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RSM-CCD optimization of factors affecting Chlorophyll extraction from leaves of *Murraya koenigii*: Enhancing the yield of Chlorophyll a

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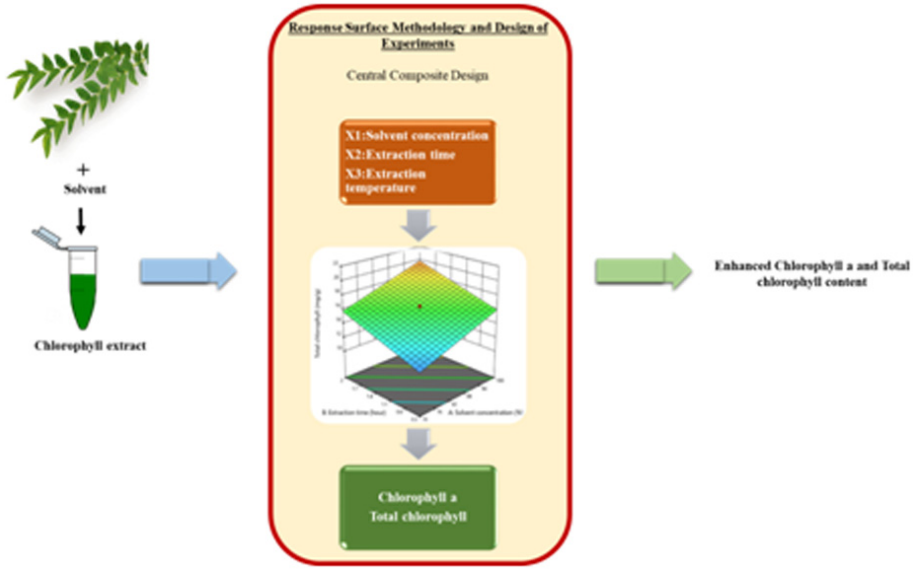
ABSTRACT

Murraya koenigii, or curry leaves (Rutaceae) is indigenous to India and is widely used in Ayurvedic medicine around the world for the treatment and prevention of a wide range of illnesses. Recent research has supported the prospective biological and pharmacological effects of its leaves, such as anticancer, antidiabetic, antioxidant, and anti-inflammatory properties. An improved comprehension of the Chlorophyll (Chl) content of curry leaves will help in relating its therapeutic potential to effective medicine. However, the extraction of pure Chl is challenging due to inefficient extraction and purification techniques. Therefore, we extracted Chl using a solid-liquid extraction method that was dependent on several factors. In this study, the factors affecting the Chl extraction process were optimized by the Response Surface methodology-Central Composite Design (RSM-CCD) approach using Design Expert 11 in relation to solvent concentration, extraction time, and extraction temperature. The responses were obtained after maximizing Chl a and total Chl content. The ideal parameters for the maximum yield of Chl predicted by the software were acetone concentration (100%), extraction time (2 hours), and extraction temperature (50°C). It is anticipated that optimized extraction of Chl will expand its use in various fields.

GRAPHICAL ABSTRACT



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1. Introduction

Humanity has used plants for medical purposes since the dawn of time (Sofowora *et al.*, 2013). Plant extracts are highly valued in many scientific fields, including Ayurvedic medicine, Unani medicine, and others, for their ability to treat various illnesses (Prasath Kumar *et al.*, 2021). Common medicinal herbs that are readily found in our homes are tulsi (*Ocimum tenuiflorum* L.), neem (*Azadirachta indica* L.), and mint (*Mentha arvensis* L.) (Yuan *et al.*, 2016). Among the popular medicinal herbs, *Murraya koenigii* L., commonly known as curry tree/sweet neem, stands out due to its extensive use in Indian culinary and traditional medicines. It is grown wild in forests and under cultivation all over India, Australia, the Andaman Islands, China, Ceylon, Burma, and the Pacific Islands (Abdelwahab and Taha, 2023). It is a member of the Rutaceae family. The leaves are an excellent source of proteins, carbs, and carbazole alkaloids in addition to being high in minerals and vitamins (Nouman *et al.*, 2015). Its possible use as a home medicine for conditions like cancer, diabetes, rheumatism, influenza, and traumatic injuries is described in the literature (Malode *et al.*, 2021). It can also be used to treat piles, inflammation, itching, new cuts, diarrhea, vomiting, bruising, and dropsy (Sarvananda and Umayangani, 2017). Curry leaves are useful in the management of nausea, indigestion, and diarrhea. Additionally, it aids in weight loss and guards against cataract development and early hair graying (Senand Email, 2021).

It has been demonstrated recently that Chloffers therapeutic benefits (Helena *et al.*, 2023). Calculating the amount of Chl present in these plants may demonstrate the significant role that Chl plays in their therapeutic qualities (Solymosi and Mysliwa-Kurdziel, 2016). The following are some of the reported advantages of Chl: It has been observed to aid in tissue growth and healing (Pangestuti and Kim, 2011). Chl is an excellent supplement for smokers as it aids in counteracting the pollutants we breathe in and consume on a daily basis. It transports magnesium to all cells and tissues effectively and aids the blood in delivering oxygen to all tissues (Pangestuti and Kim, 2011). Assimilation and chelation of calcium and other heavy minerals have also been observed to benefit from it. It has demonstrated a strong potential for enhancing the oxygen delivery system by activating red blood cells (Helena *et al.*, 2023). Chl has demonstrated the ability to counteract free radicals, which can cause harm to healthy cells, in conjunction with other vitamins like A, C and E (Hayes and Ferruzzi, 2020). In addition, body odour, urine odour, and foul breath can all be effectively eliminated by Chl. It might make carcinogens less able to attach themselves to DNA in the body's primary organs. The symptoms of calcium oxalate stones may be alleviated by Chl (Mishra *et al.*, 2011). Furthermore, it has some anti-atherogenic properties. Infected wounds can be naturally treated with it. This substance possesses antimutagenic and anticarcinogenic qualities, which could aid in shielding your body from pollutants and lessening the negative effects of medications (Vaòková *et al.*, 2018).

Determining the Chl content of typical curry leaves may aid to a better understanding of their therapeutic uses (Meher *et al.*, 2018). In addition to their added therapeutic value, they can be a cheap and readily available source of Chl. They have no detrimental effects and can be used naturally (Raju *et al.*, 2007). Curry leaves are foods that we might include in our diets. Therefore, this medicinal plant is used for investigation and is quite affordable and widely accessible. Few studies have been conducted on the extraction of Chl from curry leaves (Ahmad and Ramli, 2018). It has been challenging to extract pure Chl from natural resources since the purification processes are more complicated and require greater attention to prevent heat and light damage (Danesi *et al.*, 2004). Numerous studies have also been conducted on the optimization of variables such as solvent type, solvent concentration, solvent/leaf ratio, extraction time, extraction temperature, etc. to produce an adequate quantity of Chl from plant resources (Thao *et al.*, 2022).

Response surface methodology (RSM) is a well-established set of statistical and mathematical procedures used to analyse experimental data using an empirical model (Tran *et al.*, 2019). This was first demonstrated by Box and Wilson, who showed that a reaction to input variables or factors influencing it is connected with fundamental experimental design and analysis. This includes different ways of optimizing the factorial variables for the production of the highest or lowest response value. Factorial methods and ANOVA with more detailed modelling are used to model the response output. The most common method for validating RSM is Central Composite Design (CCD) or Box-Behnken Design (BBD). BBD covers a very small number of design points compared to the axial points of CCD, which increases the number of tests in CCD. Better results for quadratic models are produced by CCD, which encompasses all extreme circumstances. In order to concentrate on the impacts and further sensitize the model, this study's three-level factorial design allows for the summation of additional treatments. In order to maximize the extraction of Chl*a* and total Chl content, this is employed as an enhancement to the current procedures.

This study aims to optimize the factors affecting Chl extraction using the response surface methodology (RSM)

method and Design Expert 11 software to increase the extraction efficiency of Chl a from curry leaves. Moreover, the crucial roles of input factors in the process of Chl extraction were also examined.

2. Materials and methods

2.1. Sample preparation

The leaves of *Murraya koenigii* were collected on the campus of NIT Rourkela. The leaves were rinsed with distilled water three times to avoid contaminants on the leaf surface. They were blotted dry using a paper towel and stored at 4°C for subsequent analysis.

2.2. Solvent extraction

Fresh leaves (2 g) were weighed and cut into small pieces (approximately 9 –15 mm^2). The sample was ground using a mortar for 1 minute using 3 ml of acetone, and the mixture was homogenized using 10 mg of $CaCO_3$ for 2 –4 minutes until a green solution was obtained. Then, using 7 ml of acetone, the mixture was transferred to a beaker and kept for 1 hour at 40 °C. Then, the extract was centrifuged for 15 minutes at 7000 rpm to obtain the supernatant. The supernatant was filtered by Whatman filter paper of pore size (20–25 µm), and the solution obtained was evaporated by a vacuum rotor. The precipitate obtained was dissolved in an appropriate amount of acetone for subsequent experiments (Jinasena *et al.*, 2016; Thao *et al.*, 2022) (Fig. 1).

2.3. Extraction optimization

Optimization of parametric levels was done through comprehensive analysis using RSM-CCD. The complete design of experiments and statistical evaluation were conducted using Design Expert 11 software. Three factors and two responses were used in the design of the experiments in relation to solvent concentration (X1, %), extraction time (X2, hour), and extraction temperatures (X3, ^{0}C), as shown in table 1. Responses recorded were optimized for maximum Chl a (Y1) and total Chl content (Y2). By using CCD, 17 experiments with variable factor ranges were designed, and the responses were fitted to quadratic, linear, tertiary, or 2-fi models. ANOVA was used to assess the best fit model, which was further validated by the p-value, the squared correlation coefficient (R^2) both adjusted and predicted, and the lack of fit. Finally, the optimized responses were built using a statistical model.

Figure 1: Schematic illustration of chlorophyll extraction process.

Table 1

Representation of factors and responses used in response surface design of Chl extraction process.

Response Surface Methodology		
Factors	Low limit	High limit
X1:Solvent concentration	70	100
X2:Extraction time	0.5	2
X3:Extraction temperature	30	60
Responses (mg/g)	Goal	
Chl*a*	Maximize	
Total Chl	Maximize	

2.4. Determination of Chl a and total Chl content

Optical density (OD) was recorded using a spectrophotometer to estimate the amount of Chl present in individual solutions. Spectrophotometric analysis was used to ascertain the amounts of Chl *a* and *b* in acetone extracts.At 645 nm and 663 nm, respectively, the The absorption peaks were measured and the concentrations of Chl *a*, Chl *b* and total Chl were determined (Su *et al.*, 2010).

2.5. Statistical analysis

All the experiments were carried out in triplicate, and the responses were recorded in the form of the mean ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA, and Bonferroni multiple comparison tests were used for multiple statistical comparisons for experiments involving three or more groups. $P<0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Preliminary screening of Chl extraction

Preliminary screening of the extraction was limited to varying one parameter (solvent concentration, extraction time, and extraction temperature) of the Chl extraction condition. A summary of the initial experimental components has been represented in Table 2.

3.1.1.Effect of solvent concentration on Chl extraction

Acetone of varied concentrations (70 %, 80 %, 90 %, and 100 %) was used for estimating the content of Chl*a* and total Chl from the leaves of *Murraya koenigii*. The highest concentration was recorded at 100 % acetone concentration (i.e., 10.87 mg/g of Chl a and 15.68 mg/g of total Chl) (Table 2).

Table 2

Summary of the initial experimental components.

Parameter	Range	Chla concentration, mg/g	Total Chl concentration, mg/g
Solvent concentration(%)	70	6.21	10.48
	80	7.18	11.37
	90	8.54	13.74
	100	10.87	15.68
Extraction time (hr)	0.5	6.67	9.62
	1	7.19	10.59
	1.5	7.82	10.91
	2	8.43	11.46
Extraction temperature (^{0}C)	30	8.41	13.21
	40	7.48	11.58
	50	11.39	15.34
	60	7.94	11.67

3.1.2.Effect of extraction time on Chl extraction

A constant solvent concentration (acetone 100 %) was set at various extraction times ranging from 0.5-2 hr in order to determine the ideal extraction time. It was observed that the amount of Chl (i.e., 8.43 mg/g of Chl a and 11.46 mg/g of total Chl) obtained was highest at 2 hours (Table 2). A literature study reveals that the diffusion of the particles to be extracted from the raw material into the solution increases with longer extraction times because they will increase the amount of time the raw material and solvent are in contact, boosting extraction efficiency. However,

prolonging the extraction period will reduce the amount of Chl once equilibrium is reached (Abidin *et al.*, 2016).

3.1.3.Effect of extraction temperature on Chl extraction

An acetone concentration of 100 % and an extraction time of 2 hours were the set criteria for an evaluation of extraction temperature. It was noted that Chl (i.e., 11.39 mg/g of Chl*a* and 15.34 mg/g of total Chl) was maximum at 50 °C and minimum (i.e., 8.41 mg/g of Chl a and 13.21 mg/g of total Chl) at 30 °C. However, with increasing temperature, the content decreased (i.e., 7.94 mg/g of Chl *a* and 11.67 mg/g of total Chl) (Table 2). According to the report, the extraction of Chl becomes more effective as the temperature rises to 50 °C. At this temperature (50 °C), the phospholipid layer and hydrocarbon chain that makeup plant cell walls break more easily, allowing the compounds within the leaf to escape the cell wall. Elevated temperatures also contribute to the sample's solubility in the solvent, which in turn accelerates the mass transfer of the solute into the solvent and reduces the extraction time. High temperatures (> 60 °C) can cause Chl breakdown. Thus, it was determined that extraction duration of a maximum of two hours was ideal at 50 °C. Our results were consistent with the reported literature (Ahmad and Ramli, 2018).

3.2. RSM-CCD optimization of Chl extraction

The Central - Composite Design was followed in the execution of the experiments (Table 3). The target function (Y1, Chl*a* and Y2, total Chl content)'s quadratic regression equation with three components [X1, acetone conc (%); X2, time (hr); X3, temperature (^{0}C)]:

$$Y1 = 9.22 + 1.78\ X1 - 0.4402\ X2 + 1.12\ X3 + 0.5249\ X1X2 + 0.2587\ X1X3 + 0.8230\ X3X2 - 2.09\ X12 - 0.31\ X22 - 1.64\ X32 \quad (1)$$

$$Y2 = 5.15 + 0.1355\ X1 + 2.5768\ X2 - 0.0896\ X3 + 0.4587\ X1X2 + 0.2648\ X1X3 + 0.7895\ X3X2 - 1.08\ X12 - 2.41\ X22 - 1.78\ X32 \quad (2)$$

Chl*a* (Y1) and total Chl content (Y2) are shown in Tables 4 and 5, respectively, as the typical summary of the fitting model with response parameters. The model was predicted to be quadratic for both the responses Y1 and Y2, respectively. Many statistical parameters were examined, including the square correlation coefficient, lack of fit, and p-value, in order to verify the fitness of the statistical model. The prospective quadratic model was deemed significant with a 95 % confidence interval when the p-value was less than 0.001. The difference between pure error and relative

Table 3

Experimental configuration and observation of response design in terms of Chl a and total Chl content.

Run	Factor 1X1	Factor 2X2	Factor 3X3	Response 1 Y1	Response 2Y2
1	110.227	1.25	45	11.3	18.97
2	59.7731	1.25	45	6.06607	12.84
3	85	-0.0113446	45	8.01	11.85
4	70	0.5	30	7.34	14.19
5	100	0.5	60	9.42	13.74
6	70	0.5	60	6.41	10.75
7	70	2	30	9.7	16.28
8	85	1.25	70.2269	8.19	14.25
9	85	1.25	45	8.98	15.67
10	100	2	50	13.1	20.99
11	85	1.25	19.7731	11.1	18.14
12	70	2	60	6.38	13.45
13	85	1.25	45	8.98	15.17
14	100	0.5	30	12.67	17.85
15	85	2.51134	45	9.48	19.38
16	100	2	60	10.2	19.54
17	85	1.25	45	9.34	16.49

error from replicated design points is investigated in the "lack of fit tests." The lack of fit value in the current model was greater than the p-value, demonstrating the relevance and dependability of the model. The similarity of the suggested model and the experimental data to one another is indicated by the adjusted and anticipated R^2 values (Raguraman *et al.*, 2018). Better model sensitivity is indicated by an R^2 value that is closer to 1. Our model clearly relates to a low standard deviation and is statistically significant because its R^2 value was greater than 0.9 and the difference between them was less than 0.2.

According to variance analysis results, the regression model for Chl*a* content is statistically significant (Table 4). ANOVA analysis revealed the statistical significance of the regression model for Chl a content ($p < 0.0001$, $R^2 = 0.93$, adjusted $R^2 = 0.91$). Lack of fit was statistically insignificant ($p > 0.05$). This indicates the strong compatibility of the chosen model and suggests that 97 % of the response's values can be explained using the aforementioned suggested model. The model was considered significant when the difference between the adjusted R^2 and projected R^2 was less than 0.2. The ANOVA table showed that the parameter with the most significant effect (high F-value) is solvent concentration, followed by extraction temperature. However, the time period of extraction had the least effect on the response values. A high F-value (10.30) and a low p-value (<0.0001) further demonstrated the relevance of this quadratic model.

In the case of total Chl content, it was evident from the ANOVA table that the factor parameter with the maximum impact (highest F-value) was extraction time ($p<0.0001$), followed by solvent concentration. The response total Chl content showed an R^2 value of 0.95 and an adjusted R^2 value of 0.94, proving the significance of the model (Table 5).

It was observed that with an increase in solvent concentration and extraction time, total Chl yield increased (Fig. 2). These results were in good alignment with the reported literature (Maciej Serda *et al.*, 2013). Maximum extraction was attained at the highest concentration of the solvent. Also, with the increasing time period of extraction, the concentration of Chl increased. This might be due to the increased exposure period of the leaves to the solvent.

The validity of the model has been further examined by analyzing residuals vs. run plots and normal probability. The optimized model of Chl extraction from *Murraya koenegii* displays the correlation between treatment values. Fig. 3 displays the normal probability as well as the residuals vs. run plots. By comparing the experimental and predicted values, it is apparent that the model's treatment values are at the optimal level for the experimental concentration of Chl a, and they also nearly fit the regression line. The same observations also followed for total Chl content. The accuracy of the predicted model is confirmed by the linear probability plot for the residuals. Upon completion of this investigation, we infer that the ideal conditions for maximum Chl extraction are solvent concentration (acetone 100 %), extraction time (2 hours), and extraction temperature (50 °C).

Table 4

Representative model fitting summary of factors elements with response parameter (Y1: Chl a, Y2: Total Chl content) with ANOVA analysis. NS - not significant.

Factors	Y1		Y2	
	Coeff	**F-value**	**Coeff**	**F-value**
Model		55.03		81.68
Intercept	9.22		15.86	
X1	1.78	113.46	1.93	94.58
X2	-0.4402	6.91	2.03	104.62
X3	1.12	44.71	-1.35	45.83
R^2	0.9270		0.9496	
Adjusted R^2	0.9102		0.9380	
F value Lack of fit	10.30 (NS)		1.25 (NS)	
Suggested relation	QUADRATIC		QUADRATIC	

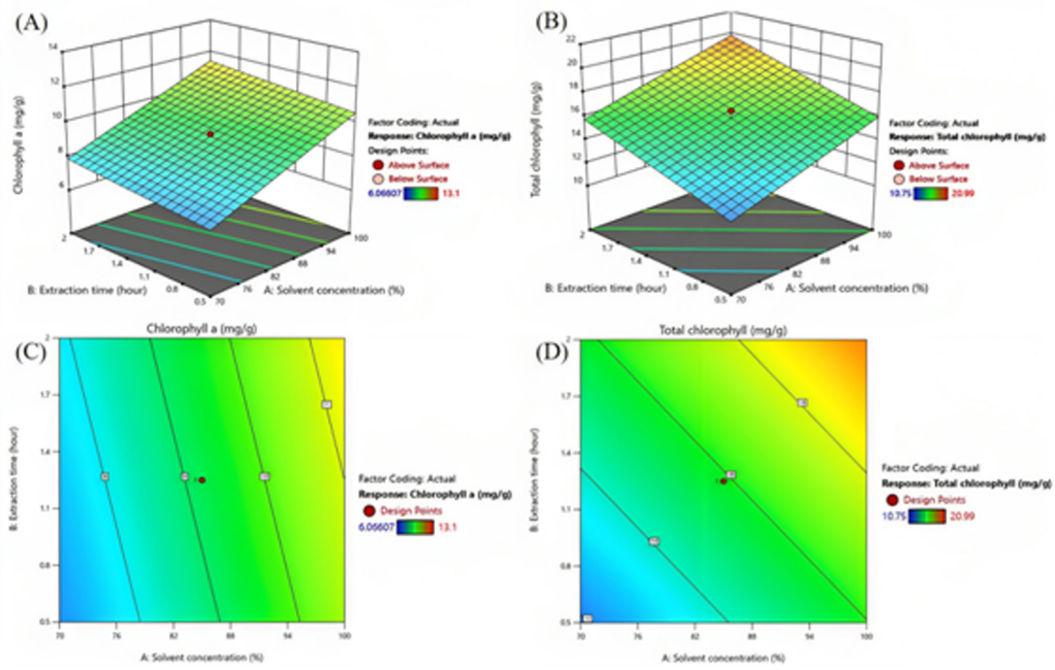

Figure 2: Three dimensional representation of response with surface plots. (A) Chl a (B) Total Chl (C) Contour plot for Chl *a* (D) Contour plot for total Chl.

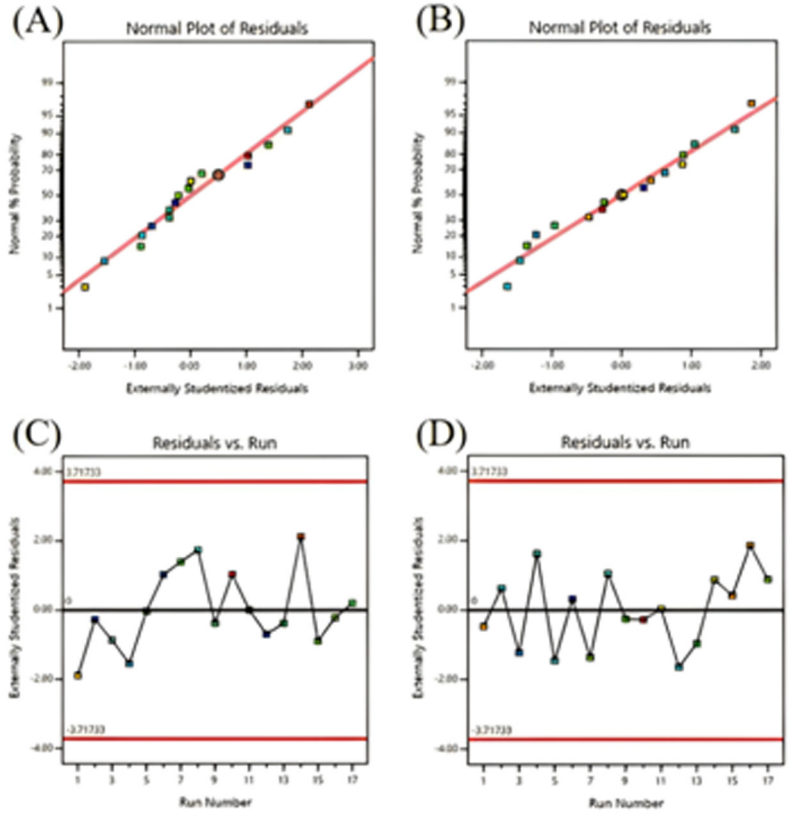

Figure 3: Residual plots (A) normal plot of residuals for Chl a (B) normal plot of residuals for total Chl (C) residual vs run number for Chl a (D) residual vs run number for total Chl.

4. Conclusion

Based on the results, it was determined that the optimal conditions for maximum yield of Chl are acetone concentration (100 %, extraction time (2 hours) and extraction temperature (50 °C). The concentration of Chl a achieved in the experiment was 11.39 mg/g, but the concentration obtained according to the model is 13.1 mg/g under these ideal conditions. This indicates that the optimal model is compatible with 98.7% expectation. The findings support scale-upgradation to extract pure Chl in case further transformation procedures are required for use in functional foods and medications. Briefly, *Murraya koenegii* leaf extract has good medical potential and can be utilized as a natural supply of Chl.

Author Contributions

SM is the first author and has contributed towards the literature survey, performed the formal analysis along with its data curation and writing of the whole manuscript. RL has contributing in performing experiments. BN is the corresponding author. She provided the idea, supervised and validated the entire process from inception to the final submission, and edited the final manuscript.

Conflicts of interest

Authors declare no conflict of interest.

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