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Determination of phytochemical markers andrographolide, eugenol and zingerone in nilavembu kudineer by RP-HPLC method

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ABSTRACT

In the present research, the reverse phase (RP)-high performance liquid chromatography (HPLC) method was developed and validated to determine phytochemical markers andrographolide, eugenol, and zingerone in nilaveembu kudineer (NK) a Siddha polyherbal formulation. There are no RP-HPLC methods reported for the simultaneous estimation of these marker compounds in any herbal formulation. In this study, the optimized chromatographic conditions were C_{18} Column (150 × 4.6 × 5 µm) mobile phase containing Acetonitrile and 0.1% Ortho Phosphoric acid 30:70, flow rate 1.0 ml/minutes, at 254 nm using a ultravoilet detector, column temperature of 40˚C and run time of 20 minutes. In the linearity study, a linear relation was observed from 25 to 200 µg/ml with a correlation coefficient of 0.9990. The optimized method is successfully applied for the estimation of these phytochemical markers in NK formulation. The suggested approach is an excellent quality control tool for the simultaneous quantitative assessment and detection of phytochemical markers present in polyherbal formulations and in traditional medicines such as ayurveda, siddha, unani, and homeopathic formulations.

INTRODUCTION

An alternative system of medicine known as a traditional system of medicine is an embodiment containing more than 45,000 species of medicinal plants [1]. Siddha medicine called the ancient Indian medicine, which is prominently followed for maintaining health. In addition to treatment of various complications, mainly relies on therapeutic efficacy using medicinal plants [2]. Siddha medicine focuses on diagnosing diseased conditions (imbalance) and healthy states (balanced condition) to maintain a holistic approach to treatment in human beings and provides a healthy lifestyle in completely eliminating the root cause of the disease. Quality control and standardization of phytochemicals are important criteria to ensure the quality and purity of herbal materials [3].

Separation and standardization of phytoconstituents present in the polyherbal formulation is a great challenge to researchers. Sophisticated methods such as high performance liquid chromatography (HPLC), high performance thin layer chromatography, gas chromatography (GC), liquid chromatography mass spectrometry, and GC mass spectrometry are available to estimate phytochemicals in medicinal plants and herbal formulations. Herbal formulations are available as solid, semisolid, and liquid dosage forms and choornam like nilaveembu kudineer (NK). NK is available as choornam (mixture of powdered herbs) and syrup form. Compared to choornam, syrup is more acceptable as there are no prepreparatory requirements like choornam. The polyherbal formulation contains many herbal plant materials prepared by powdering herbal raw materials and prepared as a decoction for the treatment of viral disorders such as dengue fever and many complications such as anti-inflammatory activity, antipyretic, digestive disorders, hepatoprotective, and so on [4].

NK is a Siddha polyherbal medicine available in liquid and powdered form called as NK choornam. NK comprises 8 to 9 herbs namely Andrographis paniculata Burm.f. (Nees) (whole plant) Kalmegh containing the major constituent

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(Andrographolide), which is a labdane derivative, *Vetiveria zizanioides* (L) Roberty (root), *Plectranthus vettiveroides*, *Cyperus rotundus* L. (rhizome), *Santalum album* L. (heart wood), Zingiber officinale Roscoe (rhizome), *Piper nigrum* L. (fruit), *Trichosanthes cucumerina* L. (whole plant), and *Mullugo cerviana* (L.) Ser. (whole plant) in equal proportions. NK consists predominantly andrographolide, eugenol and zingerone, diterpenoids, glycosides, flavonoids, and lactones [5].

Eugenol has a polar hydroxy group and polar ether group in the structure. The molecular weight of eugenol is 164 and the molecular formula is $C_{10}H_{12}O_2$ [6]. Zingerone which has a polar hydroxy group with a mol. Wt. of 194.22 g/mol and the molecular formula of zingerone is $C_{11}H_{14}O_3$. Andrographolide is a polar compound because of hydroxy and carbonyl groups attached to the rings. When carbon count increases the non-polarity increases, hence andrographolide is less polar when compared to eugenol and zingerone [7]. Zingerone is mid-polar compared with eugenol and andrographolide, whereas eugenol is more polar because of its less carbon atom and more polar hydroxy group [8]. The chemical structures of eugenol, andrographolide, and zingerone are depicted in Figure 1.

The shim-Pack C_{18} column has superior inertness, which increases column stability. It is a hybrid silica ODs column. Shim-Pack C_{18} column with 150 mm gives better R_t when compared to the published articles. Andrographolide R_t is less than 7 in reported studies due to an increase in the organic phase concentration. For zingerone, very few studies were reported; it shows a very good symmetrical peak with a retention time of 6.2 minutes. Eugenol R_t is 4.3 minutes, within 7 minutes all three compounds can be eluted with good resolution. It shows that the shim pack C_{18} column is better when compared to previous methods.

There are numerous reports available in the literature for determining andrographolide [9], eugenol [10], and zingerone [11] individually and in combination with other drugs. On the other hand, there is no reverse phase (RP)-HPLC method described for the simultaneous quantification of these marker standard compounds as well as these markers in herbal formulations, combined marker compounds in any formulation. Consecutively, the present research was proposed to develop and validate the RP-HPLC method for simultaneous determination of standard samples of andrographolide [12], eugenol [13], and zingerone in further the optimized method was planned to apply for the estimation of andrographolide, eugenol, and zingerone NK a Siddha formulation [14]. HPLC is an advanced sophisticated instrument that can detect multicomponent analysis of mixtures present in phytoconstituents in medicinal plants and polyherbal formulations. The suggested approach is an excellent quality control tool for the concurrent quantitative assessment and detection of phytochemical markers present in polyherbal formulations.

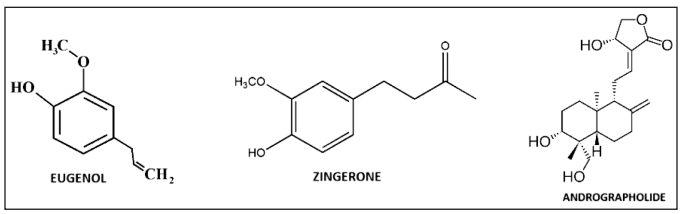

Figure 1. Structures of eugenol, zingerone, and andrographolide.

MATERIALS AND METHODS

Chemicals and reagents

Andrographolide, eugenol, and zingerone were procured from Yucca Enterprises Mumbai, India. Acetonitrile—HPLC, Methanol-HPLC, Water—HPLC, (Merck, India).

Chromatographic system and conditions

The chromatographic analysis was performed on the Shimadzu HPLC-ultravoilet (UV) system. The instrument consisted of a quaternary pump gradient LC 20AD, the injector used was an autosampler SIL 20AC, the column oven consisted of CTO 10 AS, the column is a C_{18}, 100 A°, (5 µm, 4.6 × 150 mm) and the detector was a UV detector SPD M 20 A (Shimadzu, Japan).

Preparation of standard solution of andrographolide (100 µg/ml)

About 1 mg of andrographolide was accurately weighed and transferred into a 10 ml volumetric flask containing 1 ml of HPLC grade methanol and the volume was adjusted with methanol up to 10 ml. The solution was sonicated for 5 minutes to remove air bubbles [15].

Preparation of standard solution of eugenol (100 µl/ml)

About 1 ml of eugenol (1.06 g/cm³) was exactly measured and transferred to a 10 ml volumetric flask containing 1 ml of HPLC grade methanol and made up the volume up to 10 ml with HPLC methanol [16].

Preparation of standard solution of zingerone (100 µg/ml)

Exactly weighed 1 mg of Zingerone was transferred into a 10 ml volumetric flask containing 1 ml of HPLC grade water and the volume was adjusted to 10 ml with water and sonicated for 5 minutes [17].

Fractionation of phytoconstituents from NK

NK 10 ml was taken and transferred into a separating funnel, to this 30 ml of hexane was added and gently shaken for 5 minutes and left for 30 minutes until two clear layers were formed. The hexane layer was separated and collected separately in a beaker, and labeled as hexane fraction, and kept aside. To the remaining layer, chloroform was added and extracted in the same way and the organic layer was collected separately. To the remaining layer, 10 ml of acetonitrile was added and extracted leaving behind the residual fraction. The residual fraction was evaporated on a rotary evaporator and subjected to sample preparation.

Preparation of sample solution

The sample solution was established by exactly weighing 1 mg fraction residue in a 10 ml volumetric flask and dissolved in 1 ml of HPLC grade water and the solution was

adjusted with HPLC grade water up to 10 ml, sonicated for 5 minutes to get the required concentration of 100 µg/ml.

Analytical method development

The best optimal conditions were chosen for selecting the mobile phase, establishing the column, and determining the detection wavelength, a number of factors were taken into consideration. Following multiple trials, the most successful chromatographic trial that achieved ideal peak resolution and high symmetry for the simultaneous estimation of three phytomarkers is reported in Table 1.

Validation of developed method

Validation of the developed method was established as per International Council for Harmonization (ICH) guidelines which include accuracy, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, robustness, and system suitability.

Table 1. Chromatographic conditions of the developed method.

Parameters	Value
Injection volumes	20 µl
Column temperature	40°C
Detection wavelength	254 nm
Flow rate	1 ml/minute
Mobile phase	Acetonitrile and 0.1% Ortho Phosphoric acid with a ratio of 30:70
Mode of separation	Isocratic
Run time	20 minutes

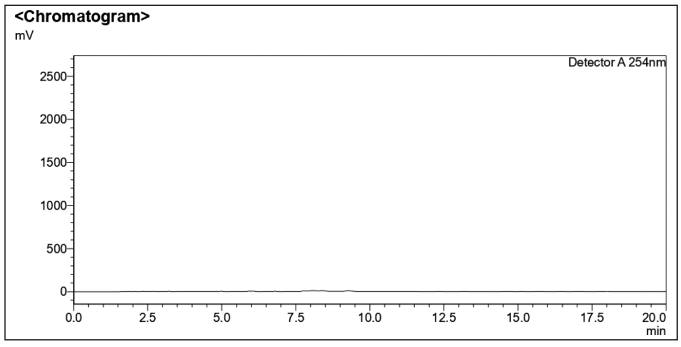

Figure 2. Chromatogram of blank.

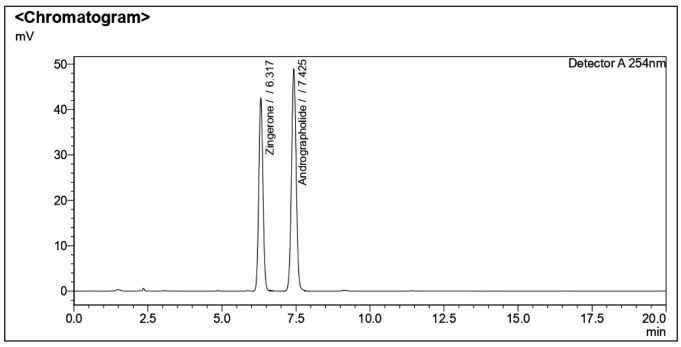

Figure 3. Standard chromatograms of andrographolide and zingerone.

Specificity

The specificity of the method was determined by injecting blank samples to demonstrate the absence of interference with the elution of phytomarkers andrographolide, zingerone, and eugenol in the polyherbal formulation.

Linearity

The linearity was tested by preparing a stock solution of three phytomarkers andrographolide, zingerone, and eugenol. Dilution was carried out to achieve a final concentration of 25–200 µg/ml for all three marker compounds. The linearity was assessed and calibration curves were established by plotting average peak area vs standard concentrations.

LOD and LOQ

The LOD and LOQ of the study were achieved by assuming the linearity of the standards and repeating the procedure three times to acquire the standard deviation (SD) of the intercept and slope of the regression equation (*S*) values. The following formulas were used to establish and determine the SD technique for LOD and LOQ:

$$LOD = 3.3 \times SD/S \text{ and } LOQ = 10 \times SD/S$$

Precision

The precision study was demonstrated by accurately weighing and preparing a stock solution (100 µg/ml) of three

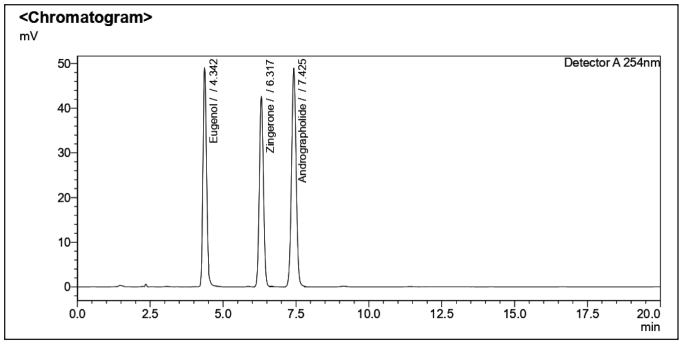

Figure 4. Standard chromatogram of andrographolide, eugenol, and zingerone.

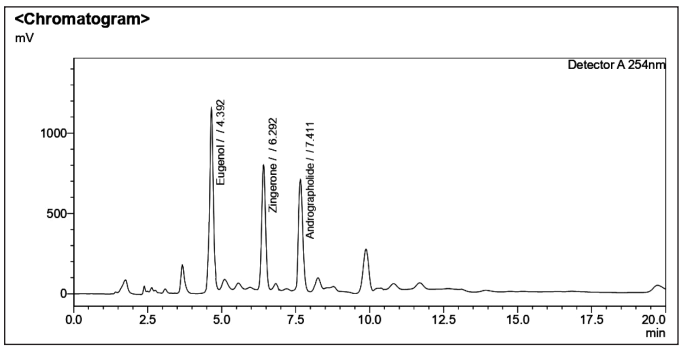

Figure 5. Sample chromatogram of andrographolide, eugenol, and zingerone.

Table 2. System suitability parameters for marker compounds.

Parameters	Eugenol	Andrographolide	Zingerone
R_t	4.342	7.425	6.225
Theoretical plates	6,880	11,083	10,181
Resolution	-	4.011	11.243
Tailing factor	1.192	1.016	1.112

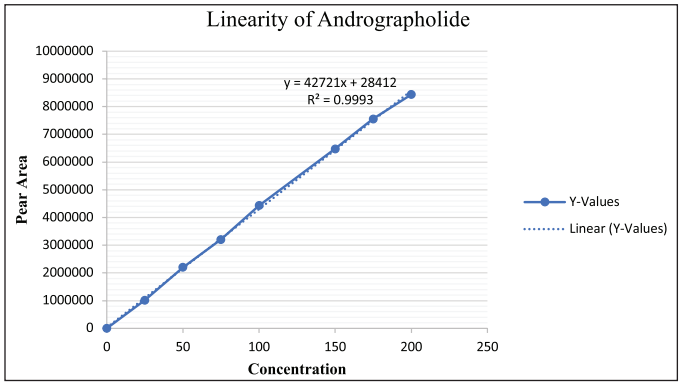

Figure 6. Linearity of andrographolide.

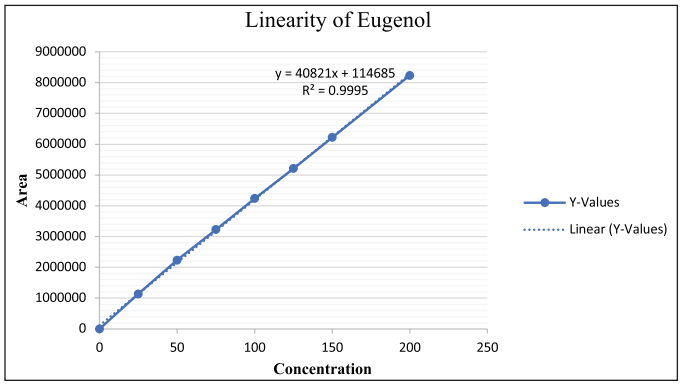

Figure 7. Linearity of eugenol.

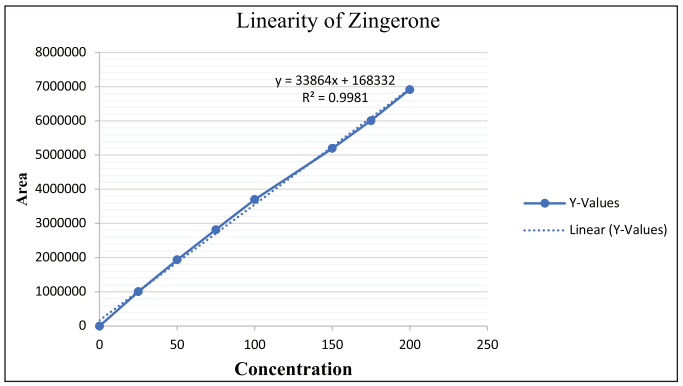

Figure 8. Linearity of zingerone.

phytomarkers. The method precision was shown by determining the sample response six times a day. The procedure outlined in the "Preparation of sample solution" section was used to prepare each sample in turn. Relative standard deviation (RSD) was established to document the findings of all precision-related data.

Table 3. LOD and LOQ data for phytochemical markers.

Parameters	Andrographolide	Eugenol	Zingerone
LOD	0.13	0.41	0.21
LOQ	0.39	1.26	0.65

Accuracy

The relative recovery, which was calculated at three different concentrations at three levels in the sample (50%, 100%, and 150%) was used to determine the accuracy of the method.

Robustness

Robustness is altering the parameters such as temperature and flow rate following deliberate conditions. By purposefully varying experimental parameters including column temperature and flow rate, the method's durability was tested. To test the method's robustness, flow rates of ± 1 ml/minutes and column temperatures of ± 40°C were used.

RESULTS

Optimization of chromatographic conditions

Mobile phase combinations containing methanol: water and acetonitrile: water in varying ratios were used in the trials but the R_t value was found to be high. These solvent combinations were used in a number of studies but they resulted in unsatisfactory peak shapes and unsuccessful separations. Finally, several ratios of ortho phosphoric acid and acetonitrile were explored for improved resolution of phytomarkers. Blank chromatogram is shown in Figure 1.

Quantification of phytomarkers

Optimized chromatographic parameters were established to estimate the amount of sample solutions to determine the phytomarkers present along with a mixture of individual standards. The blank chromatogram is shown in (Fig. 2). The mixture of standards andrographolide and zingerone (Fig. 3) standard chromatograms andrographolide, eugenol, and zingerone (Fig. 4). The sample chromatogram of NK is depicted in (Fig. 5). Based on the calibration curve, the results for each individual marker were determined.

System suitability

By extrapolating chromatographic conditions from the chromatogram of standard solutions, such as the number of theoretical plates (N), resolution (R_s), and tailing factor (T_f), the method's system suitability was established. The results are displayed in Table 2. The specified chromatographic conditions were determined by system suitability parameters to be suitable for the development and validation of the method.

Linearity data for marker compounds

The linearity of calibration curves for andrographolide, eugenol, and zingerone was established in a range of 25–200

Table 4. Precision study for Marker compounds.

Precision	% RSD					
	Andrographolide	R_t	Eugenol	R_t	Zingerone	R_t
1	4,469,695	7.415	3,099,074	4.324	3,788,524	6.18
2	4,468,266	7.386	3,095,134	4.417	3,789,563	6.33
3	4,467,239	7.412	3,088,534	4.320	3,792,518	6.34
4	4,472,154	7.405	3,097,117	4.355	3,793,585	6.34
5	4,470,072	7.411	3,085,545	4.367	3,791,988	6.34
6	4,470,190	7.403	3,093,520	4.372	3,794,133	6.34
Mean	4,469,603	7.405333	3,093,154	4.355	3,791,719	6.311667
±SD	1700.392	0.010482	5177.79	0.03096	2230.537	0.064627
% RSD	0.038043	0.141543	0.1674	0.71084	0.058827	1.023931

Table 5. Accuracy data of marker compounds.

Parameters (ppm)	% Recovery		
	Andrographolide	Eugenol	Zingerone
50	100.96	99.17	99.69
100	100.83	100.67	100.76
150	102.39	102.28	101.33
Average	101.3933	100.7067	100.5933
% RSD	0.86	1.55	0.83

Table 6. Results of robustness study.

Parameters	Deliberate changes	% RSD		
		Andrographolide	Eugenol	Zingerone
Flow rate (-)	0.9 ml	0.09	0.21	0.12
Flow rate (+)	1.1 ml	0.22	0.09	0.23
Column temperature (-)	39°C	0.32	0.51	0.71
Column temperature (+)	41°C	0.09	0.62	0.72

µg/ml. Representation of results obtained and the calibration curves are depicted in Figures 6–8.

Detection limit and LOQ

The LOD and LOQ for andrographolide are 0.13 and 0.39 µg/ml, when compared to other methods andrographolide LOD and LOQ is superior because the published article shows the average range of 0.40 to 1.82. Very few studies were reported on zingerone which shows the LOD and LOQ level from 0.2 to 0.95. Hence, the reported LOD and LOQ for zingerone are superior then other methods. Eugenol shows slightly higher LOD and LOQ values when compared to other methods. Eugenol shows the average LOD and LOQ values from 0.09 to 1.34 µg/ml. The LOD and LOQ values were determined and results obtained are shown in Table 3.

Precision

The precision was measured six times a day and the % relative standard deviation (RSD%) ranged from 0.1% to 1.0%. The results obtained from precision study are shown in Table 4.

Accuracy

The accuracy of the assay method, measured as relative recovery at three concentration levels, was 100.5%–101.39%, with all RSD% values ≤ 2%. Recovery study results are shown in Table 5.

Robustness

The robustness of the developed method was established by intentionally adjusting the experimental conditions such as flow rate from ± 1 ml/min and the column temperature was varied from ±40°C. Results obtained from the robustness study are shown in Table 6.

DISCUSSION

The research involves the development of a new, simple, rapid, and sensitive RP-HPLC approach for the synchronous determination of andrographolide [18] eugenol [19] and zingerone [20] marker compounds in NK, a Siddha polyherbal formulation. Previous research has revealed that there are numerous methods for determining andrographolide [21] eugenol and zingerone separately. However, no RP-HPLC methods were reported for the simultaneous determination of these marker compounds in any herbal formulation. The research is primarily aimed at developing a rapid, accurate, and cost-effective method involving minimal sample preparation.

Several mobile phase combinations were tried during method development and validation. The isocratic mobile phase consisting of 30:70 acetonitrile and 0.1% ortho phosphoric acid was finalized to precisely estimate all three marker chemicals. This developed method was validated through factors including system suitability, accuracy, precision, linearity, LOD, quantification, and robustness.

Utilizing variables such as the theoretical plate count (N), resolution (R_s), and tailing factor (T_f), the system suitability research was established. Each parameter was perceived to be within the recommended range. The developed method was found to be appropriate for analyzing andrographolide, eugenol, and zingerone in the system. The proposed approach is linear for all three phytomarkers in

the specified range, according to the obtained R2 > 0.999 for all standards in the linearity experiment. The least concentration required to quantify and identify the markers in the sample solution was estimated using LOD and LOQ as well. The intra-day precision study was used to determine the precision study. The study results, i.e., % RSD (2%), were within acceptable range in accordance with ICH recommendations.

Three different concentration levels of the phytomarkers were used to test their accuracy. For the compounds of interest, complete recovery of marker compounds was obtained, demonstrating the method's ability to recover markers completely. The recovery study sample preparation process was simple and rapid mainly due to the short variation time. The method was determined to be resilient for simultaneous analysis by altering flow rate and temperature, according to the robustness study findings [22].

This optimized and developed method can be used in the estimation of andrographolide, eugenol, and zingerone siddha polyherbal formulations such as NK Ayurvedic and homeopathic formulations containing these three phytomarkers. These compounds can be used as a quality control tool for the standardization and evaluation of formulations. This study can be an imperative tool in developing new methods and validating these novel methods for utilization in Herbal industries.

CONCLUSION

With the increased demand for herbal medicine and enhanced usage of herbal medicines, the development of an authentic standardization method will help sustain the quality of such predominant polyherbal preparations. The developed RP-HPLC method is suitable for the simultaneous quantification of standard phytochemical markers andrographolide, eugenol, and zingerone and also for their estimation in NK liquid siddha formulation.

LIST OF ABBREVIATIONS

NK: nilavembu kudineer; HPLC: high performance liquid chromatography; R_t: retention time; N: number of theoretical plates; T_f: Tailing factor; R_s: resolution factor; R2: regression coefficient; RSD: relative standard deviation; SD: standard deviation; LOD: limit of detection; LOQ: limit of quantification; ICH: International Council for Harmonization.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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