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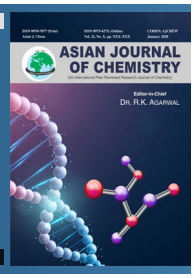

Development and Validation of UV Spectrophotometric and RP-HPLC Methods for the Estimation of Gallic Acid in Herbal Formulation of *Amalaki*

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In this study, two analytical methods *viz*. UV spectrophotometry and RP-HPLC were developed for the evaluation of *Amalaki* in the marketed herbal formulation. The amount of gallic acid estimated in the marketed formulation complies with the standard (not less than 1% w/w of gallic acid) specified in the official monograph of *Amalaki*. The developed methods showed good linearity, accuracy, precision, ruggedness, robustness, specificity, LOD and LOQ. Results of validation studies were found satisfactory with % RSD values of less than 2% indicating good specificity, validity and reliability of the developed methods. Both analytical methods are claimed to be simple, accurate and precise. Present methods can, therefore, be applied widely for the routine analysis of marketed formulations or any crude traditional preparations of *Amalaki* based on the quantitative determination of gallic acid at a reasonable cost with simple analytical set up.

Keywords: *Amalaki*, Herbal formulation, Gallic acid, UV Spectophotometry, RP-HPLC.

INTRODUCTION

Amalaki (*Emblica myrobalan* or Indian Goseberry) is an herbal drug official in the Indian Pharmacopoeia. It contains dried rhizomes of fruit pericarp of *Emblica officinalis* Gaertn. belonging to the family, Phyllanthaceae [1]. Since ancient times, it has been used in Indian Systems of Medicine (Ayurveda, Unani and Siddha) for the treatment and management of a variety of human diseases [2]. Various formulations of *Amalaki* are now available in the market, which are used extensively as antioxidant, neuroprotective and anti-inflammatory [3]. Gallic acid (not less than 1.0 % w/w) is the chief active principle of *Amalaki* [1]. It is chemically 3,4,5-trihydroxy benzoic acid, belonging to the group of plant polyphenols and occurs as a colourless crystalline powder soluble in water, methanol and ethanol [4,5]. Gallic acid and its derivatives are attributed to be responsible for the medicinal potential and health benefits of *Amalaki*.

Since *Amalaki* is an herbal drug, there is no sufficient standardized protocol (as per WHO guidelines) for its evaluation (quality, purity and potency) in medicinal preparations. Due to this fact, the analytical evaluation of *Amalaki* is not well established. Though many scientific reports are available in literature, but the methods lack consistency, adequacy and reliability [2,6-8]. In this context, it is necessary to develop new analytical methods that can be applied widely for the evaluation of *Amalaki* in traditional as well as marketed herbal formulations. In present study, an attempt is made to develop a simple and specific analytical methods for the evaluation of marketed formulation of *Amalaki* in terms of its principle ingredient *i.e.* gallic acid by UV spectrophotometry and RP-HPLC methods.

EXPERIMENTAL

Gallic acid was purchased from Yarrow Chem Products, Mumbai. *Amalaki* formulation (tablets) was purchased from the local market. HPLC grade methanol, acetonitrile and water were procured from Merck Pvt. Ltd., Mumbai, India. All other chemicals and reagents used in the study were of analytical grade.

Spectrophotometric measurements were performed on Elico SL 244 Double Beam UV-Vis spectrophotometer with quartz cuvette of 1 cm width using methanol as solvent. Chromatographic analysis was achieved on a Cyber Lab RP-HPLC

equipped with a pump, manual sampler and a UV detector. The chromatographic column was a Phenomenex Luna C18 column (250 mm × 4.6 mm i.d., 5 µm). The column temperature was maintained at 55 ºC. The mobile phase consisted of acetonitrile: water (70:30 %v/v). The separation was achieved on an isocratic mode at ambient temperature. The flow rate was 1.0 mL/min and the injection volume was 20 µL. The run time was 9.0 min. The wavelength of UV detection was set at 272 nm. Citizen Ultra Sonicator was used for sonicating the sample solutions. Digital weighing balance (Shimadzu Aux 220) was used for weighing.

Preparation of standard and sample solutions

UV method: To prepare the standard stock solution, 10 mg of gallic acid was accurately weighed and transferred into a 10 mL volumetric flask. The volume was finally made up to the mark with methanol to obtain a concentration of 1000 µg/mL. Standard solutions were prepared in the concentration range of 2-10 µg/mL by further dilutions with methanol.

Twenty tablets were accurately weighed and finely powdered. Powder equivalent to the standard stock concentration was weighed and dissolved in a 10 mL of volumetric flask using methanol to obtain a sample stock solution of 1000 µg/mL. The solution was sonicated for 20 min and filtered through Whatman filter paper. Using the stock solution, final sample solutions in the concentration range of 2-10 µg/mL were prepared by serial dilution with methanol [9].

RP-HPLC method: An accurately weighed quantity (10 mg) of gallic acid was transferred to a 10 mL volumetric flask, dissolved and diluted to the mark with acetonitrile:water (70: 30 %v/v) to obtain a standard stock solution of 1000 µg/mL. Standard solutions were prepared in the concentration range of 2-10 µg/mL by further dilutions with the same solvent.

Twenty tablets were accurately weighed and finely powdered. Powder equivalent to the standard stock concentration was weighed and dissolved in 10 mL of volumetric flask using acetonitrile:water (70:30 %v/v) to obtain a sample stock solution of 1000 µg/mL. The solution was sonicated for 20 min and filtered through Whatman filter paper. Using the stock solution, final sample solutions in the concentration range of 2-10 µg/mL were prepared by serial dilution with the same solvent [10].

Estimation of gallic acid in *Amalaki* formulation

UV method: The assay of gallic acid in the test solution (10 µg/mL) of *Amalaki* was performed by absorbance ratio method. The absorbance was measured at 272 nm against methanol as the blank.

HPLC method: The assay of gallic acid in the test solution (10 µg/mL) of *Amalaki* was performed by recording the peak area of the sample and comparing it with that of the standard. The wavelength detection was conducted at 272 nm.

RESULTS AND DISCUSSION

UV method: Based upon solubility studies, methanol was selected as a suitable solvent for spectrophometric measurements. To determine the wavelength of maximum absorbance (λ_{max}), a test solution of gallic acid with the concentration of 10 µg/mL was prepared and scanned between 300-500 nm of UV-V range using methanol as a blank. Relevant information was obtained from available literature [9] for the selection of suitable solvent and determination of wave length of absorption. A representative spectrum of gallic acid in methanol is given in Fig. 1. The spectrum indicates a broad and well-defined peak at the λ_{max} of 272 nm.

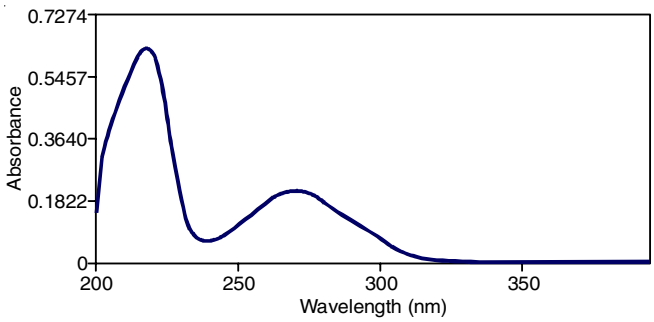

Fig. 1. Spectrum of gallic acid in methanol at λ_{max} 272 nm

HPLC method: Several trial runs were performed using C8 and C18 RP columns, various mobile phase compositions and different flow rates for the separation of gallic acid with good chromatographic parameters (retention time, resolution, peak area, theoretical plates, tailing factor, *etc.*). A C18 column (250 mm × 4.6 mm i.d., 5 µm) as a stationary phase with a mobile phase consisting of acetonitrile:water (70:30 %v/v) at a flow rate of 1.0 mL/min and a detection wavelength of 272 nm afforded the best separation with a sharp and well-resolved peak for gallic acid [10-12]. The optimized chromatograms obtained for the standard and test samples of gallic acid are displayed in Fig. 2. Results indicated that the test chromatogram matches with the standard chromatogram having the retention time (RT) of 2.31 min. The values of peak area (100286), theoretical plates (3895) and tailing factor (1.90) were found to be acceptable for the optimized chromatogram.

Method validation: The validation of developed UV and RP-HPLC methods was performed in terms of the following analytical parameters according to the ICH (Q2 R1) guidelines [13-17].

Linearity: The linearity of the methods was studied by analyzing the calibration standards of gallic acid in the concentration range of 2-10 µg/mL at 272 nm. The calibration curve was prepared by plotting average absorbance (mean ± SD) *versus* concentration for triplicate observations (n = 3). The plot of absorbance versus concentration was found linear in the range of in the range between 2-10 µg/mL. The regression equations were obtained as follows: $y = 0.0475x - 0.0031$ ($r^2 = 0.999$) and $y = 11357x + 613.8$ ($r^2 = 0.999$) for UV and HPLC methods, respectively (Fig. 3). Results implied that the developed methods were linear over the specified range.

Accuracy: To determine accuracy or percentage recovery of the developed methods, a known amount of the standard gallic acid was added to the sample solution to obtain three different concentrations. This study was performed at three concentrations levels *i.e.* 50, 100 and 150% in triplicate observations (n = 3). The percent recoveries were in the range between

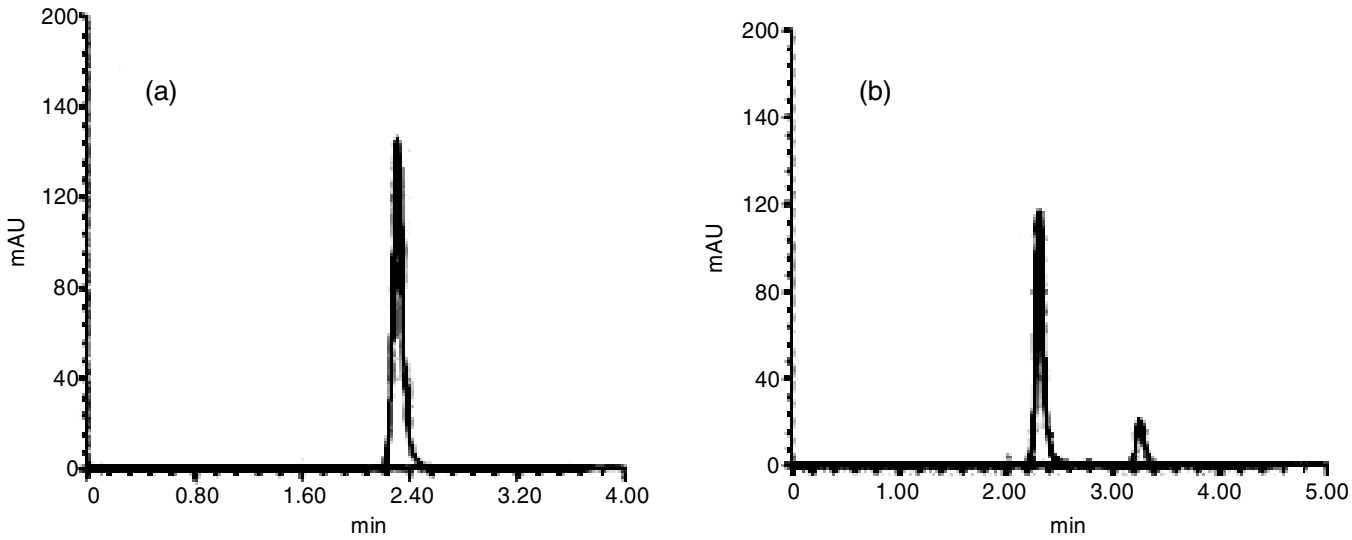

Fig. 2. Chromatogram of gallic acid (a) standard and (b) test (RT is 2.31 min at 10 µg/mL concentration)

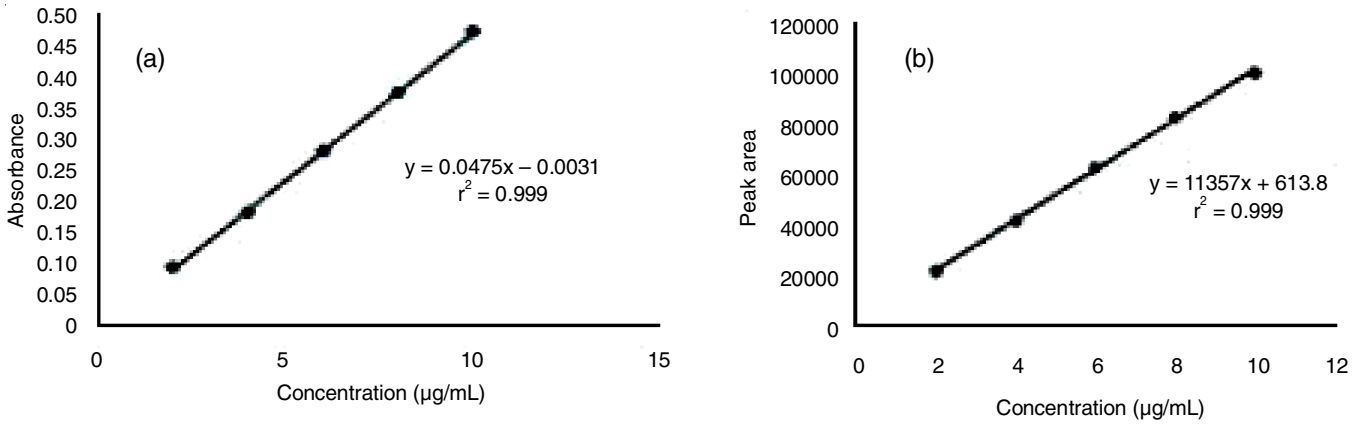

Fig. 3. Calibration curve of gallic acid (a) in UV and (b) in HPLC

38.416-76.381 and 67.827-79.869% for UV and HPLC methods, respectively. From these results, it is clear that the HPLC method is more accurate than the UV method. The RSD values were found to be satisfactory with less than 2%. The % recovery data are summarized in Table-1.

Precision: Repeatability (intra-day precision) was evaluated by injecting six replicate (n = 6) solutions of the standard concentration (6 µg/mL) on the same day. Similarly, reproducibility (inter-day precision) was determined by analyzing six samples (n = 6) of the standard concentration (6 µg/mL) by in the same laboratory, but on different day under similar experimental conditions. The RSD values were calculated. Results of precision studies were also found to be satisfactory. The % RSD values were less than 2% indicating good repeatability as well as reproducibility of both UV and HPLC methods. Results of precision studies are depicted in Table-2.

Ruggedness: Ruggedness was determined by analyzing six samples (n = 6) of the standard concentration (6 µg/mL)

TABLE-1
ACCURACY STUDIES OF GALLIC ACID AT DIFFERENT CONCENTRATIONS

Recovery level (%)	Concentration (µg/mL)		Absorbance/ Peak area*	Mean % recovery	% RSD
	Test (initial amount)	Standard (amount added)			
UV method					
50	4	2	0.14216 ± 0.002	38.416	0.170
100	4	4	0.25733 ± 0.003	60.575	0.291
150	4	6	0.38390 ± 0.002	76.381	1.010
HPLC method					
50	4	2	42315.0 ± 0.013	67.827	1.069
100	4	4	63644.0 ± 0.002	77.609	1.208
150	4	6	81375.0 ± 0.014	79.869	0.986

*Values are presented as mean ± RSD of three replicate observations (n = 3)

TABLE-2
RESULTS OF PRECISION FOR THE TWO DIFFERENT METHODS

Precision*	Repeatability (Intra-day)		Reproducibility (Inter-day)	
	Morning	Evening	Day 1	Day 2
UV method				
Absorbance#	0.26088 ± 0.002	0.27586 ± 0.004	0.26088 ± 0.002	0.29240 ± 0.017
%RSD	0.282	0.498	0.282	0.786
HPLC method				
Peak area#$	62563.4 ± 0.001	61901.8 ± 0.008	62563.4 ± 0.001	61930.0 ± 0.006
%RSD	0.121	0.826	0.121	0.599

*Values are presented as mean ± RSD of six replicate observations (n = 6); #Concentration was 6 µg/mL; $RT was 2.31 min

by two different analysts in the same laboratory under similar experimental conditions. Results of ruggedness studies are presented in Table-3. The % RSD values were less than 2%. It proves good ruggedness of the developed methods.

TABLE-3
RESULTS OF RUGGEDNESS FOR THE TWO ANALYTES

Ruggedness*	Analyst 1	Analyst 2
UV method		
Absorbance#	0.26088 ± 0.002	0.27586 ± 0.004
%RSD	0.282	0.498
HPLC method		
Peak area#$	62171.7 ± 0.004	62166.2 ± 0.003
%RSD	0.415	0.357

*Values are presented as mean ± RSD of six replicate observations (n = 6); #Concentration was 6 µg/mL; $RT was 2.31 min

Robustness: The robustness of the methods was investigated by analyzing six replicates (n = 6) of standard solution (6 µg/mL) by introducing small changes in the UV spectrometric measurements such as temperature and chromatographic conditions such as flow rate. Results of robustness studies presented in Table-4 indicate both the developed are practically robust. The % RSD values of the methods determined under robustness conditions were less than 2%.

LOD and LOQ: The limit of detection (LOD) and limit of quantification (LOQ) of the UV method were found to be 0.051 and 0.154 µg/mL, respectively. For the HPLC method, the values were found to be 0.178 and 0.540 µg/mL, respectively. The LOD and LOQ indicate that the developed methods are sensitive for the precise determination of component of interest, *i.e.* gallic acid in the marketed formulation.

Specificity: The specificity of the HPLC method was demonstrated by the separation of the analytes from other potential components such as impurities and other active principles. A volume of 20 µL of gallic acid and related components were injected and the chromatogram was recorded. No peaks were observed in the chromatogram at the desired location other than the peak due to gallic acid with retention time of 2.31 min. Results of specificity study indicate that the method is free from interferences due to excipients and/or related components present in the formulation (Fig. 4). The proposed method is, therefore, claimed to be specific for the quantitative determination of gallic acid in herbal formulations.

System suitability: System suitability was determined by injecting six replicate injections of the standard solution (6 µg/mL) of gallic acid. Results of system suitability parameters depicted in Table-5 were found within limit. The summary of validation parameters are represented in Table-5.

Estimation: In both UV and HPLC methods, the amount of gallic acid estimated in the marketed formulation of *Amalaki* was in good agreement with the label claimed. In the UV method, the percentage of gallic acid was found to be 83.34%, whereas it was 90.44% by HPLC method estimated at the concentration of 10 µg/mL (Table-6). The amount of gallic acid estimated complies with the standard (not less than 1% w/w of gallic acid) specified in the official monograph of *Amalaki* [1].

From validation studies, it is evident that the developed analytical methods are practically useful and valid. Results of validation parameters described above are satisfactory in both the methods. In all validation experiments, the % RSD values are less than 2% indicating high reliability and validity of the developed methods. Results obtained in present study are in consistent with existing literature [9,18-20]. However, the analytical performance of the HPLC method was found comparatively better than that of the UV method. It is probably due to the poor selectivity and/or sensitivity of the UV instrument as compared to HPLC instrument. There is no doubt with the method of extraction (solvent extraction by ultrasonication) and solvents [methanol in UV and acetonitrile:water (70:30 %v/v) in HPLC] used for extracting gallic acid from the marketed

TABLE-4
ROBUSTNESS DATA OF UV AND HPLC METHODS FOR GALLIC ACID

Robustness*	Parameter		
UV method	Room temperature (29 °C)	Elevated temperature (35 °C)	–
Absorbance#	0.26088 ± 0.002	0.27813 ± 0.003	–
%RSD	0.2821	0.358	–
HPLC method	Flow rate 0.8 mL/min	Flow rate 1 mL/min	Flow rate 1.2 mL/min
Peak area#$	61901.8 ± 0.008	62166.2 ± 0.003	62234.6 ± 0.006
%RSD	0.826	0.357	0.629

*Values are presented as mean ± RSD of six replicate observations (n = 6); #Concentration was 6 µg/mL; $RT was 2.31 min

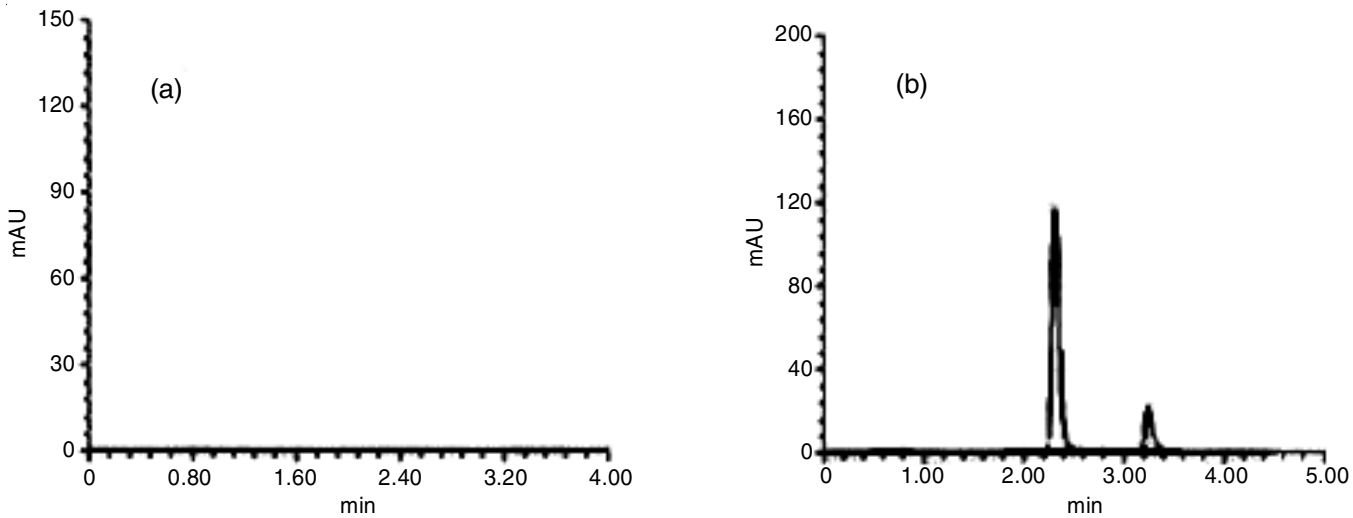

Fig. 4. Blank chromatogram (a) and sample (b) chromatogram (RT of gallic acid is 2.31 min)

TABLE-5
STATISTICAL DATA FOR METHOD VALIDATION PARAMETERS

Parameters		UV method	HPLC method
Wavelength of detection ($\lambda_{max,}$ nm)		272	272
Linearity	Beer's law limit (µg/mL)	2-10	2-10
	Slope	0.0475	11357
	Intercept	0.0031	613.8
	Coefficient of correlation	0.999	0.999
Accuracy or % recovery (%RSD)		< 2.0	< 2.0
Precision (% RSD)	Repeatability	< 2.0	< 2.0
	Reproducibility	< 2.0	< 2.0
Ruggedness (% RSD)		< 2.0	< 2.0
Robustness (% RSD)		< 2.0	< 2.0
LOD (µg/mL)		0.051	0.178
LOQ (µg/mL)		0.154	0.540
System suitability	Retention time (min)	–	2.31
	Peak area	–	62644
	Theoretical plates	–	3783
	Tailing factor	–	1.98
	Efficiency	–	37783
	Asymmetry	–	1.167

TABLE-6
APPLICATION OF THE PROPOSED UV AND HPLC METHODS FOR DETERMINATION OF THE GALLIC ACID IN MARKETED FORMULATION OF *Amalaki*

Standard absorbance/ peak area[#]	Test absorbance/ peak area[#]	Amount found (mg)	Gallic acid estimated (%)
UV method			
0.4746 ± 0.029	0.2044 ± 0.013	107.58	83.34
HPLC			
100286 ± 0.018	18194 ± 0.023	226.11	90.44

[#]Values are expressed as mean ± RSD of three replicate observations (n=3); [$]Concentration was 10 µg/mL

tablet formulation. Good separation with high resolution of the peak proves satisfactory analytical performance of the HPLC method. Moreover, higher percentage of recovery and non-interference of excipients and/or related components assure that the developed HPLC method is highly specific for the estimation of gallic acid in the *Amalaki* formulation.

Conclusion

In present work, two analytical methods were developed for the evaluation of *Amalaki* in the marketed herbal formulation by UV spectrophotometry and RP-HPLC. The developed methods were successful for the estimation of gallic acid in the marketed formulation of *Amalaki* and claimed to be simple, accurate and precise. The developed methods are also reported to be highly valid, specific, reliable and can be applied widely for the routine analysis of marketed formulations or any crude traditional preparations of *Amalaki* based on the quantitative determination of gallic acid.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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