 (where Y represents peak area and X represents concentration (µg/ml)), and the correlation coefficient was calculated to be 0.9999.

2.2 Method of preparation of nanocarriers of Bedaquiline:

A custom design was applied to observe the effects of material and process attributes on the development of NLCs of bedaquiline using JMP software V16. Extensive preformulation work was carried out to identify the parameters and their levels for the design.

The independent variables included the solid-to-liquid lipid ratio, surfactant concentration, homogenization time, and speed, while the amount of drug and total lipid content of the formulations were kept constant. The drug content in each formulation was maintained at 10% w/w of the total lipid. The dependent variables measured were drug entrapment and particle size, as outlined in Table 1. Fourteen experimental trials were conducted according to the design.

Table 1 Variables with coded levels

Independent variables	**-1**	**0**	**+1**
Solid Lipid: Liquid Lipid	70:30	80:20	90:10
Conc of SAA (%)	5	7.5	10
Homogenization Time (min)	5	7.5	10
Homogenization Speed (rpm)	5000	7500	10,000
Dependent Variables			
Particle size (nm)	Minimize		
Entrapment efficiency (%)	Maximize		

*SAA- Surfactant

Bedaquiline-loaded NLCs were prepared using the high shear homogenization method. The drug, solid lipids, and liquid lipids were accurately weighed and melted in a water bath at 70°C with continuous stirring. A surfactant solution of Tween 80 in water (1.5% w/v) was maintained separately at 70°C. The surfactant solution was gradually added to the lipid dispersion at high speed for a sufficient time as per the design. The resulting emulsion was poured into a petri dish and allowed to solidify on ice with gentle stirring for 15 minutes [17].

2.3 Evaluation of nanocarriers of bedaquiline

2.3.1 Particle size analysis

Dynamic light scattering (DLS) was used to estimate the particle size of the nano lipid carriers, utilizing a Horiba SZ-100 (Germany). The samples were dispersed in Millipore water, and the analysis was conducted at 25°C. The particle size for each trial formulation was recorded as the average of three trials ± SD.

2.3.2 Estimation of entrapment efficiency

Entrapment efficiency measures the effectiveness of

the carriers in encapsulating the drug. Approximately 5 mg of bedaquiline-loaded NLCs were dispersed in 10 ml of methanol and vortexed. The sample was filtered using a 0.45 µm syringe filter and analyzed chromatographically to estimate the total drug (Td). A separate quantity of the sample was dispersed in distilled water, centrifuged at 10,000 rpm for 30 minutes, and the supernatant was collected, diluted, and analyzed chromatographically to estimate the free drug (Fd) [18]. Three trials were conducted for the estimation, and the % entrapment efficiency was calculated using the formula:

% Entrapment efficiency = {(Td -Fd) ÷ Td} × 100

The same procedure was repeated for blank formulations in all experimental trials to account for the effects of excipients during estimation. After completing all experimental trials, model validation and optimization were performed at a significant level of $p < 0.05$. The optimized formulation (Fopt) was prepared and subjected to further analysis.

2.3.3 Powder X ray diffraction study (PXRD)

To characterize the crystalline state of the drug in the formulation, powder X-ray diffraction (Rigaku SmartLab 3kW, Japan) was performed for the pure drug and formulation Fopt using Cu Kα radiation at 25°C. The scanning was conducted at a 2θ (diffraction angle) range from 2 to 70 degrees. The diffraction pattern was recorded with a vertical goniometer [19].

2.3.4 Differential Scanning calorimetry (DSC)

Thermal analysis of the pure drug bedaquiline and formulation Fopt was performed using a differential scanning calorimeter (SHIMADZU DSC-60). A precisely weighed sample (5 mg) enclosed in an aluminum pan was heated at a predetermined scanning rate (10°C/min) from 30°C to 400°C. Dry nitrogen gas (at a flow rate of 25 mL/min) was used as the carrier gas [20].

2.3.5 Scanning electron microscopy (SEM)

The surface characteristics of the pure drug and formulation Fopt were examined using scanning electron microscopy with a gold sputtering technique (Hitachi 3400S, Japan). The powder samples were fixed onto a stub, gold was sputtered onto the samples, and the sample slides were subjected to vacuum drying. The samples were investigated at 10 kV, and photographs were taken at different magnifications [21].

2.3.6 *In vitro* drug release study

The in vitro release of the drug from formulation Fopt was estimated using the dialysis bag method (molecular weight cut-off of dialysis membrane: 12-14 kDa; pore size: 2.4 nm). The dialysis bag was pre-soaked in biorelevant media (Fed State Simulated Intestinal Fluid [FESSIF], pH 6.8) for 12 hours. The formulation Fopt (10 mg equivalent bedaquiline) dispersed in 2 mL of water was placed into the dialysis bag and immersed in a beaker containing 100 mL of FESSIF. The beaker was magnetically stirred at 100 rpm, and the temperature was maintained at 37°C. Specific volumes of aliquots were withdrawn at regular intervals and replenished with equal volumes of media. Sampling was conducted for 24 hours. The samples were analyzed at 242 nm using the established chromatographic technique. The cumulative amount of drug release was estimated. The data were analyzed according to different kinetic models: zero-order, first-order, Higuchi, and Korsmeyer-Peppas models [12, 22].

2.3.7 *Ex vivo* permeation study

Ex vivo permeation studies were performed for Fopt and the pure drug. Freshly excised goat ileum from a local slaughterhouse was used for the study. The ileum was cut into 5.5 cm pieces and washed initially with saline solution, followed by phosphate buffer (pH 7.4). A weighed quantity of 10 mg equivalent of the sample, dispersed in 1 mL of water, was introduced into the ileum and then placed in a beaker containing 50 mL of FESSIF (pH 6.8) under magnetic stirring. The temperature was maintained at 37±0.5°C with continuous aeration. At each time point, 1 mL of the sample was withdrawn at specified intervals up to 24 hours and replaced with an equal volume of phosphate buffer. The samples were analyzed chromatographically at 242 nm. A plot of cumulative permeation of bedaquiline versus time was

created. Apparent permeability (Papp) was calculated using the following equation:

$$Papp = \frac{Q}{ACT}$$

Where Q=total amount of drug permeation, A= surface area of the intestine, C=initial concentration of drug, t=total time of permeation study.[23]

The same process was repeated for pure drug and apparent permeability was compared with the optimized formulation.

2.3.8 Antimicrobial Study

The test organism studied was Mycobacterium tuberculosis (H37RV Strain, ATCC No. 27294). The anti-mycobacterial activity of the pure drug and Fopt was assessed using the microplate Alamar Blue assay (MABA). The microdilution broth technique was used to determine the minimum inhibitory concentration (MIC). Sterile 96-well microplates were initially filled with 200 µL of sterile deionized water, followed by 100 µL of Middlebrook 7H9 broth. Serial dilutions of the compounds (100 to 0.2 µg/mL) were prepared on the plates. The plates were incubated for five days at 37°C. Freshly prepared Alamar Blue reagent and 10% Tween 80 (1:1 mixture) were added to the plates and incubated for an additional 24 hours. A blue color in the wells indicated no bacterial growth, while the formation of pink color was considered growth. The minimum inhibitory concentration (MIC) was estimated [24].

The measurement of colony-forming units (CFU) was carried out for the pure drug and Fopt using Middlebrook 7H9 broth supplemented with 10% OADC at the MIC concentration, infected with the Mycobacterium strain. The agar plates were incubated for 48 hours at 37°C. After the incubation period, the CFUs were quantified [25].

2.3.9 *In silico* prediction of oral absorption of bedaquiline NLCS

GastroPlus® 9.5 was used to predict the possible absorption model of the bedaquiline NLCs in a human model. [26] The pharmacokinetics were evaluated with ADMET Predictor v8.5.0.0. [27] An absorption simulation model was created for Fopt for a 100 mg oral dose. The physicochemical properties predicted by GastroPlus® version 9.5 (Simulations Plus) and ADMET Predictor version 8.5 (Simulations Plus) were used for the simulation and are listed in Table 2.

Table 2 Physicochemical properties of Bedaquiline predicted by GastroPlus

Property	Predicted
Molecular formula	C32H31BrN2O2
Molecular weight	555.52
Predicted log P	6.1
Dosage form (human)	IR tablet
Dose (mg)	100
Dosing interval (h)	56
Mean Precipitation time	900
Diffusion coeff (cm^2/S)	0.54 X 10^{-5}
Solubility (mg/ml)	2.89X 10^{-3} at pH 8.52
Drug Particle density(g/ml)	1.2
Particle size in radius(µm)	0.065
Dosage volume (ml) (human)	250

The human physiology was represented as a series of compartments, each defined with the default values for the drug absorption process, as listed in Table 3.

The outcome of the model was evaluated for oral bioavailability and pharmacokinetics and compared with data available in the literature.

3. RESULT

4. *Screening of lipids for the preparation of bedaquiline loaded NLC*

The solubility of bedaquiline was determined in various liquid and solid lipids. Among the tested lipids, bedaquiline demonstrated the highest solubility in glyceryl behenate and palm oil, as shown in Table 4.

4.1 Evaluation of nanocarriers

A total of 14 experimental runs were generated using a custom design with three center points, employing JMP version 16 software. The experiments varied the composition, homogenization speed, and time to assess their effects on entrapment efficiency and particle size. The entrapment efficiency of the drug ranged from 56% to 87%, while the particle size ranged from 60 to 70 nm. The recorded responses are detailed in Table 5.

Table 3 Default Gastrointestinal Physiology in GastroPlus

GI Compartment	ASF	pH	Transit time (min)	Volume (ml)	Bile
Stomach	0	1.30	0.25	46.56	0
Duodenum	2.741	6	0.26	41.56	2.8
Jejunum 1	2.719	6.2	0.93	154.2	2.33
Jejunum 2	2.719	6.4	0.74	122.3	2.03
Ileum 1	2.709	6.6	0.58	94.29	1.41
Ileum 2	2.688	6.9	0.42	70.53	1.16
Ileum 3	2.677	7.4	0.29	49.83	0.14
Caecum	7.221	6.4	4.19	47.49	0
Asc Colon	21.49	6.8	12.57	50.33	0

Table 4 Solubility of bedaquiline in different lipids

Lipids	Solubility (mg/gm)
Stearic acid (S)	0.51±0.37
Glyceryl monostearate (S)	1.09±0.24
Glyceryl behenate (S)	1.65±0.19
Vitamin -E (L)	0.63±0.21
Olive Oil (L)	0.33±0.05
Palm oil (L)	0.88±1.10
Oleic acid (L)	0.13±0.10

*S and L denotes solid and liquid lipid respectively

Table 5 Evaluation of NLCs as per custom design.

Formulation code	SL: LL	%Conc of SAA	Homogenization time (min)	Homogenization speed(rpm)	% EE	Particle size(nm)
F1	1	-1	1	1	70.52±0.06	66.1±11.4
F2	0	0	0	0	62.39±0.04	68.0±14.2
F3	1	1	1	-1	73.26±0.05	68.1±25.1
F4	-1	-1	1	1	65.13±0.05	66.2±20.2
F5	1	1	0	1	70.12±0.01	66.8±13.7
F6	-1	1	1	-1	58.39±0.02	70.8±19.2
F7	0	0	0	0	58.45±0.03	69.1±12.8
F8	-1	1	-1	1	56.25±0.04	65.6±24.1
F9	1	-1	-1	1	59.38±0.07	60.5±21.7
F10	1	-1	1	-1	68.26±0.08	60.1±17.4
F11	1	1	-1	-1	81.30±0.04	60.3±10.6
F12	1	-1	-1	-1	87.16±0.03	60.5±19.3
F13	0	0	0	0	59.59±0.01	68.2±22.5
F14	-1	-1	-1	-1	68.51±0.03	69.1±15.6

The data for both responses were analyzed statistically at a significance level of $p<0.05p < 0.05p<0.05$. Model validation was conducted using an actual vs. predicted plot, as shown in Fig. 1.

The main factors affecting the responses are illustrated in the response surface diagrams, which highlight the positive impact of homogenization conditions on particle size and formulation factors on the percentage of drug entrapment, as presented in Fig. 2.

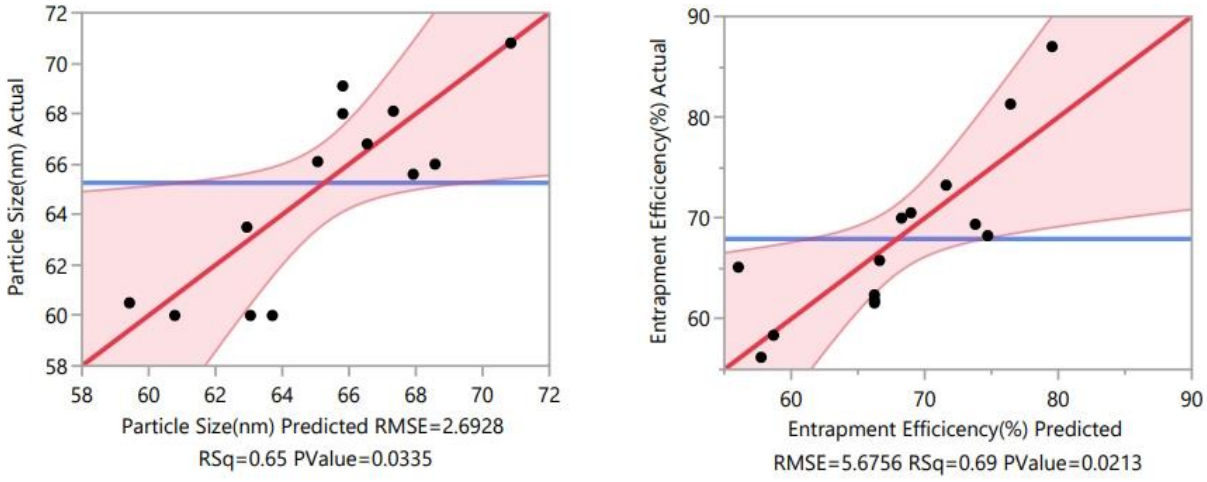

Fig.1 Predicted vs. actual plot for the responses.

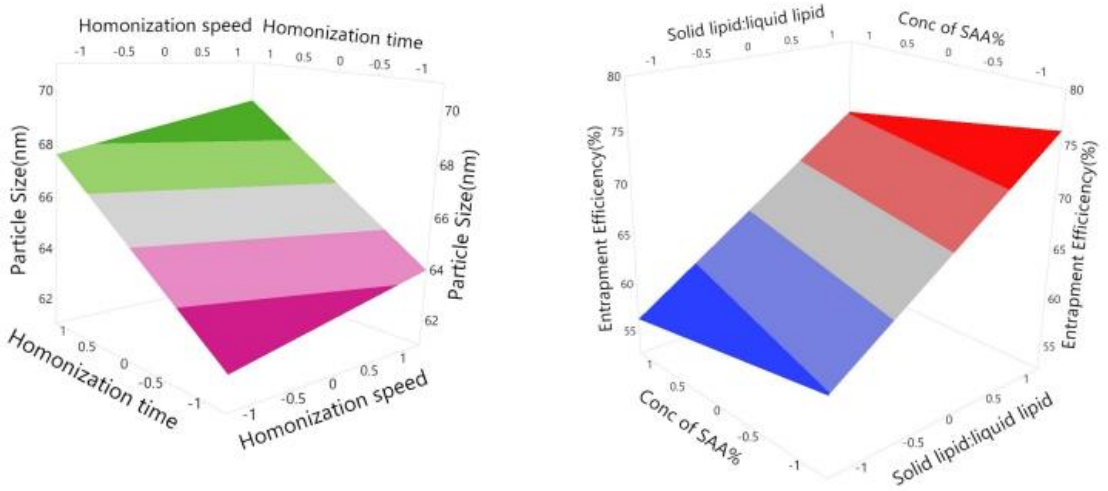

Fig.2 3D surface plots of the responses with the factors.

Mathematical optimization was carried out with a desirability factor set above 0.5. The optimization predicted a formulation with coded values for lipid ratio, surfactant concentration, homogenization time, and homogenization speed of 1:0:1:0, yielding a desirability of 0.568, as shown in Fig. 3. The prediction estimated a particle size of 66.19 nm and a drug entrapment percentage of 70.28%. The optimized NLCs were formulated according to these predicted conditions and analyzed for their responses. The formulation Fopt exhibited a particle size of 65.57 ± 11.3 nm and a drug entrapment percentage of 66.82 ± 0.89%. The percentage bias between the predicted and observed responses was less than 5%.

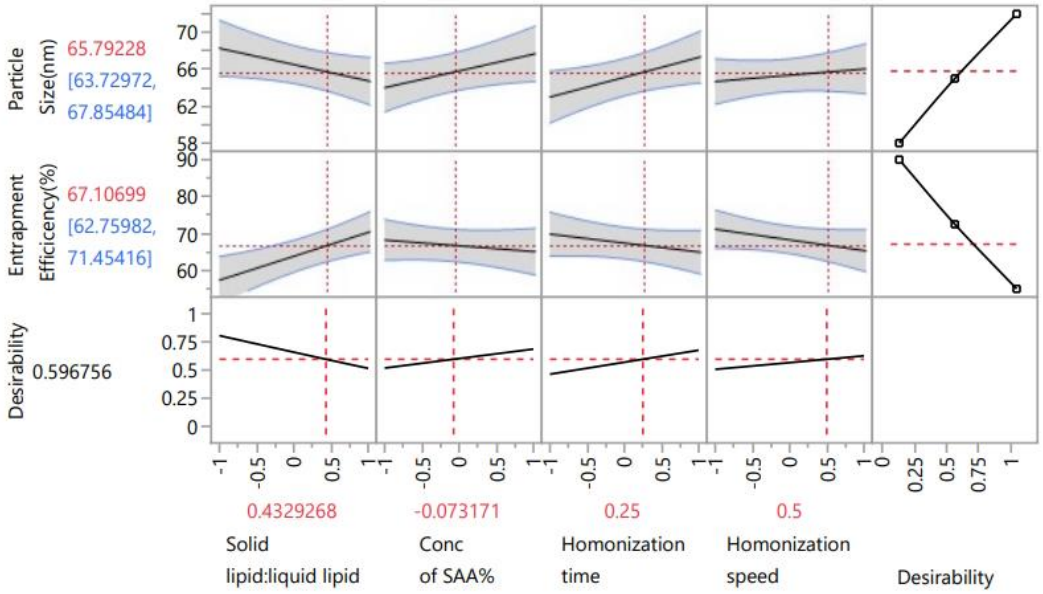

Fig.3 Prediction profiler for the custom design

4.2 PXRD

The PXRD patterns of the pure drug and Fopt are shown in Fig. 4. The pure drug exhibited high-intensity characteristic peaks at 2θ values of 6.11°, 19.36°, 21.53°, 23.18°, and 36.67°. In the formulation, the intensity of these peaks was reduced.

4.3 DSC

The thermogram of the pure drug bedaquiline showed a characteristic endothermic peak at 180°C. In contrast, the thermogram of the formulation Fopt did not exhibit a peak at the same temperature, as shown in Fig. 4.

4.4 SEM

The SEM images of the pure drug and Fopt are shown in Fig. 5. Surface characterization was performed at different magnifications, revealing the morphology of both the pure drug and the formulation.

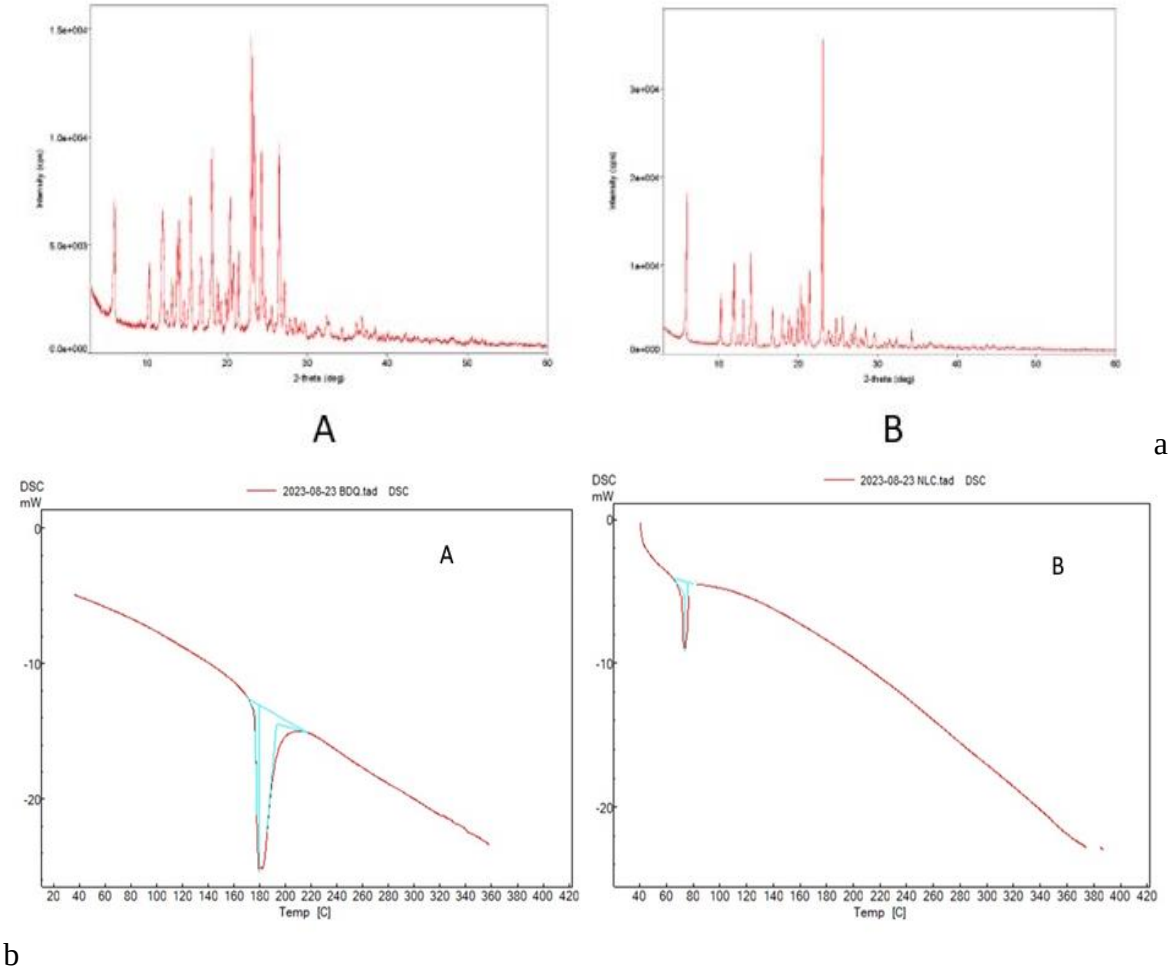

Fig.4 PXRD (a) and DSC thermogram (b) for pure drug: A Fopt: B

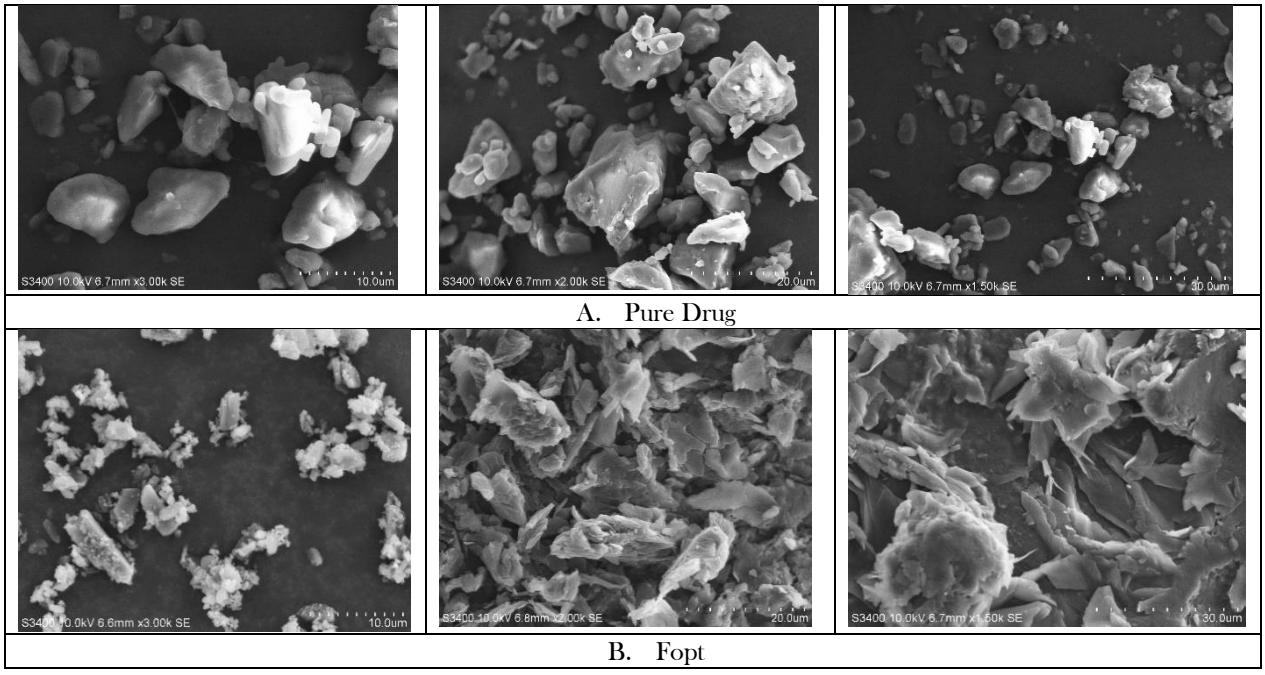

Fig.5 SEM image of pure drug (A) SEM image of Fopt (B)

4.5 In vitro drug release study

The in vitro release study was conducted over 24 hours, revealing a biphasic release profile of the drug from the nanocarriers, as shown in Fig. 6. Initially, a rapid release of the drug was observed, followed by a sustained release phase from the NLCs.

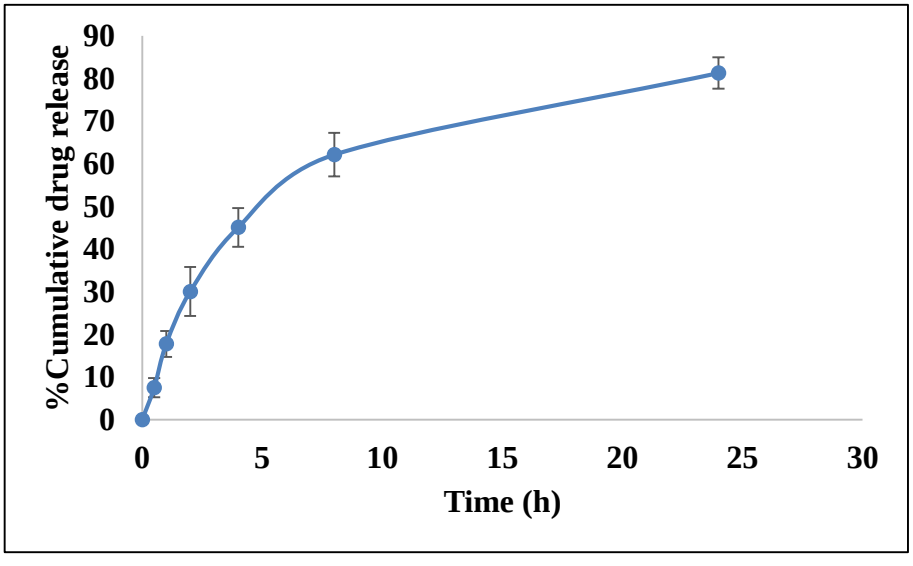

Fig.6 In vitro drug release study of Fopt (mean(n=3) ± SD)

Various kinetic models were explored for the in vitro release pattern. It was found that the regression coefficient (R^2) was highest for the Higuchi release model (R^2 = 0.999), and the release also followed non-Fickian diffusion according to the Korsmeyer-Peppas model (release exponent n = 0.744).

4.6 Ex vivo permeation study

The ex vivo permeation study was performed using the non-inverted intestinal sac method. The optimized formulation (Fopt) demonstrated 3.7 times greater potential for diffusion and permeation compared to the pure drug, as shown in Fig. 7.

4.7 Antimicrobial Study

The formulation was evaluated for antibacterial activity. The formulations proved to be effective, with significant antibacterial efficacy. The minimum inhibitory concentration (MIC) for Mycobacterium tuberculosis (Vaccine strain, H37RV strain) was found to be 1.6 µg/mL for formulation Fopt and 0.08 µg/mL for bedaquiline. After 48 hours of incubation, the colony-forming units (CFUs) for the pure drug and Fopt were found to be 35 ± 4.5 and 10 ± 2.5, respectively. A significant reduction in CFUs was observed at the MIC concentrations of the formulation compared to the pure drug.

4.8 In silico prediction of oral absorption of bedaquiline NLCs

The ADMET predictor revealed low penetration of the formulation through the blood-brain barrier (BBB), 97% inhibition of P-glycoprotein (Pgp) and breast cancer resistance protein (BCRP) transporters, and the likelihood of drug absorption in various gastrointestinal compartments, as shown in Fig. 8. The predicted outcome supports enhanced absorption of the drug through the intestine. The absorption rate in the Advanced Compartmental Absorption and Transit (ACAT) model was calculated using Log D. The predicted bioavailability (F) and pharmacokinetic parameters are presented in Table 6. The predicted plasma concentration-time graph, shown in Fig. 9, indicates very low hepatic and biliary clearance.

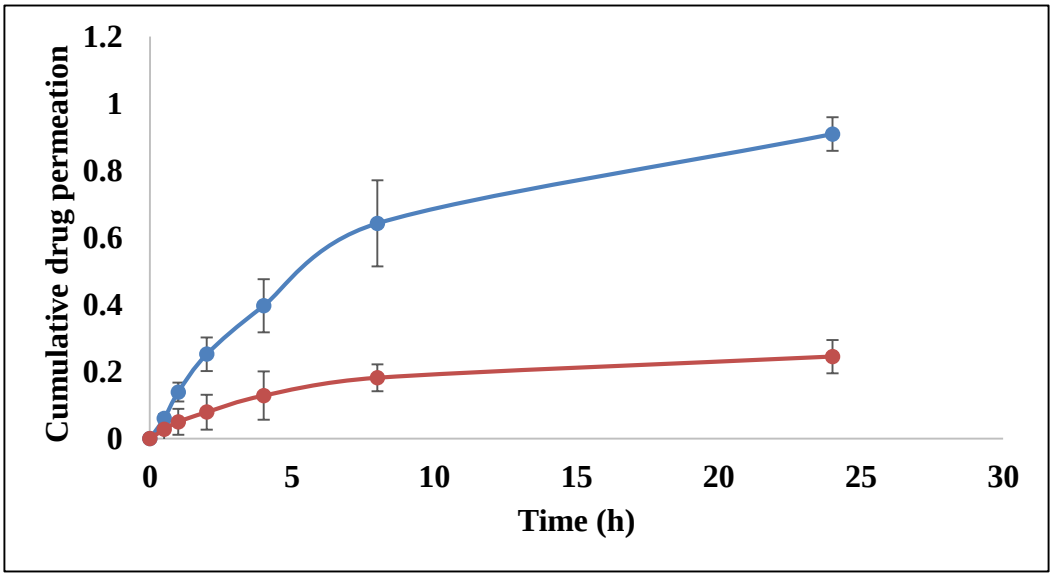

Fig.7 Ex vivo permeation study (mean(n=3) ± SD)

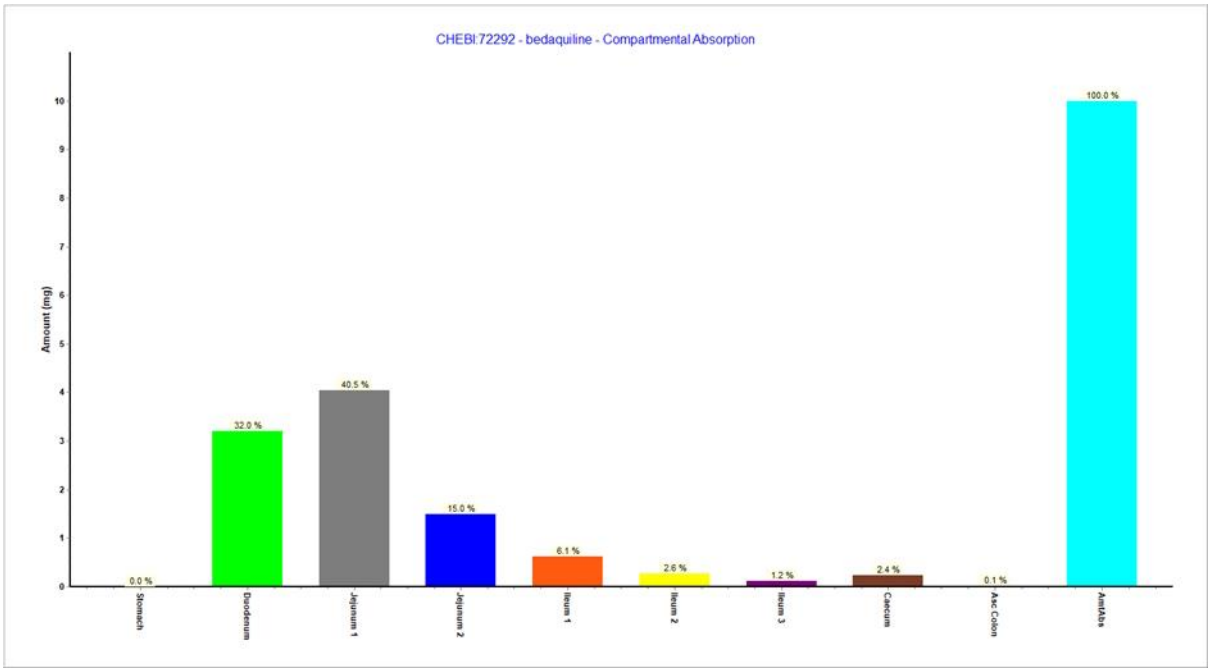

Fig.8 GastroPlus® prediction of drug absorption from Fopt in different GI compartments.

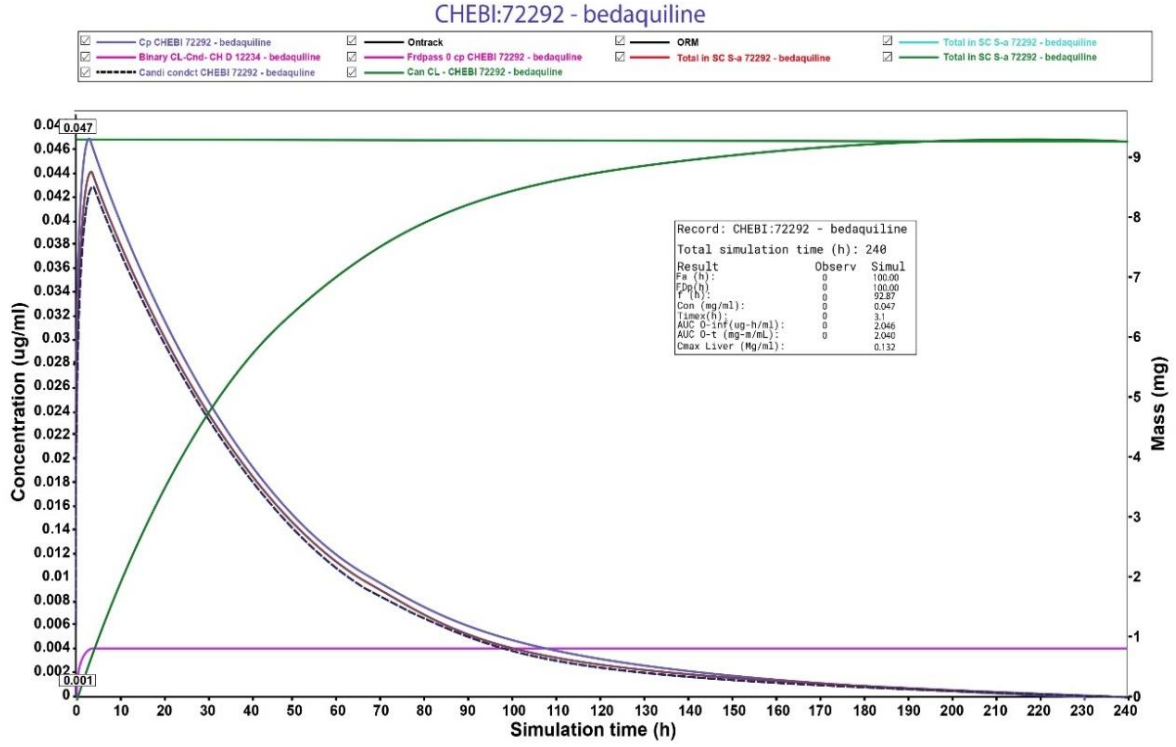

Fig.9 GastroPlus® prediction of plasma concentration of Fopt following oral administration

Table 6 Predicted bioavailability (F) and pharmacokinetics parameters

Parameters	**Predicted**	**CV%**	**Reported data**[28]
Bioavailability (F%)	92.869	1.57	*15.1*
Cmax (µg/ml)	0.468	16.079	0.24
Tmax (h)	3.1	27.202	4.3
AUC $_{0\text{-inf}}$ (µg-h/ml)	20.456	32.	43.5

5. DISCUSSION

The solubility of the drug in solid and liquid lipids is a crucial determinant for the entrapment and successful formulation of nano lipid carriers (NLCs). Lipids were screened to identify those with good affinity for the drug, ensuring high entrapment efficiency in the lipid carriers. Based on the solubility studies, glyceryl behenate and palm oil were selected as the solid and liquid lipids, respectively, for the preparation of bedaquiline-loaded nano carriers.

Bedaquiline belongs to Biopharmaceutical Classification System (BCS) class II, characterized by low solubility, a slow onset of action, and improved oral bioavailability in the presence of fatty food. Thus, the primary focus of this study was to develop a delivery system to enhance the solubility and dissolution of the drug using lipid components. Bedaquiline-loaded NLCs were proposed for this purpose. The quality objectives were to achieve high entrapment of the drug in a nanosized lipid carrier.

A custom design approach was chosen to closely monitor the effect of process and material attributes with the most optimal set of design points to meet the experimental needs for the preparation of NLCs of bedaquiline. This design generated a minimum number of trial runs, and three center points were included to avoid bias. Custom design diagnostics, such as D, G, and A efficiency, were found to be less than 100 and were 75.9, 59.2, and 72.93, respectively. These values satisfied the evaluation of the design for the diagnostics of the factors and the 14 experimental runs. The factor sensitivity analysis and response surface diagrams indicated that homogenization speed and time had a significant effect on particle size, whereas the formulation components, such as lipid ratio and surfactant concentration, had a substantial impact on drug entrapment. The optimized product exhibited less than 5% bias from the design prediction for both drug entrapment and particle size. Therefore, it can be concluded that the custom design approach can be successfully employed for the formulation of lipid nano carriers of bedaquiline and can yield nanosized formulations with high entrapment efficiency.

The PXRD pattern of the pure drug revealed its crystalline nature, with the peak intensity drastically reduced in the formulation Fopt [24]. This reduction in peak intensity might be due to the molecular dispersion of the drug in the solid and liquid lipids.

The broad endothermic peak at 180 ℃ in the DSC thermogram represents the melting point of the pure drug. The optimized formulation did not show the peak at the same temperature, indicating that the drug was completely dispersed in the lipids. The additional peak at 70 ℃ in the optimized formulation might indicate the presence of free lipids in the sample [30].

The surface morphology of the pure drug revealed its crystalline structure with angular-shaped flakes. The nanoparticles were discrete, with minimal conglomeration, and were in the nanometric range. The surfaces of the NLCs appeared irregular and rough.

The in vitro drug release study exhibited a biphasic release pattern. In the initial hours, drug release was rapid due to the displacement of the drug from the irregular outer surface of the NLCs, followed by a consistent release from the core of the lipid matrix [12]. The lipophilic nature of bedaquiline and its tight binding to the core of the NLCs

might be responsible for the consistent release of the drug. The rate kinetic of the release study indicated a case II transport mechanism, supporting the preliminary burst release from the outer crust, accompanied by diffusion through the inner core. Hence, it can be concluded that the initial accelerated and subsequent passive release of the drug from the carriers may offer promising results for better efficacy and improved oral bioavailability.

From the ex vivo study, it was found that the apparent permeability of the optimized formulation was 3.7 times greater than that of the pure drug. The nanosized formulation facilitated drug dissolution and permeation. The enhancement in permeation might be due to the availability of the solubilized drug at the site of absorption. The pure drug exhibited lower permeation due to its poor solubility.

The antimicrobial study demonstrated the efficacy of the nano lipid carrier of bedaquiline in controlling bacterial growth compared to the pure drug. The presence of lipids might have favored drug penetration through the bacterial cell wall [31], resulting in a reduction in the number of Mycobacterium colonies.

Bedaquiline exhibits poor oral bioavailability, a slow onset of action, and extensive hepatic metabolism, as reported in the literature [6]. The absorption of the drug is dissolution-rate limited. The absorption of the drug increases in the presence of lipids [6]. Therefore, the present study explored the in silico evaluation of NLCs of bedaquiline to overcome these limitations.

The ACAT absorption model was used to simulate the oral absorption of bedaquiline NLCs from the intestinal tract. The effect of bile salts on the dissolution of the drug was considered for each region of the gastrointestinal tract [32]. This effect was compared with the in vitro and ex vivo solubility and diffusion, respectively, in biorelevant media. It was predicted that the NLCs of bedaquiline might improve oral bioavailability by approximately 92% and overcome P-gp efflux transport. The time to reach maximum plasma concentration (tmax) was predicted to be significantly shorter compared to the pure drug. The pharmacokinetics (Cmax, AUC0-inf) of the drug were also found to be improved in the ACAT model for oral absorption in humans. The enhancement in pharmacokinetics and bioavailability might provide a better and safer pharmacotherapy for the treatment of TB.

Therefore, the overall study established that a nano lipid carrier of bedaquiline might ensure a safer and more effective regimen for the treatment of TB.

6. CONCLUSION

The present study aims to address the drawbacks associated with the oral administration of bedaquiline. A biocompatible platform for the drug was created using solid and liquid lipids. A statistical design was applied to develop nano-sized lipid carriers of bedaquiline using glyceryl behenate and palm oil. The optimized product demonstrated improved drug release and permeation through in vitro and ex vivo studies compared to the pure drug. The antimicrobial efficacy was significantly enhanced. The in-silico simulation for oral absorption showed promising results. The prediction indicated that both bioavailability and tmax could be improved significantly, while hepatic metabolism could be controlled to enhance the overall pharmacokinetics of the drug. Hence, the proposed approach can be explored in animal models for further confirmation of the outcomes and subsequent research on TB. It can be concluded that nano lipid carriers of bedaquiline offer a novel platform to overcome the challenges associated with its oral administration.

Abbreviations

TB	Tuberculosis
BCS	Biopharmaceutical classification system
NLCs	Nano lipid carriers
ACAT	Advanced Compartmental Absorption Transit
PEG	Polyethylene glycol

Vit E	Vitamin E
DLS	Dynamic light Scattering
TD	Total Drug
FD	Free Drug
SD	Standard deviation
Fopt	Optimized formulation
PXRD	Powder X ray diffraction study
DSC	Differential Scanning Calorimetry
SEM	Scanning electron microscopy
MABA	Microplate Alamar Blue assay
MIC	Minimum inhibitory concentration
CFU	Colony form units
R^2	Regression coefficient
GI	Gastrointestinal
ADMET	Absorption, distribution, metabolism, excretion and toxicity
ACAT	Advanced compartmental absorption and transport model

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Author contributions

SB was involved in the conceptualization, design, supervision, and analysis of the research outcomes. NR was involved in data generation and analysis. Both authors contributed to the preparation of the manuscript.

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Availability of data and materials

All data are presented in the manuscript. Queries regarding the data can be addressed to the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data supporting the conclusions are included within the article.

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تحضير وتقييم حاملات الدهون النانوية للبيداكويلين – التقييم في المختبر والتنبؤ بالسيليكو

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ملخص

الخلفية: يعاني البيداكيلين، وهو عقار قوي مضاد للسل، يستخدم في علاج السلالات المقاومة للأدوية المتعددة، من انخفاض التوافر البيولوجي عن طريق الفم، وبطء بداية التأثير العلاجي، وآثار جانبية. يقترح البحث تطوير ناقلات نانوية للدواء من خلال تحسين إطلاق الدواء وتقدير تأثيره في الامتصاص عن طريق الفم من خلال نموذج السيليكو. في البداية، تم دراسة تصميم مخصص لتقدير التركيب وتأثيرات العملية على الانحباس وحجم الجسيمات للناقلات. خضعت الناقلات النانوية لدراسة خصائص السطح، وتشكل السطح، والحرارة، وإطلاق الدواء، والنفاذ خارج الجسم الحي، ودراسة فعالية مضادات الميكروبات. في التنبؤ السيليكو بشأن التوافر البيولوجي ومعلمات الحرائك الدوائية للتركيبة المُحسّنة، تم تنفيذها من خلال برنامج GastroPlus®.

النتائج: كشفت الدراسة أن البيداكويلين المحصور في ناقلات الدهون النانوية (65.5 نانومتر) من غليسيريل بيهينات وزيت النخيل زاد بشكل فعال من معدل إطلاق الدواء بأكثر من 80٪، وأدى إلى تكثيف النشاط المضاد للميكروبات ضد المتفطرة السلية بمقدار 3.5 مرة. تم تعزيز تخلل الأمعاء بمقدار 3.7 مرة. تم توسيع النتائج التي تم الحصول عليها للتنبؤ بالتوافر الحيوي عن طريق الفم من خلال برنامج GastroPlus® وكشفت عن حقيقة أن حامل الدهون النانوية للبيداكويلين يمكن أن يكون طريقة واعدة لتحسين فعالية الدواء مع مزيد من التوطين في الأجزاء المعدية المعوية، وتحسين الحرائك الدوائية بنسبة 93٪. التوافر البيولوجي. الاستنتاج ومن ثم يمكن أن نستنتج أن الناقلات النانوية للبيداكويلين في المصفوفة الدهنية يمكن أن تكون بمثابة أداة فعالة لتعزيز فعالية البيداكويلين في علاج مرض السل.

الكلمات الدالة: حاملات الدهون النانوية، بيداكيلين، التوافر الحيوي، التخلل، في تنبؤ السيليكو.

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Analytical Approaches for Assessing Curcumin and Nicotinamide Co-Encapsulated in Liposomal Formulation: UV Spectrophotometry and HPLC Validation

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ABSTRACT

Background: The study presents two distinct analytical methods tailored for the precise determination of curcumin (CUR) and nicotinamide (NIC) within liposomal formulations, addressing the needs of researchers and analysts in the biomedical and food supplement sectors.

Method: UV spectrophotometry provides a swift and cost-effective solution for quantification, while High-Performance Liquid Chromatography (HPLC) offers enhanced specificity and sensitivity, particularly in complex matrices. Method validation, especially for HPLC, ensures reliability and suitability for rigorous analysis, advancing the field of Analytical Chemistry and strengthening development and quality assurance processes in the pharmaceutical and biotechnology industries.

Results: The encapsulation efficiencies of CUR and NIC into liposomes, primarily composed of DPPC and CHO, were found to be 30% ± 6% and 80% ± 5%, respectively. The developed analytical methods using UV spectrophotometry and reverse-phase HPLC demonstrated robustness and efficiency, allowing for the simultaneous analysis of CUR and NIC with high specificity, accuracy, and precision. Validation according to ICH Q2 guidelines revealed excellent system suitability, linearity, and robustness, with relative standard deviation consistently below 2%. Stability studies over three weeks at 4°C showed minimal changes in liposomal characteristics, indicating good stability. Furthermore, release studies at 37°C demonstrated enhanced solubility and increased release of curcumin, suggesting the potential of the liposomal formulation for drug delivery applications.

Conclusion: This study developed straightforward, time-efficient, and cost-effective analytical methods using UV spectrophotometry and reverse-phase HPLC to quantify CUR and NIC encapsulated in liposomal formulations.

Keywords: Liposomes, Analytical Methods, UV Spectrophotometric, HPLC.

1. INTRODUCTION

Natural compounds offer numerous advantages in the quest for novel antioxidants, anti-inflammatory agents, anticancer agents, and antimicrobials (1-4). They often exhibit unique mechanisms of action that can selectively target specific pathways involved in oxidation, cell growth, and immunomodulation. Moreover, natural compounds hold promise for overcoming drug resistance, a common challenge encountered in many diseases (5, 6).

Curcumin (Figure 1A), a polyphenol present in turmeric, has demonstrated promising effects across various biological systems, modulating multiple signaling pathways implicated in general health (7). On the other hand, CUR has shown antibacterial activity against both Gram-negative and Gram-positive bacteria in many studies. CUR disrupts bacterial membranes and inhibits bacterial biofilm formation, which leads to oxidative stress (8, 9).

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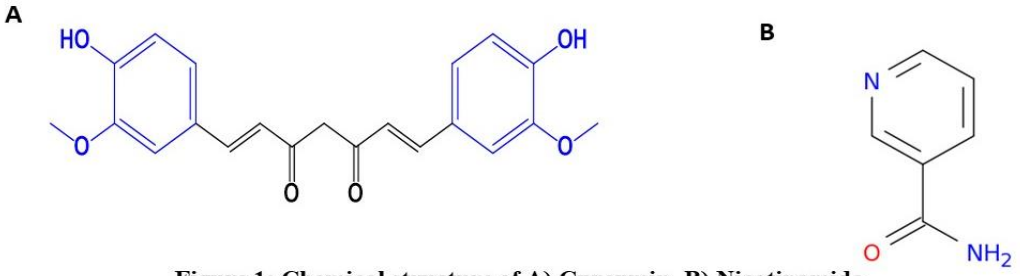


Figure 1: Chemical structure of A) Curcumin, B) Nicotinamide

Nicotinamide (Figure 1B), a derivative of Vitamin B3, has displayed notable potential in treating infectious diseases such as acne. NIC has shown promise as an adjuvant therapy when combined with other agents, enhancing their effectiveness while mitigating adverse effects (10). Its role in cellular metabolism is crucial, as it is involved in maintaining genomic stability and facilitating DNA repair processes (11-14). Nicotinamide has also demonstrated the ability to enhance the effectiveness of conventional cancer therapies, including radiation and chemotherapy. By sensitizing cancer cells to the damaging effects of these treatments, it makes them more vulnerable to therapy, potentially allowing for reduced doses (15). Additionally, NIC has the capacity to modulate the energy metabolism of cancer cells, targeting their altered metabolic pathways. NIC disrupts these metabolic adaptations, leading to energy depletion and increased susceptibility to treatment (16). Consequently, in this study, these two bioactive compounds (CUR and NIC) were combined in a liposomal nanoparticle formulation as immune-modulating supplements.

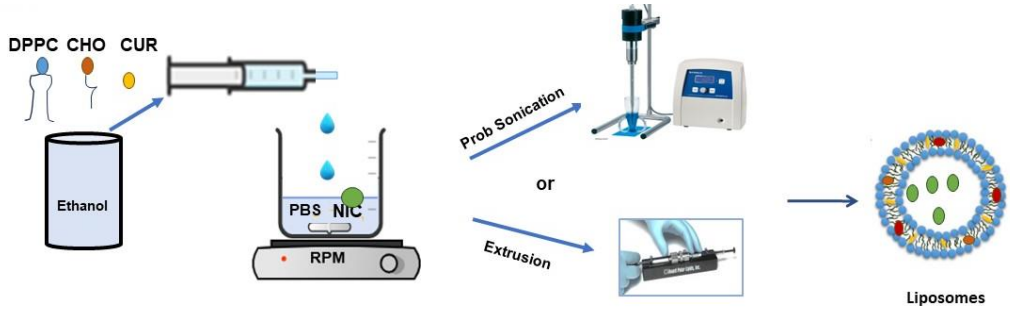


Figure 2: Schematic representation of CUR-NIC preparation by ethanol injection method

Liposomes are lipid-based vesicles that serve as carriers for both hydrophilic and hydrophobic drugs, enabling targeted delivery of therapeutic agents to specific anatomical sites. Their unique structure allows for the encapsulation of both water-soluble and lipid-soluble drugs, thereby enhancing solubility and stability (17, 18). Moreover, liposomes can protect drugs from premature degradation and metabolism, minimizing systemic toxicity and optimizing the therapeutic index (Joy et al., 2022) (19). Additionally, liposomal formulations offer the potential for incorporating targeting ligands on their surface, enabling active recognition of cancer cells. These

ligands can specifically bind to receptors overexpressed on cancer cells, promoting the internalization of liposomes and improving drug delivery to the tumor site (20).

This study aims to develop and validate fast and consistent UV-spectrophotometry and HPLC methods for the simultaneous quantification of CUR and NIC in liposome suspensions, in accordance with current official ICH guidelines. This is the first study to encapsulate a combination of the natural compounds NIC and CUR inside a liposomal delivery system to enhance their promising antioxidant and anti-inflammatory therapeutic activities. Accurate and validated analytical methods are essential for the consistent and precise quantification of encapsulated drugs in liposomal formulations. To date, no method has been reported that simultaneously quantifies these two bioactive compounds in supplement formulations.

2. MATERIALS AND METHODS

2.1. Materials:

CUR was sourced from ICT (Japan), while NIC was acquired from Sigma-Aldrich (USA). 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was obtained from Avanti Polar Lipids, Inc. (Alabaster, Alabama, USA), and cholesterol (CHO) was procured from Carbosynth (UK). Phosphate Buffer Saline (PBS) was purchased from LONZA® (USA). HPLC-grade methanol was obtained from Sigma-Aldrich (USA), whereas HPLC-grade ethanol and methanol were sourced from the Carbon Group (England). All other chemicals and solvents used were of high analytical purity, and no further treatment was conducted on any of the reagents or chemicals.

2.2. Liposomes Preparation

Liposomes were prepared using the conventional ethanol injection method. Specifically, 4 mg of DPPC, 1.0 mg of CHO, and 1.0 mg of CUR were dissolved in 0.3 mL of ethanol, which was then warmed to 40°C in a water bath. Meanwhile, NIC was dissolved in phosphate-buffered saline (PBS) and heated on a hot plate at approximately 50°C for 14 minutes with continuous stirring at 700 rpm. Subsequently, the warm ethanol solution containing lipids and CUR was swiftly injected into the NIC-containing PBS under continuous stirring and heating conditions.

The resulting liposomal suspension underwent sequential extrusion, if necessary, through a polycarbonate membrane to achieve the desired size of 100 nm, using a Mini-Extruder (Avanti Polar Lipids, Inc., USA) at a temperature of 50°C. Following extrusion, free drug molecules were removed by washing the liposomes twice with PBS solution using a dialysis bag. Finally, the liposomes were stored at 4°C to maintain stability (19).

2.2. Liposomes Characterization

2.2.1. Hydrodynamic diameter, zeta potential and polydispersity Index of liposomes.

The average particle size, zeta potential (charge), and polydispersity index (PDI) of the liposomes were determined through dynamic light scattering (DLS) experiments using a Zetasizer Nano-ZS (Malvern Instruments Ltd., Malvern, UK). Liposomal samples were appropriately diluted with deionized water (10:990 µL, v/v) to achieve a suitable counting rate. Before each measurement, all samples were equilibrated at room temperature for 30 seconds in the specimen holder of the Zetasizer. This ensured consistent and accurate measurements of particle size, zeta potential, and PDI, providing valuable insights into the characteristics and stability of the liposomal formulations (21).

2.2.2. *In vitro* Release and Stability of prepared liposomes:

The stability of the liposomes was evaluated over a storage period of 4 weeks at 4°C. At regular intervals, liposome samples (50 µL) were collected, and the mean hydrodynamic diameter and zeta potential of the loaded liposomes were determined using the previously described DLS method. All liposome samples were appropriately diluted in deionized water before measurements to ensure accurate results.

To assess the release profile of encapsulated CUR and NIC, liposome suspensions were placed in dialysis bags (molecular weight cutoff 10 kDa, Thermo Fisher Scientific) containing 1 mL PBS. These dialysis bags were then suspended in a release volume of 20 mL PBS at pH 7.4 and maintained at 37°C. At regular intervals, 200 µL of the release medium was collected for HPLC analysis, with an equivalent volume of fresh PBS buffer at the same temperature immediately added to maintain a constant release volume. Consistency in the length of the dialysis tubing was maintained across all experiments to ensure a uniform surface area for dialysis. This comprehensive approach allowed for the monitoring of liposomal stability and the assessment of drug release kinetics over the specified storage period.

2.2. Measuring of liposomal Encapsulation Efficiencies:

To assess the encapsulation efficiencies (EE%) of CUR and NIC in the liposomal formulations, 200 µL of the liposome suspension was mixed with 800 µL of methanol to disrupt the liposomes. The resulting solution was vortexed for 3 minutes and then centrifuged at 15,300 rpm for 15 minutes. This process was conducted in triplicate, and the results were recorded as the mean ± standard deviation (SD).

$$Encapsulation\ Efficiency\ (EE\%) = \frac{\text{Actual Ammount of Drug Loaded}}{\text{Total Theoritical Amount of Drug Used}}\ X\ 100\ \% \quad (1)$$

$$Loading\ efficeiency\ (EE\%) = \frac{\text{Actual ammount of drug loaded}}{\text{Total lipid and drug used}}\ X\ 100\ \% \quad (2)$$

Where:

The total amount of drug in liposomes refers to the amount of drug encapsulated within the liposomes, while the amount of free drug is the drug remaining in the supernatant after centrifugation. The total amount of drug added is the initial amount of drug introduced during liposome preparation. This methodology ensured accurate quantification of drug encapsulation within the liposomal formulations, providing insights into their efficacy and performance.

2.3. Standard solution UV system and HPLC:

Two stock solutions were prepared for CUR and NIC. The first stock standard solution of CUR (2.0 mg/mL) was prepared by dissolving 2.0 mg of CUR in 2.0 mL of methanol. The second stock standard solution of NIC (1.0 mg/mL) was prepared by dissolving 1.0 mg of NIC in 1.0 mL of either ethanol or methanol. Both solutions were subjected to ultrasonication in a water bath for 10 minutes to ensure complete dissolution. Subsequently, calibration curves were established using eight standard solutions with increasing concentrations of CUR and NIC for the linearity assay. Prior to analysis, all solutions were filtered through a 0.45 µm cellulose membrane and then injected into the HPLC system, with each measurement performed in triplicate (n=3).

This rigorous preparation and analysis protocol ensured the accuracy and reliability of the HPLC method for quantifying CUR and NIC concentrations, facilitating the establishment of robust calibration curves for subsequent sample analysis.

2.4. HPLC Conditions and parameters:

The analysis was conducted using a Shimadzu LC-2030 HPLC system equipped with a UV detector (Shimadzu Corporation, Kyoto, Japan). A Thermo Scientific™ Hypersil™ C18 HPLC column, with dimensions of 4.6 × 150 mm and a particle size of 5 µm, featuring a 100 Å pore size, was employed. The mobile phase consisted of methanol and water (80:20, v/v) with a pH of 3. Chromatographic data acquisition and integration were performed using Laboratory Solutions version 5.92. Prior to use, the mobile phase was filtered through a nylon Millipore membrane filter with a pore size of 0.2 µm and degassed to remove any trapped air. The mobile phase was pumped at a flow rate of 1.0 mL/min, and the injection

volume was set to 20 µL. The analytical column temperature was maintained at 40°C throughout the analysis. The chromatographic run time was set to 10 minutes, during which CUR and NIC were detected at a wavelength of 262 nm using the UV detector. This comprehensive setup ensured precise separation and quantification of CUR and NIC in the analyzed samples, providing accurate and reliable results for further data interpretation and analysis.

2.5. Validation of the method

The method was validated according to the International Conference on Harmonization (ICH) guidelines regarding the following parameters: specificity, linearity, detection and quantification limits, precision, and accuracy. System suitability parameters, such as the theoretical plate number, tailing factor, and resolution between CUR and NIC peaks, were evaluated.

2.5.1. Selectivity and Specificity

Analytical method specificity was determined by evaluating the blank and unloaded liposomes compared to CUR and NIC-loaded liposomes at the concentrations used in the working standard. The samples were analyzed under the same chromatographic conditions to verify the absence of interference or overlaps from excipients, phospholipids, and sucrose. The method was validated according to ICH guidelines.

2.5.2. Calibration curve and linearity range:

Six standard solutions of CUR and NIC were prepared as detailed in Table 1. Three independent calibration curves were established, and linearity was evaluated by least-squares regression analysis. The lower limit of detection (LOD) and the lower limit of quantification (LOQ) were determined from the calibration plot based on the following equations:

$$LOD = \frac{3.3\,\sigma}{S} \quad (3)$$

$$LOQ = \frac{10\sigma}{S} \quad (4)$$

where σ is the Standard Deviation of the Intercept and S is the Slope of the Calibration Plot (22) .

Table 1: System suitability results for CUR and NIC.

Parameters	Value		Limits
	CUR	NIC	
Number of theoretical plates	2200	2300	> 2000
Tailing factor	0.938	0.907	< 2

2.5.3. Assessment of method Precision

Intermediate precision, also known as inter-day precision, was evaluated by measuring samples with the same concentrations—250 µg/mL for CUR and 250 µg/mL for NIC—on three different days by two different analysts. The results were expressed as the relative standard deviation (RSD%). Repeatability, also referred to as intra-day precision, was assessed by measuring six sample solutions, each in triplicate, at the same concentrations on a single day under identical experimental conditions. The results were also expressed as RSD%. These precision studies were essential for evaluating the consistency and reliability of the analytical method across different days and analysts, as well as within the same day, providing valuable insights into the robustness and reproducibility of the HPLC method for quantifying CUR and NIC concentrations.

2.5.4. Assessment of method Accuracy

Accuracy was assessed by triplicate assays of samples with known liposome concentrations spiked with three different concentrations of CUR and NIC, as well as standard solutions at four different levels (50%, 100%, and 150%). Recovery (%) was calculated based on the differences between the measured concentration of the spiked solutions

and the expected concentration, with results expressed as the RSD% of the triplicate measurements. This accuracy evaluation was crucial for determining the reliability and correctness of the analytical method in quantifying CUR and NIC concentrations in liposomal formulations across a range of spiked concentrations.

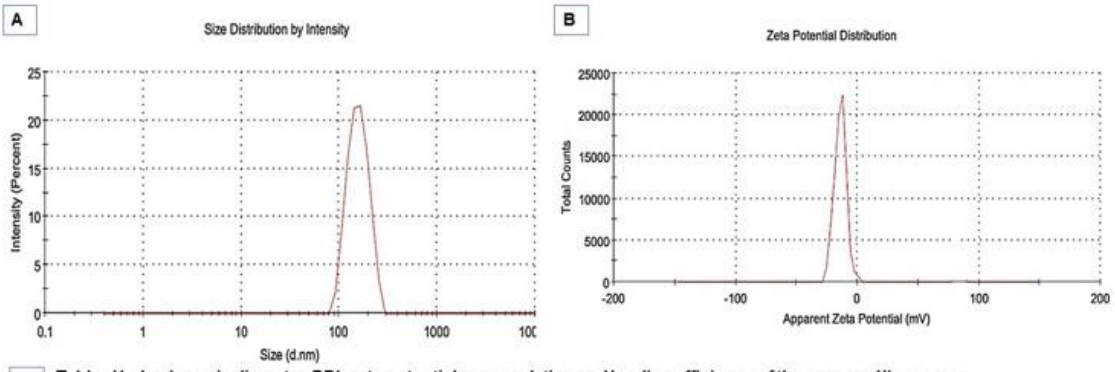
2.5.5. Assessment of method Robustness

Method Robustness was examined by measuring the sample under various method conditions to assess the impact of each variation.

3. RESULT AND DISCUSSIONS

3.1. Liposomes Characterization

In the current study, CUR and NIC were encapsulated into a liposomal formulation composed mainly of DPPC and CHO. The CUR and NIC liposomes exhibited an average particle size of 158.5 ± 6.1 nm and a polydispersity index (PDI) of less than 0.2. The zeta potential of the prepared liposomes was −16.6 ± 0.74 mV. The encapsulation efficiencies were found to be 30 ± 0.82% for CUR and 80.10 ± 0.39% for NIC, which are considered promising and high, especially for hydrophobic drugs encapsulated into the liposomal bilayer simultaneously. The experiments were conducted to develop a quick and effective method to simultaneously analyze CUR and NIC using both UV-spectrophotometry and HPLC with a UV detector (Figure 3A, 3B, 3C).



C Table : Hydrodynamic diameter, PDI, zeta potential encapsulation and loading efficiency of the prepared liposomes.

Liposomes (n=3)	Hydrodynamic diameter (nm ± SD)	Polydispersity index (PDI ± SD)	ζ-potential (mV ± SD)	Encapsulation efficiency %	Loading efficiency %
Lipo-NC-CUR	155.6±10.3	0.086±0.5	-22±5	NC: 80% CUR: 30%	NC:16% CUR: 5%

Figure 3: liposomes DLS characterization of CUR-NIC mixture (A) Size distribution by intensity (B) Apparent zeta potential (C) DLS measurements and encapsulation and loading efficiencies.

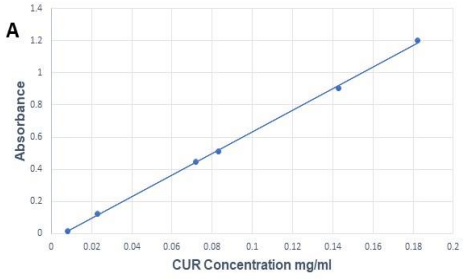
The chromatographic method employed utilizes a mobile phase consisting of methanol and water (pH 3) in an 80:20 v/v ratio, yielding symmetric peaks with retention times of 2.796 and 4.644 minutes for CUR and NIC, respectively (see Figure). These conditions facilitate swift and routine analyses. The selected chromatographic parameters include a column temperature of 40 ± 2°C, a 20 µL sample injection volume, an isocratic flow rate of 1.0 mL/min, a detection wavelength of 262 nm, and a 10-minute run time. These conditions have been deemed suitable for further procedures, including method validation.

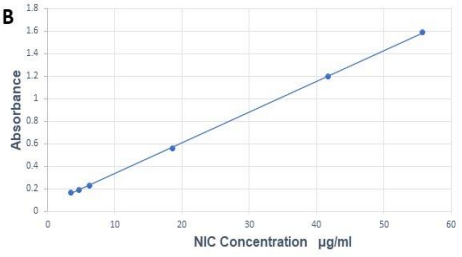
Furthermore, the chromatogram of blank liposomes shows no peaks that might interfere with the retention times of CUR and NIC, indicating that the liposome constituents do not interfere with the quantification of these drugs. This confirms the method's reliability (Figure 4A, 4B, 4C, 4D).

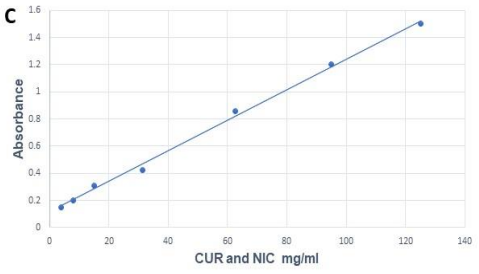
3.1.1. Validation of HPLC Method/ Limit Of Detection and Limit of Quantification:

Validation was performed following ICH Q2 guidelines. The tailing factors for both CUR and NIC peaks remained below 2 in all instances, with the number of theoretical plates exceeding 2,000, indicating the column's efficacy. The validation process included assessments of system suitability, specificity, linearity range, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, and robustness. Repeated analyses consistently placed CUR and NIC retention times at approximately 2.8 and 4.6 minutes, respectively, demonstrating commendable resolution between the two peaks. The relative standard deviation (%RSD) of recorded retention times was consistently below 2%, indicating excellent precision on the HPLC system. Overall, these results reflect the method's robustness and reliability for the simultaneous quantification of CUR and NIC (Table 1).

Linearity was assessed using calibration curves for both CUR and NIC, with both analytes showing an R^2 value of 0.999, indicating a strong correlation between concentration and peak area (Figure 4D). The LOD and LOQ for CUR were 0.0152 µg/mL and 0.0171 µg/mL, respectively. The measured LOD and LOQ values for CUR and NIC were 0.02 µg/mL and 0.025 µg/mL (LOD), and 0.06 µg/mL and 0.075 µg/mL (LOQ), respectively. Notably, no significant differences were observed when comparing the calculated and measured LOQ values on the linear calibration curve ($p < 0.05$) (Figure 4D).







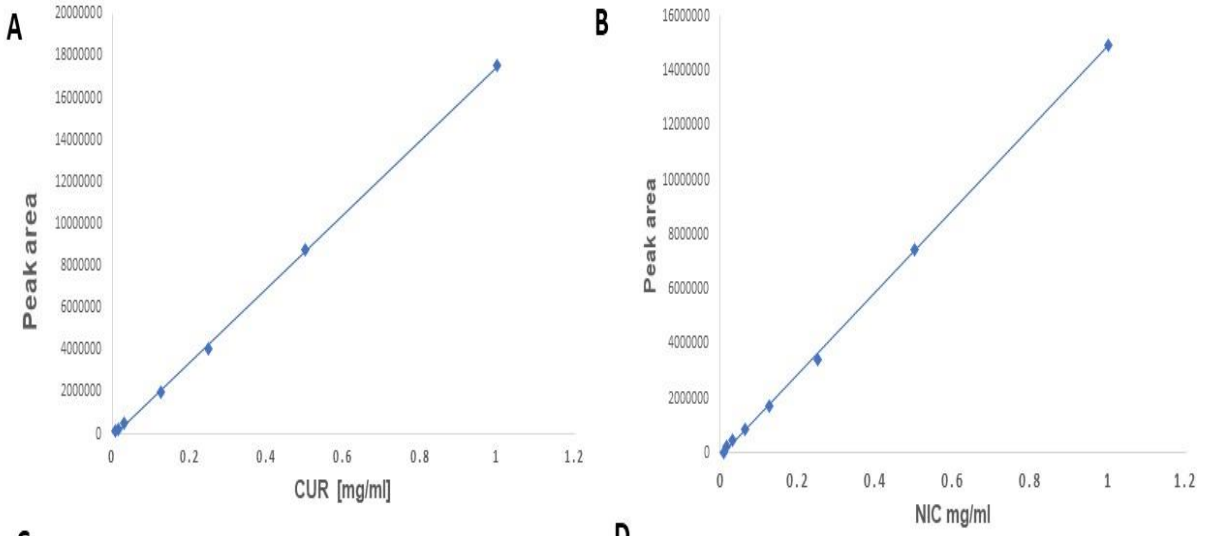
D

Parameter	CUR	NIC	CUR-NIC Ratio1:1
Calibration equation	y = 6.7166x - 0.0384	y = 0.0274x + 0.0599	y = 0.0113x + 0.1153
Determination coefficient (R^2)	R^2 = 0.9993	R^2 = 0.9998	R^2 = 0.997
Linearity range (µg/mL)	4-125 µg/ mL	3.5- 50 µg/ mL	4-125 µg/ mL
Wavelength	262	262	262
Number of points	6	6	6

Figure 4: Calibration curve of A) CUR B) NIC and C) CUR-NIC mixture (1:1) using UV spectrophotometry D) Table of the measuring parameters

3.1.2. Selectivity and Specificity:

In adherence to guidelines, specificity in the developed method is defined as the ability to distinguish the analytes without any interference. The peaks corresponding to CUR and NIC exhibited well-defined separation at distinct retention times, with no interference from additives in the liposomal formulation. This clear separation of peaks indicates the method's specificity and selectivity, underscoring its ability to differentiate CUR and NIC from each other and the lipids in the sample (Figures 5 and 6).



C

Instrument	Optimized chromatographic conditions
Detector	UV Detector (Shimadzu Corporation, Kyoto, Japan)
Column	C18 HPLC Column, 100Å, 5µm, 4.6 x 150mm
Mobile phase	Methanol: water 80:20; v/v (pH= 3)
Flow rate	1.0 mL/ min
Wavelength	262 nm
Temperature	40°C
Retention time	NIC ≈ 2.8 min
	CUR ≈ 4.7 min

D

Parameter	CUR	NIC
Calibration equation	$y = 2*10^7x - 103600$	$y = 2*10^7x - 92189$
Determination coefficient (R^2)	$R^2 = 0.9996$	$R^2 = 0.9996$
Linearity range (µg/mL)	7.813-1000	7.813-1000
Number of points	6	6
LOD (µg/mL)	0.0171	0.0152
LOQ (µg/mL)	0.052	0.046

Figure 5: A) Calibration curve of CUR B) Calibration curve of NIC C) Instrument optimized condition (1:1) using HPLC D) Table of the measuring parameters

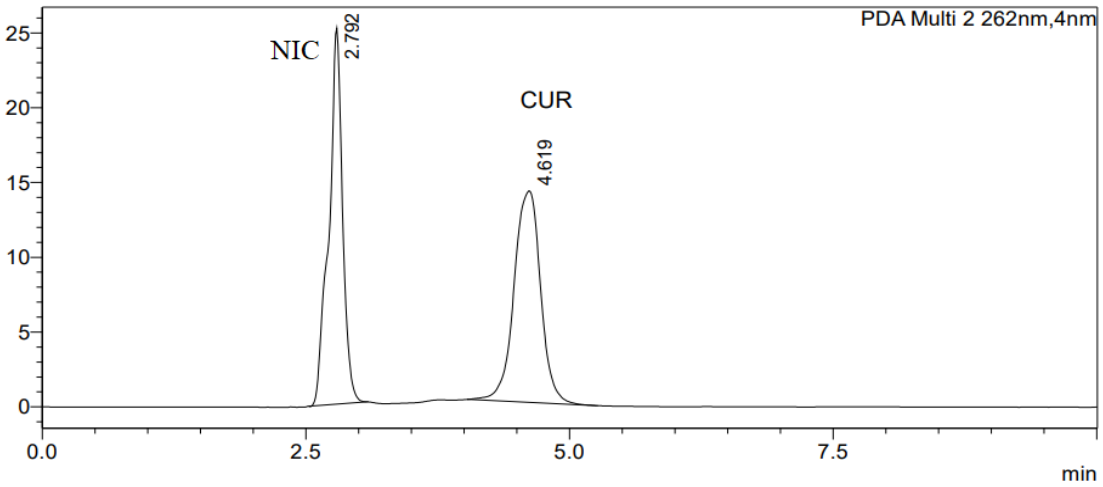


Figure 6: Representative HPLC chromatograms obtained from A) disrupted CUR and NIC loaded liposomes.

3.1.3. Accuracy and Precision:

The accuracy of the developed method was verified by measuring the recovery of standard samples. The recovery rates were acceptable, with %RSD values below 2%, indicating good accuracy. Specifically, the method achieved a recovery of 100.9% for CUR and 99.7% for NIC. These results, along with %RSD values consistently below 2% (Tables 2 and 3), confirm the method's accuracy.

Table 2: Precision study results of CUR and NIC

Sample	Injected concentration (mg/mL)	Intra- day (n=6)		Inter- day (n=6)	
		Measured concentration (mg/mL)	RSD%	Measured concentration (mg/mL)	RSD%
CUR	0.5	0.51	0.70%	0.491	0.7%
NIC	0.5	0.473	0.47%	0.509	0.6%

Table 3: Accuracy study results of CUR and NIC

Injected Sample (µg/mL)		Theoretical Concentration (µg/mL)	Measured concentration (µg/mL)	Recovery %	RSD%
CUR encapsulated into liposomes EE % = 30 %	**Standard**	**30**	**31**	**103%**	**0.24%**
	50%	15	14.06	93.73%	1.05%
	100%	30	28.83	96%	0.24%
	150%	45	40. 85	90.8%	1.5%
NIC encapsulated into liposomes EE % =80 %	**Standard**	80	80.9	101.13%	0.10%
	50%	40	39.2	98%	1.21%
	100%	80	76 ± 3.1	95%	0.11%
	150%	120	118 ± 1.9	92.5%	0.9%

3.1.4. Robustness of the method:

In accordance with recommended guidelines, the robustness of the developed HPLC analytical method was evaluated by assessing its ability to withstand minor variations in the UV wavelength (262 ± 2 nm). The method demonstrated robustness, with recovery results falling within the specified guideline range of 80–120%. Additionally, the relative standard deviation (%RSD) values remained below 2%. These findings indicate that the method is robust, even when subjected to slight variations in analytical conditions (Table 4).

Table 4: Robustness study results of CUR and NIC

Sample concentration	Conditions	Measured concentration (mg/mL)	Recovery %	RSD%
CURC (mg/mL)	**Reference**	0.50	100.%	0.27%
0.5	**260 nm**	0.486	97.2%	1.34%
	262 nm	0.5.07	101.28%	0.27%
	264 nm	0.543	108.68%	1.2%
NIC (µg/mL)	**Reference**	0.505	101%	0.6%
0.5	260 nm	0.4825	96.50%	1.29%
	262 nm	0.5064	101.28%	0.27%
	264 nm	0.529	105.8%	1.7%

3.2. Liposomes *in vitro* Stability

The stability of the prepared liposomes was investigated by measuring changes in the average particle size and zeta potential over a period of three weeks at a storage temperature of 4°C. The results demonstrated good stability of the CUR and NIC liposomes, as indicated by minimal changes in both average particle size and zeta potential at the tested temperature (Figures 7A, 7B, 7C). A release study conducted at 37°C revealed that the liposomal formulation enhanced the release of curcumin due to improved solubility (Figure 7D).

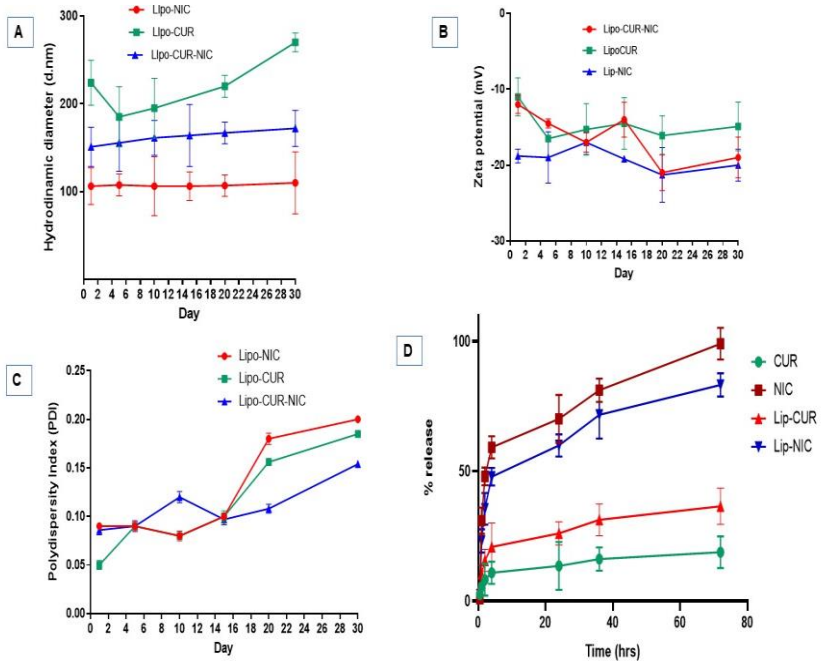


Figure 7: Colloidal stability of CUR and NIC liposomes in terms of average particle size and zeta potential changes over 4 weeks at 4 oC A) Hydrodynamic diameter B) Zeta potential C) Polydispersity index, D) In vitro release curve of a CUR and NIC loaded liposomes and free drug in phosphate buffer at 37 °C (mean ± SD, n = 3)

5. CONCLUSIONS

In this study, straightforward, time-efficient, and cost-effective analytical methods were developed using UV spectrophotometry and reverse-phase HPLC to quantify CUR and NIC encapsulated in liposomal formulations. Liposomes, widely recognized as a versatile lipid drug delivery system, provide an effective platform for encapsulating both hydrophilic and hydrophobic drugs. This approach helps to mask undesirable drug properties, improve release profiles, enhance pharmacokinetics, and serve as a promising drug carrier. The validated analytical methods were employed for the simultaneous estimation of CUR and NIC in liposome formulations containing DPPC, prepared using the ethanol injection technique, with a specific focus on accurately determining encapsulation efficiencies.

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طرق تحليلية لتقييم الكركمين والنيكوتيناميد في تركيبة الجسيمات الشحمية،اليبوسومات: القياس باسنخدام جهاز اللأشعة فوق البنفسجية و جهاز.HPLC

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ملخص

المقدمة: تقدم الدراسة طريقتين تحليليتين متميزتين مصممتين لتحديد دقيق للكركمين والنيكوتيناميد ضمن تركيبات الجسيمات الشحمية (الليبوسومات)، لتلبية احتياجات الباحثين والمحللين في قطاعات الطب الحيوي والمكملات الغذائية .

الطريقة: يوفر قياس الطيف الضوئي للأشعة فوق البنفسجية حلاً سريعًا وفعالاً من حيث التكلفة للقياس الكمي، بينما يوفر التحليل اللوني السائل عالي الأداء خصوصية وحساسية. يضمن التحقق من صحة الطريق، الموثوقية والملاءمة للتحليل الدقيق، وتطوير مجال الكيمياء التحليلية ودعم عمليات التطوير وضمان الجودة في الصناعات الدوائية والتكنولوجيا الحيوية باستخدام جهازي ، خاصة بالنسبة لجهاز. (HPLC & UV spectrophotometer))

النتائج: تم العثور على كفاءة تغليف الكركمين وفيتامين ب و في الجسيمات الشحمية، المكونة أساسًا من DPPC وCHO، لتكون 30% ± 6% و80% ± 5%، على التوالي. أظهرت الطريقة التحليلية المطورة باستخدام القياس الطيفي للأشعة فوق البنفسجية و HPLCللمرحلة العكسية المتانة والكفاءة، مما يسمح بالتحليل المتزامن لـ CUR و NICبدرجة عالية من الخصوصية والدقة والدقة. كشفت عملية التحقق من الصحة وفقًا لإرشادات ICH Q2 عن ملاءمة النظام الممتازة، والخطية، والمتانة، مع انحراف معياري نسبي أقل باستمرار من 2%. أظهرت دراسات الثبات على مدار ثلاثة أسابيع عند 4 درجات مئوية تغيرات طفيفة في خصائص الجسيمات الشحمية، مما يشير إلى ثبات جيد. علاوة على ذلك، أظهرت دراسات الإطلاق عند 37 درجة مئوية زيادة في قابلية الذوبان وزيادة إطلاق الكركمين، مما يشير إلى إمكانية استخدام تركيبة الجسيمات الشحمية في تطبيقات توصيل الأدوية.

الاستنتاج: في هذه الدراسة، تم تطوير طرق تحليلية واضحة وموفرة للوقت وفعالة من حيث التكلفة باستخدام القياس الطيفي للأشعة فوق البنفسجية و ذو الطور العكسي لتحديد كمية المغلفة في تركيبات الجسيمات الشحمية.

الكلمات الدالة: الجسيمات الشحمية، الطرق التحليلية، قياس الطيف الضوئي للأشعة فوق البنفسجية، HPLC.

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Optimized HPLC-UV Methodology for the Simultaneous Quantification of Multiple Preservatives in Jordanian Yogurt Products

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ABSTRACT

A method based on high-performance liquid chromatography with a UV detector (HPLC-UV) was developed for the simultaneous determination of sorbic acid, benzoic acid, and natamycin in yogurt. The method does not require time-consuming, labor-intensive pre-treatment processes or complicated procedures. Using a C18 150 mm × 4.6 mm, 3.0 µm column (Roc) at 25 °C, the target analytes were separated within 5 minutes with high sensitivity and selectivity. The mobile phase consisted of trifluoroacetic acid (0.1%) in water containing 100 mM sodium acetate, trifluoroacetic acid (0.1%) in acetonitrile, and trifluoroacetic acid (0.1%) in tetrahydrofuran, in a ratio of 70:20:10 (v/v). Using this mobile phase as an extraction mixture, recoveries ranged from 83.0% to 110.2% at spike levels between 2.5 µg/kg and 80.0 µg/kg. The relative standard deviations (RSDs) for these recoveries were below 10%. Intra-day precision and inter-day precision varied from 5.3% to 6.7% and 7.6% to 9.2%, respectively. Additionally, the limits of detection (LOD) were between 0.24 and 0.61 mg/L, and the limits of quantification (LOQ) ranged from 0.80 to 2.0 mg/L for sorbic acid, benzoic acid, and natamycin. Principal component analysis revealed that yogurt type had the greatest positive influence on preservative concentration, while the weight or volume of the yogurt package had the greatest negative influence.

Keywords: HPLC, Benzoic acid, Sorbic Acid, Natamycin, Preservatives, Principal component analysis (PCA).

INTRODUCTION

Yogurt is a nutritious food made from milk (lactose) that has undergone bacterial fermentation (1) and has been acidified with viable and well-defined bacteria (2). The bacteria used in the yogurt industry are generally referred to as "yogurt cultures" (3). Lactic acid bacteria ferment lactose, a disaccharide made up of galactose and glucose that accounts for about 4–5% of milk by weight (4), into lactic acid. This lactic acid interacts with milk proteins to produce yogurt, giving it its characteristic texture and tart flavor. Yogurt typically contains the bacterial cultures *Lactobacillus bulgaricus*, *Lactobacillus delbrueckii* subsp., and *Streptococcus thermophilus* (5). Additionally, other lactobacilli and bifidobacteria are sometimes added during or after the yogurt culturing process (6).

In yogurt production, milk is heated to around 85°C to denature the proteins and prevent curd formation. Afterward, the milk is cooled to approximately 45°C (7, 8). The bacterial culture is then mixed in and maintained at 45°C for four to twelve hours to allow fermentation to occur (9).

Yogurt produced following good manufacturing practices is expected to have a shelf life of about three

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days, with less than one yeast cell per gram during transportation and storage under refrigerated conditions in retail outlets (10). However, yogurt has been reported to contain yeast and mold contaminants, with yeast counts ranging from 2.39 to 5.39 log colony-forming units (CFU) per gram (11, 12). High yeast counts in milk have been attributed to factors such as inadequate heat treatment, contamination of utensils and air, the use of starter cultures prepared from previous-day milk, and temperature abuse during treatment (13, 14).

To improve yogurt consistency and extend its shelf life, additives are commonly used to prevent microbial attacks. Preservatives, technically speaking, are chemicals that inhibit microorganisms, preventing food from fermenting and spoiling without posing harm to the consumer (15). Food preservatives such as benzoic acid, sorbic acid, propionates, and dimethyl dicarbonate are used as antimicrobials, while ascorbic acid and butylated hydroxyanisole serve as antioxidants. Antibiotics like oxytetracycline, nisin, and natamycin are also employed in some cases (16).

Commonly used additives in the yogurt industry include benzoic acid, sorbic acid, and their metal derivatives (17). The presence of these additives in yogurt poses potential health risks to consumers. Therefore, permissible levels have been established to prevent the misuse of preservatives. The Joint FAO/WHO Expert Committee on Food Additives set an allowable daily intake of 5 mg/kg/day for benzoic acid and 25 mg/kg/day for sorbic acid (18, 19).

Sample preparation is often regarded as a crucial stage in the analysis of preservatives since it heavily depends on the chemical and physical properties of the contaminants. Food products with high levels of fat and protein, such as yogurt, require a complex, multi-step treatment process. Most methods for extracting these preservatives involve cumbersome, laborious, and time-consuming pre-treatment techniques, including the use of primary extraction solvents or a mixture of organic solvents (20, 21).

Developing an extraction method requires a fundamental understanding of extraction principles, including the transfer of target analytes from the sample matrix to the extracting phase (22). Factors such as selectivity, speed, and sample throughput vary depending on the extraction approach used (23). Once extracted, preservatives are detected and quantified using various analytical methods, such as gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC) coupled with ultraviolet-visible (UV-Vis) detection, and liquid chromatography coupled with mass spectrometry (LC-MS) (18, 24, 25).

In this study, we employed a simple solvent extraction followed by HPLC-UV analysis for the detection of sorbic acid, benzoic acid, and natamycin in yogurt samples.

EXPERIMENTAL

Reagents and materials

Standard solutions of benzoic acid (99.6%) and sorbic acid (99.0%) were purchased from Acros Organics (Switzerland). A standard solution of natamycin (100%) was obtained from the U.S. Pharmacopeia (Rockville, MD, USA). Acetic acid (99.8%) was sourced from Fluka (Switzerland), while sodium acetate anhydrous (extra pure, SLR) was acquired from Fisher Chemical™ (UK). HPLC-grade methanol was provided by Labscan (Ireland), and a 0.45 µm disposable nylon syringe filter (25 mm) was purchased from Shimadzu (Japan).

HPLC analysis

HPLC analysis was performed using a Shimadzu LC-2030C 3D Plus system (Kyoto, Japan). The system included a four-solvent low-pressure gradient pump, degasser, autosampler with a 200 µL sample loop, column oven, and photodiode array detector, all controlled by LabSolution software (version 5.90, Shimadzu, Japan). Separation was carried out using a Roc C18, 3.0 µm, 4.6 x 150 mm chromatographic column from RESTEK (Pennsylvania, USA).

Sample extracts were analyzed using HPLC with a mobile phase consisting of three solutions:

- **Solution A**: 0.1% trifluoroacetic acid in water containing 100 mM sodium acetate
- **Solution B**: 0.1% trifluoroacetic acid in acetonitrile
- **Solution C**: 0.1% trifluoroacetic acid in tetrahydrofuran

The mobile phase was prepared by mixing the solutions in a 70:20:10 ratio (v/v).

Sample preparation

Target analytes were extracted from the yogurt samples using a solid-liquid extraction technique. Briefly, well-homogenized samples (5 g) were weighed into a 250 mL conical flask containing 50 mL of the mobile phase, comprising solutions A, B, and C in a 70:20:10 ratio (v/v). The mixture was stirred at high speed for 3 minutes, then filtered through filter paper, followed by filtration through a 0.45 µm disposable membrane filter prior to HPLC-UV analysis.

The target analytes were quantified using the external standard calibration method at five concentration levels ranging from 2.5 to 80 mg/L in the mobile phase. Both the standard solutions and samples were analyzed in triplicate, and calibration curves for each analyte were constructed by plotting the average chromatographic peak area against the standard concentration (26).

RESULTS AND DISCUSSION

Optimization of HPLC conditions

The peaks in the chromatograms obtained from HPLC-UV were identified and confirmed by matching their spectra with those of the standard solutions. The UV spectrum of natamycin displayed three main absorption peaks between 290 and 320 nm, with 302 nm selected for the identification and detection of natamycin (27). For benzoic acid, the UV spectrum showed two broad bands around 190 nm and 230 nm. To avoid the UV absorbance cut-off of the selected mobile phase, the peak at 227 nm, which gave higher absorption than the 190 nm peak, was chosen for the detection and quantification of benzoic acid. The maximum UV absorption for sorbic acid was found between 252 and 256 nm, with 254 nm used for its detection (28).

A 40 mg/L mixture of standard solutions of benzoic acid, natamycin, and sorbic acid was used to optimize the chromatographic conditions in a single run. The analytical column was a Brownlee Analytical 5 µm C18, 250 mm × 4.6 mm, operated at 25°C. The mobile phase consisted of an isocratic acetate buffer (pH 5.6) and methanol mixture in a 60:40 v/v % ratio (29). Preliminary investigations focused on the chromatographic retention of the target analytes, examining the effect of mobile phase composition and polarity. The influence of C8 and C18 columns with varying particle sizes and lengths, at temperatures ranging from 15°C to 30°C, was also explored.

In this experiment, different combinations of trifluoroacetic acid in water (containing 100 mM sodium acetate), acetonitrile, and tetrahydrofuran were tested in different ratios—70:20:10, 65:25:10, and 60:20:20 (v/v%)—for the HPLC mobile phase to optimize the resolution of sorbic acid, benzoic acid, and natamycin and improve sensitivity. When the mobile phase solutions A, B, and C were combined in a 60:20:20 (v/v%) ratio, overlapping peaks were observed between benzoic acid and natamycin. Additionally, using solutions A, B, and C in a 65:25:10 (v/v%) ratio resulted in poorly resolved peaks (resolution Rs ≈ 1.1) with shorter retention times. The mobile phase composed of solutions A, B, and C in a 70:20:10 (v/v%) ratio provided optimal separation between the benzoic acid and sorbic acid peaks (Rs > 1.5) with short retention times under 6 minutes and was selected for subsequent analysis.

To determine the optimum column temperature, four different temperatures (15°C, 20°C, 25°C, and 30°C) were tested, using the resolution factor and peak area as key criteria. The optimal temperature was found to be 25°C, which was subsequently used for further analysis. Under these conditions, effective separation of the target analytes was achieved, with retention times for all analytes within 5 minutes. The retention times were 2.68 minutes for

sorbic acid, 3.05 minutes for benzoic acid, and 4.95 minutes for natamycin (Fig. 1).

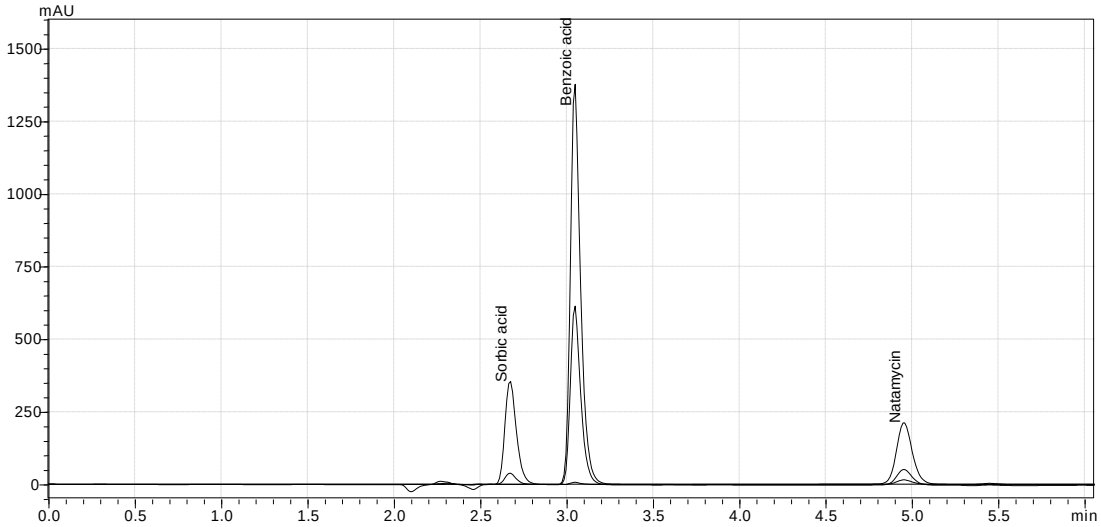

Figure 1: HPLC chromatogram of preservative standard solutions

Optimization extraction conditions

The extraction step is often considered the bottleneck of analytical methodologies and is recognized as the most critical step in sample preparation and chromatographic analysis (30, 31). The extraction technique employed must be both effective and efficient, with high sample throughput. Several methods have been used for the extraction and pre-concentration of preservatives from complex sample matrices, but these often involve multiple steps, are time-consuming, expensive, and labor-intensive (30).

When the mobile phase was used as the extraction solvent, the peak area of sorbic acid increased 1000-fold. However, there was a 54% reduction in the peak area of benzoic acid, while no significant change was observed in the peak area of natamycin (Table 1).

Table 1: Peak area of preservatives in standards mixtures extracted using different procedures method.

Preservative standards mixture	Extraction Methods	
	Mobile Phase (Peak Area)	Extraction Solvent (Peak Area)
Sorbic Acid	4401440	4525
Benzoic Acid	9838464	17265845
Natamycin	1228470	1182222

Validation of analytical methodology

The method was validated in terms of linearity, accuracy, intra- and inter-day precision, limits of quantification (LOQ), and limits of detection (LOD). Linearity was assessed using standard mixtures of the target analytes, with concentration ranges of 3.12–50.0 mg/L for sorbic acid and benzoic acid, and 2.50–40 mg/L for natamycin. As shown in Table 2, the calibration curves demonstrated good linearity, with correlation coefficients greater than 0.99, indicating a strong linear relationship between the concentration of the target analytes and the chromatographic peak response.

Table 2: Linearity range, Equation, r^2 value, LOD and LOQ of the target analytes

Preservative	Linearity Range (mg/L)	Equation	r^2	LOD (mg/L)	LOQ (mg/L)
Sorbic Acid	3.12 – 50.0	Y = (-87858) + (65255) X	0.9999	0.24	0.80
Benzoic Acid	3.12 – 50.0	Y = (-201579) + (142279) X	0.9998	0.39	1.3
Natamycin	2.50 – 40.0	Y = (-126923) + (34612) X	0.9975	0.61	2.0

The limits of detection (LOD) and quantification (LOQ) were estimated using signal-to-noise ratios of 3 and 10, respectively, from preservative-free samples (Table 2). The LODs were 0.66 mg/L for sorbic acid, 0.51 mg/L for benzoic acid, and 0.01 mg/L for natamycin.

The accuracy of the method was evaluated by calculating the average recoveries of the target analytes from diluted yogurt samples spiked with three different concentrations (5, 10, and 20 mg/L) of the preservative standard mix, each analyzed in triplicate. The recovery was calculated using Equation 1 (32):

$$\text{Recovery (\%)} = \frac{\text{Recovered Amount (mg/L)}}{\text{Added Amount (mg/L)}} \times 100 \quad (1)$$

As shown in Table 3, recoveries ranged from 83.0% to 112.8%, with relative standard deviations (RSD) between 1.3% and 9.6%. Sorbic acid exhibited the highest recoveries (97.7%–112.8%), followed by benzoic acid (89.9%–96.3%), while natamycin showed the lowest recoveries (84.9%–85.7%).

Table 3: Average recoveries and Relative standard deviation (RSD) of the target analytes

Preservative	Spiking Level (mg/L)	Yogurt	Diluted Yogurt
		Mean of Recovery (%) ± RSD (%)	
Sorbic Acid	5	112.8±6.7	83.0±7.7
	10	97.9±6.0	88.6±8.8
	20	97.7±5.3	108.1±4.9
Benzoic Acid	5	91.4±1.3	86.0±7.4
	10	89.9±8.3	110.2±5.0
	20	96.3±5.4	96.5±6.3
	5	84.9±7.4	86.0±7.4
Natamycin	10	85.7±9.3	110.2±5.0
	20	85.4±6.7	96.5±6.3

In this study, five extractions were performed in a single day by spiking preservative-free samples with 20 mg/L of the target analytes to determine intra-day precision. Inter-day precision was assessed by performing five extractions per day over three days. The calculations and results for intra-day and inter-day precision are presented in Table 4.

The intra-day precision (n = 5) ranged from 5.3% to 6.7%, while the inter-day precision ranged from 7.6% to 8.0%. The figures of merit for the analytical methodology obtained in this study were satisfactory and comply with the SANTE 11312/2021 guidelines (33).

Table 4: The intra-day precision and inter-day precision of the developed method

Preservative	Spiking Level (mg/L)	Intra-Day Precision (n = 5)[a]	Inter-Day Precision (n = 15)[a]
Sorbic Acid	20	5.3	7.6
Benzoic Acid	20	5.4	8.0
Natamycin	20	6.7	9.2

Analysis of samples

The developed method was applied to analyze local and imported yogurt samples purchased from Jordanian markets. A total of 120 dried yogurt samples were analyzed, consisting of 60 Jameed yogurt samples (locally produced in Jordan and some imported from Hungary, Turkey, Syria, and Egypt) and 20 liquid Jameed yogurt samples from Jordan. Benzoic acid was detected at varying concentrations (13.1–97.3 mg/L) in 19 samples of Jameed (4 from Jordan, 5 from Syria, and 9 from Egypt) and in 1 sample of liquid Jameed yogurt. Natamycin was found in 13 samples of dried yogurt and 1 sample of liquid Jameed yogurt. Sorbic acid was detected in 14 separate samples of dried yogurt from Jordan at concentrations ranging from 1.61–22.72 mg/L and 5.87–383.69 mg/L. No preservatives were detected in the remaining 151 yogurt samples obtained from Jordan, Hungary, and Turkey. Figure 2 displays an HPLC chromatogram of a dried yogurt sample with 30 mg/kg natamycin. The detection of benzoic acid in the yogurt correlates with the study by Mazdeh and colleagues, who detected benzoate and sorbate in concentrations ranging from 2.08–58.19 mg/kg and 3.81–246.60 mg/kg, respectively, in yogurt samples obtained in Iran (18).

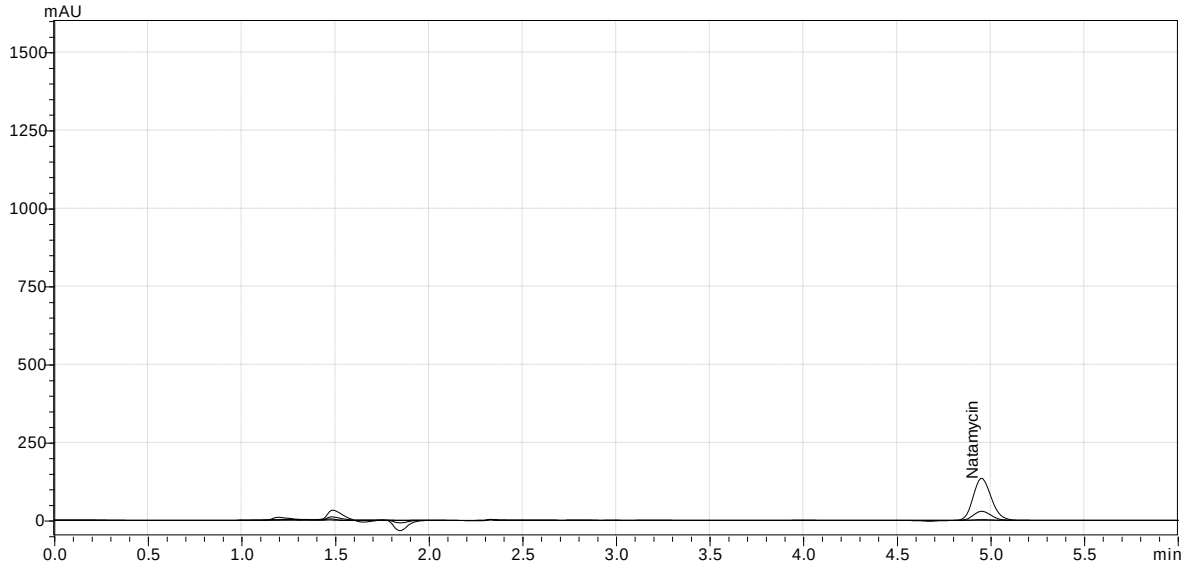

Figure 2: HPLC chromatogram of a yogurt sample containing 30 ppm natamycin.

Statistical analysis

Samples were analyzed randomly after being coded and evaluated in triplicate. Principal component analysis (PCA) was used to quantitatively analyze the relationships between the types of yogurt and the concentrations of benzoic acid, sorbic acid, and natamycin (34). Two principal components with Eigenvalues greater than 1 explained 50.4% of the variation, as shown in the Scree plot (Figure 3).

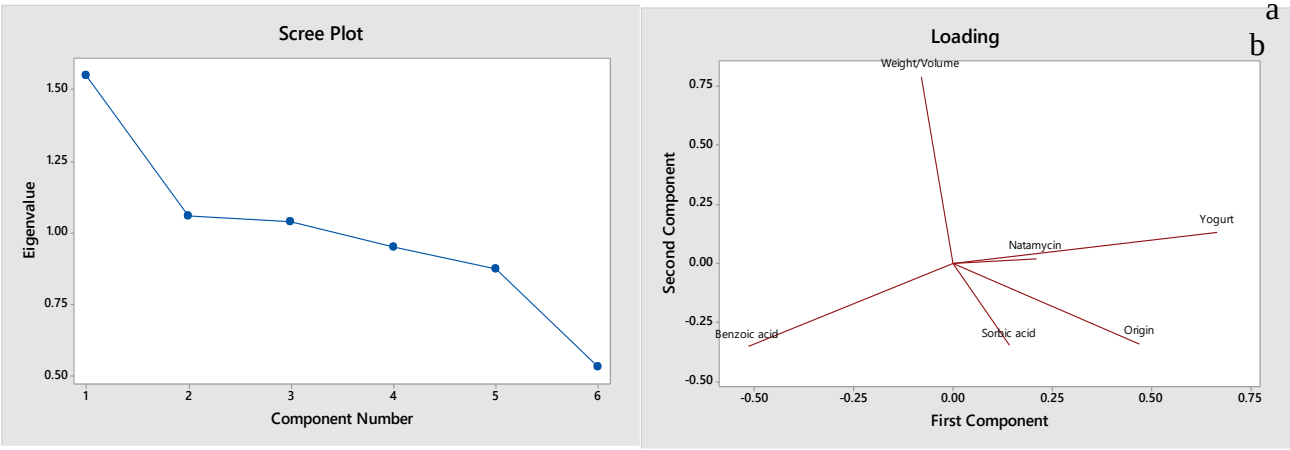

Figure 3: Scree plot and loading plot

Table 5: Eigenanalysis of the correlation matrix

Eigenvalue	1.5480	1.0580	1.0400	0.9502	0.8714	0.5324
Proportion	0.258	0.176	0.173	0.158	0.145	0.089
Cumulative	0.258	0.434	0.608	0.766	0.911	1.000
Eigenvectors						
Variable	PC1	PC2	PC3	PC4	PC5	PC6
Yogurt	0.665	0.130	–0.520	–0.132	–0.042	–0.720
Origin	0.469	–0.340	0.080	–0.602	0.291	0.459
Weight/Volume	–0.081	0.787	–0.214	–0.214	0.523	0.091
Benzoic acid	–0.517	–0.353	0.131	–0.359	0.447	–0.511
Sorbic acid	0.142	–0.350	–0.674	0.445	0.453	0.009
Natamycin	0.209	0.019	0.688	0.498	0.484	0.027

As shown in Table 5, the first three factors—type of yogurt, country of origin, and weight or volume of the yogurt—account for 60.80% of the variation in the concentration of preservatives in yogurt samples. This is also evident from the Scree plot (Figure 2a), which shows that these three components have Eigenvalues greater than 1, according to the Kaiser criterion (35). The loading plot (Figure 2b) indicates that the type of yogurt has the largest positive influence on the preservative content, followed by the country of origin. The weight or volume of the yogurt package has a weaker influence on preservative concentration. Additionally, it was observed that the

benzoic acid content has a significant negative impact on yogurt.

Table 5 also shows that the type of yogurt has a significant positive influence on the country of origin, and vice versa. The origin of the yogurt positively influences the type of yogurt, the weight or volume of the yogurt, and the concentrations of sorbic acid and natamycin. The weight or volume of the yogurt positively affects the origin and also the concentrations of sorbic acid and natamycin. Conversely, the concentration of benzoic acid is positively influenced by both the weight/volume of the yogurt and the concentration of sorbic acid. Additionally, the concentration of sorbic acid has a significant positive influence on the type of yogurt, the weight/volume of the yogurt, and the concentration of natamycin. The concentration of natamycin was found to have a large positive influence and strongly correlate with other variables. Thus, the preservative content of yogurt samples may be influenced by their source, as well as by storage and handling methods.

Comparison with previous studies

The developed method was compared with previous methods for analyzing preservatives in yogurt, as shown in Table 6, in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, and relative standard deviation.

Table 6: comparison of present study with previous studies

S/N	Method	Linearity (R^2) (mg/L)	LOD (mg/L)	LOQ (mg/L)	Recovery (%)	RSD (%)	Ref
1	Ultrasonic Extraction/HPLC	5.1 – 50 (0.9999)	10	50	91–105	8 – 8.3	(19)
2	Solvent extraction/HPLC	2 – 6 (n.r)	n.r	n.r	91.33–99.50	n.r	(36)
3	Solvent extraction/HPLC	0.01-0.8 (0.9991)	0.320	0.403	104	0.562	(37)
4	Solvent extraction/HPLC	5-40 (0.997)	0.326—.520	0.989-1.575	87.85–94.16	0.55–1.33	(18)
5	Solvent extraction/RP-HPLC	2.5-50 (0.997)	0.24–0.61	0.80–1.3	84.9–112.8	1.3-9.3	Present study

CONCLSION

The analysis of preservatives in yogurt has been facilitated by the development of a sample preparation method that is quick, easy, low-cost, effective, and efficient. The method was optimized for mobile phase composition, column type, column length, and particle size to enhance the sensitivity of HPLC-UV chromatography. It involves a single extraction step with no pre-treatment required. This method is recommended as an alternative for analyzing preservatives in food samples.

The method achieved good separation of target analytes with short retention times, using a C18 250 mm × 4.6 mm × 5 μm column at 25°C, with excellent selectivity and sensitivity. The method provided satisfactory figures of merit, including good linearity, accuracy (in terms of average recoveries), precision, and a low limit of detection, demonstrating its suitability for detecting preservatives in yogurt samples. The concentration of preservatives was found to be influenced separately and independently by the type of yogurt, origin, and weight/volume.

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طريقة HPLC-UV المحسنة لتقدير كمية المواد الحافظة المتعددة في منتجات الزبادي الأردنية في وقت واحد

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ملخص

تم تطوير طريقة تعتمد على تطبيق كروماتوغرافيا السائل عالية الأداء مع كاشف الأشعة فوق البنفسجية (HPLC-UV) لتحديد حمض السوربيك وحمض البنزويك والناتاميسين في الزبادي في وقت واحد. لم تتضمن الطريقة المطورة عمليات معالجة أولية تستغرق وقتًا طويلاً وتتطلب عمالة مكثفة أو إجراءً معقدًا. باستخدام عمود C18 150 مم 4.6 مم × 3.0 ميكرومتر (Roc) عند 25 درجة مئوية، تم فصل المحللات المستهدفة في 5 دقائق بحساسية وانتقائية عالية. يتكون الطور المتحرك من مزيج من حمض ثلاثي فلورو أسيتيك (0.1٪) في الماء يحتوي على 100 مليمول من أسيتات الصوديوم وحمض ثلاثي فلورو أسيتيك (0.1٪) في أسيتونتريل وحمض ثلاثي فلورو أسيتيك (0.1٪) في رباعي هيدروفوران (بنسبة 70:20:10، حجم / حجم). أدى استخدام الطور المتحرك كمزيج استخلاص إلى استردادات تتراوح من 83.0٪ إلى 110.2٪ عند مستويات الذروة التي تتراوح من 2.5 ميكروجرام / كجم إلى 80.0 ميكروجرام / كجم. كانت الانحرافات المعيارية النسبية (RSDs) المرتبطة بهذه الاستردادات أقل من 10٪. تراوحت النتائج الخاصة بالدقة داخل اليوم والدقة بين الأيام من 5.3٪ إلى 6.7٪ و 7.6٪ إلى 9.2٪ على التوالي. بالإضافة إلى ذلك، كانت حدود الكشف (LOD) بين 0.24 و 0.61 مجم / لتر وحدود التقدير (LOQ) بين 0.80 و 2.0 مجم / لتر لحامض السوربيك وحمض البنزويك والناتامايسين على التوالي. بناءً على تحليل المكونات الرئيسية، فإن نوع الزبادي له أكبر تأثير إيجابي على تركيز المواد الحافظة، في حين أن وزن أو حجم عبوة الزبادي له أكبر تأثير سلبي على تركيز المواد الحافظة.

الكلمات الدالة: HPLC، حمض البنزويك، حمض السوربيك، ناتامايسين، المواد الحافظة، تحليل المكونات الرئيسية (PCA).

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Evaluation of Proximate Composition, Multielement, and Bioactive Phenolics Contents of Different Coix Seed Varieties using Multivariate Analysis Techniques

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ABSTRACT

Coix lacryma-jobi L. is a plant that serves as a source of food, medicine, cosmetics, and forage in Asian countries. Due to the distinct geographic environments, Coix seed germplasm resources are extremely diverse. In this study, we evaluated the proximate composition, multi-elemental content by ICP-OES, and phenolic bioactives by UPLC-QTOF/MS of five varieties of Coix seeds from different Asian countries, including China, Thailand, Indonesia, and Malaysia. Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were used to classify the different varieties of Coix seeds. The C. lacryma-jobi var. *ma-yuen* seeds from Origin 1 (China) and Origin 2 (Thailand) contained high levels of energy, total fat, and calcium, while C. lacryma-jobi var. *agrotis* (Indonesia) and C. lacryma-jobi var. *lacryma-jobi* (Malaysia) had high levels of crude fiber and carbohydrates. Twenty-three phenolic compounds were identified. Protein, carbohydrate, crude fiber, magnesium, zinc, meliadanoside, and 3,4-dihydroxyphenethyl-3-O-β-D-glucopyranoside were the dominant variables and contributed the most to data variability in PCA. The HCA results were consistent with the PCA, classifying the samples into three groups: those rich in nutrients, those rich in phenolics, and those with a mixture of nutrients and phenolics. This comprehensive analysis provides valuable insights into the nutritional and bioactive composition of Coix seed varieties, with potential applications in nutrition, food science, and pharmaceuticals.

Keywords: *Coix lacryma-jobi*; Coix seed, mineral content, nutritional composition; phenolic compounds, multivariate analysis.

1. INTRODUCTION

Coix lacryma-jobi L., a perennial plant native to Southeast Asia, belongs to the Poaceae (or Gramineae) family and the Coix genus (1). Coix seeds, in particular, are highly nutritious and can be used in medicine (2). They contain polysaccharides, lipids, flavonoids, phenols, proteins, vitamins, and other beneficial compounds (3). Modern pharmacological research has revealed that these bioactive components possess potential in tumor inhibition, antibacterial and antiviral activities, blood lipid regulation, pancreatic protein suppression, anti-inflammatory, analgesic, and antioxidant properties (4-7).

Also known as Job's Tears, Coix lacryma-jobi originated in India and is now indigenous to Southeast Asia, including China, Japan, the Philippines, Burma, and Thailand. Numerous products are made from these seeds. After cleaning and dehulling, the mature seeds are boiled and served with rice. When used as a cooling beverage similar to barley or flour water, the pounded flour is occasionally mixed with water (8). Among the

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Garo, Karbi, and Naga tribes, Coix seeds are used to make beer from pounded grains and for ornamental purposes such as rosaries and necklaces. In China, Coix is used as a medicinal food supplement and traditional medication. In Thailand, the seeds are consumed after removing the seed covering and are also used to create a beverage sold in stores. The benefits of Job's Tears include reducing liver fat buildup, inhibiting tumor growth, preventing viral infections, decreasing the risk of allergic reactions, coronary artery disease, atherosclerosis, and osteoporosis (3, 9, 10).

Coix lacryma-jobi exhibits wide distribution across tropical and temperate regions, showcasing its remarkable ability to thrive in various environments, including wetlands and forest fringes (11). There are four varieties of Coix: C. lacryma-jobi var. *ma-yuen* (Rom. Caill.) Stapf., C. lacryma-jobi var. *lacryma-jobi*, C. lacryma-jobi var. *puellarum* (Balansa) A. Camus, and C. lacryma-jobi var. *stenocarpa* Oliv (12). C. lacryma-jobi var. *lacryma-jobi* is commonly found as a wild type, recognized for its durable shells and traditional use in crafting beads for necklaces and rosaries. The cultivated form, C. lacryma-jobi var. *ma-yuen*, has a softer shell. The genetic diversity of Coix seeds is evident in their varied shapes and colors, reflecting extensive genetic variation. This diversity may contribute to variations in Coix's chemical composition, a topic that warrants further investigation. Limited research has been conducted on the phytochemical profiles of different Coix seed varieties. To address this gap, we conducted a study on the proximate composition, multi-elemental, and chemical contents of Coix seed varieties. To the best of our knowledge, this is the first comparative study of the chemical composition of Coix seed varieties.

2. MATERIALS AND METHODS

2.1 Plant characterization, sampling, and preparation

The botanical classification of the five samples was conducted by comparing them with descriptions provided in taxonomic reviews and scientific botanical resources, such as botanical atlases and species photographs. Coix seeds originating from Indonesia, specifically Coix lacryma-jobi var. *ma-yuen* (CMY3) and C. lacryma-jobi var. *agrotis* (CA), were sourced from KWT Pantastik in Sumedang, West Java, Indonesia. C. lacryma-jobi var. *ma-yuen* (CMY1) from Origin 1 (China) and CMY2 from Origin 2 (Thailand) were purchased from different companies that claimed their seeds originated from these countries. C. lacryma-jobi var. *lacryma-jobi* (CLJ) was sourced from Murni Herbs in Perak, Malaysia (Figure 1). All samples were carefully stored in a cool, dry environment until analysis. The experimental research, including the collection of plant material, adhered to relevant institutional, national, and international guidelines and legislation.

2.2 Proximate composition

The moisture, ash, protein, lipid, and carbohydrate levels were evaluated using procedures specified in AOAC methods (13). All chemical analyses were carried out in triplicate to ensure reliability. The total carbohydrate content was determined by subtracting the combined percentages of moisture, ash, lipid, and protein from 100%.

2.3 Multielement analysis

A microwave-enclosed jar was filled with approximately 300 mg of dried powder, and 7.0 mL of 65% (v/v) HNO_3 and 1 mL of 30% (v/v) H_2O_2 solutions were added. The heating program involved four successive steps at a constant pressure of 35 bar: Step 1 for 6 minutes at 750 watts, Step 2 for 4 minutes at 750 watts, Step 3 for 8 minutes, and Step 4 for 15 minutes at 750 watts and 180°C. After digestion, the samples were cooled via ventilation for 20 minutes. The digested samples were then diluted with deionized water to a final volume of 50.0 mL. Elemental analysis was performed using ICP-OES (Perkin Elmer, Optima 8300) to determine the concentrations of lead (Pb), sodium (Na), zinc (Zn), indium (In), silver (Ag), bismuth (Bi), thallium (Tl), potassium (K), magnesium

(Mg), gallium (Ga), aluminum (Al), nickel (Ni), cobalt (Co), chromium (Cr), calcium (Ca), strontium (Sr), iron (Fe), barium (Ba), manganese (Mn), copper (Cu), and cadmium (Cd).

***C. lacryma-jobi var. ma-yuen* dehusk (Origin 1)**

***C. lacryma-jobi var. ma-yuen* dehusk (Origin 2)**

***C. lacryma-jobi var. ma-yuen* (Origin 3)**

C. lacryma-jobi var agrotis

C. lacryma-jobi var. lacryma-jobi

Figure 1: Different varieties of Coix seeds

2.4 Chromatographic analysis

The preparation of extracts for phenolic compound analysis was conducted in triplicate. Approximately 1 g of dried Coix seed powder was extracted with water and sonicated for 30 minutes. The water extracts were then dried using a freeze dryer. Each sample extract, consisting of 1 mg of dried powder, was dissolved in 1 mL of a 1:1 methanol solution and subjected to UPLC-QTOF/MS (Waters, VION Ion Mobility QTOF MS) analysis. Sample separation was performed on a Zorbax Eclipse Plus Acquity UPLC BEH C18 column (1.7 µm particle size, 2.1 mm × 50 mm). The column temperature was maintained at 40°C. Aliquots of 2 µL were injected into the UPLC-QTOF/MS at a flow rate of 0.50 mL/min. The following gradient was used for elution with A (water + 0.1% formic acid) and B (acetonitrile + 0.1% formic acid): the gradient started with 1% B for 5 minutes, increased linearly to 35% B from minutes 5 to 16, then to 100% B from minutes 16 to 18, and finally returned to 99% A from minutes 18 to 20. UPLC-QTOF/MS analysis was conducted in positive ion mode using an electrospray ionization (ESI) source. MS spectra were collected for each test sample within the mass range of 50 Da to 1500 Da. Ultra-high purity helium (He)

served as the nebulizing gas, and highly pure nitrogen (N_2) was used as the collision gas. For positive electrospray mode, the capillary voltage was set at 1500 V. The Traditional Chinese Medicine (TCM) library was used for the identification of phenolic groups.

2.5 Multivariate analysis

Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were employed for initial exploration of the samples. PCA involved evaluating principal components based on a data matrix comprising 23 variables and five observations. The data were pre-processed by autoscaling. For HCA, a dendrogram was generated using Euclidean distance and Ward's method to represent the results. All data were collected in triplicate, and analyses were conducted using the XLSTAT 2019 add-on for data analysis within Excel.

2.6 Statistical Analysis

All data collected in triplicate were presented as means and standard deviations (Std). Statistical analysis was performed using Microsoft Excel 2019 data processing software with one-way analysis of variance (ANOVA). The significance of the data was determined by Duncan's test ($\alpha = 5\%$).

3. RESULTS AND DISCUSSION

3.1 Proximate composition

Table 1 shows the nutritional composition (total ash, moisture, protein, total fat, carbohydrate, energy, and crude fiber) of Coix seeds. The proximate composition of CMY seeds exhibited higher carbohydrate and crude fiber levels compared to the other samples in this study. CMY1 and CMY2 had higher protein, total fat, and energy content. The protein content of Coix varieties ranged from 8.7% to 14.6%. This finding aligns with previous research (14), which also noted that Coix seeds contain approximately 20% protein, with the major constituent being prolamin called coixin. Another study involving NIR analysis of 41 Coix seed samples (15) revealed that the major protein component, coixin, accounted for 79% of the total protein. Coix prolamins, or coixins, are classified into two groups: α- and γ-coixins. γ-coixins consist of a single molecular weight class, while α-coixins are divided into four size classes. When prolamins were isolated from the endosperm during seed development, SDS-PAGE and Western blot analyses showed that α-coixins are synthesized earlier than γ-coixins. Polysomal RNA coupled to protein bodies extracted from mid-maturation endosperm was translated in vitro, revealing significant coixin message enrichment in these polyribosomes. cDNA clones representing α- and γ-coixins were used for electrophoresis and probing of polysomal RNAs extracted from all developmental stages. These findings confirmed that α-coixins were expressed earlier and indicated that coixin RNA accumulation in the endosperm predominantly occurs during mid-seed maturation (16).

Additionally, 17 proteins were detected, supporting the high protein content of CMY seeds. Consequently, the substantial protein content contributes to the elevated nutritional value of Coix seeds. The study demonstrated that various Coix seed types can be predicted to have differing amounts of protein, asparagine, serine, glutamine, alanine, valine, isoleucine, leucine, phenylalanine, and proline, using NIRS and chemometrics techniques (17).

CLJ exhibited the highest total ash content at 11.1%, while CMY1 had the lowest at 1.6% ($P < 0.05$). The five samples showed a range between 1.6% and 11.1%. This study's findings slightly differ from those of reference 18, where the total ash content in Coix lacryma-jobi from different parts of North-Eastern India ranged from 2.67% to 4.27%. The moisture content of the five Coix seed samples varied between 10.1% and 13.9%. CMY3 exhibited the highest moisture content at 13.9%, while CLJ had the lowest at 10.1%. These results indicate slightly higher moisture content compared to the study conducted by reference 18, which reported moisture content ranging from 9.65% to 12.9%.

CMY2 exhibited the highest total fat content at 5.2%, while the fat content across samples ranged from 0.6% to 5.2% ($P < 0.05$). CMY2 can be classified as having a high total fat content, as Coix seeds naturally contain more lipids compared to most cereals and millets. This finding aligns with research (18) that reported a range of crude fat content from 2.22% to 6.26%, with the highest fat content recorded. The moisture content of all Coix seed samples ranged from 10% to 13% by mass. CLJ had the highest total ash content. Variations in plant composition can be attributed to factors such as temperature, rainfall, sun exposure, soil composition, growth stage, and interactions with other organisms in the ecosystem (19, 20).

3.2 Multielement analysis

Investigating the composition of inorganic elements is crucial for public health, as these elements play essential roles in human and animal health while also considering the potential presence of harmful components. The study conducted a multielement analysis of Coix seeds using microwave-assisted acid digestion and ICP-OES. The concentration values for elements in Coix seeds are presented in Table 2. Notably, macroelements such as Ca, K, Na, P, and Mg are significant for both plants and animals (19). CMY1 had the highest calcium content at 11,028.00 mg/kg compared to other samples ($P < 0.05$). The calcium content in Coix seeds was higher than that found in other grains (21).

Table 1: Proximate composition of Coix seeds

Sample	Total ash g/100 g	Moisture g/100 g	Protein g/100 g	Total fat g/100 g	Carbohydrate g/100 g	Energy kcal/100 g	Crude fiber g/100 g
CMY1	1.6 ± 0.2	12.3 ± 0.1	14.6 ± 0.2	4.4 ± 1.5	67.1 ± 2.0	366.0 ± 1.5	13.1 ± 0.5
CMY2	1.9 ± 0.3	12.4 ± 0.4	13.2 ± 0.5	5.2 ± 0.7	67.3 ± 0.9	369.0 ± 0.2	1.2 ± 0.5
CMY3	8.2 ± 0.2	13.9 ± 0.1	10.1 ±0.9	0.3 ± 0.5	67.5 ± 0.5	313.0 ± 0.2	12.8 ± 0.3
CA	9.3 ± 0.1	13.4 ± 0.2	8.7 ± 0.5	0.6 ± 1.0	68.0 ± 0.9	312.0 ± 1.0	26.5 ± 0.2
CLJ	11.1 ± 0.2	10.1 ± 0.1	5.9 ± 0.2	1.3 ± 0.5	71.6 ± 0.1	322.0 ± 0.1	45.6 ± 0.2

Total samples (n=3). Data presented as mean ± standard errors. Values are significantly different by Duncan's test ($p< 0.05$).

Table 2: The concentration values obtained for Coix seeds elements

Sample	Zn (mg/kg)	P (mg/kg)	Mg (mg/kg)	Ca (mg/kg)
CMY1	47.62 ± 2.50	1271.00 ± 0.50	1414.00 ± 0.30	11028.00 ± 0.50
CMY2)	45.89 ± 1.50	1483.00 ± 2.50	1418.00 ± 0.50	ND
CMY3	52.48 ± 1.55	1593.00 ± 0.50	1423.00 ± 0.30	270.49 ± 0.50
CA	ND	ND	ND	ND
CLJ	ND	420.45 ± 0.30	622.73 ± 1.50	397.73 ± 1.60

Total samples (n=3). Data presented as mean ± standard errors. Values are significantly different by Duncan's test ($p< 0.05$).
ND: not detected

CMY3 showed the highest level of phosphorus at 1,593.00 mg/kg ($P < 0.05$). Extensive research has

investigated how *Chloris gayana* Kunth and *Coix lacryma-jobi* L. plants respond physiologically to water stress and phosphorus nourishment. Prior studies have suggested a potential link between elevated phosphorus levels and enhanced drought resistance in plants (22). Coix seeds are also rich in minerals essential for human health, such as calcium, magnesium, phosphorus, iron, zinc, and manganese (23). The mineral content varies significantly, with low concentrations in the seed shell and skin, but higher concentrations in the roots and leaves. The highest levels of phosphorus and zinc in *Coix lacryma-jobi* L. were found in the seed kernels, reaching 1.93 mg/g and 0.15 mg/g, respectively ($P < 0.05$). The highest concentrations of potassium, calcium, sodium, and magnesium were found in the leaves, with levels of 4.18 mg/g, 15.65 mg/g, 1.90 mg/g, and 5.85 mg/g, respectively ($P < 0.05$). The highest concentrations of copper, iron, and chromium were found in the roots, with levels of 0.01 mg/g, 1.46 mg/g, and 0.04 mg/g, respectively (23).

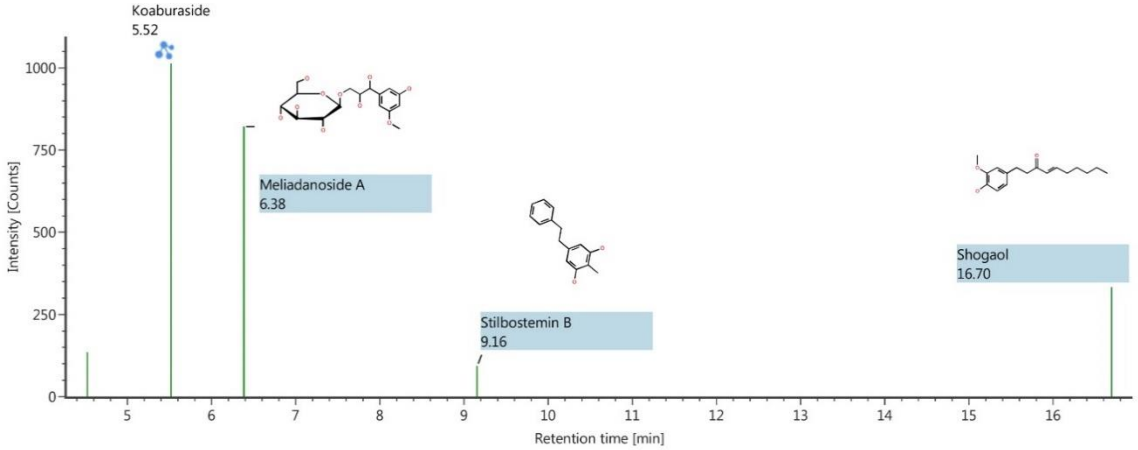
A study (24) that evaluated mercury, lead, cadmium, arsenic, chromium, and 116 pesticides in Coix seeds found that among the 123 Coix seed samples, the average levels of lead, cadmium, arsenic, and chromium were 0.0069 mg/kg, 0.0021 mg/kg, 0.0138 mg/kg, and 0.1107 mg/kg, respectively. Notably, no traces of mercury were detected in any of the Coix seed samples. These findings suggest that Coix seeds are considered safe for consumption based on the assessment of heavy metal and pesticide residue pollution indices and associated risks.

3.3 Chromatographic analysis

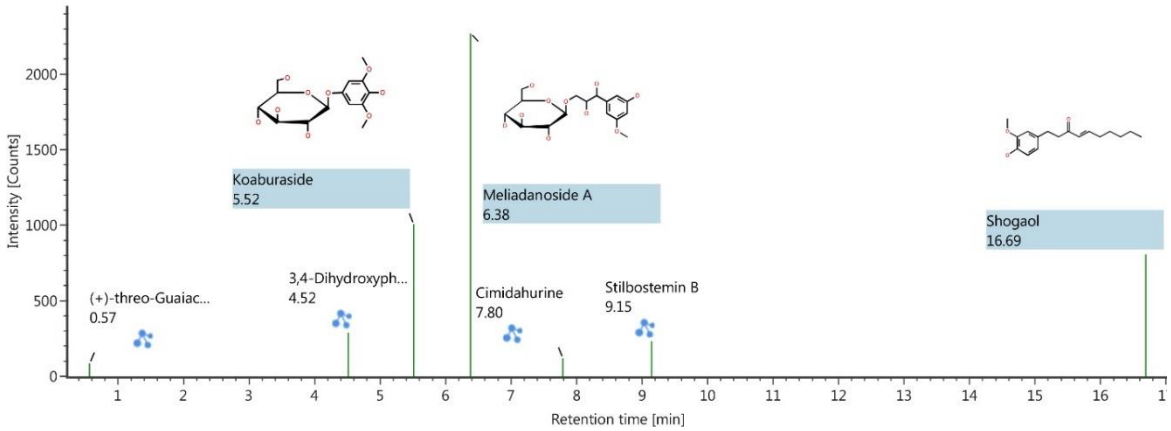
Phenolic compounds are currently being investigated in various fields, particularly in chemical and biological research aimed at discovering novel compounds with biological activities. These compounds are significant in industries such as food, chemicals, and pharmaceuticals. Figure 2 shows the components identified by UPLC/QTOF-MS in all Coix samples. Phenolic compounds identified in CMY1 and CMY2 include 3,4-dihydroxyphenothyl-3-O-β-D-glucopyranoside, koaburaside, meliadanoside A, and shogaol, each with varying ion intensities. CMY3 was found to contain abundant amounts of salidroside, meliadanoside A, mulberrofuran A, and shogaol. CLJ showed the presence of meliadanoside A and shogaol. The high abundance of 3,7-dihydroxy-2,4-dimethoxyphenanthrene-3-O-glucoside and shogaol was detected in CA ($P < 0.05$)).

Based on Table 3, the results indicate the ion response of phenolic compounds in Coix seeds. CMY3 was found to contain the most abundant phenolic compounds, while CA had the least. All Coix samples showed a significant difference in the concentration of shogaol. Previous studies on polyphenols in Coix seeds have mainly focused on the total polyphenol content and their antioxidant activity. Recent research has led to the isolation of phenolic compounds from Coix husk, including p-coumaric acid, vanillic acid, chlorogenic acid, tannic acid, catechol, and ferulic acid. Additionally, polyphenols such as p-hydroxybenzoic acid, vanillic acid, syringic acid, ferulic acid, p-coumaric acid, caffeic acid, erucic acid, vanillin, 2-hydroxyphenylacetic acid, coixol, 4-ketopinol ester, syringaresinol, and catechin have been identified in *Coix lacryma-jobi* (23).

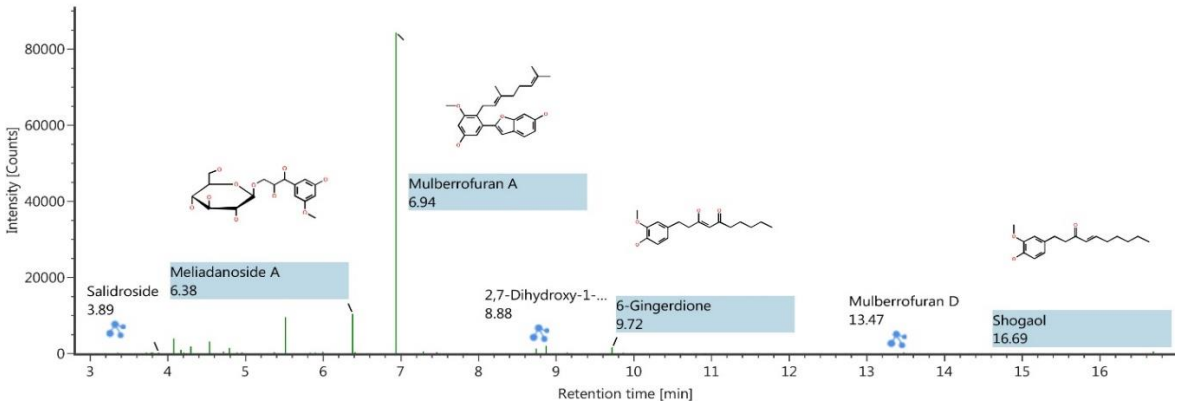
The observed differences in quality can be attributed to variations in geographical environments, soil composition, and climate conditions. Even within the same species, distinct DNA barcodes, elemental compositions, and chemical fingerprints can exhibit unique characteristics, aiding in the identification of their origins (25). Variation in geographical locations significantly influences the chemical compounds within the same species. To the best of our knowledge, there is no documented research on the chemical composition of CA.



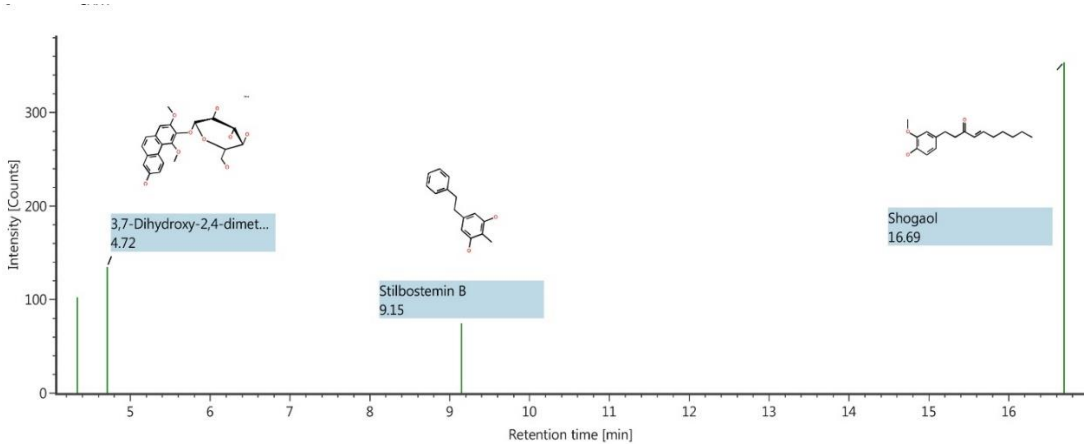
C. lacryma-jobi var. ma-yuen (CMY1)



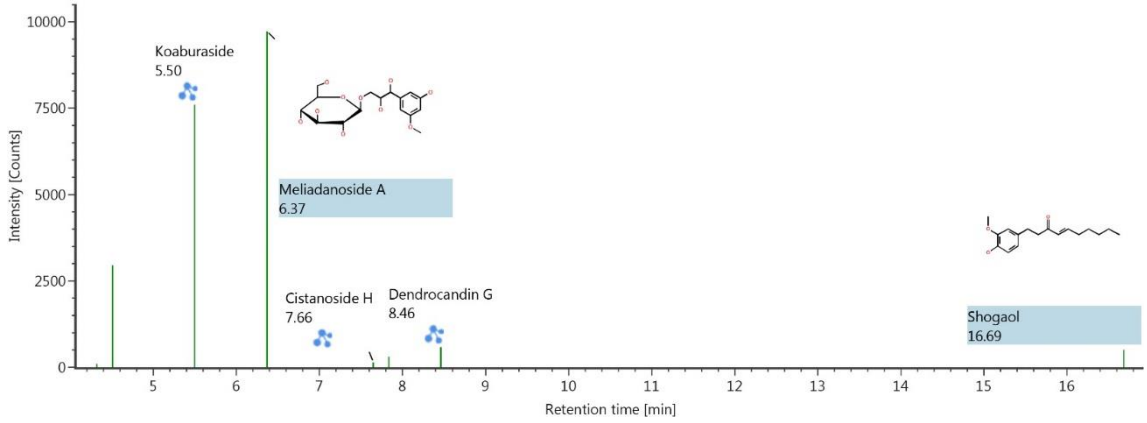
C. lacryma-jobi var. ma-yuen (CMY2)



C. lacryma-jobi var. ma-yuen (CMY3)



C. lacryma-jobi var agrotis (CA)



C. lacryma-jobi var. lacryma-jobi (CLJ)

Figure 2: Chemical component identified by UPLC/QTOF-MS in Coix seeds water extract

3.4 Multivariate analysis

Exploratory analyses, such as Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA), were employed to assess nutritional compositions, multielement content, and phenolic compounds. PCA, functioning as an exploratory technique, offers a comprehensive overview of the analyses. It enables the derivation of significant conclusions for potential decision-making based on observed outcomes, effectively reducing data set dimensionality. This results in a statistical analysis involving fewer variables while retaining critical information. Given the extensive data points, PCA was utilized as a multivariate statistical tool to evaluate outcomes across diverse variables.

The biplot obtained from PCA is shown in Figure 3. It is shown that the extracts are mainly distributed into three groups: group I–III. Two components, PC-1 and PC-2 showed, accounted for 63.73% of the total variance. From the scores plot, group I was in the positive PC-1 axis while groups II and III were in the negative PC-1 axis. Coix *C. lacryma-jobi var. ma-yuen* (Origin 1) and *C. lacryma-jobi var. ma-yuen* (Origin 2) in a group I, *C. lacryma-jobi var.*

agrotis and *C. lacryma-jobi var. lacryma-jobi* in a group II while *C. lacryma-jobi var. ma-yuen* (Origin 3) in Group III. Based on the result, those samples with similar chemical profiles were grouped near to each other, while a larger distance was observed in samples with different chemical profiles. To identify the variables responsible for significant differences between various extracts, correlation loading plots for PC-1 and PC-2 were generated. These plots reveal that Coix Thailand and *C. lacryma-jobi var. ma-yuen* (Origin 1) exhibit moderate levels of total fat, energy, and calcium, whereas *C. lacryma-jobi var. lacryma-jobi* has a lower content of these nutritional components. *C. lacryma-jobi var. ma-yuen* (Origin 3) contains the majority of the phenolic compounds. Additionally, *C. lacryma-jobi var. agrotis* and *C. lacryma-jobi var. lacryma-jobi* display moderate levels of carbohydrate, crude fiber, 3,7-dihydroxy-2,4-dimethoxyphenanthrene-3-O-glucoside, cistanoside H, and dendrocandin G.

Table 3: Phenolic compounds identified in different varieties of Coix seeds water extract

Component name	CMY(1)	CMY(2)	CMY(3)	CA	CLJ
(+)-threo-Guaiacylglycerol	-	84.0 ± 1.3	76.5 ± 0.3	-	-
1,7-Bis(4-hydroxyphenyl)-hepta-4E,6E-dien-3-one	-	-	114.0 ± 1.5	-	-
10-O-Methylprotosappanin B	-	-	190.5 ± 0.5	-	-
2,6-Bis(4-hydroxyphenyl)-3′,5-dimethoxy-3-hydroxybibenzyl	-	-	523.5 ± 0.3	-	-
2,7-Dihydroxy-1-(p-hydroxybenzyl)-4-methoxy-9,10-dihydrophenanthrene	-	-	1924.0 ± 0.2	-	-
3,4-Dihydroxyphenothyl-3-*O*-β-D-glucopyranoside	135.0 ± 2.5	288.0 ± 0.3	3078.5 ± 0.1	-	2946.5 ± 4.5
3,7-Dihydroxy-2,4-dimethoxyphenanthrene-3-*O*-glucoside	-	-	-	135.0 ± 0.5	87.0 ± 0.6
Albaspidin AA	-	-	418.5 ± 0.1	-	-
Asebotin	-	-	77.5 ± 1.0	-	-
Blestriarene B	-	-	117.0 ± 0.5	-	300.5 ± 0.7
Cimidahurine	-	117.5 ± 0.3	273.5 ± 0.3	-	-
Cistanoside H	-	-	-	-	133.0 ± 0.2
Dendrocandin G	-	-	-	-	566.0 ±0.5
Isomucronustyrene	-	-	1307.0 ± 0.1	-	-
Koaburaside	1013.0 ± 0.5	1005.0 ± 0.5	9552.0 ± 0.1	-	7599.5 ± 1.5
Kuwanon P		-	249.5 ± 2.5	-	-
Meliadanoside A	822.5 ± 0.3	2269.0 ± 0.1	10376.0 ± 4.5	-	9725.5 ± 0.5
Mulberrofuran A	-	-	84344.5 ± 6.5	-	-
Nobilin B	-	-	278.0 ± 0.5	-	-
Salidroside	-	-	3893.0 ± 0.5	-	-
Shogaol	332.0 ± 0.4	806.5 ± 1.5	453.0 ± 0.3	354.5 ± 0.3	508.5 ± 0.4
Stilbostemin B	94.5 ± 1.3	229.0 ± 4.5	173.5 ± 3.0	75.0 ± 0.2	-
Tachioside	-	-	485.5 ± 0.5	-	-

Total samples (n=3). Data presented as mean ± standard errors. Values are significantly different by Duncan's test ($p < 0.05$).

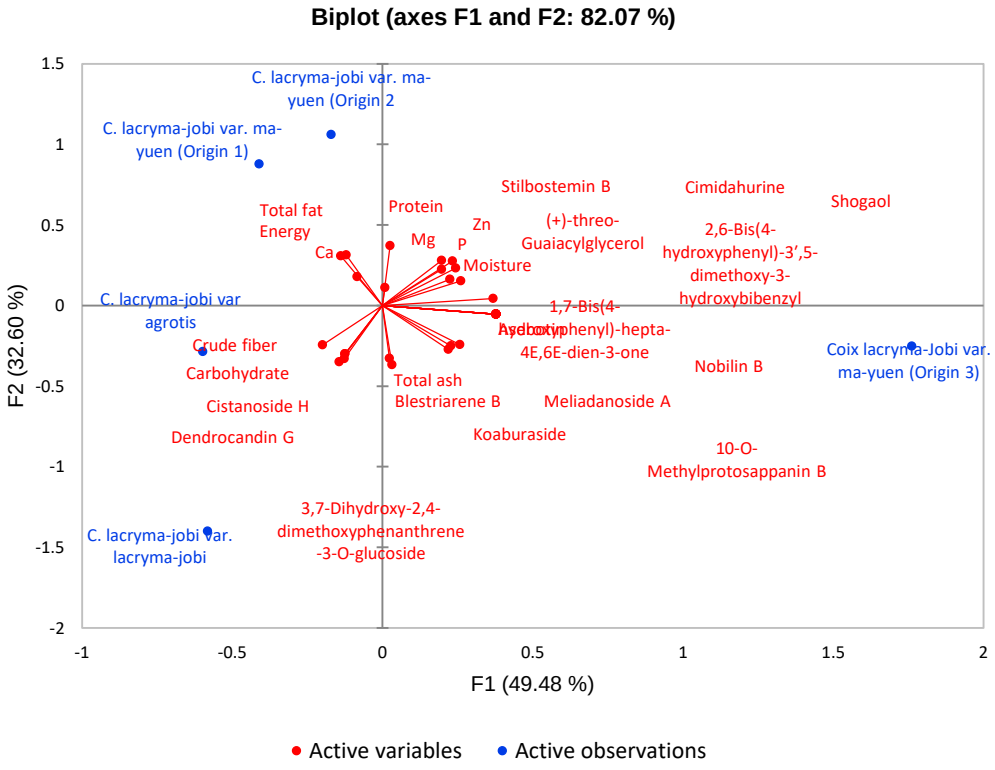


Figure 3. PC-1 and PC-2 biplot chart of Coix seeds

This variation illustrates that plants possess inherent chemical traits, leading to differences in concentrations even among samples of the same species due to factors such as climate, soil composition, and harvest timing (26). To assess the contributions of these factors to the overall variability in phenolic compounds, we employed two analytical techniques: Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA). HCA evaluates data based on their similarities and differences, effectively grouping them according to their degrees of resemblance (27). The trends in correlations identified through PCA were further validated by constructing a dendrogram using HCA. For this purpose, Ward's method was used to minimize variance within each cluster, while Euclidean distances quantified the similarities between different samples.

In today's technology-driven and fast-paced environment, trade globalization and the rapid evolution of e-commerce have led to increased product returns. This has heightened customer expectations for well-organized and efficient logistics, particularly in reverse logistics, making its role even more critical.

The HCA dendrogram shows that the samples were separated into three groups: in group I (in red) formed by *C. lacryma-jobi var. ma-yuen* from Origin 1 and Origin 2, while in group II (in blue) were *C. lacryma-jobi var. lacryma-jobi* and *C. lacryma-jobi var. agrotis* and group III (in blue) was *C. lacryma-jobi var. ma-yuen* from Origin 3. Analysis of the dendrogram (Figure 4) shows the same classification as in the PCA result.

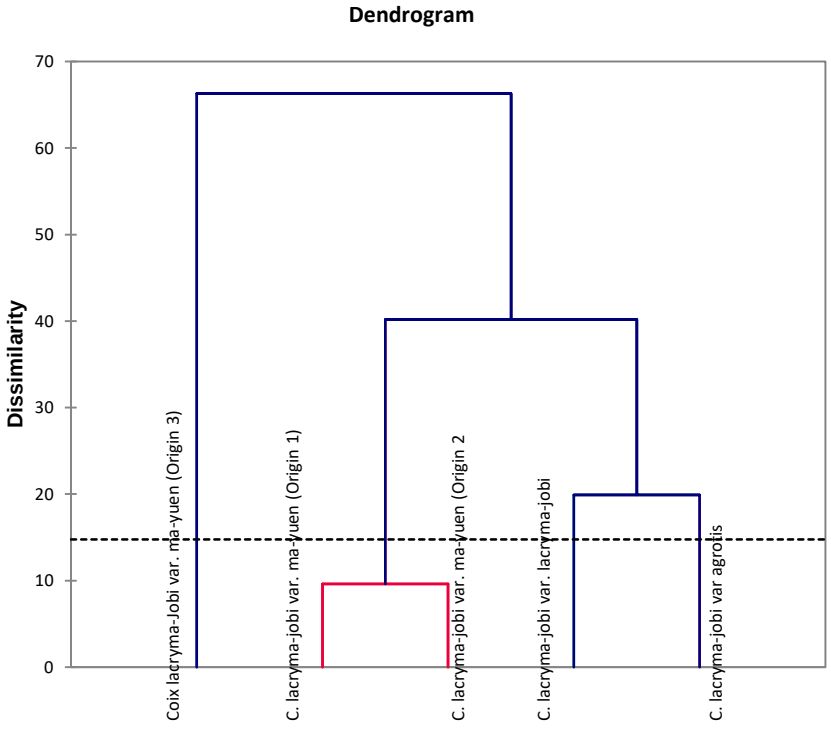


Figure 4. Dendrogram obtained from the analyzed Coix samples

4 CONCLUSIONS

In summary, the proposed methods for assessing nutritional content and microelements using ICP-OES, along with bioactive compound analysis through UPLC/QTOF MS and multivariate analysis, offer a robust framework for comprehensive component analysis of diverse Coix samples from different countries. This approach is highly relevant for studying Coix and has the potential to be applied to other edible plants as well. By addressing a gap in the existing literature on Coix seeds, this study provides valuable insights to the food composition database.

The findings reveal significant variation in the types and relative quantities of chemical constituents within Coix seeds. This establishes a foundation for broader applications, including setting quality standards and effectively utilizing these seeds. Notably, the presence of essential nutrients, potentially hazardous elements, and phenolic compounds in Coix seed samples suggests their potential for medicinal and edible purposes across various plant parts. This encourages further exploration into plant metabolic processes.

Data availability

All data generated or analyzed during this study are included in this published article.

Author Contribution statement

Izzah Hayati Yahya : Data collection and analysis. Hazrulrizawati Abd Hamid : Concept idea and review the manuscript. Ade Chandra Iwansyah: Preparation of sample and review of the manuscript

Conflict of Interest Declaration

The authors declared that there are no competing interests as defined by Nature Research, or other interests that might be perceived to influence the results and/or

discussion reported in this paper

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تقييم التركيب التقريبي والمحتوى الفينولي متعدد العناصر والفعال بيولوجيًا لأصناف مختلفة من بذور Coix باستخدام تقنيات التحليل متعدد المتغيرات

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ملخص

Coix lacryma-jobi L. هو نبات يعمل كمصدر للغذاء والدواء ومستحضرات التجميل والأعلاف في البلدان الآسيوية. نظرًا للبيئة الجغرافية المتميزة، فإن موارد الأصول الوراثية لبذور Coix واسعة للغاية ومتنوعة. في هذه الدراسة، تم تقييم التركيب التقريبي والعناصر المتعددة بواسطة ICP-OES والفينول الحيوي النشط بواسطة UPLC-QTOF/MS لخمسة أنواع من بذور Coix من دول آسيوية مختلفة. تم استخدام تحليل المكون الرئيسي (PCA) وتحليل المجموعات الهرمية (HCA) لتصنيف أنواع مختلفة من بذور Coix. ج. لاكريما جوبي فار. تحتوي بذور ma-yuen من الأصل 1 والأصل 2 على نسبة عالية من الطاقة والدهون الكلية والكالسيوم، في حين تحتوي بذور C. lacryma-jobi var. agrotis و C. lacryma-jobi var. يحتوي لاكريما جوبي على نسبة عالية من الألياف الخام والكربوهيدرات. تم تحديد ثلاثة وعشرون مركباً فينولياً. كانت البروتينات والكربوهيدرات والألياف الخام والمغنيسيوم والزنك والميليادانوسيد و4،3–ثنائي هيدروكسي فينوثيل–3–O-β-D-glucopyranoside هي المتغيرات السائدة وساهمت في أكبر تباين في البيانات في PCA. تحليل HCA يظهر نفس التصنيف كما في نتيجة PCA، حيث تم فصل العينات إلى ثلاث مجموعات، والتي كانت غنية بالمواد المغذية، الفينولات وخليط العناصر الغذائية مع الفينولات. تساهم هذه الدراسة في التطوير العلمي واستخدام أصناف مختلفة من بذور الكويكس.

الكلمات الدالة: كويكس لاكريما–جوبي، بذور جوز الهند، المحتوى المعدني، التركيب الغذائي، مركبات فينوليه.

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Nanomedicine Advancements in Cancer Therapy: A Scientific Review

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ABSTRACT

Cancer nanomedicines, characterized by submicrometer-sized formulations, aim to optimize the biodistribution of anticancer drugs by minimizing off-target effects, reducing toxicity, enhancing target site accumulation, and improving overall efficacy. Numerous nanomedicines have been developed to improve the effectiveness and safety of traditional anticancer treatments. These include formulations with carbon nanotubes, nanodiamonds, enzyme-responsive nanoparticles for controlled drug release, dendrimers as nanoparticle drug carriers, quantum dot nanocarrier systems for precise drug delivery, solid lipid nanoparticles, and polymeric nanoparticles designed for targeted drug delivery. Additionally, nanotechnology has been explored in cancer treatment through gene therapy. Despite these advances, the complex nature of carrier materials and functional integration presents challenges in preparing these candidates for clinical translation. Nanotechnology, with its unique features at the nanoscale, offers novel possibilities for developing cancer therapies while increasing efficacy and safety. Although only a few nanotherapeutics have obtained clinical approval, exciting uses for nanotechnology are on the horizon. Nanoparticles possess unique transport, biological, optical, magnetic, electrical, and thermal capabilities due to their small size within the light wavelength spectrum. This results in high surface area-to-volume ratios, allowing for the incorporation of various supporting components in addition to active medicinal substances. These properties aid in solubilization, degradation protection, delayed release, immune response evasion, tissue penetration, imaging, targeted distribution, and triggered activation. In summary, the future of nanomedicine holds promise for introducing innovative platforms in cancer treatment. The research presented underscores the potential for nanoparticles to revolutionize anticancer therapies, enhancing the overall therapeutic approach.

Keywords: Nanoparticles; Anticancer therapy; Carbon-Based Nanomaterials; Metal-Based; Lipid-Based; Polymeric Nanoparticles.

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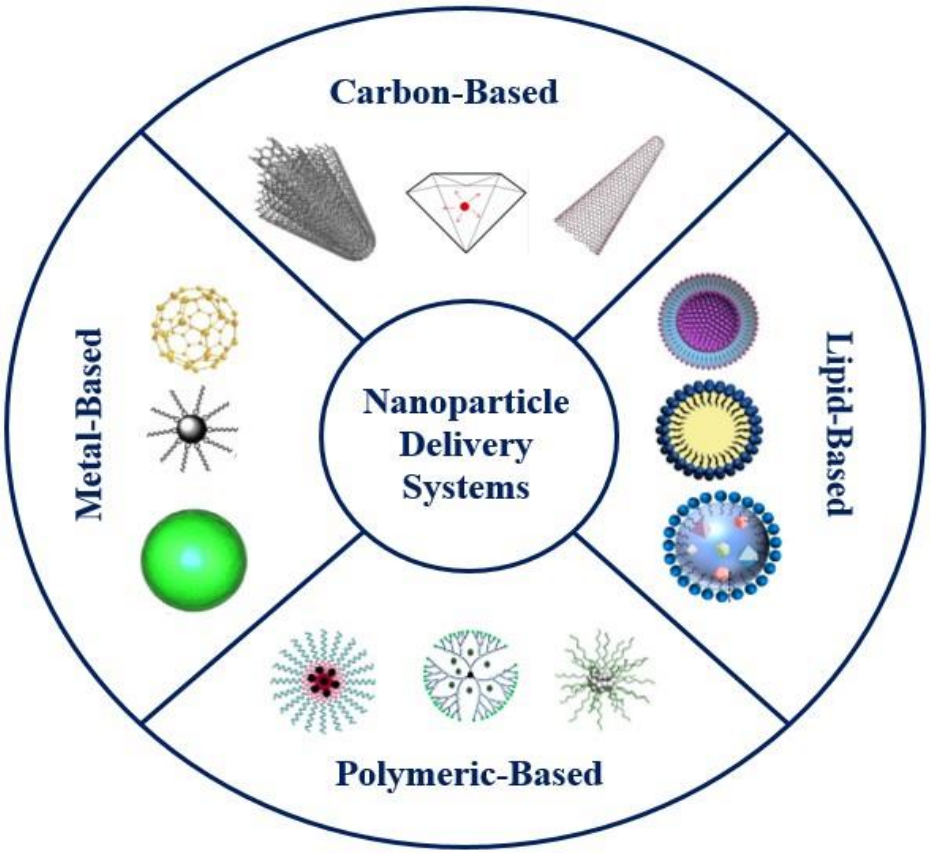

Graphical abstract

INTRODUCTION

In 2023, the United States was expected to have 1,958,310 new cancer cases and 609,820 cancer deaths [1]. Cancer treatment options include surgeries, radiation therapy, chemotherapy, immunotherapy, and targeted therapy. Surgical interventions remove cancerous tissue, radiation therapy eliminates cells, chemotherapy eradicates cells, immunotherapy boosts the immune system, and targeted therapy addresses genetic alterations. Chemotherapeutic agents are a crucial component of the diverse arsenal of cancer treatments [2,3].

Cancer treatment faces challenges such as severe side effects, drug resistance, and high economic burdens [4]. Nanotechnology, a multidisciplinary field combining chemistry, engineering, biology, and medicine, has emerged as a promising frontier in cancer research. Nanometer-sized nanoparticles interact with biomolecules on cell surfaces and inside cells, enabling effective and targeted medication delivery [5]. Several types of nanoparticles, including quantum dots, carbon nanotubes, liposomes, and gold nanoparticles, have shown promise in detecting and treating various cancers [6,7]. Recent advancements, such as bioaffinity nanoparticle probes and integrated nanodevices, hold significant potential for personalized oncology based on individual patients' molecular profiles [8] (Figure 1).

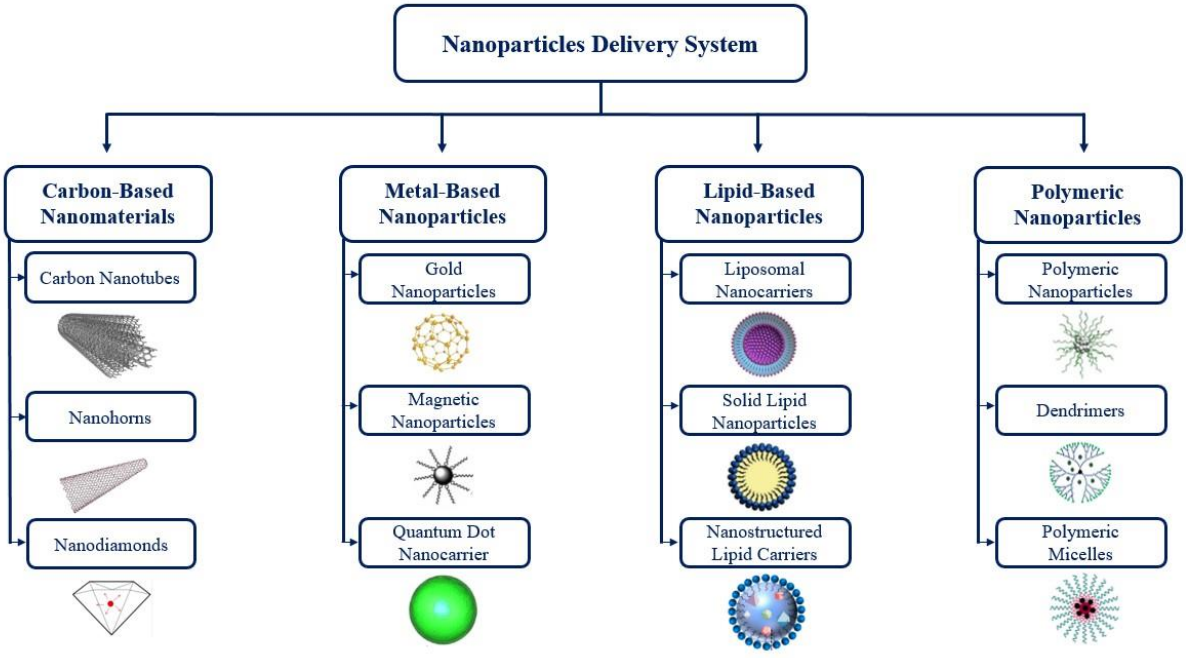

Figure 1: Comprehensive overview of nanotechnologies in cancer treatment.

While nanotechnology presents excellent prospects for cancer therapy, issues such as biocompatibility, in vivo dynamics, tumor-targeting efficiency, and cost-effectiveness must be resolved before broad clinical application [9]. Despite these challenges, the emergence of nanotechnology-based techniques offers promise for transforming cancer research by opening new paths for diagnostics and therapies [10,11]. This review traces the various nanotechnology-based cancer treatment options**Carbon-Based Nanomaterials**

Carbon-based nanostructured materials, such as nanotubes, nanohorns, and nanodiamonds, offer significant benefits in cancer treatment due to their small size and hybridized carbon atoms [12]. These materials facilitate easy functionalization, promote biocompatibility, and feature efficient drug transport, imaging, and controlled release mechanisms [12]. They also exhibit high in vivo stability, a large surface area for functionalization, and ease of penetration through biological barriers [13]. However, challenges like biocompatibility, toxicity evaluation, and regulatory hurdles remain [14]. Further research is needed to fully realize the potential of these nanoparticles in cancer care [15].

1. Nanotubes

Carbon nanotubes, with their unique optical properties, have gained popularity in cancer therapy due to their ability to convert light into heat [16]. This localized heat treatment enhances therapeutic effects and tumor specificity for nanoscale carbon catalysts [17]. The use of target-specific delivery systems in nanomedicine has redefined the field. Carbon nanotubes can be functionalized with various groups, allowing for more efficient delivery of medicines to cancerous cells [18]. They are classified into single-walled, double-walled, and multi-walled carbon nanotubes [19] (Figure 2).

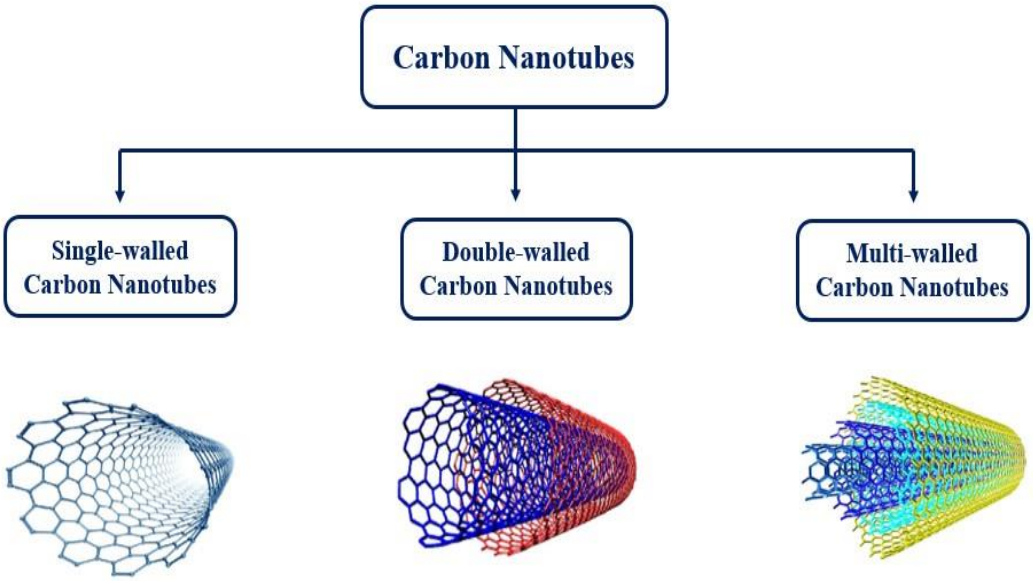

Figure 2: Classification of carbon nanotubes.

Carbon nanotubes possess various fascinating features due to their compact size and tubular form. Their electrical characteristics vary significantly between single-walled carbon nanotubes, which have a diameter of approximately 1 nm and a single graphene wall, and multi-walled carbon nanotubes, which have diameters ranging from 1 to 100 nm and multiple graphene walls [20]. These nanotubes exhibit physical and chemical characteristics such as a high aspect ratio, ultralight weight, exceptional mechanical strength, heightened electrical conductivity, and elevated thermal conductivity [21].

Carbon nanotubes play a crucial role in cancer research due to their cylindrical shapes, which are similar to rolled graphene sheets, and their excellent mechanical strength, strong electrical conductivity, and considerable thermal conductivity [20]. They serve as carriers for targeted drug delivery and imaging agents for diagnostics. Recently, carbon nanotubes have been extensively researched in various cancer treatment techniques, including drug administration, lymphatic-targeted chemotherapy, thermal therapy, photodynamic therapy, and gene therapy [21].

Despite their promising attributes, rigorous investigation into toxicity and biocompatibility is imperative before their widespread clinical use. Regulatory challenges must be addressed to ensure the safe and practical application of nanotubes in cancer therapeutics [6]. Carbon nanotubes have recently emerged as intriguing medical agents, indicating potential advancements [22]. In vitro studies have shown that multi-walled carbon nanotubes can facilitate the enzymatic cleavage-based release of the anticancer medication methotrexate in breast cells, and dendrimer-modified multi-walled carbon nanotubes can effectively deliver doxorubicin [23]. Single-walled carbon nanotubes have demonstrated efficacy as low-toxicity carriers for drug delivery in lung cancer treatment [6].

2. Nanohorns

Carbon nanohorns are a family of carbon nanomaterials with a unique ability to adsorb various molecules, making them promising candidates for controlled drug release applications [24]. They have a distinctive hexagonal stacking structure, resulting in

microporosity and mesoporosity. Structural modifications, achieved through oxidation, introduce nanoscale windows on the walls of single-walled carbon nanohorns, controlling size and concentration while enhancing microporosity and inducing mesopores [25]. The conical structure influences the electronic properties of single-walled carbon nanohorns, demonstrating semiconductor behavior depending on oxidation status and gas adsorption [26]. Carbon nanohorns exhibit unique magnetic characteristics, including temperature-activated paramagnetic susceptibility and antiferromagnetic correlations between localized electrons [26]. They can be functionalized through covalent bonding, π-π stacking, and metal nanoparticle decoration, enhancing their compatibility and enabling a wide range of applications [26].

Single-walled carbon nanohorns are versatile in cancer therapy, acting as potent anticancer nanoparticles that induce apoptosis [27]. They serve as effective drug delivery systems for chemotherapeutics, allowing controlled release and minimizing dosage. They can also be used in photothermal, photodynamic, and gene therapies, contributing to cancer diagnosis through immunosensing that targets specific biomarkers [27]. A water-dispersible nanohybrid, created by integrating carbon nanohorns with polyglycerol-gold, has been developed to release doxorubicin, enhancing cell apoptosis and tumor observation [28].

3. Nanodiamonds

Nanodiamonds are a promising platform for theranostic applications due to their ease of synthesis, small size, inertness, surface functional groups, biocompatibility, stable fluorescence, and long fluorescent lifetime [29]. These properties have accelerated their use in cancer therapy and imaging, emphasizing the rational tailoring of particle surfaces to deliver bioactive chemicals, resist aggregation, and form composite materials [30]. Nanodiamonds can be artificially manufactured using detonation, chemical vapor deposition, or high-temperature, high-pressure techniques [31]. They exhibit strong fluorescence with minimal toxicity, making them promising for drug delivery systems, fluorescent bio-labels, and multimodal theranostic systems [32]. Additionally, they are cost-effective and can be sourced from mining waste, making them economically viable for diverse biomedical applications. Nanodiamonds can traverse the blood-brain barrier, making them potential carriers for brain-targeted drug delivery [33]. They also exhibit enhanced absorption when administered from the basolateral side of cells, which is particularly beneficial for specific cell types [34].

Table 1 provides a comprehensive overview of carbon-based nanomaterials and their applications in cancer therapy.

Table 1 presents a list of carbon-based nanomaterials used in cancer therapy, including nanotubes, nanohorns, and nanodiamonds. Nanotubes, including single-walled, double-walled, and multi-walled carbon nanotubes, have unique cylindrical structures with exceptional mechanical strength, electrical, and thermal conductivity. They are utilized in drug delivery, thermal therapy, photodynamic therapy, and gene therapy. Nanohorns, conical horn-shaped nanostructures with controlled size, increased porosity, semiconductor behavior, and temperature-activated magnetic features, are used in anticancer drug delivery, targeted chemotherapy, photothermal and photodynamic cancer therapy, as well as cancer gene therapy and immunosensing. Nanodiamonds, including those produced by detonation and fluorescent nanodiamonds, are chemically stable, biocompatible, extremely hard, transparent, and highly thermally conductive. They are applied in various cancer treatments.

Table 1: A comprehensive summary of carbon-based nanomaterials and their applications in cancer therapy

Carbon-based nanomaterials	Type	Size	Structure	Characteristics	Preparation Method	Applications in Cancer	References
Nanotubes	▪ Single-walled carbon nanotubes	▪ < 1 nm in diameter	▪ Single cylindrical layer of carbon atoms arranged hexagonally.	▪ Exceptional mechanical strength. ▪ High electrical conductivity. ▪ Significant thermal conductivity.	▪ Arc discharge method ▪ Laser ablation method ▪ Chemical vapor deposition method ▪ Gas-phase catalytic method	▪ Anticancer drug delivery ▪ Lymphatic targeting chemotherapy ▪ Thermal therapy ▪ Photodynamic therapy ▪ Gene therapy	[35–39]
	▪ Double-walled carbon nanotubes	▪ 1-50 nm in diameter	▪ Two layers of graphene sheets rolled into cylindrical structures.	▪ Enhanced structural stability compared to SWCNTs. ▪ Combined properties of both inner and outer walls.			
	▪ Multi-walled carbon nanotubes	▪ 1-100 nm in diameter	▪ Multiple layers of graphene sheets arranged concentrically.	▪ Increased mechanical strength compared to SWCNTs. ▪ Enhanced thermal and electrical conductivity.			
Nanohorns	Single-walled carbon nanohorns	▪ 2–5 nm in diameter	▪ Conical horn-shaped nanostructures.	▪ Have unique internal and external pores. ▪ Controlled size and increasing porosity. ▪ Show semiconductor behavior based on their conical structure. ▪ Display temperature-activated magnetic features.	▪ CO_2 laser ablation method ▪ Arc discharge method	▪ Anticancer drug delivery ▪ Targeted chemotherapy ▪ Photothermal and Photodynamic Cancer Therapy ▪ Cancer Gene Therapy and Immunosensing	[40–42]
Nanodiamonds	▪ Detonation nanodiamonds ▪ Fluorescent nanodiamonds	1-100 nm in diameter	▪ Diamond-like crystalline structure on a nanoscale, which consists of carbon atoms arranged in a tetrahedral lattice.	▪ Chemically stable and resist reactions. ▪ Biocompatible. ▪ Extremely hard, ideal for industrial tools. ▪ Transparent. ▪ High thermal conductivity.	▪ High-pressure, high-temperature method ▪ Detonation method	▪ Anticancer drug delivery ▪ Targeted chemotherapy ▪ Photothermal and Photodynamic Cancer Therapy ▪ Cancer Gene Therapy and Immunosensing	[35,43–46]

Metal-Based Nanoparticles

Metallic nanoparticles, derived from noble metals such as gold, silver, and platinum, are increasingly studied for their potential applications in fields such as catalysis, polymer composites, disease diagnosis, sensor technology, and optoelectronic media labeling [47]. These nanoparticles are produced and stabilized using various techniques, which impact their morphology, stability, and physicochemical characteristics [48]. Metal-based nanoparticles, made of elements like gold, silver, iron, and platinum, exhibit unique physical and chemical properties due to their small size and enhanced surface area [49]. Besides their use in medicine, they are also utilized in electronics, catalysis, and environmental remediation. Metal-based nanoparticles offer targeted drug delivery, early cancer detection, and medicinal properties [50]. However, challenges include potential toxicity, biodistribution issues, uncertain immunological responses, production cost concerns, and regulatory approvals [51]. Table 2 comprehensively summarizes metal-based nanomaterials and their applications in cancer therapy.

Table 2: A comprehensive summary of metal-based nanomaterials and their applications in cancer therapy.

Metal-based nanomaterials	Size	Structure	Characteristics	Preparation Method	Applications in Cancer	References
Gold nanoparticles	▪ 5-100 nm	▪ Diverse structures, such as spheres, rods, cages, and tubes.	▪ Precise control over size and shape. ▪ Color and optical features. ▪ Fluorescence modulation. ▪ Electromagnetic field. ▪ Surface plasmon resonance and high surface area.	▪ The sol-gel micro reactors method ▪ Physical vapor deposition method ▪ Reduction method ▪ γ-Irradiation v ▪ Biosynthesis method	▪ Anticancer drug delivery ▪ Targeted chemotherapy ▪ Tumor imaging ▪ Tumor radio sensitization ▪ Tumor hyperthermia ▪ Tumor gene therapy	[52–55]
Magnetic nanoparticles	1-100 nm	▪ Diverse such as core-shell, monodisperse, composite, hollow, and clustered.	▪ Can be composed of pure metals. ▪ Offer versatile and safe theranostic properties. ▪ Easy synthesis and modification. ▪ Possess intrinsic magnetic features.	▪ Co-precipitation method ▪ Microemulsion method ▪ High-temperature method ▪ Hydrothermal method ▪ Sonochemical method	▪ Anticancer drug delivery ▪ Targeted chemotherapy ▪ Tumor imaging ▪ Tumor biosensors ▪ Tumor hyperthermia	[56–59]
Quantum dot nanocarrier	2-10 nm	▪ Core-shell design.	▪ Resist degradation for extended cellular tracking. ▪ 10–20x brighter and more stable than organic dyes. ▪ Stable fluorophore. ▪ High drug loading ▪ Chemically inert imaging ▪ Dual drug encapsulation	▪ Microemulsion method ▪ Sol-gel method ▪ Hotsolution decomposition method ▪ Hot-arrangement decay method ▪ Molecular beam epitaxy method ▪ Physical vapor deposition method ▪ Chemical vapor deposition method ▪ Physical vapor deposition method ▪ Chemical vapor deposition method ▪ Green method	▪ Anticancer drug delivery ▪ Tumor imaging ▪ Tumor diagnosis	[60–65]

Table 2 presents a summary of metal-based nanomaterials and their applications in cancer therapy. Gold nanoparticles, sized between 5-100 nm, offer precise control over size and shape, color and optical features, fluorescence modulation, electromagnetic fields, surface plasmon resonance, and high surface area. They are utilized in anticancer drug delivery, targeted chemotherapy, tumor imaging, radiosensitization, tumor hyperthermia, and tumor gene therapy. Magnetic nanoparticles, ranging from 1-100 nm, exhibit diverse structures and offer versatile and safe theranostic properties. Composed of pure metals, they are easy to synthesize and modify. Quantum dot nanocarriers, sized between 2-10 nm, feature a core-shell design, resist degradation, and are 10-20 times brighter and more stable than organic dyes. They possess stable fluorophores, high drug-loading capacity, chemically inert imaging properties, and dual drug encapsulation capabilities. These are used in anticancer drug delivery, tumor imaging, and tumor diagnosis.

1. Gold Nanoparticles

Gold nanoparticles are emerging as potent tools in cancer therapy, offering a multifaceted approach to anticancer treatment [66]. They are gaining popularity in cancer management due to their advantageous properties, including cytotoxicity against specific cancer cells, size-dependent inhibition, and tunable optical properties [67]. Due to their controlled synthesis, gold nanoparticles are also valuable in bioimaging, theranostics, and cancer treatment. Their unique physical and chemical characteristics, influenced by their diverse shapes and sizes, contribute to their versatility [68]. Recent studies highlight the impact of size, surface charge, and functional groups on cytotoxicity, making careful consideration essential for their safe and effective use in biomedical applications, particularly in cancer management [69]. Malaikolundhan et al. synthesized gold nanoparticles using Albizia lebbeck aqueous leaf extract, demonstrating promising therapeutic effects against colon cancer cells [70]. Wang et al. developed paclitaxel-conjugated gold nanoparticles, focusing on the positioning of small molecular drugs within nanoparticles [71]. This two-step drug release process emphasizes the promise of paclitaxel-gold nanoparticles as a novel method of cancer therapy [72].

2. Magnetic Nanoparticles

Magnetic nanoparticles, composed of materials like iron, cobalt, or nickel, are tiny particles with unique physical and chemical characteristics [73]. They have gained attention in fields like medicine, electronics, and environmental science due to their versatile applications [74]. They are used in biomedical applications such as Magnetic Resonance Imaging (MRI), drug delivery, data storage, sensors, and information storage, as well as in antiferromagnetic and paramagnetic nanoparticles [75]. Their magnetic manipulation through an external field provides a key advantage, and their chemical composition, size, shape, morphology, and magnetic behavior are pivotal in determining their biomedical applications [76]. Magnetic nanoparticles are a promising basis for a multimodal theranostic platform in biomedical applications. Ursachi et al. synthesized nanocomposites with a magnetic core for precise targeting, a polymeric surface shell for stability and multifunctionality, and the chemotherapeutic agent paclitaxel [77].

3. Quantum Dot Nanocarrier

Quantum dots, highly fluorescent nanocrystals, show potential in biomedical applications, particularly in cancer screening, tumor classification, and imaging, with advancements in technology enabling multifunctional probes [60]. Quantum dot nanocarriers, utilizing semiconductor nanoparticles' electronic and optical properties, offer precise drug delivery through a core-shell architecture, using various synthesis methods for design flexibility [61]. They offer high drug loading capacity, efficient surface area, targeted delivery, real-time imaging, biocompatibility, precise control, simultaneous drug delivery, and reduced side effects [64]. Challenges in quantum dot materials include potential toxicity risks, biocompatibility studies, hazardous handling, and long-term stability concerns, necessitating careful handling and consideration of potential degradation over time [62].

Quantum dot nanocarriers offer targeted cancer treatment, real-time monitoring, and combination therapy, enhancing efficacy and utilizing quantum dots' unique properties for personalized therapeutic strategies [63]. Li et al. developed nanocarriers for precise nucleus-targeted anticancer drug delivery and real-time imaging. These nanocarriers combine an enzyme-activatable peptide with mesoporous silica-coated quantum dots, enhancing antitumor activity and demonstrating superior efficacy [78]. Rezaei et al. developed a pseudohomogeneous carbon-based vehicle, chitosan-citric acid-arginine-carbon quantum dots, for efficient gene transfer into cells. This carboplex, resistant to enzyme destruction, outperforms chitosan and enables more efficient gene transfection [79].

Lipid-Based Nanoparticles

Lipid-based nanoparticles are advanced drug delivery systems for targeted therapeutic agent encapsulation, offering versatile, biocompatible platforms in various forms such as liposomes, solid nanoparticles, and nanostructured carriers [80,81]. Prepared using techniques like solvent evaporation, homogenization, and microemulsion, these nanoparticles provide high drug encapsulation efficiency, controlled release, and biocompatibility for therapeutic applications [82,83]. They show promise in cancer treatment, enhancing targeted drug delivery and minimizing systemic toxicity, thus contributing to personalized cancer therapies and advancements in oncology [84,85]. Table 3 comprehensively summarizes lipid-based nanoparticles and their applications in cancer therapy.

Table 3 presents lipid-based nanomaterials and their applications in cancer therapy. Liposomal nanocarriers, ranging from 50 to 1000 nm in size, offer high drug loading capacity, stability, and biocompatibility, mimicking natural lipid membrane structures. They can be prepared using various methods, including thin-film hydration, detergent removal, solvent injection, ethanol injection, ether injection, reverse-phase evaporation, sonication, extrusion, high-pressure homogenization, freeze-drying, supercritical reverse, microfluidic methods, and membrane contactor methods. Solid-lipid nanoparticles, sized between 50 and 1000 nm, offer controlled release and targeting capabilities with low toxicity and protection for labile drugs. They are formulated without organic solvents, allowing for flexible sterilization and versatile encapsulation. Nanostructured lipid carriers, ranging from 10 to 1000 nm in size, offer controlled release and targeting with excellent biocompatibility. They are easy to scale up and sterilize, can be formulated without organic solvents, and offer versatile encapsulation.

1.Liposomal Nanocarriers

Liposomal nanocarriers are advanced drug delivery systems with spherical structures, a hydrophilic core, and a bilayer of phospholipids, forming spontaneously when lipids are hydrated in aqueous environments [45]. Liposomes, composed of synthetic or natural phospholipids, arise spontaneously from water molecules and hydrophobic phosphate groups and are loaded with pharmaceuticals through various techniques [86]. Liposomal nanoformulations, ranging from 50-500 nm, are crucial for drug delivery in biomedical applications, with tiny, giant, and multilamellar types facilitating efficient cell uptake and tissue penetration [88]. Liposomes, with their stable, biocompatible, and degradable structure, play a crucial role in encapsulating hydrophilic drugs and influencing their pharmacokinetics and biodistribution [87, 95]. With cancer treatment approvals, liposomes offer efficient drug delivery, protection, improved bioavailability, and reduced side effects. Challenges include rapid clearance and stimulus-sensitive structures [83]. Zarrabi et al. developed intelligent biocompatible stealth nanoliposomes for targeted curcumin delivery, showing high drug entrapment efficiency and controlled release patterns. These liposomes hold promise for cancer therapy but require further validation and clinical trials [96]. Ghafari et al. developed nanoliposomes containing cisplatin, enhancing its efficacy and mitigating side effects. The modified

formulations showed improved cellular absorption and cytotoxicity, offering the potential for improved therapeutic efficacy [97].

Table 3: A comprehensive summary of lipid-based nanomaterials and their applications in cancer therapy.

Lipid-based nanomaterials	Size	Structure	Characteristics	Preparation Method	Applications in Cancer	References
Liposomal nanocarriers	▪50-1000 nm	▪Spherical	▪High drug loading ▪Stability and biocompatibility ▪Mimics natural lipid membrane structure ▪Avoids immune system ▪Amphipathicproperties liposomes can encapsulate hydrophilic and hydrophobic drugs.	▪Thin-film hydration method ▪Detergent removal method ▪Solvent injection method ▪Ethanol injection method ▪Ether injection method ▪Reverse-phase evaporation method ▪Sonication method ▪Extrusion method ▪High-pressure homogenization method ▪Freeze-drying method ▪Supercritical reverse method ▪Microfluidic methods ▪Membrane contactor method	▪Anticancer drug delivery ▪Targeted chemotherapy ▪Tumor diagnosis	[45,86–88]
Solid-lipid nanoparticles	▪50-1000 nm	▪Spherical	▪Controlled release and targeting ▪Low toxicity ▪Labile drug protection ▪Flexible sterilization ▪Formulated without organic solvents. ▪Versatile encapsulation ▪Reduced side effects	▪High shear homogenization ▪Hot homogenization ▪Cold homogenization ▪Ultrasonication method ▪Microemulsion method ▪Supercritical fluid method ▪Solvent evaporation method ▪Double emulsion method ▪Spray drying method	▪Anticancer drug delivery ▪Targeted chemotherapy ▪Tumor diagnosis	[89–91]
Nanostructured lipid carriers	▪ 10-1000 nm	▪Spherical	▪Controlled release and targeting Excellent biocompatibility ▪Easy to scaleup and sterilize ▪Formulated without organic solvents. ▪Versatile encapsulation	▪Hot homogenization ▪Cold homogenization ▪Microemulsion method ▪High-pressure homogenization ▪Solvent evaporation method ▪Phase inversion method ▪Ultrasonication method ▪Membrane contractor method	▪Anticancer drug delivery ▪Targeted chemotherapy ▪Tumor diagnosis	[92–94]

2. Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) are a submicron-sized drug delivery system composed of a solid lipid matrix, surfactants, and cosurfactants, ensuring controlled drug release and stability [89,90,98]. SLNs offer biocompatibility, biodegradability, and regulated medication release, making them promising for large-scale drug delivery systems and versatile for various routes [89,90]. SLNs show potential in cancer therapy by improving drug efficacy and overcoming the challenges of traditional chemotherapy [99]. They enhance cellular uptake, prolong drug circulation, and increase apoptosis induction [90]. Qureshi et al. developed docetaxel-incorporated lipid nanoparticles to improve their pharmacokinetic profile and solubility [100]. The nanotechnology template engineering technique demonstrated 96% incorporation efficiency and sustained release characteristics. The nanoparticles exhibited

increased anticancer activity and improved therapeutic outcomes in breast cancer treatment [100]. Smith et al. developed solid lipid nanoparticles to enhance the therapeutic effectiveness of 5-fluorouracil (5-FU) in colorectal cancer therapy [101]. The nanoparticles, loaded with unique PEGylated lipids and a surfactant mixture, showed lower IC50 values and increased tumor efficacy in HCT-116 cancer cells. This underscores the need for intelligent nano-delivery systems [101].

3. Nanostructured Lipid Carriers

Nanostructured lipid carriers, combining solid and liquid lipids, offer improved drug delivery and controlled release through high-pressure homogenization, solvent emulsification/evaporation, and microemulsion techniques [92]. Nanostructured lipid carriers are effective due to their biocompatibility, solvent-free preparation, cost-effectiveness, and controlled drug release, making them eco-friendly and cost-effective for mass production and sterilization [92]. They offer efficient drug delivery, versatility in transporting lipophilic and hydrophilic drugs, and biodegradability, making them a promising choice for environmental sustainability [93,102]. Nanostructured lipid carriers provide enhanced drug delivery, controlled release, and efficient transport in cancer treatment, outperforming complex formulation optimization and limited long-term stability for specific drugs [92,93]. Sun et al. developed biocompatible, biodegradable quercetin-nanostructured lipid carriers to improve water solubility, stability, and cellular bioavailability [103]. These carriers demonstrated increased cytotoxicity and apoptosis in breast cancer cells, indicating potential for chemoprevention [103]. Ferreira et al. optimized nanostructured lipid carriers as methotrexate carriers using hot ultrasonication [104]. The carriers exhibited robustness, a spherical shape, and 87% entrapment efficiency. They released methotrexate quickly and persistently without harming fibroblasts [104].

Polymeric Nanoparticles

Polymeric nanoparticles, composed of synthetic or natural polymers (Figure 3), offer customizable features in medication delivery systems and biocompatibility, making them safe and efficient for drug administration, as summarized in Table 4-A [105,106].

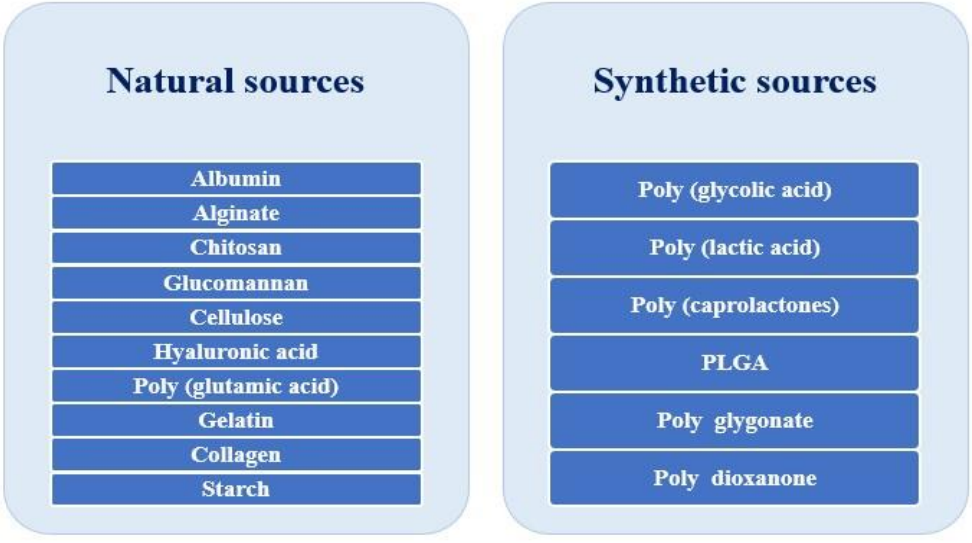

Figure 3: Commonly used polymers for cancer drug delivery.

Table 4-A: Comprehensive summary of polymeric-based nanoparticles and their applications in cancer therapy [105,106].

Table 4- B: A comprehensive summary of polymeric-based nanomaterials and their applications in cancer therapy.

Polymeric-based nanomaterials	Size	Structure	Characteristics	Preparation Method	Applications in Cancer	References
Polymeric nanoparticles	▪ 10-1000 nm	▪ Spherical, Rod-shaped, polyhedral, filamentous, star-shaped, and core-shell.	▪ Ease of surface modification ▪ Biocompatibility ▪ Versatility with natural and synthetic polymers. ▪ Enable controlled and targeted drug release ▪ High encapsulation efficiency ▪ Good stability ▪ Biodegradability ▪ The ease of surface modification ▪ Precise particle size control	▪ Solvent evaporation method ▪ Double emulsification method ▪ Emulsion diffusion method ▪ Nanoprecipitation method ▪ Coacervation method ▪ Salting out method ▪ Dialysis method ▪ Supercritical fluid method	▪ Anticancer drug delivery ▪ Targeted chemotherapy ▪ Cancer diagnosis and imaging	[107–109]
Dendrimers	1-100 nm	▪ Compact and globular structure.	▪ Hyperbranched subunits. ▪ High structural control. ▪ Well-defined architecture. ▪ Monodisperse with precise molecular weight, shape, and size. ▪ Surface functional groups for drug conjugation and inner cavities for drug entrapment. ▪ Lower glass temperatures.	▪ Divergent synthesis ▪ Convergent synthesis ▪ Hypercores and branched monomer growth ▪ Double exponential growth ▪ Lego chemistry ▪ Click chemistry	▪ Anticancer drug delivery ▪ Targeted chemotherapy ▪ Cancer diagnosis and imaging	[110–113]
Polymeric micelles	1-100 nm	▪ Spherical or globular shape, characterized by a core-shell structure	▪ Amphiphilic properties ▪ Hydrophobic core ▪ Size and shape control ▪ Enhanced drug solubility ▪ Extended blood circulation ▪ Biodistribution control ▪ Low toxicity and fast clearance ▪ No drug modification is needed	▪ Direct dissolution ▪ Solvent evaporation ▪ Dialysis	▪ Anticancer drug delivery ▪ Targeted chemotherapy ▪ Cancer diagnosis and imaging	[114–118]

Table 4-B presents a comprehensive overview of polymeric-based nanomaterials and their applications in cancer therapy. Polymeric nanoparticles, ranging from 10 to 1000 nm, have various structures and offer ease of surface modification, biocompatibility, and versatility. They enable controlled drug release with high encapsulation efficiency, stability, biodegradability, and precise particle size control. Preparation methods include solvent evaporation, double emulsification, emulsion diffusion, nanoprecipitation, coacervation, salting out, dialysis, and supercritical fluid methods. Dendrimers, sized between 1 and 100 nm, have a compact and globular structure with hyperbranched subunits. They offer high structural control, well-defined architecture, monodispersity, surface functional groups for drug conjugation, inner cavities for drug entrapment, and lower glass transition temperatures. They are used in anticancer drug delivery, targeted chemotherapy, and cancer diagnosis and imaging. Polymeric micelles, ranging from 1 to 100 nm, have a spherical or globular shape with a core-

shell structure. They possess amphiphilic properties, enabling enhanced drug solubility, extended blood circulation, biodistribution control, low toxicity, and fast clearance without drug modification.

Polymeric nanoparticles offer flexibility in medication delivery systems, allowing controlled release and targeted distribution, potentially improving the effectiveness and safety of medicinal applications in various medical contexts [119].

1. Polymeric Nanoparticles

Polymeric nanoparticles offer advanced drug delivery in cancer treatment, featuring a core-shell design for stability and controlled release facilitated by advanced techniques like nanoprecipitation and solvent evaporation [120]. They are ideal for cancer therapy due to their versatility in encapsulating various payloads, including drugs, genes, and imaging agents [121]. They enhance drug stability, improve pharmacokinetics, and reduce side effects [122]. However, challenges include complex formulations, potential toxicity, and size constraints [123]. Research has shown promising results in ovarian cancer treatment, with paclitaxel-loaded nanoparticles showing potential [124]. Additionally, PLGA-based polymeric nanoparticles are efficient delivery vehicles for various drugs [125].

2. Dendrimers

Dendrimers, highly branched macromolecules with tree-like structures, are valuable in cancer research due to their precision in design and functionality, achieved through controlled synthetic processes [111]. Made of polyamidoamine (PAMAM), dendrimers are a versatile tool in cancer treatment due to their uniform size, precise molecular weight, and ability to carry multiple functional groups [126]. They offer controlled release capabilities, enhanced solubility of drugs, and targeted delivery to specific cells. However, challenges like complex synthesis and potential toxicity at higher concentrations are drawbacks [127]. Dendrimers excel in targeted drug delivery, minimizing side effects, and aiding in cancer imaging, visualization, and combined diagnostics and therapy [128]. They also show promise in gene delivery, photodynamic therapy, and immunotherapy support. Guanglan et al. used PLA and hyaluronic acid-modified half-generation PAMAM G4.5 dendrimers as intelligent carriers for administering paclitaxel and sorafenib in liver cancer treatment [129]. Torres-Pérez et al. developed a unique one-step PAMAM dendrimer formulation loaded with methotrexate and D-glucose for triple-negative breast cancer cell lines, demonstrating that dendrimers containing methotrexate and D-glucose significantly decreased cell viability, outperforming free methotrexate [130].

3. Polymeric Micelles

Polymeric micelles, formed by self-assembling amphiphilic block copolymers, have a hydrophobic core and shell, facilitating drug solubilization in aqueous solutions, as summarized in Table 4-B [114]. With a size range of 10-100 nm, polymeric micelles offer advantages such as improved drug solubility, circulation time, and targeted drug delivery [131]. However, they also present challenges like complex synthesis and limited drug loading capacity. Polymeric micelles are extensively explored for cancer applications, including drug delivery and imaging [132]. Studies have shown their efficacy against cancer stem cells and gastrointestinal cancers [133]. Electron-stabilized polymeric micelles loaded with docetaxel show promise as a therapeutic option for advanced-stage gastrointestinal malignancies [134].

Table 4-B presents a comprehensive overview of polymeric-based nanomaterials and their applications in cancer therapy. Polymeric nanoparticles, ranging from 10 to 1000 nm, are versatile and easy to modify. They enable controlled drug release with high efficiency, stability, and biodegradability. These nanoparticles are used in anticancer drug delivery, targeted chemotherapy, and cancer diagnosis and imaging. Dendrimers, sized between 1 and 100 nm, have a compact and globular structure with hyperbranched subunits. They offer high structural control, monodispersity, and lower glass transition temperatures. Polymeric micelles, ranging from 1 to 100

nm, have a spherical or globular shape with a core-shell structure. They possess amphiphilic properties, enabling enhanced drug solubility, extended blood circulation, biodistribution control, low toxicity, and fast clearance without drug modification. Preparation methods include direct dissolution, solvent evaporation, and dialysis.

Table 4- B: A comprehensive summary of polymeric-based nanomaterials and their applications in cancer therapy.

Polymeric-based nanomaterials	Size	Structure	Characteristics	Preparation Method	Applications in Cancer	References
Polymeric nanoparticles	▪10-1000 nm	▪Spherical, Rod-shaped, polyhedral, filamentous, star-shaped, and core-shell.	▪Ease of surface modification ▪Biocompatibility ▪Versatility with natural and synthetic polymers. ▪Enable controlled and targeted drug release ▪High encapsulation efficiency ▪Good stability ▪Biodegradability ▪The ease of surface modification ▪Precise particle size control	▪Solvent evaporation method ▪Double emulsification method ▪Emulsion diffusion method ▪Nanoprecipitation method ▪Coacervation method ▪Salting out method ▪Dialysis method ▪Supercritical fluid method	▪Anticancer drug delivery ▪Targeted chemotherapy ▪Cancer diagnosis and imaging	[107–109]
Dendrimers	1-100 nm	▪Compact and globular structure.	▪Hyperbranched subunits. ▪High structural control. ▪Well-defined architecture. ▪Monodisperse with precise molecular weight, shape, and size. ▪Surface functional groups for drug conjugation and inner cavities for drug entrapment. ▪Lower glass temperatures.	▪Divergent synthesis ▪Convergent synthesis ▪Hypercores and branched monomer growth ▪Double exponential growth ▪Lego chemistry ▪Click chemistry	▪Anticancer drug delivery ▪Targeted chemotherapy ▪Cancer diagnosis and imaging	[110–113]
Polymeric micelles	1-100 nm	▪Spherical or globular shape, characterized by a core-shell structure	▪Amphiphilic properties ▪Hydrophobic core ▪Size and shape control ▪Enhanced drug solubility ▪Extended blood circulation ▪Biodistribution control ▪Low toxicity and fast clearance ▪No drug modification is needed	▪Direct dissolution ▪Solvent evaporation ▪Dialysis	▪Anticancer drug delivery ▪Targeted chemotherapy ▪Cancer diagnosis and imaging	[114–118]

CONCLUSIONS

In 2023, the United States is expected to have 1,958,310 new cancer cases and 609,820 cancer deaths. Nanotechnology, a multidisciplinary field combining chemistry, engineering, biology, and medicine, has emerged as a promising frontier in cancer research. Nanoparticles, including carbon-based nanomaterials, nanohorns, nanodiamonds, metal-based nanoparticles, gold nanoparticles, magnetic nanoparticles, quantum dot nanocarriers, lipid-based nanoparticles, solid lipid

nanoparticles (SLNs), polymeric nanoparticles, dendrimers, and polymeric micelles, have shown promise in detecting and treating various cancers. However, challenges such as biocompatibility, toxicity evaluation, and regulatory hurdles remain. Carbon nanotubes, nanohorns, and nanodiamonds offer benefits in cancer treatment due to their small size and hybridized carbon atoms. Metal-based nanoparticles, derived from noble metals like gold, silver, and platinum, provide targeted drug delivery, early cancer detection, and medicinal properties. Gold nanoparticles are emerging as potent tools in cancer therapy due to their advantageous properties, including cytotoxicity against specific cancer cells, size-dependent inhibition, and tunable optical properties. Magnetic nanoparticles have gained attention in fields such as medicine, electronics, and environmental science due to their versatile applications.

This comprehensive review combines existing knowledge on nanomaterials in cancer therapy and highlights their various applications and potential benefits in improving cancer treatment outcomes. It advances scientific knowledge by providing a thorough overview of nanomaterial properties, preparation methods, and applications, making it an invaluable resource for cancer researchers and clinicians. Furthermore, it emphasizes the importance of ongoing research and development using nanomaterials to address cancer treatment challenges, leading to advances in personalized and effective cancer therapy strategies.

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التطورات في مجال العلاج باستخدام الأدوية المحضرة باستخدام العلاج النانوي في علاج مرض السرطان: مراجعة علمية

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ملخص

العلاجات النانوية للسرطان، والمميزة بتكوينات حجمها النانومترية، تهدف إلى تحسين توزيع الأدوية المضادة للسرطان في الجسم، وتقليل التأثيرات الجانبية (والغير مرغوبة نتيجة استهداف انسجة غير سرطانية)، وتقليل السمية، وزيادة تراكم هذه العلاجات في المواقع الهدف، وتحسين الكفاءة العامة للعلاجات. لقد تم تطوير العديد من العلاجات النانوية لتحسين فعالية وسلامة العلاجات المضادة للسرطان التقليدية. تشمل هذه التطورات تكوينات تحتوي على أنابيب نانوية من الكربون، وجزيئات من الألماس نانوية الحجم، وجسيمات نانوية تستجيب للإنزيمات بالتزويد المنتظم من الدواء، و"الديندرايمرات" كحاملات للدواء على شكل جسيمات نانوية، وأنظمة حمل الدواء بجسيمات النانو النقطية لتوصيل الدواء بدقة متناهية، وجسيمات الدهون الصلبة النانوية، وجسيمات البوليمر المصممة لتوصيل الدواء بشكل مستهدف. وعلاوة على ذلك، تمت مناقشة التكنولوجيا النانوية في علاج السرطان باستخدام العلاج الجيني. على الرغم من هذه التقدمات، فإن الطبيعة المعقدة لمواد الحمل والتكامل الوظيفي تحمل العديد من الصعوبات في تحضير أنظمة ايصال السالفة الذكرهذه للتطبيق السريري. التكنولوجيا النانوية، بميزاتها الفريدة على مستوى النانو، تقدم إمكانيات جديدة لتطوير علاجات السرطان مع زيادة الكفاءة والسلامة. وبالرغم من أن عددًا قليلاً من العلاجات النانوية حصلت على الموافقة السريرية، إلا أن هناك استخدامات مثيرة للدهشة للتكنولوجيا النانوية في المستقبل. تتمتع الجسيمات النانوية بقدرات فريدة في قدراتها على النقل، والخصائص البيولوجية، والبصرية، والمغناطيسية، والكهربائية، والحرارية الخاصة بها بسبب صغر حجمها. وهذا يؤدي إلى نسب سطح كبيرة مقارنة بالحجم، مما يسمح بدمجها مع مكونات داعمة مختلفة بالإضافة إلى المواد الدوائية الفعالة. تساعد هذه الخصائص الجزيئات النانوية في عمليات التحلل، والحماية من التحلل، وتأخير إطلاق العلاجات المقصود، وتجنب الاستجابة المناعية، وتعزيز اختراق الأنسجة، والتصوير، والتوزيع المستهدف، والتفعيل المستند. وخلاصة القول، فان مستقبل العلاجات النانوية يعد واعدًا بإدخال منصات مبتكرة في علاجات السرطان المختلفة. تؤكد الأبحاث التي تم عرضها وتلخيصها على إمكانية أن تحدث الجسيمات النانوية ثورة في علاجات مكافحة السرطان، مع تعزيز النهج العلاجي العام.

الكلمات الدالة: الجسيمات النانوية؛ العلاج المضاد للسرطان؛ المواد النانوية القائمة على الكربون؛ الجسيمات النانوية القائمة على المعادن، القائمة على الدهون، البوليمرية.

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Inhibitory Effects of Polyphenols from *Equisetum ramosissimum* and *Moringa peregrina* Extracts on *Staphylococcus aureus,* Collagenase, and Tyrosinase Enzymes: *In vitro* Studies

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ABSTRACT

Background: Skin problems caused by oxidative stress lead to the activation of collagenase and tyrosinase enzymes, contributing to skin aging, discoloration, and infections. *Equisetum ramosissimum* and *Moringa peregrina* were assessed for their potential uses in treating various skin conditions.

Objective: The present research aimed to investigate the positive effects of polyphenols in *Equisetum ramosissimum* and *Moringa peregrina* extracts as potential cosmetic products for the treatment of different skin conditions.

Methods: Total phenolic and flavonoid contents, antioxidants, and anti-collagenase and anti-tyrosinase activities of plant extract mixtures (PEM) at different ratios of (*M. peregrina*: *E. ramosissimum*) were determined using standard procedures. Inhibitory effects of PEM against acne-causing *Staphylococcus aureus* (ATCC 29213) were evaluated using the diameter (cm) of the inhibition zone method. A cream formulation containing PEM was developed and characterized for stability and potential skin irritation in rats using standard procedures.

Results: The PEM at a ratio of (2:1) showed the highest total phenolic and flavonoid content (150.15 ± 2.8 mg/g, equivalent to gallic acid, and 41.5 ± 1.2 mg/g, equivalent to quercetin, respectively). Antioxidant activities for PEM (2:1) were also optimal, as determined by the DPPH and ABTS methods (IC50 = 7.06 ± 0.12 µg/mL and 53.29 ± 3.3 µg/mL, respectively). Furthermore, PEM (2:1) exhibited superior inhibitory activities against collagenase and tyrosinase enzymes (IC50 = 32.4 ± 1.19 µg/mL and 8.4 ± 1.19 µg/mL, respectively). Antimicrobial activity of PEM (2:1) tested on *S. aureus* showed the largest zone of growth inhibition (2.8 cm) at a concentration of 60 mg/mL. Studies on the PEM (2:1) cream formulation revealed that it remained stable under room conditions. Skin irritation tests on rats showed no signs of oedema or erythema after treatment.

Conclusion: The PEM with a ratio of (2:1) demonstrated optimal activity as an oxidative stress-neutralizing agent, inhibitor of enzymes responsible for skin aging and hyperpigmentation, and antibacterial agent. The cream formulation containing PEM exhibited physical stability and no detectable risk of skin irritation throughout the research procedures.

Keywords: *Equisetum ramosissimum*, *Moringa peregrina*, *Staphylococcus aureus*, collagenase, tyrosinase.

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1. INTRODUCTION

Skin aging is considered a naturally occurring process influenced by several environmental factors, such as ultraviolet radiation (UVR) and oxidative stress [1,2]. Collagen, a major component of the skin, provides structural stability, firmness, elasticity, and flexibility, all of which are essential for maintaining skin health. Collagenases are enzymes that break down collagen in the skin. As humans age, the body produces more of these enzymes, leading to the appearance of wrinkles. Therefore, it is crucial to find substances that can inhibit collagenases to slow this process and delay skin aging [3].

Skin pigmentation is also one of the most distinctive and visible personal traits. Increased melanocyte activity and melanin production, driven by tyrosinase enzymes, result in hyperpigmentation disorders, such as post-inflammatory pigmentary alteration, senile lentigo, melasma, and ephelides [4]. Tyrosinase plays a crucial role in melanogenesis; therefore, inhibiting it is considered an effective approach, alongside other therapeutic techniques, to prevent the accumulation of melanin in the skin [5,6]. Tyrosinase inhibitors function through four distinct mechanisms: competitive, noncompetitive, uncompetitive, and mixed-type inhibition [6]. Many natural substances, such as hydroquinones and deoxyarbutins, act as competitive tyrosinase inhibitors [7]. Luteolin, a key component of *Moringa oleifera* and ginseng extracts, has been shown to employ an uncompetitive inhibitory mechanism against tyrosinase [8].

Acne vulgaris is a chronic inflammatory skin disorder that arises from infections in the pilosebaceous unit [9]. Hormonal imbalances can also contribute to acne [10]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common causes of skin and soft tissue infections [11,12]. The urgent need to discover novel treatments from non-traditional sources to combat MRSA infections has been highlighted as a current challenge [13].

Cosmetics containing herbal remedies have recently gained popularity as a solution for skin problems [14]. Thus, the inhibition of collagenase and tyrosinase activities by active components derived from herbs may have beneficial effects, such as delaying the degradation of collagen and other components of the extracellular matrix [15].

The *Moringa* genus belongs to the Moringaceae family and contains thirteen species found in tropical and subtropical regions [16]. The tree *Moringa peregrina* (M. peregrina), a member of the Moringaceae family, grows naturally in Jordan [17,18]. Previous studies investigating the phytocomponents of *M. peregrina* extracts have revealed numerous bioactive compounds, including phenolic acids, volatile isothiocyanates, flavonoids, alkaloids, and glucosinolates. These compounds contribute to the plant's diverse therapeutic activities, including antioxidant, anti-inflammatory, antimicrobial, antidiabetic, and hepatoprotective effects [19]. *Moringa peregrina* extracts have demonstrated potential benefits for skin health, such as moisturizing, anti-aging, and wound-healing properties. Traditionally, *M. peregrina* leaf extract is rubbed on the skin to manage rashes and paralysis [20]. The oil of *M. peregrina* is used to treat skin conditions such as freckles, scabies, and itching [21].

Several *Moringa*-based products are available in the pharmaceutical market. Cold-pressed *Moringa* oil is found in various products intended for application to the face, skin, and hair [22]. Additionally, anti-aging creams containing virgin seed oil from *Moringa* are marketed for use in skincare, hair care, aromatherapy oils, soaps, liquid body washes, face creams, massage oils, perfumes, and deodorants [23].

The plant *Equisetum ramosissimum* (E. ramosissimum), a member of the Equisetaceae family [24], is widely distributed in Europe, North America, and Asia. Several studies have shown that *E. ramosissimum* contains various compounds, such as flavonoids, alkaloids, phenolics, saponins, tannins, triterpenoids, and phytoesters, which possess a range of biological activities [25]. Traditionally, it has been used to treat various

ailments, including urinary tract disorders, skin conditions, and for wound healing [26].

Vanithamani et al. [27] highlighted several herbal mixtures used in skincare formulations, concluding that these combinations exhibited a potential skin-protectant effect with no noticeable side effects. Consequently, the combination of *M. peregrina* and *E. ramosissimum* extracts has been suggested as a potential agent with superior effects in inhibiting several skin-related disorders, such as bacterial infections and the activities of tyrosinase and collagenase enzymes. Cosmetic formulations containing the proposed plant extract mixtures as active ingredients can be investigated for their potential use in treating or reducing various skin conditions, including aging, infections, and hyperpigmentation, using standard in-vitro methods. The present research aims to investigate the positive effects of polyphenols in *Equisetum ramosissimum* and *Moringa peregrina* extracts as potential cosmetic products for treating different skin conditions.

2. MATERIAL AND METHODS

2.1. Materials

2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Sigma Aldrich, USA. Folin-Ciocalteu reagent and potassium persulfate were also purchased from Sigma Aldrich, USA. 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH•) was obtained from Sisco Research Laboratories Pvt. Ltd., India. Dimethyl sulfoxide and gallic acid were purchased from GCC, UK, and quercetin was purchased from Santa Cruz Biotechnology, USA.

Emulsifying wax, Germall Plus, glycerin, isopropyl myristate, lanolin, mineral oil, lavender oil, and allantoin were obtained from LabChem Laboratory Chemicals, USA. Collagenase activity kits (ab196999) and tyrosinase activity kits (ab252899) were sourced from Abcam®, UK. All other organic solvents and materials were of HPLC, analytical, or pharmaceutical grade

2.2. Animals

For the skin irritation test, three healthy male Wistar rats weighing 250 ± 15 g were housed and acclimated at the Laboratory Animal Research Unit, Applied Science University, Amman, Jordan. The Applied Science University Ethics Committee granted clearance for this investigation (Clearance Number: 2023-PHA-32).

The rats were kept in separate cages with controlled temperature (20 ± 3°C), humidity (50 ± 15%), and a photoperiod cycle of 12 hours of light and 12 hours of darkness. They were fed a standard laboratory diet and had unlimited access to water.

2.3. Methods

2.3.1. Collection and processing of crude plant material

Moringa peregrina dried leaves and *E. ramosissimum* dried aerial parts were purchased from a local shop in Amman, Jordan. *M. peregrina* dried leaves were milled using an electric mill, while *E. ramosissimum* dried aerial parts were milled using a commercial hammer milling machine. The resulting powders of *M. peregrina* and *E. ramosissimum* were dried and stored separately in airtight jars at room temperature for further experiments.

2.3.2. Plant extraction methods

A total of 200 g of *M. peregrina* dried leaves or *E. ramosissimum* dried aerial parts was soaked in 1000 mL of pure ethanol for 48 hours. The resulting extract was then filtered using a Büchner funnel and dried using a rotary evaporator. The extract was left to dry completely for 48 hours in a fume hood and then stored at 4°C for subsequent tests. Different ratios of *M. peregrina* and *E. ramosissimum* plant extract mixtures (PEM) (2:1, 1:2, 1:1 w/w) were prepared from the dried extracts.

2.3.3. Phytochemical analysis of plant extract

2.3.3.1. Total phenolic content (TPC)

The Folin-Ciocalteu method, as reported by Lohvina et al. [28], was used to determine the TPC of each extract and mixture ratio. Ethanol was used to create a stock solution of each extract combination at a concentration of 2 mg/mL.

A 0.1 mL aliquot of Folin-Ciocalteu reagent, 1.6 mL of distilled water, and 0.3 mL of a 20% Na_2CO_3 aqueous

solution were mixed with 0.2 mL of the stock solution. The mixture was allowed to sit at room temperature for 1 hour in the dark, after which the UV absorption was measured at 750 nm.

Gallic acid (GA) was used as a reference standard at concentrations ranging from 100 to 6.25 µg/mL to construct a calibration curve. The TPC of the PEMs was calculated as mg/g dry extract equivalent of GA.

2.3.3.2. Total flavonoid content (TFC)

The aluminium chloride assay, as described by Chang et al. [29], was used to assess the TFC. Ethanol was used to create a stock solution of each extract combination at a concentration of 2 mg/mL. Then, 0.5 mL of the stock solution was combined with 0.1 mL of 10% aluminium chloride, 2.8 mL of distilled water, and 0.1 mL of 1M sodium acetate. The mixtures were allowed to sit at room temperature for 30 minutes. UV absorption was then measured at 415 nm.

Quercetin (QE) was used as a reference standard at concentrations of 25, 50, and 100 µg/mL to construct a calibration curve. The TFC of the PEMs was calculated as mg/g dry extract equivalent of QE.

2.3.4. The antioxidant activity of plant mixture extract

2.3.4.1. DPPH free radical scavenging activity assay

The 2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH•) scavenging activity was determined as described by Nurzaman et al. [30]. Briefly, 2 mL of each extract mixture, at concentrations ranging from 100 to 6.25 µg/mL, was mixed with 2 mL of DPPH• (0.1 mM DPPH in pure ethanol, 1:1 v/v), stirred vigorously, and incubated for 30 minutes in a dark place at room temperature. The UV absorbance was measured at 517 nm.

The percentage of DPPH scavenging activity was calculated using Equation 1. The IC50 values of the PEMs were compared with the IC50 of ascorbic acid (Vitamin C) as a reference antioxidant compound.

$$\%\text{DPPH scavenging activity} = \frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100\% \quad \text{-----(1)}$$

2.3.4.2. ABTS scavenging activity assay

The 2.2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation (ABTS•+) scavenging activity was determined as described by Re et al. [31]. Briefly, a solution of ABTS•+ was prepared by mixing 2.45 mM potassium persulfate solution (6.6 mg of potassium persulfate in 10 mL of water) with a 7 mM stock solution of ABTS (36 mg of ABTS in 10 mL of water). Ethanol was added as a diluent until the prepared ABTS•+ solution reached an absorbance value of 0.7 at 734 nm.

A stock solution of each extract mixture at different concentrations, ranging from 200 to 1.56 µg/mL, was prepared in ethanol. A 1 mL aliquot of each sample was added to 2 mL of the ABTS•+ solution. The absorbance was measured at 734 nm after 1 hour of incubation in a dark room. Ascorbic acid (Vitamin C) at a concentration range of 100 to 1.56 µg/mL was used as a reference compound [31].

The percentage of ABTS scavenging activity was calculated using Equation 2. The IC50 values of the PEMs were compared with the IC50 of ascorbic acid (Vitamin C) as a reference antioxidant agent.

$$\%\text{ ABTS scavenging activity} = \frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100\% \quad \text{----------(2)}$$

2.3.5. Collagenase inhibition activity

The inhibition of plant extracts on collagenase enzyme (EC 3.4.24.3) was tested using the reference kit and the protocol provided by the supplier. The enzyme's activity was noted as 0.35 U/mL. In the experiment, collagenase enzyme solution vials were briefly centrifuged before use, and Collagenase Assay Buffer (pH = 7.6) was brought to room temperature. The collagenase substrate, synthetic peptide (FALGPA), was diluted with buffer. The inhibitor used was 1,10-Phenanthroline (1 M). A 96-well plate was employed for measurement.

Briefly, PEM (2 µL) at different concentrations were combined with buffer (88 µL) and collagenase enzyme (10 µL). A positive control contained enzyme (10 µL) and buffer

(90 µL). A substrate mixture (collagenase substrate: buffer, 4:6) was freshly prepared (100 µL) and added to each well. After 15 minutes of dark incubation at 37°C, UV absorbance was measured at 345 nm using a microplate reader. The percentage inhibition of collagenase enzyme activity by the PEMs was calculated using Equation 3.

$$\% \text{ Collagenase inhibition activity} = \frac{Abs\ control - Abs\ sample}{Abs\ control} x100\% \quad \text{------(3)}$$

2.3.6. Tyrosinase inhibition activity

The inhibition of plant extracts on tyrosinase enzyme activity was tested using the reference kit and protocol provided by the supplier. In this experiment, tyrosinase enzyme solution vials were briefly centrifuged. Sample wells containing PEM (2 µL) at different concentrations were combined with 3 µL of tyrosinase, 5 µL of enhancer, 10 µL of substrate, and 80 µL of assay buffer (pH = 7.6). The percentage inhibition of tyrosinase enzyme activity by the PEMs was calculated using Equation 4.

$$\% \text{ Tyrosinase inhibition activity} = \frac{Abs\ control - Abs\ sample}{Abs\ control} x100\% \quad \text{---(4)}$$

2.3.7. Anti-bacterial activity

Different ratios of PEMs were evaluated for growth inhibition activity on *Staphylococcus aureus* (ATCC 29213) using the method developed by Bauer-Kirby et al. (1966). For this experiment, Mueller-Hinton broth (Oxoid Ltd., UK) was used to cultivate *S. aureus*, which was then incubated overnight at 37ºC.

To test the antibacterial activity, an aliquot of 100 µL of PEM (2:1) at concentrations of 60, 30, and 10 mg/mL in water was transferred into each well. Plates were then incubated at 37ºC for 24 hours. The diameter (cm) of the inhibition zone was used for comparison. Tetracycline (30 µg) was used as a positive control.

2.3.8. Cream formulation and evaluation

PEM (2:1) was formulated as an oil-in-water emulsion using the formula in Table 1, as described by Ubaydee et al. [32]. Briefly, emulsifying wax, mineral oil, isopropyl myristate, phenoxyethanol, and lanolin were melted at 50°C and mixed to create the oil phase. PEM (2:1), allantoin, glycerin, and Germall plus were dissolved in deionized water to create the aqueous phase. The water phase was warmed to 50°C to dissolve all the components. The aqueous phase was gradually added to the oil phase with gentle agitation once both phases reached the same temperature. The mixture was continuously agitated until the temperature decreased to 35°C. Finally, lavender oil was added to the cream formulation. After gradually stirring the emulsion until it became homogenous, the cream was allowed to cool to room temperature.

Table 1: List of ingredients in the cream formulation with PMS (2:1)

Ingredient	Master formula (%w/w)	Uses
Isopropyl myristate	4 %	Lubricant and emollient
Mineral oil	12 %	Lubricant
Emulsifying wax	6 %	Non anionic emulsifier
Lanolin	1%	Emollient
Glycerin	3 %	Humectants
Allantoin	0.2 %	Healing agent
PEM (2:1)	1.5 %	Active ingredient
Water	71.8 %	Solvent
Lavender oil	q s	Flavoring agent
Germall plus	0.5	Preservative

2.3.8.1. Stability tests

The stability study of the prepared cream formulation was conducted as described by Bora et al. (2019) [33] over a one-month period under different conditions, including room temperature (25±2°C), refrigeration (4±2°C), and accelerated temperature (40±2°C). The physicochemical parameters (homogeneity, odor, color, and pH) were observed periodically. About 0.5 g of the cream was weighed and dissolved in 50 mL of distilled water, and its pH was measured [34]. The homogeneity of the freshly prepared cream formulation was determined using a centrifugation test [35]. This test was performed by placing a 5 g sample in a centrifuge tube and spinning it at 4000 rpm for 20 minutes, after which the appearance and phase separation were observed. Both color and odor properties were determined organoleptically by the research group.

2.3.8.2. Rheological study

The rheology of the prepared cream was tested using a rheometer (Physica MCR 302, Anton Paar) according to the method by Helgesen [36]. All measurements were carried out at a temperature of (25±1°C), using spindle Cp 50. About 0.5 g of the cream was loaded between concentric cylinders, and the flow curve was constructed by plotting controlled shear rate versus controlled shear stress. In addition, the viscosity–temperature curve was plotted [37].

2.3.8.3. Skin irritation/corrosive potential test

The evaluation of the PMS-cream formulation, intended for topical use, for its potential to cause skin irritation or corrosion, as well as the reversibility of dermal effects, was performed in accordance with the OECD Guideline for Acute Dermal Irritation/Corrosion [38]. Briefly, 3 healthy male Wistar rats were examined as follows: Rat #1 (negative control with no treatment), and Rats #2 and #3 were topically treated with the PMS-cream formulation. Prior to the experiment, the rats' fur was carefully trimmed from the dorsal part of their trunks with an electric clipper while they were held in a humane manner. The animals were ready for the cream formulation to be applied once it was confirmed that their skin was healthy and undamaged. A dosage of about 0.5 g of the prepared cream was applied to an area of about 6 cm^2, and the region was covered with a gauze patch. In accordance with the guidelines, one patch was applied for the first test and removed after 3 minutes. An hour later, a second patch was applied and removed after 3 minutes if no significant skin response was observed. A third patch was then applied and left in place for 4 hours. Upon removing the patch, the animals were checked visually for signs of erythema and edema. The appearance of cutaneous responses was assessed. A 4-hour exposure session on a different animal confirmed the negative reaction. Following the removal of the patch, the cutaneous reaction was assessed immediately, after 1 hour, and again 24 hours later, as recommended by Draize's dermal irritation scoring model [38] (Table 2).

Table 2. Draize dermal irritation scoring system [38].

Erythema and Eschar Formation	Value	Edema Formation	Value
No erythema	0	No edema	0
Very slight erythema (barely perceptible)	1	Very slight edema (barely perceptible)	1
Well-defined erythema	2	Slight edema (edges of area well defined by definite raising)	2
Moderate to severe erythema	3	Moderate edema (raised approximately 1 mm)	3

2.3.8.4. Antibacterial activity of the PEM-cream formula

The procedure used for the evaluation of the antimicrobial activity of PEM against *S. aureus* was also utilized to evaluate the antimicrobial activity of the prepared PEM-cream formulation on *E. coli* (ATCC 25922), *S. aureus*, and *P. aeruginosa* (ATCC 27853). The prepared PEM-cream formula containing Germall Plus preservative (T1), the PEM-cream formula containing Germall Plus and phenoxyethanol preservatives (T2), Germall Plus preservative (Pre1), and phenoxyethanol preservative (Pre2) were all tested for their antimicrobial preservative activities. The findings were compared to the reference positive control (Tetracycline) and the negative controls (1% DMSO / 1% Germall Plus) [39].

2.3.9. Statistical analysis

All statistical analyses were performed using Microsoft Excel. The results were presented as mean ± SD, and all experiments were conducted in triplicate.

3. RESULTS

3.1. Extraction yield

The resulting dry extracts were sticky with a dark green color. The extraction yield percentage was calculated for each plant, showing 31.3% for *M. peregrina* and 28.8% for *E. ramosissimum*.

3.2. Total phenolic content (TPC)

The total phenolic content for PEM (2:1) showed the highest value (150.15 ± 2.8 mg GAE/g), followed by PEM (1:1) and PEM (1:2) (133.25 ± 5.5 and 105.55 ± 1.9 mg GAE/g), respectively.

3.3. Total flavonoid content (TFC)

The total flavonoid content for PEM (2:1) also showed the highest value (41.5 ± 1.2 mg QE/g), followed by PEM (1:1) and PEM (1:2) (32.11 ± 0.8 and 26.3 ± 3 mg QE/g), respectively.

3.4. DPPH free radical scavenging activity

Using the DPPH antioxidant assay, the calculated IC50 for PEM (2:1) showed the highest antioxidant activity (7.06 ± 0.12 µg/mL), followed by PEM (1:1) and PEM (1:2) (10.18 ± 0.5 and 18.03 ± 0.28 µg/mL), respectively.

3.5. ABTS free radical scavenging activity

Using the ABTS free radical scavenging method, the calculated IC50 for PEM (2:1) showed the highest radical scavenging activity (53.29 ± 3.3 µg/mL), followed by PEM (1:1) and PEM (1:2) (67.4 ± 5.4 and 192.43 ± 13 µg/mL), respectively.

3.6. Anti-collagenase activity

The percentage inhibition and IC50 for collagenase enzyme activity using PEMs were calculated as shown in Figure 1. The findings revealed that the IC50 for PEM (2:1) was 32.4 ± 1.19 µg/mL, which was similar to the other samples: PEM (1:1) and PEM (1:2) were 33 ± 2.2 µg/mL and 33.3 ± 1.46 µg/mL, respectively. However, this activity increased significantly at higher concentrations, with comparable activities among the tested samples.

3.7. Anti-tyrosinase activity

The percentage inhibition and IC50 of tyrosinase enzyme activity using the PEMs were calculated as shown in Figure 2. The findings revealed that the IC50 for PEM (1:2) was 10.1 ± 0.4 µg/mL, which is lower than the other samples: PEM (2:1) and PEM (1:1) were 40.3 ± 2.3 µg/mL and 46.5 ± 0.9 µg/mL, respectively. However, this activity increased significantly, showing comparable differences among the tested samples at higher concentrations. Additionally, the results indicated that PEM (2:1) exhibited higher enzyme inhibition activity at concentrations greater than 60 µg/mL compared to the other samples tested.

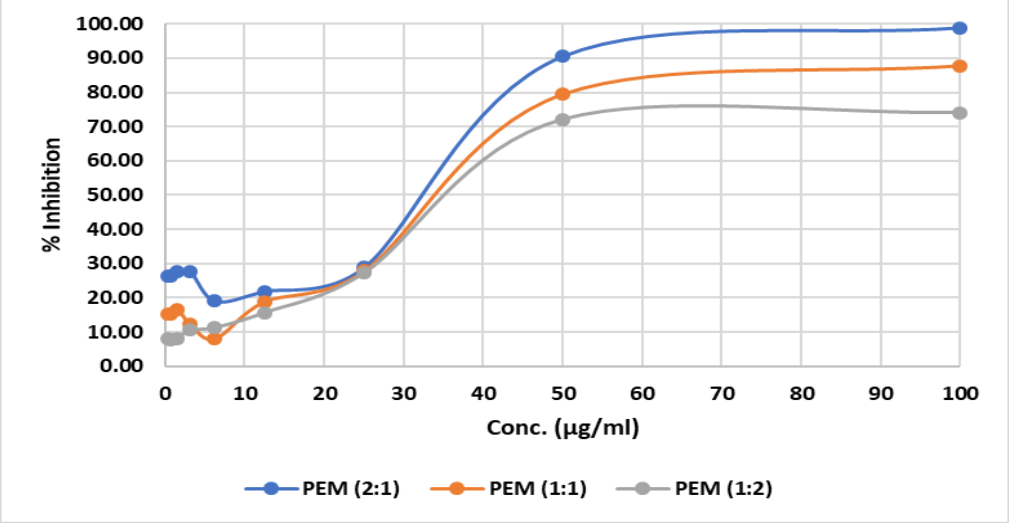

Fig. 1: The % inhibition on collagenase enzyme activity by PEMs

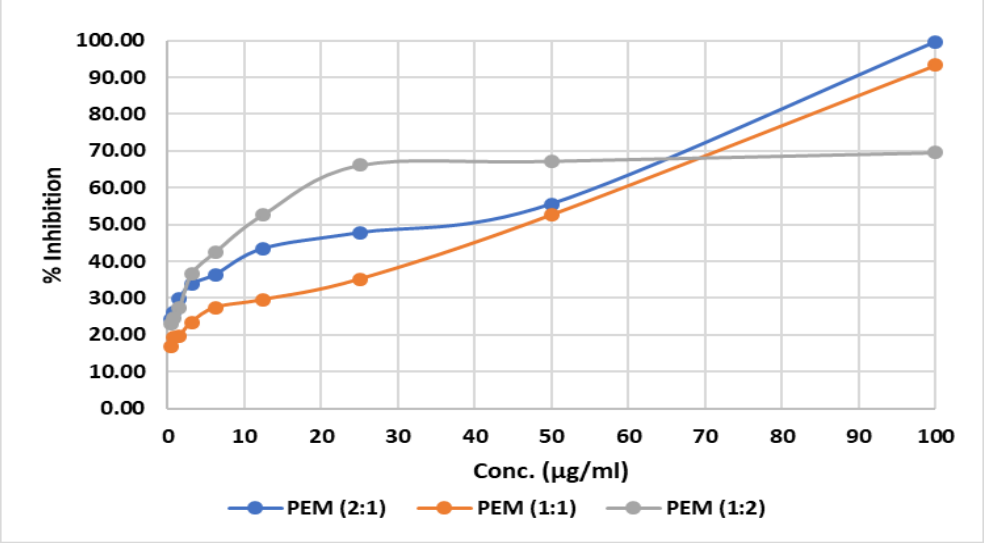

Fig. 2: The % inhibition on tyrosinase enzyme activity by PEMs

3.8. Antibacterial activity

Figure 3 shows the measured diameters of the inhibition zones (cm) for PEM (2:1) at three different concentrations against S. aureus. The findings revealed inhibition zone diameters of 2.3 cm, 2.55 cm, and 2.8 cm for PEM (2:1) at concentrations of 10 mg/mL, 30 mg/mL, and 60 mg/mL, respectively. In comparison, the positive control, Tetracycline, yielded an inhibition zone diameter of 1.45 cm. The tested PEM samples demonstrated superior antibacterial activity against S. aureus.

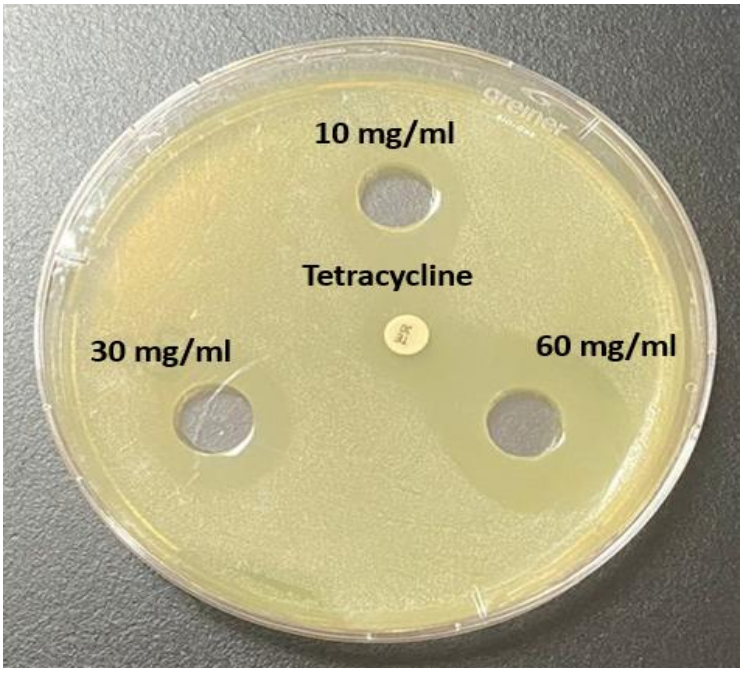

Fig. 3. Zone of inhibition (cm) of PEM (2:1) at different concentrations and tetracycline against *S. aureus*.

3.9. Evaluation of Cream formula

3.9.1. Stability studies

Over the study period, the PEM-cream formulations stored at room temperature (25°C) or in the refrigerator (4°C) exhibited no changes in physical characteristics. The results indicated that the PEM-cream formulations remained homogeneous with no signs of separation. They retained a pleasant lavender odor, a light green color, and a pH of 5.33, which is within the compatible skin pH range (4.5-6). In contrast, the cream formulation stored in the oven at 40°C developed a crust on the surface, indicating instability at this temperature.

3.9.2. Rheological study

Figure 4 shows the rheological pattern of the cream formulations, with a plot of shear stress versus shear rate. Figure 4.A illustrates a pseudoplastic rheological flow, characterized by a non-linear relationship between shear rate and shear stress. A shear-thinning or pseudoplastic system is the most common type of time-independent non-Newtonian fluid behavior.

The effect of shear rate on the viscosity of the PEM-cream formulations is presented in Figure 4.B. The findings revealed that as the shear rate increases, the viscosity of the formulations decreases. Thus, the rheological study results indicate that the PEM-cream formulation has good spreadability and homogeneity.

3.9.3. Skin irritation/corrosive potential

The interpretation of skin irritation and corrosive potential was based on Draize's Dermal Irritation Scoring Model [38], as described in Tables 2 and 3. Results indicated that the application of the formula on the rats' skin, serving as a model for human skin, showed no signs of irritation, erythema, or redness over a 24-hour period. This suggests that the prepared PEM-containing formulations can be safely applied to the skin (Figure 5).

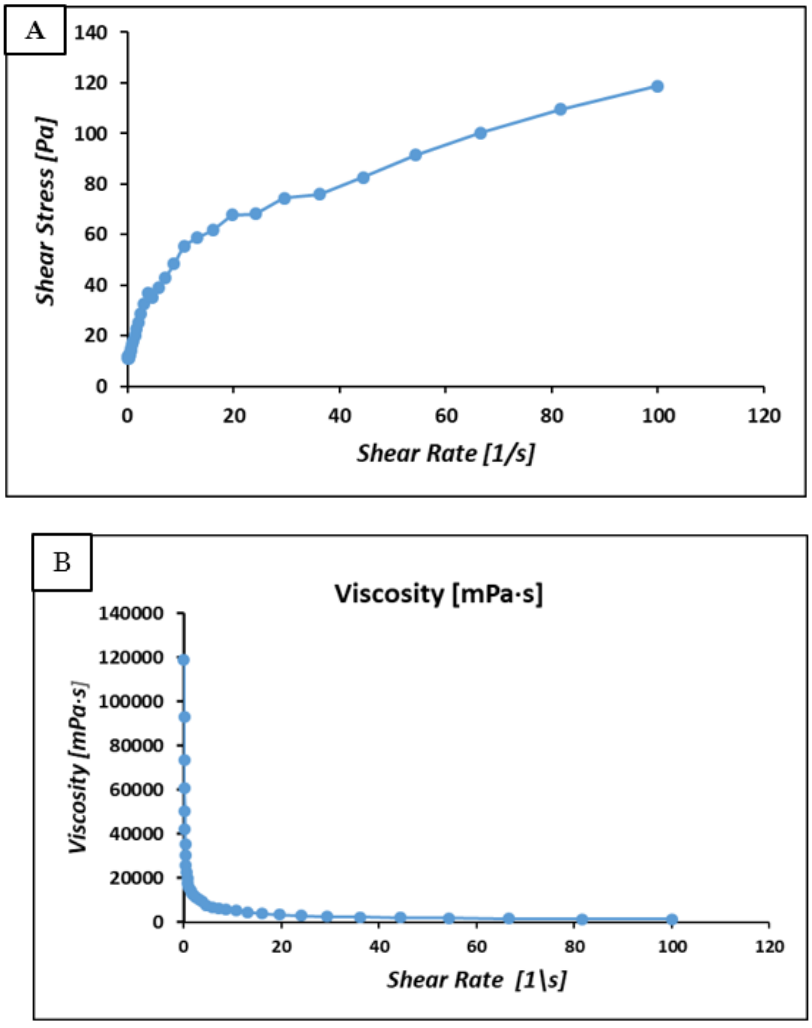

Fig. 4.1 Shear stress versus shear rate plot of cream showed non- Newtonian flow. (B) The effect of shear rate on the viscosity of the cream plant extract.

Table 3. Dermal Responses Observed in Individual Rats

Erythema			
Wistar Rat (1) control, Rat (2) test	**Evaluation after removal of test substance**		
	0 minutes	**60 minutes**	**24 hours**
(1) Control	0	0	0
(2) Test	0	0	0
Edema			
Wistar Rat (1) control. Rat (2) test	**Evaluation after removal of test substance**		
	0 minutes	**60 minutes**	**24 hours**
(1) Control	0	0	0
(2) Test	0	0	0

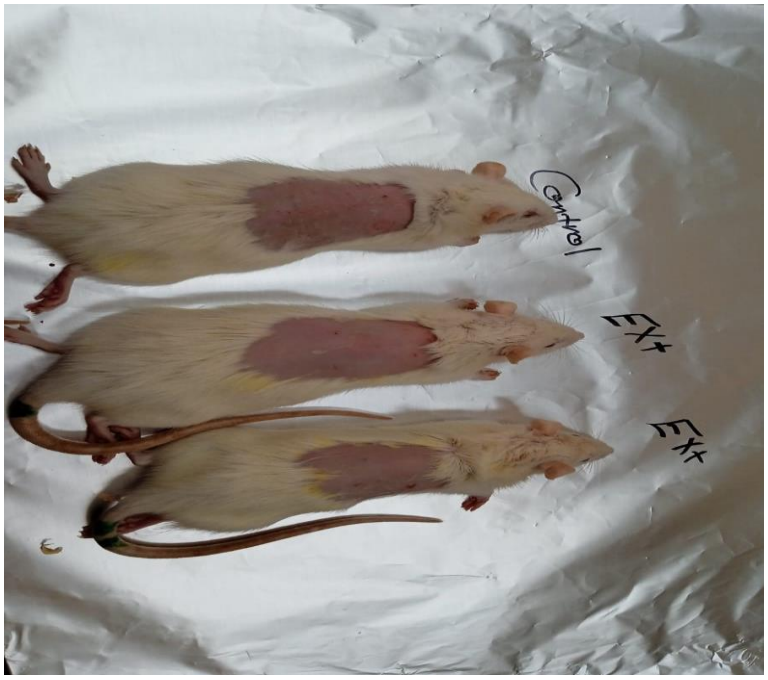

Fig. 5. Results of irritation test (Negative control with no treatment and duplicates with PEM-cream formulations after 24 hours).

3.9.4. Antibacterial activity for the PEM-cream formula

The antimicrobial activities of the prepared T1 and T2 formulas, as well as the Pre 1 and Pre 2 samples, are shown in Table 4. The findings suggest that incorporating a second preservative into the PEM-cream formula is unnecessary, as both T1 and T2 formulas (Figure 6D, E, F) demonstrated comparable antimicrobial effects to the positive control (Figure 6A, B, C). In contrast, Pre 2 (Figure 6D, E, F) showed no inhibition against the three bacterial strains and was therefore excluded from the final formula.

Table 4. Zone of inhibition (cm) for bacterial growth against different bacterial strains by DMSO (negative control), Tetracycline (positive control), T1, T2, Pre 1, and Pre 2.

Samples	*P. aeroginosa*	*E. coli*	*S. aureus*
DMSO	0 cm	0 cm	0 cm
Tetractcline	1 cm	2.9 cm	1.1 cm
T1	2.9 cm	3.2 cm	3.2 cm
T2	2.7 cm	3.2 cm	3.4 cm
Pre 1	0.6 cm	1.8 cm	1.2 cm
Pre 2	0 cm	0 cm	0 cm

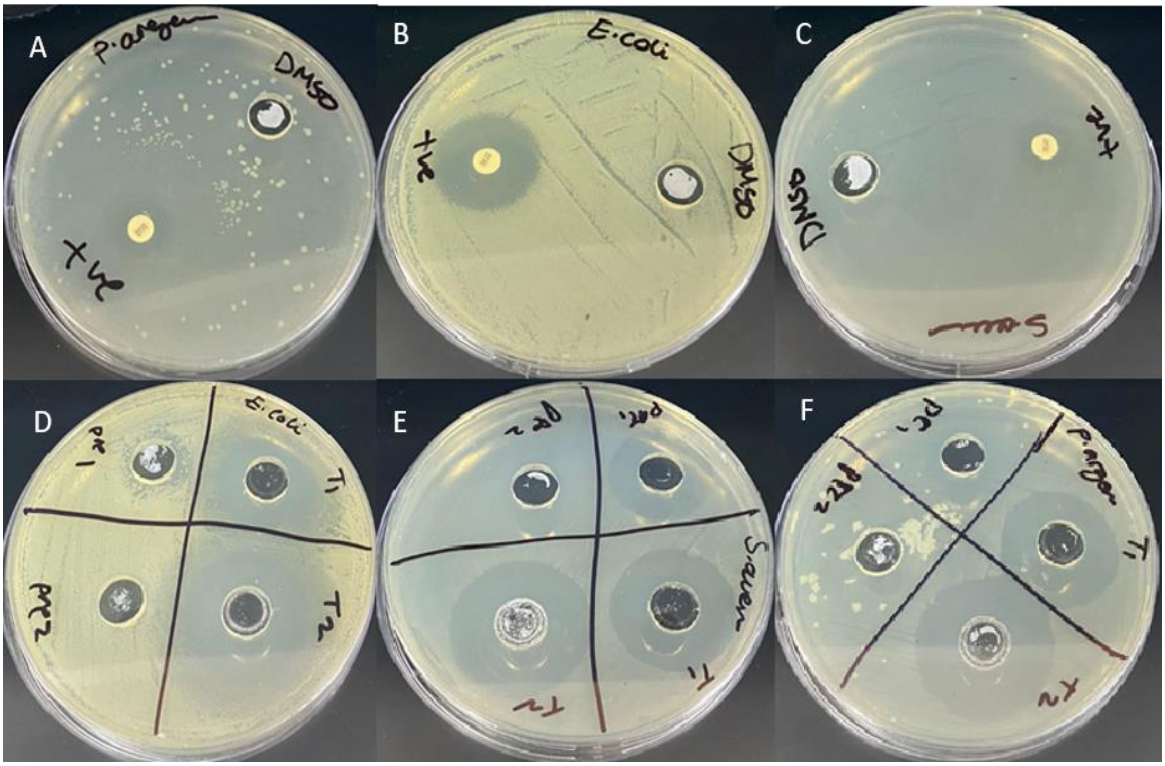

Fig. 6. Inhibition of bacterial growth against different bacterial strains by DMSO (negative control), andTetracycline (positive control) (A-C), T1, T2, Pre 1, and Pre 2 (D-F).

A: Tetracycline vs. DMSO against *P. aeruginosa,* B: Tetracycline vs. DMSO against *E. coli*, C: Tetracycline vs. DMSO against *S. aureus*, D: T1, T2, Pre 1, and Pre 2 against *E. coli,* E: T1, T2, Pre 1, and Pre 2 against *S. aureus,* F: T1, T2, Pre 1, and Pre 2 against *P. aeruginosa*.

4. DISCUSSION

Herbal medicine in skincare products is currently one of the most significant areas in the pharmaceutical industry [40,41]. Several modern herbs, such as Green Tea (Camellia sinensis), Liquorice (Glycyrrhiza glabra), and Centella Asiatica, have recently been introduced to the field of skincare [42]. Traditionally, M. peregrina and E. ramosissimum have been used to treat various skin conditions, including moisturizing, anti-aging, and wound-healing [21]. Furthermore, these plant extracts have been previously studied for their antimicrobial, anti-collagenase, and anti-tyrosinase activities [25,43]. To our knowledge, no previous work has been performed on the combination of M. peregrina and E. ramosissimum extracts.

The findings of the current study revealed that the PEM (2:1) mixture ratio exhibited the highest total phenolic content, flavonoid content, and antioxidant activities. This data is consistent with previous studies [16, 25]. M. peregrina's bioactive compounds and therapeutic effects were studied by Dehshahri et al. [19], who highlighted its bioactive compounds, including phenolic acids, isothiocyanates, flavonoids, alkaloids, and glucosinolates, which contribute to its diverse therapeutic effects such as antioxidant, anti-inflammatory, antimicrobial, antidiabetic, and hepatoprotective activities. The phytochemical analysis

of E. ramosissimum conducted by Savaya et al. [25] revealed the presence of flavonoids, phenolic acids, alkaloids, and saponins. A study by Parham [44] identified diverse compounds in E. ramosissimum, including flavonoids, alkaloids, phenolics, saponins, tannins, triterpenoids, and phyto-esters. Specific compounds like Myricetin, Quercetin, Kaempferol, and Kaempferol-3-O-glycoside were found to be the most abundant [45,46].

The antioxidant activities of the PEM were investigated using the DPPH and ABTS assays as generators of free radicals, which mimic reactive oxygen species (ROS) and reactive nitrogen species (RNS) under controlled laboratory conditions. These radicals impact biological systems and play a role in counteracting damage caused by oxidative stress and lipid peroxidation [47]. In this study, lower IC50 values against DPPH• and ABTS•+ radicals were obtained for PEM (2:1), which confirms our previous findings. The antioxidant activities of M. peregrina extract were previously tested by Hasan et al. [48], who found the extract to display significant scavenging activity against DPPH free radicals. Consistent with our research, Al-Bayati et al. [49] demonstrated notable free radical scavenging ability using the DPPH method in methanolic extracts of E. ramosissimum.

As expected, PEM (2:1) showed antimicrobial effects on S. aureus, as indicated by the measured inhibition zone using the well diffusion method. The antibacterial activity of M. peregrina ethanol extract was previously tested by Majali et al. [50], who found a correlation between extract concentration and inhibition zone against bacterial growth for E. coli, S. aureus, and K. pneumoniae. The antimicrobial potential of E. ramosissimum was illustrated by Karak [51] against various bacterial strains, revealing potential antimicrobial benefits for the extract. Similarly, Savaya et al. [25] examined E. ramosissimum extracts for phenolic content, antioxidants, and antibacterial effects against Propionibacterium acnes. The aqueous-methanol extract displayed potent antioxidant and antimicrobial effects, while other extracts exhibited varying levels of activity.

Studies on the tyrosinase enzyme inhibition effect revealed that PEM (2:1) was effective in a concentration-dependent manner. These data were expected, as the inhibitory effects of E. ramosissimum extracts on mushroom tyrosinase were previously examined [25]. Previous studies have shown that phytochemical compounds found in M. peregrina, including β-sitosterol, can potentially act as anti-tyrosinase agents [52]. Other plant extracts containing β-sitosterol, such as Arbutus andrachne L., have also shown potent inhibitory effects against tyrosinase [52]. Additionally, Prommaban et al. [53] demonstrated that β-sitosterol displays inhibitory effects on mushroom tyrosinase enzymes by suppressing the oxidation process of L-3,4-dihydroxyphenylalanine (L-DOPA), a reaction catalyzed by tyrosinase.

Studies on the collagenase enzyme inhibition effect revealed that PEM (2:1) was effective in a concentration-dependent manner. These data are consistent with a previous study by Da Costa et al. [54], who found that E. ramosissimum promotes collagen synthesis and inhibits enzymes related to skin aging, suggesting its potential use as a cosmeceutical ingredient. Additionally, several anti-collagenase compounds were identified in M. peregrina, such as lupeol acetate [55], which was tested for its ability to bind to matrix metalloproteinase-1 (MMP-1), a collagenase enzyme [56], and showed considerable results.

A cream formula was prepared using the emulsification method, incorporating the plant extract PEM (2:1) as an active ingredient at 1.5%. The prepared formula exhibited good stability at room temperature, as evidenced by its physicochemical appearance. Furthermore, the cream displayed pseudoplastic rheological behavior, meaning that it becomes easier to spread when subjected to rubbing force, contributing to its good spreadability and homogeneity. The antimicrobial test performed on the prepared cream formula revealed that the preservatives added to the formula were effective.

A skin irritation test was performed using a rat model. The results showed that this formula did not induce edema

or erythema, indicating that the cream's components are safe for application on skin models, pending further research.

5. CONCLUSION

Findings from the current study indicate that this plant extract mixture holds potential as an active ingredient for incorporation into pharmaceutical formulations for skin care. Its superior antioxidant activity, along with its inhibitory effects on tyrosinase and collagenase enzymes, and its antibacterial effect on the tested bacterial strains, underscore its promise. Furthermore, the PEM-cream formula demonstrated acceptable stability at room temperature and showed no risk of skin irritation, as confirmed by both in vitro and in vivo tests.

ABBREVIATIONS

PEM: Plant extract mixture; TPC: Total phenolic content; TFC: Total flavonoid content; ABTS: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid; DPPH: 2,2-Diphenyl-1-picrylhydrazyl Radical; QE: Quercetin; GA: Gallic acid; IC_{50}: Half inhibitory concentration; MRSA: Methicillin resistant *Staphylococcus aureus*; UVR: Ultraviolet radiation; MMP-1: Matrix metalloproteinase-1; SD: Standard deviation.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the study. They were also involved in material preparation, data collection, and manuscript writing. All authors have read and approved the final manuscript.

CONFLICTS OF INTERESTS

The authors report no conflicts of interest in this work.

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التأثيرات المثبطة للبوليفينول من مستخلصات Equisetum ramosissimum و Moringa peregrina على المكورات العنقودية الذهبية والكولاجيناز وإنزيمات التيروزيناز: دراسات مخبرية تجريدية

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ملخص

خلفية: تؤدي مشاكل الجلد الناجمة عن الإجهاد التأكسدي إلى تنشيط إنزيمات الكولاجيناز والتيروزيناز ، والتي يمكن أن تسهم في شيخوخة الجلد وتغير لونه والالتهابات. سابقا تم تقييم النباتات Equisetum ramosissimum و Moringa peregrina لاستخداماتهما في حالات جلدية مختلفة. الهدف: يهدف البحث الحالي إلى التحقيق في التأثير الإيجابي للبوليفينول في مستخلصات Equisetum ramosissimum و Moringa peregrina كمنتج تجميلي محتمل يستخدم لعلاج مشاكل الجلد المختلفة. الطرق: تم تحديد المحتويات الكلية للفينول والفلافونويد ومضادات الأكسدة ومضادات الكولاجيناز ومضادات التيروزيناز لمخاليط المستخلصات النباتية (PEM) بنسب مختلفة من (M. peregrina: E. ramosissimum) باستخدام الإجراءات المعيارية. تم تقييم التأثيرات المثبطة ل ((PEMضد البكتيريا المسببة لحب الشباب المكورات العنقودية الذهبية ((ATCC 29213باستخدام طريقة قياس قطر منطقة التثبيط (سم). تمت صياغة تركيبة كريمية تحتوي على PEM والتحقق من ثباتها وتاثيها على تهيج جلد الفئران في المختبر. النتائج: أظهرمزيج PEM بنسبة (2: 1) على محتوى إجمالي من الفينول والفلافونويد (150.15 ± 2.8 مجم / جم مكافئ لحمض الغال) ، و (41.5 ± 1.2 مجم / جم مكافئ للكيرسيتين) ، على التوالي. كانت الأنشطة المضادة للأكسدة (2: 1) PEMكما تم الحصول عليها باستخدام طرق DPPH و IC50= 0.12 ± 7.06) ABTS ميكروغرام / مل و 3.3±53.29 = IC50 ميكروغرام / مل) ، على التوالي. علاوة على ذلك ، أظهر (2: 1) PEMأنشطة تثبيط متفوقة ضد إنزيمات الكولاجيناز والتيروزينات (= IC50 1.19 ± 32.4 ميكروغرام / مل و IC50=8.4±1.19 ميكروغرام / مل) ، على التوالي. أظهر النشاط المضاد للميكروبات PEM (2: 1)الذي تم اختباره على S. aureus أكبرقطر منطقة تثبيط النمو (2.8 سم) ، بتركيز 60 مجم / مل. كشفت الدراسات التي أجريت على تركيبة كريم (2: 1) PEM أنها ثابتة فيزيائيا في ظروف الغرفة. أظهر اختبار تهيج الجلد على الفئران عدم ظهور الوذمة أو الحمامي بعد العلاج. الخلاصة: أظهر PEM بنسبة (2: 1) الأنشطة المثلى كعامل تحييد الإجهاد التأكسدي ، ومثبط للإنزيمات المسببة لاكتساب الجلد وفرط التصبغ ، وكذلك مع تأثير مضاد للبكتيريا. كما أظهرت تركيبة كريمية تحتوي على PEM ثباتا فيزيائيا مع عدم وجود أي خطرا محتمل لتأثير تهيج الجلد خلال فترة إجراءات البحث.

الكلمات الدالة: نبات ذيل الفرس ، نبات المورينغا، المكورات العنقودية الذهبية، كولاجيناز ، تيروزيناز .

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Senna alata: Phytochemistry, Antioxidant, Thrombolytic, Anti-inflammatory, Cytotoxicity, Antibacterial activity, and GC-MS analysis

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ABSTRACT

Objective: Nepal's medicinal herbs are rich in cultural importance and have several uses. Senna alata, a plant belonging to the Leguminosae family, is prized for its aesthetic and therapeutic qualities. The goal of the study was to extract Senna alata leaves using several solvent macerations.

Methods: The study aims to evaluate the phytochemistry, total phenolic and flavonoid levels, antioxidant qualities in vitro, anti-inflammatory effects, cytotoxicity, anti-thrombolytic potential, and antibacterial activity, a variety of methodologies were employed.

Results: The extractive values of Senna alata were determined as 1.58%, 0.78%, and 5.92% in hexane, ethyl acetate, and methanol, respectively. GC-MS analysis revealed major compounds such as 3-Methylmannoside, Neophytadiene, Campesterol, and Vitamin E in the leaf extract. Qualitative phytochemical screening confirmed the presence of tannins, carbohydrates, flavonoids, cardiac glycosides, glycosides, and saponins in the methanol extract. The total phenolic and flavonoid contents were 46.36±4.5 mg GAE/g and 480.4±3.055 QE/g of dried extract, respectively. The extract exhibited significant antioxidant and anti-inflammatory activities, with IC50 values of 29.81 and 9.93, respectively. Additionally, it demonstrated cytotoxic activity with an LC50 value of 767.85 in the brine shrimp bioassay. In terms of thrombolytic activity, the extract showed clot lysis percentages of 7.89% and 10.13% at concentrations of 10 mg/ml and 25 mg/ml, respectively.

Conclusion: The methanolic extract of Senna alata leaves displayed therapeutic potential, including antioxidant, anti-inflammatory, cytotoxic, thrombolytic, and antibacterial effects. The presence of several bioactive compounds, as confirmed by GC-MS analysis, further supports the plant's potential for therapeutic use.

Keywords: *Senna alata*, phytochemical screening, antioxidant activity, anti-inflammatory activity, thrombolytic activity, cytotoxic activity

INTRODUCTION

Due to a lack of research and scientific validation, herbal medicines are still not widely accepted by the medical community [1]. Antibiotic resistance is an issue in today's world because of the widespread use of commercial antibiotics to treat infectious diseases [2].

The immune system's natural reaction to harm caused by microbial, chemical, and physical agents is inflammation. Excessive swelling leads too many acute and long-term illnesses such as autoimmune diseases,

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illnesses of the circulatory system, malignancies, and metabolic and neurological disorders [3].

An entire class of extremely reactive molecules produced by the metabolism of oxygen is known as reactive oxygen species (ROS). Normally occurring physiological quantities of ROS are necessary for cellular functions. Antioxidants, both natural and synthetic, can help the body reduce oxidative damage brought on by reactive oxygen species [3].*Senna alata* contains chemical components that have been shown to have a variety of pharmacological activities. However, the real effects are yet unknown, thus more research is needed to investigate its medical benefits [4].

Meantime, some studies reported that the generation of reactive oxygen species (ROS) caused inflammation which has been related to the pathogenesis factors for various diseases in patients [5]. Hence, this complication may be prevented through the scavenge ROS by the free radical scavenging mechanism. Most bioactive phytoconstituents like; phenolic, alkaloid, and flavonoid compounds curing the endogenous cells and cellular proteins through the free radical scavenging activity [6-8]. Such preventive effects are important for inflammation, cytotoxicity, and microbial disease which is also due to oxidative processes.

Senna alata is commonly known as a candle tree or ringworm brush [1]. It is locally known as the Agasti plant and is used in various religious rituals. *Senna alata* is a significant flowering plant that is both decorative and medicinal [9]. Foliage of plants is utilized as a pungent, expectorant, vermicide, purgative, and as well as in the handling of fungal illnesses. *Cassia alata* leaf extract may have cytotoxic, analgesic, antibacterial, anti-inflammatory, and fungicidal properties [1, 9].

The search for new antimicrobial active agents obtained by using plant extracts of *Senna alata* has led to the discovery of many clinically useful drugs, which help to solve the problems of antibiotic resistance exhibited by pathogenic microorganisms [2]. *Senna alata* contains anti-inflammatory agents; hence can be a sensible and successful research approach in the hunt for novel anti-inflammatory medications. These plant extracts may led to the concentration of scientific effort on finding reliable and efficient sources of antioxidants that prevent the cellular and tissue damage caused by oxidative stress [3].

In the search for new drugs, the search for bioactive plant components from natural sources is always a deciding factor. Research on *Senn aalata* leaves will improve the proper use of this plant in various medical conditions as an alternative treatment plan and will help to find possible therapeutic agents for specific diseases. The purpose of this study is to look into the in vitro antioxidant, antibacterial, anti-inflammatory, thrombolytic, cytotoxic effects, phytochemical profiling, and GC-MS analysis of *Senna alata* leaf extract.

MATERIAL AND METHODS

Drugs and chemicals

Methanol, Ethyl acetate, and Hexane were purchased from Merck Life Science Pvt. Ltd., while the Vincristine Sulphate was obtained from the Neon Laboratories, Limited, India. Standard reference drugs Diclofenac and Ciprofloxacin were obtained from Saheka India and Arati Drugs India respectively. Ascorbic acid, Gallic acid, Quercetin, and DPPH was purchased from Hi-Media, India. Every reagent and chemical of analytical are used for this research.

Study plant material

Senna alata leaves were gathered from Chandragiri-06, Kathmandu, Nepal. Plant herbarium was authenticated by a taxonomist from National Herbarium and Plant Laboratories, Kathmandu, Nepal. A voucher specimen (Acc. No. 02-1221- 2020) was deposited in the herbarium of Manmohan Memorial Institute of Health Science for future reference.

Plant extract preparation and extract percentage yield

Fresh water was used to clean the plant leaves completely. After that, the leaves were dried and

sliced into little bits and introduced to successive maceration techniques in which 3 different solvents were used according to the polarity of the solvents. The method involved the use of non-polar solvent ethyl acetate and n-hexane and polar solvent methanol for extraction of active constituents from the powdered leaves. For this, a known amount of powdered sample was taken in a beaker. First, hexane, then ethyl acetate, and finally methanol was used for extraction in the polarity order using the maceration process. Ultimately, a table fan was used to concentrate the extracted material at room temperature after it had been moved to a stainless-steel plate. Dried extracts were stored in borosilicate glass vials and subjected to different investigations. Then obtained extract was filtered with Whatman No. 1 filter paper. The filtrate was evaporated by a rota evaporator under reduced pressure (60 mmHg) at 40ºC and stored at 4ºC. The extract yield percentage can be determined using the formula below:

Extract % yield = (Weight of dried extracts / Weight of plants sample) ^{x}100

GC-MS analysis

The chemical components present in the methanolic extract that demonstrated the different biological activities were identified by performing a GC-MS analysis on the extract. The Nepal Academy of Science and Technology in Khumaltar, Lalitpur, conducted the GC-MS. For the GC-MS examination of plant material, GCMS QP2010 (Shimadzu, Kyoto, Japan) equipped with RTx-5MS fused silica capillary column of 30m length X 0.25 mm diameter X 0.25µm film thickness. Helium (>99.99% purity) with 36.2 cm/sec linear velocity was employed as carrier gas. The system was configured using 3.9 ml/min of total flow rate, 0.95 ml/min of column flow, and 3.0 ml/min purge flow. The volume of the injected sample was 1µl. The injector was set in splitless mode having 280ºC of temperature. Starting at 100ºC, the oven temperature increased to 250ºC at 15ºC/min with a 1-minute pause. It then increased to 280ºC at 30ºC/min with a 1-min hold, then it increased once more from 280ºC to 300ºC at 15ºC/min with an 11-minute hold. With solvent cut-off duration of 3.5 minutes, the ion source and interface temperatures were set at 200ºC and 280ºC, respectively. 20 minutes were spent on the mass range scan, which covered 40 to 500 m/z. By comparing the mass spectra of the compounds with information from the NIST 08 mass spectral collection, the compounds were identified.

Qualitative phytochemical analysis

The *Senna alata* leaves extracts undergo phytochemical analysis for the detection of plant secondary metabolites like alkaloid, flavonoid, tannin, carbohydrate, anthraquinone, saponins and protein [10, 11, 34].

Quantitative phytochemical analysis

Using the Folin-Ciocalteu technique, the total phenolic content of *Senna alata* extracts was determined [12] using certain adjustments, and the results were expressed as gallic acid (GA) equivalents in milligrams (mg) per gram of dry leaf extract (mg GAE/g).

Using the aluminum chloride method, the total flavonoid content was evaluated [13] using a few minor adjustments, and the total flavonoid were expressed as milligrams of quercetin equivalents (QE) per gram of extract from dried leaves (mg QE/g).

DPPH free radical scavenging assays

The previous approach was applied to evaluate the *Senna alata* leaves extract's capacity to scavenge DPPH free radicals [14]. To put it briefly, 2 mL of DPPH solution (60 µM) was added to 2 mL of ethanolic and aqueous extracts at varying concentrations (0.1-100 µg/ml). Next, incubate in the dark at 25 ºC for 30 minutes. For the positive control, ascorbic acid (AA) was used. [15, 16]. IC_{50} value of the sample containing plant extracts—that is, the

concentration required to scavenge 50% radicals was also determined. Each sample's free radical inhibition activity was calculated using the following formula:

DPPH free radical scavenging activity (%)

$$= [(A_{control\ 517} - A_{Sample\ 517})/ A_{control\ 517}] \times 100$$

Where, $A_{control\ 517}$ is the absorbance control $A_{sample\ 517}$ is the plant extract sample absorbance

In vitro thrombolytic activity

For assessing the clot-dissolving activity of *Senna alata*, a plant extract, compared to positive (Streptokinase) and negative control (water). Blood samples water collected from 21 healthy volunteers and distributed into pre-weighed micro centrifuge tubes. Following clot formation, serum was extracted, and the weight of the clot was measured. *Senna alata* extract, streptokinase, and water were added separately to different tubes. Following 90 minutes incubation, after removing any fluid that had leaked, the tubes underwent another weight measurement. As a percentage of clot lysis, the weight difference between before and after clot lysis was computed.

The experimental setup allowed the evaluation of *Senna alata* effectiveness in dissolving blood clots and comparing it to the controls [17].

$$\% \text{ clot lysis} = \frac{W_3 - W_2}{W_2 - W_1} X\ 100$$

Where, Clot weight = W_2-W_1

W_1 = weight of tube alone, W_2 = weight of clot containing tube, W_3 = final weight of tube with test

Brine Shrimp Lethality Bioassay

In this experiment, the lethality test for brine shrimp was utilized to assess the cytotoxic potential of a plant extract [18]. Six different concentrations of the extract were tested, ranging from 800 µg/ml, 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml. After a 24-hour exposure, the number of surviving shrimps was recorded. Larvae showing no movement were considered dead. Negative control using Dimethyl sulfoxide and a Vincristine sulfate as reference standard were included. To make sure famine was not the cause of the observed death, they were compared with control group.

The toxicity of the plant exracts was determined by calculating the median lethal concentration (LC_{50}) using probit analysis, as described by Finney (Singleton & Rossi, 1965). The Brine Shrimp lethality bioassay offers several advantages, including its rapidity, low cost, simplicity, and the ability to use a large number of organisms for statistical validation. It also requires minimal sample volume (2-20 mg or less) and does not necessitate animal serum, which is typically needed for other cytotoxicity assays.

Mortality % = (No. of Dead larvae / Total no. of Larvae) x 100%

Antibacterial activity

Evaluation of antibacterial activity

To evaluate antibacterial activity, the agar well method was employed. In the method test, organisms were gathered, isolated as pure cultures, and standardized using the 0.5 M Mac-Farland standard [19].

Microorganism culture

It was decided that selectable microorganisms may be used for the investigation of antibacterial characteristics. The ATCC culture was obtained from the MMIHS laboratory, while the clinical isolates were obtained from the Natural Product Research Laboratory, Thapathali, Kathmandu. The creatures being assessed were:

Gram positive: *Staphylococcus aureus* (ATCC 6538P), *Bacillus subtilis* (ATCC6051)

Gram negative: *E.coli* (ATCC 8739), *Klebsiellapneumoniae* (ATCC700603)

Mueller Hilton Agar (MHA) petri plates that had just been made were used to cultivate the obtained bacteria. The organism was allowed to develop in peptone water broth for the purpose of standardization prior to the test. Before being injected into the petri plates, the organisms in peptone water broth were cultured for 4-5 hours.

Standard Preparation

The antimicrobial evaluation standard employed in the experiment consisted of (320, 160, 80, 40) µg/ml of a solution of Gentamycin and Azithromycin dissolved in 1% DMSO.

Sample preparation

Different concentration of Normal extract solution (320, 160, 80, 40) µg/ml was dissolved in DMSO solution.

Test Procedure

The bore well diffusion method was employed for the test. According to the manufacturer's directions, MHA agar was made. The agar was prepared and 15 minutes of autoclaving at 15 pounds of pressure. In a sterile laminar hood, sterile petri dishes were filled with agar. The sun could set. For the test method, bores were prepared using an 8mm well borer. Using a sanitized swab stick, the bacteria were extracted from the peptone water broth and swabbed in the petri plates. Using a micropipette with sterile tips, 100 g/L of the extracts were collected. For 5 different concentrations of the test extracts, 5 holes were punched into each plate. Standard and blank underwent a similar process. Following a 24-hour incubation period, the zones of inhibition were identified on the plates.

Statistical analysis

The mean ± standard deviation of the data was displayed. Using the regression line equation in Microsoft Excel (2007), the standard calibration curve for gallic acid and quercetin was created to estimate the concentration of phenolic and flavonoid compounds. The findings were displayed in the table and picture. SPSS version 16 was used to do the statistical analysis.

RESULTS

Qualitative phytochemical analysis

Numerous phytoconstituents, including tannin, flavonoids, saponin, carbohydrates, terpenoids, and cardiac glycosides, were found in methanol extracts using qualitative phytochemical screening; these results are shown in Table 1.

Table 1: Phytochemical screening of water and ethanol extract of *Senna alata leaves*.

Phytochemical constituents	Specific tests	Result
		Methanol extract
Alkaloid	Mayer's test	-
	Wagner test	-
	Hager’s test	-
	Dragendroff’s test	-
Carbohydrate	Molish's test	+
	Benedict's test	+
Glycoside	Modified Borntrager test (Anthraquinones)	-
	Killer killiani test (Cardiac glycoside)	+
Saponin	Foam test	+
Phenol	Ferric chloride test	+
Flavonoid	Alkaline reagent test	+
	Shinoda test	+
	Zn-HCl test	+
Tannin	Gelatin test	+
	Ferric Chloride test	+
Terpenoid	Salkowaski test	-
	Copper acetate test	-

+: Presence, -: Absence

GC-MS analysis

GC-MS analysis of the *Senna alata* methanol leaf extracts revealed that the presence of the 3-methylmannoside, neophytadiene, squalene, campesterol, stigmasterol, alpha-tocospiro (Table 2).

Table 2: GC –MS analysis

S.N	Name of Compound	Molecular formula	Reported Activity
1	3-Methylmannoside	$C_7H_{14}O_6$	Plant growth regulator/ regulate plant growth by modulating glycoconjugation to lectins in plants
2	Neophytadiene	$C_{20}H_{38}$	Antimicrobial, additive for liquid cigarette
3	Squalene	$C_{30}H_{50}$	Antioxidant
4	Campesterol	$C_{28}H_{48}O$	Anticancer, Antimicrobial, anti-inflammatory
5	Stigmasterol	$C_{29}H_{48}O$	Anticancer, Antiinflammatory
6	Alpha.Tocospiro	$C_{29}H_{50}O_4$	Cytotoxicity against human A549 cells by SRB assay.
			Antimicrobacterial activity against Mycobacterium tuberculosis H37Rv

Quantitative Phytochemical analysis

The methanol leaf extracts of *Senna alata* exhibited the highest percentage of extraction yield (5.92%). Highest phenol content was observed in methanol extract (46.36±4.5) mg GAE/gm dry extract weight while highest flavonoid content was found in methanol extract (480.4±3.055) mg QE/gm dry extract weight (Table 3).

Table 3: Extraction yield (%) of three solvents of *Senna alata* leaves, total phenolic and flavonoids content.

Extract	Extraction yield (%)	Phenols (mg GAE/g dry extract weight)	Flavonoids (mg QE/g dry extract weight)
Methanol extract	5.92	46.36±4.5	480.4±3.055
Hexane extract	1.54	44.89±4.49	476.17±4.33
Ethyl acetate	0.78	40.66±0.36	435.77±4.81
Values calculated from the mean of three times experiment and represented as mean ± S.D			

DPPH radical scavenging activity

Compared to conventional ascorbic acid, the methanol extracts of *Senna alata* leaves (IC_{50} value 29.81 µg/mL) demonstrated good DPPH free radical scavenging action (Table 4, Fig. 1).

Table 4: IC_{50} value and DPPH free radical scavenging activity of both *Senna alata* extracts at varying concentrations.

Extract/ Standard	% activity of DPPH scavenging					IC_{50} µg/mL
	5 µg/mL	10 µg/mL	15 µg/mL	20 µg/mL	25 µg/mL	
Methanol extract	15.13±0.0093	24.65±0.0063	29.28±0.0094	37.33±0.0290	42.83±0.0049	29.81
Ascorbic acid	41.19±0.0090	61.69±0.0012	75.59±0.0050	92.56±0.0050	97.07±0.0080	6.12
Values calculated from the mean of three times experiment and represented as mean ± standard deviation (n=3).						

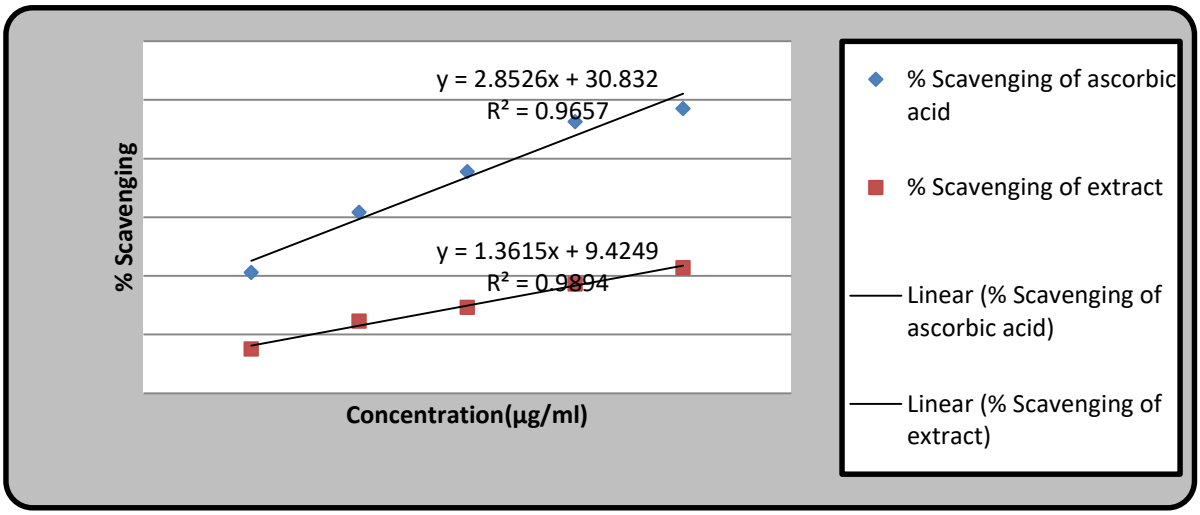

Fig. 1. Antioxidant activity by using DPPH.

Thrombolytic activity

This study found that the percentage of clot lysis was 7.89% at 10 mg/ml and 10.13 % at 25 mg/ml. In a similar vein, clot lysis with conventional streptokinase was found to be 40.77% (Table 5, Fig. 2).

Table 5: Percentage clot lysis of extract.

S.N	Concentration(mg/ml)	% clot lysis
Extract 1	10	7.89
Extract 2	25	10.13
Streptokinase	30,000 I.U	40.77
Negative control	D/W	2.03

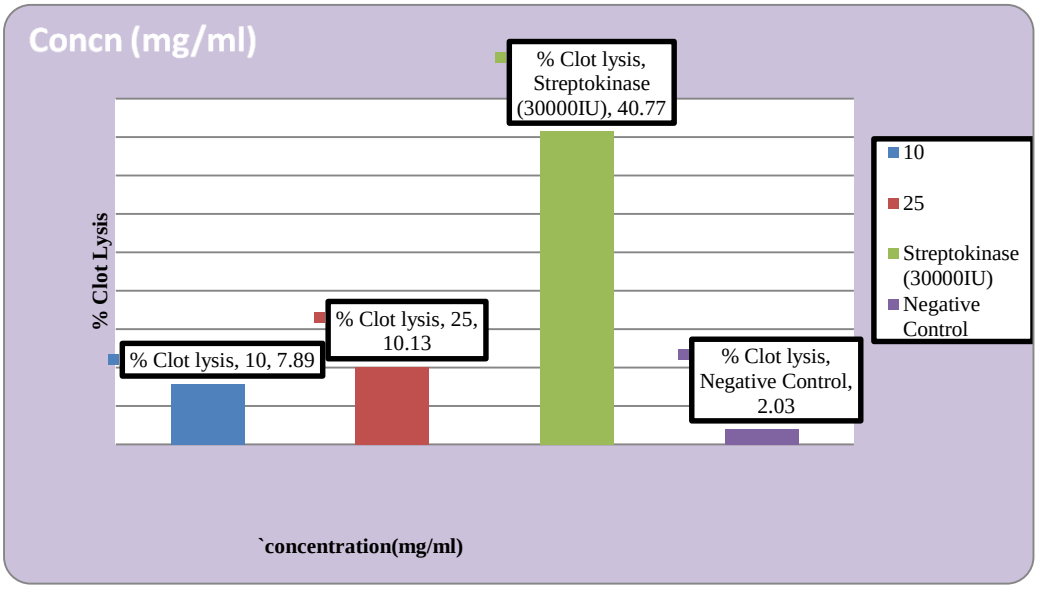

Fig. 2. Thrombolytic activity of the extract.

Anti-inflammatory activity

Human red blood cell (HRBC) membrane stabilizing method assessed the in vivo anti-inflammatory properties of *Senna alata* extracts. As a standard, Diclofenac Sodium was used. The results obtained by studying in vivo anti-inflammatory activity are tabulated in Table 6 (Fig. 3, 4, 5).

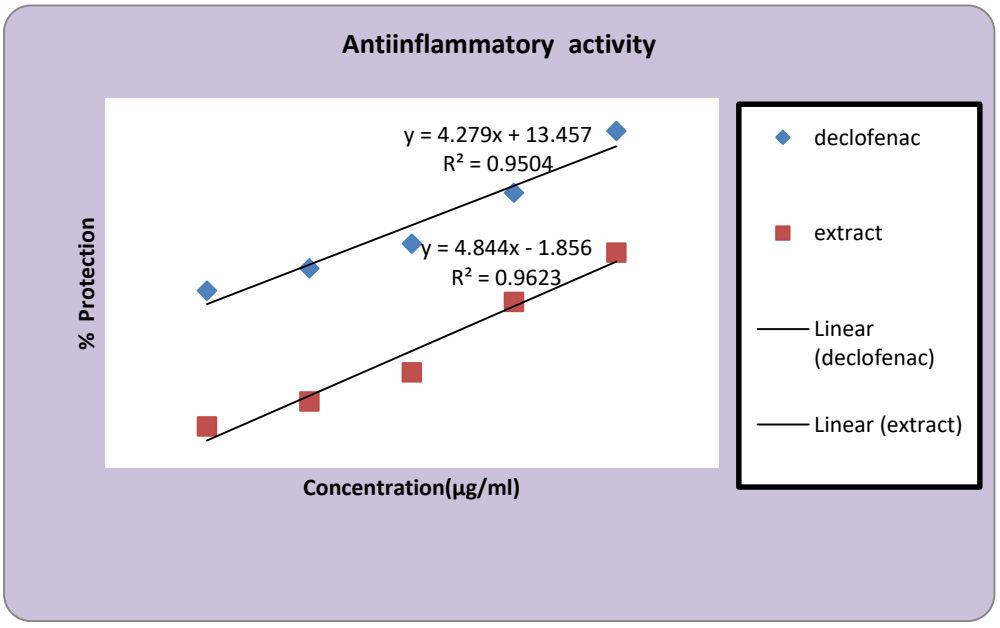

Fig. 3. Anti-inflammatory activity of standard and extract.

Table 6: Percentage protection and percentage hemolysis of extract and standard.

Concentration(µg/ml)	Percentage Protection		% hemolysis	
	Diclofenac	Extract	Diclofenac	Extract
10	19.21	4.5	80.79	95.46
20	21.62	7.4	78.38	92.75
40	24.32	10.32	75.68	89.62
80	29.81	18.16	70.19	77.90
100	36.51	23.32	63.49	75.17

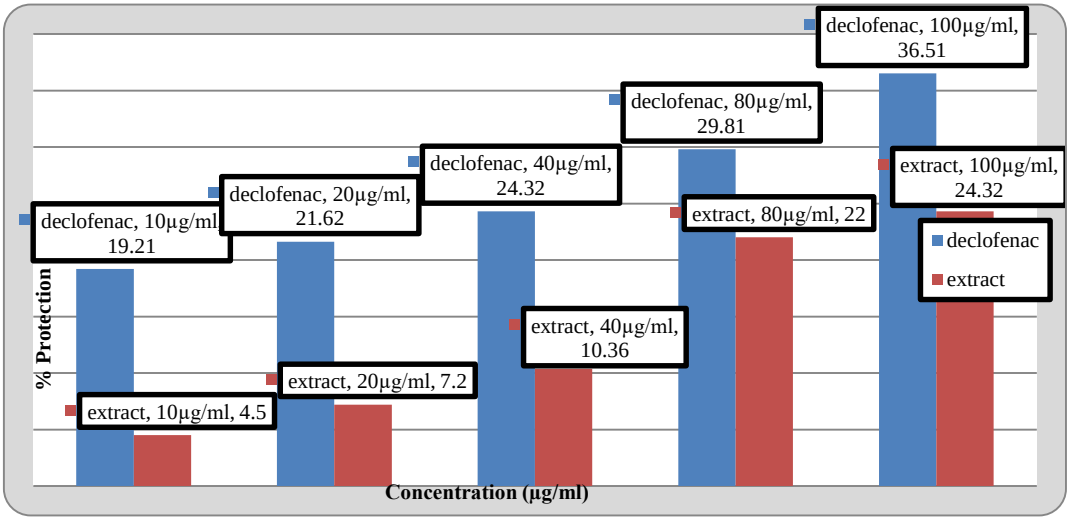

Fig. 4. Anti-inflammatory activity of the *Senna alata* extract.

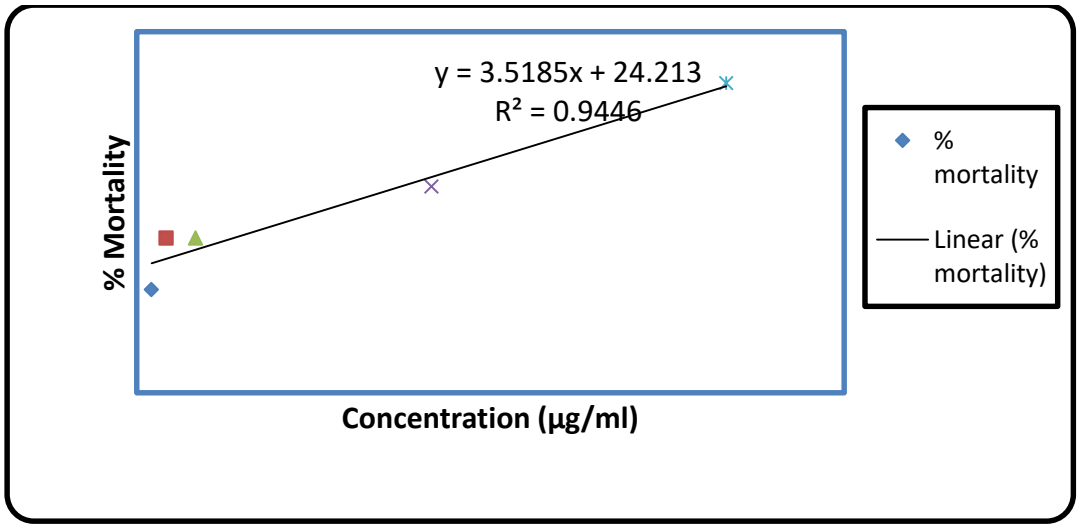

Fig. 5. Cytotoxic activity of vincristine Sulphate

Cytotoxic activity

Vincristine sulfate had an LC_{50} value of 7.32 (µg/ml), while *Senna alata*'s methanolic extract had an LC_{50} value of 767.85 (µg/ml) (Table 7, 8, 9) (Fig. 5, 6).

Table 7: EC50 values for Diclofenac and extract.

Name	EC50
Diclofenac sodium	8.54
Extract	9.93

Table 8: Percentage mortality by standard Vincristine Sulphate

Concn (µg/ml)	% mortality	LC_{50} value
0.25	20	7.32
0.5	30	
1	30	
5	40	
10	60	

Table 9: Percentage mortality of brine shrimp by extract.

Concentration (µg/ml)	% Mortality	LC_{50} (µg/ml)
50	0	767.85
100	0	
200	10	
400	30	
800	50	

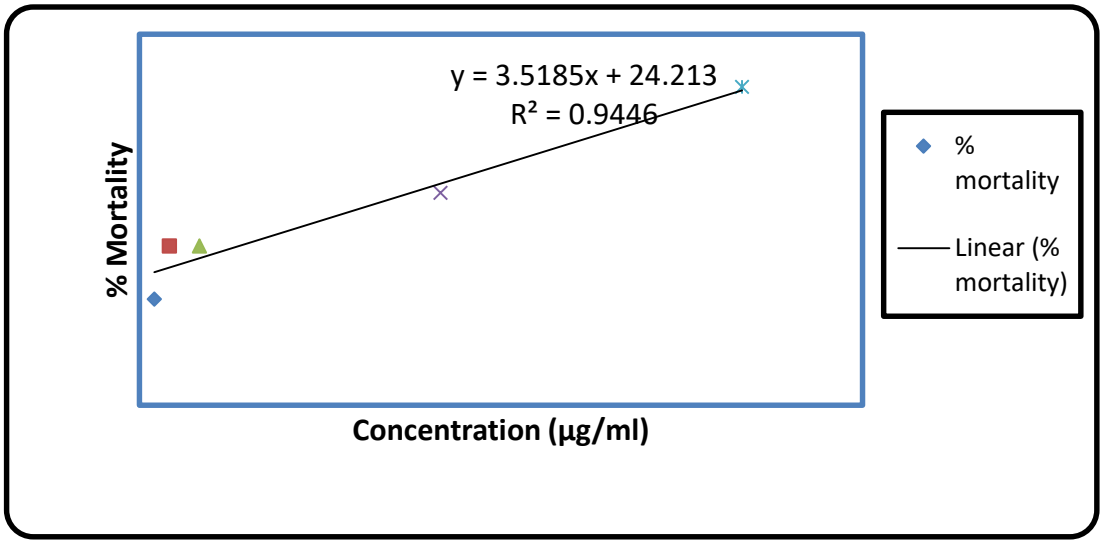

Fig. 5. Cytotoxic activity of vincristine Sulphate

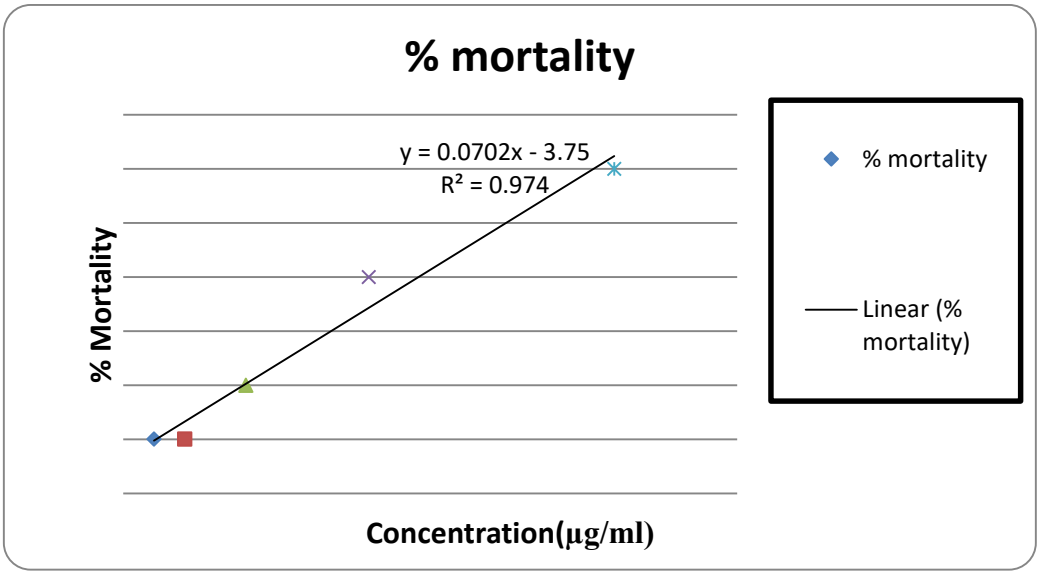

Fig. 6. Cytotoxic activity of *Senna alata* extract

Antibacterial activity

Table 10 displays the methanolic extract's antibacterial activity. While the extract does not demonstrate activity against *E. coli*, it does demonstrate activity against *S. aureus*, *B. subtilis*, and *K. pneumoniae* (Fig. 7, 8, 9).

Table 10 Antibacterial activity

Sample	Concn (µg/ml)	Inhibition zones of antibacterial screening (mm)			
		S. aureus	*B. subtilis*	*K. pneumoniae*	*E. coli*
Azithromycin	40	5	10	-	-
	80	7	12	-	-
	160	12	19	-	-
	320	14	21	-	-
Gentamycin	40	-	-	3	8
	80	-	-	3	9
	160	-	-	4	13
	320	-	-	7	15
Extract	40	2	-	-	-
	80	3	1	-	-
	160	4	2	1	-
	320	6	4	2	-

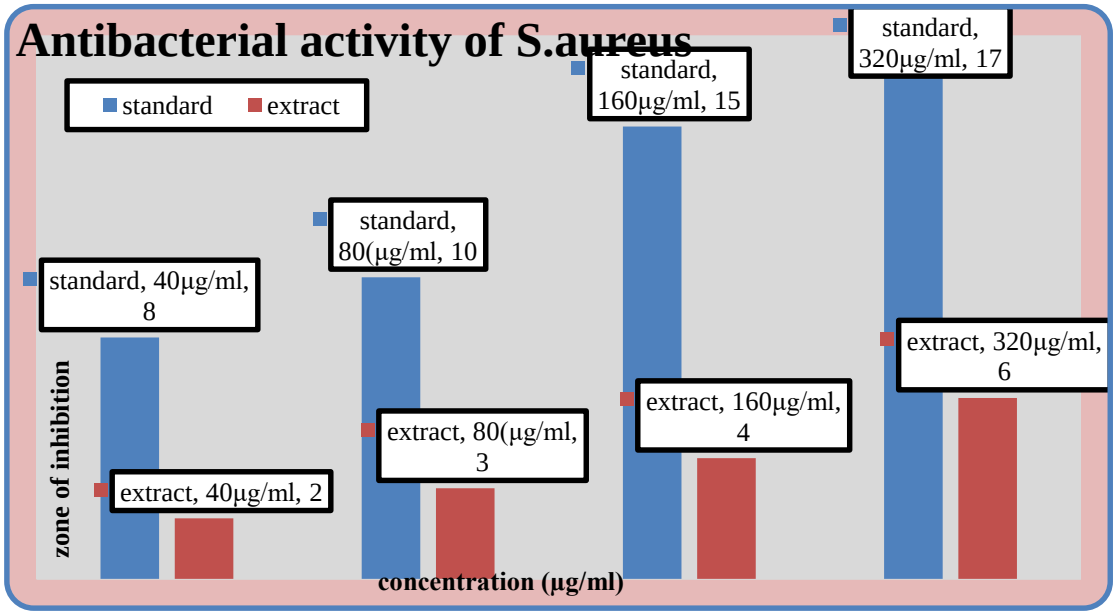

Fig. 7. ***Antibacterial activity of Senna alata extract against S.aureus***

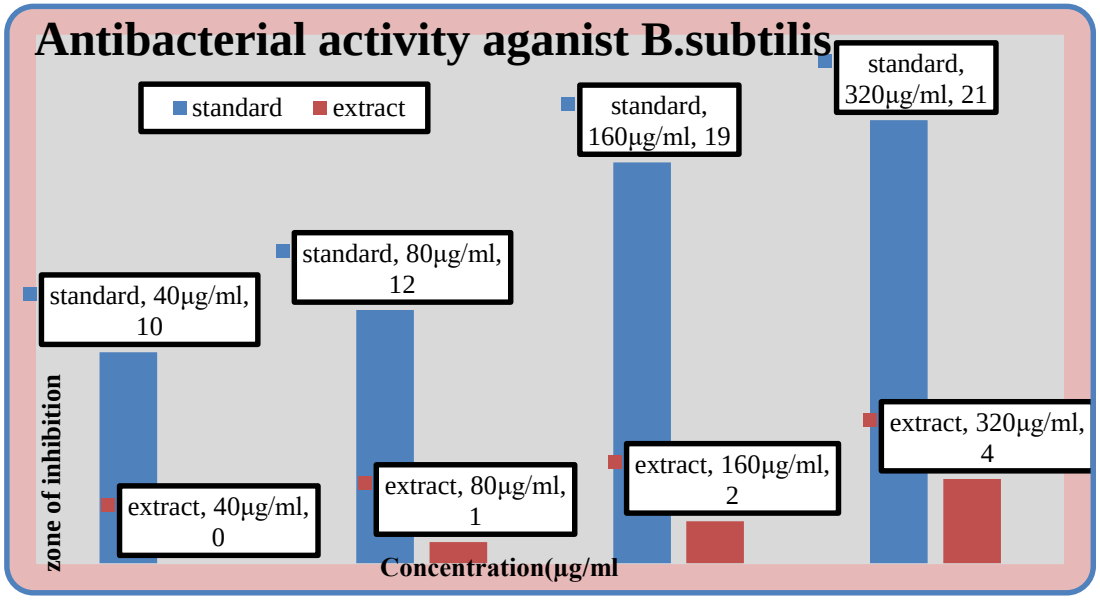

Fig. 8. Antibacterial activityof *Senna alata* extract against *B.subtilis.*

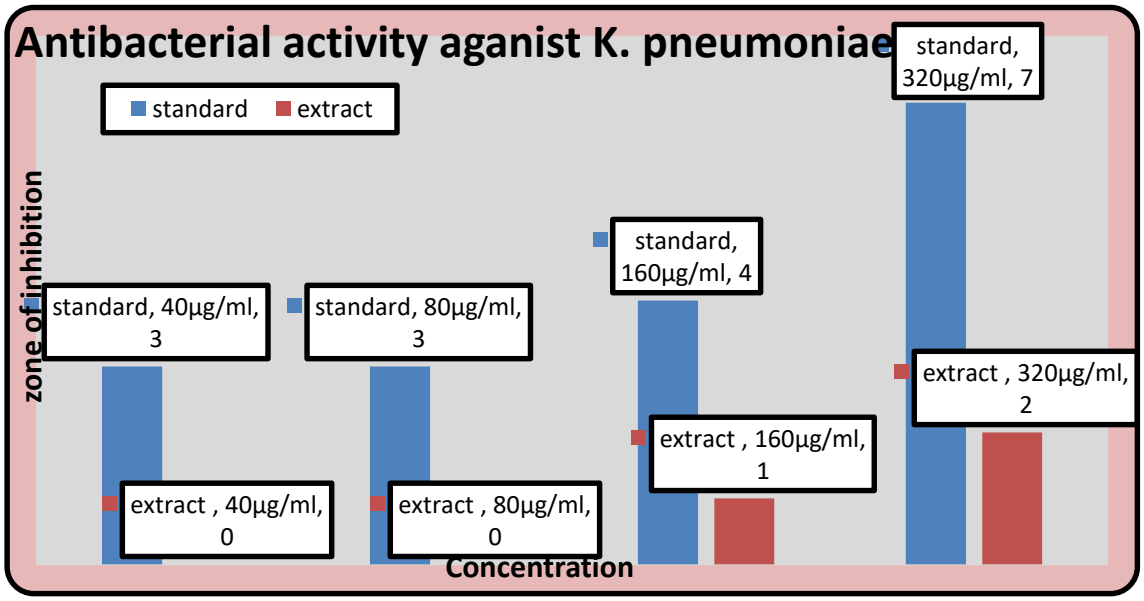

Fig. 9. Antibacterial activity of *Senna alata* extract against *K .pneumoniae*.

DISCUSSION

It is widely accepted that natural products are the most valuable source of lead compounds for innovative drug development in the pharmaceutical industry. Bioactive plant components are used as drug candidates or drug substitutes to treat various human diseases [20]. The selection of *Senna alata for* this communication was based on its limited scientific research, and traditional and ethnomedicinal uses. *In vitro* antioxidant, *antibacterial*, *in vitro* anti-inflammatory, thrombolytic, cytotoxic activities, phytochemical profiling and *Senna alata* leafmethanolic extract was subjected to GC-MS analysis.

The percentage yield was highest in methanol, at 5.92%. In a previous study, the methanol extract of Senna alata yielded 8.32% [21]. This was mostly impacted by various cultivation circumstances, including climate, plant location, and harvest times. The solvent's polarity has an impact on the phytochemicals that are recovered in the extracts.

The phytochemical analysis of *Senna alata* extracts showed the occurrence of a variety of chemical components that may have different pharmacological effects, such as flavonoids, carbohydrates, tannins, saponins, and cardiac glycoside. An earlier investigation found that the *Senna alata* plant's root and leaf extracts have antimicrobial properties in Nigeria revealing that saponins, alkaloids, flavonoids, anthraquinone, tannins, phenols, and glycosides are present in *Senna alata* [18]. Because they contain phenols and flavonoids, plant secondary metabolites have anti-inflammatory and antioxidant properties. They also have a favorable correlation with as antibacterial, cytotoxicity, and anti-inflammatory activity [18, 31].

The total phenolic content was determined using Folin-Ciocalteu's technique in using a standard agent as gallic acid. The leaves of *Senna alata* had the highest quantity of total phenols (42.76±2.13 mg GAE/g dry extract weight) in the methanol extract. Using quercetin as a reference, the

total flavonoid concentration was determined using the aluminum chloride colorimetric test. Of the two methanol extracts with flavonoid content, this one has a high concentration of flavonoids (34.97±2.86 mg QE/g dry extract weight). It demonstrates how important a role the solvent system plays in the solubility of various chemical components. It has been demonstrated that higher polarity solvents remove phenolic chemicals from the entire plant more effectively than lower polarity solvents [22]. This result agrees with the previous study [23]. The similar previous study performed on the methanol extract of this plant showed 41.6±0.41 mg GAE/g and 31.9±0.63 QE/g of the dried extract [21].

The various samples' DPPH radical scavenging ability was tested at various doses (at 5, 10, 15, 20, and 25 µg/ml) methanol extract revealed the concentration-dependent radical scavenging activity. Ascorbic acid's IC_{50} value in the DPPH scavenging method was 6.62 (µg/ml), while the plant extract's IC_{50} value was 29.81 (µg/ml). Previous study conducted by J. Sujatha, and S. Asokan showed that the IC_{50} value was 24.56 µg/ml [24]. According to this study, the IC_{50} value decreased as the phenolic and flavonoid concentration increased. The plant samples' antioxidant activity could be attributed to the presence of these chemical ingredients [25]. It has been demonstrated that plants with flavonoid and phenolic compounds can scavenge free radicals in living things [24]. Plant metabolites known for their phenolic and flavonoid components are widely distributed and exhibit a variety of pharmacological properties, including antibacterial, antioxidant, hepatoprotective, antidiabetic, and antimutagenic properties [26, 27]. The majority of compounds classified as antioxidants are derived from plants as secondary metabolites, such as phenolic compounds (flavonoids, phenolic acids, tocopherols, etc.) [25]. Because they can scavenge reactive oxygen species such as superoxide free radicals, singlet oxygen, and hydroxyl radicals, phenolic compounds have the potential to be antioxidants [28, 32]. The many functional hydroxyl groups found in flavonoids mediate their antioxidant action by scavenging dangerous free radicals and chelating metal ions to prevent the generation of dangerous radicals that damage vital biomolecules. Lipid peroxidation is oxidative stress's most frequent side effect. Through a variety of mechanisms, flavonoids play a significant role in lipid peroxidation against oxidative damage [29, 33].

This study showed that the lethal concentration (LC_{50} value) for the *Senna alata* leaves extract was found to be 767.85 µg/ml and the highest mortality percentage was 30 % at the concentration of 800 µg/ml. In the earlier research carried out by M.A. Awal et al, it was found that the toxicity effect of ethanolic leaf and seed extract of *Cassia alata* and found promising activity, rated that LC_{50} value of 4.31µg/ml for seed and 5.29µg/ml for leaf [18]. The phytochemicals present in plants such as alkaloids, flavonoids are believed to have an anti-cancer activity that can inhibit either initiation or progression of the tumors. The absence of alkaloids may be causes for relatively lower value of cytotoxicity activities [35, 36].

In the anti-inflammatory activity, the percentage protection at 100µg/ml was found to be 36.51% and 23.32% for the standard drug Diclofenac sodium and extract respectively. It was discovered that the thrombolytic activity at a concentration of 10 mg/ml was 7.89% and at 25 mg/ml it was 10.13 %. In the earlier investigation carried out by Adnan Mannan et al., the extract of cassia seed showed 37.92% clot lysis when the amount of 100µl/ml [17].

The zone of inhibition's existence verifies the test substance's capacity to impede growth. The largest zone of inhibition was found to be against *Streptococcus aureus* i.e. 6mm and for *Bacillus subtilis* it was found to be 4 mm. In addition, the extract did not show any action against the E. *coli* and for *Klebsiella pneumoniae*, and it was found to be 2mm. In the previous study conducted by AA. Ogunjobi and M.A Abiala, the methanol extracts of *Senna alata* powder inhibited the growth of *Staphylococcus aureus*, and *Bacillus subtilis* with inhibition zone diameters of 15 mm and 12mm respectively [30].

The results of the current study on *Senna alata* included higher concentrations of phenolic and flavonoid components as well as potent thrombolytic, antibacterial, anti-inflammatory, and antioxidant properties. Considering that it might be a strong candidate for the creation of an innovative oral medicinal substance.

CONCLUSIONS

In summary, the methanol extraction of Senna alata leaves yielded substantial amounts of extract, along with a significant concentration of flavonoids and phenolic compounds exhibiting antioxidant activity. The GC-MS analysis identified 3-methylmannoside as having the highest area ratio in Senna alata. This study suggests potential antibacterial, thrombolytic, cytotoxic, and anti-inflammatory properties of the methanol extract. These findings support the need for further scientific validation and research to explore the therapeutic benefits of this medicinal plant in combating microorganisms, inflammatory diseases, and diseases related to harmful cell proliferation.

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Author contributions

DK and BR conceived and designed the experiments. DK, BP, PJ, and AA performed the experiments. BP, DPK, BR, and SP analyzed the data. DK, PJ, BP, and AA wrote the manuscript. BP, DPK, BR, and SP reviewed the manuscript. SP and BP critically revised the manuscript and provided intellectual input. DPK and BR supervised the project.

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Data Availability Statement

All relevant data is in the paper and any query regarding the findings of this study is obtainable from the corresponding author upon request.

Conflict of interest

The authors declare no conflict of interest.

Approval of the research protocol by an Institutional Reviewer Board and the approval number

The study was conducted with utmost care and showed no signs of endangering people or the environment. Ethical clearance was obtained from the Institute Review Committee of the Department of Pharmacy, Manmohan Memorial Institute of Health Sciences, Kathmandu (Approval No: MMIHS-BP-2018).

Informed Consent

Before participation, informed consent was properly obtained from the willing human blood donors. Additionally, municipal regulations were consulted to secure permission for research on the subject plant.

Registry and the Registration No. of the study/trial N/A

Animal Studies N/A

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Senna alata الكيمياء النباتية، ومضادات الأكسدة، والتحليل، ومضادات الالتهاب، والسمية الخلوية، والنشاط المضاد للبكتيريا، وتحليل الكتلة الطيفية للغاز

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ملخص

الهدف: تتمتع الأعشاب الطبية في نيبال بأهمية ثقافية كبيرة ولها استخدامات متعددة. يعتبر نبات السنا ألاتا، وهو نبات ينتمي إلى عائلة البقوليات، ذو قيمة عالية لخصائصه الجمالية والعلاجية. وكان هدف الدراسة هو استخراج أوراق السنا ألاتا من خلال عدة نقعات بالمذيبات.

الطرق: لتقييم الكيمياء النباتية، ومستويات الفينول الكلية والفلافونويد، والخصائص المضادة للأكسدة في المختبر، والتأثيرات المضادة للالتهابات، والسمية الخلوية، والإمكانات المضادة للتخثر، والنشاط المضاد للبكتيريا، استخدمت هذه الدراسة مجموعة متنوعة من المنهجيات.

النتائج: تم تحديد القيم الاستخراجية للسنا ألاتا على أنها 1.58٪ و 0.78٪ و 5.92٪ في الهكسان، وأسيتات الإيثيل، والميثانول، على التوالي. كشف تحليل كروماتوغرافيا الغاز–مطياف الكتلة عن مركبات رئيسية مثل 3–ميثيل مانوسايد، ونيوفيتادين، وكامبيستيرول، وفيتامين هـ في مستخلص الأوراق. وقد أثبت الفحص الكيميائي النباتي النوعي محتوى التانين والكربوهيدرات والفلافونويدات والجليكوسيدات القلبية والجليكوسيدات والسابونين في مستخلص الميثانول. وكانت القيم الفينولية الكلية والفلافونويدية 46.36±4.5 ملغ / GAE جم و 480.4±3.055 / QE جم من المستخلص المجفف على التوالي. أظهر المستخلص أنشطة مضادة للأكسدة ومضادة للالتهابات بشكل ملحوظ، مع قيم IC50 29.81 و 9.93 على التوالي. وعلاوة على ذلك، فقد أظهر نشاطًا سامًا للخلايا بقيمة LC50 767.85 في اختبار الروبيان الملحي. من حيث النشاط التحللي للخثرة، أظهر المستخلص نسب انحلال الجلطات 7.89٪ و 10.13٪ عند تركيزات 10 ملغ / مل و 25 ملغ / مل على التوالي.

الاستنتاج: وفي الختام، أظهر المستخلص الميثانولي لنبات السنا ألاتا إمكانات علاجية، بما في ذلك التأثيرات المضادة للأكسدة والالتهابات والسمية الخلوية ومضادات التخثر والبكتيريا. كما أكد وجود العديد من المواد الكيميائية عن طريق تحليل كروماتوغرافيا الغاز–مطياف الكتلة على إمكانات النبات للاستخدام العلاجي.

الكلمات الدالة: *Senna alata*، الفحص الكيميائي النباتي، النشاط المضاد للأكسدة، النشاط المضاد للالتهابات، النشاط المضاد للتخثر، النشاط السام للخلايا.

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Integrating Artificial Intelligence and Advanced Genomic Technologies in Unraveling Autism Spectrum Disorder and Gastrointestinal Comorbidities: A Multidisciplinary Approach to Precision Medicine

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ABSTRACT

This article explores the potential impact of Artificial Intelligence (AI), Machine Learning (ML), CRISPR-Cas9 gene editing, and single-cell RNA sequencing on improving our understanding and management of Autism Spectrum Disorder (ASD) and its gastrointestinal (GI) comorbidities. It examines how these technologies illuminate the complex interplay between the gut and the brain, identifying specific enzyme deficiencies and microbial imbalances linked to GI symptoms in ASD. By leveraging AI and ML, personalized intervention strategies are developed through the analysis of genomic, proteomic, and environmental data, enhancing our ability to predict and address GI issues in ASD. Additionally, CRISPR-Cas9 gene editing holds promise for correcting genetic abnormalities related to enzyme production, potentially offering precise treatments. Single-cell RNA sequencing provides critical insights into the cellular diversity of the ASD gut, uncovering new therapeutic targets. The article highlights the transformative potential of these technologies while addressing the associated challenges and ethical considerations. It underscores the necessity of a multidisciplinary approach to fully harness their benefits and discusses the significant progress and emerging trends in the field, emphasizing the role of technological advancements in advancing precision medicine for ASD and its GI comorbidities.

Keywords: Autism Spectrum Disorders; Enzymatic Dysfunction; Dietary Interventions; Gastrointestinal Comorbidities; Personalized Nutrition Therapy.

INTRODUCTION:

Autism Spectrum Disorder (ASD) and its gastrointestinal (GI) comorbidities present significant healthcare challenges [1-3], affecting a large number of individuals worldwide. Recent advancements in Artificial Intelligence (AI), Machine Learning (ML), CRISPR-Cas9 gene editing, and single-cell RNA sequencing mark a notable progression in precision medicine [4, 5]. These technologies enhance our understanding and management of these complex conditions. This article offers a comprehensive analysis of how these innovations contribute to elucidating the intricate relationship between the gut and the brain in individuals with ASD, as illustrated in Figure 1.

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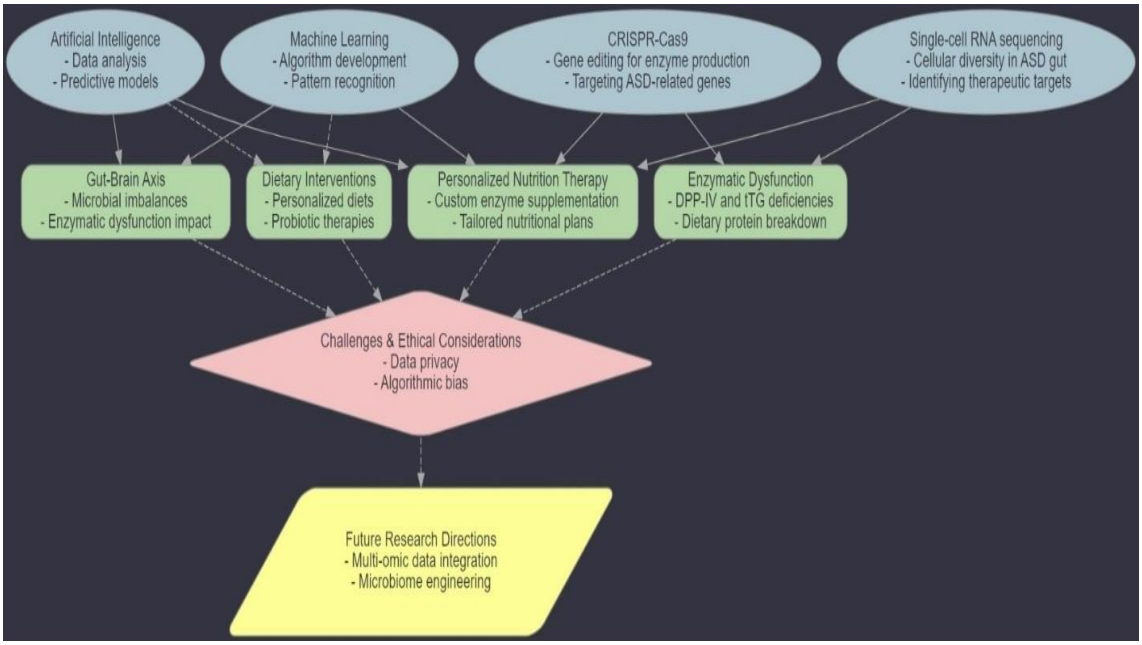

Figure 1 Role of various technologies in understanding the complex relationship between the gut and the brain in individuals with Autism Spectrum Disorder.

It highlights key findings related to specific enzyme deficiencies and microbial imbalances associated with GI symptoms in patients with ASD. The use of AI and ML to analyze genomic, proteomic, and environmental data has led to the development of personalized intervention strategies tailored to the unique characteristics of individuals with ASD. The effectiveness of these interventions hinges on a deep understanding of the genetic and cellular mechanisms underlying ASD, which is facilitated by CRISPR-Cas9 and single-cell technologies. This review offers a comprehensive analysis of recent advancements, addresses the challenges encountered in translational research, and proposes potential future directions. These advanced technologies hold the promise of transforming diagnostic and therapeutic approaches, offering customized solutions that extend beyond conventional methods, as illustrated in Figure 2.

This review aims to provide a critical analysis of technological advancements in the study of ASD and GI comorbidities. It advocates for the continued exploration and application of AI, ML, and genetic technologies. Collaborative efforts from various scientific disciplines are necessary to address the remaining challenges and fully leverage these technologies to improve patient care in ASD.

Autism and Gastrointestinal (GI) Comorbidities

The integration of AI and ML into the diagnosis of ASD and GI disturbances has marked a significant shift towards precision medicine [4]. AI and ML methodologies focus on creating and utilizing algorithms capable of learning from data to make predictions or decisions. AI models are developed using extensive datasets that include genomic, proteomic, and environmental information to study the relationship between ASD and GI comorbidities [6, 7]. These models use supervised learning to recognize patterns and connections between genetic markers or environmental factors and the occurrence or intensity of ASD and its associated GI symptoms [8, 9]. ML methods, including deep learning, are particularly valuable for analyzing complex, multi-dimensional data, enabling the discovery of subtle biomarkers and risk factors that might elude human analysts [10-12].

Figure 2 Illustrating the integration of AI, ML, CRISPR-Cas9, and single-cell RNA sequencing in advancing the understanding and treatment of Autism Spectrum Disorder (ASD) and its gastrointestinal (GI) comorbidities, highlighting the pathway from technological advancements to personalized interventions.

These technologies have elucidated the complex interplay between neurological development and GI health, identifying key enzymatic and microbial factors underlying ASD-related GI symptoms [7, 13]. By analyzing enzyme activity profiles and gut microbiota composition with enhanced precision, AI and ML are uncovering specific enzyme deficiencies that impair the breakdown of dietary proteins such as gluten and casein in individuals with ASD [14, 15], leading to the accumulation of neuroactive peptides. Advanced AI models utilize genomic and proteomic data to identify enzyme gene expressions linked to these digestive inefficiencies [6, 7]. ML algorithms also map changes in gut microbiota, highlighting microbial imbalances that exacerbate GI symptoms and influence neurodevelopment through the gut-brain axis [16]. These models provide predictive insights, enabling the identification of individuals at risk for specific GI conditions and the development of targeted interventions.

Innovative applications extend to predictive algorithms that integrate clinical, genomic, and environmental data to forecast the emergence of GI symptoms, facilitating early intervention. For example, ML analyses of stool samples have identified microbial signatures predictive of constipation in ASD [16], guiding the development of personalized probiotic therapies. AI and ML have greatly improved our understanding and management of ASD and its related GI comorbidities. Through the application of these technologies in examining clinical, genomic, and environmental data, we have successfully predicted GI symptoms and tailored interventions for individuals with ASD. Table 1 presents the predictive insights from AI and ML analyses, underscoring their crucial role in developing targeted therapeutic strategies.

Table 1 AI and ML Predictive Outcomes for ASD Interventions. Summarizes predictive outcomes based on clinical, genomic, and environmental data, their implications for tailored ASD interventions.

Data Type	Predictive Outcome	Implications for Intervention	References
Clinical	Assessing the Risk of GI Conditions in ASD	Early, personalized interventions improving outcomes.	[17]
Genomic	GI symptom emergence based on ASD genetic profiles	Genetically tailored dietary and therapeutic strategies.	[18]
Environmental	GI symptom triggers in ASD from environmental factors	Custom environmental management to mitigate symptoms.	[19]
Clinical + Genomic	ASD diagnosis/prognosis and GI disturbances prediction	Timely, integrated interventions for ASD and GI health.	[20]
Genomic + Environmental	Enzyme deficiencies and microbial imbalances risk in ASD	Proactive diet and lifestyle management for ASD GI health.	[21]
Multifaceted (Clinical, Genomic, Environmental)	Personalized therapy outcomes for ASD, including dietary and probiotic efficacy	Precision care plans optimizing dietary and therapeutic interventions.	[22]

Furthermore, the application of AI and ML extends beyond diagnostic and therapeutic interventions to include genetic editing insights from CRISPR-Cas9 and cellular-level understanding through single-cell RNA sequencing, offering a comprehensive view of the ASD-GI connection [23-26]. This approach enhances the potential for personalized care, leveraging in-depth knowledge of individual enzymatic and microbial profiles to customize dietary and therapeutic strategies, thereby improving the quality of life for individuals with ASD.

Enzymatic Mechanisms in ASD

Research into ASD and its associated enzymatic dysfunctions is being advanced through the integration of AI and ML technologies [7]. This integration is leading to significant progress in understanding the complexities of ASD. These new methods are improving our knowledge of the genetic and microbial factors that affect enzyme activity and are paving the way for the development of new interventions and diagnostic tools. A notable development is the use of AI algorithms to analyze outcomes from CRISPR-Cas9 gene editing experiments that target genes involved in enzyme production essential for ASD [27]. CRISPR-Cas9 gene editing is a highly accurate and adaptable method for making specific changes to the DNA of organisms [28]. The technique involves creating a short RNA sequence (guide RNA) that corresponds to the DNA sequence intended for editing [29]. The Cas9 enzyme, guided by the RNA molecule, cleaves the DNA at a precise location, enabling the modification of DNA sequences [30]. CRISPR-Cas9 is used

in ASD research to correct or introduce mutations in genes linked to enzymatic production or regulation [31]. This helps understand the genetic basis of ASD and GI comorbidities and facilitates the creation of therapeutic approaches.

Additionally, single-cell RNA sequencing (scRNA-seq) is an effective technique for examining the gene expression patterns of individual cells [32]. The process involves isolating single cells, reverse-transcribing their RNA into cDNA, and sequencing the cDNA to identify RNA molecules in each cell [33]. By analyzing profiles from numerous cells of individuals with ASD, scRNA-seq enables researchers to reveal variations in cells within the GI tract and brain [34], identify distinct cell types and states related to the disorder, and find novel targets for treatment.

By simulating genetic alterations in enzyme activity, researchers can investigate gene therapy strategies to address the underlying causes of digestive and neurological symptoms associated with ASD. Modifying genes related to the production of dipeptidyl peptidase-IV (DPP-IV) has the potential to enhance the metabolic breakdown of gluten and casein [35], thereby reducing their adverse effects on individuals with ASD. This thorough mapping enables the identification of specific cell types involved in enzymatic dysfunctions and abnormal gut permeability [36]. Accurate identification of cellular targets for therapeutic intervention has the potential to restore gut health and alleviate symptoms associated with ASD [37]. In the field of metabolomics, AI and ML models are being used to analyze the metabolic pathways affected in the gut microbiome of individuals with ASD [7, 38]. These models identify specific metabolic signatures linked to enzyme deficiencies, guiding the development of targeted interventions such as dietary modifications, supplements, or microbiome engineering. This approach aims to restore equilibrium to metabolic pathways, thereby mitigating the GI and behavioral symptoms associated with ASD.

In addition, AI is being used in therapeutic interventions, such as the creation of virtual reality (VR) platforms that replicate social interactions for individuals with ASD [39, 40]. These AI-driven VR systems dynamically adjust to users' reactions, offering personalized behavioral therapy to improve social skills and alleviate anxiety. Moreover, AI is transforming ASD diagnostics by utilizing tools that analyze intricate patterns in behavior, genetics, and facial expressions [41]. The goal of these tools is to identify ASD at an early stage, as shown in Figure 3.

Allowing for prompt interventions in dietary management and therapy, this has the potential to mitigate the severity of symptoms associated with enzymatic dysfunctions.

Gluten, Casein, and Enzymatic Dysfunction: Evidence and Insights

In the field of ASD research, the integration of AI and ML, along with CRISPR-Cas9 gene editing, scRNA-seq, metabolomics, microbiome analysis, and VR interventions, has significantly improved our understanding and approaches to managing the enzymatic digestion of dietary proteins like gluten and casein [42-44]. The utilization of AI and ML technologies has played a crucial role in analyzing the intricate genetic and biochemical aspects linked to ASD [7, 45]. By analyzing extensive genomic and proteomic datasets, these technologies have identified genetic variations and enzyme deficiencies, particularly in Dipeptidyl Peptidase-IV (DPP-IV), an essential enzyme for breaking down dietary proteins like gluten and casein, commonly found in wheat and dairy products. DPP-IV facilitates protein digestion by removing dipeptides from the N-terminus of polypeptides, which is crucial for their breakdown and absorption. If DPP-IV activity is lacking or blocked, it may cause incomplete breakdown of gluten and casein, leading to the creation and buildup of peptide fragments with potential neuroactive effects. Similarly, Tissue Transglutaminase (tTG), an enzyme that alters gluten peptides, can enhance their immunogenicity and initiate an autoimmune reaction in genetically susceptible individuals [46, 47], thereby hindering the digestion of gluten and casein.

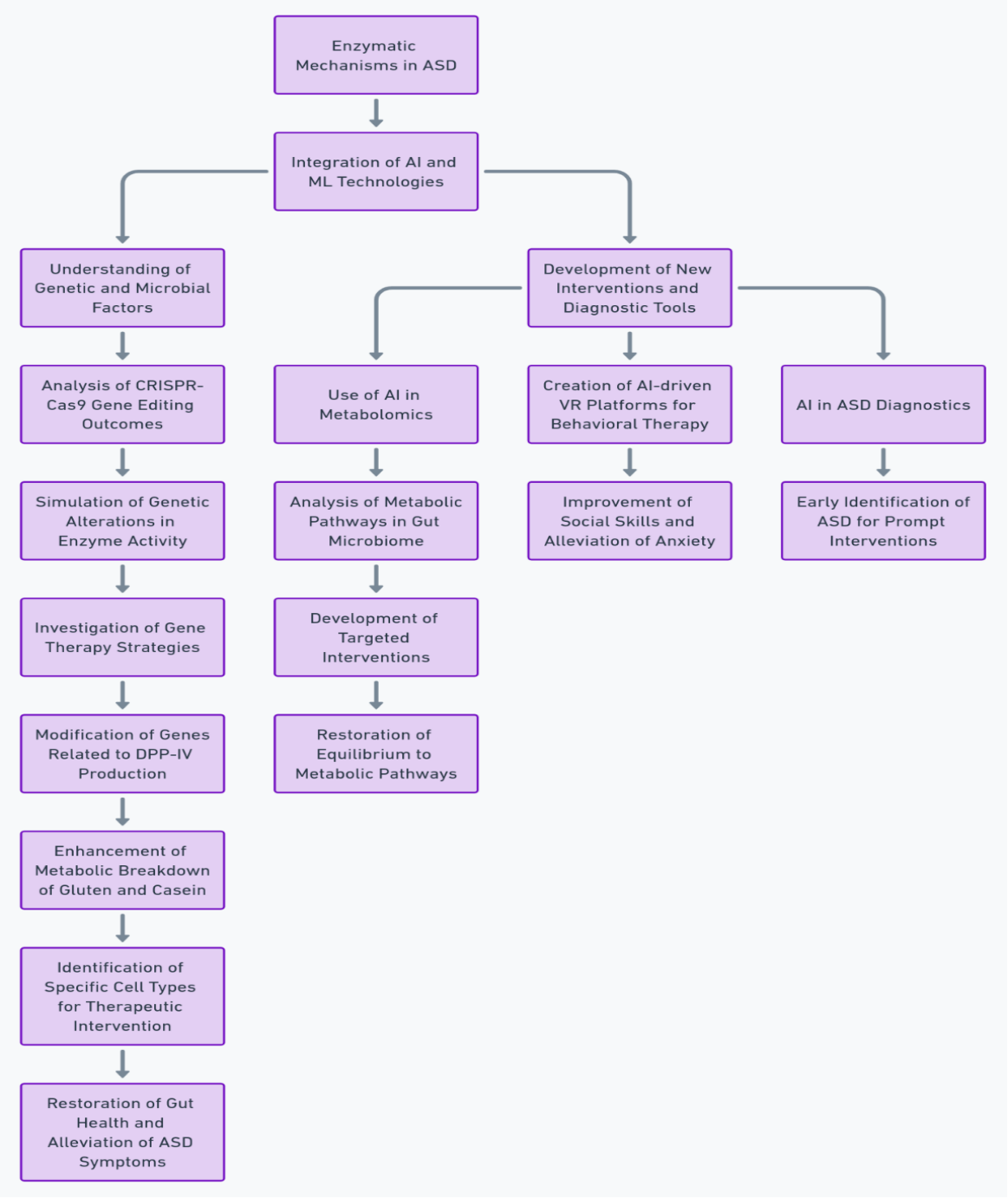

Figure 3 The diagram demonstrates the incorporation of artificial intelligence and machine learning in studying enzymatic dysfunctions in ASD, emphasizing progress in genetic analysis, therapeutic interventions, and diagnostic tools development.

A comprehensive understanding of these mechanisms allows for accurate predictions of individual susceptibilities to dietary proteins and enables the customization of dietary interventions that could potentially alleviate symptoms associated with ASD. CRISPR-Cas9 gene editing holds promise for the future of ASD treatment by addressing the underlying genetic causes of enzymatic dysfunctions [48]. Through the modification of genes associated with enzyme production, CRISPR-Cas9 presents a promising opportunity to address metabolic pathways impacted by ASD, potentially alleviating the dietary effects on the disorder's symptoms.

The utilization of scRNA-seq technology has yielded significant findings regarding the cellular diversity within the gut of individuals with ASD [49]. Understanding this level of detail is essential for developing targeted therapies aimed at restoring normal gut function and potentially alleviating ASD symptoms. Additionally, studies on metabolomics and the microbiome have successfully charted the distinct metabolic and microbial characteristics of individuals with ASD [50, 51]. These findings demonstrate the connection between variations in gut microbiota and metabolic profiles and the manifestation of dietary sensitivities and symptoms, as shown in Figure 4.

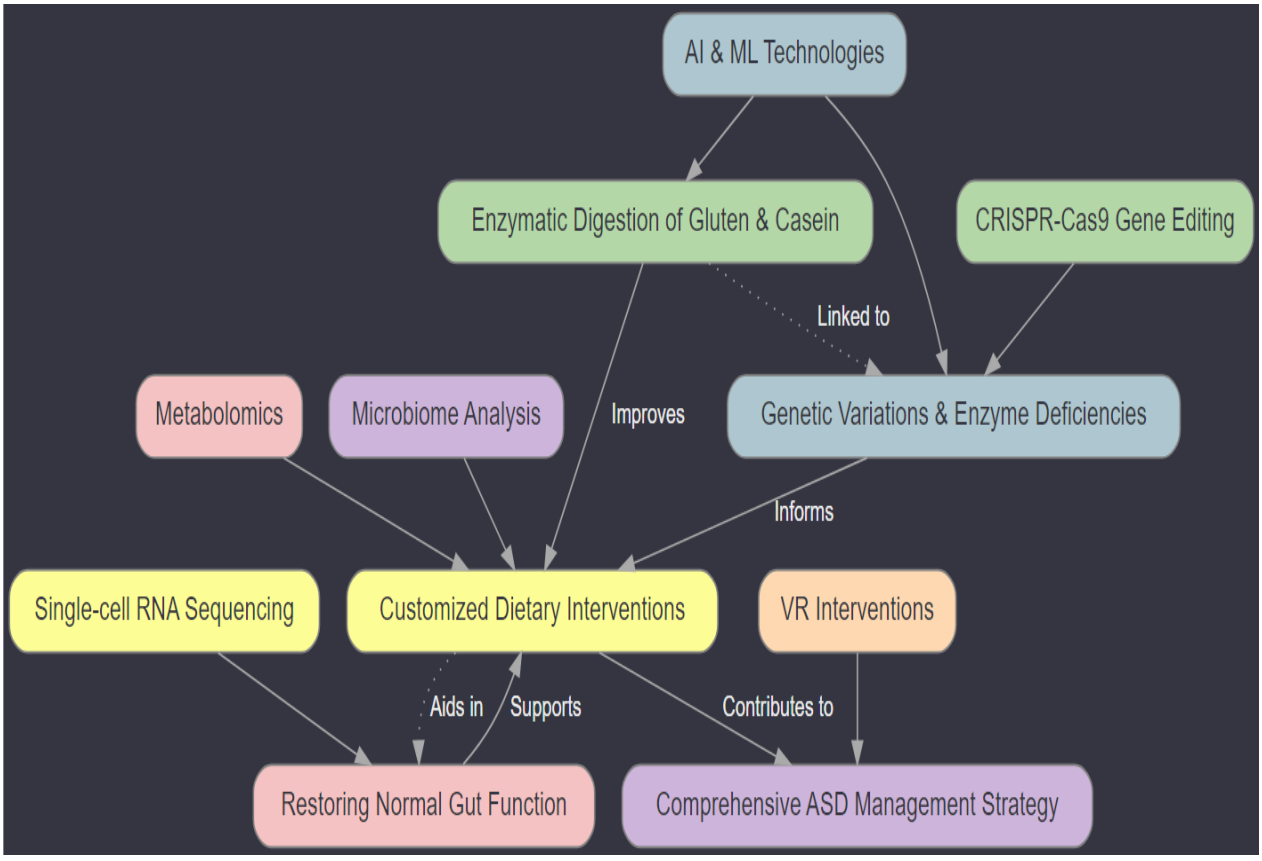

Figure 4 Elucidating the Mechanisms of Gluten and Casein Digestion in ASD Through the Integration of Artificial Intelligence, Genomic Technologies, and Virtual Reality Interventions

Furthermore, VR technologies have been employed to develop immersive social and cognitive training programs tailored to individuals with ASD [52, 53]. These programs are personalized using AI to address the specific requirements of each user. While these interventions significantly impact a comprehensive ASD management strategy by improving quality of life and functional outcomes, they are not directly related to dietary protein metabolism.

The Gut-Brain Axis: Enzymatic and Microbial Interactions

Researchers are currently investigating the use of CRISPR-Cas9 gene editing to target specific gene modifications, such as those related to zonulin [54], a protein responsible for regulating gut permeability. These studies aim to address gut barrier integrity issues [55, 56] that could be associated with ASD. ScRNA-seq has revealed the presence of overactive microglia in the brain and distinct epithelial and immune cell subsets in the gut, suggesting their potential significance in the pathophysiology of ASD [57, 58].

Metabolomics approaches have uncovered distinct metabolic byproducts, such as modified profiles of short-chain fatty acids (SCFAs), which can serve as potential biomarkers for gut dysbiosis or malabsorption in individuals with ASD [59, 60]. Microbiome analyses have identified imbalances in certain bacterial strains, including a decrease in *Faecalibacterium prausnitzii* and an increase in *Clostridium difficile* [61, 62]. These findings provide specific targets for probiotic interventions. AI and ML have effectively analyzed these data streams, identifying genetic polymorphisms and enzyme deficiencies, such as those found in the DPP-IV enzyme, that are associated with ASD symptoms [63, 64].

Nutritional Considerations and Enzymatic Interventions

The management of nutrition in Autism Spectrum Disorder (ASD) extends beyond conventional methods by incorporating advanced technologies such as CRISPR-Cas9 gene editing, scRNA-seq, metabolomics, microbiome analysis, and AI and ML algorithms. These technologies significantly improve the customization of dietary and enzyme supplementation strategies, enabling a precision medicine approach to alleviate symptoms associated with ASD.

CRISPR-Cas9 gene editing shows promise in addressing genetic variations that impact the efficiency of enzymes involved in breaking down dietary proteins like gluten and casein [65]. These proteins are frequently linked to the exacerbation of ASD symptoms. This approach has the potential to address dietary sensitivities at their genetic root by precisely modifying genes associated with enzyme production.

ScRNA-seq technology provides valuable insights into the cellular landscape of the gut and its interaction with dietary components [66]. By identifying distinct cell types and states that play a role in the enzymatic environment of the gut and its effects on gut health, scRNA-seq offers crucial insights into how individual cell mechanisms impact the efficacy of dietary changes and enzyme supplementation in individuals with ASD [67].

Metabolomics and microbiome analysis enhance our understanding of the biochemical and microbial context in which dietary interventions function [68, 69]. Metabolomics can detect distinct metabolic signatures that reveal how individuals with ASD metabolize different types of food [70, 71]. This information is valuable for tailoring personalized diet plans. Microbiome analysis provides insights into the gut bacterial profiles associated with ASD [72, 73], which can guide the selection of probiotics or dietary adjustments aimed at restoring a healthy balance of gut flora.

AI and ML are at the forefront of integrating diverse data streams to predict individual responses to dietary interventions [74, 75]. By comprehensively analyzing genetic, cellular, metabolic, and microbial information, these technologies facilitate the creation of personalized nutrition strategies.

Challenges and limitations of research

Studying enzyme functions and utilizing AI in ASD treatment present numerous challenges and constraints, along with ethical considerations crucial for directing these scientific pursuits conscientiously. Enzyme activity variability across the ASD population poses a significant research challenge, making it difficult to establish standardized treatment protocols. This variability affects both the metabolic breakdown of dietary components and the effectiveness of enzyme supplementation, necessitating personalized intervention strategies. Additionally, the diverse range of dietary responses underscores the complexity of customizing dietary adjustments, requiring a thorough understanding of each patient's specific enzymatic profile and dietary sensitivities [76].

The integration of AI and ML in ASD research adds another layer of complexity. While these technologies have the potential to transform the diagnosis and treatment of ASD and its GI comorbidities, they also raise ethical concerns regarding data privacy [77], informed consent [78], and algorithmic bias [79]. It is essential to prioritize the confidentiality and security of sensitive health information by adhering to data protection regulations and ethical standards. Informed consent procedures should clearly communicate the use of AI in both research and clinical environments, outlining the associated risks and benefits. Furthermore, algorithmic bias poses a significant ethical dilemma [80-82], necessitating careful attention to ensure AI models are trained on diverse datasets to avoid perpetuating inequalities in diagnosis and treatment.

The implementation of AI-driven insights and genetic editing technologies such as CRISPR-Cas9 in clinical settings also encounters challenges related to effectiveness, safety, and the ethical implications of gene editing [83-85]. Thorough safety assessments and ethical discussions are crucial regarding genetic modifications in therapeutic applications due to the possibility of unintended consequences.

Future Directions in Autism Spectrum Disorder research

The future of ASD research is shifting towards a precision medicine paradigm that incorporates AI, ML, and genetic editing technologies. Future research is likely to utilize multi-omic data integration, incorporating genomic, proteomic, metabolomic, and microbiome data to understand the intricate causes of ASD and its GI issues. The primary emphasis will be on enhancing AI and ML algorithms to accurately predict individual responses to dietary and enzyme supplementation strategies, facilitating personalized treatment plans. Advancements in CRISPR-Cas9 gene editing will focus on exploring corrective interventions for enzyme deficiencies, with attention to safety, efficacy, and ethical considerations. Microbiome engineering will become a crucial therapeutic approach, aimed at improving gut health by precisely adjusting gut flora based on AI-driven assessments. Single-cell technologies will enhance our understanding of the cellular basis of ASD, providing new therapeutic targets and in-depth mechanistic insights. Amidst these advancements, establishing robust ethical and regulatory frameworks will be essential to address privacy, consent, and equity issues, ensuring the ethical use of these innovative technologies. Overall, the combination of these new methods will likely mark a new phase in ASD research and treatment, characterized by a focus on tailored, precise interventions.

CONCLUSION

The combination of AI, ML, CRISPR-Cas9 gene editing, and single-cell RNA sequencing represents significant progress in the understanding and management of ASD and its GI comorbidities. This article highlights the critical role of these technologies in elucidating the intricate relationship between the gut and the brain, revealing genetic, enzymatic, and microbial factors associated with ASD. AI and ML have been instrumental in customizing interventions through the analysis of extensive datasets, allowing for the identification

of enzyme deficiencies and microbial imbalances linked to GI symptoms in individuals with ASD. CRISPR-Cas9 holds promise for developing targeted therapies by directly addressing enzyme deficiencies. Additionally, single-cell RNA sequencing has unveiled cellular heterogeneity in the ASD gut, offering new avenues for therapeutic strategies. The variability in enzyme activity and dietary responses among individuals with ASD underscores the need for a highly individualized treatment approach, while also raising important ethical issues related to data privacy, informed consent, and algorithmic bias. Future research should focus on leveraging these technologies, with an emphasis on integrating multi-omic data to better understand the complexities of ASD and its GI comorbidities. The potential for precision medicine in ASD is promising, offering the opportunity to enhance the quality of life for affected individuals and their families by overcoming existing barriers and addressing ethical concerns. Advanced technologies in ASD research illuminate complex biological processes and enable personalized interventions.

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دمج الذكاء الاصطناعي والتقنيات الجينومية المتقدمة في استكشاف اضطراب طيف التوحد والمشكلات المعدية المعوية المصاحبة: نهج متعدد نحو التشخيص الدقيق

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ملخص

هذا المقال يسلط الضوء على التأثير المحتمل للذكاء الاصطناعي والتعلم الآلي وتقنية تحرير الجينات كريسبر–كاس9 وتسلسل الحمض النووي الريبوزي للخلايا الفردية على تحسين الفهم والإدارة لاضطراب طيف التوحد ومشكلات الجهاز الهضمي المصاحبة له. يتناول هذا البحث الية كشف هذه التقنيات للعلاقة المعقدة بين الأمعاء والدماغ، مشيرًا إلى نقص في إنزيمات محددة وخلل في التوازن الميكروبي مرتبط بأعراض الجهاز الهضمي في اضطراب طيف التوحد حيث انه من خلال استخدام الذكاء الاصطناعي والتعلم الآلي، يتم تطوير استراتيجيات تدخّل مُصممة خصيصًا عبر تحليل البيانات الجينومية والبروتيومية والبيئية، مما يعزز قدرتنا على التنبؤ بمشاكل الجهاز الهضمي في اضطراب طيف التوحد ومعالجتها. بالإضافة إلى ذلك، تعد تقنية تحرير الجينات كريسبر–كاس9 طريقة مبشرة لتصحيح العيوب الجينية المرتبطة بإنتاج الإنزيمات، ما يتيح الحصول على علاجات محددة. يوفر تسلسل الحمض النووي الريبوزي للخلايا الفردية رؤى قيمة حول تنوع الخلايا في أمعاء مرضى اضطراب طيف التوحد، كاشفاً عن اتجاهات علاجية جديدة. يبرز هذا البحث إلامكانات الواعدة لهذه التقنيات واهميتها مع الإشارة أيضًا إلى التحديات والمسائل الأخلاقية المتعلقة باستخدامها، مؤكدًا على أهمية النهج المتعدد التخصصات للاستفادة الكاملة من مزاياها لعلاج مرضى التوحد.

الكلمات الدالة: اضطرابات طيف التوحد؛ الخلل الوظيفي الإنزيمي؛ التدخلات الغذائية؛ الاضطرابات المعوية المرتبطة؛ العلاج الغذائي المُفَصَّل.

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Fostering Healthier Choices: Empowering Pharmacy Students to Bridge the Food Label Gap in Lebanon

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ABSTRACT

Background: Non-communicable diseases are a significant public health issue in Lebanon, making it crucial to promote preventive measures such as informed dietary choices. Food labels play a key role in this, but there is a disconnect between knowledge and utilization, even among healthcare professionals. This study aimed to investigate this gap among Lebanese pharmacy students by assessing their nutrition knowledge, usage of food labels, and the factors influencing their label use.

Materials and Methods: This cross-sectional study examined the knowledge gap among 81 pharmacy students in Beirut. A validated questionnaire assessed demographics, nutrition knowledge, label features influencing purchase decisions, and barriers to label use.

Results: Despite having high overall nutrition knowledge (mean score of 82%), students reported inconsistent use of food labels. Gender did not significantly influence the prioritization of label features. However, students in higher academic years were more likely to pay attention to production dates ($p < 0.001$). Additionally, there was a positive correlation between the frequency of label use and knowledge scores.

Conclusion: This study highlights a persistent knowledge-practice gap in food label utilization among pharmacy students. Educational interventions tailored to address specific knowledge gaps and perceived barriers are necessary.

Keywords: Food literacy; knowledge-behavior gap; nutrition education; pharmacy students; public health.

INTRODUCTION

In the heart of Lebanon, beneath the whispering cedars and vibrant kitchens, a silent yet formidable threat looms: non-communicable diseases (NCDs). These insidious invaders claim a staggering 91% of lives and affect every corner of society. Among them, osteoporosis emerges as a particularly pervasive menace, affecting 70% of the elderly, surpassing even familiar foes like hypertension and diabetes. These statistics paint a poignant picture of families navigating fragility, where laughter is often laced with unspoken anxieties. Understanding and combating this silent enemy is a crucial call to action (1,2).

Chronic diseases leave a lasting impact on people's lives, causing both physical and emotional distress. Cigarette smoke curls around conversations, diabetes undermines strength, and obesity casts a long shadow over communities. Inactivity becomes a silent enemy, while hypertension steals the rhythm of laughter. These diseases are not unique to individuals; they represent a shared challenge for our community. We can learn from each other's stories and work together for a healthier future (3). NCDs lead to premature deaths, disabilities, and decreased productivity, placing a significant burden on the healthcare system (4).

In this context, addressing NCDs requires innovative and cost-effective solutions tailored to the Lebanese population. Nutrition plays a crucial role in improving

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overall well-being, and understanding food labels is essential for making informed dietary choices. Pharmacists emerge as key players in this endeavor. They not only manage adverse food-drug interactions but also provide counseling and health education to patients. Their vital role extends to offering public health advice and guidance for chronic conditions such as obesity, diabetes, undernutrition, and even parenteral nutrition support (5).

Today, pharmacists serve as easily accessible points of contact for many health concerns, acting as a bridge between the public and essential healthcare advice (6,7). With one-third of all pharmacy sales involving nutritional products or nutraceuticals, a robust understanding of nutrition has become imperative for pharmacists (8,9). As future healthcare professionals, they are likely to encounter questions not only about prescription drugs but also about navigating the complex world of dietary supplements and making informed nutritional choices. Moreover, the increasingly evident connections between diets and diseases underscore the critical importance of equipping pharmacists with relevant expertise in nutrition(10).

This is where pharmacy students step into the spotlight, as they prepare to become the next generation of healthcare advisors. It is crucial to provide them with sufficient knowledge of nutrition so they can effectively guide their patients. Evaluating their understanding of food labels and the impact these labels have on dietary decisions can reveal areas that need improvement (11,12). However, a disconnect exists between theoretical knowledge and practical application (13). Studies suggest that healthcare professionals, including pharmacy students, often demonstrate limited utilization of food labels when making dietary decisions, despite their understanding of nutrition (14,15).

OBJECTIVES

This study aims to investigate the gap between knowledge and behavior regarding food label use among pharmacy students in Lebanon. We will achieve this by focusing on the following key areas:

Prevalence of Label Use: Assess how frequently pharmacy students in Lebanon utilize food labels to inform their dietary choices and explore the factors influencing their label use patterns.

Knowledge-Behavior Disconnect: Evaluate the relationship between pharmacy students' understanding of nutrition and their ability to apply that knowledge when interpreting food labels. This includes identifying specific knowledge areas critical for effective label interpretation.

Barriers to Label Utilization: Identify the obstacles that prevent pharmacy students from fully utilizing food labels to make informed dietary decisions.

Ultimately, the goal of this investigation is to contribute to a healthier future for Lebanon. This can be achieved by enhancing the knowledge and skills of future pharmacists and providing valuable insights into the factors that influence dietary decisions in the country. By empowering pharmacy students to become effective advisors on healthy food choices, we can take a crucial step toward preventive healthcare and address the challenges presented by NCDs. The significance of this journey extends beyond Lebanon's borders, offering valuable lessons for other nations facing similar challenges in dealing with NCDs. By understanding the intricate link between nutrition knowledge, the utilization of food labels, and individual dietary choices, we can pave the way for a healthier future.

METHODS

Study Design and Participants:

This cross-sectional study was conducted from March to July 2023 with pharmacy students enrolled at LIU University in Lebanon. To ensure a representative sample across academic years, we employed stratified random sampling based on the year of study. We obtained a list of enrolled BS pharmacy students from university records and randomly selected participants within each year-stratum in proportion to

their representation in the population.

Before participating, potential subjects were informed about the study objectives and provided with an informed consent form outlining their rights and responsibilities. Those who agreed to participate then completed a self-administered online questionnaire designed to assess their food label use and comprehension.

Instrument Development: Food Label Use and Comprehension Questionnaire

A 35-item questionnaire, included in the Appendix, was developed to assess pharmacy students' use and comprehension of food labels. The questionnaire was modified from validated instruments used in previous studies on food label use (16,17), with a particular focus on pharmacy students and the Lebanese context. This adjustment involved extracting key components from the original tools and integrating aspects relevant to pharmacy education and the Lebanese market. The questionnaire comprised the following:

Demographics: This part collected background information about the participants, including age, gender, and academic year.

Food Label Use Habits: This part evaluated how frequently participants read food labels, where they learned to read food labels, and if they had any health conditions requiring them to pay close attention to labels.

Perceived Barriers to Label Use: This part delved into reasons why participants might not always read food labels, including unattractive design, complex language, doubts about information accuracy, and personal beliefs about health and ingredients.

Information Prioritized on Food Labels: This part assessed which information participants prioritized on food labels using a format that allowed them to select their most important choices (e.g., expiry date, ingredients list, nutritional content, health claims).

Additional Factors Influencing Food Choices: This part investigated other factors influencing participants' food choices beyond food labels, such as price, product packaging, advertisements, and Ministry of Health approvals.

The questionnaire used a combination of multiple-choice and Likert scale questions (ranging from strongly agree to strongly disagree).

Sample Size:

We used a stratified random sampling approach to ensure a representative sample of pharmacy students across academic years. A power analysis conducted using G*Power formula with Cohen's d effect size of 0.3, a confidence level of 95%, and a power of 80%, indicated that a minimum sample size of 40 students per stratum was needed for subgroup analysis. This sample size was designed to achieve a margin of error of +/- 5% (assuming a normal distribution). Although our goal was to recruit this number, we were able to enroll a total of 81 students.

Given the program size (approximately 300 students enrolled per academic year), the number of students recruited varied each year: 12 from the second year, 63 from the third year, 5 from the fourth year, and 1 from the fifth year. While this exceeds the minimum recommended sample size overall, the distribution across years may limit the precision of detecting subgroup effects as initially anticipated in our subgroup analyses.

Inclusion and Exclusion Criteria:

- Inclusion criteria: Lebanese students enrolled in the BS pharmacy program at LIU university, aged 18 years or older.
- Exclusion criteria: To ensure that the findings are applicable to the target population of pharmacy students at our university, exchange students from other universities were excluded from participating. This decision was made in acknowledgment of potential differences in the pharmacy education curriculum and program focus across institutions. Additionally, individuals with visual impairments that would significantly hinder their ability to read food labels without assistance were also excluded to ensure that the data collected accurately reflects

participants understanding gained from reading food labels. It is important to note that these exclusion criteria were pre-defined and distinct from participants who opted not to take part or provided incomplete questionnaire responses.

Variables and Measurements:

- Reasons for reading or not reading labels: Participants were asked about their motivations for using each label element.
- Most important nutritional data: Participants indicated which aspect of the food label information (such as calorie content, fat content, nutrient percentages) they find most valuable for making informed choices.
- Understanding of food labels: Participants rated their confidence in understanding food labels on a 5-point Likert scale.

Questionnaire Validation:

Content validity was established through the Delphi expert inquiry method involving registered dietitians and public health researchers with expertise in nutrition and food labeling in the Lebanese context. Cronbach's Alpha (0.9039) and Spearman-Brown Coefficient (0.9495) indicate excellent internal consistency and reliability, respectively (18,19). The Mean Inter-Item Correlation (0.3333) within an acceptable range suggests reasonable commonality among items (20).

Statistical Analysis:

To assess statistical significance, we set alpha (α) at 0.05. The data were analyzed using SPSS software version 26. Descriptive statistics (mean, standard deviation, frequencies, and percentages) were used to summarize participant characteristics and their responses to questionnaire items. Bivariate analyses were conducted to explore relationships between variables. Chi-square tests assessed associations between categorical variables, such as gender and attention to specific label features. T-tests were used to assess differences in mean scores between groups, such as gender and academic year. Pearson correlation coefficients were calculated to evaluate linear relationships between continuous variables, including nutrition knowledge scores and food label use scores. Spearman correlation coefficients were computed to assess potential non-linear relationships between variables.

RESULTS:

As shown in Table 1, more than two-thirds of the participants at the Beirut campus are young females aged 20-30 years (73% female). Most are moderately active (49%) and rarely follow specific diets (82%). Academically, 78% are in their third year, with smaller groups in other years. Geographically, the majority of participants are from Beirut (67%), with smaller contingents from other campuses. This profile indicates a relatively homogeneous group of young, active students in their mid-twenties.

Expiration dates are the most important information on labels, with 70.4% of consumers prioritizing them. Nutritional aspects also carry significant weight, with key considerations being calories per serving (35.8%), total fat (23.5%), and sugar (27.2%). Halal claims influence purchasing decisions for 40.7% of participants. Consumers primarily seek indicators of "good" health when evaluating product claims. The most influential factors are "without sugar" (48.1%), followed by "high fiber" (37%) and "no trans-fat" (29.6%). Claims related to lower fat content are also significant, such as "low fat/light" (17.3%) and "reduced fat" (21%). Interestingly, aspects like "organic" (23.5%) and "natural product" (39.5%) attract moderate attention, while more specific labels like "lactose-free" (9.9%) or "probiotic" (7.4%) seem to have less influence (Figure 2).

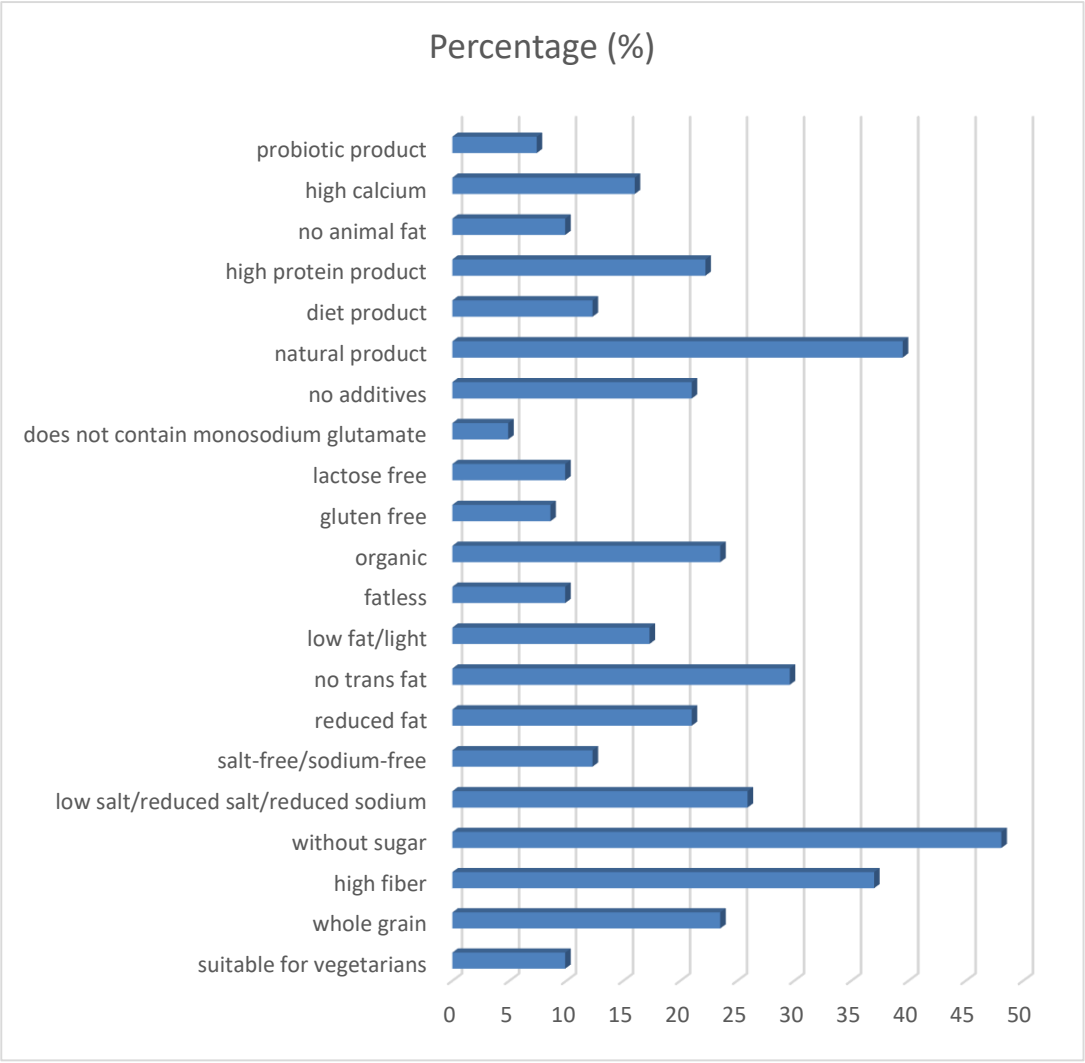

Figure 2. Health perception factors influencing purchasing decisions

Table 2 presents the frequency and percentage distribution of participants' responses regarding their habits of reading food labels, sources of learning how to read labels, health problems that require attention when reading labels, and reasons for not reading nutritional labels.

To explore student preferences for various product information attributes on food labels, T-tests were conducted to assess potential differences based on gender and academic year (Table 3). Pearson's and Spearman's correlation coefficients were calculated to investigate the strength and direction of the relationships between these preferences and other variables. Table 4 displays the correlation between knowledge scores derived from the questionnaire and other relevant variables, such as age, frequency of label use, and source of learning.

Table 1. Demographics and Background of the Study Group

Variables	Category	Frequency	Percent(%)
Age	<20	25	30.9
	20-30	56	69.1
Sex	Male	22	27.2
	Female	59	72.8
Educational year	Second year	12	14.8
	Third year	63	77.8
	Fourth year	5	6.2
	Fifth year	1	1.2
Campus	Akkar	6	7.4
	Beirut	54	66.7
	Mount Lebanon	1	1.2
	Nabatieh	7	8.7
	Saida	5	6.2
	Tripoli	4	4.9
	Tyre	4	4.9
Physical Activity	Active	32	39.5
	Moderately Active	40	49.4
	Inactive	9	11.1
On a specific diet	No	66	81.5
	Yes	15	18.5

Table 2. Unveiling the Reality of Food Label Utilization in Pharmacy Students

Variables		Frequency	Percent (%)
Reading the food label when buying foodstuff	Rarely	14	17.3
	Most of the times	23	28.4
	Never	6	7.4
	Sometimes	29	35.8
	Always	9	11.1
If you know how to read food labels, where did you learn it	Dietician	10	12.3
	friend/family	9	11.1
	other	12	14.8
	school	5	6.2
	TV/Magazine/newspaper/internet	14	17.3
	University	31	38.3
Do you have a health problem that requires attention when reading a food label?	No	67	82.7
	Yes	14	17.3
Which of the following is/are the reasons why you don't read the nutritional labels of foods?	food labels are unattractive	9	11.1
	I believe the information on the food label is incorrect	3	3.7
	I don't mind because I'm healthy and at a good weight	31	38.3
	I know what's in the product I bought	20	24.7
	I'm afraid to learn the ingredients of the foods I consume	2	2.5
	The language on the product label is too small for me to read	5	6.2
	Words that are difficult to understand are utilized in food label information	11	13.5

Table 3. Statistical Analysis of Pharmacy Students' Priorities for Product Information Attributes

Attribute	Production Date	Expiry Date	Origin	Shelf Life	Amount
Gender (p-value)	0.506	0.467	0.153	0.155	0.954
Academic year (p-value)	<0.001*	0.944	0.070	0.894	0.718

Table 4. Correlation Between Knowledge Scores and Predictor Variables

Predictor Variable	Age	Gender	Frequency of Label Use	Source of Learning
Pearson Correlation	0.223	0.056	0.321	0.248
p-value	0.097	0.424	<0.001*	0.003*

DISCUSSION

Empowering individuals to make informed food choices is a crucial aspect of public health initiatives. Pharmacy students, in particular, play a significant role in this landscape, as they have the potential to become future advisors who can effectively translate their knowledge into practical dietary guidance. However, bridging the gap between knowledge and behavior remains a persistent challenge. This discussion aims to explore the underlying complexities of this disconnect, investigating the hidden factors and various influences that impact consumers' engagement with food labels. It specifically focuses on pharmacy students as a key population that can benefit from targeted interventions.

Pharmacy students have demonstrated a moderate understanding of food labels (59.4%), but they rarely apply this knowledge when making purchasing decisions (27.4%). This knowledge-behavior gap is concerning, especially considering their future role as healthcare advisors who are expected to guide others in making healthy food choices. As highlighted by Hameed et al. (21), there is a concerning gap between the knowledge gained in educational programs and its application in clinical practice among nurses in Pakistan. This issue is not unique and aligns with observations by Vasli et al. (22) regarding similar challenges in applying knowledge from programs like CPE. These findings emphasize the need to bridge this gap by improving healthcare educational programs to ensure the effective translation of knowledge into practical skills. Further investigation is needed to understand this disconnect.

We employed a stratified random sampling approach to ensure that students from all academic years were included in the study. However, despite achieving a high participation rate (78%), it was primarily concentrated among third-year students. This skewed distribution may limit the generalizability of our findings to the entire pharmacy student population at our university. For future studies employing stratified random sampling, considering additional stratification criteria beyond academic year, such as program specialization or campus location, could improve representativeness. Moreover, implementing targeted recruitment strategies for each stratum and adjusting the sample size based on actual student distribution data could enhance the representativeness of the sample.

Several factors influenced the frequency of label use. As shown in Table 2, lack of awareness, perceived difficulty, and low perceived relevance were significant barriers for 17.3% of participants. This highlights the need for targeted interventions to increase awareness and simplify label interpretation. Conversely, it is important to examine the factors contributing to consistent label use, reported by 28.4% of participants, to promote this positive behavior. Interestingly, 7.4% of participants completely avoided labels due to reasons such as distrust, complexity, or health conditions. This underscores the importance of initiatives that promote labeling transparency, use

simplified language, and provide support for individuals with specific needs, as suggested in previous research (23). Additionally, halal claims were reported by 40.7% of participants as a factor influencing purchasing decisions, highlighting the significance of considering religious dietary needs for a substantial portion of consumers.

University education played a crucial role in label literacy for 38.3% of participants, highlighting its importance in pharmacy curricula (Table 2). However, the influence of dietitians (12.3%), friends/family (11.1%), and media (17.3%) underscores the potential of informal networks and accessible resources in fostering label literacy. This finding is consistent with previous research conducted by Chau et al. (24). Further investigation into these diverse pathways could provide valuable insights for developing comprehensive educational strategies.

To enhance the credibility of our recommendations for expanding label utilization, recent research supports the identified areas of focus. According to Grunert et al. (25), consumer understanding and use of nutrition labeling are critical factors. Advocating for broader recognition of label-informed choices beyond participants with health conditions (17.3%) aligns with efforts to address misconceptions about good health not requiring label guidance (38.3%). Additionally, challenging assumptions about product familiarity (24.7%) and encouraging wider adoption for preventive health are supported by Grunert et al.'s insights into factors influencing consumer behavior regarding nutrition labels. This reference reinforces the importance of our proposed strategies for maximizing the impact of nutritional information on consumer choices.

Unattractive label design (11.1%), distrustful interpretations (3.7%), and complex terminology (13.5%) necessitate industry collaboration to create clearer, user-friendly labels that inspire trust and empower consumers, especially those hesitant to confront potentially negative information (2.5%). According to Fraser (26), "packaging design greatly impacts consumer perceptions and purchasing choices, underscoring the importance of collaborative efforts between the food industry and design experts in crafting engaging and informative labels."

While knowledge of food labels is important, student preferences for specific label information also influence purchasing decisions. Table 3 presents the statistical analysis of these preferences. Interestingly, a moderate positive correlation was found between student preferences and production date (Pearson's $r = 0.718$, p-value < 0.001), indicating that students who prioritize fresher products pay more attention to this information. The analysis also revealed no significant gender differences in prioritizing any specific attribute (Table 3).

One of the main findings of our study is the development of a knowledge scoring system to assess pharmacy students' understanding of food labels. This scoring system was derived from the study questionnaire, which examined different aspects of food label understanding, such as students' knowledge of key label components. Each question was assigned a specific point value depending on the complexity of the information being tested. The maximum achievable score on the questionnaire was 40, allowing us to assess a comprehensive understanding of core food label interpretation concepts. Table 4 displays the correlation between these scores and other relevant variables. The score indicated a statistically significant positive correlation (p-value < 0.001) with the frequency of label utilization. Students who regularly reference labels exhibit a better grasp of the information presented. Conversely, those who seldom consult labels may benefit from interventions aimed at improving their ability to interpret food labels. This finding highlights the potential of educational approaches that encourage label use to enhance food literacy among pharmacy students. Interestingly, while the frequency of label use showed the most robust positive correlation (p-value < 0.001), the source of learning also emerged as significant (p-value $= 0.003$). This implies that exploring diverse learning methods could be beneficial for students looking to

enhance their understanding of food labels.

This analysis examined the importance of various product attributes in purchasing decisions, considering factors such as gender, academic year, and physical activity levels. While not all factors showed statistically significant associations, the results provide valuable insights into how consumers prioritize different product features. The findings of this study have substantial practical implications for pharmacy education and public health initiatives. The study identified a gap between knowledge and behavior among pharmacy students, highlighting the need for targeted educational interventions that enhance cognitive understanding and promote practical application. By incorporating these insights into curricula and collaborating with industry experts, pharmacy students can be empowered to become effective advisors, bridging the knowledge-behavior gap. Furthermore, the findings regarding influential factors in consumer behavior can guide the refinement of public health strategies aimed at promoting informed food choices. The statistical analyses offer valuable information for developing tailored interventions, such as gender-specific marketing and improvements in label design.

Limitations of the study

This study has provided valuable insights into the knowledge-behavior gap related to food label use among Lebanese pharmacy students. However, some limitations are worth noting. Despite a well-designed survey instrument, the study relied on self-reported data, which can be susceptible to bias. Additionally, focusing on a single university limits the generalizability of the findings to a broader population of pharmacy students. To enhance the robustness of future research, incorporating objective measures, such as eye-tracking technology to gauge actual label-reading behavior, could be beneficial. Furthermore, employing a longitudinal design would allow for investigating causal relationships between educational interventions and changes in label-use practices. Despite these limitations, this study lays a solid foundation for further exploration. By addressing these constraints and incorporating feedback from various stakeholders (including educators, healthcare professionals, and consumers) in future studies, we can gain a more comprehensive understanding and develop targeted interventions to close the knowledge-behavior gap and encourage healthier dietary decisions among pharmacy students and others.

CONCLUSION

In conclusion, bridging the knowledge-behavior gap concerning food labels requires a comprehensive and multi-faceted approach. Our findings highlight the potential of tailored educational interventions to enhance students' understanding and utilization of food labels. Easily accessible resources, combined with improved label design considerations, can significantly empower pharmacy students as future advisors on healthy food choices. Given the evolving landscape of the food industry, collaborative efforts between academia and industry stakeholders are crucial. This synergistic collaboration is key to fostering informed consumer choices among pharmacy students and advancing public health outcomes on a broader scale. As we look to the future, these insights lay the groundwork for transformative initiatives that can positively impact the intersection of nutrition knowledge and consumer behavior.

Conflicts of Interests

The authors declare that there are no conflicts of interest.

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Ethical considerations

Ethical clearance for this study was obtained from the LIU SOP research committee (2023RC-033-LIUSOP).

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تعزيز الخيارات الصحية: تمكين طلاب الصيدلة من سد فجوة الملصقات الغذائية في لبنان

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ملخص

المشكلة: تمثل الأمراض غير المعدية مشكلة صحية عامة مهمة في لبنان، مما يجعل من الضروري تعزيز التدابير الوقائية مثل الخيارات الغذائية المستنيرة. تلعب الملصقات الغذائية دورًا رئيسيًا في هذا، ولكن هناك انفصال بين المعرفة والاستخدام، حتى بين المتخصصين في الرعاية الصحية.

المنهج التجريبي: بحثت هذه الدراسة المقطعية هذه الفجوة بين 81 طالب صيدلة في بيروت. قام استبيان تم التحقق من صحته بتقييم التركيبة السكانية والمعرفة التغذوية وميزات الملصق التي تؤثر على قرارات الشراء والعوائق التي تحول دون استخدام الملصق.

النتائج الرئيسية: على الرغم من المعرفة الغذائية الشاملة العالية (متوسط الدرجات 82%)، أظهر الطلاب استخدامًا غير متسق للملصقات الغذائية. وكشف التحليل الإحصائي عن اختلافات كبيرة على أساس الجنس في الاهتمام بخصائص منتج معين. والجدير بالذكر أن اختبارات T أظهرت اختلافات بين الجنسين في الأهمية المرتبطة بتاريخ الإنتاج (ع = 0.255) وكمية المنتج (ع = 0.542). وعلاوة على ذلك، أشارت معاملات ارتباط بيرسون إلى وجود علاقة إيجابية بين العام الدراسي والاهتمام بتواريخ الإنتاج0.56 = r) ، (p <0.001، مما يشير إلى زيادة الوعي بمستويات التعليم العالي.

الاستنتاج: تسلط هذه الدراسة الضوء على الفجوة المستمرة بين المعرفة والممارسة في استخدام الملصقات الغذائية بين طلاب الصيدلة. ومن الضروري إجراء تدخلات تعليمية مصممة لمعالجة فجوات معرفية محددة والحواجز المتصورة.

الكلمات الدالة: محو الأمية الغذائية؛ الفجوة بين المعرفة والسلوك؛ التثقيف الغذائي؛ طلاب الصيدلة؛ الصحة العامة.

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Chemical Composition of Essential Oils Total Phenols and Antioxidant Activity of Achillea fragrantissima and A. santolina Grown in Syria

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ABSTRACT

The aim of this study is to investigate the active components in the essential oils and determine the total phenol content and antioxidant activity of flowers of *Achillea fragrantissima* and *A. santolina* collected from Al-Kalamoon (Damascus countryside, Syria). Flower oils were extracted and analyzed using gas chromatography-mass spectrometry (GC-MS). Three extracts were prepared using distilled water, methanol, and chloroform. Total phenol content and antioxidant activity were determined for the essential oils as well as for the aqueous, methanolic, and chloroformic extracts. The results revealed the presence of 20 components in the essential oil of *A. fragrantissima*. The major compounds identified were beta-thujone (39.63%), santolina alcohol (15.54%), artemisia ketone (15%), and alpha-thujone (10.58%). Sixteen components were identified in the essential oil of *A. santolina*, with the primary compounds being camphor (49.13%), eucalyptol (17.13%), and terpine-4-ol (8.29%). The essential oil and aqueous, methanolic, and chloroformic extracts of *A. santolina* contained 414.2, 1388.4, 2084.2, and 965.7 mg of TAE/g of dry extract, respectively. In interaction with 2,2-Diphenyl-1-picrylhydrazyl (DPPH), the IC50 values were 105, 120, and 110 µg/L for the aqueous, methanolic, and chloroformic extracts of *A. fragrantissima*, respectively, and 720 and 320 µg/L for the aqueous and methanolic extracts of *A. santolina*, respectively. The essential oils of *A. fragrantissima* and *A. santolina*, as well as the chloroformic extract of *A. santolina*, did not show antioxidant activity. The study demonstrated that the aqueous and methanolic extracts of *A. fragrantissima* exhibit good free radical scavenging activity.

Keywords: *Achillea fragrantissima, A. santolina*, essential oil, total phenols, antioxidant acivity.

1. INTRODUCTION

The genus *Achillea*, belonging to the Asteraceae family, comprises approximately 115 species predominantly found in the temperate regions of the Northern Hemisphere, particularly in northern Africa, southeastern Europe, and southwestern Asia [1]. *Achillea fragrantissima* (Forssk.) Sch. Bip. is a small shrub that grows to a height of 30-60 cm. The leaves are oval with shallow, toothed edges, and the flower heads are disc-shaped terminals composed of yellow tubular flowers [2]. It is commonly used as an antispasmodic to treat rheumatic pain, acute cough, and to lower blood sugar levels [3]. Terpine-4-ol, linalool, carvone, β-phellandrene, artemisia ketone, and α-thujone have been identified as important bioactive compounds in the essential oil of *A. fragrantissima* [4] [5] [1]. Studies have shown that the essential oil can inhibit the growth of many Gram-positive and Gram-negative bacteria [5]. In addition, the aqueous extract has demonstrated cytotoxic activity against cell lines [6] and antitumor activity [7].

A. santolina L. is a herbaceous plant with yellow tubular flowers arranged in disc-shaped inflorescences. The leaves are deeply divided transversely several times,

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giving them a worm-like shape [8]. It is commonly used to treat toothaches, as a tonic and carminative, for the treatment of colic, kidney stones, diabetes, and dysentery [2]. The major compounds of the essential oil of *A. santolina* are camphor, α-pinene, linalool, and fragranyl acetate [9] [10] [1] [11]. Other identified compounds include santolin, stigmasterol, clionasterol, salvigenin, eupatorin, poriferasterol, leukodin, artemistin, and 6,7,3',4'-tetramethoxy-5-hydroxy flavone [12] [8]. Studies have demonstrated its hypoglycemic effect [13] and its ability to inhibit the growth of *Candida albicans* [2]. Medicinal plants are known for their therapeutic and antioxidant effects [14] [15] [16].

This research investigates the chemical composition of essential oils by GC-MS. The phenol content and antioxidant activity of the aqueous, methanolic, and chloroformic extracts, as well as the essential oils of *A. fragrantissima* and *A. santolina*, were also measured.

2.METHODOLOGY

2.1 Materials

All chemicals and reagents used in the study were of analytical grade. Solvents were purchased from Panreac, Spain; Folin-Ciocalteu reagent from Merck KGaA, Germany; anhydrous sodium carbonate from AvonChem, United Kingdom; tannic acid from Rhône-Poulenc LTD Prolabo; 2,2-Diphenyl-1-picrylhydrazyl (DPPH) from Tokyo Chemical Industry; and butylated hydroxytoluene (BHT) from Titan Biotech.

2.2 Instruments

Gas chromatography instrument: GC-plus MS2010 (Shimadzu), sensitive electronic balance of Shimadzu AX200 (Japan), UV-VIS Spectrophotometer ((T80+), PG Instruments, United Kingdom), rotatory evaporator (RV 10 digital IKA, Germany).

2.3 Sourcing and preparation of extracts

Flowering aerial parts were collected from AL-Kalamoon (Damascus countryside, Syria) between May and June (2018-2020). The identification of the plant was verified by the Department of Plant Biology, Faculty of Science, Damascus University. The plant material was dried at room temperature for 10 days in the shade, then powdered and stored in closed containers until extraction. Essential oil was extracted from 500 g of the plant material using the steam distillation method [17]. Powdered flowers (30 g) were placed in a Soxhlet apparatus, and extraction was performed by adding 250 mL of solvent (methanol or chloroform) at 60°C for 4 hours. The extract was then filtered through filter paper [18]. Flowers (30 g) were also extracted with 200 mL of freshly distilled water using a condenser distillation method [19] for 90 minutes. The extracts were concentrated using a rotary vacuum evaporator, and the extraction yield was calculated using the following equation [10]: Extraction yield % = (weight of dry extract/ weight of dry powdered plant material) × 100.

2.4 GC-Ms analysis

The analysis of the volatile oil components was conducted according to the method described in [17], with some modifications. A gas chromatography instrument with a mass spectrometer detector (GC-MS), GCMS-QP2010 Plus (Shimadzu, Kyoto, Japan) was used. The column was an OPTIMA 5 (100% dimethylpolysiloxane USP G1, G2, G38) with a film thickness of 0.25 µm, a column length of 25 m, and a diameter of 0.25 mm. The carrier gas was helium at 54 kilopascals (KPa) pressure. The split ratio was set at 1:30. The injector was automatic, with the injector temperature set at 250°C. The injection mode was split, and the injected sample volume was 1 µL.

Thermal program: The oven temperature was programmed from 80°C to 240°C at a rate of 3°C/min, with a total program time of 62 minutes. The mass spectrometer was operated with an ion source temperature of 200°C and an ionization voltage of 70 eV. Identification

of volatile oil components was performed using the Wiley electronic library.

2. 3 Determination of the total phenolic content in plant extracts

The method published by Abdeltaif et al. was applied to determine the total phenolic content in the samplesaliquot of extract was mixed with 1.58 mL of distilled water, 300 µL of 20% sodium carbonate, and 100 µL of Folin-Ciocalteu reagent. A blanked simultaneously, containing 2 mL of ethanol. The samples were mixed and then left in a dark place at room temperature for 45 minutes. The absorbance was measured at 765 nm. Total phenols were quantified using a calibration curve of tannic acid in ethanol, within the concentration range of 0-500 mg/L (standard curve equation: y=0.0011x + 0.0698 R2=0.9961). Means were calculated from three parallel analyses.

2.4 Evaluation the DPPH scavenging activity

The free radical scavenging activity was measured using a modified version of the method described by . Eight concentrations for each extract and essential oil were prepared (25, 50, 75, 100, 125, 250, 500, and 750 µg/mL in methanol). A series of BHT concentrations (1.5, 3.1, 6.25, 12.5, 25, 50, 75, and 100 µg/L in methanol) were also prepared. The DPPH solution in methanol was prepared at a concentration of 0.04 g/L. The reaction was initiated by adding 0.5 mL of the sample (BHT or extract) to 3.5 mL of DPPH solution. A control was prepared by adding 0.5 mL of methanol to 3.5 mL of DPPH solution. The reaction mixture was incubated in the dark at room temperature for 30 minutes. After incubation, the absorbance was measured at 517 nm using a UV-visible spectrophotometer (Model: T80+, PG Instrument Ltd, United Kingdom). A decrease in absorbance indicates greater radical scavenging activity. Each measurement was repeated three times, and the average was calculated. A calibration curve was generated, and the percentage of scavenging activity was calculated using the following equation:

DPPH scavenging activity
(%) = (Acontrol – Asample) / Acontrol × 100.

Where:
Acontrol: Absorbance of control
(Ethanol + DPPH)
Asample: The absorbance of the sample
(Extract + DPPH).

Butylated hydroxytoluene (BHT) was used as the positive control, and IC_{50} was calculated. Means were calculated from three parallel analyses.

3.RESULTS AND DISCUSSION

3.1 GC-Ms analysis

The essential oil yield from *A. fragrantissima* is 2.4%, wheres that from *A. santolina* is 0.533%.

20 Components were identified in the essential oil of *A. fragrantissima* (Figure1.a). The major compounds found are β-thujone (39.63%), followed by santolina alcohol (15.54%), artemisia ketone (15%), and α-thujone (10.58%) (Table1) (Figure2).

This composition closely resembles the oil studied in wild-grown Egypt [1]. In contrast, essential oil from plants cultivated in Jordan has a different composition, mainly containing terpene-4-ol, linalool, and carvone [4], Which shows the difference in the chemical composition of essential oil depending on the region in which the plant grows and whether it is cultivated or wild-grown.

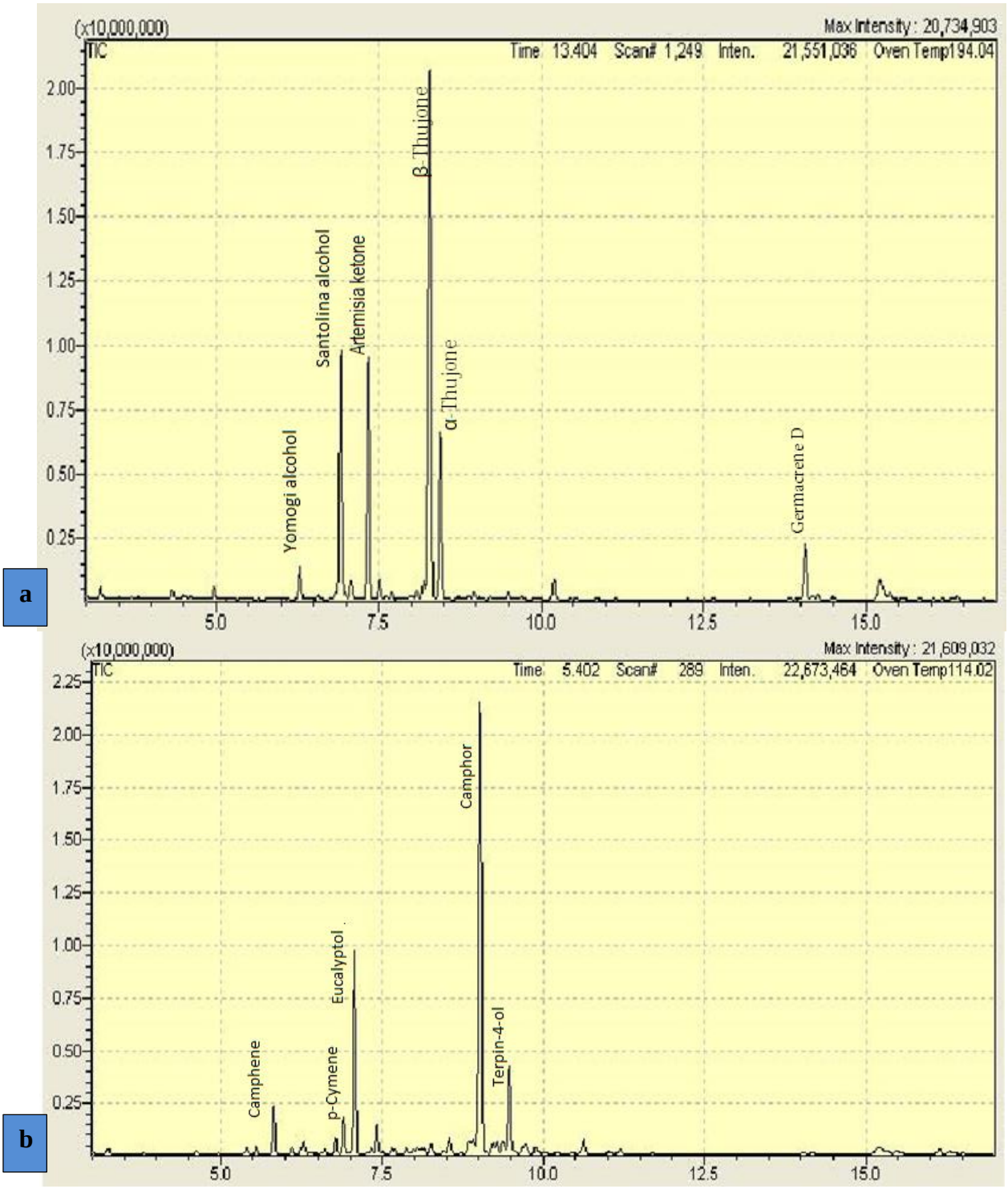

Figure1. a. Chromatogram of the essential oils a. the essential oils *A. fragrantissima*, b. the essential oils *A. santolina.*

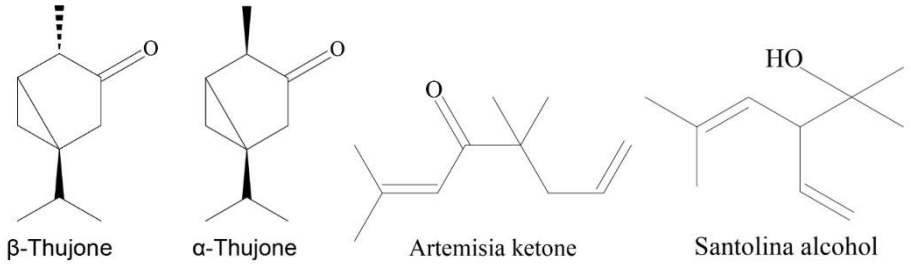

Figure2. Chemical structure of β-thujone, α-thujone, santolina alcohol, and artemisia ketone.

16 Components were identified in the essential oil of *A. santolina* (Figure1.b). The primary compounds are camphor (49.13%), eucalyptol (17.13%), and terpine-4-ol (8.29%) (Table1).

In comparison with other studies on the composition of essential oils, the essential oil studied in Iran contained camphor and eucalyptol, while terpene-4-ol and camphene were absent, and other compounds were present. For example, linalool [9]. Another study conducted in Iran also revealed a different composition, with the essential oil mainly containing fragranyl acetate and fragranol, along with small amounts of camphor, eucalyptol, and terpene-4-ol [10]. This composition is similar to that of the essential oil of the wild plant studied in Egypt [1]. The difference in chemical composition can be explained by the variation of genotypes.

Table 1. Composition of the essential oil of A. fragrantissima, and A. santolina

A. fragrantissima			*A. santolina*		
Component	**RT**	**Percentage**	**Component**	**RT**	**Percentage**
Butanol	3.230	0.67	α-Pinene	5.543	0.54
Methanone	4.325	0.51	Champhene	5.823	3.78
Citronellal	4.505	0.25	Yomogi alcohol	6.288	0.98
Camphene	4.965	0.65	-Terpineneα	6.783	1.32
Yomogi alcohol	6.290	1.76	p-Cymene	6.903	3.03
Iso-amyliso-butyrate	6.562	0.25	Eucalyptol (1.8-Cineole)	7.074	17.13
Santolina alcohol	6.914	15.54	-Terpeneγ	7.424	2.21
Propanoic acid	7.074	1.03	Trans-Sabinene hydrate	8.158	2.50
Artemisia ketone	7.339	15.00	-Thujoneβ	8.265	0.85
Butanoic acid	7.510	1.44	p-Menth-2-en-ol	8.850	1.47
Epoxylinalol	7.617	0.13	Trans-Pinocarveol	8.904	2.54
Artemisia alcohol	7.700	0.40	Camphor	9.026	49.13
Iso-cetronellol	7.800	0.13	-Fenchyl alcoholβ	9.281	1.10
β-Thujone	8.288	39.63	Borneol	9.380	1.26
α-Thujone	8.452	10.58	Terpin-4-ol	9.475	8.29
Artemisyl acetate	8.968	0.34	Myrtanol	9.719	3.31
Norborineol	10.199	1.24			
Germacrene D	14.063	3.94			
Spathulenol	15.367	0.48			
Rosifoliol	16.394	0.19			

3.3 *Yield of extraction*

The yield of extraction ranged from 13.4% in the methanolic extract of *A. fragrantissima* to 4.87% in the aqueous extract of *A. santolina* (Table 2).

3.3 Determination of the total phenolic content

Phenolic compounds ranged from 345.5 µg Tannic acid equivalents/g dry plant in the essential oil of A. fragrantissima to 2084.2 µg Tannic acid equivalents/g dry plant in the methanolic extract of *A. santolina* (Table 2).

In A. fragrantissima, the aqueous extract contains the highest amount of phenols, followed by the chloroform extract, then the methanolic extract, and finally the essential oil. This indicates the presence of both polar and non-polar phenols. Regarding A. santolina, the methanolic extract contains the highest concentration of phenols. The aqueous extract is followed by the chloroform extract, and finally the essential oil, indicating the polar nature of phenols. The following chart displays the total phenol content of both plants.

The inhibitory Concentration (IC_{50}) ranged from 720 µg/L in the aqueous extract of *A. santolina* to 105 µg/L in the aqueous extract of *A. fragrantissima*, whereas BHT had IC_{50} value 16 µg/L (Table 2) [24].

Antioxidant activity was assessed by calculating the IC_{50} of the tested extracts. The results indicate that the methanolic extract of *A. fragrantissima* has the smallest value, followed by the two chloroform extracts, followed by the methanolic extract of the same plant, followed by the methanolic extract, and finally the aqueous extract of *A. santolina*. Note that it was not possible to calculate the IC_{50} values within the studied concentrations for both the essential oils and the chloroform extract of *A. santolina*.

Aqueous extract of *A. fragrantissima* contains the largest content of phenols, and have the highest antioxidant activity. As for the *A. santolina*, methanolic extract contains the largest amount of phenols and its antioxidant activity is greater than that of the other extracts.

The antioxidant activity of different extracts varies according to the composition of the extracts, which may give an idea about the nature of the compounds that have an antioxidant effect. Aqueous, methanolic, and chloroform extracts of *A. fragrantissima* have similar antioxidant activities. This results indicates that the plant contains polar and non-polar antioxidant compounds. As for *A. santolina*, the chloroformic extract did not show antioxidant activity at the concentrations studied, while the aqueous and methanolic extracts have antioxidant activities that can be attributed to the polar compounds present in the plant.

Table 2. Total Phenol Content, and IC_{50} value IC_{50} value *A. santolina*, *A. fragrantissima*, and positive control

	Yield %		**Total Phenols (mg tannic acid equivalents/ g dry extract)**		**IC_{50} (µg/L)**	
	A. santolina	***A. fragrantissima***	***A. santolina***	***A. fragrantissima***	***A. santolina***	***A. fragrantissima***
Methanolic extract	12.46	13.4	2084.2±8.58	657.7±22.5	350±0.1	120±0.1
Chloroform extract	6.76	6.7	965.7±12.6	1402.7±27.81	-	110±0.2
Aqueous extract	4.87	5.1	1388.4±9.55	1490.3±9.43	720±0.2	105±0.3
Essential oil	0.533	2.4	414.2±22.8	345.5±6.12	-	-
BHT						16±0.1

Values are mean ±standard deviation

CONCLUSION

It is concluded that the aqueous, methanolic, and chloroformic extracts of *A. fragrantissima* exhibit antioxidant activity, whereas the essential oil contains compounds, the most prominent of which are β-thujone, santolina alcohol, artemisia ketone, and α-thujone. The aqueous and methanolic extracts of *A. santolina* flowers have demonstrated antioxidant effects, suggesting their potential utility in combating free radicals. In contrast, the chloroformic extract did not exhibit effectiveness at the concentrations tested. The study showed that aqueous and methanolic extracts of *A. fragrantissima* have good free radical scavenging activity. Therefore, extracts are useful for preventing diseases caused by oxidation and free radicals.

Disclosure statement: Conflict of Interest: The authors declare that there are no conflicts of interest.

Compliance with Ethical Standards: This article does not contain any studies involving human or animal subjects.

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التركيب الكيميائي للزيوت الأساسية الفينولات الكلية والفعالية المضادة للأكسدة لـ *Achillea fragrantissima* و *A. santolina* النامي في سوريا

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ملخص

الهدف من هذه الدراسة هو تحري المكونات الفعالة في الزيوت الأساسية وتحديد المحتوى الفينولي الكلي والفعالية المضاة للأكسدة لأزهار نبات *Achillea fragrantissima* و*A. santolina* التي جمعت من القلمون (ريف دمشق، سوريا). استخلاص الزيت الأساسي لأزهار النباتين وحللت باستخدام جهاز الكروماتوغرافيا الغازية ومطياف الكتلة (GC-MS). حضرت ثلاثة خلاصات باستخدام الماء المقطر والميثانول والكلوروفورم. حدد المحتوى الفينولي الكلي و الفعالية المضادات للأكسدة في الخلاصات. أظهرت النتائج وجود 20 مكوناً في الزيت الأساسي لنبات *A. fragrantissima*. المركبات الرئيسية الموجودة هي بيتا ثوجون (39.63%)، يليها كحول السانتولينا (15.54%)، كيتون الأرتيميسيا (15%)، وألفا ثوجون (10.58%). تم التعرف على 16 مكوناً في الزيت العطري لنبات *A. santolina*. المركبات الرئيسية هي الكافور (49.13%)، والأوكالبتول (17.13%)، والتيربين-4-أول (8.29%). احتوى الزيت الأساسي والخلاصات المائية والميثانولية والكلوروفورمية لـ *A. santolina* على (414.2، 1388.4، 2084.2، و965.7 مغ من TAE/غ من الخلاصة الجافة)، على التوالي. في التفاعل مع 2،2-ثنائي فينيل-1- بيكريل هيدرازيل، كانت قيمة التراكيز المثبطة لنصف الجذور الحرةهي (105، 120، و110 مكغ/لتر) للخلاصات المائية والميثانولية والكلوروفورمية لنبات*A. fragrantissima* على التوالي، و(720 و320 مكغ/لتر). بالنسبة للخلاصات المائية والميثانولية لـ *A. santolina*، على التوالي، لم يظهر الزيت الأساسي لكل من *A. fragrantissima* و*A. santolina* و الخلاصة الكلوروفورمية لـ *A. santolina* فعالية مضادة للأكسدة. أظهرت الدراسة أن الخلاصات المائية والميثانولية لنبات *A. fragrantissima* تمتلك فعالية جيدة في كبح الجذور الحرة.

الكلمات الدالة: *Achillea fragrantissima*، *A. santolina* ، زيت عطري، الفنولات الكلية، الفعالية المضادة للأكسدة.

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Assessment of QTc-interval Prolonging Medication Utilization and Associated Potential Drug-Drug Interactions in Hospitalized Cardiac Patients: A Cross-Sectional Study in Cardiology

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ABSTRACT

Background: Several medications are linked to QTc-interval prolongation and torsades de pointes (TdP), a risk that is more common among hospitalized patients due to polypharmacy and associated QTc-interval-prolonging drug-drug interactions (QTc-pDDIs).

Objective: This study aimed to identify the prevalence of QTc-interval-prolonging drug (QTc-Drug) utilization and QTc-pDDIs among postoperative cardiac patients admitted to the National Institute of Cardiovascular Diseases (NICVD).

Method: We conducted a cross-sectional study at the NICVD, reviewing patients' medication charts for the use of QTc-Drugs and QTc-pDDIs. The CredibleMeds list was used to identify drugs associated with QTc-interval prolongation, while Micromedex Drug-Int.® and Lexicomp Interact® were utilized to screen for QTc-pDDIs.

Results: A total of 384 patients, with an average age of 48.9 ± 13.9 years, were included in the study. On average, patients used 10.3 ± 1.7 medications. Of the 3,956 medications prescribed, 22.9% were QTc-Drugs. The most frequently used QTc-Drug classes were diuretics (69.3%), anti-emetics (61.5%), and proton pump inhibitors (51.0%). Overall, 99.7% of patients received at least one QTc-Drug. The most frequent QTc-pDDI was ciprofloxacin-domperidone (7.6%), classified as major by Micromedex and a category B interaction by Lexicomp.

Conclusion: The prevalence of QTc-Drugs was very high among postoperative cardiac patients, with nearly all patients (99.7%) receiving at least one QTc-Drug. The most common QTc-pDDI was ciprofloxacin-domperidone (7.6%), identified as a major interaction by Micromedex and a category B interaction by Lexicomp. Category X (contraindicated) QTc-pDDIs should be avoided in hospitalized patients.

Keywords: QTc-interval prolonging drugs; Polypharmacy; Torsade de pointes; Potential drug-drug interaction; cardiovascular diseases; Pakistan.

INTRODUCTION

Each year, around six million people die from sudden cardiac death (SCD) related to ventricular tachyarrhythmias [1]. A prolonged QTc interval is associated with torsades de pointes (TdP), a potentially life-threatening polymorphic ventricular tachycardia [2]. While cardiovascular arrest due to TdP is rare, it can be devastating for hospitalized patients. QTc prolongation is defined as a value greater than 460 ms for men and 470 ms for women [3].

Several factors can cause the QTc interval to be prolonged, with polypharmacy playing a significant role

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[4]. Polypharmacy is defined as the concurrent use of five or more medications, while hyper-polypharmacy refers to the use of ten or more medications simultaneously [5]. In addition to polypharmacy, potential drug-drug interactions associated with QTc-interval prolongation (QTc-pDDIs) are a risk factor for prolonged QTc intervals. The risk of TdP is significantly higher when a QTc-interval-prolonging drug is involved in potential drug-drug interactions (pDDIs) [6]. QTc-Drugs may cause pharmacodynamic interactions with additional therapies, exacerbating the prolongation of the QTc interval [7]. Adding new drugs to a patient's ongoing treatment regimen may increase the risk of pDDIs in hospitalized patients [8, 9]. Beyond QTc-interval prolongation and the consequent risk of TdP, pDDIs can result in serious outcomes, which may prompt consultants to take risks due to the lack of evidence supporting adverse effects [10].

There is a relationship between geriatric patients, females, electrolyte imbalances, bradycardia, hereditary heart abnormalities, and the likelihood of developing QTc-interval prolongation. Several drugs can prolong the QTc interval, a phenomenon known as drug-induced QTc-interval prolongation [11]. The Arizona Center for Education and Research on Therapeutics (AZCERT) has identified over 220 drugs linked to QTc-interval prolongation on CredibleMeds®. To demonstrate the level of assurance regarding TdP risk, AZCERT has categorized QTc-interval-prolonging medications into four groups: known risk of TdP, possible risk of TdP, conditional risk of TdP, and drugs to avoid in congenital long QT syndrome (cLQTS) patients. It has been identified that more than 50 drugs are associated with a known risk of TdP [1].

Prescribing drugs that prolong QTc intervals requires prescribers to weigh the risks against the potential therapeutic benefits. Dhanani and colleagues highlighted the importance of clinical pharmacists in prescribing and managing medications across a clinical healthcare system [12]. Managing daily clinical risks is challenging due to the lack of specific guidelines for reducing QTc-interval prolongation. According to Tisdale et al. and Vandael et al., a risk score can be used to predict the risk of QTc prolongation, with scoring systems developed based on observational studies. Tisdale et al. classified QTc prolongation risk groups into low, moderate, and high by calculating a summated risk score [13]. Vandael et al. developed the RISQ-PATH score, based on a systematic review of observational studies, which aids in clinical decision-making and facilitates the prescribing process for QTc-drugs [14].

There is relatively little published data about QTc-pDDIs and QTc-Drugs. In cardiac tertiary care units, only a few studies have focused on overall pDDIs or interactions that may lead to QTc-interval prolongation [15]. In developing countries, particularly Pakistan, data on this topic are limited. Therefore, research in this demographic is recommended. Additionally, many limitations in relevant studies, such as small sample sizes, retrospective approaches, a restricted scope focusing solely on pDDIs, and the use of diagnostic instruments, suggest that more thorough investigations are needed [4].

Thus, this study evaluated the prevalence of QTc-Drug usage, QTc-pDDIs, and the AZCERT classification of drugs associated with QTc-interval prolongation.

METHODOLOGY

Setting and Design of Study

A cross-sectional study was conducted over six months at the NICVD in Karachi, Pakistan, from November 2021 to April 2022. We collected data using approved data collection forms from patient records, including information on patients' demographics, diagnoses, and prescribed medications. All patients in our study were aged 18 years or older and had received at least two medications, regardless of the route of administration. Patients under 18 years of age were excluded from the study.

The sample size was calculated using Daniel's formula, $n = Z^2P\ (1 - P)/d^2$ [16]. A total of 384 patients were included in our study based on this formula. Ethical

approval for our study was obtained from the Ethical Review Committee (ERC), Clinical Research Department, NICVD, Karachi, Pakistan (ERC-117/2021).

Data Analysis

Each patient's list of dispensed medications was analyzed for QTc-pDDIs using Micromedex DrugReax® and Lexicomp Interact® [17]. Drugs associated with QTc-interval prolongation were assessed using the AZCERT QTc-Drugs lists and were categorized based on their TdP risks [18]. Data were collected in an MS Excel™ spreadsheet, which was checked for accuracy and completeness by the co-supervisor. All statistical analyses were performed using SPSS (version 25.0).

RESULTS

Among the 384 patients, 70.1% were male. The mean age of the patients was 48.9 ± 13.9 years, with the majority (46.4%) aged between 46 and 60 years. Patients with a single co-morbidity accounted for 34.9%, those with more than one co-morbidity accounted for 35.9%, and those without any known co-morbidity comprised 29.2%. The most common surgery was Coronary Artery Bypass Grafting (CABG), performed in 54.2% of the patients (Table 1).

Table 1: Baseline Characteristics of total patients

Variables	**% (n)**
Gender	
Male	70.1 (269)
Female	29.9 (115)
Age	
Mean±SD	48.9 ± 13.9
18-30 years	12.8 (49)
31-45 years	21.1 (81)
46-60 years	46.4 (178)
Above 60 years	19.8 (76)
Co-Morbidities	
Single Co-morbidity	34.9 (134)
More than one co-morbidity	35.9 (138)
NKCM (No Known Co-Morbidities)	29.2 (112)
Commonest Procedures/Surgeries	
Coronary artery bypass grafting (CABG)	54.2 (208)
Mitral Valve Replacement (MVR)	15.1 (58)
Aortic Valve Replacement (AVR)	10.2 (39)
Double Valve Replacement (DVR)	5.5 (21)
Atrial Septal Defect (ASD) Closure	5 (19)
Wound Debridement	6.5 (25)
Excision of Myxoma	1.8 (7)

The study analyzed 3,956 drugs prescribed to 384 patients, of which 906 (22.9%) were identified as QTc-Drugs, with a mean of 2.36 ± 0.85 per patient. The minimum number of drugs administered to patients was five, while the maximum was fifteen. The average number of medications administered per patient was 10.3 ± 1.7, as shown in Table 2. Overall, 99.7% of patients received at least one QTc-Drug of any class, with 16.1% receiving a single QTc-Drug, 39.6% receiving two QTc-Drugs, 35.7% receiving three QTc-Drugs, 8.1% receiving four QTc-Drugs, and 0.3% receiving five QTc-Drugs (Table 2).

Table 2:Polypharmacy and Types of QTc-Drugs as per CredibleMeds Classification [27].

Variables	% (n)
Polypharmacy	
Total Drugs (n=384)	3956
Min – Max (Mean ± SD)	5 - 15 (10.31 ± 1.7)
QTc-Drugs	
Total (n=384)	22.9 (906)
Min – Max (Mean ± SD)	0 - 5 (2.36 ± 0.85)
Known risk of TdP	
Total (n=384)	34.5 (313)
Min – Max (Mean ± SD)	0 - 2 (0.82 ± 0.58)
Possible risk of TdP	
Total (n=384)	2.9 (26)
Min – Max (Mean ± SD)	0 - 2 (0.07 ± 0.27)
Conditional risk of TdP	
Total (n=384)	60.9 (552)
Min – Max (Mean ± SD)	0 - 3 (1.41 ± 0.59)
Drugs to avoid for cLQTS patients	
Total (n=384)	1.6 (15)
Min – Max (Mean ± SD)	0 - 2 (0.04 ± 0.2)
No. of QTc-Drugs per patient	
1	16.1 (62)
2	39.6 (152)
3	35.7 (137)
4	8.1 (31)
5	0.3 (1)
Patients received QTc-Drugs	99.7 (383)

Table 3 presents the prevalence of QTc-Drugs along with their associated TdP risks. The most frequently used QTc-Drugs in postoperative cardiac patients were furosemide (69.3%), domperidone (61.5%), omeprazole (51%), ciprofloxacin (14.3%), tramadol (6.3%), and amiodarone (4.7%). This table provides valuable insights into the prevalence of drug utilization and the TdP risk classifications across different therapeutic categories, aiding healthcare providers in making well-informed decisions regarding medication selection for their patients.

Table 3: Drug Classification of QT Drugs and CredibleMeds Category [27]

Drug Class	QTc-Drugs	% (n)	TdP Risk Category
Diuretic	Furosemide	69.3 (266)	Conditional risk of TdP
Gastrointestinal Agent/Anti-Emetic	Domperidone	61.5 (236)	Known risk of TdP
	Ondansetron	0.3 (1)	Known risk of TdP
Proton Pump Inhibitor	Omeprazole	51 (196)	Conditional risk of TdP
Antibiotics	Ciprofloxacin	14.3 (55)	Known risk of TdP
	Moxifloxacin	0.8 (3)	Known risk of TdP
	Piperacillin-Tazobactam	0.8 (3)	Conditional risk of TdP
	Co-trimoxazole	0.8 (3)	Drugs to avoid for cLQTS patients
Anti-Arrhythmic	Amiodarone	4.7 (18)	Known risk of TdP
Opioid Analgesic	Tramadol	6.3 (24)	Possible risk of TdP
Bronchodilator	Salbutamol (Albuterol)	2.6 (10)	Drugs to avoid for cLQTS patients
Beta-Agonist	Adrenaline	1 (4)	Drugs to avoid for cLQTS patients
	Norepinephrine	0.5 (2)	Drugs to avoid for cLQTS patients
Calcium Channel Blocker	Diltiazem	0.3 (1)	Conditional risk of TdP
Hyperpolarization-activated Cyclic Nucleotide-gated (HCN Blocker)	Ivabradine	0.3 (1)	Conditional risk of TdP
Antipsychotic	Levosulpiride	0.3 (1)	Known risk of TdP
	Quetiapine	0.8 (3)	Conditional risk of TdP
	Risperidone	0.3 (1)	Conditional risk of TdP
Anticonvulsant	Levetiracetam	0.3 (1)	Possible risk of TdP

Table 4 shows the drug-drug interaction pairs (Micromedex DrugReax®) involved in QTc-pDDIs and their associated TdP risks. The most frequent pairs were domperidone-ciprofloxacin (7.6%), domperidone-amlodipine (4.4%), domperidone-amiodarone (1.6%), and amiodarone-ciprofloxacin (0.8%).

Table 5 presents the drug-drug interaction pairs (Lexicomp Interact®) involved in QTc-pDDIs and their TdP risks. The most frequent pairs were domperidone-ciprofloxacin (7.6%), domperidone-salbutamol (1.8%), domperidone-amiodarone (1.6%), and amiodarone-ciprofloxacin (0.8%). Category X (contraindicated) pairs, such as domperidone-amiodarone and domperidone-verapamil, were labeled as major interactions in Micromedex DrugReax®.

Table 4:QTc-pDDIs pairs as per Micromedex Screening

QTc-pDDIs	% (n)	Severity	Onset	Documentation	Mechanism
Domperidone-Ciprofloxacin	7.6 (29)	Major	Not Specified	Fair	Pharmacokinetic
Domperidone-Amlodipine	4.4 (17)	Major	Not Specified	Fair	Pharmacokinetic
Domperidone-Amiodarone	1.6 (6)	Major	Not Specified	Fair	Pharmacokinetic
Domperidone-Quetiapine	0.8 (3)	Major	Not Specified	Fair	Pharmacodynamic
Amiodarone-Ciprofloxacin	0.8 (3)	Major	Not Specified	Fair	Pharmacodynamic
Domperidone-Diltiazem	0.3 (1)	Major	Not Specified	Fair	Pharmacokinetic
Domperidone-Ticagrelor	0.3 (1)	Major	Not Specified	Fair	Pharmacokinetic
Domperidone-Alprazolam	0.3 (1)	Major	Not Specified	Fair	Pharmacokinetic
Domperidone-Verapamil	0.3 (1)	Major	Not Specified	Fair	Pharmacokinetic

Table 5: QTc-pDDIs pairs as per Lexicomp Screening

QTc-pDDIs	% (n)	Risk Rating	Mechanism
Domperidone-Ciprofloxacin	7.6 (29)	B	Pharmacokinetic
Domperidone-Salbutamol	1.8 (7)	B	Pharmacokinetic
Domperidone-Amiodarone	1.6 (6)	X	Pharmacokinetic
Domperidone-Quetiapine	0.8 (3)	D	Pharmacodynamic
Amiodarone-Ciprofloxacin	0.8 (3)	C	Pharmacodynamic
Domperidone-Diltiazem	0.3 (1)	X	Pharmacokinetic
Domperidone-Verapamil	0.3 (1)	X	Pharmacokinetic
Furosemide-Ivabradine	0.3 (1)	C	Pharmacodynamic
Amiodarone-Salbutamol	0.3 (1)	C	Pharmacodynamic

DISCUSSION

According to this study, postoperative cardiac inpatients exhibited a high prevalence of QTc-interval prolongation. Our research aimed to determine both the prevalence of QTc-Drugs and QTc-pDDI prescribing patterns simultaneously. Multiple comorbid conditions likely contribute to the high prevalence of QTc-interval prolongation risk factors. Postoperative patients frequently experience polypharmacy, which may be attributed to comorbid illnesses [19]. Our findings suggest that polypharmacy may be associated with the use of drugs that prolong the QTc interval and with QTc-pDDIs. Postoperative patients are highly likely to experience QTc-pDDIs, which can lead to severe arrhythmias and sudden death [20]. Our analysis revealed that most patients frequently used QTc-Drugs, increasing the risk of TdP and

prolonged QTc intervals.

Additionally, a study conducted in a medical ward found extensive use of QTc-Drugs [21]. Many postoperative inpatients suffer from chronic illnesses that may necessitate long-term medication use. To minimize risks, it is essential to consider alternative treatments for patients on QTc-Drugs [18]. Data on QTc-interval prolongation risk factors in postoperative patients are limited. However, several studies have documented QTc-interval prolongation in intensive care units (ICUs), where there is a relatively high prevalence of QTc-Drugs [3, 13, 22].

A study indicated a significant correlation between polypharmacy and QTc-interval prolongation [23]. There was also a substantial correlation between QTc-pDDIs and drug classes such as antimicrobials and anti-emetics. The combination of these medications with others may increase the risk of QTc-interval prolongation and subsequent TdP [18]. Among the drug-interacting pairs, the most common was domperidone-ciprofloxacin (a pharmacokinetic interaction), and the most critical interactions were with amiodarone, domperidone, and ondansetron, known to cause TdP. Patients experiencing these interactions must be closely monitored to prevent potentially harmful effects [24].

The findings of this study suggest that appropriate measures should be taken for postoperative cardiac patients to avoid adverse outcomes caused by QTc-Drugs and QTc-pDDIs. Electrocardiographic (ECG) monitoring, including manual measurement of the QTc interval, should be a standard part of clinical practice, especially for patients with risk factors and those taking QTc-prolonging medications. It is recommended to perform an ECG 8-12 hours after initiating or increasing a high-risk QTc-prolonging medication [25]. Drug selection for these patients should be based on a risk-benefit analysis [26]. Pharmacists should be well-educated about QTc-interval prolongation when conducting drug reviews. Implementing pharmacist-driven QTc-interval monitoring can help reduce the risk of QTc-interval prolongation [1].

CONCLUSION

In postoperative cardiac patients, there is a high prevalence of QTc-Drugs. QTc-pDDIs categorized as Category X should be avoided in these patients. Healthcare professionals, especially clinical pharmacists, must understand ECG interpretation, QTc-pDDIs, and their associated risks. Risk assessment tools should be implemented to reduce the risk of QTc prolongation and TdP.

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تقييم استخدام الأدوية التي تطيل فترة QTc والتفاعلات المحتملة بين الأدوية المرتبطة بها لدى المرضى القلبية المقيمين في المستشفى: دراسة عرضية في مجال القلب

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ملخص

الخلفية :ترتبط العديد من الأدوية بإطالة فترة QTc وتورساد دي بوانت(TdP) ، ويكون ذلك أكثر شيوعًا بين المرضى المقيمين في المستشفى بسبب التعددية الدوائية والتفاعلات بين الأدوية التي تؤدي إلى إطالة فترة.(QTc-pDDIs) QTc

الهدف :صُممت هذه الدراسة لتحديد انتشار استخدام أدوية إطالة فترة (QTc-Drugs) QTc والتفاعلات بين الأدوية التي تؤدي إلى إطالة فترة (QTc-pDDIs) QTc بين المرضى القلبية بعد الجراحة الذين تم إدخالهم إلى المعهد الوطني للأمراض القلبية الوعائية.(NICVD)

الطريقة :أجرينا دراسة عرضية في المعهد الوطني للأمراض القلبية الوعائية .(NICVD) تم مراجعة سجلات أدوية المرضى للتحقق من استخدام أدوية إطالة فترة (QTc-Drugs) QTc والتفاعلات بين الأدوية التي تؤدي إلى إطالة فترة .(QTc-pDDIs) QTc تم استخدام قوائم CredibleMeds لتحديد الأدوية المتعلقة بإطالة فترة .QTc وتم استخدام ®Micromedex Drug-Int. و ®Lexicomp Interactلفحص التفاعلات بين الأدوية.

النتائج :شملت الدراسة 384 مريضًا بمتوسط عمر 48.9 ± 13.9 عامًا. في المتوسط، استخدم المرضى 10.3 ± 1.7 أدوية. من بين 3956 دواءً موصوفًا، كانت 22.9% منها أدوية إطالة فترة .(QTc-Drugs) QTc كانت الفئات الأكثر استخدامًا من أدوية QTcهي المدرات بنسبة 69.3%، ومضادات القيء بنسبة 61.5%، ومثبطات مضخة البروتون بنسبة 51.0%. بشكل عام، تلقى 99.7% من المرضى دواءً واحدًا على الأقل من أدوية .QTc كانت التفاعلات الأكثر شيوعًا بين أدوية QTc هي السيبروفلوكساسين-دومبيريدون بنسبة 7.6%، والتي تم تصنيفها كأدوية رئيسية بواسطة Micromedex وتصنيف B بواسطة.Lexicomp

الاستنتاج :كان انتشار أدوية إطالة فترة (QTc-Drugs) QTc مرتفعًا جدًا بين المرضى القلبية بعد الجراحة. تلقى تقريبًا جميع المرضى (99.7%) دواءً واحدًا على الأقل من أدويةQTc ، وكانت التفاعل بين الأدوية الأكثر شيوعًا هو السيبروفلوكساسين-دومبيريدون بنسبة 7.6%، والذي تم تصنيفه كدواء رئيسي بواسطة Micromedex وتصنيف B بواسطة .Lexicomp يجب تجنب التفاعلات بين الأدوية من الفئة X (الممنوعة) في المرضى المقيمين بالمستشفى.

الكلمات الدالة: أدوية إطالة فترة QTc؛ التعددية الدوائية؛ تورساد دي بوانت؛ التفاعلات المحتملة بين الأدوية؛ الأمراض القلبية الوعائية؛ باكستان.

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Phenolic Compounds and Antioxidant Activity of *Chiliadenus montanus* (Vhal.) Brullo. grown *in vitro*

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ABSTRACT

This study explores the in vitro cultivation of *Chiliadenus montanus* (Vhal.) Brullo (Asteraceae), focusing on callus multiplication, in vitro seed germination, phenolic compound production, and antioxidant activity. Callus induction was optimized, followed by multiplication using Murashige and Skoog (MS) media supplemented with 1.0 mg·L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) and 2.0 mg·L^{-1} 6-Benzylaminopurine (BAP). The highest in vitro germination rate of *C. montanus* seeds (11.6 ± 2.22%) was achieved using half-strength MS media supplemented with 0.5 mg·L^{-1} gibberellic acid (GA_3) and 1.0 mg·L^{-1} BAP. Methanol extracts from wild and in vitro samples were analyzed for Terpinen-4-ol, Eucalyptol (1,8-Cineole), and total phenolic content. In vitro microshoots exhibited an elevated Terpinen-4-ol concentration (0.01 ± 0.003 mg/g) compared to wild plants, while the concentrations of Eucalyptol (0.06 ± 0.001 mg/g) were similar in both microshoots and wild plants. Phenolic compound analysis revealed maximum levels in wild plants (30.67 ± 2.82 gallic acid equivalents [GAE]), followed by microshoots (22.81 ± 0.65 GAE), and the lowest in callus (6.37 ± 0.27 GAE). Antioxidant properties, evaluated via the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, indicated superior radical scavenging in wild plants (Inhibitory Concentration 50 [IC_{50}] 32.13 ± 0.83 µg/ml) compared to greenhouse plants (IC_{50} 221.04 ± 1.34 µg/ml). *C. montanus* emerges as a potential natural antioxidant source. In conclusion, an effective in vitro production system for phenolic compounds in *C. montanus* was established, offering a sustainable alternative to wild plant harvesting. The study highlights the potential benefits of *C. montanus* as a reservoir of bioactive substances and emphasizes the importance of in vitro cultivation for sustainable resource utilization.

Keywords: callus culture, microshoot, medicinal plant, *Chiliadenus montanus*, antioxidant activity, phenolic compounds.

1. INTRODUCTION

Chiliadenus montanus (Vhal.) Brullo, also known as *Jasonia montana*, *Varthemia montana* (Vahl.) Boiss., is an herbaceous plant of the Asteraceae family has been shown to contain diverse volatile organic compounds (VOCs) such as Alloaromadendrene, cis-Myrtanol, p-Cymene, α-pinene and β-Cyclocitral [1]. Phenolic compounds, recognized as prominent natural secondary metabolites in plants due to their significant physiological and biochemical activities, are exemplified by gallic acid (GA) [2]. These polyphenolic metabolites are known for their numerous roles, including antioxidant [3], antineoplastic [4], and apoptosis-inducing properties [5]. The antioxidative potential of phenolic compounds, by neutralizing oxygen radicals, addresses redox damage and associated health risks within cells [6,7].

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Chiliadenus montanus, commonly referred to as Haneida, is a traditional medicinal plant indigenous to Jordan, Egypt, Palestine, and Saudi Arabia [8]. *C. montanus* is widely used in Mediterranean traditional healing practices for conditions such as diarrhea, kidney problems, stomachaches, and chest ailments [9-11]. Scientific investigations have confirmed its pharmacological activity, including antitumor [12], free radical scavenging [13], antiviral [14], cholestasis-reducing, and hypoglycemic properties [15], further emphasizing its biomedical significance [16].

In vitro cultivation of plants emerges as a crucial bioengineering tool for micropropagation, serving as a sustainable resource for management and conservation [17], as well as for the synthesis of natural products [18,19]. The induced formation of callus, an unorganized mass of cells, is a pivotal stage in in vitro culture, offering advantages such as reliable production of therapeutic metabolites without impacting wild plant populations [20,21]. In our previous studies on *C. montanus*, an efficient protocol for rapid micropropagation and secondary metabolite production was documented for the first time [1]. There is limited research on the phytochemical [10,22] and biological activities [12-16] of *C. montanus* extracts; therefore, this work enriches the understanding of the total phenolic content and antioxidant activity present in micropropagated plants.

This study was conducted to achieve the following objectives: to initiate in vitro cultivation of *C. montanus* using different hormone combinations for callus multiplication and in vitro germination of *C. montanus* seeds; to determine the volatile components Terpinen-4-ol and Eucalyptol (1,8-Cineole) using gas chromatography-mass spectrometry (GC-MS) analysis in micropropagated samples, and compare these outcomes with those of wild and greenhouse plants; to compare the total phenolic compounds, such as gallic acid, in wild and in vitro cultures; and to evaluate antioxidant properties using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

2. MATERIALS AND METHODS

2.1. Plant material and sterilization

C. montanus seeds were harvested from indigenous plants located in the Al-Kamsheh region within the Zarqa governorate, Jordan (32°07′28.4″ N; 35°53′22.1″ E) in November 2020 (Figure 1, A). After collection, the seeds underwent meticulous germination under controlled greenhouse conditions at the Agriculture Institute, The University of Jordan (Figure 1, B). Leaves obtained from the wild plants underwent a systematic disinfection procedure, involving a 30-second exposure to 70% (v/v) ethanol within a laminar airflow environment (ESCO Labculture® Class II Type A2 Biological Safety Cabinet, Esco Micro Pte. Ltd., Singapore). Following this, the plant material was subjected to a one-minute treatment with a 1% (v/v) solution of sodium hypochlorite (NaOCl) enriched with a small quantity of Tween® 20. Post-treatment, the explants underwent rigorous washing, involving six cycles with sterilized distilled water.

2.2. Callus Induction and Culture Medium

In our previous published paper, we provided detailed information on the specific media compositions used for callus culture induction. optimal culture conditions were observed when disinfected leaves were aseptically cultured on (MS) media enriched with 0.5 $mg \cdot L^{-1}$ (2, 4-D) and 1.5 $mg \cdot L^{-1}$6-Furfurylaminopurne (Kin) , along with 30 g sucrose per liter, 100 $mg \cdot L^{-1}$ myo-inositol. The pH of the medium was ranged between 5.7-5.8, and 6 g/l agar (Bacto-agar, Difco, India) was added following established protocols [1]. The resulting medium was subjected to autoclaving at 121 ◦C and 1.06 $kg \cdot cm^{-2}$ for 15 minutes, and subsequently, 25 mL aliquots were distributed to Petri dishes. The aseptically treated explants were horizontally positioned on the medium and maintained in complete darkness at 25 ± 2 ◦C for eight weeks.

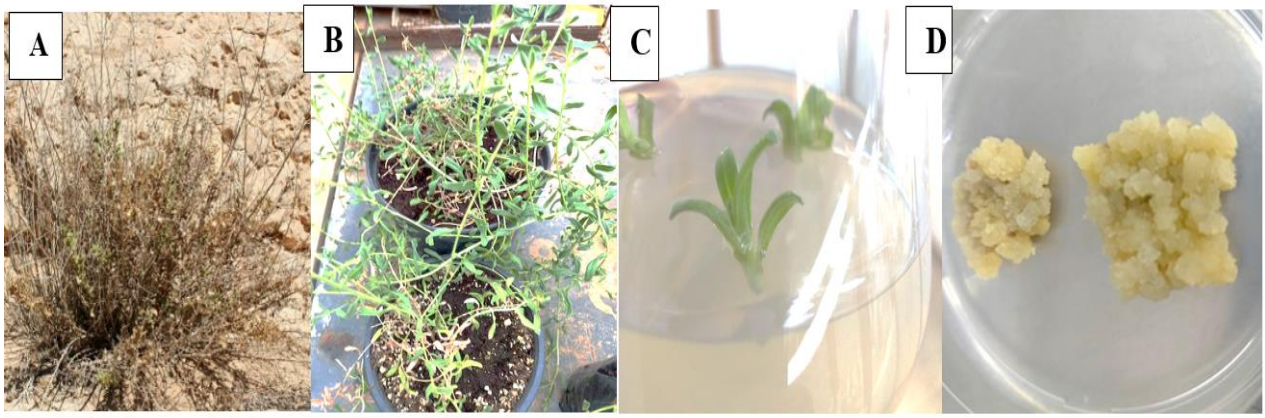

Figure 1. (a) Wild plant (WP) *C. montanus* (Vahl.) Brullo in the natural site; (b) Green house plant of *C. montanus*; (c) Germinated plant *in vitro*, and (d) callus culture.

2.3. Callus Multiplication

Following callus induction, 0.5 g of callus was transffered to (MS) media supplemented with varying combinations of (BAP), Thidiazuron (TDZ), or (Kin) at concentrations of 0.5, 1, and 2 mg·L^{-1}, along with 1.0 mg·L^{-1} (2, 4-D). Incubation occurred in total absence of light at 24 °C, resulting in the proliferation of calli, which were then utilized in subsequent experimental procedures. Employing a completely randomized design (CRD), each experimental condition was replicated 10 times (each replicate comprised two 0.5 g callus pieces within individual culture plates). Upon completion of the incubation period, measurements were taken for callus diameter, fresh weight, texture, and color. Statistical analyses were conducted utilizing SAS software (version 9, SAS Inc., Cary, NC, USA), employing analysis of variance (ANOVA) for each experiment. Mean separation was performed at a significance level of 0.05 using Tukey's Honestly Significant Difference (HSD) test. The growth regulators utilized in this study were sourced from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. The optimal callus multiplication medium, determined by superior callus growth characterized by increased fresh weight and morphological alterations, was subsequently employed for callus proliferation.

2.4. *In vitro* Shoot Germination

To determine the optimal basal medium formulation for maximizing seed germination, four distinct formulations were assessed: (1) Half-strength (MS) medium supplemented with 15 g sucrose, 2.2 g MS salts, 0.5 mg·L^{-1} (GA_3), and 1 mg· L^{-1} (BAP).(2) Full-strength MS medium containing 30 g sucrose, 4.4 g MS salts, and 2 mg· L^{-1} GA_3.(3) Full-strength MS medium supplemented with 0.1 mg· L^{-1} α-naphthaleneacetic acid (NAA) and 1.0 mg· L^{-1} BAP.(4) Full-strength MS medium supplemented with 0.5 mg· L^{-1} (NAA) and 2.0 mg· L^{-1} BAP. Prior to solidification with 8 g agar (Bacto-agar, Difco, India), the pH of each medium was adjusted to 5.8. Petri dishes containing inoculated seeds were incubated in a culture room under a 16-hour photoperiod with an irradiance of 56 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by cool white fluorescent lamps (Philips, Germany) at 25 ± 2°C. A CRD was employed, with each treatment replicated 10 times, and each replicate comprising ten seeds in a Petri dish. Following a 4-week incubation period, the percentage of seed germination was calculated using the formula: [Seed germination percentage] = [Number of seeds germinated] \ [Total number of seeds] times [100%]. Data were analyzed statistically as described in callus culture experiments.

2.5. Preparation of Extracts

This study utilized four distinct samples of *C. montanus* plant extracts, including extracts from the wild plant collected in November 2020 from Alkamsheh, Zarqa, one-month-old microshoots cultivated in shoot maintenance media (comprising half-strength MS media, 0.5 $mg \cdot L^{-1}$ GA^3, 1.0 $mg \cdot L^{-1}$ BAP, and 0.1 $mg \cdot L^{-1}$ NAA), callus grown in callus maintenance media (consisting of full-strength MS media, 0.5 $mg \cdot L^{-1}$ 2, 4-D, and 1.5 $mg \cdot L^{-1}$ Kin), and greenhouse plants (Figure 1).

The preparation of all plant extracts involved soaking 1.3 g of dried plant samples in 100 ml of HPLC-grade methanol (80%, technical quality, Algol, Espoo, Finland), with subsequent agitation in darkness for one week. Following filtration through a 110 mm filter paper (Whatmann), the filtrate obtained was moved into a 250 ml round-bottom flask for each material. Methanol was then evaporated from the filtrate using a rotavapor (Heidoph machine LABORATA 4000, Germany) operating at 60 rpm and 62 °C until complete evaporation, leaving only the extract in the flask. The net weight of the derived concentrate was determined and subsequently solubilized using 10 ml of methanol.

2.6. Determination of Terpinen-4-ol and Eucalyptol (1, 8-Cineole) Content

Identification of the key compounds, Terpinen-4-ol and Eucalyptol (1, 8-Cineole), within the essential oil of *C. montanus* from the four samples were performed using gas chromatography-mass spectrometry (GC-MS), as described previously [1]. GC-MS analyses were conducted utilizing a Varian chrompack CP-3800 C/MS/MS-200 instrument (Saturn, The Netherlands), equipped with a DP-5 (5% diphenyl, 95% dimethyl polysiloxane) GC capillary column (30 m × 0.25 mm × 0.25 µm film thickness). Peak identifications, along with specific retention times, were determined employing a mixture of (C8–C20) alkanes in hexane (Sigma Aldrich, Saint Louis, MI, USA), which was injected under the same conditions outlined for GC-MS analysis. Retention indices were subsequently calculated. External reference standards for Terpinen-4-ol and Eucalyptol (1, 8-Cineole) were procured from Sigma Standard SAFC supply solution, USA. The identification process involved matching the recorded mass spectrum of these compounds with the NIST library of mass spectral database (National Institute of Standards and Technology, Standard Reference Data Program, Gaithersburg, MD 2089, USA). The relative abundance of each identified compound was determined by dividing the peak area of the specific compound by the total area encompassing all identified peaks [24]. To quantify the area under the peak, a standard curve was employed. Each plant sample was assayed by GC-MS in three replicates.

2.7. Assessment of Total Phenolic Content

The Folin–Ciocalteau assay, as outlined by Singleton et al. (1999), was employed to assess the total phenolic content within extracts obtained from callus, wild plant, and microshoots of *C. montanus*. briefly, 0.5 ml of each methanol concentrate (mg/ml) was aliquoted into a 10 ml volumetric flask. Subsequently, 2.5 ml of deionized water was added, followed by the introduction of 2.5 ml Folin–Ciocalteu reagent 0.2 N (Sigma-Aldrich Chemie, Stieinheim, Germany), with thorough mixing for 3 minutes utilizing a Vortex (IKA Genius350Hz Vortex Mixer Genius3 50Hz 500 - 2500rpm). Following this, 0.5 ml of 10% sodium carbonate Na_2Co_3 (Labosi, Paris, France) was incorporated, and the volume was adjusted to 10 ml with deionized water, followed by incubation in darkness for two hours. The resultant green-blue complex was spectrophotometrically measured at 765 nm (Model UVD-2950; Labomed Inc., Los Angeles, CA, USA), and the wavelength was standardized to 765 nm using a blank sample containing 0.5 ml of methanol without extract. a calibration curve was generated using gallic acid serving as the standard (Figure 2).

The overall phenolic compound contents (mg/g) were

quantified as gallic acid equivalent (GAE) per gram of extract, utilizing the regression model derived from the established standard curve:

[y = 0.0741x - 0.0114, \ R^2 = 0.9955]. In this context, Y represents absorbance, and x denotes gallic acid concentration in ppm. All measurements were conducted in triplicate.

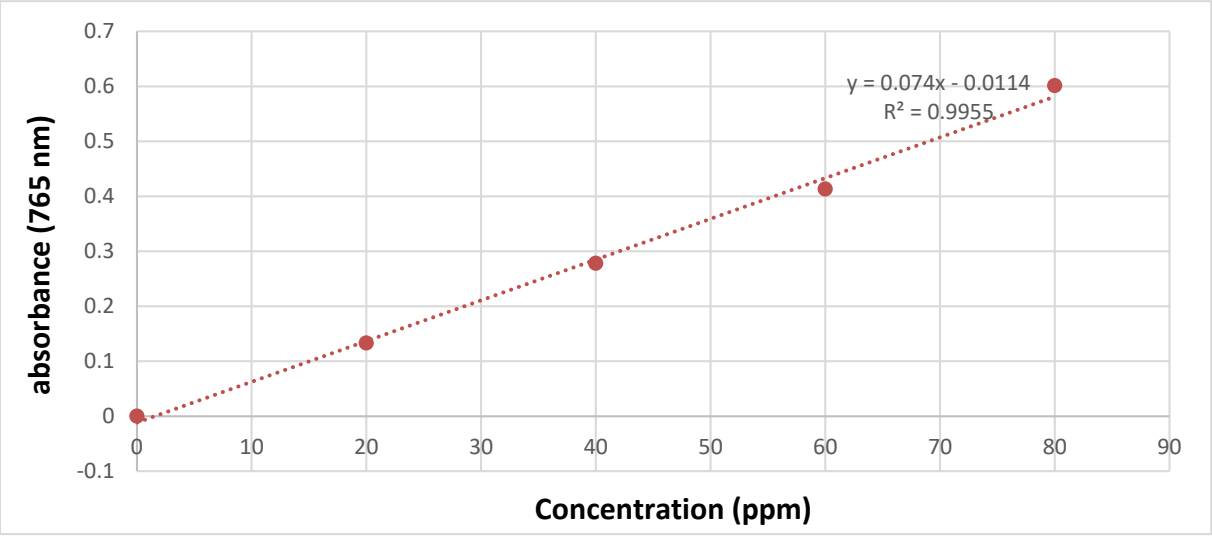

Figure 2: Gallic acid calibration curve using concentrations: 0, 20, 40, 60, and 80 ppm. The regression model was derived by correlating absorbance values at 765 nm with gallic acid concentration.

2.8. Assessing antioxidant Activity Measurement through DPPH Radical Scavenging assay

The assessment of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging was conducted to evaluate the antioxidant capacity in the methanol extract from four plant samples, following the experimental procedure outlined by Piątkowska et al. (2022) with a modification of 30-minutes of exposure at room temperature in the dark, deviating from the original 24-hour duration. Methanolic DPPH was prepared by dissolving 24 milligrams of DPPH (Sigma Aldrich, Saint Louis, MI, USA) in 100 ml of methanol HPLC grade 80%. Subsequently, 100 µL of various concentrations (12.5-400 µg/ml) of the plant extracts, along with positive and negative controls, were introduced to 100 µL of methanolic DPPH in a 96-well microplate. Ascorbic acid served as the positive control in the experimental setup. Initially, 10 mg of ascorbic acid powder was solubilized in 1 mL of PBS (phosphate-buffered saline), followed by mixing thoroughly. Subsequently, triplicate volumes of 100 µL each were prepared for ascorbic acid concentrations of 2, 6, 10, 17, 22 µg/ml. The mixture was homogenized using a pipette tip, and following 30-minutes exposure in darkness at room temperature, absorbance values were quantified at 517 nm using a UV-VIS spectrophotometer in a microplate reader (BIO-TEK-UQANT-MXQ200). The percentage of DPPH radical inhibition was computed using the formula: Inhibition % = [(Abs control - Abs sample/standard) / (Abs control)] x 100, where Abs control is the absorbance of the control reaction (0.1 ml of methanolic DPPH), and Abs sample is the absorbance of the sample reaction (0.1 ml sample dispersed in methanolic DPPH).

The DPPH scavenging activity of the extracts was represented through the IC_{50} value, indicating the essential

oil concentration required to scavenge 50% of DPPH free radicals, in accordance with the approach outlined by Brand-Williams et al. (1995). Determination of IC_{50} for each sample involved plotting percentages of inhibition against sample concentrations, using a nonlinear regression model through GraphPad Prism 8. The experiment was conducted in triplicate for robustness and reliability.

3. RESULTS AND DISCUSSION

3.1. Initiation of *C. montanus* cultures

C. montanus green house plants were grown from seeds sourced from (WP). Microshoots, derived from *in vitro* germinated seeds, were subcultured every four weeks to generate extracts. Leaves from wild plants were cultured for two months, producing friable callus utilized for extract preparation (Figure 1 a-d).

3.2. Callus multiplication

Callus proliferation was achieved by subjecting 0.5 g of fresh callus to a blend of phytohormones, as detailed in Table 1. Optimal results, including the maximum fresh weight (1.59 g) and diameter of the callus (3.08 cm) exhibiting a yellow color and friable texture, were noted on MS media enhanced with 1.0 mg·L^{-1} 2, 4-D and 2.0 mg·L^{-1} BAP. Subsequently, MS media enriched with 1.0 mg·L^{-1} 2,4-D and 2.0 mg·L^{-1} Kin yielded friable and yellow-brown callus, presenting a fresh mass of 1.45 g and a callus diameter of 2.6 cm. Other callus induction intervention displayed limited growth, featuring pale brown, compact-textured calli. The synergistic influence of cytokinins and auxins played a pivotal role in fostering callus formation [28]. Various factors, such as explant source, medium composition, and *in vitro* culture conditions (temperature, light, humidity), contribute to the intricacies of callus induction [29].

In the context of *A. adenophora* (Asteraceae), leaves served as explants, and effective callus induction was attained among of 0.5 mg·L^{-1} 2,4-D and 2.0 mg·L^{-1} BAP [30]. Similarly, optimal callus development was achieved in *Dieffenbachia* cv. Camouflage leaf explants through treatment of 1.0 mg·L^{-1} 2, 4-D and 2.0 mg·L^{-1} BAP [31]. Successful callus initiation in *Ruta graveolens* was demonstrated on MS media fortified with 1.0 mg·L^{-1} 2, 4-D and 1.5 mg·L^{-1} Kin [32]. The *Elephantopus scaber* plant, affiliated to the Asteraceae family, necessitated a synthesis of 2,4D and Kin for successful callus induction [33].

Table 1. The influence of 1.0 mg·L^{-1} 2, 4-D in combination with different concentrations of BAP, TDZ, or Kin (0.5, 1, 2 mg·L^{-1}) on callus multiplication from leaf explants of *C. montanus* (Vhal.) Brullo was assessed four weeks post-induction.

Growth regulators(mg·L^{-1})	Callus weight (g)	Callus diameter (cm)	Callus color	Callus texture
Free	0.10 g	0.36 g	Brown	Compact
1.0 (2,4-D) +0.5(kin)	1.19 d	2.12 cd	Yellow	Friable
1.0 (2,4-D) +1.0(kin)	1.15 d	2.06 d	Yellow+brown	Compact
1.0 (2,4-D) +2.0(kin)	1.46 b	2.60 b	Yellow+light brown	Friable
1.0 (2,4-D) +0.5(BAP)	1.27 c	2.25 c	Yellow+light brown	Friable
1.0 (2,4-D) +1.0(BAP)	1.14 d	2.06 d	Yellow+light brown	Friable
1.0 (2,4-D) +2.0 (BAP)	1.59 a*	3.08 a*	Yellow	Friable
1.0 (2,4-D) +0.5 (TDZ)	0.76 f	1.08 f	Yellow+brown	Compact
1.0 (2,4-D) +1.0 (TDZ)	0.87 e	1.56 e	Yellow+brown	Compact
1.0 (2,4-D) +2.0 (TDZ)	0.71 f	1.04 f	Yellow+brown	Compact

* Mean values with different letters are significantly different according to Tukey's HSD test at $p < 0.05$.

3.3. *In vitro* seeds germination

In the present study, the highest *in vitro* germination of *C. montnus* seeds (11.6 ± 2.22 %) was achieved using half strength (MS) media supplemented with 0.5 mg·L^{-1} (GA_3), and 1 mg·L^{-1} (BAP). Very low seed germination rates were obsreved with the following media; (MS media + 2.0 mg·L^{-1} GA_3) (5.2± 0.79 %), (MS with 0.1 mg·L^{-1} NAA and 1.0 mg·L^{-1} BAP) (2.3±0.67 %) and (0.9± 0.47 %) recorded for seeds inoculated in (MS media + 0.5 mg·L^{-1} NAA and 2.0 mg·L^{-1} BAP) (Table 2).

Table 2. Effect of different media types on seed germination (n = 10) after four weeks of *in vitro* culture of *C. montanus*.

Medium	Seeds germination percentage (%)
MS 1⁄2 +0.5 mg·L^{-1} GA_3+1.0 mg·L^{-1} BAP	11.6 ± 2.22 a*
MS +2.0 mg·L^{-1} GA_3	***5.20 ±0.79 b***
MS +0.1 mg·L^{-1} NAA+1.0 mg·L^{-1} BAP	***2.30 ±0.67 c***
MS +0.5 mg·L^{-1} NAA+2.0 mg·L^{-1} BAP	***0.90 ±0.47c***

* Mean values with different letters are significantly different according to tukey's hsd test at $p < 0.05$ ± standard deviations.

Even today, there are no reports concerning in vitro seed germination of C. montanus or other members of Chiliadenus species. This delay could be related to the poor in vitro seed germination percentage encountered during our study. The Murashige and Skoog formulation is the most commonly used medium in plant tissue culture experiments [34]. In vitro germination experiments for some Asteraceae species observed that the seeds of Centaurea arifolia were very difficult to germinate, with a germination percentage of around 25% after three weeks when seeds were inoculated on half strength (MS) media [35].

This result is in agreement with Okay and Günöz (2015) who showed that germination percentage of *C. tchihatcheffii* seeds were quite low. Yüzbaşıoğlu, et al. (2012) compared different types of MS media with or without BAP, NAA, IBA, and 2,4-D at different concentrations and combinations, and show that half strength (MS) media was the best for seed germination. The low germination percentage of *C. montanus* seeds could be related to deep dormancy resulting from physiological factors such as hormones or other environmental factors [37]. The failure of full-strength MS media could be linked to osmotic pressure, thus the need for low nutrients [38]

3.4. Determination of Terpinen-4-ol and Eucalyptol (1, 8-Cineole) Content

A calibration curve was established for Terpinen-4-ol and Eucalyptol (1,8-Cineole) reference standard solutions, facilitating the quantification of Terpinen-4-ol and Eucalyptol content in volatile oils obtained from wild, microshoot, and callus samples (Figure 3). The elution times for Terpinen-4-ol and Eucalyptol were 19.8 min and 10.4 min, respectively (Figure 4, 5). A three-point linear calibration curve spanning the range of 0.005-0.02 µg/mL (0.005, 0.01, and 0.02 ppm) resulted in r2 values of 0.9878 and 0.997 for Terpinen-4-ol and Eucalyptol, respectively.

Microshoots exhibited a higher Terpinen-4-ol content at 0.01 ±0.003 mg/g compared to the minimal concentration in wild plants at 0.001 ± 0.0002 mg/g, while callus samples showed no detectable Terpinen-4-ol. Regarding Eucalyptol concentration, *in vitro* microshoots demonstrated a comparable level (0.06 ±0.001 mg/g) to wild plants, whereas callus tissues exhibited significantly lower potential for Eucalyptol production at 0.003 ±0.0002 mg/g (Table 3).

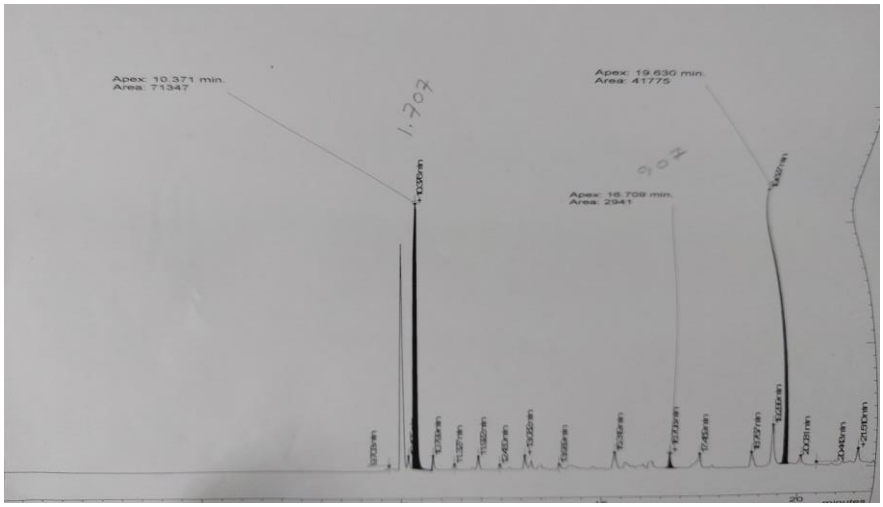

Figure 3. GC-MS chromatogram for standard Eucalyptol and Terpinen-4-ol.

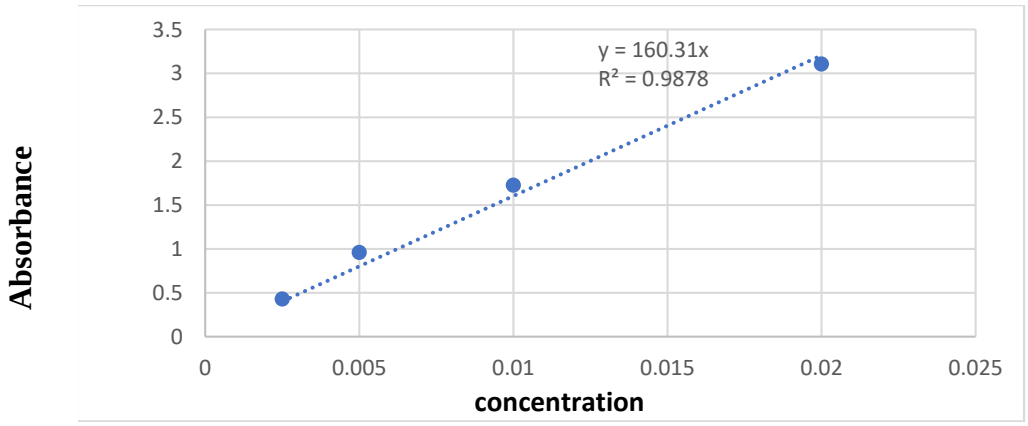

Figure 4: Standard calibration curve of Terpinen-4-ol.

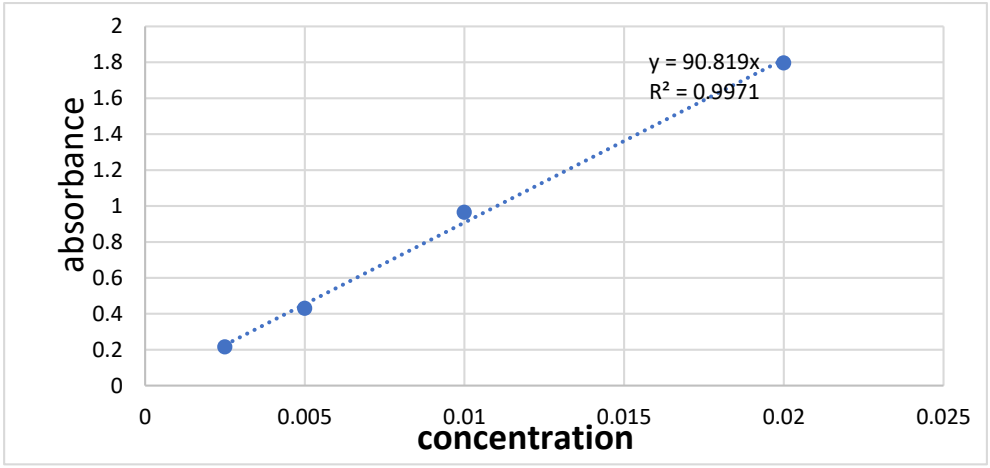

Figure 5. Standard calibration curve of Eucalyptol (1, 8-Cineole).

Table 3. Average Terpinen-4-ol and Eucalyptol (1, 8 Cineol) content (ppm) of different *C. montanus* plant extracts.

Extracts	Concentration (ppm)	
	Terpinen-4-ol	Eucalyptol(1, 8 Cineol)
Callus	0.0 ±0 c	0.003 ±0.0002 b
Wild Plant	0.001 ± 0.0002 b	0.06 +0.001 a
Microshoot	0.01 ±0.003 a	0.06±0.001 a

* Mean values with different letters are significantly different according to Tukey's HSD test at $p < 0.05$ ± standard deviations.

Zito et al. (2013) investigated the volatile oils derived from the leaves and flowers of the wild plant *Chiliadenus lopadusanus* through hydrodistillation and GC-MS analysis, revealing a notable concentration of 1,8-cineole (3.8%). In the Jordan Valley, essential oils from *Chiliadenus iphionoides* were extracted using hydrodistillation, and subsequent GC-MS analysis identified 44 components, with 1,8-cineole (8.4%) and camphor (3.7%) as the principal constituents [40].

Variations in essential oil content among different plant samples may be attributed to the auxins or cytokinins employed in in vitro culture media [41]. Various factors impact the chemical constituents of essential oils in the field, including abiotic factors such as water, light, temperature, soil pH, and salinity, as well as biotic factors encompassing soil microorganisms [42, 43]. Postharvest handling also plays a role in the quantity and quality of secondary metabolite production, with research indicating that drying plant material before extraction enhances oil yield [44, 45]. Moreover, the extraction procedure itself can impact the chemical constituents of the extracted essential oil [46].

Genetic factors exert a significant influence on the observed variations between wild plants and those cultivated in vitro [47]. In vitro cultivation techniques frequently introduce genetic alterations, arising from the manipulation of tissue culture conditions and hormone combinations [48]. These alterations induce changes in gene expression, metabolic pathways, and, consequently, the biosynthesis of secondary metabolites such as Terpinen-4-ol and Eucalyptol. Wild plants have developed within specific ecological habitats, resulting in a natural genetic makeup. Conversely, in vitro cultivation enforces artificial growth conditions [49].

In vitro propagation techniques often involve clonal propagation methods, which may reduce genetic diversity compared to sexually reproducing wild populations [50]. This reduction in genetic variability can significantly influence the secondary metabolite profile of in vitro cultivated plants relative to their wild counterparts [51].

3.5. Total Phenolic Content

Distinct concentrations of phenolic compounds, expressed as gallic acid, were obtained through methanol extracts from wild, green house and *in vitro* samples (callus and microshoots) of *C. montanus*. The wild plant exhibited the highest yield of phenolic compounds, reaching 30.67 ±2.82 gallic acid equivalent (GAE) per gram of dry mass, followed by *in vitro* microshoots at 22.81± 0.65 of GAE per gram of dry mass, with the lowest amount recorded in callus at 6.37 ±0.27 of GAE per gram of dry weight (Table 4). Analogous findings were reported by Al Khateeb et al. (2012), who observed that wild *Cichorium pumilum* Jacq. produced more phenolic compounds compared to *in vitro* samples. Barral-Martinez et al. (2021) also documented similar results, noting that wild *Achillea millefolium* contained 110 mg/g dry extract of phenolic compounds using heat-assisted extraction. Additionally, Eissa et al. (2013) indicated a total phenolic content of 107.4 mg/g sample (GAE) in hydroalcoholic extracts of wild *C. montanus* using the Folin-Ciocalteau method.

Table 4. Average total phenolic content GAE (mg/g) of different *C. montanus* plant extracts.

Extracts	Phenolic Content
Callus	6.37 ±0.27 d
Wild	30.67 ±2.82 a
Microshoot	22.81± 0.65 c
Green house	26.38 ±2.11 b

* Mean values with different letters are significantly different according to Tukey's HSD test at $p < 0.05$± standard deviations.

Discrepancies in gallic acid equivalent (GAE) values obtained from methanolic extracts between wild, greenhouse, and in vitro cultures may be associated with variations in the chemical constituents of plant samples, likely influenced by environmental conditions [56]. The elevated concentration of total phenolic content could be explained by differences in growth conditions, extraction methods, and solvents used during extraction [46].

Understanding the interaction between genetic and environmental factors is essential for explaining the mechanisms of phenol content variation in both wild and in vitro cultured plants [57]. Genetic factors include genotypic variation among plant populations as well as differential expression of genes involved in phenolic compound biosynthesis [47]. Environmental factors, on the other hand, affect phenol content by influencing plant metabolism and enzymatic activities. Factors such as light intensity, temperature fluctuations, nutrient status, and soil composition profoundly influence phenol content by affecting plant physiological responses and metabolic pathways [58].

3.6. Antioxidant Activity

In experimental conditions, reference ascorbic acid was examined at six varying concentrations, demonstrating scavenging effects on DPPH radicals, as indicated by its IC50 value (18.71±0.41 µg/ml) (Figure.6)

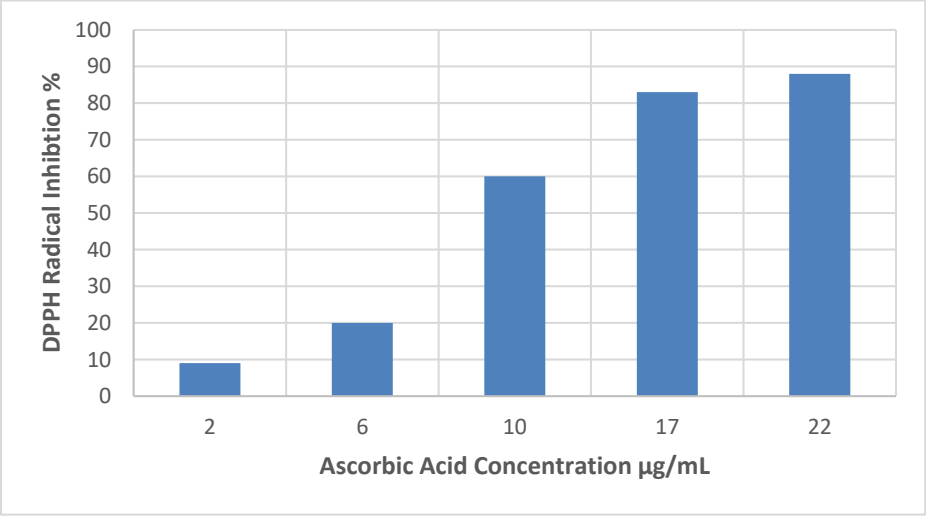

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Figure 6. Ascorbic acid's inhibition of DPPH radicals. The reported are triplicates mean values.

In the current study, the antioxidant functionality of methanolic extracts from various samples of *C. montanus*, including wild, greenhouse-grown, and in vitro samples (callus and microshoots), was investigated using the DPPH scavenging assay. The findings are presented in Table 5. The antioxidant potential was assessed using the DPPH assay as described by Gulcin and Alwasel (2023). In this assay, the stable DPPH free radical, initially deep violet-

purple, reacts with the methanol plant extract, causing a reduction and conversion to the more stable product DPPH-H, resulting in a pale yellow or colorless solution. The discoloration was measured at an absorbance of 517 nm using a spectrophotometer (Figure 7) [60].

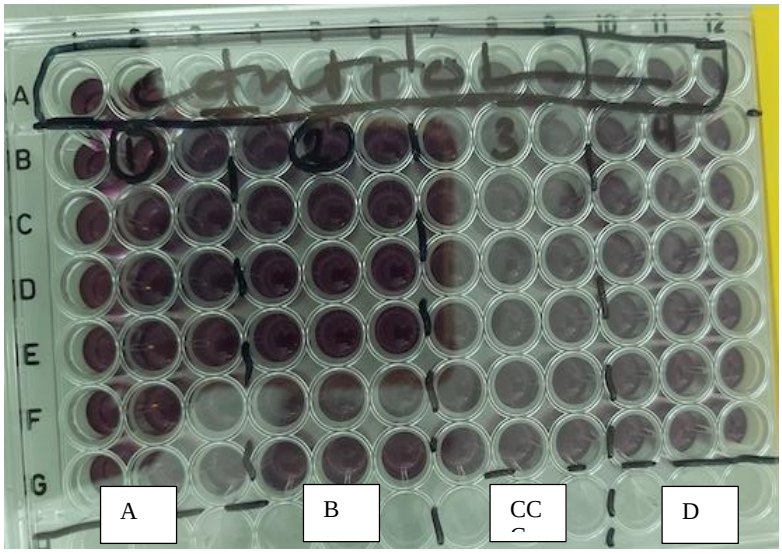

Figure 7: 96 well microplates, showing the changes in *C. montanus* methanolic extract color. A) callus, B) microshoots, C) green-house plant, D) wild plant.

A decreased IC50 value corresponds to heightened antioxidant activity, representing the compound's effectiveness in inhibiting the DPPH free radical by 50%. The same equation employed in the previous DPPH method was employed to determine the free radical scavenging capacity IC50. The results demonstrate significant variation in free radical scavenging activity among different *C. montanus* plant samples (Table 5). The DPPH activity of wild *C. montanus* methanolic extract (IC50 32.13 ±0.83 µg/ml) exhibited greater effectiveness compared to greenhouse-grown plants (IC50 221.04 ±1.34 µg/ml), while *in vitro* samples (microshoots and callus) showed low activity.

Table 5. Antioxidant activity represented as IC_{50} values (µg/ml) of *C. montanus* methanolic extract, values are mean of three replicates.

Extracts	**IC_{50} µg/ml**
Callus	>8000 ±5.2 c
Wild	32.13 ±0.83 a
Microshoot	>8000 ±5.2 c
Green-house plant	221.04 ±1.34 b
Ascorbic acid (standard)	18.71±0.41

* Mean values with different letters are significantly different according to tukey's hsd test at $p < 0.05$± standard deviations.

This finding was analogous to results obtained previously by Eissa et al., 2014, who have studied the antioxidant activity of *C. montanus* essential oil using ORAC assay. Eissa et al. (2014) founds that *C. montanus* have scavenging activity (39±0.18 µg/ml). Researches showed that using methanol extract produce the maximum DPPH radical scavenging activity compared to water extract [61]. The methanolic extracts of *Grammosciadium platycarpum* (Asteraceae) displayed (IC50 45±2.1 µg/ml) high level of antioxidant scavenging activity of DPPH [62]. Antioxidant activity of *Pluchea indica* (Asteraceae) was determined using DPPH assay (16.66 ±0.08µg/ml) [63].

The difference in antioxidant activity of phenolic compounds between in vitro-cultured plants and wild plants can be attributed to various factors, including altered growth conditions, stress responses, and genetic variations [46,47]. In vitro conditions may lack certain environmental stresses that contribute to the development of phenolic compounds in wild plants [64]. Additionally, the tissue culture process can influence gene expression patterns, leading to variations in secondary metabolite production [65,66].

Finally, considering that our experiments were conducted using only a low dose of the compounds (1.3 g/100 ml methanol) for the antioxidant evaluation of phenolic compounds, it is recommended to conduct further studies at higher doses using different techniques, such as the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) assay, Ferric Reducing Antioxidant Power (FRAP) assay, Total Antioxidant Capacity (TAC) assay, and Oxygen Radical Absorbance Capacity (ORAC) assay, to obtain a comprehensive understanding of the antioxidant potential of plant extracts.

4. CONCLUSION

Conclusively, this study successfully established a productive in vitro production system for phenolic compounds in *Chiliadenus montanus*. The callus culture multiplication method was optimized, and in vitro shoots exhibited elevated concentrations of Terpinen-4-ol. Phenolic compound analysis revealed varying amounts between wild and in vitro cultures, with the highest phenolic content observed in wild plants. The antioxidative potential, assessed through the DPPH radical scavenging assay, indicated superior radical scavenging activity in wild plants compared to greenhouse-grown plants. *C. montanus* emerges as a potential natural antioxidant source, providing a sustainable alternative to wild plant harvesting. The study offers valuable insights into callus tissue culture, phenolic compound synthesis, and antioxidant activities in *C. montanus*, suggesting a promising technique for in vitro culture and bioactive compound synthesis**.**

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المركبات الفينولية والنشاط المضاد للأكسدة في نبات الهنيدة البري *Chiliadenus montanus* (Vhal.) Brullo.

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ملخص

تستكشف هذه الدراسة زراعة نبات الهنيدة البري (العائلة النجمية) *Chiliadenus montanus* (Vhal.) Brullo مركزة على تكاثر الكالوس ،إنتاج مركبات الفينولية ،والنشاط المضاد للأكسدة. تم تحسين تحفيز الكالوس، عن طريق زراعة فرعية في وسط موراشيغ وسكوج المزود ب1.0 ملغم/ لتر حمض 2,4-ثنائي كلورو فينسكي الاسيتيك (2,4-D) و2.0 ملغم/ لترحمض 6–بنزيل أمينو بيورين (BAP). تم تحليل استخراجات الميثانول من النباتات البرية وعينات الاكثار الدقيق لتيربينين–4–أول ،يوكاليبتول (1،8–سينيول) ومحتوى الفينول الكلي . أظهرت البراعم الدقيقة تركيزًا مرتفعًا للتيربينين–4–أول (0.003± 0.01 ملغ/غ) مقارنة بالنباتات البرية، بينما كانت لديها مستويات متماثلة من اليوكالبتول (1،8–سينيول) (0.001± 0.06 ملغ/غ). أظهر تحليل المركبات الفينولية أعلى مستويات في النباتات البرية (2.82± 30.67 مكافئ حمض الجاليك) تلتها البراعم الدقيقة في الانابيب (0.65 ±22.81 مكافئ حمض الجاليك) وأدنى كمية في الكالوس (0.27± 6.37 مكافئ حمض الجاليك). أشارت الخصائص المضادة للأكسدة ،المقيمة من خلال اختبار (1,1–diphenyl-2- picrylhydrazyl DPPH)، الى فعالية فائقة في النباتات البرية (0.83± 32.13 IC50ميكروغم/مل) مقارنة بالنباتات الدفيئة (1.34± 221.04 IC50ميكروغم/مل) يظهر *C. montanus* مصدرًا محتملاً لمضادات الأكسدة الطبيعية ويعمل بمثابة مزيل فعال للجذور الحرة. في الختام، تم العثور على نظام فعال لانتاج المركبات الفينولية في المختبر لنبات *C. montanus*، مما يقدم بديلاً مستداماً لاستخدام النباتات البرية. تسلط الدراسة الضوء على الفوائد المحتملة لنبات *C. montanus*، كمخزن للمواد الحيوية الفعالة وتؤكد أهمية زراعته في المختبر للاستفادة المستدامة من الموارد.

الكلمات الدالة: زراعة الكالوس، البراعم الدقيقة، النبات الطبي، النشاط المضاد للأكسدة، المركبات الفينولية، *Chiliadenus montanus*.

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Social Determinants of Health in Pharmacy Practice

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ABSTRACT

This narrative review explores the role of pharmacy practice in addressing social determinants of health and its potential to mitigate major public health issues in Iraq. It focuses on the key concepts of social determinants of health, their impact on public health, and their implications for the community. The paper defines social determinants of health, examines the connection between these determinants and pharmacy practice, and highlights the role of pharmacists in addressing these determinants. Social determinants of health, such as agriculture and food production, employment, education, and housing, can significantly influence the health status of individuals and communities. By understanding these factors, healthcare practitioners can shift their focus toward prevention rather than solely relying on medication, particularly for those in low-income situations. This approach can reduce the overall need for medications. The review proposes a framework for integrating pharmacy practice into public health strategies, emphasizing how social determinants of health shape this integration. Additionally, it suggests that pharmacists can contribute to improving public health by fostering preventive care within the community.

Keywords: Pharmacy practice, public health, social determinants

INTRODUCTION

Social determinants of health are impacts on health related to the conditions in which individuals are born, grow up, live, and work. They are designed to identify ways to create social and physical environments that promote good health for all [1]. The World Health Organization (WHO) defines social determinants of health as "the circumstances in which people are born, grow up, work and age, as well as the systems put in place to deal with illness" [2]. For example, poor health or lack of education can lead to loss of employment opportunities, which in turn constrains income. Reduced income limits access to healthcare and nutritious food, increasing hardship. Hardship causes stress, which can lead to unhealthy coping mechanisms such as substance abuse and overeating of unhealthy food [3]. There are many types of social determinants of health that will be discussed, including housing, agriculture and food production, education, and employment status. Housing is related to housing instability, a term describing the continuum from homelessness [4]. Some individuals may not be homeless but live in unstable conditions such as exposure to allergens or pests, poor sanitation, and substandard housing structures. Additionally, severe rent burden also contributes to housing instability, while homelessness leads to poor health, with affected individuals being more likely to experience infectious diseases and chronic conditions such as cardiovascular disease and chronic pulmonary disease [5]. Therefore, homeless or unstably housed individuals are likely to be hospitalized more frequently and require more attention and care than patients with stable living arrangements [6].

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Agriculture and food production also play an important role in health. A good agricultural system is essential for the well-being and health of individuals, as the production of staple foods such as grains and vegetables is crucial for growth, energy, and maintaining good health. However, studies show that low-income and underserved populations often consume junk food and face limitations in accessing healthy food, such as fresh fruits and vegetables [7, 8]. Additionally, health can also impact agriculture, as people's health status influences the production of agricultural products. In agricultural communities, poor health decreases work performance and reduces income and productivity, which can eventually lead to further health issues. Therefore, interactions between agriculture and health provide opportunities for both sectors to collaborate in finding solutions to their respective problems [9]. Health and education are closely linked, with better education contributing to the development of healthier children over their lifetime. School-Based Health Centers (SBHCs), established by The Community Preventive Services Taskforce, are an initiative aimed at improving health and education among children from less fortunate families [10]. SBHCs enhance access to healthcare by providing convenient locations and services where students typically spend their time [11], and by helping to care for children who might otherwise miss school due to health issues [12]. Furthermore, SBHCs are uniquely positioned to integrate prevention into their clinical care, effectively addressing a full range of health determinants. They also serve as resources for group and classroom health education, school-wide screenings and prevention programs, and support other school staff in creating a positive school climate [13].

Lastly, a crucial factor in shaping people's social position is employment and working conditions. The Employment Conditions Knowledge Network (EMCONET) has developed models and measures to clarify how different types of jobs, conditions of underemployment, and the threat of unemployment affect social position and health outcomes.

Employment and working conditions are crucial factors in shaping people's social position and can significantly affect workers' health [14]. In addition, limited job availability or poor job quality may diminish or negate any positive effects of employment. To improve employment outcomes, it is important for service providers to consider the interaction between a client's physical health and their employment status, and to assess their physical functioning. However, further research is necessary to better understand this interaction [15].

Impact of social determinants on health inequality

Health inequality refers to the differences in health levels or the distribution of health determinants between different groups within the population [16]. Housing is one of the social determinants contributing to health inequality. This is primarily due to variations in housing quality, housing costs, and the role of housing in social life. Many people do not live in homes that are comfortable, warm, and affordable. Today, housing costs are exceedingly high and often unaffordable for the poor [2]. Consequently, some individuals are forced into homelessness or live in places not intended for human habitation. As a result, they are vulnerable to morbidity and mortality due to exposure to extreme temperatures. Even those who can afford housing costs still face challenges in maintaining their health and well-being. Their mental health is often affected by stress and anxiety related to housing payments. Some are also compelled to live in low-quality housing with poor conditions, which can be detrimental to their health [17]. In contrast, individuals with better socioeconomic status can afford high housing costs without experiencing financial strain or worrying about housing payments [18]. This clearly demonstrates that housing contributes to health inequality between the poor and the wealthy.

Agriculture and food production systems also contribute to gaps in health status. Generally, food supply impacts health inequality through macro, long-term effects on domestic and global food prices. Environmental Factors

such as climate change, soil conditions, and depletion of water and fossil fuels cause long-term reductions in agricultural supply and substantial price impacts. This results in inadequate food supply for people [7, 8]. Additionally, policies that do not ban the use of antibiotics in animal husbandry or harmful chemicals in pesticides, as well as the prevalence of processed foods high in saturated fats, trans fats, free sugars, and salt, contribute to unsafe food conditions [19]. Many young individuals prefer consuming processed foods, which are unhealthy and may lead to obesity or other diseases. Consequently, there is a gap in health status between younger and older people.

Furthermore, health inequality is also influenced by differences in education levels. Individuals with higher education levels generally have better health knowledge compared to those with lower education levels. This knowledge enables them to live healthier lives through practices such as maintaining better hygiene and having a balanced diet [20]. Moreover, highly educated individuals are more likely to secure higher-paying jobs, which allows them to afford healthier food and supplements that are often more expensive. Therefore, it is evident that education also contributes to health inequality [2].

Furthermore, employment also contributes to health inequality. Developed countries tend to have more standard forms of employment, whereas developing countries often have a higher prevalence of informal and unhealthy employment types, such as hazardous child labor and forced sex work. These conditions can adversely affect the health of workers, making them more susceptible to diseases. For example, sex workers are at higher risk of sexually transmitted diseases, including AIDS [21]. People in developed countries typically earn more money, which allows them to better maintain their health and afford treatments when they become ill, compared to those in developing countries. In developed nations, fewer people are employed in the agricultural sector, while in poorer countries, a large proportion of workers are involved in agriculture, often earning only a meager income.

Workers in the agricultural sector experience very different health consequences and potential health interventions compared to those working in the service and industrial sectors [22]. The level of development of health systems is influenced by the health benefits provided by employers. In low-income countries, employers in informal and insecure forms of employment often do not provide health services to their employees, unlike employers in developed countries [21].

Connection between social determinant of health and pharmacy practice

The connection between social determinants of health and pharmacy practice was evaluated based on the role of pharmacists in public health and preventive care. Globally, over the past decades, the roles of pharmacists have undergone significant changes, resulting in a more diverse and highly relevant profession [23]. The original focus of pharmacy practice has shifted from merely compounding and supplying medicine to providing services, information, and ultimately administering patient care [24]. This evolution in the pharmacy profession has become known as pharmaceutical care. To embrace this new responsibility successfully, pharmacists need to develop different skills, attitudes, and behavioral understandings [45]. Adopting these criteria will enable pharmacists to optimize their roles in healthcare provision [25]. Pharmaceutical public health is a relatively new field for pharmacists, and currently, only a portion of community pharmacies globally are practicing population health and preventive care [26]. Healthcare practitioners, especially pharmacists, can reach out to patients and encourage lifestyle changes that promote better health. For patients with chronic diseases, practitioners can offer guidance in managing their illnesses in ways that fit their dynamic lives and reduce potential severity. A significant reduction in the nation's health burden could occur if the pharmacy profession contributes more to the public health sector, such as disease prevention and promotion of self-management and aftercare [27]. Additionally, non-health-

related curricula also play a vital role in seeking initiatives to improve healthcare systems [28]

Health-system pharmacist's role towards the social determinants of health

The health status of individuals and communities can be affected by many interrelated factors known as social determinants of health, such as food production, employment, education, and housing [29]. By understanding each social determinant of health, effective and suitable initiatives can be taken to address community health problems. This involves first identifying the problems and then categorizing them according to the different social determinants of health, so that specific initiatives can be designed to address the issues associated with each category. Housing is one such social determinant, and health conditions like respiratory diseases due to poor housing should be classified under this category [30]. Different types of housing have varying structures, which result in different indoor environments and housing conditions, ultimately leading to diverse respiratory outcomes [31, 32]. Therefore, pharmacists should also be involved in supporting individuals suffering from chronic diseases, including behavioral health problems, through supportive housing initiatives. Previous studies have demonstrated that such interventions reduced hospitalizations, emergency visits, and long-term care utilization; in some cases, these interventions also reduced overall costs and improved health outcomes [33, 34]. Another study showed that pharmacists addressed social obstacles related to the cost of basic necessities, including housing concerns and food assistance, as well as issues with transportation [35]. Employment status is another critical social determinant of health. Unemployed individuals face many health challenges, including stress-related conditions that increase the risk of chronic diseases such as cardiovascular disease. Some may also develop mental health issues, such as depression and anxiety This is because unemployment will result in the loss of stable source of income and causes financial stringency. Some unemployed workers who have a family to support or who are burdened with significant debt experience greater stress-related conditions [36]. Therefore, information on stress-related diseases, such as cardiovascular diseases, should be widely disseminated by pharmacists so that patients are aware of the symptoms and can seek early treatment or learn stress management techniques to prevent such conditions. This information can be spread through social media to reach a larger audience [37]. A study by Levit et al. (2022) conducted pharmacist-led social determinants of health interventions during the COVID-19 pandemic, which supported 21.4% of patients with community resources, including affordable and accessible grocery stores and medicines, and offered assistance in finding new employment [38].

Education is another crucial social determinant of health. Adequate knowledge on maintaining health, such as having a balanced diet and practicing good hygiene, is essential [39]. Educational interventions by pharmacists have been effective in improving patients' knowledge and awareness about medicine use and disease management, particularly when combined with successful patient counseling [40]. Previous studies have also shown the impact of pharmacists' interventions on patient education during the COVID-19 pandemic, including awareness about exercise, COVID testing, and vaccines [38, 46].

Lastly, food production is also a social determinant of health, and issues related to health conditions caused by pesticide use in food production should be classified under this category [41]. Health conditions associated with pesticide exposure include birth defects, hearing loss, cancer, and infertility, as well as acute symptoms such as weakness, vomiting, seizures, breathing difficulties, loss of appetite, and nosebleeds, among others. Additionally, highly processed foods, which are high in saturated fats, sugar, and sodium, increase the risk of obesity and other chronic diseases [42]. Therefore, initiatives to improve access to healthy food, such as food pharmacies, clinics, and hospitals that dispense healthy food, as well as doctor-

prescribed food covered by the healthcare system, are important to reduce healthcare costs [43]. Table 1 presents the impact of pharmacists' interventions on social determinants of health

Table 1: The impact of pharmacists' intervention on social determinants of health

Author	Aim	Findings
Foster et al. 2022[35]	Implementation and evaluation of social determinants of health	- 33% of unsafe housing conditions - Social obstacles related to the cost of basic requirements like housing concerns and food assistance, as well as issues with transportation
Livet et al. 2021[38]	Pharmacist-led social determinants of health interventions during COVID pandemic.	- Patient education (71.4%) including exercising, COVID testing, spread, and vaccine. - Community resources (21.4%) including grocery stores, medicines that are affordable and accessible, and offered resources to assist patients in finding new employment. - Care coordination (7.14%) involves transferring the patient to a different pharmacy that provides drug delivery services.
Foster et al. 2023[44]	The feasibility of a community pharmacy on the screening and referral program on health-related social need.	- Out of the 86 pharmacists who completed screening on social need, 24.4% of them carried out an intervention and made a referral. - The intervention on social need was identified, 31% on neighborhood and built environment, and 30% on economic stability issues which were the most prominent social determinants of health domains.

CONCLUSION

This review explored the social determinants of health, emphasizing the connections between these determinants and pharmacy practice to design a framework for addressing health issues. Additionally, it provides ideas for effective initiatives in pharmacy practice to promote health equity. The role of healthcare practitioners should be expanded to actively contribute to public health. Understanding the social determinants of health and social inequality can help pharmacists focus more on prevention rather than solely on medication, especially for those with low-income jobs and disadvantaged individuals, thereby potentially reducing the need for medications. By identifying characteristics associated with negative health events, pharmacists can intervene and tailor care approaches to prevent such events from occurring or worsening, which could help reduce complications and the overall burden on patients.

Compliance with Ethical Standards

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العوامل الاجتماعية المحددة للصحة في ممارسة الصيدلة

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ملخص

تقدم هذه المراجعة السردية نظرة على دور ممارسة الصيدلة في العوامل الاجتماعية المحددة للصحة من خلال تقديم التدخلات اللازمة لتقليل المشاكل الصحية العامة الرئيسية في العراق. حيث تركز المراجعة على المفهوم الرئيسي للعوامل الاجتماعية المحددة للصحة، وتأثيرها على الصحة العامة، والروابط والتداعيات في المجتمع. ان هذا البحث يصف جوانب العوامل الاجتماعية المحددة للصحة، والارتباط بين العوامل الاجتماعية المحددة للصحة وممارسة الصيدلة، مع تسليط الضوء على دور الصيدلي تجاه هذه العوامل. ومع ذلك، يمكن أن يتأثر الوضع الصحي للأفراد والمجتمعات بالعديد من العوامل المترابطة التي تسمى العوامل الاجتماعية المحددة للصحة، مثل الزراعة وإنتاج الغذاء، التوظيف، التعليم، والإسكان. إن فهم تأثير العوامل الاجتماعية المحددة للصحة يساعد العاملين في مجال الرعاية الصحية على التركيز بشكل أكبر على الوقاية بدلاً من العلاج، خاصة لأولئك الذين يعملون في وظائف منخفضة الدخل والأشخاص العاطلين عن العمل، وبالتالي تقليل الحاجة إلى الأدوية. تقترح هذه المراجعة إطاراً لمساهمة ممارسة الصيدلة في الصحة العامة التي تتحدد من خلال العوامل الاجتماعية المحددة للصحة. بالإضافة إلى ذلك، يمكن للصيادلة المساعدة في تحسين الصحة العامة من خلال إنشاء رعاية وقائية في المجتمع.

الكلمات الدالة: ممارسة الصيدلة، الصحة العامة، العوامل الاجتماعية المحددة.

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المجلة الأردنية في العلوم الصيدلانية

تعريف بالمجلة الأردنية في العلوم الصيدلانية

تأسست المجلة الأردنية في العلوم الصيدلانية بقرار لجنة البحث العلمي/ وزارة التعليم العالي والبحث العلمي رقم 367/2/10 تاريخ 2007/1/11 بشأن إصدار "**المجلة الأردنية في العلوم الصيدلانية**" ضمن إصدارات المجلات الأردنية الوطنية، وهي مجلة علمية عالمية متخصصة ومحكمة، وتصدر بدعم من صندوق دعم البحث العلمي والجامعة الأردنية تعنى بنشر البحوث العلمية الأصيلة المقدمة إليها للنشر في كافة مجالات العلوم الصيدلانية والعلوم الأخرى المرتبطة بها. وتصدر عن عمادة البحث العلمي وضمان الجودة في الجامعة الأردنية باسم الجامعات الأردنية كافة، خدمة للمتخصصين والباحثين والمهتمين في هذه المجالات من داخل الأردن وخارجه. وهي مجلة تصدر أربع مرات في العام أعتبارا من 2021، ومواعيد صدورها (آذار وحزيران وأيلول وكانون أول) من كل عام.

وباسمي وباسم أعضاء هيئة التحرير نود أن نشكر الزملاء الذين أسهموا بإرسال أبحاثهم إلى مجلتنا وتمكنا من إخراج العدد الأول. ونأمل من جميع الزملاء بإرسال ملاحظاتهم الإيجابية إلينا لنتمكن من النهوض بمجلتكم بالشكل الذي يليق بها.

وهذه دعوة إلى كافة الزملاء لإرسال اسهاماتهم العلمية من الأبحاث الأصيلة إلى عنوان المجلة.

والله ولي التوفيق

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