Simultaneous detection of Homovanillic acid (HVA) and Vanillyl mandelic acid (VMA) in urine sample by HPLC method

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ABSTRACT

Objective: The research was carried out to develop a validated analytical method for assessing the biogenic amines metabolites Vanillyl mandelic acid (VMA) and Homovanillic acid (HVA) simultaneously in biological fluids by High performance liquid chromatography- Photodiode Array (HPLC-PDA).

Method: An HPLC technique was developed and validated for the measurement of VMA and HVA in artificial urine samples. The chromatographic separation was achieved on a Kromasil C8, 5µm (125 X 4.6 mm) column at 25^{0}C with combination of mobile phase of acetonitrile: 0.1 % o-phosphoric acid (30:70 v/v), flow rate of 0.9 mL/min using an Shimadzu HPLC system with LC Solutions liquid chromatography software. Different mobile phases were used on a trial and error basis to separate these two metabolites. Validation of ICH parameters in terms of linearity, accuracy, repeatability, precision, and robustness of methodology was performed.

Result: The separation of vanillyl mandelic acid and homovanillic acid was achieved using a high-resolution HPLC technique. The linearity range for VMA and HVA was selected from 10 µg/mL to 60 µg/mL, and were found to be linear, with coefficients of determination (r^2) of 0.996 and 0.990 for both metabolites, respectively. The detection limits for VMA and HVA were 1.96 µg/mL and 1.31 µg/mL, respectively, with quantification limits of 5.96 µg/mL and 3.97 µg/mL. For repeatability, precision, and robustness parameters, the results demonstrated a low percent RSD value.

Conclusion: A validated HPLC-PDA approach was developed for quantifying Vanillyl mandelic acid and Homovanillic acid in urine samples.

Keywords: Homovanillic acid, Vanillyl mandelic acid, HPLC-PDA, method validation, biogenic metabolites, urine sample, chromatographic separation

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Abbreviations:

VMA Vanillyl mandelic acid

HVA Homovanillic acid

HPLC-PDA High performance liquid chromatography- Photodiode Array

MAO Monoamine

PNMT Phenylethanolamine N-methyltransferase

PD Parkinson's disease

AD Alzhemer,s disease

HMMA 4-hydroxy-3-methoxymandelic acid

LOD Limit of detection

LOQ Limit of quantification

HPLC MS High performance liquid chromatography mass spectroscopy

HPLC ED High performance liquid chromatography electrochemical spectroscopy

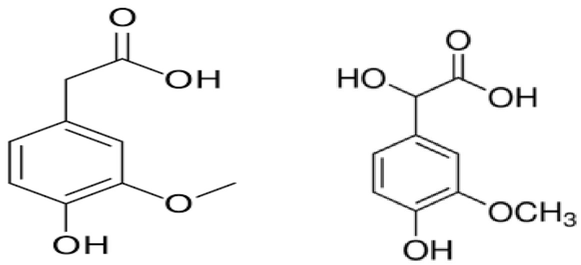
1. INTRODUCTION

Homovanillic acid and vanillylmandelic acid , are compounds excreted in urine [1]. Because HVA is the primary metabolite of dopamine, it can be utilized as a marker of dopamine metabolism, particularly in relation to reward systems, emotional responses, personality trait expression, and psychopathological phenomena. HVA has been discovered to be a neuroendocrine marker of behavioural problems and drug misuse risk in recent investigations [2].

Because urine is highly complex containing a variety of potentially interfering substances, problems with lack of specificity are likely to be just as serious [3]. Because VMA is the predominant end metabolite, it's been used to diagnose pheochromocytoma for a long time [4, 5]. However, the MAO enzyme is required for VMA production, which occurs not only in the adrenal medulla but also in the sympathetic nervous system. Furthermore, because the PNMT enzyme is missing, the sympathetic nervous system is unable to create epinephrine and so contributes only normetanephrine (from norepinephrine) but not metanephrine to the serum level (from epinephrine). As a result, the proportionate rise in VMA levels in the presence of a pheochromocytoma is significantly less dramatic than the rise in metanephrine levels, and urine VMA levels have a poor sensitivity (below 65 percent in some series) [6, 7]. Lower levels of these biogenic amines metabolites in healthy patients, on the other hand, may contribute to the advancement of neurological diseases such as PD, AD, and depression [8, 9].

HVA together with 4-hydroxy-3-methoxymandelic acid (HMMA) can be utilised as a prognostic indicator in malignant pheochromocytoma, is useful in identifying normotensive pheochromocytoma, and is a good combination for neuroblastoma detection. A simple, reliable, and accurate assay that concurrently detects HVA and VMA in urine is needed. With its improved ease, specificity, and sensitivity over colorimetric approaches, HPLC with photodiode array (PDA) detector is ideally suited to this analytical methodology [10].

Similar research articles using sophisticated, costly, and extremely advanced instrumentation setup have been described previously. Despite the fact that in clinical laboratories, HPLC is a commonly used method for assessing HVA and VMA in urine samples, it usually necessitates expensive equipment and time-consuming sample preparation [11, 12]. The proposed study was to develop and present a new easy, economic, less sophisticated HPLC instrumentation setup for measuring HVA and VMA in urine samples.



Homovanillic acid ($C_9H_{10}O_4$)
Vanillyl mandelic acid ($C_9H_{10}O_5$)

Figure 1: Structure of metabolites

2. MATERIALS AND METHODS

2.1 Chemicals and solvents

Sigma Aldrich USA provided VMA and HVA, which were used as working standards. On a dry weight basis, it has 97 percent and 98 percent (w/w) purity of VMA and HVA, respectively.

Merck life sciences private limited, Mumbai, India, provided acetonitrile (HPLC quality), methanol (99.5c/o HPLC grade, Loba Chemie), O-phosphoric acid (38 percent AR grade, Loba Chemie), glacial acetic acid (70 percent AR grade, SRL Chemical), and formic acid (98-99 percent AR grade, Research lab). Standards were dissolved in Millipore-purified distilled water (Merck, Direct O_2).

For animal study IAEC permission was granted with approval number (DYPIPSR/IAEC/Oct/2021-22/P-05)

2.2 Instruments and apparatus

LC Solutions Liquid Chromatography Shimadzu HPLC system, UV detector, Kromasil C8, 5µm (250 X 4.6 mm) analytical column, LC Solutions software for data processing and evaluation, weighing balance (Shimadzu ATX224), pH meter (ELICO LI 120) and Fast clean ultrasonic cleaner (Wensar WUC) along with grade A certified glassware (borosil) were used for the study. Sample injector with 20 µL capacity was used.

2.3 Liquid chromatography

The separation was achieved in low pressure gradient mode using a Kromasil C8, 5 µm (250 X 4.6 mm) analytical column. Sonication was employed to degas a mixture of acetonitrile: 0.1 % o-phosphoric acid (pH 2) in the ratio (30:70 v/v). The flow rate and column temperature were held constant throughout the experiment, at 0.9 mL/min and 25^{o}C, respectively.

2.3.1 Optimization of chromatographic conditions

Based on available literature on HPLC analysis, various mobile phases such as acetonitrile, formic acid, methanol, and orthophosphoric acid were explored. The injected volume was 20 µL, and the wavelength of 279 nm was discovered by UV and subsequently validated based on previous study data [13, 14]. In standard solutions, both VMA and HVA show distinct separation at particular retention duration.

2.4 Preparation of standards and sample solutions

2.4.1 Preparation of 0.1% solution of orthophosphoric acid

In 100 ml of deionized water, a 100 µL solution of orthophosphoric acid was added.

2.4.2 Standard Homovanillic acid stock solution

A standard stock solution of HVA was made by carefully weighing 1 milligram of HVA and placing it in a sterile Eppendorf tube. 1 mL distilled water was added to the mixture before it was sonicated to dissolve the metabolite. The volume was prepared using 1mg/ml distilled water.

2.4.3 Vanillyl mandelic acid standard stock solution

A standard stock solution of VMA was made by carefully weighing 1 milligram of VMA into a sterile Eppendorf tube. To dissolve the metabolite, volume was adjusted with 1 ml distilled water, which was then sonicated. The volume was prepared using 1mg/ml distilled water.

2.4.4 Standard mixture preparation

By transferring 1000 µg of each HVA and VMA standard to 1000 µL of sterile Eppendorf tube, both standards were combined. To make a mixture of 1mg/ml (HVA) and 1mg/ml (VMA), the volume was made with distilled water.

2.5 Method optimization and development

A mixture of working standard solutions containing 1000 µg/mL of HVA and 1000 µg/mLof VMA was used for the separation of two metabolites and the development of new procedures.

2.6 Determination of analytical wavelength

HVA and VMA working standard solutions of 1 µg/mL were made, and these solutions were scanned in the 200-400 nm range with a UV-spectrophotometer. Each metabolite's spectra's maximum wavelength was found.

2.7 Optimization of HPLC Method

2.7.1 Optimization of mobile phase

The mobile phases were made using a variety of solvents, including acetonitrile, formic acid, methanol, orthophosphoric acid, and water, and each combination was tested to ensure that both metabolites were separated. For degassing, all solvents were filtered and sonicated before being blended in appropriate proportions.

2.7.2 Optimization of flow rate

Flow rate optimization was carried out by experimenting with flow rates of 1, 0.9, 0.7, and 0.5 mL/min. The column was saturated with mobile phase after being conditioned with acetonitrile. Other chromatographic parameters were kept constant for varied flow rates, such as the Kromasil C8, 5µm (250 X 4.6) mm analytical column, ambient temperature of 25^{0}C, and analytical wavelength. With varied eluent flow rates, separation of both metabolites was observed.

2.8 Solution stability

Analytical solution stability refers to the ability of the analyte to remain stable over time and at the required temperature. A standard mix solution and a standard dissolved in artificial urine solution were made, as previously stated. The cumulative percent RSD was computed after injecting a mixed standard solution and an artificial urine solution at varying time intervals.

2.9 Analysis of Mixed standard solutions

2.9.1 Analysis of Mixed standard metabolite solution

A mixed standard solution of 50 µg/mL HVA and 50 µg/mL VMA was introduced into the HPLC apparatus. For retention periods, standard areas, average tailing factor, and theoretical plate number, we determined the percent RSD.

2.9.2 Analysis of artificial urine sample

The experiment employed six samples from a single sample of artificial urine. After introducing the solutions into the HPLC apparatus, the percent RSD was computed.

2.10 Method validation

To validate newly generated method parameters, the ICH guidelines (ICH guidelines Q2A; Q2B) were followed.

2.10.1 Linearity range

1 mg/mL VMA and HVA were made from a standard mixture of stock solutions. The mixed standard stock solution was then further diluted to generate working standards at concentrations ranging from 10, 20, 30, 40, 50, 60 µg/ml. The system was injected with solutions, and the range was determined using a linearity analysis. The linearity of the graph of peak area vs. concentration over increasing metabolite concentrations was studied. As previously reported, standard solutions of combination VMA and HVA were created.

2.10.2 System precision

The system precision of an analytical method refers to the degree of reproducibility of results in a set of tests performed by a single session operator with identical reagents and equipment. Percent RSD was used to calculate retention periods, standard areas, average tailing factor, and the number of theoretical plates.

2.10.3 Method precision

The method precision of an analytical procedure describes the level of agreement between multiple samplings of the same homogeneous sample under specified conditions. The experiment included six trials from a single batch of samples. The percent RSD for six VMA and HVA assays was determined in triplicate using a standard metabolite preparation (6 injections).

2.10.4 Detection limit (LOD) and quantitation limit (LOQ)

The detection and quantitation limits were calculated using a mixed standard solution at a suitably low concentration level, based on the response's standard deviation and slope formula, respectively, and were set at 3 and 10 times the signal-to-noise ratio. 1883

LOD = 3.3 σ/S; LOQ = 10 σ/S

where σ = the standard deviation of the response

S = the slope of the calibration curve

2.10.5 Accuracy

To ensure the approach's accuracy, recovery studies were conducted using the traditional addition method at concentration levels of 80, 100, 120 percent. In triplicate, known quantities of VMA and HVA were spiked in artificial urine samples at three different levels. The solutions were prepared and analysed using the recommended method. After that, the % metabolite recovery was determined for both metabolites.

2.10.6 Robustness

An analytical procedure's robustness is a measure of its ability to remain unaffected by modest but purposeful changes in method parameters, and it represents its dependability in routine use. The robustness of the result was examined by comparing the fluctuations in the result when the mobile phase ratio was altered from 25 to 35 percent acetonitrile and 75 to 65 percent 0.1 percent of-phosphoric acid. The wavelength was changed from 277 to 281 nm.

2.10. Ruggedness

The method's robustness was proven by two independent analysts evaluating six samples of a single artificial urine containing metabolite under identical operational and environmental situations.

3. RESULT AND OBSERVATION:

3.1 Determination of absorption maximum for metabolite

A UV spectrophotometer was used to study the spectra of both metabolites, and the λmax of homovanillic acid and vanillyl mandelic acid was found at 279 nm. According to the absorbance spectra, both metabolites had substantial absorbance at 279 nm. As a result, the analytical wavelength (max) was set at 279 nm.(Figure 1A and 1B).

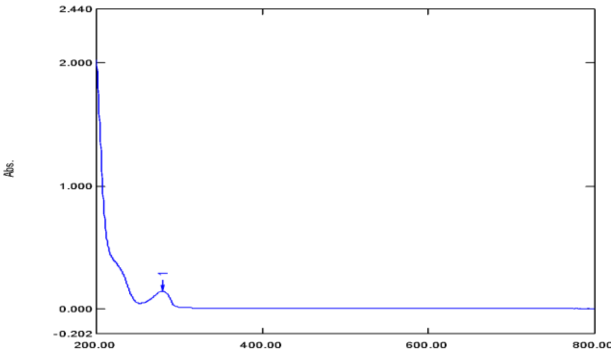


Figure 1a: Homovanillic acid λmax at 279 nm

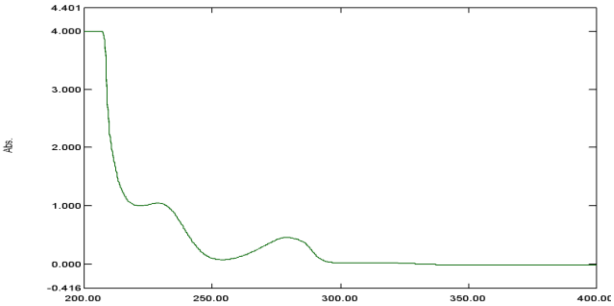


Figure 1b: Vanillyl mandelic acid λmax at 279 nm

3.2 Development of method and optimization of mobile phase

Homovanillic acid and Vanillyl mandelic acid, with pKa 3.74 and 3.11, respectively, require a pH of 2 or below and an acidic environment to remain protonated in solution. The chromatograph was developed in different solvent systems after being injected with a solution of standard HVA and VMA. At flow rates of 1,0.9,0.7 mL/min, the first solvent system of methanol: 0.2 percent ortho-phosphoric acid (5:95; 10:90 percent v/v) with pH 4.2 shows no separation, while the second solvent system of acetonitrile:0.1 percent formic acid (10:90 percent v/v) shows only one peak with no separation of two metabolites. At low pressure gradient mode, the mobile phase combination of acetonitrile and 0.1 percent ortho-phosphoric acid in a 30: 70 percent v/v ratio at a flow rate of 0.9 mL/min was able to properly separate VMA and HVA peaks, with peaks visible in the chromatogram. VMA and HVA elution retention times were determined to be 3.7 and 5.2 minutes, respectively. As a result of this positive conclusion, the use of the HPLC method for subsequent analysis has been confirmed. Figure 2 depicts the development of a method for separating VMA and HVA utilising an improved mobile phase.

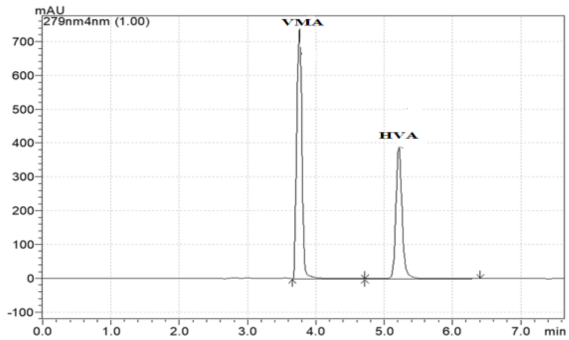


Figure 2: Chromatogram of Vanillyl mandelic acid and Homovanillic acid at 279 nm

3.3 Solution stability

The stability of the known area of homovanillic acid and vanillyl mandelic acid in analytical solution was validated by assessing the sample after 4, 8, 12, and 16 hours of storage at 25°C. For sample solution stability, the cumulative percent RSD for homovanillic acid and vanillyl mandelic acid peak regions were within the acceptable range. As a result, the sample solution was found to be stable at 25°C for up to 16 hours. The results of the VMA and HVA solution stability tests are shown in Table 1 and Figure 2.

3.4 Analysis of Mixed standard solutions

3.4.1 Analysis of Mixed standard metabolite solution

Both standards were injected in 6 replicates into the HPLC equipment. The mean, SD, and percent RSD for the VMA and HVA peaks were calculated. Table 2 shows that the % RSD for both peak area and retention time was less than 2.0.

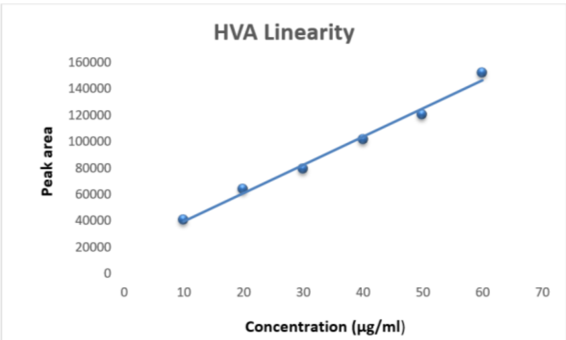
3.4.2 Analysis of laboratory standard metabolite solutions

Three samples from a single batch were evaluated using the test procedure. Three samples were examined, and the percent RSD was less than 2.0. The data for analysing laboratory batches of samples can be found in Table 3.

3.5 Method validation

3.5.1 Assessment of linearity range

IThe linearity of a mixture of Vanillyl mandelic acid and Homovanillic acid was determined in the range of 10 µg/ml to 60 µg/ml. A graph with concentration on the X axis and mean peak areas on the Y axis was used to calculate the correlation coefficient. The findings show that as both metabolite concentrations rise, the peak area grows in lockstep, suggesting a linear relationship. Also larger than 0.99 was the regression coefficient (r^2). The linear range of detectability was substantially within the range of higher and lower linear metabolite concentrations, as predicted by Beer's law. Table 4 and picture 3 summarise observations from VMA and HVA linearity investigations.



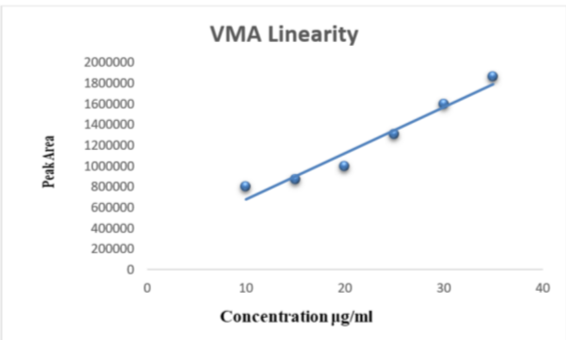


Figure 3: Linearity studies of VMA and HVA.

3.5.2 System precision

Six replicate injections of standard solution were injected using the HPLC equipment. Table 5 shows that the analytical system's precision is satisfactory.

3.5.3 Method precision

Six samples of single batch metabolite standard solutions were produced and evaluated using the proposed approach. The data is shown in Table 6. The % RSD figures indicate that the method has appropriate precision.

3.5.4 Detection limit (LOD) and Quantitation limit (LOQ)

VMA had a detection limit of 1.96 µg/mL, while HVA had a detection limit of 1.31 µg/mL. Similarly, the quantitation limit for VMA and HVA was 5.96 µg/mL and 3.97 µg/mL, respectively.

3.5.5 Specificity

There was no interference from the diluent (distilled water), mobile phase at the retention time of the analyte peak. There was no interference at the VMA and HVA peak retention time. The method is intended to determine VMA and HVA in artificial urine (figure 2).

3.5.6 Accuracy (recovery studies)

VMA and HVA recoveries were 90.31- 99.83 percent and 92.88- 96.75 percent, respectively, when low, medium, and high concentration standards were spiked in artificial urine samples (80, 100, and 120 percent) shown in fig 4 (Table 7).

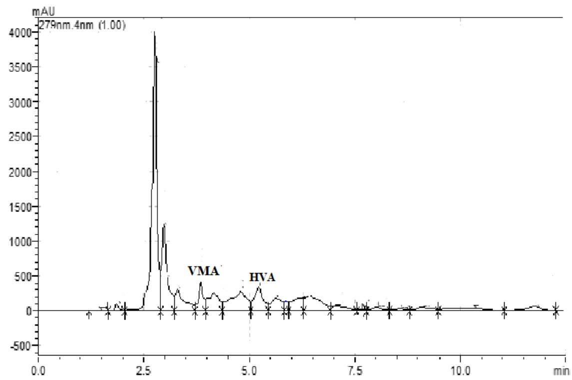


Figure 4: Chromatogram of Vanillyl mandelic acid (3.7 min) and Homovanillic acid (5.2 min) in rat urine sample, Flow rate: 0.9ml/min

3.5.6 Robustness

Small but deliberate adjustments to the procedure parameters were done to test the reliability of the created approach. The percent RSD for method variables such as detection wavelength and mobile phase ratio was lower than the standard limits, as shown in Table 8.

3.5.7 Ruggedness

In the ruggedness experiment, the metabolites VMA and HVA were estimated using HPLC I and HPLC II, respectively. There were no significant differences between the HPLC I and HPLC II VMA and HVA tests. Percent RSD values were less than 2 and met the acceptance criteria. (refer to table 9).

4. DISCUSSION

The HPLC-PDA was chosen for developing a new and easy method for quantifying vanillyl mandelic acid and homovanillic acid in urine samples. Different mobile phase combinations were used, with the best one being chosen for good separation of these two chromatograms.

Biogenic amines are brain metabolites that are discharged through urine and control mental behaviour patterns. They have long been the subject of neuropsychopharmacological research [15, 16]. The quantities of vanillyl mandelic acid and homovanillic acid in the brain have been demonstrated to fluctuate in neurological disorders such as PD, AD, schizophrenia, depression, and panic disorder [17].It underlines the need of developing simple, sensitive, and repeatable methods for detecting bioamine metabolites in urine samples. Advanced methods [18] can identify these substances at lower concentrations, but they require costly equipment and solvents. We were able to discover vanillyl mandelic acid at 5.96 µg/mL and homovanillic acid at 3.97 µg/mL at lower concentrations using inexpensive chemicals and HPLC-PDA. It has the same sensitivity as the more expensive HPLC-MS approach.

Using metabolite concentrations, the recovery values in samples extracted using various sample preparation procedures were evaluated. The reference values of Vanillyl mandelic acid 3.3 µg/mL and Homovanillic acid 1.1 µg/mL l were chosen and identified using HPLC-ED [19].

Since the amount of these metabolites identified in the urine of control rat samples is approximately equal Strong, symmetrical peaks that were clearly resolved revealed the separation of VMA and HVA from other components in the urine collected [19]. The approach's linearity is confirmed by calibration curves with a regression coefficient greater than 0.99. The method's great sensitivity is demonstrated by low LOD and LOQ readings along with efficient chemical consumption.

Good recovery values were obtained, which are comparable to those obtained using HPLC-MS techniques and are within ICH guidelines [20, 21]. The percent RSD readings for repeatability and intra- and inter-day precision studies show that the approach is accurate and repeatable at all three concentration levels. The method's resilience is demonstrated by the low % RSD values obtained for deliberate changes in the wavelength and ratio of the mobile phase

5. CONCLUSION

For the simultaneous isolation and quantification of vanillyl mandelic acid and homovanillic acid in rat urine samples, a sensitive, precise, and efficient HPLC-PDA approach with a low pressure gradient mode was developed. The procedure was carried out with low-cost chemicals and reagents and was rather straightforward. Other monoamines, as well as their metabolites, can be used.

6. FUNDING SOURCE

There was no financial support for the research from any agency.

7. CONFLICT OF INTEREST

The research was conducted as part of a PhD project. There is no potential for a conflict of interest.

8. AUTHORS CONTRIBUTIONS

Vrushali Bhalchim: Literature review, Conceptualization, Experimental work, Manuscript writing & editing.

Vaishali Undale: Conceptualization, Critical reviews.

Sunil Shewale: Conceptualization, Data analysis and curation.

Shubham Padole: Experimental work.

Pallavi Phadatare: Manuscript drafting.

Ashlesha Mali: Manuscript drafting

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