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PRISMA statement of preferred reporting items for systematic reviews and meta-analyses (Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 2009; 6(7): e1000097.) (http://www.prisma-statement.org/);

STARD checklist for the reporting of studies of diagnostic accuracy (Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, et al., for the STARD Group. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. Ann Intern Med 2003;138:40-4.) (http://www.stard-statement.org/);

STROBE statement, a checklist of items that should be included in reports of observational studies (http://www.strobe-statement.org/);

MOOSE guidelines for meta-analysis and systemic reviews of observational studies (Stroup DF, Berlin JA, Morton SC, et al. Meta-analysis of observational studies in epidemiology: a proposal for reporting Meta-analysis of observational Studies in Epidemiology (MOOSE) group. JAMA 2000; 283: 2008-12).

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Results: The results of the study should be stated, with tables/figures given in numerical order; the results should be evaluated according to the statistical analysis methods applied. See General Guidelines for details about the preparation of visual material.

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Conclusion: The conclusion of the study should be highlighted.

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LETTER TO EDITOR

Administration of Potential Medications for COVID-19 Treatment Through Feeding Tube

COVID-19 Tedavisi için Kullanılan Potansiyel İlaçların Beslenme Tüpünden Uygulanması

Burcu KELLECİ ÇAKIR*, Oğuzhan FIRAT, Kutay DEMİRKAN

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Key words: Clinical nutrition, COVID-19, clinical pharmacy, medication administration
Anahtar kelimeler: Beslenme tedavisi, COVID-19, klinik eczacılık, ilaç uygulama

Dear Editor,

The evidence behind Coronavirus disease-2019 (COVID-19) medical treatment is growing tremendously,[1] thus, alternative administration routes of these medications should be considered especially for patients who are intubated with feeding tube (FT) or for elderly and pediatric patients with swallowing difficulties.

Administration of drugs via FT with inappropriate administration techniques can increase, decrease, or delay the effect of drugs or either clog feeding tubes. Larger diameter tubes are used in adults, thus, tube clogging is a relatively more common concern in children.[2] We believe that a quick overview of this possible alternative administration for potential drugs used for COVID-19 treatment will be useful for healthcare professionals, especially in countries where alternative dosage forms are unavailable.

Various antiviral agents are used for COVID-19 treatment. Favipravir, one of those antiviral agents, can be crushed and mixed with water to be administered via FT.[3] In practice, before and after each drug administration through an FT, tube should be flushed with at least 15 mL of water. If the oral suspension dosage form is not available, opening up the oseltamivir capsule is recommended. Since remdesivir is only available in a parenteral dosage form, it cannot be administered via FT.[4]

Even though hydroxychloroquine with/without azithromycin treatment is not preferred in current clinical practice, it is still recommended in some local treatment algorithms of COVID-19. Hydroxychloroquine is available in film coated tablet form. Crushing the tablets is recommended by the manufacturer to be avaoided; however; the literature recommended that tablets may be crushed if other option is unavailable.[4] Azithromycin is available in tablet, capsule, and oral suspension dosage forms. Oral suspension is preferred if available. Otherwise, opening the capsules/tablets is approproate and mixing them with water for FT administration.[4]

When oral anticoagulants or acetylsalicylic acid are chosen for thromboprophylaxis of COVID-19, opening or crushing the dabigatran capsules is not recommended as it results in a 75% increase in their absorption.[5] In addition, rivaroxaban may be crushed and suspended in 50 mL water prior to administration. The location distal end point of the FT should be in the stomach for better bioavailability.[5] Apixaban may also be crushed and suspended with 60 mL water or apple juice. Crushed tablets are stable up to 4 hours in water. Edoxaban may be crushed and suspended in 60-90 mL of water and should be administered immediately.[5]

No specific usage is recommended for aspirin or other non-steroidal antiinflammatory drugs for COVID-19 treatment in the guidelines. However, crushing the enteric-coated tablets containing acetylsalicylic acid may adversely affect the

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formulation properties, alter the bioavailability, or clog the tube, therefore administration of conventional tablets via FT should be considered.[5]

Corticosteroid therapy is mostly considered in patients with severe pneumonia and those who are in the intensive care unit who need oxygen support/mechanical ventilation. Treatment should be planned for short term and with low doses. Dexamethasone and methylprednisolone tablets may disperse in water quickly without the need of crushing and therefore can be administered through FT. The information on administration of prednisolone tablets through FT is not available. Therefore, parenteral application should be considered as better treatment option.

Total daily sorbitol amount over 20 g might cause diarrhea, therefore if liquid dosage form of any of medication is preferred, the amount of its sorbitol ingredient needs to be considered (2). Diarrhea is also one of the symptoms of COVID-19. Thus, if clinicians are unaware of sorbitol related diarrhea, this might mislead their clinical judgment.[1]

None of these medications is confirmed for COVID-19 treatment yet, thus many countries published various treatment algorithms. Therefore, administration of these medications through FT should be the part of these possible treatment algorithms or guidelines. Furthermore, clinicians should be aware that inappropriate administration of these drugs through FT may result in treatment failure.

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ORIGINAL ARTICLE

Strategic Analysis of the Turkish Over-the-Counter Drugs and Non-pharmaceutical Products Market

Türkiye Tezgah Üstü İlaç ve İlaç Dışı Ürün Pazarının Stratejik Analizi

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ABSTRACT

Objectives: The over-the-counter (OTC) drug (i.e., non-prescription drugs) market is growing significantly on a global scale. Our study reviews strategies for OTCs, together with other non-pharmaceutical products, such as herbal products, dietary supplements, and other healthcare products. The aim of this study is to analyze the expanded OTC industry to offer possible strategic solutions for existing problems.

Materials and Methods: We utilized integrated SWOT and Fuzzy Analytic Network Process analyses, together with quantitative analysis covering industry professionals' perspectives.

Results: Our findings showed that the most suitable market strategies are WO2 (i.e., to use information and digital technologies, including mobile applications and social media, to reduce marketing costs), SO2 (i.e., to promote self-medication/self-care to grow the OTC market and invest in information and communication technologies for this purpose), and ST2 (i.e., to improve health literacy and increase access to accurate and understandable information via alternative channels, such as the internet and social media). These key strategies are closely related to the utilization of digital technologies. Other strategies, such as SO1 (i.e., to encourage pharmacists to provide consulting for OTC products, which carry high profitability) and ST1 (i.e., to undertake stakeholder training programs to ensure production quality and introduce safe use to improve community health), were examined in detail, and their outcomes were interpreted in this study.

Conclusion: Given the impact of digital transformation, the same strategies can be implemented for other emerging OTC markets. This study underlines the importance of the OTC sector as one of the main drivers for improving community health and reducing health costs.

Key words: Over-the-counter drugs, non-prescription drugs, non-pharmaceutical products, SWOT analysis, Fuzzy Analytic Network Process

ÖZ

Amaç: Global olarak tezgah üstü ilaç (OTC) pazarı önemli ölçüde büyümektedir. Bu bağlamda çalışmamızda, reçetesiz ilaçların yanı sıra, bitkisel ürünler, gıda takviyeleri ve diğer sağlık ürünleri gibi ilaç dışı ürünler de stratejik olarak değerlendirilmiştir. Bu çalışmanın amacı, genişletilmiş OTC endüstrisinin mevcut problemlerine olası stratejik çözümler sunmak için analizini yapmaktır.

Gereç ve Yöntemler: Bu çalışmada entegre SWOT ve Bulanık Analitik Ağ Prosesi analizlerinin yanı sıra sektör profesyonellerinin bakış açılarını kapsayan nicel analizler yapılmıştır.

Bulgular: Çalışmadan elde ettiğimiz bulgular en uygun pazar stratejilerinin WO2 (sosyal medya ve mobil uygulamalar dahil bilişim ve dijital teknolojileri pazarlama maliyetlerini düşürmek için kullanmak), SO2 (OTC pazarının büyümesi için self-medikasyon/kişisel bakımı teşvik etmek ve bu amaçla bilgi ve iletişim teknolojilerine yatırım yapmak), ST2 (sağlık okuryazarlığını geliştirmek için internet ve sosyal medya gibi alternatif kanallar aracılığıyla doğru ve anlaşılır bilgilere erişimi artırmak) olduğunu göstermiştir. Bu kilit stratejiler dijital teknolojilerin kullanımı ile yakından ilgilidir. Ayrıca bu çalışmada SO1 (OTC ürünleri daha yüksek karlılık taşıdığı için eczacıyı danışmanlık yapmaya teşvik etmek), ST1 (üretimde kaliteyi sağlamak ve toplum sağlığını iyileştirmeye yönelik güvenli ilaç kullanımını tanıtmak için, paydaşlara eğitim programları düzenlemek) gibi diğer stratejiler ayrıntılı olarak incelenmiş ve sonuçları yorumlanmıştır.

Sonuç: Dijital dönüşümün etkisi düşünüldüğünde, aynı stratejiler gelişmekte olan diğer pazarlar için de uygulanabilir. Bu çalışma, OTC sektörünün toplum sağlığını geliştiren ve buna bağlı olarak sağlık maliyetini azaltan paydaşlardan biri olarak önemini vurgulamıştır.

Anahtar kelimeler: Tezgah üstü ilaçlar, reçetesiz ilaçlar, ilaç dışı ürünler, SWOT analizi, Bulanık Analitik Ağ Prosesi

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INTRODUCTION

Over-the-counter (OTC) drugs are sold directly to the consumer without a prescription because they are known to be safe and effective following long-term clinical use. However, similar to regular prescription drugs, OTC drugs may have adverse effects, and they can be misused and abused for their active substances.[1-4]

Non-pharmaceutical products include a wide range of products, such as vitamins, herbal products, dietary supplements, biocidal products licensed by the Ministry of Health (MoH), certain medical devices in pharmaceutical form, medical infant formulas, cosmetics and dermo-cosmetics, and baby food. These products are used in self-care/self-medication and complementary therapy but have not been evaluated as prescription drugs because of their active metabolites.

Regulations applied by the MoH on non-prescription drugs are rather cumbersome. Pricing decisions for non-pharmaceutical products licensed by the Ministry of Agriculture and Forestry, for example, are simpler and easier to process than those for pharmaceutical products. This issue represents an advantage to non-pharmaceutical product manufacturers.

In Turkey, registration files must be submitted according to Common Technical Documents, similar to EU regulations. Some products are licensed by the MoH, while others are licensed by the Ministry of Agriculture and Forestry. Processes for the same task may differ in terms of duration and other registration requirements.[5]

In 2018, the Turkish pharmaceuticals market increased by 26.1% to 30.94 billion TL compared with that in 2017. The reasons behind this growth involve price escalation, sales distribution, volume, and new products. The Pharmaceutical Manufacturers Association of Turkey (IEIS) reported that non-pharmaceutical products described as medicinal products made up approximately 31.5% of the growth in 2018 and were valued at 2.4 billion TL.[6] These medicinal products are examined in our study.

Unfortunately, the data available do not cover certain products, such as sports nutrition, homeopathic medicinal products, and some herbal products. Therefore, a major problem for all stakeholders, including manufacturing companies, is data quality. This problem contributes to the difficulty of understanding and interpreting the OTC market dynamics of Turkey.[7,8]

The aim of the present study is to analyze Turkey's expanded OTC industry to offer possible strategic solutions for existing problems, including poor data quality and lack of regulation. Thus, the related market is evaluated, and strategies to grow this market are proposed.

MATERIALS AND METHODS

Data collection

The data collected consisted of literature and quantitative field research. Only a limited number of studies on OTC/non-pharmaceutical products/self-medication are available in Turkey (Table 1), and none of them include a strategic market analysis via SWOT-Analytic Network Process (ANP)/Analytic Hierarchy Process (AHP). The available directly related studies only include our previous integrated SWOT and PESTEL analysis[7] and a qualitative analysis of the Turkish OTC market.[8] Our previous qualitative analysis was performed using in-depth semi-structured interviews of marketing professionals representing OTC and non-pharmaceutical products manufacturers, as well as OTC consultants, in Turkey.[8]

Besides the literature review (Table 1), data were collected from several reports and resources, such as the Turkish Statistical Institute, IEIS, and IMS Health.

The collected data (i.e., related literature, reports, quantitative field research) were used to prepare the SWOT matrix. All data from the literature, except field research, are shown in the SWOT matrix as L. The findings obtained from the online survey are shown as S (Table 2).

Quantitative survey data

This study received ethical approval (decision no: 2019/26-13, date: 25.02.2019) from the Biruni University Ethics Board. The quantitative assessment included an online survey. The IEIS, AIFD, TİSD, and other related institutes were informed about our study, but only the IEIS agreed to participate. Thus, this survey was pilot-tested on a group of IEIS experts before it was conducted on a group of OTC professionals. Data

Table 1. Results of the literature search [9-20]

References	Related topic
Özcelikay et al.[9]	OTC, pharmacists and patients' perspectives
Yüksel[10]	OTC, pharmacists' perspectives
Gül et al.[11]	Role of pharmacists, OTC purchases
Kirgiz[12]	Regulations, advertising, self-medication
Sencan and Uyar[13]	Advertising, pharmacist and industry perspectives
Lionis et al.[14]	OTC prescribing, rational drug use
Gülpınar and Özçelikay[5]	Regulations, pharmacist and industry perspectives
Atikeler and Özçelikay[15]	Regulations, pricing
Oztora et al.[16]	Self-medication
Okyay and Erdoğan[18]	Self-medication
Oral and Özçelikay[19]	Policies, regulations, ethics
Memisoglu[8]	OTC marketing
Memisoglu[7]	OTC SWOT-PESTEL
Cavaco et al.[17]	Self-medication
Gülpınar et al.[20]	Pharmacists' perspectives about non-pharmaceutical products

OTC: Over-the-counter

collection was conducted between March and June 2019. The main data were collected from a purposive sample, and the target audience included functional managers and senior executives of the OTC industry. The designed questionnaire, which included a SWOT segment, was sent to 55 members, most of whom are manufacturers, through the IEIS. The online questionnaire was also sent to 40 OTC professionals with similar qualifications via LinkedIn. In total, 42 participants responded to the questionnaire. Six questionnaires were excluded from the analysis because they included missing answers. Thus, a total of 36 valid questionnaires were included in the sample. This number of participants is considered acceptable because the participants were specifically selected for purposive sampling.

Statistical analysis

The results were evaluated using advanced statistical tools. The latter incorporated the use of AHP/ANP/ Fuzzy ANP (FANP) methods, which do not require a large number of samples to be statistically significant.[21-23] Because the participants of the survey were experts with deep knowledge and experience on the topic of interest, we believe that all 36 respondents are qualified and sufficient to provide the required information for the study.

SWOT-FANP

SWOT, as a decision-making tool, enables the subjective examination of companies, industries, and even countries. However, SWOT presents some disadvantages, such as a lack of weighting factors and ambiguity. Besides SWOT, the FANP is used in this study.

The AHP, which was introduced by Saaty,[24] is a flexible and effective mechanism for complex decision-making that can help decision-makers set priorities and make the best decisions. The ANP, which is a generalization of AHP, enables the analysis of the interactions of decision criteria. Its basic structures are networks, which undergo interactions and feedback within and between clusters to solve sophisticated decision problems.

ANP and AHP are used for multi-criteria decision-making. However, the former is more appropriate for solving complicated problems because it allows the analyst to capture the complex structures of real interconnections and make predictions with greater accuracy. Saaty suggested the use of ANP to solve the problem of dependence among alternatives or criteria.[25,26]

The ANP method is feasible for fuzzy decision-making problems because it has relatively fewer limitations compared with FANP. Determining the weights and effects of alternative strategic criteria quantitatively by using SWOT alone is impossible. Therefore, ANP/FANP should be combined with SWOT to improve the insufficiency of SWOT data.[27] Thus, FANP is the preferred research method in our study.

FANP consists of eight steps.

Step 1. Identify SWOT factors and sub-factors. Determine alternative strategies according to the SWOT sub-factors.

Step 2. Develop a matrix using all factors and sub-factors and form a fuzzy scale of 1-9 (W_1, i.e., matrix calculation).

Table 2. SWOT matrix for the Turkish OTC industry

Strengths (S)	**Weaknesses (W)**
S1 increasing self-medication/self-care (L)	W1 healthcare professionals' negative perception of herbal products and dietary supplements (L)
S2 aging population (L)	W2 information pollution caused by companies (S)
S3 increasing government support for minimizing reimbursement (L)	W3 lack of education of related stakeholders (S)
S4 absolute population growth and increased migration (S)	W4 lack of OTC regulations and certain classifications (L)
S5 increasing consumer awareness (S)	W5 non-regular market; difficulty obtaining clear data (L)
S6 pharmacies as distribution channels (L)	W6 rising costs (S)
S7 qualified people in the OTC industry (S)	W7 lack of direct-to-consumer advertising for non-prescription drugs (S)
S8 possible synergistic effects of non-pharmaceutical products (S)	W8 absence of pharmacist's role as a consultant (L)
S9 greater profitability compared with prescription drugs for the pharmacist (S)	W9 unwillingness of healthcare professionals to recommend and provide prescriptions (S)
Opportunities (O)	**Threats (T)**
O1 rapid market growth (S) O2 rapid developments in information and communication technologies (S) O3 R&D and innovation (S) O4 direct-to-consumer advertising (L) O5 pharmacists as consultants/salespersons (L) O6 company mergers and acquisitions (L)	T1 economic crisis, exchange rate fluctuations (L) T2 poor-quality production for some non-pharmaceutical products (L) T3 poor health literacy (L) T4 media disinformation (S) T5 lack of direct-to-consumer advertising for non-prescription drugs (S) T6 market penetration difficulties, highly competitive environments (S)

OTC: Over-the-counter, S: Survey findings, L: Literature findings

Step 3. Determine the inner dependence matrix of each SWOT factor with a fuzzy scale of 1-9 with respect to other factors by using a schematic representation of inner dependence among the SWOT factors (W_2, i.e., matrix calculation).

Step 4. Determine the interdependent priorities of the SWOT (W_{SWOT} factors=$W_1 \times W_2$).

Step 5. Determine the local importance degrees of the SWOT sub-factors with a fuzzy scale of 1-9 (W_{SWOT} sub-factors, local).

Step 6. Determine the global importance degrees of the SWOT sub-factors (W_3 global sub-factors of SWOT=$W_{factors} \times W_{relative}$ sub-factors of SWOT).

Step 7. Determine the importance degrees of the strategic options with respect to each SWOT sub-factor with a fuzzy scale of 1-9 (W_4).

Step 8. Determine the overall priorities of the strategic options considering internal relations among SWOT factors ($W_{alternatives}=W_4 \times W_3$ global sub-factors).

Saaty[28] reported that the acceptable limit of the consistency ratio (CR) is 0.10 or less. Thus, the CR of the matrix was checked.[28] The AHP template developed by SCB Associates Ltd. was used for statistical analysis.

A schematic structure of the SWOT analysis was established. The related SWOT criteria and sub-criteria are shown in Figure 1. Strategies were prioritized on the basis of the FANP approach.

RESULTS

The demographics of the participants were summarized in Table 3. The respondents comprised 66.7% males and 33.3% females. Among the 36 respondents, 69.5% were aged 31-50 years, 22.2% were aged 51-60 years, and 8.3% were aged 26-30 years. Experience could be divided into two major groups of 6-10 years (33.4%) and <21 years (27.8%). The majority of the participants worked in the marketing and sales department of the OTC industry (47.2%), and others worked in the regulatory affairs department (25%). The sample also included executives (16.7%) and managers from the medical department (11.1%) of

Table 3. Demographic characteristics of the participants

		Percentage %	Frequency n
Gender	Female	33.3	12
	Male	66.7	24
Age	26-30	8.3	3
	31-40	30.6	11
	41-50	38.9	14
	51-60	22.2	8
Years of experience	6-10	33.4	12
	11-15	19.4	7
	16-20	19.4	7
	21<	27.8	10
Department	Regulatory affairs/ market access	25	9
	Marketing	33.3	12
	Sales	13.9	5
	Medical	11.1	4
	Executives	16.7	6

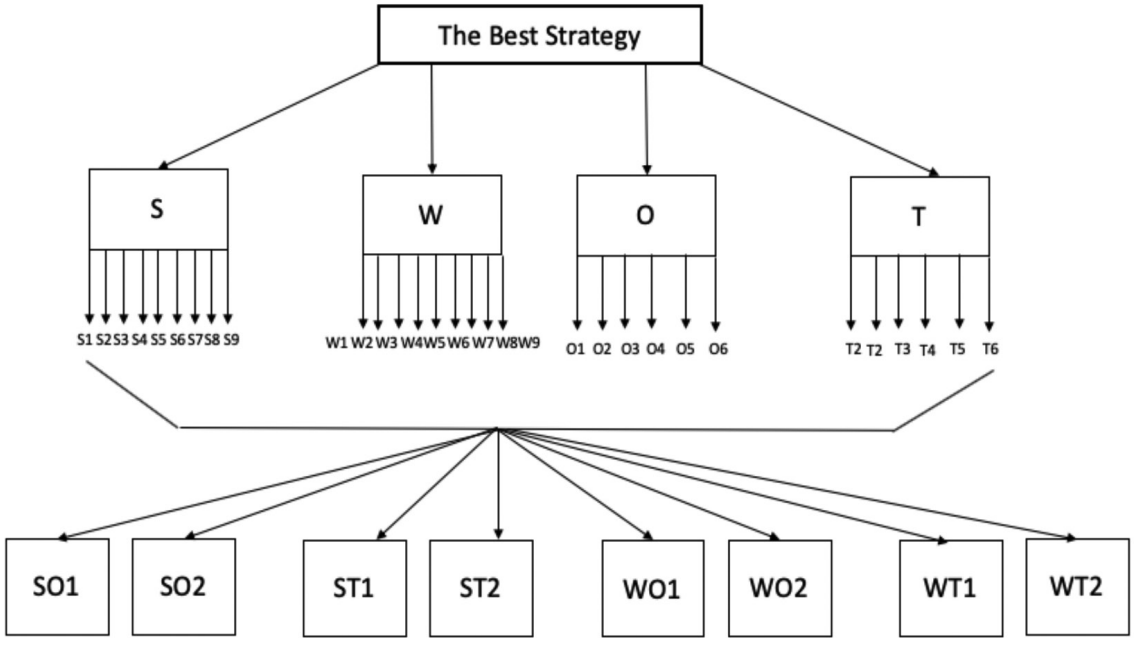

Figure 1. FANP model for the selection of the best strategies
FANP: Fuzzy Analytic Network Process

the OTC sector. All participants were functional managers or senior executives. Unlike in our previous study,[8] both marketing professionals and managers specializing in other departments of the companies participated in our study.

Findings from the survey and other sources are consolidated in the SWOT matrix, as shown in Table 2.

Each SWOT element was checked for consistency. The CR was calculated to be less than 0.1 (10%), which is acceptable. Thus, reexamination was unnecessary because passing the consistency theory.

The fuzzy linguistic variables are shown in Table 4.

The weighting factor of FANP is essential to achieve a strategic choice. The impact of each group was analyzed on all other factors by using pairwise comparisons to obtain relative-importance weights (Table 5). Some required questions, such as "How important is strength when it is compared with a weakness?" and "How important is an opportunity when it is compared with a threat?" were used.

In this study, all possible criteria and interactions were considered (Table 6). The following table summarizes possible strategies for the Turkish OTC industry, and each strategy is individually examined (Table 7).

Table 4. Linguistic variables

	Saaty's scale	TFN	Definition of TFN		
			Bottom	Medium	Top
Equally preferred	1	1	1	1	1
Equally to moderately preferred	2	2	1	3/2	3/2
Moderately preferred	3	3	1	2	2
Moderately to strongly preferred	4	4	3	7/2	4
Strongly preferred	5	5	3	4	9/2
Strongly to very strongly preferred	6	6	3	9/2	5
Very strongly preferred	7	7	5	11/2	6
Very strongly to extremely preferred	8	8	5	6	7
Extremely preferred	9	9	5	7	9

TFN: Triangular fuzzy number

SO1: The profitability of OTC products will increase the consultation capacity of the pharmacist. Thus, companies may need to involve pharmacists in their marketing strategies. This strategy maybe effective if the prejudice of pharmacists against OTC drugs and non-pharmaceutical products is reduced and knowledge about these products is increased. Therefore, improving the quality of training and supporting training with digital technologies are important.

SO2: Self-medication and self-care are increasing rapidly on a global scale.[29] In line with these developments, the promotion of self-medication/self-care may be expected to grow the OTC market in Turkey. The expansion of health and wellness trends will also grow the market. The government should support OTC products because these products do not require reimbursement. Therefore, support for these products may be a good strategy to support self-medication. However, increasing the health literacy of the public is necessary to avoid the incidence of serious adverse reactions.

ST1: Poor-quality products of some non-pharmaceuticals present a great threat to the OTC industry. Education and training are vital in the eco-system. As one will increase the qualified personnel in the OTC industry, production quality will increase via the application of good manufacturing practice standards and inevitably lead to the safe use of these products.

ST2: Assuming that companies provide correct information, access to this information and diversification of channel sources, such as the internet, can increase health literacy. Increased use of advertising and various information technologies will also promote self-medication.

WO1: Poor-quality production can be overcome by investing in innovation. Companies should prioritize innovation and spend on R&D. As the active metabolites of non-prescription drugs are well-known, the available innovations are somehow limited. The main innovation practices of OTCs are combination products, different dosage forms, and line extension.

Differentiation from competitors is an essential factor in building a successful brand and increasing market share. According to our survey findings, innovation is best when using advanced technology and creative communication strategies (72.2%). Sector participants in our survey also emphasized efficacy, quality, and corporate reputation (42.9%) as key factors in building a strong brand.

WO2: In times of economic volatility, all pharma companies and consumers strive to survive. External factors, such as

Table 5. Pairwise comparison of SWOT groups without interdependencies

	S	W	O	T	TFN importance of SWOT factors		
					Bottom	Medium	Top
Strengths (S)	1.000	3.000	2.000	2.000	0.250	0.352	0.352
Weaknesses (W)	-	1.000	0.500	0.500	0.250	0.166	0.166
Opportunities (O)	-	-	1.000	1.000	0.250	0.241	0.241
Threats (T)	-	-	-	1	0.250	0.241	0.241

TFN: Triangular fuzzy number

the economic crisis and exchange rate fluctuations, could increase costs for companies. As companies cannot directly intervene in macro factors, such as economic crises, they can instead develop counter-strategies by introducing cost-saving measures. They may, for example, switch from traditional to digital media, thereby taking advantage of the benefits of social media. At this point, effective content management will gain importance for OTC companies.

WT1: Advertising restrictions may be effective in improving the negative perspectives of physicians and pharmacists. Advertising control is important for patient safety, especially when literacy and/or educational levels vary among the public. In our study, the participants agreed that environmental, economic, and social sustainability, which also cover the safety of non-pharmaceutical products (86.1%), should be among the priorities of pharma companies (88.8%).

Direct-to-consumer advertising for prescription and non-prescription drugs is banned in Turkey[30] but freely available for non-pharmaceutical products. No specific regulation regarding the use of social media by companies is yet available. The

Table 6. Importance of the criteria and sub-criteria of the SWOT analysis

SWOT groups-criteria	Importance of the SWOT criteria	SWOT sub-criteria	Local importance of SWOT sub-criterion	The overall importance of SWOT sub-criterion
Strengths (S)	0.250-0.352-0.352	12S1	0.26-0.284-0.308	0.065-0.1-0.108
		S2	0.087-0.125-0.118	0.022-0.044-0.042
		S3	0.134-0.095-0.09	0.034-0.033-0.032
		S4	0.148-0.161-0.164	0.037-0.057-0.058
		S5	0.085-0.099-0.096	0.021-0.035-0.034
		S6	0.08-0.08-0.076	0.02-0.028-0.027
		S7	0.069-0.054-0.051	0.017-0.019-0.018
		S8	0.071-0.051-0.048	0.018-0.018-0.017
		S9	0.066-0.051-0.048	0.017-0.018-0.017
Weaknesses (W)	0.250-0.166-0.166	14W1	0.103-0.131-0.127	0.026-0.022-0.021
		W2	0.103-0.137-0.132	0.026-0.023-0.022
		W3	0.103-0.09-0.087	0.026-0.015-0.014
		W4	0.237-0.235-0.258	0.059-0.039-0.043
		W5	0.089-0.1-0.093	0.022-0.017-0.015
		W6	0.133-0.131-0.132	0.033-0.022-0.022
		W7	0.078-0.064-0.062	0.02-0.011-0.01
		W8	0.078-0.057-0.054	0.02-0.009-0.009
		W9	0.078-0.057-0.054	0.02-0.009-0.009
Opportunities (O)	0.250-0.241-0.241	28O1	0.281-0.416-0.44	0.07-0.1-0.106
		O2	0.18-0.205-0.202	0.045-0.049-0.049
		O3	0.159-0.149-0.144	0.04-0.036-0.035
		O4	0.14-0.094-0.088	0.035-0.023-0.021
		O5	0.114-0.065-0.059	0.029-0.016-0.014
		O6	0.127-0.071-0.066	0.032-0.017-0.016
Threats (T)	0.250-0.241-0.241	29T1	0.549-0.492-0.516	0.137-0.119-0.124
		T2	0.093-0.105-0.1	0.023-0.025-0.024
		T3	0.093-0.105-0.1	0.023-0.025-0.024
		T4	0.088-0.099-0.095	0.022-0.024-0.023
		T5	0.088-0.099-0.095	0.022-0.024-0.023
		T6	0.088-0.099-0.095	0.022-0.024-0.023

lack of direct-to-consumer advertising can be turned into an advantage for public health, especially among groups with low health literacy. This strategy may be a strong point for Turkey, where nearly half of the population has limited health literacy.[31]

Companies should prioritize the disclosure of accurate information to target consumer groups. In Turkey, new regulations, as well as a proper classification for OTCs, are necessary. OTC ads should also be organized in this context.

According to Table 8, 9, after prioritizing the defined strategies, we can conclude that WO2, which has the highest weight of 0.163, is the best market strategy. Other potential strategies include SO2 (weight, 0.161) and ST2 (weight 0.160). In terms of weight, these three strategies highly similar to each other.

DISCUSSION

The survey results revealed that the strengths of the OTC industry are multifold and highly variable. Increased consumer awareness and a tendency to self-medicate are among the more noteworthy findings. Self-medication/self-care presents a number of important advantages, such as reduced government spending.[29] However, this same benefit may also pose a serious threat to this market. OTC drugs have potential risks, such as misdiagnosis, drug misuse and abuse, and polypharmacy-induced drug-drug interactions, especially in elderly patients.

Pharmacovigilance, also known as drug safety, for prescription drugs has been implemented since 2005. However, this regulation has yet to be established clearly for other types

Table 7. Strategies for the Turkish OTC industry

SO strategies	**WO strategies**
SO1 to encourage pharmacists to provide consulting as OTC products carry higher profitability	WO1 to invest in R&D and innovation to overcome poor-quality manufacturing and increase the available variety of medical treatments
SO2 to promote self-medication/self-care to grow the OTC market and invest in information and communication technologies for this purpose	WO2 to use IT and digital technologies, including social media and mobile applications, to reduce marketing costs
ST strategies	**WT strategies**
ST1 to undertake stakeholder training programs to ensure production quality and introduce safe use for improved community health	WT1 to limit direct-to-consumer advertising for non-pharmaceutical products to improve negative views of physicians and pharmacists. This limitation may be transformed into an advantage for communities with low health literacy
ST2 To improve health literacy and increase access to accurate and understandable information via alternative channels, such as the internet and social media	-

OTC: Over-the-counter

Table 8. Elements of the fuzzy matrix W~ 4

B values	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**W1**	**W2**	**W3**	**W4**	**W5**	**W6**
SO1	0.143	0.103	0.139	0.098	0.392	0.143	0.095	0.143	0.334	0.143	0.107	0.143	0.115	0.354	0.076
SO2	0.143	0.103	0.297	0.105	0.105	0.143	0.413	0.143	0.107	0.143	0.107	0.143	0.138	0.106	0.076
WO1	0.143	0.132	0.113	0.098	0.098	0.143	0.095	0.143	0.082	0.143	0.107	0.143	0.115	0.1	0.411
WO2	0.143	0.132	0.113	0.105	0.098	0.143	0.095	0.143	0.082	0.143	0.1	0.143	0.115	0.1	0.076
ST1	0.143	0.32	0.113	0.105	0.105	0.143	0.103	0.143	0.101	0.143	0.107	0.143	0.138	0.106	0.21
ST2	0.143	0.108	0.113	0.392	0.098	0.143	0.103	0.143	0.088	0.143	0.1	0.143	0.264	0.1	0.076
WT1	0.143	0.103	0.113	0.098	0.105	0.143	0.095	0.143	0.206	0.143	0.373	0.143	0.115	0.135	0.076
B values	**W7**	**W8**	**W9**	**O1**	**O2**	**O3**	**O4**	**O5**	**O6**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
SO1	0.113	0.113	0.189	0.111	0.077	0.093	0.115	0.264	0.143	0.111	0.113	0.113	0.143	0.119	0.113
SO2	0.113	0.113	0.126	0.333	0.077	0.093	0.137	0.115	0.143	0.111	0.113	0.113	0.143	0.137	0.297
WO1	0.139	0.113	0.103	0.111	0.077	0.434	0.115	0.115	0.143	0.111	0.297	0.113	0.143	0.119	0.113
WO2	0.113	0.139	0.103	0.111	0.361	0.101	0.115	0.115	0.143	0.333	0.113	0.113	0.143	0.137	0.139
ST1	0.297	0.113	0.154	0.111	0.077	0.093	0.115	0.137	0.143	0.111	0.139	0.139	0.143	0.118	0.113
ST2	0.113	0.297	0.137	0.111	0.251	0.093	0.137	0.115	0.143	0.111	0.113	0.297	0.143	0.137	0.113
WT1	0.113	0.113	0.189	0.111	0.077	0.093	0.264	0.137	0.143	0.111	0.113	0.113	0.143	0.233	0.113

Table 8. Continued

M values	S1	S2	S3	S4	S5	S6	S7	S8	S9	W1	W2	W3	W4	W5	W6
SO1	0.121	0.076	0.117	0.077	0.426	0.155	0.072	0.117	0.382	0.167	0.109	0.142	0.093	0.400	0.067
SO2	0.199	0.090	0.337	0.123	0.104	0.118	0.440	0.236	0.094	0.188	0.081	0.117	0.109	0.082	0.067
WO1	0.106	0.166	0.083	0.074	0.065	0.114	0.072	0.105	0.060	0.117	0.103	0.118	0.093	0.078	0.429
WO2	0.134	0.113	0.093	0.137	0.065	0.239	0.078	0.105	0.060	0.176	0.082	0.164	0.093	0.079	0.067
ST1	0.123	0.373	0.093	0.084	0.133	0.125	0.125	0.154	0.101	0.117	0.122	0.118	0.178	0.133	0.236
ST2	0.197	0.109	0.185	0.429	0.079	0.125	0.140	0.177	0.077	0.117	0.082	0.111	0.339	0.075	0.067
WT1	0.120	0.073	0.093	0.077	0.127	0.125	0.072	0.106	0.225	0.117	0.421	0.231	0.093	0.153	0.067
M values	**W7**	**W8**	**W9**	**O1**	**O2**	**O3**	**O4**	**O5**	**O6**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
SO1	0.091	0.092	0.221	0.133	0.069	0.079	0.076	0.325	0.125	0.100	0.091	0.091	0.100	0.091	0.100
SO2	0.091	0.092	0.115	0.398	0.069	0.079	0.121	0.088	0.188	0.100	0.091	0.091	0.147	0.147	0.336
WO1	0.191	0.092	0.069	0.094	0.069	0.457	0.087	0.088	0.125	0.100	0.353	0.091	0.100	0.091	0.100
WO2	0.091	0.154	0.069	0.094	0.384	0.150	0.104	0.088	0.125	0.400	0.104	0.091	0.147	0.147	0.122
ST1	0.352	0.102	0.148	0.094	0.069	0.079	0.103	0.135	0.188	0.100	0.177	0.182	0.147	0.091	0.114
ST2	0.091	0.366	0.110	0.094	0.272	0.079	0.171	0.088	0.125	0.100	0.091	0.364	0.213	0.147	0.114
WT1	0.091	0.102	0.268	0.094	0.069	0.079	0.337	0.187	0.125	0.100	0.091	0.091	0.147	0.286	0.114
T values	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**W1**	**W2**	**W3**	**W4**	**W5**	**W6**
SO1	0.121	0.071	0.115	0.074	0.451	0.155	0.069	0.117	0.397	0.167	0.104	0.142	0.090	0.419	0.062
SO2	0.199	0.086	0.355	0.117	0.099	0.118	0.465	0.236	0.090	0.188	0.077	0.114	0.108	0.079	0.062
WO1	0.106	0.160	0.080	0.071	0.062	0.114	0.069	0.105	0.057	0.117	0.098	0.116	0.090	0.075	0.443
WO2	0.134	0.108	0.090	0.130	0.062	0.239	0.075	0.105	0.057	0.176	0.079	0.202	0.090	0.077	0.062
ST1	0.123	0.377	0.090	0.080	0.127	0.125	0.120	0.154	0.097	0.117	0.116	0.116	0.175	0.128	0.244
ST2	0.197	0.133	0.176	0.455	0.076	0.125	0.133	0.177	0.072	0.117	0.078	0.110	0.357	0.073	0.062
WT1	0.120	0.064	0.094	0.073	0.122	0.125	0.069	0.106	0.231	0.117	0.447	0.199	0.090	0.150	0.062
T values	**W7**	**W8**	**W9**	**O1**	**O2**	**O3**	**O4**	**O5**	**O6**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
SO1	0.088	0.088	0.228	0.127	0.064	0.075	0.073	0.342	0.125	0.095	0.088	0.087	0.100	0.088	0.097
SO2	0.088	0.088	0.114	0.426	0.064	0.075	0.119	0.085	0.188	0.095	0.088	0.087	0.147	0.145	0.358
WO1	0.187	0.088	0.067	0.089	0.064	0.480	0.084	0.085	0.125	0.095	0.375	0.087	0.100	0.088	0.097
WO2	0.088	0.151	0.067	0.089	0.393	0.143	0.101	0.085	0.125	0.429	0.101	0.087	0.147	0.145	0.159
ST1	0.374	0.099	0.147	0.089	0.064	0.075	0.100	0.133	0.188	0.095	0.173	0.178	0.147	0.088	0.097
ST2	0.088	0.389	0.109	0.089	0.285	0.075	0.168	0.085	0.125	0.095	0.088	0.386	0.213	0.145	0.097
WT1	0.088	0.099	0.266	0.089	0.064	0.075	0.355	0.184	0.125	0.095	0.088	0.087	0.147	0.299	0.097

of drugs. For example, healthcare professionals can only report hepatotoxicity and nephrotoxicity to the MoH for herbal medicines. The concept of pharmacovigilance should cover all types of products in Turkey. Indeed, in our study, 86.1% of the participants agreed that the vigilance system should be extended to non-pharmaceutical products.

The environmental impacts of non-pharmaceutical products remain unknown. Although some global companies attach importance to sustainability, the government should promote campaigns related to the ecological footprint, climate change awareness, and eco-pharmacovigilance. According to our survey, 88.8% of the participants confirmed that environmental, economic, and social sustainability should be among the priorities of companies.

In this study, we stress the significant role of pharmacists. Pharmacists are the most accessible healthcare professionals and can improve medication adherence and decrease self-medication risk and cost.[29,32,33] Thus, pharmaceutical companies

Table 9. Priorities of the alternative strategies

Strategy	Weight	Ranking/priority
SO1	0.131	4
SO2	**0.161**	**2**
WO1	0.130	6
WO2	**0.163**	**1**
ST1	0.131	5
ST2	**0.160**	**3**
WT1	0.126	7

should liaise with pharmacists to develop marketing strategies that could result in safe and ethically correct results to build consumer awareness.

The pharmaceutical industry has high-quality production capabilities, but not all non-pharmaceutical products are manufactured with high-quality standards. Kotecki[34] found that medical factors, such as the active ingredients of the products, clinical studies, and information obtained from scientific references, are quite effective in supporting pharmacists' OTC product decisions. Therefore, companies should focus on manufacturing quality and evidence-based information in efforts to improve community health and develop a good impression for healthcare professionals.[34]

Unlike in previous SWOT analyses of the OTC market, Dzeparoski et al.[35] underlined qualified personnel as a strength factor and market growth as an opportunity. These results are similar to our findings. An earlier study on the SWOT analyses of traditional Chinese medicine reported government policy support as an opportunity,[36] similar to our results.

The first three strategies highlighted in our research are related to the advantages of utilizing digital technologies. Technology is crucial for innovation and differentiation in this competitive environment.[37] In this context, companies should adopt the rapid development of information and communication technologies to educate all stakeholders. While the internet can be a highly effective channel with which to reach the consumer and, thus, increase their awareness, one must also be aware of the dangers of information pollution. Hence, companies should conduct full diligence to protect the public from information pollution.

Turkish legislation prohibits the sale of prescription and non-prescription drugs via the internet or any other electronic media.[38] Companies should utilize digital communication options by complying with relevant laws and regulations.

Study limitations

The definition of the term "OTC" is ambiguous in Turkey because of the lack of regulation of some drugs and the slow progress of the diversification of sales channels. Thus, our study refers to all products sold in pharmacies, including non-prescription drugs and non-pharmaceutical products but excluding prescription drugs, as OTC products.[7,8]

Our study is limited by its small sample size, which prohibits quantitative analyses. The questionnaire was sent to IEIS members and some experts with similar qualifications via LinkedIn. Only 36 participants completed all of the questions.

The AHP, ANP, and FANP methods used in our study do not require a large number of samples to be statistically significant.[21-23] Additionally, the participants of the survey were experts with deep knowledge and experience on the OTC and pharmaceutical industry. All 36 responders were qualified to provide the required information for this study.

CONCLUSION

Our study is among the first detailed strategic studies to use integrated SWOT and FANP to analyze the Turkish OTC market.

We strongly believe that the transformation of the OTC industry can contribute to the health of society. Companies must allocate a budget for training as part of their marketing activities. The training program should be directed to all stakeholders, such as company professionals, physicians, pharmacists, and consumers.

Companies should also increase the production quality of non-pharmaceutical products and expand their portfolio with innovative products. In addition, they should avoid aggressive and misleading advertising to improve the health literacy of consumers.

Our main finding is that the utilization of digital technologies is within the scope of priority strategies for the OTC market. Our study has clearly shown that all of the best possible strategies (i.e., WO2, SO2, ST2) highlight the importance of investing in digital technologies. The development of social media, websites, microsites, and, in particular, the related content management, are indispensable for utilizing the most appropriate strategies. The widespread utilization of digital technologies in many areas, starting from R&D, the supply chain, and production to marketing and corporate communications, will accelerate the development of OTC companies. The effective use of digital platforms will also contribute to increasing health literacy in various communities and raise awareness of OTC consumption and self-medication among consumers.

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ORIGINAL ARTICLE

Granny Smith Apple Extract Lowers Inflammation and Improves Antioxidant Status in L-arginine-induced Exocrine Pancreatic Dysfunction in Rats

Granny Smith Elma Ekstresi, Sıçanlarda L-arginin Kaynaklı Ekzokrin Pankreas Bozukluğunda İnflamasyonu Azaltır ve Antioksidan Durumunu İyileştirir

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ABSTRACT

Objectives: Granny Smith is a cultivated hybrid variety of apple with a high antioxidant content relative to all other species of apple. Acute pancreatitis (AP) is an instantly emerging inflammatory condition with a high mortality rate. The preferred treatment is restricted to symptomatic relief and supportive care. The present study was undertaken to evaluate the favorable effects of Granny Smith apple extract (GSAE) as a prophylactic treatment for L-arginine-induced AP in rats.

Materials and Methods: Male Sprague Dawley rats were divided in to five groups (n=6): Normal control (saline), disease control (a single dose of L-arginine 2.5 g/kg I.P.), positive control (pelatonin 10 mg/kg I.P.), and GSAE I and II (200 mg/kg and 400 mg/kg, orally, respectively). All groups were treated for 7 days. At the end of the study, blood samples were collected from the retro-orbital plexus, serum separated, and subjected to estimation of biomarker enzymes such as amylase, lipase, antioxidant enzymes, etc. The animals were then sacrificed, and the pancreas was isolated and subjected to estimation of tissue biomarkers, DNA fragmentation assay, and histopathological studies.

Results: Serum levels of amylase and lipase were significantly ($p<0.001$) reduced in L-arginine-treated rats. Similar results were also observed with tissue inflammatory markers such as malondialdehyde, nitrate, etc. There was a dramatic increase ($p<0.001$) in the overall antioxidant enzyme levels when compared with disease control rats. Histopathological examination of pancreatic tissue showed an intact structural feature of acinar cells in the extract-treated group of rats, which was further in pact with the intact DNA found in the DNA fragmentation assay.

Conclusion: Thus, GSAE treatment was found to be beneficial in lowering the inflammatory conditions of AP by improving the overall antioxidant levels, and a further investigation into its exact molecular mechanism is needed.

Key words: Granny Smith apple, L-arginine, free radicals, pancreatitis

ÖZ

Amaç: Granny Smith, diğer elma türlerine göre yüksek antioksidan içeriğine sahip, yetiştirilmiş bir melez elma çeşididir. Akut pankreatit (AP), yüksek ölüm oranına sahip, anında ortaya çıkan bir enflamatuvar süreçtir. Tercih edilen tedavi, semptomatik rahatlama ve destekleyici bakım ile sınırlıdır. Bu çalışma, Granny Smith elma özütünün (GSAE) sıçanlarda L-arginin kaynaklı AP için profilaktik bir tedavi olarak olumlu etkilerini değerlendirmek için yapılmıştır.

Gereç ve Yöntemler: Erkek Sprague Dawley sıçanları beş gruba ayrıldı (n=6): Normal kontrol (salin), hastalık kontrol (tek doz L-arginin 2,5 g/kg I.P.), pozitif kontrol (pelatonin 10 mg/kg I.P.) ve GSAE I ve II (sırasıyla 200 mg/kg ve 400 mg/kg ağızdan). Tüm gruplar 7 gün tedavi edildi. Çalışmanın sonunda, retro-orbital pleksustan kan örnekleri alındı, serum ayrıldı ve amilaz, lipaz, antioksidan enzimler gibi biyobelirteç enzimler analiz edildi. Hayvanlar daha sonra öldürüldü ve pankreas izole edildi. Doku biyobelirteçlerinin analizi, DNA fragmantasyon analizi ve histopatolojik çalışmalara tabi tutulmuştur.

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Bulgular: Serum amilaz ve lipaz seviyeleri, L-arginin ile tedavi edilen sıçanlarda önemli ölçüde azaldı (p<0,001). Malondialdehit, nitrat, vb. gibi doku enflamatuvar belirteçlerinde de benzer sonuçlar gözlendi. Hastalık kontrol sıçanlarına kıyasla genel antioksidan enzim seviyelerinde dramatik bir artış vardı (p<0,001). Pankreas dokusunun histopatolojik incelemesi, DNA fragmantasyon analizinde bulunan sağlam DNA ile daha da anlaşılan, ekstraktla muamele edilmiş sıçan grubundaki asiner hücrelerin sağlam bir yapısal özelliğini gösterdi.

Sonuç: GSAE tedavisinin, genel antioksidan seviyelerini iyileştirerek AP'nin enflamatuvar koşullarını geriletmede faydalı olduğu bulunmuştur ve kesin moleküler mekanizmanın aydınlatılmasına yönelik daha ileri bir araştırmaya ihtiyaç vardır.

Anahtar kelimeler: Granny Smith elma, L-arginin, serbest radikaller, pankreatit

INTRODUCTION

Acute pancreatitis (AP) is a perilous condition, and there has been a spike in its incidence worldwide.[1] AP is a mild, self-limiting inflammatory condition of the exocrine pancreatic tissue caused by activation of stress signals and a disparity of protective mechanisms in the pancreatic tissue. The initial symptoms include persistent and recurring epigastric pain, nausea, vomiting, weight loss, fever, chills, and shock, which, if left undiagnosed or untreated, may eventually lead to severe AP, where the pancreatic tissue is displaced by fibrotic cells, and the association of additional organ manifestations leads to mortality.[2]

Almost 80% of reported pancreatitis cases have familial incidence,[3] and the major causes of mortality include cardiovascular and respiratory collapse.[4] No specific therapies have been reported for the management of AP, and treatment is primarily based on supportive care to prevent hypoxemia, fluid resuscitation, and a critical component of the disease, malnutrition.

Despite many explorations in regard with pathogenesis and the treatment modalities developed, the pathogenesis of AP still remains vague. The most widely accepted hypothesis is the involvement of oxidative stress and premature activation of zymogens, followed by autodigestion of the tissue and successive activation of local and systemic inflammation.[5] The autodigestive process of acinar cells stimulates an inflammatory response (neutrophil and macrophage infiltration, release of cytokines, interleukins 1, 6, and 8, and other inflammatory mediators) within the pancreatic parenchyma.[2]

Based upon the above hypothesis, a number of experimental animal studies have proposed the administration of antioxidant compounds that deplete reactive oxygen species and show a beneficial effect in the treatment of AP.[6] Many medicinal plants have yielded favorable outcomes in the management of the disease course.[7] Suggested preventive mechanisms include the presence of phenolics and other phytochemical antioxidants in the eradication and neutralization of free radicals.[8]

Several epidemiological studies suggest that increased dietary intake of fruits and vegetables is associated with a decreased risk of disease incidence.[9] One of the well-known and most produced fruits worldwide is the apple (*Malus x domesticus*). Apples play a significant role in the dietand are consumed in various forms. They belong to the family *Rosaceae* and contain high amounts of fiber, pectin, potassium, and vitamins A and C. They also contain different classes of phenolics such as flavonols, dihydro chalcones, p-hydroxy benzoic acid, etc. Many different varieties of apples are available, and the focus of our study is on Granny Smith apples.

The Granny Smith apple is a hybrid variety of *Malus domesticus* and *Malus slyvesterus* propagated by Maria Smith in Australia, hence the name. It is reported to have the second highest amount of flavonoids and procyanidins[10] among all the varieties of apples. The fruits are crisp, tart, and juicy with a light green, hard skin and a long storage life. The low amount of ethylene production facilitates their preservation, when compared with other apple varieties.[11] Granny Smith apple is the preferred fruit in healthy weight loss regimens because of their wealth of dietary fiber and potassium and low caloric content.[12] The objective of the current study is to evaluate the shielding potential of Granny Smith apple extract (GSAE) on experimentally induced AP by L-arginine and its potential to overcome malnutrition.

MATERIALS AND METHODS

Chemicals

Sodium phosphate buffer, sodium chloride, starch, chloroform, ethanol, O-dianisidine, phosphate buffer, ethanol, hydrogen peroxide, potassium dihydrogen phosphate, trichloro acetic acid (TCA), dinitro phenyl hydrazine (DNPH) reagent, butanol, ethylene diamine tetra acetic acid (EDTA), pyridine, potassium chloride, sodium dodecyl sulfate, thiobarbituric acid (TBA), 5,5-dithio bis 2-nitrobenzoic acid (DTNB), thiourea, N-(1-naphthyl) ethylene diamide dihydrochloride, and formaldehyde, were purchased from Sigma-Aldrich Inc., Mumbai, India. β-NADH, hexadecyltrimethylammonium bromide (HTAB), and sodium pyruvate were purchased from SD Fine Ltd. Mumbai, India. Commercial kits for estimation of amylase, C-reactive protein from Akray health care Pvt Ltd., Hyderabad, India and Lipase kit from Aggape diagnostics Pvt Ltd. Hyderabad, India. DNA isolation kit from Bioartis Pvt Ltd. Hyderabad, India. L-arginine and melatonin were purchased from Sigma-Aldrich Pvt Ltd. Mumbai, India.

Plant material and extraction

Fresh Granny Smith apples were purchased in the month of March 2017 from the local market in Hyderabad, India. The samples were authenticated for their variety with voucher no.1002 by the Department of Botany, S.V. University, Andhra Pradesh, India. The core of the apple was removed, and the rest of the apple was blended to obtain fresh juice with pulp. The obtained pulp was subjected to extraction with 95% ethanol in an orbital shaker for 24 h. The extracts were filtered and

evaporated to dryness with the help of a rotary evaporator. The resultant dry extracts were stored at a cool temperature and resuspended in normal saline immediately before the use.

Animals

In the present study, male Sprague Dawley rats were chosen with a body weight range of 150-200 g. They were housed in pathogen-free polypropylene cages and acclimated for one week before the onset of the study. The temperature was maintained at 25˚C±1˚C with 55% humidity. Rats were controlled constantly with a 12:12 h light/dark cycle and were givena standard pellet diet with water *ad libitum*. All the experiments carried out were approved by the Institutional Animal Ethics Committee (1448/PO/Re/S/11/CPCSEA/07/2016) as per CPCSEA guidelines.

Acute toxicity studies

The acute toxicity study was conducted according to the fixed dose method of OECD guideline 420.[13] GSAE was dissolved in normal saline and administered orally as per the prescribed doses mentioned in the guidelines, and observations were conducted for the next 14 days. Animals given the test extracts did not manifest any significant abnormal signs, behavioral changes, changes in body weight or macroscopic findings at any time during the observation period. At the end of the study period, no mortality or lethality was observed, and hence, the LD50 was found to be above 2000 mg/kg. Thus 1/10th and 1/5th of the LD50 dose was selected for the present study.

Preparation of L-arginine solution

A 20% L-arginine solution was prepared in normal saline, and the pH was adjusted to 7.0. The solution was filtered through a syringe tube filter in a tissue culture hood before administration to rats at a dose of 2.5 g/kg body weight.[14] The prepared solution was used for the induction of AP and was administered intraperitoneally.

Study protocol

The present study involved randomization of animals in to five groups with six animals in each. Group I was considered as the control group and received normal saline p.o. daily for 7 days. Group II, the disease control group, received a single dose of freshly prepared L-arginine solution at a dose level of 2.5 g/kg b.w. on day 5 of the experiment. Group III, a positive-control group, received melatonin at a dose of 10 mg/kg I.P. for 7 days.[15,16] Groups IV and V received oral administration of GSAE at doses of 200 mg/kg and 400 mg/kg, respectively. Groups III, IV, and V were induced with AP by single-dose administration of L-arginine (2.5 g/kg b.w.) at an interval of 1 h after administration of the extracts on day 5 of the study.[17]

At the end of the experimental period, the animals were anesthetized under light ether, and blood samples were obtained from the retro-orbital plexus. Further, these samples were used for evaluation of pancreatic, inflammatory, and antioxidant biomarker enzymes. The rats were then decapitated for isolation of the pancreas. Isolated pancreas were divided into portions for tissue inflammatory marker evaluation, DNA fragmentation assays, and histopathological investigations.

Estimation of biomarker enzymes

Estimation of amylase

Serum amylase was estimated with a commercial kit manufactured by Akray Healthcare Pvt. Ltd. Briefly, 1000 µL of amylase mono reagent was mixed with 20 µL of serum and incubated for 60 seconds. The absorbance was read at 405 nm. Amylase activity was reported in U/L, where one unit was described as the amount of amylase required to generate 1 µ mole of p-nitrophenol per minute at 25˚C.

Estimation of lipase

Serum lipase was analyzed by using the commercial kit from Aggape Diagnostics Ltd. According to the manufacturer's instructions, 1000 µL of reagent 1 was mixed with 20 µL of serum and incubated at 37˚C for 5 minutes. To this mixture, 250 µL of reagent 2 was added and held at room temperature for 2 minutes. The absorbance of the resultant mixture was read at 580 nm. Lipase activity was reported in U/L, where one unit was described as the amount of lipase required to generate 1 µ mole of methyl resorufin at 37˚C.

Estimation of superoxide dismutase (SOD)

Cold water and a chloroform/ethanol mixture (15:1 ratio) were added to an equal quantity of packed cells, and the mixture was centrifuged at 2000 rpm for 20 minutes. A 0.1 mL aliquot of the supernatant was separated and 0.88 mL of riboflavin and 60 µL of O-dianisidine were added. The absorbance was measured at 460 nm.[18] The SOD activity was calculated from a standard curve and expressed in mg/protein/min.

Estimation of glutathione

A tissue homogenate was prepared in 0.1 M phosphate buffer. To the homogenate 20% TCA and 1 mM EDTA were added in equal volumes and allowed to stand for 5 min, which was then centrifuged at 2000 rpm for 10 min. 200 µL of the supernatant was separated and 1.5 mL of DTNB reagent was added. The absorbance was measured at 412 nm.[19] The values obtained were articulated as mg/dL which was calculated against a standard curve and the amount of glutathione reduced is directly proportional to the production of 5-thio 2-nitrobenzoic acid from DTNB.

Estimation of catalase

To 0.1 mL of serum, 2.5 mL of phosphate buffer was added, and the mixture was incubated for 30 min at 25˚C. The blend was transferred into a cuvette, and 650 µL of hydrogen peroxide solution was added to initiate the reaction. The alteration in absorbance was measured at 240 nm for 3 min.[20] The catalase activity was expressed in µmoL H_2O_2/mg protein/minute, which was calculated against the total amount of protein lysed by the enzyme to degrade 1 µmol hydrogen peroxide per minute.

Estimation of vitamin C

The blood plasma was separated, and 0.6% TCA was added to a volume of 0.5 mL plasma to bring the total volume to 1.5 mL. The mixture was centrifuged for 20 min at 3500 rpm. The clear supernatant was collected, and to it an equal volume of

DNPH reagent (2% DNPH and 4% thiourea in 9N H_2SO_4) was added and incubated for 30 min at room temperature. The resultant mixture was then read at an absorbance of 530 nm.[21] The amount of vitamin C was calculated from a standard curve obtained by taking ascorbic acid as the reference standard and was expressed in mg/dL.

Estimation of lactate dehydrogenase (LDH)

To 0.1 mL of tissue homogenate or serum, 3 mL of LDH reagent (2.8 mL of 0.13 mM β-NADH and 0.1 mL of 34 mM sodium pyruvate) was added and incubated at 37°C for 5 minutes. The absorbance of the mixture was tested at 340 nm every minute for 3 minutes. The ΔA activity was measured and represented as U/L.[22] The LDH activity was expressed as U/L where one unit was articulated as the reduction of 1 μ mole of pyruvate to L-lactate in 1 minute at 37°C and pH 7.5.

Estimation of myeloperoxidase (MPO)

Pancreatic MPO was estimated according to Bradley et al.[23] The homogenized tissue was suspended in a mixture containing 50 mM phosphate buffer (pH 6.0) containing 0.5% HTAB and sonicated in an ice bath for 10 minutes. The suspensions were freeze-thawed three times, and the resultant mixture was centrifuged at 40,000x g for 15 min. The pellet thus obtained was mixed with 2.9 mL of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/mL of O-dianisidine hydrochloride and 0.0005% of hydrogen peroxide and checked for absorbance at 460 nm. One unit of MPO was defined as the quantity that degraded 1 μ/mol of H_2O_2 per minute, and the activity was expressed as units/mg of protein.[23]

Estimation of malondialdehyde (MDA)

The levels of MDA were estimated using 0.4 mL of tissue homogenate to which a reaction mixture of 1.5 mL, containing TBA (0.8%), acetic acid (20%), and distilled water was added and incubated for 1 h at 95°C in a water bath. Following incubation, the mixture was cooled, and to it 5 mL of butanol: pyridine mixture (15:1) was added and centrifuged at 3.000 rpm for 10 minutes. The clear supernatant was collected and checked for absorbance at 532 nm against a blank containing a butanol:pyridine mixture. The quantity of MDA was calculated by a standard graph preparation using 1,1,3,3-tetramethoxypropane in the concentration range of 1-10 nmol in 1 mL distilled water. The results are articulated as nmol of MDA/mg protein.[24]

Tissue nitrite levels

The tissue nitrite levels were estimated according to Green et al.[25] with slight modifications. Briefly, the homogenized pancreatic tissue was centrifuged at 11,000 g for 15 min at 4°C. The obtained supernatant (100 μL) was mixed with 100 μL of Griess reagent and incubated at room temperature for 10 min. The absorbance was checked at 540 nm. Nitrite levels were standardized with using sodium nitrite, and the results obtained were expressed as micromoles of nitrate/nitrite.

Estimation of C-reactive protein (CRP)

Serum CRP levels were estimated by using a commercial kit from Akray Healthcare Ltd. Quantitative estimation was performed by preparing a series of dilutions of the test serum in normal saline (e.g., 1:2, 1:4, 1:8, etc.), to which one drop of CRP latex reagent was added. The formation of agglutination on the glass slide was taken as the highest titer for CRP and represented as a factor of 6 with units in micrograms per milliliter.

DNA fragmentation assay

The DNA fragmentation assay was performed according to the method of Basnakian and James[26] using agarose gel electrophoresis. Briefly, DNA from pancreatic tissue was isolated by using a commercial available DNA Isolation kit by Bioartis Pvt Ltd. The isolated DNA pellet was air dried and resuspended in Tris-EDTA buffer (pH 8.0) containing 1mM EDTA. The resuspended DNA was loaded on to the agarose gel electrophoresis for analysis.

Histopathological studies

The isolated pancreases were fixed in formal in and subjected to histopathological studies. The pancreatic tissues were washed and fixed in paraffin blocks, which were sliced in 5-μm sections and stained with hematoxylin and eosin and then evaluated under a microscope with dark field background changes in the pancreatic tissue. Pancreatic tissue injury was reviewed for degeneration of acinar cells, edema, interstitial inflammation, and hemorrhage.

Statistical analysis

All the results are articulated as mean ± standard error of mean. Statistical analysis of the data was performed by One-Way ANOVA followed by Dunett's multiple comparison test using GraphPad Prism 5 software with the threshold for statistical significance at $p<0.001$.

RESULTS

Effect of GSAE on AP-induced enzyme production

To examine the effect of GSAE on the development and severity of AP, rats were pretreated with GSAE (200 mg/kg and 400 mg/kg) in the respective groups as described in the experimental design. A few hours after induction of AP, rats in the control and positive-control groups were still active; whereas rats in disease control group were lethargic, with decreased motor activity, reduced reflex action, and reduced intake of food and water.

The rats in disease control group showed a significant increase ($p<0.001$) in the levels of amylase when compared with the control group rats. Rats treated with melatonin showed marked reduced levels of serum amylase, revealing its protective action. Rats pretreated with GSAE (200 mg/kg and 400 mg/kg b.w.) showed a significant dose-dependent decrease in serum amylase levels ($p<0.001$) when compared with the L-arginine control group rats (Table 1).

Similar changes were also observed for serum lipase levels. Rats of the disease control group showed a significant increase ($p<0.001$) in lipase levels when compared with those of the control group; whereas the melatonin and GSAE-

treated rats showed a dose-dependent significant decrease ($p<0.001$) in the lipase enzyme levels when compared with those of the L-arginine control group (Table 1).

Effect of GSAE on antioxidant enzymes in AP

There was a significant decrease ($p<0.001$) in the SOD, catalase, glutathione, and vitamin C levels in rats of the disease control group when compared with those of the control group. In contrast, the levels were significantly increased in rats treated with melatonin and in the GSAE group of rats ($p<0.001$) when compared with rats of the disease control group, and no significant change was observed in GSAE group rats when compared with control group rats (Table 2).

Table 1. Changes in the pancreatic biomarkers in rats treated with L-arginine and GSAE

Group	Amylase (IU/L)	Lipase (U/L)
Normal control	14.17±0.12#	16.17±0.98#
Disease control	330.5±3.23*	83.17±1.14*
Positive control (melatonin 10 mg/kg)	98.8±2.24*#	34.45±1.46*#
GSAE I (200 mg/kg)	144±1.57*#	44.5±1.21*#
GSAE II (400 mg/kg)	83.17±1.54*#	37.5±0.56*#

Values are expressed in mean ± SEM (n=6); *,#$p<0.001$ when compared with the normal control and disease control group, respectively. GSAE: Granny Smith apple extract, SEM: Standard error of mean

Effect of GSAE on other inflammatory mediators in AP

Treatment with L-arginine increased the levels of nitrate, MDA, LDH, CRP, and MPO significantly ($p<0.001$) in comparison with positive control and control group rats, indicating the incidence of pancreatic damage and inflammation. In contrast, melatonin and GSAE treatment reduced the levels of nitrate, MDA, LDH, CRP, and MPO significantly ($p<0.001$) in a dose-dependent manner. However, the levels were higher in the GSAE group of rats when compared with the control group (Table 3).

Histopathological studies

The isolated pancreas was subjected to histopathological study using hematoxylin and eosin staining. Normal pancreatic architecture was seen in the control group of rats (Figure 1A), whereas L-arginine treatment showed inflammatory changes with vacuolar degeneration and extensive damage to acinar cells (Figure 1B). The positive-control group treated with melatonin showed normal architecture (Figure 1C). Rats treated with GSAE showed reduced inflammatory changes with no degeneration and maintenance of normal structural design (Figure 1D, E).

DNA fragmentation assay

One of the major criteria for DNA fragmentation apoptosis. In the present study, control group rats showed intact DNA (Figure 2A) when compared with L-arginine control group rats, where a smear pattern was observed, representing extensive damage to DNA (Figure 2B). The melatonin and GSAE-treated groups of rats showed intact DNA, indicating their protective effects against DNA damage (Figure 2C-E).

Table 2. Changes in the antioxidant biomarkers in rats treated with L-arginine and GSAE

Groups	SOD (mg/protein/min)	Catalase (μM of H_2O_2/mg/protein/minute)	Glutathione (mg/dL)	Vitamin C (mg/dL)
Normal control	18.5±0.43#	48.18±0.31#	2236±0.96#	95.73±0.35#
Disease control	6.167±0.31*	22.7±0.29*	575.8±1.50*	21.35±0.58*
Positive control (melatonin 10 mg/kg)	61.23±1.72*#	47.14±0.23#	3124±3.55*#	212.34±0.68*#
GSAE I (200 mg/kg)	31.5±0.34*#	42.0±0.15#	2304±0.88*#	212.7±0.70*#
GSAE II (400 mg/kg)	42.5±0.43*#	47.48±1.16#	2999±0.79*#	228.8±1.81*#

Values are expressed in mean ± SEM (n=6); *,#$p<0.001$ when compared with the normal control and disease control groups, respectively. GSAE: Granny Smith apple extract, SEM: Standard error of mean, SOD: Superoxide dismutase

Table 3. Changes in inflammatory mediators in rats treated with L-arginine and GSAE

Groups	MDA (mM/dL/h)	Nitrate (μM/g)	CRP (μg/mL)	MPO (μM of peroxide/min)	LDH (U/L)
Normal control	14.89±0.34#	11.08±0.21#	486.8±2.37#	4.032±0.06#	23.4±0.54#
Disease control	86.87±0.88*	35.05±0.15*	19601±2.46*	25.69±0.33*	127.5±0.39*
Positive control (melatonin 10 mg/kg)	35.56±0.99*#	14.34±0.88#	697±4.35*#	6.45±0.46#	45.56±0.90*#
GSAE I (200 mg/kg)	37.3±0.20*#	17.14±0.19#	1258±3.57*#	10.44±0.37#	52.57±0.67*#
GSAE II (400 mg/kg)	24.77±0.36*#	13.67±0.12#	1021±2.97*#	7.902±0.28#	43.73±1.28*#

Values are expressed in mean ± SEM (n=6); *,#$p<0.001$ when compared with normal control and disease control group, respectively. GSAE: Granny Smith apple extract, SEM: Standard error of mean, MDA: Malondialdehyde, CRP: C-reactive protein, MPO: Myeloperoxidase, LDH: Lactate dehydrogenase

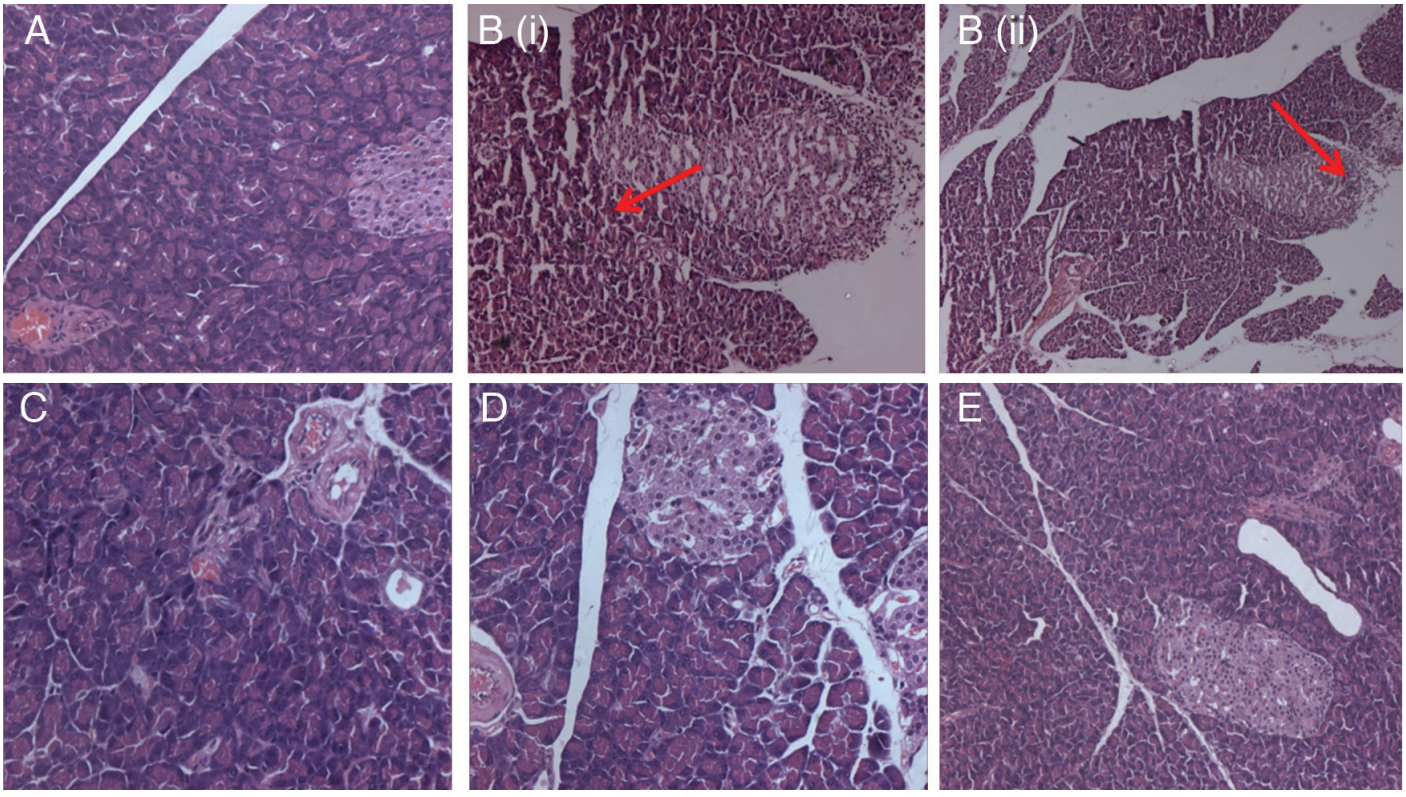

Figure 1. Histopathological changes in the pancreas. A) Normal control group rats with no damage to acinar cells; B (i) and B (ii) disease control group: L-arginine treated rats with vacuolar degeneration and extensive damage of the acinar cells with infiltration of leucocytes-red arrow; C) positive-control melatonin group of rats with normal architecture of acinar cells; D, E) GSAE-treated rats showing mild damage to the normal echotexture of acinar cells

GSAE: Granny Smith apple extract

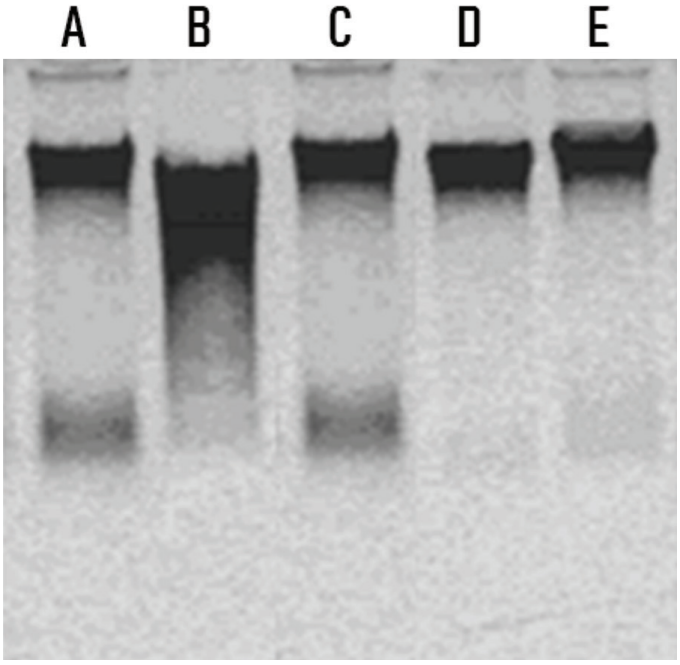

Figure 2. Gel image of DNA fragmentation assay of the pancreatic tissue. A) Group I: Normal control pancreas; B) group II: Disease control group pancreas showing fragmented DNA in the form of a smear indicating extensive damage; C) group III: Positive-control melatonin-treated group showing intact DNA; D, E) group IV, V: GSAE-treated pancreas showing intact DNA

GSAE: Granny Smith apple extract

DISCUSSION

The results of the study indicate a protective effect of GSAE on experimentally induced AP in rats by L-arginine. Administration of GSAE exhibited beneficial effects by reducing oxidative and nitrosative stress and modulating the inflammatory process.

L-arginine, an essential amino acid, was used for induction of AP in the present study, which is reported as a highly reproducible, non-invasive model of AP that produces dose-dependent acinar necrosis.[27] Further, incessant administration of L-arginine for an extended period may also induce chronic pancreatitis.[28]

The chief indicative markers for the diagnosis of AP include serum amylase and lipase due to their direct release into the circulation, which is accredited to enzymatic activation in pancreatic acinar cells. After the initial attack, the levels of these enzymes usually increase within 4-8 h and peak at 24 h.[29] Increased serum lipase levels are considered as a more reliable marker than those of amylase.[30] In the present study, rats treated with L-arginine showed a significant elevation of serum amylase and lipase levels, which was observed as acinar cell necrosis (devoid of changes in the Islets of Langerhans) as observed in the histopathological study, indicating the development of AP, which is in accordance with previous reports.[31] By comparison, treatment with GSAE decreased the elevated levels of lipase and amylase significantly in a dose-dependent manner. The reduction of enzyme levels by GSAE is

consistent with a previously reported *in vitro* study on inhibition of lipase, α-amylase, and α-glucosidase enzymes.[30,32] The results of the GSAE extract groups are synchronous with that of the positive-control group treated with melatonin, which also reduced the elevated levels of amylase and lipase radically, in accordance with previous reports.[31]

The pathogenesis of AP implicated the generation of free radicals and activation of inflammatory mediators, which contributed to the unfavorable effects.[33] Lipid peroxidation provoked by oxidative stress and altered glutathione metabolism has been reported to take place early in the disease course. The rate of MDA production directly signifies lipid membrane peroxidation, which indirectly reflects the association of free-radical generation in mitochondria.[34] On the other hand, MPO in the circulation indicates its release from activated neutrophils portraying dominant proinflammatory properties.[35] Additionally, the severity and stage of AP show a relationship with the levels of blood MPO. Further, MPO levels correlate with CRP levels. Many studies have reported that these events were neutralized by administration of antioxidants with a favorable effect on reduction of reactive oxygen species.[36] Melatonin, a renowned antioxidant, lowered the levels of MDA, MPO, and CRP, in agreement with previous studies.[31] Similar effects were observed to that of GSAE administration, where the elevated levels of MDA, MPO, and CRP were reduced dose dependently, owing to the eradication of reactive oxygen species and halting of the lipid peroxidation process and inflammatory cytokine release, which has also been observed as a decreased amount of acinar cell damage in histopathological studies of the pancreatic tissue. The Granny Smith apple stands out as an antioxidant-rich fruit among all apple varieties. The presence of two major metabolite compounds, rutin and catechin, streng then the antioxidative properties of the fruit.[37] The presence of these flavonoid compounds along with other compounds such as quercetin and procyanidins contribute to the total antioxidant capacity of many plant products.[38,39] Further, Granny Smith peels have demonstrated antioxidant activity in the total oxy-radical scavenging assay and have shown the capacity to inhibit HepG2 cell proliferation.[40]

The presence of LDH has been reported in tissues under hypoxia conditions, which is a major event in inflammatory processes.[41] Increased LDH levels were observed in the L-arginine group of rats, and the opposite was observed in melatonin-and GSAE-treated rats in a dose-dependent manner.

Damage by oxidative and nitrosative stress is mitigated by antioxidant resistance enzymes like SOD, glutathione, catalase, and vitamin C. L-arginine-treated rats showed decreased levels of SOD, glutathione, catalase, and vitamin C, indicating the involvement of reactive oxygen species.[42] Melatonin and GSAE administration increased the levels of SOD, glutathione, catalase, and vitamin C, suggesting that it can exert protective effects by modulating defense mechanisms. The favorable effects of GSAE could be due to its antioxidant activity, which is in accordance with the *in vitro* antioxidant capacity reported by Tzanakis et al.[43] and Saxena et al.[44] Additionally, there was an increase in the levels of nitrite, indicating the involvement of released inducible nitric oxide synthase (iNOS) from inflammatory cytokines, i.e., the release of iNOS is directly proportional to nitrite levels in the plasma. NO and its metabolic products have a key role in inflammatory processes.[45] NO combines with superoxide to form peroxynitrite, a highly reactive oxidant that damages the cell by lipid membrane and sulfhydryl oxidation.[46] Administration of antioxidants decreased the release of NO indirectly, indicating its beneficial effect on nitrosative stress.[36] GSAE administration reduced the levels of nitrite dose dependently. Granny smith is one of the varieties of apple containing richest polyphenols. Procyanidin B2, catechin, flavanols, quercetin, and vitamin C have been isolated from the whole fruit.[47] The antioxidant activity of Granny Smith apples has been ascribed to the presence of these polyphenolic compounds.[47] Also, Lotito and Frei[48] reported on the antioxidant capacity of apple polyphenolics in human plasma along with their favorable effects on the prevention of many diseases. These facts suggest a role for the eradication of reactive oxygen and nitrogen species in the protection against AP. Furthermore, phenolics have been reported to restrain the NO-induced proinflammatory reaction by obstructing the levels of expression of iNOS dose dependently.[49]

Accumulating evidence suggests that apoptosis plays a significant role in relation to the severity of AP. The balance between apoptosis and necrosis plays a key role in defense mechanisms against AP and the resolution of disease severity.[50] DNA fragmentation is considered as the universal criterion for the detection of apoptosis.[51] Mervi et al.[52] suggested that inhibition of polyamine synthesis has an effect on protein synthesis; in turn, nucleic acid synthesis is restricted. Further, catabolism of proteins is highly active in acinar cells of the pancreas, and it is probable that the overdose of L-arginine induced necrosis or degradation in these cells initially. Mitochondrial damage initiates the process of apoptosis as a consequence of high calcium loads. The cellular damage in pancreatitis is mostly associated with damage to mitochondria along with succession of the disease in most animal models and in Humans,[53] which is in accordance with the results of the current study, where smear pattern of DNA was observed in L-arginine-treated disease control rats. Oxidative stress and protein oxidation in the cells could cause abnormal cross linking and cleavage of DNA leading to death of the cell.[54] Acinar cells from the pancreas of rats treated with melatonin and GSAE showed intact DNA, signifying protective effects against stress-mediated DNA damage.

CONCLUSION

The current study suggests that GSAE exerts beneficial effects on L-arginine-induced AP by eradicating stress markers and augmenting antioxidant status. Chemical constituents such as polyphenols and flavonoid compounds present in the Granny Smith apple are responsible for its favorable effects. Further investigations are required to evaluate the exact chemical constituents and their molecular mechanism of action on the disease profile.

The present study was carried out to evaluate the prophylactic action of GSAE on AP. The amount of damage to the pancreas with L-arginine administration was evidenced with increased levels of amylase and lipase and reduction in the levels of antioxidative markers with increased levels of inflammatory markers. The histopathological findings with acinar cell necrosis were also in sync with the biochemical markers. The administration of GSAE prophylactically reversed the diseased conditions by improving antioxidant status and maintaining the normal echo texture of the pancreatic acinar cells. The shielding effect was rational in a dose-dependent manner. Further investigation of its molecular mechanism with extraction of each constituent is needed to determine the precise therapeutic potential of GSAE.

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ORIGINAL ARTICLE

Cardioprotective Effect of *Marsdenia tenacissima* and *Sansevieria roxburghiana* in Doxorubicin-induced Cardiotoxicity in Rats *in vivo*: The Role of Dresgenin and Lupeol

Sıçanlarda Doksorubisin Kaynaklı Kardiyotoksisitede *Marsdenia tenacissima* ve *Sansevieria roxburghiana*'nın *in vivo* Kardiyoprotektif Etkisi: Dresgenin ve Lupeol'ün Rolü

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ABSTRACT

Objectives: The major adverse effect of doxorubicin (DOX) in cancer treatment is cardiac toxicity. Murva is a controversial plant used in the Ayurvedic system, which consist of more than 12 medicinal plant roots found in different parts of India. *Marsdenia tenacissima* (MT) is an acceptable source in Murva, whereas *Sansevieria roxburghiana* (SR) Schult & Schult.f. (*S. zeylanica* Roxb.) are also considered as Murva in West Bengal, India. The present study focused on the evaluation of the cardioprotective mechanism as well as the *in vivo* cardioprotective potential of methanol extracts of MT and SR on rats by using *in silico* methods.

Materials and Methods: A total of 48 rats were divided into 8 groups with 6 in each group. DOX 20 mg/kg, intraperitoneally (i.p.) was administered to all rats on the 13th day, with the exception of group 1. Group 2 was the disease control, group 3 was the treated with the standard drug propronolol, and groups 4 to 5 were treated with two lower doses of methanol extract of MT (MEMT) and methanol extract of SR (MESR), whereas group 7 received higher dose combinations of both extracts for 14 continuous days. Blood and tissue antioxidant levels as well as cardiac enzymes were measured at the end of the study. Damage to cellular functional units was analyzed by histopathological study. Dresgenin from MT similarly lupeol from SR were taken as ligands for the target peroxisome proliferator activated receptors (PPARα) protein to find out the mechanism of action. High-performance thin layer chromatography (HPTLC) fingerprinting was performed to determine the number of phytoconstituents present in both extracts.

Results: The combination that showed the most significant ($p<0.001$) effect on altered cardiac enzymes and antioxidant enzyme levels in both blood and tissues also corrected the extreme damage in cellular functional units. Dresgenin and lupeol showed binding scores of -8.2 (kcal/mol) and -9 (kcal/mol), respectively, with PPARα. HPTLC reports revealed that 17 and 12 peaks were found at 254 nm for dresgenin and lupeol, respectively.

Conclusion: The study results concluded that the combination of MESR and MEMT and that of MESR and MEMT exerted cardioprotective activity via binding of dresgenin and lupeol to PPARα. The order of efficacy was the extract combination > MESR > MEMT.

Key words: Doxorubicin, cardioprotective, molecular docking, cardiac enzyme, HPTLC

ÖZ

Amaç: Doksorubisinin (DOX) kanser tedavisinde en önemli yan etkisi kardiyak toksisitedir. Murva, Hindistan'ın farklı bölgelerinde bulunan 12'den fazla tıbbi bitki kökünden oluşan Ayurvedik sistemde kullanılan tartışmalı bir bitkidir. *Marsdenia tenacissima* (MT), Murva'da kabul edilebilir bir kaynak iken, *Sansevieria roxburghiana* (SR) Schult & Schult.f. (*S. zeylanica* Roxb.) da Batı Bengal, Hindistan'da Murva olarak kabul edilmektedir. Bu çalışmada, MT ve SR metanol ekstraktlarının *in siliko* yöntemler kullanılarak *in vivo* kardiyoprotektif potansiyelinin yanı sıra kardiyoprotektif mekanizmanın değerlendirilmesi amaçlanmıştır.

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Gereç ve Yöntemler: Her grupta 6 tane olmak üzere toplam 48 sıçan 8 gruba ayrıldı. DOX 20 mg/kg intraperitoneal (i.p.) olarak grup 1 hariç tüm sıçanlara 13. günde uygulandı. Grup 2 hastalık kontrolü, grup 3 standart ilaç propronolol ve 4'ten 5'e kadar olan gruplar, iki farklı düşük dozda MT metanol özütü (MEMT) ve SR metanol özütü (MESR) ile tedavi edilirken, grup 6, 14 ardışık gün boyunca her iki özütün daha yüksek doz kombinasyonlarını aldı. Çalışmanın sonunda kan ve doku antioksidan seviyeleri ile kardiyak enzimler ölçüldü. Hücresel fonksiyonel birimlere verilen hasar histopatolojik çalışma ile değerlendirildi.

MT'den Dresgenin benzer şekilde SR'den elde edilen lupeol, etki mekanizmasını bulmak için hedef peroksizom proliferatör aktive reseptörler (PPARα) proteini için ligand olarak alındı. Her iki ekstraktta bulunan bitki bileşenlerinin sayısını belirlemek için yüksek performanslı ince tabaka kromatografisi (HPTLC) parmak izi yapıldı.

Bulgular: Hem kanda hem de dokularda değişen kardiyak enzimler ve antioksidan enzim düzeyleri üzerinde en önemli etkiyi ($p<0,001$) gösteren kombinasyon, hücresel fonksiyonel birimlerdeki aşırı hasarı da düzeltti. Dresgenin ve lupeol, PPARα ile sırasıyla -8,2 (kcal/mol) ve -9 (kcal/mol) bağlanma skorları gösterdi. HPTLC raporları, sırasıyla dresgenin ve lupeol için 254 nm'de 17 ve 12 pik bulunduğunu gösterdi.

Sonuç: Çalışma sonunda, MESR ve MEMT ile MESR ve MEMT kombinasyonunun, dresgenin ve lupeolün PPARα'ya bağlanması yoluyla kardiyoprotektif aktivite sergilediği sonucuna varmıştır. Etkinlik sırası özüt kombinasyonu > MESR > MEMT idi.

Anahtar kelimeler: Doksorubisin, kardiyoprotektif, moleküler yerleştirme, kardiyak enzim, HPTLC

INTRODUCTION

Doxorubicin (DOX), an anthracycline antibiotic used for the treatment of various neoplastic disorders, shows severe organ toxicity when used clinically. Cardiotoxicity is the major fatal event that occurs in pediatric and adult patients at the normal therapeutic dose and is characterized by irreversible damage of cardiac muscle leading to a major cause of chemotherapy-associated morbidity and mortality. Even though several less toxic derivatives of DOX are available for chemotherapy, cardiotoxicity induction is taken as the major concern, and the use of traditional anticancer drugs is preferred. Cardioprotective adjuvants such as leucovorin, mesna, angiotensin receptor blockers, and beta blockers are available and are administered along with DOX to reduce cardiotoxicity. These adjuvants exhibit marked cardioprotection and do not compromise the anticancer activity of DOX.[1] Hence, a new strategy was developed in pharmaceutical industries to establish a formulation with extended cardioprotective activity without compromising the cancer chemotherapeutic efficacy of DOX. Under this concept, recently, medicinal herbs and their formulations received greater attention on the treatments of various lives threatening disease because of their efficacy and rapid curative properties. Among the available herbal preparations, Ayurvedic formulations have been placed at the top of the list for many thousands of years due to their low toxicity and wide acceptability.[2] One such Ayurvedic plant is Murva. It is a controversial drug, containing more than 12 medicinal plants roots found in different parts of India. *Marsdenia tenacissima* (MT) is an acceptable source of Murva, whereas *Sansevieria roxburghiana* (SR) Schult and Schult.f. (*S. zeylanica* Roxb.) are considered as Murva in West Bengal, India. The remaining plants in Murva, their scientific names, and their source locations are *Helicteres isora L* (Sterculiaceae) from Punjab, *Maerua arneria* (Capparaceae) from Bihar, *Chonomorpha fragrans* (Apocynaceae) from Kerala, *Clematis triloba* (Ranunculaceae), *Bauhinia tomentosa* (Leguminoseae) and SR Schult & Schult.f. (*S. zeylanica* Roxb.) from West Bengal, *Wattakaka volubilis* (Linn. f.) Stapf (Asclepidaceae) and *Salvadora persica L* (Salvadoraceae) from South India, *Argyreia nerova* (Convolvulaceae), *Maerua oblongifolia* (Capparaceae), and *Dregea volubilis* (Apocynaceae) from other regions of India.[3] Traditionally, Murva is used for the treatment of anemia, diabetes, stomach disorders, typhoid, cough, fever, and urinary tract infections.[4] Based on a review of the literature, MT is traditionally used for heart diseases,[3,5] similarly, SR is used as a cardiotonic.[6] However, the effects of these drugs on the hearts of animals have not been tested experimentally. Therefore, this study aimed to evaluate the cardioprotective effect of extracts of SR and MT on DOX-induced cardiotoxicity in rats.

MATERIALS AND METHODS

Experimental animals

Wistar albino rats (200-250 g) were obtained from the animal housing facility of St. Joseph's College of Pharmacy, Cherthala, Kerala, India and then acclimatized for 1 week under standard controlled conditions (12 hours light/12 hours darkness, at 25°C). The study protocol (SJCP/IAEC/2018-4/35) was approved by the Institutional Animal Ethics Committee (IAEC), St. Joseph's College of Pharmacy Cherthala, Kerala, India.

Plant materials, drugs, and chemicals

Roots and rhizomes of SR and MT were collected from Kerala in the month of October 2018. MT was identified and authenticated by Dr. K. Madhava Chetty, Department of Botany, Sri Venkateswara University Tirupathi A.P. A herbarium specimen was deposited at the Department of Botany, Sri Venkteswara University (voucher number 1132). SR was identified and authenticated by Dr. Jose Mathew, Assistant Professor, Department of Botany, Santana Dharma College, Alappuzha. The herbarium specimen (no: AAM001) was deposited at the Department of Botany, Santana Dharma College, Alappuzha, Kerala, India.

DOX was procured from Dabur Pharmaceuticals Ltd., New Delhi, India. Propranolol was purchased from Cipla Ltd., India. Lactate dehydrogenase (LDH), choline kinase (CK), aspartate transaminase, and other assay kits were purchased from Accurex biomedical Pvt. Ltd., India. All other chemicals used during the study were of analytical grade.

Extraction

The roots of MT and the rhizome and roots of SR were cleaned and dried at room temperature (shade dried). Approximately 300

g of defatted, coarse powered drug was successively extracted in a Soxhlet apparatus with methanol (70-80˚C for 48 hours). Methanol extract of MT (MEMT) and SR (MESR) and aqueous extract of MT and SR were collected with a rotary evaporator followed by drying and storage in an airtight container for experimental purposes.

Molecular docking

The protein selected for the cardioprotective study was peroxisome proliferator activated receptors (PPARα) with PDB ID: 1K7L. Three-dimensional (3D) structures were downloaded from a protein data bank (www.rcsb.org). Protein was prepared by eliminating water and small molecules by using Pymol software. Chemical constituents, such as tenasogenin, cissogenin, tenacigenin-C, tenacigenoside, and dresgenin from MT and 6-methyl-1-octanol, diethyl phthalate, methyl hexadecanoate, 3, 3-dimethylhexanal, and lupeol from SR, were selected as the ligands. PubChem was used to retrieve the 3D structures of ligands in SDF format, and Openbabel 2.3.2 was used to convert them to PDB format. Ligands, important chemical constituents, and targets in PDB format were loaded into autodock vina PyRx. The binding energy with the least RMSD (upper and lower) were selected and expressed in kcal/mol. At the first dock, the pdb.qt files for protein and ligand were prepared.[7,8] The ligands and targets in pdb.qt format were loaded into Pymol for visualization. From the visualization, the number of hydrogen bonds and sequence of amino acids to which the ligand bound were obtained.

Design of the cardioprotective activity study

A total of 48 Wistar rats of both sexes weighing 180-220 g were divided into eight groups containing six animals in each. Standard and test drugs were administered to the respective groups of animals once daily for 14 consecutive days.[9-11] Group 1, the normal control, was treated with distilled water orally. Group 2 served as the disease control and received DOX 20 mg/kg intraperitoneally (i.p.) only. Group 3 received the standard propranolol 10 mg/kg orally and DOX 20 mg/kg, i.p. on the 13th day. Groups 4 and 5 received 100 mg/kg and 200 mg/kg of MEMT orally and DOX 20 mg/kg i.p. on the 13th day. Groups 6 and 7 received 50 mg/kg and 100 mg/kg MESR orally and DOX 20 mg/kg, i.p. on the 13th day. Group 8 received the combination of 100 mg/kg of MESR and 200 mg/kg MEMT orally and DOX 20 mg/kg i.p. on the 13th day. All animals were challenged by using single-dose administration of DOX 20 mg/kg i.p. on the 13th day, except group 1 animals. After 48 hours of DOX administration, blood was collected, and animals were sacrificed for isolation of the vital organs such as the liver, kidney, and heart for histopathological studies. Blood and liver antioxidant markers such as superoxide dismutase (SOD), reduced glutathione (GSH), and malondialdehyde (MDA) levels and cardiac enzymes such as CK-MB, and LDH 1 were estimated with Accurex biomedical Pvt. Ltd., India using a semi autoanalyzer.

Histopathology study

Organs such as the heart, liver, and kidneys were isolated immediately after the animal was sacrificed, washed with ice-cold normal saline, trimmed, and placed in 10% formaldehyde. The organs were sectioned and stained with haematoxylin and eosin. The structures were examined under a light microscope at 10X and 40X magnification by a pathologist blinded to the groups under study.[12]

Estimation of tissue antioxidant levels

Isolated hearts were divided in to two portions for the preparation of homogenates: 10% (w/v) homogenate in potassium chloride (0.15 M) and 10% (w/v) homogenate in 0.25% (w/v) sucrose in phosphate buffer (5 M pH 7.4). Both homogenates were centrifuged at 8000x g for 10 minutes. The supernatant from the first homogenate was used for the estimation of MDA, and the supernatant from the second homogenate was used for the estimation of SOD and GSH.[13] All estimations were conducted according to the manufacturer's manual of reagents by Accurex biomedical Pvt. Ltd., India.

High-performance thin layer chromatography HPTLC analysis

HPTLC fingerprinting analysis was performed with CAMAG LINOMAT 5, where 2 µL of sample was applied by using a Hamilton syringe on a $60F_{254}$ TLC plate as a band length of 5 mm. Later, it was kept in a TLC developing chamber, which was saturated with solvent vapor (mobile phase) of toluene: ethyl acetate: methanol (7:3:1). The plate was then dried with hot air, placed in a photo documentation chamber, and scanned at 254 nm, 366 nm, and 550 nm, following derivatization with anisaldehyde-sulfuric acid reagent.[14]

Statistical analysis

In vivo data were expressed as the mean ± standard error of the mean of six values. The difference between experimental groups was compared with the negative control and normal control by One-Way ANOVA followed by Newman-Keul's multiple comparison test, where $p<0.05$ implied significance.

RESULTS

Docking scores, binding energies, hydrogen bonds, and binding sites were obtained from the various isolated chemical constituents of MT and SR used as ligands for the PPARα receptor, and the results are presented in Table 1, 2. Dresgenin from MT and lupeol from SR showed higher docking scores of -8.2 (kcal/mol) and -9.1 (kcal/mol) respectively. Visualization of dresgenin with the PPARα receptor is shown in Figure 1, in which three hydrogen bonds were found at the 213ALA, 231GLY, and 216LYS positions. Similarly, visualization of lupeol on the PPARα receptor is shown in Figure 2, in which two hydrogen bonds were seen at the 214 TYR and 213 ALA positions.

Table 3 illustrates the effect of MEMT, MESR, and the combination of MEMT and MESR on cardiac enzymes of DOX-induced cardiotoxicity in rats. CK-MB and LDH 1 were increased in DOX control group rats, but rats treated with the combination of 100 mg/kg of MESR and 200 mg/kg of MEMT showed significantly ($p<0.001$) reduced levels of cardiac enzymes. Similarly, 50 and 100 mg/kg of MESR also significantly ($p<0.001$) reduced the CK-MB and LDH 1 enzymes, whereas 100 mg/kg MEMT ($p<0.01$) and 50 mg/kg of MESR ($p<0.05$) showed a less significant

Table 1. Docking scores of various ligands on the PPARα receptor

Sl. no	*Marsdenia tenacissima*		*Sansevieria roxburghiana*	
	Ligand	Docking score (kcal/mol)	Ligand	Docking score (kcal/mol)
1	Tenasogenin	-7.2	Lupeol	-9.1
2	Cissogenin	-7.1	6-methyl-1-octanol	-6.8
3	Tenacigenin-C	-6.8	Diethyl phthalate	-5.7
4	Tenacigenoside	-4.1	Methyl hexadecanoate	-5.5
5	Dresgenin	-8.2	3,3-dimethylhexanal	-4.6

PPAR: Peroxisome proliferator activated receptors

Table 2. Binding energy, hydrogen bonds, and binding sites of the targets

Ligands	PPAR alpha receptor		
	Binding score (kcal/mol)	Hydrogen bonds	Binding site
Dresgenin	-8.2	3	213ALA, 231GLY, and 216LYS
Lupeol	-9.1	2	214 TYR and 213 ALA

PPAR: Peroxisome proliferator activated receptors

Table 3. Effect of MEMT and MESR on serum cardiac marker enzymes

Treatment/parameters	CK-MB	LDH 1
Normal control	1035.67±3.91	1545.5± 3.56
DOX (20 mg/kg)	1920.17±5.78	3592.67±5.07
Propranolol (10 mg/kg)	1033±6.39	1633.83±14.11
MEMT (100 mg/kg)	1893±4.95[c]	3556.83±3.03[c]
MEMT (200 mg/kg)	1878.33±3.16[b]	3539.5±3.04[b]
MESR (50 mg/kg)	1803.83±13.87[c]	2892.17±14.72[c]
MESR (100 mg/kg)	1505.33±11.33[c]	2309±8.83[c]
MEMT (200 mg/kg) + MESR (100 mg/kg)	1104.5±6.49[c]	1961.83±12.01[c]

All values are expressed as mean ± SEM (n=6), One-Way ANOVA followed by Newman-Keul's multiple comparison test. [a]p<0.05, [b]p<0.01, [c]p<0.001 as compared with the doxorubicin group
DOX: Doxorubicin, MEMT: Methanol extract *Marsdenia tenacissima*, MESR: Methanol extract *Sansevieria roxburghiana*, CK: Choline kinase, LDH: Lactate dehydrogenase, SEM: Standard error of the mean

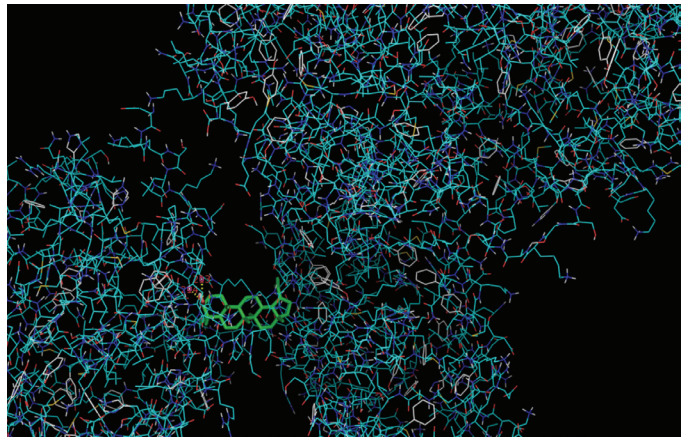


Figure 1. Visualization of docking in Pymol: PPARα with lupeol
PPAR: Peroxisome proliferator activated receptors

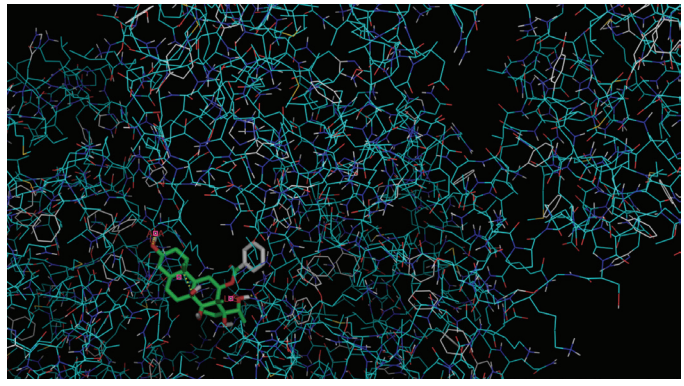


Figure 2. Visualization of docking in Pymol: PPARα with dresgenin
PPAR: Peroxisome proliferator activated receptors

effect on reduction of CK-MB and LDH 1 when compared with disease control animals. The standard drug propranolol showed a highly significant (p<0.001) reducing effect on the elevated levels of cardiac enzymes in DOX-induced cardiotoxicity in rats.

The effect of MEMT and MESR on tissue (heart) antioxidant enzymes of cardiotoxicity-induced rats are shown in Table 4. There was an increased level of MDA and a decreased level of SOD and GSH only in the 20 mg/kg DOX-treated disease control group. Both the doses of orally administered MESR and the combination of MESR + MEMT showed a highly significant (p<0.001) effect on the reduction of MDA as well as enhancement of SOD and GSH found with 14 days of treatment. In the case of MEMT, the 100 mg/kg dose showed a less significant (p<0.01)

effect on the increase in SOD, but the effect on the reduction of MDA and increase in the GSH level in the heart was non-significant (p>0.05), whereas 200 mg/kg of MEMT showed a significant alteration of the re-establishment of cardiac antioxidant enzyme levels with respect to that of normal rats.

The effects of a 14-day, single-dose oral feeding of MEMT and MESR on blood antioxidant enzymes of cardiotoxicity-induced rats are shown in Table 5. The levels of GSH and SOD of the disease control group were lower than those of normal controls (p<0.001). The MDA levels in the blood were higher than those of the normal control group (p<0.001). Treatment with 100 or 200 mg/kg of MEMT and 50 mg/kg or 100 mg/kg of MESR significantly (p<0.001) altered and nearly normalized MDA, SOD, and GSH levels, whereas the combination of both extracts showed a marked reversal of DOX-induced cardiotoxicity in rats.

Figure 3 and Table 6 represent the HPTLC fingerprint of MESR, which was applied in track 1 and was viewed at 254 nm. A total of 17 peaks were found with R_f values ranging from 0.01 to 0.86.

Figure 4 and Table 7 represent the HPTLC fingerprint of MESR, which was applied in track 2 and was viewed at 254 nm. A total of 12 peaks were found with R_f values ranging from 0.01 to 0.84.

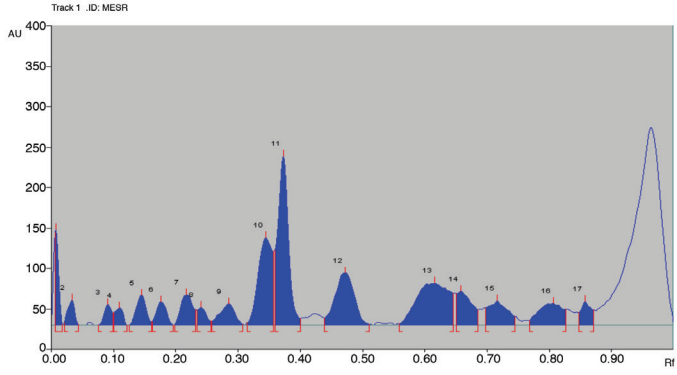


Figure 3. HPTLC fingerprint of MESR at 254 nm

HPTLC: High-performance thin layer chromatography, MESR: Methanol extract *Sansevieria roxburghiana*

Table 4. Effect of MEMT and MESR on tissue antioxidant enzymes of cardiotoxicity- induced rats

Treatment/parameter	SOD (U/mg protein)	MDA (nmol/g of protein)	GSH (mmol/g protein)
Normal control	45.28±0.15	48.47±0.44	10.58±0.39
DOX (20 mg/kg)	26.24±0.21	138.84±0.23	5.47±0.09
Propranolol (10 mg/kg)	37.36±0.38	45.20±0.64	9.99±0.30
MEMT (100 mg/kg)	27.24±0.18[b]	137.78±0.34[d]	6.00±0.07[d]
MEMT (200 mg/kg)	29.75±0.17[c]	136.87±0.23[b]	6.22±0.02[a]
MESR (50 mg/kg)	29.996±0.13[c]	80.4±0.297[c]	6.51±0.02[b]
MESR (100 mg/kg)	32.92±0.18[c]	68.77±0.74[c]	7.52±0.08[c]
MESR (100 mg/kg) + MEMT (200 mg/kg)	36.65±0.09[c]	53.54±0.51[c]	9.79±0.27[c]

All the values were expressed as mean ± SEM (n=6), One-Way ANOVA followed by Newman-Keul's multiple comparison test. [a]p<0.05, [b]p<0.01, [c]p<0.001, and [d]NS p>0.05 as compared with the doxorubicin group
DOX: Doxorubicin, MEMT: Methanol extract *Marsdenia tenacissima*, MESR: Methanol extract *Sansevieria roxburghiana*, SOD: Superoxide dismutase, MDA: Malondialdehyde, GSH: Glutathione, SEM: Standard error of the mean

Table 5. Effect of MEMT and MESR on blood antioxidant enzymes of cardiotoxicity-induced rats

Treatment/Parameter	SOD (U/mL serum)	MDA (nmol/mL)	GSH (U/L)
Normal control	15.21±0.5	8.47±0.44	70.48±0.37
DOX (20 mg/kg)	1.04±0.21	38.84±0.23	25.44±0.19
Propranolol (10 mg/Kg)	12.3±0.31	15.20±0.64	69.95±1.30
MEMT (100 mg/kg)	7.21±0.18[b]	17.78±0.34[d]	56.11±0.08[d]
MEMT (200 mg/kg)	9.55±0.27[c]	16.87±0.23[b]	65.4±0.52[a]
MESR (50 mg/kg)	10.1±0.15[c]	13±0.297[c]	67.55±0.12[b]
MESR (100 mg/kg)	12.12±0.08[c]	11±0.74[c]	69.58±2.08[c]
MESR (100 mg/kg) + MEMT (200 mg/kg)	14.69±0.23[c]	8.54±0.51[c]	99.79±1.38[c]

All values are expressed as mean ± SEM (n=6), One-Way ANOVA followed by Newman-Keul's multiple comparison test. [a]p<0.05, [b]p<0.01, [c]p<0.001, and [d]NS p>0.05 as compared with the doxorubicin group
DOX: Doxorubicin, MEMT: Methanol extract *Marsdenia tenacissima*, MESR: Methanol extract *Sansevieria roxburghiana*, SOD: Superoxide dismutase, MDA: Malondialdehyde, GSH: Glutathione, SEM: Standard error of the mean

A photograph of a TLC plate with methanol extracts of SR and MT is shown in Figure 5. The bands were formed with respect to the track where the sample was applied.

Histopathology reports of vital organs such as the livers and hearts of mice are presented in Table 8. Organ damage to various cellular functional units was reported in organ samples of the disease control as well as the lower-dose treatment of MEMT and MESR group animals, but it was normalized in the higher dose treatment groups and in mice treated with the combination of 100 mg/kg of MESR and 200 mg/kg of

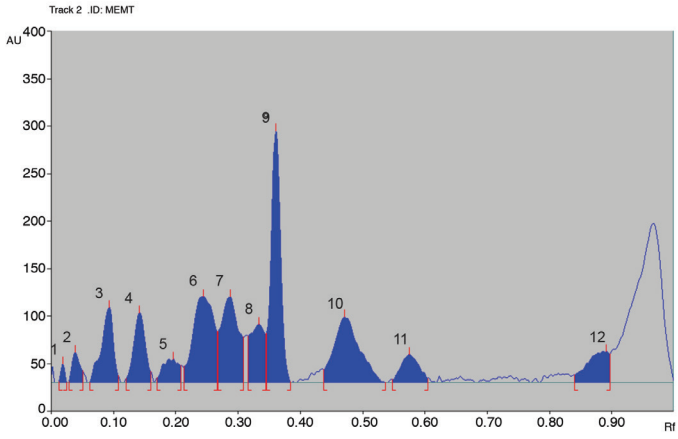


Figure 4. HPTLC of MEMT at 254 nm

HPTLC: High-performance thin layer chromatography, MEMT: Methanol extract *Marsdenia tenacissima*

Figure 5. TLC plate photograph of MEMT and MESR at 254 nm

TLC: Thin layer chromatography, MEMT: Methanol extract *Marsdenia tenacissima*, MESR: Methanol extract *Sansevieria roxburghiana*

Table 6. R_f values of MESR at 254 nm

Peak	Start position	Start height	Max position	Max height	Max %	End position	End height	Area	Area %	Assigned substance
1	0.01 R_f	114.4 AU	0.01 R_f	117.8 AU	12.99%	0.02 R_f	1.1 AU	614.2 AU	3.63%	Unknown"
2	0.02 R_f	2.9 AU	0.03 R_f	31.1 AU	3.43%	0.04 R_f	0.6 AU	314.0 AU	1.85%	Unknown"
3	0.07 R_f	0.1 AU	0.09 R_f	25.5 AU	2.81%	0.10 R_f	15.0 AU	279.1 AU	1.65%	Unknown"
4	0.10 R_f	15.2 AU	0.11 R_f	20.9 AU	2.31%	0.12 R_f	0.0 AU	259.4 AU	1.53%	Unknown"
5	0.13 R_f	0.2 AU	0.14 R_f	36.8 AU	4.06%	0.16 R_f	3.9 AU	529.4 AU	3.13%	Unknown"
6	0.16 R_f	4.3 AU	0.18 R_f	28.8 AU	3.17%	0.20 R_f	0.2 AU	426.4 AU	2.52%	Unknown"
7	0.20 R_f	0.4 AU	0.22 R_f	37.5 AU	4.13%	0.23 R_f	18.9 AU	630.1 AU	3.72%	Unknown"
8	0.23 R_f	18.8 AU	0.24 R_f	21.7 AU	2.39%	0.26 R_f	4.6 AU	283.8 AU	1.68%	Unknown"
9	0.26 R_f	4.9 AU	0.28 R_f	26.3 AU	2.90%	0.31 R_f	1.1 AU	572.5 AU	3.38%	Unknown"
10	0.32 R_f	2.9 AU	0.34 R_f	108.1 AU	11.91%	0.36 R_f	30.8 AU	2262.4 AU	13.36%	Unknown"
11	0.36 R_f	92.4 AU	0.37 R_f	208.7 AU	23.01%	0.40 R_f	7.9 AU	3269.0 AU	19.30%	Unknown"
12	0.44 R_f	8.7 AU	0.47 R_f	64.6 AU	7.12%	0.51 R_f	1.1 AU	1842.0 AU	10.87%	Unknown"
13	0.56 R_f	1.3 AU	0.62 R_f	51.6 AU	5.69%	0.65 R_f	39.4 AU	2486.2 AU	14.68%	Unknown"
14	0.65 R_f	39.1 AU	0.66 R_f	41.6 AU	4.58%	0.69 R_f	18.8 AU	938.5 AU	5.54%	Unknown"
15	0.70 R_f	21.2 AU	0.72 R_f	29.7 AU	3.27%	0.75 R_f	10.7 AU	844.4 AU	4.99%	Unknown"
16	0.77 R_f	5.8 AU	0.81 R_f	26.9 AU	2.96%	0.83 R_f	19.2 AU	943.9 AU	5.57%	Unknown"
17	0.85 R_f	15.1 AU	0.86 R_f	29.6 AU	3.26%	0.87 R_f	18.7 AU	443.7 AU	2.62%	Unknown"

MESR: Methanol extract *Sansevieria roxburghiana*

MEMT. The combination of MEMT and MESR showed greater protection of tissues than individual extract treatment against DOX-induced cardiotoxicity in rats.

DISCUSSION

DOX is an anticancer drug belonging to the anthracycline antibiotics and widely used for various hematological and solid tumors. Cardiotoxicity is a major adverse effect caused by DOX via free-radical production, calcium overloading, mitochondrial dysfunction, and peroxynitrite formation. The cumulative effects of these mechanism lead to altered gene and protein expression followed by cardiomyocyte death. This can be assessed by evaluation of the isoenzymes CK-MB and LDH 1 in serum. They are the cardiac marker enzymes where LDH activity was found to be high in patients' serum within 10 hours of acute myocardial infarction. Similarly, CK-MB may also be undetectable in normal people or may be found in a small fraction in the blood, but if any myocardial muscle insults occur, its level will be elevated in the serum. Therefore, both CK-MB and LDH 1 are reliable cardiac-specific markers used for diagnosis of cardiotoxicity symptoms.[15] The present study results revealed that treatment with MEMT, MESR, and the combination of both extracts normalized cardiac marker enzyme changes in rats.

Oxidative stress (OS) is the most commonly reported adverse effect of a few anticancer drugs such as anthracyclines, cisplatin, and cyclophosphamide. It may occur either directly or indirectly during chemotherapy, but by this mechanism, only few chemotherapeutic agents are cytotoxic to cancerous cells. The generated OS acts on non-targeted normal tissue, leading to tissue injury. DOX also causes OS by the proposed mechanism, the formation of reactive oxygen species (ROS), when the drug accumulates in cellular mitochondria, which leads to the production of redox imbalance followed by sequential generation of superoxide radicals that result in oxidative tissue injury. The second proposed mechanism is that the developed ROS attenuate cardiotoxicity via deletion of Topoisomerase 2β from cardiomyocytes. However, an anticancer drug with antioxidant properties can prevent OS-induced cellular damage and indirectly block ROS and the interaction of the drug with "Top2β".[16] The present study also maintained the tissue antioxidant enzyme level in DOX-induced cardiotoxicity in rats.

PPARs are nuclear receptors that exist in three isoforms: PPARα, PPARγ and PPARβ. They control cellular physiology and pathology and also regulate tissue metabolic homeostasis of skeletal muscle, adipose tissue, intestinal tissue, and the cardiovascular system, which are frequently involved in many inflammatory processes. The α, β/δ forms of PPAR are present in the heart. Apart from their metabolic functions, they are involved in the regulation of circadian rhythms, extracellular matrix remodeling, OS, and tissue inflammation. Cardiac dysfunction is due to loss of PPARα caused by OS, which affects the myosin molecule. Generally, cardioprotective drugs act as agonists of PPARα and reduce the inflammatory condition, increase adiponectin expression on cardiac muscle, and reduce the efficiency of the heart which may be due to increased expression of cardiac UCP3 mRNA. Researchers found that cardiovascular PPARα expression in conditions of cardiomyocyte hypertrophy reduce inflammation by activating inflammatory signaling pathways and also have antioxidative effects. Arrhythmogenic right ventricular dysplasia is due to functional abnormalities of PPAR, a rare genetic disease characterized by a progressive fibro fatty infiltration, decreased PPARα, and increased PPARγ expression in the right ventricle.[17] In order to elucidate the molecular mechanism of the cardioprotective nature of these plant extracts, a few isolated plant constituents were tested with the target protein PPARα, where dresgenin from MT and lupeol from SR showed the high

Table 7. R_f value of MEMT at 254 nm

Peak	Start position	Start height	Max position	Max height	Max %	End position	End height	Area	Area %	Assigned substance
1	0.01 R_f	0.5 AU	0.02 R_f	19.3 AU	2.23%	0.03 R_f	0.3 AU	108.0 AU	0.60%	Unknown"
2	0.03 R_f	1.0 AU	0.04 R_f	31.7 AU	3.66%	0.05 R_f	12.1 AU	368.1 AU	2.04%	Unknown"
3	0.06 R_f	0.4 AU	0.09 R_f	79.0 AU	9.13%	0.11 R_f	6.9 AU	1360.0 AU	7.55%	Unknown"
4	0.12 R_f	4.0 AU	0.14 R_f	73.5 AU	8.49%	0.16 R_f	11.7 AU	1246.6 AU	6.92%	Unknown"
5	0.17 R_f	4.8 AU	0.20 R_f	24.3 AU	2.81%	0.21 R_f	17.7 AU	585.2 AU	3.25%	Unknown"
6	0.21 R_f	17.1 AU	0.24 R_f	90.6 AU	10.47%	0.27 R_f	53.3 AU	2768.6 AU	15.37%	Unknown"
7	0.27 R_f	53.5 AU	0.29 R_f	89.9 AU	10.39%	0.31 R_f	47.8 AU	2322.6 AU	12.89%	Unknown"
8	0.32 R_f	49.2 AU	0.33 R_f	61.4 AU	7.09%	0.34 R_f	50.9 AU	1321.5 AU	7.34%	Unknown"
9	0.35 R_f	52.0 AU	0.36 R_f	265.1 AU	30.64%	0.38 R_f	0.8 AU	3466.0 AU	19.24%	Unknown"
10	0.44 R_f	13.6 AU	0.47 R_f	68.7 AU	7.94%	0.54 R_f	0.0 AU	2589.6 AU	14.38%	Unknown"
11	0.55 R_f	2.9 AU	0.57 R_f	29.2 AU	3.38%	0.60 R_f	4.4 AU	793.5 AU	4.41%	Unknown"
12	0.84 R_f	8.1 AU	0.89 R_f	32.5 AU	3.76%	0.90 R_f	29.8 AU	1082.6 AU	6.01%	Unknown"

MEMT: Methanol extract *Marsdenia tenacissima*

Table 8. Histopathology reports of the cardioprotective study

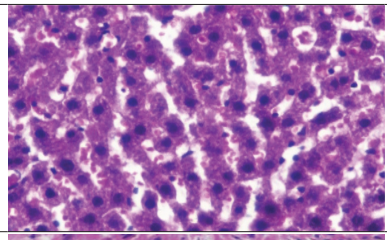
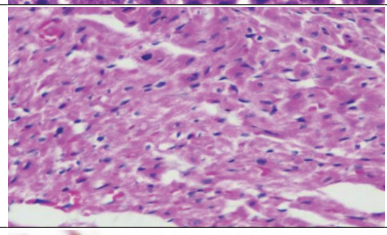
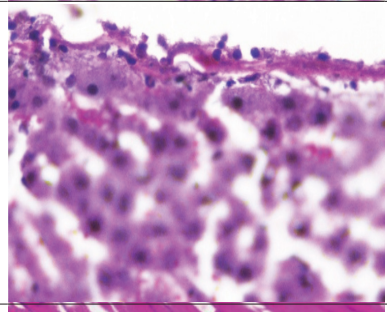
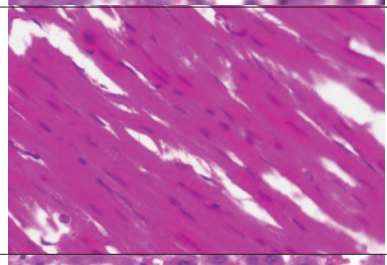
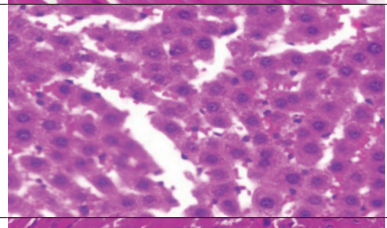
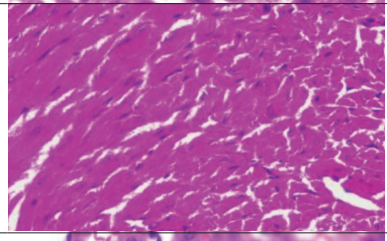
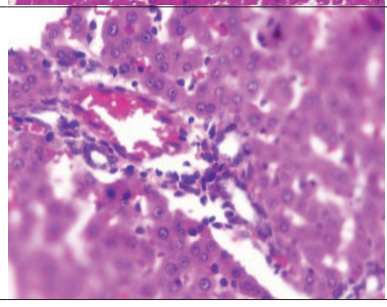
Sample code	Group details	Image	Histopathology report
NCL	Normal control (liver)		Section showed that all the cellular functional units were within normal limits
NCH	Normal control (heart)		Section showed that all the cellular functional units within normal limits
DCL	Disease control (liver)		Section showed the infiltrates with mononuclear, capsular, diffuse and mild alteration in cellular functional units
DCH	Disease control (heart)		Section showed the infiltrates with mononuclear, myocardial, multifocal and minimal damage to the cellular units
PL	Propranolol (10 mg/kg) (liver)		Section showed that the cellular functional units were within normal limits
PH	Propranolol (10 mg/kg) (heart)		Section showed that the cellular functional units were within normal limits
MTLL	MEMT (100 mg/kg) (liver)		Section showed that infiltrates with mononuclear, capsular, diffuse and mild alteration in cellular functional units

Table 8. Continued

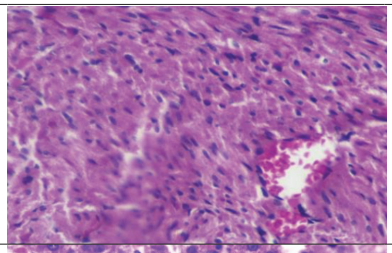
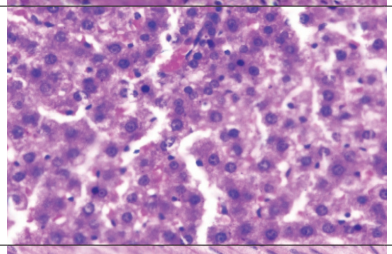
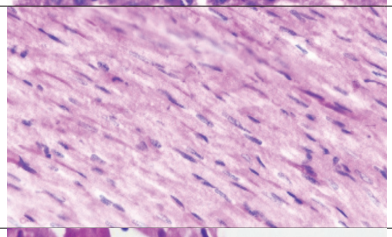
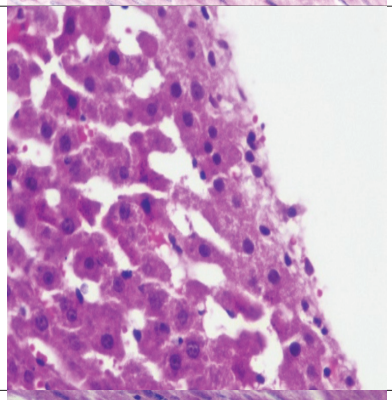
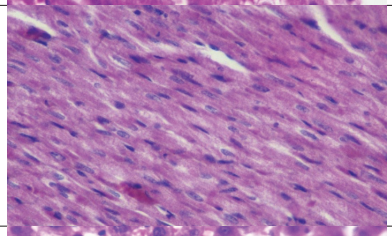
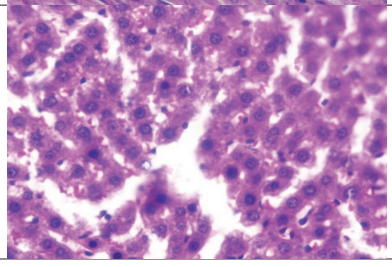
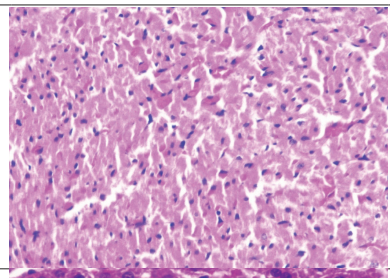
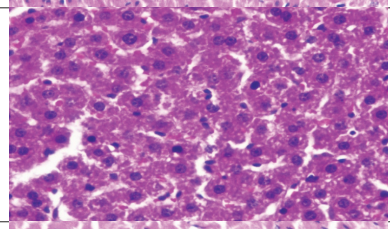
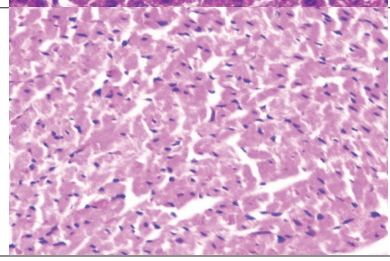
Sample code	Group details	Image	Histopathology report
MTLH	MEMT (100 mg/kg) (heart)		Section showed that the cellular functional units were within normal limits
MTHL	MEMT (200 mg/kg) (liver)		Section showed that the cellular functional units were within normal limits
MTHH	MEMT (200 mg/kg) (heart)		Section showed that the cellular functional units were within normal limits
SRLL	MESR (50 mg/kg) (liver)		Section showed the infiltrates with mononuclear, capsular, diffuse and minimal damage to the cellular functional units
SRLH	MESR (50 mg/kg) (heart)		Section showed that the cellular functional units were within normal limits
SRHL	MESR (100 mg/kg) (liver)		Section showed that the cellular functional units were within normal limits

Table 8. Continued

Sample code	Group details	Image	Histopathology report
SRHH	MESR (100 mg/kg) (heart)		Section showed that the cellular functional units were within normal limits
CML	100 mg/kg of MESR & 200 mg/kg of MEMT (liver)		Section showed that the cellular functional units were within normal limits
CMH	100 mg/kg of MESR & 200 mg/kg of MEMT (heart)		Section showed the cellular functional units were within normal limits

MEMT: Methanol extract *Marsdenia tenacissima*, MESR: Methanol extract *Sansevieria roxburghiana*

affinity toward the target protein, and these phytoconstituents protected the myocardium from toxic agents.

HPTLC fingerprinting of natural drugs may encourage the recognition of natural products, and it is suited to the delivery of core scaffolds for forthcoming drugs. Hence, there will be further developments in the use of novel analytical techniques in natural product drug discovery campaigns.[14] The qualitative analysis of MEMT and MESR through HPTLC confirmed the existence of many secondary metabolites. Traditional therapeutic uses of this species are due to the pre-existences of these metabolites. Therefore, the present study adds value to the medicinal importance of this Morva species.

Histopathological reports revealed treatment-related microscopic changes in the livers and hearts of rats. Infiltrates with mononuclear, capsular, diffuse, and minimal damage to the cellular functional units were found in animals that received 50 mg/kg of MESR, whereas the infiltrates with mononuclear, myocardial, multifocal, and minimal damage to the cellular units were found in disease control animals. All the incidences were within the normal range in other extract-treated and control animals. It can, therefore, be concluded that all histological changes observed were normalized by extract treatment.

CONCLUSION

The cardioprotective study results suggest a correlation between antioxidant enzymes and the degree of damage caused by DOX. By increasing the activities of antioxidant enzymes that combat free-radical damage, MT exerts antioxidant effects that can be useful in the treatment of cancer. Similarly, ethyl acetate extract of SR possess significant antioxidant as well as anticancer properties.

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ORIGINAL ARTICLE

Evaluation of Prescribing Patterns of Antibiotics Using Selected Indicators for Antimicrobial Use in Hospitals and the Access, Watch, Reserve (AWaRe) Classification by the World Health Organization

Hastanelerde Antimikrobiyal Kullanımı ile İlgili Seçilmiş Göstergeler ve Dünya Sağlık Örgütü Tarafından Tanımlanan AWaRe Sınıflandırması Kullanılarak Antibiyotik Reçeteleme Kalıplarının Değerlendirilmesi

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ABSTRACT

Objectives: Antibiotic resistance poses a significant threat to the world, and irrational use of antibiotics is a major contributing factor. Evaluation of antimicrobial use is underway with the help of indicators and the World Health Organization (WHO) classification of antibiotics into Access, Watch, and Reserve (AWaRe) categories. We aimed to evaluate the prescribing pattern of antibiotics using the AWaRe classification by the WHO and selected indicators for antimicrobial use in hospitals.

Materials and Methods: A total of 1.000 prescriptions were analyzed during the study for antibiotic prescribing patterns. Antibiotic consumption was calculated using defined daily dose (DDD) methodology. The prescribing pattern was evaluated using the WHO classification of antibiotics into the categories AWaRe and using selected indicators (hospital and prescribing) for antimicrobial use in hospitals.

Results: A total of 1.128 antibiotics were prescribed during the study. The 19-44 age group was prescribed a high number of antibiotics (n=510). Females were prescribed a high number of antibiotics compared with males (n=602). Azithromycin was the most commonly consumed antibiotic (14.97 DDD/1000/day). Four antibiotics from the Access category and five from the Watch category were prescribed in the study. The Watch category of antibiotics were consumed in a high number. There were no standard treatment guidelines in the hospital. In all, 98.0% of antibiotics were consistent with the hospital formulary and prescribed under generic names. The average number of antibiotics prescribed per patient was 1.12. The average duration of antimicrobial treatment was 5.24 days. The percentage of patients prescribed antimicrobials for pneumonia in accordance with treatment guidelines was 13.28%.

Conclusion: Irrational use of antibiotics exists in hospitals. There is a need to maintain standard treatment guidelines in the hospital because it prevents irrational use of antibiotics.

Key words: Access, Watch, Reserve, indicator, prescribing, antibiotic, evaluation, hospital, WHO

ÖZ

Amaç: Antibiyotik direnci dünya için önemli bir tehdit oluşturmaktadır ve akılcı olmayan antibiyotik kullanımı bu duruma önemli katkıda bulunan faktördür. Antimikrobiyal kullanımının değerlendirilmesi çeşitli göstergeler ve Dünya Sağlık Örgütü'nün (DSÖ) antibiyotiklerin "Erişim, İzleme ve Rezerv [*Access, Watch ve Reserve* (AWaRe)]" kategorilerine göre sınıflandırılmasının kullanılmasıyla devam etmektedir. DSÖ'nün AWaRe sınıflandırmasını kullanarak antibiyotik reçeteleme modelini ve hastanelerde antimikrobiyal kullanımı için seçilen göstergeleri değerlendirmeyi amaçladık.

Gereç ve Yöntemler: Çalışma süresinde antibiyotik reçeteleme modelleri için toplam 1,000 reçete analiz edildi. Antibiyotik tüketimi, tanımlanan günlük doz metodolojisi (DDD) kullanılarak hesaplandı. Reçeteleme paterni, DSÖ antibiyotik sınıflandırması kullanılarak AWaRe kategorilerine göre ve hastanelerde antimikrobiyal kullanımı ile ilgili seçilen göstergeler (hastane ve reçete yazma) kullanılarak değerlendirildi.

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Bulgular: Çalışma sırasında toplam 1,128 antibiyotik reçete edildi. On dokuz-kırk dört yaş grubuna fazla sayıda antibiyotik reçete edildi (n=510). Kadınlara erkeklere (n=602) kıyasla çok sayıda antibiyotik reçete edildi. Azitromisin en sık tüketilen antibiyotiktir (14,97 DDD/1000/gün). Çalışmada Access kategorisinden dört ve Watch kategorisinden beş antibiyotik reçete edildi. Watch kategorisindeki antibiyotikler yüksek sayıda tüketildi. Hastanede standart tedavi yönergeleri yoktu. Toplamda, antibiyotiklerin %98,0'ı hastane formüleriyle uyumluydu ve jenerik isimler altında reçete edildi. Hasta başına reçete edilen ortalama antibiyotik sayısı 1,12 idi. Ortalama antimikrobiyal tedavi süresi 5,24 gündü. Tedavi kılavuzlarına uygun olarak pnömoni için antimikrobiyal reçete edilen hastaların yüzdesi %13,28 idi.

Sonuç: Hastanelerde akılcı olmayan antibiyotik kullanımı mevcuttur. Mantıksız antibiyotik kullanımını engellediği için hastanede standart tedavi kurallarının sürdürülmesine ihtiyaç vardır.

Anahtar kelimeler: Erişim, Saat, Rezerv, gösterge, reçeteleme, antibiyotik, değerlendirme, hastane, DSÖ

INTRODUCTION

Antibiotic resistance poses a significant threat to global public health[1,2] and was given special mention as a serious threat to public health, economic growth, and global economic stability.[3] Increased antibiotic resistance rates may lead to prolonged hospitalization and duration of treatment, as well as increased treatment costs and mortality.[4] The major contributing factor to this resistance is inappropriate or irrational use of antibiotics. Irrespective of the alarming increase in resistance, there is an increased irrational prescribing practice of antibiotics across different regions.[5-13] In 2017, the World Health Organization (WHO) commissioned comprehensive reviews on antibiotic use for specific infections in order to update the Essential Medicines List.[14] The expert committee then formulated the Access, Watch, Reserve (AWaRe) classification of antibiotics with the goals of better accessibility and clinical outcomes, a decreased probability of antimicrobial resistance, and safeguarding the effectiveness of last-resort antibiotics.[15] Access group of antibiotics are first and second choices for empirical treatment of 21 common or severe clinical syndromes. The Access group of antibiotics are a core set of antibiotics and should always be made available in every place at an appropriate quality, dose, duration, formulation, and price. The Watch group includes antibiotics with higher toxicity concerns or resistance potential compared with the Access group. The Watch group antibiotics assist the development of tools for stewardship at the local, national, and global levels. The Reserve group antibiotics are last-resort options and are used for specific patients and clinical settings in case of failure of other alternatives. Prioritizing this group as key targets of high-intensity national and international stewardship programs preserves their effectiveness.[15] Thus, the AWaRe index help to estimate the relative use of narrow-spectrum and broad-spectrum antibiotics. The Strengthening Pharmaceutical Systems (SPS) Program also developed selected indicators for investigating antimicrobial use in hospitals, which complements the existing WHO indicators of outpatient antimicrobial use. These indicators provide a simple tool for fast and assuredly figuring out critical aspects of antimicrobial use and to recognize problems with antibiotic use in hospitals.[16] So, we aimed to evaluate the prescribing pattern of antibiotics using the WHO AWaRe classification and selected indicators for investigating antimicrobial use in hospitals using the SPS Program.

MATERIALS AND METHODS

A descriptive, cross-sectional study was conducted on 1.000 patients with various diseases in a tertiary care hospital, for a duration of six months (08/01/2019 to 01/31/2020). The study was approved by the Institutional Human Ethics Committee (VIPT/IEC/61/2019). Prescriptions containing at least one antibiotic, prescribed to patients of all ages, and in various departments were included in the study. Prescriptions without antibiotics were excluded from the study. Simple random sampling was used to select prescriptions. The estimated sample size was 651 (margin of error 5%, confidence level 99%, population size 324,000, and response distribution 50%). However, we collected data for 1.000 prescriptions. The aim of the study was explained clearly to the patients and an informed consent form obtained from willing patients. Sociodemographic data including age and gender, and clinical details including name of the department, diagnosis, name of the antibiotic, dose, indication etc. were collected from the patient's prescription.

The WHO AWaRe classification (2019) was used to evaluate the rational use of antibiotics. Selected indicators for antimicrobial use for hospitals (hospital indicators and prescribing indicators) developed by the SPS Program was used to investigate antimicrobial use. The defined daily dose (DDD) per 1000 inhabitants per day was calculated using the following formula. Descriptive statistics (mean and standard deviation) were calculated using Minitab (version 18.0).

$$\text{DDD}/1000/\text{ day} = \frac{\text{Total number of dosage units prescribed} * \text{Dosage strength} * 1000}{DDD * Duration\ of\ the\ study * Total\ sample\ size}$$

RESULTS

A total of 1.128 antibiotics were prescribed during the study. The mean age of the patients in our study was 33.04±18.59 years. Patients in the 19-44 age group were prescribed with a high number of antibiotics (n=510, 45.21%) (Table 1). Females were prescribed with a higher percentage of antibiotics than males (53.47% vs. 46.63%, respectively, Table 2). The general medicine department consumed a higher proportion of antibiotics (36.79%, Table 3).

The DDD for azithromycin was high relative to that of other antibiotics (14.97 DDD/1000/day, Table 4). Four antibiotics from the Access category and five from the Watch category of the AWaRe classification were prescribed (Table 5). Standard

Table 1. Age-wise distribution of antibiotics in patients

S. no.	Name of the antibiotic	1-18 years	19-44 years	45-63 years	≥64 years	Total (%)
1	Amoxicillin + clavulanic acid	131	122	51	18	322 (28.54)
2	Cefixime	43	110	64	19	236 (20.92)
3	Azithromycin	42	107	57	25	231 (20.47)
4	Metronidazole	21	67	34	13	135 (11.96)
5	Ciprofloxacin	8	39	17	2	66 (5.85)
6	Ofloxacin	11	29	13	3	56 (4.96)
7	Amoxicillin	21	21	8	2	52 (4.60)
8	Doxycycline	3	9	6	1	19 (1.68)
9	Norfloxacin	2	6	3	0	11 (0.97)
	n (%)	282 (25.00)	510 (45.21)	253 (22.43)	83 (7.35)	1128

n: Number, %: Percentage

Table 2. Gender-wise distribution of antibiotics

S. no.	Name of the antibiotic	Males	Females	Total
1	Amoxicillin + clavulanic acid	135	187	322
2	Cefixime	112	124	236
3	Azithromycin	108	123	231
4	Metronidazole	75	60	135
5	Ciprofloxacin	30	36	66
6	Ofloxacin	31	25	56
7	Amoxicillin	26	26	52
8	Doxycycline	6	13	19
9	Norfloxacin	3	8	11
	n (%)	526 (46.63)	602 (53.37)	1128

n: Number, %: Percentage

Table 3. Department-wise distribution of antibiotics

S. no.	Name of the antibiotic	G.M.	ENT	Ortho	Ped	Pul	Others
1	Amoxicillin + clavulanic acid	94	124	14	35	22	33
2	Cefixime	82	40	68	7	8	31
3	Azithromycin	90	27	2	22	60	30
4	Metronidazole	83	19	2	11	0	20
5	Ciprofloxacin	28	25	3	0	1	9
6	Ofloxacin	15	11	0	7	1	22
7	Amoxicillin	14	21	2	5	3	7
8	Doxycycline	2	0	11	0	0	6
9	Norfloxacin	7	1	0	0	1	2
	n (%)	415 (36.79)	268 (23.75)	102 (9.04)	87 (7.71)	96 (8.51)	160 (14.18)

n: Number, %: Percentage, G.M.: General medicine, ENT: Ear, nose, throat, Ortho: Orthopedics, Ped: Pediatrics, Pul: Pulmonology, Others: Dermatology, general surgery, endocrinology, gastroenterology, nephrology, neurology, urology, dental, gynecology

treatment guidelines for infectious diseases and essential medicines were listed in the hospital. The average number of days that a set of key antimicrobials was out of stock was 3.2 days/month. In all, 98.3% of key antimicrobials were available on the day of the study (Table 6). The average number of antibiotics prescribed per hospitalization was 1.12. In all, 98% of antimicrobials were consistent with the hospital formulary list and were prescribed by their generic names. In all, 13.28% of antimicrobials for pneumonia patients were prescribed in accordance with standard treatment guidelines (Table 7).

DISCUSSION

We observed a high antibiotic prescribing rate in the 19-44 patient age group. Interestingly, the rate of antibiotic prescription in the elderly was low (7.35%). In general, the elderly are more vulnerable to infections, and thus a higher number of antibiotics are expected to be prescribed for them. Overprescription of antibiotics for the elderly is a common practice, and the physicians here were the exception to that, as was evident from Table 1. Females were prescribed a higher number of antibiotics than males (53.37% vs. 46.3%, respectively). Relatively speaking, females are less exposed to external environments than males; however, in our study, females were prone to more infections. The general medicine department covers a wide variety of diseases. Hence, the general medicine department consumed a higher percentage of antibiotics (36.79%).

The commonly prescribed antibiotics in our study were amoxicillin + clavulanic acid (n=322). Cefixime (n=236) and azithromycin (n=231) were the next most widely prescribed antibiotics. Atif et al.[11] reported ceftriaxone as the most

Table 4. Defined daily dose of each antibiotic along with the ATC code

S. no.	Name of the antibiotic	ATC code	DDD	DDD/1000/day
1	Amoxicillin + clavulanic acid	J01CR02	1.5 g	8.64
2	Cefixime	J01DD08	0.4 g	9.17
3	Azithromycin	J01FA10	0.3 g	14.97
4	Metronidazole	P01AB01	2 g	3.15
5	Ciprofloxacin	J01MA02	1 g	2.56
6	Ofloxacin	J01MA01	0.4 g	2.17
7	Amoxicillin	J01CA04	1.5 g	1.34
8	Doxycycline	J01AA02	0.1 g	1.47
9	Norfloxacin	J01MA06	0.8 g	0.42
	Total	-	-	43.89

ATC: Anatomic, therapeutic, chemical, DDD: Defined daily dose

Table 5. Categorization of antibiotics according to AWaRe classification by the WHO

S. no.	Name of the antibiotic	Class of antibiotic	AWaRe category	Listed in EML
1	Amoxicillin + clavulanic acid	Beta lactam-beta lactamase inhibitor	Access	Yes
2	Metronidazole	Imidazole	Access	Yes
3	Amoxicillin	Penicillins	Access	Yes
4	Doxycycline	Tetracycline	Access	Yes
5	Cefixime	Third generation cephalosporin	Watch	Yes
6	Azithromycin	Macrolide	Watch	Yes
7	Ciprofloxacin	Fluoroquinolone	Watch	Yes
8	Ofloxacin	Fluoroquinolone	Watch	No
9	Norfloxacin	Fluoroquinolone	Watch	No

AWaRe: Access, Watch, Reserve, EML: Essential medicines list, WHO: World Health Organization

Table 6. Hospital indicators for antimicrobial use in the hospital

S. no.	Name of the indicator	Result
1	Existence of standard treatment guidelines for infectious diseases	No
2	Existence of approved hospital formulary list or essential medicines list	Yes
3	Availability of a set of key antimicrobials in the hospital stores on the day of the study	98.30%
4	Average number of days that a key antimicrobial was out of stock	3.2 days/month

Table 7. Prescribing indicators for antimicrobial use in the hospital

S. no.	Name of the indicator	Result
1	Percentage of antimicrobials prescribed consistent with the hospital formulary list	98.00%
2	Average duration (in days) of prescribed antimicrobial treatment	5.24±1.35
3	Percentage of antimicrobials prescribed by generic name	98.00%
4	Average number of antibiotics prescribed per hospitalization	1.12
5	Percentage of patients with pneumonia who are prescribed antimicrobials in accordance with standard treatment guidelines	13.28%

commonly prescribed antibiotic (71.8%). The most frequently prescribed antibiotic class was cephalosporins (81.5%), while the most frequent antibiotic combination was ciprofloxacin + metronidazole (52.1%). A repeated point prevalence survey on the appropriateness of antimicrobial prescribing reported that penicillins with beta-lactamase inhibitors were the most frequently prescribed antibiotics (30%), which was in close agreement with the results of our study.[6]

Azithromycin (14.97 DDD/1000/day) was the most commonly prescribed antibiotic, followed by cefixime (9.17 DDD/1000/day) and amoxicillin and clavulanic acid (8.64 DDD/1000/day). Similar to our study, Mule et al.[17] reported higher consumption of azithromycin (107.83 DDD/1000/day) in their research. In contrast, a population-based study on trends of antibiotic use in Korea reported penicillins (mean consumption 4.52 DDD/1000/day) as a commonly used antibiotic subgroup, followed by second-generation cephalosporins (4.47 DDD/1000/day), macrolides (3.32 DDD/1000/day), and fluoroquinolones (2.75 DDD/1000/day).[18] Another study on antibiotic consumption in pediatric patients reported high consumption of penicillins (271.22 DDD/1000/day), followed by cephalosporins (98.46 DDD/1000/day) and macrolides (72.70 DDD/1000/day) in the pulmonology department.[19] Bansal et al.[20] reported higher consumption of ceftriaxone (143.22 DDD/1000 patient-days), followed by doxycycline (85.02 DDD/1000 patient-days) and azithromycin (66.37 DDD/1000 patient days, oral; 59.37 DDD/1000 patient days per oral).

We observed azithromycin as a drug of choice for upper respiratory tract infections. However, according to the WHO model list of essential medicines, azithromycin belongs to the Watch category and is the first-choice antibiotic for sexually transmitted infections such as gonorrhea, as well as cholera,[21] amoxicillin and clavulanic acid were prescribed for pneumonia, urinary tract infections, and otitis media in our study. According to the WHO model list of essential medicines list, amoxicillin and clavulanic acid belong to the Access category. It is the preferred first-choice antibiotic for community-acquired pneumonia, skin and soft-tissue infections, lower urinary tract

infections, hospital-acquired pneumonia, and COPD. It is the second-choice antibiotic for bone and joint infections, otitis media, and surgical prophylaxis.[21] Cefixime was prescribed for bone and joint infections, chronic suppurative otitis media, and urinary tract infection. However, according to the WHO model list, cefixime belongs to Watch group antibiotics and is preferred as the second choice for acute diarrhea/dysentery and gonorrhea.[21]

We observed the absence of standard treatment guidelines for infectious diseases in the hospital. However, there was an approved hospital formulary list or essential medicines list in the hospital. A study by Atif et al.[10] reported a similar result, whereas Shahbazi et al.[7] reported contrasting results. Irrational prescribing or inappropriate prescribing of antibiotics is a crucial contributing factor to antimicrobial resistance. Standard Treatment Guidelines allow prescribers to follow the standard, avoid irrational prescribing, and provide quality patient care without any compromise. They also prevent unnecessary drug reactions and out-of-pocket expenditures to the patient and promote a faster recovery for the patient. The Treatment Guidelines for Antimicrobial Use in common syndromes, 2019 by the Indian Council of Medical Research[22] offer guidelines for antimicrobial use in common infectious diseases with dose, frequency of administration, duration, and monitoring antimicrobial use. They are available free of charge. Framed according to the Indian scenario, if followed, they help in preventing irrational or inappropriate antimicrobial use.

The main drawback was the absence of standard treatment guidelines in the hospital. Although the remaining indicators are satisfactory, prescribing without standards is worrying. According to the Indian Council for Medical Research,[22] the preferred antimicrobial agent for pelvic inflammatory disease, and alternative antibiotic for typhoid fever, bacterial sinusitis was cefixime. However, in our study, cefixime was also prescribed for throat infection, upper respiratory tract infection, fever, chronic otitis media, etc. Ofloxacin was indicated for epididymo-orchitis,[22] whereas it was prescribed for topical ulcer, alcoholic gastritis, and perianal infection. Likewise, standard treatment guidelines can prevent inappropriate prescribing practices.

In all, 98.3% of key antimicrobials were available in the hospital stores on the day of our study. Atif et al.[10], Shahbazi et al.[7], and Woldu et al.[23] reported a lower percentage of key antimicrobial availability in the hospital stores on the day of their study (93.8%, 90.1%, and 78.5% respectively). The availability of key antimicrobials all the time is essential because the practitioners will start prescribing antimicrobials that are not indicated for the disease, or they may prescribe branded forms of critical antimicrobials for purchase from outside the hospital. Branded types of drugs are more economical and increase the out-of-pocket expenditures for the patient.

The average number of days that a set of essential antimicrobials was out of stock in our study was 3.2 days/month. Atif et al.[10] reported a similar result (3.3 days/month). However, Shahbazi et al.[7] and Woldu et al.[23] reported a high average number of out-of-stock days for essential antimicrobials (6.78 days/month and 15-45 days over a 12-month period). This indicator provides information about healthcare capacity and practices to maintain inventory control, procurement, and correct distribution.[10]

The average number of antibiotics prescribed per hospitalization in our study was 1.12. Atif et al.[10], Shahbazi et al.[7], and Osama and Ibrahim[24] reported a higher average number of antibiotics than our study (2.35, 2.85, and 2.7, respectively). Antibiotics should be prescribed whenever needed and appropriate. However, in real situations, patients are unaware of antimicrobial resistance and influenced by false beliefs, and behavioral factors often cause them to ask the physician to prescribe an antibiotic or think that the physician is not competant if he/she does not prescribe an antibiotic. Patient awareness of antimicrobial resistance due to the irrational use of antibiotics can prevent these circumstances.

In our study, 98.00% of antibiotic prescription was consistent with the formulary list. Two other studies reported similar results.[10,11] In contrast, Shahbazi et al.[7] reported 100% consistency in prescription with the hospital formulary list. The hospital formulary list optimizes medication use. Lack of awareness of the formulary list among physicians, a deficiency of the listed antibiotics, or prescribing brand names instead of generic names may cause non-adherence to such hospital policy.[22] Physicians will not prescribe the medication if they are unaware of the formulary list. This results in a waste of healthcare resources because the stocked drugs reach their expiry dates and become useless.

In our study, 98.00% of antibiotics were prescribed using the generic name. This prescribing practice was far better than those reported by Atif et al.[10], Green et al.[25], and Shahbazi et al.[7] (52.5%, 88%, and 13.18%, respectively). Prescribing drugs by their generic names is essential in developing countries because it lessens the economic burden on poor people. Patients' misconceptions about generic drugs versus brand drugs allow easy exploitation and make them prefer branded drugs over generic drugs. Moroever, prescribing generic drugs often prevents confusion surrounding multiple names assigned to the same product.[16] Patients are also habituated to buy the drug with the same brand name only, although the same drug is available in generic form or under another brand. There is a need to strengthen the awareness of generic drugs and their availability among patients. In India, the central government set up a "Jan Aushadhi" scheme wherein pharmacies will sell generic drugs and all medicines so that pharmacies are affordable for the people.

The mean duration of antimicrobial treatment prescription was 5.24 days, and similar results were reported by Atif et al.[10] and Shahbazi et al.[7] (5.4 days and 5.65 days, respectively). The duration of antibiotic treatment varies according to the severity of the disease and the nature of the drug. Since there is no consensus on the optimal duration of therapy for the majority of infectious diseases, it is better to treat for at least 7-10 days. A short course of treatment may lead to antimicrobial-resistant

microbes. At the same time, prolonged exposure increases the risk of adverse drug reactions, antimicrobial resistance, and also unwanted expenditure on antibiotics.[16] The percentage of pneumonia patients prescribed antimicrobials according to standard treatment guidelines was 13.28%. Shahbazi et al.[7] reported 19.23% for the same figure. However, in our study, there was no use of standard treatment guidelines. Green et al.[25] also reported that pneumonia patients in their study were prescribed antibiotics without any standard treatment guidelines.

Four antibiotics from the Access category and five antibiotics from the Watch category were prescribed in our study. The WHO's AWaRe classification specified that the antibiotics consumed from the Access group should be at least 60%.[26] In our study, 46.80% of antibiotics from the Access category was prescribed. Watch group antibiotics accounted for 53.19 % of the total antibiotics prescribed. This indicates the overuse of Watch group antibiotics. A study on pediatric antibiotic prescription in China also reported a similar practice of overuse.[27] A pediatric survey reported varied consumption of AWaRe antibiotics among countries. Access group antibiotic consumption for children in Slovenia accounted for 61.2%, whereas in China, it was 7.8%. Watch group antibiotic consumption for children is highest in Iran (77.3%), whereas it is lowest in Finland (23.0%). In neonates, Singapore Access group antibiotics accounted for 100% of all those prescribed, whereas China registered the lowest consumption of Access group (24.2%).[28]

Study limitations

The study has a few limitations. One of the hospital indicators, Expenditure on antimicrobials as a percentage of total hospital medicine costs, was not calculated due to administrative policies in the hospital. One of the prescribing indicators, the average cost of antimicrobials prescribed per hospitalization, was not calculated due to organizational policies. We collected data from outpatient departments only, so we are unable to calculate two prescribing indicators i.e., antimicrobials used in surgical prophylaxis and the average number of antibiotic doses administered for cesarean sections.

CONCLUSION

Our study observed irrational prescribing practices. Strict implementation of the use of standard treatment guidelines prevents inappropriate prescribing. Drugs should be prescribed by their generic names, and the percentage of antibiotics prescribed consistent with the hospital formulary should reach 100% for better results.

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ORIGINAL ARTICLE

Characteristic Comparison of an Intraoral Thin Film Containing Astaxanthin Nanoemulsion Using Sodium Alginate and Gelatin Polymers

Sodyum Aljinat ve Jelatin Polimerleri Kullanılmış Nanoemülsiyon Astaksantin İçeren İntraoral İnce Filmin Karakteristiğinin Karşılaştırılması

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ABSTRACT

Objectives: The present study was conducted to compare the characteristics of a thin film containing an astaxanthin-loaded nanoemulsion (TFANE) using two kinds of natural polymers, namely sodium alginate and gelatin.

Materials and Methods: An astaxanthin nanoemulsion was prepared by using the self-nanoemulsifying method, followed by incorporation into a polymer matrix system by the solvent casting method to form TFANE. A characteristic comparison between the sodium alginate and gelatin matrix systems was carried out by comparing the physical and mechanical film properties. At the end of the study, *in vitro* dissolution tests were also assessed.

Results: An intraoral film with good physical and mechanical properties containing an astaxanthin-loaded nanoemulsion was developed successfully using a natural polymer matrix system. The film, made from a gelatin matrix system containing an astaxanthin nanoemulsion, was more flexible and harder than films made from a sodium alginate matrix system, where all of the films have ideal characteristics for intraoral delivery. The dissolution test results showed that, with both sodium alginate and gelatin, more than 90% of the drug was released at 15 minutes.

Conclusion: Gelatin as a natural polymer appears to be promising for the preparation of an intraoral thin film delivery system.

Key words: Astaxanthin, nanoemulsion, thin film, solvent casting method

ÖZ

Amaç: Bu çalışma, astaksantin yüklü nanoemülsiyon (TFANE) içeren ince filmin özelliklerini sodyum aljinat ve jelatin olmak üzere iki çeşit doğal polimer kullanılarak karşılaştırmak amacıyla yapılmıştır.

Gereç ve Yöntemler: Astaksantin nanoemülsiyon, kendiliğinden nanoemülsifikasyon yöntemi kullanılarak hazırlandı, ardından TFANE elde etmek için çözücü döküm yöntemi ile polimerler matris sistemine dahil edildi. Sodyum aljinat ve jelatin matris sistemlerinin karşılaştırması, fiziksel ve mekanik film özellikleri karşılaştırılarak gerçekleştirilmiştir. Çalışmanın sonunda *in vitro* çözünme testleri de değerlendirildi.

Bulgular: İyi fiziksel ve mekanik özelliklere sahip astaksantin yüklü nanoemülsiyon içeren intraoral film, doğal polimer matris sistemi kullanılarak başarıyla geliştirilmiştir. Tüm filmlerin intraoral ilaç taşınımı için ideal özelliklere sahip olduğu; astaksantin nanoemülsiyon içeren jelatin matris sisteminden yapılan filmin, sodyum aljinat matris sisteminden yapılan filmlerden daha esnek ve daha sert olduğu tespit edilmiştir. Çözünme testi sonuçları, hem sodyum aljinat hem de jelatinin 15 dakikada ilacın %90'ından daha fazla salındığını gösterdi.

Sonuç: Doğal bir polimer olarak jelatin, intraoral ince film ilaç taşıyıcı sisteminin hazırlanması için umut verici görünmektedir.

Anahtar kelimeler: Astaksantin, nanoemülsiyon, ince film, çözücü döküm yöntemi

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INTRODUCTION

Astaxanthin is a lipophilic pigment with a reddish color, synthesized naturally by algae or plants. As a member of the xanthophyll group of compounds, which comprises oxygenated derivatives of carotenes, astaxanthin contains conjugated double bonds, hydroxyl groups, and ketone groups and possesses both lipophilic and hydrophilic properties. Its unique structure gives astaxanthin strong antioxidant power and superior biological activity to that of other antioxidants, owing to its ability to forms linkages with the cell membrane.[1-3]

In humans, the bioavailability of carotenoids is low and variable (10%-50% of a given dose), due to their low solubility in gastrointestinal tract juices, leading to poor absorption in the small intestine.[3] Another factor that lowers the bioavailability of astaxanthin is its degradation in the gastrointestinal tract and the possibility of first-pass metabolism. A pharmacokinetic study by Choi et al.[1] showed that the hepatic and gastrointestinal elimination extraction ratios of astaxanthin were 0.490 and 0.901, respectively. The value of the elimination extraction ratio ranges between 0 and 1, where a value close to 1 indicates that the drug is eliminated by the intended organ.[1]

To overcome these drawbacks, an astaxanthin nanoemulsion was developed. A nanoemulsion preparation may offer an improvement in dissolution and absorption rates, while also improving the drug release profile.[4,5] Furthermore, to facilitate its use in patients; the astaxanthin nanoemulsion was incorporated into a polymer matrix system to create a thin film for intraoral use. This research was extended to develop a new dosage form to maximize the use of astaxanthin. In this research, astaxanthin was encapsulated in oil in an oil-in-water nanoemulsion system. This nanoemulsion was developed by the self-nanoemulsifying method. Then, two different natural polymers, sodium alginate and gelatin, were selected to obtain the best film matrix that was able to incorporate the astaxanthin nanoemulsion. Both physical and mechanical evaluations of thin film containing an astaxanthin-loaded nanoemulsion (TFANE) were performed, including pH and viscosity of film-forming mixtures, film thickness, film weight uniformity, film disintegration time, tensile strength, percent elongation, and film morphology.

MATERIALS AND METHODS

Materials

Astaxanthin (Astareal® L10) was purchased from Fuji Chemical Industries (Japan). Sunflower oil was purchased from Jan Dekker International (The Netherlands). Polyoxy-35-castor oil (Kolliphor® RH40) was purchased from BASF (Indonesia). Polyethylene glycol 400 (PEG 400) was purchased from Merck (Indonesia). Sodium alginate was purchased from Merck (Indonesia). Poly [butyl methacrylate, (2-dimethylaminoethyl) methacrylate, methyl methacrylate] 1:2:1 (Eudragit® EPO) was purchased from Evonik Industries (Thailand). Gelatin was purchased from Global Capsules Ltd (Bangladesh). All other chemicals used were of pharmaceutical grade.

Preparation of the astaxanthin nanoemulsion

The astaxanthin nanoemulsion was prepared by using the self-nanoemulsifying method with the optimized ratio of oil phase to surfactant to co-surfactant determined in a previous study.[6] Forty milligrams of astaxanthin was added to the 1-gram mixture of oil phase (sunflower oil), surfactant (Kolliphor® RH40), and co-surfactant (PEG 400) in the ratio 1:8:1, respectively. This mixture was then mixed with a mixing speed of 100 rpm for 30 minutes using a magnetic stirrer (IKA® C-MAG HS7), followed by sonication for 1 hour (Krisbow®). A nanoemulsion was formed by addition of deionized water with mild stirring.

Optimization of thin film preparation

In this study, sodium alginate and gelatin were used as thin film-forming polymers, with PEG 400 as a plasticizer. The experiment to optimize both the polymer and the plasticizer concentration that produced the best thin-layer preparation was designed by using Design-Expert® Version 12 Software with the Simple Lattice Design method. A thin film was formed by pouring the wet mixture (WME) into a petri dish with a flat, clean surface of diameter 10 cm and dried for 48 hours at ambient temperature (30˚C±5˚C). After drying, the film thickness and film disintegration time were evaluated. The experimental design results from the software are given in Table 1.

Preparation of TFANE

A quantity of 1.25 g of the astaxanthin nanoemulsion was dispersed slowly into a mixture of the optimized polymer matrix

Table 1. Experimental designs of thin film preparation

Formula	Components			
	Sodium Alginate (g)	Gelatin (g)	PEG 400 (g)	Deionized water
F1	0.125	-	0.625	Add up to 25 mL
F2	0.281	-	0.500	
F3	0.438	-	0.375	
F4	0.594	-	0.250	
F5	0.750	-	0.125	
F6	0.438	-	0.375	
F7	0.125	-	0.625	
F8	0.750	-	0.125	
F9	-	0.25	0.250	
F10	-	0.25	0.250	
F11	-	1.00	0.156	
F12	-	0.75	0.188	
F13	-	1.25	0.125	
F14	-	0.75	0.188	
F15	-	1.25	0.125	
F16	-	0.50	0.219	

PEG: Polyethylene glycol

system and 0.01 g Eudragit® EPO (in 2.5 mL ethanol 96%). The final mixing was performed by adding deionized water up to 25 mL and mixing with a magnetic stirrer (IKA® C-MAG HS7) in 100 rpm for 1 hour. A thin film was formed by pouring this WME onto the flat, clean surface of a petri dish with a diameter of 10 cm and dried for 48 hours at ambient temperature (30˚C±5˚C). Then, the TFANE was cut into a 3x3 cm square.

Physical, chemical, and mechanical characterizations of TFANE

Visual observation and pH determination

Visual observation included observation of the color, odor, and clarity of the WME. The pH of the WME was determined by using a calibrated pH meter (Mettler® Toledo).

Film thickness and weight uniformity

Film thickness was determined by using a micrometer (Mitutoyo®) at three different locations on the film. Meanwhile, the weight uniformity of the thin film was determined by weighing six pieces of thin film (with a size of 3x3 cm) using an analytical balance (Mettler Toledo XS204). It is important to know these parameters because they are directly related to the accuracy of doses in the film. The thickness requirement for thin film dosage form must be in the range of 0.005 to 0.2 mm.[7]

Film disintegration time

The film disintegration time was determined visually in a petri dish containing 10 mL of phosphate buffer, pH 6.8 at 37˚C with shaking every 10 s. Disintegration time is the time at which the film begins to break or collapse. The disintegration time of a good thin film is less than 60 s.[7]

Tensile strength and percent elongation

Mechanical stress tests of TFANE were performed by using a universal testing machine (Oriented UCT-5T). Dry film was cut into pieces of uniform size using a sharp-bladed cutting mold. Film (with area exposed to the stress of 25 mm x 4 mm) was sandwiched between two machine jaws. The load was given to the film gradually (at a speed of 30 mm/minute) and automatically until the film shredded. The test was carried out at 23˚C and 50% relative humidity. Tensile strength is calculated by the applied load at rupture divided by the cross-sectional area of the film. Percent elongation is defined as a strain of the film. Strain is basically the deformation of the strip divided by the original dimension of the sample.

Film morphology

The film morphology of TFANE was examined by scanning electron microscopy (SEM). The sample was sized according to the specimen container, followed by smearing with silver paste at several points before the sample was placed. The sample was dried at 20˚C. The sample was fine coated as a voltage of 1.2 kV, current of 6-7.5 mA, and air pressure of 0.2 torrs for 4 minutes to obtain a sample with a thickness of approximately 400 Å.

Assay of astaxanthin in TFANE

The assay was carried out by dissolving the TFANE (with a size of 3x3 cm) in a volumetric flask containing 10 mL of phosphate buffer pH 6.8 for 30 minutes. Then, the absorbance was measured by ultraviolet-visible spectrophotometry (Genesys™ 10S) at a maximum wavelength of 472 nm. The astaxanthin concentration in the TFANE was calculated by estimating the astaxanthin content in the individual film. The limit of the assay is 85%-115%.[8]

In vitro dissolution test

In vitro dissolution tests were performed using a USP 41 apparatus 2, paddle apparatus. Nine hundred milliliters of phosphate buffer (pH 6.8) was used and maintained at 37˚C ±5˚C, and the paddle was set at 50 rpm. A film sample of 9 cm^2 (3x3 cm) was cut and added to the medium. Five milliliters of samples were removed at predetermined time points at 1; 2; 3; 4; 5; 10; 15; and 20 minutes, and the same amount was replaced with fresh buffer. The withdrawn samples were filtered and analyzed using a spectrophotometer at a wavelength of 472 nm. The percentage release was calculated, and the relationship between time and percentage release was plotted.

Statistical analysis was not used in this research.

RESULTS AND DISCUSSION

Preparation of the astaxanthin nanoemulsion

Self-nanoemulsifying dosage forms are anhydrous homogenous liquid mixtures consisting of an oil, surfactant, drug, and co-surfactant, which spontaneously form an oil-in-water nanoemulsion upon dilution with water under gentle stirring.[4,5] Adding a surfactant and co-surfactant to such systems enhances drug dissolution and formulation dispersibility during dilution with the aqueous medium of GIT. During dilution with water, the active substance dissolves in the oil phase and/or surfactant, which forms a film between the oil and water phases.[4] The appropriate type and ratio of the oil phase, surfactant, and co-surfactant are critical parameters in the formation of nanoemulsion. Based on our previous study, the best ratio between sunflower oil as the oil phase, Kolliphor® RH40 as the surfactant, and PEG 400 as the co-surfactant was 1:8:1, respectively.[6] Our results showed that the astaxanthin nanoemulsion had droplet sizes in the nano-range (26-27 nm) with a polydispersity index less than 0.5 (0.2-0.3) and a zeta potential value more than (-20) mV.

Optimization of thin film preparation

In the preliminary study, prior to formulation of the astaxanthin nanoemulsion in the polymer matrix systems, optimization of polymers and plasticizer concentrations were carried out by using Design-Expert® version 12 Software with Simple Lattice Design method. This software is a tool to determine the optimal variations in polymers and plasticizer concentrations in a thin film preparation. Using this software will produce eight experimental designs for each of the natural polymers that were used. Critical evaluations including film disintegration time and film thickness were carried out to find the best thin film characteristics. The results of the evaluation in the preliminary screening of thin film-matrix systems are given in Table 2. All of the formulas had good characteristics of both disintegration

time and thickness (Table 2). The best characteristic of thin film from both sodium alginate and gelatin were determined by using Simplex Lattice Design modeling. The film thickness and film disintegration time parameters were used to determine the optimum film formulation to be used in the TFANE preparation. The results of data analysis from the model are presented in Figure 1.

Based on the contour plots in Figure 1, it can be seen that the effects of application of sodium alginate and gelatin in the matrix systems were similar. Although the shape of the

Table 2. The results of preliminary screening on optimization of polymer matrix systems

Parameters	Formula							
	F1	F2	F3	F4	F5	F6	F7	F8
Film thickness (mm)*	0.137±0.001	0.159±0.001	0.182±0.001	0.192±0.001	0.203±0.001	0.178±0.001	0.131±0.001	0.205±0.001
	F9	F10	F11	F12	F13	F14	F15	F16
	0.132±0.001	0.135±0.001	0.171±0.001	0.152±0.001	0.199±0.002	0.145±0.002	0.197±0.001	0.144±0.002
	F1	F2	F3	F4	F5	F6	F7	F8
Film disintegration time (s)*	28.78±0.015	35.43±0.015	44.3±0.020	51.55±0.015	56.32±0.020	42.17±0.020	23.94±0.021	59.33±0.015
	F9	F10	F11	F12	F13	F14	F15	F16
	23.03±0.015	24.58±0.021	49.68±0.030	43.89±0.035	58.42±0.020	45.93±0.025	58.00±0.010	33.96±0.020

*Values are given as the mean ± standard deviation (n=3)

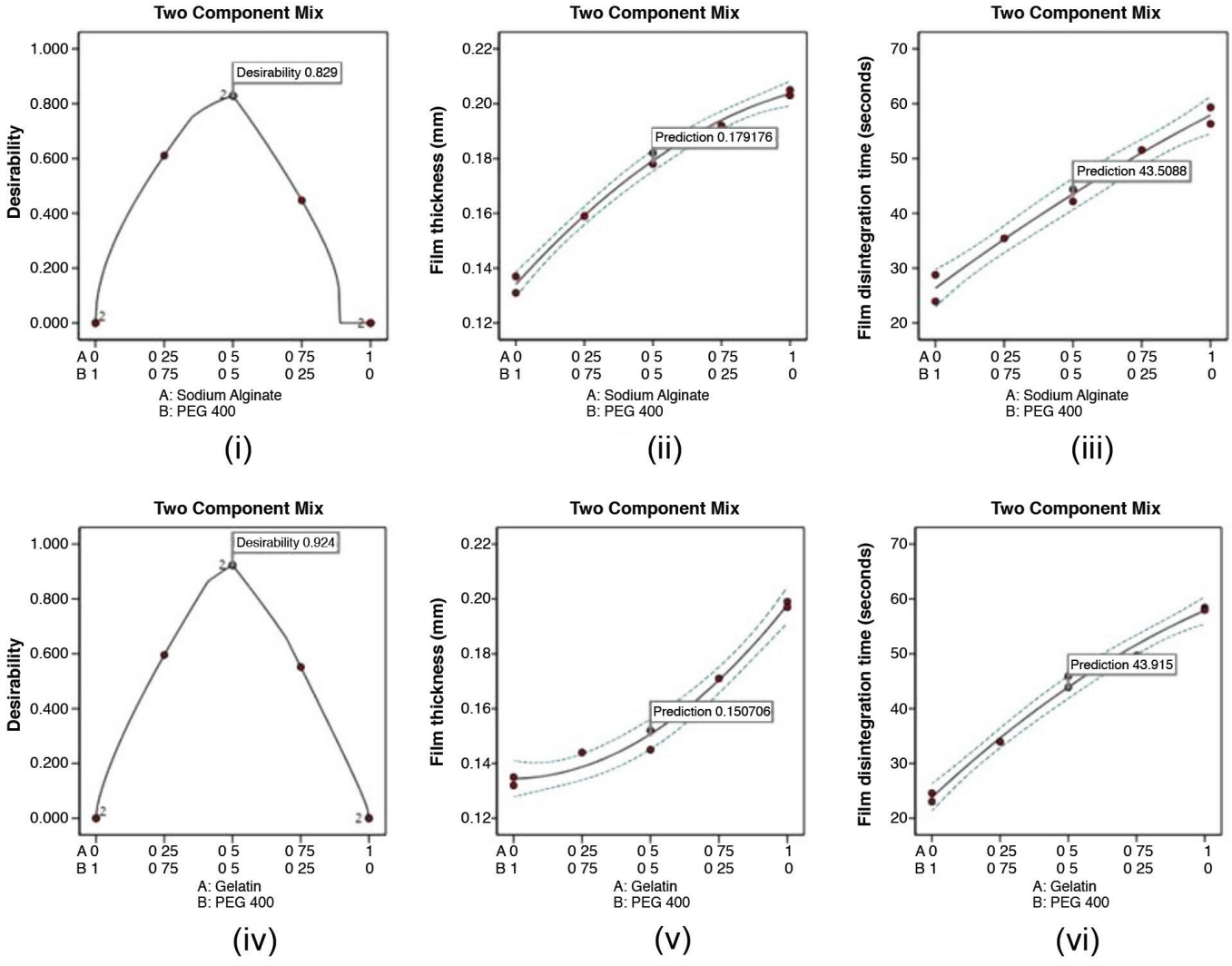

Figure 1. Data analysis of thin film optimization using Simplex Lattice Design modeling. (i)-(iii) for sodium alginate and (iv)-(vi) for gelatin

PEG: Polyethylene glycol

contour plot in film thickness was different, sodium alginate has a convex quadratic shape, whereas gelatin had a concave quadratic shape. The higher of the two polymer concentrations, the longer of the film disintegration time needed and the greater the film thickness. In contrast to the effects of PEG on film thickness and disintegration time, with the higher PEG concentration, a thinner film was produced and the film disintegrated faster. The polymer concentration is an important factor in the development of the thin film. The integrity of fast-dissolving oral films is dependent upon the nature of the selected polymer and its concentration. Different polymers are employed to modulate the diverse properties of films.[9,10] PEG also has good film-forming properties either alone or in combination with other polymers.[11] The disintegration rate of the polymers is decreased by increasing the molecular weight and its concentration of the polymer film matrix system.[12,13] In thin film development, mechanical properties such as tensile strength and percent elongation are improved by adding a plasticizer to the formulations.[12] The mechanical properties of a thin film depends on the plasticizer concentration;[13] thus, proper selection of a plasticizer is very important as improper selection may cause cracking and splitting of the film.[12,14]

The desirability value in Figure 1 (i) showed the highest value (0.829) in a mixture of sodium alginate and PEG 400 with a film thickness of approximately 0.179176 mm and a film disintegration time of approximately 43.5088 s, whereas Figure 1 (iv) shows the highest value (0.924) in a mixture of gelatin and PEG 400 with a film thickness of approximately 0.150706 mm and a film disintegration time of approximately 43.915 s. The desirability value can range from zero (outside of the limits) to one (at the goal). Desirability is simply a mathematical method to determine the optimum (closed to one).[15] Thus, it can be concluded that the two best polymer matrix systems for the preparation of TFANE were mixtures containing 1.75% (w/v) sodium alginate and 1.5% (w/v) PEG 400 and those comprising 3% (w/v) gelatin and 0.75% (w/v) PEG 400.

Preparation of TFANE

The film properties of astaxanthin nanoemulsions prepared with both sodium alginate and gelatin are presented in Table 3. In this study, the films prepared using a 10-mm-diameter petri dish showed good weight homogeneity. All films showed a disintegration time of less than 60 s, which related to ease of drug release from the matrix system. The ideal intraoral film should have the following mechanical properties: High tensile strength and high percent elongation. The astaxanthin nanoemulsion incorporated into the gelatin matrix system had a higher tensile strength and higher percent elongation values than the sodium alginate matrix system (Table 3). Tensile strength is the maximum stress applied to a point at which the film breaks, whereas percent elongation indicates the ability to stretch when a stress is applied. Hard and brittle films demonstrate high tensile strength,[16] which means that the film made from sodium alginate was relatively smoother than that made from gelatin. The percent elongation of the gelatin matrix system was greater than that of the sodium alginate matrix system, which means that the film made from gelatin was more flexible than that made from sodium alginate.

Referring to a study conducted by Lakshmi et al.[17], Eudragit® EPO was selected as the second polymer because the film made from this polymer showed good tensile strength. Other studies have also shown that Eudragit® EPO has taste-masking properties to prevent a negative impact on patient compliance, which is a major consideration when developing an oral formulation.[18,19]

Visual observation of the TFANE was conducted by observing its organoleptic properties. The TFANE was orange in color, odorless, with a smooth surface, and transparent (Figure 2). The morphology of the surface film was observed by SEM (Figure 3). Clear differences were observed between the TFANE containing sodium alginate and gelatin matrix systems, in which the film made from sodium alginate showed a grainier texture than that made from gelatin.

At the end of the study, *in vitro* dissolution tests were performed to compare the drug release profiles of the sodium alginate and gelatin matrix systems. The plotted curves of percentage release over time are shown in Figure 4. The films formed by sodium alginate and gelatin released >90% of the drug within 15 minutes. These results indicate that there was no difference

Table 3. Physical and mechanical properties of TFANE

Parameters	Polymer matrix systems	
	1.75% (w/v) sodium alginate + 1.5% (w/v) PEG 400	3% (w/v) gelatin + 0.75% (w/v) PEG 400
Visual properties of WME	Orange, clear, and odorless	Orange, clear, and odorless
pH of WME*	6.56±0.05	6.80±0.01
Film thickness (mm)*	0.196±0.001	0.184±0.008
Weight uniformity/sheet 3x3 cm (g)*	0.221±0.002	0.202±0.007
Film disintegration time (s)*	48.69±0.10	47.64±0.70
Tensile strength (MPa)*	2.01±0.16	5.33±0.40
Percent elongation (%)*	12.76±1.17	77.15±7.29
Assay of astaxanthin (%)*	98.85±0.54	98.73±0.47

*Values are given as mean ± standard deviation (n=6), TFANE: Thin film containing an astaxanthin-loaded nanoemulsion, PEG: Polyethylene glycol, WME: Wet mixture

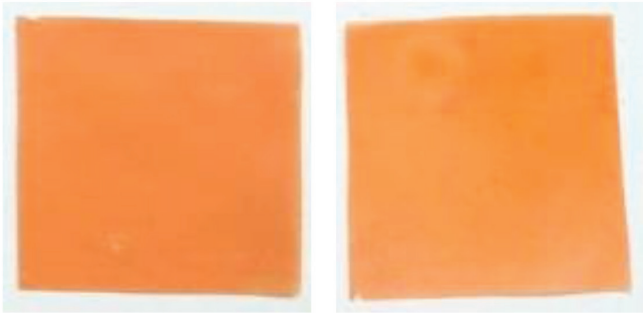

Figure 2. Visual observation of TFANE. (A) TFANE was contained the sodium alginate matrix system and (B) TFANE was contained the gelatin matrix system

TFANE: Thin film containing an astaxanthin-loaded nanoemulsion

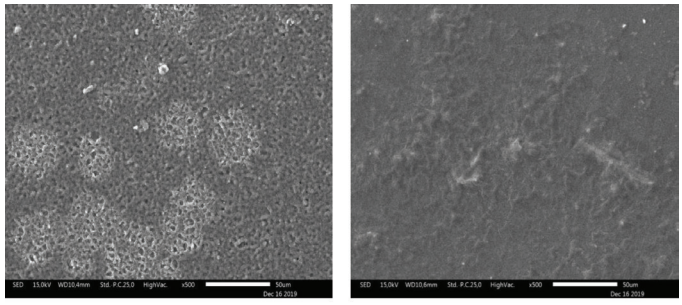

Figure 3. The morphology of TFANE at 500x magnification. (A) TFANE was contained the sodium alginate matrix system, and (B) TFANE was contained the gelatin matrix system

TFANE: Thin film containing an astaxanthin-loaded nanoemulsion

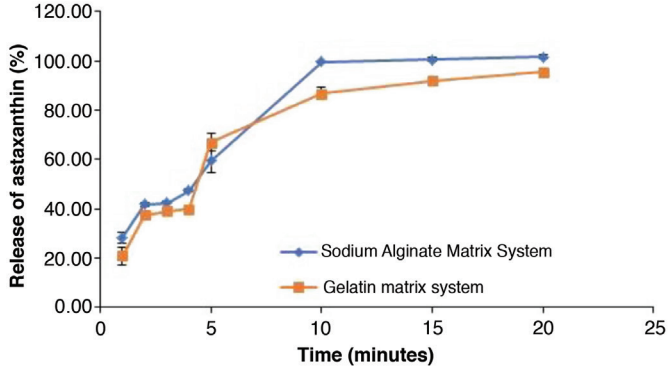

Figure 4. *In vitro* drug release from TFANE prepared using sodium alginate and gelatin polymers

TFANE: Thin film containing an astaxanthin-loaded nanoemulsion

regarding drug release between films made from sodium alginate and those made from gelatin.

CONCLUSION

An intraoral film containing an astaxanthin-loaded nanoemulsion with good physical and mechanical properties was successfully developed using a natural polymer matrix system. The film made from a gelatin matrix system containing astaxanthin nanoemulsion was more flexible and harder than film made from a sodium alginate matrix system, whereas all of the films had the characteristics that made them ideal for intraoral delivery. There was no difference regarding drug release from films made by sodium alginate or gelatin. Gelatin as a natural polymer appears to be promising for the preparation of an intraoral thin film delivery system.

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Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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ORIGINAL ARTICLE

A Novel Analytical Method for the Simultaneous Estimation of Remogliflozin and Metformin Hydrochloride by UPLC/PDA in Bulk and Formulation Application to the Estimation of Product Traces

Bulk ve Formülasyon Uygulamalarında Eser Ürünlerin Kestirimi için UPLC/PDA ile Remogliflozin ve Metformin Hidroklorürü Eşzamanlı Belirleyebilen Yeni Bir Analitik Yöntem

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ABSTRACT

Objectives: A selective and novel method has been optimized for the evaluation of remogliflozin and metformin hydrochloride in bulk and in the formulation and cleaning of samples by UPLC-PDA in bulk and formulation and product traces.

Materials and Methods: The principle analytes were eluted with phosphate buffer (pH: 4.5): acetonitrile (60:40%, v/v) as the mobile phase using the Spherisorb C18, 5 µm, 4.6 mm x 150 mm analytical column with a 1.0 mL/min flow rate and a 10 µL sample volume at 245 nm in a photodiode array detector.

Results: The retention times of remogliflozin and metformin hydrochloride were 3.017 min and 5.011 min with a total run time of 8 min. The curve indicates that the correlation coefficient (r^2) was superior with a value of 1.000 in the linear range of 10 ng/mL-100.0 ng/mL for remogliflozin and 50 ng/mL-500.0 ng/mL for metformin hydrochloride. The correlation coefficient (r^2) for metformin hydrochloride was found to be 1.000. The lower limits of quantification and detection for remogliflozin and metformin hydrochloride were found to be 10 ng/mL and 50 ng/mL, and 5 ng/mL and 10 ng/mL, respectively.

Conclusion: The developed method was validated and applied to the bulk drug estimation and drug formulation and cleaning samples. All the results obtained with this method was accurate and precise.

Key words: Remogliflozin, metformin hydrochloride, bulk drug, formulation, cleaning samples, UPLC-PDA

ÖZ

Amaç: UPLC-PDA ile yığında ve formülasyon uygulamalarında remogliflozin ve metformin hidroklorürün eş zamanlı tayini ve örnek temizliğinin belirlenmesi için seçici ve yeni bir yöntem optimize edilmiştir.

Gereç ve Yöntemler: Temel analitler, hareketli faz olarak fosfat tamponu (pH: 4,5): asetonitril (60: 40%, v/v) ile, Spherisorb C18, 5 µm, 4,6 mm x 150 mm analitik kolon kullanılarak, 1,0 mL/dk akış hızında fotodiyot array dedektörü ile 245 nm'de 10 uL örnek hacmi ile elüe edilmiştir.

Bulgular: Remogliflozin ve metformin hidroklorürün alıkonma süreleri sırasıyla 3,017 dakika ve 5,011 dakikaydı ve toplam çalışma süresi 8 dakikaydı. Eğri, korelasyon katsayısının (r^2), remogliflozin için 10 ng/mL-100,0 ng/mL ve metformin hidroklorür için 50 ng/mL-500,0 ng/mL doğrusal aralıkta 1,000 değeriyle üstün olduğunu göstermektedir. Metformin hidroklorür için korelasyon katsayısı (r^2) 1,000 olarak bulundu. Remogliflozin ve metformin hidroklorür için alt kantifikasyon ve saptama sınırları sırasıyla 10 ng/mL ile 50 ng/mL ve 5 ng/mL ile 10 ng/mL olarak bulunmuştur.

Sonuç: Geliştirilen yöntem valide edilmiş ve yığında, ilaç formülasyonunda ve temizleme numunelerinde ilaç belirlenmesi için uygulanmıştır. Bu yöntemle elde edilen tüm sonuçlar doğru ve kesindir.

Anahtar kelimeler: Remogliflozin, metformin hidroklorür, yığın ilaç, formülasyon, temizleme numuneleri, UPLC-PDA

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INTRODUCTION

Remogliflozin etabonate [5-methyl-4-(4-(1-methylethoxy) benzyl)-1-(1-methylethyl)-1H-pyrazol-3-yl 6-O-(ethoxycarbonyl)-β-D-glucopyranoside] is a pro-drug of remogliflozin. It belongs to the glifozin class of drugs. This drug is primarily used in cases of non-alcoholic steatohepatitis and type-2 diabetes. Remogliflozin inhibits the sodium-glucose transport proteins, which are responsible for glucose reabsorption in the kidney. Metformin (N,N-dimethylimido dicarbonimidicdiamide) is used to lower blood sugar in those with type 2 diabetes. It is also used to treat polycystic ovary syndrome. Metformin is a dimethyl biguanide that reduces elevated blood glucose levels primarily by reducing hepatic glucose production and improving peripheral tissue sensitivity to insulin.[1]

Based on a literature survey, there are no existing analytical methods for this new formulation, i.e., remogliflozin and metformin hydrochloride. Several methods have been developed for other gliflozin drugs, such as dapagliflozin, empagliflozin, and canagliflozin, with other combination of gliptins such as saxagliptin and linagliptin and with biguanides such as metformin.[2-20] For the remogliflozin and metformin hydrochloride combination, there was a lack of sensitive analytical methods for the identification and quantification in bulk and in formulations. Moreover, there was no sensitive analytical method with the 10 ng/mL sensitivity necessary to quantify the product traces left in manufacturing areas after a product changeover.

MATERIALS AND METHODS

Remogliflozin (Figure 1), metformin hydrochloride (Figure 2), and high-purity acetonitrile were from (J.T. Baker, Phillipsburg, NJ, USA); water was from a (Milli-Q system, Millipore, Bedford, MA, USA); potassium dihydrogen phosphate and sodium dihydrogen phosphate were from (Merck Pvt. Ltd, Worli, Mumbai); and ortho phosphoric acid was from (Merck Pvt. Ltd, Worli, Mumbai). The formulation was provided by the Yountus Life Sciences, Andhra Pradesh, India.

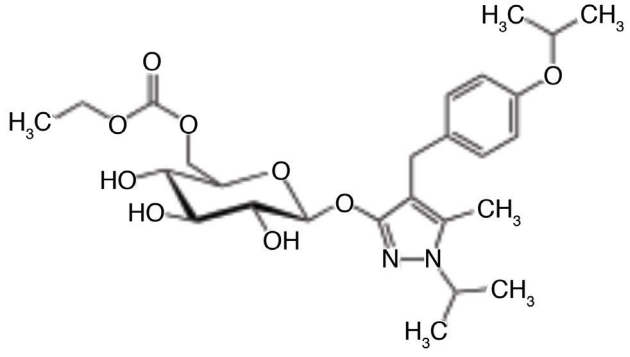

Figure 1. Remogliflozin

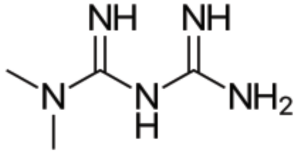

Figure 2. Metformin

Preparation of standard solutions

Metformin hydrochloride and remogliflozin etabonate standard stock solutions were prepared by placing 25.38 mg and 126.92 mg, respectively in 25 mL volumetric flasks and then adding 10 mL diluent and sonicating for 3 minutes. Then, the volume was adjusted to 25 mL with diluent. From the stock 25 mL, 1 mL was removed to a 1000 mL volumetric flask and the volume adjusted to 25 mL with diluent. From this 1000 mL, 1 mL was removed to a 10 mL volumetric flask and the volume adjusted to the mark with diluent to obtain a 100 ng/mL solution of remogliflozin and a 500 ng/mL solution of metformin hydrochloride.

Preparation of buffer (pH 4.5)

Potassium dihydrogen phosphate (13.9 g) and disodium hydrogen phosphate (35.04 g) were weighed precisely and added to a 1000 mL beaker. Water (500 mL) was added and stirred with a glass rod to completely dissolve the salts, and then the volume was adjusted to 1000 mL with water. The prepared buffer solution was adjusted to pH to 4.5 with dilute ortho phosphoric acid.

Preparation of the mobile phase

From the 1000 mL buffer, 600 mL buffer was removed and added to a 1000 mL mobile-phase bottle. Acetonitrile (400 mL) was added to the buffer and the buffer degassed to prepare 1000 mL of mobile phase.

Preparation of diluent

The diluent was prepared by adding 2000 mL of water to a 4000 mL mobile-phase bottle and then adding 2000 mL of methanol and degassing to obtain 4000 mL of diluent.

Optimization of chromatographic conditions

After a series of trials, the final chromatographic conditions were determined as follows. The mobile phase was a buffer with pH 4.5 and acetonitrile (60:40% v/v), and the stationary phase was a Spherisorb C_{18} column with dimensions 5 µm, 4.6 mm x 150 mm to obtain the best peak shape. The separation of remogliflozin and metformin hydrochloride was good at 245 nm with a column temperature of 25°C, a sample compartment temperature of 10°C, a flow rate of 1.0 mL/min, and a sample volume of 10 µL.

Assay sample preparation

One tablet (REMO-M) containing remogliflozin 100 mg and metformin 500 mg was added to a 1000 mL volumetric flask, dissolved in diluent, and the volume adjusted to 1000 mL. This preparation was considered as the stock solution. From the stock solution, 1 mL was removed and added to a 1000 mL volumetric flask and the volume adjusted to the mark with

diluent to obtain 100 ng/mL of remogliflozin and 500 ng/mL of metformin hydrochloride.

Validation of the analytical method

Validation was performed for the developed method within stringent limits to test the efficiency of this method.[1,2]

To verify that the system produced consistent results with the optimized method, the standard was injected 6 times with the criteria of % relative standard deviation (RSD) for retention time (RT) and area not more than (NMT) 2.0%, the oretical plates not less than (NLT) 3000 plates, tailing factor NMT 1.5, and resolution NLT 4.

Selectivity

To verify the method validation in terms of selectivity and exactness, triplicate preparations of 100% concentration, i.e., 100 ng/mL of remogliflozin and 500 ng/mL of metformin hydrochloride, were injected. Then, one blank was also injected to test for carryover. The limit of specificity is that it should pass the system suitability criteria, and there should not be an RT shift for any of the three preparations.

Precision

After passing the specificity and system suitability criteria, the method was verified for system precision and method precision with the limit of % RSD for the RT and area NMT 2%. The intermediate precision was verified on the next day with another column by setting the limit as % RSD for the RT and NMT 2% for the area.

Accuracy and recovery

To verify the method accuracy, triplicate preparations were prepared at 80%, 100%, and 120% of the 100% concentrations (100 ng/mL for remogliflozin and 500 ng/mL for metformin hydrochloride) by spiking the standard into the diluent. The percent recovery was calculated with acceptance criteria of 95%-105%.

Linearity

The method linearity was verified with 5 dilutions of the 100% concentration: 10 ng/mL, 20 ng/mL, 50 ng/mL, 75 ng/mL, and 100 ng/mL for remogliflozin and 50 ng/mL, 100 ng/mL, 250 ng/mL, 375 ng/mL, and 500 ng/mL for metformin hydrochloride. The acceptance criterion of the regression coefficient (R^2) was NLT 0.99.

Robustness

To verify the method efficiency when minor changes occurred in optimized method parameters such as mobile-phase composition, column temperature and flow, and buffer pH, these parameters were tested with the criteria that they should pass the system suitability criteria.

Lower level of quantification (LOQ)

By considering the 10% concentration of the target concentration, the sample was injected into the system with the acceptance criteria S/N ratio NLT 10. From the lower LOQ, preparations of different concentrations were injected to identify the detectability with the acceptance criteria 3:1, and the minimum detectability was five times out of six injections from the same concentration.

Lower level of quantification precision

LOQ precision was verified with the limit NMT 2.0% for the RT and area.

Assessment of stability of the standard and mobile phase

The prepared mobile phase and standard preparations were verified for stability up to 72 hours.

Degradation behavior

To test the developed method for stability indicating method the formulation sample was subjected to acid and base, and thermal, photo, and peroxide degradation were carried with the aim of detection of degradants in the chromatogram. Acid degradation was carried out by adding 20 mL of 0.1N HCL to the stock solution, and from that 1 mL was removed and added to a 1000 mL volumetric flask and the volume adjusted to the mark. In the same way, 2 mL 1N NaOH was added to test for base degradation. To test for thermal degradation, the sample was subjected to heat at 105°C for 3 hours and the sample prepared as per the assay procedure. For photo degradation, the sample was exposed to ultraviolet light with an intensity NLT 2000 lux power for 6 hours and the sample prepared as per the assay procedure. For peroxide degradation, 2 mL H_2O_2 were added to the stock 1000 mL volumetric flask, 1 mL was removed and added to a 1000 mL flask, the volume adjusted to the mark with the diluent, and the sample was injected.

Filter compatibility

To evaluate the impact of polyvinylidene fluoride (PVDF) and Nylon filters on the assay results, the samples were analyzed after passage through the filters.

Recovery of the Swabs from the stainless steel (SS) and glass and epoxy plate

Due to the high sensitivity (nanogram level) of the developed method, it can be used in cleaning method validation or for surface cleaning sample quantification at the time of product changeover in the manufacturing area. Hence, the method applicability for the quantification of surface cleaning samples in the manufacturing area was verified. Three surfaces (SS, glass, epoxy) were selected based on the manufacturing area designs as per the cGmp. Sterile swabs were taken and the recovery verified from the SS plate, glass plate, and epoxy plate with the acceptance criteria NLT 90% with the LOQ concentration (10 ng/mL remogliflozin and 50 ng/mL metformin). The recovery was calculated by pouring the 1 mL sample before the final concentration (after the first dilution in 1000 mL) of the standard preparation on the plates. After drying, the swab was added to a 10 mL volumetric flask and the volume adjusted to the mark with diluent.

Statistical analysis

The data were processed through the Q Sight software, and the results were calculated as mean and ± SD for the accuracy and

the RSD was calculated for the precision. The coefficient of regression was also calculated in the linearity parameter.

RESULTS

Clear separation and good resolution without any carryover was achieved with this method as shown in Figure 3-6. The system suitability acceptance criteria were also found to be satisfactory as shown in Table 1, 2. For the system precision parameters, the % RSD of RT and area for remogliflozin and metformin hydrochloride achieved 0.02% and 0.03%, and 0.01% and 0.03% as shown in Table 3 against the limit NMT 2.0%. For the method precision parameters, the %RSD of RT and area for remogliflozin and metformin hydrochloride achieved 0.03% and 0.02%, and 0.02% and 0.05% against the limit NMT 2.0% as shown in Table 4. The linearity parameter was quantified by peak area vs. concentration methodology. Different concentrations from 10 ng/mL to 100 ng/mL standard solutions for remogliflozin and from 50 ng/mL to 100 ng/mL were prepared and injected into the system. The recovery for 80%, 100%, and 120% was more than 99% against the acceptance criteria of 95%-105% as shown in Table 5 and Figure 7-9. The calculated regression coefficient for remogliflozin and metformin hydrochloride was 1.000 as shown in Figure 10, 11. To evaluate the method's capability of producing precise results with minor variations in flow, mobile-phase composition, pH, and column temperature variations, a test for robustness was performed. The results are shown in the Table 6. The results prove that the method was stable to produce consistent results with minor variations of the method parameters. The compatibility of the filters was verified with PVDF and Nylon filters. The assay for remogliflozin and metformin hydrochloride was more accurate (100.2% for remogliflozin and 99.7% for metformin hydrochloride) with the PVDF filter compared with the Nylon filter (99.8% for remogliflozin, 98.9% for metformin hydrochloride) as shown in Table 7. To demonstrate that the

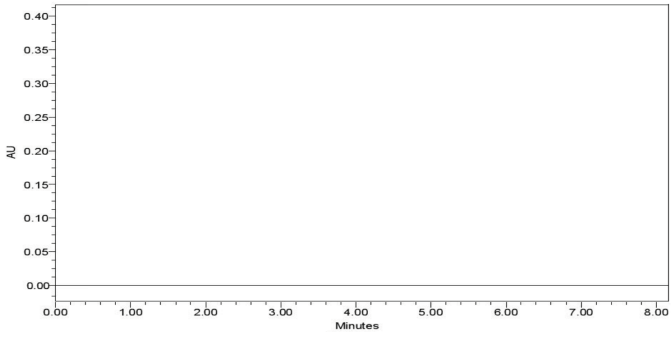

Figure 3. Blank chromatogram

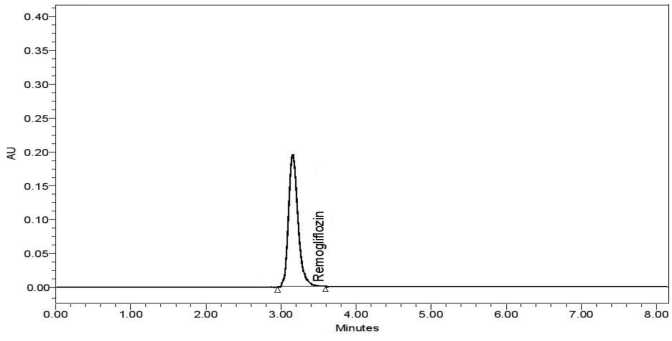

Figure 4. Specificity chromatogram of remogliflozin

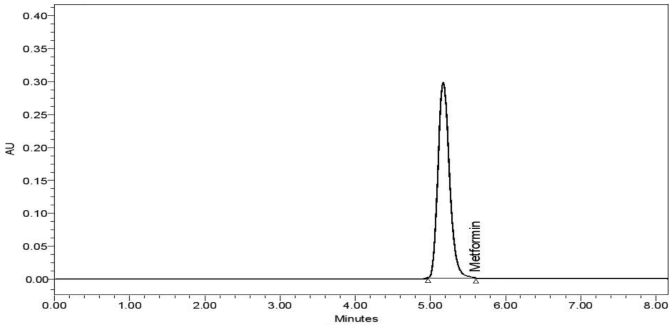

Figure 5. Specificity chromatogram of metformin

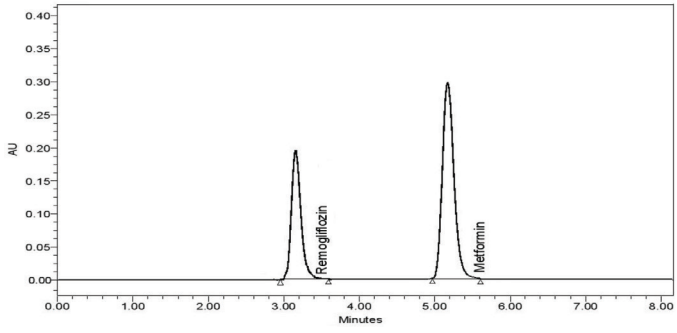

Figure 6. System suitability chromatogram of remogliflozin and metformin

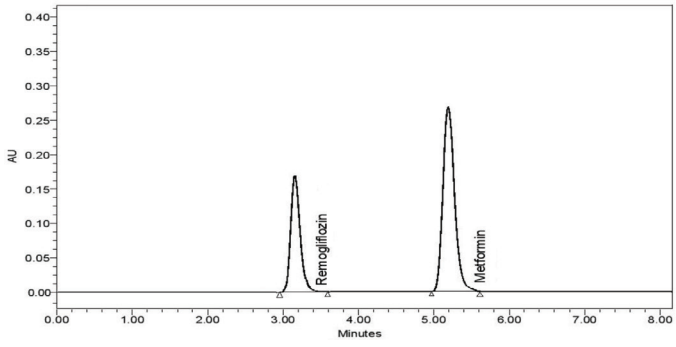

Figure 7. 80% accuracy level chromatogram of remogliflozin and metformin

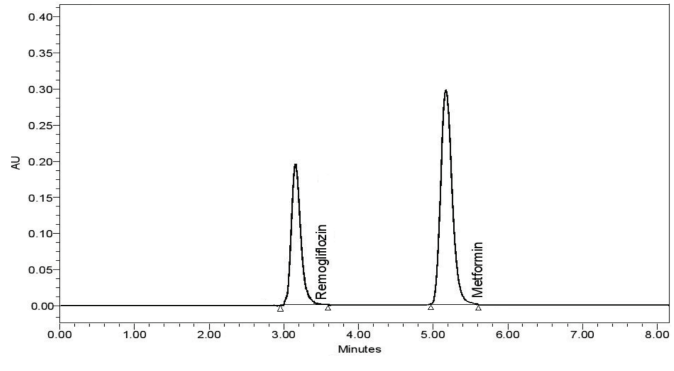

Figure 8. 100% accuracy level chromatogram of remogliflozin and metformin

Table 1. Specificity data

S. no	Injection	Remogliflozin	RT	Area	Metformin	RT	Area
01	Blank	Not detected	NA	NA	Not detected	NA	NA
02	01	Detected	3.018	983652	Detected	5.011	1215689
03	02	Detected	3.017	983259	Detected	5.012	1215697
04	03	Detected	3.018	983452	Detected	5.011	1215986

RT: Retention time

Table 2. System suitability data

Parameter	Remogliflozin	Metformin
Retention time	3.017	5.011
Area	983717	1216101
Asymmetry	0.8	1.1
Theoretical plates	6200	7800
Resolution	5.4	
% RSD of area	0.02	0.03

RSD: Relative standard deviation

Table 3. System precision data

Drug name	Remogliflozin		Metformin	
Injection	RT	Area	RT	Area
01	3.018	983251	5.011	1215641
02	3.017	983652	5.012	1216121
03	3.018	983569	5.011	1215624
04	3.018	983569	5.011	1215698
05	3.017	983957	5.012	1215564
06	3.018	983267	5.011	1216521
Average	3.018	983544	5.011	1215862
SD	0.0005	263.0821	0.0005	380.1435
% RSD	0.02	0.03	0.01	0.03

RSD: Relative standard deviation, SD: Standard deviation, RT: Retention time

method was stable, acid degradation was carried out, and the degradants were identified at 4.019 min and 6.017 min as shown in Figure 12. In base degradation, the degradants were detected at 4.516 min and 5.802 min and 7.224 min as shown in Figure 13. In light degradation, the degradants were detected at 3.681 min and 5.844 min and 6.192 min as shown in Figure 14. In thermal degradation, the degradants were detected in 3.841 min and 4.412 min and 5.942 min and 6.454 min as shown in the Figure 15. In the peroxide stress condition, the degradants occurred at 3.642 min and 4.235 min and 6.94 min and 7.421 min as shown in Figure 16. The LOQ for remogliflozin was 10 ng/mL and 50 ng/mL with S/N ratios of 11.8 and 10.8 as shown in Table 8. The LOQ precision was also performed to evaluate the repeatability at the lower end of the quantification range. The obtained % RSD of the area for remogliflozin and metformin hydrochloride

Table 4. Method precision data

Drug name	Remogliflozin		Metformin	
Injection	RT	Area	RT	Area
01	3.016	983958	5.012	1215632
02	3.015	983587	5.011	1216985
03	3.017	983695	5.012	1215896
04	3.016	983895	5.013	1215348
05	3.015	983958	5.012	1215835
06	3.017	983689	5.011	1215798
Average	3.016	983797	5.012	1215916
SD	0.0009	159.7586	0.0008	559.8081
% RSD	0.03	0.02	0.02	0.05

RSD: Relative standard deviation, SD: Standard deviation, RT: Retention time

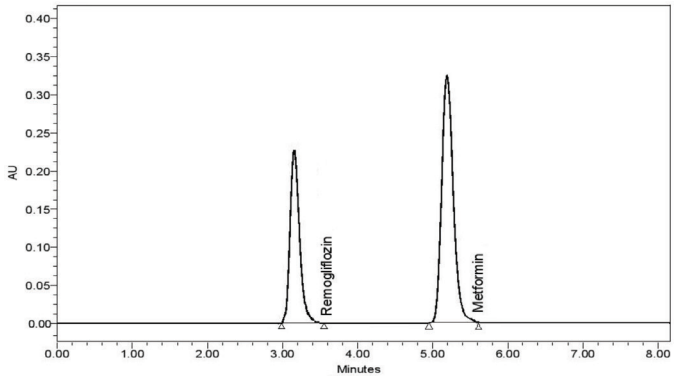

Figure 9. 120% accuracy level chromatogram of remogliflozin and metformin

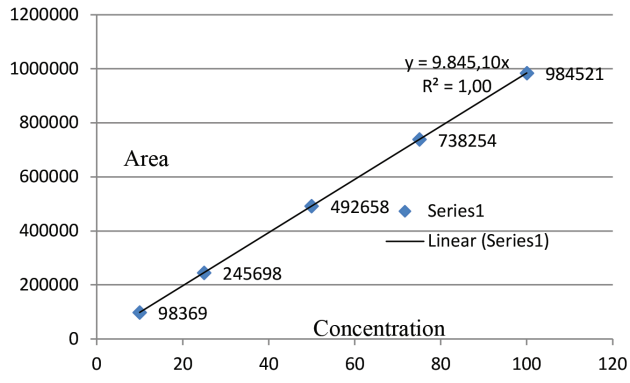

Figure 10. Linearity graph of remogliflozin

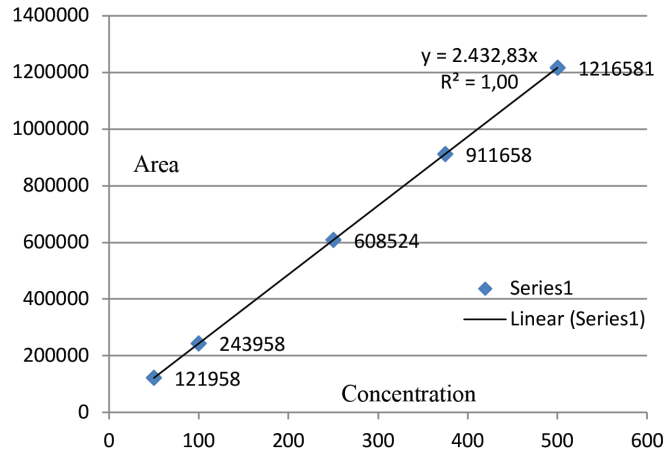

Figure 11. Linearity graph of metformin

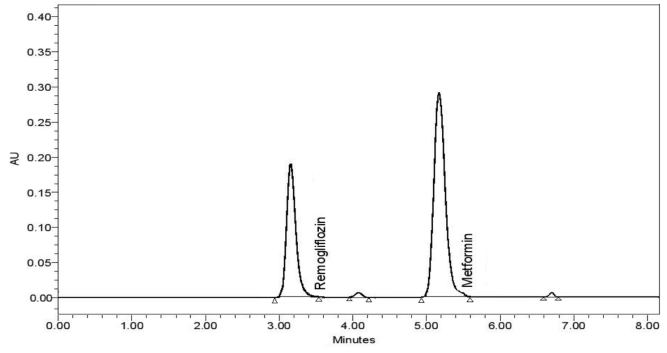

Figure 12. Acid degradation chromatogram

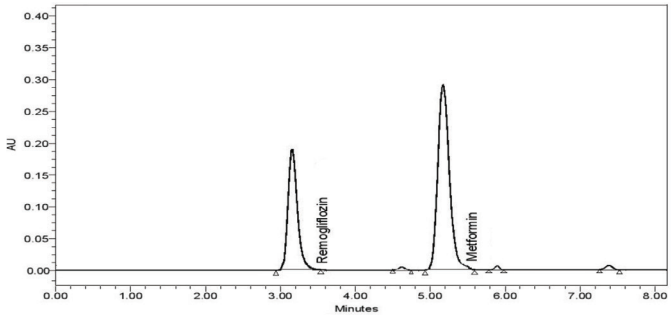

Figure 13. Base degradation chromatogram

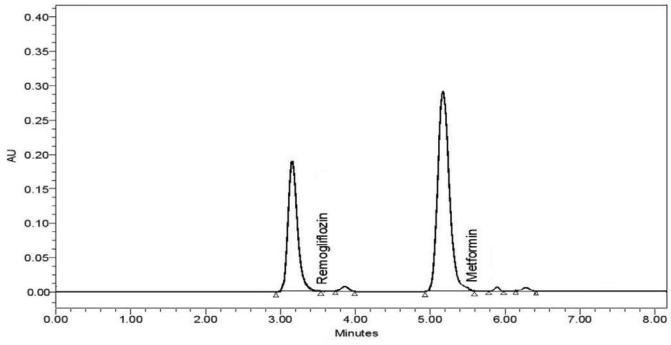

Figure 14. Photo degradation chromatogram

was 0.03 and 0.18% as shown in Table 9. The lower limit of detection (LOD) for remogliflozin was 5 ng/mL and 10.0 ng/mL with an S/N ratio of 3.8 and 3.5 as shown in Table 10, and clear detection is shown in Figure 17. For the intermediate precision parameter, the % RSD of area for remogliflozin and metformin hydrochloride achieved on day-1 was 0.03% and 0.02 and on the next day 0.06% and 0.02% against the limit NMT 2.0% as shown in Table 11. Solution and mobile-phase stability were established, and it was confirmed that the solution and mobile phase were stable for 72 hours as per the data furnished in Table 12. The purity angle and purity threshold were good as shown in Table 13. From these results, we can conclude that the method was stable. The method was verified for robustness as well as interday and intraday precision. The LOQ and LOD were identified by injecting the lower concentrations with the S/N ratio criteria, and the drugs were detected six times out of six injections. The obtained % RSD showing the capability of

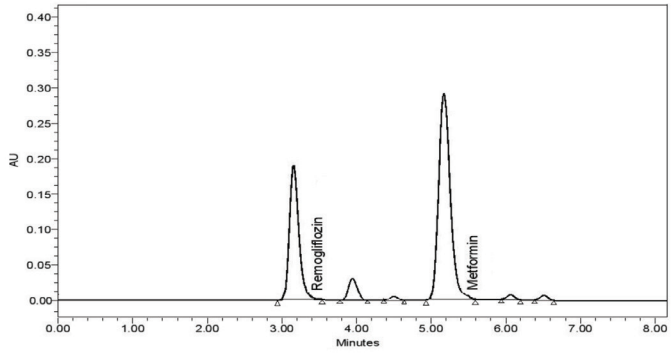

Figure 15. Thermal degradation chromatogram

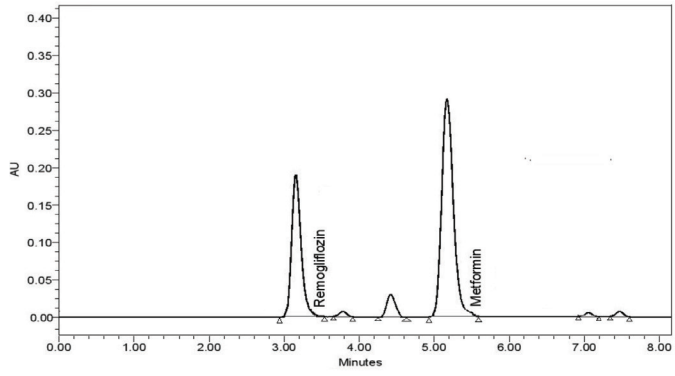

Figure 16. Peroxide degradation chromatogram

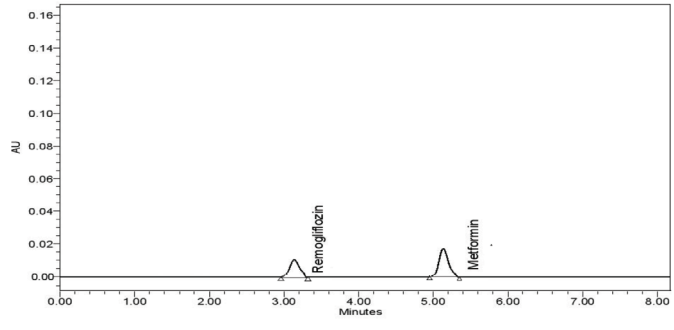

Figure 17. Limit of detection chromatogram

also quantifying the activities at lower concentrations. Then, the method was applied for recovery on a SS plate, a glass plate and an epoxy plate with the aim of recovery NLT 90% to prove its utility in cleaning method validation. The obtained average recovery for remogliflozin and metformin hydrochloride was above 94% as shown in Table 14-16.

DISCUSSION

During method optimization, organic solvents were initially used as the mobile phase with water in varying composition. However, neither compound was detected. Then, buffer was used with organic solvent such as acetonitrile in different ratios and at varying pH with the Spherisorb C_{18}, 5 µm,

Table 5. Accuracy and recovery data

S. no	Drug name	% Level spiking	Spiked amount (ng)	Area	Recovered amount (ng)	% Recovery	% CV
01	Remogliflozin	80	80.06	786851	80.00	99.9	0.10
			80.14	785695	79.88	99.7	
			80.22	785968	80.22	99.6	
02		100	100.08	983561	100.00	99.9	0.04
			99.90	982564	99.90	100.0	
			100.00	983651	100.01	100.0	
03		120	120.01	1178952	119.86	99.9	0.19
			120.17	1176951	119.66	99.6	
			120.09	1175689	119.53	99.5	
01	Metformin	80	403.32	974258	402.73	99.9	0.03
			403.40	975121	403.08	99.9	
			403.32	974568	402.85	99.9	
02		100	504.25	1216495	502.86	99.7	0.13
			504.21	1219585	504.14	100.0	
			504.25	1220214	504.40	100.0	
03		120	605.02	1459889	603.47	99.7	0.10
			605.09	1462315	604.47	99.9	
			605.17	1454898	601.41	99.7	

Table 6. Robustness data

Condition	Value	Remogliflozin				Metformin		
		RT	Area	Asymmetry	Resolution	RT	Area	Asymmetry
Flow	0.8 mL/min	3.112	984526	0.84	5.2	5.112	1218987	1.12
	1.0 mL/min	3.018	983625	0.81	5.4	5.011	1214658	1.10
	1.2 mL/min	2.997	982652	0.80	5.1	4.998	1214236	1.14
Mobile phase composition (buffer:acetonitrile)	55/35 v/v	3.201	984265	0.82	5.3	5.042	1210565	1.13
	60/40 v/v	3.016	983584	0.81	5.4	5.012	1215987	1.11
	65/45 v/v	2.895	982674	0.84	5.3	5.001	1201985	1.19
pH	4.0	2.965	984652	0.82	5.5	5.125	1219875	1.13
	4.5	3.017	983875	0.80	5.4	5.012	1215897	1.10
	5.0	2.912	982159	0.83	5.3	4.958	1219837	1.17
Column temperature	23	3.124	983121	0.82	5.3	5.064	1219856	1.12
	25	3.016	983898	0.80	5.4	5.011	1215648	1.10
	27	2.986	983687	0.85	5.3	4.985	1219765	1.15

RT: Retention time

Table 7. Filter compatibility

Drug name	0.2 µm PVDF filter assay	0.2 µm Nylon filter assay
Remogliflozin	100.2%	99.8
Metformin	99.7%	98.9
Difference	0.4% for remogliflozin, 0.8% for metformin	
Suitability	PVDF 0.2 µM filter	

PVDF: Polyvinylidene fluoride

Table 8. Limit of quantitation

Drug name	Area	LOQ	S/N ratio
Remogliflozin	98526	10 ng/mL	11.8
Metformin	122652	50 ng/mL	10.8

LOQ: Limit of quantitation, S/N: Signal to noise

Table 9. Limit of quantitation precision

Drug name	Remogliflozin		Metformin	
Injection	RT	Area	RT	Area
01	3.017	98537	5.013	122561
02	3.015	98579	5.012	122565
03	3.018	98567	5.012	122869
04	3.017	98521	5.011	122875
05	3.017	98585	5.011	122856
06	3.018	98596	5.011	122359
Average	3.017	98564	5.012	122681
SD	0.0011	29.24665	0.0008	216.8616
% RSD	0.04	0.03	0.02	0.18

RT: Retention time, RSD: Relative standard deviation, SD: Standard deviation

Table 10. Limit of detection

Drug name	Area	LOD	S/N ratio
Remogliflozin	49263	5 ng/mL	3.8
Metformin	24530	10 ng/mL	3.5

LOD: Limit of detection, S/N: Signal to noise

4.6 mm x 150 column. Finally, the method was found to be optimized with the conditions of mobile phase [buffer pH 4.5 and acetonitrile (60:40% v/v), wavelength 245 nm, flow rate of 1.0 mL/min, column temperature of 25˚C, sample compartment temperature of 10˚C, and sample volume of 10 µL]. With this method, both active compounds, i.e., remogliflozin and metformin hydrochloride eluted at 3.017 min and 5.011 min with good resolution and symmetry. Following method optimization, the method was validated as per ICH guidelines. As per the results obtained in the method validation, there was no interference of the blank or carryover problem, even at the LOQ. Both the LOQ and LOD of this method were verified practically in the instrument with S/N ratio criteria. The results were found to be satisfactory. The method was applied to degraded samples to verify its usefulness within the shelf-life period (stability indicating nature). The method detected degradants successfully in all the degradation conditions. As the method was highly sensitive, it was applied to the quantification of cleaning samples of manufacturing area surfaces with the criteria of recovery NLT 90%. Based on the results of recovery from SS, glass, and epoxy plates, this method has proven its capability to analyze cleaning validation samples at the time of products changeover in the manufacturing area.

CONCLUSION

Based on the results obtained in the current study, the developed method was very sensitive, accurate, linear, and economical. Due to the short duration of the chromatographic program, more samples can be analyzed within a short period, which will be helpful in the industry at a time when multiple products are manufactured continuously. The method met all the predefined acceptance criteria. With this method, the sample of bulk and formulation samples and surface cleaning samples can be analyzed. As the method is capable of detecting degradant formulations, bulk shelf-life samples can also be analyzed by using this method.

Table 11. Ruggedness data

Drug name	Injection	Day-1	Day-2	Drug name	Day-1	Day-2
Remogliflozin	01	983562	983256	Metformin	1216525	1215698
	02	984452	983265		1216956	1215669
	03	983652	983598		1215985	1215985
	04	983598	983645		1215152	1215678
	05	983675	983759		1214985	1215345
	06	983656	983458		1216256	1215985
Average		983766	983497		1215977	1215727
Standard deviation		338.7662	206.9661		774.7693	239.0219
% RSD		0.03	0.02		0.06	0.02

RSD: Relative standard deviation

Table 12. Standard and mobile-phase stability

Drug name	Remogliflozin		Metformin	
Injection	RT	Area	RT	Area
Initial	3.018	983251	5.011	1215641
12	3.015	983165	5.011	1215591
24	3.017	982991	5.012	1215232
36	3.017	982854	5.012	1214985
48	3.016	982718	5.012	1214568
72	3.015	982568	5.011	1214121
Average	3.016	982925	5.012	1215023
SD	0.0012	262.1334	0.0005	595.0297
% RSD	0.04	0.03	0.01	0.05

RT: Retention time, RSD: Relative standard deviation, SD: Standard deviation

Table 13. Degradation study on drug product data

Drug name	Condition	Peak area	% Recovery	% Degradation	Purity angle	Purity threshold
Remogliflozin	Undegraded	985652	100.2	-	-	-
	Acid	980125	99.6	0.6	0.211	1.221
	Base	981002	99.7	0.5	0.201	1.212
	Photo	979758	99.6	0.6	0.214	1.298
	Thermal	935654	95.1	5.1	0.944	1.720
	Peroxide	925452	94.1	6.1	0.984	1.611
Metformin	Undegraded	1209568	100.0	-	-	-
	Acid	1206521	99.7	0.3	0.116	1.141
	Base	1195681	98.9	1.1	0.311	1.351
	Photo	1195282	98.8	1.2	0.329	1.324
	Thermal	1186525	98.1	1.9	0.365	1.285
	Peroxide	1176521	97.3	2.7	0.485	1.261

Table 14. Recovery on stainless steel plate of 100% spiking

Drug name	Amount spiked (ng/mL)	Recovery (ng/mL)	% Recovery
Remogliflozin	10.17	9.66	95.0
Metformin	50.48	48.83	96.7

Table 15. Recovery on glass plate of 100% spiking

Drug name	Amount spiked (ng/mL)	Recovery (ng/mL)	% Recovery
Remogliflozin	10.32	9.77	94.6
Metformin	50.88	48.71	95.7

Table 16. Recovery on epoxy plate of 100% spiking

Drug name	Amount spiked (ng/mL)	Recovery (ng/mL)	% Recovery
Remogliflozin	10.13	9.71	95.9
Metformin	50.40	48.16	95.5

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ORIGINAL ARTICLE

Development and Validation of an HPLC Method Using an Experimental Design for Analysis of Amlodipine Besylate and Enalapril Maleate in a Fixed-dose Combination

Amlodipin Besilat ve Enalapril Maleatın Sabit Dozlu Kombinasyondan Analizi için Deney Tasarımı Yoluyla Bir YBSK Yöntemi Geliştirilmesi ve Validasyonu

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ABSTRACT

Objectives: The aim of this study was to develop and optimize a simple, cost-effective, and robust high-performance liquid chromatography (HPLC) method by taking an experimental design approach to the assay and dissolution analysis of amlodipine besylate and enalapril maleate from a fixed-dose combination tablet.

Materials and Methods: The chromatographic analysis was performed on a C18 column (4.6x250 mm id., particle size of 5 µm). The injection volume was 5 µL, and the detection wavelength was 215 nm. A Box-Behnken design was used to test the robustness of the method. The flow rate (1, 1.2, and 1.4 mL/min), column temperature (25˚C, 30˚C, and 35˚C), methanol ratio of the mobile phase (5, 10, and 15%), and pH of the mobile phase (2.8, 3, and 3.2) were selected as independent variables. The method was validated according to International Conference on Harmonization guidelines. Dissolution of the tablets was performed by using USP apparatus 2 and analyzed using the optimized HPLC method. Multivariate linear regression analysis and ANOVA were used in the statistical evaluation.

Results: Linear models were fitted for all variables. The flow rate was the most significant factor affecting the APIs' concentrations. The optimized method included the following parameters: Column temperature of 25˚C, 10% methanol as the mobile phase, pH of 2.95, and flow rate of 1.205 mL/min. Retention times were 3.8 min and 7.9 min for enalapril and amlodipine, respectively. The method was found to be linear in the range of 0.8-24 µg/mL (R^2 >0.999) and 1.6-48 µg/mL (R^2 >0.999) for amlodipine and enalapril, respectively. Both APIs were dissolved more than 85% within 10 min.

Conclusion: The experimental design was proved as a useful tool for the determination and separation of enalapril maleate and amlodipine besylate in dosage forms. The optimized method can be used for *in vitro* performance and quality control tests of fixed-dose tablet combinations containing enalapril maleate and amlodipine besylate.

Key words: Amlodipine, enalapril, design of experiment, HPLC, fixed-dose combination

ÖZ

Amaç: Bu çalışmanın amacı, amlodipin besilat ve enalapril maleat içeren sabit dozlu kombinasyon tabletinden disolüsyon ve miktar tayini analizi için deney tasarımı yaklaşımı ile basit, ekonomik ve sağlam bir yüksek basınçlı sıvı kromatografisi (YBSK) yönteminin geliştirilmesi ve optimizasyonudur.

Gereç ve Yöntemler: Kromatografik analiz C18 kolonda (4,6x250 mm id., 5 µm partikül çapı) gerçekleştirilmiştir. Enjeksiyon hacmi 5 µL ve dalga boyu 215 nm'dir. Yöntemin sağlamlığının test edilmesinde Box-Behnken tasarımı kullanılmıştır. Akış hızı (1, 1,2, ve 1,4 mL/dk), kolon sıcaklığı (25°C,

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30°C ve 35°C), hareketli fazdaki metanol oranı (%5, %10 ve %15) ve hareketli fazın pH'sı (2,8, 3 ve 3,2) bağımsız değişkenler olarak seçilmiştir. Yöntemin validasyonu ICH kılavuzlarına göre gerçekleştirilmiştir. Tabletlerin çözünme hızı deneyleri USP cihaz 2 kullanılarak 75 devir/dk hızda gerçekleştirilmiştir. Çözünme hızı çalışması 0,1 N HCl'da 37±0,5°C'de yapılmış ve optimize edilen YBSK yöntemi ile analiz edilmiştir. İstatistiksel değerlendirmede çok değişkenli doğrusal regresyon analizi ve ANOVA testi kullanılmıştır.

Bulgular: Tüm değişkenler için doğrusal modeller kullanılmıştır. Etkin madde konsantrasyonlarını etkileyen en anlamlı faktör akış hızıdır. Optimize edilen yöntem şu parametreleri içermektedir: 25°C kolon sıcaklığı, hareketli fazda %10 metanol oranı, 2,95 hareketli faz pH'sı ve 1,205 mL/dk akış hızı. Alıkonma zamanları enalapril ve amlodipine için sırasıyla 3,8 dk ve 7,9 dk olarak bulunmuştur. Yöntem amlodipin ve enalapril için sırasıyla 0,8-24 µg/mL (R^2 >0,999) ve 1,6-48 (R^2 >0,999) µg/mL aralıkta doğrusal bulunmuştur. Her iki etkin madde de 10 dakika içinde %85'ten fazla çözünmüştür.

Sonuç: Enalapril maleat ve amlodipin besilatın dozaj formlarından analizinde deney tasarımı faydalı bir yaklaşım olarak görülmüştür. Optimize edilen yöntemin enalapril ve amlodipin içeren bir sabit dozlu kombinasyonun *in vitro* performansı ve kalite kontrol testlerinde kullanılabileceği gösterilmiştir.

Anahtar kelimeler: Amlodipin, enalapril, deney tasarımı, YBSK, sabit dozlu kombinasyon

INTRODUCTION

At the early stages of the treatment of hypertension, it can be useful to choose monotherapy to observe the effect and the side effects of the drug. However, monotherapy can be insufficient to reach the target blood pressure in a majority of patients.[1-3] A greater therapeutic benefit can be achieved with two or even more antihypertensive drugs.[4] Therefore, fixed-dose combinations (FDCs) are frequently used in cardiovascular diseases such as hypertension. In order to develop an FDC product including two drugs, certain conditions must be met. For instance, a synergistic effect can be observed using two drugs together, or a side effect related to a drug may be eliminated using the other drug concurrently.[5] In the treatment of hypertension, there is a synergistic effect between calcium channel blockers (CCBs) and angiotensin-converting enzyme inhibitors (ACEIs). In addition, ACEIs such as enalapril prevent peripheral edema caused by CCBs such as amlodipine.[6]

Amlodipine is a long-acting CCB that inhibits the transmembrane influx of calcium ions into vascular smooth muscle and cardiac muscle. It is indicated for the treatment of hypertension and coronary artery disease when used alone or in combination with another antihypertensive agent.[7] Amlodipine is given orally as besylate in general, but doses are calculated in terms of amlodipine base. A dose of 6.94 mg of amlodipine besylate is equivalent to 5 mg of amlodipine base. The recommended dose of amlodipine is 5-10 mg once daily.[8] Since amlodipine is a weak base, it exhibits high solubility in physiological pH values. Although the bioavailability of amlodipine is approximately 60%-65%, it is defined as a highly permeable drug because of the 90%-95% excretion rate as an inactive metabolite in the urine Shohin et al.[9] Amlodipine is a class 1 drug according to the Biopharmaceutics Classification System (BCS).[9-11]

Enalapril is the ethyl ester of enalaprilat, an ACEI indicated for the treatment of hypertension and heart failure. Enalapril is available as maleate salt in the drug market. Enalapril maleate is a white crystalline powder sparingly soluble in water. Although the solubility is 25 mg/mL at pH 3.5, it increases to 200 mg/mL at pH 7.0. It is defined as BCS class 3 with high solubility but low permeability properties.[12]

There are high-performance liquid chromatography (HPLC) methods recommended in United States Pharmacopeia (USP42) for analysis of amlodipine besylate[13] and enalapril maleate,[14] separately and a few liquid chromatography methods are available in the literature for analyses of amlodipine,[15] and enalapril,[16,17] individually or in combination with other drugs.[18-23] However, these methods are not suitable for the separation of amlodipine and enalapril in the same dosage unit. Nevertheless, there are three published articles for HPLC analysis of amlodipine besylate and enalapril maleate together in dosage forms.[24-26] However these methods contain a high ratio of organic solvents in the mobile phase, which is environmentally inappropriate according to the green chemistry approach. An important principle of green chemistry is to reduce toxic organic solvents and to consume safer chemicals.[27,28] Relating to the green analytical chemistry approach, Korany et al.[27] recommended reducing the acetonitrile amount in the methods and using multiparameter methods such as design of experiment (DOE) instead of the one factor at a time (OFAT) approach.[28] In the method developed by Chaudhari[24], the mobile phase contains 50% acetonitrile and 40% methanol and a higher injection volume (20 µL), which increases the consumption of mobile phase and the linearity range was comparatively narrow (0.5-6 µg/mL and 0.5-8 µg/mL for enalapril and amlodipine, respectively). In another method, the mobile phase includes 60% acetonitrile, the injection volume was 20 µL, and the linearity range was not suitable for lower concentrations (20-100 µg/mL), which might be essential for the initial points of the dissolution tests.[25] In the method developed by Masih et al.[26], 50% 1N HCl and 50% methanol were included in the mobile phase, and the injection volume was 10 µL. Additionally, none of the studies include the application of DOE in robustness testing in validation for amlodipine besylate and enalapril maleate. Furthermore, there is no dissolution analysis of enalapril and amlodipine in the combined dosage form in the literature.

DOE is a well-defined mathematical methodology to demonstrate how to obtain maximum reliable and valuable scientific information by performing minimal experiments.[29] In this technique, the effects of multiple variations on one or more responses can be investigated at the same time, instead of changing OFAT. Although conventional developmental approaches are mainly empirical and are often conducted using the changing OFAT method, DOE provides the facility of performing systematic and multivariate experiments in order to entirely understand the process and to assess the statistical significance of the variables.[30,31] By creating experimental

matrix, DOE allows faster visualization and determination of more factors at a time.[32] Besides, in OFAT approach factors are evaluated independently, so it is assumed that the factors do not influence each other. However, the potential interactions between the factors can be identified using the appropriate DOE model.[33,34] In the pharmaceutical field, DOE helps to understand the effects of the critical formulation and process variables on the final product.[35,36] DOE can be used for factor screening and characterization of a new system or optimization of a characterized system. Factors are independent variables that might affect the results of critical responses. For instance, in an analytical method development process, the flow rate can be an independent factor that has potential effects on the peak area of the analyte. In a screening design it is aimed to investigate numerous factors that might affect the response and to discover the factor which has the most significant influence on the responses.[37] On the other hand, in an optimization process, the main objective of which is to define the optimal conditions and settings for the factors.[38] In case more than one factor must be examined, the multivariate optimization designs can be reasonable in order to evaluate different factors at the same time and to determine if interactions exist between factors.[37,38]

In analytical chemistry, DOE can be used for chromatographic analytical method development to optimize the sampling preparational, column, detector, instrumental, or environmental factors.[31,39] Similarly, analytical method validation parameters such as accuracy, linearity, precision, or robustness can be performed by experimental design approaches.[29,40-46] Using DOE in validation studies is recommended in the International Conference on Harmonization (ICH) guidelines.[27,47] There have been many studies in which DOE was applied to robustness.[31,32,43,48,49] Experimental design targeting robustness is a good approach to fully understand the factors with effects on the responses and provide maximum information about the method in a short time. Robustness should be built into methods in the pre-validation stages; otherwise, a robustness test performed too late has a risk of obtaining inappropriate results which can cause redevelopment and revalidation.[50] Therefore, a robustness test in the earlier stage of the method development process leads to a saving of effort, time, and money. Experimental data obtained from early stages can aid in performance method evaluation and can be used to guide further method development.[51]

Optimization can be performed by using response surface methodology (RSM) designs such as the Box-Behnken design (BBD) and the central composite design (CCD).[49,52] The BBD is a second-order design that allows investigation of numerous factors with three levels. It is preferable to the CCD because it prevents an unrealistic extreme scenario by creating the experimental matrix without containing extreme points in the same experiment.[33,52] BBD is used in analytical method optimization in many studies.[6,48,53-65]

In this study, a simple, rapid and robust HPLC method with photodiode array (PDA) detection at 215 nm was developed for the determination and separation of amlodipine besylate and enalapril maleate in FDC tablets. This method, which is available for assay and dissolution studies, was fast, environmentally friendly, and more cost-effective than the earlier published methods.[24-26] In this study, DOE was adapted to the robustness parameter of the analytical method for determining amlodipine and enalapril together. DOE principles were used in the method development of amlodipine and enalapril for the first time. The validation of the method was performed according to the ICH Q2 (R1) guideline.[47] The BBD was used for the optimization of the method. The optimized HPLC method was applied to dissolution and assay analysis of an in-house FDC tablet including amlodipine and enalapril.

MATERIALS AND METHODS

Materials and reagents

HPLC-grade methanol, o-phosphoric acid and hydrochloric acid 37% were obtained from Merck, Germany. Amlodipine besylate (Hetero Drugs, India) and enalapril maleate (Zheijiang Huahai, China) were kindly gifted by Nobel Pharma, Turkey.

The FDC tablet contains 6.94 mg of amlodipine besylate and 10 mg of enalapril maleate as APIs.

Apparatus

The HPLC system was a Shimadzu chromatographic system (Japan) with LC-20AD pump, SPD-M20A PDA detector at a wavelength of 215 nm, a reversed phase C18 column (4.6x250 mm id., particle size of 5 µm) from Waters® (USA). The HPLC system was controlled by LC Solution Software. Design Expert® Version 9 (Stat-Ease Inc, USA) was used for the experimental design and statistical analysis of data. A pH meter (PASS1 P11-BNC-Bante, England) was used to control the aqueous buffer. Dissolution test was performed with Pharmatest® Dissolution System (Germany).

Chromatographic conditions

The mobile phase was a mixture of methanol and water (pH adjusted to 3.0 with o-phosphoric acid) in the proportion of 10:90 (v:v). The injection volume of the samples was 5 µL. The flow rate was 1.2 mL/min. The detector wavelength was 215 nm and the column temperature was 30°C.

Preparation of standard solutions

The standard solution was prepared according to the following process: 6.94 mg of amlodipine besylate (equivalent to 5 mg amlodipine base) and 10 mg of enalapril maleate were weighed and transferred to a 50 mL volumetric flask and diluted to the appropriate volume with 0.1N HCl. This solution included 0.1 mg/mL of amlodipine base and 0.2 mg/mL of enalapril maleate. The calculations were performed considering amlodipine base and enalapril as maleate salts because of the dose proportionality in market products.

Calibration procedure

Calibration series were prepared in volumetric flasks by the appropriate dilution of standard solution with 0.1N HCl. The calibration curve was plotted with eight concentrations in the

range of 0.8-24 µg/mL for amlodipine and 1.6-48 µg/mL for enalapril (as maleate). The experiments were performed in three replicates for each level. The linearity of the calibration curve was evaluated by the linear regression statistics of concentrations against peak area.

Statistical analysis

Experimental design

Experimental plan, data analysis and optimization process were executed in Design Expert® Version 9 by using the BBD. The BBD is a three-level and multi-factor design which is a combination of 2K factorial and balanced incomplete block designs. In this study, four factors with three levels for each were determined as given in Table 1.

The significant factors in the model were determined by multivariate linear regression analysis and ANOVA F-test and its lack of fit with a confidence interval of 95% for each response. Significant factors were determined by the probability level that the p value is less than 0.05 and one-factor graphs.

Assay in FDC tablets

The FDC tablet containing amlodipine besylate and enalapril maleate was prepared by using direct compression method. For assay of the tablets, 10 tablets for each product were selected at random and weighed. Then these tablets were powdered, and a quantity of the powder (equivalent to 5 mg of amlodipine and 10 mg of enalapril maleate) was accurately weighed and transferred to a 50 mL volumetric flask. A 30 mL volume of diluent solution (0.1N HCl) was added and mixed for 15 min in magnetic stirrer. Then, it was diluted with the same solution to the volume and mixed in an ultrasonic bath for 10 min. A 4 mL volume of this solution was transferred to a 25 mL volumetric flask and diluted to the volume using the same solvent and was held in an ultrasonic bath for 5 min. The samples were filtered through a syringe tip filter of 0.45-µm pore size and then analyzed using HPLC.

Dissolution studies

Dissolution studies were performed using USP apparatus II (paddle method) in 0.1N HCl (pH 1.2). The dissolution volume was 900 mL, and the temperature was 37°C±0.5°C. The paddle rotational speed was 75 rpm. Samples (2 mL) were withdrawn at 10, 20, 30, 45, and 60 min, and the same amount of fresh media was replaced. The samples were filtered through 0.45-µm membrane filters to vials and analyzed by the optimized HPLC method. The dissolution profiles were evaluated as the cumulative drug dissolved (%) over time. All experiments were performed in n=3 and the cumulative amounts were evaluated as the mean ± standard deviation (SD).

Table 1. Experimental design

Factors	Low level	Nominal level	High level
Methanol ratio in the mobile phase (%)	5	10	15
Flow rate (mL/min)	1.0	1.2	1.4
pH of the mobile phase	2.8	3.0	3.2
Column temperature (°C)	25	30	35

RESULTS AND DISCUSSION

The chromatograms of diluent (blank) and those obtained from the standard solutions of amlodipine and enalapril are given in Figure 1, 2 respectively. The initial method provided good separation in a short time of 3.8 min for enalapril and 7.9 min for amlodipine. This level of separation is acceptable in a conventional method development process. A robustness study with DOE was also performed.

Robustness with DOE principles

According to the ICH Q2 (R1), in a robust method, small variations in certain method parameters do not affect the reliability and results of the method.[47] These small variations are important for the pharmaceutical industry in terms of the transfer of the analytical method from research and development to the quality control laboratory or from one company to another. In other words, it is the indication of the strength of the method.[51] In order to assess the concurrent influences of the changes in factors on the defined responses, a multivariate analysis by DOE is recommended in robustness studies.[43] DOE is used in analytical method development for two main purposes: To determine the most significant factor influencing the response of the study and to discover the optimized value of the factors for best results for the response.[37]

The DOE plan in a robustness test includes the following stages:[31]

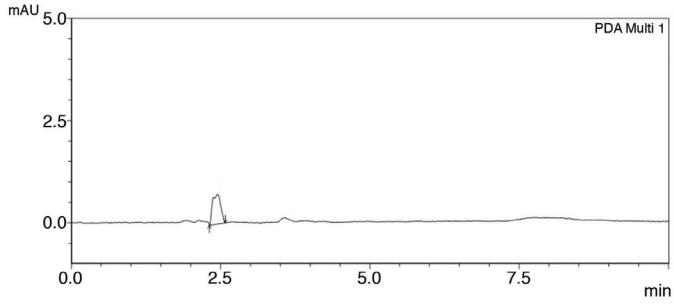

Figure 1. Chromatogram of the placebo (blank medium) for specificity testing

PDA: Photodiode array

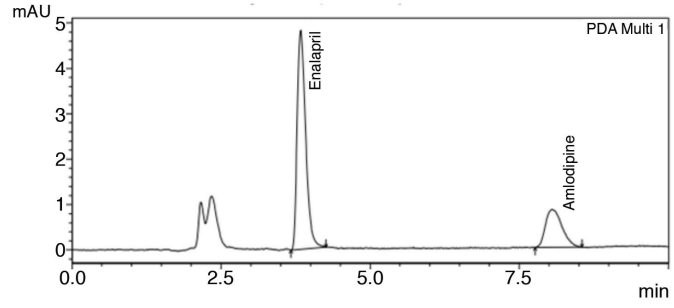

Figure 2. Chromatogram of enalapril (8 µg/mL, as maleate) and amlodipine (4 µg/mL) in the initial method

PDA: Photodiode array

Selection of factors and their levels

Robustness studies are an excellent opportunity to apply statistical experimental design to provide data-based control of the method.[51] Since there are many factors that might affect the method, it is vital to choose the right factors. In robustness studies of liquid chromatography, the most frequently preferred factors are the pH of the mobile phase, analysis time, flow rate, column type, temperature, composition of the mobile phase, detection wavelength, chosen filters, or the variations in sample preparation such as dilution, shaking time, or heating temperature.[39,51] It should be noted that there are no absolute truths in selecting factors in a DOE process; the chosen factors should comply with the purpose. According to ICH Q2 (R1), the following variations were recommended for the robustness test of HPLC methods: 1) pH of the mobile phase, 2) composition of the mobile phase, 3) column type, 4) temperature, and 5) flow rate. Except for the column type, all recommended factors (mobile phase ratio, pH, flow rate, and column temperature) were investigated in this study. The chosen factors and their pre-defined levels have the potential to affect the method depending on the analyst, laboratory or equipment, and environmental conditions.[47]

After selecting the factors, it is necessary to define their levels. In a two-level model such as Plackett-Burman Design (PBD) or two-level factorial designs, a maximum and a minimum limit are required for the factor values. In three-level designs, additional middle values, which generally represent the target or the expected value, are added to the design. Defining the levels is a critical step in experimental design. Particularly in two-level designs in which inappropriate levels were used, inaccurate and low-quality results can be obtained.[33] In order to avoid this problem, a three-level BBD design is preferred. The levels of the factors are usually defined symmetrically around the nominal level, which is the middle level in a three-level design. The interval chosen between the levels is generally decided according to the operator's personal experiences or anticipated changes from one laboratory to another. For example, if the developed method will be transferred to another laboratory, the pH can be measured using a pH meter with a small deviation, so pH should be considered as critical. The pH of a solution varies with a deviation of 0.02 with a confidence limit of 95%.[50] Therefore, this limit is acceptable for the pH in a robustness test. The interval of pH was ±0.02 in this study. The levels of column temperature were decided ±5°C as recommended in the article by Vander-Heyden et al.[50], which was aimed to guide a robustness parameter in method development. The levels of other factors, selected as 5% for mobile phase composition and 0.2 mL/min for flow rate, were in agreement with previous similar studies.[32,43,65]

Defining responses to be investigated

In the HPLC studies where robustness was investigated by DOE, various responses such as peak area, peak height, determined concentration, retention time, tailing factor, theoretical plate number, and resolution were used. The most important selection criterion for a response to use in factor evaluation is ease of measurement.[39] Additionally, using a large number of responses can lead to confusion when interpreting the results. Therefore, API concentrations calculated from the peak areas were selected as responses in this study.

Choosing an experimental design

A suitable experimental design should be selected based on the aim of the study. In case a large number of factors might affect the method, the aim can be to discard some factors that have no significant effect on the response. For this purpose, a screening design such as PBD can be used. On the other hand, if the main objective is to investigate the effects of the relatively lower number of factors deeply, or optimize the most effective factors, optimization designs should be preferred.[31] Generally, optimization is carried out following determination of the most significant factors by screening design. In case there is a factor known to be highly effective in the separation (such a flow rate or temperature), optimization designs can be preferred directly.[37] In this study, factors that may affect the results, such as the column temperature, flow rate, and composition of the mobile phase, were chosen with the purpose of performing an optimization. Another reason for choosing an RSM design is to observe any interaction between the factors.

The most used RSM designs are CCD and BBD. BBD requires the fewest experiments among the RSM designs because it does not contain values that are maximum or minimum values in the experimental matrix.[33] Since BBD requires fewer experiments, and the experimental matrix does not contain the highest or lowest level in the combination, this experimental design prevents an unrealistic extreme scenario. Therefore, the experiment number, time, and cost are reduced. BBD can evaluate the linear and non-linear effects of factors.[34,66] Thus, BBD was selected for the experimental plan, data analysis and optimization process using the Design Expert® Version 9 software.

Execution of experiments

Experimental executions were computed by Design Expert Software. Robustness was assessed by using BBD with 29 runs. Experimental design and calculated concentrations of enalapril (as maleate) and amlodipine and the corresponding responses are given in Table 2.

Statistical evaluation of responses and their interpretations

The best fit model was linear for all factors and their responses. In the literature, linear analysis is frequently indicated and recommended in robustness tests.[29,30] Therefore, our results were as expected. Linear models are used to show the main effects of factors.

The equation model for Y_1 (enalapril concentration) and Y_2 (amlodipine concentration) was as follows:

$$Y_1=32.32+0.079X_1-5.32X_2+0.11X_3+0.51X_4 \quad \text{(Equation 1)}$$

$$Y_2=16.19+0.12X_1-2.72X_2+0.020X_3+0.021X_4 \quad \text{(Equation 2)}$$

Where, X_1 is column temperature, X_2 is flow rate, X_3 is the methanol ratio in the mobile phase, and X_4 is the pH of the mobile phase.

The ANOVA results are given in Table 3. The significant effects showed a p value less than 0.05, a low SD (CV %), and a high adjusted R-square (adj R^2) value indicating a good relationship between the experimental data and those of the fitted model. The predicted R-square (pred R^2) value was in agreement with the adj R^2 for all responses.

The one-factor graphs (Figure 3, 4) demonstrated that the flow rate was the most significant factor on the responses; inverse proportionality was found ($p<0.05$). It was revealed that the

Table 2. Experimental plan for robustness and calculated responses

	Factors				Responses	
Run	Column temperature (°C)	Flow rate (mL/min)	Methanol ratio (%)	Mobile phase pH	Amlodipine concentration (µg/mL)	Enalapril maleate concentration (µg/mL)
1	30	1.2	5	3.2	15.888	32.058
2	30	1.2	10	3.0	16.171	32.090
3	35	1.4	10	3.0	13.729	27.696
4	25	1.0	10	3.0	18.749	37.797
5	30	1.2	10	3.0	15.991	31.951
6	25	1.2	5	3.0	15.998	31.954
7	30	1.4	10	3.2	13.837	28.039
8	35	1.2	15	3.0	16.102	32.001
9	30	1.2	15	2.8	15.954	31.684
10	25	1.2	15	3.0	16.047	32.003
11	25	1.2	10	3.2	16.051	32.185
12	35	1.2	5	3.0	16.078	31.909
13	25	1.4	10	3.0	13.022	27.539
14	30	1.4	5	3.0	13.822	27.465
15	30	1.0	5	3.0	19.209	38.283
16	30	1.2	15	3.2	16.084	32.385
17	30	1.2	10	3.0	16.059	31.844
18	35	1.2	10	2.8	16.045	31.391
19	35	1.2	10	3.2	16.099	32.295
20	30	1.2	10	3.0	16.083	31.960
21	30	1.2	5	2.8	16.137	31.772
22	35	1.0	10	3.0	19.132	38.345
23	30	1.2	10	3.0	16.094	31.998
24	30	1.4	15	3.0	13.868	27.869
25	25	1.2	10	2.8	15.920	31.214
26	30	1.0	15	3.0	19.321	38.836
27	30	1.4	10	2.8	13.721	26.818
28	30	1.0	10	2.8	19.084	36.981
29	30	1.0	10	3.2	19.149	39.053

Table 3. ANOVA results

Responses	± SD	Mean	CV %	Press	R^2	Adj R^2	Pred R^2	Adeq precision	p value
Amlodipine	0.24	16.19	1.51	2.21	0.984	0.982	0.976	55.91	<0.0001
Enalapril maleate	0.59	32.32	1.82	12.69	0.976	0.972	0.964	47.76	<0.0001

SD: Standard deviation, CV: Cardiovascular, Adj R^2: Adjusted R-square

most critical factor in robustness is the flow rate. The methanol ratio in mobile phase, temperature, and pH had no significant effect on the calculated concentrations of amlodipine and enalapril in defined levels. Kovacs et al.[30] have evaluated the same factors in their robustness test with different responses such as peak asymmetry and retention time. They found that the proportion of methanol in the mobile phase had a significant effect on the retention time of strontium ranelate. Similarly, Dhumal et al.[32] found that the proportion of methanol in the mobile phase and the flow rate had a negative effect, while the pH had a positive effect on the peak area and the determined tapentadol concentration. In another study, in which the same factors and different responses (tailing factor, retention time and theoretical plate) were used, the most effective factors were found to be the methanol composition and pH.[45] However, the significance of factors depends on the APIs and chromatographic conditions. If we had defined our levels more broadly for other factors (methanol ratio, temperature, and pH) or if we had assessed more responses such as tailing factor or resolution we might have observed a meaningful effect with other factors. However, this was not considered to be an error in the design because the DOE is specific to the purpose. In this study, we would like to see how possible rational changes would affect the analytical results, rather than creating a design space based on the extreme values of factors.

Two-way interactions between independent variables were found to be insignificant ($p>0.05$). Therefore, a simple screening design, such as a PBD, which is the most popular design in robustness evaluation, might be used in this study.[37] However, since PBD is a two-level design, it can cause inaccurate statistical evaluations when unsuitable factor levels are selected or when there might be an interaction between the factors. If an experimental model is needed to determine tolerable variations, an optimization design is recommended by Sahu et al.[31] For this reason, as discussed before, we preferred a BBD that contained a third level (target middle level) and provided more information about the method. There have been similar studies with other drugs in which calculated drug concentrations were the only response and flow rate was the only significant factor in the response.[43,46]

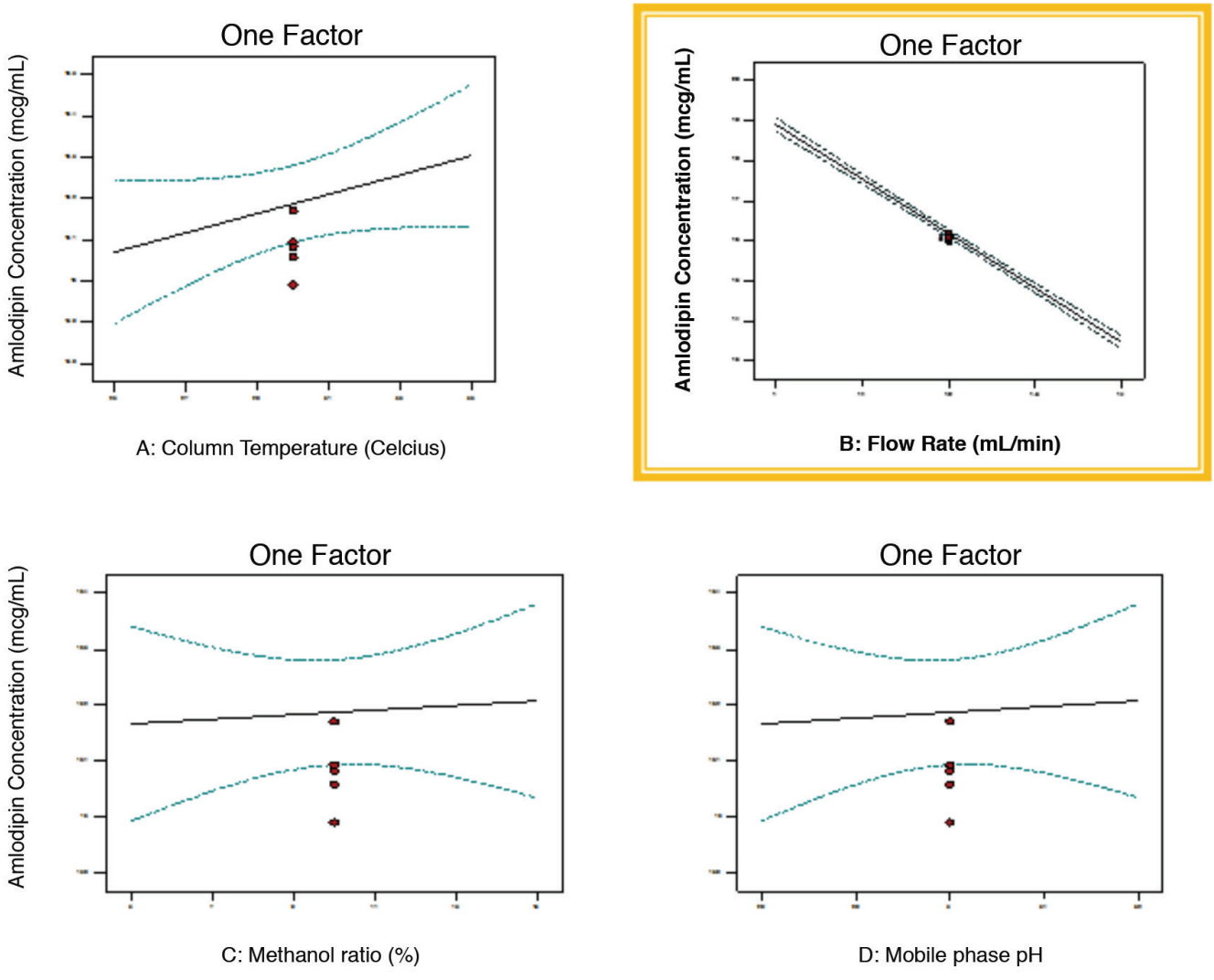

Figure 3. A-D) One-factor graphs of the main effects of the factors on amlodipine concentration

Optimization

Following linear model fitting, an optimization run was performed, and factor settings were defined using the prediction spreadsheet of the software (Figure 5). The final optimized parameters were a flow rate of 1.205 mL/min, pH of 2.95, and column temperature of 25°C. The factors described in the optimization were very close to the nominal levels in the BBD design. Non-etheless, these minor changes caused a better peak shape for amlodipine and a lower tailing factor (from 1.417 to 1.164, $p<0.05$) (Figure 6). Retention times were not changed in the method with 3.8 min and 7.9 min for enalapril and amlodipine, respectively.

The optimized method was validated based on international guidelines.

Linearity

The linearity of the peak area versus concentration was shown in the range of 0.8-24 µg/mL for amlodipine and 1.6-48 µg/mL for enalapril (as maleate). Linearity results were given in Table 4. The linearity range was kept wider than the previously published methods.[24-26] The lower concentrations are considered for the first minutes of the dissolution study, and higher values are for the assay.

Accuracy

Accuracy was demonstrated using six different solutions, containing 1.39, 2.78, 5.56, 12, 16, and 19.2 µg/mL of amlodipine and 2.78, 5.56, 11.12, 24, 32, and 38.4 µg/mL of enalapril maleate. Recovery values were obtained within the range of 98.6%-101.6%. The low value of relative standard deviation (RSD) less than 1% indicates that the proposed method is accurate. Results are presented in Table 5.

Table 4. Calibration data for amlodipine and enalapril maleate (n=3 for each level) for the optimized method

APIs	Equation	R^2
Amlodipine	y=4253.2x-796.1	0.9998
Enalapril maleate	y=6272.4x-1177.1	0.9995

R^2: R-square

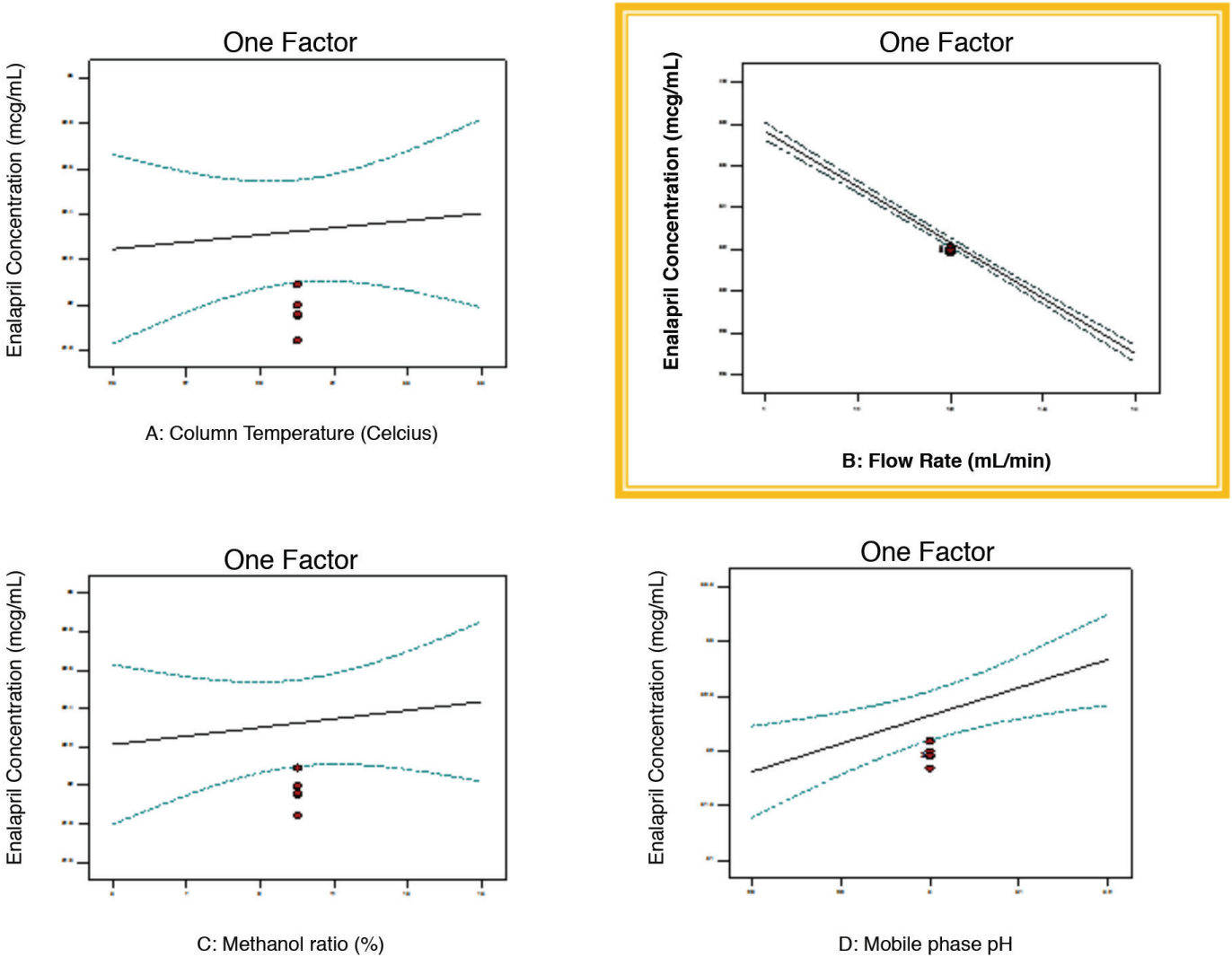

Figure 4. A-D) One-factor graphs of the main effects of the factors on enalapril concentration

Repeatability

Repeatability is also termed intraday precision and provides information about the precision under the same operating conditions in a short time interval.[47] Repeatability was assessed using 10 determinations of the solutions including 16 µg/mL of amlodipine and 32 µg/mL of enalapril maleate. The recovery values were 99.9±0.31% and 100±0.07% for amlodipine and enalapril maleate, respectively.

The RSDs were 0.307% and 0.0711% for amlodipine and enalapril maleate, respectively.

Intermediate precision

Intermediate precision was assessed using the interday variations. Two different concentrations (4 and 16 µg/mL for amlodipine and 8 and 32 µg/mL for enalapril maleate) were analyzed on three consecutive days. The RSD values of interday precision were less than 1%, confirming the method precision. The results are given in Table 6.

The low RSD value for intermediate precision and repeatability of the method as well as within-day and day-to-day variation suggested that the method was precise within the range of measurement.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated based on the SD of the response and the slope by using the equations below:

$$LOD = \frac{3.3 \times \sigma}{S} \quad \text{(Equation 3)}$$

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

where σ is the SD of the response, and S is the slope of the calibration curve. According to the equations, LOD values were 0.0631 µg/mL and 0.0424 µg/mL and LOQ were 0.19 µg/mL and 0.129 µg/mL for amlodipine and enalapril maleate, respectively.

The LOD and LOQ results suggested that the method was highly sensitive.

Stability

The drugs dissolved in 0.1N HCl were stable when stored at 25˚C for 72 hours. After 72 hours, drug recovery values were 99.7% for amlodipine and 99.4% for enalapril maleate.

Assay in tablets

The optimized method was used for the assay of amlodipine and enalapril in FDC tablets. An additional peak from excipients was not observed. The results were in the range of the labeled amount ±5% for both drugs (Table 7).

Dissolution

Dissolution was performed with the in-house FDC tablet by using USP apparatus II in 0.1N HCl. 0.1N HCl was selected as the model dissolution medium. The proposed HPLC method was available for dissolution of FDC tablets. Both amlodipine and enalapril were dissolved more than 85% within 10 min. Dissolution profiles of amlodipine and enalapril were given in Figure 7. The dissolution media of 0.1N HCl replaces the artificial stomach medium that is frequently used with the purpose of formulation development and quality control. For

Table 5. Accuracy results for amlodipine and enalapril maleate (n=3 for each level)

	Concentration (µg/mL)	Recovery (% ± SE)	RSD (%)
Amlodipine	1.39	99.0±0.70	0.68
	2.78	98.6±1.60	1.59
	5.56	100.0±0.40	0.42
	12.0	100.1±0.30	0.27
	16.0	99.7±0.16	0.16
	19.2	101.1±0.40	0.40
Enalapril maleate	2.78	100.4±0.60	0.64
	5.56	99.6±0.10	0.08
	11.12	100.6±0.10	0.10
	24.0	100.0±0.20	0.19
	32.0	99.7±0.25	0.26
	38.4	101.6±0.30	0.28

SE: Standard error, RSD: Relative standard deviation

Table 6. Interday precision results of amlodipine and enalapril maleate (n=3)

	Concentration (µg/mL)	1st day (% ± SE)	2nd day (% ± SE)	3rd day (% ± SE)	RSD (%)
Amlodipine	4.0	99.0±0.04	98.3±0.02	99.0±0.02	0.754
	16.0	99.9±0.06	99.4±0.04	99.7±0.03	0.248
Enalapril maleate	8.0	99.3±0.02	99.1±0.02	99.0±0.10	0.816
	32.0	99.8±0.02	99.8±0.02	100.0±0.02	0.111

SE: Standard error, RSD: Relative standard deviation

Table 7. Assay for FDC tablets (n=3)

	Labeled amount (mg/tablet)	Observed amount (mg/tablet)	RSD (%)
Amlodipine	5.00	4.95±0.03	0.52
Enalapril maleate	10.00	10.17±0.06	0.63

FDC: Fixed-dose combination, RSD: Relative standard deviation

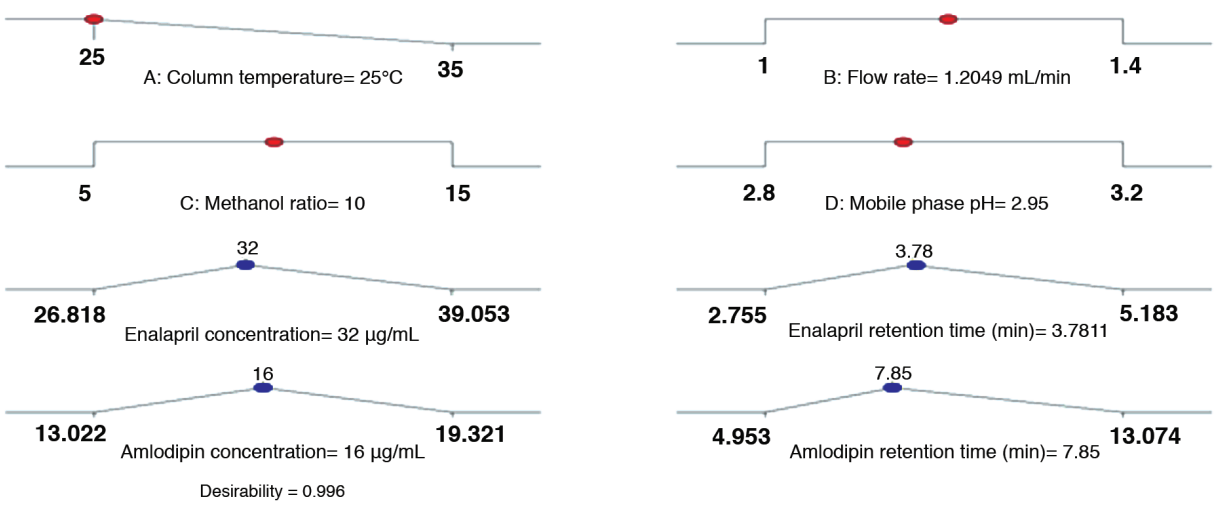

Figure 5. Optimization conditions of independent variables according to the Design Expert® Software

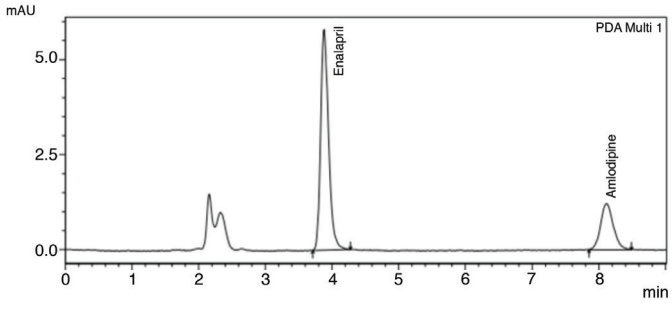

Figure 6. Chromatograms of enalapril (8 µg/mL, as maleate) and amlodipine (4 µg/mL) in the optimized method

PDA: Photodiode array

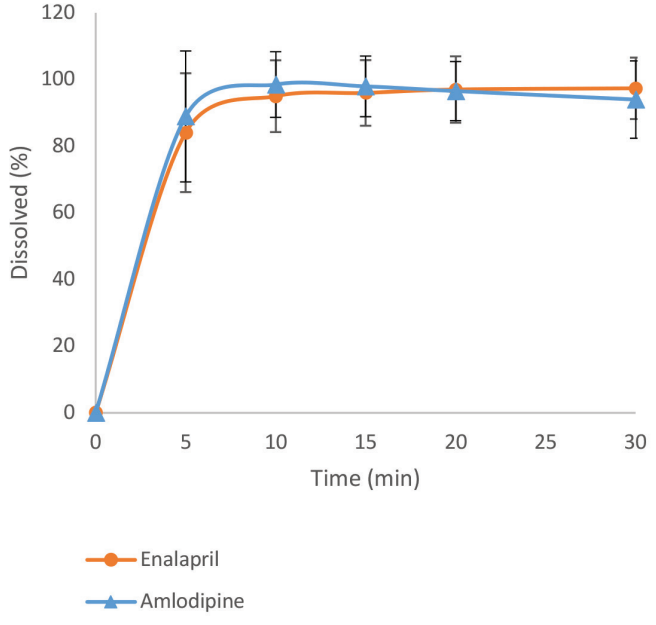

Figure 7. Dissolution results of amlodipine and enalapril in an in-house FDC product (n=3)

FDC: Fixed-dose combination

using this analytical method for other dissolution media such as pH 4.5 or pH 6.8 there might be small modifications in chromatographic conditions.

CONCLUSION

In conclusion, an accurate, precise, specific, and environmentally appropriate HPLC method was developed and validated for amlodipine besylate and enalapril maleate in the typical dosage unit. The BBD, an optimization design, was used to evaluate the operational factors in a robustness test, and validation was performed according to international guidelines. The developed method was more economic and suitable for green chemistry with less solvent consumption, which improved column performance. The method was applied to assay and dissolution studies and was found suitable for quality control tests and in vitro performance of pharmaceutical dosage forms for a fixed-dose tablet combination containing amlodipine besylate and enalapril maleate for the treatment of hypertension.

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ORIGINAL ARTICLE

Preclinical Study on the Hepatoprotective Effect of Pollen Extract of *Pinus brutia* Ten. (Red Pine) in Mice and Phenolic Acid Analysis

Pinus brutia Ten. (Kızılçam) Polen Ekstresinin Karaciğer Koruyucu Etkisinin Preklinik Olarak Araştırılması ve Fenolik Asit Analizleri

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ABSTRACT

Objectives: Many agents, including those from herbal sources, have been sought as preventives or cures for hepatotoxicity. The pollen of *Pinus brutia* Ten., known as red pine (Pinaceae), is used against liver diseases in Anatolian folk medicine.

Materials and Methods: In the current study, pollen ethanol extract of *P. brutia* was investigated for its possible hepatoprotective activity using a mouse model of CCl_4-induced hepatotoxicity. Swiss albino mice were divided into five groups, and extract-treated groups were compared with a silymarin-treated group as the reference. The extract was tested at 100, 200, and 300 mg/kg (b.w.). Phenolic acids were analyzed using high-performance column chromatography (HPLC) in the extracts as pollens are usually known to be rich in phenolics.

Results: Our data revealed that the extract displayed the best hepatoprotection at a dose of 100 mg/kg when compared with silymarin (Legalon®), the reference drug. HPLC analysis indicated presence of protocatechuic acid (0.176 mg/g extract), p-hydroxybenzoic acid (0.001 mg/g extract), vanillic acid (VA) (0.537 mg/g extract), syringic acid (0.050 mg/g extract), and tr-cinnamic acid (0.310 mg/g extract), while the major phenolic acid was VA.

Conclusion: The outcomes of this study allow us to conclude that red pine pollen extract can serve as a promising hepatoprotective agent. Among the phenolic acids analyzed in the pollen extract, vanillic acid as the major one besides some other phenolic acids detected seems to be responsible for its remarkable hepatoprotective effect.

Key words: *Pinus brutia*, red pine, pollen, hepatoprotective activity, HPLC

ÖZ

Amaç: Hepatotoksisiteyi önlemek veya iyileştirmek için bitkisel kaynaklar dahil olmak üzere birçok bileşik araştırılmaktadır. Kızılçam olarak bilinen *Pinus brutia* Ten. bitkisinin polenleri Anadolu'da halk arasında karaciğer rahatsızlıklarında kullanılmaktadır.

Gereç ve Yöntemler: Bu çalışmada, *P. brutia* polen ekstresinin farelerde CCl_4 ile indüklenen hepatotoksisite modeli üzerindeki muhtemel hepatoprotektif etkisi araştırılmıştır. Swiss albino fareler 5 gruba ayrılmış ve ekstrakt uygulanan gruplar referans olarak silimarin (Legalon®) kullanılan grup ile karşılaştırılmıştır. Ekstreler 100, 200 ve 300 mg/kg (v.a.) konsantrasyonlarında çalışılmıştır. Polenlerin genellikle fenolik açıdan zengin olduğu bilindiğinden, ekstrede bulunan fenolik asitler yüksek performanslı sıvı kromatografisi (HPLC) kullanılarak analiz edilmiştir.

Bulgular: Sonuç olarak, ekstrenin referans ilaç olan silimarin (Legalon®) ile karşılaştırıldığında, en etkin hepatoprotektif etkiyi 100 mg/kg'lik dozda gösterdiği belirlenmiştir. HPLC analizi ekstrede protokateşik asit (0,176 mg/g ekstre), p-hidroksibenzoik asit (0,001 mg/g ekstre), vanilik asit (VA) (0,537 mg/g ekstre), siringik asit (0,050 mg/g ekstre), ve tr-sinnamik asit (0,310 mg/g ekstre) bulunduğunu, en yüksek miktardaki fenolik asidin ise VA olduğunu ortaya koymuştur.

Sonuç: Çalışmamız neticesinde, kızılçam polen ekstresinin umut verici bir hepatoprotektif ajan olarak kullanılabileceği sonucuna varılmıştır. Polen ekstresinde analiz edilen fenolik asitler arasında, majör olan vanilik asidin yanı sıra bazı diğer fenolik asitlerin ekstrenin gösterdiği hepatoprotektif etkiden sorumlu olduğu düşünülmektedir.

Anahtar kelimeler: *Pinus brutia*, kızılçam, polen, hepatoprotektif aktivite, HPLC

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INTRODUCTION

The liver is one of the most important organs that regulate metabolic functions, hormones, and defense mechanisms in the body. On the other hand, the liver is exposed to many threats, such as alcohol, viruses, and xenobiotics; hence, protection of the liver is essential to the maintenance of liver function.[1,2] The genus *Pinus* (Pinaceae) contains approximately 80 species with a worldwide distribution.[3] In Turkey, *Pinus* contains seven species, *Pinus pinaster* Aiton, *P. brutia* Ten., *P. halepensis* Mill., *P. pinea* L., *P. sylvestris* L., *P. nigra* J.F.Arnold, and *P. radiata* D.Don.[4] *P. brutia* Ten. (red pine) is spread out over Eastern Mediterranean countries such as Turkey, Greece, and Cyprus; Black Sea countries such as Ukraine and Georgia; and the Caucasus countries.[5] Different parts of *P. brutia*, such as the bark, resin, tar, and cones, are used to treat asthma, bronchitis, cancer, diabetes, diarrhea, pneumonia, and tuberculosis in Turkish folk medicine.[6-9]

Pollens from many kinds of plants have been used as food traditionally for many years, even since pre-historic times.[10-16] Pine pollen, which is the male spores of *Pinus*, have been used to protect the liver, combat senility and fatigue, treat gastrointestinal dysfunction, improve sexual function, and increase cerebral-cardiac blood vessel function for many years.[17,18] The relevant literature survey shows that most previous studies on *P. brutia* were conducted on its bark. On the other hand, *P. brutia* bark contains some phenolic compounds, such as 4-hydroxybenzoic acid, resveratrol, gentisic acid, vanillin, vanillic acid (VA), catechin hydrate, p-coumaric acid (p-COU), ferulic acid (FA), protocatechuic acid (proCA), gallic acid (GA), myricetin, naringenin, caffeic acid (CA), luteolin, and kaempferol.[19] On the other hand, there have been a few studies on the phytochemistry and biological activity on the pollens of *P. brutia*.[20-26]

There have been several studies describing the hepatoprotective effect of various pollens, such as bee pollen and that of chestnut, canola, *P. massoniana*, *Schisandra chinensis*, etc.[27-32] However, no reports on the hepatoprotective effect of pollen from *P. brutia* are available to date, although it is used against liver diseases around Muğla province in the Aegean region of Turkey (personal communication). Taking this information into account, we aimed to perform the present study in order to evaluate anecdotal claims of the hepatoprotective use of red pine pollen ethanol extract using carbon tetrachloride (CCl_4)-induced liver damage in mice and to identify major phenolic acids in the extract using high-performance column chromatography (HPLC).

MATERIALS AND METHODS

Experimental

Plant material

The pollen of *P. brutia* trees was collected from the forest area belonging to the General Directorate of Forestry, Ministry of Agriculture and Forestry, in the vicinity of Antalya province, Turkey in June 2015.

Preparation of the extract

Air-dried pollens (51.15 g) of *P. brutia* were macerated with 800 mL ethanol (80%) for 24 h twice at room temperature. The ethanol macerate was filtrated and evaporated to dryness *in vacuo*. The yield of the crude extract was 24.98% (w/w).

High-performance liquid chromatography analysis

Chemicals used for HPLC (methanol and formic acid) analysis were of chromatographic grade (Sigma-Aldrich, St. Louis, MO, USA). Phenolic acid standards, e.g., GA, proCA, p-OHBA (4-hydroxybenzoic acid), VA, CA, chlorogenic acid, syringic acid (SA), p-COU, FA, o-coumaric acid, rosmarinic acid, and trans-cinnamic acid (tr-CIN) used in HPLC analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Merck (GmbH, Darmstadt, Germany). Analysis of phenolic acids in the extract was carried out with an Agilent 1.100 series auto-sampler system from Agilent, GL Sciences Inc. (Waldbronn, Germany) equipped with a system controller, a DAD detector (G 1315B, 280 nm), and a quaternary LC pump (G1311A). The separation was carried out with a Zorbax Eclipse XDB-C18 column (150 mm, 4.6 mm i.d., and 5 µm particle size) (Agilent, Waldbronn, Germany) with the column temperature set at 25°C. Chromatographic separation was carried out using two solvent systems: A) methanol:water:formic acid (10:88:2, v/v/v) and B) methanol:water:formic acid (90:8:2, v/v/v), as reported elsewhere.[33] The analyses were performed by using a linear gradient program. The conditions were 100% A from 0 to 15 min, 100% A from 15 to 20 min, 85% A from 20 to 30 min, 50% A from 30 to 35 min 0% A from 36 to 42 min, and returned to 100% A. The flow rate was 1 mL/min, and the injection volume was 10 µL. Signals were detected at 280 nm. The extract was dissolved in a mixture of methanol and water (1:1, v/v) and injected into the HPLC.

Each compound was identified by its retention time and by spiking with the standards under the same conditions. The identities of phenolic acids were also confirmed with a photodiode array detector by comparison with the ultraviolet spectra of standards in the wavelength range of 220-320 nm. Each compound was quantified according to the peak area measurements, which were reported in calibration curves of the corresponding standards. Data are reported as means ± standard deviations of three independent analyses.

Animals

Swiss albino mice of either sex (50 to 70 g) were maintained under standard animal housing conditions fed with commercial mice chow and allowed water *ad libitum*. The experimental protocol was approved by an Institutional Ethic Committee constituted by PMAS Arid Agriculture University Rawalpindi for the animal study.

Hepatoprotective activity

Forty mice were divided into eight groups of five each (n=5). Group 1: The control group received 0.5 mL of saline (0.9%, v/v) in water. Group 2: Animals of this group received 0.5 mL of olive oil (0.5%). Group 3: Animals of this group received ethanol (0.2%, v/v). All of these animals received doses once per day for

the entire period (7 days) by i.p. injection, respectively. Animals of group 4 were administrated i.p. with CCl_4 dissolved in olive oil at a dose of 0.5 mL/kg/day body weight. Animals of groups 4 to 8 were administrated *i.p.* with CCl_4 dissolved in olive oil at a dose of 0.5 mL/kg/day body weight (b.w.).

Animals of group 5 were fed with silymarin dissolved in ethanol at a dose of 50 mg/kg/day. Animals of group 6 were fed with the pollen ethanol extract at dose of 100 mg/kg once per day by gavage, while animals of group 7 and 8 were fed with the extract at doses of 200 and 300 mg/kg, respectively, once *per* day by gavage. At the end of the experiments, all mice were sacrificed, serum was collected, the livers were removed, and washed with ice-cold physiological saline.

Acute oral toxicity study

An acute toxicity study was conducted for a selected suitable dose of plant extracts.[34] Approximately 100 to 300 mg of dried pollen extract was dissolved in 5 mL of ethanol, and 1 mL of each dose was given to animals by gavage.

Biochemical analysis

Organs were homogenized in 0.1 M Tris HCl buffer (pH 7.4) to give a 10% homogenate. This homogenate was used for the estimation of triglycerides, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol by using commercial kits (Randox Laboratory) and the enzymatic method of Bierman.[35] The enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP); total bilirubin; red blood cell count (RBC); white blood cell count (WBC); and platelet levels were estimated by using their respective diagnostic kits and an auto-analyzer (Merck). The body weights of animals were calculated by measuring the weight before and after treatment with the extract.

Statistical analysis

All values are expressed as mean ± standard deviation. One-Way ANOVA was used to determine the consequences of different treatments by using the computer software GraphPad Prism 5.0.

RESULTS AND DISCUSSION

HPLC analysis

The pollen ethanol extract was analyzed by HPLC, which led to identification and quantification of the following phenolic acids: proCA (0.176 mg/g extract), p-hydroxybenzoic acid (0.001 mg/g extract), VA (0.537 mg/g extract), SA (0.050 mg/g extract), and tr-CIN acid (0.310 mg/g extract). The major phenolic acid was found to be VA (Figure 1).

Results of liver enzymes, lipid profiles, and blood cells

When the effects of the extract on mouse lipid profiles were examined, the pollen extract was found to reduce triglycerides and total cholesterol levels significantly at the 100 mg/kg dose. Nevertheless, the extract also reduced the HDL cholesterol level (Table 1). Although the extract applied at the 200 and 300 mg/kg doses decreased the triglyceride and cholesterol levels, the activities were lower than that of the 100 mg/kg extract. The extract at a dose of 100 mg/kg exhibited a greater reducing effect than that of silymarin.

Our data indicated that plasma levels of AST and ALT enzymes were notably elevated in rats treated with CCl_4. The ALT, AST, ALP, and bilirubin levels were diminished drastically with extract at the 100 mg/kg, 200 mg/kg, and 300 mg/kg doses (Table 2). The activity of the extract on these enzymes and proteins was higher than that of the reference drug, silymarin. Considering the pollen extract treatment on blood cells, the counts of reduced RBC and WBC was increased, so the extract presented a similar effect to that of silymarin (Table 3).

Histopathologic findings

Histopathologic data displayed that livers from the healthy mouse group showed normal hepatocyte structures. However, after administration of CCl_4, complete loss of liver architecture was observed, whereas the damaging effects of CCl_4 were reversed by treatment with the pollen extract (Figure 2). The recovery of tissue was significant when it was treated with the 300 mg/kg dose of the pollen extract, which indicated that tissue regeneration was dose-dependent (Figure 2e), while similar results were obtained when the hepatoprotective agent was used, i.e., silymarin (Figure 2b).

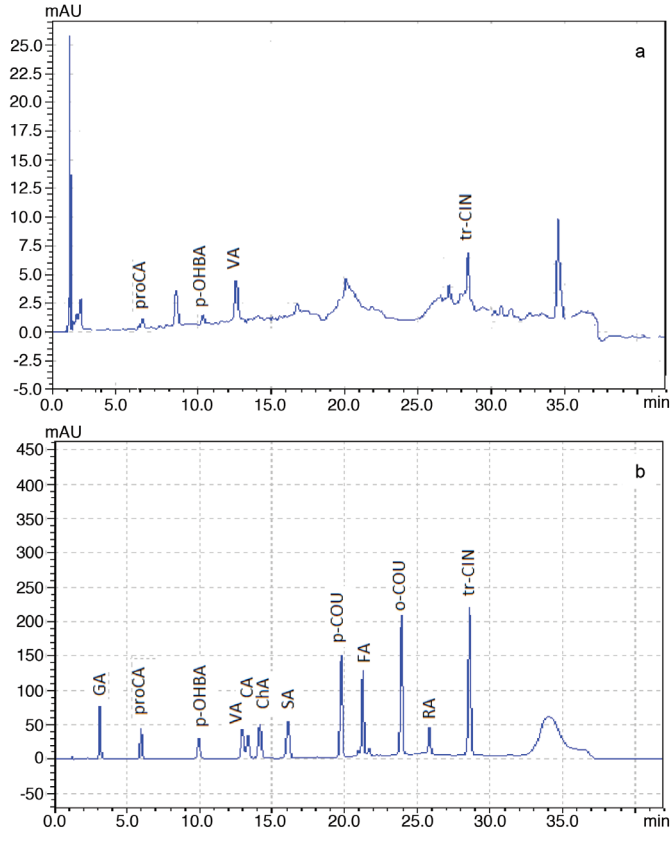

Figure 1. HPLC chromatograms of the pollen extract (a) and standard phenolic acids (b)

HPLC: High-performance column chromatography, GA: Gallic acid, proCA: Protocatechuic acid, VA: Vanillic acid, SA: Syringic acid, p-OHBA: 4-hydroxybenzoic acid, p-COU: P-coumaric acid, tr-CIN: Trans-cinnamic acid

Liver damage induced by CCl_4 in rats is one of the most preferable experimental models for the study of hepatoprotection. Several studies have been performed to determine the hepatoprotective or lipid-lowering effects of the various aforementioned pollen extracts as the liver is known to play the foremost role in lipid transformations.[27-32] Pollen grains are the tiny male particles released from trees, weeds, and grasses. The main function of pollen grains is to fertilize other parts of plants. An early study on a flower pollen extract (0.4 mL/100 g b.w.), in which the name of the extract was mentioned as cernitins, was described to possess a hepatoprotective effect on livers damaged by alcohol by reducing serum AST and ALT levels.

Another pollen extract from a flower, whose scientific or local name was not indicated, was reported to exert a

Table 1. Effects of red pine pollen extract on the lipid profiles of mice

Group	Treatment	Triglycerides (mg/dL)	Cholesterol (mg/dL)	HDL (mg/dL)
1	Normal (vehicle)	81.15±1.22	65.38±2.34	59.24±2.36
2	Olive oil group	75.27±2.51	71.34±1.34	45.43±1.23
3	Ethanol group	82.35±2.37*	66.24±2.14*	54.26±1.45*
4	CCl_4 + olive oil	142.35±2.37	132.35±0.39	112.35±1.34
5	Silymarin + olive oil	89.31±1.32	74.12±1.25	62.17±0.38
6	Pollen extract at 100 mg/kg	82.31±1.27*	59.23±2.14*	47.26±1.45*
7	Pollen extract at 200 mg/kg	91.38±2.76	88.34±1.32	51.24±2.35
8	Pollen extract at 300 mg/kg	98.35±1.52	89.65±0.57	52.78±1.45

*Significant ($p<0.05$) values vs. control/normal and expressed as mean ± SD, n=5
CCl_4: Carbon tetrachloride, HDL: High-density lipoprotein, SD: Standard deviation

Table 2. Effects of red pine pollen extract on liver enzyme and proteins in mice

Group	Treatment	ALT (U/L)	AST (U/L)	ALP (U/L)	Bilirubin (mg/dL)
1	Normal (vehicle)	81.15±1.21	65.38±2.34	59.24±2.36	0.146±0.028
2	Olive oil group	75.27±1.51	62.54±1.33	45.43±1.25	0.245±0.051
3	Ethanol group	62.35±2.37*	56.24±2.14*	44.26±1.45*	0.691±0.596
4	CCl_4 + olive oil	162.35±2.37	152.35±0.39	142.35±1.34	1.289±0.19
5	Silymarin + olive oil	89.31±1.32	84.12±1.25	72.17±0.38	0.571±0.22
6	Pollen extract at 100 mg/kg	68.31±1.27*	69.23±2.14*	67.26±1.45*	0.169±0.33
7	Pollen extract at 200 mg/kg	61.38±2.76	58.34±1.32	61.24±2.35	0.186±0.02
8	Pollen extract at 300 mg/kg	57.48±1.53	61.54±0.78	62.35±2.57	0.192±0.01

*Significant ($p<0.05$) values vs control/normal and expressed as mean ± SD, n=5
ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, SD: Standard deviation, CCl_4: Carbon tetrachloride

Table 3. Effects of red pine pollen extract on blood cell parameters in mice

Group	Treatment	RBCs ($10^6/mm^3$)	WBCs ($10^3/mm^3$)	Platelets ($10^3/mm^3$)
1	Normal (vehicle)	4.65±0.31	5.38±1.34	259.24±1.36
2	Olive oil group	4.87±0.51	6.24±0.33	245.23±1.26
3	Ethanol group	3.25±0.37	3.64±0.14	234.26±1.45
4	CCl_4 + olive oil	1.25±0.37	1.55±0.39	132.35±1.54
5	Silymarin + olive oil	3.91±0.32	4.42±0.25	242.15±1.38
6	Pollen extract at 100 mg/kg	4.81±0.27*	5.93±0.14	243.56±2.15
7	Pollen extract at 200 mg/kg	4.68±0.76	4.84±0.32	241.22±1.37
8	Pollen extract at 300 mg/kg	4.98±0.53	4.91±0.35	248.12±0.58

*Significant ($p<0.05$) values vs. control/normal and expressed as mean ± SD, n=5
RBC: Red blood cell, WBC: White blood cell, SD: Standard deviation, CCl_4: Carbon tetrachloride

hepatoprotective effect via normalization of AST, ALT, and ALP levels as well as hypolipidemic and hypocholesterolemic activity in testosterone-androgenized rats.[36] The same extract was also shown to have a protective effect in a paracetamol-induced hepatotoxicity model in mice along with hypolipidemic effect.[27]

Bee pollen from China was previously demonstrated effective in decreasing the amount of lipofuscin (fine yellow-brown pigment granules composed of lipid-containing residues of lysosomal digestion) in cardiac muscle, liver, and brain as well as adrenal gland cells in NIH mice.[37] Additionally, bee pollen extract with a rich polyphenol content from Poland was tested for its anti-atherogenic effect in apolipoprotein E-knockout mice at two doses of 0.1 and 1 g/kg body weight (b.w.) for 16 weeks.[32] The extract led to a decrease in triglyceride and LDL cholesterol levels and displayed complete protection of the coronary arteries at 1 g/kg b.w. The effect was speculated to correlate with the polyphenol content of the pollen extract, which was supported by histopathological data on the cardiac vessels. In another study, a strong hepatoprotective effect of a pollen ethanol (70%) extract prepared from *Phoenix canariensis* hort. ex Chabaud as one of the palm species was shown in adult male Wistar albino rats.[38] The pollen extract was found to contain isorhamnetin-3-O-rutinoside and rutin as the phenolic compounds, which were concluded to contribute to its hepatoprotective effect. Yildiz et al.[29] studied the hepatoprotective effect of chestnut bee pollens collected from the western Black Sea region of Turkey at doses of 200 and 400 mg/kg/day through CCl_4-induced liver damage in Sprague-Dawley rats. Particularly, bee pollen extract led to a significant decrease in AST and ALT levels at a dose of 400 mg/kg, whereas silybinin administered at a dose of 50 mg/kg in rats revealed a better hepatoprotective effect as compared with that of bee pollen extract at 200 mg/kg. Phytochemical analysis of chestnut pollen pointed to the presence of total phenolic compounds (28.87 mg GA equivalent/g), total flavonoids (8.07 mg quercetin equivalent/g), total anthocyanins (92.71 mg cyanidin-3-glucose equivalent/kg), and total carotenoids (29 mg β-carotene equivalent/100 g). Since the antioxidant activity of the extract in that study was also consistent with its hepatoprotective effect, the phenolic compounds analyzed in the extract were considered to contribute to its antioxidant and hepatoprotective effects. Similarly, the pollen extract of *Schisandra chinensis* of Chinese origin was reported to exert strong antioxidant and hepatoprotective effects against hepatotoxicity induced by CCl_4, which is consistent with our data.[28] Recently, Taishan *Pinus massoniana* pollen extract was shown to exert marked hepatoprotection in CCl_4-induced oxidative stress in the liver of rats tested at doses of 100, 200, and 400 mg/kg b.w., where AST, ALT, ALP, lactic dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase levels were significantly reduced.[31] The strong hepatoprotective action of this pollen extract was concluded to be a result of its polysaccharide content, which was described as an acidic heteropolysaccharide with glucose

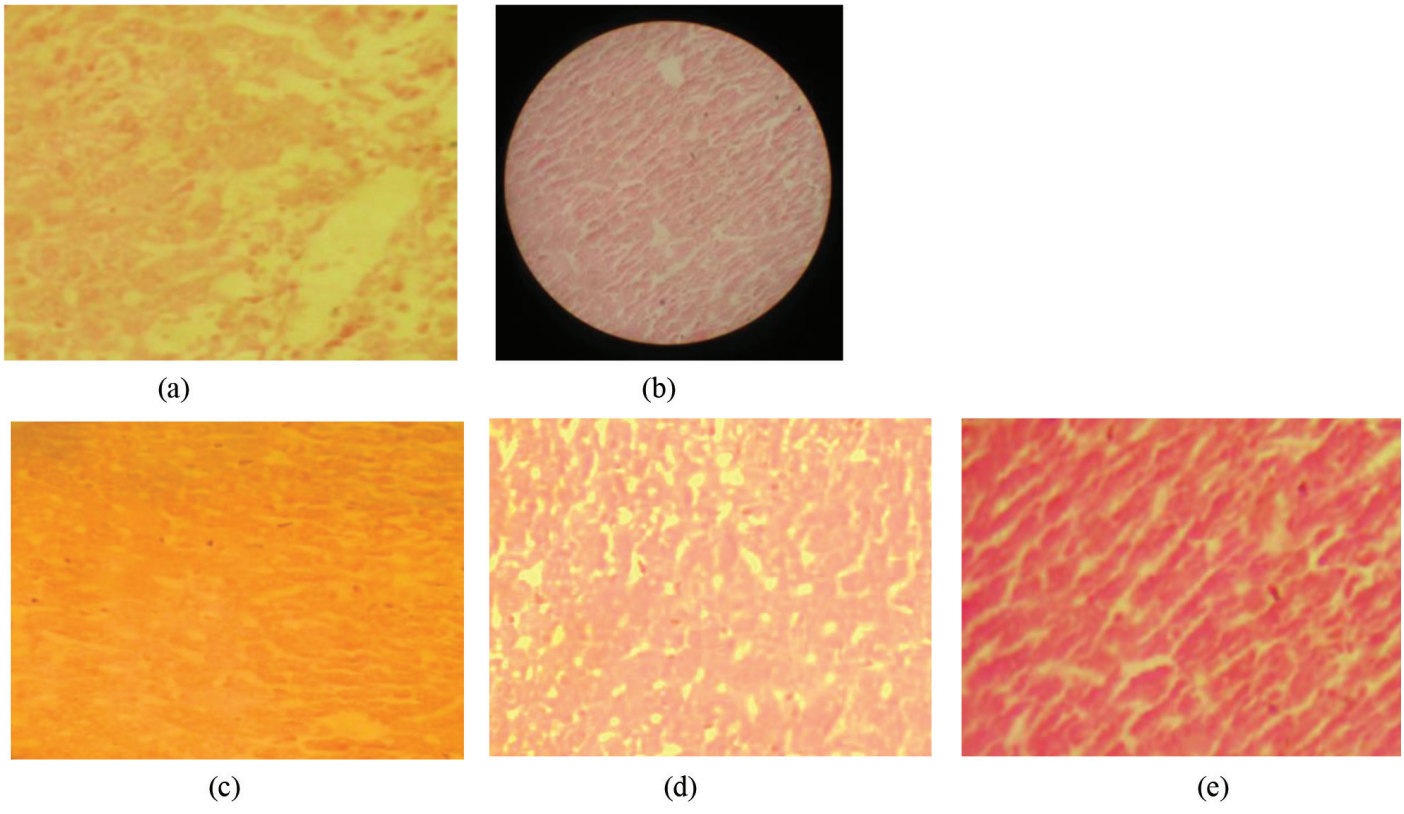

Figure 2. Histopathological results with cellular organization by red pine pollen extract (a) in CCl_4-induced liver damage; (b) by silymarin (c) pollen extract at 100 mg/kg; (d) pollen extract at 200 mg/kg; (e) pollen extract at 300 mg/kg

CCl_4: Carbon tetrachloride

and arabinose as the key constituent monosaccharides. On the other hand, Rzepecka-Stojko et al.[32] mentioned a positive correlation between polyphenols present in bee pollen and its hepatoprotective and other biological activities.

In another study on honeybee products including chestnut honey, pollen, propolis, and royal jelly, their hepatoprotective activity was investigated using a CCl_4-induced model in rats.[39] Recovery of hepatotoxicity was observed by measuring AST and ALT levels as well as oxidative stress parameters such as MDA, SOD, and catalase (CAT). The use of bee pollen due to its discernible bioactivities was also suggested to be beneficial not only for human health but also for animal health (up to 20 g/kg diet) for production and health patterns of livestock.[40]

On the other hand, VA was detected as the major phenolic compound in the extract along with some other phenolic acids. In fact, VA was reported to have a strong hepatoprotective activity in a number of plant or mushroom extracts.[41] For instance, VA and SA were reported to be the active constituents in edible mushroom *Lentinula edodes* (shiitake) in concanavalin A-induced liver injury in mice.[42] In another study, *L. edodes*, rich in VA and SA, was shown to exert strong hepatoprotection in mice with acute and chronic liver injury induced by CCl_4, which is in good agreement with our findings.[43] The phenolic composition of a Taiwanese mushroom species, *Xylaria nigripes*, with a high amount of epicatechin, catechin, and p-COU, was interpreted to be related to its activity against *in vivo* CCl_4-induced hepatotoxicity by Song et al.[44]

Consistently, the leaf methanol extract of *Capparis spinosa* of Tunusian origin, found to contain rutin, resveratrol, coumarin, epicatechin, luteolin, catechin, kaempferol, VA, and GA, led to a notable decrease in serum ALT, AST, and LDH levels in CCl_4-induced acute liver damage, as well as in the amount of hepatic MDA formation, whereas it raised the activities of SOD, CAT, and GPx, and repaired injury that occurred in the liver.[45] In a similar study, a strong hepatoprotective effect was observed with the hot aqueous extract prepared from the leaves of *Asparagus albus* in male Wistar rats by Serairi-Beji et al.[46], where some phenolic acids, e.g., GA, VA, and 3,4-dimethoxybenzoic acid, along with several flavonoids, e.g., catechin, rutin, and quercetin, were identified through HPLC. The authors commented that the hepatoprotective effect of the extract was correlated with its polyphenolic content. A remarkable *in vivo* hepatoprotection was caused by *Artocarpus lakoocha* fruits which contain chromatotropic, gallic, vanillic, cinnamic, and FAs as well as quercetin and kaempferol, which is consistent with the findings of our study.[47] A *in vivo* study parallel to ours was conducted on the hepatoprotective effect induced by thioacetamide of the ethanol extract of *Prunus amygdalus* stem and leaves from Egypt.[48] Analysis of the extract using LC-DAD-ESI-MS in negative ion mode indicated the presence of a number of phenolics, including VA and homovanillic acid, which were correlated to hepatoprotection by the plant. Actually, all these previous studies have underlined a considerable contribution of VA to the hepatoprotective activity of a number of plants, which may also lead us to propose that VA might be the major compound responsible for the hepatoprotective effect of red pine pollen extract.

CONCLUSION

Red pine pollen extract exhibited remarkable and dose-dependent hepatoprotection against CCl_4-induced liver damage in mice. Phenolic compounds, VA in particular, present in the pollen extract could be responsible for its notable hepatoprotective effect. We conclude that red pine pollen extract has the potential to serve as a promising hepatoprotective agent.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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ORIGINAL ARTICLE

Prophylaxis and Therapeutic Ability of Inactivated Dermatophytic Vaccine Against Dermatophytosis in the Rabbits as an Animal Model

Bir Hayvan Modeli Olarak Tavşanlarda Dermatofitoza Karşı İnaktive Dermatofitik Aşının Profilaksi ve Terapötik Yeteneği

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ABSTRACT

Objectives: Dermatophytosis is a group of cutaneous diseases widely distributed in human and animals. It causes serious infection in some human cases and economic losses in farm animals. The primary aim of this study is to conduct an investigation of prophylaxis and a potential therapeutic vaccine against dermatophytosis.

Materials and Methods: The rabbit was chosen as an animal model of dermatophytosis for a case control study conducted in two parts. Inactivated cells of *Trichophyton mentagrophytes* were prepared for use as a vaccine. The prophylaxis part included vaccination of rabbits with the prepared vaccine either alone or with Freund's adjuvant, followed by infection with the same fungus. The second part included treatment of infected rabbits with an inactivated vaccine.

Results: The prepared vaccine showed prophylactic ability against infection with *T. mentagrophytes* for more than 6 months without requiringan adjuvant and also revealed at herapeutic ability in infected animals after a short time (16 days), compared with the control group.

Conclusion: Inactivated vaccine gives animals durable protection and shortens the treatmenttime for infection with dermatophytosis.

Key words: Dermatophytosis, *Trichophyton mentagrophytes*, vaccine, prophylaxis, rabbit

ÖZ

Amaç: Dermatofitoz, insan ve hayvanlarda yaygın olarak bulunan bir grup deri hastalığıdır. Bazı olgularda ciddi enfeksiyonlara ve çiftlik hayvanlarında ekonomik kayıplara neden olur. Bu çalışmanın temel amacı, profilaksi ve dermatofitoza karşı potansiyel bir terapötik aşı araştırması yapmaktır.

Gereç ve Yöntemler: Tavşan, iki bölümden oluşan bir olgu kontrol çalışması için dermatofitozun hayvan modeli olarak seçilmiştir. *Trichophyton mentagrophytes*'in etkisizleştirilmiş hücreleri, aşı olarak kullanılmak üzere hazırlandı. Profilaksi kısmı, tavşanların hazırlanan aşı ile tek başına veya Freund adjuvanı ile aşılanmasını ve ardından aynı mantarla enfeksiyonu içermiştir. İkinci kısım, enfekte tavşanların etkisizleştirilmiş bir aşı ile tedavisini içeriyordu.

Bulgular: Hazırlanan aşı, *T. mentagrophytes* ile enfeksiyona karşı 6 aydan fazla süreyle, bir adjuvana ihtiyaç duymadan profilaktik yetenek gösterdi ve ayrıca enfekte hayvanlarda, kontrol grubuna kıyasla kısa bir süre sonra (16 gün) terapötik yetenek gösterdi.

Sonuç: İnaktive aşı, hayvanlara kalıcı koruma sağlar ve dermatofitoz ile enfeksiyon için tedavi süresini kısaltır.

Anahtar kelimeler: Dermatofitoz, *Trichophyton mentagrophytes*, aşı, profilaksi, tavşan

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INTRODUCTION

Nowadays, fungal infections are widely distributed and associated with serious mortality and morbidity rates all over the world.[1] Most of these infections, especially the systemic types, are usually diagnosed too late to begin treatment.[2] Thus, prophylaxis by vaccination against the most common fungal infections should take priority to limit the incidence of such diseases. For decades, antifungal vaccines have been considered impractical by most international scientific societies.[1,3,4] Most attention has been focused on the development of vaccines against viral and bacterial infections.[1,3] The reasons are that fungal infections usually show low incidence rates, and some of them are not widely distributed in comparison with bacterial and viral infections.[1] Weakness of the immune system in most patients with fungal infections was also believed to decrease the efficacy of vaccines against fungi.[3] However, to date, no vaccine had been licensed for use against a fungal infection in humans.[4,5] Recently, this view has changed due growing interest in limiting a common type of fungal infection after its incidence increased, especially in immunocompromised patients or those with other predisposing factors.[1,6] Several studies approved the suitability of vaccine development against common fungal infections such as those caused by *Aspergillus* spp., *Candida* spp., *Paracoccidioides brasiliensis*, *Sporothrix* spp., *Cryptococcus* spp., *Coccidioides* spp., *Histoplasma* spp., and *Blastomyces* spp.[5,6]

Dermatophytosis is one of several skin diseases common in both humans and animals.[7,8] It is caused by a special group of keratinophyllic fungi called dermatophytes.[7] Although dermatophytosis is restricted to within a cutaneous layer of the skin, a systemic distribution in humans has been registered by many case studies.[9-11] This development in the pathogenesis of dermatophytes directed specialists to consider dermatophytosis as a serious disease demanding increased attention. In animals, dermatophytosis is considered a very important disease due to its effect on the economic value of animal breeding.[12] Although an effective vaccine against dermatophytosis is not a novel idea, research is ongoing for the ideal one with good prophylactic and therapeutic activity. Various components of dermatophytes have been evaluated as vaccines against dermatophytosis but have not received approval for commercial use in humans.[13,14] However, these studies are still at an experimental level, even though a few of them are used commercially for various animals such as dogs, cats, bovines, and guinea pigs.[13-26]

Herein, rabbits were chosen as a model to investigate the suitability of a vaccine produced from *Trichophyton mentagrophytes* for prophylaxis and treatment of dermatophytosis.

MATERIALS AND METHODS

Fungal isolate

T. mentagrophytes was isolated from the tinea corporis of a 56-year old male for use in experimental infection of rabbits. The isolate was diagnosed by polymerase chain reaction (PCR) amplification and sequencing of the internal transcribed spacer region using the primer pair ITS1 (forward) and ITS4 (reverse).[27] The fungal genome was extracted by using the FavorPrep™ Fungi/Yeast Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., Taiwan).The PCR mixture was prepared in a total volume of 20 µL, including 5 µL of AccuPower® PCR Premix (Bioneer, South Korea), 1 µL of each primer, 1 µL of template DNA, and sterile deionized distilled water. A negative control containing all reagent sexcept template DNA was also included. PCR cycling was initiated at 94˚C for 2 min, followed by 35 cycles of denaturation at 94˚C for 35 seconds, annealing at 52˚C for 30 seconds, and extension at 72˚C for 60 seconds, with a final extension at 72˚C for 6 min. Sequencing of PCR the product was performed by Bioneer Company (South Korea). The fungal species was diagnosed after comparison of the obtained sequences with that recorded in GenBank by using the BLAST program.

Vaccine preparation

The antifungal vaccine was prepared from inactivated fungal cells of isolated from *T. mentagrophytes*. 100 mL of sterilized distilled water containing about $8x10^8$ mature fungal cells [grown for 1 week at 30˚C on Sabouraud's Dextrose agar (Himedia, India)] in each mL of D.W. was prepared

The aqueous fungal suspension was heated at 70˚C for 3 hours in a water bath.[28] To ensure inactivation of fungal cells, 0.1 mL of the treated fungal suspension was cultured on Sabouraud's Dextrose agar and incubated at 30˚C for 2 weeks. The absence of growth is an indicator of successful inactivation. The prepared suspension was stored at 4˚C until subsequent use as a vaccine. Freund's adjuvant of inactive *Mycobacterium tuberculosis* was chosen asthe adjuvant. It was used in two forms: initially as complete Freund's adjuvant and subsequently as incomplete Freund's adjuvant for the remainder of the experimental period.

Animals

A case control study was performed on rabbits to investigate the prophylactic and therapeutic potential of the dermatophytic vaccine. Ethical approval for this study was obtained from the Ethical Scientific Committee of the College of Medicine, University of Karbala, no. 504, on June 2, 2020. A total of 18 healthy rabbits weighing 2.5-3.5 kg were used in this study. For the prophylaxis study, 12 healthy rabbits were divided into four groups with 3 rabbits in each group. The first group was injected subcutaneously with 1 mL of vaccine only, the second with a mixture of 1 mL of vaccine and 0.1 mL of Freund's adjuvant (Wahag Al-Dna, Baghdad), the third with 0.1 mL of Freund's adjuvant only, and the fourth with neither vaccine nor adjuvant. Groups were infected later with isolated *T. mentagrophytes*, and the development of dermatophytosis was followed up. Clinical changes atthe infection site inall groups were observed for 6 months.

For the treatment study, six non-vaccinated rabbits infected with *T. mentagrophytes* were divided into two groups of three. The first group was treated with 1 mL of prepared dermatophytic vaccine by subcutaneous injection once daily for 16 days. The second group, used as a control, was left untreated. Clinical changes in infected lesions were followed up for approximately 3 months.

Infection of animals

An inoculum solution containing 8x10^8 cells/mL of *T. mentagrophytes* was prepared by mixing an amount of fungal mycelium grown on Sabouraud's Dextrose agar (Himedia, India) for 1 week at 30˚C in sterilized normal saline. Counting was performed by the hemocytometer method.[29] About 5-7 cm of the neck area of each rabbit was shaved by a mechanical method to remove covering hairs from the skin. A few drops of prepared fungal suspension were inoculated onto the shaved area with some pressure and spread on the skin surface by hand. Infection development was followed up for more than 3 weeks, when lesions were evaluated clinically as dermatophytosis infection.

Statistical analysis

All test data were expressed as mean ± standard deviation. The values were analyzed statistically by One-Way ANOVA with Microsoft Windows Excel, version 10. The threshold for significance was set at $p>0.05$.

RESULTS

The rabbit was chosen as a model to study the prophylactic and treatment efficacy of an antidermatophytic vaccine against dermatophytosis. In the prophylaxis control study, the first two groups of rabbits treated with the prepared vaccine and the vaccine together with Freund's adjuvant showed resistance to infection with *T. mentagrophytes* for more than 6 months, and there were no clinical features of infection or serious inflammatory responses to the adjuvant. Thus, the vaccine alone showed a successful immunization effect against dermatophytosis without the need for adjuvant. Mean while, rabbits treated with Freund's adjuvant only and control (untreated) rabbits revealed clinical features of dermatophytosis after 16 days from the start of infection (Table 1, Figure 1). In the second study, treatment of infected rabbits with the prepared vaccine was shown to be completely curative after 8 days. The number of lesions decreased gradually after vaccination until complete healing. Rabbits in the control group revealed no signs of cure, even after 3 months (Table 2, Figure 2).

DISCUSSION

The control of opportunistic fungal infections has been met with challenges due to an increase in the occurrence of these infections among a wide range of patients, especially immunocompromised individuals, cancer patients, those in long-term treatment, and premature infants.[6] The development of resistance to antifungal agents is also associated with difficulty in their control.[4,30] Thus, a vaccine against infections by many pathogenic fungi is considered the best option to enhance the efficiency of the immune system.[6,30] This has been taken seriously as an issue since our understanding of immunity toward pathogenic fungi has improved and the incidence and mortality rate of fungal infections has increased.[6] Moreover, successful discovery of an antifungal vaccine will play an important role in limiting the use of chemotherapy or antifungal agents for the control of fungal infections.[4] The main effective role of antifungal vaccines in the human body is to elevate the stimulation of immune system components against invasive fungi. Humoral immunity is the component of the immune system most affected by vaccine.[7,31] This type of activation will provide protection to immunocompromised patients, especially after activation of antibody production.[1] Activation of cellular immunity represented by a Th1 response with induction of IL-12 and IFN-γ is also required from an effective vaccine.[30]

Antifungal vaccine is usually prepared from living or inactivated whole cells, or from one component of fungi such as cell wall components, cytoplasmic extracts, and genetic recombinant proteins.[32] Extensive studies have been performed to develop an effective vaccine against fungal infections in both man and animals.[4,5] Although some vaccines are available for use in animals, researchers are still looking for a perfect vaccine. A satisfactory result has been achieved from vaccines used to immunize against various fungal diseases such as candidiasis,

Table 1. Infection period of vaccinated rabbits

Group	Infection period (days)
Vaccine only	None*
Vaccine with adjuvant	None*
Adjuvant only	16
Control	16

*Significant difference between groups at $p<0.05$

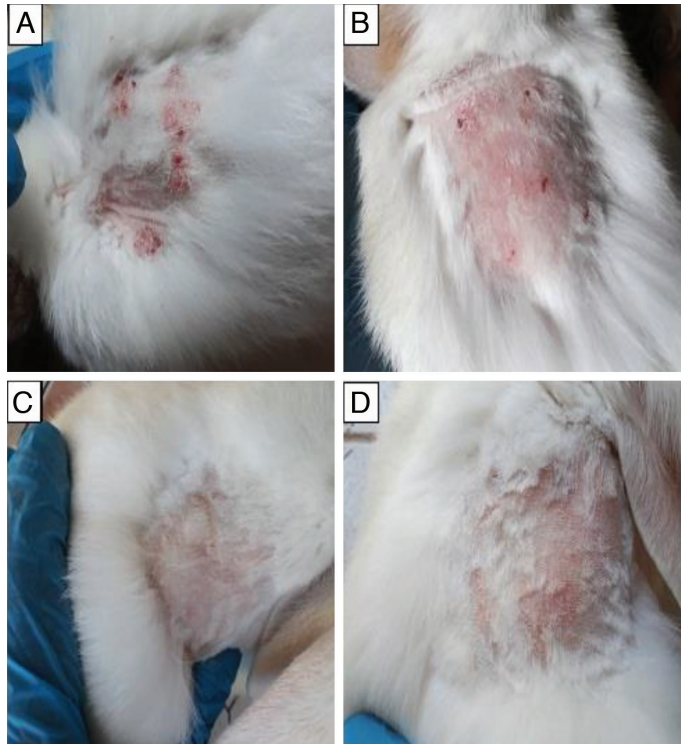

Figure 1. Infected rabbit with *Trichophyton mentagrophytes* after vaccination. A) Control rabbit with dermatophytosis lesions (red with granulated skin). B) Vaccinated rabbit with ad juvant only, which showed the same infectious features as the control. C) Vaccinated rabbit with vaccine ony without any lesions of dermatophytosis for more than 6 months. D) Vaccination with vaccine and adjuvant without any lesions of dermatophytosis for more than 6 months

blastomycosis, coccidioidomycosis, histoplasmosis, and paracoccidioidomycosis.[4] However, the United States Food and Drug Administration (US FDA) has not yet licensed any vaccine for commercial use.[4,5,32]

Dermatophytosis is a typical common disease in the cutaneous layer of the skin of different parts of the human or animal body.[8] Control of this disease has recently gained attention after elevation of the drug-resistance rate in many causative dermatophytes[33] and also because treatment of dermatophytosis, especially in animals, is usually expensive and time-consuming.[7] Thus, the development of an anti-dermatophytic vaccine may introduce a solution to decrease these disadvantages and also to limit the transfer of dermatophytes between humans and animals.[7,34] However, increasing the immune response against dermatophytosis can be a key to limiting the toxicity and virulence effects of this disease.[35] Recently, many studies have attempted to enhance the prophylactic action of the antifungal vaccine by stimulating cellular immunity to increase the immunization rate against dermatophytosis.[8] This has been achieved by using specific antigens of a dermatophyte, especially those from *Trichophyton* spp., to provide stronger immunization than it can gain from inactive vaccine.[7-8] Although no vaccine has a license for commercial use against dermatophytosis, some countries, such as Norway, immunize their cattle with a vaccine against *Trichophyton verrucosum* as a strategy to control dermatophytosis.[7] The company Biocan M plus in the Czech Republic also produces an unlicensed vaccine from inactivated *Microsporumcanis* for the treatment of dogs against dermatophytosis.[26]

Table 2. Treatment period of infected rabbits after vaccination

Group	No of lesions before treatment	Treatment period (days)
Vaccine only	5-20	8*
Control	6-12	None

*Significant difference between groups at $p<0.05$

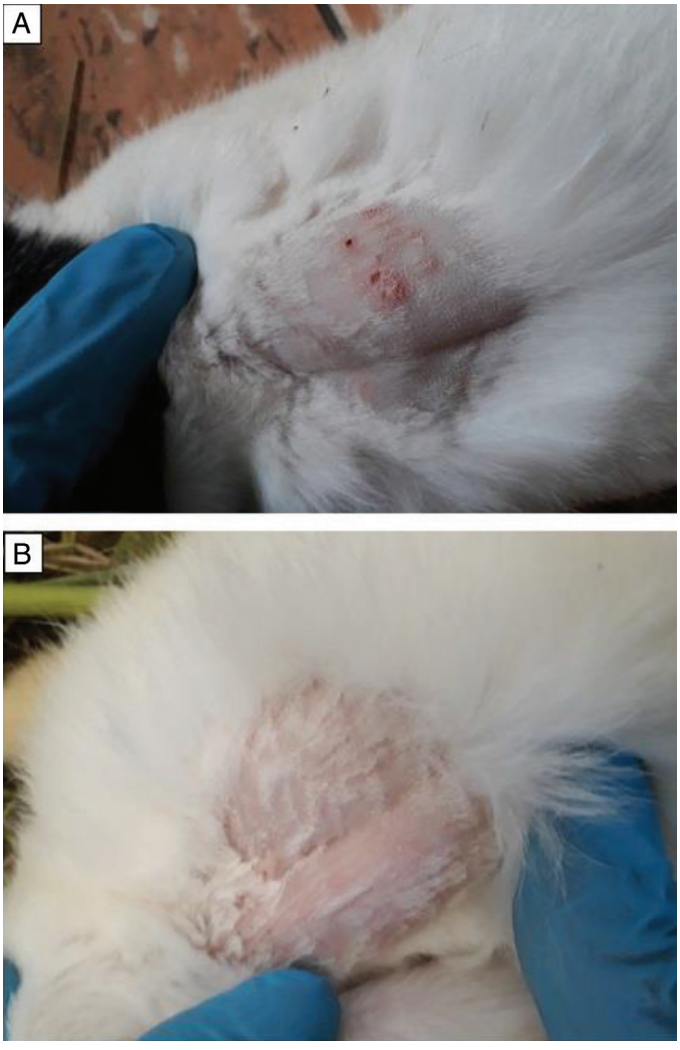

Figure 2. Treatment of infected rabbit with inactivated vaccine. A) Rabbit with dermatophytosis lesions before treatment. B) Rabbit with cure of infection after 8 days of treatment

Our prepared vaccine provided prophylaxis to rabbits from dermatophytic infection for more than 6 months. Other studies failed to achieve this period;for example, the study of DeBoer and Moriello[21] found that dermatophytosis lesions developed in cats vaccinated with killed *M. canis* cell wall after a 16-week challenge with other infected cats. The rabbit is often preferred for use as an animal model for fungal infection over small mammals due to the ease of observation of changes in fungal lesions.[34] Vaccination of rabbits with culture filtrate antigens of one dermatophyte species was found to provide immunization against six other species, as indicated by a positive skin test.[24] Subcutaneous injection of rabbits with heat-killed *Trichophyton purpureum* suspended in Freund's adjuvant also provided protection against infection bythe same fungus for more than 17 months.[36] Vaccination with heat-inactivated macroconidia ($6\text{-}24x10^6$ cell/mL) of *T. mentagrophytes* and *M. canis* protected rabbits and guinea pigs against infection with a virulent isolate of *T. mentagrophytes*.[15] Other animals have also shown resistance to infection with dermatophytes after immunization with antifungal vaccine. Vaccination of guinea pigs with *Trichophyton equinum* vaccine increased resistance to *M. canis* compared with non-vaccinated controls.[24]

In this study, vaccination was performed by inactive cells of *T. mentagrophytes* either alone or with adjuvant. This type of vaccine can exhibit a better outcome in some cases than from vaccination of animals with a specific component of dermatophytes.[7,13,18,19,21,25,37] Intramuscular injection with a live vaccine of *T. verrucosum* was found useful to protect calves from dermatophytosis,[18] while purified recombinant keratinolytic metalloprotease failed to protect guinea pigs against infection with *M. canis*.[13] A freeze-dried preparation of live vaccine of *T. verrucosum* was also used successfully to protect calves against experimental dermatophytosis.[25] Cats vaccinated with killed cell wall of *M. canis* showed efficiency at stimulating production of a high titer of anti-dermatophyte IgG and a weak cell-mediated response.[21] A vaccine of whole cells of live and killed *Aspergillus fumigatus* also provided variable protection against aspergillosis in a mouse model.[37] However, intra- or subcutaneous injection of whole cell or crude extract

of dermatophytes has the ability to introduce greater protection in animals than when introduced by other routes.[19]

According to the results of this study, there was no difference between the use of prepared vaccine alone and with Freund's adjuvant. Both vaccinated groups of rabbits were resistant to infection by *T. mentagrophytes* for more than 6 months, while rabbits treated with only Freund's adjuvant showed infection after 16 days. This indicates that the presence of Freund's adjuvant had no effect on the prophylactic efficacy of prepared vaccine to stimulate the immune system. Westhoff et al.[20] also found a similar result when they tested the prophylactic activity of non-adjuvanted inactivated vaccine in cats prepared from some strains of dermatophytes. In fact, the main reason to use Freund's adjuvant in this study was to increase potential immunologic stimulation by theprepared vaccine. Many studies have investigated the efficacy of antidermatophytic vaccines in animals after mixing them with adjuvant. Pier[23] found that a suspension of killed *Trichophyton equinum* with adjuvant showed effective prophylaxisin horses and guinea pigs against infection with the same fungus or with other species of dermatophytes. Adjuvanted secreted compounds of *M. canis* with monophosphoryl lipid-A revealed partial protection against infection with the same fungus in guinea pigs.[14] In general, adjuvant, which contains one or more complex compounds, is preferred for use with a vaccine of a single antigen that has a weak ability to stimulate the immune response.[4,36] Recently, researchers have attempted to develop an antifungal vaccine from purified, recombinant, or synthetic antigen, which all require adjuvant to obtain suitable protection against infection with pathogenic fungi.[1,4,36] Unlike vaccines prepared with inactivated organisms, vaccines made from asingle antigen always have problems with purity and production.[1] Freund's adjuvant, which contains heat-killed *Mycobacterium tuberculosis* emulsified in mineral oil, is commonly used for accelerating new vaccine testing to stimulate immunity against various infections in animal experiments.[1,4,31] This adjuvant role may maintain continual vaccine-stimulated immunity for a long period of time. By lengthening fungal antigen release into the injection site.[38] Cellular immunity, such as that mediated by T-helper cells is usually elicited by Freund's adjuvant, which can also stimulate humoral immunity.[1,4,31,36] However, the combination of a vaccine with an adjuvant is used to achieve many purposes, including an increased immunological response through stimulation of various immunologic pathways; alteration of the immune response to a specific infection; and allowing the useof a small vaccine dose.[31]

In the second part of this study, the antifungal vaccine preparation showed therapeutic activity against dermatophytosis in rabbits in a short time (8 days), compared with the untreated group. In another study, a filtered culture of *T. verrucosum* was prepared as an injected vaccine with adjuvant for treatment of cows and buffaloes with dermatophytosis, and it showed effective results 10 days after injection.[39] In a placebo-controlled, double-blind study, a mixed aqueous preparation of inactivated vaccine from six dermatophyte species exhibited better curative action for cats with dermatophytosis, especially those with a first infection or ata young age.[17] In activated vaccine of five species of dermatophytes without adjuvant was also used in another control study for treatment of cats with dermatophytosis, but no significant differences were observed between treated cats and a control group.[20] Some companies are attempting to produce an effective prophylaxis and treatment vaccine against dermatophytosis in animals, but their work is still not licensed by the FDA. The Micanfin (Biocan-M®) vaccine manufactured by Bioveta (Czech Republic), which is composed of inactivated *M. canis*, is used commercially to immunize cats and dogs against dermatophytosis.[16,40] Erman Or et al.[40] also found that the Micanfin product has therapeutic action against dermatophytosis in cats after two vaccination doses with a 21-day interval, while another study showed that this vaccine needed 20-30 days for treatment of cats with dermatophytosis.[16] Meanwhile, Chansiripornchai and Suanpairintr[22] found that treatment of a male cat with dermatophytosis with the Micanfin vaccine reduced infectious lesions and regrowth of hair 14 days after the first injection. Nedosekov et al.[15] performed a clinical trial to evaluate the ability of the heat-inactivated vaccine of *T. mentagrophytes* and *M. canis* called Funhikanifel to immunize dogs and cats against experimental dermatophytosis. A single vaccination was followed by recovery of 27% of dogs, while double vaccination cured 96.8% of all animals.

The development of new vaccines for dermatophytosis in humanshas faced many challenges. First, the majority of fungal infections affect immunocompromised patients (this is not the case with dermatophytosis). This can be resolved by choosing a vaccine with the ability to elicit humoral immunity. Second, vaccine preparation has become expensive, especially for those prepared from recombinant antigens. Third, the new vaccine may act on the normal flora in the human body when it is used against diseases caused by one of them, for example candidiasis. Fourth, the use of an antigen similar toone present in the host that can induce an unnecessary autoimmune response and fifth, some types of vaccine either with or without adjuvant may not induce adequate immunization in some individuals.[1,6,31,32,37]

CONCLUSION

Although the search continues for a suitable vaccine against dermatophytosis, no one has a license from a regulatory organization such as the FDA for use in the commercial field. A new preparation of vaccine from inactivated *T. mentagrophytes* showed effective prophylaxis and treatment results against dermatophytosis in rabbits. Long-term protection and a short treatment time are the most significant results obtained from this study.

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ORIGINAL ARTICLE

Hepatoprotective Activity of Gentisic Acid on 5-Fluorouracil-induced Hepatotoxicity in Wistar Rats

Wistar Sıçanlarında Gentisik Asidin 5-Florourasil Kaynaklı Hepatotoksisite Üzerindeki Hepatoprotektif Aktivitesi

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ABSTRACT

Objectives: 5-Fluorouracil (5-FU) is a very potent and effective antineoplastic drug that has been widely used for the management of various types of cancer. However, the clinical use of 5-FU is often associated with severe toxicities including hepatotoxicity, which limit its therapeutic use as a potent anticancer agent. The present study aimed to evaluate the hepatoprotective activity of a plant phenolic acid, gentisic acid (GA) (2,5-dihyroxybenzoic acid), against hepatotoxicity induced by 5-FU administration in Wistar rats.

Materials and Methods: The rats were randomly divided into six groups, with six rats per group. Among these, group I and II served as normal control and 5-FU control groups, respectively. The rats in these groups received distilled water (1 mL/kg) for 14 days by oral route. Groups III, IV, V, and VI served as test groups and received GA at doses of 3, 10, 30, and 100 mg/kg body weight, respectively, via oral route for 14 days. From Day 9 onwards, all the groups, except group I, received intraperitoneal dose of 5-FU (20 mg/kg body weight) for five days up to day 14. At the end of the study, the rats were sacrificed, blood was withdrawn for biochemical estimations, and hepatic tissues were excised for histopathological evaluations.

Results: Administration of 5-FU at a dose of 20 mg/kg body weight resulted in a significant increase in the serum levels of hepatic biomarkers, including aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, direct bilirubin, and total bilirubin. In comparison to these, 5-FU treatment resulted in a reduction in total protein content (TPC). This was accompanied by significant histopathological changes in the hepatic tissues of the rats, indicating severe hepatotoxicity. Pre- and co-administration of GA with 5-FU at doses of 30 and 100 mg/kg body weight for 14 days resulted in a dose-dependent amelioration of the 5-FU induced alterations in the biochemical and histopathological parameters.

Conclusion: The results of the study highlighted the potential of GA as a hepatoprotective agent for the prevention of 5-FU-induced hepatotoxicity.

Key words: 5-Fluorouracil, gentisic acid, 2,5-dihyroxybenzoic acid, hepatotoxicity, plant phenolics, hepatoprotective, cancer chemotherapy

ÖZ

Amaç: 5-Florourasil (5-FU), çeşitli kanser türlerinin tedavisinde yaygın olarak kullanılan çok güçlü ve etkili bir antineoplastik ilaçtır. Bununla birlikte, 5-FU'nun klinik kullanımı genellikle, güçlü bir antikanser ajan olarak terapötik kullanımını sınırlayan hepatotoksisite dahil olmak üzere ciddi toksisitelerle ilişkilidir. Bu çalışmada, Wistar sıçanlarında 5-FU uygulaması ile uyarılan hepatotoksisiteye karşı bir bitki fenolik asiti olan gentisik asidin (GA) (2,5-dihiroksibenzoik asit) hepatoprotektif aktivitesini değerlendirmeyi amaçladık.

Gereç ve Yöntemler: Sıçanlar rastgele olarak grup başına altı sıçan olacak şekilde altı gruba ayrıldı. Bunlar arasında grup I ve II, sırasıyla normal kontrol ve 5-FU kontrol grupları olarak belirlendi. Bu gruplardaki sıçanlar 14 gün boyunca oral yolla damıtılmış su (1 mL/kg) aldı. Gruplar III, IV, V ve VI test grupları olarak görev yaptı ve 14 gün boyunca oral yoldan sırasıyla 3, 10, 30 ve 100 mg/kg vücut ağırlığı dozlarında GA aldı. Dokuzuncu günden itibaren, grup I hariç tüm gruplar, 14. güne kadar beş gün boyunca 5-FU (20 mg/kg vücut ağırlığı) intraperitoneal doz aldı. Çalışmanın sonunda, biyokimyasal analiz için kanları alındıktan sonra sıçanlar sakrifiye edildi ve hepatik dokular histopatolojik değerlendirmeler için eksize edildi.

Bulgular: 20 mg/kg vücut ağırlığı dozunda 5-FU uygulaması aspartat aminotransferaz, alanin aminotransferaz, alkalen fosfataz, bilirubin ve toplam bilirubin dahil olmak üzere hepatik biyobelirteçlerin serum seviyelerinde önemli bir artışa neden olmuştur. Bunlarla karşılaştırıldığında, 5-FU tedavisi toplam protein içeriğinde bir azalmaya yol açtı. Buna, sıçanların hepatik dokularında şiddetli hepatotoksisiteyi gösteren önemli histopatolojik değişiklikler eşlik etti. GA'nın 14 gün boyunca 30 ve 100 mg/kg vücut ağırlığı dozlarında 5-FU ile önceden ve birlikte uygulanması, biyokimyasal ve histopatolojik parametrelerde 5-FU kaynaklı değişikliklerde doza bağlı bir iyileşme ile sonuçlanmıştır.

Sonuç: Çalışmanın sonuçları, GA'nın 5-FU ile indüklenen hepatotoksisitenin önlenmesi için bir hepatoprotektif ajan olarak potansiyelini vurguladı.

Anahtar kelimeler: 5-Florourasil, gentisik asit, 2,5-dihiroksibenzoik asit, hepatotoksisite, bitki fenolikleri, hepatoprotektif, kanser kemoterapisi

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INTRODUCTION

Cancer is one of the leading causes of death worldwide. It accounted for ~10 million deaths in the year 2020. Chemotherapy is a standard cancer therapy that is used either alone or in combination with surgery or radiation therapy (neoadjuvant and adjuvant therapy). Despite its success in killing cancer cells, chemotherapy-induced hepatotoxicity and impairment of liver function limit its application. The adverse clinical complications associated with chemotherapy often require dose reduction or withdrawal of chemotherapeutic agents, thereby limiting their therapeutic potential as effective antineoplastic agents. Thus, the negative aspects associated with the administration of chemotherapeutic drugs limit their clinical application, regardless of their contribution in the improvement of patient survival rate.[1,2]

5-Fluorouracil (5-FU) is a fluorinated pyrimidine analog, which is widely used in the management of various types of cancer, including stomach, breast, head and neck, colorectal, and genitourinary cancers.[3] It is used either alone or in combination with other drugs. 5-FU generally acts via incorporation into both DNA and RNA. The administration 5-FU results in the incorporation of deoxyuridine triphosphate and fluorodeoxyuridine triphosphate molecules into DNA via replacement of reduced thymidine triphosphate (TTP).[4] Incorporation of fluorodeoxyuridylate and deoxyuridylate in DNA is accompanied by initiation of excision-repair process, which results in the breakage of DNA strand owing to the absence of TTP. This is generally mediated via blockade of the enzyme activity of thymidylate synthase. The insertion of 5-FU into RNA severely affects the functioning as well as processing of RNA, which further results in severe toxicities.[5] 5-FU chemotherapy has been reported to exhibit severe systemic toxicities, including hepatotoxicity, in clinical practice.[6] Generally, 5-FU is eliminated from the body via hepatic metabolism. Dihydropyrimidine dehydrogenase enzyme found in the liver is the key enzyme that catalyzes the rate-limiting step in 5-FU catabolism. Toxic intermediates produced during the metabolism of 5-FU are majorly responsible for liver injury.[7] Various *in vitro* and *in vivo* studies have demonstrated that administration of 5-FU is accompanied by induction of oxidative stress in the liver, which consequently results in the structural and functional disruption of hepatocytes. Thus, devising strategies to reduce 5-FU associated hepatotoxicity might prove beneficial for improving the overall clinical outcomes of this chemotherapeutic agent.[8-10]

Since the primordial era, plants have been successfully used as therapeutic agents to cure various ailments. Currently, sincere efforts are being invested to harness the therapeutic potential of the phytoconstituents, having antiapoptotic, antioxidant, and antiinflammatory properties, to reduce the cancer chemotherapy-induced drug toxicities.[11,12] Several preclinical studies have reported beneficial effects of daily consumption of fruits and vegetables, particularly in decreasing the risk of neoplasm. This effect is majorly contributed by the presence of various essential nutrients, predominantly phenolics, in fruits and vegetables.[13] Phenolic acids are the most extensively distributed secondary metabolites in the plant kingdom and about 10,000 structures of plant phenolics are known. These compounds have been widely investigated and several preclinical studies have established their importance for human well-being.[14,15]

Gentisic acid (GA) is a phenolic compound that has been shown to exert beneficial effects on human health. Various pharmacological activities have been reported for GA including analgesic, antiarthritic, antiinflammatory, antimutagenic, anticancer, antirheumatic, and antispasmodic activities. In addition to these, it is also known to exert antiparkinsonian, antifungal, iron chelation, and siderophoric effects. Evidences are available for its antihyperlipidemic activity, protective activity against cyclophosphamide-induced genotoxicity, and inhibitory activity against fibroblast growth factor. Besides these, antioxidant effects of GA have been reported in both *in vivo* and *in vitro* studies.[16-28] In a previous study, GA has been shown to ameliorate cyclophosphamide-induced hepatotoxicity *in vivo*. The results of the study showed that both pre- and co-treatment with GA at doses of 50 and 100 mg/kg body weight ameliorated the cyclophosphamide-induced increase in malondialdehyde levels. This was accompanied by normalization of the levels of all oxidative stress biomarkers, including glutathione peroxidase, glutathione reductase, glutathione, catalase, and quinone reductase. GA administration also resulted in a reduction in DNA fragmentation and formation of micronuclei. Additionally, pre- and co-treatment with GA ameliorated the cyclophosphamide-induced increase in the hepatic biomarkers, such as alanine aminotransferase (ALT), lactate dehydrogenase, and aspartate aminotransferase (AST).[29] Currently, no reports are available for the protective effects of GA against the hepatotoxicity induced by anticancer agents including 5-FU.

The present study aimed to evaluate the protective activity of GA against 5-FU-induced hepatotoxicity. The study involved quantitative estimation of the enzymes, involved in hepatic function, in the serum. Histopathological investigations were carried out to evaluate the ultrastructural alterations in the hepatic tissue arising due to 5-FU treatment.

MATERIALS AND METHODS

Chemicals and kits

GA was procured from Sigma-Aldrich Chemicals, USA. 5-FU (fiveflurd) was purchased from GlaxoSmithKline Pharmaceuticals Ltd. Mumbai, Maharashtra, Analytical grade solvents and chemicals used in the study were obtained from Oswal Chemicals, New neeta Chemicals and Loba Chemie Pvt. Ltd., Pune, Maharashtra, India. Diagnostic kits used for biochemical analysis were procured from Biolab Diagnostics Pvt. Ltd., Kiran Enterprises, Pune, India.

Animals

For *in vivo* experiments, 8-12 weeks old adult Wistar rats (200-250 g) from either sex were used. These rats were

purchased from the National Institute of Biosciences, Pune, Maharashtra. Upon arrival, the rats were caged in groups of 5-6 rats in standard polypropylene cages having a wire mesh lid. The rats were maintained under standard environmental conditions, at a temperature of 25°C±2°C with 45%-55% relative humidity and 12/12 h light/dark cycle, in the institutional animal house facility. The animals had free access to standard pelleted chow (Nutrivet Life Sciences, Pune, India) and water during the entire course of the study. The animals were acclimatized upto 04 days prior to the experimental procedures. All experimental procedures were performed during the day time, between 12:00 and 16:00 h. The animals were transferred from the animal house facility to the experimental laboratory one hour prior to the experiments.

Ethical clearance

All the experimental procedures included in the study were carried out in compliance with the Institutional Animal Ethical Committee (IAEC) Guidelines given as per the Committee for the Purpose of Control and Supervision of Experiments on Animals, New Delhi, India (Section-15 of the Prevention of Cruelty to Animals Act, 1960; Ministry of environment and forest, Government of India). The experimental protocols were approved by IAEC of Modern College of Pharmacy, Yamunanagar Nigdi, Pune-411044 (proposal no: MCP/IAEC/004/2017; date: 07/11/2017).

Preparation of drug solutions

GA stock solutions, at concentrations of 3, 10, 30, and 100 mg/mL, were prepared by dissolving desired amount of GA in distilled water. For 5-FU, a stock solution of 20 mg/mL was prepared by dissolving required amount of 5-FU in distilled water. The selection of stock solutions was made according to the required dose of administration.

Experimental design

The rats were randomly divided into six groups, with six rats per group. Group I and II served as normal control and 5-FU control groups, respectively. The rats in these two groups received distilled water (1 mL/kg body weight) orally for 14 days. Groups III, IV, V, and VI served as test groups and received oral doses of GA at concentrations of 3, 10, 30, and 100 mg/kg body weight, respectively, for 14 days. From Day 9 onwards, all groups, except group I, received intraperitoneal dose of 5-FU (20 mg/kg body weight) for five days up to day 14.

At the end of the experiment, the rats were sacrificed by cervical dislocation. Blood samples were obtained by cardiac puncture and collected in serum separation tubes. The serum was obtained from the collected samples by centrifugation at 3000 rpm at 20°C for 20 min. Post centrifugation, the serum was carefully transferred into Eppendorf tubes and stored at -20°C. These sera samples were used for the assessment of hepatic biomarkers. Following this, the livers were excised, washed using ice-cold saline solution, dried, and stored at -80°C to -20°C until used for further analysis.[29]

Estimation of hepatic biomarkers

The levels of alkaline phosphatase (ALP), AST, ALT, total bilirubin (TB), direct bilirubin (DB), and total protein content (TPC) in the collected serum samples were estimated using standard biochemical estimation kits. These assays involved spectrophotometric measurements that were performed using a utlraviolet (UV)-2600 UV-visible spectrophotometer (Shimadzu Corporation).

Histopathological studies

For fixation, the whole intact liver specimens were treated with formalin (10%, v/v) for 24 hours. Following this, the samples were embedded in paraffin. A rotary microtome (Biocraft) was used to obtain representative coronal slices (5 µm thickness) of organs. Further, these coronal slices were stained with hematoxylin for 8 min, followed by staining with eosin for 3 min Luna.[30] Permanent slides were prepared using these thin sections. These slides were visualized under a lens with 45x magnification power using a digital trinocular microscope (Olympus CX-21-TR), available at the institutional imaging facility. The photomicrographs were captured with the help of Magnuspro eyepiece camera software.

Statistical analysis

The results were expressed as mean ± standard error of mean. For comparison between the groups, One-Way ANOVA was performed, followed by Tukey's Kramer Multiple Comparison test using Instat Graph Pad software (version 3).

RESULTS

Estimation of hepatic biomarkers

In the present study, the levels of serum hepatic biomarkers, including ALP, AST, ALT, TB, DB, and TPC, were estimated using biochemical assays. As shown in Figure 1, 2, the administration of 5-FU resulted in a significant ($p<0.001$) increase in the serum levels of ALP, AST, ALT, TB, and DB levels; however, a significant reduction in TPC levels was observed in the 5-FU control group as compared to the normal control group. In comparison to this, pre- and co-treatment with GA at the doses of 30 and 100 mg/kg body weight resulted in a dose-dependent amelioration of the alterations in the hepatic biomarkers, with statistical significance of $p<0.01$ and 0.001, respectively (Figure 1, 2).

Histopathological studies of hepatic tissue

Histopathological investigation of the hepatic tissues for the normal control group showed the presence of normal hepatocytes and central vein. In comparison to this, liver specimen for 5-FU control group rats were characterized by marked reactive changes, suggestive of hydropic degeneration (HD) of the hepatocytes and focal necrosis at the central vein zone along with the disruption of the hepatic central vein (HCV). The pre- and co-treatment with GA resulted in a dose-dependent amelioration of these histopathological alterations. As shown in Figure 3, GA administration at doses of 30 and 100 mg/kg body weight showed moderate and marked improvement, respectively, in the histopathological alterations of the liver tissues.

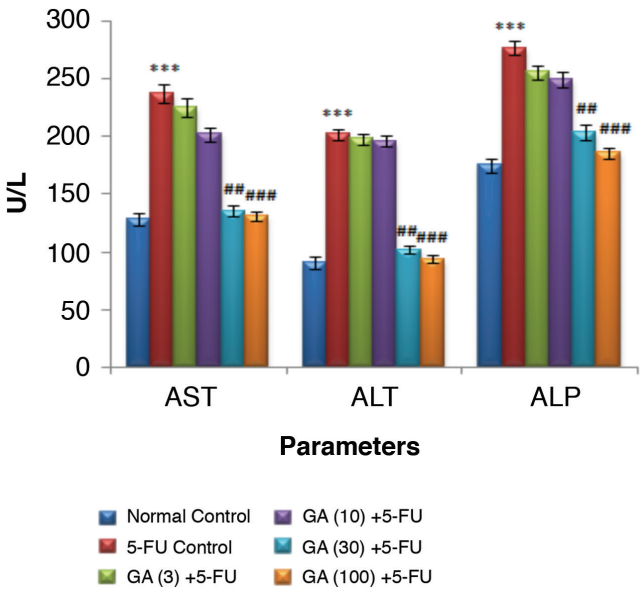

Figure 1. Effect of GA on serum levels of AST, ALT and ALP in 5-FU-induced hepatotoxicity in rats. Results were expressed as mean ± SEM (n=6). Comparison between the groups was carried out using One-Way ANOVA, followed by Tukey's Kramer Multiple Comparison test.

***p<0.001 as compared to normal control, ##p<0.01, ###p<0.001 as compared to 5-FU induced control group, SEM: Standard error of mean, GA: Gentisic acid, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, 5-FU: 5-Fluorouracil

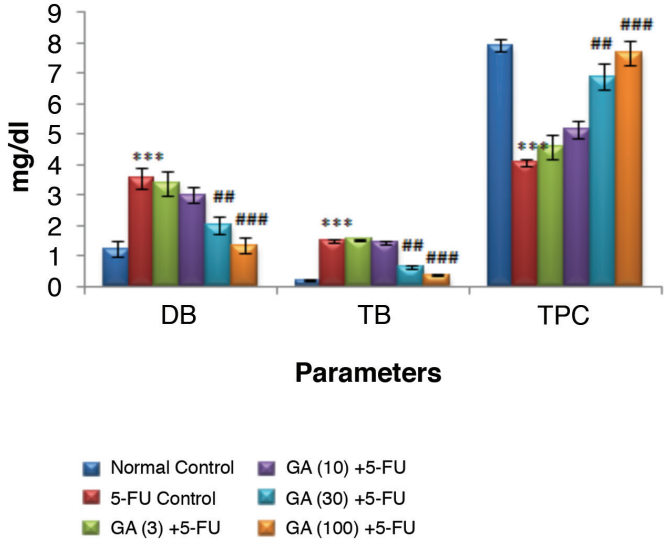

Figure 2. Effect of GA on serum levels of DB, TB, and TPC in 5-FU-induced hepatotoxicity in rats. Results were expressed as mean ± SEM (n=6). Comparison between the groups was performed using One-Way ANOVA, followed by Tukey's Kramer Multiple Comparison test.

***p<0.001 as compared to normal control, ##p<0.01, ###p<0.001 as compared to 5-FU-induced control, GA: Gentisic acid, DB: Direct bilirubin, TB: Total bilirubin, TPC: Total protein content, 5-FU: 5-Fluorouracil, SEM: Standard error of mean

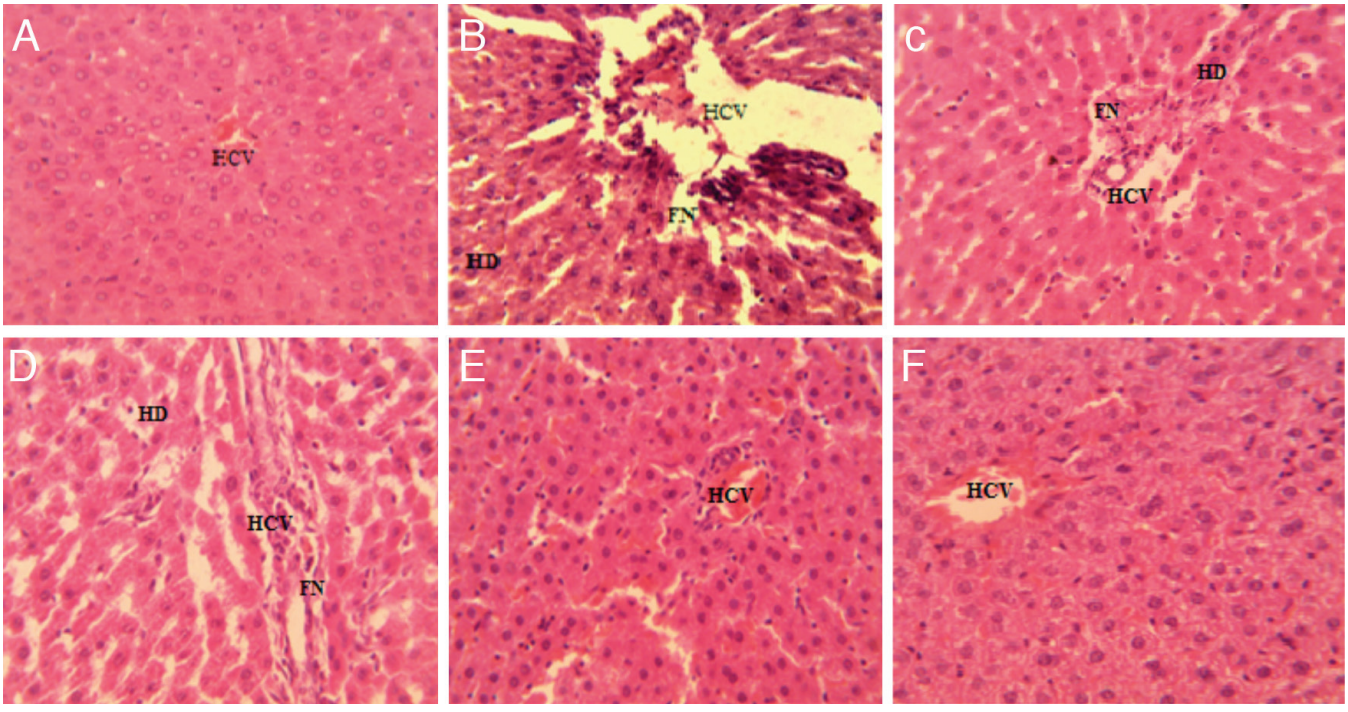

Figure 3. Histopathological investigations for effect of GA on 5-FU-induced hepatotoxicity representative photomicrographs (H & E stain) for liver sections of: (A) Normal control rats showing normal liver architecture with normal hepatic central vein (HCV) and hepatocytes; (B) 5-FU control rats showing marked reactive changes, suggestive of hydropic degeneration (HD) of the hepatocytes as well as focal necrosis (FN) at central vein zone with the disruption of the HCV; (C) GA (3 mg/kg body weight) + 5-FU and (D) GA (10 mg/kg body weight) + 5-FU group rats showing reactive changes similar to 5-FU control with no improvement; (E) GA (30 mg/kg body weight) + 5-FU rats showing a moderate reduction in the reactive changes caused by 5-FU; (F) GA (100 mg/kg body weight) + 5-FU showing marked amelioration of histological alterations caused by 5-FU. Photographs were taken under 45x magnification power using a trinocular microscope (Olympus CX-21-TR) with Magnuspro eyepiece camera software

GA: Gentisic acid, 5-FU: 5-Fluorouracil, H & E: Hematoxylin and eosin

DISCUSSION

The liver is the central organ involved in the detoxification and clearance of waste products. Frequent administration of high doses of chemotherapeutic agents for cancer treatment has been shown to be associated with hepatotoxicity.[31] 5-FU is an important drug that has been widely used for cancer treatment. Since 5-FU is mainly metabolized in the liver, its administration is often associated with hepatotoxicity. The toxic metabolites produced by 5-FU tend to initiate hepatic injury resulting in severe hepatotoxicity, which limits the chemotherapeutic utility of 5-FU as an efficacious anticancer agent. 5-FU induced hepatotoxicity generally involves increase in apoptosis, oxidative stress, and inflammatory reactions.[29] In the present study; the levels of different hepatic biomarkers were evaluated to confirm 5-FU-induced hepatotoxicity.

Generally, serum transaminases such as AST, ALT, and ALP are used as important indicators of hepatic damage.[32] Among these, ALT is an important cytosolic enzyme that is more specific for the liver. AST is generally located in the hepatic mitochondria. The obstruction or inflammation of the biliary tract has been shown to result in an increase in ALP activity in plasma.[33] Increased levels of ALP in the blood are majorly contributed by leakage of these transaminases from the hepatocytes into the circulation, indicating liver damage or dysfunction.[32] In the present study, administration of 5-FU resulted in a significant increase in the levels of hepatic biomarkers, including ALP, AST, ALT, DB, and TB, as compared to the normal control group. Besides this, 5-FU treated group showed decrease in TPC. All these results suggested that 5-FU administration was associated with severe hepatotoxicity. These results were in agreement with the previous studies.[29] In the treatment groups of the present study, pre- and co-treatment with GA resulted in the reversal of transaminase levels to normal. These results suggested hepatoprotective activity of GA, which was probably mediated by the reduction of hepatic injury and inflammation. Total and DB levels are generally used as indicators for the normal functioning of the liver. An increase in total and DB levels has been found to be associated with hepatic disorders, indicating severe hepatic damage leading to jaundice.[34] In the present study, GA administration resulted in the reduction in the elevated levels of bilirubin that were induced by 5-FU treatment. Thus, all these results indicated the hepatoprotective potential of GA, highlighting its usefulness in ameliorating the side effects arising from the use of 5-FU therapy for various hepatic disorders.

TPC in serum provides an estimate for the total number of proteins present in the body fluids. Since the liver is involved in the synthesis of various proteins in the body, low protein content is used as an important marker for hepatic damage and dysfunction in various hepatic disorders.[35] In the present study, pre- and co-treatment with GA resulted in an overall improvement in the TPC post 5-FU administration. These results further established the hepatoprotective activity of GA mediated by the reduction of hepatic damage and dysfunction. Thus, all these observations highlighted the potential of GA as a protective agent to ameliorate 5-FU-induced hepatotoxicity.[36]

Histophathological examination is one of the most important investigation that is required to establish the protective role of drugs against vital organ toxicities.[35] In order to confirm the results obtained for the biochemical estimations, histopathological studies were also performed for the hepatic tissue excised from the rats at the end of the study. In the present study, significant histopathological changes were observed in the hepatic tissues of the 5-FU control group as compared to the normal control group. These changes provided visual evidences for the hepatotoxicity induced by 5-FU administration. These results were in agreement with the alterations reported for hepatic biomarkers in the biochemical assay. Treatment with 5-FU resulted in HD and necrosis of the hepatocytes with the HCV disruption. Similar histopathological alterations have been reported in several previous studies.[37] 5-FU-induced hepatoxicity generally involves hepatic inflammation, apoptosis, and oxidative stress. GA has been previously reported to possess antiinflammatory and antioxidant properties, which might be responsible for this hepatoprotective effect of GA against 5-FU-induced hepatotoxicity. In a previous study, GA was shown to exert hepatoprotective effect against cyclophosphamide-induced hepatotoxicity. This hepatoprotective activity involved restoration of hepatic antioxidant levels and reduction of micronuclei formation and DNA fragmentation.[28] A similar mechanism might also be responsible for GA mediated amelioration of 5-FU-induced hepatotoxicity. A better understanding regarding the underlying mechanism involved in the hepatoprotective activity of GA would ensure true utilization of this protective agent.

CONCLUSION

In the present study, administration of 5-FU exhibited severe hepatotoxicity which was confirmed by severe alterations in both biochemical and histopathological parameters. Further, pre- and co-treatment with GA resulted in the attenuation of 5-FU-induced hepatotoxicity. GA administration ameliorated 5-FU-induced hepatic alterations in a significant and dose-dependent manner. The chemoprotective potential of GA might be attributed to its antiinflammatory and antioxidant properties. All these results provided strong evidence to support the hypothesis that pre- and co-administration of GA could overcome 5-FU chemotherapy-induced toxicities. Thus, the use of GA might prove beneficial for the well-being of cancer patients, both during and after the chemotherapy. In addition to this, it might also enhance the overall life expectancy of the cancer patients. In order to utilize the true potential of GA as a protective agent, future studies must unravel the mechanism of action of GA and optimize the correct dose for human use. The conclusions drawn from the present study can be efficiently utilized to design suitable clinical studies to evaluate GA efficacy and safety in humans.

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ORIGINAL ARTICLE

Voltametric Determination of Zoledronic Acid in a Pharmaceutical Formulation

Farmasötik Bir Formülasyonda Zoledronik Asidin Voltametrik Tayini

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ABSTRACT

Objectives: The aim of this study is to study the electroactivity of zoledronic acid (ZOL), optimize the parameters affecting voltametric analysis of ZOL, and make a comparison between voltametric methods used to assay ZOL.

Materials and Methods: Three voltametric methods, cyclic voltammetry (CV), square wave voltammetry (SWV), and differential pulse voltammetry (DPV), were used to determine the concentrations of ZOL solutions (0.25^{-1}.2 $mg.mL^{-1}$). Britton-Robinson universal buffer solutions (BRB) were used as supporting electrolytes with a glassy carbon working electrode.

Results: The calibration plots were linear in the range from 0.20 to 1.2 $mg.mL^{-1}$ for differential DPV and CV and from 0.09 to 1.2 $mg.mL^{-1}$ for SWV. DPV showed the highest correlation coefficient R^2 value of 0.993 and the lowest limit of detection (LOD) of 37.2 $\mu g.mL^{-1}$. Furthermore, DPV exhibited the highest precision with the lowest relative standard deviations (RSD) values. For a commercial product of ZOL, DPV showed the best accuracy and precision with 102.32% recovery and 2.88% RSD.

Conclusion: ZOL is an electroactive compound. The pH of the BRB supporting the electrolyte is important for ZOL electroactivity. DPV is the recommended method for voltametric analysis of ZOL because of its high-performance regarding accuracy, precision, and LOD compared with other studied methods.

Key words: Zoledronic acid, electroanalytical methods, voltammetry, pharmaceutical compound analysis

ÖZ

Amaç: Bu çalışmanın amacı zoledronik asidin (ZOL) elektroaktivitesini incelemek, ZOL'nin voltametrik analizini etkileyen parametreleri optimize etmek ve ZOL'yi test etmek için kullanılan voltametrik yöntemler arasında bir karşılaştırma yapmaktır.

Gereç ve Yöntemler: ZOL solüsyonlarının konsantrasyonlarını ($0,25^{-1}$,2 $mg.mL^{-1}$) belirlemek için üç voltametrik yöntem, dönüşümlü voltametri (CV), kare dalga voltametri (SWV) ve diferansiyel puls voltametri kullanıldı (DPV). Britton-Robinson evrensel tampon çözeltileri (BRB) destekleyici elektrolitler olarak camsı karbon çalışma elektrodu ile kullanıldı.

Bulgular: Kalibrasyon grafikleri, DPV ve CV için 0,20 ile 1,2 $mg.mL^{-1}$ aralığında ve SWV için 0,09 ile 1,2 $mg.mL^{-1}$ aralığında doğrusaldı. DPV ile en yüksek korelasyon katsayısı R^2 değeri 0,993 ve en düşük saptama sınırı (LOD) 37,2 $\mu g.mL^{-1}$ elde edildi. Ayrıca, DPV, en düşük bağıl standart sapma (RSD) değerleriyle en yüksek hassasiyeti sergilemiştir. ZOL'nin ticari bir ürünü için DPV, %102,32 geri kazanım ve %2,88 RSD ile en iyi doğruluğu ve hassasiyeti gösterdi.

Sonuç: ZOL, elektroaktif bir bileşiktir. Elektroliti destekleyen BRB'nin pH değeri, ZOL elektroaktivitesi için önemlidir. DPV, incelenen diğer yöntemlerle karşılaştırıldığında doğruluk, hassasiyet ve LOD ile ilgili yüksek performansı nedeniyle ZOL'nin voltametrik analizi için önerilen yöntemdir.

Anahtar kelimeler: Zoledronik asit, elektroanalitik yöntemler, voltametri, farmasötik bileşik analizi

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INTRODUCTION

Zoledronic acid (ZOL), which is known by the IUBAC name (1-hydroxy-2-imidazole-1-yl-phosphonoethyl) phosphonic acid (Figure 1), is a bone resorption inhibitor compound used principally for the treatment osteoporosis, Paget's bone disease, and malignant hypercalcemia.[1-3] The pharmacologic action of ZOL bisphosphonate is to inhibit bone resorption by acting on osteoclasts or on osteoclast precursors to inhibit osteoclastic activity and skeletal calcium release induced by tumors.[2]

Several methods have been used for analysis of pharmaceutical compounds, such as chromatographic, spectroscopic, and electrochemical methods. Most standard methods essentially rely on chromatography, especially high-performance liquid chromatography (HPLC). On the other hand, chromatographic methods have high operation and instrumentation costs; furthermore, chromatographic methods are not green analytical methods because of the large amount of organic solvents used in them.

Electroanalytical methods are better alternatives to spectroscopic and chromatographic methods, because they offer advantages such as low instrumentation and operation costs, short analysis times, and simplicity.

Electroanalytical methods such as voltametric, potentiometric, and polarographic methods have been widely applied to pharmaceutical compound analysis.[4-8] These techniques show reliable results with respect to accuracy, precision, sensitivity, and selectivity.[4,5]

Several research studies have worked on ZOL determination. Most of these studies used HPLC to study ZOL in different media, such as pharmaceutical products,[9,10] human urine and blood plasma,[11] human bone,[12,13] and murine bone.[14] Legay et al.[15] developed a radioimmunoassay method for ZOL determination in human serum, plasma, and urine. Amin et al.[16] developed a switchable fluorescence probe for ZOL determination in human serum. In the present work, we study the electroactivity of ZOL and the parameters affecting voltametric analysis of ZOL, such as the working electrode and supporting electrolyte. In addition, we establish a comparison between voltametric methods used for ZOL assay in a pure pharmaceutical formulation and the final product solution.

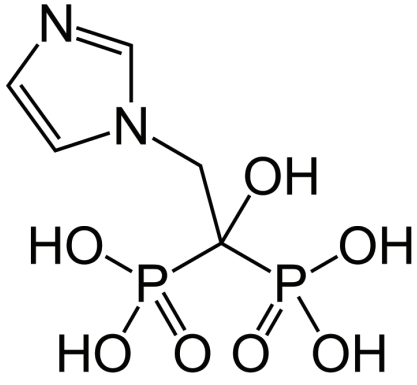

Figure 1. Structure of zoledronic acid

MATERIALS AND METHODS

Materials

Commercial product of ZOL (ZOL HIKMA® 4 mg/5 mL concentrate for solution for infusion) and ZOL standard material were provided by Hikma pharmaceutical company (Jordan). Potassium nitrate (KNO_3 ACS reagent) was from Fluka, sodium sulfate anhydrous (Na_2SO_4) was from Janssen Chemica, and citric acid anhydrous was from Al-Saggaf pharma.

Britton-Robinson universal buffer solutions (BRB) pH (2.1-11.6) were prepared by mixing certain amounts of boric acid, acetic acid, and phosphoric acid, then sodium hydroxide (0.20 M) was used to adjust the pH of the mixture.

Phosphate buffer pH 6.8 (NaH_2PO_4/H_3PO_4) supporting electrolyte was prepared by dissolving 24 g of $NaH_2PO_4 \times H_2O$ in 800 mL of deionized water, and then pH 6.8 was reached by adding 85% H_3PO_4. After that, deionized water was used to complete the volume to 1.0 L.

Sodium citrate buffer (pH 3) supporting electrolyte solution was prepared by mixing 42.5 g of citric acid with 800 mL of deionized water, and then pH 3 was reached by adding NaOH 20% w/v to the solution.

Standard ZOL solutions

Standard stock solutions of ZOL were prepared by dissolving a certain quantity of standard ZOL powder in supporting electrolyte solutions. Working solutions of ZOL standard were prepared by diluting the standard stock solution with the supporting electrolyte. ZOL HIKMA® 4 mg/5 mL concentrate for the solution for the infusion vial was diluted in BRB buffer solution pH 10.52 to reach the required concentration. All voltametric analysis methods were performed in triplicate at each concentration.

Apparatus

A potentiostat (Metrohm Autolab) PGSTAT 204 was used for voltametric determination of ZOL samples. Glassy carbon (GC), gold and platinum were used as the working electrodes in this study, with platinum as the counter electrode and Ag/AgCl (3M KCl) as the reference electrode.

Statistical analysis

Several statistical analyses were conducted in the present work according to ICH guidelines. The statistical analysis included linearity, range, limits of detection and quantitation, and interday and intraday relative standard deviation (RSD), in addition to the recovery study.

RESULTS AND DISCUSSION

Optimization of voltametric analysis parameters

Several supporting electrolytes have been studied for voltametric assay of ZOL in a pharmaceutical formulation. The results of supporting electrolyte studies demonstrate the significance of this parameter, since ZOL did not show electroactivity with several supporting electrolytes, KNO_3 1.0 M, phosphate buffer pH 6.8, and sodium citrate buffer pH 3.0,

even with different kinds of working electrodes, such as GC, Pt, and Au electrodes. ZOL exhibited electroactivity when BRB was used as the supporting electrolyte with pH 5.94-10.52 and GC as the working electrode (Figure 2), where BRB buffer of pH 10.52 showed the highest performance. Cyclic voltammograms (CV) of ZOL in (Figure 3) indicated that ZOL is electroactive with an irreversible anodic peak current at 1.48V.

Voltametric method study

Three voltametric methods were applied to the ZOL assay in a pharmaceutical formulation: CV, square wave voltammetry (SWV), and differential pulse voltammetry (DPV). ZOL solutions (0.25-1.2 mg.mL^{-1}) were studied with BRB buffer, pH 10.52 as the supporting electrolyte and a GC working electrode. An anodic current potential of 1.48V was selected for the ZOL assay since it showed the highest correlation between SOL concentration and current in all studied voltametric methods (Table 1, Figure 3-5).

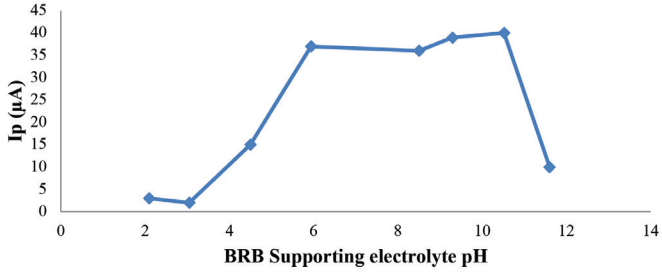

Figure 2. Effect of BRB supporting electrolyte pH on anodic peak current of cyclic voltametric analysis of ZOL (1.0 mg.mL^{-1}). Scan rate 0.1 V.s^{-1}, GC working electrode

BRB: Britton-Robinson universal buffer, ZOL: Zoledronic acid, GC: Glassy carbon

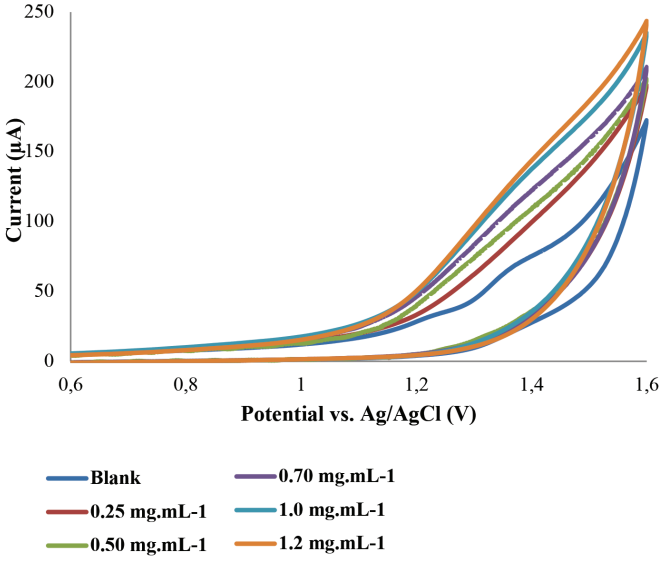

Figure 3. CV study of ZOL (0.25-1.2 mg.mL^{-1}), GC working electrode, BRB pH 10.52 supporting electrolyte, scan rate 0.1V.s^{-1}

CV: Cyclic voltammetry, ZOL: Zoledronic acid, BRB: Britton-Robinson universal buffer, GC: Glassy carbon

Linearity and range

The linearity of the studied voltametric methods was evaluated from the ZOL standard regression line according to ICH guidelines. Each concentration of ZOL was analyzed in triplicate. According to Table 1, DPV has the highest R^2 value of 0.993 compared with other studied methods. The current values plateaued at concentrations higher than (1.2 mg.mL^{-1}) for all studied methods. These results make the dynamic range of DPV and CV (0.20-1.2 mg.mL^{-1}) and (0.09-1.2 mg.mL^{-1}) for SWV.

Detection and quantitation limits

The limits of detection and quantitation for voltametric analysis of ZOL were evaluated based on the SD of the blank (BRB buffer of pH 10.52), where:

Limit of detection (LOD) = $y_B + 3S_B$

Limit of quantitation (LOQ) = $y_B + 10S_B$

S_B: Standard deviation of the blank

$y_{B:}$ Anodic current response of the blank

According to the results in Table 1, DPV shows the lowest LOD of 37.2 µg.mL^{-1} and SWV the lowest LOQ of 87.5 µg.mL^{-1}.

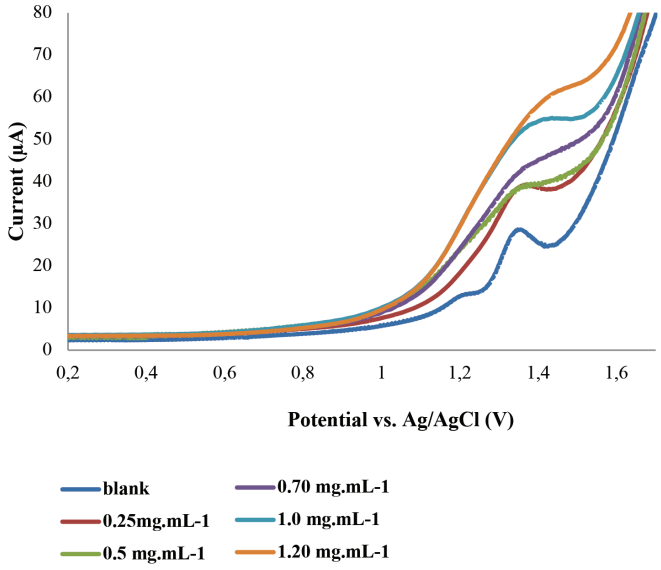

Figure 4. SWV study of ZOL (0.25-1.2 mg.mL^{-1}), GC working electrode, BRB pH 10.52 supporting electrolyte

SWV: Square wave voltammetry, ZOL: Zoledronic acid, GC: Glassy carbon, BRB: Britton-Robinson universal buffer

Table 1. Linearity of ZOL (0.25-1.20 mg.mL^{-1}, BRB pH 10.53) GC electrode

Method	Linear regression	R^2	LOD (µg.mL^{-1})	LOQ (µg.mL^{-1})
CV	y=51.178x+116.2	0.991	84.0	196
DPV	y=6.0346x+12.625	0.993	37.2	202
SWV	y=23.935x+31.843	0.961	41.7	87.5

ZOL: Zoledronic acid, GC: Glassy carbon, BRB: Britton-Robinson universal buffer, CV: Cyclic voltammetry, DPV: Differential pulse voltammetry, SWV: Square wave voltammetry, LOD: Limit of detection, LOQ: Limit of quantitation

The slopes of the linear regression calibration curves indicate that CV has the highest sensitivity compared with other methods (Table 1).

Precision

Each concentration of ZOL was assayed in triplicate. The SD of each point on the calibration curves indicated higher precision at high ZOL concentrations for all studied methods. The repeatability and reproducibility of voltametric analysis of ZOL are shown in (Table 2). All studied voltametric methods showed low intraday and interday RSD, which reflected high precision, where DPV exhibited the highest precision with the lowest RSD values (Table 2).

Accuracy

A commercial preparation of ZOL (ZOL HIKMA® 4 mg/5 mL) concentrate for solution for infusion was used to study the accuracy of voltametric methods. Table 3 shows the recovery and RSD of 0.40 mg.mL^{-1} ZOL using voltametric methods. DPV shows the best accuracy and precision of 102.32% recovery and 2.88% RSD, respectively.

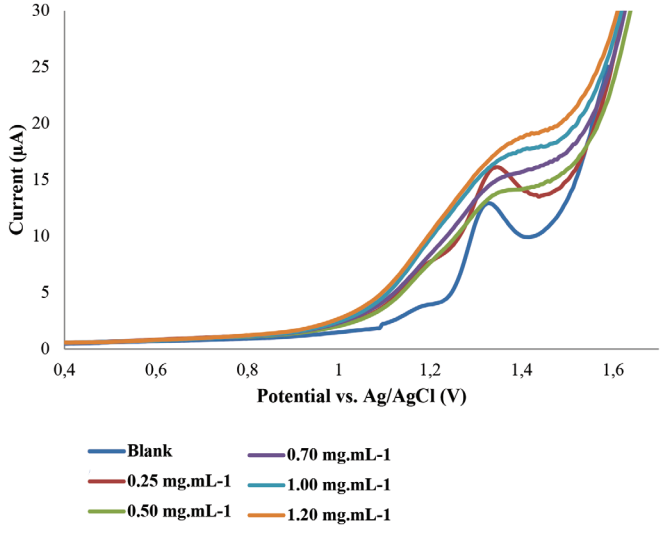

Figure 5. DPV study of ZOL (0.25-1.2 mg.mL^{-1}), GC working electrode, BRB pH 10.52 supporting electrolyte

DPV: Differential pulse voltammetry, ZOL: Zoledronic acid, GC: Glassy carbon, BRB: Britton-Robinson universal buffer

Table 2. Precision of ZOL (1.00 mg.mL^{-1}, BRB pH 10.53) GC electrode

Method	Intraday RSD %	Interday RSD %
CV	0.476	0.652
DPV	0.286	0.344
SWV	0.733	0.854

Intraday RSD %: Relative standard deviation on the same day of triplicate measurements (repeatability). Interday RSD %: Three consecutive days' measurements relative standard deviation (reproducibility). ZOL: Zoledronic acid, GC: Glassy carbon, BRB: Britton-Robinson universal buffer, RSD: Relative standard deviation, CV: Cyclic voltammetry, DPV: Differential pulse voltammetry, SWV: Square wave voltammetry

A comparison has been established between different methods used for ZOL determination and the present method according to precision, accuracy and LOD (Table 4). It is clear from Table 4 that most chromatographic methods have better detection limits than those used in the present study. On the other hand, the present study shows comparable recovery and RSD values.

Table 3. Recovery and precision of commercial preparation of ZOL (ZOL HIKMA® 4 mg/5 mL) GC electrode (BRB pH 10.53)

Method		0.40 mg.mL^{-1}
CV	Found ± SD	0.3874±0.0158
	Recovery %	96.86
	RSD %	4.08
DPV	Found ± SD	0.4092±0.0117
	Recovery %	102.32
	RSD %	2.88
SWV	Found ± SD	0.4162±0.0144
	Recovery %	104.06
	RSD %	3.46

Recovery: Obtained ZOL/added ZOL *100%. ZOL: Zoledronic acid, GC: Glassy carbon, BRB: Britton-Robinson universal buffer, CV: Cyclic voltammetry, DPV: Differential pulse voltammetry, SWV: Square wave voltammetry, RSD: Relative standard deviation, SD: Standard deviation

Table 4. Comparison of detection limit, precision, and recovery for determination of zoledronic acid between the present work and other used methods

Method	LOD	Precision	Recovery
RP-HPLC UV detector[9]	0.04 µg.mL^{-1}	2.5%	101%
RP-HPLC evaporative light scattering detection[10]	0.9 µg.mL^{-1}	0.4%-0.8%	98%-102%
HPLC-tandem mass spectrometry[12]	3.4 ng.mL^{-1}	0.52%-8.7%	97%-101.9%
RP-HPLC Hydroxyapatite-based Nanoparticles[13]	200 µg.mL^{-1}	0.32%-1.15%	99.01%-100.8%
Switchable fluorescence probe[16]	0.011 µg.mL^{-1}	2.70%	92.2%-104.0%
DPV, GC electrode (present work)	37.2 µg.mL^{-1}	0.286%-0.344%	102.32%

RP-HPLC: Reversed-phase high-performance liquid chromatography, UV: Ultraviolet, DPV: Differential pulse voltammetry, GC: Glassy carbon, LOD: Limit of detection

CONCLUSION

Voltametric analysis indicated that ZOL is an electroactive compound. CV of ZOL exhibited an irreversible anodic peak current at 1.48V. Optimization of voltametric analysis parameters indicated the significance of the supporting electrolyte pH and the type of working electrode. CV, SWV, and DPV have been applied for voltametric analysis of ZOL, But DPV is the recommended method for voltametric analysis of ZOL because of its high-performance regarding accuracy, precision, and LOD compared with other studied methods.

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ORIGINAL ARTICLE

Development and Evaluation of Solid Witepsol Nanoparticles for Gene Delivery

Gen Taşınımı için Katı Witepsol Nanopartiküllerinin Geliştirilmesi ve Değerlendirilmesi

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■*: We are terribly sorry to announce that Selen İsar passed away on the date of 9 December 2020. We thank her for her contributions.*

ABSTRACT

Objectives: Gene therapy approaches have become increasingly attractive in the medical, pharmaceutical, and biotechnological industries due to their applicability in the treatment of diseases with no effective conventional therapy. Non-viral delivery using cationic solid lipid nanoparticles (cSLNs) can be useful to introduce large nucleic acids to target cells. A careful selection of components and their amounts is critical to obtain a successful delivery system. In this study, solid Witepsol nanoparticles were formulated, characterized, and evaluated *in vitro* for gene delivery purposes.

Materials and Methods: Solid Witepsol nanoparticles were formulated through the microemulsion dilution technique using two grades of Witepsol and three surfactants, namely Cremephor RH40, Kolliphor HS15, and Peceol. Dimethyldioctadecylammonium bromide was incorporated into the system as a cationic lipid. Twelve combinations of these ingredients were formulated. The obtained nanoparticles were then evaluated for particle size, zeta potential, DNA binding and protection ability, cytotoxicity, and transfection ability.

Results: Particle sizes of the prepared cationic cSLNs were between 13.43±0.06 and 68.80±0.78 nm. Their zeta potential, which is important for DNA binding efficiency, was determined at >+40 mV. Gel retardation assays revealed that the obtained cSLNs can form a compact complex with plasmid DNA (pDNA) encoding green fluorescent protein and that this complex can protect pDNA from DNase I-mediated degradation. Cytotoxicity evaluation of nanoparticles was performed on the L929 cell line. *In vitro* transfection data revealed that solid Witepsol nanoparticles could effectively transfect fibroblasts.

Conclusion: Our findings indicate that solid Witepsol nanoparticles prepared using the microemulsion dilution technique are promising non-viral delivery systems for gene therapy.

Key words: Gene delivery, Witepsol, solid lipid nanoparticle, transfection, pDNA

ÖZ

Amaç: Etkili bir geleneksel tedavinin uygulanamadığı hastalıkların tedavisinde gen tedavisi yaklaşımları, uygulanabilirliği nedeniyle tıp, ilaç ve biyoteknoloji endüstrilerinde giderek daha çekici hale gelmiştir. Katyonik katı lipid nanopartiküller (cSLN) kullanılarak viral olmayan uygulama, büyük nükleik asitlerin hedef hücrelerin içine alınması için faydalı olabilir. Etkin bir taşıyıcı sistem elde etmek için bileşenlerin ve miktarlarının dikkatli bir şekilde seçilmesi çok önemlidir. Bu çalışmada, katı Witepsol nanopartikülleri, gen taşınması amacıyla *in vitro* formüle edilmiş, karakterize edilmiş ve değerlendirilmiştir.

Gereç ve Yöntemler: Katı Witepsol nanopartikülleri, iki farklı Witepsol ve üç yüzey aktif madde (Cremephor RH40, Kolliphor HS15 ve Peceol) kullanılarak mikroemülsiyon dilüsyon tekniği ile formüle edildi. Dimetildioktadesilamonyum bromür, sisteme katyonik bir lipid olarak dahil edildi. Bu bileşenlerin on iki kombinasyonu formüle edildi. Elde edilen nanopartiküller daha sonra partikül boyutu, zeta potansiyeli, DNA bağlanma ve koruma kabiliyeti, sitotoksisite ve transfeksiyon kabiliyeti açısından değerlendirildi.

Bulgular: Hazırlanan katyonik cSLN partikül boyutları 13,43±0,06 ile 68,80±0,78 nm arasındaydı. DNA bağlanma etkinliği için önemli olan zeta potansiyelleri >+40 mV olarak belirlendi. Jel retardasyon testleri, elde edilen cSLN'lerin yeşil floresan proteini kodlayan plazmit DNA (pDNA) ile kompakt bir kompleks oluşturabildiğini ve bu kompleksin pDNA'yı DNaz-I aracılı bozunmadan koruyabildiğini ortaya koydu. Nanopartiküllerin sitotoksisite değerlendirmesi, L929 hücre hattında gerçekleştirildi. *In vitro* transfeksiyon verileri, katı Witepsol nanopartiküllerinin fibroblastları etkili bir şekilde transfekte edebildiğini ortaya çıkardı.

Sonuç: Bu çalışma, mikroemülsiyon-dilüsyon tekniği ile hazırlanan katı witepsol nanopartiküllerin viral olmayan gen terapisi için umut verici sistemler olduğunu göstermektedir.

Anahtar kelimeler: Gen taşınımı, Witepsol, katı lipid nanopartikül, transfeksiyon, pDNA

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INTRODUCTION

Genetic treatment approaches show promise for diseases ranging from cancer to inherited illnesses. The use of naked nucleic acids is not therapeutically effective due to their hydrophilic character, anionic charge, and high molecular weight, which hinder them from crossing cell membranes.[1,2] To overcome these challenges, viral or non-viral gene delivery systems are used. Production of non-viral delivery systems that can carry large amounts of genetic material, have low toxicity and a low risk for immune responses, and be easily produced is one of the research areas of pharmaceutical sciences.[3,4]

In recent years, lipid-based carrier systems, such as liposomes, emulsions, nanostructured lipid carriers, and solid lipid nanoparticles (SLNs), have gained interest as delivery systems for gene therapy. Of these, SLNs have emerged to the forefront as promising systems in the field of non-viral gene delivery owing to their various advantages.[5] Specifically, SLNs are suitable for large scale production and exhibit long-term stability. They are usually prepared by using non-toxic, biodegradable lipids.[6,7] SLNs can be produced in nanosize and with cationic properties, which enable binding to nucleic acids and increase cellular influx and/or intracellular transition due to electrostatic interactions.[8] The size and charge of SLNs can be modified by changing production strategies based on the purpose of production.[9]

Essential components for SLN formulations include lipids, emulsifiers, and an aqueous phase. The melting point of lipids is usually higher than room and body temperatures. Witepsol, which is mainly used in pharmaceutical research and development as an excipient, is a lipid that can be used for SLN formulation. A range of grades of Witepsol that comprise different proportions of triglycerides, diglycerides, and monoglycerides are commercially available to meet the requirements of pharmaceutical formulation and production and biopharmacy. Witepsol grades are classified in categories, namely H, W, S, and E. For example, Witepsol H series lipids consist mostly of triglycerides with a proportion of, at most, 15% diglycerides and not more than 1% monoglycerides. They are characterized by hydroxyl values up to 15 and have a small gap between the melting and solidification temperatures. By contrast, Witepsol W series lipids consist of a mixture of triglycerides (65%-80%), diglycerides (10%-35%), and monoglycerides (1%-5%). Their hydroxyl values are between 20-50 and exhibit a bigger difference between melting and solidification points.[10,11] Because of the diversity in their composition, Witepsol grades exhibit different characteristics; therefore, the selection of the most suitable grade during formulation development must be conducted according to these properties.[11,12]

Surfactants used in this study include Kolliphor HS15 (macrogol-15-hydroxystearate) and Cremephor RH40 (macrogolglycerol hydroxystearate). They are non-ionic oil-in-water (o/w) solubilizing and emulsifying agents with hydrophilic-lipophilic balance (HLB) values of 14-16, making them suitable for o/w emulsion formulations.[11,13] However, they exhibit different emulsifying capacity depending on the formulation components.

The aim of this study was to develop stable, solid Witepsol nanoparticles suitable for gene delivery. Therefore, the microemulsion dilution technique was employed and various formulation parameters were evaluated to produce non-toxic, biocompatible, cationic SLNs, with an optimal size and surface properties, which can be used in gene delivery. The obtained SLNs were first characterized and then complexed with plasmid DNA (pDNA). Then, protection potential against DNase I, cytotoxicity, and transfection ability were investigated. According to our findings, this study is one of the first on the development of solid Witepsol nanoparticles using the microemulsion dilution technique for gene delivery.

MATERIALS AND METHODS

Materials

Witepsol H35 [WH35; a mixture of triglycerides (65%-80%), diglycerides (10%-35%), and monoglycerides (1%-5%)] and Witepsol W35 (WW35; a mixture of triglycerides with a portion of, at most, 15% diglycerides and not more than 1% monoglycerides) were obtained from IOI Oleo GmbH (Germany). Cremophor RH40 (CRH40) and Kolliphor HS15 (KHS15) were donated by Baden Aniline and Soda Factory (BASF, Germany). Peceol (Pec) was a gift from Gattefosse (France). Dimethyldioctadecylammonium bromide (DDAB) was purchased from Sigma-Aldrich Co. (USA). Ethanol was purchased from Merck Co. (Germany). L929 murine skin fibroblast and CoS-7 African green monkey fibroblast cell lines were obtained from the American Type Culture Collection (USA). The alamarBlue cell proliferation assay kit was purchased from Thermo Fisher Scientific (USA). Agarose was purchased from Sigma-Aldrich Co. (USA). pEGFP-C1 plasmid DNA and maxiprep plasmid DNA purification kits were purchased from Invitrogen (USA). DNase I was purchased from Fermentas, Thermo Fischer Scientific (USA). Ultrapure water (UPH_2O) was used for all experiments.

Plasmid DNA

The model plasmid pEGFP-C1 (Invitrogen, USA), which encodes the green fluorescent protein (GFP) under the CMV promoter (pDNA), was amplified in *Escherichia coli* DH5α. The Maxiprep DNA purification kit (Invitrogen, USA) was used for plasmid purification. Plasmid integrity was evaluated by restriction enzyme digestion and visualized using a horizontal electrophoresis system. Furthermore, the purity and concentration of the plasmid were evaluated by measuring its absorbance at 260/280 nm. Finally, purified plasmid DNA was diluted to 100 µg/mL, aliquoted, and stored at -20°C until use.

Formulation of solid Witepsol nanoparticles

The microemulsion dilution technique was used to develop SLNs.[2] First, Witepsol was used as an internal oil phase to obtain an o/w microemulsion system. CRH40 or KHS15 and Pec (2:1, w:w) were used as surfactants (S) and ethanol as the co-surfactant (CoS). The cationic lipid DDAB was added into the solid lipid phase to achieve cationic microemulsion.[14] SLNs prepared with DDAB were abbreviated as cSLN. Compositions of the designed SLNs are listed in Table 1. All components

were weighted as that the amount of solid lipids were 50 mg per formulation and heated to 50°C (10°C above the melting temperature of Witepsol). Previously warmed UPH_2O at an equivalent temperature was then added onto the lipid and S:CoS (1:1, w:w) mixture with nearly 2% of total weight increase per addition. Transparent regions were indicated on the phase diagram to determine the o/w microemulsion formation area. A microemulsion consisting of 4% oil, 36% S:CoS, and 60% water was selected for further studies for all developed SLNs. The acquired hot o/w microemulsion was then rapidly dispersed in 8 mL UPH_2O (0°C-4°C), stirring at 1000 rpm at a ratio of 1:5 (v/v). Once hot microemulsion droplets were applied to cold water, SLNs were formed. The concentration for the final SLNs was 7.5 mg/mL for all formulations, with respect to the amounts of solid lipids.

Characterization of solid Witepsol nanoparticles

Particle size, polydispersity index (PDI), and zeta potential values of SLNs, cSLNs, and cSLN:pDNA complexes, with various formulation parameters, were measured using dynamic light scattering [(DLS), Zetasizer Nano ZS, Malvern Instruments Ltd., UK)].[15] Disposable polystyrene micro cuvettes were used to measure particle size and PDI for each sample. The zeta potential was measured in standard zeta cuvettes and calculated using the software by employing the Smoluchowski equation. Measurements were repeated at least three times for each sample.

Stability of cationic, solid Witepsol nanoparticles

Particle size and zeta potential measurements of cSLNs were followed in terms of stability for 3 months. The measurements were performed at various time intervals using DLS (Zeta sizer Nano ZS, Malvern Instruments Ltd., UK). Samples were stored at 4°C during this period.

Gel retardation assay

The cSLN:pDNA complexes were generated through electrostatic interactions between cationic nanoparticles and anionic pDNA.[16] The complex formation ability of pDNA with cSLN was determined through 1% (w/v) agarose gel electrophoresis. The purity and concentration of the pDNA were spectrophotometrically assayed at 260 and 280 nm, respectively, using Nanovette (Beckman Coulter, USA). The ratio of absorbance at 260/280 nm was 1.752 (~1.8), indicating pDNA purity.[17] A stock solution of pDNA (100 µg/mL) was prepared in nuclease-free UPH_2O. The cSLN:pDNA complexes were prepared by incubating pDNA (100 µg/mL) with cSLNs for 30 min at 25°C on a benchtop shaker for increasing cSLN:pDNA ratios [0.5:1, 1:1, 2:1, and 3:1 (v/v)] to enable binding of pDNA to cSLNs electrostatically. Glycerol (2%) was added to each sample and electrophoresis was performed at 100 V for 60 min. Then, ethidium bromide solution (500 ng/mL) was used to stain the gel. The stained pDNA bands were then visualized under the ultraviolet transilluminator (Vilber Lourmat, France) to determine the optimal pDNA:cSLN ratio. Naked pDNA was used as a control.

In vitro DNase I protection assay

The protection of pDNA by cSLNs was evaluated using the *in vitro* DNase I protection assay. For this purpose, DNase I was added to freshly prepared cSLN:pDNA systems to get 1 IU DNase I/2.5 µg pDNA as the final concentration and incubated at 37°C for 30 min. To terminate the enzymatic reaction and to obtain the release of pDNA, SDS (1%, v/v) was added. The integrity of pDNA was visualized by horizontal gel electrophoresis.[18]

Cytotoxicity analysis

Cytotoxicity evaluation of the formulations was performed on L929 cell lines. Dulbecco's modified Eagle's medium

Table 1. Investigated formulation parameters and particle characterization results of Witepsol nanoparticles

Code	Solid lipid		DDAB	Surfactant		pDNA
	WH35	WW35		CRH40:Pec (2:1, w:w)	KHS15:Pec (2:1, w:w)	
SLN_1	+	-	-	+	-	-
SLN_2	+	-	-	-	+	-
SLN_3	-	+	-	+	-	-
SLN_4	-	+	-	-	+	-
$cSLN_1$	+	-	+	+	-	-
$cSLN_2$	+	-	+	-	+	-
$cSLN_3$	-	+	+	+	-	-
$cSLN_4$	-	+	+	-	+	-
*$cSLN_1$:pDNA	+	-	+	+	-	+
*$cSLN_2$:pDNA	+	-	+	-	+	+
*$cSLN_3$:pDNA	-	+	+	+	-	+
*$cSLN_4$:pDNA	-	+	+	-	+	+

*cSLN:pDNA, cSLN formulations complexed with pDNA. cSLN: Cationic solid lipid nanoparticle, SLN: Solid lipid nanoparticle, pDNA: Plasmid DNA, DDAB: Dimethyldioctadecylammonium bromide, Pec: Peceol

supplemented with 10% fetal bovine serum and 2 mM L-glutamine was used as culture medium. Penicillin and streptomycin (100 UI/mL penicillin, 100 µg/mL streptomycin) were added to prevent bacterial contamination.

Briefly, L929 cells were plated in 96-well plates at a density of $5x10^3$ cells per well in 100 µL medium and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2. Then, the medium was renewed, the cells were treated with increasing concentrations of cSLNs (225, 450, 675, 900, and 1125 µg/mL, with respect to solid lipids), incubated for 24 h, and washed two times with poly (butylene succinate) (PBS) (pH: 7.4). The alamarBlue cell viability assay was used to evaluate the proportion of viable cells. Cell viability was determined by normalizing the fluorescence of media between treated and untreated cells.[19]

Transfection studies

The transfection ability of the developed formulations is important for the transport of therapeutic genes into the cells. For this study, CoS-7 cells were cultured in 6-well plates at a density of $5x10^4$ cells/mL and incubated until 70% confluency. The medium was then removed and the cells were washed with PBS (pH: 7.4). Fresh growth medium (500 µL) was instantly added to prevent cells from desiccation. Predetermined doses of the cSLN:pDNA complex (2:1, v/v), which contains 2.5 µg of pDNA, were applied to the cells based on cytotoxicity test results and incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2. Then, the medium containing the SLN suspension was replaced with fresh growth medium and cells were incubated for 48 h to increase GFP production. Transfection efficiency was determined c under a fluorescence microscope (Olympus, Japan).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc., USA). Results are expressed as means ± standard error of mean. Cytotoxicity results were analyzed using Student's t-test. The significance level was set at $p<0.05$.

RESULTS AND DISCUSSION

Due to the rapid progress in molecular biology and genetics, interest in using nanoparticles as vehicles in drug and gene delivery systems has increased. In this regard, the use of SLNs is important. During the last decade, SLNs have mostly been used as drug delivery systems.[20-22] Even cationic SLNs, developed using cationic lipids, can be used as gene carrying systems; however, instances of their application in gene therapy are few.[23-25] Solid lipids with relatively higher melting points, such as glyceryl behenate, behenic acid, and stearic acid (melting point >60°C), were used as the solid lipid matrix in the preparation of SLNs.[9,14] However, in this study, Witepsol, which has a melting point of approximately 37°C, has been used. The low melting point of the solid lipid matrix brings several advantages. First, the energy requirement for large scale production is decreases, thereby reducing the production cost of nanoparticle carriers and providing an advantage to enter the pharmaceutical market.[23] Furthermore, following endocytosis, the genetic material-loaded SLNs presumably encounter consecutive pH drop in the endosome and lysosome due to the acidic character of endosomal media.[26] At this step, the low melting point of Witepsol, which is close to body temperature, might facilitate the release of the genetic material into the cytoplasm compared with lipids with high melting points.[2] Here, we aimed to produce SLNs using Witepsol in the solid matrix. For this purpose, plotting pseudo-ternary phase diagrams, which is the initial process of SLN production using the microemulsion dilution method, was performed at the lipid melting temperature.[27]

Preparation of pseudo-ternary phase diagrams

For the formation of the o/w microemulsion system, the HLB of the system was adjusted between 10 and 12 with non-toxic surfactants. One of the surfactants used in these formulations is Pec. It is a readily dispersible, solubilizing agent, consisting of a mixture of mono- and diglycerides of oleic acid. Because the HLB value of Pec is quite low (approximately 3), the HLB value of surfactant mixtures was increased by using CRH40 and KHS15 to allow for the formation of an o/w microemulsion. The pseudo-ternary phase diagrams constituted by titration with UPH_2O into the oil (Witepsol), S (CRH40:Pec or KHS15:Pec, 2:1, w:w), and CoS (ethanol) mixtures are presented in Figure 1. According to the construction of the phase diagram, the final formulations were selected providing microemulsion requirements, such as being o/w type and transparent, as well as having a high proportion of the solid lipid and a low proportion of S:CoS.[28,29] Because the o/w microemulsion formation areas are similar for all formulations, the same ratios were selected to compare the effect of solid lipids (WH35/WW35) and S:CoS (CRH40:Pec/KHS15:Pec). Thus, the o/w microemulsions consisting 4 wt % solid lipid, 36 wt % S:CoS, and 60 wt % water were prepared and used for SLN preparation for all formulations.

Physicochemical characterization

To examine the particle size and zeta potential of nanoparticles, DLS measurements were performed. As shown in Table 2, the obtained solid Witepsol nanoparticles are in the nanometer size range (13.43-80.49 nm). Complex formation with pDNA increased the particle size of the system. SLNs without cationic lipids had a zeta potential between -2.4 and 13.7 mV. When CRH40 was used as the surfactant, the zeta potential values of nanoparticles were +13.7 and +12.7 mV for SLN_1 and SLN_3, respectively. By contrast, when KHS15 was used as the surfactant, the zeta potential decreased to -0.5 and -2.4 for SLN_2 and SLN_4, respectively, which is attributable to the glycerol group in CRH40. Furthermore, following DDAB incorporation, the zeta potential of cSLNs was >40 mV for all formulations. These results were as expected due to the cationic amphiphilic character of DDAB, which is commonly used in non-viral gene delivery studies.[30] Then, the final zeta potential of the formulations was decreased by complexing with pDNA; however, it remained >30 mV for all four formulations. The obtained positive zeta potential of the formulations is

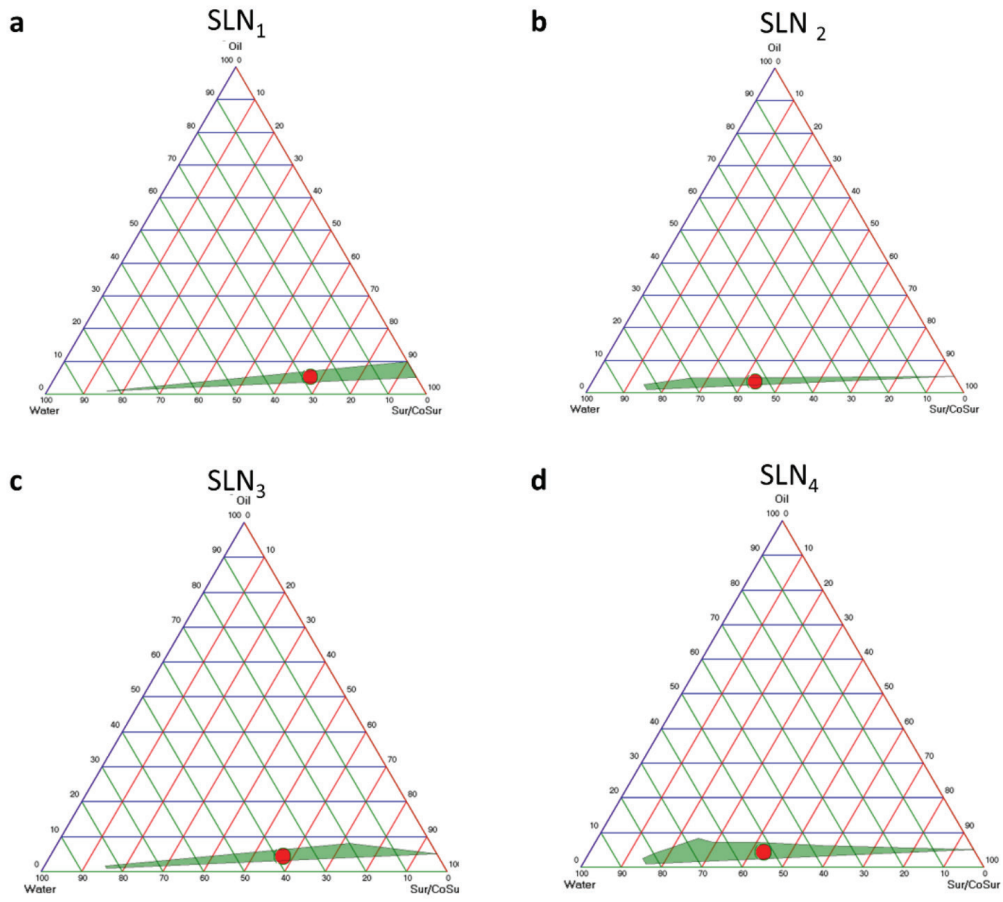

Figure 1. (a-d) Pseudo-ternary phase diagrams for different formulations, namely SLN_1, SLN_2, SLN_3, and SLN_4. The green area represents the transparent o/w microemulsion formation region

SLN: Solid lipid nanoparticle

Table 2. Particle size, PDI, and zeta potential values of SLN formulations

Code	Particle size (nm ± SD)	PDI	ZP (mV ± SD)
SLN_1	23.84±0.06	0.08	13.7±0.6
SLN_2	61.90±0.39	0.13	-0.5±0.5
SLN_3	23.42±0.01	0.04	12.7±4.7
SLN_4	47.87±0.28	0.19	-2.4±0.5
$cSLN_1$	13.43±0.06	0.62	43.8±1.8
$cSLN_2$	23.45±1.60	0.91	47.7±0.7
$cSLN_3$	37.57±0.49	1.00	42.2±2.7
$cSLN_4$	68.80±0.78	0.36	41.5±1.7
*$cSLN_1$:pDNA	54.44±0.54	0.60	32.4±2.5
*$cSLN_2$:pDNA	80.49±1.29	0.52	41.8±3.2
*$cSLN_3$:pDNA	37.82±0.09	0.61	34.0±1.6
*$cSLN_4$:pDNA	69.50±0.45	0.57	34.4±8.8

*cSLN:pDNA, cSLN formulations complexed with pDNA, (n=3). PDI: Polydispersity index, cSLN: Cationic solid lipid nanoparticle, SLN: Solid lipid nanoparticle, SD: Standard deviation, ZP: Zeta potential, pDNA: Plasmid DNA

important for achieving an interaction between the negatively charged nucleic acid and cell membrane for transfection.[31,32] The PDI values of the produced SLNs increased after DDAB was incorporated into the system as a cationic lipid. However, polydispersity of the formulation decreased after complex formation with pDNA occurred. Particle sizes of the final cSLN:pDNA vector systems were <100 nm, which is necessary for i.v. injection. Moreover, they are small enough to provide stability to the dispersal system owing to Brownian motion.[33]

The physicochemical stability of the developed cSLNs was evaluated. For this experiment, cSLNs were stored at 4°C and particle size and zeta potential were measured for up to 90 d (Table 3). The results revealed that particle size and zeta potential of cSLNs remained stable for 21 d. Particle size of cSLNs increased dramatically within 90 d. The particle size of only $cSLN_3$ remained at less than 100 nm. Thus, the developed formulations can be used for complexation with pDNA for at least 21 d because their particle size is <100 nm and zeta potential is >40 mV.[34]

Gel retardation assay

The gel retardation assay is used to evaluate the nucleic acid-binding ability of cationic nanoparticles.[16] cSLNs were evaluated for their complex formation ability with pDNA using the agarose

gel retardation assay to identify the optimal cSLN:pDNA ratio. The migration of naked pDNA and cSLN:pDNA complexes for different Witepsol nanoparticles in agarose gel is shown in Figure 2. The obtained cSLNs showed pDNA binding ability and the migration of pDNA in agarose gel stopped when the ratio of cSLN:pDNA reached 2:1 (v/v) for all cSLNs.

Protection ability of nanoparticles against DNase I degradation

The agarose gel photograph of cSLN:pDNA complexes incubated with DNase I is shown in Figure 3. Lane 1 contains the untreated plasmid (negative control). As seen in lane 2, naked pDNA was completely digested by DNase I. Lanes 3-5 indicate the protection of the cSLN formulations against DNase I at three cSLN:pDNA ratios, namely 1:1, 2:1, and 3:1 (v:v). This evidence confirmed that the obtained cSLNs efficiently protected the pDNA from DNase I-mediated degradation.[35]

Cytotoxicity

The cytotoxicity assay was performed to identify non-toxic doses of the obtained nanoparticles before transfection. The results

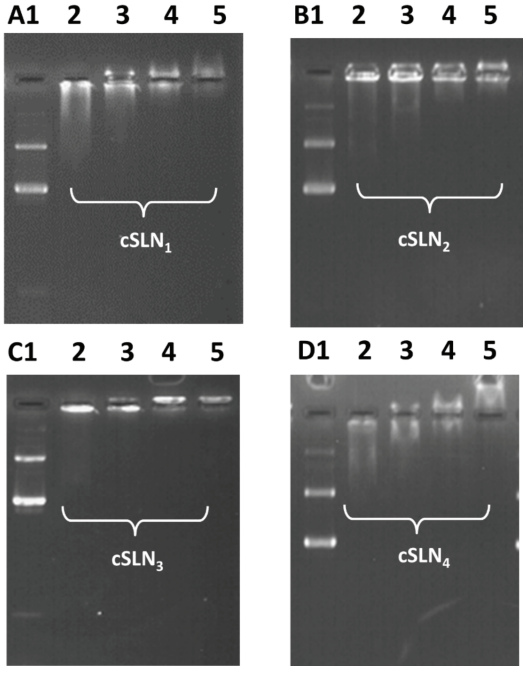

Figure 2. Agarose gel photograph of complexes containing a constant amount of pDNA and increasing amounts of $cSLN_1$, $cSLN_2$, $cSLN_3$, or $cSLN_4$ at 0.5:1, 1:1, 2:1, and 3:1 (v/v), respectively

pDNA: Plasmid DNA, cSLN: Cationic solid lipid nanoparticle

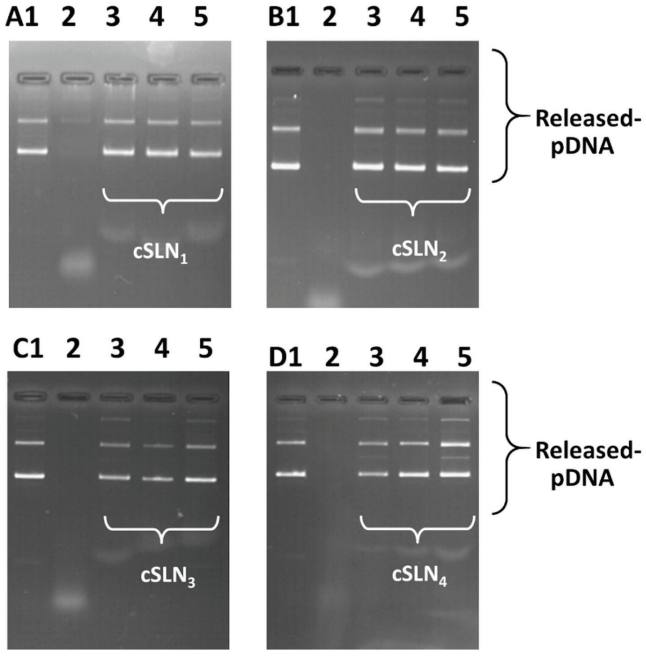

Figure 3. DNase I protection of cSLN:pDNA complexes. (1: Control pDNA; 2: Naked pDNA incubated with DNase I; 3-5: pDNA released from cSLN:pDNA complexes following incubation with DNase I for cSLN:pDNA ratios 1:1, 2:1, and 3:1 (v:v), respectively. (Lanes A1-5 for $cSLN_1$, lanes B1-5 for $cSLN_2$, lanes C1-5 for $cSLN_3$, and lanes D1-5 for $cSLN_4$)

cSLN: Cationic solid lipid nanoparticle, pDNA: Plasmid DNA

Table 3. The physicochemical stability of cSLNs at 4°C

Code	Particle size (nm ± SD)				
	Day 0	Day 7	Day 14	Day 21	Day 90
$cSLN_1$	13.43±0.06	10.96±0.33	10.02±0.18	12.68±0.12	125.0±44.52
$cSLN_2$	23.45±1.60	22.83±0.25	21.49±1.37	22.34±0.33	109.1±26.94
$cSLN_3$	37.57±0.49	35.49±3.23	39.26±8.31	36.71±2.25	73.58±3.55
$cSLN_4$	68.80±0.78	73.46±9.56	77.30±3.63	70.45±0.39	453.0±43.20
Code	**Zeta potential (mV ± SD)**				
	Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 90**
$cSLN_1$	43.8±1.8	37.0±0.8	37.0±0.7	37.8±4.30	43.9±1.3
$cSLN_2$	47.7±0.7	40.0±10.0	40.0±10.0	43.5±0.49	42.7±1.8
$cSLN_3$	42.2±2.7	40.5±0.3	40.5±0.3	41.0±4.17	42.0±1.5
$cSLN_4$	41.5±1.7	44.6±2.3	44.6±2.3	44.5±2.48	44.7±2.4

cSLN: Cationic solid lipid nanoparticle, SD: Standard deviation

obtained from the alamarBlue cytotoxicity assay on the L929 cell line supported that all excipients used in this study have low cytotoxicity. Significant cytotoxicity was not determined on L929 cells in at concentrations ranging from 225 to 1125 µg/mL. The applied doses showed >70% cell viability, which is considered the minimum reference cell viability dose for further transfection studies.[33] Furthermore, concentration dependent cytotoxicity was observed for all formulations (Figure 4).

Cellular uptake studies

The uptake of Witepsol nanoparticles in the CoS-7 cell line was determined using the pEGFP-C1 plasmid and visually investigated under a fluorescence microscope (Figure 5). Green shows the fluorescence signal from GFP. According to transfection results, all obtained formulations have transfection ability.[36,37] Since the developed nanoparticles showed efficient transfection in cell culture, they can be used for different genetic diseases by loading disease specific therapeutic genes.

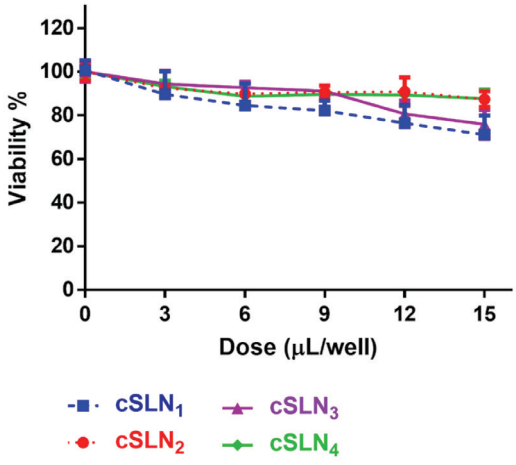

Figure 4. Viability of L929 cells exposed to cSLN formulations. Each condition was tested in at least four replicates for 24 h

cSLN: Cationic solid lipid nanoparticle

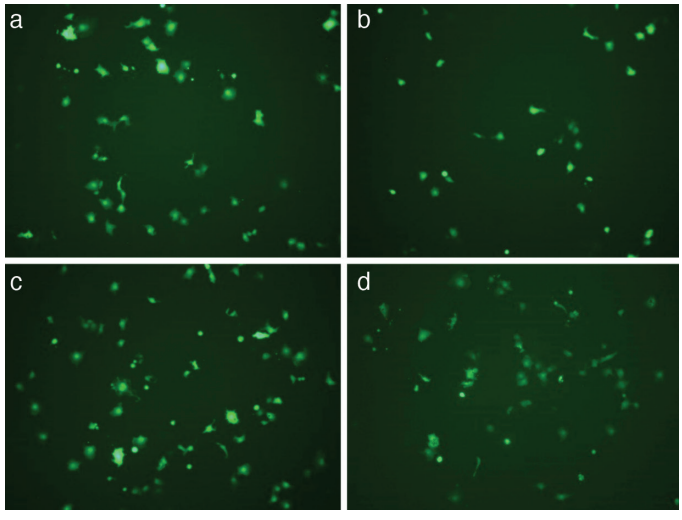

Figure 5. Images of GFP-positive cells following cSLN:pEGFP-C1 application, observed using inverted fluorescence microscopy after 48 h of transfection for $cSLN_1$ (a), $cSLN_2$ (b), $cSLN_3$ (c), and $cSLN_4$ (d) pEGFP-C1 complexes

GFP: Green fluorescent protein, cSLN: Cationic solid lipid nanoparticle

CONCLUSION

We prepared and characterized solid Witepsol nanoparticles. The obtained cationic nanoparticles exhibited pDNA binding and protection ability. The characterization of formulations in terms of particle size, zeta potential, and PDI revealed that the obtained nanoparticles are in the nanometer size range. Cytotoxicity results confirmed the suitability of the nanoparticles for gene delivery. Transfection studies showed that the developed nanoparticles can carry genetic material into cells efficiently. Thus, solid Witepsol nanoparticles can be considered promising delivery vehicles for non-viral gene therapy.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

Development and Rheological Evaluation of DEET (N,N-DiethyL-3-Methylbenzamide) Microparticles Loaded Hydrogel For Topical Application

Topikal Uygulama İçin DEET (N,N-Dietil-3-Metilbenzamid) Mikropartikül Yüklenmiş Hidrojel Geliştirilmesi ve Reolojik Değerlendirilmesi

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ABSTRACT

Objectives: N,N-Diethyl-3-methylbenzamide (DEET) is a broad-spectrum insect repellent that can easily permeate through the skin and can cause undesirable effects, especially in children and pregnant women. The objective of this research was to formulate and evaluate DEET-encapsulated microparticles containing a hydrogel designed to reduce skin permeation and prolong drug release.

Materials and Methods: The formulation design was based on the independent formulation variables of the concentration of chitosan and sodium tripolyphosphate using a simple factorial design experiment. DEET-loaded microparticles were developed and incorporated into a hydrogel. The size of the microparticles was analyzed using the Zetasizer Nano® particle size analyzer, and the surface morphology, using field emission scanning electron microscopy. Drug release from the microparticles was determined by the dialysis bag method. A rheological evaluation of the formulated gel was performed using a Thermo Haake Rheometer. The *in vitro* permeation of the formulation was performed using a synthetic Strat-M® membrane.

Results: The size of the microparticles ranged from 0.45 to 8.3 µm, and the encapsulation efficiencies were >50% for all the formulations. The drug-release curves showed no initial burst release from the microparticle formulation. Instead, a slow and controlled drug release was observed over 24 hours that followed Higuchi kinetics. The cumulative amount of DEET permeated (over 24 h) from the DEET solution (control), and the formulation was 211.6±19.5 µg/cm^2 and 4.07±0.08 µg/cm^2, respectively.

Conclusion: A significantly low DEET permeation from the microparticle formulations indicated minimal absorption of the drug into the body and thus, reduced systemic toxicity. Thixotropic evaluation of the hydrogel formulation demonstrated a hysteresis loop that fitted closely to the Herschel-Bulkley rheological model, ensuring an effortless application and prolonged retention on the skin. Hence, it can be concluded that the developed formulation is an effective delivery approach for controlled insect repellent activity with reduced skin absorption.

Key words: Transdermal, DEET, microparticles, permeation, hydrogel

ÖZ

Amaç: N, N-Dietil-3-metilbenzamid (DEET), özellikle çocuklarda ve hamile kadınlarda deride kolaylıkla nüfuz edebilen ve istenmeyen etkilere neden olabilen geniş spektrumlu bir böcek kovucudur. Bu araştırmanın amacı, deri nüfuzunu azaltmak ve ilaç salınımını uzatmak için tasarlanmış bir hidrojel içeren DEET kapsüllü mikropartikülleri formüle etmek ve değerlendirmektir.

Gereç ve Yöntemler: Formülasyon tasarımı, basit bir faktöriyel tasarım deneyi kullanılarak kitosan ve sodyum tripolifosfat konsantrasyonunun bağımsız formülasyon değişkenlerine dayanıyordu. DEET yüklü mikropartiküller geliştirildi ve bir hidrojele disperse edildi. Mikropartiküllerin boyutu, Zetasizer Nano® partikül boyutu analizörü ve alan emisyonu taramalı elektron mikroskobu kullanılarak yüzey morfolojisi kullanılarak analiz edildi. Mikropartiküllerden ilaç salımı, diyaliz torbası yöntemi ile belirlendi. Formüle edilmiş jelin reolojik bir değerlendirmesi, bir Thermo Haake Reometre kullanılarak gerçekleştirildi. Formülasyonun *in vitro* geçirgenliği, sentetik bir Strat-M® membran kullanılarak gerçekleştirildi.

Bulgular: Mikropartiküllerin boyutu 0,45 ile 8,3 µm arasında değişiyordu ve enkapsülasyon etkinliği tüm formülasyonlar için >%50 olarak tespit edildi. İlaç salım profilleri, mikropartikül formülasyonundan ilk patlama etkisi göstermedi. Bunun yerine, Higuchi kinetiğini izleyen 24 saat içinde

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yavaş ve kontrollü bir ilaç salımı gözlemlendi. DEET solüsyonundan (kontrol) geçirilen kümülatif DEET miktarı (24 saatin üzerinde) ve formülasyon sırasıyla 211,6±19,5 μg/cm^2 ve 4,07±0,08 μg/cm^2 idi.

Sonuç: Mikropartikül formülasyonlarından önemli ölçüde düşük bir DEET geçirgenliği, ilacın vücuda minimal absorpsiyonu ve dolayısıyla sistemik toksisitenin azaldığı tespit edildi. Hidrojel formülasyonunun tiksotropik değerlendirmesi, Herschel-Bulkley reolojik modeline yakından uyan, kolay uygulama ve deride uzun süreli tutunma sağlayan bir histerezis döngüsü gösterdi. Bu nedenle, geliştirilen formülasyonun, azaltılmış deri absorpsiyonu ile kontrollü böcek kovucu aktivite için etkili bir uygulama yaklaşımı olduğu sonucuna varılabilir.

Anahtar kelimeler: Transdermal, DEET, mikropartiküller, nüfuz etme, hidrojel

INTRODUCTION

N,N-Diethyl-3-methylbenzamide (DEET) is a well-known broad-spectrum insect repellent that has been shown to be effective against a variety of insects, including mosquitos, bugs, and mites.[1] The unique odor of this insect repellent allows it to act from a certain distance, not too far from the applied surface. Therefore, DEET must remain on the skin surface, instead of getting absorbed into the skin, to ensure ideal activity. However, DEET can easily permeate through the skin and can enter the systemic circulation owing to its low molecular weight [(MW), 191.27 Da] and a favorable octanol/water partition coefficient (log P) of 2.1. Generally, compounds having MW <500 Da and log P values in the range of 1-3 can easily permeate through the skin. Such compounds are considered ideal for transdermal delivery.[2] Although DEET is considered to be safe for humans, but its entry into the systemic circulation is unnecessary. Moreover, most of the commercially available DEET preparations provide protection for only 1-4 hours, probably due to its rapid absorption by the skin. Topical application of polymeric microparticles on the skin allows slow release of the encapsulated active drugs, which can further prolong the therapeutic effect of the drug. In addition to this, the large size of microparticles might impede the rapid skin permeation, reducing any unwanted effects.[3] Currently, various strategies involving the use of formulations, like liposomes and microparticles, are being explored to address the issue of dermal absorption of DEET.[4] The present study aimed to develop a chitosan-based biocompatible microparticle formulation of DEET to allow controlled release of the drug. Chitosan microparticles have been shown to offer several advantages such as biocompatibility, enhanced stability, and a simple production process devoid of any requirement for organic solvents or high temperature.[5,6] DEET microparticle formulations were incorporated into a hydrogel base and rheological evaluations were performed. Rheological studies are very important for the evaluation of newly synthesized topical formulations; however, these measurements are mostly limited to viscosity determination. An ideal gel must be thixotropic i.e., when a gel formulation is applied onto the skin it should easily transform into a sol consistency, and post application it should regain its original gel form. Thixotropic property of gel allows the formulation to spread easily onto the skin surface, such that it will remain on the skin for a sufficient duration and exert its therapeutic effect. Previous studies have reported that particles with size >300 nm are unable to penetrate through the stratum corneum, the outermost layer of the skin.[7] Chitosan-based nanoparticles or microparticles have been shown to allow a controlled release of the encapsulated contents assisted by the slow erosion of the polymer.[8] In the present study, DEET encapsulated chitosan microparticles were synthesized to achieve a controlled release of DEET while ensuring lower skin permeation. These chitosan-based microparticles were further assessed for their rheological properties, *in vitro* drug release, and permeation ability. Factorial design was employed to obtain an appropriate mathematical model for optimizing the composition of the formulation.

MATERIALS AND METHODS

Materials

DEET, chitosan (low MW), and sodium tripolyphosphate (TPP) were purchased from Sigma-Aldrich, USA. Strat-M® was procured from Merck Millipore, Germany. Acetic acid and triethanolamine were obtained from Chemiz (M) Sdn. Bhd., Malaysia. Methanol high performance liquid chromatography [(HPLC) grade] was purchased from Fisher Scientific, UK. Other chemicals used in the study were of analytical grade.

Methodology

Formulation of DEET containing microparticles

DEET-loaded chitosan microparticles were prepared using ionic gelation method.[9] Chitosan (0.4% w/v) solution was prepared in dilute acetic acid (2%v/v). The solution was stirred at 500-1000 rpm using a magnetic stirrer to ensure complete dissolution of chitosan. The pH of chitosan solution was adjusted to 5.0 and 1 mg of DEET was added to this solution. This was followed by dropwise addition of the cross-linker TPP (0.1% w/v), with continuous stirring at 700 rpm. To remove excess reagents, the micro-particulate suspension was centrifuged at 6000 rpm for 30 mins.

Optimization of formulation by factorial design using design-expert software

The complexity in the pharmaceutical formulations are evaluated using established tools like factorial design.[9] The present study was based on independent formulation variables, selected using simple two-level factorial designs of experiments.[2] Design-expert software version 11 (Stat-Ease. Inc) was used to design the experimental runs. Two-level factorial design and three variables (factors) were applied, with a minimum of 5 runs. For analysis, chitosan and TPP concentrations were used as variable (independent), while particle size, encapsulation efficiency (EE), and drug release of the developed formulation were used as the dependant factor. Further, 2x2 factorial design and statistical design of the responses were applied to the optimized formulations.

Development and validation of HPLC method for DEET analysis

A simple and economical method based on reversed-phase HPLC (RP-HPLC) was employed for accurate quantification of DEET present in the microparticles. HPLC system (Perkin Elmer, USA) equipped with an auto-injector, a quaternary pump, and a diode array detector was used. HPLC analysis was performed on a reversed-phase column (Hypersil Gold C18, 5 µm, 250 mm×4.6 mm) maintained at 25˚C with a mobile phase comprising of methanol:water (70:30) at a flow rate of 1.2 mL/min. For DEET detection, the wavelength was set at 210 nm. The retention time of DEET was found to be 6.1 minutes.

Fourier-transform infrared spectroscopy (FTIR) analysis

The drug-excipient compatibility studies were performed using attenuated total reflectance-FTIR [(ATR)-FTIR, Thermo Fisher Scientific, USA] over the range of 400-4000 cm^{-1}.[10] FTIR plays an important role in pre-formulation phase of drug development and provides information regarding any possible interactions between active drug and used additives. FTIR spectra of DEET, TPP, chitosan, and the DEET microparticles loaded hydrogel were recorded using ATR-FTIR spectrophotometer equipped with OMNIC software version 9.2.

Particle size and surface morphology analyses

The particle size analysis was performed using Zeta sizer Nano® (Malvern Instruments, Malvern, UK). For measurement, the suspension was diluted using deionized water.[11] Surface morphology of the microparticles was analyzed using a field emission-scanning electron microscope [(FE-SEM), Quanta 400F, FEI, USA] at a voltage of 5 kV. For sample preparation, the microparticle suspension was placed on a double-sided carbon tape, mounted onto an imaging stub, and allowed to dry overnight at room temperature.

Encapsulation efficiency

The EE of the formulation was determined using the centrifugation technique. The drug-loaded microparticle suspension was centrifuged at 6000 rpm for 30 mins. The supernatant was collected in a separate tube and filtered using a nylon syringe filter (0.45 µm). The drug content in the supernatant, representing the unencapsulated drug, was analyzed using HPLC. The EE was calculated using below mentioned formula.[12]

$$\% \text{ EE} = \frac{\text{Total amount of drug added to the formulation-Unencapsulated drug}}{\text{Total amount of drug added to the formulation}} \times 100$$

In vitro drug release studies for DEET microparticles

Drug release from the microparticles was determined by dialysis bag method.[13] The formulation was re-dispersed in phosphate buffered saline (PBS) at pH 5.0 and placed in a dialysis bag (MWCO 14000 Da). Both ends of the bag were tied and it was placed in a bottle containing PBS maintained at 37˚C with continuous stirring at 100 rpm. At predetermined time intervals, the release medium was removed and replaced with an equal volume of fresh buffer. The amount of drug present in the release medium was quantified using HPLC. Various zero and first order kinetic models, like Higuchi, Korsmeyer-Peppas, and the Hixson-Crowell model, were used to evaluate the drug release mechanism of DEET microparticles.[14]

Formulation of microparticles loaded hydrogel and its rheological evaluation

A hydrogel was prepared by dissolving Carbopol 940 (1% w/v) in purified water. After 24 h of swelling, triethanolamine was added to the hydrogel to adjust the pH to 5.0 and achieve the desired consistency.[15] DEET microparticles were added to the gel and mixed continuously using a magnetic stirrer to obtain homogeneous preparation. Rheological studies for viscosity and thixotropy measurements were performed using Thermo Haake Rheometer (Thermo Fisher Scientific, USA). Commercially available DEET cream formulation was used as control.[16,17] The data were analyzed using various rheological models, such as Bingham, Casson, and Herschel-Bulkey model.[18]

In vitro permeation of DEET across synthetic Strat-M® membrane

In vitro permeation of the formulation was performed using synthetic Strat-M® membrane.[19] Static Franz diffusion cells having donor and receptor capacities of approximately 1 and 2 mL, respectively, were used for the permeation experiments. Strat-M® membrane was placed between the donor and receptor compartment. The formulation was loaded into the donor compartment, while the receptor compartment was filled with PBS at pH 7.4. The solution in the receptor compartment was stirred continuously using a magnetic stirring rod. The samples were collected from the receptor compartments at predetermined time points up to 24 hours and immediately replaced with equal volume of fresh receptor medium.[20] To generate permeation profile curves, the cumulative amount of drug permeated (µg/cm^2) was plotted as a function of time. The steady state flux (J), representing the amount of drug permeated per unit area, was determined from the linear plot.[21]

Statistical analysis

Statistical analyses were performed using Graphpad Prism 7 software. Student's t-test was used to evaluate statistical significance between two groups. For more number of groups, statistical analysis involved One-Way ANOVA and post-hoc Tukey-honestly significant difference. $P<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Optimization of microparticle preparation using design expert software

Design expert software was used to design the experiments for optimizing microparticle formulations.[22] To study the significance of the concentration of chitosan and TPP in the development of DEET microparticles, three responses including particle size, entrapment efficiency, and drug release were considered. In the present study, two factorial design and statistical design of the responses were applied to the optimized formulations. Statistical design was considered to be significant for $p<0.05$. The two factorial design showed that the optimized formulations were characterized by two concentrations i.e.,

low and high concentrations. Statistical analysis for three responses was performed and it was characterized by two coefficients, positive and negative. The positive response indicated an increase in the parameters (particle size, entrapment efficiency, and drug release). In comparison to this, the negative sign suggested a decrease in the parameters. Chitosan and TPP were considered as two factors, while their concentrations represented two levels. The statistical analysis suggested that chitosan and TPP concentrations had no significant effect on the particle size and entrapment efficiency ($p>0.05$), for the selected concentrations. However, chitosan and TPP concentrations showed significant effect on drug release of DEET microparticles ($p=0.038$) (Figure 1). Therefore, the release of DEET from the microparticles, developed using ionic gelation technique, could be modulated by changing the concentration of chitosan and TPP.

Particle size and surface morphology analyses

DEET microparticles, prepared using different ratios of chitosan: TPP, were characterized by an average particle size of 0.45-8.3 µm (Table 1). Among five formulations, DM-3 (chitosan: TPP, 4:1) showed the lowest polydispersity index (PDI) <0.5 and average particle size of 0.45 µm. Thus, it was selected as the optimized formulation. Previous studies have reported that the particles with size >300 nm pose difficulty in permeating through the skin.[7] FE-SEM analysis of the optimized formulation showed that the microparticles were spherical in shape with smooth surface (Figure 2). In addition to this, the microparticles were sufficiently separated and showed no signs of aggregation, indicating physical stability of the formulation.

Encapsulation efficiency

The EE of the formulations varied in terms of the concentration of chitosan and TPP. The formulations DM-1, DM-2, DM-3, DM-4, and DM-5 showed EE of 57.56±0.41, 55.79±0.53, 50.07±2.09, 56.64±1.32, and 57.34±0.34%, respectively. The highest EE was observed for the formulation DM-1. This was possibly attributed to high proportion of chitosan and TPP, which would have further resulted in strong electrostatic interactions between the molecules.[22] The availability of larger number of amino groups at higher concentrations of chitosan results in a decrease in the intermolecular distance and increase in the cross-linking density.[6]

FTIR analysis

As shown in Figure 3, the spectrum for DEET showed two bands at 2971.27 cm^{-1} and 2933.59 cm^{-1} corresponding to CH_3 asymmetric stretch and symmetric stretch, respectively. The band present at 1626.39 cm^{-1} might be contributed by C=O stretching of the amide bond.[23] The peak at 1583.77 cm^{-1} was contributed by C-C stretching of aromatic ring. The presence of C-C stretching was observed at 1427.59 cm^{-1}.[24] In case of chitosan, the peak at 3354.85 cm^{-1} was contributed by-NH stretching vibration. The band at 2875.90 cm^{-1} represented-CH

Table 1. Average particle size and polydispersity index of polymeric DEET microparticles

Formulation code	Chitosan:TPP (mass ratio)	Average particle size (nm)	PDI
DM-1	4:4	8258.7±366.3	0.70
DM-2	1:4	6525.7±182.7	0.73
DM-3	4:1	446.5±18.6	0.50
DM-4	1:1	4950±172.5	0.80
DM-5	2.5:2.5	5385.7±129.1	0.79

DEET: N,N-Diethyl-3-methylbenzamide, TPP: Tripolyphosphate, PDI: Polydispersity index

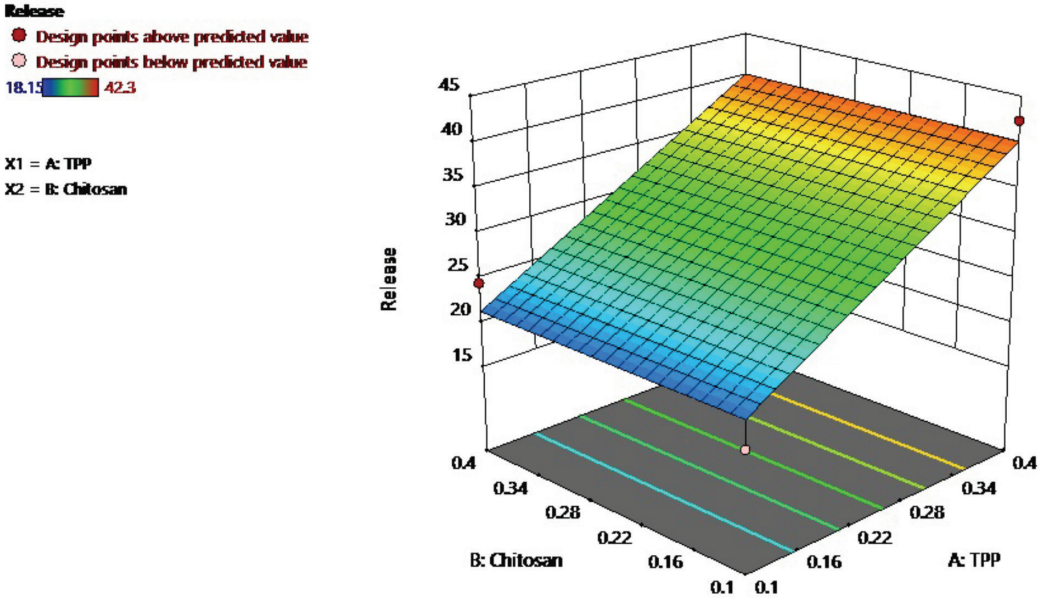

Figure 1. Contour plot (phase diagram) for drug release from the developed DEET microparticles
DEET: N,N-Diethyl-3-methylbenzamide

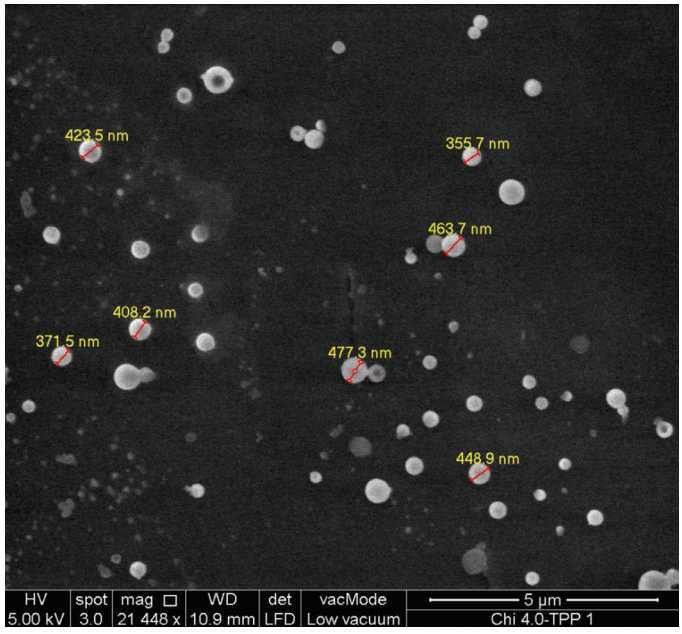

Figure 2. FE-SEM image of optimised polymeric DEET microparticles (chitosan:TPP mass ratio of 4:1)

FE-SEM: Field emission-scanning electron microscope, DEET: N,N-Diethyl-3-methylbenzamide, TPP: Tripolyphosphate

stretching and band at 1645.99 cm^{-1} indicated C=O (carbonyl) corresponding to the amide group from-CONH. In addition to this, a band corresponding to amine (NH_2) group was also observed seen at 1588.73 cm^{-1}. TPP showed two characteristic peaks at 1209.59 and 1136.47 cm^{-1} corresponding to phosphate (P=O) symmetric and anti-symmetric stretching vibrations (O-P=O), respectively. Similar peaks were recorded for chitosan-TPP microparticles, indicating no incompatibility between DEET and the excipients used.

In vitro drug release

The drug release curves for DEET microparticles showed that there was no initial burst release of the drug from the formulation. Instead, a slow and controlled drug release was observed over time (Figure 4). This might be attributed to homogenous encapsulation of DEET into the microparticles, which was supported by the low PDI value of <0.5. In general, the microparticles with poor encapsulation are characterized by drug deposition on the surface that might result in initial burst release of the drug. Besides this, the concentration of TPP might also have some effect on drug release from microparticles. In the present study, the formulation DM-1 showed a lower drug release (38.03%±1.97%) as compared to the formulation DM-3 (42.30%±4.84%), over a period of 24 hour. This might be contributed by higher TPP content of

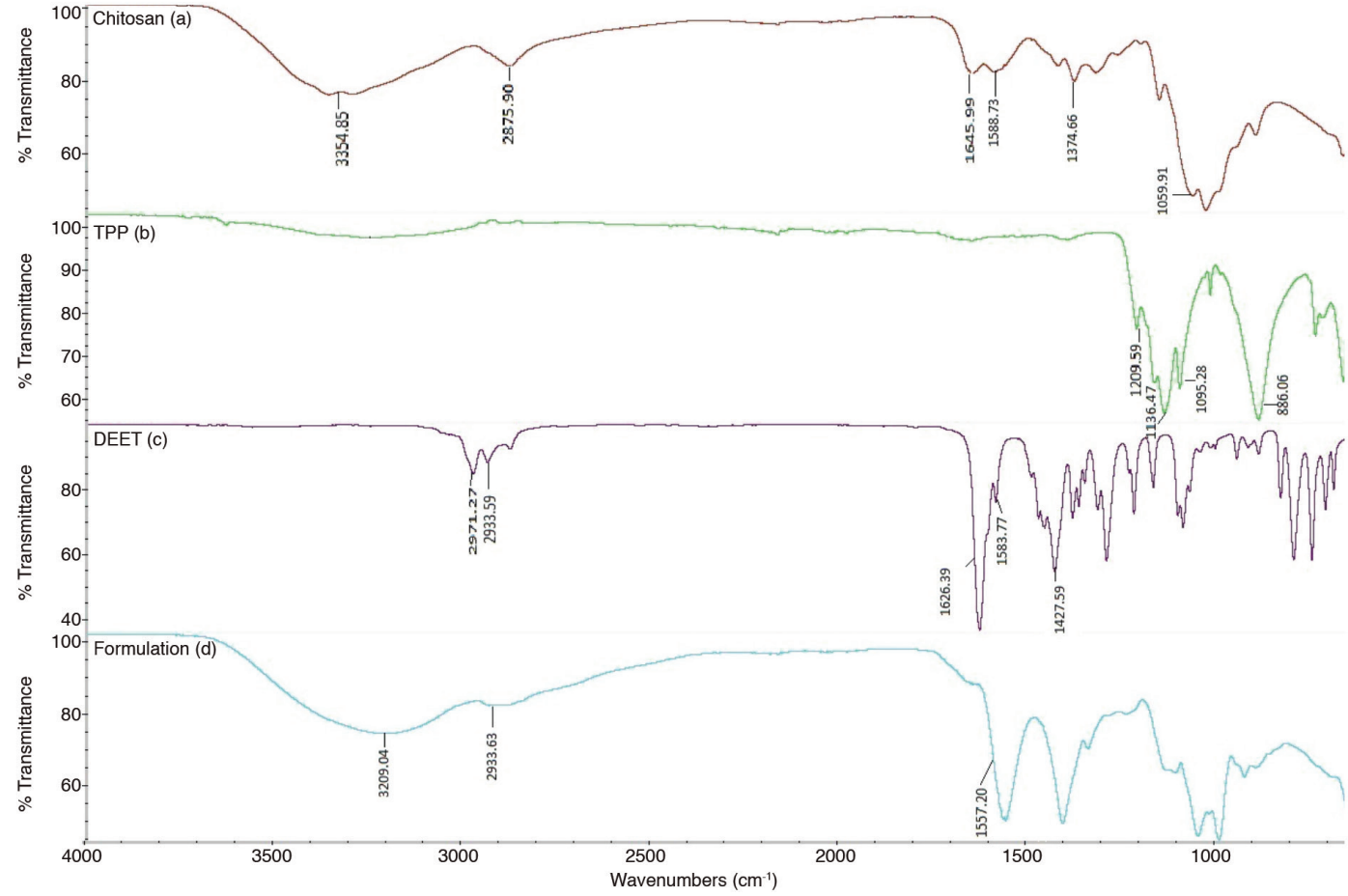

Figure 3. FTIR spectrum for (a) chitosan, (b) TPP, (c) DEET, and (d) formulation

FTIR: Fourier-transform infrared spectroscopy, DEET: N,N-Diethyl-3-methylbenzamide, TPP: Tripolyphosphate

DM-1 as compared to DM-3. The drug release from chitosan-TPP microparticles was found to decrease with an increase in the concentration of crosslinking agent (TPP).[8] Several studies have previously reported that smaller microparticles exhibit larger surface area, resulting in faster swelling and drug release. Chitosan has been previously shown to absorb the release medium, that assists in the swelling and penetration of the medium inside the polymer matrix.[25] The drug release from the microparticles might be attributed to diffusion of the drug through the swollen polymer. Higuchi model correlation coefficient provided best fit with R^2 value 0.9586. This suggested involvement of a diffusion mechanism wherein the drug diffuses constantly from the polymer matrix while maintaining a perfect sink condition.[26,27]

In vitro permeation of DEET across synthetic Strat-M® membrane

In vitro permeation experiments were performed using Static Franz diffusion cells with synthetic Strat-M® membrane placed between the donor and receptor compartments. Strat-M® membrane is widely used for the permeation studies and the obtained results have been shown to be comparable to the human skin.[28] Strat-M® comprises of two layers of polyether sulfone, followed by one layer of porous polyolefin. In addition to its close similarity with the skin, Strat-M® usage is devoid of any special requirements of storage and safety, that are usually associated with biological membranes. Permeation experiments were conducted for pure DEET solution (control) and the optimized formulation. Over a period of 24 h, the cumulative amounts of DEET permeated from the control solution and DEET formulation were found to be 211.6±19.5 µg/cm^2 and 4.07±0.08 µg/cm^2, respectively (Figure 5). A significant difference ($p<0.05$) was observed between the cumulative drug permeation for the control and the formulation. Flux values of 9.61±0.94 µg cm^{-2} h^{-1} and 0.12±0.001 µg cm^{-2} h^{-1} were recorded for the control DEET solution and microparticles loaded hydrogel, respectively. The flux of the optimized formulation was significantly lower as compared to the control ($p<0.05$). All these results showed that the formulation allowed minimal permeation of the drug into the skin, thereby ensuring a possible reduction in the systemic toxicity.

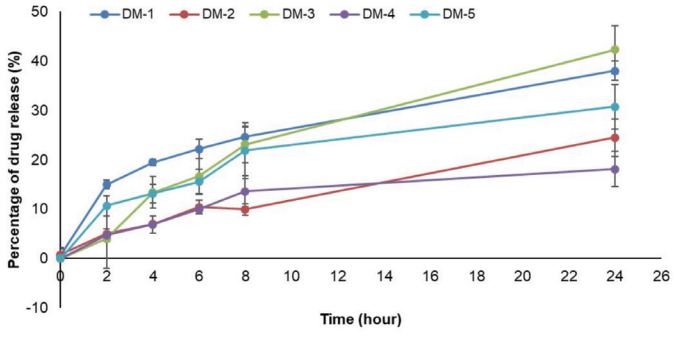

Figure 4. Profile for drug release from DEET microparticles (chitosan:TPP mass ratio of 4:1) at pH 7.4. Mean ± SD, (n=3)

DEET: N,N-Diethyl-3-methylbenzamide, TPP: Tripolyphosphate, SD: Standard deviation

Rheological studies of the formulation

As shown in Figure 6, a hysteresis loop demonstrated the thixotropic behavior of the hydrogel (Figure 6). The intrinsic mechanism contributing to thixotropic behavior of the gel was based on the three-dimensional structure of the molecules that were capable of forming hydrogen bonds. When a shearing stress was applied, reduction in these interactions resulted in a decrease in viscosity, turning the gel into a sol state (ascending curve). Post the removal of shearing stress, the gel regained its conformation slowly (descending curve). The restructuring of bonds between the molecules was attributed to Brownian motion of the molecules, which could be linked to the amount of shear stress applied.[18] It is important to evaluate the thixotropic property of a semisolid preparation, designed for topical application, as it reflects the ease of application of the product on the skin. The sol form of the product allows effortless application on the skin. Once the shear stress is removed, the product regains its initial gel conformation, which is better retained on the skin. The best-fit rheological model was determined by assessing R^2 values. All the samples followed Herschel-Bulkley model. The rheological parameters corresponding to the Herschel-Bulkey model are summarized in Table 2. Yield stress is the minimum stress required to induce the flow of a material. It is an important factor used for the characterization of semisolid preparation. It usually affects the spreadability and retention of the formulations. High value of yield stress demonstrates a reduction in the spreadability and enhancement in the retention capacity of the preparation, and vice-versa.[29] In the present study, the yield stress for the formulation and control DEETcream were found to be 26.39 and 6.474 Pa, respectively. This indicated that the standard cream was more spreadable as compared to the optimized formulation. Substantial yield strength was required to break the microgel network present in the hydrogel, thus contributing to the higher yield stress observed for the hydrogel as compared to the cream.[16] Degree of shear thinning behavior of semisolid preparations can be analyzed by looking

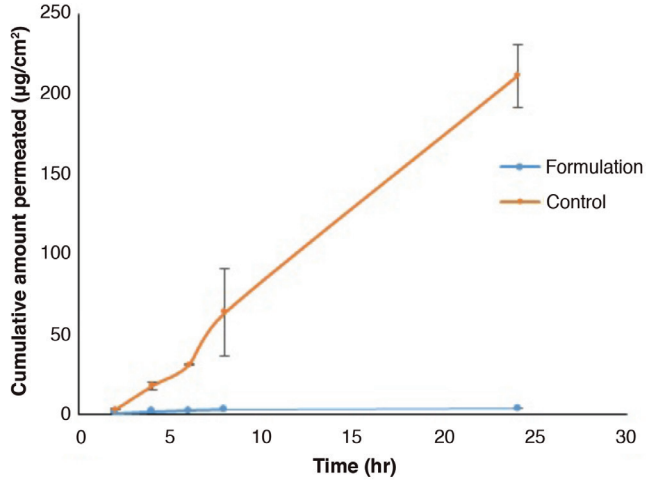

Figure 5. Cumulative amount of DEET permeated µg/cm^2 over time (hr) for control (DEET solution) and the formulation. Mean ± SD, (n=3)

DEET: N,N-Diethyl-3-methylbenzamide, SD: Standard deviation

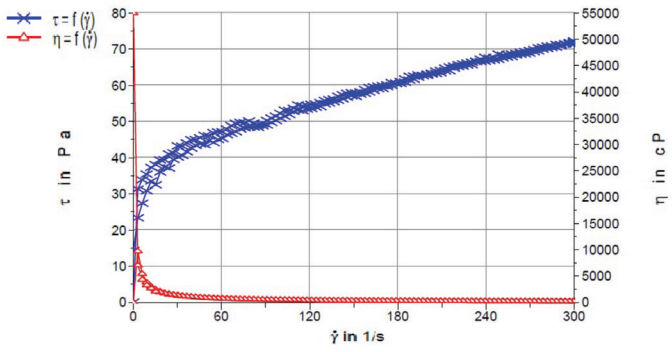

Figure 6. Rheogram of the hydrogel formulation containing DEET mocroparticles showing thixotropic behaviour

DEET: N,N-Diethyl-3-methylbenzamide

Table 2. Rheological parameters corresponding to the Herschel-Bulkey model for the standard cream, and the formulation

Formulations	Yield stress (Pa)	Flow index (n)
Standard cream	6.47±1.37	0.275±0.01
Hydrogel formulation	26.39±4.9	0.791±0.26

at the flow index values, n. In the present study, all the samples were characterized by n <1. These results suggested that all the samples exhibited a non-Newtonian pseudoplastic behavior.[30]

CONCLUSION

DEET microparticles loaded hydrogel was successfully formulated and evaluated *in vitro.* The formulation permitted topical delivery of DEET with controlled drug release, which followed Higuchi kinetic model. The rheological evaluations demonstrated the suitability of the hydrogel for topical application. Ease of application and prolonged drug retention on the skin balanced with high cosmetic acceptability, highlighted the utility of this gel as a promising drug carrier. *In vitro* drug permeation studies provided evidence for low permeation of DEET from the hydrogel as compared to the control. Thus, all these results suggested that DEET microparticle loaded hydrogel could be used as an effective strategy to ensure controlled and effective insect repellent activity of DEET. Future studies focusing on *ex vivo* and *in vivo* evaluation of this formulation are required to support these preliminary findings.

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Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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ORIGINAL ARTICLE

Assessment of Protective Effects of Methanolic Extract of *Salvia verbenaca* Roots Against Oxidative Damage Induced by Hydrogen Peroxide

Hidrojen Peroksitin Neden Olduğu Oksidatif Hasara Karşı *Salvia verbenaca* Köklerinin Metanol Ekstresinin Koruyucu Etkilerinin Değerlendirilmesi

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University of 20 Aout 1955, Department of Natural Science and Life, Skikda, Algeria

ABSTRACT

Objectives: *Salvia verbenaca* is a medicinal plant that has been traditionally used in Algeria for the treatment of wounds and emptied abscesses. The present study aimed to evaluate the cytotoxicity of methanolic extract of *S. verbenaca* roots and explore its ability to bestow protection against oxidative damage induced by H_2O_2 (200 µM).

Materials and Methods: The cytotoxic effects and protective properties of *S. verbenaca* on human monocytic leukemia cells (THP-1) was studied using thiazolyl blue tetrazolium bromide assay. The protective effects of the extract against H_2O_2-induced oxidative damage was evaluated using single cell gel electrophoresis (comet) assay and 2,7-dichlorodihydrofluorescien diacetate (H2DCFDA) assay.

Results: *S. verbenaca* extract was found to be non-cytotoxic at concentrations <500 µg/mL. However, the use of 500 and 1000 µg/mL of the extract decreasedcell viability. H2DCFDA assay provided evidence for anti-oxidative properties of *S. verbenaca*. Addition of *S. verbenaca* (1 and 10 µg/mL) resulted in significant reductionin H_2O_2-induced reactive oxygen species (ROS) production. Further, comet assay showed that addition of the extract resulted in a significant reductionin the length and % DNA content of comet tail. Additionally,nuclei in the cells also appeared to be devoid ofdegradation.

Conclusion: The use of *S. verbenaca* root extract conferred protection against H_2O_2-induced ROS production and DNA breakage *in vitro*.

Key words: *Salvia verbenaca* root, THP-1 cells, reactive oxygen specices production, comet assay

ÖZ

Amaç: *Salvia verbenaca*, Cezayir'de geleneksel olarak yaraların ve boşalan apselerin tedavisinde kullanılan tıbbi bir bitkidir. Bu çalışma, *S. verbenaca* köklerinin metanol ekstresinin sitotoksisitesini değerlendirmeyi ve H_2O_2 (200 µM) ile indüklenen oksidatif hasara karşı koruyucu etkisini araştırmayı amaçlamaktadır.

Gereç ve Yöntemler: *S. verbenaca*'nın insan monositik lösemi hücrelerindeki (THP-1) sitotoksik etkileri ve koruyucu özellikleri tiyazolil mavi tetrazolyum bromür testi ile belirlendi. Ekstrenin H_2O_2 kaynaklı oksidatif hasara karşı koruyucu etkileri, tek hücre jel elektroforezi (comet) testi ve 2,7-diklorodihidroflorescien diasetat (H2DCFDA testi) kullanılarak değerlendirildi.

Bulgular: *S. verbenaca* ekstresinin <500 µg/mL konsantrasyonlarında sitotoksik olmadığı bulundu. Bununla birlikte, 500 ve 1000 µg/mL ekstrenin kullanılması hücre canlılığını azaltmıştır. H2DCFDA testi, *S. verbenaca*'nın antioksidan özellikleri için kanıt sağlamıştır. *S. verbenaca* (1 ve 10 µg/mL) ilavesi, H_2O_2 ile indüklenen reaktif oksijen türlerinin (ROS) üretiminde önemli derecede azalma ile sonuçlandı. Ayrıca, comet analizi, ekstrenin eklenmesinin comet kuyruğunun uzunluğunda ve % DNA içeriğinde önemli bir azalmaya neden olduğunu gösterdi. Ek olarak, hücredeki çekirdeklerde degradasyon olmadığı görüldü.

Sonuç: *S. verbenaca* kökü ekstresinin kullanılması, *in vitro* olarak H_2O_2 kaynaklı ROS üretimine ve DNA kırılmasına karşı koruma sağladı.

Anahtar kelimeler: *Salvia verbenaca* kökü, THP-1 hücreleri, reaktif oksijen türlerinin üretimi, comet testi

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INTRODUCTION

Plants of the *Salvia* genus are widely known for their applications in food, cosmetics, and pharmaceutical industries.[1] *Salvia* is the largest genus of the Lamiaceae family with ~1000 species distributed all across the globe, particularly in the Mediterranean basin, South-East Asia, and Central and South America.[2] Essential oils and concentrates from *Salvia* species are endowed with numerous therapeutic properties, including antimicrobial, hypoglycemic, antiphlogistic, antituberculous, and anti-inflammatory properties. The use of these oils also aids in cancer prevention.[3-5] In Algeria, 18 species of *Salvia* genus are known. Among these, *Salvia verbenaca*, commonly known as Meryiamia or khiyata, is used in combination with other medicinal herbs to treat cold.[6] Flowering leaves and tops of this plant exhibit stomachic, stimulating,[7] tonic, vulnerary, and antirheumatic effects.[8]

S. verbenaca is used to prepare tonics and stimulating infusions.[9] Freshly chopped leaves of this plant are applied as a poultice on the infected wounds and emptied abscesses to facilitate healing.[10]

Oxidative stress is generally induced by the generation of reactive oxygen species (ROS), which principally react with proteins, lipids, and DNA.[11] ROS are known to promote decrease in glutathione (GSH), changes in hormones, oxidative DNA damage, genetic transformation, DNA chain rupture, and chromosomal modifications.[12]

Oxidative damage to DNA poses a serious problem as DNA cannot be resynthesized or corrected. ROS mainly include free radicals like hydroxyl radicals ($^{\bullet}OH$), superoxide anions ($O_2^{\bullet-}$) and non-radical molecules like hydrogen peroxide (H_2O_2). Primarily, ROS are generated from oxidative metabolism in mitochondria. The other endogenous sources of ROS include inflammatory cells and peroxisomes.[13] ROS can attach easily to DNA, which is mainly mediated via higher reactivity of ROS with strong nucleophilic sites present on nucleobases. Various mutations, like base alterations or base adhesion, can be produced via interaction of mutagenic agents with DNA bases or deoxyribose sugar.[14] Moreover, oxidative damage to DNA might induce mutations, resulting in activation of oncogenes or inactivation of tumor suppressor genes as well as alterations in gene expression.[15]

Several studies have explored the biological activity of the aerial parts of *Salvia* species. The present study aimed to evaluate biological activities of *S. verbenaca* root extract. This is the first report for *in vitro* evaluation of cytotoxic and protective effects of the methanolic extract of *S. verbenaca* roots against H_2O_2-induced oxidative stress.

MATERIAL AND METHODS

Salvia verbenaca as plant sample

S. verbenaca plants were collected from Oued Abid-Batna. The roots of the plants were dried for 15 days at room temperature and converted into fine powder using a blender.

Extraction

For extraction, 100 mg of powdered roots were macerated in a hydroalcoholic mixture (MeOH:H_2O, 7:3 v/v) for 24 h. The extraction process was repeated twice. The hydroalcoholic extracts were mixed and concentrated using a rotary vacuum evaporator. The crude extract was weighed and stored at 4°C until further use.[16]

Cell culture

Human monocytic leukemia cell line THP-1 (Rockville, MD, USA) was grown in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µg/mL streptomycin, and 50 U/mL penicillin (Sigma, Milan, Italy). The cells were cultured in T75 flasks at cell density of $5x10^5$ cells/mL and maintained at 37°C in a humidified chamber under 5% CO_2.

For assay, THP-1 cells were seeded in 24- or 96-well plates and treated with H_2O_2 (200 µM) for 24h either alone or in the presence of the extract. The extracts were added to the culture medium 30 mins prior to the addition of H_2O_2. Stock solution (50 mg/mL) of the extract was prepared by dissolving extract in dimethyl sulfoxide (DMSO) (1%).

Evaluation of cytotoxicity of plant extract

To evaluate the cytotoxic effects of *S. verbenaca*, THP-1 cells were treated with six concentrations of the extract (1, 10, 50, 100, 500, and 1000 µg/mL) and cytotoxicity was measured using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Generally, MTT assay involves conversion of yellow tetrazolium salt to purple formazan crystals in viable cells by the action of NAD(P)H-dependent oxidoreductase enzymes. The resulting insoluble formazan crystals are dissolved using DMSO and cell viability/proliferation is measured in terms of absorbance of purple colored solution at 570 nm.

The concentration at which the root extract was non-cytotoxicity was identified and used for further studies. To evaluate the protective effects of *S. verbenaca*, THP-1 cells were seeded in a 96-well plate at a cell density of $5x10^4$ cells/mL and treated with H_2O_2 (200 µM) for 24 h both in the presence and absence of the extract. Further, the cells were washed with phosphate buffer solution (PBS), transferred intoa fresh 96-well plate, and incubated with MTT (0.5 mg/mL) for 4 h at 37°C. The formazan crystals were dissolved using100 µL of acidic isopropanol (0.04 M HCl in absolute isopropanol) and the cells were incubated for 1 h.[17] The absorbance of the samples was measured at 540 nm using a microplate spectrophotometer (Tecan Italia, CologNoMonzese, Italy). The cell viability (%) was estimated using the formula, [(absorbance of treated cells/absorbance of untreated cells) ×100]. The experiment was performedin triplicates, with three wells per treatment (n=9).

Evaluation of ROS production

The production of ROS was evaluated using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) a non-fluorescent probe which crosses the plasma membrane before being cleaved into DCFH by intracellular esterases. DCFH can then be oxidized to fluorescent DCF by ROS.

In a 12-well plate, the cells were seeded at a density of $5x10^5$ cells/mL and treated with *S. verbenaca* extract (1 and 10 µg/mL), both in the presence and absence of H_2O_2. The cells were incubated for 24 h at 37˚C. After 24 h, the cells were washed twice with PBS and ROS production was estimated using H2DCFDA (5 µM). The cells were incubated at 37˚C for 30 min in a humidified incubator under 5% CO_2. Following this, the cells were suspended again in 200 mL PBS containing 0.1 M K_2PO_4 and 0.5% Triton X-100. The samples were transferred into a 96-well plate with transparent bottom.[18] The oxidation of H2DCFDA was estimated in terms of fluorescence using a microplate reader, with excitation-emission filters fixed at 485 and 480 nm, respectively. The amount of ROS produced was expressed in term of percentage of fluorescence intensity of THP-1 cells. The experiment was performed in triplicates, with three wells per treatment (n=9).

Evaluation of DNA breakage

H_2O_2-induced DNA breakage was assessed using comet assay, according to the protocol described by Di Pietro et al.[19] The comet assay works on the principle that undamaged DNA migrates in the gel at a slow rate and remains within the confines of the nucleoid when current is applied. Thus, it appears as an intact comet head. In comparison to this, the broken DNA migrates at a faster rate and forms a comet-like tail. The fluorescent intensity and shape of this tail can be used to measure level of damage.[20] DNA breakage can be quantified by measuring the length and % DNA in the tail of each comet. In a 96-well plate, THP-1 cells were seeded at a density of $5x10^4$ cells/mL and treated with *S. verbenaca* extract with and without H_2O_2. After 24 h, the cells were washed twice with PBS. Following this, the cells were mixed with 0.5% low melting point agarose at 37˚C to achieve a concentration of $10x10^4$ cells/mL. Further, 50 µL of this cell suspension was loaded on to pre-coated glass slides and immediately covered with covers slips. These slides were incubated at 4˚C for 15 min. Further, the covers slips were carefully removed and additional 50 µL of low melting agarose (0.5%) was added onto the previously coated cell layer. The samples were allowed to solidify in ice surface for 5 min. The slides were immersed into lysis solution (100 mM EDTA, 2.5 M NaCl, 10 mM Tris, 1% Triton X-100, pH 10, 4˚C) for 1 h. Further, the slides were incubated in an electrophoresis solution (10 N NaOH, 200 mM EDTA, pH >13) for 20 min to ensure unwinding of DNA. Post winding, the DNA was subjected to electrophoresis in a similar buffer for 30 min (25 V, 300 mA).

After electrophoresis, the slides were washed with neutralization buffer (0.4 M Tris, pH 7.5), three times for 5 min each. The slides were stained with ethidium bromide and analyzed at 10x40 magnification using an epifluorescence microscope DM IRB (Leica Microsystem, Heidelberg, Mannheim, Germany) with an integrated digital camera (Canon Power Shot S50, Milan, Italy).

Statistical analyses

All statistical analyses were performed using One-Way ANOVA. The difference between the means of the group was analyzed by Tukey's post-hoc test using SPSS software. The results were reported as the mean ± standard error and the analysis was considered significant for $p<0.05$.

RESULTS

Cytotoxicity

MTT assay was used to evaluate the cytotoxic effects of six concentrations of *S. verbenaca* root extract. As shown in Figure 1, the treatment of THP-1 cells with 200 µM of H_2O_2 resulted in a significant reduction in cell viability ($p<0.05$). After 24 h of treatment with H_2O_2 (200 µM), only 67% of the cells were viable. For *S. verbenaca* extract, four concentrations (1, 10, 50 and 100 µg/mL) did not show any significant cytotoxicity. However, use of 500 and 1000 µg/mL of the extract resulted in a significant decrease in cell viability. *S. verbenaca* extract at 1000 µg/mL concentration resulted in 70% cell death ($p<0.001$). Thus, *S. verbenaca* was found to be safe at doses ≤100 µg/mL.

Production of ROS

H_2O_2-induced oxidative stress was evaluated in terms of intracellular levels of ROS, assessed using H2DCFDA assay. As shown in Figure 2 the treatment of THP-1 cells with H_2O_2 (200 µM) alone promoted the production of ROS ($p<0.001$). To evaluate the antioxidative effect of *S. verbenaca* extract, the cells were treated with 1 and 10 µg/mL of the extract along with H_2O_2. The presence of the extract resulted in a significant reduction in the intracellular levels of ROS as compared to the cells treated with H_2O_2 alone. The protective effect of the root extract was found to be more pronounced at the concentration of 10 µg/mL. The use of *S. verbenaca* extract alone did not show any augmentation in the intracellular ROS levels (Figure 2).

DNA breakage

Comet assay was used to evaluate H_2O_2-induced DNA breakage as well as the ability of *S. verbenaca* extract to protect THP-1 cells against this DNA damage (Figure 3). DNA damage was measured in terms of the amount of DNA present in the comet head and tail. As shown in Figure 3, the treatment of THP-1 cells with H_2O_2 for 24 h resulted in a significant increase in DNA

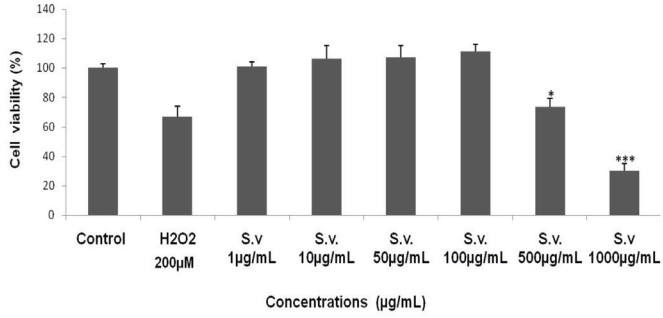

Figure 1. Effect of methanolic extract obtained from *Salvia verbenaca* roots (S.v) on the cell viability of THP-1 cells studied using MTT assay. No cytotoxic effects were observed for the root extractat concentrations <500 µg/mL, however, higher concentrations (500 and 1000 µg/mL) of the extract resulted in significant cytotoxicity. Data presented as mean ± SE (n=9).*$p<0.05$ and ***$p<0.001$ compared tocontrol cells

MTT: 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide, SE: Standard error

breakage ($p<0.001$). As expected, THP-1 cells treated only with *S. verbenaca* root extract (1 and 10 µg/mL) did not promote any increase in the comet parameters ($p>0.05$). These cells showed no significant changes in the length and % DNA ofthe tail as compared to the control.

Interestingly, *S. verbenaca* resulted in a significant ($p<0.001$) and dose-dependent reduction in DNA breakage induced by H_2O_2 in THP-1 cells.

THP-1 cells treated with H_2O_2 alone were characterized by fragmented head and apparent tail (comet aspect). However, treatment of the cells with the extract (1 and 10 µg/mL) resulted in lower fragmentation and the cells displayed a spherical aspect. Thus, *S. verbenaca* root extract exhibited ability to protect THP-1 cells against DNA breakage induced by H_2O_2 (Figure 4).

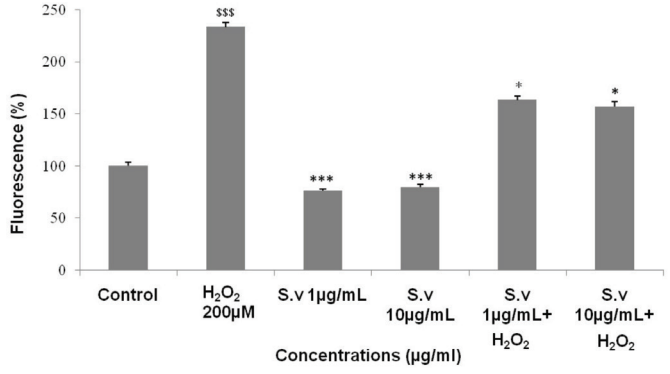

Figure 2. Effect of methanolic extract of *Salvia verbenaca* roots (S.v) on the intracellular levels of ROS induced by H_2O_2 in THP-1 cells studied using H2DCFDA assay. THP-1 cells ($5x10^5$ cells/mL/12-well plate) were incubated with *S. verbenaca* (1 and 10 µg/mL) with or without H_2O_2 (200 µM) for 24 h. Data presented as mean±SE (n=9). $^{\$\$\$}p<0.001$, H_2O_2 compared to control, $^{***}p<0.001$ and $^{*}p<0.05$ *S. verbenaca* treated groups compared to H_2O_2

ROS: Reactive oxygen species, H2DCFDA: 2,7-dichlorodihydrofluorescien diacetate, SE: Standard error

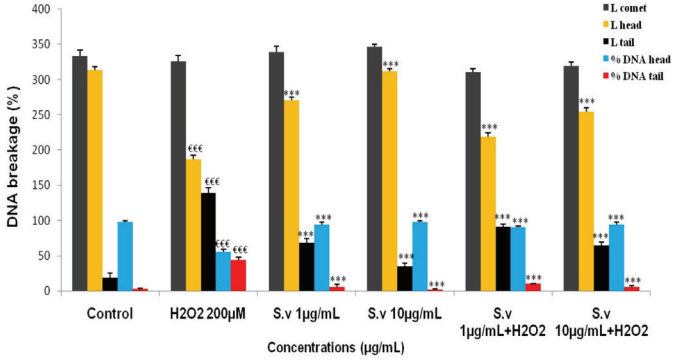

Figure 3. Effect of methanolic extract of *Salvia verbenaca* roots (S.v) on the level of DNA damage induced by H_2O_2 in THP-1 cells studied using comet assay. THP-1 cells ($5x10^4$ cells/mL) were incubated with *S. verbenaca* (1 and 10 µg/mL) with or without H_2O_2 (200 µM) for 24 h. DNA damage was expressed in terms of % of DNA content in comet head and tail. Data presented as mean±SE (n>50). $^{\$\$\$}p<0.001$, H_2O_2 compared to control, $^{***}p<0.001$ and $^{*}p<0.05$ *S. verbenaca* treated groups compared to H_2O_2

SE: Standard error

DISCUSSION

The present study aimed toevaluate the ability of *S. verbenaca* root extract to ameliorate H_2O_2-induced ROS generation and DNA breakage. To establish the protective effects of the extract, THP-1 cells were incubated with 1 and 10 µg/mL of the extract for 24 h. *S. verbenaca* extract (at concentrations <500 µg/mL) was found to non-cytotoxic and promoted cell growth, as indicated by cell viability of >100%. Interestingly, *S. verbenaca* at concentrations >500 µg/mL was found to be more cytotoxic as compared to H_2O_2. In fact, the use of 1000 µg/mL of the extract resulted in 30% viability. Thus, all these results indicated anticancerous activity of higher doses of *S. verbenaca* toward THP-1 cells. Several previous studies have reported cytotoxic effects of various *Salvia* species, however, the concentration at which these extracts exert cytotoxic effects is species dependent.

Poyraz et al.[21] evaluated the cytotoxic activities of *S. aethiopis* L. and *S. ceratophylla* L. in mouse embryonic fibroblast cell line

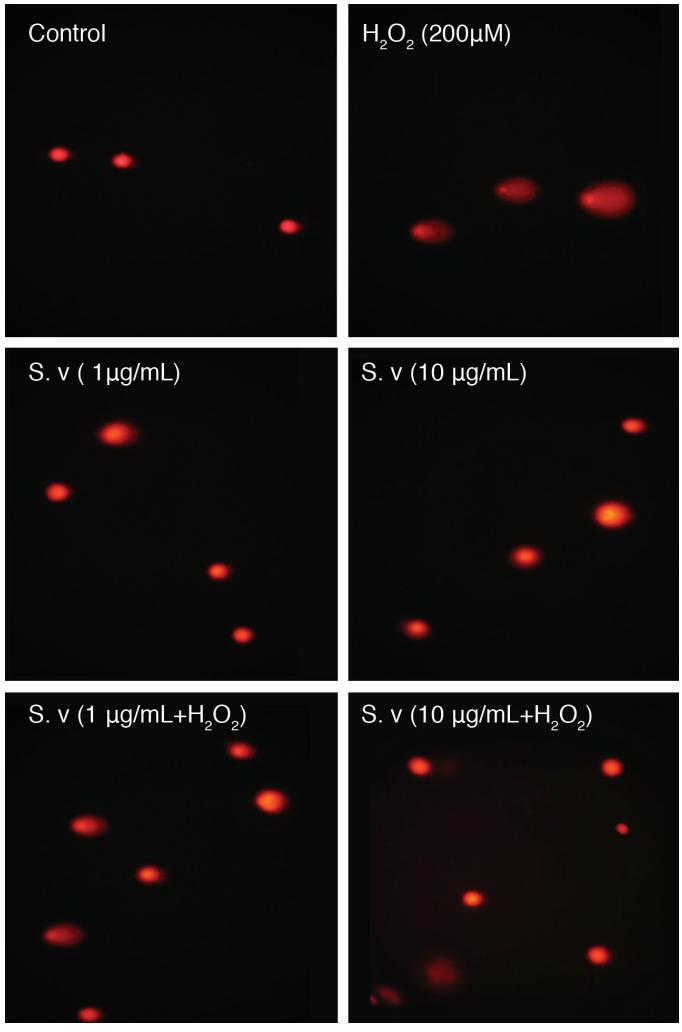

Figure 4. Fluorescence photomicrographs for DNA breakage in THP-1 cells studied using comet assay (original magnification 10x40). H_2O_2-treated cells exhibited high levels of DNA breakage (comet tail-like appearance), *S. verbenaca*-treated cells showed no DNA degradation (spherical mass), the cells treated with H_2O_2 and *S. verbenaca* (1 and 10 µg/mL) showedlow to very low levels of DNA breakage

(NIH/3T3) using MTT assay. The use of methanol and ethyl acetate extracts for both species resulted in a significant dose- and time-dependent increase intoxicity. However, the ethyl acetate extracts were found to be more cytotoxic.

Gateva et al.[22] reported the cytotoxic effects of *S. officinalis* extract at the concentration of 100 µg/mL in *Hordeum vulgare* root meristematic cells ($p<0.05$). Additionally, *S. officinalis* extract was shown to exert cytotoxic effect on human lymphocytes, especially at the concentrations of 50 and 100 µg/mL ($p<0.01$). *In vivo* study conducted by Vujošević and Blagojević[23] reported cytotoxic effects of *S. officinalis* extract, at the concentration of 100 µL/kg, toward mammalian bone marrow.

The ability of *S. verbenaca* extract to reduce H_2O_2-induced ROS generation was measured using H2DCFDA assay. H_2O_2 was used as a positive control. H_2O_2 is widely used as a model for ROS production. It generates hydroxyl radicals (•OH) in the presence of transition metal ions. Generally, H_2O_2 is delivered endogenously via certain physiological processes during oxidative phosphorylation.[24] It can enter the nucleus and interact with DNA.[25] These radicals can attack the sugar residues present in the DNA backbone, prompting single strand breaks. Additionally, these radicals can change purines and pyrimidines to their hydroxyl derivatives, for example 8-hydroxyguanine.[26]

The present study showed that the treatment of the cells with *S. verbenaca* extract (1 and 10 µg/mL) resulted in a significant reduction in the intracellular levels of ROS produced by H_2O_2 ($p<0.05$). The protective effects of *S. verbenaca* (1 and 10 µg/mL) against ROS production were found to be similar both in the presence or absence of H_2O_2. For both concentrations, *S. verbenaca* showed no cytotoxicity and promoted cell viability. The ability of *S. verbenaca* extract to reduce ROS production might be attributed to its antioxidant activity. In aprevious study, Nassar et al.[27] reported the presence of high antioxidant activity in *S. verbenaca* even at low concentrations. Similar results were reported in several studies involving *Salvia* species. Chang et al.[28] reported that the root extracts of *S. miltiorrhiza* of *Salvia* genus exhibited antiapoptotic and antioxidant effects. This root extract was endowed with cancer preventing properties. *S. miltiorrhiza* extract mediated reduction in ROS generation was achieved by inhibition of oxidases, decrease in superoxide production, inhibition of oxidative alteration of low-density lipoprotein, and promotion of mitochondrial oxidative stress. This was accompanied by an increase in the enzymatic activity of antioxidant enzymes, such as GSH peroxidase, MnSOD, and catalase.[28]

It has been previously shown that the use of sage induced an increase in the amount of antioxidant enzyme GSH in Caco-2 and HepG2 cells.[29,30] This antioxidant enzyme reduced ROS production and provided protection against H_2O_2-induced cytotoxicity in Caco-2 cells.[29]

Comet assay was used to measure H_2O_2 mediated DNA breakage in THP-1 cells. The assay also investigated the ability of *S. verbenaca* extract to protect the cells against this H_2O_2-induced DNA breakage. The treatment of the cells with H_2O_2 for 24 h resulted in a significant increase in DNA breakage and THP-1 nuclei were found to be highly fragmented. In H_2O_2 treated cells, DNA fragmentation was indicated by the augmentation of tail length and % DNA content in the comet tail as compared to the untreated cells. Interestingly, no DNA breakage was observed in the cells treated with the extract, both in the presence and absence of H_2O_2. All these results suggested that the extract was endowed with excellent ability to protect the cell nuclei against DNA breakage. The efficacy of the extract (1 and 10 µg/mL) was indicated by a significant decrease in the tail length and % DNA content in each nucleus ($p<0.001$). In addition to this, all cell nuclei were characterized by a nearly spherical shape having low breakage. These results were in concordance with the findings of Bani Hani and Bayachou.[31] The study showed that the incubation of HEK-293 cells with 100 µM/L of H_2O_2 for 3 h resulted in a significant increase in the intrinsic cellular DNA oxidation. However, the use of 100 µL of *S. fruticose* extract in the presence of 100 µM/L of H_2O_2 for 3 h resulted in a significant decrease in the intrinsic cellular DNA oxidation. These results suggested that *S. fruticose* extract might enhance the activity of DNA repair machinery.

Several *in vitro* studies have previously suggested that *Salvia* species are endowed with antimutagenic, antidiabetic, antiangiogenic, and gastroprotective properties.[32-35]

The major polyphenols found in *Salvia* species include rosmarinic acid, caffeic acid, carnosol, and carnosic acid.[36] In another study, Fotovvat et al.[37] reported the occurrence of five phenolic compounds (rosmarinic acid, salvianolic acid A, salvianolic acid B, carnosic acid, and caffeic acid) at different concentration in the roots of 41 populations of 27 *Salvia* species. However, rosmarinic and caffeic acids were found to be most abundant among these five compounds. Renzulli et al.[38] reported that rosmarinic acid present insage offered cytoprotective effect against *in vitro* cell damage induced by ochratoxin A and aflatoxin B1. Rosmarinic acid in sageacted via inhibition of toxin-induced ROS production and DNA and protein synthesis. *In vitro* study conducted by Tumur et al.[39] showed that rosmarinic acid could reduce the cell viability of HNSCC tumoral cell line. Rosmarinic acid regulated the proliferation of the cells by blocking the signaling pathway of epidermal growth factor and increased ROS levels.[39] In another study, carnosic acid and carnosol were found to inhibit ROS production and secretion of human leukocyte elastase. In addition to this, both polyphenols could attenuate the generation of proinflammatory leukotrienes in intact PMNL.[40]

Currently, very limited information is available regarding the chemical composition of *S. verbenaca* roots and its biological activities.

CONCLUSION

In the present study, *S. verbenaca* root extract was found to exhibit protective activity against H_2O_2-induced oxidative damage. The treatment of THP-1 cells with 1 and 10 µg/mL of the extract resulted in amelioration of H_2O_2-induced cytotoxicity, ROS production, and DNA breakage. Since *S. verbenaca* belongs to

the Lamiaceae family, the DNA protective effects of the extract could be attributed to its antioxidant activity. Additionally, the polyphenols present in this plant might further contribute to this protective ability. Future studies aimed at the identification of secondary metabolites present in *S. verbenaca* roots might provide better understanding regarding its protective ability.

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ORIGINAL ARTICLE

Development of Polylactic Acid and Bovine Serum Albumin-layered-coated Chitosan Microneedles Using Novel Bees Wax Mould

Yeni Balmumu Kalıbı Kullanılarak Polilaktik Asit ve Sığır Serum Albümin Tabakalı Kaplanmış Kitosan Mikroiğnelerinin Geliştirilmesi

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ABSTRACT

Objectives: This work illustrates a novel method of fabrication of polymeric microneedle (MN) construct using bees wax as mould and development of coated polymeric MNs for drug delivery.

Materials and Methods: A novel method of MN fabrication using bees wax as mould was established. The porous chitosan MN arrays were fabricated and coated with polylactic acid (PLA). The optimized MN arrays were coated with bovine serum albumin (BSA). The MNs were subjected to physiochemical and tensile strength characterization, followed by drug release study. The skin penetration and irritation study were performed *in vivo* in Wistar Albino rats.

Results: The constructed MN arrays contain MNs with 0.9 mm length, 600 µm width at the base, 30-60 µm diameter at the tip, and 1.5 mm distance between 2 needles. These MNs patch was having good mechanical strength (0.72 N/needle) and tensile strength 15.23 Mpa. The MN array patch had 6.26% swelling index and 98.5% drug release was observed on the 50th hr. Good penetration and no skin irritation was observed for optimized MN batch.

Conclusion: Polymeric MN arrays were successfully developed using bees wax mould and were successfully coated with PLA to deliver the BSA through skin epidermis layer.

Key words: Microneedles, transdermal drug delivery, coated microneedles, microneedle mould, bees wax, polylactic acid

ÖZ

Amaç: Bu çalışma, arı balmumunun kalıp olarak kullanıldığı polimerik mikroiğnelerin (MN) üretimine ilişkin yeni bir yöntemi ve ilaç taşınımı için kaplanmış polimerik MN'lerin geliştirilmesini amaçlamaktadır.

Gereç ve Yöntemler: Kalıp olarak arı balmumu kullanan yeni bir MN üretim yöntemi oluşturulmuştur. Gözenekli kitosan MN dizileri üretilmiş ve polilaktik asit (PLA) ile kaplanmıştır. Optimize edilmiş MN dizileri, sığır serum albümini (BSA) ile kaplandı. MN'ler, fizyokimyasal ve gerilme mukavemeti karakterizasyonuna tabi tutulmuş, ardından ilaç salımı çalışması yapılmıştır. Deriye nüfuz etme ve tahriş çalışması, Wistar Albino sıçanlarında *in vivo* koşullarda gerçekleştirilmiştir.

Bulgular: Oluşturulan MN dizileri, 0,9 mm uzunluğunda, tabanda 600 µm genişliğinde, uçta 30-60 µm çapında ve 2 iğne arasında 1,5 mm mesafeli MN'ler içerir. Bu MN yaması, iyi mekanik mukavemete (0,72 N/iğne) ve 15,23 Mpa gerilme mukavemetine sahipti. MN dizisi yaması, 50. saatte %6,26 şişme indeksine sahipti ve %98,5 ilaç salımı gözlendi. Optimize edilmiş MN grubu için iyi penetrasyon elde edilirken ve deri tahrişi gözlenmedi.

Sonuç: Polimerik MN dizileri, arı balmumu kalıbı kullanılarak başarılı bir şekilde geliştirildi ve BSA'yı deri epidermisi katmanından iletmek için başarıyla PLA ile kaplandı.

Anahtar kelimeler: Mikroiğneler, transdermal ilaç dağıtımı, kaplanmış mikroiğneler, mikroiğneli kalıp, balmumu, polilaktik asit

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INTRODUCTION

Microneedles (MNs) are structures, which are up to 2 mm in length, with thickness in few microns. MNs pierce the skin without pain and deliver drugs on the epidermis. MNs allow delivery of hydrophilic and lipophilic drugs and macro molecular therapeutics through the micro channels that are physically formed by the MN, while disrupting the stratum corneum. MNs do not produce pain because they enter the dermis layer without stimulating the sensory nerves.[1] The first generations of MNs were prepared from metals, organic polymers, glass, silicones as they were used to create micropores into the skin to facilitate drug, vaccine, or protein diffusion in the skin.[2] The first patent on MNs-based drug delivery was filed in US in 1971. At that time, MNs were referred to as "puncturing projections". However, the first successful attempt of MNs development was in the 1990s, as silicon MNs successfully facilitated the delivery of calcein through the human skin. MNs have shown effectiveness in delivering many therapeutic molecules through biological membranes, including sclera, skin, and mucosal tissue.[3,4] MNs arrays are based on combining advantages of the non-invasive and invasive systems and the elimination of their drawbacks.[5] Solid MNs show the increase in skin permeability for the compounds with size ranging from small molecules to larger molecules, as with proteins to nanoparticles.[6-9] The successful delivery of insulin[10] oligonucleotides, desmopressin, human growth hormone,[11-13] and the immune response from transportation of DNA and protein antigens.[14,15] Most MNs reported were prepared from silicon[16,17] or metal.[18,19] Silicon is mostly used as a common microelectronics industry substrate, but it is costly, fragile, and is an untested biocompatible material. There are many metals that are cost effective, possess good strength, and known to be biocompatible,[20] which are the preferred choice for hollow MNs, as it needs good mechanical strength. Research on polymer MNs is recently being explored extensively, as they provide inexpensive and biocompatible materials that offer good strength due to polymer viscoelasticity.[21-23] In addition, earlier MN fabrication methods were expensive and time consuming because of the clean room-intensive process.[24]

As an innovative approach of MN fabrication, this study aimed to formulate MNs patch using biodegradable polymers and recyclable mould-based fabrication methods. The polymeric MNs are cost effective, biologically safe, have novel features (such as biocompatibility, dissolvable, swellable, and biodegradable), without cross contamination, and precise in large scale production; and have therefore gained importance in recent times. Dissolvable MNs were also reported and they were well-known to be prepared from many biocompatible materials, such as the biopolymer hyaluronic acid. Generally, the popular polymers used in fabrication of MNs are carboxymethyl cellulose, hydroxy propyl cellulose, polylactic acid (PLA), polyglycolic acid, poly lactic-co-glycolic acid, and poly (vinyl) alcohol, poly- vinylpyrrolidone.[25-29] In this study, PLA-coated chitosan MN array patches were prepared using novel wax-based mould fabrication. The MNs were tested for their mechanical, physicochemical, release of medicament, and swelling properties. The wax-based mould provides a unique advantage of melting and refabricating a mould multiple times to precisely develop reproducible MN.[30,31]

MATERIALS AND METHODS

Materials

Chitosan (MW: 190-310 kDa, degree of deacetylation: 85%), bovine serum albumin (BSA), dichloromethane, acetic acid, and bees wax (MP 63˚C) were purchased from High Media, Dorset, UK. Phosphate buffer saline (PBS) solution was obtained from Fischer scientific. PLA (MW: 60 kDA) was procured from Sigma-Aldrich, Darmstadt, Germany. All chemicals used in this study were of analytical grade. MN roller was purchased from ZGTS Derma Roller® (1 mm), Medsorimpex, sewak park, New Delhi.

Preparation of mould

Wax-based mould is a simple, economic, less time consuming, and innovative technique to prepare MNs. In this technique, the MN moulds were developed using a bee's wax. The bee's wax (MP: 63˚C) was melted and mixed. The liquid wax preparation was poured into a Petri plate and allowed to cool and solidify at room temperature.[32] The array was prepared by impressing the Derma Roller® (1 mm needle length) on the surface of the solidified wax. The prepared wax mould was placed in a vacuum oven at (-500 mmHg) for 20 min at (37˚C) for removal of dust particles and wax debris.

Fabrication of MN patch

The 0.5%-3% w/v chitosan dissolved in 1% v/v acetic acid solution was used for fabrication of MNs. The prepared gel was rested overnight and spread over the prepared MN wax mould uniformly. Before the spread, care was taken to avoid formation of air bubbles. After few minutes, the MN array was placed in a vacuum oven for 1 day at 37˚C and -550 mmHg. After completion of the 1-day period, the MN array mould was removed from the oven and the air bubbles generated were scrapped using a glass rod and dried for another 1 day at room temperature. After drying, the MNs patches were pulled out of the wax mould and dried in a hot air oven at 70˚C for 5 hrs. After complete drying of the MNs, the coating was done with PLA solution prepared by dissolving 500 mg of PLA in 5 mL of dichloromethane. The MN patch was dipped 20 times in the PLA solution until complete coating.[33] After PLA coating, whole MN patch was dried at 60˚C to completely remove the traces of dichloromethane. This PLA-coated chitosan MN patch was again coated with BSA by dipping 20 times in 10 mg/mL BSA solution (BSA was used as a representative protein for macromolecules). The MN patches were prepared in several batches (A to G batches), as shown in (Table 1).

Characterization of optimized MN array patch

Physical examination of MN array by microscope and scanning electron microscopy (SEM)

MN arrays were observed under compound microscope for preliminary morphological examination. MN arrays were initially

mounted on circular disc and morphologically characterized by SEM (Hitachi S-2460N, Germany) in a high vacuum using Everhart Thornley detector at 10^{-5} Torr and 15kV. Each sample was coated with gold using K550 Emitech Sputter coater (Gatan Inc., Pleasanton, CA). A computer software (XT Microscope control, Quanta Oregon, USA) was used to analyze the SEM images.[34]

Mechanical testing of MNs arrays

Mechanical strength of MNs

Mechanical strength of MN was studied with a displacement force test station (Model 921A, Tricor Systems Inc., Elgin, IL, USA). MN array (1x3 cm with 108 MN) was attached to the mount and an axial force was applied at a rate of 1 mm/s. The MN array was mounted against a flat, rigid surface perpendicular to the axis of the mount movement. The test station measured the compression force needed to move the mount as a function of distance. The study was done in triplicate (n=3).

Tensile strength of MN arrays

Tensile strength of MN arrays was determined using a texture analyzer (TA.XT plus, Stable Micro System, United Kingdom). The tensile strength was determined based on maximum load at the time of film rupture. Three strips of MN array were cut (1x5 cm) and used for this test. The thickness and breadth of strips were checked and noted at three different sites and the average value was taken for calculation. The study was done in triplicate (n=3).

$$\text{Tensile strength (MPa)} = \frac{(\text{Load at break in gm}) \times 100}{(\text{Original width in mm})\,(\text{Original thickness in mm})} \quad (1)$$

Physicochemical characterization of MNs array

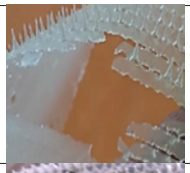
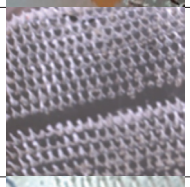
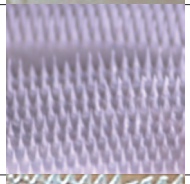
Determination of standard and released BSA from MN array by SDS-PAGE

SDS-PAGE was used for determination of standard BSA (66 kD) and BSA released from MN array from Franz diffusion cell, according to the method described by Badhe et al.[35] The resolving gel used was 9% and staining was done with Coomassie brilliant blue stain.

Determination of chemical integrity of MNs

Fourier-transform infrared spectroscopy (FTIR) absorption spectrum of Chitosan, PLA, BSA, and BSA coated on PLA-coated

Table 1. MN batches prepared on the wax mould

Batch	Image	Composition	Observation
(A)		0.5% w/v chitosan 1% v/v acetic acid solution	Arrays were brittle (*TS - 4.18 Mpa) and needles formed were thread-like with very less mechanical strength -0.08 N/needle)
(B)		1% w/v chitosan in 1% in acetic acid solution	Microneedles were formed but short length (0.7 mm) and less mechanical strength (0.15 N/needle)
(C)		2% w/v chitosan in 1% v/v acetic acid solution	Microneedles formed with optimum length but with less mechanical strength (0.28 N/needle)
(D)		3% w/v chitosan in 1% v/v acetic acid solution	Microneedles are formed with proper length and shape but with poor mechanical strength (0.53 N/needle)
(E)		3% w/v chitosan in 1% v/v acetic acid MN coated with PLA	MNs arrays were formed with good length and strength (0.70 N/needle) of needles
(F)		3% w/v chitosan in 1% acetic acid MN coated with PLA and BSA	Needles were obtained with good length and mechanical strength (0.72 N/needle)

*TS: Tensile strength, MN: Microneedle, PLA: Polylactic acid, BSA: Bovine serum albumin

chitosan MN was analyzed using a FTIR spectrophotometer (8400S Shimadzu, Japan) over the range 4000-600 cm^{-1}. The baseline correction was performed using dried potassium bromide. Subsequently, the spectrum of mixture of analyte and potassium bromide was recorded and the peaks belonging to major functional groups were recorded.

Thermodynamic evaluation of MNs arrays

Differential scanning calorimetry (DSC) analysis was performed for Chitosan, PLA, PLA-coated chitosan MN, and BSA coated on PLA-coated chitosan MN array using DSC7 (PerkinElmer, Germany). Sample weights were taken in the range of 5-10 mg. All samples were analyzed in scanning mode from 25°C to 350°C at a heating rate of 10°C/min. Dry nitrogen gas was purged in during the DSC analysis.

Evaluation of the degree of crystallinity

X-ray diffraction (XRD) spectra for chitosan, PLA, and BSA coated on PLA-coated chitosan MN arrays were recorded using Brucker D8 Advanced X-ray diffractometer (PDXL2 software, Tokyo, Japan) using Cu K 2α rays at a voltage of 40 kV and current of 25 mA. Samples were scanned at the rate of 2°ɵ from 10 to 60°ɵ.

Determination of viscosity of wax mixture

A Rheometer (RVDV-II, Brookfield, USA) was used to study the viscosity of wax and chitosan gel. The temperature control was achieved using heated plate, allowing the gradual increase of temperature from 20°C to 80°C.

Determination of swelling index of MNs array

A MN patch of 1 cm^2 (36 MN) size from the optimized batch was weighed and placed on a pre-weighed cover slip. It was placed in a petridish and 10 mL of distilled water was added. After 10 min, the cover slip was removed and excess water was wiped off carefully using a tissue paper and weighed. Weight increase due to absorption of water and swelling of patch was determined by calculating the difference between initial and final weight.

The percentage swelling index was calculated using the following equation (2):

$$\% \text{ Swelling index} = (Wt - Wo / Wo) \times 100 \quad (2)$$

Where Wt is the final weight of the swollen film after time t, Wo is the initial weight of the film at zero time. Due to scarcity of the optimized sample, statistical analysis was not performed.

BSA release study from microneedle array

Standard calibration curve of BSA was prepared by dissolving 10 mg of BSA in 100 mL PBS buffer (pH 7.4) to yield 100 µg/mL stock solution. From the stock solution, serial dilutions were made: 2, 4, 6, 8, 10, 20, 30, 40, and 50 µg/mL with PBS (pH 7.4). From each dilution, 1 mL of the solution was pipetted out and 2 drops of biuret reagent was added, followed by dilution of the solution up to 3 mL with PBS. The reaction of BSA with biuret reagent generates a pink or purple coloration, which is observed and analyzed in a ultraviolet-visible spectrophotometer (Shimadzu, Japan 1700) against PBS as blank at 540 nm λ_{max}. The absorbance values obtained were used to prepare the standard calibration curve of BSA.

The drug release studies of BSA-coated MN arrays were performed with Franz diffusion cell apparatus using 45 mL of PBS (pH 7.4) as a dissolution medium at 37°C±0.5°C. The speed of the magnetic stirrer was adjusted to 100 rpm. The MN arrays were inserted into the shaved rat skin fixed to the receiver compartment. From this compartment, 1 mL of the medium was collected at a specific time interval and analyzed for BSA content, following the same protocol (biuret test) used for preparing the BSA standard calibration curve. An equivalent volume (1 mL) of the fresh PBS was added to Franz diffusion cell apparatus each time to make up the loss due to sampling. Due to scarcity of the optimized sample, statistical analysis was not performed.

Skin irritation study

Skin irritation study of MNs arrays was performed to determine whether the prepared MN arrays can cause any irritation to the rat's skin (Animal Ethical Committee approval no DYPIPSR/IAEC/Nov./18-19/P-09). MN arrays (1 cm x 3 cm) were applied using gentle pressure to shaved back skin of Wistar Albino rats (180-220 gm) and secured for 24 hrs with a medical adhesive tape. After 24 hrs, MN arrays were removed and the rats were monitored for any sign of irritation on the rat's skin or any other adverse effect. The test site was analyzed for 7 days after removal of the MN. As the study involves only visual inspection of any reaction, no statistical evaluation was performed.

RESULTS AND DISCUSSION

Fabrication of wax mould

The bees wax was melted at 63°C and allowed to settle in petridish for 30 min. The impression of Derma Roller® (1 mm needle length) was easily done to obtain the MN mould. For fabrication of MNs, four different concentrations of chitosan were used. The various batches that were tried are given in Table 1, Figure 1.

Optimization batches of MN patch

The optimized batch (batch D) was selected as 3% w/v chitosan prepared in 1% v/v acetic acid solution based on the mechanical strength and morphology of MNs (Table 1). This optimized batch was further coated with PLA (batch E), followed by BSA (batch F).[36-39]

Physical characterization of MN

Physical examination of MN array using SEM

SEM

The normal and SEM images of plain chitosan MN, PLA-coated chitosan MN, and BSA coated on PLA-coated MN arrays are shown in (Figure 2). It can be clearly observed that 0.5% chitosan MN appear thread-like and has very less mechanical strength (Figure 2a). However, 3% chitosan MN are formed nicely, but with a porous structure inside (Figure 2b). When these 3% chitosan MNs were coated with PLA, the MN became

stronger with improved mechanical strength (Figure 2c) and Figure 2d suggests the uniform coating of BSA over PLA coat.[40] Moreover, the SEM images suggest that MNs are 0.9 mm in height, 600 µm in width at base, and 30-60 µm at the tip diameter and that the distance between 2 MN was 1.5 mm.[41]

Mechanical strength of MNs arrays

Mechanical strength of MN needs must be sufficient to sustain the force applied during pressing of MN array patch into the skin.[42] This insertion compression force might lead to bending or breaking of MNs. The reported mechanical strength for

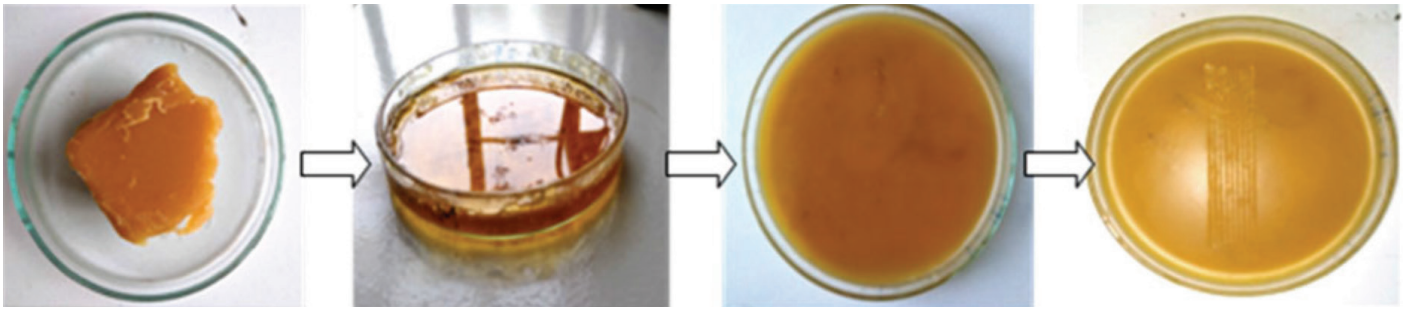


Figure 1. Fabrication of wax-based MN array mould
MN: Microneedle

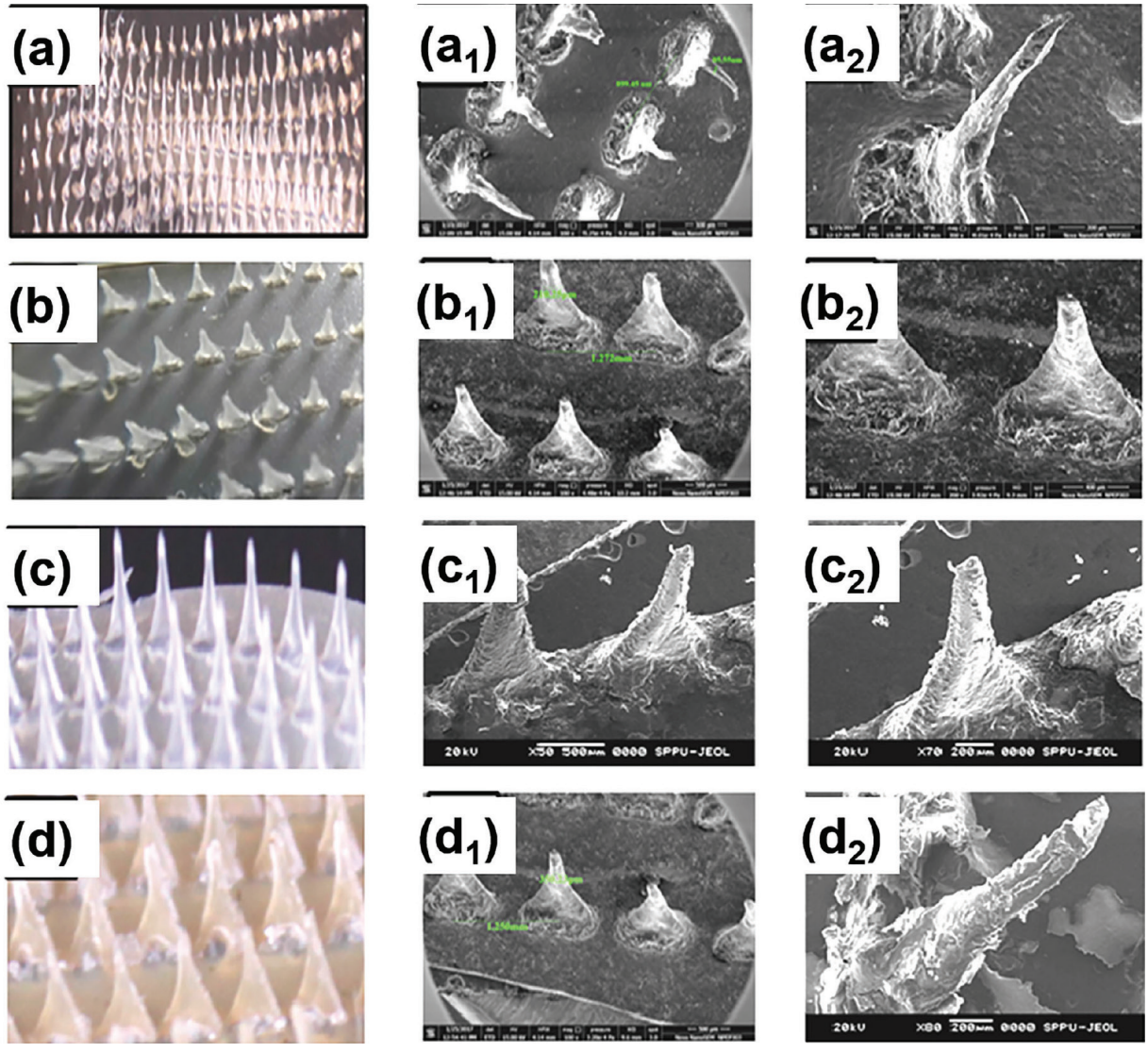


Figure 2. SEM images of (a, a_1, and a_2) 0.5_ chitosan MN (b, b_1, and b_2) 3_ chitosan MN (c, c_1, and c_2) PLA-coated chitosan MN (d, d_1, and d_2), and BSA coated on PLA-coated chitosan MN array
SEM: Scanning electron microscopy, MN: Microneedle, PLA: Polylactic acid, BSA: Bovine serum albumin

efficient chitosan MNs was 0.50 N/needle.[43] and the mechanical strength of BSA and PLA-coated chitosan MN was 0.72 N/needle.

Tensile strength of MNs arrays

Tensile strength of MN arrays is an important property, since it defines the integrity of the patch and capacity of the patch to survive the physical stress. The previously reported tensile strength for chitosan film is 11.23 Mpa[44] and that of BSA and PLA-coated MN array patch shows 15.23 Mpa.

Spectral and thermal analysis of MNs arrays

FTIR spectroscopy

The FTIR spectrum of chitosan (Figure 3a) showed important bands of the characteristics functional groups, which were recorded in the infrared range (4000-6000 cm^{-1}). The infrared spectra for chitosan showed a stretching vibration band at 3419.42 cm^{-1} for OH group and 1064.79 cm^{-1} for -C-O of CH_2OH group. The spectra also showed NH bend at 1643.41 cm^{-1} and NH stretch at 3354.15 cm^{-1} for amine group. FTIR spectra of PLA (Figure 3b) showed 2839.73 cm^{-1} and 2910.68 cm^{-1} for C-H stretch and 1491.02 cm^{-1} C-H bending vibrations in CH_3. Also, OH stretch at 3464.23 cm^{-1}, C=O stretch at 1757.84 cm^{-1}, and C-O-C stretching vibration were observed at 1350.32 cm^{-1}. FTIR spectra of plain BSA (Figure 3c) showed C=O stretch vibrations of the peptide linkages at 1667.21 cm^{-1}, N-H bending vibration for amide II at 1537.91 cm^{-1}, and N-H bending vibration at 3292.60 cm^{-1}. FTIR spectra of BSA and PLA-coated chitosan MN (Figure 3d) showed all the characteristic peak of chitosan, such as NH bend (amine) at 1635.41 cm^{-1} and NH stretch at 3249.20 cm^{-1}, 2930.93 cm^{-1} for C-H stretch and 1498.74 cm^{-1} C-H bending vibrations in CH_3 for PLA and BSA, N-H bending vibration for amide II at 1525.74 cm^{-1}, and C-N stretching/bending vibration at 1166.97 cm^{-1}/2330.34 cm^{-1} for BSA. Thus, all the molecules retained their functional group and no interaction was observed between them.

Thermal analysis of MN array

DSC thermogram of PLA (Figure 4a) shows endothermic peaks at 56˚C and 105˚C, which are related to glass transition (GT) and moisture loss, as well as broad endothermic peak at 170˚C, followed by broad exothermic peak at 220˚C (melting), followed by degradation of PLA. These values match closely with previously reported value.[45] DSC thermogram of Chitosan (Figure 4b) shows endothermic peak at 90˚C, which is related to moisture loss, endothermic peak at 240˚C, followed by exothermic peak at 280˚C, which corresponds to the degradation of chitosan. These values match closely with previously reported values.[46] The DSC thermogram of PLA-coated chitosan MN (Figure 4c) shows small endothermic peak at 60˚C for GT of PLA, endothermic peak of water loss at 100˚C, and endothermic peak at 230˚C, followed by exothermic peak at 270˚C, which represents the degradation of PLA and chitosan, respectively. The DSC thermogram of BSA and PLA-coated MN (Figure 4d) shows a short endothermic peak for GT of PLA at 63˚C and a short endothermic peak at 100˚C, which is related to water loss and degradation of BSA. It also shows a small exothermic peak at 215˚C corresponding to the degradation of PLA and a broad endothermic peak at 215˚C, followed by 260˚C broad exothermic peak corresponding to chitosan and PLA degradation.

XRD spectral analysis

The diffractogram of chitosan shows sharp peak at 22˚ɵ, 26.5˚ɵ, and 33˚ɵ and the broad peak shows chitosan slightly

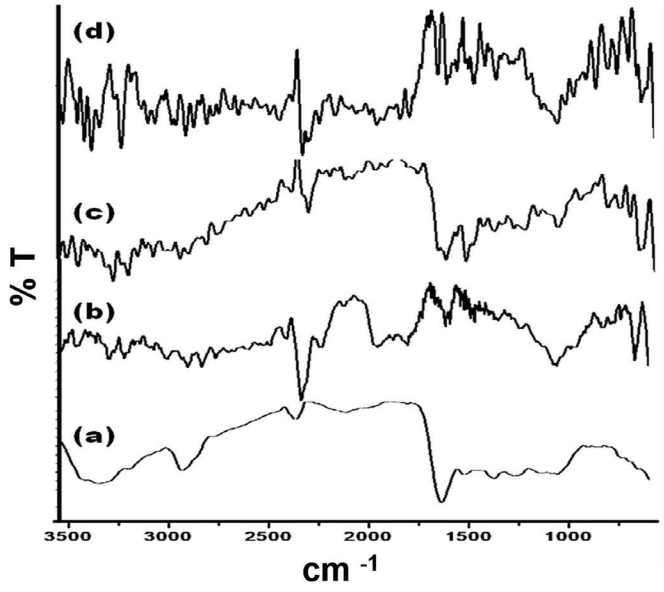


Figure 3. FTIR spectra of a) chitosan b) PLA c) BSA, and d) BSA coated on PLA-coated chitosan MN array

FTIR: Fourier-transform infrared spectroscopy, PLA: Polylactic acid, MN: Microneedle, BSA: Bovine serum albumin

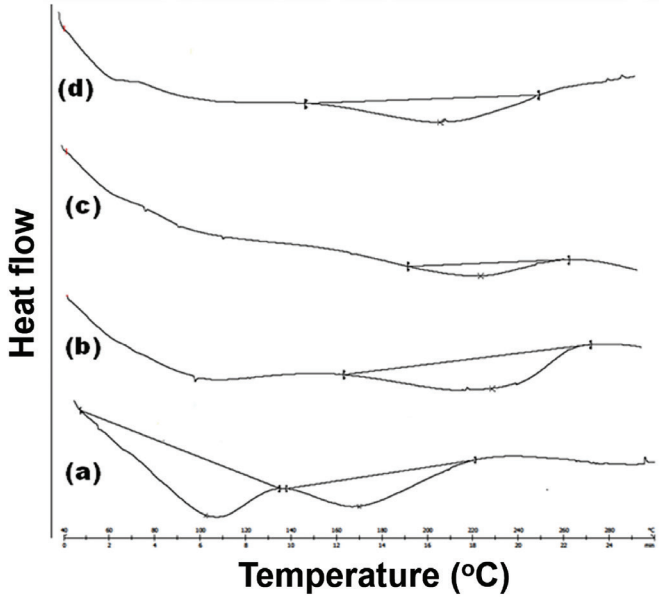


Figure 4. DSC thermogram of a) PLA b) chitosan c) PLA-coated chitosan MN d) BSA coated on PLA-coated chitosan MN array

DSC: Differential scanning calorimetry, PLA: Polylactic acid, MN: Microneedle, BSA: Bovine serum albumin

crystalline in nature (Figure 5a). These observations match with previous reports.[21,46] The diffractogram of PLA shows a broad peak at 16.55°ɵ and 30°ɵ. It suggests that PLA used in this study is amorphous in nature (Figure 5b) this observation match with reported value[47] and supports the DSC data, which lacks sharp endothermic peak of GT temperature at 60°C. The diffractogram of BSA coated on PLA-coated chitosan MN array batch (Figure 5c) showed sharp peak at 22°ɵ, 24°ɵ, and 32°ɵ, emerging from a broad peak corresponding to chitosan and broadness of the overall diffractogram corresponding to PLA, which suggests that coating of chitosan MN does not hamper crystallinity to the MN array. This is an important observation, as it explains the improved mechanical properties of the MN array.

Drug release profile of optimized batch

The *in vitro* drug release study was performed in order to ensure a release of drug in selected dissolution medium. The drug release profile was determined in PBS (pH 7.4) and 98.5% BSA was released within 50 hours from the rat skin (Figure 6).[48]

Determination of viscosity of wax and gel

The change of viscosity with increasing temperature gradient was performed for bee's wax and chitosan gel. The viscosity decreased with increase in temperature. Thus, both gel and wax come under the Newtonian flow behavior.

Swelling index of MNs arrays

Figure 7 shows the swelling behavior of the BSA and PLA-coated MN array. It was observed that the maximum swelling of 6.79% was observed at 30 minutes. The weight decreased after 30 min might be due to the dissolution of BSA.

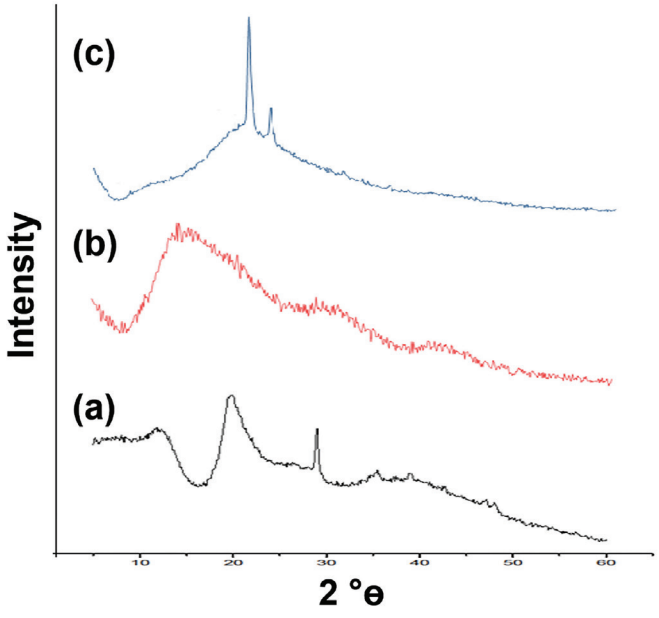


Figure 5. XRD spectral analysis of a) chitosan b) PLA c) BSA coated on PLA-coated chitosan MN array

XRD: X-ray diffraction, PLA: Polylactic acid, MN: Microneedle, BSA: Bovine serum albumin

In vivo tolerance study

After removal of MN and careful observation for the next 7 days, it was noted that there was no sign of irritation and any adverse effect due to MN (Figure 8).

CONCLUSION

The successful fabrication of MN was performed using chitosan polymer and bees wax mould. Selected MN batch was subjected to coating with PLA and BSA, followed by morphological, mechanical, and drug release studies. Based on SEM characterization of MN formulation; the coated MNs had 0.9 mm length, 600 µm width at the base, 1.5 mm distance between 2 needles, and 30-60 µm tip diameter. The optimized MN batch showed the percentage BSA release of 98.5% in 50 hours. It also showed good mechanical strength (0.72 N/needle), tensile strength (15.23 Mpa) and maximum swelling of 6.79%.

The results obtained from various studies performed for PLA- and BSA-coated-layered chitosan MNs possessed desired mechanical strength, tensile strength, swelling index, and drug release. SEM, XRD, and DSC studies established the physicochemical properties of MN.

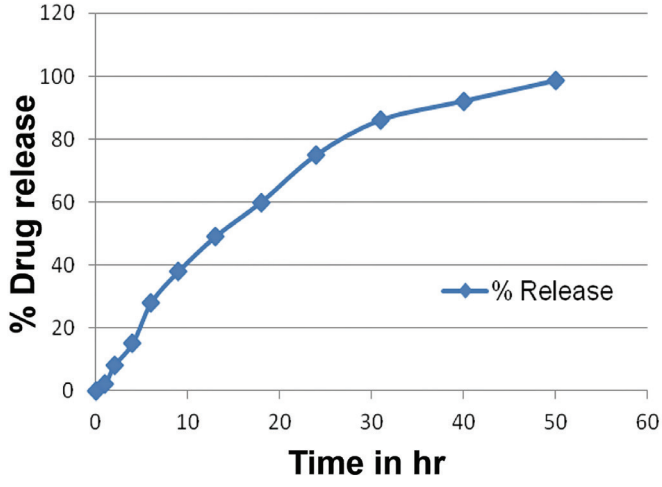


Figure 6. Drug release of optimized batch

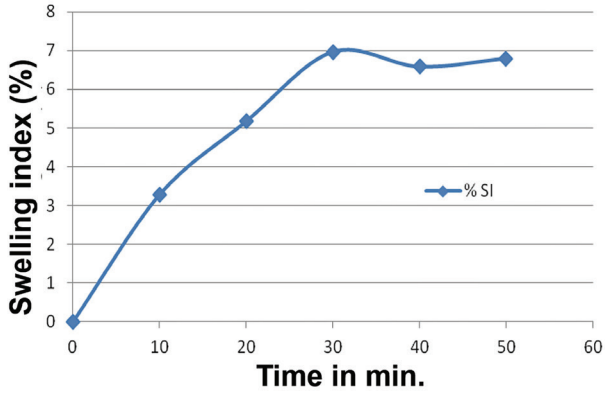


Figure 7. Swelling index of BSA and PLA-coated MN array

PLA: Polylactic acid, MN: Microneedle, BSA: Bovine serum albumin, SI: Swelling index

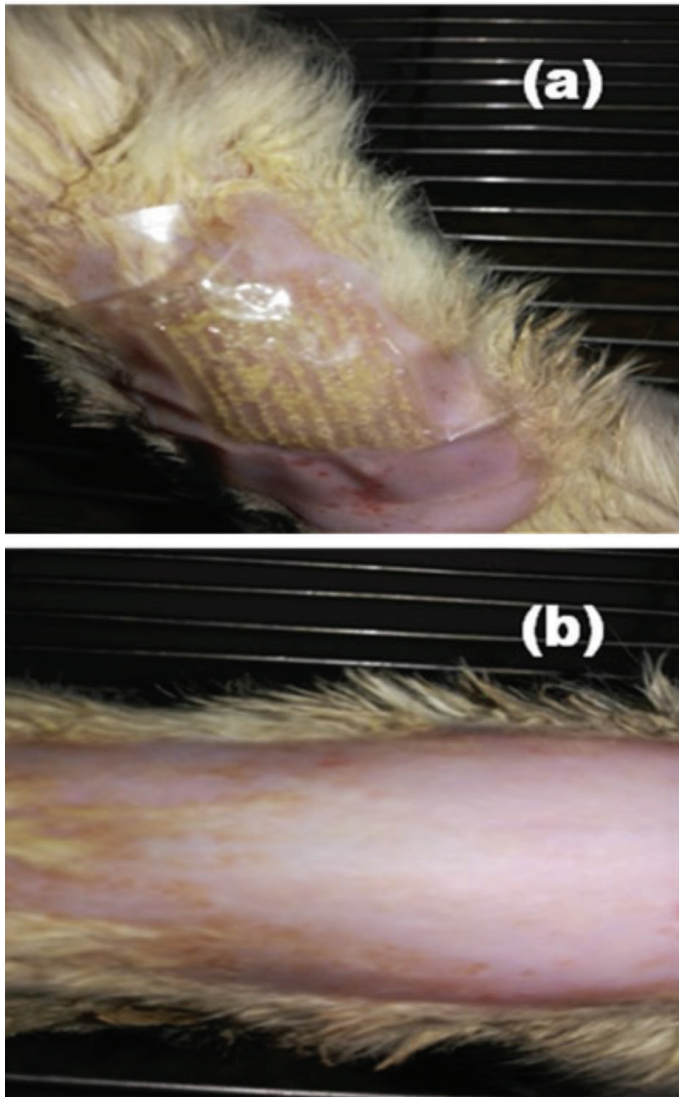


Figure 8. Skin irritation study a) microneedle array patch inserted in the dorsal skin of rat, b) dorsal skin of rat after seven days of MN array patch removal

MN: Microneedle

Thus, it is concluded that the study of fabrication of novel wax-based mould (which can be melted and re-casted multiple times) and development of BSA and PLA-coated chitosan MN was successfully attempted. The MN patches easily pierced the skin with gentle application of force. It showed significant amount of drug release into the dermis. Furthermore, it is proposed that the wax-based mould technique for the development of MN patch and the developed coated polymeric MNs can be tested for its drugs, macromolecules, and vaccines delivery potential, as a pain less and effective drug delivery system.

The same MN arrays can act as a time-controlled delivery system, since porous chitosan will be exposed after the dissolution of two layers (BSA and PLA). Thus, chitosan MN can be loaded with the drugs to be released after a particular time. Even PLA can be doped with certain medicament to obtain sustained drug delivery after dissolution of the BSA layer. BSA can be substituted with vaccines or DNA/RNA to achieve the immediate release. Thus, each layer of MN will provide the platform for a time-bound drug delivery system.

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REVIEW

Mechanistic Biomarkers in Toxicology

Toksikolojide Mekanistik Biyobelirteçler

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ABSTRACT

Biomarkers are important parameters that are reliable, applicable, reproducible, and generally inexpensive. All biomarkers have a significant role in human health, especially mechanistic biomarkers, which are the most important for the prevention of toxic effects and diseases. They demonstrate the possibility of diagnosis, prognosis, recurrence, and spread of disease. Furthermore, they show the exposure levels to numerous chemical, biological, and physical agents. To date, the development and application of biomarkers require the knowledge of mechanisms underlying their production. Therefore, the present study focused on the possible mechanistic biomarkers.

Key words: Mechanistic, biomarker, toxicology

ÖZ

Biyobelirteçler önemli, güvenilir, uygulanabilir, tekrarlanabilir ve genelikle maliyeti uygun parametrelerdir. Biyobelirteçlerin insan sağlığı açısından önemli rolü olmakla birlikte özellikle mekanistik biyobelirteçlerin toksik etki ve hastalıklardan korunmada önemi büyüktür. Biyobelirteçler hastalıkların tanısını, gidişatını, tekrarlama ve yayılma olasılığını, tedavinin etkinliğini gösterebilir. İlave olarak birçok kimyasal, biyolojik ve fiziksel ajanlara maruz kalma düzeyini gösterir. Bugün biyobelirteç geliştirilmesi ve uygulanması, bunların oluşumunun altında yatan mekanizma bilgilerini gerektirir. Bu nedenle bu yazıda olası mekanistik biyobelirteçler üzerinde yoğunlaşılmıştır.

Anahtar kelimeler: Mekanistik, biyobelirteç, toksikoloji

INTRODUCTION

The National Institutes of Health defines a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes or pharmacological responses to a therapeutic agent".[1,2] Parameters, such as glomerular filtration rate or recorded blood pressure at different time intervals, are examples of biomarkers. Generations of epidemiologists, physicians, and scientists have used various biomarkers to study human diseases. Biomarkers have been used in the management and diagnosis of cardiovascular diseases, infections, genetic disorders, cancer, and others.[3] The time course of injury and underlying molecular mechanisms are reflected by the measuremment of biomarkers. Accurate diagnosis, prognosis, and treatment regime can be applied to a patient by analyzing different biomarkers.[2] Periodic surveillance of biomarkers also serves as a tool to determine whether a treatment protocol or daily dietary habits are improving the patient's condition.[2] The periodic follow-up of biomarkers will provide the health care personnel with important information about the efficacy or toxicity of the treatment regime and act as a border for clinical trials, with the final goal of treating the patients with safe and effective medical therapies.[2]

Biomarkers are generally classified as biomarkers of (i) exposure, (ii) effect, and (iii) susceptibility.[2,4] Exposure biomarkers are considered early markers, which result from the interaction between a chemical agent and a target molecule.[4] Therefore, these biomarkers are essential and valuable in collaboration with biomarkers of early disease detection to develop personalized medical treatment strategies.[2] The biomarkers of effect are considered late markers. They are used to measure the burden of injury or damage caused by different agents on the target organ. These biomarkers are also employed to objectively and accurately measure the overall health status of patients, usually after being exposed to an agent or disease.[4] Susceptibility biomarkers are used as a guide to inherent or gained ability of the body to respond

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to difficulties. These markers are the result of exposure to diseases or chemicals.[4,5]

Biomarker development requires detailed and considerable knowledge. Studies about the pathogenesis of diseases, molecular changes, and alterations in biochemical pathways underlying toxic effects should be conducted. Such mechanistic information causes the formation of mechanistic biomarkers. Mechanistic biomarkers cover exposure, effect, and susceptibility biomarkers and lead to the generation of new ones.[6,7] Therefore, they have the highest potential for assisting with clinical decision making. The best example of a mechanistic marker is a genetic trait index, which is commonly used for the diagnosis of certain diseases. Mechanistic biomarkers provide information regarding patient prognosis and the probability of response to various treatment options; however, it is not used for follow-up of progression nor response to an applied medical therapy.[6,8] Numerous biochemical processes, such as oxidative stress, alterations in biotransformation, alteration in protective and repair systems, and organelle damage, are the mechanistic information that lead to detectable biomarkers.

Oxidative stress biomarkers

In 1985, Helmust Sies defined "oxidative stress as a disturbance in the prooxidant-antioxidant balance in favor of the former".[9] Oxidants are mostly produced by cellular metabolism. The antioxidant system of the body quickly eliminates small amounts of oxidants, but in certain cases, they cause remarkably profound damage to macromolecules (proteins, lipids, DNA, and carbohydrates) (Figure 1). Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide radicals, and hydroxyl radicals, are common by-products of metabolic activities.[2,10] ROS are synthesized during mitochondrial respiration, inflammation, immune system activity, and other processes.[2,11-13] The increase in oxidative stress level increases the production of ROS through Fenton reaction (reduction of iron by superoxide).[2,11,14] Excess amounts of ROS interfere with the physiological activity of mitochondria and result in adenosine triphosphate (ATP) depletion. An increase in oxidative stress levels has also been associated with numerous diseases or toxicities. A substantial amount of evidence reveals the association between oxidative stress and different diseases, such as cancer, diabetes, infections, cardiovascular and neurodegenerative diseases, and the aging process (Figure 2).[2,12,15]

Markers of oxidative stress are used to evaluate the nature and effect of ROS. The measurement of ROS may be a useful marker, but such method is unstable; their detection requires invasive methods, and the results may lack specificity. Thus, scientists measure the by-products of the reaction of ROS with other biomolecules that are more stable. Surrogate markers include nitrite and nitrate levels, products of lipid peroxidation, and levels of oxidized proteins.[11] Figure 1 shows the effect of ROS on macromolecules and several end products.

Lipid peroxidation is a cascade of reactions due to ROS attack on lipids in the cell membrane, and it has been implicated in various diseases, such as hypertension, Alzheimer's disease, cancers, and other disorders.[16-19] The burden of lipid peroxidation can be measured by analyzing thiobarbituric acid, N-epsilon-hexanoyl-lysine, malondialdehyde, 4-hydroxy-nominal, and F2-isoprostane 15(S)-8-iso-prostaglandin F2α, which are by-products of lipid peroxidation.[17-20]

Antioxidants

The human body is equipped with different antioxidant systems that serve as a counterbalance to the effect of oxidants. The antioxidant defense involves several strategies, namely, enzymatic and non-enzymatic mechanisms. Enzymatic mechanisms, such as superoxide dismutase (SOD), and non-enzymatic defense systems, protect cells against free radicals and ROS. Antioxidants, including alpha-tocopherol, scavenge oxidants (which damage cell membranes and cause lipid peroxidation) or ascorbate-trap ROS.[15]

Glutathione (GSH) is a three-peptide molecule that contains cysteine, glycine, and glutamate, and it is the most critical molecule of the antioxidant system. GSH plays a significant role in the detoxification of aggressive electrophilic molecules, such as radicals, epoxides, and halides, by conjugation reactions. GSH is the major thiol in the body and a perfect reductant molecule that prevents oxidative damage.[21] The ratio of reduced-to-oxidized GSH indicates the redox balance of the cell. This redox balance is an indicator of the overall health of cells.[22] Dysregulations in GSH synthesis and its concentration are considered important biomarkers in the diagnosis of diseases, such as human immunodeficiency virus, cancer, inflammation, tuberculosis, Alzheimer's disease, and numerous others.[23-25] The evaluation of the GSH pathway will reflect the status of the antioxidant system, which may elucidate various underlying pathology etiologies. Among the enzymes that participate in the antioxidant system, GSH peroxidases (GSH-Pxs) consist of four enzymes (Table 1),[26,27] all of which contain selenium. These GSH-Pxs are hydrogen and lipid peroxide scavengers. Hydrogen peroxide is produced during cell metabolic processes, and its amount increases under oxidative stress.[15]

Table 1. Different GPX enzymes

The enzyme	Location
Glutathione peroxidases I, neutralizes hydrogen peroxide and protects hemoglobin from oxidative damage[28,29]	Cell cytosol
Glutathione peroxidases II; this isoenzyme level increases in different cancers such as prostate, hepatocellular, and breast cancers[29,30]	Cell cytosol, especially in the gastrointestinal tract
Glutathione peroxidases III; It is a glycoprotein[29,30]	Plasma
Glutathione peroxidases IV; It is activated in case of free radical damage, serum cholesterol, and lipoproteins[29]	Mitochondria

GPX: Glutathione peroxidase

Another essential enzyme in the antioxidant system is SOD, which eliminates superoxide radicals.[31] SOD has three

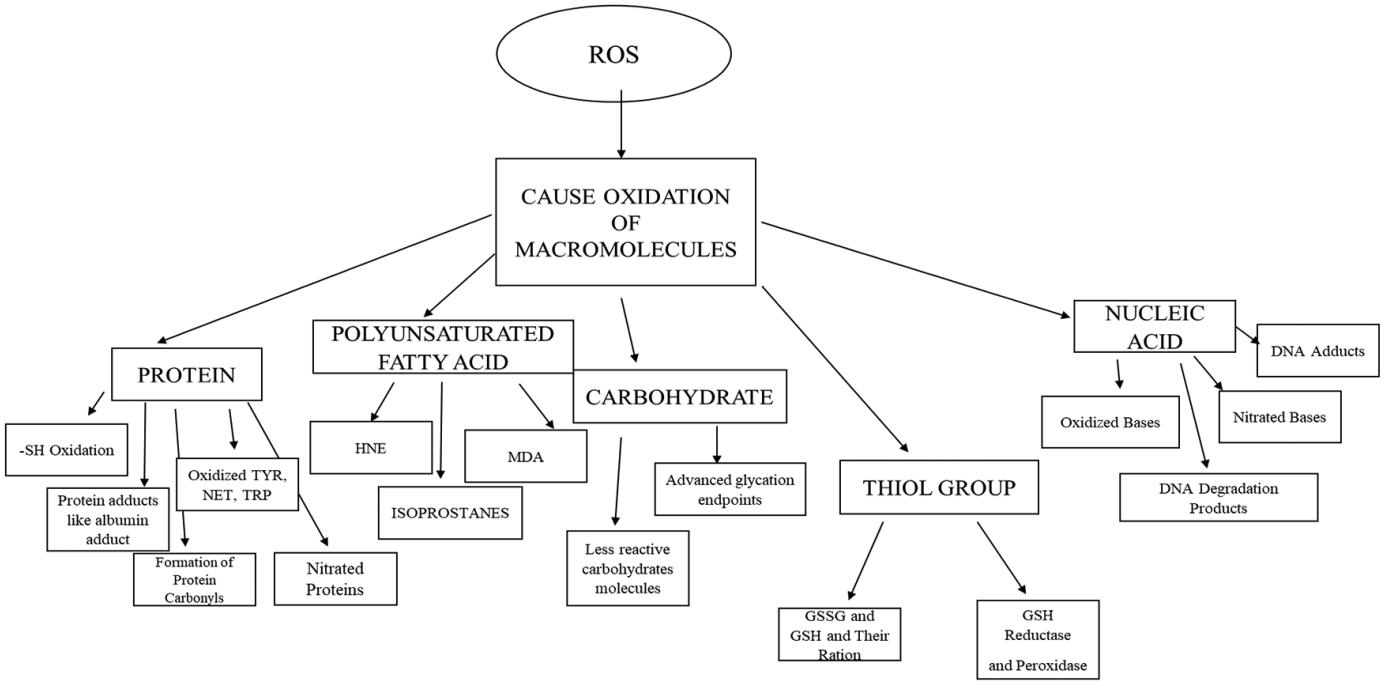

Figure 1. ROS cause the oxidation of macromolecules. As a result of this oxidation, the end products of the oxidation process have been mentioned. These end products are used as biomarkers to detect the presence of oxidative damages (drawn by authors)

ROS: Reactive oxygen species

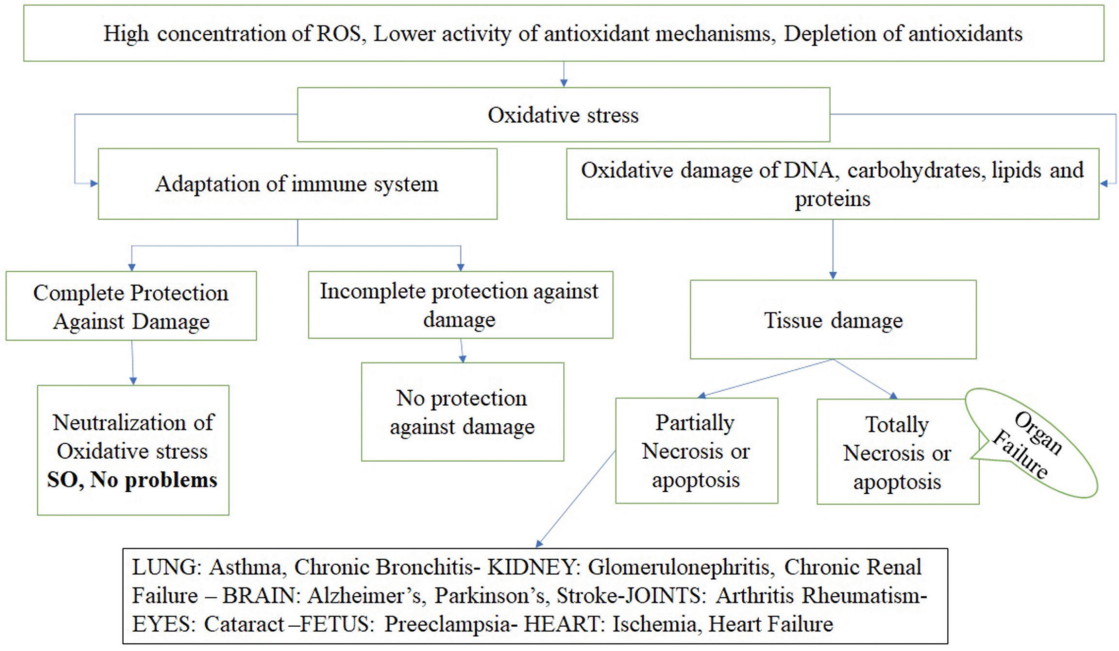

Figure 2. Oxidative stress in the general pathogenesis of diseases (drawn by authors)

metalloenzyme forms, namely, cytoplasmic Cu/Zn-SOD, mitochondrial Mn-SOD, and extracellular EC-SOD, all of which require cofactors (Cu or Mn) for their activity.[32,33] Hydrogen peroxide formation results from the neutralization of superoxide. Catalase and GSH-Px enzymes then catalyze this H_2O_2. Given that all these enzymes depend on each other, fluctuations in their levels will affect the overall antioxidant system.[15,34]

Biomarkers related to biotransformation

Biotransformation is the process of enzymatic transformation of xenobiotics to excretable metabolites. However, in certain cases, the metabolites may be toxic and reactive electrophiles. These toxic metabolites lead to cell damage or cell death. The measurement of active metabolites (such as morphine as the active metabolite of codeine biotransformation), determining

the effect of reactive metabolites on macromolecules and the analysis of end products (such as of mercapturic acid or hippuric acid in urine samples), and measurement of enzyme activity, which is responsible for xenobiotics metabolism, are several of the biomarkers related to biotransformation.[2]

The effect of xenobiotics or toxins is dependent on their metabolism, which is controlled by the action of enzymes. Any modification in the activity of these enzymes results in a change in the fate of xenobiotics. Metabolization can be altered by enzyme induction or inhibition. Enzyme induction or inhibition has been studied as a biomarker for the measurement of responses to environmental pollutants, exposure to various drugs, or drug interactions.[35] Chronic alcohol usage results in the induction of the 2E1 enzyme. The induction of this enzyme will alter the fate of specific drugs that are metabolized by it.[36] Organophosphate pesticides reversibly or irreversibly bind to and inhibit cholinesterase. This inhibition prevents neurotransmitter (acetylcholine) degradation.[37] Quinidine is a potent CYP2D6 inhibitor.[38] In polypharmacy, the inhibition or induction of enzymes is very important. The first or second drug interferes with the other drug's biotransformation, and the outcome is either the toxicity or absence of a therapeutic activity.[39]

Differences in genetic traits that cause differences in the expression and activity of enzymes are the primary cause of susceptibility to various diseases. The mutations and alterations in genes can be detected in 1% of populations, a phenomenon called genetic polymorphism. Polymorphisms in phase I and II biotransformation enzymes or DNA repair enzymes can be biomarkers. The polymorphisms of GSH S-transferase, N-acetyl transferase, and CYP1A2, 2A6, 2D6, and 2E1 have been studied[2] in various conditions; as an example, the polymorphism of CYP2C9 causes the patient to need less doses of warfarin, which will increase the susceptibiltiy of the patient to increased risk of bleeding and in the case of CYP2C19, the increased risk of anticonvulsant side effects.[40] People who are CYP2D6 polymorphic need high doses of fluoxetine to show the same plasma levels as those with normal CYP2D6.[41]

Furthermore, protein expression and function can be altered as a result of molecular response to signals, post-translational modifications, and other factors.[40] Additionally, the measurement of the parent-compound-to-metabolite (metabolite ratio) ratio is considered an applicable and practicable biomarker. The measurment of metabolic ratio is a valuable indicator of the metabolism rate. If the ratio is high, the patient is a poor metabolizer, and vice versa. Codeine is converted to morphine by CYP2D6 to show its analgesic effect. However, in poor metabolizers, codeine is a poor analgesic.[42]

DNA

DNA damage is a sign of several disorders, such as colon cancer, chronic renal damage, and aging-related problems.[43,44]

DNA damage can be caused by endogenous agents, such as various metabolic by-products, and environmental factors, such as ultraviolet and ionizing radiation.[45,46] ROS also cause DNA damage. The degree of DNA damage can be used as a biomarker to assess the oxidative stress in various conditions, such as pancreatic and mammary cancers and the damage from ionizing radiation that is used for radiotherapy of cancer patients.[46]

Certain compounds undergo bioactivation reactions that result in the production of potentially carcinogenic metabolites. These metabolites are carcinogenic because they react with the DNA and form DNA adducts. The metabolism of benzo (alpha) pyrene results in the formation of a cation radical, which forms DNA adducts, during exposure to tobacco smoke or coal.[47] In addition, reactive oxygen and nitrogen species can directly interact with DNA.[48] This interaction causes the oxidation of DNA and produces DNA adducts. The most important of these oxidative DNA adducts are 8-hydroxydeoxyguanosine (8-OHdG), thymine glycol, hydroxymethyl uracil, 8-hydroxydeoxyadenine, and formylamidopyrimidine. The measurement of oxidized bases from urine samples is a good indicator of the oxidative damage of nuclear DNA, which occurs during carcinogenesis, and an important prognostic factor for certain cancers.[48]

Damage to mitochondrial DNA is mainly caused by oxidative stress damage. The DNA repair systems are incomplete in the mitochondria, which increases the susceptibility and mitochondrial dysfunction. Therefore, this damage directly interferes with oxidative phosphorylation and results in the induction of apoptosis and cell death. The increased levels of 8-OHdG in biological samples can be a surrogate marker for mitochondrial DNA damage.[48]

DNA repair systems have an important role in repairing DNA damage at first sight. If the DNA repair systems are defective or overwhelmed, the risk of cancer and various diseases related to aging increases.[48]

Cofactors: Nicotinamide (NAM) adenine dinucleotide (NAD)⁺ and NAM adenine dinucleotide phosphate (NADP)⁺

Cofactors mediate a wide range of biological reactions. NAD [reduced form: NAD(H)], NADP [reduced form: NADP(H)], and ATP are important mechanistic biomarkers. NAD^+ was first discovered in 1906.[49-52] NAD^+ and NADH play an important role in various metabolic processes, such as glycolysis, mitochondrial oxidative phosphorylation, oxidation of fatty acids, citric acid cycle, and other oxidation-reduction (redox) reactions.[51-53] Its effects determine the circadian change.[50,51] Fluctuations in the NAD^+ level have a significant effect on cell function and metabolism. As shown in Figure 3, NAD^+, as a co-substrate for three important enzymatic activities [sirtuin, poly (ADP-ribose) polymerases (PARPs), and redox enzymes], has gained attention recently. CD 38/CD157 are ectoenzymes that consume mitochondrial NAD^+ and degrade it to cyclic ADP ribose and NAM. The CD38 activity increases with age, resulting in the increased NAD^+ consumption and depletion of NAD^+ reserves. CD38 is overexpressed in chronic inflammation and chronic lymphocytic leukemia, whereas mitochondrial NAD^+ is depleted in these diseases. PARPs play a role in epigenetics, DNA repair, and chronic inflammation. An increase in the expression of PARPs results in NAD^+ consumption and reduction of the NAD^+ pool. Sirtuin is an important factor that increases the life span

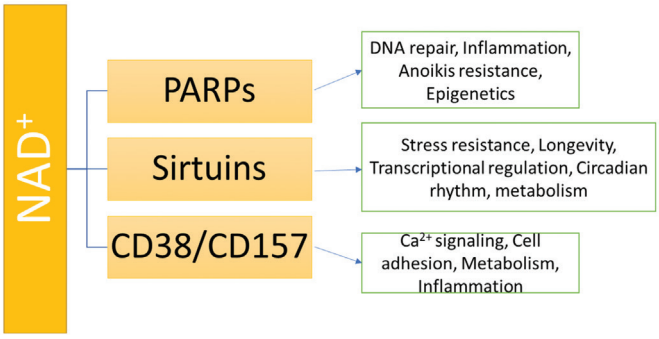

Figure 3. NAD^+ is a co-enzyme for the function of PARPs, sirtuins, and cyclic ADP-ribose synthases (CD38/CD157). Fluctuations in the NAD+ level affect the biological processes that are dependent on these enzymes[51]

NAD: Nicotinamide adenine dinucleotide, PARPs: Poly (ADP-ribose) polymerases, ADP: Adenine dinucleotide phosphate

of cells. Sirtuin pool decreases by aging along with the NAD^+ pool. Thus, increasing the NAD^+ pool enhances the life cycle of cells.[54] Sirtuin acts as a tumor suppressor by regulating transcription, programming the metabolic pathways of cells, and increasing cell resistance against oxidative stress.[51,52] Through these enzymes, NAD^+ affects the energy balance, stress response, and cellular homeostasis.[50,54,55] Fluctuations in NAD^+ levels result in fluctuations in protein levels, which are dependent on NAD^+, and thus, these proteins are significant in carcinogenesis.[51]

The increase in NAD^+ levels possibly reduces the risk of cancer, but this increase leads to the increased activity of PARP enzymes. PARPs promote the protection and repair of DNA, especially in cancer cells. PARPs cause the overexpression of inflammatory genes, which are responsible for the increased incidence of hormone-dependent tumors.[51] Sirtuin is sensitive to the fluctuation in NAD^+ levels. Different sirtuin isoforms act as tumor suppressors by altering transcription and rescheduling the cell metabolic activity.[51]

Aging is an essential factor in decreasing the NAD^+ synthesis. Aging means implies the susceptibility to chronic inflammation, circadian rhythm changes, and fluctuations in microRNA gene expression. All the factors mentioned above decrease the activity of NAM phosphoribosyltransferase (Nampt), which is an important enzyme in NAD^+ synthesis. Nampt is a rate-limiting enzyme in the NAD^+ salvage biosynthesis pathway from NAM. The decrease in Nampt activity results in the reduced synthesis of NAD^+, increased NAD^+ degradation, and increased risk of age-related diseases.[50]

$NADP^+$ is formed by the addition of phosphate to NAD^+. $NADP^+$ and NADPH are critical cofactors, fighting against oxidative stress and playing a role in the synthesis of nucleic acids, fatty acids, and cholesterol.[53-55]

Thus, these redox couples act as a substrate for the majority of enzymes. They play an active part in cellular redox homeostasis. The deficiency in any of them disrupts this homeostasis, which results in oxidative stress, disease onset, and energy impairment.

Polyamines: Ornithine decarboxylase (ODC)

Polyamines are small, cationic amines derived from amino acids. They are required for healthy cell growth; however, they are also involved in cancer cell proliferation.[1,56-59] Putrescine, spermidine, and spermine are the main polyamines in eukaryotes and prokaryotes.[56,58] Dietary or endogenous polyamines produced by the gut microbiota and those that are synthesized in the cytoplasm are the chief sources for all cells and tissues.[57,60] Given their significance in cell function, their levels are strictly regulated by maintaining a balance between synthesis, degradation, and uptake. ODC plays a critical role in the biosynthesis of polyamines. Increased levels of ODC enzyme in blood has been reported in regenerating tissues and in cancer.[56,57]

Along with chemical cancer promoters, which result in ODC increase, several environmental and genetic factors, such ultraviolet light, can result in increased *ODC* gene expression. ODC levels have been reported to increase in skin, lung, and prostate cancers.[56,57,60-62]

s-Adenosylmethionine, which forms acetylated polyamines, is another enzyme in polyamine synthesis. Both the parent polyamines and acetylated derivatives (e.g., N_1-acetyl spermidine, N_8-acetylspermidine, N_1-acetylspermine, and N_1, N_{12}-diacetylspermine) can be detected in urine and have been associated with cancer.[60]

Tumor cells with a high polyamine production show an increased synthesis of proteinases and cathepsins, which destroy the surrounding tissue. These cells also induce hypoxia, which results in the increased uptake of polyamines by cells and results in an increased proliferation rate.[56,58]

Pteridine pathway: Folate and neopterin

Pteridines are bicyclic nitrogenous ring system pyrazino-(2,3-d)-pyrimidine derivatives that bear small substituents, such as neopterin and biopterin, and are called unconjugated pteridines. The derivatives with a large residues, such as folic acid and riboflavin, are called conjugated pteridines.[63]

Several crucial cellular mechanisms depend on folate as the source of 1-carbon in DNA synthesis and methylation of protein. Thus, folate plays a significant role in DNA synthesis.[64-68] Dihydrofolate reductase and thymidylate synthase have been used as targets in chemotherapy, thus rendering conjugated pteridines as good candidate biomarkers.[69] Folate deficiency leads to different disorders and diseases.[70]

Among folate derivatives, 5-methyltetrahydrofolate (5-methyl THF) is found in circulation, and it acts as a co-substrate in the conversion of homocysteine to methionine (Figure 4)[71]. DNA mutations and strand breakage can also be the result of an increase in the replacement of uracil instead of thymidine. These events occur due to the decrease in 5,10-methyl THF.[66,72] Moreover, the decreased levels of 5-methyl THF will lead to the reduced levels of s-adenosylmethionine, which will cause the activation of oncogenesis and increase DNA damage.[73-75] For this reason, folate level can be a useful biomarker in predicting or diagnosing cancer.[64,66]

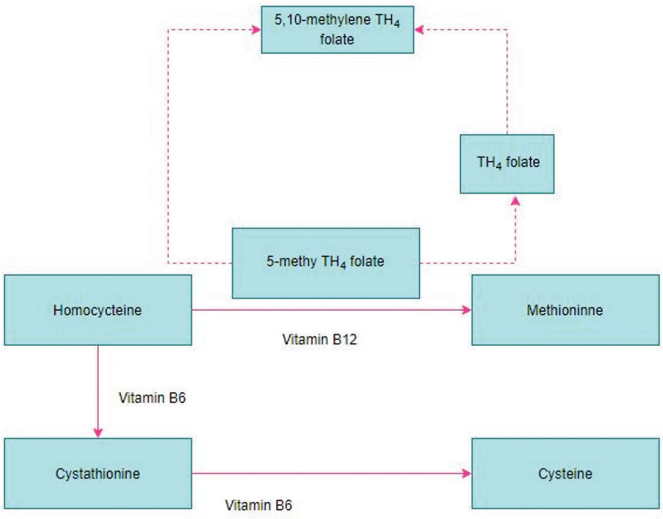

Figure 4. Conversion of homocysteine to methionine[71]

The relationship between folate and cancer is directly related to its dosage. Low doses of folate increase the risk of cancer. On the other hand, high doses of folate will reversely inhibit dihydrofolate reductase. Another risk factor for carcinogenesis is the circulation of the unreduced form of folate. Antifolate medications have been used widely in cancer therapy to inhibit single-carbon metabolism, which is necessary for cell proliferation in cancerous tissue.[66,70] Other agents, such as chronic alcohol usage,[76] antacids,[77] and general anesthetics,[78] cause depletion or alteration in folate levels.

Additionally, diseases, such as Crohn's disease, celiac disease, and several kind of cancers, result in folate depletion. The evaluation of folate levels is important in patients who have been on long-term diuretic therapies, including those using furosemide and amiloride. These medications increase the elimination of folate.[70]

The measurement of folate levels in the process of testing new therapeutic agents is considered a vital biomarker because of the essential role of folate in DNA biosynthesis and red blood cell synthesis. The depletion of folate levels increases the rate of cardiovascular and neuronal disorders.[73]

Unconjugated pteridines and their derivatives act as intermediates in metabolism, and their biological concentrations have shown changes in various disease processes. Unconjugated pteridines can be measured in the serum, cerebrospinal fluid, and urine.[79] Neopterin, as an unconjugated pteridine, is one of the early biomarkers for cancer, systemic diseases, infectious and/or inflammatory diseases such as HIV, rheumatoid arthritis, Behçet disease, and acute myocardial infarction. Neopterin became popular among scientists because it is highly fluorescent, and it can be synthesized easily by gamma interferon-activated macrophages and monocytes.[11,80,81] 7,8-dihydroneopterin, a form of neopterin produced by macrophages, acts as a radical scavenger and inhibits free radicals that are formed during lipid and protein oxidation. 7,8-dihydroneopterin is a hydroxyl, superoxide, and peroxyl scavenger. Surveillance of neopterin in body samples is a good indicator of the levels of free radicals in tissues and cells.[82] The Austrian government has been using neopterin screening to test donated blood to ensure their safety.[83] Neopterin screening can also be used to predict a patient's inflammatory status.[84-86]

CONCLUSION

In general, biomarkers are used to measure the response of biological systems. In the field of toxicology, biomarkers are practical tools to understand the mechanisms of toxicity. They are also useful in risk management and assessment. From the toxicological aspect, biomarkers play an important role in the prevention and reduction of harmful effects of different chemicals and agents. Mechanistic biomarkers have been used as a tool in diagnosis, treatment, and monitoring of the treatment course of different diseases, such as cancers, Alzheimer's disease, immunological disorders, and other pathologies.

In conclusion, reliable and applicable proper biomarkers that accord with ethical rules are beneficial for human health. Nevertheless, further research is still needed to define ideal biomarkers for different fields of life sciences.

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