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Research Article

A NOVEL REVERSE-PHASE ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD WITH GRADIENT ELUTION FOR SIMULTANEOUS DETERMINATION OF DOLUTEGRAVIR, LAMIVUDINE AND TENOFOVIR DISOPROXIL FUMARATE IN FIXED-DOSE COMBINATION

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ABSTRACT

Objective: To develop a sensitive and rapid reverse phase ultra-performance liquid chromatographic method for the simultaneous determination of dolutegravir (DTG), lamivudine (LAM), and tenofovir disoproxil fumarate (TDF) with improved separation and stability-indicating features.

Methods: Chromatographic separation was performed on an acquity bridged ethyl siloxane/silica hybrid C18 (50 × 2.1 mm, 1.7 µm) column at 40°C using 0.05% trifluoroacetic acid in water (pH 3.0) and methanol as mobile phase, with a flow rate of 0.4 mL/min and ultraviolet detection at 265 nm. The method validation was performed per ICH guidelines and included stress degradation studies.

Results: Effective separation was achieved; retention times were 1.25 min for LAM, 2.87 min for TDF, and 3.65 min for DTG. Supporting validation, the limits of linearity were set at 15–90 µg/mL for both LAM and tenofovir, and 2.5–15 µg/mL for DTG, with R_2 values exceeding 0.999.

Conclusion: The proposed technique provides high accuracy, precision, sensitivity, and specificity in detecting multiple antiretroviral drugs in fixed-dose drug combinations. Compared to previously published methods, the gradient elution profile offers better peak height, greater peak splitting, as well as decreased total time required for analysis. The method's stability-indicating features, confirmed by forced degradation studies, demonstrate the method's value for pharmaceutical analysis quality control and stability testing.

Keywords: Dolutegravir, Lamivudine, Tenofovir disoproxil fumarate, Reverse phase ultra-performance liquid chromatographic, Gradient elution, Stability-indicating method, Method validation.

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INTRODUCTION

The advent and widespread use of antiretroviral therapy have vastly improved the clinical management of human immunodeficiency virus (HIV) infection in recent decades. Enhanced patient adherence and therapeutic outcomes to treatment have been made possible through the use of fixed-dose combinations (FDCs). The World Health Organization also provides guidelines on the treatment of HIV where the combination of dolutegravir (DTG), lamivudine (LAM), and tenofovir disoproxil fumarate (TDF) is among the most efficacious ones [1,2].

Also known as 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2(1H)-one, LAM is a nucleoside reverse transcriptase inhibitor selective for HIV and hepatitis B virus infection [3]. Viral DNA is synthesized in infected cells, and LAM acts by incorporating it into the growing DNA chain and causing chain termination. An oxathiolane ring constitutes LAM's structure, which appears as a white to off-white crystalline solid with high water solubility [4].

TDF is described as 9- [(R) -2-[[bis[[(isopropoxycarbonyl)oxy] methoxy] phosphinyl] methoxy] propyl] adenine fumarate. It is a prodrug of a medication called tenofovir. Tenofovir diphosphate, which results from hydrolysis and subsequent phosphorylation of TDF, competes with deoxyadenosine 5'-triphosphate, a natural substrate of HIV reverse transcriptase, resulting in viral DNA chain elongation prevention. TDF is characterized by possessing better oral bioavailability than its parent compound [5].

DTG belongs to the class of integrase strand transfer inhibitors (INSTI). Its chemical name is (4R,12aS)-9-{[(2,4-difluorophenyl) methyl] carbamoyl} -4-methyl-6,8-dioxo-3,4,6,8,12,12a-hexahydro-2H-pyrido [1',2':4,5]pyrazino [2,1-b][1,3] oxazin-7-olate sodium. DTG works by binding to the integrase active site of the HIV-1 integrating enzyme, blocking retroviral DNA integration strand transfer crucial for HIV replication [6]. It is more effective at achieving viral suppression with less likelihood of developing resistance compared to earlier marketed INSTIs [7].

The individual or combined determination of these antiretroviral drugs has already been reported in the literature using several analytical methods. High-performance liquid chromatography (HPLC), high-performance thin-layer chromatography, and liquid chromatography-mass spectrometry are examples [8-11]. However, the majority of methods report these techniques have long analysis times, complicated mobile phase compositions, and low sensitivity or selectivity needed for routine quality control uses, which are all considerable limitations [12].

Ultra-performance liquid chromatography (UPLC) has pronounced benefits compared to regular HPLC, including better resolution and sensitivity, as well as greatly decreased analysis time. All of these factors make UPLC a very effective technique in pharmaceuticals when analyzing multi-component formulations like antiretroviral FDCs [13,14].

To the best of our knowledge, there are limited reports on the simultaneous determination of DTG, LAM, and TDF using UPLC with gradient elution. Therefore, we aimed to develop a rapid, sensitive, and stability-indicating UPLC method for the simultaneous estimation of these three antiretroviral drugs in pharmaceutical formulations.

The present study presents a novel approach with optimized gradient elution parameters to achieve superior chromatographic performance while ensuring compatibility with forced degradation studies.

There are very few methods in the literature where DTG, LAM, and TDF were analyzed simultaneously using UPLC with gradient elution. This is the rationale for our attempt to create a precise and sensitive UPLC method that is stability indicative for the simultaneous quantification of these three anti-viral agents in pharmaceutical formulations [15].

MATERIALS AND METHODS

Materials

Mylan Laboratories Ltd., India, supplied the pharmaceutical grade reference standards DTG sodium (99.8% purity), LAM (99.5% purity), and TDF (99.7% purity) as gift samples. The FDC tablets containing 50 mg DTG, 300 mg LAM, and 300 mg of TDF disoproxil were purchased from a pharmacy in Bangalore, India. Methanol and acetonitrile to the HPLC grade were supplied by Merck, India. Sigma-Aldrich, India supplied hydrochloric acid, sodium hydroxide, hydrogen peroxide, orthophosphoric acid, and trifluoroacetic acid in analytical grade. Deionized water from a Millipore Milli-Q water purification system served as standards.

Instrumentation

A waters acquity UPLC H-Class system with a quaternary solvent manager, sample manager, column manager, and a photodiode array (PDA) detector was used to develop and validate the analytical method. Empower 3 was used for data acquisition and processing. Other equipment includes an analytical balance (Mettler Toledo), pH meter (Eutech Instruments), sonicator (Bandelin Sonorex), and hotplate with a magnetic stirrer (IKA).

Chromatographic conditions

Methanol with 0.05% trifluoroacetic acid (pH 3.0 with triethylamine) was used as A and methanol was used as B. Gradient elution was used to achieve methanol chromatography and separation on water acquity bridged ethyl siloxane/silica hybrid (BEH) C18 column (50×2.1 mm, 1.7 µm) [16,17]. The following gradient program was used: 0–1.0 min (20% B), 1.0–2.5 min (20–45% B), 2.5–4.0 min (45–65% B), 4.0–4.5 min (65–20% B), 4.5–5.0 min (20% B) for re-equilibration. During the entire process, the flow rate was kept at 0.4 mL/min, the column temperature at 40°C, with a detection wavelength of 265 nm. The injection volume was 2 µL and the total run time was 5 min [18].

Standard stock solutions

A primary stock solution was created containing LAM (3 mg/mL), TDF (3 mg/mL), and DTG (0.5 mg/mL) by adding 300 mg of LAM and TDF as well as 50 mg of DTG into individual 100 mL volumetric flasks. Each of the standards was made up with 70 mL of the diluent (methanol: water, 70:30 v/v) and sonicated for 10 min and finally made up to the mark with the same diluent [19].

Preparation of working standard solution

About 5 mL of each primary stock solution containing 300 µg/mL of LAM, 300 µg/mL of TDF, and 50 µg/mL of DTG was taken. The solutions were added one-by-one into a 50 mL volumetric flask to obtain a final volume and subsequently diluted with the diluent which resulted in the working standard solution [20].

Preparation of sample solution

Twenty tablets were accurately weighed and finely powdered. One tablet containing 50 mg DTG, 300 mg LAM, and 300 mg TDF was dipped and stirred into a 100 mL volumetric flask containing 100 mL of water. A phenomenal 70 mL of diluent was included, and sonication was carried out for 20 min even without shaking the solution. Afterward, the solution was cooled to room temperature and diluted with the diluent to achieve the desired concentration. The solution was filtered through a filter funnel and then passed through a 0.22 µm nylon syringe filter [21].

This solution was prepared by extracting a 5 mL aliquot and diluting it in a 50 mL volumetric flask. The desired concentration of 300 µg/mL of LAM, 300 µg/mL of TDF, and 50 µg/mL of DTG was achieved by using the diluent to further purify the sample solution [22].

Statistical analysis

All experimental results were presented as mean±standard deviation (SD) of at least three replicate determinations. Statistical analysis was performed using Microsoft Excel 2019. One-way analysis of variance was used to evaluate the significance of differences between groups, with $p<0.05$ considered statistically significant [21,22].

RESULTS AND DISCUSSION

Method development and optimization

The main objective of this study was to develop a robust reverse-phase ultra-performance liquid chromatographic (RP-UPLC) method that is rapid and sensitive for the simultaneous quantification of DTG, LAM, and TDF. Method development started with a column selection, where stationary phases with sub-2 µm particles were chosen to exploit the benefits of UPLC technology.

Table 1: System suitability results

Parameter	Results			Acceptance criteria
	Lamivudine	Tenofovir	Dolutegravir	
RSD of peak area* (%)	0.85±0.07	0.92±0.08	0.64±0.05	≤2.0
RSD of retention time* (%)	0.32±0.03	0.29±0.02	0.25±0.02	≤1.0
USP tailing factor	1.08	1.15	1.03	≤2.0
USP plate count*	15873±245	16249±315	18625±382	>5000
USP resolution*	7.82±0.18	4.56±0.12	-	>2.0

RSD: Relative standard deviation, *Values represent mean±SD (n=6)

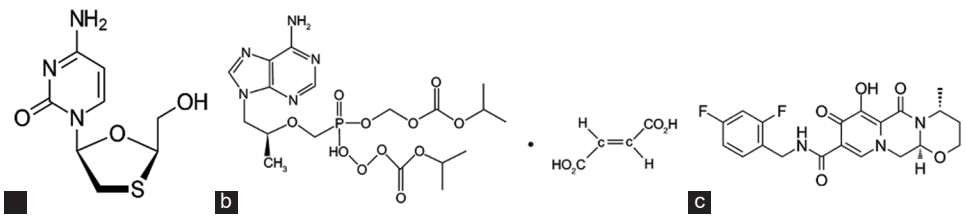

Fig. 1: Structures of (a) lamivudine (b) tenofovir disoproxil fumarate (c) dolutegravir

Several stationary phases were evaluated, such as C18, C8, and phenyl columns. The acquity BEH C18 column (50 × 2.1 mm, 1.7 µm) had the best peak shape and resolution for all three analytes, therefore, it was selected for further optimization [20,21].

The mobile phase composition was one of the most critical parameters that required extensive optimization due to the diverse physicochemical properties of the target analytes. Initial attempts at isocratic elution with acetonitrile or methanol buffers of varying pH proved unsatisfactory in separating all three components within a reasonable time.

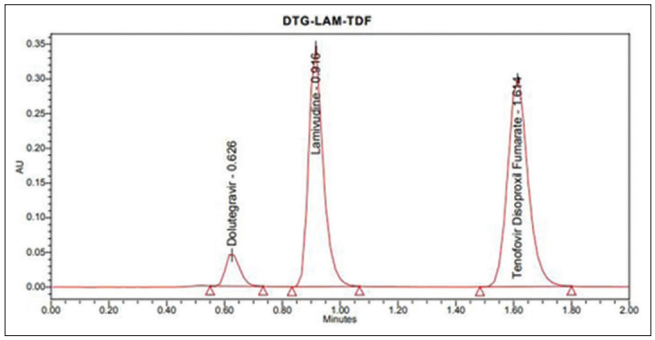

Fig. 2: System suitability chromatograms

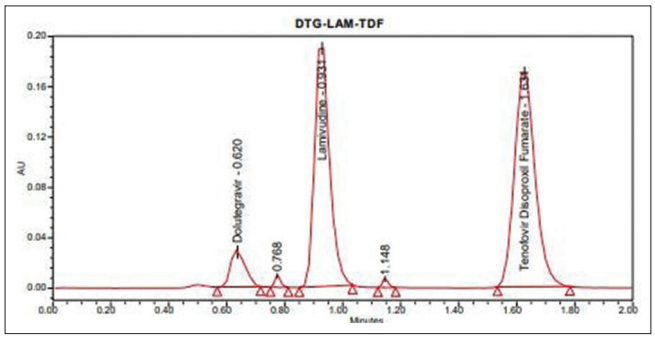

Fig. 3: Chromatograms of acid degradation study

Table 2: Regression equation parameters

Parameter	Lamivudine	Tenofovir	Dolutegravir
Linearity range (µg/mL)	15–90	15–90	2.5–15
Correlation coefficient (r^2)	0.9997	0.9998	0.9995
Slope	18259	20478	32547
Y-intercept	4852.3	5124.8	1035.7
% Y-intercept	1.78	1.65	1.82

To achieve the best separation in the least amount of analysis time, gradient elution was explored next. Of the buffer systems tried (ammonium acetate, potassium dihydrogen phosphate, and trifluoroacetic acid), 0.05% trifluoroacetic acid (pH 3.0) was selected for providing sharp peak shapes with minimal tailing for all three analytes.

The gradient program was modified to provide the best resolution possible with the least amount of runtime. The final gradient permitted LAM to elute at 1.25 min, TDF at 2.87, and DTG at 3.65, with a total run time of 5 min including re-equilibration [22].

Flow rate and column temperature were changed to optimize backpressure and efficiency. A flow rate of 0.4 mL/min and a column temperature of 40°C was the best equilibrium of resolution, peak shape, and system pressure.

Choosing the detection wavelength was done considering the maximum ultraviolet (UV) absorption of the three drugs. Even though the individual compounds have differing absorption maxima (LAM 272 nm, tenofovir 259, and DTG 260) 265 nm was chosen as the detection wavelength since it proved sufficient sensitivity for all three analytes.

As for the optimized method of resolution, it was evident all other peaks were resolved with LAM (the most polar) being first, followed by TDF, and DTG (the least polar).

Method validation

The validation for the developed RP-UPLC method was issued using the ICH Q2 (R1) guidelines for the following parameters: specificity, linearity and range, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness.

System suitability

The system suitability criteria were verified to confirm that the chromatographic system was valid before proceeding to validation studies. The standard solution was subjected to six replicate injections, calculating the retention time, peak area, and other relevant values. The results, which have been summarized in Table 1, clearly indicate that all system suitability criteria were satisfied confirming the goodness of the chromatographic system as one from which valid analytical results can be obtained [23]. The chromatograms are shown in Fig. 2.

Specificity

No interference at the retention times of the three analytes from blocking components was observed. This indicates that the method is specific. The result of the peak purity test carried out through the PDA detector showed that all peaks of the analyte in the sample solution were indeed homogeneous [24].

Linearity and range

Standard solutions were prepared for LAM and TDF at concentrations of 15–90 µg/mL and for DTG at 2.5–15 µg/mL. Peak areas were used to

Table 3: Accuracy data

Parameter (%)	Amount added (µg)	Amount recovered* (µg)	% Recovery*	Mean % recovery
Lamivudine				
50 level	30	30.25±0.32	100.83±0.32	100.25
100 level	60	59.85±0.48	99.75±0.17	99.75
150 level	90	90.15±0.65	100.17±0.21	100.17
Tenofovir				
50 level	30	30.38±0.51	101.27±0.24	101.27
100 level	60	60.79±0.29	101.32±0.44	101.32
150 level	90	89.52±0.16	99.47±0.51	99.47
Dolutegravir				
50 level	5	4.94±0.35	98.80±0.18	98.80
100 level	10	9.87±0.41	98.74±0.59	98.74
150 level	15	14.96±0.56	99.73±0.22	99.73

*Values represent mean±SD (n=3)

calculate the concentration of each analyte. Each of the three analytes displayed exceptional linearity over the utilized ranges, achieving correlation coefficients (r^2) in excess of 0.999. The corresponding values of the regression equations are shown in Table 2.

Accuracy
To assess accuracy, recovery studies at three different levels (50%, 100%, and 150% of the target concentration) using the standard addition approach were undertaken. Known quantities of standards were added to pre-analyzed sample solutions and the percentage recoveries were calculated [25]. The results showed, excellent accuracy [Table 3]with mean recoveries from 98.74% to 101.32%, meeting the acceptance range of 98.0–102.0%.

Precision
Precision was assessed on three levels: repeatability (intra-day precision), intermediate precision (inter-day precision), and then reproducibility. Results presented as RSD of peak areas demonstrate excellent sufficient precision across all levels with RSD values far below the acceptance criterion of 2.0% [11,12]. These results are presented in Table 4.

LOD and LOQ
The methods for both detection and quantification limits were computed using the signal-to-noise ratio approach. For defining LOD, the concentration that yields a noise-to-signal ratio of 3:1 is acceptable while for LOQ, a noise-to-signal ratio of 10:1 serves best. The calculated values of LOD were 0.82 µg/mL, 0.75 µg/mL, and 0.14 µg/mL for, respectively, LAM, TDF, and DTG. The respective values of LOQ for the same compounds were 2.47 µg/mL, 2.26 µg/mL, and 0.42 µg/mL.

Table 4: Results for precision

Parameter	Lamivudine	Tenofovir	Dolutegravir
Repeatability			
RSD of retention time (%)	0.32±0.03	0.29±0.02	0.25±0.02
RSD of peak area (%)	0.85±0.07	0.92±0.08	0.64±0.05
Reproducibility			
RSD of retention time (%)	0.36±0.04	0.31±0.05	0.28±0.03
RSD of peak area (%)	0.89±0.02	0.97±0.02	0.68±0.04
Intermediate precision			
RSD of retention time (%)	0.38±0.01	0.33±0.03	0.29±0.01
RSD of peak area (%)	0.93±0.02	1.02±0.01	0.72±0.06

RSD: Relative standard deviation, *Values represent mean±SD (n=6)

Table 5: Assay results

Drug	Labeled amount (mg/tab)	Amount found (mg/tab)	% Assay
Lamivudine	300	301.26±1.85	100.42±0.62
Tenofovir	300	299.58±1.73	99.86±0.58
Dolutegravir	50	49.78±0.35	99.56±0.70

*Values represent mean±SD (n=6)

Robustness
Robustness was evaluated by deliberately changing the method flow rate (±0.05 mL/min), column temperature (±2°C), and mobile phase composition (±2% absolute of organic modifier). Results indicated that the changes made to the chromatographic conditions did not majorly alter the retention time, peak area, or resolution between peaks, confirming the method's robustness [16,17].

Solution stability
The standard and sample solutions were assessed for stability at room temperature for up to 48 h. An analysis was also done at 0, 12, 24, and 48 h. The peak area did not change, nor were any additional peaks formed, demonstrating that both the standard and sample solutions remained stable for at least 48 h at room temperature [20,21].

Mobile phase stability
Mobile phase stability was assessed by analysis of standard solution with mobile phase freshly prepared and with mobile phase that was stored for 48 h at room temperature. There were no notable changes in retention time, peak shape, or peak area which confirms mobile phase stability for at least 48 h [22].

Analysis of marketed formulation
The validated method was used to perform analysis on the commercially available FDC tablets of DTG 50 mg, LAM 300 mg, and TDF 300 mg. The procedure was performed in six replicates and the results are shown in Table 5. The obtained results for the assay were between 99.56% and 100.42%, indicating excellent accuracy and precision of the method for routine quality control analysis.

Forced degradation studies
The purpose of carrying out forced degradation studies is to assess the method's ability to indicate the stability of the substance alongside the possible degradation products. The samples went through several stress steps which included hydrolysis by acid, base hydrolysis, thermal degradation, degradation through oxidation, neutral hydrolysis, as well as photolytic degradation [23].

Acid degradation
Treating the sample solution with 5 mL of 0.5 N HCl, followed by heating the solution at 70°C for 6 h while stirring, led to the formation of 13.2% degradation products for LAM, 11.8% for TDF, and 8.5% for DTG. Major degradation products were created and noticed at retention periods of 0.92 min and 2.15 min and were well separated from the primary peaks as seen in Fig. 3. Afterward the sample is neutralized with 0.5N NaOH and diluted to volume with the diluent of choice [24].

Base degradation
The sample solution was prepared by treating with 5 mL of 0.5 N NaOH solutions and subsequently heated to 70° centigrade for a duration of

Table 6: Results of the forced degradation study

Stress condition	% Assay of active ingredient			% Degradation		
	Lamivudine	Tenofovir	Dolutegravir	Lamivudine	Tenofovir	Dolutegravir
Acid	86.8±0.92	88.2±0.85	91.5±0.78	13.2±0.92	11.8±0.51	8.5±0.85
Alkali	84.4±0.24	82.8±0.78	87.7±0.21	15.6±0.78	17.2±0.29	12.3±0.56
Peroxide	89.3±0.41	85.5±0.78	90.8±0.25	10.7±0.63	14.5±0.36	9.2±0.64
Reduction	87.5±0.62	86.3±0.78	89.6±0.62	12.5±0.14	13.7±0.42	10.4±0.71
Thermal	91.6±0.59	90.4±0.78	92.2±0.71	8.4±0.19	9.6±0.56	7.8±0.55
Photo	97.7±0.81	96.5±0.78	98.2±0.54	2.3±0.26	3.5±0.74	1.8±0.81
Hydrolysis	98.8±0.12	98.2±0.78	99.1±0.43	1.2±0.32	1.8±0.81	0.9±0.29

*Values represent mean±SD (n=3)

6 h. The sample solution was neutralized with hydrochloric acid and brought to volume with diluent. The stressed sample demonstrated degradation of 15.6% for LAM, 17.2% for TDF, and 12.3% for DTG. Three major degradation products were observed at retention times of 0.85 min, 1.98 min, and 3.25 and possessed no interference with the main peaks in the chromatogram.

Oxidative degradation
The sample solution was treated with 5 mL of 3% hydrogen peroxide and heated at 70°C for 3 h. The stressed sample showed 10.7% degradation for LAM, 14.5% for TDF, and 9.2% for DTG. Two major degradation products were observed at retention times of 1.45 min and 3.05 min, well resolved from the main peaks [25].

Thermal degradation
Sample powder was subjected to dry heat at 80° centigrade for a period of 24 h in a hot air oven. The stressed sample showed a valve of 8.4% degradation for LAM, 9.6% for TDF, and 7% for DTG. One major degradation product was observed at a retention time of 2.35 min and was well resolved from the main peaks [25].

Photolytic degradation
A sample was subjected to UV irradiation for 24 h. The stressed sample exhibited 2.3% degradation of LAM, 3.5% of TDF, and 1.8% of DTG. No significant degradation products were formed.

Neutral hydrolysis
Water was added to the sample and heated to 70°C for 6 h. The stressed sample demonstrated 1.2% degradation of LAM, 1.8% of TDF, and 0.9% of DTG. No significant degradation products were formed [25].

Forced degradation study results were reported in Table 6. All degradation products were well resolved from the main peaks which confirms the stability indicating property of the method.

CONCLUSION

A new, fast, and precise RP-UPLC method for the simultaneous determination of DTG, LAM, and TDF in pharmaceutical formulations was developed and validated. The method was performed on an acquity BEH C18 column at 0.05% trifluoroacetic acid, methanol (pH = 3.0), using gradient elution. The newly developed technique has a reduced analysis time of 5 min as compared to the 10–15 min spent on conventional HPLC methods, has improved resolution between all peaks, and has enhanced sensitivity. Validation was conducted following the ICH guidelines and the method demonstrated specificity, linearity, accuracy, precision, and robustness, which were all classified as excellent. Studies conducted on forced degradation confirmed that the method's stability-indicating capability was effective, as all of the degradation products were well resolved from the main peaks. The method was able to successfully separate and quantify the three antiretroviral drugs in the presence of degradation products formed under various stress conditions. The validated technique can be used for quality control measurements of DTG, LAM, and TDF in FDC tablets. Furthermore, they can be used in stability studies. The simplicity in sample preparation and short analysis period make the technique fit for high-throughput analysis in pharmaceutical laboratories.

AUTHOR'S CONTRIBUTION

Gampa Nagamalleswari: Conceptualization, methodology, validation, formal analysis, investigation, data curation, writing - original draft. MS Uma Shankar: Supervision, project administration, resources, writing - review and editing.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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