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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/);

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Keywords: A list of minimum , but no more than 5 key words must follow the abstract. Key words in English should be consistent with "Medical Subject Headings (MESH)" (www.nlm.nih.gov/mesh/MBrowser.html). Turkish key words should be direct translations of the terms in MESH.

Original research articles should have the following sections:

Introduction: Should consist of a brief explanation of the topic and indicate the objective of the study, supported by information from the literature.

Materials and Methods: The study plan should be clearly described, indicating whether the study is randomized or not, whether it is retrospective or prospective, the number of trials, the characteristics, and the statistical methods used.

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.

Discussion: The study results should be discussed in terms of their favorable and unfavorable aspects and they should be compared with the literature. The conclusion of the study should be highlighted.

Study Limitations: Limitations of the study should be discussed. In addition, an evaluation of the implications of the obtained findings/results for future research should be outlined.

Conclusion: The conclusion of the study should be highlighted.

Acknowledgements: Any technical or financial support or editorial contributions (statistical analysis, English/Turkish evaluation) towards the study should appear at the end of the article.

References: Authors are responsible for the accuracy of the references. See General Guidelines for details about the usage and formatting required.

Review Articles

Review articles can address any aspect of clinical or laboratory pharmaceuticals. Review articles must provide critical analyses of contemporary evidence and provide directions of or future research. Most review articles are commissioned, but other review submissions are also welcome. Before sending a review, discussion with the editor is recommended.

Reviews articles analyze topics in depth, independently and objectively. The first chapter should include the title in Turkish and English, an unstructured summary and key words. Source of all citations should be indicated. The entire text should not exceed 25 pages (A, formatted as specified above).

Turkish Journal of
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CONTENTS

Original Articles

1 Knowledge Assessment During the Medication Process Use by Older Patients on Clinical Routine: A Pilot Study
Margarida ESPÍRITO-SANTO, Tânia NASCIMENTO, Ezequiel PINTO, M. Dulce ESTÊVÃO

8 Use of Herbal Tea/Herbal Preparations for Children with Symptoms of Viral Upper Respiratory Infections
Methiye MANCAK KARAKUŞ, Anıl TAPISIZ, Nazmi MUTLU KARAKAŞ, Melis DENİZ, Ufuk KOCA ÇALIŞKAN

16 *In Vitro* Anti-Leishmanial Activity of Glucosinolate Fraction from *Alyssum linifolium* Steph. ex Willd (Brassicaceae)
Fatemeh MIRZAEE, Roghiyeh FARIDNIA, Mahdi FAKHAR, Hamed KALANI, Somayeh SHAHANI5

23 Resveratrol-Loaded Microsponge Gel for Wound Healing: *In Vitro* and *In Vivo* Characterization
Vinita Chandrakant PATOLE, Devyani AWARI, Shilpa CHAUDHARI

35 Investigation of Antimicrobial Activity of Some Ethylparaben Hydrazide-Hydrazone Derivatives
Ufuk İNCE, M. İhsan HAN

39 Development and Validation of SI/RS-UHPLC-PDA Method for Olmesartan Medoxomil and Metoprolol Succinate-Related Substance
Nirmal THAKKER, Gajanan SHINDE, Abhay DHARAMSI, Vishnu CHOUDHARI

48 Stability Evaluation of the Biosimilar Monoclonal Antibody Using Analytical Techniques
Deniz DEMİRHAN

Review

58 Folate-Mediated Paclitaxel Nanodelivery Systems: A Comprehensive Review
Ashwini K. BAWANKULE, Amol A. TATODE, Pranali S. PATIL, Milind J. UMEKAR

Turk J Pharm Sci 2023;20(1):1-7
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ORIGINAL ARTICLE

Knowledge Assessment During the Medication Process Use by Older Patients on Clinical Routine: A Pilot Study

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ABSTRACT

Objectives: The consumption of medicines has been increasing over the last decades. The lack of medication knowledge (MK) may affect the process of medication use and, consequently, may lead to negative health outcomes. This study carried out a pilot study using a new tool to assess MK in older patients in a daily clinical practice.

Materials and Methods: An exploratory cross-sectional study was conducted, including older patients (≥65 years), taking two or more medicines, followed in a regional clinic. Data were collected during a structured interview, which included an algorithm for assessing MK regarding the identification of the medicines and its use and storage conditions. Health literacy and treatment adherence were also assessed.

Results: The study enrolled 49 patients, mainly between 65 and 75 years (n: 33; 67.3%) and polymedicated (n: 40; 81.6%), taking a mean of 6.9 ± 2.8 medicines *per* day. A lack of MK (score <50%) was observed in 15 (30.6%) participant patients. "Drug strength" and "storage conditions" were the items which presented the lowest score. MK was positively correlated with higher scores for health literacy and treatment adherence. Younger patients (age <65 years old) also had a higher MK score.

Conclusion: This study showed that the applied tool could evaluate the MK of the participants and identified specific gaps regarding MK within the process of medicine use. Further studies, with more participants, will allow the confirmation of these findings and will stimulate the development of specific strategies to improve MK, thus contributing to better health outcomes.

Key words: Older patients, health literacy, patient medication knowledge, treatment adherence

INTRODUCTION

Aging and the global increase in life expectancy of populations lead to a greater number of health problems and, consequently, to a higher number of prescribed medicines, especially in the older population and in patients diagnosed with chronic diseases. This situation conduces to an increased risk of drug adverse events and potential drug-drug interactions.[1-3]

Patients' medication knowledge (MK) may influence the process of how the medicines are taken, potentially leading to incorrect use and lower effectiveness. Thus, MK may be a relevant factor to bear in mind, when assessing the use of medicines by the patients and their adherence to the prescribed therapy, which, consequently, have a substantial impact on health outcomes.[4]

According to García Delgado et al.[5], the knowledge that a patient has about the medication can be defined as the amount of information that the patient acquires regarding that medication, which is required to use medicines properly, including the proper process of use (regimen, dosage, duration of treatment, and route of administration), the therapeutic objectives (indication and effectiveness), the security (adverse effects, precautions, contraindications, and interactions), and the conservation.

Frequent changes in medication (prescriptions, doses or new medicines) and the inability of patients may have to denominate the medicines used may contribute to reduce MK.[6-8] Also, medicines, which have only recently become available on the market, seem to lead to more knowledge gaps, as suggested

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by a medication review study in patients taking non-vitamin K antagonist oral anticoagulants.[7] Additionally, low MK has been correlated with non-adherence to the medication and the consequent risk of relapse as reported in a prospective study held in patients diagnosed with inflammatory bowel disease.[9] Also, a lack of MK has been identified as a factor contributing to poor disease management for chronic diseases.[10]

It is not frequent to assess the MK during a patient care process nor during the evaluation of medication use. Despite MK not being a systematically systematically assessed parameter in daily practice, some studies, which included this assessment, revealed a high prevalence of patients with low levels of MK.[8,11-13] An increased rate of inadequate MK (72%) was identified among Spanish community pharmacies costumers in a cross-sectional study. The lowest knowledge score was determined in the "medication safety" item (12.6% and 15.3% for "contraindications" and "side effects," respectively).[8]

There are a few tools available to assess patients´ MK, but the most commonly used tools, which are structured interviews and specific questionnaires, are only available in English.[14] However, studies that analyse their validity and reliability are still missing so that they can be used with confidence.[6,13,15,16]

In 2009, García Delgado et al.[5] proposed a questionnaire to assess patients' MK, which includes four dimensions (therapeutic goal, medicines use process, safety, and conservation). This tool is reported as reliable, presenting a Cronbach´s alfa value of 0.68.[8] This questionnaire was applied to a group of patients followed up in a Spanish community pharmacy, taking one or more medicines. Some predictive factors for low patient MK score were identified and included the use of several associated medicines, unqualified workers and caregivers, and the inability to identify the name of the medicines.[8] This questionnaire was used as a starting point to produce a cultural adaptation to the European Portuguese language, designated as Patient Knowledge about their Medications, Conocimiento del Paciente sobre sus Medicamentos; in Portugal; CPM-ES-ES. However, the authors mentioned that further studies are needed to demonstrate the equivalence of the psychometric properties (reliability and validity) of the Portuguese version, so it could be used in pharmaceutical care research projects in Portugal.[17]

Given the inexistence of a range of tools properly certified to be applied to the Portuguese population directly in clinical practice, the aim of this study was to perform out a pilot study employing a new tool to perform the assessment of older patients' MK in a daily clinical practice.

MATERIALS AND METHODS

Study design

We conducted a descriptive, cross-sectional study, in an outpatient diabetes clinic in the municipality of Faro (Algarve, Portugal). This clinic is integrated into a regional association of patients with diabetes (in Portuguese, AEDMADA - Associação para o Estudo da Diabetes Mellitus e Apoio ao Diabético do Algarve).

Patient selection

Patients were recruited for the study during their routine, previously scheduled, and consultation with a physician. Before the consultation, following the usual procedure at the clinic, the date of the consultation was confirmed with each patient by phone, and patients were specifically asked to bring with them all medicines and food supplements they were taking at date.

During the consultation, patients were invited to be a part of the study. Those who accepted filled and signed all necessary informed consent forms. Data collection occurred at the end of the consultation and was registered anonymously. Patients with alterations in cognitive abilities that could hinder understanding the study aim were excluded.

Study recruitment was performed for three months (January-March 2018), using a convenience sample, according to patient acceptance.

Approval for this study was previously obtained from the clinic's administration board.

This study only included patients with 65 or more years old.

Medication knowledge assessment

The assessment of patient MK was carried out through a structured interview using an algorithm specifically developed for this purpose, that considers six different parameters; the name of the medicines, the strength of the drugs, the therapeutic indication, the timing of administration, dosing intervals, and the storage conditions (Table 1). All six parameters were evaluated individually for each drug used by the patients.

For each patient, the average percentage of the six parameters considered in the MK assessment was determined and then used to obtain the score for medication knowledge (SMK) according to the following formula:

$$SMK = \frac{\sum_{i=1}^{6} MK_i}{total\ number\ of\ medicines} \times 100$$

The patient was considered as having "lack of MK", when the obtained score was less than 50%.

Furthermore, for each patient, data regarding socio-demographic characteristics, the clinical profile, and the number of physicians following the patient at the moment of the interview were collected using an appropriate form created for this purpose. Health literacy was assessed throughout the Short Assessment of Health Literacy - Portuguese language (SAHL-PT),[18] using a tool where patients were flagged as "low health literacy" when reached a score ≤14 (out of 18). WHO ATC index was used to perform medication classification (https:// www. whocc.no/atc_ddd_index/). Treatment adherence was also assessed using the Haynes Sackett method.[19] In this test, patients with scores between 80% and 100% were considered "adherents to the therapeutic".

Ethical consideration

Ethical approval of this study was obtained from the Cranfield University Research Ethics Committee (reference:

CURES/840/2016). All data were collected anonymously without any identification of the participants.

Statistical analysis

IBM-SPSS software, version 26.0 (SPSS Inc, Chicago, Illinois), and AMOS 24.0 (SPSS Inc, Chicago, Illinois) were used to analyse all the collected data. Quantitative variables are presented as minimum, maximum, mean, standard deviation, and median. The qualitative data are described by counts (n) and the respective percentages (%). Adherence to normal distribution was assessed with Kolmogorov-Smirnov's test. Parametric (Student's *t*-test, Pearson correlation coefficient) or non-parametric (chi-*square*, Mann-Whitney's *U* test, Spearman's correlation coefficient) procedures were used for analysing associations or group differences. Statistical significance for all procedures was considered when $p<0.05$.

RESULTS

A sample of 49 patients was included in this study, of whom 27 (55.1%) were male. The mean age was 73.22 ± 5.72 years, with 16 (32.7%) over 75 years old. Patients were mainly retired (n: 44; 89.8%) and only 4 (8.1%) were living alone. More than half of the patients (n: 27; 55.1%) only concluded up to 6 years of schooling (Table 2).

Patients were taking a mean of 6.9 ± 2.8 medicines and 7.7 ± 3.3 daily units *per* day, and 346 medicines. Most of them were using 5 or more medicines *per* day (81.6%), which can be considered polymedication (Table 2). Only 6 patients (12.2%) were using food supplements.

Medicines most frequently taken were those acting on the alimentary tract and metabolism (group A), cardiovascular system (group C) and nervous system (group N) (Table 3).

Low health literacy was flagged in 27 (55.1%) patients with an average score of 13.3 ± 3.6, and a prevalence of non-adherence to treatment in 9 patients (18.4%).

Regarding the use of medicines, 15 patients (30.6%) showed a lack of MK (score <50%), but an average prevalence of 58.5 ± 15.2% for correct information about the medicines currently used was determined.

Despite most patients (n: 46; 94%) stated being able to read the packaging, the name of the medicine was identified only in less than half of the medicines used (n: 21; 41.9%) (Table 4).

For 279 (80.6%) medicines, patients could not indicate the drug's strength, and for 83 (24.0%) medicines, patients did not know either their therapeutic indication or presented the wrong one. Problems related to the administration time were identified in 32 (9.2%) medicines, and in 25 (7.2%) of the analyzed medicines, difficulties related to the number of units were also pointed. Incorrect storage conditions were mentioned in relation to 207 (59.8%) medicines (Table 4).

The "administration time" and "number of units" were the items with the highest knowledge rate, followed by the item "therapeutic indication." On the opposite, the items presenting the lowest knowledge rate were "drug´s strength" and "storage conditions" (Table 4).

Older patients, presenting fewer years of schooling, revealed significantly lower MK than younger patients (p=0.049) (Table 5). Patients with 12 years of schooling had significantly more

Table 1. Parameters for the assessment of patients' medication knowledge and the respective possible results and indicators

Parameters	Results	Indicators
Medicine´s name* What is your medicine name?	☑ Knows medicine's name ☒ Does not know medicine´s name	Rate (%) of medicines whose name was correctly identified
Drug´s strength* What is your medicine strength?	☑ Knows drug's strength ☒ Does not know drug's strength	Rate (%) of medicines whose drug's strength was correctly identified
Therapeutic indication* For what purpose, do you take this medicine?	☑ Knows the correct therapeutic indication ☑ The information on the therapeutic indication is not complete ☒ Does not know the correct therapeutic indication ☒ Does not know the therapeutic indication	Rate (%) of medicines whose therapeutic indication was correctly identified (answer 1 or 4)
Timing of administration* At what time of the day, do you take your medicine?	☑ Knows the correct time for the administration of the medicine ☒ Does not know the correct time for the administration of the medicine	Rate (%) of medicines whose administration time was correctly identified
Dosage intervals* How often do you take your medicine?	☑ Knows the correct time for dosage intervals ☒ Does not know the correct time for dosage intervals	Rate (%) of medicines whose dosage intervals were correctly identified
Storage conditions* Where do you keep your medicines at home?	☑ Knows the correct storage conditions ☑ Lack of information on medication storage ☒ Does not know the correct storage conditions	Rate (%) of medicines whose storage conditions were correctly identified

*All parameters described in the current table were also analyzed for food supplements, whenever applicable.

Table 2. Socio-demographic characterization of the patients enrolled in the study

	N (%)
Gender	
Female	22 (44.9)
Male	27 (55.1)
Age (mean age, minimum, maximum)	
Mean age	73.22 ± 5.72
Minimum	66
Maximum	88
Marital status	
Married/committed	38 (77.6)
Widower	6 (12.2)
Divorced	5 (10.2)
Living with	
Alone and autonomous	3 (6.1)
Alone with support	1 (2.0)
Other family members	45 (91.8)
Schooling	
Can read or write without formal education	1 (2.0)
Cannot read or write	2 (4.1)
4 Years	22 (44.9)
6 Years	2 (4.1)
9 Years	11 (22.4)
Professional/technological course	5 (10.2)
12 years	3 (6.1)
Higher education	3 (6.1)
Use of medicines	
(<2 medicines)	0 (0.0)
(2-4 medicines)	9 (18.4)
≥5 medicines	40 (81.6)
Use of food supplements (mean age, minimum, maximum)	
Number of patients	6 (12.2)
Mean	0.12 ± 0.3
Maximum	0
Minimum	1
Professional situation	
Retired	44 (89.8)
Employed	1 (2.0)
Retired with activity	3 (6.1)
Without professional activity	1 (2.0)

Table 3. Medicines anatomical therapeutic chemical classification (level 1) consumed by the participants in the study

ATC classification (level 1)	N (%)
A - Alimentary tract and metabolism	114 (32.9)
B - Blood and blood forming organs	22 (6.4)
C - Cardiovascular system	135 (39.0)
G - Genito urinary system and sex hormones	11 (3.2)
H - Systemic hormonal preparations, *excl.* sex hormones and insulins	5 (1.4)
J – Anti-infectives for systemic use	2 (0.6)
M - Musculo-skeletal system	12 (3.5)
N - Nervous system	33 (9.5)
R - Respiratory system	6 (1.7)
S - Sensory organs	1 (0.3)
Total	**346 (100)**

N: Number of medicines *per* ATC classification, ATC: Anatomical therapeutic chemical

Table 4. Characterization of patients´ medication knowledge

	N (%)
Name	
Knows the name of the medicine	145 (41.9)
Does not know the name of the medicine	201 (58.1)
Drug's strength	
Knows the drug's strength	67 (19.4)
Does not know the drug's strength	279 (80.6)
Therapeutic indication	
Knows the correct therapeutic indication	220 (63.6)
Does not know the correct therapeutic indication	16 (4.6)
Does not know the therapeutic indication	67 (19.4)
The information on the therapeutic indication is not complete	43 (12.4)
Administration time	
Knows the correct administration time	314 (90.8)
Indicates an incorrect administration time	32 (9.2)
Units number	
Indicates the correct number of units *per* day	321 (92.8)
Indicates an incorrect number of units *per* day	25 (7.2)
Storage conditions	
Indicates the correct storage conditions	112 (32.4)
Indicates incorrect storage conditions	207 (59.8)
Lack of information on medication storage	27 (7.8)

MK than those with 4 years of schooling (72.5 ± 13.6 *vs.* 55.4 ± 12.4; p=0.036).

No significant differences were observed for MK score in relation to the remain socio-demographic variables, number of medicines used *per* patient and patient´s medication adherence (p>0.05).

Polymedicated patients (5 or more medicines) exhibited an increased score of MK (mean of 60.4 ± 14.7 *vs.* 50.3 ± 15.0), although differences were statistically non-significant (p>0.05) in relation to patients taking less than 5 medicines.

An increased score for health literacy (p<0.05) and treatment adherence (p>0.05) was achieved for patients showing a higher MK (Table 5).

A lower MK (score <50%) was determined for the medicines belonging to the C group (drugs acting in the cardiovascular system) and A groups (drugs acting on alimentary tract and metabolism) compared to other therapeutic groups, but no significant differences were observed between all different groups (p>0.05).

Additionally, patients being followed by 2 or more physicians presented a higher but not statistically significant (p>0.05) MK score.

DISCUSSION

Some methodologies are emerging for evaluating MK, although each one includes different evaluation parameters, making it difficult to compare the obtained results. By the time this study was conducted, for the Portuguese population, only one validated tool was available to assess MK. However, that tool only considers one drug *per* patient and the results of the psychometric tests were shown to be inadequate possibly due to the sample size.[20]

Using the methodology presented in the current study, about a third of the patients enrolled (n: 15; 30.6%) showed a lack of MK while another cross-sectional study that applied the questionnaire (PKM-PT-PT) and that was carried out in community pharmacies in the Lisbon Metropolitan area (Portugal) identified 65.9% of patients without appropriate MK. Although, in the current study, patients revealed a higher rate of MK, items such as "therapeutic goal" (70.9%) and "process of use" (36.7%) were the items with higher scores and results were similar in both studies. Likewise, the item with the lowest rate was "storage conditions", which also had similar rates of knowledge in both studies. However, note that the patients' MK was assessed only for a medicine they were taking,[9] while in the present study, all medicines prescribed were considered for each patient and so the comparisons between the obtained results should be interpreted with caution.

The results obtained in the current study indicate a lower prevalence (n: 15; 30.6%) of inappropriate MK compared to the score determined by Romero-Sanchez et al.[8], in a study enrolling community pharmacy users, where an inadequate MK was determined in 71.9% of the patients, with 65.7% having no MK and 6.2% insufficient MK. The interpretation of these results should take in consider that different tools were used to assess MK, using different weighting factors for the various parameters analyzed, which may contribute to the different results reported.

One factor previously correlated with inappropriate MK is the lack of knowledge that patients revealed in relation to the name of the medicine they use,[8] which was also verified in about 60% of the medicines used by the patients in this study.

Although medicines included in groups A and C are the most used by this group of patients, a particular lack of MK associated with these groups was observed, which is a surprising and worrying situation as we could expect that patients know more about the medicines they use regularly, highlighting the need of improving patients' knowledge.

Medication adherence has been considered as a factor which may limit the improvement of chronic disease (*e.g.* diabetes, hypertension) control. Participants in the current study presented a 18.4% prevalence for medication non-adherence, adding to the observed lack of MK, will contribute to aggravate health outcomes with negative impacts on disease control.[21-23]

Education level has been considered a relevant factor to achieve positive health results, given its ability to influence treatment adherence and patient self-care.[19,24] In a study conducted in Portugal with university students, the level of MK identified was low for self-medication.[25] The results obtained in this study have shown that patients with lower schooling levels tend to present low MK scores, as described in the previous section, which reinforces the need to pay additional attention to those patients to help them achieve the desired positive outcomes with the prescribed medication.

In this study, a failure in the identification of drugs (name and drug strength) was clearly observed, which may represent a risk in case they should provide information about the medication they are taking (for example, to the other physicians or in any health service). Therefore, it could be useful, in the future, to draw up the list of medicines or to develop appropriate

Table 5. Patients' health literacy, treatment adherence, and medication knowledge scores

	Health literacy		Treatment adherence (%)		Age (years)	
	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median
Lack of MK	11.0 ± 4.1	10	87.8 ± 13.6	88.9	74.7 ± 4.9	73
Appropriate MK	14.3 ± 2.9	15	90.9 ± 15.9	99.2	72.6 ± 6.0	71
p value	0.006		0.160		0.049	

Statistical significance was considered when p<0.0505 (Mann-Whitney *U* test). MK: Medication knowledge, SD: Standard deviation

tools (information technology tools or others) for each patient, considering information from different sources (*e.g.,* hospital, community pharmacies), so they can carry it with them, whenever they access health services or whenever they must identify their medicines.[26,27]

The signaling of patients with low MK scores may allow the identification of patients, who could benefit from participating in therapeutic education programs. An improvement in MK was achieved for patients under polymedication through the provision of a counseling session after being referred by physicians to medication review service, provided by pharmacists, due of signaling problems with therapeutic adherence or knowledge.[28]

Farsaei et al.[29] have revealed that progress could be achieved in disease management, including a significant metabolic improvement, through the implementation of an educational program (interventional approach, conducted by pharmacists), which addressed several topics, such as medicines, therapeutic adherence, daily self-care records, and pill box usage.[24]

A more recent systematic review and meta-analysis, which evaluated patient-centred outcomes reported in studies searching for interventions to increase treatment adherence, has shown that MK assessment may be an important tool to be used in clinical services such as medication reviews.[23]

Also, in the management of chronic diseases, such as Parkinson's disease, MK can have a relevant influence on physical and social performance, impacting the health-related quality of life.[30]

Study limitations

The current study has some strengths and limitations. The main strength is that the assessment of MK was applied to all medicines used by patients. Although this methodology may be time-consuming and exhaustive, it allows to specifically identify the items in which the patients have more difficulties regarding their MK.

The obtained results show that for each patient, the knowledge varies with the medicines they are taking, which may compromise their full benefits.

One limitation of MK assessment carried out in this study is that the questionnaire did not include questions related to drug safety (side effects, interactions, and contraindications). In the future, this MK tool should include questions related with this item, using a perceptible format that allows to assess patients' knowledge about the safety of the medication.

Our sample size was low, as we were unable to recruit additional patients, and the methodology we used to assess MK was not previously validated. Additionally, the descriptive nature of this work does not allow us to establish causation, but despite these limitations and shortcomings, the use of a novel tool in a population that is scarcely studied with regard to the subject of this paper leads us to assert that the data we gathered was an important starting point, which will soon lead us to conduct further research in this matter.

CONCLUSION

The results obtained in the current study gave us access to detailed information about MK of each medicine used by the enrolled patients, which seems to be useful in the future for scheduling pharmaceutical interventions and customizing the needs for each patient according to inappropriate points in the use of each medicine.

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The authors acknowledge the AEDMADA clinic for allowing the conduct of this study in their facilities and all subjects, who accepted to participate in this study.

Ethics

Ethics Committee Approval: Ethical approval of this study was obtained from the Cranfield University Research Ethics Committee (reference: CURES/840/2016).

Informed Consent: Those who accepted filled and signed all necessary informed consent forms.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: M.E-S., Design: M.E-S., T.N., Data Collection or Processing: M.E-S., Analysis or Interpretation: E.P., M.D.E., Literature Search: M.E-S., Writing: M.E-S., M.D.E.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Use of Herbal Tea/Herbal Preparations for Children with Symptoms of Viral Upper Respiratory Infections

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ABSTRACT

Objectives: Respiratory tract infections (RTIs) are common in children. To treat the symptoms of simple health problems, individuals look for natural healing methods that can be easily prepared at home. The aim of this study was to determine the plants and herbal products used by the parents of children suffering from viral upper respiratory tract symptoms by questionaire. In the study, applications and products, other than plants used by families for their children were also investigated.

Materials and Methods: This study is a cross-sectional survey conducted at Faculty of Medicine, Gazi University (Ankara, Türkiye). A questionnaire form was used by scanning the existing literature and reviewed with the patients face to face by the researchers. The data obtained from the study were analyzed with the Statistical Package for the Social Sciences (SPSS) statistical program.

Results: About half of the participants reported that they used non-chemical drug practices for their children with upper RTIs. The most common practice was to prepare herbal tea (30.5%), followed by mandarin/orange and/or their juice (26.9%) for oral application. The most used herbal tea for upper RTIs is with linden (*Tilia* sp.). Patients who used linden usually prepared it as tea, in other words by infusion, and served their children 1-2 cups/1-3 times a week. Except for herbal tea, the participants mostly used honey (19.0%) for their children's symptoms.

Conclusion: Where available, appropriate doses and dosage forms of herbal supplement products with scientifically proven efficacy and safety should be determined for the pediatric population. Parents should use these products based on the recommendations of their pediatrician.

Key words: Pediatric, symptoms, respiratory tract infection, herbal tea, cross-sectional survey

INTRODUCTION

Respiratory tract infections (RTIs) are one of the most common diseases in children. They mostly have a viral origin and disturb the larynx, throat, trachea, mouth, and nose. The most common symptoms are cough, fever, nasal congestion, runny nose, and sore throat.[1] RTIs can be frustrating for both children and their parents, affecting daily activities as well and respiratory symptoms.[2]

To treat the symptoms of simple health problems, individuals look for approaches that they can easily apply at home.[3] These methods, which are included in complementary and alternative medicine (CAM) applications, are used in all age groups, including children.[4] Complementary and alternative medicinal practices, especially herbals are frequently used for managing symptoms of RTIs in children.[5,6] Studies in pediatric patients showed that herbal remedies are often used to treat coughs and colds.[7,8]

Although many plants are used for RTI symptoms, studies on the safe use of these plants in children are incomplete. There are ethical and moral limitations. Moreover, children have different physiology than adults and scientific data explaining the effectiveness of herbal products, their interaction with drugs and their side effects are lacking for all age groups.[9,10]

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The aim of this study was to determine the plant drugs and herbal products used by the families of children mostly suffering from viral upper respiratory tract symptoms. In this study, applications and products other than plants used by families for their children were also investigated. In this large-scale research, types of teas, preparation techniques of the teas, the herbs used in the teas, their preparation, the quantity and frequency of applications of those teas are examined.

MATERIALS AND METHODS

The research was conducted as a cross-sectional survey at the Department of Pediatric Infectious Disease in General Pediatrics at the Faculty of Medicine, Gazi University between July 1st 2020 and September 1st 2020. The approval was obtained for this study from the Ethics Committee at Gazi University (12.05.2020-E.54135). All the participants voluntarily participated in the study. The research was conducted with the families of the patients, who applied to the hospital with the symptoms of viral upper RTI. The questionnaire was prepared based on the relevant literature and applied to the patients with face-to-face by the researchers. Cough, rhinorrhea, nasal congestion, sore throat, and fever were determined as symptoms of upper RTI and their presence in children was questioned. In the first part of the questionnaire, the descriptive characteristics of the participants and their children were examined and 250 individuals were included in the study. Since the amount of the salary can change in numbers in our country, respondents were asked to categorize their family income as "very low", "low", "middle", "good" or "excellent". In the next sections, herbals and non-pharmacological applications used for upper respiratory tract symptoms are presented. In addition to questions with two options such as "yes" and "no," "multiple response" questions, where more than one option can be selected were also included. The preparation technique, frequency and amount of used herbal teas were also examined. Herbal tea preparation techniques were classified as infusion, decoction, and maceration. The infusion is defined as brewing with boiling water, decoction is adding cold water and boiling with it, and maceration is adding water, keeping it at room temperature by shaking occasionally.

Statistical analysis

The data obtained from the study were analyzed with the Statistical Package for the Social Sciences (SPSS) 26.0 statistical program. The frequency of participant responses is shown in the accompanying tables and figures. Cross tables were created to correlate the responses and chi-square tests were conducted. The level of significance for all statistical analyzes was accepted as $p<0.05$.

RESULTS

Characteristics of participants and children

The characteristics of the participants and children are shown in detail in Table 1. A total of 250 individuals participated in this study, in which 72.0% were mothers, 25.2% were fathers, and

Table 1. Characteristics of participants and children (n: 250)

Characteristics	Number (n)	Percentage (%)
Child's gender		
Girl	130	52.0
Boy	120	48.0
Child's age		
0-2	58	23.2
3-6	70	28.0
7-11	59	23.6
12-17	63	25.2
***Child's symptoms**		
Cough	162	25.8
Rhinorrhea	129	20.5
Nasal congestion	119	18.9
Fever	92	14.6
Sore throat	84	13.4
Other	43	6.8
Children having chronic diseases		
Yes	36	14.4
No	214	85.6
Children having a regular medication intake		
Yes	42	16.8
No	208	83.2
Participant's relationship to child		
Mother	180	72.0
Father	63	25.2
Other	7	2.8
Participant's age		
18-29	56	22.4
30-44	163	65.2
45-59	28	11.2
≥60	3	1.2
Participant's education		
Illiterate	6	2.4
Literate	4	1.6
Primary school graduate	33	13.2
Secondary school graduate	48	19.2
High school graduate	74	29.6
University graduate	73	29.2
Master's and PhD	12	4.8
Family income		
Very low	2	0.8
Low	15	6.0
Middle	161	64.4
Good	68	27.2
Excellent	4	1.6
Residence		
City center	163	65.2
District	83	33.2
Other	4	1.6

*Multiple response

2.8% were other individuals. Most individuals (65.2%) between the ages of 30 and 44 and more than half of the participants (65.2%) reside in the city center, moreover, about 65% of respondents described their family income as "middle". As for the education of the participants 29.6% of the participants are high school graduates, 29.2% are university graduates and 4.8% are postgraduate.

The participated children were mostly between the ages of 3 and 6 (28.0%), 52% of the children were girls and 48% were boys, who generally did not have chronic illnesses or did not take regular medications. The most common upper respiratory tract symptoms in children were cough (25.8%) followed by rhinorrhea (20.5%).

Use of non-pharmaceutical products

Non-drug applications and products used are given in Table 2 and symbolized in Figure 1. The number of participants who applied or did not apply anything other than medication for their child's upper RTI symptoms was close to. 47.2% of the participants reported that they used non-drug therapeutic approaches for their children with upper RTI symptoms. These applications were herbal tea, honey milk, vitamin supplements, mandarin/orange and/or their juice, and Vicks® rub, and steam application. The most common non-drug practice was to drink herbal tea (30.5%). The rate of use of herbal and/or herbal products, which was asked as a different question, was 34%. Participants reported that they mostly used honey (19.0%) for their children's symptoms, apart from herbal tea as a product. Differently, among the frequently used products were grape molasses (11.4%), vitamins (6.9%), carob molasses (5.9%), and fish oil (5.2%).

Use of herbal tea and products

The details about the application of herbal teas and products by the participants are given in Tables 3 and 4. Participants (34%) used herbal tea/product for their children's symptoms, used them most often at the onset of diseases (61.2%). Herbal use declined after the disease progressed. Most participants did not use the prescribed medicine and herbal products together (89.6%).

Participants applied herbs mostly by brewing or boiling. The most used herbal hot drink was reported as linden infusion/decoction (44.2%) for upper RTI symptoms, followed by mint-lemon (28.6%) infusion/decoction. Individuals, who used linden for their children reported that they usually prepared it by infusion and they made their children drink 1 to 2 cups, usually 1 to 3 times a week. However, they prepared mint-lemon by decoction and made them drink 1-2 cups. The other reported herbs were sage, cinnamon-ginger, winter tea, chamomile, quince, fennel, rosehip, marshmallow, green tea, and pomegranate flower.

The data associated with herbal use and characteristic features of the children/participants are presented in Table 5. There was a statistically significant relationship between the age of the children and herbals use. Children between the ages of 7 and 11 highly consumed herbal teas ($p<0.05$). However, herbal usage was low in children with chronic diseases and taking regular medication. Analysis showed that parents between 30 and 44 years old and mostly mothers used herbs/herbals for their children. Among those parents, there were university graduates had a middle-income and lived in the city center. However, the relationship between herbs usage and parents' gender, age, education, and income level was not statistically significant ($p>0.05$). Participants who used herbs for themselves were more probably to use herbs for their children. Individuals who did not use herbs for themselves generally did not use herbs for their children (40.8%) either. The rate of the participants, who both themselves and their children used herbal medicine, was 31.2%.

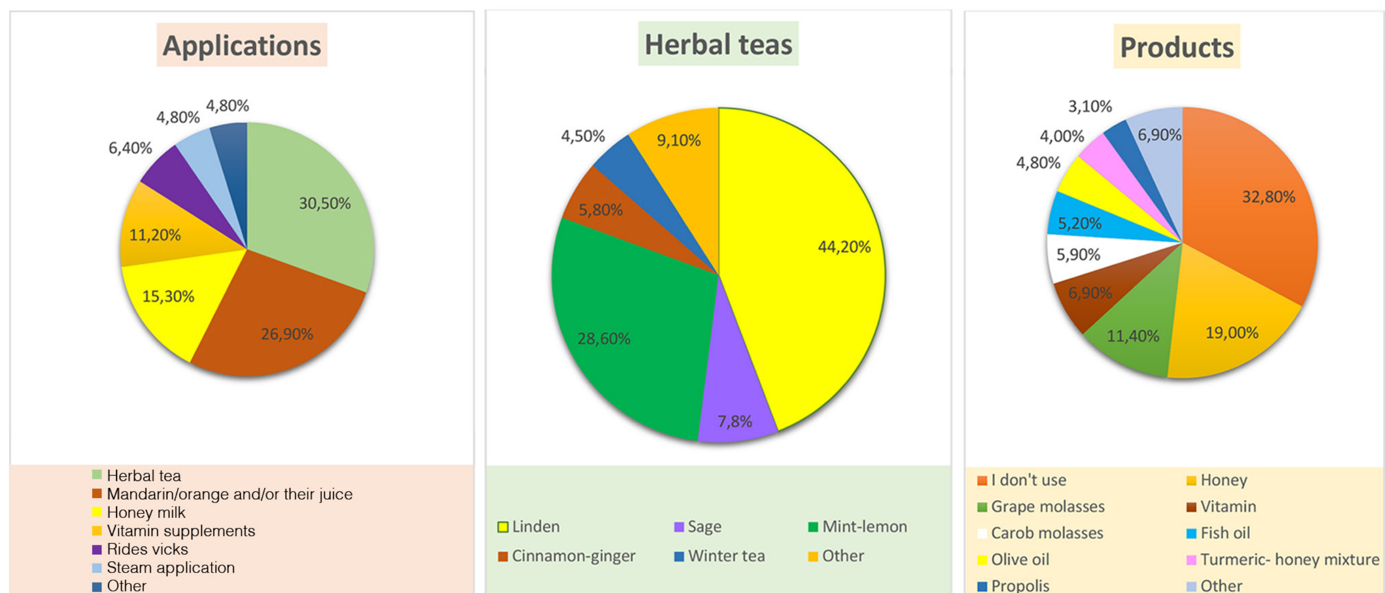

Figure 1. Use of non-pharmaceutical products

Participants reported that they learned the use of herbals mostly from relatives, family elders, neighbors, and friends (60.5%). The number of participants who received advice from health professionals was quite low (15.5%). For this reason, the rate of use of herbal products sold in pharmacies (Umca® and Sambucol®) was also very little (1.5%). Almost half of the individuals obtained the herbs from herbalists or spice shops. Apart from that, they were mostly obtained from village/hometown and market/supermarkets. The number of individuals who bought the herbs from the pharmacy was quite low (5%). Individuals using herbals preferred them because they thought they were useful and less harmful than chemicals. About half of the participants who did not use herbals reported that they considered them ineffective (49%). Some of them described herbs as expensive (1.9%) and harmful (13.5%), additionally, 4.8% of the participants reported that their family doctor said they should not use herbal medicine because they were already taking medicine.

Table 2. Use of herbal and non-pharmaceutical products

	Number (n)	Percentage (%)
Before applying to the hospital for your child, did you apply anything other than medication for upper respiratory tract infection symptoms?		
Yes	118	47.2
No	132	52.8
*Before taking your child to the hospital, what applications did you use for upper respiratory tract infection symptoms other than medication?		
Herbal tea	76	30.5
Mandarin/orange and/or their juices	67	26.9
Honey milk	38	15.3
Vitamin supplements	28	11.2
Rides vicks	16	6.4
Steam application	12	4.8
Other	12	4.8
Do you use herbal tea/product for upper respiratory tract infection symptoms in your child?		
Yes	85	34.0
No	165	66.0
Linden (*Tilia* sp.)	68	44.2
Mint- lemon (*Mentha* sp.-*Citrus limonum*)	44	28.6
Sage (*Salvia* sp.)	12	7.8
Cinnamon- ginger (*Cinnamomum* sp.-*Zingiber officinalis*)	9	5.8
Winter tea	7	4.5
Chamomile (*Matricaria recutita*)	3	1.9
Quince (*Cydonia oblonga*)	3	1.9
Fennel (*Foeniculum* sp.)	2	1.3
Marshmallow (*Althaea* sp.)	1	0.6
*What products do you use for your child other than herbal tea?		
I do not use	138	32.8
Honey	80	19.0
Grape molasses	48	11.4
Vitamin	29	6.9
Carob molasses	25	5.9
Fish oil	22	5.2
Olive oil	20	4.8
Turmeric- honey mixture	17	4.0
Propolis	13	3.1
Black cumin oil	5	1.2
Mulberry molasses	4	1.0
Black radish- honey mixture	4	1.0
Umca® (*Pelargonium sidoides*)	4	1.0
Ginger- honey mixture	2	0.5
Sambucol® (*Sambucus nigra*)	2	0.5
Germ oil	1	0.2
Other	7	1.6

*Multiple response

DISCUSSION

The use of herbs and herbal products in pediatric populations has been studied mostly under the heading of CAM. Studies have focused more on determining for what kind of symptoms the CAM are used in the general pediatric population. In Italy, CAM is mostly used to treat ear, nose, and throat pathologies. The most used methods are phytotherapy and homeopathy.[11] In southwest England, 50% of the children had used complementary medicine who had upper RTIs.[12] As well as in the United States of America, CAM practices are also frequently used for respiratory diseases in children. More than 50% of users have been using vitamin supplements and over 40% have been using herbal treatments. *Aloe vera*, chamomile tea, echinacea, garlic, and ginger were among the most consumed herbs.[13]

Among the studies investigating herbal use in specific pediatric populations, studies in children having respiratory tract disorders are relatively few. In Australia, it has been reported that parents of children being affected by acute respiratory infections commonly use chest lotions/herbal liniments, lemon and honey-mixed drinks, only honey, and probiotics as treatments. However, herbals were used in children, less than these applications. Elderflower, echinacea, ginger, mint, turmeric, and herbal combinations were among the herbals used. Parent's educational status played a role in the use of CAM in children.[14] In Türkiye, 77.2% of mothers who applied to the emergency department with respiratory system complaints in their children reported that they applied CAM to their children. The most common practice was the use of herbal products and the plants were as follows: linden (50.9%), mint-

Table 3. Herbal tea/product usage details

	Number (n)	Percentage (%)
*When do you use herbal tea/product for upper respiratory tract infection in your child?		
Before disease	20	16.5
When the disease started	74	61.2
When the disease progresses	8	6.6
After stopping the medicine	6	5.0
With the medicine	11	9.1
Other	2	1.7
Do you give your child any other medicines while using the herbal tea/product for upper respiratory tract infection?		
Yes	26	10.4
No	224	89.6
*From whom/where did you learn that you can use herbal tea/product for your child's upper respiratory tract infection?		
Relatives, family elders	54	38.0
Neighbor, friend	32	22.5
I have made up my own mind	18	12.7
Internet, television	14	9.9
Doctor	13	9.2
Pharmacist	6	4.2
Other healthcare professionals	3	2.1
Other	2	1.4
*Where do you buy the herbs, which you prepare the herbal tea?		
Herbalist, spice	58	47.9
Village/hometown	25	20.7
Market, supermarket	23	19.0
I picked the plant myself	6	5.0
Pharmacy	6	5.0
Internet, television	3	2.5
*Why do you use herbal tea/product?		
I think it is		
Useful	133	57.6
More harmless then chemical drugs	61	26.4
Easily accessible	24	10.4
Cheap	7	3.0
Other	6	2.6
Do you use a herbal tea/product for yourself when you feel sick?		
Yes	141	56.4
No	109	43.6

*Multiple response

lemon (40.8%), and carob (29.4%).[15] In another study, conducted to determine the approaches of mothers to their children having cough, it was determined that 72.8% of mothers benefited from herbals such as linden and mint. The same study indicated that practices such as eating tangerine/orange fruits, drinking milk with honey, applying vics on all over the body were applied for healing.[16] In Saudi children, the herbal medicine use rate for acute lower respiratory tract disease was 59.3%. Sesame oil, fenugreek, olive oil, and dates were also often used.[17]

Analysis of the results showed that children's families used various non-drug practices for their children with viral upper respiratory tract symptoms. It has been determined that the applications and products used by families for their children are easily accessible and applicable at home. The results obtained in this study are similar to the results of previous studies.[15,16]

Previous studies have shown that female parents tend to use more CAM and herbs for their children; even, some studies were conducted with mothers only.[11,15,16] Similarly, in this study, herbs were mostly used by mother, moreover, similar to previous studies, participants who used herbs were mostly university graduates.[11,14] Although this suggested that the participants with a higher education level researched herbs more, additionally, the participants reported that they learned the information mostly from their family members. Participants residing in the city center used herbs more. Considering that the participants mostly obtained herbs from herbalists, this may have been due to their easier access to herbalists in the city center.

The survey results showed that honey is the most used product of all non-pharmacological therapeutic approaches. In addition to its use alone, honey has been used as a mixture by adding it to plants. Previous studies have also shown that honey is often used for respiratory problems in children, especially for cough. Due to its antibacterial, antimicrobial, and topical soothing properties, honey has been suggested as a potential treatment for coughs and colds.[18] A Cochrane review compared the effectiveness of honey for acute cough in children with the effectiveness of diphenhydramine, dextromethorphan, and salbutamol. Results showed that honey reduced the cough-time better than placebo and salbutamol. For cough symptoms, honey was approximately equally effective with dextromethorphan, while it was more effective than diphenhydramine, control, and placebo. There was no difference between honey and others in terms of adverse effects.[19] However, honey is not recommended in children younger than 1 year old as it may cause infantile botulism.[20]

Linden was the most applied herb for children's symptoms in our study. According to the European Medicines Agency (EMA), linden flowers can be used to relieve cold symptoms.[21] Commission E approved the use of linden for cough and bronchitis. According to Physician Desk Reference (PDR), linden flowers can be used for colds of the respiratory tract due to their diaphoretic effect and for febrile colds and infectious diseases, where sweating treatment is required.[22] However, the indications stated in the monographs are based on traditional use and are not supported by clinical studies. EMA recommends the preparation and use of tea from linden flowers as an infusion, while according to the PDR, it can be prepared both as an infusion and a decoction. Although it is reported in the monographs that linden has no serious side effects, EMA does not recommend the use of linden in children under 4 years of age due to insufficient data. In our study, it was found that the participants prepared linden by both infusion and decoction methods, mostly by infusion. Moreover, this study used linden in children under 4 years old, contrary to what is stated in EMA.

Table 4. Herbs and usage information

Herbs	Number of users for child				Preparation technique (n)			Frequency of use (n)				Amount of usage (n)			
	0-2 age	3-6 age	7-11 age	12-17 age	Infusion	Decoction	Maceration	Every day	Throughout the disease	1-3 *per* week	4-6 *per* week	1-2 cups a day	3 cups a day	4 or more cups a day	Other
Linden	13	21	21	13	50	18	0	11	26	28	3	64	1	0	3
Sage	2	4	0	6	10	2	0	1	6	5	0	12	0	0	0
Mint- lemon	6	15	12	11	16	28	0	5	15	22	2	39	4	0	1
Cinnamon-ginger	1	2	3	3	5	3	1	1	4	4	0	8	0	1	0
Winter tea	0	2	1	4	4	2	1	1	3	3	0	6	1	0	0
Chamomile	0	0	1	2	1	1	1	0	0	3	0	3	0	0	0
Marshmallow	0	1	0	0	1	0	0	0	1	0	0	1	0	0	0
Quince	0	1	0	2	2	1	0	0	1	2	0	3	0	0	0
Fennel	1	0	0	1	2	0	0	1	1	0	0	1	0	0	1

n: Number

Table 5. Cross-table of herbal tea/product use

		Use of herbal tea/product		Significance p value
		Yes (%)	No (%)	
Child's age	0-2	5.6%	17.6%	
	3-6	10.4%	17.6%	
	7-11	10.8%	12.8%	$p<0.05$
	12-17	7.2%	18.0%	
Child's chronic disease	Yes	4.8%	9.6%	
	No	29.2%	56.4%	$p>0.05$
Child's regular medication intake of	Yes	4.8%	12.0%	
	No	29.2%	54.0%	$p>0.05$
Participant's relationship to child	Mother	25.6%	46.4%	
	Father	7.2%	18.0%	$p>0.05$
	Other	1.2%	1.6%	
Participant's age	18-29	6.8%	15.6%	
	30-44	24.0%	41.2%	
	45-59	3.2%	8.0%	$p>0.05$
	≥60	0.0%	1.2%	
Participant's education	Illiterate	0.4%	2.0%	
	Literate	1.2%	0.4%	
	Primary school graduate	3.6%	9.6%	
	Secondary school graduate	5.2%	14.0%	$p>0.05$
	High school graduate	9.6%	20.0%	
	University graduate	10.8%	18.4%	
	Postgraduate	3.2%	1.6%	
Family income	Too bad	0.4%	0.4%	
	Bad	2.8%	3.2%	
	Middle	20.8%	43.6%	$p>0.05$
	Good	10.0%	17.2%	
	Excellent	0.0%	1.6%	
Family residence	City center	24.0%	41.2%	
	District	10.0%	23.2%	$p>0.05$
	Other	0.0%	1.6%	
Herbal tea/product use for the participant's own	Yes	31.2%	25.2%	$p=0.00$
	No	2.8%	40.8%	

Individuals stated that they mostly used herbal teas or products for their children at the onset of illness. Herbal use declined after the disease progressed. This shows that parents turn to herbs as a first and simple remedy, when their child starts showing symptoms. When the disease progresses, they prefer using the drugs that their doctors prescribe. Generally, they do not prefer to use the prescribed drug and the herbs together. The age of the child and the use of herbs by the families themselves were effective in the use of herbs in children. Participants who used herbs generally used them because they thought they were beneficial, while those who did not use them generally thought they were ineffective.

Study limitations

This study was planned and the permissions were obtained before the pandemic but conducted during the Coronavirus disease-2019 (COVID-19) pandemic conditions. Therefore, the number of participants was limited to 250 due to the closures of the clinics from time to time. Moreover, the variation of participants was mostly from the city, since not many parents made to the city hospital due to the pandemics. Since the study was conducted during the first 6 months of the pandemic, parents' habits might be the same as before pandemics through their children.

CONCLUSION

This study is valuable for public health and clinicians in terms of presenting the data on herbal use applied to children in detail at a university hospital. Most of the parents unconsciously used CAM for their children with the information they learned from relatives, family elders, neighbors, and friends. They did not have enough information about the preparation technique of the herbs, the frequency, duration, and amount of use. As a conclusion, appropriate doses and dosage forms of herbal supplement products with scientifically proven efficacy and safety should be determined for the pediatric population. Parents should be informed about herbs that can be used in

the pediatric population. Due to the pandemic, parents might have turned to natural resources as there is no proven specific treatment for COVID-19 or *vice versa*. However, they might have hesitated to use herbs/herbal products because there is not enough information about the COVID-19 and effects on children. Furthere questionairs/studies should be applied to examine the change in parent's behaviors on herbal usage of their children. Parents should use these products under the supervision of their pediatrician with phytotherapy or natural medicine knowledge, moreover might be consultants of pharmacists.

Ethics

Ethics Committee Approval: The approval was obtained for this study from the Ethics Committee at Gazi University (12.05.2020-E.54135).

Informed Consent: All the participants voluntarily participated in the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: M.M.K., A.T., N.M.K., M.D., U.K.C., Design: M.M.K., A.T., N.M.K., M.D., U.K.C., Data Collection or Processing: M.M.K., A.T., N.M.K., M.D., U.K.C., Analysis or Interpretation: M.M.K., U.K.C., Literature Search: M.M.K., U.K.C., Writing: M.M.K., A.T., N.M.K., M.D., U.K.C.

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ORIGINAL ARTICLE

In Vitro Anti-Leishmanial Activity of Glucosinolate Fraction from *Alyssum linifolium* Steph. ex Willd (Brassicaceae)

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ABSTRACT

Objectives: The intracellular parasitic protozoan, *Leishmania* spp., causes several forms of diseases in humans. Cytotoxicity and emergence of new strains resistance to the current anti-leishmanial drugs have encouraged researchers to focus on new resources. Glucosinolates (GSL) are found mainly in the Brassicaceae family with potential cytotoxic and anti-parasitic properties. The present study reports *in vitro* antileishmanial activity of the GSL fraction from *Alyssum linifolium* seeds against *Leishmania major*.

Materials and Methods: The GSL fraction was prepared by ion-exchange and reversed-phase chromatography. For the assessment of antileishmanial activity, the promastigotes and amastigotes of *L. major* were treated with different concentrations of the fraction (75-625 µg/mL).

Results: The IC_{50} was 245 µg/mL for anti-promastigote effect of the GSL fraction and 250 µg/mL for its anti-amastigote effect that had a significant difference ($p<0.05$) with both glucantime and amphotericin B. The selectivity index of the GSL fraction (15.8), to glucantime and amphotericin B, was greater than 10, indicating the selective effect of this fraction against *L. major* amastigotes. Glucoiberverin was the major constituent of the GSL fraction characterized using nuclear magnetic resonance and electron ionization-mass spectrometry spectra. Based on gas chromatography-mass spectrometry data, iberverin and iberverin nitrile, the hydrolysis constituents from glucoiberverin, included 76.91% of the total seed volatiles.

Conclusion: The results suggest that GSLs like glucoiberverin could be considered a new promising candidate for further studies on antileishmanial activity.

Key words: *Alyssum linifolium*, glucosinolate, Brassicaceae, antileishmanial activity, *Leishmania major*

INTRODUCTION

The genus *Alyssum* contains about 175 species and belongs to the Brassicaceae (previously Cruciferae) family. Its main habitat is in temperate and mountainous regions of Asia, Africa, America, and Europe. 27 *Alyssum* species are distributed in Iran that their seeds are known as "qodume / ku:doʊme/" and are used for medicinal purposes.[1,2] Flax-leaf *Alyssum* (*Alyssum linifolium* Steph. ex Willd.) is an annual weedy plant commonly distributed in semi-arid areas of Iran.[3] Traditionally, mucilage of the plant seeds is used for respiratory diseases such as cough and hoarseness.[4] Several bioactive glucosinolates (GSLs),[5] phenolic compounds[6], and fatty acids[7] have been identified from *Alyssum* species with potential cytotoxic,[5] neuroprotective,[7] anti-parasitic,[8] antioxidant,[5] and antimicrobial[6] activities.

GSLs and their enzymatic hydrolysis products are groups of compounds with sulphur atoms found abundantly in seeds and other parts of Brassicaceae plants. Recently, the promising

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chemoprotective, anti-tumor,[9] anti-parasitic,[10] and biopesticide activities of GSL compounds have been reported.[11]

The anti-parasitic activities of GSLs and their hydrolysis products (such as isothiocyanates) have previously been demonstrated, including the nematicidal[12] and antiprotozoal[13] effects. Based on the literature reviews, no study has yet examined the effect of GSLs against *Leishmania* species. The intracellular parasitic protozoan, *Leishmania* spp. are mostly distributed in North Africa, Middle East, and Central Asia. The female sandflies that are infected by *Leishmania* spp. can disseminate the parasites. It causes several forms of diseases in humans such as cutaneous, mucocutaneous, and visceral leishmaniasis.[14] The major clinical manifestations of leishmaniasis include fever, jaundice, dyspnea, and hematologic disorders.[15] Due to the high cytotoxicity and drug resistance to the available medications for treating leishmaniasis,[16] recent investigations have been focused on the new resources such as active ingredients from medicinal plants.

According to anti-parasitic potential of Brassicaceae plants and the widespread distribution of *Alyssum linifolium* in Iran, we evaluated the anti-leishmanial activity of the GSL fraction from *A. linifolium* seeds in both promastigote and amastigote stages of *Leishmania major* parasites. The purification and identification of the major GSL constituents of *A. linifolium* seeds were performed by chromatographic techniques (ion-exchange and reversed-phase chromatography), electron ionization-mass spectrometry (EI-MS) and nuclear magnetic resonance (NMR) spectroscopy. For further investigation of the volatile constituents in *A. linifolium* seeds (isothiocyanates and nitriles), the gas chromatography-mass spectrometry (GC-MS) method was performed.

MATERIALS AND METHODS

Experimental instruments

Ultraviolet-visible spectrophotometer: PG Instruments (T80+ series, Lutterworth, UK). EI-MS: (Agilent Technologies, Santa Clara, USA). NMR spectroscopy (^{1}H- and ^{13}C-NMR): Bruker Advance III Spectrophometer, (Billerica, MA, USA).

Preparation of glucosinolate fraction and identification of the major glucosinolates

The seeds of *A. linifolium* were collected and authenticated by Dr. Yousef Ajani from Karaj, Iran (May 2019). The voucher herbarium specimen (105795) was deposited at the Research Institute of Forests and Rangelands, Tehran, Iran. The GSL fraction was prepared according to our previous study.[17] Briefly, the ground seeds were extracted with hot methanol and the resulting extract was loaded onto the DEAE Sephadex A-25 column. The sulfatase (S9626, Sigma) solution was added to the column for the desulfation of GSLs. For further purification, the GSL fraction was loaded onto a vacuum-liquid chromatography (VLC) column packed with C18-reversed phase silica gel and eluted with aqueous CH_3CN. The separation of GSLs was achieved using high performance liquid chromatography (HPLC). The data from EI-MS and ^{1}H-^{13}C-NMR spectroscopy were used for the structural analysis.

HPLC analysis

The semi-preparative purification of the desulfo-GSL fraction was performed on Knauer equipment including the WellChrom pump K-1800 and C18 gravity-SB column (250 × 20 mm). Separation of the desulfo-GSLs was carried out using water (A) and acetonitrile (B) programed as follows: 0-1 min, 1% B; 1-25 min, linear gradient up to 22% B; 25-29 min, linear gradient drops down to 1% B; flow rate: 8 mL/min; injection volume: 5 mL; wavelength: 229 nm.[18]

Determination of total glucosinolate content

The quantification was done according to Jezek et al.[19] In this method, GSLs are hydrolyzed by sodium hydroxide to release 1-thioglucose and the latter compound reacts with a potassium ferricyanide solution. The absorbance of the solution was measured at 420 nm using a spectrophotometer. Various concentrations of sinigrin (0-1 mg/mL) were applied for plotting the standard curve.[19]

Volatile fraction preparation

Hydrodistillation was achieved using a Clevenger-type apparatus. *A. linifolium* seeds were used for the extraction of volatile compounds. Hydrodistillation was achieved for 3 h and the volatiles were trapped by pentane. Anhydrous sodium sulfate was used to dry the volatile fraction. The concentrated fraction was analyzed using GC-MS.[20] An Agilent 6890 gas chromatograph consisting of a BPX-5 MS capillary column (30 m × 0.25 mm) was employed under the following conditions; temperature gradient: 50°C: for 5 min, 240°C at 3°C/min, 300°C at 15°C/min; carrier gas: helium, flow rate: 0.5 mL/min. MS mode: EI; detector voltage: 70 eV; mass range: 40 to 500 *m/z*.

Parasite culture

The promastigotes of *L. major* (MRHO/IR/75/ER) were cultured in NNN medium, then sub-cultured in RPMI-1640 medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum, 100 µg streptomycin, and 100 IU penicillin (Gibco, Scotland, UK).[21]

Anti-leishmanial activity

In vitro anti-promastigote activity assay

The evaluation was carried out for 72 hours incubation in 96 well plates.[22] The 2-fold serial dilutions of the GSL fraction were prepared at the concentrations of 15-125 µg/100 µL of RPMI-1640 medium. As positive controls, amphotericin B and glucantime were diluted serially in two-fold steps at concentrations of 0.98-7.8 and 140-18.700 µg *per* 100 µL of RPMI, respectively. Next, 100 µL of each concentration of GSL fraction or standard drugs was added to the wells in triplicate. Finally, 100 µL of RPMI-1640 medium supplemented with 1×10^5 promastigotes, was added to wells (final volume of 200 µL). All above concentrations were calculated *per* mL for IC_{50}. The number of 3 untreated wells containing the parasite was used as the negative control. After incubation (72 h), the amount of 20 µL of each well was mixed with an equal volume of 2% formaldehyde solution in phosphate-buffered saline (PBS). The promastigotes/mL were calculated in a hemocytometer under a light microscope (400× magnification) by mixing 20 µL

of each well content with an equal volume of 2% formaldehyde solution in PBS, pH 7.2. The following formula was used for measuring the death rate (DR):

DR (%) = [(NC - DT)/NC] x100

Here, NC and DT denote the promastigotes number in negative control and treated wells, respectively.

In vitro anti-amastigote assay

The macrophage cell line (murine J774A.1) from the Pasteur Institute (Tehran, Iran) was cultured in RPMI medium and added to a 96 well plate (2 × 10^5 cells/well) containing 200 µL of RPMI medium. After the incubation (5 h), the supernatants were discarded and 200 µL of RPMI containing the promastigotes was added to each well at a ratio of 1:10 (cell:promastigote) and incubated (24 h). RPMI was used for removing free parasites. Then, 200 µL of different concentrations of reference drugs or GSL fraction (as described in promastigotes treatment) was added to each well and incubated (72 h). The number of 3 untreated wells were used as negative controls. Next, the supernatants were removed, and the cells were incubated with 50 mL MTT (Sigma, Lyon, France) solution (stock solution: 5 mg/mL in PBS). After the incubation, dimethyl sulfoxide (100 mL) was added to wells, rotated for 30 min and centrifuged (700 × g, 5 min). The supernatants were transferred to the new plates and scanned at 570 nm for measuring the optical absorbance using a well spectrophotometer (BioTek, Winooski, VT, USA). The cell DR was measured by the formula below: 1-(AT/AC) ×100

where, AT means the absorbance of wells treated with different concentrations of reference drugs or GSL fraction; AC means the absorbance of negative control wells.[22]

Cytotoxicity and selectivity index (SI) evaluation

The DR and SI were determined for each concentration of reference drugs or GSL fraction according to our previous study. The SI >10 represents the safety of the medication.[21]

Statistical analysis

The IC_{50} and CC_{50} were analyzed by GraphPad Prism software (version v6). The IBM SPSS v20 software was used for two-tailed *t*-test analysis.

RESULTS

Identification of the major GSLs

The GSL fraction, obtained from anion exchange chromatography, was purified using VLC. The number of 5 fractions (A1-A5) were resulted. Based on HPLC peaks, the fraction A3 showed two peaks (Figure 1), where its major desulfo-GSL constituent was isolated by semi-preparative HPLC and identified as 3-methyl thiopropyl-GSL (glucoiberverin) (Figure 2) using NMR and EI-MS spectroscopy. ^{1}H-NMR: 1.90 (2H, *m*, CH_2-2'), 2.1 (3H, *s*, S-CH_3), 2.55 (2H, *m*, CH_2-3'), 2.66 (2H, *t*, $J_{1',2'}$ = 7.2, CH_2-1'), 3.2-3.4 (4H, *m*, H-2,3,4,5), 3.58 (1H, *dd*, $J_{5,6a}$= 5.2, $J_{6a,6b}$= 17.6, H-6a), 3.77 (1H, *dd*, $J_{5,6b}$= 3.7, $J_{6a,6b}$= 17.6 H-6b), 4.82 (1H, *d*, $J_{1,2}$= 9.6, H-1). ^{13}C-NMR: 14.56 (C-4'), 26.75 (C-2'), 30.77 (C-3'), 32.97 (C-1'), 61.66 (C-6), 69.98 (C-4), 72.87 (C-2), 78.16 (C-3), 80.24 (C-5), 81.53 (C-1), 152 (C-7). EI-MS: The major mass fragments (*m/z*): 147 (R-N=C=S), 115 (R-CN), 72(CH_2NCS^+), 61 ($CH_3SCH_2^+$), 45 (CH3S$^+$).[23]

Total glucosinolate content

The total GSL content was calculated as 23.28 ± 0.7 mmol equivalent of sinigrin/kg of dry sample by the following equation: y= 0.361x + 0.164, R^2= 0.9916.

Identification of the volatile compounds using GC-MS

Based on the results, 76.91% of the total volatiles were identified as glucoiberverin hydrolysis products (Figure 3). The major compounds were recognized as 4-methyl thiobutyl nitrile (62.91%) and 3-methyl thiopropyl isothiocyanates (iberverin 14.01%), which were characterized by their retention time and MS spectra (Table 1).

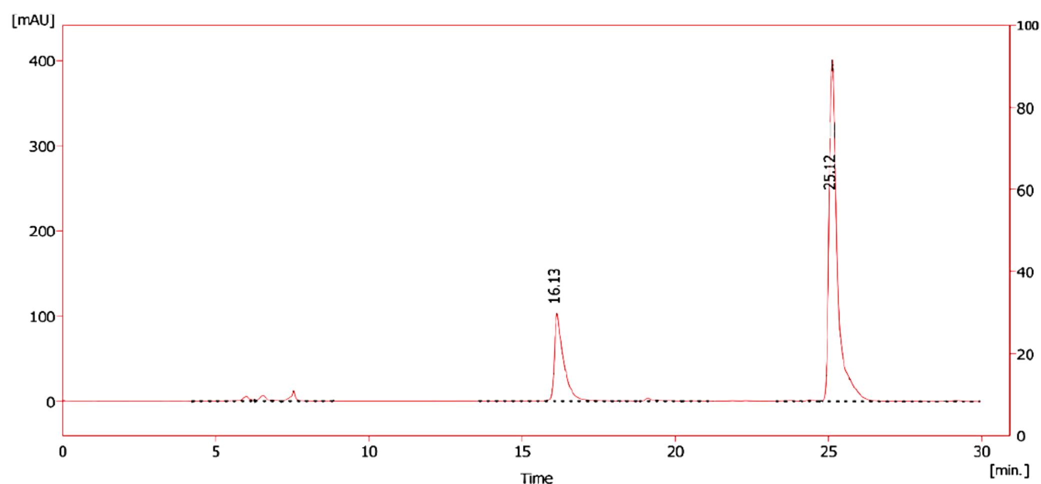

Figure 1. HPLC profile of desulfo-glucosinolates isolated from seeds of *Alyssum linifolium*. The peak in Rt of 25.12 min was identified as glucoiberverin according to NMR and mass spectrometry data

HPLC: High performance liquid chromatography, Rt: Retention time, NMR: Nuclear magnetic resonance

Antileishmanial activity

GSL fraction-treated promastigotes

The highest DR for the GSL fraction was found at a concentration of 75 µg/mL (20%). The concentrations of 39 and 4.9 µg/mL of amphotericin B showed the highest (73.8%) and lowest DR (38.4%), respectively. The highest DR for glucantime was 68.2% at a concentration of 93.500 mg/mL and the lowest was 24.5% at a concentration of 700 µg/mL. A significant difference ($p<0.05$) was observed between the concentrations of the GSL fraction and standard drugs (amphotericin B and glucantime). The IC_{50} of amphotericin B and glucantime were calculated 8 and 95 µg/mL, respectively. This was determined 245 µg/mL for the GSL fraction (Figure 4).

GSL fraction-treated amastigotes

The concentrations of 625 and 75 µg/mL of GSL fraction showed the highest (59.3%) and lowest (31%) death rate, respectively. The highest DR for amphotericin B was 78% at a concentration of 39 mg/mL and the lowest was 41.8% at a concentration of 4.9 µg/mL. For glucantime, the highest and lowest death rates were 70.2% (93.500 µg/mL) and 26.8% (700 µg/mL), respectively. No significant difference was observed between different concentrations of the GSL fraction and standard drugs ($p>0.05$). IC_{50} was also 5.5 µg/mL for amphotericin B, 165 µg/mL for glucantime and 250 µg/mL for GSL fraction (Figure 5).

Cytotoxicity test and selectivity index

Cytotoxic concentration 50 (CC_{50}) and SI were 105 µg/mL and 19.09 for amphotericin B, 2650 µg/mL, and 16.06 for glucantime, and 3950 µg/mL and 15.8 for the GSL fraction, respectively.

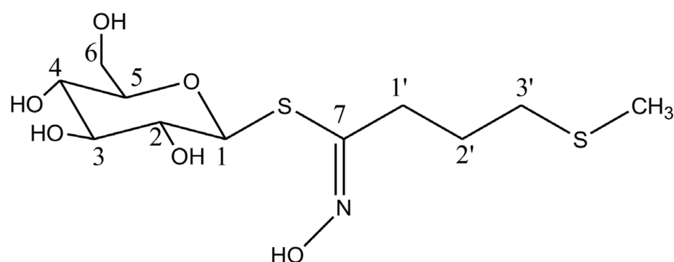

Figure 2. The structure of the purified desulfo-glucoiberverin

DISCUSSION

This study reports the isolation of the main GSL constituent from *A. linifolium* seeds by ion-exchange and reversed-phase chromatography and identification of the isolated compound using EI-MS and NMR spectra for the first time. The major constituent of the GSL fraction was identified as glucoiberverin, a methyl thioglucosinolate, which was previously identified in some other *Alyssum* species such as *A. sibiricum* (as the main GSL constituents) and *A. peltarioides*.[24] NMR and MS data of the isolated compound were confirmed with the published spectroscopic studies.[25,26] A survey of the literature revealed that the methylthio and methylsulphinyl GSLs are found to be the common constituents in *Alyssum* species[27,28] and the present results agree with these findings. The chemical characterization of the volatile fraction from *A.*

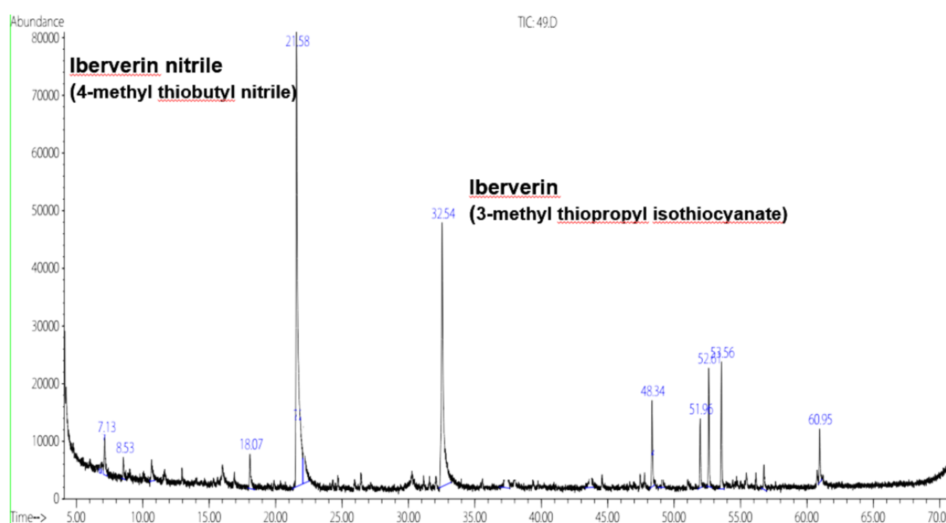

Figure 3. GC chromatogram of the volatile fraction from *Alyssum linifolium* seeds
GC: Gas chromatography

Table 1. ***Alyssum linifolium* seed volatiles identified from GC-MS analysis**

Compounds	R_t (min)	KI	Area (%)	MS spectral data (*m/z*)
4- Methyl thiobutyl nitrile	21.58	1128	62.91	115 (M^+), 74, 68, 61 (bp)
3- Methyl thiopropyl isothiocyanate	32.54	1359	14.01	147 (M^+), 101 (bp), 72, 61, 41

R_t: Retention time, KI: Kovats index, GC-MS: Gas chromatography-mass spectrometry, bp: Is indicated as the base peak (100%)

linifolium seeds showed 4-methyl thiobutyl nitrile (iberverin nitrile, 62.91%) and 3-methyl thiopropyl isothiocyanate (iberverin, 14.01%) that are normally the hydrolysis products of glucoiberverin.[29]

The GSL fraction from *A. linifolium* seeds exhibited a considerable inhibitory effect against *L. major* promastigotes (45.7%) with a significant SI value of 15.8 compared to amphotericin B (19.09). Literature surveys report the anti-parasitic properties of Brassicaceae plants and their GSL constituents.[30,31] Based on Montazeri et al.[8], the hydroalcoholic extracts of some Brassicaceae plants, including *Alyssum homalocarpum*, provided considerable *in vitro* anti-toxoplasma activities. Spectrophotometric determination showed GSLs (24.37 mmol/kg dry weight) in *A. homalocarpum* seeds. Calzada et al.[13] reported that the benzyl GSL isolated from *Lepidium virginicum* (Brassicaceae) root, possessed significant anti-amoebic activity against *Entamoeba histolytica* trophozoites (IC_{50}: 100.1 μg/mL).[13]

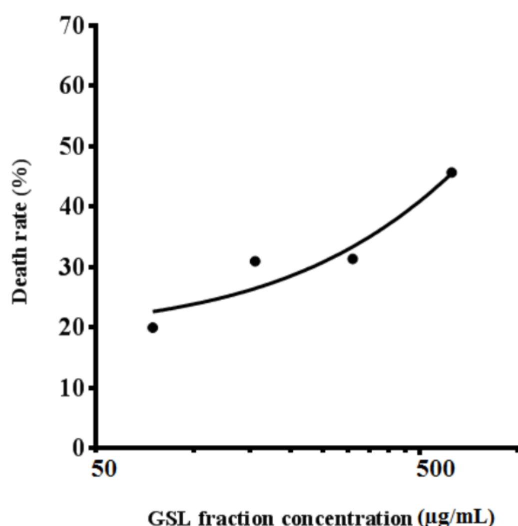

Figure 4. Treated promastigotes with different concentrations of GSL fraction from *Alyssum linifolium* seeds. The resulted IC_{50} was 245 μg/mL
GSL: Glucosinolate

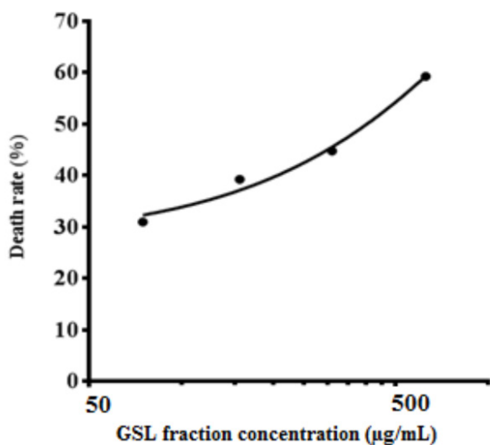

Figure 5. Treated amastigotes with different concentrations of GSL fraction from *Alyssum linifolium* seeds. The resulted IC_{50} was 250 μg/mL
GSL: Glucosinolate

The *in vitro* nematocidal activity of some GSL constituents from seeds of Brassicaceae plants was evaluated by Lazzeri et al.[12] The hydrolysis products of GSLs such as sinigrin, gluconapin, and glucotropaeolin showed lethal effects on the larval stage of *H. schachtii.*

Miltefosine, the first oral treatment of leishmaniasis, induces cell death in parasites by releasing Cyt c from mitochondria.[32] Recent investigations showed that GSLs and their hydrolysis products (isothiocyanates, ITCs) induce apoptosis, mitochondrial release of Cyt c and induce the activation of different cellular proteases such as caspases upon apoptotic stimuli.[33] Therefore, it is hypothesized that GSLs and their hydrolysis products may cause the parasite death through the induction of apoptosis, and this issue needs further investigation.

Infection of cells with *Leishmania donovani* parasites leads to an increase in anti-apoptotic Bcl-2 expression, therefore, the Bcl-2 inhibition results in eliminating of the parasites.[34] Based on Aghaei et al.[35], downregulation of anti-apoptotic Bcl-2 protein in macrophages infected with *Leishmania* parasite, promotes the apoptosis process of infected-macrophages through the changes in the mitochondrial membrane and releasing Cyt c, resulting in the parasite death. The Bcl-2 expression is also altered by aliphatic GSLs such as sulforaphane through the downregulation of anti-apoptotic Bcl-2 proteins.[36] Therefore, this could be considered a potential mechanism of the parasite death induced by GSLs.

Different *Leishmania* species increase the activation of NF-κB in the host cell, resulting in the survival of the parasite and increasing the intracellular parasite burden. Therefore, NF-κB may be regarded as a target for treating leishmaniasis.[37] It has been reported that ITCs of Brassicaceae plants can inhibit NF-κB-mediated inflammatory process[38] and this could be considered a potential antileishmanial effect of *A. linifolium.*

The cell division cycle (CDC) proteins and mitogen-activated protein kinases (MAPK) play important roles in CDC and apoptosis.[39] Based on the recent investigation, the CDC2-related kinase (*CRK3*) genes are involved in the life cycles (promastigote and amastigote stage) of *Leishmania* parasites. It appears that *CRK3* is active in the G2 cell cycle of parasites and regulates the mitosis.[40] It was reported that benzyl isothiocyanate (BITC) and phenethyl ITC decrease the cyclin-dependent kinase 1, CDC2 and CDC25C proteins and suppress G2/M phases to induce apoptosis.[39,41] Therefore, the CRK3 inhibitor could be considered a novel antileishmanial drug.

The involvement of ITCs has been demonstrated in the activation of MAPK protein kinase and induction of apoptosis. Moreover, BITC induce the activation of two important MAPK signaling pathways, JNK and p38 MAPK, and promote apoptosis.[5] Researchers observed that these two signaling pathways are involved in *Leishmania* disease.[42] Moreover, several types of MAPK are involved in the life stages of *Leishmania* parasites.[43] Therefore, ITCs and BITC can be considered the effective anti-leishmanial compounds.

Based on the present results, it seems that GSL fraction from *A. linifolium* seeds might affect the *L. major* parasites through the above pathways.

CONCLUSION

Based on our findings, the GSL fraction from *A. linifolium* seeds have antileishmanial activity against *L. major*. Glucoiberverin was identified as the main GSL constituent of *A. linifolium* seeds. Due to anti-parasitic activities of GSLs, these compounds could be considered promising candidates for developing new antileishmanial drugs. Consequently, it needs more investigations on the inhibitory effects of GSLs such as glucoiberverin against *L. major* in animal models and more studies should be performed to identify the molecular pathways related to the efficacy of these compounds on *Leishmania* parasite.

Ethics

Ethics Committee Approval: IR.MAZUMS.REC.1397.297 and IR.MAZUMS.REC.1398.1069.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: S.S., Design: S.S., M.F., Data Collection or Processing: F.M., R.F., H.K., Analysis or Interpretation: S.S., M.F., Literature Search: F.M., R.F., Writing: F.M., R.F., S.S., H.K.

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ORIGINAL ARTICLE

Resveratrol-Loaded Microsponge Gel for Wound Healing: *In Vitro* and *In Vivo* Characterization

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ABSTRACT

Objectives: The study was aimed to formulate resveratrol (RSV) loaded microsponges to deliver drug at the wound site and incorporate it in the *Moringa oleifera* Lam. (Moringaceae) gel base to provide an appropriate moist environment for wound management. RSV, a stilbenoid that activates sirtuins and cell-signaling regulators involved in the process of wound healing.

Materials and Methods: Microsponges were prepared by oil in oil emulsion solvent diffusion method by optimizing the independent variables; drug: polymer ratio and volume of internal phase solvent and their effects on entrapment efficiency and particle size. Formulation batches were evaluated for drug content, production yield, entrapment efficiency, and *in vitro* drug release. The microsponges were further incorporated into *M. oleifera* gum gel, which was then evaluated for spreadability, viscosity, *ex vivo* diffusion study and *in vivo* studies using an excision wound model in rats.

Results: Scanning electron microscopy revealed spherical and porous nature of the microsponges *in vitro*-release study of the optimized batch of RSV microsponges showed 80.88% drug release within 8 h. Differential scanning calorimetry results revealed no drug and polymer interaction during the formation of microsponges. An *ex vivo* diffusion study through goat skin revealed sustained release of RSV through porous microsponges embedded in the gel base at the wound site. An *in vivo* study performed using an excision wound model showed wound healing and closure within day 8. Histopathology showed increased re-epithelization and reduced ulceration in RSV microsponge gel-treated group compared with sham operated.

Conclusion: RSV microsponge gel delivered the drug at the wound site and the gel base provided a moist environment and influenced cell adhesion, thereby promoting faster wound healing.

Key words: Resveratrol, microsponges, wound healing, *Moringa oleifera* gum, excision wound model

INTRODUCTION

Microsponges are polymeric drug delivery systems composed of porous structure.[1,2] These are tiny porous, sponge-like spherical particles with a surface area of 5 to 150 mm. The major advantages of microsponges are good entrapment efficiency with good stability at high pH and temperature. Due to their porous structure, they can extend the drug release.[3] Emulsion solvent diffusion, suspension polymerization, or oil in oil emulsion solvent diffusion methods are used for the formulation of microsponges.[4] Microsponges encapsulate the drug and this technique of microencapsulation helps control drug release rates and prolong the release time.[5]

To formulate microsponges, one of the preferred polymers is Eudragit RL 100 to control the drug release of the formulation. Eudragit RL 100 is methacrylic acid esters possessing hydrophilic properties due to the presence of more amounts of quaternary ammonium groups compared to Eudragit RS 100. This nature of Eudragit RL 100 helps improve the water uptake capacity, which is required for the rapid absorption of exudates from wound, maintaining its ability to preserve water required for wound healing.[6] The cationic nature of Eudragit RL helps it interact strongly with the negatively charged mucins *via* electrostatic attraction, increasing its bio-adhesivity.[7] Also, Eudragit RL 100 is reported to permit water vapor and oxygen permeation, which is required for wound healing.[8]

Resveratrol (RSV) (3,5,40-trihydroxy-*trans*-stilbene), a natural polyphenolic compound present in grape skin, peanuts, and red wine.[9,10] It belongs to Biopharmaceutical Classification System

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Class II and exhibits low solubility and high permeability.[11] It is a non-flavonoid polyphenolic compound.[12,13] The compound was first isolated from the root of *Reynoutria japonica* Hutt. (previously *Polygonum cuspidatum* Siebold & Zucc.) from Polygonaceae, a plant used in traditional Chinese and Japanese medicine.[14] RSV was studied for its different activities *viz.* anti-inflammatory, immunomodulatory, cardioprotective, antioxidant, anticancer, and for promoting vascular endothelial function.[15,16] It stimulates endothelial nitric oxide (NO) synthase activity and facilitates vascular endothelial growth factor (VEGF) expression,[17] thus providing vascular protection and improving the blood supply.[18]

Lakshmanan et al.[18] and co-workers reported RSV-loaded nanofibrous scaffolds accelerated the wound healing process by regenerating dermal tissue. In a study conducted by Poornima and Korrapati[19], composite nanofibrous scaffolds loaded with RSV and ferulic acid showed 100% wound closure within 15 days. RSV potentiates the activity of antioxidant enzymes superoxide dismutase and glutathione peroxidase and thus has a positive influence on wound healing.[20] RSV-loaded microparticles distributed in collagen laminin matrix scaffold showed improved wound healing without a serious inflammatory response.[21] Zhao et al.[22] and co-workers reported in their study that the direct local application of RSV in dorsal skin wound bed tissue activated AMP-activated protein kinase (AMPK) signaling pathway, resulting in rapid wound healing through effective vascularization.[22] Considering the beneficial effects of RSV in the wound healing process, the study was conducted to formulate RSV microsponges to promote wound healing.[9]

Moringa oleifera Lam. (Moringaceae) gum is natural gum obtained from the exudates of the plant. It is a hydrophilic plant polymer acting as an emulsifier, gelling agent, suspending agent, thickener, and stabilizer.[23] Being a natural gum, it is biocompatible, non-toxic, environmental friendly, and economical to cost and biodegradable in nature.[24] It has antimicrobial activities against various strains of bacteria.[25] Arabinogalactan, a polysaccharide present in the *M. oleifera* gum, has been reported to stimulate cell proliferation, which in turn promotes tissue reepithelialization and reorganization of the tissues at the wound site.[26,27]

The current scenario focuses on treating the wound at an economical cost with easy applicability and no pain to the patient. Most of the microorganisms are resistant toward the current synthetic drug and therefore a need arise to explore natural drugs with minimum dose and maximum effect. The synthetic gel formulations for wound healing cause burning sensation, produce rashes on the skin, and even damage the skin around the wound area. Effective wound healing requires that the active ingredients should be delivered in high concentration at the target site or the contact time of the active ingredient on the surface of the skin or within epidermis should be increased, thereby preventing its penetration inside the skin as well eliminating the skin aliments associated with the synthetic agents. To achieve this, porous microsponges loaded with active ingredients prove to be the best choice,[2] which can be incorporated into gels, creams, lotions, and powders. Microsponges also help modify the drug release rate, thus reducing the frequency of application of the dosage form and improving patient compliance. Controlling the rate of moisture at the wound site locally, through the application of gel is an important criterion required for faster wound healing.[4] Hence, this work was carried out with an aim to formulate RSV-loaded microsponges incorporated into the gel base of *M. oleifera* gum to make the drug available at the wound site.

The hypothesis of the research work is to achieve the dual advantage of sustained release of the drug due to its entrapment, in the porous structure of the microsponges as well as the benefits of *M. oleifera* gel base for efficient wound healing without any irritation and damage to the skin around the wound site.

MATERIALS AND METHODS

Materials

Herbo Nutra Chemical Supplier, New Delhi, India, supplied RSV. Evonik India Pvt. Ltd. Mumbai, India gifted Eudragit RL 100. *M. oleifera* gum were collected from a local market. Magnesium stearate, acetone, *n*-hexane, methyl paraben, propyl paraben, propylene glycol, and triethanolamine (TEA) were purchased from Loba Chemie, Mumbai, India.

Methods

Preparation of microsponges

Oil in oil emulsion solvent diffusion method was used for the preparation of microsponges.[28] The internal phase consisted of RSV, Eudragit RL 100, magnesium stearate (Loba Chemie, Mumbai, India), and acetone (Loba Chemie, Mumbai, India). Appropriate ratios of drug and polymer were dissolved in acetone, magnesium stearate (3% w/v of solvent) was added to it, which was then sonicated in ultrasonic bath 70 kHz frequency for 20 to 25 min (Bio-Techniques, Mumbai, India) to get a homogenous dispersion. Magnesium sterate in appropriate concentration acts as a droplet stabilizer in the formulation of microsponges, it reduces interfacial tension between light liquid paraffin and formed microparticles of Eudragit RL 100 and thus prevents flocculation resulting in the formation of stable discrete microsponges.[29] The obtained internal phase solution was then poured drop wise into cold liquid paraffin (external phase) and stirred at 800 rpm for 1 h using an overhead mechanical stirrer (EMTEK Instruments, Mumbai, India). Lastly, the solidified microsponges were filtered and washed with *n*-hexane to get the porous rigid structure, air dried at room temperature for 12 h and stored in desiccators for further study.[29,30]

Selection of formulation parameters

In the preliminary trials, the effect of formulation parameters; the ratio of drug: polymer (1:2, 1:3, and 1:4), the volume of acetone (5, 7.5 and 10 mL), the volume of light liquid paraffin (15 mL and 30 mL), volume of magnesium stearate (1.5, 3, and 5% w/v of internal phase solvent), and stirring speed (600 and 800 *rpm*) for a period of 90 min were evaluated on the formulation aspect of the microsponges.

Experimental design

Based on the preliminary results, it was found that the concentration of drug: polymer ratio (X1) and volume of internal phase solvent (X2) were the critical parameters governing the drug entrapment efficiency (Y1) and particle size (Y2). To further optimize these parameters, 3^2 full factorial design (Design Expert 11.0., Stat-Ease Inc., Minneapolis) was adopted to optimize the microsponge formulation (Table 1).

Determination of λ_{max} in ultraviolet (UV) spectroscopy

For the preparation of the stock solution (1000 µg/mL), 10 mg RSV was dissolved in 10 mL methanol. Stock solution (0.1 mL) was further diluted with methanol to get 10 µg/mL. The spectrum was scanned over the range of 200-400 nm. The standard calibration curve for RSV was then plotted at different drug concentrations (0.2-10 µg/mL) absorbance was measured using UV spectroscopy.

Characterization of microsponges

Production yield

Production yield was calculated using equation (1) and carried out in triplicate.[31]

$$Production\ yield = \frac{Practical\ mass\ of\ microsponges}{Therotical\ mass\ (polymer + drug)} \times 100 \quad (1)$$

Drug content and entrapment efficiency

RSV microsponges (10 mg) were dispersed in methanol (5 mL), followed by shaking for 10 min using vortex mixer and the final volume made of 10 mL using methanol. The resulted solution was filtered, diluted and the concentration of RSV was determined spectrophotometrically using a UV spectrophotometer (1800, Shimadzu, Japan) at λ_{max} 305.80 nm against a blank (methanol),[32] and tests were performed in triplicate using equations (2) and (3):

$$Drug\ content = \frac{Amount\ of\ drug\ in\ microsponges}{Amount of Microsponges} \times 100 \quad (2)$$

$$Drug\ entrapment\ efficiency = \frac{Actual\ drug\ content}{Theoretical\ drug\ content} \times 100 \quad (3)$$

Particle size

The particle size of RSV loaded microsponges was evaluated by optical microscope and repeated thrice to calculate the mean particle size. Approximately 50 microsponge particles were randomly measured. Edmondson's equation was used to estimate the average particle size of the microsponges:

$$Dmean = \sum \frac{nd}{n} \quad (4)$$

Where n: number of microsponges counted

D: Mean size range

In vitro drug release study

In vitro release studies of RSV-loaded microsponges were carried out in USP dissolution test apparatus (type II paddle) (Electrolab India PVT.LTD). An accurately weighed amount of RSV loaded microsponges (100 mg) were placed in the dissolution test apparatus containing 900 mL phosphate buffer pH 7.4 maintained at 37 ± 0.5°C. An aliquot (2 mL) of sample was withdrawn at definite time intervals for a predetermined time of 8 h. The samples were analyzed for the drug content spectrophotometrically at λ_{max} 305.80 nm. Each time after withdrawal of the sample, the aliquots were replaced with the same buffer solution pH to maintain sink condition.[30]

Scanning electron microscopy (SEM) and differential scanning calorimetric analysis of RSV microsponges

The morphology of the RSV microsponges was assessed by SEM (JEOL, JSM-6360-A, Tokyo, Japan)[33,34] and the thermal analysis was carried out using Differential Scanning Calorimeter (Mettler Star SW 12.10, Mumbai, India).[35]

Table 1. Formulation of microsponges using 3^2 factorial design

Formulation code	Drug: polymer (X_1)	Acetone volume (mL) (X_2)	Magnesium stearate (mg)	Actual drug content (%)	Production yield (%)	Entrapment efficiency (%) (Y_1)	Particle size (µm) (Y_2)
F1	3	7.5	225	35.12 ± 1.43	66.58 ± 1.41	72.71 ± 1.92	547 ± 1.50
F2	2	5	225	27.12 ± 1.43	56.11 ± 0.76	59.36 ± 1.35	564 ± 1.80
F3	2	10	150	23.31 ± 1.25	82.13 ± 1.38	88.27 ± 0.88	432 ± 1.77
F4	4	7.5	300	36.95 ± 0.96	74.55 ± 2.48	79.55 ± 1.29	581 ± 1.94
F5	3	10	300	34.32 ± 2.32	84.04 ± 1.72	87.93 ± 2.36	460 ± 2.47
F6	4	5	150	38.61 ± 1.75	62.93 ± 1.45	68.26 ± 1.34	586 ± 1.03
F7	2	7.5	300	25.09 ± 1.26	70.89 ± 1.2	75.2 ± 1.50	518 ± 2.55
F8	3	5	225	36.25 ± 1.34	60.42 ± 1.62	65.13 ± 1.11	576 ± 3.01
F9	4	10	150	36.05 ± 1.4	87.41 ± 1.17	91.75 ± 1.69	481 ± 0.90

Each value is the mean of three observations

Formulation of microsponge-loaded gel

M. oleifera gum (4%, w/w) was soaked in water for a period of 24 h, followed by drying at room temperature. The dried gum was washed with acetone to remove any impurities and then passed through sieve number 45. The purified powdered *M. oleifera* gum was soaked in water and the mixture was stirred at 600 rpm to obtain a uniform dispersion.[23] To the obtained homogenous dispersion, methyl paraben (1%), propyl paraben (0.05%), and propylene glycol (5%), were added. The pH of the *M. oleifera* gel base was adjusted by slow addition of triethanolamine, followed by the incorporation of RSV microsponges into the gel base. Microsponges equivalent to 4% (w/w) of RSV were added into the three batches of the gel base.

Characterization of microsponge-loaded gel

The gel formulations were characterized for their visual appearance, color, odor, and pH.[36]

Measurement of pH

The pH of the RSV microsponge gel was evaluated with a digital pH meter (Digital Instrument Corporation, Ahmedabad, Gujrat). The readings were recorded as the mean of three readings.[37]

Measurement of viscosity

Brookfield Viscometer (Brookfield Engineering Corporation, Ametek, Mumbai, India) was used to measure the viscosity of the RSV microsponge gel. The gel was placed in a beaker and viscosity was measured using spindle number 64 at 10 rpm after a period of three minutes. The readings were recorded as the mean of three readings.[38]

Texture profile analysis

A Texture Analyzer (CTX, Brookfield, Ametek, Mumbai, India) was used to conduct the texture profile analysis of RSV-loaded microsponge gel. The sample holder of a cone analytical probe (TA3/100) (30 mm diameter, 60°C) was completely filled with gel, followed by forcing the cone down into sample holder (1 mm/s and depth of 10 mm). Once, the trigger force of 5 g was achieved, the cone started to pierce the sample at a speed of 2 mm/s to a depth of 25 mm. After achieving the penetration up to a specified distance, the probe (cone) departed from the sample. The obtained force-time plot was used to determine the hardness and adhesiveness of the gel.[39]

Ex vivo diffusion study

The goat skin membrane was obtained from a local slaughter house and washed with water, scalpel was used to remove non-dermatome skin. Then, the skin membrane was soaked in phosphate buffer pH 7.4 for 24 h and then placed on the Franz diffusion cell (DBK diffusion cell, Peliyagoda, India). A predetermined amount of microsponges loaded gel (1 g) was placed on the donor side. The receptor medium was filled with phosphate buffer pH 7.4, maintained at 37 ± 0.5°C and continuously stirred at 300 rpm. To assess the amount of drug diffused, samples (2 mL) were collected from the receiver compartment at specific time intervals. An equal volume of fresh phosphate buffer pH 7.4 solution was used to replace the solution to maintain the sink condition. Collected samples were evaluated by UV spectrophotometer at λ_{max} 305.80 nm. The readings were recorded as the mean of three readings.[35,36,40,41]

In vivo studies

Healthy Albino Wistar rats of either sex weighing between 180 and 220 g were used for the study. The study protocol was approved by the Institutional Animal Ethics Committee (approval no: 1249/P0/Re/S/09/CPCSEA) and IAEC protocol number RSCPR/IAEC/2019/06. Animals were procured from the National Institute of Bioscience, Pune and house in the animal house of JSPM's Rajarshi Shahu College of Pharmacy and Research, Tathwade, Pune, maintained at 10-12 h light and dark cycle, provided with 23 ± 2°C temperature and 44-50% humidity with food and water *ad libitum* during the study.

Excision wound model

The rats were divided into four groups containing 6 rats each (n: 6). Group I sham-operated group, group II represents the placebo control group received *M. oleifera* gel base, group III is the treatment control group received RSV-loaded microsponge gel and group IV represents the standard control group received Megaheal gel-colloidal silver (ARISTO Pharmaceuticals Pvt. Ltd, Mumbai, India).

The dorsal furs of animals were removed using depilatory cream [Veet hair removal cream, Reckitt Benckiser (India) Ltd.] and anesthetized using pentobarbital sodium (40 mg/kg, *i.p.*, body weight). An impression was made and wound approximately 12 mm and 2 mm depth of full thickness was marked and created using forceps, surgical blade, and scissors.[42,43]

Treatments were started immediately after creating the wound on day 0 by daily application of above-mentioned gel formulations on the wounded area. Day 0 is considered as a wounding day, when the wound was created first. One g of each formulated gels and marketed gel mentioned above was applied once daily from day 0 until complete healing to the respective groups.

Measurements of wound contraction

The wounded area was monitored and measured using vernier caliper and the percentage contraction after every 4th day was calculated. The initial size of the wound was considered 100% using the following equation (4).[42]

$$\%\,Wound\ contraction = \frac{Initial\ wound\ area - Specific\ day\ wound\ area}{Initial\ wound\ area} \times 100 \quad (5)$$

Histology of wound granulating tissue

All the rats were anesthetized and specimen samples from the healed wound tissues were collected from each group and stored in 10% (v/v) formalin solution to conduct the histological examination.[44]

Statistical analysis

All results of wound closure were presented as mean ± standard deviation and analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test using Graph Pad Prism 5.0 to determine the statistical significance ($p \leq 0.001$) were considered as statistically significant.

RESULTS AND DISCUSSION

Preparation of microsponges

Oil in oil emulsion solvent diffusion method was used for formulating the microsponges. The results from the preliminary trial batches indicated the appropriate parameters required for the formulation of microsponges were the concentration of light liquid paraffin (30 mL), concentration of magnesium stearate (3%, w/v) and stirring speed of 800 rpm for a period of 90 min. The critical parameters affecting the drug entrapment efficiency (Y1) and the particle size (Y2) of the microsponges were identified as drug: polymer ratio (X1) and the volume of solvent (acetone) as an internal phase (X2), which were further optimized using the 3^2 full factorial design. An optimization technique was used to get desired concentration of drug: polymer ratio and volume of internal phase solvent to formulate microsponges with desired characteristics as shown in Table 1.

Determination of λ_{max} in UV

A solution of 10 µg/mL of RSV in methanol was scanned in the range of 200-400 nm. UV scan of the drug is shown below (Figure 1). The λ_{max} of the RSV was found to be 305.80 nm. The calibration curve for RSV was linear in the range of 2-10 µg/mL confirming the Beer-Lambert's law with the regression co-efficient value (0.9994)

Drug entrapment efficiency

The drug entrapment efficiency of RSV-loaded microsponge formulations ranged from 59.36 ± 1.35% to 91.75 ± 1.69%.

Formulation F9 showed an entrapment efficiency of 91.75%. The ratio of polymer concentration and the volume of acetone in the formulation F9 was 1:4 and 10 mL, respectively. With the increase in the concentration of polymer, entrapment efficiency was increased due to more amount of polymer available to entrap the drug. Also, as the volume of the internal phase was increased, viscosity was decreased, resulting in uniform mixing of drug and polymer, forming a matrix that in turn enhanced the drug entrapment efficiency of the microsponges. As the concentration of polymer and the volume of the internal phase decreased, entrapment efficiency also decreased and was found to be 59.36%.

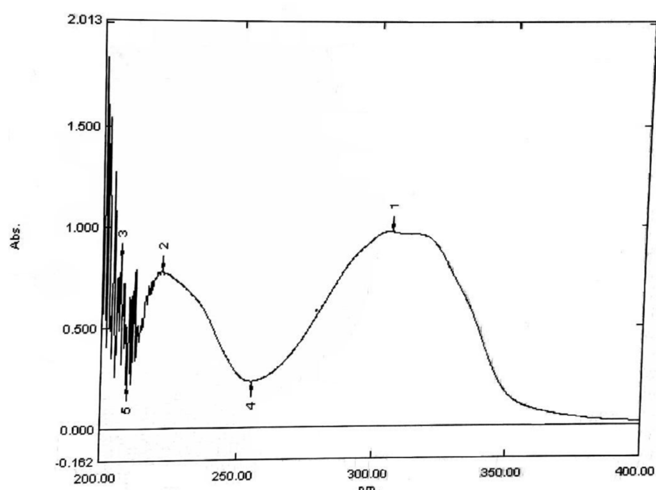

Figure 1. Ultraviolet spectrum of resveratrol

The statistical analysis by Design-Expert software (Design Expert 11.0., Stat-Ease Inc., Minneapolis) indicated the effect of factors influencing the entrapment efficiency. The effect of both independent variables (X1 and X2) on entrapment efficiency (Y1) was given by the following equation:

$$Y1 = 30.48944 + 2.80X1 + 5.00867X2 \quad (6)$$

A linear regression equation was obtained for the response of drug entrapment efficiency (Y1), which indicated a positive effect of X1 and X2 on the production of microsponges with a good fit R^2 value 0.979 and significant F value 103.63 and p value <0.0001.

From equation 5 and Figure 2a; the factor X1 showed a positive value, indicating a high drug entrapment efficiency in microsponges with the higher concentration of polymer. Also, with the increase in the polymer concentration, the diffusion rate of internal solvent from the microsponges decreased. This in turn led to the formation of a concentrated solution resulting in more time for droplet formation with increased precipitation of the drug in microsponges leading to increased entrapment efficiency.

A positive effect of X2 was observed on entrapment efficiency. The high volume of internal solvent was attributed to better solubilization of the drug in the internal solvent, resulting in enhanced entrapment efficiency of the microsponges.

Particle size

Polymer concentration and volume of the internal phase solvent are the major attributes determining the particle size of microsponges. The particle size of the microsponge formulation ranged from 432 to 586 µm.

The decrease in particle size could be attributed to the viscosity of the emulsion formed during processing. With the increase in the volume of the internal phase, the viscosity of emulsion decreased, resulting in reduction in globule size of emulsion droplets, leading to the formation of smaller particles and *vice versa*.[45]

The effect of (X1) and (X2) on the particle size of microsponges was explained by the following equation:

$$Y2 = +549.11 + 22.33X1 - 58.53X2 + 6.75X1X2 - 0.6667X_1^2 - 32.17X_2^2 \quad (7)$$

Equation (6) is a quadratic regression equation for the response Y2 with R^2 value 0.9899 and significant F value 59.08, p value <0.0034.

According to equation (6) and Figure 2; X1 showed a positive influence on particle size due to higher concentration of polymer, resulting in increased particle size due to more amount of the polymer available to entrap the drug. X2 exhibited negative influence on particle size indicating that, with the increase in the volume of internal phase, the viscosity of emulsion decreased, resulting in a reduction in globule size of emulsion droplets,

leading to the formation of smaller particles.

The interaction terms, *e.g.* X1 and X2, indicating the combined effect on the concentration of polymer and volume of internal solvent, have a positive effect on particle size. The reason for this could be an increase in the viscosity of the internal solvent. It was observed that with the increase in the concentration of polymer, the emulsion globules can hardly be subdivided into smaller particles.[32] The individual and the combined effects of the factors X1 and X2 on the response Y1 and Y2 responses are explained in the 3D surface graphs (Figure 2).

Optimization of microsponge formulation

The numerical optimization method was used to optimize the microsponge formulation. The desirability plot obtained indicates the optimum conditions needed for the formulation of microsponges with desired attributes. The optimized formulation (F5) showed minimal particle size (460 μm), higher drug entrapment efficiency (87.93 ± 2.369%) with desirability value of 0.915577.

SEM of RSV microsponges

SEM of the optimized batch of RSV microsponges (F5) showed uniform, spherical shape particle at 35X magnification, and a porous surface at 3000X magnification (Figure 3).

Differential scanning calorimetry

RSV exhibited a single sharp endothermic peak at 263˚C. RSV-loaded microsponges of batch F5 showed a broad endothermic peak at 215.90˚C and physical mixture of RSV and Eudragit RL 100 at 247˚C, respectively, Figure 4. RSV peak was not observed in RSV-loaded microsponge formulation due to encapsulation of the drug in matrix form of microsponges.[46] These results demonstrated no interaction between drug and polymer during the formation of microsponges.

In vitro drug release study of microsponges

In vitro drug release of formulations F4, F6, and F9 containing high polymer concentrations showed delayed release of drug (84.54%, 82.25%, and 79.05%, respectively within, 8 h). Due to the high amount of polymer, the escape of drug required more time to escape from the pores of the microsponge. Low concentrations of polymer in formulation F2, F3, and F7 resulted in rapid release of drug (96.87%, 94.59%, and 90.48% within 8 h) from microsponges. This fast drug release at the wound site was not satisfactory as sustained release of the drug is desirable for wound healing.[47] Formulation F1, F5, and F8 released 90.02%, 80.88%, and 86.82% drug within 8 h from the microsponges. Formulation F5 showed sustained

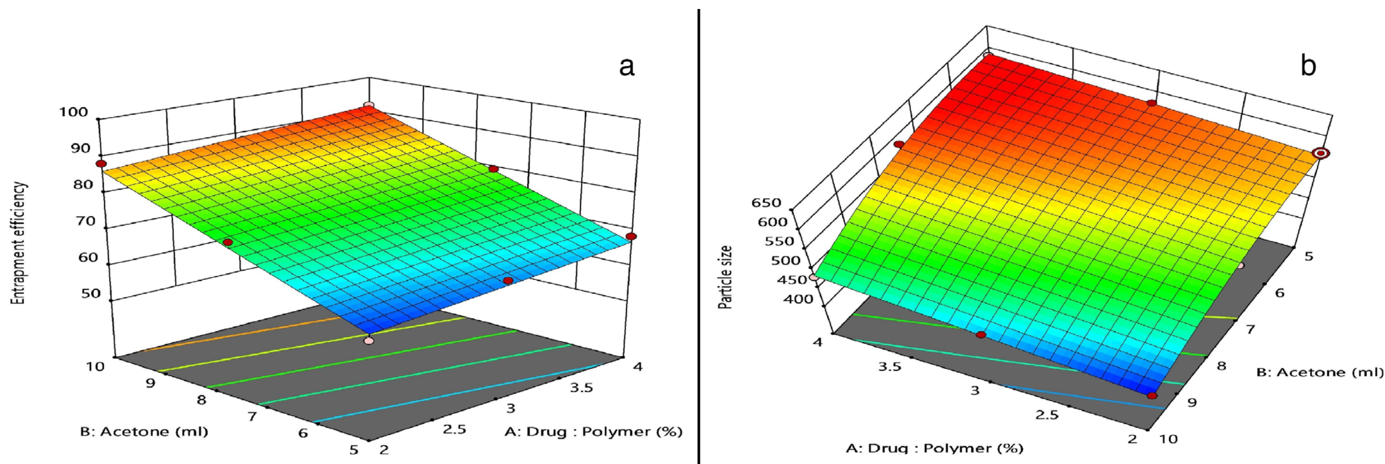

Figure 2. Three dimensional surface plots of (a) entrapment efficiency, (b) particle size

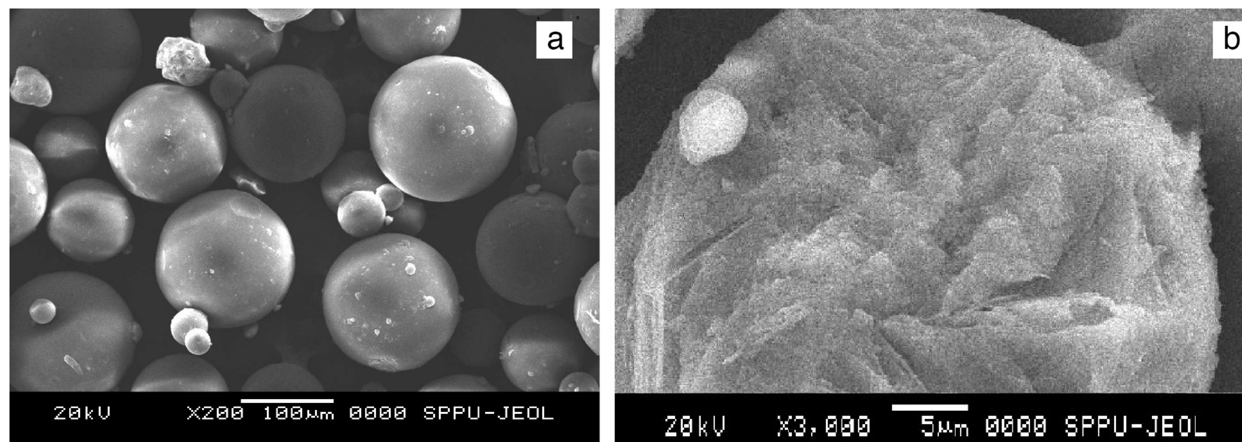

Figure 3. Scanning electron microscopy of resveratrol microsponges (a) 200x magnification, (b) 3000x magnification

drug release; thus, selected for further study in Figure 5. As the formulation F5 had a higher amount of drug: polymer ratio. The greater number of binding sites on the surface of Eudragit RL 100 was available due to the presence of ammonium groups leading to the stronger interaction of the drug with the polymer, which in turn prolonged the drug release.[48]

Evaluation of RSV microsponge-loaded M. oleifera gel

RSV microsponge-loaded *M. oleifera gel* was smooth, free from grainy particles with a pH close to 7.2 similar to wound pH,[49] indicating easy applicability of the gel to the wound site without any discomfort and irritation to the patient after application on wound area. The appropriate viscosity 15392 ± 5.567 exhibited by the gel indicated the easy of applicability and retention of gel at the wound site. Texture profile analysis of gel showed hardness (firmness) 273 g, adhesiveness 1.4 mJ, and adhesive force 79 g, Figure 6 indicating the easy spreadability of RSV-loaded microsponge gel. The *in vitro* drug diffusion studies of RSV microsponge-loaded *M. oleifera* gel showed 23.17% release in 3 h, 57.97% in 6 h and 68.98% in 8 h, respectively, thus indicating sustained release of RSV through the gel matrix of the polymer. The reason for this could be the swelling of the gel, which increased the diffusional path length, thereby sustaining the drug release.

In vivo study

Wound closure and contraction were assessed by image analysis visually (Figure 7). The wound area and % contraction after topical application of all gel formulations (*M. oleifera* gel base, RSV loaded microsponge gel, and standard gel) are presented in Table 2.

During treatment, the formulations were found to show its

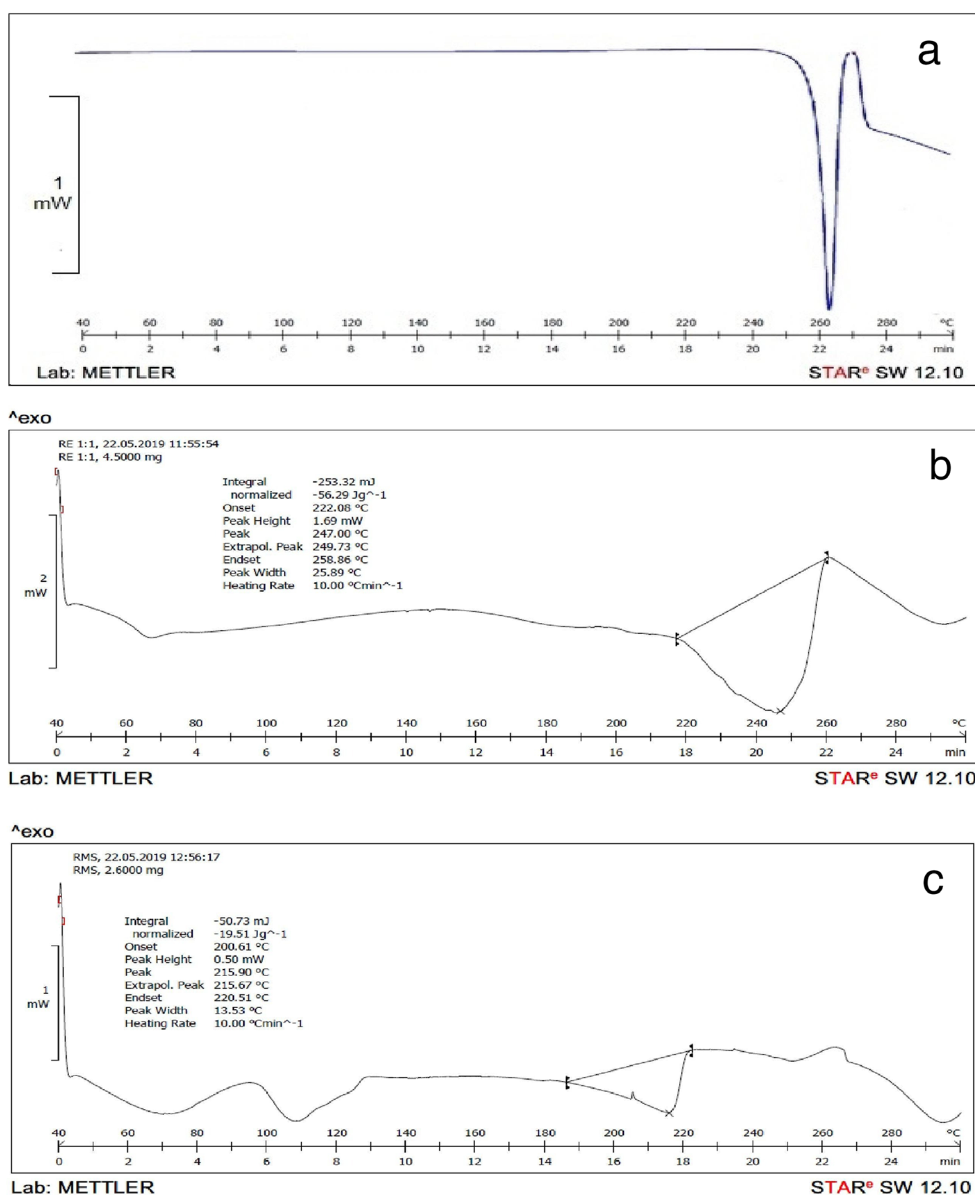

Figure 4. Differential scanning calorimetry thermograms of (a) resveratrol, (b) physical mixture, and (c) resveratrol loaded microsponges

preliminary effect from day 4 to day 16 (Figure 7). Group I (sham-operated) showed a slight wound healing even after 16 days of study. The percent wound closure of group I on days 0, 4, 8, 12, and 16 was 0%, 4.41%, 6.08%, 10.25%, and 17.66%, respectively. Group II referred as placebo-treated group received (*M. oleifera* gel base) exerted significant ($p<0.001$) increase in % wound closure on days 4, 8, 12, and 16 which was found to be 14.83%, 30.41%, 37.41%, and 45.83%, respectively, compared with sham-operated group. After a wound, the physiological reparative process of the body initiates, which involves movement of adjacent epithelial cells to the injured area. Arabinogalactan, a polysaccharide present in *M. oleifera*, is reported to influence this integrins recognition, thus influencing cell adhesion and affecting the healing process. The cell adhesion and the higher replication ability of the epithelial cells led to a faster stratification of the tissue and faster wound healing. Hence, on day 16, the percent wound closure in group II (*M. oleifera* gel base) was 45.83% compared to group I (sham-operated), which was 17.66% ($p<0.001$).

Gel containing RSV microsponges (group III) showed its maximum significant effect ($p<0.001$) by wound contraction with respect to the sham-operated group that proportionally confers the healing process and showed similar contraction as that of the standard control (group IV) on days 12 and 16. As *per* the rate of epithelization concerned, the RSV-loaded microsponge gel displayed its contributory role in accelerating epithelization ($p<0.001$) as compared to sham-operated group I and standard control group IV.

Many researchers have reported the role of polyphenolic compounds in promoting wound healing. The important events involved in the wound healing process are inflammation; cell proliferation and cell migration. Sirtuins (SIRTs) are NAD^+-dependent histone deacetylases which exhibit anti-inflammatory activity and stimulate cell proliferation and cell migration.[50] RSV acts as an activator of SIRTs, which act as one of the therapeutic strategies to enhance, wound healing. The activation of SIRTs by RSV suppresses the stimulation of TNF-α, which is an important cytokine causing inflammation and inactivates nuclear factor-κB, a transcription factor that is a major regulator of proinflammatory cytokine expression thus exhibiting anti-inflammatory effects.[51,52] The activation of SIRTs by RSV enhances the production of nitric oxide, which is involved in re-epithelialization, neovascularization, and collagen synthesis.[53] NO production also accelerates wound closure by recreation of keratinocyte proliferation.[54]

Group III showed significant ($p<0.001$) increase in percent wound closure at days 4, 8, 12, and 16, which was found to be 70.58%, 98.33%, 99.33%, and 99.41%, respectively, when compared with sham operated and standard control group. Reason for this could be the *M. oleifera* gel base, which provided suitable moist environment required for wound management. In the moist

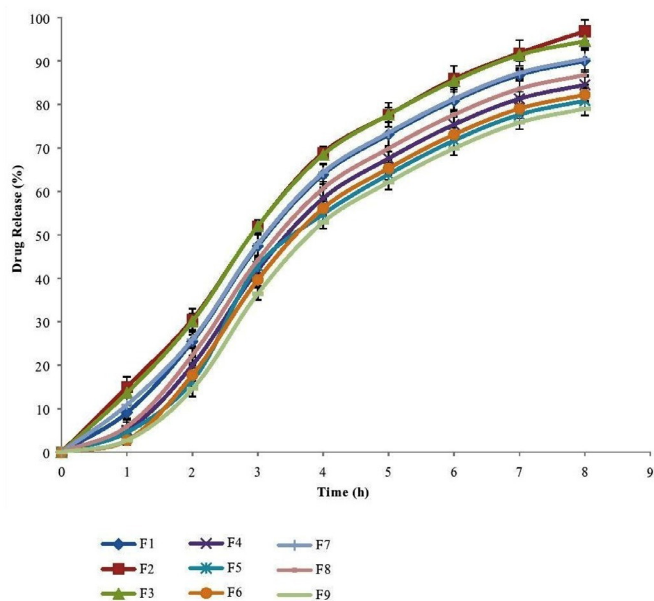

Figure 5. *In vitro* drug release of resveratrol-loaded microsponges

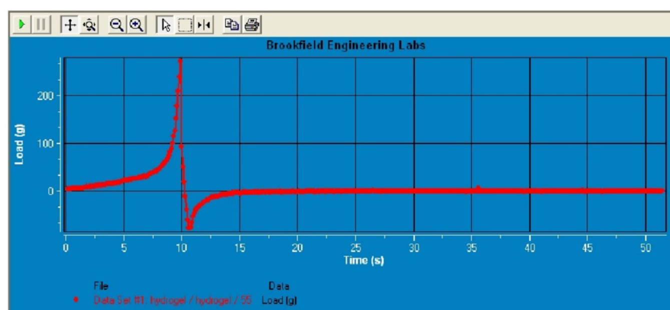

Figure 6. Spreadability of resveratrol-loaded microsponge gel

Table 2. Effect of resveratrol loaded microsponge gel on wound closure and % wound contraction in excision wound model

	Group I		Group II		Group III		Group IV	
	Area (cm)	% contraction	Area (cm)	% contraction	Area (cm)	% contraction	Area (cm)	% contraction
Day 0	1.200 ± 0.008	0	1.200 ± 0.007	0	1.200 ± 0.003	0	1.200 ± 0.008	0
Day 4	1.147 ± 0.032	4.41	1.022 ± 0.008***	14.83	0.353 ± 0.009***	70.58	0.194 ± 0.006***	83.83
Day 8	1.127 ± 0.040	6.08	0.835 ± 0.009***	30.41	0.020 ± 0.009***	98.33	0.008 ± 0.008***	99.33
Day 12	1.077 ± 0.061	10.25	0.751 ± 0.024***	37.41	0.008 ± 0.009***	99.33	0.007 ± 0.008***	99.41
Day 16	0.988 ± 0.015	17.66	0.650 ± 0.007***	45.83	0.007 ± 0.008***	99.41	0.008 ± 0.008***	99.33

All results of wound closure were presented as mean ± standard deviation. ***($p<0.001$) to determine statistical significance

environment the epithelial cells migrate more readily compared to dry ones; also the growth factors are active, readily available, and synthesized in moist environments.[4] Arabinogalactan, a polysaccharide present in the *M. oleifera* gum, has been reported to stimulate cell proliferation, which in turn promotes tissue re-epithelialization and reorganization of the tissues at the wound site, thus, promoting faster wound healing.[26,27] The prolonged release of RSV through gel and further through microsponges formulated with Eudragit RL 100 indicated the retention of the drug in the porous structure of microsponges to slowly release it at the target site to heal wound. The mucoadhesive and the hydrophilic properties of Eudragit RL 100 permitted the retention of the RSV microsponges at the wound site, ensuring the availability of higher concentration of drug at the wound area of the skin as well as maintaining its ability to preserve the moisture required for wound healing. The solubility of RSV in phosphate buffer pH 7.4 might have also favored faster drug diffusion in wound area to achieve faster healing. Hence, the topical application of gel containing RSV microsponges in an excision wound model in rats successfully closed and healed the wound within day 8. It was observed that on day 4, drying of the wound was observed and the wound area was reduced to 0.353 ± 0.009 in group III. It was further observed that, on day 8, the wound was completely dried and the wound area was further reduced to 0.020 ± 0.009. In control sham group I, the area of the wound was 1.127 ± 0.040 even on day 8. In the present study, RSV-loaded microsponge gel showed prolonged drug release and retain on the skin to accelerate the wound healing process followed by wound contraction within 8-12 days, by reducing scar formation. Thus, fulfilling the hypothesis of the use of microsponge technology, where a higher concentration of entrapped RSV is available at the wound area of the skin with

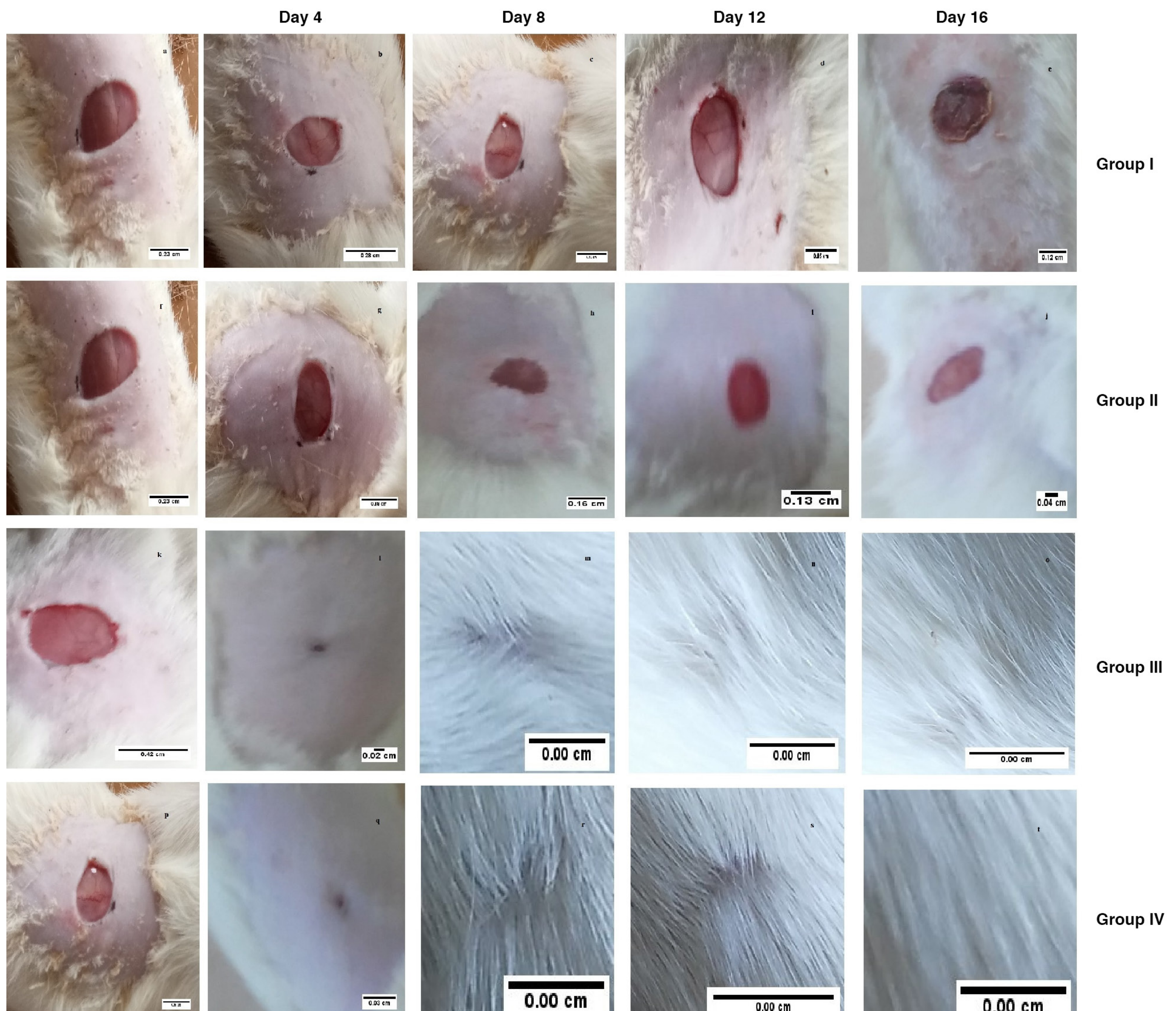

Figure 7. Photographs of wounds in rats in group I (sham-operated): (a) wound created on day 0, (b) wound on day 4, (c) wound on day 8, (d) wound on day 12 and (e) wound on day 16; group II (placebo): (f) wound created on day 0, (g) wound on day 4, (h) wound on day, (i) wound on day

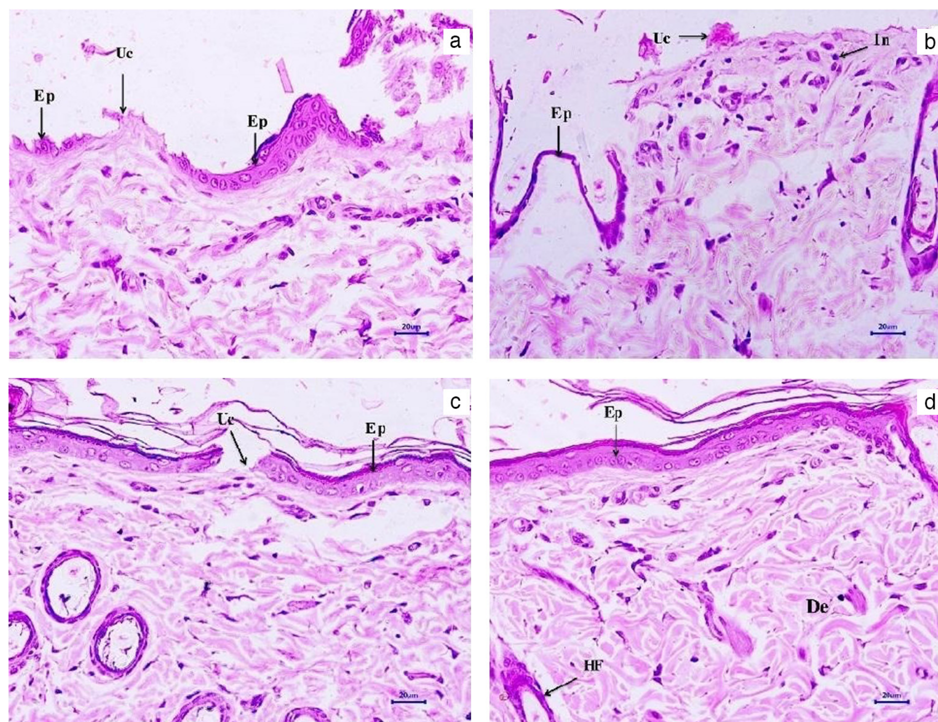

Figure 8. Histopathological sections of wound on day 16 of (a). Sham-operated, (b) placebo, (c) resveratrol-loaded microsponge gel, (d) standard gel

sustain release and the benefits of *M. oleifera* gel for efficient wound healing with no irritation and damage to the skin around the wound.

Histopathology

A histopathological study was conducted to observe any pathological changes in rat skin during application of formulated gels. The tissue samples of excision wounds were given for histopathological studies on day 16 (Figures 8a-d).

Platelet coagulation, cytokine and pro-inflammatory mediators, erythrocytes, blood vessels, fibroblast, mast cell, fibrin thread, ulceration, and development of newly formed epithelium in the sham-operated group (group I) was observed Figure 8b. In the placebo, treated skin (group II) showed slight ulceration and inflammation. Start of fibroplasia-collagen synthesis and deposition and arrival of neutrophils, macrophages, and slight formation of epithelial bridge with newly formed epithelium were observed Figure 8c. Group III showed a rapid reduction in ulceration with reduced inflammatory cells, fibroplasia-collagen synthesis, granulation tissue formation, matrix formation, and collagen fiber deposition, regenerated epithelium. The integrity of the basement membrane was preserved. Healing by a process of epithelization was observed in group III Figure 8c. In the standard group IV, Figure 8d reveals epithelization with angiogenesis, regeneration of the epidermis, dermis, hair follicles, and reduced inflammatory cells with no ulceration. Collagen fibrin cross linking and scar maturation was seen.

These results are in accordance with the previous studies, stating that RSV increases the synthesis of collagen fibers, increases granulation by enhancing angiogenesis, scar formation, and improvement in wound healing.[9,55,56]

CONCLUSION

In conclusion, RSV-loaded microsponge gel was successfully formulated and developed for wound healing. Microsponges were prepared by oil in oil emulsion solvent diffusion method using Eudragit RL 100. Microsponges-released drug in a sustained manner at the wound site through pores and *M. oleifera* gum gel provided a moist environment in the later stages of wound healing. This unique combination of RSV-loaded microsponges in *M. oleifera* gel demonstrated rapid wound healing and could be considered as easy to apply with no pain dosage form and a potential alternative to the current synthetic agents used for treating wound healing, thus improving the patient compliance and reducing the global burden of wound care.

Ethics

Ethics Committee Approval: The study protocol was approved by the Institutional Animal Ethics Committee (approval no: 1249/P0/Re/S/09/CPCSEA) and IAEC protocol number RSCPR/IAEC/2019/06.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: D.A., Concept: V.P., Design: V.P., Data Collection or Processing: V.P., Analysis or Interpretation: V.P., Literature Search: V.P., S.C., Writing: V.P., S.C.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Investigation of Antimicrobial Activity of Some Ethylparaben Hydrazide-Hydrazone Derivatives

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ABSTRACT

Objectives: The development of antimicrobial molecules discussed with considerable achievement over the past decades provided many classes of semisynthetic or synthetic compounds. Resistance to many antimicrobial agents requires the discovery of novel molecules.

Materials and Methods: In this study, ten ethylparaben hydrazide-hydrazone derivatives, the previously reported, were evaluated for their *in vitro* antibacterial and antifungal activities. The microbroth dilution method was used for the determination of the minimum inhibitory concentration (MIC) values of the novel molecules.

Results: The antimicrobial activities of the molecules were found in a wide range with MIC values of 2-256 µg/mL. The synthesized compounds showed good to moderate antimicrobial activity compared with the standards. Among the synthesized molecules, compound 3g showed the best antimicrobial activity at 2 µg/mL against *Staphylococcus aureus* strain (ATCC 29213).

Conclusion: Ethylparaben hydrazide-hydrazone compounds in our study were found to have antimicrobial activities. Ethylparaben is currently used as an antibacterial agent and preservative for preparations. These studies are necessary since they detect the relationship between the substitutions and activity.

Key words: Antimicrobial activity, ethylparaben, hydrazide-hydrazone, microbroth dilution method, *in vitro*

INTRODUCTION

Since the beginning of the last century, various antimicrobial molecules have been systematically introduced for use, both experimentally and by trial and error. Because of the exceptional genetic plasticity of the microorganisms, misuse, and world population, resistance to bacterial strains has appeared and has radiated throughout the world.[1] Today, antibiotic resistance has become a big clinical and public health problem and resistance rates are climbing dangerously worldwide. Meanwhile, minimizing toxicity and development of drug resistance, an optimal antimicrobial dose ensures enough drugs to access a clinical response. Better methods to pursue and rapidly adjust antimicrobial dosing must understand, although current approaches to antimicrobial dose optimization address fixed variability.[2] Resulting in high morbidity and mortality reports, the antibiotic treatment diversity is restricted for existing hard-to-treat multidrug-resistant bacterial infections.[3] For human and veterinary pathogens, antibiotic-resistant genes constituting the environmental "resistome" get transferred.[4] On developing new antimicrobial drugs, all scientists, governments, health sectors, and societies must take the necessary precautions and support investigations.

Hydrazide-hydrazone compounds are molecules that result in the formation of a Schiff base on the structure by the reaction of hydrazides with various aldehydes and ketones. It is known that hydrazide-hydrazones have various pharmacological activities.[5] According to the literature above, it was shown that hydrazide-hydrazone compounds have antimicrobial activity.[6-12]

Meanwhile, we tested our compounds for their antimicrobial activity. In this study, ten ethylparaben hydrazide-hydrazone derivatives, previously reported, were tested for their antibacterial and antifungal activity using various microorganism strains.

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MATERIALS AND METHODS

Antimicrobial activity tests

The synthesized ten compounds were investigated for their potential antimicrobial activities against *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922) (Gram-positive and Gram-negative bacteria, respectively), *Candida albicans* (ATCC 10231) (fungus), and the clinical isolates of these microorganisms. The study was conducted according to the Clinical Laboratory Standards Institute (CLSI) M100-S28 protocol for bacteria[13] and CLSI M27-A3 protocol for fungi.[14] In the study, cation-adjusted Mueller Hinton Broth and RPMI-1640 media were used for the determination of potential antibacterial and antifungal activities, respectively.

The compunds (1 mg) were dissolved in 0.976 mL of 10% dimethyl sulfoxide (DMSO) with a final concentration of 1024 μg/mL and the serial dilutions of each compound in the range of 2-512 μg/mL were prepared in 96 well microplates, after placing broth medium in each well. The suspension of each microorganism was prepared using McFarland: 0.5 standard and as result, 10^5 cfu/mL densities were reached. Microplates containing bacteria and fungus were incubated for 16-20 hours at 37°C and 48 h at 35°C, respectively. The reference antimicrobials were tested against these microorganisms at the same time. Besides, growth control of microorganisms and sterilization control of the media were tested. Antimicrobial activity of DMSO, which was used as a solvent in the study, was also tested. The wells with the lowest concentration with no microbial growth were determined as the minimum inhibition concentrations (MIC). The detection was made by visual evaluation using dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT).[15] Each test was repeated 3 times.

Chemistry

Synthesis of all compounds was reported in our previous study.[16] The chemical route for the synthesis of compounds is shown in Figure 1.

RESULTS AND DISCUSSIONS

Several ethylparaben hydrazide-hydrazone derivatives (3a-j) were synthesized and characterized (Figure 1 and Table 1). These compounds were previously studied and evaluated for their anticancer activity. In this study, all compounds were evaluated for antimicrobial activity.

The minimum inhibitory concentration (MIC) values determined for each substance and reference antimicrobial agents because of the experiment are shown in Table 2. MIC values of the compounds were compared to reference antimicrobials (*i.e.* ampicillin, gentamicin, and vancomycin for antibacterial; fluconazole for antifungal activity). The antimicrobial activity of 10% DMSO used as a solvent could not be determined. According to the result of the study, among the compounds with the best MIC value was compound 3g. The MIC of compound 3g on *S. aureus* (ATCC 29213) strain was 2 μg/mL, which is equivalent to MIC of the ampicillin, the reference antibiotic. Compound 3g was shown to have the best antibacterial activity among the compounds in our study. All compounds except compound 3g had antimicrobial activity in the range of 64-256 μg/mL. Therefore, other compounds in the study were also found to have moderate antimicrobial activity. We determined that compound 3b showed the highest antifungal activity. The MIC value of compound 3b on *C. albicans* (ATCC 10231) and its clinical isolate was determined to be 64 and 64 μg/mL, respectively. Other compounds other than 3b had antifungal activity in the range of 128-256 μg/mL.

Among our compounds, a bis-3,5-trifluoromethyl substituent (compound 3g) in this position increased the antibacterial activity against *S. aureus* compared to those of other synthesized compounds. It was observed that other substituents did not have effect on the activity. In the literature, there are many studies investigating the antimicrobial activities of synthesized chemical compounds. In one of these studies, Noshiranzadeh et al.[17] evaluated the antibacterial activity of some new

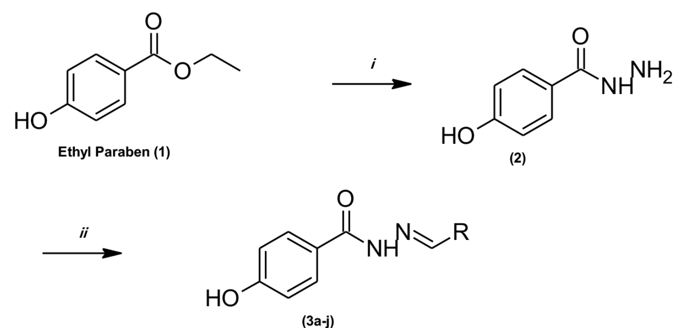

Figure 1. Synthesis route of hydrazide-hydrazone derivatives (3a-j) (*i*): $NH_2NH_2 \cdot H_2O/C_2H5OH$, (*ii*) C_2H_5OH/glacial CH_3COOH/R-CHO

Table 1. Substituents of compounds 3a-j

Compounds	-R	Compounds	-R
3a		3f	
3b		3g	
3c		3h	
3d		3i	
3e		3j	

Table 2. *In vitro* MICs (µg/mL) observed of the compounds and reference antimicrobial drugs

Compounds	Microorganisms					
	S.a.	S.a.*	E.c.	E.c.*	C.a.	C.a.*
3a	256	256	128	128	256	128
3b	256	256	128	128	64	64
3c	256	256	256	128	128	128
3d	256	256	256	128	128	128
3e	256	256	256	128	128	64
3f	256	256	128	64	256	256
3g	**2**	256	128	128	256	256
3h	256	256	256	256	256	256
3i	256	256	256	128	256	128
3j	256	256	256	128	256	256
Ampicillin	2	32	16	16	-	-
Gentamicin	1	8	1	16	-	-
Vancomycin	1	4	-	-	-	-
Fluconazole	-	-	-	-	1	1

S.a.: *Staphylococcus aureus* ATCC 29213, S.a.*: *Staphylococcus aureus* isolate (MRSA), E.c.: *Escherichia coli* ATCC 25922; E.c.*: *Escherichia coli* isolate (contains broad spectrum β-lactamase enzyme -GSB-), C.a: *Candida albicans* ATCC 10231, C.a.*: *Candida albicans* isolate

hydrazide-hydrazones of lactic acid. In that study, which used the microbroth dilution method, it was stated that MIC values of the compounds were 64-128 µg/mL against some bacterial strains. In another study, Abdelrahman et al.[18] tested the *in vitro* antibacterial activity of novel hydrazide-hydrazon derivates and found that some compounds exhibited better antibacterial activity compared to ampicillin and ciprofloxacin, respectively. For example; a compound in the study (MIC: 0.49 µg/mL) exceeded MIC of ampicillin (0.98 µg/mL), that was the reference agent against *S. pneumoniae.*[18]

In summary, the compounds in our study showed varying levels of antimicrobial activity. However, MIC value of the compound 3g, which captures the reference antibiotic, makes this compound stand out among others. Although studies in the literature show that hydrazide-hydrazone derivatives have variable antimicrobial MIC values, our study with these studies supports the idea that these derivatives are promising antimicrobial molecule candidates for the future.

CONCLUSION

In this study, ten ethylparaben hydrazide-hydrazone derivatives were screened for their antibacterial and antifungal activities. Among the synthesized molecules, compound 3g showed the best antimicrobial activity at 2 µg/mL MIC value to *S. aureus* strain (ATCC 29213). MIC value of compound 3b on *C. albicans* (ATCC 10231) and its clinical isolate was determined to be 64 and 64 µg/mL, respectively. Ethylparaben is currently used as an antibacterial agent and preservative for preparations. As these studies are necessary to understand the relationship between the substitutions and activity, which can lead to the design and synthesis of more potent antimicrobial compounds, which can occur in the therapeutic use.

Ethics

Ethics Committee Approval: Not applicable.

Informed Consent: Not required.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: U.İ., M.İ.H., Design: U.İ., M.İ.H., Data Collection or Processing: U.İ., M.İ.H., Analysis or Interpretation: U.İ., M.İ.H., Literature Search: U.İ., M.İ.H., Writing: U.İ., M.İ.H.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Development and Validation of SI/RS-UHPLC-PDA Method for Olmesartan Medoxomil and Metoprolol Succinate-Related Substance

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ABSTRACT

Objectives: Olmesartan medoxomil (OLM) and metoprolol succinate (MPS) in fixed-dose combination (FDC) tablet formulation prescribed extensively. Stability indicating (SI) method for impurities and related substance (RS) test quantitates the amount of these analytes in formulation; the manuscript presents SI/RS-ultra-high performance liquid chromatography-photodiode array (UHPLC-PDA) method for OLM and MPS and their impurities.

Materials and Methods: Well-resolved separation of all analytes was achieved with gradient elution on a Shimadzu on Shimpack GIST-C18 (100 mm x 2.1 mm, 2 μm) column maintained at 25°C. Mobile phase-A consist of 0.1% orthophosphoric acid in water and mobile phase-B was acetonitrile at a flow rate of 0.4 mL/min, data integrated at 225 nm and 16 min of short runtime for satisfactory elution of all peaks.

Results: The proposed SI/RS-UHPLC-PDA method was developed and validated as *per* International Conference on Harmonisation (ICH) of Technical Requirements guidelines. The system suitability test complied by all eluted peaks of the interest with acceptable linearity, recovery, and precision. Specificity, robustness, and method sensitivity parameters were determined; all the parameters were found to be within the limits. All the impurities and stress-degraded peaks were well resolved.

Conclusion: The proposed method was found to be simple, fast, linear, and accurate. Further, the method is precise, robust, and specific; suitable for routine IPQC during active pharmaceutical ingredient manufacturing, stability and impurity profiling studies of the titled bulk analytes. Furthermore, the method can be extended to assess the levels of impurities formed during life cycle of new FDCs of titled analytes.

Key words: SI/RS-UHPLC-PDA, related substances, impurities, stability studies, and gradient elution

INTRODUCTION

Olmesartan medoxomil (OLM) is chemically (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl 5-(2-hydroxypropan-2-yl)-2-propyl-3-[[4-[2-(2*H*-tetrazol-5-yl) phenyl] phenyl] methyl] imidazole-4-carboxylate (Figure 1a). Metoprolol succinate (MPS) is a chemically butanedioic acid: 1-[4-(2-methoxyethyl) phenoxy]-3-(propan-2-ylamino) propan-2-ol (Figure 1b). OLM is an angiotensin II type 1 [AT (1)] receptor antagonist. It inhibits actions of angiotensin II and was administered once daily. OLM recommended in the dosage range of 10-40 mg to adult patients for treatment of hypertension.[1] MPS a β1-selective adrenoceptor blocking agent preferred in arrhythmia, hypertension, angina pectoris, and myocardial infraction. Extended-release tablets for controlled and predictable release of MPS achieved by once-daily oral administration.[2] These active pharmaceutical ingredients (APIs) are official in Indian Pharmacopoeia (IP) and British Pharmacopoeia (BP) (IP 2010; BP 2010). For MPS, Impurity A (Figure 1c) is reported in official books. Correspondingly, for OLM impurity B (Figure 1d), impurity C (Figure 1e), impurity D (Figure 1f) and dimer impurity (Figure 1g) reported in official books. The International Council for Harmonisation (ICH) of Technical Requirements tripartite guidelines specify limits on impurity levels in APIs and their dosage. There is no need for

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testing impurity unless there is generation of impurity as part of drug degradation in dosages as *per* ICH guidelines (ICH Q1 2021, ICH Q3 2021).[3,4] To support institutional research product development and stability studies and achieve faster quantitation and evaluations of combined formulation from stability and process samples, there was a need of stability indicating/related substance-ultra-high performance liquid chromatography-photodiode array (SI/RS-UHPLC-PDA) method. Literature survey reveals that there are various methods available for estimation of OLM.[5-19] Various methods are available for individual estimation of MPS.[20-24] Various ultraviolet spectrophotometric, thin-layer chromatography (TLC), and HPLC methods are available for estimation of MPS along with other drugs and OLM.[25-37]

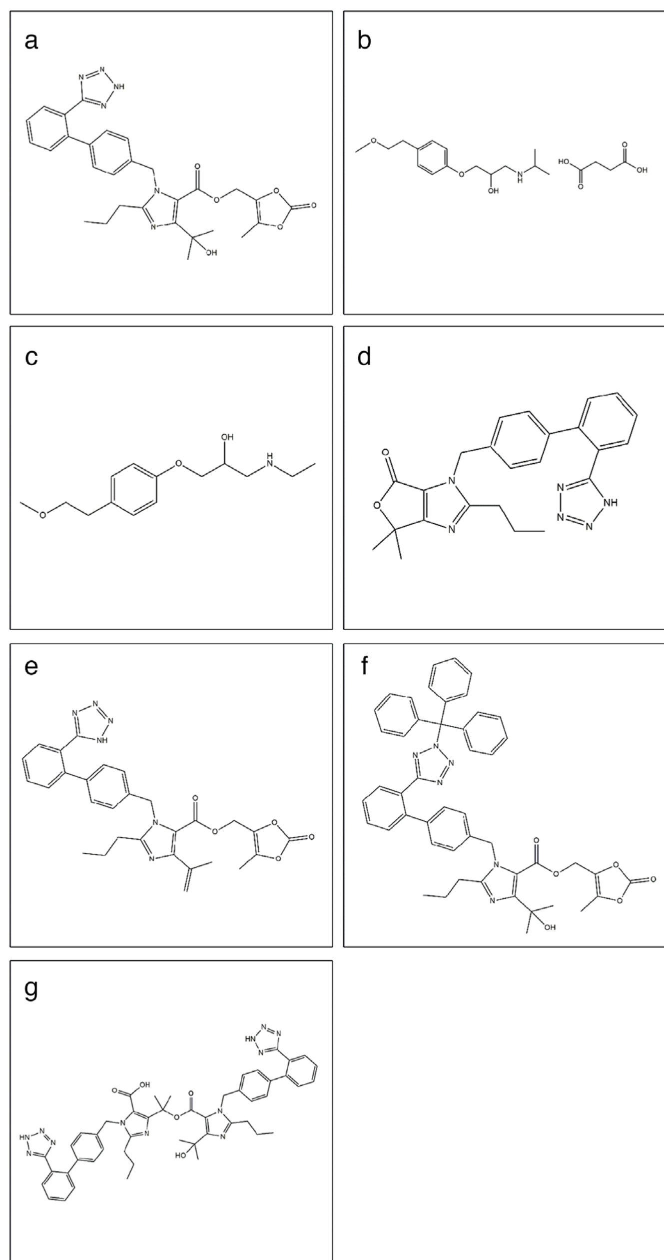

Figure 1. Structures of a) olmesartan, b) metoprolol succinate, c) MPS Imp-A, d) OLM Imp-B, e) OLM Imp-C, f) OLM Imp-D, g) OLM Imp dimer

OLM: Olmesartan medoxomil, MPS: Metoprolol succinate, Imp: Impurity

The literature survey also reveals that there is no impurity profiling UHPLC method reported for estimation of OLM and MPS. Therefore, SI/RS-UHPLC-PDA method development and validation for these analytes from formulations along with impurities and stress degradation products undertaken. Proposed method is a simple, fast quantification and identification method for OLM and MPS along with their impurities/RS. The proposed analytical methods are beneficial in achieving time and other resource efficiency. The method was developed and validated as *per* ICH guidelines.[38]

MATERIALS AND METHODS

Chemicals, reagents, and instrumentation

The drug samples of OLM (assay-99.81%) and MPS (assay-99.77%) were gifted by Cadila Healthcare Ltd., Ahmedabad, India. Impurities were gratis gifted by Piramal Healthcare Limited. HPLC grade solvents and analytical-grade reagents and chemical used in presented research work were purchased from Sisco Research Lab Pvt Ltd., Mumbai. Method development and validation work carried out on Shimadzu N-Series UHPLC instrument. Data were integrated using Shimadzu LabSolutions software version 6.89. The column used was Shimadzu Shimpack GIST-C18 (100 mm x 2.1 mm, 2 µm) and the injection volume was 5 µL using an autosampler (LC40AD). Mobile phase flow rate was 0.4 mL/min with online degassed. Fixed-dose combination tablets containing 20 mg OLM and 25 mg MPS, manufactured by Glenmark Pharmaceuticals Limited was used.

Standard solution preparation

About 100 mg OLM and 125 mg MPS were transferred into separate 100 mL VFs containing 50 mL of diluent (water: acetonitrile; 50:50% v/v). Analytes dissolved by 5 min sonication and diluent were used to makeup volume to get first standard stock solutions (SSS). 2 mL of these SSS transferred separately into 100 mL VFs; volume made up to get second SSS with the same solvent system. Combined OLM and MPS solution was prepared by transferring 2 mL from each of first SSS of analytes. Further 5 mL of above second SSS was transferred into 100 mL of volumetric flask; volume made up to the mark with same solvent system. 100 µg/mL SSS of impurity A of MPS as well as all impurities of OLM were prepared individually in diluent and used to spike solutions of actives.

Sample preparation

Weight of 20 intact tablets were recorded and tablets crushed to get powder, form this tablet powder equivalent to 100 mg OLM (125 mg MPS) transferred to 100 mL volumetric flask. To the flask, 70 mL of diluent was added and analytes dissolved by sonication for 20 min. Volume made up to the mark with diluent and mixed well. The solution was filtered through a 0.45 µm polyvinylidene fluoride (PVDF) syringe filter by discarding the first 3 mL filtrate.

Preparation and treatment of mobile phase

Mobile phase-A contains 1 mL of orthophosphoric acid (OPA) in 1000 mL of HPLC grade distilled water and sonicated for 15

min; filtered through 0.45 μ filter. Correspondingly, acetonitrile used as mobile phase-B.

Method validation

Validation of the optimized chromatographic method was carried out as *per* ICH guidelines for stability, impurity, and analytical method validation. After multiple initial method development trials with different mobile phase compositions and different gradient programs, efficient separation and resolution of the degraded products and spiked impurities were achieved on a Shimadzu Shim-pack GIST-C18 (100 mm x 2.1 mm, 2 μm) column maintained at 25°C and data processed at isopiestic wavelength of 225 nm. Mobile phase-A consist of 0.1% OPA in water and mobile phase-B consist of acetonitrile with gradient elution at a flow rate of 0.4 mL/min. The instrument used was a Shimadzu N-Series UHPLC. Method validation was performed for various parameters such as linearity, method sensitivity [limit of detection (LOD) and limit of quantitation (LOQ)], precision, accuracy, specificity (formulation specificity, stress degradation and impurity spiking) and robustness. To support validation data for formulation studies, filter compatibility studies were also conducted using 0.45 μ PVDF and nylon membrane filters. Solution stability studies were performed at room temperature and 5°C. Standard mixture was injected in six replicates to perform system suitability test (SST) of analytes before start of each validation experiments and by determining relative standard deviation (RSD) % of the peak area, which was always <5% throughout the validation studies. Method validation parameters studied are as follows:

Filter compatibility studies

Filter compatibility studies were performed using a standard solution for 0.45 μ nylon and 0.45 μ PVDF membrane filters. % Assay of filtered standard against the control centrifuged standard was calculated by discarding first 3 mL filtrate.

Linearity method sensitivity and specificity

Linearity assessed visually and by using a lack-of-fit test; interval between the upper and the lower levels of the analyte considered as the method range. Furthermore, method linearity was evaluated from the LOQ level to 150% of specification level. Slope, intercept, correlation coefficient, and Y% intercept bias were calculated. For method specificity, chromatographic peak interference from blank, placebo, and impurities at the retention time of both analyte peaks and stress-degraded products in stressed samples were observed for accepted resolution. Purity of the analyte peaks for each of these conditions was assessed by the peak purity test. To evaluate the peak purity criteria peak purity index and peak purity threshold values generated by the software system were noted and interpreted. Peak purity index value less than peak purity threshold values of relevant peak indicate that the peak is pure.

Method sensitivity study

Solution was prepared at 0.03 ppm to 100 ppm for all impurities and injected for determination of LOD and LOQ, respectively, as method sensitivity parameters. These parameters were estimated based on S/N ratio (LOD: S/N >3; LOQ: S/N >10). As part of this study, LOQ precision was performed by injecting 6 replicates and RSD% was determined.

Method precision

Six different sets were prepared by spiking all the impurities at 100% level (5 ppm) in the API at sample concentration level (1000 ppm OLM + 1250 ppm MPS) and RSD% was determined.

Accuracy (recovery)

Method recovery was evaluated by a standard spiking technique. Known amounts of standard impurities were spiked in API and placebo mixture preparation at 50%, 100%, and 150% levels. Accuracy studies were performed in triplicate.

Forced degradation and specificity

Forced degradation study on formulation was conducted in a sample solution state. For acid stress, 5 mL of tablet stock solutions were transferred into 25 mL of volumetric flask and 2.5 mL of 0.01 N HCl was added and the solutions were subjected to stress at 60°C for 10 min. Stressed sample was neutralized with 2.5 mL of 0.01 N NaOH. Similarly, a solution for base stress was prepared. For oxidation stress, hydrogen peroxide (3%) was used and sample was subjected to stress at room temperature for 2 h. For thermal stress, tablet formulation was kept at 60°C for 1 week and for humidity stress tablets were exposed to 75% RH at 60°C for 1 week. Heat and humidity stress samples were appropriately extracted, filtered, and diluted as *per* sample preparation procedure and used for the study. Peak purity of all stressed samples was checked for specificity.

Robustness

Robustness was performed for method parameters such as flow rate, column oven temperature, and concentration of OPA in mobile phase buffer. System suitability parameters were reported for the conditions.

Solution stability studies

Solution stability studies were performed at room temperature and at 5°C temperature in mix standard solution for 4 hrs. Further study extended to 24 h at 5°C; results of the study were analysed against fresh standard.

RESULT AND DISCUSSION

Method development

The stepwise process was adopted for logical and scientific analytical method development. Development efforts undertaken are presented along with the reasoning. Method development was started considering the HPLC method as a base method. Initial trial condition comprised 0.1 OPA as mobile phase-A and acetonitrile as mobile phase-B with a gradient elution. Various trials with C18 columns with length 50 mm, 75 mm, and 100 mm were taken under various gradient conditions. With shorter columns of 50 mm and 75 mm, known impurities were getting merged in to the tailing of main peak (Figure 2). A reasonable and acceptable separation was achieved with 100 mm column. Hence, various gradient trials were taken and the final optimized method parameters are given in Table 1. SST parameters are

presented in Table 2, while optimized chromatographs of OLM, MPS, and their impurities are presented in Figure 3.

Method validation

Proposed RP-HPLC method for RS of both the titled drugs was validated for various parameters as described in procedure section. Efforts were also directed toward the separation of the stress degraded products of both the analytes. Results for various validation parameters are described as follows.

Filter compatibility, linearity, method sensitivity, and precision

Filter compatibility studies for PVDF filter and nylon filter were studied as described in procedure section. Study data results of PVDF-filtered solutions were close to standard assay results compared to nylon-filtered solution. Therefore, based on the study data PVDF filter was selected for all further validation studies.

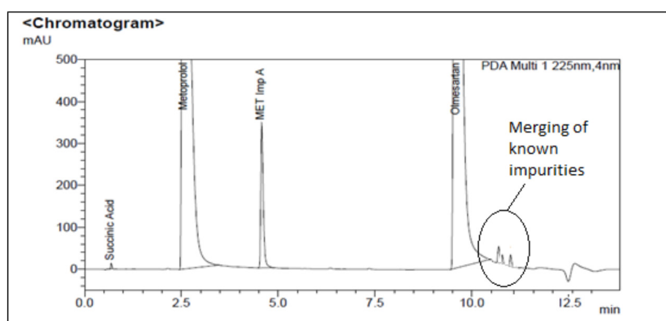

Figure 2. Trial 1 chromatogram of impurity spiked standard

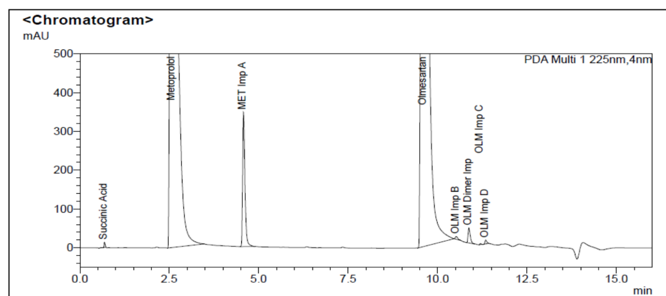

Figure 3. Optimised chromatograph of impurity spiked standards

Linearity of the proposed method was studied as described in procedure section by spiking impurities in analyte solutions. Chromatographs were acquired using optimized chromatographic conditions and the data integrated using system software to generate linearity equation values (slope and intercept). One of the indicators of the linearity, coefficient of correlation (r) generated by system software was noted; the values for the analytes of the current interest were always more than 0.999. Linearity data were used to determine method sensitivity values (LOD and LOQ); relevant precision values, intermediate precision, precision at LOQ, and repeatability were determined. All the parameter values are depicted in Table 3.

Accuracy (recovery)

Accuracy was estimated by recovery studies as described. As presented in Table 3, amount of impurities was spiked at the given recovery level with respect to test the concentration of OLM (1.0 mg/mL) and MPS (1.25 mg/mL). Data indicate that the recovery values and RSD% were always within the limit at the three levels of accuracy for the impurities in Table 3.

Forced degradation and specificity

Results of acid, base, oxidation, heat, and humidity stress degradation study on formulation solutions are shown in Table 4. Specificity was performed by checking interference from blank and placebo at the retention of main peak and impurities (Figure 3); no interference was observed during the forced degradation studies (Figures 4-8). Peak purity data for the stress conditions for both analytes shows that peak purity index values were always less than peak purity threshold. Results of stress degradation display that the OLM is very sensitive to acid and base stress (Table 4).

Robustness

Robustness was performed for changes in optimized chromatographic method parameters such as MP flow rate, column oven temperature, and concentration of OPA in mobile phase buffer. Resolution was the most important parameter considered for the study. Robustness study data are presented in Table 5 and indicate that the proposed SI/RS-RP-HPLC method is robust and small variation within the experimental limits does not affect the results in Table 3.

Table 1. Optimized chromatographic conditions

Sr. no.	Optimized chromatographic conditions		Gradient program (time and mobile phase composition)		
	Parameters	Details	Time (min)	Mobile phase-A	Mobile phase-B
1.	Column	Shimadzu Shimpack GIST-C18 (100 mm x 2.1 mm, 2 μm)	0.01	80	20
2.	Mobile phase-A	0.1% orthophosphoric acid in water	5.0	65	35
3.	Mobile phase-B	Acetonitrile	7.5	65	35
	Mobile phase program	Gradient	10.0	45	55
4.	Column temperature	25°C	12.5	45	55
5.	Injection volume	5 μL	13.5	80	20
6.	Flow rate	0.4 mL/minute	16.0	80	20

Table 2. System suitability parameters of OLM, MPS and impurities

Peak	Name	Retention time	Area	Area%	Resolution (Rs >1.5)	Tailing (T <2.0)	TP (TP >2000)	K' (k' >2)
1	Succinic acid	0.688	26825	0.058	-	1.33	2959	2.44
2	Metoprolol	2.576	18008242	38.812	8.258	1.83	2680	11.88
3	ME Imp-A	4.572	1560580	3.363	7.763	1.57	23552	21.86
4	Olmesartan	9.604	26568380	57.261	23.408	1.79	15281	47.02
5	OLM Imp-B	10.520	32047	0.069	4.181	1.27	109125	51.60
6	OLM dimer Imp	10.872	162154	0.349	2.920	1.55	146291	53.36
7	OLM Imp-C	11.196	7016	0.015	3.438	1.20	358621	54.98
8	OLM Imp-D	11.344	33524	0.072	1.720	1.34	217633	55.72
Total			46398767	100.000	-	-	-	-

OLM: Olmesartan medoxomil, MPS: Metoprolol succinate, Imp: Impurity

Table 3. Linearity, LOD, LOQ, precision, and accuracy data of drugs and impurities

Analytes → Parameter ↓	MPS impurity Imp-A	OLM impurities			
		Imp-B	Dimer impurity	Imp-C	Imp-D
LOQ (ppm)	0.05	0.05	0.05	0.65	0.15
LOD (ppm)	0.03	0.03	0.03	0.25	0.05
Range (ppm)	0.05-7.5	0.05-7.5	0.05-7.5	0.65-7.5	0.15-7.5
Slope (b)	313459.7	6274.8	32228.2	1447.3	6869.4
Intercept (a)	-3182.4	877.8	1083.2	-222.6	-1158.8
Correlation coefficient (r)	0.99993	0.99976	0.99983	0.99977	0.99957
Y% intercept @ 100% level	-0.20	2.74	0.67	-3.17	-3.46
Precision of repeatability (% RSD)[#]	0.65	0.93	1.11	1.98	0.87
Intermediate precision[#] precision (RSD%)[#]	0.99	0.87	1.34	1.45	1.32
Precision at LOQ (RSD%)[#]	1.45	1.83	1.67	2.54	2.11
Recovery at level[€] and recovery limit	**% Accuracy data for impurities (± RSD%)**				
50% (**RSD% ≤5.00%)[$]**	98.6 ± 1.81	98.7 ± 1.57	99.1 ± 1.32	98.1 ± 1.36	98.2 ± 1.22
100% (**RSD% ≤5.00%)[$]**	99.0 ± 0.92	98.5 ± 1.07	98.7 ± 1.01	99.1 ± 1.12	98.7 ± 1.38
150% (**RSD% ≤5.00%)[$]**	98.7 ± 1.23	99.2 ± 0.88	99.3 ± 1.26	98.6 ± 0.82	99.4 ± 0.91

[#]Average RSD% for six determinations, [€]Amount spiked with respect to test concentration of OLM (1.0 mg/mL) and MPS (1.25 mg/mL), [$]Mean ± RSD% for three determinations. OLM: Olmesartan medoxomil, MPS: Metoprolol succinate, LOD: Limit of detection, LOQ: Limit of quantitation, RSD: Relative standard deviation

Solution stability studies

Solution stability studies were performed at room temperature and 5˚C temperature in mix standard solutions. Percent of impurities in solution was determined at each of the time points; results of the study are shown in Table 6.

CONCLUSION

SI/RS-UHPLC-PDA method for estimation of impurities of OLM and MPS in tablet formulation was developed and validated. All system suitability and peak purity parameters of analyte peaks during stressed and stability studies were in an acceptable range. Linearity of the developed method was near 1.0 within the specified range. RSD% was found to be less than 2% for repeatability. % Recovery of all impurities was found to be within 95%-105% across all levels with RSD% values always less than 2. The said method can go to an LOD level as low as 0.03 ppm, which is otherwise 2.2 ppm for the reported methods. This ultimately results in a lower linear range of as low as

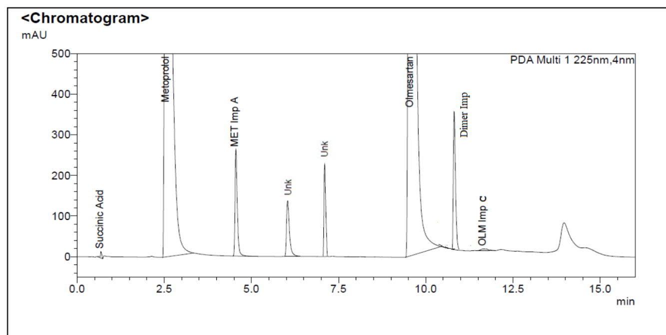

Figure 4. Stress degradation chromatographs of OLM and MPS tablet solution - acid stress

OLM: Olmesartan medoxomil, MPS: Metoprolol succinate

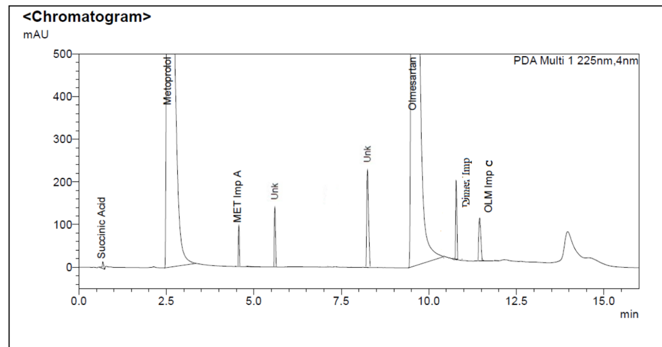

Figure 7. Stress degradation chromatographs of OLM and MPS tablet - heat stress

OLM: Olmesartan medoxomil, MPS: Metoprolol succinate

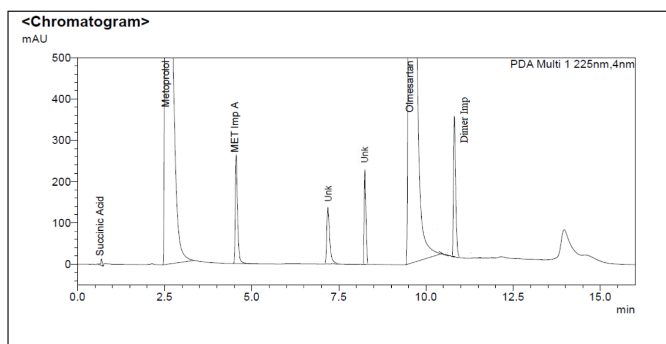

Figure 5. Stress degradation chromatographs of OLM and MPS tablet solution - base stress

OLM: Olmesartan medoxomil, MPS: Metoprolol succinate

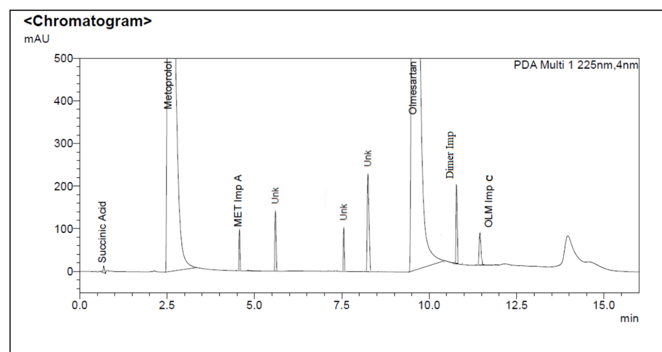

Figure 8. Humidity stress degradation chromatographs of OLM and MPS tablet

OLM: Olmesartan medoxomil, MPS: Metoprolol succinate

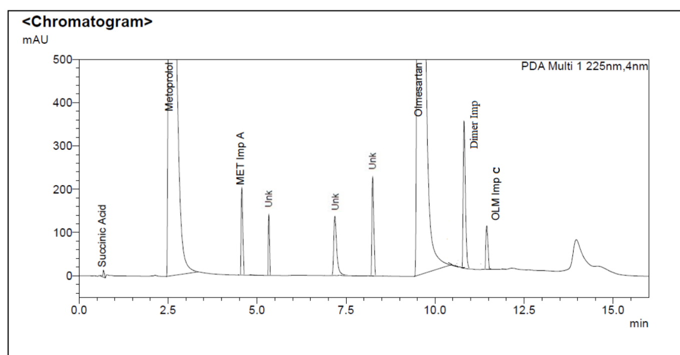

Figure 6. Stress degradation chromatographs of OLM and MPS tablet - oxidative stress

OLM: Olmesartan medoxomil, MPS: Metoprolol succinate

Table 4. Forced degradation study results

Stress conditions	Total degradation (%)	Peak purity index for OLM	Peak purity threshold for OLM	Peak purity index for MPS	Peak purity threshold for MPS	Peak purity results
As such condition	3.06	0.999991	0.999978	1.000000	0.999987	Pass
Acid stress	13.42	0.999984	0.999910	1.000000	0.999956	Pass
Base stress	17.91	0.999990	0.999938	0.999993	0.999978	Pass
Peroxide stress	10.22	0.999978	0.999937	0.999995	0.999983	Pass
Heat stress	5.11	0.999999	0.999983	0.999998	0.999972	Pass
Humidity stress	6.02	0.999996	0.999902	1.000000	0.999967	Pass

OLM: Olmesartan medoxomil, MPS: Metoprolol succinate

Table 5. Robustness data

Parameter Variations→ *Analytes↓	Adjacent peaks resolution (R_s) values						
	Optimised chromatographic conditions	Flow rate (mL/min)		Temperature (ºC)		OPA composition of MP (% v/v)	
		0.38	0.42	23	27	0.09	0.11
MPS	NA	NA	NA	NA	NA	NA	NA
MPS Imp-A	7.8	7.9	7.6	7.9	7.8	7.8	7.8
OLM	23.4	23.2	23.5	23.3	23.5	23.3	23.5
OLM Imp-B	4.2	4.0	4.1	4.2	4.1	4.2	4.3
OLM dimer Imp	2.9	2.9	2.7	2.8	3.0	2.9	2.9
OLM Imp-C	3.4	3.5	3.2	3.4	3.3	3.1	3.3
OLM Imp-D	1.7	1.7	1.6	1.7	1.7	1.7	1.7

*Imp: Impurity, OLM: Olmesartan medoxomil, MPS: Metoprolol succinate, NA: Not applicable

Table 6. Solution stability data, n: 3

Time point and storage conditions	Imp contents (% w/w)					Other unknown total
	MPS	OLM				
	Imp-A	Imp-B	Dimer	Imp-C	Imp-D	Total (Unk)
Initial	3.37	0.07	0.35	0.02	0.07	0.12
1 hour (R_t)	3.65	0.08	0.46	0.02	0.07	0.19
2 hour (R_t)	4.27	0.09	0.55	0.03	0.08	0.31
4 hour (R_t)	5.12	0.09	0.86	0.02	0.07	0.45
1 hour (5˚C)	3.38	0.07	0.37	0.02	0.08	0.13
2 hour (5˚C)	3.35	0.08	0.36	0.03	0.07	0.14
4 hour (5˚C)	3.38	0.08	0.38	0.02	0.08	0.13
8 hour (5˚C)	3.39	0.07	0.35	0.03	0.08	0.13
16 hour (5˚C)	3.37	0.08	0.36	0.02	0.07	0.14
24 hour (5˚C)	3.38	0.08	0.35	0.03	0.08	0.14
24 hour (5˚C)	3.38	0.08	0.35	0.03	0.08	0.14

OLM: Olmesartan medoxomil, MPS: Metoprolol succinate, R_t: Retention time, Imp: Impurity

0.05-7.5 ppm. These results indicate that the developed method is fast, accurate, precise, and specific. It can be used in the routine quality control of API manufacturing and formulations. Resolution between actives and impurities was more than 2.5. USP S/N achieved more than 3 for LOD and more than 10 in LOQ preparation. Total run time *per* sample analysis was 16 min, which ultimately reduced the overall analysis time and cost of analysis.

Ethics

Ethics Committee Approval: Not applicable.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: N.T., A.D., Design: N.T., G.S., Data Collection or Processing: N.T., Analysis or Interpretation: N.T., V.C., Literature Search: N.T., G.S., V.C., Writing: N.T., V.C.

Conflict of Interest: No conflict of interest was declared by the authors.

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ORIGINAL ARTICLE

Stability Evaluation of the Biosimilar Monoclonal Antibody Using Analytical Techniques

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ABSTRACT

Objectives: Determination of the drug substance (DS) and drug product (DP) stability is especially important for biosimilar monoclonal antibodies since it can affect the quality, efficacy, and safety of the drugs. The main objective of this study was to determine the stability of the biosimilar candidate (TUR01) using state-of-the-art (current) analytical techniques.

Materials and Methods: Analytical techniques used in this study were isoelectric focusing on capillary electrophoresis, capillary electrophoresis-sodium dodecyl sulfate, size exclusion chromatography-ultra-high performance liquid chromatography, binding affinity, and physicochemical and microbiological tests. DS was kept in polyethylene terephthalate copolyester, glycol modified (PETG) bottles at ≤-65.0°C and 5.0 ± 3.0°C for 18 months, where the pre-filled syringe stability study was conducted at 5.0 ± 3.0°C for 24 months and 25.0 ± 2.0°C/60% ± 5 relative humidity (RH) for 6 months. The accelerated condition for DS was accepted as 5.0 ± 3.0°C, while it was 25.0 ± 2.0°C for the DP.

Results: The results indicated that TUR01 DS was stable when it was stored under long-term storage conditions at ≤-65°C and at 5 ± 3°C at least 18 months. Also, TUR01 DP was stable at 5 ± 3°C for 24 months and at 25 ± 2°C with 60.5% RH for 2 months without any significant changes.

Conclusion: State-of-the-art analytical techniques proved to be invaluable tools for evaluate the stability of the TUR01 DS and drug product.

Key words: Biosimilar monoclonal antibody, drug substance and drug product stability, analytical techniques, and stability indicating methods

INTRODUCTION

Biosimilars are a fast licensure pathway, which provides access for patients to reach life-saving medications through lower healthcare costs. In this study, a biosimilar monoclonal antibody (mAb) candidate (TUR01), which can serve as a tumor necrosis factor alpha (TNF-α) inhibitor, was developed. TUR01 is a fully human mAb, immunoglobulin isotype G subclass 1 molecule produced by Chinese hamster ovary cells. The proven mechanism of TUR01 is through blocking pro-inflammatory activity. By neutralizing soluble TNFα, it can inhibit the inflammatory response. The possible indications for this biosimilar are psoriasis, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, hidradenitis suppurativa, uveitis, and juvenile idiopathic arthritis.[1,2]

For marketing authorization, the stability of biosimilars must be demonstrated due to the International Conference on Harmonisation of Technical Requirements (ICH) for registration of pharmaceuticals for human use, especially based on ICHQ1A, ICHQ5C, ICHQ5E, and ICHQ6B.[3] The ICHQ5C (stability testing of biotechnological/biological products) specifically focuses on the biotechnological drugs considering their distinguishing properties. Based on this guidance, the stability protocol must include the testing to judge the potency, purity, molecular characterization, and product characteristics during the stability period. The stability testing needs to be conducted both for drug substance (DS) and drug product (DP). Although most of the mAbs are in the same formulation buffer in DS or DP forms, their packaging material and storage temperatures can be different. DS can be kept in bottles or bags before primary packaging, which differs depending on the route of administration. Most of the proteins can be stable at very low temperatures such as -80°C for very long periods (5 years). At lower temperatures such as -80°C, the drugs can be kept in plastic material rather than glass material due to the glass

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breakage at low temperatures. For this reason, it is more cost-effective for manufacturers to keep the DS as long as possible until sending it to the primary packaging. For both DS and DP, the stability analysis was conducted at two different temperatures for stability, one normal and one accelarated condition (≤-65.0°C and 5.0 ± 3.0°C for DS and 5.0 ± 3.0°C and 25.0 ± 2.0°C/60% ± 5 relative humidity (RH) for DP) due to ICH guidelines. Accelerated studies can enlighten us for longer periods before conducting a prolonged study.

During the shipping and storage of the mAbs, many stress factors can cause physical or chemical instability. For this reason, the stability period of biosimilars at different temperatures and storage conditions must be demonstrated. Physical instability can cause adsorption to the surface, which can lead to unfolding and aggregation. Chemical instability can cause degradation through asparagine deamidation, oxidation, and aspartic acid isomerization, and so on.[3] In addition to the physical and chemical stability, biological assessment has high importance in providing the potential efficacy of mAbs. Based on the mechanism of action, binding and/or any other biological assessment needs to be conducted throughout the shelf life of the study.[4]

Physicochemical and functional analyses with orthogonal analytical techniques need to be applied to understand whether there is any change in the primary, secondary, and higher-order structure during the extended periods for any developed biosimilars to determine the life span of the drug and storage conditions.[4,5] Determination of the stability period is extremely important since drug instability can affect the quality, efficacy, and safety of the monoclonal antibody.[6,7] Additionally, stability data become very valuable in the incidents, where the cold chain is broken[7,8] throughout the warehouse storage, distribution, and usage periods. Any changes that can impact the quality need to be monitored during the stability period.[9-11]

The main objective of this study was to determine the stability of TUR01 using state-of-the-art analytical techniques including isoelectric focusing capillary electrophoresis (icIEF), capillary electrophoresis-sodium dodecyl sulfate (CE-SDS), size exclusion chromatography-ultra-high performance liquid chromatography (SEC-UPLC), binding affinity, physicochemical and microbiological tests, as demonstrated in Figure 1. The physicochemical and microbiological tests used here are based on the pharmacopeia methods, whereas others are based on the in-house developed product-specific monographs. For microbiological tests, endotoxin and bioburden were followed during the stability study. In terms of physicochemical tests, appearance, color, opalescence, pH, osmolality, sub-visible particulates, and extractable volume determination were used during the stability period. The stability study was conducted in the same formulation buffer but in two different containers, both in polyethylene terephthalate copolyester, glycol modified (PETG) Nalgene bottles and glass type I pre-filled syringes as DS and DP forms, respectively. The bottles were kept at ≤-65.0°C and 5.0 ± 3.0°C for 18 months, whether the pre-filled syringe stability study was conducted at 5.0 ± 3.0°C for 24 months and 25.0 ± 2.0°C/60% ± 5 RH for 6 months. The accelerated condition for DS is accepted as 5.0 ± 3.0°C whether it is 25.0 ± 2.0°C for the DP. This study was designed to demonstrate if any subvisible particles, degradation or aggregation occur during the stability period affecting the safety and integrity of the product. Furthermore, biological assessment with SPR was conducted to measure the binding of the biosimilar to TNF-α, which directly shows the changes in the efficacy of the drug.

MATERIALS AND METHODS

Appearance

The appearance was evaluated by visual inspection according to the pharmacopeia methods, Ph. Eur. 2.9.20.[12]

pH

pH was measured by potentiometric determination according to the Ph. Eur. 2.2.3.[12]

Color

Visual inspection according to the method described in Ph. Eur. 2.2.2. was used to determine the degree of coloration.[12]

Opalescence

A turbidimeter was used to evaluate the clarity and degree of opalescence according to the method described in Ph. Eur. 2.2.1.[12]

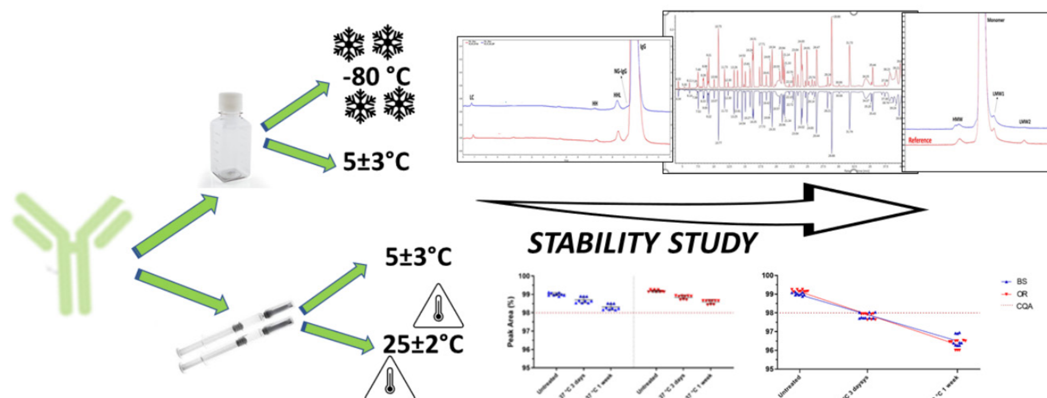

Figure 1. Overview of the conducted stability study for biosimilar TNF-α inhibitor

Osmolality

The osmolality is evaluated by an osmometer (Advanced Instruments, OsmoTECH) according to the method described in Ph. Eur. 2.2.35.[12]

Sub-visible particulates

The light obscuration particle count test was used to evaluate the sub-visible particles according to the Ph. Eur. 2.9.19 method.[12]

Extractable volume

The gravimetric volume determination method was used to measure the extractable volume due to Ph. Eur. 2.9.17.[12]

Bioburden

Bioburden was evaluated by the total aerobic microbial count method using membrane filtration due to Ph. Eur. 2.6.12.[12]

Bacterial endotoxin

Bacterial endotoxin was determined using *Limulus amebocyte* lysate kinetic turbidimetric technique, in accordance with Ph. Eur. 2.6.14 (equivalent to USP <85>). The results are expressed as EU/mL.[12]

Sterility

Sterility was evaluated by membrane filtration test according to the method described in the Ph. Eur. 2.6.1.[12]

Protein content

Protein content was measured in a nanodrop (Thermo Scientific, Nanodrop One) using optical densitometry method. Firstly, blank measurement was done with 2.2 mL formulation buffer. Then, protein sample was mixed and 2.2 mL of the mixed sample was measured by a nanodrop. This procedure was repeated thrice and the average value was taken. The absorption of the samples was determined at 280 nm. Results were reported as mg/mL.

Isoform profile and isoform abundance measurement

Isoform profile and isoform abundance were measured by capillary isoelectric focusing (protein simple, iCE3) using ultraviolet (UV) detection at 280 nm. Both the reference and samples were diluted to 1 mg/mL in deionized water, with a final volume of at least 20 µL. Master mix was prepared due to the suppliers' protocol and added to both reference and samples before vortexing for 3 s. All the samples were centrifuged at 10,000 rpm for 3 min before transferring to the vials for analysis on iCE3. Pre-focusing was done at 1.5 kV for 1 min and focusing was at 3 kV for 6 min. Pharmalyte ampholytes at pH 3-10 and pH 8-10.5 were used for generation of a pH gradient. The pI markers (7.9 and 10.0) were used to determine the charge variants isoelectric pH (pI) values.

Electrophoretic purity determination

Electrophoretic purity was determined using a PA800 Plus Capillary Electrophoresis System (Sciex, PA800 plus). Species are separated based on their hydrodynamic size while passing through the capillary and detected by a photodiode array detector. Both the reference and samples were diluted with deionized water to a final concentration of 2 mg/mL in a 0.5 mL tube. An immunoglobulin isotype G (IgG) control standard was aliquoted at room temperature and prepared in a 0.5 mL tube. The prepared samples were vortexed approximately 5 s prior to centrifugation for 30 s at 14000 rpm. The samples were incubated at 70°C for about 10 min followed by cooling for about 3 min. Reference and blank samples were transferred to 200 µL volume microvials. All injections were made in triplicate tubes. For non-reduced conditions, a final concentration of 500 mM iodoacetamide (Sigma Aldrich, USA) and for reduced conditions, β-mercaptoethanol (Merck, USA) were used. The analysis was conducted under 15 kV for 40 min and 220 nm was used for the main electropherogram wavelength.

Monomer and aggregate determination

Monomer and aggregate values were measured at 280 nm by SEC (Waters Acquity H-Class Bio UPLC with UV detector). Both reference and samples were diluted to 2.50 mg/mL with formulation buffer followed by vortexing 3 s. Then, the samples were injected into the system. The injection was done into a BEH SEC 200 (4.6 x 30 cm, 200 Å) column with a flow rate of 0.25 mL/min was used for the separation. The mobile phase was at pH 7.4 with 20 mM phosphate (Sigma, Germany) and 188 mM sodium chloride (Merck, Germany).

Biological activity - binding kinetics

The KD value (relates to the concentration of antibody) for binding to the soluble antigen was determined in a single-cycle assay format using surface plasmon resonance (SPR) with Biacore T200 (Cytiva). CM5 S series sensor chipand amine coupling kit (GE Healthcare, Cat ID BR-1000-50) was used. The biosimilar antibody was captured *via* a protein A/G (Pierce, Cat ID 21186) immobilized surface matrix, and several concentrations of antigen were injected consecutively. Before injection, soluble antigen was diluted from 200 nM to 2.5 nM in 1X HBS-EP+ running buffer and mixed thoroughly. Multiple diluents were prepared. Reference and samples were also diluted to 2.5 µg/mL using 1X HBS-EP+ buffer. A vortex was applied after each step. The binding of antigen to TUR01 was measured and fitted using a 1:1 kinetic binding model. A relative binding affinity was calculated by comparing the values obtained for the sample with respect to the reference.

RESULTS

In this study, the stability period and stability-indicating methods were investigated for both DS and DP of a recently developed biosimilar mAb functioning as a TNF-α inhibitor. DS stability was conducted for 18 months in PETG Nalgene bottles both at ≤-65.0°C and 5.0 ± 3.0°C, whereas DP stability was conducted for 24 months in type I glass pre-filled syringes at 5.0 ± 3.0°C and at 25.0 ± 2.0°C/60% ± 5 RH. The determined stability methods according to the European Pharmacopeia for both DS and DP are listed in Table 1. The critical quality attributes of the developed inhibitor were determined according to the physicochemical and functional properties of the 18 commercially available originators. The acceptance criteria

for shelf-life were defined due to these set critical quality attributes, as specified in Table 1.

For this particular molecule, the pH should be between 5 and 5.4 and osmolality needs to be between 285 and 340 mOsm/kg. Isoform profile with icIEF was designated as an identity test. The critical quality range for the protein content was defined as 45-55 mg/mL and the biological activity for the biosimilars in terms of comparative KD needs to be between 80-120%. Monomer amounts including the shelf life of both DS and DP should be over 98% and the IgG percentage should be over 95%.

Drug substance stability

Although most of the mAb are relatively stable at ≤-65.0°C, there are some circumstances that mAbs can be unstable. To determine the stability of TUR0, appearance, acidic and basic variants, protein content, biological activity, monomers, and

Table 1. Stability test methods, their acceptance criteria, and information regarding the methods used

Test type	Acceptance criteria	Reference method
Physicochemical Tests		
Appearance	Without visible particles	Visual inspection Ph. Eur. 2.9.20
Color	Not more intensely coloured than reference solution B7	Visual inspection Ph. Eur. 2.2.2
Opalescence	Not more opalescent than reference solution IV	Nephelometry Ph. Eur. 2.2.1
pH	5.0 – 5.4	Potentiometric determination Ph. Eur. 2.2.3
Osmolality	285-340 mOsm/kg	Osmometry Ph. Eur. 2.2.35
Sub-visible particulates		Light obscuration particle count test Ph. Eur. 2.9.19
≥10 µm	NMT 6000 particles/container	
≥25 µm	NMT 600 particles/container	
Extractable volume	0.79-0.84 mL	Volume determination (gravimetric) Ph. Eur. 2.9.17
Identity		
Isoform profile	Comparable to Ref. Std. Report pI range and % peak areas	icIEF In -house monograph
Assay		
Protein content	45.0-55.0 mg/mL	Absorbance at 280 nm (OD) In -house monograph
Biological activity: binding by biacore	80-120% (KD of sample/KD of reference material)	TNF-α binding using surface plasmon resonance In -house monograph
PURITY		
Size exclusion (Monomer, HMW species/ aggregates, LMW species	Monomer IgG: ≥98%	SE-UPLC In -house monograph
	HMW/aggregates ≤2%	
	Report value for LMW	
Electrophoretic purity (reducing)	≥ 95.0% IgG (heavy and light chains)	Reducing CE-SDS PAGE In -house monograph
Electrophoretic purity (non-reducing)	Report values for IgG LC, HH, HHL, and NG-IgG	Non-reducing CE-SDS PAGE In -house monograph
Microbiological tests		
Sterility	No growth	Membrane filtration Ph. Eur. 2.6.1
Bacterial endotoxins	≤0.2 EU/mg	LAL test: chromogenic kinetic method Ph. Eur. 2.6.14, USP <85>

aggregates, bioburden, and endotoxin were monitored for 18 months. Ultra-high performance liquid chromatography-size exclusion chromatography (SEC-UPLC), the protein contents, and binding activity were defined as the stability- indicating factors for drug substance. Table 2 shows all the data at months 0, 3, 6, 9, 12, and 18 at ≤-65.0˚C.

The stability of the DS was also followed at 5.0 ± 3.0˚C for 18 months and all the specifications were measured at months 0, 1, 2, 3, 6, 9, 12, and 18. Table 3 shows all the measurements for 18 months.

As indicated in Table 2 and Table 3, there was not any change in the appearance of the molecule for 18 months and it has passed. The isoform profile proves the similarity of the DS during 18 months to the reference standard. Figure 1 shows the acidic, basic variants and the main peak of DS at 0 months; at ≤-65.0˚C, 18 months; at 5.0 ± 3.0˚C, 18 months with the reference standard. The lowest main peak was 73.34% at ≤-65.0˚C whereas it was 71.95% at 5.0 ± 3.0˚C. At lower temperature, the monomer amount was 99% the lowest, although it was 98% at 5.0 ± 3.0˚C at the end of stability period. At both temperatures, endotoxin amount was lower than 0,1 EU/mg at the end and 18 months and bioburden was zero.

In all our detailed analyzes, we have demonstrated that our biosimilar DS is stable at ≤-65.0˚C and 5.0 ± 3.0˚C for 18 months in Nalgene PETG bottles.

Drug product stability

Liquid pharmaceuticals are generally being kept at 5.0 ± 3.0˚C due to the ease of reaching 5.0 ± 3.0˚C refrigerators at both pharmacies, hospitals, and houses. For this reason, it is critical to check stability during the shelf-life of the DP at 5.0 ± 3.0˚C. To see the stability of TUR01 drug product, protein content, appearance, color, opalescence, sub-visible particulates, pH, osmolality, extractable volume, acidic and basic variants, protein content, biological activity, monomers and aggregates, low molecular fragments, sterility, and endotoxin were followed for 24 months. Table 4 shows all the data at months 0, 1, 2, 3, 6, 9, 12, 18, and 24 at 5.0 ± 3.0˚C.

In addition to this, DP stability was followed at 25.0 ± 2.0˚C/60% ± 5 RH to see how long TUR01 can stay at room temperature. Similar specifications were checked, which are tabulated in Table 5.

DP stability results at 5.0 ± 3.0˚C for 24 months did not show any critical change, except the minor changes toward the end of 24 months. Most of the tests, including protein content,

Table 2. Drug substance stability results at ≤-65.0˚C for 18 months

Test methods	Months					
	0	3	6	9	12	18
Appearance	Pass	Pass	Pass	Pass	Pass	Pass
Isoform profile icIEF	Similar to reference standard	Similar to reference standard	Similar to reference standard	Similar to reference standard	Similar to reference standard	Similar to reference standard
pI Range	AP 2: 8.66	AP 2: 8.66	AP 2: 8.66	AP 2: 8.68	AP 2: 8.67	AP 2: 8.67
	AP 1: 8.78	AP 1: 8.79	AP 1: 8.77	AP 1: 8.79	AP 1: 8.77	AP 1: 8.78
	MP: 8.88	MP: 8.89	MP: 8.87	MP: 8.90	MP: 8.87	MP: 8.88
	BP 1: 8.96	BP 1: 8.96	BP 1: 8.95	BP 1: 8.97	BP 1: 8.95	BP 1: 8.95
	BP 2: 8.98	BP 2: 8.99	BP 2: 8.97	BP 2: 9.01	BP 2: 8.98	BP 2: 8.98
% Peak areas	AP 2: 5.80	AP 2: 5.73	AP 2: 5.83	AP 2: 5.08	AP 2: 5.66	AP 2: 5.74
	AP 1: 17.12	AP 1: 16.77	AP 1: 18.03	AP 1: 17.43	AP 1: 17.22	AP 1: 17.48
	MP: 74.44	MP: 74.80	MP: 73.34	MP: 75.07	MP: 74.12	MP: 74.00
	BP 1: 1.50	BP 1: 1.71	BP 1: 1.79	BP 1: 1.70	BP 1: 1.98	BP 1: 1.66
	BP 2: 1.13	BP 2: 0.99	BP 2: 1.00	BP 2: 0.72	BP 2: 1.02	BP 2: 1.12
Protein content	48.2	48.3	47.5	47.9	47.7	47.5
Biological activity	91	100	102	100	87	97
SE-UPLC	**Monomer** 99	99	99	99	99	99
	HMW 1	1	1	1	1	1
	LMW 0.51	0.53	0.49	0.61	0.58	0.44
Bioburden	0	NT	NT	NT	NT	0
Endotoxin	0.01	NT	NT	NT	NT	≤ 0.1 EU/mg

NT: Not tested, AP: Acidic peak, BP: Basic peak, MP: Main peak

opalescence, pH, and osmolality did not show significant change. There was variability in subvisible particulate measurement, which was due to the variability caused from the equipment itself. However, we started to see critical changes at 25.0 ± 2.0°C after 3 months as expected. Especially, monomer amount decreased to 97.40% and 96.20%, while aggregates and low molecular fragments were increased. Figure 1 demonstrates the SEC analysis results showing the changes from month 0 to month 6.

The non-reduced CE-SDS shown in Figure 2 also confirms the SEC results. As the aging happens at 25.0 ± 2.0°C, total IgG decreases, and the amount of HC, HH, and HHL increases, which explains the increase in low molecular weight fragments. In addition to non-reduced CE-SDS analysis, reduced CE-SDS analysis was carried out for 6 months, as shown in Figure 3. Although there was not any change at cold temperatures in the amount of total HC and LC, there was a small decrease in the total HC and LC at 25.0 ± 2.0°C from 99.41% to 98.90% (Figures 4, 5).

As a result, we have shown that our formulated DS is stable at ≤-65.0°C and 5.0 ± 3.0°C for at least 18 months and our formulated DP is stable in syringes at 5.0 ± 3.0°C for 24 months. It was also shown that DP stability at 25.0 ± 2.0°C/60% ± 5 RH starts decreasing after 2 months.

DISCUSSION

In this study, a deep investigation was conducted to understand the stability of TUR01 DS and drug product. Both accelerated and prolonged stability were studied at different temperatures. In this way, it was aimed to cover a larger range of temperatures for TUR01 to understand its behavior.

Different physical and chemical instabilities can occur for monoclonal antibodies during their storage, transportation, and administration. The approval and marketing of the biosimilars requires an extensive comparability study with the reference products. In addition to the comparability, the stability studies become extensively important since different modifications can be observed on the biosimilar molecule due to the variability of the cell line, cell culture conditions, passage number of the cells, and post-translational modifications. Due to these differences, extensive stability study is required by the regulatory agencies before the approval and marketing of the biosimilars.[13]

Table 3. Drug substance stability results at 5.0 ± 3.0°C for 18 months

Test methods		Months							
		0	1	2	3	6	9	12	18
Appearance		Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Isoform profile icIEF		Similar to reference standard	Similar to reference standard	Similar to reference standard	Similar to reference standard	Similar to reference standard	Similar to reference standard	Similar to reference standard	Similar to reference standard
pI Range		AP 2: 8.66	AP 2: 8.67	AP 2: 8.67	AP 2: 8.67	AP 2: 8.67	AP 2: 8.68	AP 2: 8.67	AP 2: 8.67
		AP 1: 8.78	AP 1: 8.78	AP 1: 8.77	AP 1: 8.78	AP 1: 8.77	AP 1: 8.79	AP 1: 8.78	AP 1: 8.78
		MP: 8.88	MP: 8.88	MP: 8.88	MP: 8.89	MP: 8.87	MP: 8.90	MP: 8.88	MP: 8.88
		BP 1: 8.96	BP 1: 8.96	BP 1: 8.96	BP 1: 8.96	BP 1: 8.95	BP 1: 8.97	BP 1: 8.95	BP 1: 8.95
		BP 2: 8.98	BP 2: 8.99	BP 2: 8.99	BP 2: 9.00	BP 2: 8.98	BP2: 9.00	BP 2: 8.98	BP 2: 8.98
% Peak area		AP 2: 5.80	AP 2: 5.69	AP 2: 4.90	AP 2: 5.29	AP 2: 5.38	AP 2: 5.32	AP 2: 6.42	AP 2: 5.18
		AP 1: 17.12	AP 1: 17.73	AP 1: 17.56	AP 1: 17.74	AP 1: 17.11	AP 1: 18.01	AP 1: 18.21	AP 1: 18.55
		MP: 74.44	MP: 73.32	MP: 74.15	MP: 74.29	MP: 74.77	MP: 73.80	MP: 71.95	MP: 72.74
		BP 1: 1.50	BP 1: 2.02	BP 1: 2.18	BP 1: 1.82	BP 1: 1.73	BP 1: 2.03	BP 1: 2.20	BP 1: 2.27
		BP 2: 1.13	BP 2: 1.24	BP 2: 1.20	BP 2: 0.86	BP 2: 1.02	BP2: 0.85	BP 2: 1.22	BP 2: 1.27
Protein content		48.2	48.5	49.0	48.5	47.6	48.9	48.0	49.3
Biological activity		91	94	96	105	112	97	99	104
SEC	Monomer	99	98	98	98	98	98	98	98
	HMW	1	1	1	1	1	1	1	1
	LMW	0.51	0.50	0.48	0.57	0.59	0.83	0.75	0.71
Bioburden		0	NT	NT	NT	NT	NT	NT	0
Endotoxin		0.01	NT	NT	NT	NT	NT	NT	≤0.1 EU/mg

NT: Not tested, AP: Acidic peak, BP: Basic peak, MP: Main peak

Temperature changes during processing, storage or transportation can cause perturbations to the monoclonal antibody. Therefore, it is crucial to have information at different temperatures for a certain time.[14] Not only high temperatures, but also low temperatures can also cause changes in protein conformation. In particular, freeze-thaw cycles have shown an impact on the mAb aggregation in the literature.[15-19]

At ≤-65.0°C, it is proven that TUR01 is stable in Nalgene bottles for 18 months. Any significant changes have not been observed

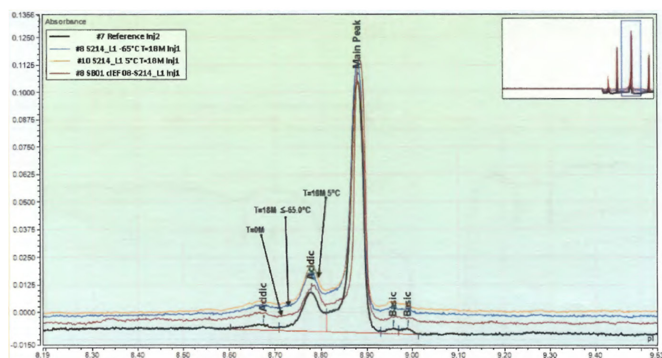

Figure 2. Comparison of DRS to DS at 0 month, DS at 5.0 ± 3.0°C at 18 months and DS ≤-65.0°C at 18 months

DS: Drug substance, Drug reference standard

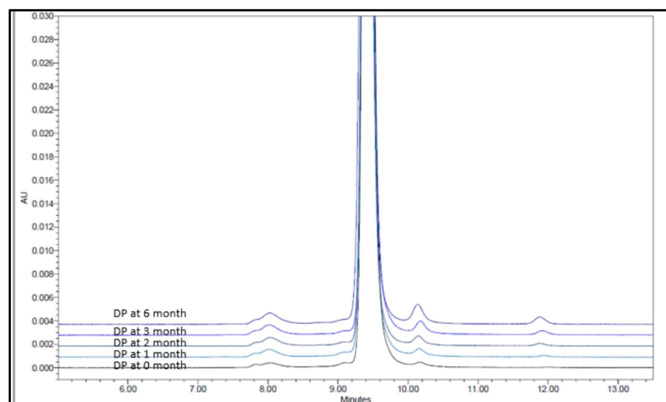

Figure 3. Size exclusion chromatography analysis results of drug product at months 0, 1, 2, 3, and 6 at 25.0 ± 2.0°C

during 18 months in the product characteristics. However, there has been only a slight change in the main peak at 5.0 ± 3.0°C for 18 months (ICE data). This slight decrease was still in the range of critical quality attributes showing the stability of TUR01 DS.

In the primary packaging, we have also proven the stability of the TUR01 DP at 5.0 ± 3.0°C for 24 months. There has been a slight decrease in the main peak, while a slight increase was observed in the acidic peak after 6 months. All the ICE data have demonstrated that the product characteristics have been in the range of critical quality attributes. However, there have been also a slight increase in the aggregate and low molecular weight, but all the values remained in the range even after 24 months at +2-8°C. The stability study of TUR01 DP at 25.0 ± 2.0°C was conducted for 6 months and it has been proven that the product is stable only for 2 months at room temperature. After 2 months, a decrease in the monomer was observed, while aggregates and low molecular weight species were increasing. After month 2, the main peak started to significantly decrease too.

During the stability study, there were also some oscillations in subvisible particulate and relative binding values. The subvisible particulate ≥10 μm needs to be lower than 6000 particles *per* container. The values measured in this study, were too low compared to 6000. There was some variability in the measurements due to the sensitivity of the equipment. At all stability time points, the relative binding affinity was calculated by comparing the values obtained for the sample with respect to the reference. At each sampling point, biosimilar and the reference KD values were measured, and the relative value was calculated. The difference between the stability points was due to the variability in the measurements.

All this data proves to us that TUR01 can be kept at 5.0 ± 3.0°C for 24 months and it does not get degraded at room temperature for 2 months. In case the cold chain is broken for 2 months, it can be still safe to use this drug product.

CONCLUSION

The results obtained to date for TUR01 DS indicate that the DS is stable, when stored under long-term storage conditions

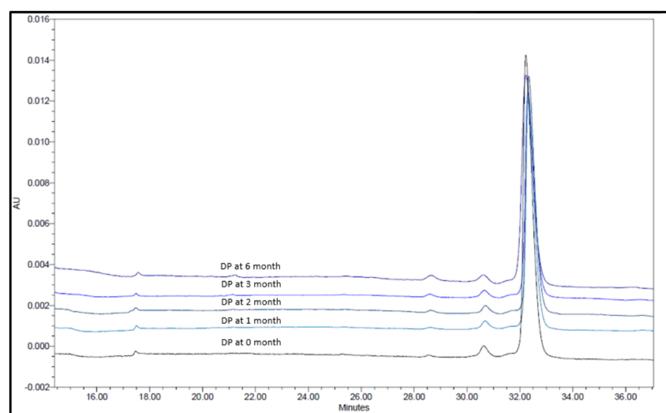

Figure 4. Non-reduced capillary electrophoresis-sodium dodecyl sulfate analysis results of drug products at months 0, 1, 2, 3, and 6 at 25.0 ± 2.0°C

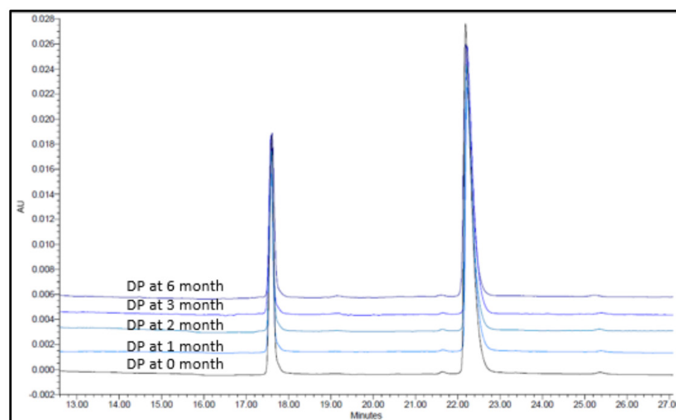

Figure 5. Reduced capillary electrophoresis-sodium dodecyl sulfate analysis results of drug products at months 0, 1, 2, 3, and 6 at 25.0 ± 2.0°C

Table 4. Drug product stability results at 5.0 ± 3.0°C for 24 months

Test		Testing interval (months)								
		0 M	1 M	2 M	3 M	6 M	9 M	12 M	18 M	24 M
Protein content		49.9	49.8	49.6	50.1	49.9	49.7	51.5	49.7	50.5
Appearance		Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Color		B8	B8	B8	B8	B8	B8	B8	B8	B8
Opalescence		8.3	9.6	10	8.9	8.3	10.1	10.7	10.2	9.5
Sub-visible particulates										
≥10 µm		147	92	83	137	228	245	49	235	13
≥25 µm		3	3	4	4	5	124	0	3	1
pH		5.3	5.3	5.3	5.3	5.3	5.3	5.3	5.3	5.3
Osmolality		299	300	316	313	317	318	313	311	316
Extractable volume		0.8	NT	NT	NT	0.8	NT	NT	NT	NT
SEC	Monomer	99.10%	98.90%	98.80%	98.70%	98.50%	98.30%	98.00%	98.00%	98.00%
	Aggregate	0.57%	0.70%	0.78%	0.84%	0.98%	1.09%	1.28%	1.28%	1.20%
	LMW	0.34%	0.43%	0.40%	0.47%	0.47%	0.60%	0.79%	0.70%	0.80%
Isoform profile icIEF		Comparable to ref.	Comparable to ref.	Comparable to ref.	Comparable to ref.	Comparable to ref.	Comparable to ref.	Comparable to ref.	Comparable to ref.	Comparable to ref.
pI range		AP: 8.66-8.77	AP: 8.66-8.76	AP: 8.68-8.79	AP: 8.67-8.77	AP: 8.66-8.77	AP: 8.67-8.76	AP: 8.66-8.77	AP: 8.67-8.78	AP: 8.68-8.80
		MP: 8.87	MP: 8.86	MP: 8.88	MP: 8.87	MP: 8.87	MP: 8.87	MP: 8.86	MP: 8.88	MP: 8.90
		BP: 8.95	BP: 8.94	BP: 8.97	BP: 8.95	BP: 8.94	BP: 8.95	BP: 8.94	BP: 8.98	BP: 8.97
% Peak area		AP: 22.28%	AP: 22.09%	AP: 21.01%	AP: 23.06%	AP: 22.90%	AP: 24.80%	AP: 18.56%	AP: 23.07%	AP: 24.62%
		MP: 75.15%	MP: 74.92%	MP: 76.28%	MP: 73.89%	MP: 74.29%	MP: 72.14%	MP: 76.66%	MP: 72.95%	MP: 71.71%
		BP: 2.57%	BP: 3.00%	BP: 2.72%	BP: 3.05%	BP: 2.82%	BP: 3.06%	BP: 4.78%	BP: 3.99%	BP: 3.69%
CE-SDS (red) (HC + LC)		99.41%	99.46%	99.47%	99.43%	99.48%	99.49%	99.48%	99.54%	99.58%
CE-SDS (Non-Red)	IgG	95.28%	95.84%	95.71%	95.23%	95.95%	95.73%	95.73%	94.82%	95.12%
	LC	0.62%	0.77%	0.68%	0.42%	0.60%	0.76%	0.90%	0.68%	0.69%
	HH	0.33%	0.30%	0.40%	0.46%	0.45%	0.32%	0.60%	0.39%	0.27%
	HHL	2.97%	2.49%	2.51%	2.79%	2.45%	2.57%	2.08%	2.87%	2.50%
	NG-IgG	0.79%	0.60%	0.70%	1.10%	0.55%	0.61%	0.69%	1.24%	1.42%
Biacore		110.50%	104.60%	108.10%	103.40%	116.10%	111.20%	96.20%	91.80%	82.80%
Endotoxins		0.0074	0.0011	0.0068	0.001	0.0048	0.001	0.0025	NT	0.026
Sterility		No growth	NT	NT	NT	No growth	NT	NT	NT	No growth

NT: Not tested, AP: Acidic peak, BP: Basic peak, MP: Main peak

Table 5. Drug product stability results at 25.0 ± 2.0°C/60% ± 5 RH

Test		Testing interval (months)				
		0 M	1 M	2 M	3 M	6 M
Protein content		49.9	49.8	49.9	49.2	50.7
Appearance		Pass	Pass	Pass	Pass	Pass
Color		B8	B8	B8	B8	B8
Opalescence		8.3	9.4	9.3	8.4	8.5
Sub-visible particulates						
≥10 µm		147	73	84	49	92
≥25 µm		3	1	1	0	0
pH		5.3	5.3	5.3	5.3	5.3
Osmolality		299	300	316	313	319
SEC	Monomer	99.10%	98.20%	98.00%	97.40%	96.20%
	Aggregates	0.57%	1.06%	1.09%	1.30%	1.53%
	LMW	0.34%	0.78%	0.90%	1.33%	2.25%
Extractable volume		0.8	NT	NT	NT	NT
Isoform profile icIEF		Comparable to reference pI	Comparable to reference pI	Comparable to reference pI	Comparable to reference pI	Comparable to reference pI
pI range		AP: 8.66-8.77	AP: 8.65-8.76	AP: 8.68-8.79	AP: 8.67-8.78	AP: 8.54-8.77
		MP: 8.87	MP: 8.85	MP: 8.89	MP: 8.87	MP: 8.87
		BP: 8.95	BP: 8.93	BP: 8.97	BP: 8.95	BP: 8.94
% Peak areas		AP: 22.28%	AP: 22.63%	AP: 26.19%	AP: 29.33%	AP: 38.78%
		MP: 75.15%	MP: 74.40%	MP: 69.21%	MP: 65.53%	MP: 55.49%
		BP: 2.57%	BP: 2.98%	BP: 4.61%	BP: 5.14%	BP: 5.74%
CE-SDS (red) (HC+LC)		99.41%	99.43%	99.39%	99.25%	98.90%
CE-SDS (Non-Red)	IgG	95.28%	95.54%	94.83%	93.89%	93.17%
	LC	0.62%	0.62%	1.00%	0.57%	0.87%
	HC	ND	ND	ND	ND	0.68%
	HH	0.33%	0.45%	0.64%	0.91%	1.54%
	HHL	2.97%	2.57%	2.51%	3.16%	2.60%
	NG-IgG	0.79%	0.81%	1.03%	1.47%	1.15%
Biacore		110.50%	109.70%	114.90%	107.90%	118.00%
Endotoxins		0.0074	0.0075	NT	0.001	0.0045
Sterility		No growth	NT	NT	NT	No growth

NT: Not tested, AP: Acidic peak, BP: Basic peak, MP: Main peak

≤-65°C for at least 18 months. TUR01 DS in Nalgene PETG bottles stability under short-term storage conditions at 5 ± 3°C is stable also at least 18 months. In this study, it is proved that the TUR01 DP is stable at 5 ± 3°C for 24 months and at 25 ± 2°C/60.5% RH for 2 months.

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Ethics

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REVIEW

Folate-Mediated Paclitaxel Nanodelivery Systems: A Comprehensive Review

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ABSTRACT

Paclitaxel (PTX) is used as a viable cancer medication in the chemotherapy of breast, ovarian, lung, bladder, neck, head, and esophageal tumors. The focus of this review is to survey various folate-targeting PTX-loaded nanopreparations in both research and clinical applications. There are diverse nanopreparations, including liposomes, micelles, polymeric nanopreparations, lipid nanopreparations, lipoprotein nanocarriers, and other inorganic nanopreparations for folate-associated PTX tumor targeting. Here, the folate targeting PTX-loaded nanopreparations, which have promising results in the constructive treatment of cancer by reducing toxic side-effects and/or improving effectiveness, was mainly reviewed.

Key words: Paclitaxel, anticancer, folate receptor, tumor targeting, nanopreparations

INTRODUCTION

Cancer is a huge group of diseases in which a part of the body can be affected. As *per* World Health Organization (WHO), globally cancer is the most common cause of death. In 2020, about 10 million deaths are caused by cancer. About 0.685 million deaths occur due to breast cancer, 0.83 million are due to liver cancer, 0.769 million are due to stomach cancer, while 1.80 million are due to lung cancer. Also, about a population of 2.26 million are being affected by breast cancer, 1.41 million from prostate cancer, 1.93 million from colorectal cancer, 1.20 million from non-melanoma skin cancer, and 1.09 million from stomach cancer.[1] Nowadays, cancer can be treated by surgery, photodynamic therapy, radiotherapy, photothermal therapy, and by using chemotherapeutic agents.[2] Chemotherapy includes the usage of various drugs for killing purpose of cancerous cells, but along with the affected cells, they kill healthy cells and, thus, cause toxicity. This toxicity is because to less targeting of the cancerous cells, and therefore, there is a need to develop the chemotherapeutics for effective targeting of cancerous cells, either by active targeting or by passive targeting. The active targeting is achieved by incorporating a molecule or ligand that can bind to overexpressed receptors on the targeted cancerous cells.[3]

Amongst the taxane group of drugs, paclitaxel (PTX) is the first to be used as a chemotherapeutic agent.[4] PTX is a diterpenoid available as a white crystalline powder, isolated from the bark of *Taxus brevifolia* Nutt. (Taxaceae), known as the Northwest Pacific yew tree, with a melting point of ~210°C having formula $C_{47}H_{51}NO_{14}$ and it was first revealed by "Mrs. Manroe E. Wall and Mansukh C. Wani".[5] The chemotherapeutic agent, PTX has a huge spectrum of activity over several cancers such as metastatic breast cancer, non-small-cell lung cancer (NSCLC), AIDS-related Kaposi's sarcoma, refractory ovarian cancer, head and neck malignancies,[6] malignant lymphoma, and lymphoblastic leukemia.[7] It exerts a cytotoxic effect by inhibiting late G2 or mitosis phases of the cell division through stabilization of microtubule.[4]

PTX is highly hydrophobic and poorly soluble in water (~0.4 μg/mL), thus, to enhance its solubility and make it bioavailable, the commercial formulation Taxol® was formulated. Taxol® is the parenteral solution containing 6 mg/mL PTX in a combination of polyoxyethylated castor oil (Cremophor EL) and dehydrated ethanol at a ratio of 1:1 v/v. Before *i.v.* administration, the above solution is diluted 5 to 20 fold with 0.9% sodium chloride injection or with other aqueous *i.v.* solutions.[8] Cremophor EL causes severe side effects such as neurotoxicity, hypersensitivity

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reaction, nephrotoxicity, and cardiotoxicity[9]. Besides, it also affects endothelial and vascular muscles causing vasodilation, labored breathing, lethargy, and hypotension.[8] To minimize the side effect of Taxol®, it is given with pretreatment using corticosteroids (*e.g.* dexamethasone), diphenhydramine, and H_2- receptor antagonists (*e.g.* cimetidine and ranitidine).[10] The major problem that arises for successful chemotherapy is due to the toxic effect of conventional surfactant used and the availability of the drug. Thus, the successful chemotherapy is mainly based upon the development of a novel delivery system.[11] For enhancing the solubility and tumor targeting of PTX, several investigations were done including liposomes, microspheres, nanoparticles (NP), polymeric micelles,[10] CD complexes,[6] nanospheres, emulsions, and polymeric conjugates.[11]

Site-specific drug delivery to a target organ, tissue or cell is known as drug targeting. The therapeutic effect of the drug is to enhance by either delivering the drug to the target site or by reducing the drug delivery to the site other than the target site.[12] Besides, the binding of an active targeting moiety or cancer cell-specific ligand to the surface of a drug can boost the uptake of drug in tumor cells, hence, it also enhances the therapeutic efficacy and reduces the side effects.[13] Among the variety of methods, ligand-mediated targeting of cancerous cells by targeting the receptors overexpressed on tumor cells was found to be most effective in complimenting therapeutic effectiveness and lowering the side effects.[14] Ligand-mediated targeting is achieved by chemically modifying the drug with tumor-targeting signaling molecules such as transferring, sugar, peptides, folic acid (FA), and antibody.[15] As a tumor targeting-ligand, FA has various advantages; FA has a high binding attraction towards folate receptors (FR) and these receptors are expressed in large numbers on various tumor cells of the brain, ovary, lungs, kidney, myelogenous cells, and breast. Along with organic compounds, FA has a high compatibility in aqueous solvents, while also low immunogenic. Due to its low molecular weight, it can be chemically modified easily and has low cost.[16]

FA and FR

FA is a naturally occurring vitamin B9 and is also the synthetic form of folate. In several metabolic pathways, FA is required for the one-carbon reaction. It helps making DNA and genetic material by biosynthesizing nucleotide bases, thus, FA is consumed in larger amounts by proliferating cells.[17] FA shows a dual mechanism for tumor specificity, as because FR is mainly expressed on the outside of the apical membrane of epithelial cells, it is inaccessible to the chemicals, which are formed in the blood cells and becomes inaccessible to the drug in circulation hence it provides local targeting. Upon transformation of epithelial cell, the polarity of cell loss and thus FR, is accessible to the drug in circulation. These all make FA a popular ligand for targeting.[18]

FR is a glycosylphosphatidylinositol-anchored membrane glycoprotein and known as "high-affinity membrane folate-binding protein" having an apparent molecular weight of 38-40 kDa.[19] FR exists in three isoforms, *e.g.* hFRα, hFRβ, and hFRγ. Out of these isoforms, hFRα is expressed in a large amount in an expansive range of cancerous cells of the uterus, ovary, cervix, breast, kidney, testis, colon, brain, and pituitary gland, at the same time, hFRb leukemias, and activated macrophages.[20] There is almost no expression of FR in healthy cells, whereas it is highly expressed in undifferentiated metastatic cancer sites (Figure 1).[21]

Folate-mediated PTX NPs

A. PTX prodrug NPs

Because of limited lipophilicity and poor aqueous solubility formulation of lipid-based delivery system for PTX is difficult. To overcome this complication, Stevens et al.[22] synthesized a prodrug of PTX that is "PTX-7-carbonyl-cholesterol (Tax-Chol)" with enhanced lipophilicity, also incorporated into lipid nanoparticles (LNs) formulation containing "folate-polyethyleneglycol-cholesterol (f-PEG-Chol)" as a ligand that targets FR. The drug-to-lipid ratio of FR-targeted LN formulation was 1:20. The resulting LNs had a smaller particle size (130 nm), higher entrapment efficiency (>90%), exhibited excellent colloidal stability, and *in vitro* therapeutic activity against tumor cells. The LNs displayed a better uptake and cytotoxicity in FR-targeted and FR-positive KB-cells and M109-cells than FR non-targeted cells. Also, the *in vivo* FR-targeted LNs notably augmented the antitumor activity along with animal survival compared to non-targeted LNs and Cremophor EL containing PTX formulation used for treating FR-positive M109 tumors bearing mice. The PTX pro-drug (Tax-Chol) incorporated LNs formulations show a greater potential for treating FR positive tumors cell than parent drug formulation.[22]

A conjugation of PTX-poly-ethyl ethylene phosphate (PTX-PEEP) with FA (PTX-PEEP-FA) forms a novel prodrug that is soluble in water, has also been reported for targeted PTX delivery.[23] The steps involved in the synthesis of this prodrug are that, firstly, the amphiphilic pro-drug PTX-PEEP was formed by a ring opening polymerization reaction of 2-ethoxy-2-oxo-1,3,2-dioxaphospholane monomer, in which the catalysis of stannous octoate [$Sn(Oct)_2$] was started at PTX. In the next step, the

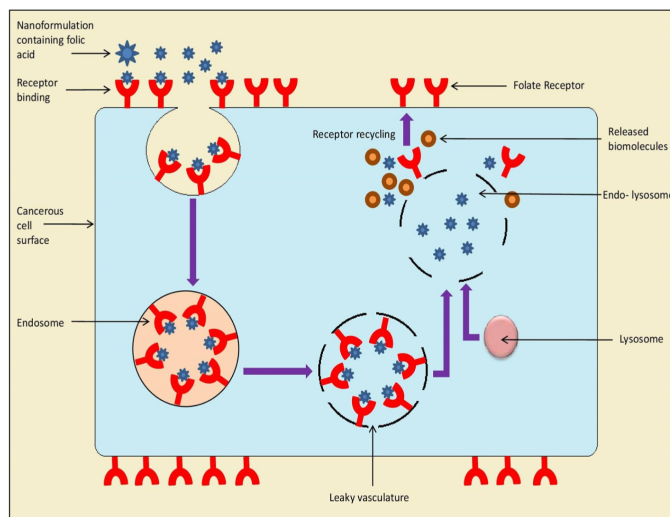

Figure 1. Receptor-mediated endocytosis of a drug conjugated to folates

process of esterification leads to form covalent conjugate of FA with PTX-PEEP. This conjugate is known as PTX-PEEP-FA amphiphilic polymeric pro-drug and is biodegradable. In an aqueous solution, the resulting PTX-PEEP-FA pro-drug was self-assembled and converted into micelles. The micelles had PTX in the hydrophobic core and PEEP in a hydrophilic coat, that as confirmed under transmission electron microscopy (TEM) and dynamic light scattering analysis (DLS). The micellar formulation was found to have smaller particle size (<130 nm), greater stability during systemic circulation, and exhibited better *in vitro* sustained-release behavior as compared to free doxicyclin (DOX) or PTX. The phosphoesterase-I degrade PEEP chain therefore the polyphosphoester-based pro-drug displayed lesser cytotoxicity of the parent drug up to the degradation of PEEP. The surface FA moiety enhances the selectivity, targeting, and efficiency of drug delivery, which were assessed *via* live-cell imaging system, by observing the cellular uptake of DOX-loaded PTX-PEEP-FA micelles for HeLa and KB cells, respectively. The endocytosis process, which is mediated by the FR, accelerates the cellular uptake of the drug formulation; hence, PTX-PEEP-FA micelles is the promising formulation for the targeted drug release intracellularly.

B. Copolymeric NPs

The copolymeric micelles are formed, when amphiphilic copolymer having both polar and non-polar segments is exposed to an aqueous environment it get self-assemble and forms a core and shell structure. As they contain both polar and non-polar portions, they may become effective targeting carriers for various water-soluble and water-insoluble amphiphilic drugs and genes to cancer cells.[24] The hydrophobicity and non-targeting nature of the drug-like PTX need to encapsulate in functionalize polymeric micelles for better therapeutic activity. The polymeric NPs can be prepared by covalent coupling[25] and physical encapsulation.[26] Physical encapsulation has the advantage like maximum drug loading efficiency of NPs, but it also has disadvantages such as easy leaking tendency at the time of delivery to the target site.[27] However, the NPs cannot achieve an adequately high concentration of drug in the cells of the tumor. For effective targeting and reducing side effects, introducing targeting moieties such as FA into NPs are required.

1. Poly-lactide (PLA) NPs

PLA is a matrix material used mostly for the formulation of polymeric NPs because of its biodegradability and safety. Wang et al.[28] effectively targeted the poorly water-soluble PTX to cancer cells by developing the folate associated hybrid polymeric NPs (FD-NPs). These FD-NPs were composed of monomethoxy-PEG-*b*-poly-lactide-PTX (MPEG-PLA-PTX) and D-R-tocopheryl PEG 1000 succinate folate (TPGS-FOL). As it remains an amphiphilic polymer the MPEG-PLA-PTX may self-assemble into NPs, even after PTX is in chemical conjugation with MPEG-PLA molecule. PTX could be delivered by physical encapsulation and chemical conjugation from FD-NPs. TPGS is a non-ionic water-soluble PEG-derivative of natural vitamin E and is one of the Food and Drug Administration (FDA) approved safe pharmaceutical adjuvants used in drug formulation. The usage of TPGS in a formulation of NPs can enhance the drug loading as well as absorption of drug-like PTX.[28]

Besides, Xiong et al.[29] developed folate-conjugated interfacially crosslinked biodegradable micelles composed of poly(ethylene glycol)-*b*-poly(acryloyl carbonate)-*b*-poly(D, L-lactide) (PEG-PAC-PLA) and FA-PEG-PLA block copolymers for the delivery of PTX *via* receptors into KB-cells. In these crosslinked biodegradable micelles, the PEG-PAC-PLA was produced to crosslink micelles at an interface in presence of ultraviolet (UV) radiation, while FA-PEG-PLA was used to target cancer cells that over expresses FR. The crosslinked micelles were found to have better physicochemical properties and stability compared to non-crosslinked controls. Likewise, it displayed sustained-release properties at low micelles concentration. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was conducted to evaluate the toxicity of crosslinked micelles in KB-cells and it was confirmed that increasing the concentration of folate in crosslinked or non-crosslinked micelles enhances the toxicity of PTX. The FA conjugated crosslinked micelles were evaluated for their cellular uptake by flow cytometry on KB-cells. The folate-decorated fluorescein isothiocyanate labeled linked micelles were found to have significant greater cellular uptake compared to micelles, which did not have folate ligands, revealing that the FA-conjugated crosslinked micelles of PEG-PLA are a promising way to target cancer therapy.[29]

Thu et al.[30] also prepared folate decorated PTX-loaded PLA-tocopheryl PEG 1000 succinate (TPGS) NPs (Fol-PTX-PLA–TPGS NPs) by a solvent evaporation or modified emulsification method. For the formulation of NPs, the ring-opening method was used to synthesize PLA-TPGS copolymer, and folate was attached covalently to TPGS (TPGS-Fol). The confirmation of NPs was done by DLS method, fourier-transform infrared (FTIR), and field emission scanning electron microscopy (SEM). In physical appearance, the NPs were seen to be spherical in shape having 50 nm size and showed a narrow size distribution. In the *in vitro* study on the HeLa cell line, the Fol-PTX-PLA-TPGS NPs demonstrated better targeting efficiency than free PTX and PTX-PLA-TPGS NPs. Also the *in vivo* analysis was performed on the colorectal tumor-bearing nude mice to investigate the inhibition activity of NPs on tumor growth, and it was found that Fol-PTX-PLA-TPGS NPs inhibit tumor growth most efficiently compared to only PTX and PTX-PLA-TPGS NPs. The results of both *in vitro* and *in vivo* analysis clearly indicated that the use of folate as a targeting agent effectively enhances delivery of PTX at the targeted site.[30]

2. Poly(lactide-co-glycolic acid) (PLGA) NPs

Due to many advantages of it in manufacturing of nanodelivery systems, PLGA is known as the best biodegradable copolymer. In the body, it produces non-toxic products of lactic and glycolic acids by hydrolysis, and at the end it produces carbon dioxide and water. As the body effectively deals with these degradants, PLGA is less prone to cause the systemic toxicity. In this view, He et al.[31] and a co-worker have attempted to synthesize the amphiphilic copolymer, FA conjugated poly(ethylene glycol)-

poly(lactic-co-glycolic acid) (FA-PEG-PLGA) NPs for treating FR overexpressing tumor cells. FA-PEG-PLGA NPs are expressed as excellent carriers for combinational therapy of PTX and cisplatin (cis-diaminodichloro platinum, CDDP). As the NPs formed by conjugation of FA show active targeting and more uptake of NPs at target site, this improves the efficacy of PTX and CDDP and reduces the side effects associated with it. [31]

3. Polyacrylamide NPs

The monomer of polyacrylamide is used in the preparation of thermal sensitive, pH-sensitive, and water-swellable preparation. In response, Seow et al.[10] fabricated a new amphiphilic copolymer, cholesterol-grafted poly(*N*-isopropylacrylamide-co-N, *N*-dimethylacrylamide-co-undecenoic acid) [P(NIPA-DMA-UA)-g-cholesterol]. The folate decorated P(NIPA-DMA-UA)-g-cholesterol conjugate was formed by conjugation of folate to the polar segment of the prepared copolymer. The ^{1}H-NMR technique was used to confirm the synthesis of the polymer and it also shows a lower critical micelle concentration (CMC) of ~ 20 mg/mL. In the presence of cholesterol, the polymer self-assembled and formed micelles *via* membrane dialysis techniques. A highly hydrophobic drug such as PTX was encapsulated into the hydrophobic cores of these micelles, thus, the solubility of PTX in water significantly increased. The lower consolute temperature and particle size of the micelles containing the drug depended on external pH values. Similarly, the micelles released PTX more rapidly at a pH 5.0 *i.e.*, in an acidic environment than normal extracellular pH 7.4, thus it was confirmly shows pH-responsive thermal sensitivity.

The *in vitro* cytotoxicity assay was performed to determine therapeutic potential against FR overexpressing KB cells, which provided evidence that the PTX-loaded functionalized micelles more effectively killed KB cells due to the FR-assisted endocytosis process. In the prepared copolymer, no significant cytotoxicity was assessed with the polymeric carriers without drug and the targeted micellar formulation exhibited potential targeting efficiency and intracellular delivery.[10]

4. Pluronic NPs

Because of its amphiphilic nature and triblock structure pluronic is most widely used in the preparation of polymeric NPs. The triblock structure [poly(ethylene oxide) (PEO)-poly(propylene oxide) (PPO)-PEO] is made up of hydrophilic PEO blocks and hydrophobic PPO blocks. Zhang et al.[32] (2011) developed mixed micelles with FA functionalized pluronic P123/F127 and PTX encapsulated in it (FPF-PTX). All these were tested *in vivo* and *in vitro* for selective targeting using pluronic P123/F127 mixed micelles loaded with PTX (PF-PTX) and taxol as control. The size of particles of the prepared FPF-PTX micelles was decreased up to 20 nm and found to a spherical shape with a higher entrapment efficiency. In an *in vitro* study of cellular uptake, it was investigated that the FPF-PTX micelles show cellular uptake in time-dependent way and was more due to the endocytosis mediated by FR compared to PF-PTX. The effect of FPF-PTX on cell apoptosis, cytotoxicity, and cell-division cycle arrest was studied in KB and KBv cells and it confirmed that the prepared FPF-PTX was founded more efficient than Taxol® and PF-PTX. In the pharmacokinetic study, it was also shown that the bioavailability of FPF-PTX NPs in rats was 3 fold greater than that of Taxol®.

The *in vivo* study revealed that the antitumor efficiency of FPF-PTX group was more effective in KBv multi-drug resistant (MDR) tumor-bearing BALB/c mice than those of the Taxol® and PF-PTX treated groups. The additive effect of MDR inverting ability of pluronic block copolymers and active targeting by FA and FR, the therapeutic efficacy of FPF-PTX was enhanced.[32]

C. Other nanoparticles

1. Albumin/albumin moiety NPs

The folate-conjugated chemotherapeutic showed poor remedial adequacy caused by limited blood circulation or suboptimal pharmacokinetics. The use of albumin to enhance the pharmacokinetics of the drug is an optimistic approach; furthermore, it improves the circulation time of the drug through blood and accumulation of drug in tumors. Albumin is a protein present abundantly in plasma and is the important carrier for delivery of drugs derived from endogenous and exogenous substances.[33] Conjugating drugs to albumin[34] and albumin-binding moieties[35] are the most prosperous approaches as they enhance the efficiency of delivery of antitumor drugs and lower the side effects. Longer PTX blood circulation time and improved pharmacokinetic properties of PTX were reported by decorating the folate to PTX-loaded biodegradable bovine serum albumin (PTX-BSA-NPs)[34] and Evans blue (EB) an albumin-binding moiety conjugated to FA-PTX.[35] The small molecule EB exhibits a greater binding affinity toward blood circulating albumin. The formation of bifunctional prodrug by binding of albumin and albumin binding moieties to folate for both active and passive targeted PTX delivery results in high antitumor activity and lower toxicity.

BSA magnetic nanocomposites have also been reported for PTX delivery and tumor diagnosis. A simple modification process was used to develop BSA magnetic nanocomposites of PTX with carboxymethyl cellulose (CMC) (PTX-BSA-CMC-FA) and chitosan (CS) (PTX-BSA-CS-FA). The BSA-CMC-FA and BSA-CS-FA conjugates were prepared by an esterification reaction of FA to CMC and CS, respectively. The nickel ferrite ($NiFe_2O_4$) nanocores (NFs) PTX-NFs-BSA-CMC-FA and PTX-NFs-BSA-CS-FA were prepared *via* thermolysis of nickel acetylacetonate and PTX loading by the diffusion process. Irrespective of FA-modified surface the fabricated multifunctional nanoconjugates demonstrated better dispersibility, excellent transversal R2 relaxation rate, along with FR targeted and magnetically guided functions. Tumor diagnosis and tumor inhibition rate of PTX-NFs-BSA-CMC-FA, and PTX-NFs-BSA-CS-FA nanoconjugates were effectively enhanced by the application of an external magnetic field.[36]

Besides, Chen et al.[37] generated lipoprotein-mimicking nanocomplex for dual targeting therapy through the electrostatic attraction of FA modified BSA (FB) and LNs loaded with PTX (PTX-LNP). The thin-film hydration method was used to prepare

PTX-LNP and the FB complex was prepared by conjugation of FA with BSA. SPARC-albumin interaction leads to increased gp60-mediated transendothelial transport along with accumulation of the drug in tumor cells thus BSA provides specific targeting to tumor cell, hence, it is employed as a protein in lipoprotein mimicking nanocomplex FB-PTX-LNP. Further, the conjugated FA to BSA accomplished the active dual targeting delivery. *In vitro* cytotoxicity assay was performed against MCF-7 and HepG2 cells and the study revealed that FB-PTX-LNP and BSA-PTX-LNP exhibited considerably a more cytotoxic effect compared to PTX-LNP. Flow cytometry analysis was performed to determine cellular uptake of the drug by MCF-7 cells and it indicated that FB-coumarin-6-LNP get quickly uptaken compared to BSA-coumarin-6-LNP and coumarin-6-LNP. In the *in vivo* analysis on mice bearing MDA-MB-231 tumor, it appeared that FB-PTX-LNP indicated better ability to target tumor cells with promising anti-tumor activity. In preparation of dual-targeted PTX-loaded protein lipid nanocomplex FB-PTX-LNP, BSA, and FA both play a considerably important role.[37]

2. Heparin NPs

Heparin is a versatile natural polymer that commonly attaches to angiogenic growth factors and was used to initiate the self-assembly of nanomicelles from designated amphiphilic molecules of peptide.[38] Heparin also improved response to drug and survival period in patients taking chemotherapy for cancer,[39] and inhibited the tumor growth related to binding of growth factors.[40] In response, Wang et al.[41] developed a novel drug delivery system that enhanced efficiency and decreased the adverse effects of PTX by fabricating heparin-FA-PTX (HFT), a ternary conjugate loaded with extra PTX (T). *In vitro* cytotoxicity study was done on FR-positive human head and neck tumor cell line KB-3-1, and the study indicates that the HFT-T NPs exhibit higher cytotoxicity as compared to free PTX. In a xenograft model of subcutaneous KB-3-1, HFT-T NPs were found to selectively target the FR overexpressing tumor tissue and extraordinarily increase the antitumor efficiency of PTX. The same results were exhibited in average tumor-volume evaluation, HFT-T treated and free PTX-treated mice group average tumor volume was 92.9 ± 78.2 mm^3 and 1670.30 ± 286.10 mm^3, respectively. The PTX tumor recurrence was not observed in HFT-T-conjugated NP treatment, which indicated that the tumor was inhibited more effectively by HFT-T NPs from developing resistance to the drug. No significant acute systemic toxicity was found in the xenograft model. All these results lead us to believe that using ternary-structured NPs (HFT-T), PTX can be delivered to FR-overexpressed tumor cells is a favorable strategy to boost the efficacy of chemotherapy and lower the adverse effects.[41]

The inhibitory effect of heparin on tumor growth is investigated so the use of a heparin-based self-assembled, folate-conjugated heparin-poly(β-benzyl-laspartate) (HP) amphiphilic copolymer containing nanoparticulate system for PTX delivery. The folate-PEG-conjugated HP (FPHP) NPs have a greater ability to serve as potential carriers for PTX targeting in cancer therapy compared to the PTX-loaded HP and PTX-loaded folate-HP. NPs which was formed get recognized easily and effectively by the FR because the PEG spacer between the heparin backbone and the folate ligand of the FPHP-PTX NPs increases the targeting moiety length.[42]

3. Chitosan NPs

In recent years, numerous micelles of CS PTX have been studied, such as amphiphilic carboxymethyl CS-quercetin PTX micelles,[43] *N*-octyl-*N*-(2-carboxylbenzoyl) CS PTX micelles,[44] α-tocopherol succinate-modified CS PTX micelles,[45] and *N*-succinyl-palmitoyl-CS PTX micelles.[46] But higher toxicity on normal cells the use of these PTX micelles was greatly constrained. To increase cancer cell targeting and minimize the side effects of PTX, Wang et al.[43] introduced a modified biodegradable micellar delivery of PTX *via* deoxycholic acid-*o*-carboxymethylated CS-FA conjugate (DOMC-FA). *o*-Carboxymethylated chitosan (OCMC) is a type of carboxymethylated derivative of CS and deoxycholic acid (DOCA) is amphiphilic natural bile acids. However, DOCA and OCMC interaction induces the self-associated self-assemble micelles for hydrophobic drugs. The DOMC-FA micelles loaded with PTX were effectively prepared and referred as a novel system for drug targeting. The covalently-bonded FA is employed as a ligand for cell membrane targeting and for improving DOMC-FA-PTX NP endocytosis through the FR. The commercially available injections of PTX (Taxol®), plain micelles, and folate conjugated micelles were tested for their cytotoxicity and ability to target tumor cells and were confirmed by studies on cellular uptake, morphological changes, apoptosis, and MTT assay in MCF-7 cells with overexpression FR. The positive results of this formulation confirmed that the DOMC-FA micelles loaded with PTX are beneficial for targeting and reducing the side effects of PTX.[43]

To increase the efficiency and decrease toxicity of PTX, Cheng et al.[47] also developed an amphiphilic injection system for PTX (FACC-PTX micelles) using a biocompatible and biodegradable FA-cholesterol-chitosan (FACC) polymer conjugates. The aminoacylation reaction of the primary amino group of CS leads to synthesize FACC polymeric conjugate and the dialysis method was used to prepare FACC-PTX micelles. FACC polymer had a critical concentration 64.13 low µg/mL and could self-built in an aqueous environment. In the *in vitro* release study the micelles of FACC-PTX showed that the drug release at the tumor site, where the environment is weak acidic was higher and at the normal environment of cells was low. Hence, all these results indicated that the formulation was less toxic. By *in vitro* cytotoxicity study against HeLa (FR-positive) and A549 (FR-negative cells), the results of cytotoxicity and targeting efficiency of FACC-PTX micelles were found to be significantly optimistic compared with Taxol®.[47]

The octadecyl quaternized lysine-modified chitosan (OQLCS) is a derivative of CS, soluble in water and organic solvent, has an amino group for functional group attachment and is easily reconstituted in liposomes. Based on OQLCS and cholesterol, Zhao et al.[35] synthesized PTX or calcein-loaded folic acid-modified TAT peptide-conjugated polymeric liposomes (PTX

loaded FA-TATp-PLs). 11% Feed ratio of PTX to FA-TATp-PLs conjugate achieved a drug loading of 9.55%, and encapsulation efficiency of 86.83%. The particle size of PTX-FA-TATp-PLs and the cellular uptake of PLs were directly proportional to each other. *In vitro* study revealed that the PTX-loaded FA-TATp-PLs showed 80% drug released in two weeks and indicated that FA-TATp-PLs displayed more ability to endocytosis in both KB cells with overexpression of FR and in FR deficient A549 cells compared to PLs. *In vitro* cytotoxicity study was conducted on KB cells (FR-positive) and the PTX-loaded FA-TATp-PLs showed higher cytotoxicity than PTX containing FA-PLs, Taxol®, and PLs. Similarly, as compared to Taxol®, the FA-TATp-PLs loaded with PTX exert promising antitumor activity under *in vivo* study conducted on the mice bearing nasopharyngeal tumor. Due to its high efficacy for delivery of TAT peptide and FA target specificity, in the future, this formulation will become a promising therapy for tumor targeting.[35]

4. Graphene oxide (GO) NPs

GO is a graphite derivative with exceptional biocompatibility, having electronic flexibility, large specific surface area, mobility, better thermal conductivity, and mechanical strength that allows it to facilitate chemical modification and functionalization.[48] Recently, it has been largely used in drug and gene delivery, photothermal cancer therapy, and as biosensors.[49]

Vinothini et al.[50] prepared GO through modified Hummers method. A novel GO-methyl acrylate-folic acid- PTX (GO-MA-FA-PTX) nanocarrier was fabricated *via* conjugation of the targeting ligand, FA, and methyl acrylate (MA) with the GO surface *via* ether and amide linkage. PTX was attached through hydrophobic interaction and *π-π* stacking on the GO-MA-FA carrier surface. The FT-IR and XRD analyses confirmed the conjugation and structural modification (GO-MA-FA-PTX) by indicating a chemical change in the GO structure. The SEM, TEM, and atomic force microscopy images confirm the surface modification of GO. On thermogravimetric analysis (TGA), it was confirmed that the GO and PTX-loaded GO-MA-FA-PTX nanocarrier demonstrated better stability. In the *in vitro* study, PTX release was higher in the acidic microenvironment as compare with the physiological compartment. MTT assay was done on MDA-MB-231 human cell line of breast cancer to determine the cytoxicity of the prepared nanocarriers and it was confirmed that the GO-MA-FA-PTX had 39% cytotoxicity. Likewise, an *in vivo* study was performed on rats suffering with DMBA-induced breast cancer and on treatment with GO-MA-FA-PTX, it was seen that the nanocarriers increased the deplected level of mitochondrial citric acid enzymes to normal. This result reveals that the GO-MA-FA-PTX nanocarrier was a significantly more potent and specific targeted delivery system for an anticancer drug.[50]

5. Hydroxyapatite (HAp) NPs

HAp is a biocompatible, biodegradable material and it has been used as a delivery system for several drugs and therapeutic agents. Venkatasubbu et al.[51] reported the synthesis of FA decorated PEG functionalized HAp NPs (Hap-PEG-FA) for the successful delivery of the anticancer drug PTX. UV spectroscopy and TGA confirmed the conjugation and structural modification of HAp-PEG-FA NPs by comparing the changes in the chemical structure of a pure component. The TEM images confirmed the absence or presence of residual components in NPs. The FT spectroscopic analysis indicated the functionalization of NPs with a polymer and its chemical adsorption. In *in vitro* study, PTX was rapidly released in the initial stage and then followed a slow, steady, and controlled release. All these results revealed that use of HAp in the formulation of drug delivery system makes it a promising one.[51]

6. Hyaluronic acid (HA) NPs

HA is a non-toxic, natural, and biodegradable polysaccharide. In biotechnological and biomedical fields, it has wide applications and this is because of its powerful affinity towards the markers, which make the cell surface specific, such as glycoprotein CD44 and receptors for motility mediated by HA, which are overexpressed mostly on various types of malignant solid tumor surface.[52] Recently, HA is also used as dual receptor targeting strategy for effective drug delivery. The most important benefit of dual targeting nanocarrier systems is to overcome MDR. Dual targeting therapy can be developed using FA targeting to folate and HA targeting to CD44 receptors. As both of these receptors are overexpressed on malignant tumor cells, it is possible to develop a dual-targeting drug delivery system. A similar dual targeting system was developed by a Chinese scientist Yanhua Liu et al.[53], using FA, HA, and C18 conjugates. MTT assay was performed on MCF-7 and A-549 cell lines with three samples *i.e.* taxol solution, FA-C18, and HA-FA-C18 micelles, and it was indicated that the cytotoxicity of taxol solution was much lower compared to conjugated micelles. Also, the pharmacokinetic study stated that conjugated micelles exhibit much longer circulation compared to taxol solution. This study suggests that these conjugates are biodegradable, biocompatible, and dual-targeting nanostructure carriers for delivery of hydrophobic anticancer drugs intercellularly.[11] In 2014, similar conjugates were evaluated for comparison of single targeting and dual-targeting micelles for eliminating multidrug resistance using MCF-7 and MCF-7/Adr cells. The efflux of the drug mediated by the P-gp transporter is a crucial reason for the resistance of PTX. The result of the study showed that targeting micelles significantly increased the drug uptake in drug resistance cells as compared to taxol solution. Furthermore, *in vitro* cytotoxicity study and intracellular uptake studies demonstrated that CD44 and FAR dual-mediated endocytosis played a vital role in overcoming MDR. These studies indicated that targeting therapy is a promising therapy for overcoming MDR for PTX drug delivery.[53]

7. Cyclodextrin NPs

Amphiphilic CDs and their derivatives are one of the promising tools for the delivery of nanoscale drug molecules. It is widely used in designing various novel functionalize materials for biomedical applications due to its biocompatibility, unique inclusion capability, and powerful functionalization capacity.[54] In aqueous solutions, the amphiphilic CDs get self-modified

and, hence, it has a great ability to interact with biological membranes.[55]

Erdoğar et al.[15] performed esterification and altered the CD derivative at the primary and secondary phases by substitution of C_6 alkyl chains and developed FCD-1 and FCD-2 folate conjugated CDs. Each derivative (FCD-1 and FCD-2) carries one folate residue on the substituted face at the termination of the C_6 linker chain, which was joined to the mother amphiphilic CD to give effective targeting efficiency to FR overexpressed on cancer cells. The optimized PTX loaded, actively targeted NP formulation was obtained through a specific modifications using 3^2 factorial designs. In water, the prepared FCD-1 and FCD-2 derivatives can self-organize in NPs having size (smaller than 100 nm) with narrow size distribution and in this carrier, up to 60% PTX should be encapsulated by the nanoprecipitation method. The PTX-loaded FCD-1 and FCD-2 NPs were more stable than the other nanoparticulate systems and delayed the drug release even more. No cytotoxicity of the blank NPs was found against L929 cells. PTX-loaded NPs exhibited a more anticancer efficacy because of the good interaction with the FR-positive T-47D and ZR-75-1 human breast cancer cells. Therefore, these novel folate conjugated CD NPs are considered a promising formulations for effective and safe delivery of PTX with a folate dependent mechanism.[15]

8. Gene therapy

In the efforts of the development of folate conjugated chemotherapeutics, development has been made in the field of folate-targeted gene therapy, in which both viral and non-viral vectors have been examined.[12] Gene therapy is introduced as an effective method for treating ovarian cancer and contains small interfering RNA (siRNA).[56] Relapse and resistance are commonly seen obstacles in ovarian cancer treatment, which are attempted to be overcome by various siRNA combination therapies, currently being studied.[57] Jones et al.[58] incorporates targeted delivery of siRNA and PTX to FR overexpress ovarian cancer cells through the tri-block copolymer micelleplexes consisting of PEI- graft-polycaprolactone-block-poly(ethylene glycol) (PEI-g-PCL-*b*-PEG-Fol) that overcome toll-like receptor 4 (TLR4)-driven chemotherapy resistance. The optimized targeted delivery of siRNA micelleplexes was explored by altering different molecular weights of PEG as well as different grafting degrees of the (g-PCL-b-PEG-Fol) chains to PEI. Western blotting and flow cytometry analysis demonstrated the effective delivery of siRNA *via* PEI-g-PCL-b-PEG-Fol conjugates, which is responsible for efficient protein destruction of TLR4. TLR4-mediated chemotherapy resistance is overcome by destruction of TLR4 within SKOV 3 cells, which makes them sensitive toward PTX treatment and increases apoptosis.[58]

CONCLUSION

PTX is the anticancer drug found to be most effective against a variety of cancers such as NSCLC, refractory ovarian cancer, metastatic breast cancer, head and neck malignancies, AIDS-related Kaposi's sarcoma, malignant lymphoma, and lymphoblastic leukemia. Apart from the effectivity that was found to be toxic due to Cremophor EL and ethanol used in the formulation, as solvent hence to overcome the formulation-related problem researchers innovate some nanodelivery systems for delivering PTX, such as lipid-based formulations, polymeric NPs, inorganic NPs, polymer conjugates, carbon nanotubes, CD NPs, and nanocrystals. To overcome PTX-related problems such as its low solubility, pharmacokinetic profile, and targeting, researchers found some targeting moiety and targeting sites like FA and FRs which are overexpressed in cancerous cells. This review contains overview about a various folate targeted PTX containing nanodelivery systems such as PTX pro-drug NPs includes Tax-Chol prodrug and water-soluble polymeric prodrug, copolymeric NPs includes PLA NPs, PLGA NPs, polyacrylamide NPs, pluoronic NPs, and there are some other NPs such as albumin/albumin moieties NPs, heparin NPs, CS NPs, GO NPs, HAp NPs, HA NPs, CD NPs, and gene therapy along with it, the review also focuses on ongoing research on targeting therapy for PTX (Table 1).

Table 1. Folate-mediated paclitaxel nanodelivery system

S. no	Formulation category	Formulation (ligand)	Reference
A. PTX Pro-drug NPs			
1.	Tax-Chol prodrug	f-PEG-Chol	22
2.	Water-soluble polymeric prodrug	PTX-PEEP-FA	23
B. Copolymeric NPs			
1.	Poly(lactide) PLA NPs	MPEG-PLA-PTX & TPGS-Fol	28
		PEG-PAC-PLA & FA-PEG-PLA	29
		Fol-PTX-PLA-TPGS	30
2.	Poly(lactide-co-glycolic acid) (PLGA) NPs	FA-PEG-PLGA	31
3.	Polyacrylamide NPs	f-P(NIPA-DMA-UA-g-Cholesterol	10
4.	Pluronic NPs	FPF-PTX	32
C. Other nanoparticles			
1.	Albumin/albumin moieties NPs	PTX-BSA NPs	36
		EB-FA-PTX	
		PTX-NFs-BSA-CS-FA	
		PTX-NFs-BSA-CMC-FA	
2.	Heparin NPs	HFT-T	41
		PTX-FPHP-PTX NPs	42
3.	Chitosan NPs	DOMC-FA/PTX	43
		FACC-PTX micelles	47
		PTX loaded FA-TATp-PLs	35
4.	Graphene oxide (GO) NPs	GO-MA/FA-PTX	50
5.	Hydroxyapatite NPs	HAp-PEG-FA	51
6.	Hyaluronic acid NPs	FA-HA-PTX	11
7.	Cyclodextrin NPs	PTX loaded FCD-1 & FCD-2 NPs	15
8.	Gene therapy	siRNA & PTX *via* PEI-g-PCL-b-PEG-Fol	58

PTX: Paclitaxel, NPs: Nanoparticles

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