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RESEARCH ARTICLE

Novel Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) Method for the Quantification of Apigenin in Ocimum Basilicum Linn Seeds (Tukmaria)

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Abstract: ***Background*:** Ocimum basilicum L. seeds, is commonly also known as Takhmaria in Gujarat. It is a common constituent present in fruits, *plant*-derived beverages, vegetables, wheat, sprouts, and some seasonings.

***Objective*:** A simple, specific, precise, accurate, and sensitive method for the quantification of apigenin by reverse-phase high-performance liquid chromatography (RP-HPLC) was developed and validated.

***Methods*:** Analysis was carried out on Inertsil ODS-3V-C18 column (250 mm × 4.6 mm i.d, 5 µm) as stationary phase and methanol-acetonitrile (55:45 v/v) as a mobile phase at a flow rate of 1.0 mL/ min. Detection was carried out at 340 nm. The retention time of apigenin was found to be 8.30 min. The proposed method was validated according to ICH Guidelines, Q2 (R1).

***Results*:** The developed method showed good linearity in the range of 10-50 µg/mL with a correlation coefficient (R2= 0.9998). The LOD and LOQ were found to be 1.23 µg/mL and 4.05 µg/mL, respectively. The percentage recovery for apigenin was found to be 97.75-100.5%. All validation parameters were found within acceptable limits and demonstrated good reliability in the quantification of apigenin.

***Conclusion*:** Thus, the newly developed and validated method can be successfully applied for the quantification of apigenin from seeds of O. basilicum L. and can also be applied for the standardization of polyherbal formulation containing O. basilicum seeds. Assay results showed good recovery when statistically compared with the high-performance thin-layer chromatography (HPTLC) method.

Keywords: Apigenin, ocimum basilicum seeds, tukmaria, rp-hplc, methanolic extract, quantification.

1. INTRODUCTION

Apigenin (4, 5, 7-trihydroxyflavone) is a natural product belonging to the flavone subclass of flavonoid and is the aglycone of several naturally occurring glycosides [1]. It is a common constituent present in fruits, plant-derived beverages, vegetables, wheat, sprouts and some seasonings. Some of the popular and abundant sources of apigenin include chamomile tea [2] grapefruits, onions, oranges and some spices such as parsley [3] and is also found in higher levels (relative to other foods) in yarrow, celery, basil, tarragon, cilantro, liquorice, foxglove, coneflower, flax, passion flower, horehound, spearmint, red wine, beer [4] and is an active ingredient in the memory herb *Gingko biloba* [5].

Apigenin is an anticancer agent and displayed good cytotoxicity activity against numerous human cancer cells, including prostate cancer, colon carcinoma and breast cancer, without any mutagenic activity [6]. There are numerous data available on anti-inflammatory, anti-viral, and purgative properties of apigenin in the literature [7].

Ocimum basilicum Linn. (Lamiaceae), commonly known as sweet basil or Tukmaria, is an important medicinal plant found throughout India. Various medicinal properties are attributed to the different part of the plant, including antimicrobial [8], anti-inflammatory [9], antioxidant [10], antiulcerogenic [11], cardiac stimulant [12], chemomodulatory [13], hepatoprotective [14] and hypoglycaemic and hypoli-

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pidemic activities [15], antioxidant, aphrodisiac, diuretic and antidysenteric action [16] and many more [17, 18].

Various phytoconstituents like ursolic acid, oleanolic acid, eugenol, methyl eugenol, mono-sesquiterpenes, isopropyl palmitate, linalool, methyl chavicol, bergamotene, germacrene D, δ-cadinene, γ-cadinene, β-selinene, and spathulenol were isolated from *O. basilicum* leaves [19]. D-xylose, L-arabinose, r-rhamnose, and D-galacturonic acid, galactose and glucose were isolated from *O. basilicum* seeds [20]. The presence of other phytoconstituents was reported from the methanolic extract of seeds, including carbohydrate, saponins, phenolics, flavonoids and, seed Mucilage [21]. In our previous study, methanolic extract of *O. basilicum* seeds had a good concentration of flavonoids. By isolation of methanolic extract of *O. basilicum* seeds, a yellow crystalline compound was obtained and was confirmed as apigenin by various spectral characterization methods [22].

Literature survey revealed that a reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed for the simultaneous estimation luteolin and apigenin in herb of *Achillea* millefolium L [23]. However, RP-HPLC method has not been reported yet for the quantification of apigenin in *O. basilicum* seeds. In our previous study, we have reported a novel HPTLC method for the estimation of apigenin in *O. basilicum* seeds [24]. This paper describes a novel, accurate, precise, sensitive, selective, and robust RP-HPLC method for the quantification of apigenin in plant samples of *O. basilicum* seeds.

2. EXPERIMENTAL

2.1. Materials

The *O. basilicum* seeds were collected from the local market, Ahmedabad, Gujarat and were authenticated (Head P.G Center in Botany, Smt. S. M. Panchal Science College, Talod, Gujarat). Analytically pure (≥98 % estimated by HPLC) Apigenin (Sigma-Aldrich, Bangalore, India), HPLC grade water and acetonitrile (Chem Dyes Chemicals Pvt. Ltd., Rajkot, India), and Nylon (0.45 µm-0.47 µm) membrane filters (Gelman Laboratory, Mumbai, India) were used for the present study.

2.2. Instruments

A HPLC system consists of Spectra physics P-1000 Pump. Inertsil ODS-3V $-C_{18}$ column (250 × 4.6 mm i.d., with 5 µm particle size), Rheodyne 7725 Injector, UV-100 Detector, Peak ABC (Dual Chanel) software was used for the method development. Analytical balance of Shimadzu Aux 120 (Gottingen, Germany) was used.

2.3. Methods

2.3.1. Preparation of Standard Stock Solutions

Precisely weighed apigenin (10 mg) was dissolved in methanol into a 10 mL volumetric flask and diluted up to the mark with methanol to get a standard stock solution (1000 µg/mL) of apigenin.

2.3.2. Preparation of Sample Solution

Air-dried powder (100 gm) of *O. basilicum* seeds sample was defatted by refluxing with 250 mL petroleum ether (60-80°C) for 4 h. The residue was air-dried and subjected to Soxhlet extraction using methanol for 6 h and filtered through Whatman filter paper No 41. This procedure was repeated three times to get complete extraction from the powder. Methanolic extracts were combined and evaporated using a vacuum-assisted rotary evaporator at 40 °C. Dried extract (10 mg) was dissolved in 10 mL of methanol. From this solution, 1 mL was diluted up to 10 mL of methanol, which was further used for chromatographic analysis.

2.3.3. Chromatographic Condition

The chromatographic separation was obtained using the Inertsil ODS-3V-C_{18} column. The HPLC system was operated in isocratic mode with the mobile phase consists of acetonitrile: water (55:45, v/v), at 1 mL/min flow rate. The mobile phase was filtered using nylon (0.45 µm-0.47 µm) membrane filters and was degassed before use. The measurement was carried out at 340 nm using peak ABC (Dual Chanel) software. Sample of 20 µL was injected with a run time of 15 min at 25 ± 2 °C temperature.

2.4. Method Validation

The method was validated according to the International Conference on Harmonization guidelines for validation of analytical procedures Q2 (R1) [25].

2.4.1. System Suitability Studies

System suitability was established by injecting six replicates of standard solution of apigenin and the RSD of retention time, asymmetry and theoretical plates were determined.

2.4.2. Specificity

Analytical method's specificity is its ability to accurately and specifically determine the analyte in the presence of components expected to be present in the sample matrix. Chromatograms of standard (30 µg/mL) and sample (methanolic extract *O. basilicum* seeds) solutions of apigenin were compared. The peak purity of apigenin was assessed by comparing the spectra at three different levels, i.e. peak start (s), peak apex (m) and peak end (e) positions of the chromatogram.

2.4.3. Linearity and Range

Aliquots (0.1, 0.2, 0.3, 0.4 and 0.5 mL) from stock solutions were transferred in different 10 mL volumetric flasks and volume was adjusted up to the mark using mobile phase to obtain a concentration of 10-50 µg/mL. Each solution was injected under the operating chromatographic conditions. Calibration curves were plotted using peak areas versus concentrations, and the regression equation was obtained. A calibration curve was repeated five times.

2.4.4. Accuracy (Recovery)

The standard addition method was used to study the accuracy of the method. It was determined by calculating the

recovery of apigenin using 80, 100, 120 % level. A known amount of standard solutions of apigenin (0.16, 0.2, 0.24 mL) were added to three different volumetric flasks of 10 mL capacity containing 0.2 mL sample stock solution. The volume was adjusted up to mark with methanol. Percentage recovery was calculated by injecting each solution three times, and % recovery was calculated with the help of the regression equation.

2.4.5. Precision

The repeatability of the method was checked by analyzing (n = 6) apigenin solutions (30 µg/mL each) and the response were recorded.

The intra-day (3 times on the same day) and inter-day (3 different days over a period of 1 week) precisions of the proposed method were checked by measuring the responses for 3 different concentrations of apigenin (10, 30 and 50 µg/mL). The solution was injected and a peak area was obtained. The assay values were calculated. The RSD was reported.

2.4.6. LOD and LOQ

The Limit of detection (LOD) and Limit of quantitation (LOQ) was calculated by the equation,

LOD = 3.3 x (SD/Slope)

LOQ = 10 x (SD/Slope)

Where,

SD = Standard deviation of the Y-intercepts of the calibration curve.

Slope = Mean slope of the calibration curve.

2.4.7. Robustness

Deliberate but slight variation in the method parameters for analyzing the area of apigenin was done for the robustness study. Different chromatographic conditions like flow rate (± 0.2 mL/min), mobile phase composition (± 5%,) and wavelength (± 3 nm), were used to determine robustness of the method. The assay values were calculated. The RSD of the assay was reported.

2.5. Estimation of Apigenin from Methanolic Extract of O. basilicum Seeds Using Developed RP-HPLC Method

The chromatographic peaks of apigenin in methanolic extract of *O. basilicum* seeds were compared with a retention time of standard apigenin and peak area was obtained. The amount of apigenin present in the methanolic extract of *O. basilicum* seeds was analyzed using a regression equation.

3. RESULTS

3.1. System-Suitability Parameters

The results of system-suitability test parameters were listed in (Table **1**). RSD for all parameters was found to be less than 2 %.

3.2. Method Development and Chromatographic Conditions

The UV spectrum of the standard solution of apigenin (100 µg/mL) was scanned using the range of 200-400 nm with methanol as a blank. Maximum absorbance was observed at 340 nm, as shown in Fig. **1** and further determination of apigenin was carried out at 340 nm.

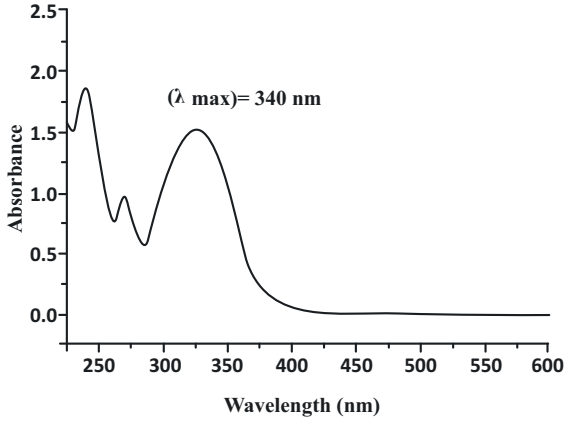

Fig. (1). UV Spectrum of Apigenin (100 µg/mL) in methanol.

Table 1. Results of system suitability test parameters (n = 6).

No.	Retention Time, Min	Asymmetry	Theoretical Plates
1	8.32	1.61	4413
2	8.31	1.62	4453
3	8.28	1.59	4408
4	8.30	1.60	4465
5	8.31	1.61	4467
6	8.32	1.62	4468
Mean	8.30	1.60	4445.66
SD	0.015	0.011	27.81
RSD	0.18	0.68	0.62

RP-HPLC method carried out in this study was aimed at developing a chromatographic system, capable of eluting and resolving flavonoid components from the methanolic extract of *O. basilicum* seeds. The nature of the stationary phase for separation was selected on the basis of the chemistry of the compound. Various columns with different stationary phases were tried. From the different trials, a column with a C_{18} stationary phase was selected, which gave proper retention, good theoretical plates. During method development, different compositions of mobile phases were tried to develop an optimum mobile phase which separates apigenin from plant extract containing other phytoconstituents with good resolution. From the different tried mobile phases, the mobile phase comprised of water: acetonitrile (55: 45, v/v) was found to be satisfactory.

Under the optimized experimental conditions, the developed chromatographic method indicated a presence of apigenin in standard and in methanolic extract of *O. basilicum* seeds, as shown in Figs. **2** and **3**.

3.3. Specificity

The study has shown that the apigenin peak was free from the sample matrix, as the peak purity index of > 0.99 was obtained as shown in Fig. (**4**) and value reported in (Table **2**).

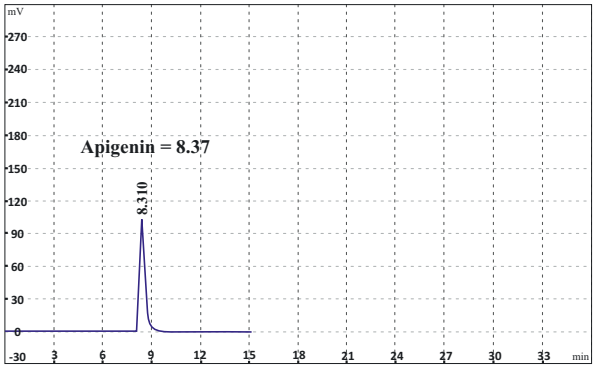

Fig. (2). RP-HPLC chromatogram of standard apigenin (30 µg/mL). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

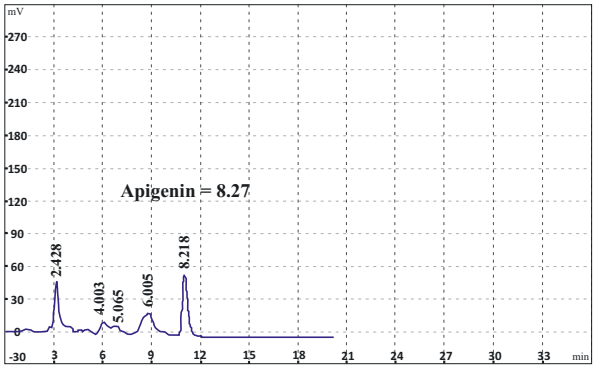

Fig. (3). RP-HPLC chromatogram of apigenin in methanolic extract of *O. basilicum* seed. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

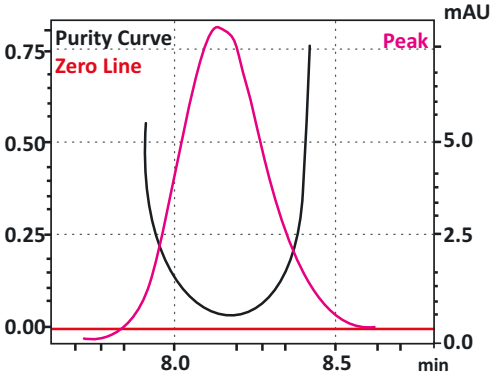

Fig. (4). Peak purity spectra of apigenin isolated from *O. basilicum* Seed. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

Table 2. Peak purity data of apigenin.

Standard/ Sample	Peak Purity Index
Standard apigenin	0.999
Apigenin from methanolic extract of O. basilicum seed	0.997

3.4. Linearity

A linear correlation was attained between peak area and concentration of apigenin in the range of 10-50 µg/mL, as shown in Fig. **5**. The linearity of the method was proved by the value of the regression coefficient (R^2). Linearity data are described in Table **3**.

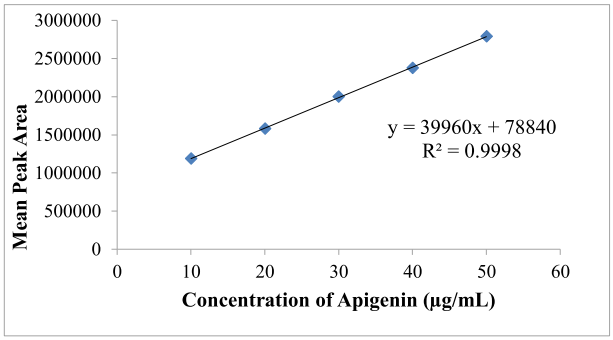

Fig. (5). Linearity curve of standard apigenin (10-50 µg/mL).

Table 3. Linearity data of apigenin (n=5).

Conc. (mg/mL)	Area (mean ± SD)	RSD
10	1189746 ± 3151.71	0.26
20	1579862 ± 6785.47	0.42
30	1999856 ± 10882.64	0.54
40	2377852 ± 16543.74	0.69
50	2788765 ± 18260.11	0.65

3.5. Accuracy (Recovery)

Recovery of the apigenin was obtained in the range of 99.03-100.69 % by standard addition method, indicating the accuracy of the method, as shown in Table **4**. The RSD for the accuracy of apigenin was 0.32-0.50%.

3.6. Precision

The results of repeatability are as shown in Table **5**. The RSD for repeatability of apigenin was 0.91. The results of intra-day precision and inter-day precision are shown in Table **6**. The RSD for intra-day precision and inter-day precision were found to be in the range of 0.26-0.54 % and 0.65-0.97 %, respectively apigenin, and were found to be less than 2%. These results demonstrate that methods develop here, achieve a high degree of precision.

Table 4. Results of recovery study.

% Level	Conc. Added (µg/mL)	Sample Conc. (µg/mL)	Total Amount Recovered (µg/mL)	Mean Peak Area • ± SD	Recovery	RSD
0	0	20	0	1579862 ± 6631	99.03	0.41
80	16	20	15.64	2245786 ± 4341	101.31	0.19
100	20	20	19.62	2377843 ± 6624	99.44	0.28
120	24	20	24.01	2558721 ± 8546	100.69	0.33

Table 5. Results of repeatability (n=6).

Apigenin Conc. (µg/mL)	1	2	3	4	5	6	SD	RSD
30	1999866	1997654	1989764	1978453	1965973	1965973	18084	0.91

Table 6. Results of intra-day and Inter- day precision (n=3).

	Intra-day precision			Inter-day precision		
Apigenin Conc. (µg/mL)	Mean Peak Area ± SD	Mean Conc ± SD	RSD	Mean Peak Area ± SD	Mean Conc ± SD	RSD
10	1189759 ±3151.71	9.82 ± 3.2	0.26	1189756 ± 11876.5	9.57 ± 3.8	0.65
30	1999872± 10882.65	30.23 ± 4.3	0.54	1999866 ± 13453.8	28.7 ± 7.4	0.84
50	2788749 ± 8260.11	49.58 ± 2.1	0.28	2788769 ± 156783.8	51.34 ± 6.3	0.97
Mean RSD	-	0.36	-	-	0.82	-

Table 7. Results of robustness studies (n=3).

Chromatographic Condition		Assay ± SD	RSD
Flow rate ± 0.2 mL/min	0.8	98.34 ± 0.75	0.76
	1 (optimum)	99.34 ± 0.82	0.82
	1.2	97.23 ± 0.86	0.88
Mobile phase composition ± 5%	(50: 50 % v/v)	98.34 ± 0.96	0.97
	(45: 55 % v/v) (optimum)	100.34 ± 0.79	0.79
	(55: 45 % v/v)	98. 45 ± 1.05	1.06
Wave-length nm ± 3 nm	337	99.20 ± 0.47	0.47
	340 (optimum)	100.67 ± 0.67	0.66
	343	99.61 ± 0.88	0.88

3.7. Limit of Detection and Limit of Quantification

The LOD was found to be 1.23 µg/mL while the LOQ was found to be 4.05 µg/mL, respectively, which indicates the sensitivity of the method.

3.8. Robustness

Results of robustness studies of RP- HPLC method are shown in Table **7**. The method was found to be robust in terms of variation in composition and flow rate of the mobile phase and wavelength, as RSD for all parameters was found

Table 8. Assay results of methanolic extract of O. basilicum seed (n=3).

Sample	Apigenin Content (%w/w)	SD	RSD
Methanolic fraction	0.78	3.5	0.25

Table 9. Summary of validation parameters of developed HPLC method.

Sr. No.	Parameters	Results
1	Linearity (µg/mL)	10–50
2	Linear regression equation	y = 39960x + 78840
3	Regression coefficient (R2)	0.9998
4	Accuracy	97.75-100.5%
5	Precision (RSD) Repeatability Intraday	0.91 0.26-0.54
6	Interday	0.65-0.97
7	Limit of Detection (µg/mL)	1.23
8	Limit of Quantification (µg/mL)	4.05
9	Robustness	Robust
10	Specificity	Specific

Table 10. Statistical comparison between two analytical methods.

Parameters	Quantification of Apigenin	
	HPLC	**HPTLC**
Linearity	100–600 (ng/band)	10–50 (µg/mL)
Regression coefficients (R2)	0.9952	0.9998
Accuracy	97.75-100.4 %	97.75-100.5%
Precision (% RSD) **Intraday** **Interday**	 0.73-1.02 0.95-0.1.20	 0.2-0.54 0.65-0.97
LOD	3.72 (ng/band)	1.23 (µg/mL)
LOQ	12.30 (ng/band)	4.05 (µg/mL)
Apigenin ± S.D, % (n=3)	0.69 ± 2.3	0.78 ± 3.5
Tabulated t-value	4.303	
Calculated t-value	16.23	

to be less than 2 %. The low value of RSD indicates the robustness of the method.

3.9. Quantification of Apigenin in Methanolic Seeds Extract of O. basilicum by Developed HPLC Method

Developed and validated HPLC method was successfully applied for quantification of apigenin from the methanolic extract of *O. basilicum* seeds. The content of apigenin in methanolic extract of *O. basilicum* seeds was found 0.78% *w/w,* as shown in Table **8**. The summary of validation parameter is shown in Table **9**.

4. DISCUSSION

In this study, a novel RP-HPLC method for quantification of apigenin in *O. basilicum* seeds has been established and validated, which is reported for the first time. RP- HPLC method was validated in terms of system suitability, linearity, accuracy, precision, limit of detection, limit of

quantification, specificity and robustness. System suitability test showed that critical parameters such as retention time, asymmetry, and a number of theoretical plates met the acceptance criteria for all the experimental days, which indicates the system is suitable. Apigenin showed good linearity (R^2=0.9998) with LOD and LOQ 1.23 µg/mL and 4.05 µg/mL, respectively, which indicate the sensitivity of the method. The spiked extract with standard compound ensured that there is no interference of sample matrix on peak response of analyte of interest with good recovery (97.75 - 100.05%). The chromatogram of apigenin was found satisfactory with good resolution and better reproducibility for the determination of apigenin from the methanolic extract of *O. basilicum* seeds. Robustness was evaluated to ensure that the HPLC method is insensitive to small changes in the experimental conditions. In this study, the wavelength, mobile phase composition, and flow rate were changed. The content of apigenin was found to be 0.78% w/w, which indicates seeds containing a good amount of apigenin. The assay results for apigenin, in methanolic extract of *O. basilicum* seeds obtained using HPLC and HPTLC, were compared statistically by applying the paired t-test. The calculated t-value for apigenin (16.23) is greater than the tabulated t-value (4.303) at the 95% confidence interval. Therefore, a significant difference was found in the content of apigenin determined by the proposed HPLC and HPTLC methods. Results were discussed in Table **10**.

From the above results, it was found that the HPLC method is more accurate, precise, sensitive, and selective for estimation of apigenin from *O. basilicum* seeds.

CONCLUSION

The developed and validated HPLC method enable to quantify apigenin from the methanolic extract of *O. basilicum* seeds. The spiked extract with standard compound ensured that there is no interference of the sample matrix on the peak response of the analyte of interest. From the above study, it can be concluded that the developed HPLC technique is fast, specific, precise and accurate; hence it can be employed for the determination of apigenin in plant samples. The developed method can also use for the marker-based standardization of herbal formulations. No interference of any impurities was found; hence the analytical method is found to be specific.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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