Data Article

Analytical method cross validation by HPLC for identification of five markers and quantification of one marker in Synacinn™ formulations and its in vivo bone marrow micronucleus test data

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A B S T R A C T

A HPLC method has been validated for identifying five markers (gallic acid, rosmarinic acid, catechin, andrographolide and curcumin) and quantifying curcumin in Synacinn™ formulation. The validation (bracketed strengths of 10 mg/mL and 100 mg/mL) involved assessment of selectivity, precision, Limit of Detection (LOD), Limit of Quantification (LOQ), linearity, accuracy, stability in diluent and formulation stability. Meanwhile, in vivo bone marrow micronucleus test data was presented to evaluate the toxicity potential of Synacinn™ to cause clastogenicity and/or disruption of the mitotic apparatus, as measured by its ability to induce micronucleated polychromatic erythrocytes (MN PCE) in Sprague Dawley rat bone marrow. The test was conducted in two phases viz., Phase I

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(Dose Range Finding experiment) and Phase II (Definitive experiment). Phase I was conducted to assess general toxicity and bone marrow cytotoxicity of Synacinn™, and to select the doses for the definitive experiment. In-life observations included mortality, clinical signs of toxicity and body weight. Bone marrow samples were collected and extracted from the femur bone using fetal bovine serum. The pellet obtained after the centrifugation was used for preparing bone marrow smears to evaluate the number of immature and mature erythrocytes.

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### Specifications Table

| Subject | Chemistry, Biological sciences |
|---------|-------------------------------|
| Specific subject area | Analytical Chemistry, Biochemistry, Genetics and Molecular Biology |
| Type of data | Table |
| How data were acquired | HPLC analysis of Synacinn™ and five markers was performed using HPLC Waters (ADTL/EQ/AR-003, ADTL/EQ/AR-004 and ADTL/EQ/AR-005) system. Individual animal body were weighed prior to dosing on Day 1 for all the animals and on Day 3 (prior to sacrifice). In microscopic analysis, a fluorescent microscope with medium magnification was used. |
| Data format | Raw Analyzed |
| Parameters for data collection | The validation involved assessment of selectivity, precision, Limit of Detection (LOD), Limit of Quantification (LOQ), linearity, accuracy, stability in diluent and formulation stability. The data collection in the Phase I and II of in vivo experiments included mortality/moribundity, body weight and clinical signs. Microscopic analysis included evaluation of bone marrow toxicity (determination of the proportion of immature erythrocytes-PCE/E ratio) and Micronucleated PCEs (MN PCEs) counts. |
| Description of data collection | All data collection and processing were performed by the data acquisition system associated with the Empower 3 (Build 3471). The processed data was compiled in the Microsoft® Excel software and further Mean, SD, relative standard deviation (% RSD), and Percentage Relative Error (% RE) values were calculated. A reduction in the proportion of immature cells among total (immature + mature) when compared with the respective vehicle control was considered as a measure of bone marrow toxicity. In the determination of MN PCEs, ≥ 4000 PCEs were scored per animal for the presence of micronuclei. The unit of scoring was micronucleated cell, (not the micronucleus) thus, the occasional cell with more than one micronucleus was counted as one MN PCE. |
| Data source location | Aurigene Pharmaceutical Services Limited Bollaram Road, Miyapur Hyderabad - 500 049, Telangana, India. |
| Data accessibility | With the article |

### Value of the Data

• These data provide information on the analytical method cross validation and stability determination by HPLC identification of five markers and quantification of one marker in Synacinn™ formulations of bracketed strengths 10 and 100 mg/mL. The data also provide information on the clastogenicity potential of Synacinn™ when tested up to the maximum tolerated dose level of 2000 mg/kg/day in Sprague Dawley rat bone marrow.
• These data might be useful as a reference for researchers who want to identify and quantify the bioactive markers in complex polyherbal formulations and its toxicity potential by in vivo study.

• Synacinn™ is a mixture of five herbs which are Orthosiphon stamineus (OS), Syzygium polyanthum (SP), Andrographis paniculata (AP), Cinnamomum zealanicum (CZ) and Curcuma xanthorrhiza (CX). It is standardized with selected five markers based on the major active constituents of the polyherbs (OS-rosmarinic acid, AP-andrographolide, SP-gallic acid, CZ-catechin and CX-curcumin) and going for clinical trial to be prescribed as botanical medicine for diabetes. Synacinn™ is currently approved by National Pharmaceutical Regulatory Agency (NPRA), Malaysia as traditional medicine with a general health claim. These data are valuable to establish the safety pharmacology for Synacinn™ as per requirement by NPRA, Malaysia in accordance to international pharmaceutical regulatory agencies such as European Medicines Agency (EMA) and Food and Drug Administration (FDA).

1. Data Description

Data of the HPLC validation in this article present the identification method of rosmarinic acid, andrographolide, gallic acid, catechin and curcumin and quantification of a marker (curcumin) in Synacinn™. Data on the LOD and LOQ of the five markers are tabulated in Table 1. The system suitability data of three HPLC systems are presented in Table 2. Repeatability and method precision data of curcumin and identified markers in 10 and 100 mg/mL formulations are tabulated in Tables 3, 4 and 5. Concentration based detector response linearity was established in the range of 50 to 150% of the nominal analyte concentration of quantified marker (curcumin) at 0.01 mg/mL and Synacinn at 5 mg/mL. Table 6 shows the linearity data of curcumin, while Table 7 shows the data of peak area for all selected markers in standard solution and Synacinn™ at 10 mg/mL. A linearity curve of curcumin (Fig. 1) are plotted with a linear response observed at 254 nm and correlation coefficient (r) of 0.995. Figs. 2–4 and 5 (raw data provided in Supplementary Tables 1S–4S) show the chromatograms of standard solutions and Synacinn™ (10 mg/mL) at 254 nm and 280 nm. As for the accuracy test, recovery of curcumin was calculated and the mean percentage recovery at 80, 100 and 120% of accuracy level are tabulated in Table 8. The stability of Synacinn™ and curcumin in diluent was evaluated at respective analytical concentration for 0, 4 and 24 h, at control room temperature (CRT) and 2–8 °C. Data on the stability in diluent of curcumin and identified markers for 10 and 100 mg/mL formulations are tabulated in Tables 9 and 10. Formulation stability of Synacinn™ and curcumin was established at CRT and 2–8 °C for 0, 6 and 24 h. Tables 11 and 12 present data on the formulation stability of curcumin and identified marker in 10 and 100 mg/mL formulation.

As for the in vivo test, 300, 600, 1000 and 2000 mg/kg/day were administered orally by gavage to rats on two consecutive days in the Phase I. In the Phase II, Synacinn™ was administered at the dose levels of 500, 1000 and 2000 mg/kg/day for low (G7), mid (G8) and high (G9) dose group rats, respectively for 2 consecutive days. Similarly, the rats in the vehicle control (G6) group received vehicle alone for 2 consecutive days. The dose volume administered

| Marker         | LOD (ng/mL) | LOQ (ng/mL) | S/N LOD (µg/mL) | S/N LOQ (µg/mL) |
|----------------|-------------|-------------|-----------------|-----------------|
| Gallic acid    | 12.672      | 38.4        | 4.3             | 13.0            |
| Catechin       | 92.4        | 280         | 3.7             | 11.8            |
| Rosmarinic acid| 5.28        | 16          | 3.9             | 11.0            |
| Andrographolide| 10.56       | 32          | 2.5             | 9.2             |
| Curcumin       | 105.6       | 320         | 3.4             | 9.6             |

Note: LOD = Limit of Detection; LOQ = Limit of Quantification; S/N = Signal to noise ratio.
Fig. 1. Linearity curve of curcumin at 254 nm.

Fig. 2. Chromatogram of standard solution at 254 nm.

Fig. 3. Chromatogram of standard solution at 280 nm.
was at a constant volume of 20 mL/kg. The positive control (G10) group, received cyclophosphamide monohydrate (25 mg/kg) as a single intraperitoneal injection only on day 2 of dosing at dose volume of 10 mL/kg. Smears were fixed using methanol and stained with May Grunwald Giemsa for evaluating the number of immature and mature erythrocytes which served as an indicator of bone marrow toxicity. Acridine Orange stained smears were used for enumerating the micronucleated immature erythrocytes. Data on the mortality of all animals at Phase I and II of bone marrow micronucleus test are summarized in Table 13. Clinical signs of toxicity, body
Table 3
Repeatability and method precision data of curcumin.

| Injection | Area- Repeatability 10 mg/mL | Area- Repeatability 100 mg/mL |
|-----------|-------------------------------|-----------------------------|
| 1         | 196779                        | 765595                      |
| 2         | 199993                        | 763289                      |
| 3         | 193178                        | 768303                      |
| 4         | 199951                        | 767792                      |
| 5         | 199058                        | 771918                      |
| Mean      | 197791.8                      | 767379.4                    |
| SD        | 2890.6575                     | 3222.4843                   |
| %RSD      | 1.50                          | 0.40                         |

| Area- Method precision |
|------------------------|
| Determination          | 10 mg/mL | 100 mg/mL |
| 1                      | 230095   | 906295    |
| 2                      | 229202   | 915413    |
| 3                      | 226826   | 763289    |
| 4                      | 223778   | 767792    |
| 5                      | 214480   | 771918    |
| Mean                   | 224876.2 | 853180.2  |
| SD                     | 6305.4967| 78077.1892|
| %RSD                   | 2.80     | 9.20       |

Table 4
Repeatability data of identified markers in 10 mg/mL and 100 mg/mL formulations.

| Injection | Retention Time (min)-10 mg/mL formulations | Retention Time (min)-100 mg/mL formulations |
|-----------|---------------------------------------------|---------------------------------------------|
|           | Gallic Acid | Catechin | Rosmarinic Acid | Andrographolide | Curcumin |
| 1         | 5.220       | 12.016   | 19.806          | 21.418          | 26.275   |
| 2         | 5.219       | 12.009   | 19.792          | 21.403          | 26.254   |
| 3         | 5.224       | 12.015   | 19.805          | 21.416          | 26.269   |
| 4         | 5.229       | 12.021   | 19.811          | 21.421          | 26.272   |
| 5         | 5.225       | 12.029   | 19.809          | 21.419          | 26.280   |
| Mean      | 5.223       | 12.018   | 19.805          | 21.415          | 26.270   |
| SD        | 0.0040      | 0.0075   | 0.0074          | 0.0072          | 0.0098   |
| %RSD      | 0.10        | 0.10     | 0.00            | 0.00            | 0.00     |

| Injection | Gallic Acid | Catechin | Rosmarinic Acid | Andrographolide | Curcumin |
|-----------|-------------|----------|-----------------|-----------------|----------|
| 1         | 5.086       | 12.189   | 19.788          | 21.395          | 26.278   |
| 2         | 5.097       | 12.204   | 19.783          | 21.388          | 26.264   |
| 3         | 5.098       | 12.194   | 19.790          | 21.399          | 26.273   |
| 4         | 5.091       | 12.177   | 19.779          | 21.388          | 26.263   |
| 5         | 5.101       | 12.206   | 19.795          | 21.402          | 26.281   |
| Mean      | 5.095       | 12.194   | 19.787          | 21.394          | 26.272   |
| SD        | 0.0060      | 0.0118   | 0.0062          | 0.0063          | 0.0081   |
| %RSD      | 0.10        | 0.10     | 0.00            | 0.00            | 0.00     |

weight and the percentage of body weight gained by animals (male and female) at Phase I are presented in Tables 14, 15 and 16 while Tables 18, 19 and 20 show data on the clinical signs of toxicity, body weight and the percentage of body weight gained by animals (male) at Phase II, respectively. Polychromatic Erythrocytes (PCE)/Erythrocytes (E) and PCE/ Normochromatic Erythrocytes (NCE) ratio are calculated and tabulated in Table 17 for Phase I and Table 21 for Phase II. Micronucleated (MN) PCE counts data on males for Phase II are tabulated in Table 22.
Table 5
Method precision data of identified markers in 10 and 100 mg/mL formulations.

| Determination | Gallic Acid | Catechin | Rosmarinic Acid | Andrographolide | Curcumin |
|---------------|-------------|----------|-----------------|-----------------|----------|
| 1             | 5.269       | 12.168   | 19.831          | 21.445          | 26.312   |
| 2             | 5.279       | 12.170   | 19.828          | 21.440          | 26.309   |
| 3             | 5.272       | 12.159   | 19.822          | 21.437          | 26.303   |
| 4             | 5.260       | 12.166   | 19.823          | 21.437          | 26.313   |
| 5             | 5.286       | 12.185   | 19.830          | 21.442          | 26.317   |
| Mean          | 5.273       | 12.170   | 19.827          | 21.440          | 26.311   |
| SD            | 0.0099      | 0.0096   | 0.0041          | 0.0034          | 0.0052   |
| %RSD          | 0.20        | 0.10     | 0.00            | 0.00            | 0.00     |

| Determination | Gallic Acid | Catechin | Rosmarinic Acid | Andrographolide | Curcumin |
|---------------|-------------|----------|-----------------|-----------------|----------|
| 1             | 5.197       | 11.955   | 19.721          | 21.333          | 26.140   |
| 2             | 5.281       | 11.869   | 19.728          | 21.340          | 26.140   |
| 3             | 5.208       | 11.858   | 19.733          | 21.353          | 26.146   |
| 4             | 5.203       | 11.864   | 19.718          | 21.327          | 26.150   |
| 5             | 5.233       | 11.902   | 19.722          | 21.344          | 26.137   |
| Mean          | 5.224       | 11.890   | 19.724          | 21.339          | 26.143   |
| SD            | 0.0345      | 0.0403   | 0.0060          | 0.0100          | 0.0053   |
| %RSD          | 0.70        | 0.30     | 0.00            | 0.00            | 0.00     |

Table 6
Linearity data of curcumin.

| Level | Final conc. (ppm) | Area  | Average area |
|-------|-------------------|-------|--------------|
| 50 %  | 5.24              | 570991| 571856       |
| 75%   | 7.86              | 787207| 787391       |
| 100%  | 10.48             | 970047| 968539       |
| 125%  | 13.10             | 113868| 1136999      |
| 150%  | 15.72             | 125035| 1253603      |

Table 7
Data of peak area for standard solution and Synacinn™ (10 mg/mL).

| Standard             | Retention time | Area       | USP Plate Count | USP Tailing |
|----------------------|----------------|------------|-----------------|-------------|
| Gallic acid          | 5.422          | 942219     | 905             | 0.8         |
| Rosmarinic acid      | 19.806         | 411738     | 153797          | 1.1         |
| Andrographolide      | 21.394         | 179535     | 129482          | 1.1         |
| Curcumin             | 25.963         | 698251     | 67632           | 0.9         |
| Catechin             | 12.135         | 322254     | 4448            | 0.8         |

| 10 mg/mL Synacinn™   | Retention time | Area       | USP Plate Count | USP Tailing |
|----------------------|----------------|------------|-----------------|-------------|
| Gallic acid          | 5.220          | 10225     | 3740            | 1.2         |
| Rosmarinic acid      | 19.806         | 230922    | 198983          | 1.1         |
| Andrographolide      | 21.418         | 19258     | 224324          | 1.8         |
| Curcumin             | 26.275         | 56035     | 111546          | 0.8         |
| Catechin             | 12.016         | 69070     | 21012           | 0.9         |
Fig. 4. Chromatogram of 10 mg/mL Synacinn™ formulation at 254 nm.

Fig. 5. Chromatogram of 10 mg/mL Synacinn™ formulation at 280 nm.

Table 8
Data of accuracy test in 10 and 100 mg/mL formulations.

| Accuracy Level | Formulation (mg/mL) | Marker Spiked Amount (mg/mL) | Amount Recovered (mg/mL) | % Recovery | Mean % recovery |
|----------------|---------------------|-----------------------------|--------------------------|------------|----------------|
| 80 %           | 10 mg/mL            | 0.19635                     | 0.158                    | 80.4       | 80.8           |
|                |                     | 0.19849                     | 0.157                    | 79.0       |                |
|                |                     | 0.19682                     | 0.163                    | 83.0       |                |
|                | 100 mg/mL           | 0.20077                     | 0.185                    | 92.1       | 94.4           |
|                |                     | 0.20041                     | 0.196                    | 97.7       |                |
|                |                     | 0.20033                     | 0.187                    | 93.4       |                |
| 100 %          | 10 mg/mL            | 0.19778                     | 0.162                    | 82.0       | 80.9           |
|                |                     | 0.19597                     | 0.154                    | 78.3       |                |
|                |                     | 0.19776                     | 0.163                    | 82.5       |                |
|                | 100 mg/mL           | 0.20088                     | 0.199                    | 99.0       | 99.6           |
|                |                     | 0.20004                     | 0.204                    | 101.9      |                |
|                |                     | 0.20035                     | 0.196                    | 98.0       |                |
| 120 %          | 10 mg/mL            | 0.19846                     | 0.160                    | 80.8       | 82.9           |
|                |                     | 0.19696                     | 0.164                    | 83.1       |                |
|                |                     | 0.19798                     | 0.168                    | 84.7       |                |
|                | 100 mg/mL           | 0.20029                     | 0.191                    | 95.3       | 96.9           |
|                |                     | 0.19926                     | 0.201                    | 100.8      |                |
|                |                     | 0.20080                     | 0.190                    | 94.7       |                |
Table 9
Stability in diluent data of curcumin (spiked with marker).

| Formulation strength | Time Interval | Storage Conditions | Area (10 mg/mL) | Area (100 mg/mL) | %RE (10 mg/mL) | %RE (100 mg/mL) |
|----------------------|---------------|-------------------|-----------------|-----------------|---------------|----------------|
|                      |               |                   |                 |                 |               |               |
| 10 mg/mL             |               |                   |                 |                 |               |               |
| Initial              | NA            |                   | NA              | NA              |               |               |
| 4 h                  | CRT           | 436913            | 1.3             | 812301          | 1.7           | 813172        |
| 2–8 °C               |               | 421643            | −2.3            | 813172          | 1.8           | 820708        |
| 24 h                 | CRT           | 370405            | −14.1           | 820708          | 2.8           | 805783        |
| 2–8 °C               |               | 379948            | −11.9           | 805783          | 0.9           |               |

Note: NA = Not applicable.

Table 10
Stability in diluent data of identified markers in 10 mg/mL and 100 mg/mL formulations.

| Formulation strength: 10 mg/mL | Retention Time (min) |
|--------------------------------|-----------------------|
|                                 | Gallic Acid | Catechin | Rosmarinic Acid | Andrographolide | Curcumin |
| Time Interval                   | Storage Conditions  |          |                |                |          |
| Initial                         | CRT         | 5.293    | 12.174         | 19.823         | 21.448   | 26.307   |
| 4 h                             | CRT         | 5.264    | 12.024         | 19.805         | 21.415   | 26.273   |
| 2–8 °C                          | CRT         | 5.263    | 12.154         | 19.820         | 21.430   | 26.301   |
| 24 h                            | CRT         | 5.230    | 12.013         | 19.804         | 21.415   | 26.270   |
| 2–8 °C                          | CRT         | 5.397    | 12.579         | 19.884         | 21.493   | 26.399   |

| Formulation strength: 100 mg/mL | Retention Time (min) |
|---------------------------------|-----------------------|
|                                 | Gallic Acid | Catechin | Rosmarinic Acid | Andrographolide | Curcumin |
| Time Interval                   | Storage Conditions  |          |                |                |          |
| Initial                         | CRT         | 5.232    | 11.906         | 19.738         | 21.346   | 26.164   |
| 4 h                             | CRT         | 5.100    | 12.179         | 19.782         | 21.384   | 26.251   |
| 2–8 °C                          | CRT         | 5.142    | 11.908         | 19.727         | 21.335   | 26.144   |
| 24 h                            | CRT         | 5.090    | 12.216         | 19.798         | 21.402   | 26.281   |
| 2–8 °C                          | CRT         | 5.045    | 12.220         | 19.779         | 21.393   | 26.258   |
|                                 | CRT         | 5.064    | 12.056         | 19.771         | 21.390   | 26.351   |

2. Materials

Synacinn™ powder was provided by Proliv Life Sciences Sdn Bhd. Andrographolide (sc-205594A), catechin (sc-204673A), curcumin (sc-200509A) and gallic acid (sc-205704A) were purchased from Santa Cruz Biotechnology, while rosmarinic acid (sc-202796A) was purchased from Chengdu Biopurify Phyto Chemicals Ltd. Positive control for the in vivo experiment was cyclophosphamide monohydrate.

2.1. Chemicals

Water was used as vehicle in the analysis. Methanol (HPLC grade, B. No.: SC9SF69266), formic acid (Analytical grade, B. No.: D301671, B307940) and TKA water were used as mobile phase.

2.2. Vehicle blank preparation

5 mL of vehicle was pipetted out and transferred into 10 mL volumetric flask. About 3 mL of diluent (mixture of methanol and water in 1:1, v/v) was added to the flask and sonicated to
Table 11: Formulation stability data of curcumin.

|           | 10 mg/mL          | 100 mg/mL         |
|-----------|-------------------|-------------------|
|           | Area   | %RSD | Area   | %RSD |
| Top       | 601937 | 1.1  | 903303 | 0.8  |
| Middle    | 592679 |       | 916837 |       |
| Bottom    | 605371 |       | 907192 |       |
| 6 h at CRT| 10 mg/mL | Area | %RSD | 10 mg/mL | Area | %RSD |
| Top       | 165802 | 34.9 | 768893 | 1.2  |
| Middle    | 329657 |       | 754177 |       |
| Bottom    | 337153 |       | 771080 |       |
| 6 h at 2–8 °C| 10 mg/mL | Area | %RSD | 10 mg/mL | Area | %RSD |
| Top       | 530088 | 2.1  | 890944 | 0.3  |
| Middle    | 508599 |       | 895680 |       |
| Bottom    | 521408 |       | 891761 |       |
| 24 h at CRT| 10 mg/mL | Area | %RSD | 10 mg/mL | Area | %RSD |
| Top       | 220319 | 4.4  | 709355 | 4.9  |
| Middle    | 228658 |       | 745256 |       |
| Bottom    | 209569 |       | 676360 |       |
| 24 h at 2–8 °C| 10 mg/mL | Area | %RSD | 10 mg/mL | Area | %RSD |
| Top       | 476384 | 2.5  | 764066 | 1.0  |
| Middle    | 454207 |       | 755590 |       |
| Bottom    | 470949 |       | 749008 |       |

dissolve. The solution was made up to the mark with diluent, mixed well, and injected once into HPLC. Then, the chromatograms were recorded.

2.3. Solution preparation

2.3.1. 0.2 mg/mL of marker stock
Each marker was weighed at 2mg and transferred into five separate 10 ml volumetric flasks. Then, 5 ml of methanol was added to each flask and dissolved completely. The volume was made up to 10 ml with water and mixed well.

2.3.2. 0.01 mg/mL of markers solution
Each of the marker stock solutions was pipetted out (1.0 mL) and transferred into 20 mL volumetric flask. The volume was made up to 20 mL with diluent and mixed well.

2.3.3. Identification solution
Synacinn™ powder was weighed at 50 mg and transferred into a 10 mL volumetric flask. Then, 5 mL of diluent was added. Each of the marker stock solutions was spiked (0.5 mL) into the 10 mL volumetric flask. The volume was made up to 10 mL with water and mixed well.

2.3.4. 10 mg/mL formulation
10 mg/mL formulation was pipetted out (5 mL) in triplicate and transferred into three separate 10 mL volumetric flasks. About 3 mL of diluent was added to each flask and sonicated to dissolve. These solutions were made up to the mark with diluent and mixed well and injected once into HPLC. Then, the chromatograms were recorded.
### Table 12
Formulation stability data of identified marker in 10 and 100 mg/mL formulation.

| Time/Layer      | Gallic Acid | Catechin | Rosmarinic Acid | Andrographolide | Curcumin |
|-----------------|-------------|----------|-----------------|-----------------|----------|
| **Retention Time (min) – Initial** |
| Dose            | 10          | 100      | 10              | 100             | 100      |
| Top             | 5.274       | 5.283    | 12.162          | 11.913          | 9.822    |
| Middle          | 5.266       | 5.217    | 12.159          | 11.889          | 9.823    |
| Bottom          | 5.256       | 5.307    | 12.159          | 11.924          | 9.822    |
| Mean            | 5.265       | 5.269    | 12.160          | 11.909          | 9.822    |
| SD              | 0.009       | 0.0466   | 0.0017          | 0.0179          | 0.006    |
| %RSD            | 0.2         | 0.9      | 0.0             | 0.2             | 0.0      |
| **Retention Time (min)-6 h at CRT** |
| Dose            | 10          | 100      | 10              | 100             | 100      |
| Top             | 5.282       | 5.032    | 12.169          | 11.805          | 9.822    |
| Middle          | 5.286       | 5.176    | 12.177          | 11.894          | 9.822    |
| Bottom          | 5.294       | 5.172    | 12.183          | 11.933          | 9.83     |
| Mean            | 5.287       | 5.127    | 12.176          | 11.877          | 9.825    |
| SD              | 0.0061      | 0.082    | 0.007           | 0.0656          | 0.0046   |
| %RSD            | 0.1         | 1.6      | 0.1             | 0.6             | 0.0      |
| **Retention Time (min)-6 h at 2–8 °C** |
| Dose            | 10          | 100      | 10              | 100             | 100      |
| Top             | 5.267       | 5.245    | 12.162          | 11.891          | 9.821    |
| Middle          | 5.287       | 5.245    | 12.174          | 11.807          | 9.832    |
| Bottom          | 5.257       | 5.285    | 12.142          | 11.949          | 9.818    |
| Mean            | 5.270       | 5.258    | 12.159          | 11.882          | 9.824    |
| SD              | 0.0153      | 0.0231   | 0.0162          | 0.0714          | 0.0074   |
| %RSD            | 0.3         | 0.4      | 0.1             | 0.6             | 0.0      |
| **Retention Time (min)-24 h at CRT** |
| Dose            | 10          | 100      | 10              | 100             | 100      |
| Top             | 5.236       | 5.059    | 12.031          | 12.181          | 9.797    |
| Middle          | 5.239       | 5.072    | 12.010          | 12.169          | 9.797    |
| Bottom          | 5.221       | 5.073    | 12.039          | 12.186          | 9.817    |
| Mean            | 5.232       | 5.068    | 12.027          | 12.179          | 9.804    |
| SD              | 0.0096      | 0.0078   | 0.015           | 0.0087          | 0.0115   |
| %RSD            | 0.2         | 0.2      | 0.1             | 0.1             | 0.1      |
| **Retention Time (min)-24 h at 2–8 °C** |
| Dose            | 10          | 100      | 10              | 100             | 100      |
| Top             | 5.243       | 5.087    | 12.018          | 12.173          | 9.806    |
| Middle          | 5.232       | 5.084    | 12.024          | 12.167          | 9.807    |
| Bottom          | 5.218       | 5.095    | 12.024          | 12.172          | 9.807    |
| Mean            | 5.231       | 5.089    | 12.022          | 12.171          | 9.807    |
| SD              | 0.0125      | 0.0057   | 0.0035          | 0.0032          | 0.0006   |
| %RSD            | 0.2         | 0.1      | 0.0             | 0.0             | 0.0      |

### 2.3.5. 100 mg/mL formulation

100 mg/mL formulation was pipetted out (1 mL) in triplicate and transferred into three separate 20 mL volumetric flasks. About 6 mL of diluent was added to each flask and sonicated to dissolve. These solutions were made up to the mark with diluent, mixed well and injected once into HPLC. Then, the chromatograms were recorded.
Table 13
Summary of mortality at phase I and phase II.

| Group No. | Mortality/Moribundity Incidence | Day of Death |
|-----------|---------------------------------|--------------|
|           | Male | Female |                      |
| G1        | 0/3  | 0/3    | −                     |
| G2        | 0/3  | 0/3    | −                     |
| G3        | 0/3  | 0/3    | −                     |
| G4        | 0/3  | 0/3    | −                     |
| G5        | 0/3  | 0/3    | −                     |
| G6        | 0/6  | −      | −                     |
| G7        | 0/6  | −      | −                     |
| G8        | 0/6  | −      | −                     |
| G9        | 0/6  | −      | −                     |
| G10       | 0/6  | −      | −                     |

G1=Vehicle Control: 0 mg/kg/day; G2=Synacinn™: 300 mg/kg/day; G3=Synacinn™: 600 mg/kg/day; G4= Synacinn™: 1000 mg/kg/day; G5= Synacinn™: 2000 mg/kg/day; G6=Vehicle control: 0 mg/kg/day; G7=Synacinn™: 500 mg/kg/day; G8=Synacinn™: 1000 mg/kg/day; G9=Synacinn™: 2000 mg/kg/day; G10= Cyclophosphamide monohydrate: 25 mg/kg; - =Not applicable.

Table 14
Individual animal clinical signs data at phase I.

| Group No. | A # | Day 1 | Day 2 | Day 3 |
|-----------|-----|-------|-------|-------|
|           |     | Pre dose | Post dose | Pre dose | Post dose | Pre-Necropsy |
|           |     | M  F | M  F | M  F | M  F | M  F |
| G1        | 1 N | N   | N   | N   | N   | N   |
|           | 2 N | N   | N   | N   | N   | N   |
|           | 3 N | N   | N   | N   | N   | N   |
|           | 4 N | N   | N   | N   | N   | N   |
|           | 5 N | N   | N   | N   | N   | N   |
|           | 6 N | N   | N   | N   | N   | N   |
| G2        | 7 N | N   | N   | N   | N   | N   |
|           | 8 N | N   | N   | N   | N   | N   |
|           | 9 N | N   | N   | N   | N   | N   |
| G3        | 10 N | N   | N   | N   | N   | N   |
|           | 11 N | N   | N   | N   | N   | N   |
|           | 12 N | N   | N   | N   | N   | N   |
| G4        | 13 N | N   | N   | N   | N   | N   |
|           | 14 N | N   | N   | N   | N   | N   |
|           | 15 N | N   | N   | N   | N   | N   |

A#= Animal number; N= Normal; M= Male; F= Female

2.4. Buffer preparation

Buffer was prepared by adding 5 mL of formic acid into 995 mL of methanol and sonicated for 5 min to degas the buffer. The buffer was stored at room temperature and used within 30 days from the date of preparation.
Table 15
Individual animal body weights (g) data at Phase I.

### Day 1 (Male)

| A#   | G1    | A#   | G2    | A#   | G3    | A#   | G4    | A#   | G5    |
|------|-------|------|-------|------|-------|------|-------|------|-------|
| 1    | 196.78| 4    | 197.71| 7    | 198.67| 10   | 200.60| 13   | 206.79|
| 2    | 209.21| 5    | 205.40| 8    | 208.41| 11   | 206.41| 14   | 201.91|
| 3    | 215.03| 6    | 222.38| 9    | 220.21| 12   | 222.48| 15   | 200.81|
| Mean | 207.01| 6    | 208.50| 8    | 209.10| 12   | 209.83| 15   | 213.10|
| SD   | 9.32  | 5    | 12.623| 10   | 10.786| 11.334| 11.334| 11.334| 15.356|

### Day 3 (Male)

| A#   | G1    | A#   | G2    | A#   | G3    | A#   | G4    | A#   | G5    |
|------|-------|------|-------|------|-------|------|-------|------|-------|
| 1    | 209.30| 4    | 215.25| 7    | 218.66| 10   | 214.44| 13   | 217.70|
| 2    | 229.37| 5    | 220.32| 8    | 222.31| 11   | 226.42| 14   | 212.60|
| 3    | 228.39| 6    | 240.40| 9    | 242.49| 12   | 238.60| 15   | 250.80|
| Mean | 222.35| 6    | 225.32| 9    | 227.82| 12   | 226.49| 15   | 227.03|
| SD   | 11.315| 13.301| 13.839| 13.839| 13.839| 13.839| 13.839| 13.839| 20.740|

### Day 1 (Female)

| A#   | G1    | A#   | G2    | A#   | G3    | A#   | G4    | A#   | G5    |
|------|-------|------|-------|------|-------|------|-------|------|-------|
| 16   | 162.21| 19   | 152.22| 22   | 157.01| 25   | 152.20| 28   | 158.59|
| 17   | 166.97| 20   | 167.40| 23   | 159.72| 26   | 160.49| 29   | 159.37|
| 18   | 172.35| 21   | 169.57| 24   | 182.22| 27   | 175.31| 30   | 183.82|
| Mean | 167.18| 21   | 163.06| 24   | 166.32| 27   | 162.62| 28   | 167.26|
| SD   | 5.073 | 9.453| 13.839| 13.839| 13.839| 13.839| 13.839| 13.839| 14.347|

### Day 3 (Female)

| A#   | G1    | A#   | G2    | A#   | G3    | A#   | G4    | A#   | G5    |
|------|-------|------|-------|------|-------|------|-------|------|-------|
| 16   | 170.86| 19   | 163.62| 22   | 166.71| 25   | 163.06| 28   | 170.85|
| 17   | 176.70| 20   | 175.93| 23   | 166.87| 26   | 172.04| 29   | 166.27|
| 18   | 177.69| 21   | 180.91| 24   | 180.41| 27   | 188.42| 30   | 192.59|
| Mean | 175.08| 21   | 173.49| 24   | 171.33| 27   | 174.51| 30   | 176.57|
| SD   | 3.691 | 8.900| 7.864 | 7.864 | 7.864 | 7.864 | 7.864 | 7.864 | 14.061|

*A#= Animal number; G1=Vehicle control: 0 mg/kg/day; G2=Synacinn™: 300 mg/kg/day; G3=Synacinn™: 600 mg/kg/day; G4= Synacinn™: 1000 mg/kg/day; G5= Synacinn™: 2000 mg/kg/day.

Table 16
Individual animal body weights gain (%) data at Phase I.

### Day 1–3 (Male)

| A#   | G1    | A#   | G2    | A#   | G3    | A#   | G4    | A#   | G5    |
|------|-------|------|-------|------|-------|------|-------|------|-------|
| 1    | 6.36  | 4    | 8.87  | 7    | 10.06 | 10   | 6.90  | 13   | 5.28  |
| 2    | 9.64  | 5    | 7.26  | 8    | 6.67  | 11   | 9.69  | 14   | 5.29  |
| 3    | 6.21  | 6    | 8.10  | 9    | 10.12 | 12   | 7.25  | 15   | 8.76  |
| Mean | 7.40  | 6    | 8.08  | 8    | 8.95  | 12   | 7.95  | 15   | 6.44  |
| SD   | 1.938 | 1.217| 1.975 | 1.975| 1.975| 1.975| 1.975| 1.975| 2.006 |

### Day 1–3 (Female)

| A#   | G1    | A#   | G2    | A#   | G3    | A#   | G4    | A#   | G5    |
|------|-------|------|-------|------|-------|------|-------|------|-------|
| 16   | 5.33  | 19   | 7.49  | 22   | 6.18  | 25   | 7.14  | 28   | 7.73  |
| 17   | 5.83  | 20   | 5.10  | 23   | 4.48  | 26   | 7.20  | 29   | 4.33  |
| 18   | 3.10  | 21   | 6.99  | 24   | −0.99 | 27   | 7.48  | 30   | 4.77  |
| Mean | 4.75  | 6.43 | 3.22  | 13.747| 1.811| 1.849|
| SD   | 1.453 | 1.217| 1.975 | 1.975| 1.975| 1.975| 1.975| 1.975| 2.006 |

*A#: Animal number; G1=Vehicle control: 0 mg/kg/day; G2=Synacinn™: 300 mg/kg/day; G3=Synacinn™: 600 mg/kg/day; G4= Synacinn™: 1000 mg/kg/day; G5= Synacinn™: 2000 mg/kg/day.
Table 17

Individual animal PCE/E and PCE/NCE ratio at Phase I.

| Group | A# | G1 | G2 | G3 | G4 | G5 |
|-------|----|----|----|----|----|----|
|       | M  | F  | M  | F  | M  | F  |
| PCE   |    |    |    |    |    |    |
| NCE   |    |    |    |    |    |    |
| Total |    |    |    |    |    |    |
| E     |    |    |    |    |    |    |
|       | M  | F  | M  | F  | M  | F  |
|       |    |    |    |    |    |    |
|       | M  | F  | M  | F  |    |    |
| Mean  | 364| 339| 184| 207| 548| 545|
| SD    | 24.2| 20.4| 16.4| 78.0| 36.1| 64.9|
|       |    |    |    |    |    |    |
|       | 4  |    |    |    |    |    |
| Mean  | 366| 356| 195| 202| 561| 558|
| SD    | 13.1| 4.0| 23.4| 6.0| 36.5| 10.0|
|       |    |    |    |    |    |    |
|       | 7  |    |    |    |    |    |
| Mean  | 347| 346| 161| 190| 509| 536|
| SD    | 22.0| 29.5| 20.0| 27.8| 11.7| 26.0|
|       |    |    |    |    |    |    |
|       | 10 |    |    |    |    |    |
| Mean  | 385| 371| 182| 193| 567| 564|
| SD    | 20.8| 79.2| 20.0| 32.3| 39.7| 108.0|
|       |    |    |    |    |    |    |
|       | 13 |    |    |    |    |    |
| Mean  | 371| 328| 180| 184| 551| 512|
| SD    | 21.2| 15.6| 24.2| 15.1| 43.0| 17.1|

A# = Animal number; M = Male; F = Female; PCE = Polychromatonic Erythrocytes; NCE = Normochromatonic Erythrocytes; E = Erythrocytes; G1 = Vehicle control: 0 mg/kg/day; G2 = Synacinn™: 300 mg/kg/day; G3 = Synacinn™: 600 mg/kg/day; G4 = Synacinn™: 1000 mg/kg/day; G5 = Synacinn™: 2000 mg/kg/day.

2.5. Mobile phase preparation

Mobile Phase A was prepared by mixing 900 mL of water and 100 mL of 0.5% formic acid in methanol, and sonicate for 5 min. For mobile Phase B, 900 mL of 0.5% formic acid in methanol and 100 mL of water were mixed, and sonicated for 5 min.

2.6. High-Performance Liquid Chromatography (HPLC)

HPLC analysis of Synacinn™ and five markers was performed using HPLC Waters (ADTL/EQ/AR-003, ADTL/EQ/AR-004 and ADTL/EQ/AR-005) system. Column used was Zodiac C18 (250 × 4.6) mm with diameter of 5μm. The gradient flow for Synacinn™ were (minutes/% mobile phase B): 0/5, 12/20, 15/50, 20/80, 25/80, 32/20, 32.1/5 and 35/5%. The flow rate and column
Individual animal clinical signs data on males at Phase II.

| Group No. | A # | Pre dose | Post dose | Pre dose | Post dose | Pre Necropsy |
|-----------|-----|----------|-----------|----------|-----------|--------------|
| G6        | 35  | N        | N         | N        | N         | N            |
|           | 36  | N        | N         | N        | N         | N            |
|           | 37  | N        | N         | N        | N         | N            |
|           | 38  | N        | N         | N        | N         | N            |
|           | 39  | N        | N         | N        | N         | N            |
|           | 40  | N        | N         | N        | N         | N            |
| G7        | 41  | N        | N         | N        | N         | N            |
|           | 42  | N        | N         | N        | N         | N            |
|           | 43  | N        | N         | N        | N         | N            |
|           | 44  | N        | N         | N        | N         | N            |
|           | 45  | N        | N         | N        | N         | N            |
|           | 46  | N        | N         | N        | N         | N            |
| G8        | 47  | N        | N         | N        | N         | N            |
|           | 48  | N        | N         | N        | N         | N            |
|           | 49  | N        | N         | N        | N         | N            |
|           | 50  | N        | N         | N        | N         | N            |
|           | 51  | N        | N         | N        | N         | N            |
|           | 52  | N        | N         | N        | N         | N            |
| G9        | 53  | N        | N         | N        | N         | N            |
|           | 54  | N        | N         | N        | N         | N            |
|           | 55  | N        | N         | N        | N         | N            |
|           | 56  | N        | N         | N        | N         | N            |
|           | 57  | N        | N         | N        | N         | N            |
|           | 58  | N        | N         | N        | N         | N            |
| G10       | 59  | N        | NA        | N        | N         | N            |
|           | 60  | N        | NA        | N        | N         | N            |
|           | 61  | N        | NA        | N        | N         | N            |
|           | 62  | N        | NA        | N        | N         | N            |
|           | 63  | N        | NA        | N        | N         | N            |
|           | 64  | N        | NA        | N        | N         | N            |

A# = Animal number; N = Normal, NA = Not Applicable; G6 = Vehicle control: 0 mg/kg/day; G7 = Synacinn™: 500 mg/kg/day; G8 = Synacinn™: 1000 mg/kg/day; G9 = Synacinn™: 2000 mg/kg/day; G10 = Cyclophosphamide monohydrate: 25 mg/kg.

temperature were 1.0 min/mL and 35 °C±5 °C, respectively. All biomarkers were detected at the wavelength of 254 nm, except for catechin, 280 nm, with injection volume of 50μL. The retention time of each markers was as following: Gallic acid (5 minutes), Catechin (12 min), Rosmarinic acid (19 min), Andrographolide (21 min) and Curcumin (26 min). The total run time was 35 min.

2.7. Validation methods

This cross validation method was designed based on the United States Food and Drug Administration’s Guidance for Industry: Analytical procedures and methods validation for drugs and biologics (USFDA, 2015) and the International Council for Harmonisation (ICH Q2[R1]) guideline [1-3].

2.7.1. Test sample preparation

The following procedure was used to prepare the test samples for specificity, method precision repeatability, stability in diluent and formulation stability. Formulation solution was
accurately transferred into suitable volumetric flask (sampling of formulation was done under continuous stirring). Methanol was added about 50% of the final volume to each flask. The volume of each flask was made up to the mark with water. The final solutions were centrifuged at 5000 rpm for 5 min. Then, the supernatant solution was transferred into HPLC vials and injected into HPLC and the chromatogram was recorded.

### 2.7.2. System suitability test

All samples were run in three HPLC systems (ADTL/EQ/AR-003, ADTL/EQ/AR-004 and ADTL/EQ/AR-005) for system suitability test. Diluent blank solution was injected to ensure that no significant interference was observed in the retention time window of five markers peak. Standard solution was injected into HPLC for five times and the chromatograms were recorded. Identification solution was injected once into HPLC and the chromatogram was recorded.

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### Table 19

| A # | G6 | A# | G7 | A# | G8 | A# | G9 | A# | G10 |
|-----|----|----|----|----|----|----|----|----|-----|
| 35  | 287.05 | 41 | 293.99 | 47 | 297.51 | 53 | 298.85 | 59 | 318.07 |
| 36  | 306.75 | 42 | 313.51 | 48 | 309.14 | 54 | 307.93 | 60 | 308.43 |
| 37  | 318.08 | 43 | 316.15 | 49 | 322.13 | 55 | 311.21 | 61 | 322.48 |
| 38  | 321.24 | 44 | 334.92 | 50 | 323.79 | 56 | 323.39 | 62 | 319.89 |
| 39  | 332.27 | 45 | 336.32 | 51 | 328.16 | 57 | 337.17 | 63 | 337.10 |
| 40  | 372.22 | 46 | 353.66 | 52 | 352.82 | 58 | 350.02 | 64 | 344.09 |
| Mean | 322.94 | 324.76 | 322.26 | 321.43 | 323.34 |
| SD  | 28.609 | 21.069 | 18.739 | 19.316 | 10.655 |

- **A=#**: Animal number; **G6**: Vehicle control: 0 mg/kg/day; **G7**: Synacinn™: 500 mg/kg/day; **G8**: Synacinn™: 1000 mg/kg/day; **G9**: Synacinn™: 2000 mg/kg/day; **G10**: Cyclophosphamide monohydrate: 25 mg/kg.

### Table 20

| A # | G6 | A# | G7 | A# | G8 | A# | G9 | A# | G10 |
|-----|----|----|----|----|----|----|----|----|-----|
| 35  | 3.00 | 41 | 12.07 | 47 | 3.46 | 53 | 2.93 | 59 | 2.35 |
| 36  | 3.96 | 42 | 4.56 | 48 | 4.68 | 54 | 3.89 | 60 | 2.33 |
| 37  | 2.59 | 43 | 2.13 | 49 | 0.88 | 55 | –3.14 | 61 | 3.44 |
| 38  | 4.61 | 44 | 3.03 | 50 | 4.72 | 56 | 6.13 | 62 | 1.94 |
| 39  | 4.68 | 45 | 3.59 | 51 | 2.61 | 57 | 2.78 | 63 | 2.76 |
| 40  | 4.69 | 46 | 2.24 | 52 | 4.53 | 58 | 4.45 | 64 | 3.03 |
| Mean | 3.92 | 4.60 | 3.48 | 2.84 | 2.64 |
| SD  | 0.923 | 3.767 | 1.523 | 3.171 | 0.543 |

- **A=#**: Animal number; **G6**: Vehicle control: 0 mg/kg/day; **G7**: Synacinn™: 500 mg/kg/day; **G8**: Synacinn™: 1000 mg/kg/day; **G9**: Synacinn™: 2000 mg/kg/day; **G10**: Cyclophosphamide monohydrate: 25 mg/kg.
### Table 21

Individual animal PCE/E and PCE/NCE ratio data on males at Phase II.

| Group No. | Slide Code | A# | PCE   | NCE   | Total E | PCE/E | PCE/NCE |
|-----------|------------|----|-------|-------|---------|--------|---------|
| G6        | P3–4       | 35 | 314   | 214   | 528     | 0.59   | 1.47    |
|           | A4–4       | 36 | 318   | 202   | 520     | 0.61   | 1.57    |
|           | E3–4       | 37 | 351   | 222   | 573     | 0.61   | 1.58    |
|           | Q3–4       | 38 | 288   | 216   | 504     | 0.57   | 1.33    |
|           | B4–4       | 39 | 324   | 184   | 508     | 0.64   | 1.76    |
|           | S3–4       | 40 | 348   | 252   | 600     | 0.58   | 1.38    |

| Mean | 324 | 215 | 539 | 0.60 | 1.52 |
| SD   | 23.4 | 22.6 | 38.8 | 0.025 | 0.156 |

| G7 | F3–4 | 41 | 332 | 176 | 508 | 0.65 | 1.89 |
|    | C4–4 | 42 | 350 | 202 | 552 | 0.63 | 1.73 |
|    | M3–4 | 43 | 358 | 192 | 550 | 0.65 | 1.86 |
|    | Y3–4 | 44 | 358 | 206 | 564 | 0.63 | 1.74 |
|    | A3–4 | 45 | 336 | 184 | 520 | 0.65 | 1.83 |
|    | W3–4 | 46 | 284 | 224 | 508 | 0.56 | 1.27 |

| Mean | 336 | 197 | 534 | 0.63 | 1.72 |
| SD   | 27.9 | 17.1 | 24.6 | 0.035 | 0.230 |

| G8 | N3–4 | 47 | 350 | 212 | 562 | 0.62 | 1.65 |
|    | T3–4 | 48 | 354 | 260 | 614 | 0.58 | 1.36 |
|    | G3–4 | 49 | 264 | 256 | 520 | 0.51 | 1.03 |
|    | X3–4 | 50 | 312 | 198 | 510 | 0.61 | 1.58 |
|    | O3–4 | 51 | 300 | 214 | 514 | 0.58 | 1.40 |
|    | Z3–4 | 52 | 306 | 196 | 502 | 0.61 | 1.56 |

| Mean | 314 | 223 | 537 | 0.59 | 1.43 |
| SD   | 33.6 | 28.3 | 43.2 | 0.040 | 0.225 |

| G9 | B3–4 | 53 | 314 | 206 | 520 | 0.60 | 1.52 |
|    | D4–4 | 54 | 364 | 216 | 580 | 0.63 | 1.69 |
|    | U3–4 | 55 | 326 | 255 | 581 | 0.56 | 1.28 |
|    | H3–4 | 56 | 334 | 200 | 534 | 0.63 | 1.67 |
|    | V3–4 | 57 | 330 | 226 | 556 | 0.59 | 1.46 |
|    | C3–4 | 58 | 350 | 248 | 598 | 0.59 | 1.41 |

| Mean | 336 | 225 | 562 | 0.60 | 1.51 |
| SD   | 17.9 | 22.3 | 30.2 | 0.027 | 0.157 |

| G10 | I3–4 | 59 | 245 | 258 | 503 | 0.49 | 0.95 |
|     | J3–4 | 60 | 258 | 334 | 592 | 0.44 | 0.77 |
|     | L3–4 | 61 | 256 | 285 | 541 | 0.47 | 0.90 |
|     | D3–4 | 62 | 250 | 253 | 503 | 0.50 | 0.99 |
|     | R3–4 | 63 | 259 | 256 | 515 | 0.50 | 1.01 |
|     | K3–4 | 64 | 258 | 252 | 510 | 0.51 | 1.02 |

| Mean | 254 | 273 | 527 | 0.49 | 0.94 |
| SD   | 5.6 | 32.3 | 34.7 | 0.026 | 0.094 |

A# = Animal Number; PCE= Polychromatic Erythrocytes; NCE= Normochromatic Erythrocytes; E= Erythrocytes; G6=Vehicle control: 0 mg/kg/day; G7=Synacinn™: 500 mg/kg/day; G8=Synacinn™: 1000 mg/kg/day; G9= Synacinn™: 2000 mg/kg/day; G10= Cyclophosphamide monohydrate: 25 mg/kg.

2.7.3. Selectivity

Each individual marker was prepared at concentration of 0.01 mg/mL. Standard solution was used as selectivity sample solution (10 mg/mL stability in diluent initial sample was considered as selectivity solution). Diluent blank, individual marker solutions and selectivity solution were injected into HPLC and the chromatogram was recorded.
### Table 22
Individual animal MN PCE counts data on males at Phase II.

| Group No. | Slide Code | A#  | Total PCE Screened | MN PCE | MN PCE/1000 |
|-----------|------------|-----|--------------------|--------|-------------|
| G6        | P3–1       | 35  | 4130               | 2      | 0.48        |
|           | A4–1       | 36  | 4102               | 4      | 0.98        |
|           | E3–1       | 37  | 4165               | 4      | 0.96        |
|           | Q3–1       | 38  | 4313               | 5      | 1.16        |
|           | B4–1       | 39  | 4022               | 6      | 1.49        |
|           | S3–3       | 40  | 4050               | 4      | 0.99        |

**Mean** 4130  4.2  1.01

**SD** 103.5  1.33  0.328

| G7        | F3–3       | 41  | 4011               | 4      | 1.00        |
|           | C4–3       | 42  | 4042               | 4      | 0.99        |
|           | M3–3       | 43  | 4081               | 4      | 0.98        |
|           | Y3–3       | 44  | 4192               | 2      | 0.48        |
|           | A3–3       | 45  | 5359               | 4      | 0.75        |
|           | W3–3       | 46  | 4050               | 3      | 0.74        |

**Mean** 4289  3.5  0.82

**SD** 527.8  0.84  0.207

| G8        | N3–3       | 47  | 4055               | 3      | 0.74        |
|           | T3–3       | 48  | 4173               | 4      | 0.96        |
|           | G3–3       | 49  | 4091               | 3      | 0.73        |
|           | X3–3       | 50  | 4248               | 6      | 1.41        |
|           | O3–2       | 51  | 4053               | 3      | 0.74        |
|           | Z3–3       | 52  | 4813               | 4      | 0.83        |

**Mean** 4239  3.8  0.90

**SD** 291.3  1.17  0.264

| G9        | B3–3       | 53  | 4463               | 5      | 1.12        |
|           | D4–2       | 54  | 4074               | 4      | 0.98        |
|           | U3–2       | 55  | 4050               | 2      | 0.49        |
|           | H3–2       | 56  | 4371               | 3      | 0.69        |
|           | V3–3       | 57  | 4019               | 3      | 0.75        |
|           | C3–3       | 58  | 4068               | 6      | 1.47        |

**Mean** 4174  3.8  0.92

**SD** 191.3  1.47  0.350

| G10       | I3–3       | 59  | 4539               | 78     | 17.18       |
|           | J3–2       | 60  | 4071               | 83     | 20.39       |
|           | L3–3       | 61  | 4191               | 112    | 26.72       |
|           | D3–2       | 62  | 4205               | 116    | 27.59       |
|           | R3–3       | 63  | 4049               | 79     | 19.51       |
|           | K3–1       | 64  | 4054               | 71     | 17.51       |

**Mean** 4185  89.8  21.48

**SD** 186.8  19.16  4.563

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A# = Animal Number; MN PCE = Micronucleated Polychromatic Erythrocytes; PCE = Polychromatic Erythrocytes; G6 = Vehicle control: 0 mg/kg/day; G7 = Synacinn™: 500 mg/kg/day; G8 = Synacinn™: 1000 mg/kg/day; G9 = Synacinn™: 2000 mg/kg/day; G10 = Cyclophosphamide monohydrate: 25 mg/kg.

2.7.4. Limit of Detection and Limit of Quantification (LOD & LOQ)

The LOD and LOQ of quantified marker was established by diluting the solution with nominal concentration of 10 μg/mL to get signal to noise ratio of about 10 for LOQ and about 3 for LOD.

2.7.5. Precision

2.7.5.1. Repeatability. A repeatability solution was prepared by diluting the formulation to the nominal analyte concentration of quantified marker at 0.01 mg/mL and Synacinn™ at 5 mg/mL as described in test sample preparation. A vehicle blank solution was injected, followed by
### Table 23
Phase I (dose range finding experiment).

| Group No. | Treatment   | Group color code | Dose (mg/kg/day) | Dose strength (mg/mL) | Dose Volume (mL/kg) | No. of Rats | Sex  | Rat Numbers |
|-----------|-------------|------------------|------------------|-----------------------|---------------------|-------------|------|-------------|
| G1        | Vehicle Control | White   | 0               | 0                     | 20                  | 3           | Male | 1–3         |
|           |             |        |                 |                       |                     | 3           | Female | 16–18       |
| G2        | Synacinn™ | Green   | 300             | 30                    | 10                  | 3           | Male | 4–6         |
|           |             |        |                 |                       |                     | 3           | Female | 19–21       |
| G3        | Blue        | 600    | 60              | 10                    |                     | 3           | Male | 7–9         |
|           |             |        |                 |                       |                     | 3           | Female | 22–24       |
| G4        | Yellow      | 1000   | 100             | 10                    |                     | 3           | Male | 10–12       |
|           |             |        |                 |                       |                     | 3           | Female | 25–27       |
| G5        | Red         | 2000   | 100             | 20                    |                     | 3           | Male | 13–15       |
|           |             |        |                 |                       |                     | 3           | Female | 28–30       |

**Extra Animals**

Male 31–32
Female 33–34

### Table 24
Phase II (definitive experiment).

| Group No. | Treatment   | Group color code | Dose (mg/kg/day) | Dose strength (mg/mL) | Dose Volume (mL/kg) | No. of Rats | Rat Numbers |
|-----------|-------------|------------------|------------------|-----------------------|---------------------|-------------|-------------|
| G6        | Vehicle Control | White   | 0               | 0                     | 20                  | 6           | 35–40       |
| G7        | Synacinn™ | Green   | 500             | 25                    | 20                  | 6           | 41–46       |
| G8        | Blue        | 1000   | 50              | 20                    |                     | 6           | 47–52       |
| G9        | Orange      | 2000   | 100             | 20                    |                     | 6           | 53–58       |
| G10       | Cyclophosphamide monohydrate # | Red   | 25              | 2.5                   | 10                  | 6           | 59–64       |

#: Administered as a single intraperitoneal injection on Day 2 of Dosing

Injection of repeatability solution for five times. The percentage relative standard deviation (% RSD) was calculated for area of one marker from five replicate injections using the following formula.

\[
\% \text{RSD} = \frac{\text{Standard deviation} \times 100}{\text{Mean of } n \text{ values}}
\]

#### 2.7.5.2. Method precision
Five independent method precision solutions were prepared by diluting the formulations to the nominal analyte concentration of quantified marker at 0.01 mg/mL and Synacinn™ at 5 mg/mL, as described in test sample preparation. The vehicle blank solution was injected, followed by injection of each method precision solution once. The % RSD was calculated for area from five determinations of quantified marker.

#### 2.7.6. Linearity
Linearity of concentration based detector response was established in the range of 50 to 150% of the nominal analyte concentration of quantified marker at 0.01 mg/mL in presence of Synacinn™ at 5 mg/mL analyte concentration. A diluent blank was injected, followed by injection of each linearity solution in triplicate. The mean area at each concentration was calculated. The calibration graph of concentration versus mean area was plotted with calculated correlation coefficient (r), slope and intercept.
2.7.7. Accuracy

Accuracy of the method was evaluated as percentage recovery. Synacinn™ at analyte concentration was spiked with one marker at 0.01 mg/mL to the vehicle at 80, 100, and 120% of solution concentration in triplicate. The spiked samples were diluted to obtain the required analyte concentration. The Synacinn™ accuracy was prepared at 80, 100 and 120% of analyte concentration and injected once along with the spiked samples at each level. A vehicle blank was injected, followed by injection of each accuracy test solution once. The recovery of quantified marker was calculated by considering the amount spiked concentration. The percentage recovery for each determination was calculated using the following formulas.

\[
\% \text{ of Marker spiked (mg/mL)} = \frac{\text{Weight of marker} \times \text{Vol. of marker} \times \text{Total dilution}}{\text{Marker dilution} \times \text{Sample dilution} \times \text{Weight of Synacinn™}}
\]

\[
\text{Amount recovered (mg/mL)} = \frac{\text{Area of accuracy solution} \times \text{Conc. of standard} \times \text{Dilution}}{\text{Mean area of standard} \times \text{Weight of Synacinn™}}
\]

\[
\text{Percentage Recovery} = \frac{\text{Amount Recovered} \times 100}{\% \text{ of Marker spiked}}
\]

2.7.8. Stability in diluent

The stability solution of quantified marker was prepared at a concentration of 0.01 mg/mL and divided into three parts immediately after preparation. The first part was injected once after injecting the vehicle blank. The second part was stored at 2-8 °C in a refrigerator, while the third part was stored at controlled room temperature. These stored solutions were injected once at 4 h and 24 h. The % assay was calculated for each set of samples. The stability of one marker in solution stored at 2-8 °C and controlled room temperature was evaluated as percentage relative error (% RE). The % assay and % RE were calculated using the following formula:

\[
\text{Drug content (mg/mL)} = \frac{\text{[A2} \times \text{C1} \times \text{D]}}{\text{[A1]}}
\]

\[
\% \text{ Assay} = \frac{\text{[Drug Content} \times 100]}{\text{Label claim}}
\]

Where,

\[
\text{A1} = \text{Mean peak area of system suitability}
\]

\[
\text{A2} = \text{Peak area of test sample}
\]

\[
\text{C1} = \text{Concentration of standard solution (mg/mL)}
\]

\[
\text{D} = \text{Dilution factor}
\]

\[
\text{Percentage Relative Error (% RE)} = \frac{(\text{Area of stored sample} - \text{Area of initial sample}) \times 100}{\text{Area of initial sample}}
\]

2.7.9. Formulation stability

Vehicle blank and samples were diluted to the nominal analyte concentration as described in Test Sample Preparation. The vehicle blank and samples were injected at different time intervals. The % assay for initial samples and samples stored at CRT and 2-8 °C was calculated by drawing samples from top, middle and bottom layers of the formulations. Homogeneity of quantified marker was evaluated as percentage relative standard deviation (%RSD). The five markers were identified in unspiked 10 and 100 mg/mL formulations.

2.8. Study design of in vivo experiment

2.8.1. Grouping and allocation of animals

Healthy rats were grouped and allocated to their respective treatment groups using stratified randomized design using Microsoft Excel®. It was ensured that mean body weights of each group before the start of the treatment are not significantly different from each other (variation were less than 20% of the mean body weight for each sex). The experiment was conducted in two phases, i.e. Phase I and Phase II according to OECD guidelines and ICH S2 (R1) [4,5].
2.8.2. Phase I: Dose range finding experiment

The dose range finding experiment was carried out in 3 male and 3 female rats at the doses of 300, 600, 1000 and 2000 mg/kg/day along with a vehicle control with the objective to assess general toxicity and bone marrow cytotoxicity of the test item. This data served as a basis for the dose selection for the Phase-II (Definitive experiment). Phase I experimental details are given in the table below:

Synacin™ was administered to Sprague Dawley rats by oral gavage for two consecutive days at an interval of approximately 24 h. The dose volumes administered was at an equipotitive of 10 mL/kg body weight (300, 600 and 1000 mg/kg/day) and 20 mL/kg body weight (vehicle control and 2000 mg/kg/day). The animals were sacrificed approximately 24 h from the last treatment. The observations included mortality/moribundity, body weight and clinical signs. The femur bone marrow was aspirated, smears prepared and stained. The ratio of PCE: total erythrocytes was determined. Based on these findings, doses of 500, 1000 and 2000 mg/kg/day were selected for Phase II (Definitive) of the study.

2.8.3. Phase II: Definitive experiment

Phase II was conducted with the objective to assess clastogenicity of the test item. Two days oral dosing regime separated by approximately 24 h was followed for treatment of animals and observations included mortality/moribundity, body weight and clinical signs. Microscopic analysis of the slides included bone marrow toxicity evaluation (determination of the proportion of immature erythrocytes-PCE/E ratio) and MNPCE counts.

2.8.4. Dose administration, duration of treatment and dosing procedure

Each animal within a dose group received the vehicle or test item by oral gavage. Individual dose volumes were calculated based on the Day 1 body weight for each phase. Duration of treatment was once daily for two consecutive days under fed conditions for both the phases of the study. Dose administration was carried out using stainless steel gavage needle fitted onto a disposable plastic syringe from a calibrated batch. Positive control was administered as a single intraperitoneal injection on Day 2 of dosing at 25 mg/kg only in Phase II of the study. Care was taken to avoid unintentional aspiration of the formulation into the airways during dosing.

2.9. Observations

2.9.1. Mortality

All animals were observed for mortality/moribundity twice daily i.e., once in the morning and once in the evening.

2.9.2. Clinical signs of toxicity

A routine clinical examination was performed twice daily (pre dose and post dose) for all the experimental animals. The post dose observations were carried out at least 0.5 h after the dose administration and completed within 2 h post dose for each animal.

2.9.3. Body weight

Individual animal body weights were recorded prior to dosing on Day 1 for all the animals and on Day 3 (prior to sacrifice).

2.10. Bone marrow evaluation

2.10.1. Animal sacrifice and bone marrow collection

Animals were sacrificed by CO₂ asphyxiation approximately 18–24 h post second dosing. The femurs were isolated from each animal for bone marrow collection. The epiphyses of the femur
were cut open and the bone marrow were flushed with fetal bovine serum into a centrifuge tube. The bone marrow cells were pelleted by centrifugation at approximately 1000 rpm for 5 min at room temperature. Supernatant was drawn off, leaving a small amount of fetal bovine serum with the remaining cell pellet. A homogeneous suspension of bone marrow cells was prepared and 5–10 μL of the bone marrow suspension was spread onto a clean glass slide. Smears were fixed using methanol. From each animal, two slides were prepared for Phase I and four slides for Phase II, respectively. All the slides were coded before subjecting to analysis for both the Phases of the Study.

2.10.2. Bone marrow toxicity: determination of proportion of immature erythrocytes
Bone marrow toxicity was evaluated by determination of the proportion of immature erythrocytes (PCEs) to total erythrocytes (immature + mature). A reduction in the proportion of immature cells among total (immature + mature) when compared with the respective vehicle control was considered as a measure of bone marrow toxicity. In order to assess the proportion of PCEs to Total Erythrocytes, methanol fixed slides were stained with May Grunwald's Giemsa and at least 500 erythrocytes from each animal were evaluated for both Phase I and II.

2.10.3. Determination of micronucleated PCEs (MN PCEs)
Methanol fixed slides obtained from Phase II main group animals were stained with Acridine Orange for the estimation of MN PCEs. Using a fluorescent microscope and medium magnification, an area of acceptable quality was selected where the cells were well spread and stained. Using oil immersion, ≥ 4000 PCEs were scored per animal for the presence of micronuclei. The unit of scoring was micronucleated cell, not the micronucleus; thus, the occasional cell with more than one micronucleus was counted as one MN PCE, not two. The Acridine Orange staining method is temporary and therefore all smears stained with acridine orange were discarded following completion of the experiment. From the observations, the following were determined for each animal; Total RBC/erythrocytes scored, number of PCEs differentiated, number of PCE with micronuclei, mean and SD of PCE with micronuclei, and ratio of PCE: Total RBC.

2.11. Statistical analysis
Male and female animal data was considered separately for analysis, as applicable. Body weight, percent body weight change, number of PCEs, total erythrocytes, PCE/E ratio and the frequency of MN PCEs for each animal, and the mean and standard deviation for each group were calculated. Body weight, percent body weight change and proportion of Immature Erythrocytes among total erythrocytes for different groups were analyzed by one-way analysis of variance (ANOVA). If ANOVA indicates a significant difference (p < 0.05) between different groups, a paired comparison was done by Dunnet's test. The number of MN PCEs in each treatment group was compared with the MN PCE in concurrent control group by 2 × 2 contingency Chi square test. All analyses and comparisons were evaluated at the 5% (p < 0.05) level using SigmaPlot®, Version 12.5.

Ethics Statement
All experiments were conducted with the approved procedures of the Institutional Animal Ethics Committee (IAEC) (Reference No.: APSL/SE/007-18/08-2020).

CRediT Author Statement
Siti Nurazwa Zainol: Investigation; Anis Fadhлина: Writing - original draft, Writing - review & editing; Sri Vijaya Rentala: Project administration; Renuka Pillai: Project administra-
tion; **Manjula Yalaka**: Formal analysis, Investigation; **Indu Bansal**: Formal analysis, Investigation; **Earati Surender**: Formal analysis, Investigation; **Leela Krishna Vatsavai**: Formal analysis, Investigation; **Rajesh Eswarappa**: Formal analysis, Investigation; **Hassan Fahmi Ismail**: Writing - review & editing; **Fadzilah Adibah Abdul Majid**: Conceptualization, Supervision.

**Declaration of Competing Interest**

The following authors; Sri Vijaya Rentala, Renuka Pillai, Manjula Yalaka, Indu Bansal, Earati Surender, Leela Krishna Vatsavai, and Rajesh Eswarappa are affiliated to Aurigene Pharmaceutical Services Limited. All authors declare that the article content was composed in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Supplementary Materials**

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.dib.2021.107001.

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