Data Article

Synacinn™: Bacterial reverse mutation test data in five histidine-requiring strains of *Salmonella Typhimurium*

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**Abstract**

The present data described the analysis of mutagenicity in Synacinn™ by assessing the point mutations occurring due to Synacinn™ exposure to five tester strains of *Salmonella typhimurium* (TA1537, TA1535, TA98, TA100 and TA102), in the presence or absence of an exogenous mammalian metabolic activation system (S9). It was conducted in two Phases - Phase I (Dose Range Finding experiment-DRF) and Phase II (Mutagenicity Assay 1 and 2). DRF and Mutagenicity Assay 1 was conducted employing plate incorporation method, while Mutagenicity Assay 2 was performed using pre-incubation method. Formulation analysis pertaining to Synacinn™ was performed for both Mutagenicity Assay 1 and 2. Dose formulations were prepared fresh on each day of the experiment. Adventol 50% v/v in purified water was selected as a suitable vehicle based on the preliminary solubility test. Based on the Phase I analysis, 5 mg/plate was selected as the highest concentration of Synacinn™ followed by lower concentrations...
of 2.5, 1.25, 0.625 and 0.313 mg/plate for the Mutagenicity Assays. Genetic integrity of all the tester strains used was confirmed by performing genotyping before their use. All the data acceptability criteria were fulfilled confirming the validity of the test.

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### Specifications Table

| Subject | Biological sciences |
|---------|---------------------|
| Specific subject area | Microbiology: Applied Microbiology |
| Type of data | Table |
| How data were acquired | Revertant colonies grown on Minimal Agar plates were counted manually. Background bacterial lawn was observed using microscope and scored. |
| Data format | Raw |
| Parameters for data collection | After incubation for 48-72 hours (DRF and Mutagenicity Assay 1 and 2), plates were removed from the incubator and each treatment plate was assessed for precipitation and cytotoxicity. |
| Description of data collection | Precipitation was graded based on the amount of visible precipitate on the plate. Cytotoxicity was assessed in terms of diminution of background bacterial lawn, presence of micro colonies and/or reduction in the number of revertant colonies. The fold increase in revertant colony counts for each test item treatment versus the vehicle control group was determined to assess the mutagenic potential of Synacinn™. |
| 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 mutagenicity potential of Synacinn™ using five tester strains of Salmonella typhimurium (TA1537, TA1535, TA98, TA100 and TA102) in the absence and presence of an exogenous metabolic activation system (S9).
- These data are useful for researchers who want to determine the mutagenicity potential of their polyherbal formulations.
- Synacinn™ is currently approved by National Pharmaceutical Regulatory Agency (NPRA), Malaysia as traditional medicine with a general health claim. Data on the mutagenicity potential of Synacinn™ are valuable to establish the safety pharmacology for Synacinn™ and can be used in future for the development process and approval of Synacinn™ as a prescription botanical medicine for diabetes.

### 1. Data Description

All markers identified in Synacinn™ formulations (Mutagenicity assay 1 and 2: raw data as in Supplementary Table 1S) was presented in Table 1 for all dose strengths (6.25 mg/mL, 12.5 mg/mL, 25 mg/mL and 50 mg/mL). Table 2 shows the peak area of catechin from HPLC analysis of top, middle and bottom layers of Synacinn™ formulations in triplicate. In the Dose Range
Table 1
Identification (Retention Time) of markers in Synacinn™ formulation.

| Formulation dose | Retention time (Mutagenicity Assay 1) |
|------------------|--------------------------------------|
|                  | Gallic acid | Catechin | Rosmarinic acid | Andrographolide | Curcumin       |
| 6.25-25mg/mL     | 5.212-5.328 | 11.668-11.910 | 19.862-19.915 | 21.533-21.651 | 26.732-26.958 |

Table 2
Peak area of catechin in Synacinn™ formulation analysis.

| Formulation layers | Area (Mutagenicity Assay 1) |
|--------------------|-----------------------------|
|                    | 6.25 mg/mL | 12.5 mg/mL | 25 mg/mL | 50 mg/mL |
| Top-1              | 50992    | 53350     | 42421   | 39714   |
| Top-2              | 56718    | 46548     | 43463   | 39219   |
| Top-3              | 47900    | 55111     | 40496   | 40956   |
| Middle-1           | 43940    | 64415     | 42195   | 40151   |
| Middle-2           | 58521    | 48852     | 41151   | 39475   |
| Middle-3           | 52010    | 57305     | 41949   | 38905   |
| Bottom-1           | 55076    | 56279     | 41881   | 40662   |
| Bottom-2           | 51249    | 49904     | 43131   | 39586   |
| Bottom-3           | 48223    | 53091     | 42998   | 39996   |
| Mean Area          | 51625.4  | 53872.8   | 42087.2 | 38851.6 |
| SD                 | 4617.9217| 5311.9905 | 906.6083| 3018.9915|
| %RSD               | 8.9      | 9.9       | 2.2     | 7.8     |

| Formulation layers | Area (Mutagenicity Assay 2) |
|--------------------|-----------------------------|
|                    | 6.25 mg/mL | 12.5 mg/mL | 25 mg/mL | 50 mg/mL |
| Top-1              | 45426     | 53383     | 35934   | 36531   |
| Top-2              | 45522     | 51243     | 34962   | 37525   |
| Top-3              | 45784     | 53550     | 37015   | 35712   |
| Middle-1           | 46193     | 50536     | 37896   | 36889   |
| Middle-2           | 46473     | 49688     | 39045   | 37029   |
| Middle-3           | 46819     | 51242     | 37510   | 36664   |
| Bottom-1           | 46586     | 50209     | 37135   | 37147   |
| Bottom-2           | 44474     | 52464     | 38408   | 35607   |
| Bottom-3           | 45015     | 50287     | 36925   | 36165   |
| Mean Area          | 45810.2   | 51400.2   | 37203.3 | 36585.4 |
| SD                 | 778.9826  | 1417.3561 | 1233.3481| 651.2646|
| %RSD               | 1.7       | 2.8       | 3.3     | 1.8     |

Finding experiment, Synacinn™ was tested for cytotoxicity and precipitation in the tester strain TA100 at concentrations ranging from 0.039 to 5 mg/plate (Table 3). No signs of precipitation or cytotoxicity (in terms of reduction of revertant colony count and or diminution of bacterial background lawn) was observed up to the highest tested concentration of 5 mg/plate. Hence, 5 mg/plate was selected as the highest dose for Phase II, followed by 4 lower concentrations separated by factor of 2. Data on the revertant colony counts and observation on the precipitation and background lawn of five selected strains obtained in Phase I and Phase II (Mutagenicity Assay 1 & 2) with or without the presence of an exogenous metabolic activation system (S9) are presented in Tables 4–13. Each of the S. typhimurium strains (TA1537, TA1535, TA98, TA100 and TA102) data is individually presented in the Tables 4–13.
### Table 3
Data of dose range finding experiment by plate incorporation method.

| Test Item          | Treatment | DRF - Plate Incorporation | Organism: TA100 |
|--------------------|-----------|---------------------------|-----------------|
|                    |           | No. of Revertants         |                 |
|                    | Untreated control | 198 203 184 | 195 9.8 | NR, NP  |
|                    | Without S9 |                          |                 |
| Adventol (50% v/v) | 0         | 178 206 211              | 198 17.8 | NR, NP  |
| Synacin<sup>TM</sup> (mg/plate) | 0.039 | 218 246 237 | 234 14.3 | NR, NP  |
|                    | 0.078     | 205 252 209              | 222 26.1 | NR, NP  |
|                    | 0.156     | 221 243 238              | 234 11.5 | NR, NP  |
|                    | 0.313     | 247 257 225              | 243 16.4 | NR, NP  |
|                    | 0.625     | 251 205 209              | 222 25.5 | NR, NP  |
| Sodium azide       | 1.25      | 240 219 233              | 231 10.7 | NR, NP  |
|                    | 2.5       | 219 232 213              | 221 9.7  | NR, NP  |
|                    | 5         | 199 226 234              | 220 18.3 | NR, NP  |
|                    | With S9   |                          |                 |
| Adventol (50% v/v) | 0         | 187 234 191              | 204 26.1 | NR, NP  |
| Synacin<sup>TM</sup> (mg/plate) | 0.039 | 248 202 201 | 217 26.9 | NR, NP  |
|                    | 0.078     | 261 220 230              | 237 21.4 | NR, NP  |
|                    | 0.156     | 238 230 212              | 227 13.3 | NR, NP  |
|                    | 0.313     | 201 198 200              | 200 1.5   | NR, NP  |
|                    | 0.625     | 227 273 207              | 236 33.8 | NR, NP  |
| Sodium azide       | 1.25      | 195 236 228              | 220 21.7 | NR, NP  |
|                    | 2.5       | 238 237 221              | 232 9.5   | NR, NP  |
|                    | 5         | 224 231 208              | 221 11.8 | NR, NP  |
| 2AA (μg/plate)     | 5         | 1550 1350 1456           | 1452 100.1 | NR, NP  |

2AA: 2-Aminoanthracene; NR: No reduction in bacterial background lawn; NP: No precipitation

### Table 4
Mutagenicity assay 1 by plate incorporation method on TA1537.

| Test Item          | Treatment | Mutagenicity assay 1 - Plate Incorporation | Organism: TA1537 |
|--------------------|-----------|-------------------------------------------|-----------------|
|                    |           | No. of Revertants                         |                 |
|                    | Untreated control | 12 18 10 | 13 4.2 | NR, NP  |
|                    | Without S9 |                          |                 |
| Adventol (50% v/v) | 0         | 11 11 14 | 12 1.7 | NR, NP  |
| Synacin<sup>TM</sup> (mg/plate) | 0.313 | 11 8 8 | 9 1.7 | NR, NP  |
|                    | 0.625     | 6 10 5 | 7 2.6 | NR, NP  |
|                    | 1.25      | 13 6 14 | 11 4.4 | NR, NP  |
|                    | 2.5       | 10 8 13 | 10 2.5 | NR, NP  |
|                    | 5         | 18 8 15 | 14 5.1 | NR, NP  |
| ICR-191 (μg/plate) | 1         | 182 165 173 | 173 8.5 | NR, NP  |
|                    | With S9   |                          |                 |
| Adventol (50% v/v) | 0         | 12 13 17 | 14 2.6 | NR, NP  |
| Synacin<sup>TM</sup> (mg/plate) | 0.313 | 16 11 11 | 13 2.9 | NR, NP  |
|                    | 0.625     | 14 14 10 | 13 2.3 | NR, NP  |
|                    | 1.25      | 12 13 12 | 12 0.6 | NR, NP  |
|                    | 2.5       | 16 8 10 | 11 4.2 | NR, NP  |
|                    | 5         | 10 10 11 | 10 0.6 | NR, NP  |
| 2AA (μg/plate)     | 5         | 252 216 208 | 225 23.4 | NR, NP  |

2AA: 2-Aminoanthracene; NR: No reduction in bacterial background lawn; NP: No precipitation
### Table 5
Mutagenicity assay 1 by plate incorporation method on TA1535.

| Test Item                | Treatment | R1 | R2 | R3 | Mean | SD  | Observation | Fold increase |
|--------------------------|-----------|----|----|----|------|-----|-------------|---------------|
| Untreated control        | -         | 23 | 13 | 17 | 18   | 5.0 | NR, NP      | -             |
| **Without S9**           |           |    |    |    |      |     |             |               |
| Adventol (50% v/v)       | 0         | 19 | 14 | 20 | 18   | 3.2 | NR, NP      | -             |
| Synacinn™ (mg/plate)     | 0.313     | 17 | 26 | 15 | 19   | 5.9 | NR, NP      | 1.06          |
|                          | 0.625     | 27 | 25 | 12 | 21   | 8.1 | NR, NP      | 1.17          |
|                          | 1.25      | 28 | 26 | 11 | 22   | 9.3 | NR, NP      | 1.22          |
|                          | 2.5       | 22 | 14 | 17 | 18   | 4.0 | NR, NP      | 1.00          |
| Sodium azide (μg/plate)  | 5         | 960| 1120| 1026| 1035 | 80.4| NR, NP      | 57.50         |
| **With S9**              |           |    |    |    |      |     |             |               |
| Adventol (50% v/v)       | 0         | 17 | 9  | 15 | 14   | 4.2 | NR, NP      | -             |
| Synacinn™ (mg/plate)     | 0.313     | 10 | 8  | 19 | 12   | 5.9 | NR, NP      | 0.86          |
|                          | 0.625     | 11 | 10 | 11 | 11   | 0.6 | NR, NP      | 0.79          |
|                          | 1.25      | 6  | 14 | 12 | 11   | 4.2 | NR, NP      | 0.79          |
|                          | 2.5       | 12 | 5  | 9  | 9    | 3.5 | NR, NP      | 0.64          |
|                          | 5         | 10 | 10 | 8  | 9    | 1.2 | NR, NP      | 0.64          |
| 2AA (μg/plate)           | 5         | 169| 258| 196| 208  | 45.6| NR, NP      | 11.56         |

2AA: 2-Aminoanthracene; NR: No reduction in bacterial background lawn; NP: No precipitation

### Table 6
Mutagenicity assay 1 by plate incorporation method on TA98.

| Test Item                | Treatment | R1 | R2 | R3 | Mean | SD  | Observation | Fold increase |
|--------------------------|-----------|----|----|----|------|-----|-------------|---------------|
| Untreated control        | -         | 46 | 63 | 52 | 54   | 8.6 | NR, NP      | -             |
| **Without S9**           |           |    |    |    |      |     |             |               |
| Adventol (50% v/v)       | 0         | 68 | 49 | 57 | 58   | 9.5 | NR, NP      | -             |
| Synacinn™ (mg/plate)     | 0.313     | 69 | 81 | 58 | 69   | 11.5| NR, NP      | 1.19          |
|                          | 0.625     | 82 | 62 | 72 | 72   | 10.0| NR, NP      | 1.24          |
|                          | 1.25      | 81 | 64 | 50 | 65   | 15.5| NR, NP      | 1.12          |
|                          | 2.5       | 79 | 43 | 61 | 61   | 18.0| NR, NP      | 1.05          |
| 2-Nitrofluorene (μg/plate)| 5         | 76 | 57 | 51 | 61   | 13.1| NR, NP      | 1.05          |
| **With S9**              |           |    |    |    |      |     |             |               |
| Adventol (50% v/v)       | 0         | 49 | 52 | 50 | 50   | 1.5 | NR, NP      | -             |
| Synacinn™ (mg/plate)     | 0.313     | 59 | 52 | 51 | 54   | 4.4 | NR, NP      | 1.08          |
|                          | 0.625     | 56 | 62 | 42 | 53   | 10.3| NR, NP      | 1.06          |
|                          | 1.25      | 65 | 46 | 58 | 56   | 9.6 | NR, NP      | 1.12          |
|                          | 2.5       | 63 | 48 | 45 | 52   | 9.6 | NR, NP      | 1.04          |
| 2AA (μg/plate)           | 5         | 1462| 1650| 1572| 1561 | 94.5| NR, NP      | 28.91         |

2AA: 2-Aminoanthracene; NR: No reduction in bacterial background lawn; NP: No precipitation
Table 7
Mutagenicity assay 1 by plate incorporation method on TA100.

| Test Item                      | Treatment | No. of Revertants | Mean | SD  | Observation | Fold increase |
|--------------------------------|-----------|-------------------|------|-----|-------------|---------------|
| Untreated control              | -         | 178, 197, 194     | 190  | 10.2| NR, NP      | -             |

**Without S9**

| Test Item                      | Treatment | No. of Revertants | Mean | SD  | Observation | Fold increase |
|--------------------------------|-----------|-------------------|------|-----|-------------|---------------|
| Adventol (50% v/v)             | 0         | 226, 189, 178     | 198  | 25.1| NR, NP      | -             |
| Synacinn™ (mg/plate)           | 0.313     | 181, 224, 215     | 207  | 22.7| NR, NP      | 1.05          |
| Synacinn™ (mg/plate)           | 0.625     | 153, 186, 191     | 177  | 20.6| NR, NP      | 0.89          |
| Synacinn™ (mg/plate)           | 1.25      | 163, 223, 184     | 190  | 30.4| NR, NP      | 0.96          |
| Synacinn™ (mg/plate)           | 2.5       | 185, 185, 203     | 191  | 10.4| NR, NP      | 0.96          |
| Synacinn™ (mg/plate)           | 5         | 187, 188, 182     | 186  | 3.2 | NR, NP      | 0.94          |
| Sodium azide (µg/plate)        | 5         | 179, 211, 186     | 192  | 16.8| NR, NP      | 1.01          |
| Sodium azide (µg/plate)        | 5         | 1650, 1580, 1768  | 1666 | 95.0| NR, NP      | 8.77          |

**With S9**

| Test Item                      | Treatment | No. of Revertants | Mean | SD  | Observation | Fold increase |
|--------------------------------|-----------|-------------------|------|-----|-------------|---------------|
| Adventol (50% v/v)             | 0         | 213, 225, 235     | 224  | 11.0| NR, NP      | -             |
| Synacinn™ (mg/plate)           | 0.313     | 214, 220, 211     | 215  | 4.6 | NR, NP      | 0.96          |
| Synacinn™ (mg/plate)           | 0.625     | 198, 207, 186     | 197  | 10.5| NR, NP      | 0.88          |
| Synacinn™ (mg/plate)           | 1.25      | 201, 221, 208     | 210  | 10.1| NR, NP      | 0.94          |
| Synacinn™ (mg/plate)           | 2.5       | 223, 230, 209     | 221  | 10.7| NR, NP      | 0.99          |
| Synacinn™ (mg/plate)           | 5         | 207, 219, 220     | 215  | 7.2 | NR, NP      | 0.96          |
| Sodium azide (µg/plate)        | 5         | 2636, 2260, 2060  | 2319 | 292.4| NR, NP      | 12.21         |

2AA: 2-Aminoanthracene; NR: No reduction in bacterial background lawn; NP: No precipitation

## 2. Experimental Design, Materials and Methods

### 2.1. Formulation analysis

Synacinn™ powder was provided by Proliv Life Sciences Sdn Bhd and standardized with five selected markers as follows: rosmarinic acid – 0.41% w/w, andrographolide – 0.25% w/w, catechin – 1.48% w/w, curcumin – 0.11% w/w and gallic acid – 1.56% w/w. 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. All test items were stored at room temperature protected from light. Identification of Synacinn™ markers was done for the formulation analysis by comparing the retention time of each marker in all the dose strengths against the identification solution. Only one marker (catechin) was quantified by reporting the peak area and Percentage Relative Standard Deviation (%RSD = Standard Deviation/ Mean x 100; acceptance limit of %RSD ≤ 20%) to evaluate homogeneity of the formulations [1]. All formulations (6.25, 12.5, 25, 50 mg/mL) were pipetted out from top, middle and bottom layer in triplicates, injected once into HPLC and chromatograms were recorded.

#### 2.1.1. Marker stock solution preparation

Each marker was weighed (2 mg) transferred into five separate 10 mL volumetric flask. 5 mL of methanol was added to each flask to dissolve completely and made up the volume to 10 mL with water and mix well.

#### 2.1.2. Marker solution preparation (0.01 mg/mL)

Each of the marker stock solution 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.
Table 8
Mutagenicity assay 1 by plate incorporation method on TA102.

| Test Item          | Treatment | R1   | R2   | R3   | Mean | SD  | Observation | Fold increase |
|--------------------|-----------|------|------|------|------|-----|-------------|---------------|
| Untreated control  | -         | 464  | 474  | 481  | 473  | 8.5 | NR, NP      | -             |

Without S9

| Test Item          | Treatment | R1   | R2   | R3   | Mean | SD  | Observation | Fold increase |
|--------------------|-----------|------|------|------|------|-----|-------------|---------------|
| Adventol (50% v/v) | 0         | 561  | 535  | 517  | 538  | 22.1| NR, NP      | -             |
| Synacinn™ (mg/plate)| 0.313     | 477  | 474  | 495  | 482  | 11.4| NR, NP      | 0.90          |
|                   | 0.625     | 494  | 550  | 537  | 527  | 29.3| NR, NP      | 0.98          |
|                   | 1.25      | 543  | 551  | 530  | 541  | 10.6| NR, NP      | 1.01          |
|                   | 2.5       | 510  | 539  | 476  | 508  | 31.5| NR, NP      | 0.94          |
|                   | 5         | 502  | 528  | 550  | 527  | 24.0| NR, NP      | 0.98          |
| Ametycin (μg/plate)| 0.5       | 2110 | 1980 | 1896 | 1995 | 107.8| NR, NP      | 4.22          |

With S9

| Test Item          | Treatment | R1   | R2   | R3   | Mean | SD  | Observation | Fold increase |
|--------------------|-----------|------|------|------|------|-----|-------------|---------------|
| Adventol (50% v/v) | 0         | 517  | 498  | 540  | 518  | 21.0| NR, NP      | -             |
| Synacinn™ (mg/plate)| 0.313     | 499  | 512  | 530  | 514  | 15.6| NR, NP      | 0.99          |
|                   | 0.625     | 552  | 560  | 440  | 517  | 67.1| NR, NP      | 1.00          |
|                   | 1.25      | 544  | 541  | 582  | 556  | 22.9| NR, NP      | 1.07          |
|                   | 2.5       | 507  | 486  | 520  | 504  | 17.2| NR, NP      | 0.97          |
|                   | 5         | 465  | 538  | 480  | 494  | 38.6| NR, NP      | 0.95          |
| 2AA (μg/plate)    | 10        | 2012 | 1996 | 1890 | 1966 | 66.3| NR, NP      | 4.16          |

2AA: 2-Aminoanthracene; NR: No reduction in bacterial background lawn; NP: No precipitation

Table 9
Mutagenicity assay 2 by pre-incubation method on TA1537.

| Test Item          | Treatment | R1   | R2   | R3   | Mean | SD  | Observation | Fold increase |
|--------------------|-----------|------|------|------|------|-----|-------------|---------------|
| Untreated control  | -         | 4    | 4    | 9    | 6    | 2.9 | NR, NP      | -             |

Without S9

| Test Item          | Treatment | R1   | R2   | R3   | Mean | SD  | Observation | Fold increase |
|--------------------|-----------|------|------|------|------|-----|-------------|---------------|
| Adventol (50% v/v) | 0         | 8    | 7    | 4    | 6    | 2.1 | NR, NP      | -             |
| Synacinn™ (mg/plate)| 0.313     | 6    | 10   | 3    | 6    | 3.5 | NR, NP      | 1.00          |
|                   | 0.625     | 5    | 4    | 6    | 5    | 1.0 | NR, NP      | 0.83          |
|                   | 2.5       | 9    | 5    | 3    | 6    | 3.1 | NR, NP      | 1.00          |
|                   | 5         | 9    | 7    | 5    | 7    | 2.0 | NR, NP      | 1.17          |
| ICR-191 (μg/plate)| 1         | 210  | 225  | 197  | 211  | 14.0| NR, NP      | 35.17         |

With S9

| Test Item          | Treatment | R1   | R2   | R3   | Mean | SD  | Observation | Fold increase |
|--------------------|-----------|------|------|------|------|-----|-------------|---------------|
| Adventol (50% v/v) | 0         | 14   | 7    | 8    | 10   | 3.8 | NR, NP      | -             |
| Synacinn™ (mg/plate)| 0.313     | 9    | 14   | 8    | 10   | 3.2 | NR, NP      | 1.00          |
|                   | 0.625     | 5    | 11   | 8    | 8    | 3.0 | NR, NP      | 0.80          |
|                   | 2.5       | 9    | 10   | 6    | 8    | 2.1 | NR, NP      | 0.80          |
|                   | 5         | 11   | 4    | 7    | 7    | 3.5 | NR, NP      | 0.70          |
| 2AA (μg/plate)    | 5         | 188  | 214  | 175  | 192  | 19.9| NR, NP      | 32.00         |

2AA: 2-Aminoanthracene; NR: No reduction in bacterial background lawn; NP: No precipitation

2.1.3. Identification solution preparation

Synacinn was weighed (50 mg) and transferred into 10 mL volumetric flask. 5 mL of diluent was added and each of the marker stock solution was spiked (0.5 mL) into 10 mL volumetric flask. The volume was made up to 10 mL with diluent and mixed well. The solution was
### Table 10
Mutagenicity assay 2 by pre-incubation method on TA1535.

| Test Item                  | Treatment | R1 | R2 | R3 | Mean | SD  | Observation | Fold increase |
|----------------------------|-----------|----|----|----|------|-----|-------------|---------------|
| Untreated control          | -         | 18 | 18 | 14 | 17   | 2.3 | NR, NP      | -             |
| Adventol (50% v/v)         | 0         | 10 | 20 | 20 | 17   | 5.8 | NR, NP      | -             |
| Synacinn™ (mg/plate)       | 0.313     | 16 | 17 | 20 | 18   | 2.1 | NR, NP      | 1.06          |
|                            | 0.625     | 13 | 13 | 9  | 12   | 2.3 | NR, NP      | 0.71          |
|                            | 1.25      | 12 | 17 | 11 | 13   | 3.2 | NR, NP      | 0.76          |
|                            | 2.5       | 13 | 11 | 27 | 17   | 8.7 | NR, NP      | 1.00          |
| Sodium azide (μg/plate)    | 5         | 17 | 13 | 14 | 15   | 2.1 | NR, NP      | 0.88          |

**Without S9**

| Test Item                  | Treatment | R1 | R2 | R3 | Mean | SD  | Observation | Fold increase |
|----------------------------|-----------|----|----|----|------|-----|-------------|---------------|
| Adventol (50% v/v)         | 0         | 7  | 7  | 9  | 8    | 1.2 | NR, NP      | -             |
| Synacinn™ (mg/plate)       | 0.313     | 5  | 10 | 10 | 8    | 2.9 | NR, NP      | 1.00          |
|                            | 0.625     | 11 | 7  | 7  | 8    | 2.3 | NR, NP      | 1.00          |
|                            | 1.25      | 6  | 7  | 12 | 8    | 3.2 | NR, NP      | 1.00          |
|                            | 2.5       | 13 | 6  | 8  | 9    | 3.6 | NR, NP      | 1.13          |
| Sodium azide (μg/plate)    | 5         | 12 | 14 | 14 | 10   | 4.7 | NR, NP      | 1.25          |

**With S9**

| Test Item                  | Treatment | R1 | R2 | R3 | Mean | SD  | Observation | Fold increase |
|----------------------------|-----------|----|----|----|------|-----|-------------|---------------|
| Adventol (50% v/v)         | 0         | 7  | 7  | 9  | 8    | 1.2 | NR, NP      | -             |
| Synacinn™ (mg/plate)       | 0.313     | 5  | 10 | 10 | 8    | 2.9 | NR, NP      | 1.00          |
|                            | 0.625     | 11 | 7  | 7  | 8    | 2.3 | NR, NP      | 1.00          |
|                            | 1.25      | 6  | 7  | 12 | 8    | 3.2 | NR, NP      | 1.00          |
|                            | 2.5       | 13 | 6  | 8  | 9    | 3.6 | NR, NP      | 1.13          |
| Sodium azide (μg/plate)    | 5         | 12 | 14 | 14 | 10   | 4.7 | NR, NP      | 1.25          |

| Test Item                  | Treatment | R1 | R2 | R3 | Mean | SD  | Observation | Fold increase |
|----------------------------|-----------|----|----|----|------|-----|-------------|---------------|
| Adventol (50% v/v)         | 0         | 59 | 60 | 71 | 63   | 6.7 | NR, NP      | -             |
| Synacinn™ (mg/plate)       | 0.313     | 47 | 64 | 61 | 57   | 9.1 | NR, NP      | 0.90          |
|                            | 0.625     | 40 | 53 | 45 | 46   | 6.6 | NR, NP      | 0.73          |
|                            | 1.25      | 42 | 53 | 64 | 53   | 11.0| NR, NP      | 0.84          |
|                            | 2.5       | 38 | 55 | 43 | 45   | 8.7 | NR, NP      | 0.71          |
| 2-Nitrofluorene (μg/plate) | 5         | 61 | 90 | 75 | 75   | 14.5| NR, NP      | 1.19          |

| Test Item                  | Treatment | R1 | R2 | R3 | Mean | SD  | Observation | Fold increase |
|----------------------------|-----------|----|----|----|------|-----|-------------|---------------|
| Adventol (50% v/v)         | 0         | 47 | 39 | 35 | 40   | 6.1 | NR, NP      | -             |
| Synacinn™ (mg/plate)       | 0.313     | 27 | 44 | 37 | 36   | 8.5 | NR, NP      | 0.90          |
|                            | 0.625     | 34 | 33 | 35 | 34   | 1.0 | NR, NP      | 0.85          |
|                            | 1.25      | 39 | 32 | 40 | 37   | 4.4 | NR, NP      | 0.93          |
|                            | 2.5       | 30 | 39 | 35 | 35   | 4.5 | NR, NP      | 0.88          |
|                            | 5         | 46 | 32 | 45 | 41   | 7.8 | NR, NP      | 1.03          |

2AA: 2-Aminoanthracene; NR: No reduction in bacterial background lawn; NP: No precipitation

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2AA: 2-Aminoanthracene; NR: No reduction in bacterial background lawn; NP: No precipitation

...centrifuged at 5000 rpm × 3360 g for 5 minutes. The supernatant solution was transferred into HPLC vials and injected into HPLC...
Table 12
Mutagenicity assay 2 by pre-incubation method on TA100.

| Test Item                  | Treatment | No. of Revertants | Mean | SD | Observation | Fold increase |
|----------------------------|-----------|-------------------|------|----|-------------|---------------|
| Untreated control          |           | R1 | R2 | R3 |           |               |
| Adventol (50% v/v)         | 0         | 181| 172| 158| 170       | 11.6          | NR, NP        |
| Synacinn™ (mg/plate)       | 0.313     | 126| 184| 188| 166       | 34.7          | NR, NP        | 0.98          |
|                            | 0.625     | 160| 143| 142| 148       | 10.1          | NR, NP        | 0.87          |
|                            | 1.25      | 192| 140| 140| 157       | 30.0          | NR, NP        | 0.92          |
|                            | 2.5       | 175| 157| 153| 162       | 11.7          | NR, NP        | 0.95          |
|                            | 5         | 180| 128| 152| 153       | 26.0          | NR, NP        | 0.90          |
| Sodium azide (μg/plate)    | 5         | 157| 180| 174| 170       | 11.9          | NR, NP        | 1.26          |
| 2AA (μg/plate)             |           | 1478| 1528| 1618| 1541   | 70.9          | NR, NP        | 11.41         |
| (Without S9)               |           |     |    |    |          |               |
| Adventol (50% v/v)         | 0         | 192| 178| 169| 180       | 11.6          | NR, NP        | -             |
| Synacinn™ (mg/plate)       | 0.313     | 199| 187| 163| 183       | 18.3          | NR, NP        | 1.02          |
|                            | 0.625     | 177| 197| 125| 166       | 37.2          | NR, NP        | 0.92          |
|                            | 1.25      | 160| 161| 162| 161       | 1.0           | NR, NP        | 0.89          |
|                            | 2.5       | 158| 171| 172| 167       | 7.8           | NR, NP        | 0.93          |
|                            | 5         | 171| 177| 153| 167       | 12.5          | NR, NP        | 0.93          |
| Sodium azide (μg/plate)    | 5         | 1688| 1854| 1724| 1755   | 87.3          | NR, NP        | 13.00         |
| (With S9)                  |           |     |    |    |          |               |

2AA: 2-Aminoanthracene; NR: No reduction in bacterial background lawn; NP: No precipitation

2.1.4. High-Performance Liquid Chromatography (HPLC)

HPLC analysis of Synacinn™ and five markers was performed using Waters Alliance™ HPLC system (Waters, USA). Methanol and water in 1:1 (v/v) ratio was used as diluent. Column used was Zodiac C18 (250 × 4.6 mm; Zodiac Life Sciences, India) 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% (A: Water: 0.5% formic acid in MeOH: 90:10; B: Water: 0.5% formic acid in MeOH: 10:90). The flow rate and column 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 total run time was 35 minutes.

2.2. Test System

Salmonella typhimurium strains of TA1537, TA1535, TA98, TA100 and TA102 were used in this experiment as per the test guidelines OECD 471 and ICH S2 (R1) [2,3]. Each tester strain was characterized and confirmed on their genotypes. The integrity of the tester strains was tested by verifying its histidine requirement, sensitivity to UV radiation, resistance to ampicillin/ tetracycline and rfa mutation. Only qualified batches of strains were employed in the experiments. All tester strains were maintained as frozen permanent stocks (Cryovial working stocks) and stored in ultra-deep freezer at approximately -70°C. Frozen aliquots of bacterial culture were thawed and a fixed inoculum was added to a flask containing Oxoid Nutrient Broth (ONB-2). Inoculated flask was incubated overnight (15-16 h) in an incubator equipped with a shaker at 37°C with shaking [110 revolutions per minute (rpm)]. These overnight grown cultures containing 10⁹ CFU/mL were used for the assay conduct. Optical density was determined using spectrophotometer at 650 nm. Actual cell titers were determined by viable count on nutrient agar plates for each assay and recorded as raw data.
2.2.1. Vehicle selection

Preliminary test was conducted to select appropriate vehicle for the experiment and to assess test item solubility. For vehicle selection, small amount of test item was taken to achieve 50 mg/mL concentration. Test item solubility was checked in different vehicles in preferential order starting with water, DMSO and 50% adventol. Test item did not form clear solution in any of the tested solvents. The formulations were vortexed for 5-10 minutes and centrifuged at 1000 rpm for 5 minutes to remove debris or non-active polysaccharides from purified formulation (supernatant) containing selected markers [4]. The supernatant from each formulation was carefully collected and submitted for analytical method feasibility. Formulation prepared in adventol 50% was most suitable for identification of all the five markers. Therefore, based on the analytical method feasibility, adventol 50 % v/v in purified water was preferred over water and DMSO and selected vehicle for this experiment. Moreover, adventol (99% ethanol) is one of the recommended solvents, biocompatible to Salmonella tester strains [5,6]. Adventol (manufactured by Advent Chembio Private Limited) was stored at room temperature.

2.3. Metabolic Activation System

2.3.1. Mammalian Liver Post Mitochondrial Fraction (S9)

The mammalian liver post mitochondrial fraction (S9) used for metabolic activation was obtained from Molecular Toxicology Incorporated, USA (MolToxTM S9), where it was prepared from male Sprague Dawley rats induced with Aroclor 1254. Batches of MolToxTM S9 were stored frozen at -70 ± 10°C and thawed just prior to use. Each batch was checked by the manufacturer for sterility, protein content, ability to convert known promutagens to bacterial mutagens and cytochrome P 450 catalysed enzyme activities (alkoxyresorufin O dealkylase activities) [7–9].

2.3.2. Preparation of S9 Mix

S9 mix was prepared freshly for each assay by mixing commercially procured S9 fraction with the required cofactors in the ratio of 1:9 which corresponds to 10% v/v of S9 in the

Table 13
Mutagenicity assay 2 by pre-incubation method on TA102.

| Test Item       | Treatment | R1  | R2  | R3  | No. of Revertants | Organism: | TA102 |
|-----------------|-----------|-----|-----|-----|-------------------|-----------|-------|
|                 |           |     |     |     |                   |           |       |
| Untreated control | -         | 469 | 336 | 472 | 426               |            |       |

|           |            |     |     |     |          |                   |       |
|------------------------------------------|-----------------|-----|-----|-----|-----------|-----------|-------|
| Without S9                                |                   |     |     |     |          |                   |       |
| Adventol (50% v/v)                       | 0                 | 459 | 432 | 439 | 441       | 10.7      | NR, NP |
| Synacinn<sup>TM</sup> (mg/plate)         | 0.313             | 479 | 489 | 451 | 473       | 19.7      | NR, NP |
| Synacinn<sup>TM</sup> (mg/plate)         | 0.625             | 462 | 458 | 432 | 451       | 16.3      | NR, NP |
| Synacinn<sup>TM</sup> (mg/plate)         | 1.25              | 451 | 488 | 477 | 472       | 19.0      | NR, NP |
| Synacinn<sup>TM</sup> (mg/plate)         | 2.5               | 470 | 410 | 452 | 444       | 30.8      | NR, NP |
| Synacinn<sup>TM</sup> (mg/plate)         | 5                 | 459 | 471 | 444 | 458       | 13.5      | NR, NP |
| Ametacin (μg/plate)                      | 0.5               | 1782| 1988| 2058| 1943      | 143.5     | NR, NP |

|           |            |     |     |     |          |                   |       |
|------------------------------------------|-----------------|-----|-----|-----|-----------|-----------|-------|
| With S9                                   |                   |     |     |     |          |                   |       |
| Adventol (50% v/v)                       | 0                 | 371 | 359 | 427 | 386       | 36.3      | NR, NP |
| Synacinn<sup>TM</sup> (mg/plate)         | 0.313             | 432 | 381 | 427 | 413       | 28.1      | NR, NP |
| Synacinn<sup>TM</sup> (mg/plate)         | 0.625             | 312 | 387 | 388 | 362       | 43.6      | NR, NP |
| Synacinn<sup>TM</sup> (mg/plate)         | 1.25              | 400 | 389 | 408 | 399       | 9.5       | NR, NP |
| Synacinn<sup>TM</sup> (mg/plate)         | 2.5               | 401 | 378 | 397 | 392       | 12.3      | NR, NP |
| Synacinn<sup>TM</sup> (mg/plate)         | 5                 | 429 | 398 | 414 | 414       | 15.5      | NR, NP |
| 2AA (μg/plate)                           | 10                | 1688| 1498| 1824| 1670      | 163.7     | NR, NP |

2AA: 2-Aminooanthracene; NR: No reduction in bacterial background lawn; NP: No precipitation
final mixture. Cofactor solution were prepared, filtered and aliquoted as per the requirement and stored approximately at -20 °C. Once prepared, S9 mix was maintained on ice throughout its use during the experiment and left over was discarded. The composition of the cofactors used is given in the following Table 14:

| Name                                                        | Quantity/L |
|-------------------------------------------------------------|------------|
| D-Glucose-6-phosphate                                       | 1.6 g      |
| Nicotinamide adenine dinucleotide phosphate (NADP)          | 3.5 g      |
| Magnesium chloride (MgCl₂)                                  | 1.8 g      |
| Potassium chloride (KCl)                                    | 2.7 g      |
| Sodium phosphate, dibasic (Na₂HPO₄)                        | 11.4 g     |
| Sodium phosphate, monobasic (NaH₂PO₄.H₂O)                  | 2.8 g      |
| Water                                                       | q.s to make up 1 L |

2.4. Positive Controls Information

The details of the positive controls used were given in the following Table 15:

| Chemical name & CAS No.         | Source          | Concentration (μg/plate) | Solvent     | Use         |
|---------------------------------|-----------------|--------------------------|-------------|-------------|
| 2-Nitrofluorene (607-57-8)      | Sigma Aldrich   | 5                        | DMSO        | TA98        |
| Sodium azide (26628-22-8)      | Sigma Aldrich   | 5                        | DMSO        | TA100       |
| 2-Aminoanthracene (613-13-8)   | Sigma Aldrich   | 5                        | DMSO        | TA102       |
| ICR-191 (17070-45-0)           | Sigma Aldrich   | 1                        | DMSO        | TA1535      |
| Ametecin (Mitomycin C) (50-07-7)| Chempure        | 0.5                      | Sterile water| TA1537     |
| 2-Aminoanthracene (613-13-8)   | Sigma Aldrich   | 5                        | DMSO        | TA98        |

2.5. Plating procedure

2.5.1. Plate incorporation method

In this method, the plating was achieved by the following sequence of additions to 2 mL of molten agar (supplemented with 10% v/v, 0.5 mM Histidine-Biotin solution) maintained at 45 ± 2 °C for treatment without metabolic activation:

0.05/0.1 mL of Synacin™ or positive or vehicle control solution
0.5 mL of Phosphate buffer solution
0.1 mL of bacterial culture

These additions were followed by rapid mixing and pouring on to pre-labelled minimal glucose agar plates. When the agar is set, the plates were inverted and incubated. In case of treatment in presence of metabolic activation, 0.5 mL of S9 cofactor mix was used instead of Phosphate buffer. For untreated (i.e. organism) control, 0.1 mL of respective tester strain was added
to 2 mL of top agar followed by rapid mixing and pouring on to pre-labelled minimal glucose agar plates. All the treated plates were incubated at 37 ± 1°C for 48 to 72 hours and evaluated at the end of incubation.

2.5.2. Pre-incubation method

For Pre-incubation method, a pre-incubation step was included in which, test item solution or control solution, S9 mix or phosphate buffer and bacterial culture were mixed and incubated for 20 minutes at 37 ± 1°C in a shaking water bath at 75 rpm, then 2 mL of molten agar maintained at 45 ± 2°C was added to this mixture. The plating and incubation procedure was as described in the routine plate incorporation procedure.

2.6. Study design

This study was conducted in two phases viz., Phase I-Dose Range Finding experiment (DRF), Phase II-Mutagenicity Assay 1 and 2, with the reference to the guidelines on theAssessment of Genotoxicity of Herbal Substances/Preparations (EMEA/HMPC/107079/2007), OECD 471 and ICH S2 (R1) [2,3,10].

2.6.1. Phase I- Dose Range Finding study (DRF)

This phase was designed to assess the cytotoxicity and precipitation with an objective to select test concentrations for the Phase II-Mutagenicity Assay. DRF was conducted with plate incorporation method using TA100 both with and without metabolic activation (10% S9 cofactor mix) at 8 test concentrations ranging from 0.039 to 5 mg/plate separated by factor of 2. Each control (untreated/vehicle/positive) and test item concentrations will be run in triplicate plates.

2.6.2. Phase II- Mutagenicity assay 1 & 2

The objective of this phase was to evaluate the mutagenic potential of the test item.

Mutagenicity assays were carried out using S. typhimurium tester strains TA1535, TA1537, TA98, TA100 and TA102. This experiment was conducted both with and without metabolic activation system (10% S9 cofactor mix) using five test concentrations. Each control (untreated/vehicle/positive) and test item concentrations were run in triplicate plates. Mutagenicity assay 1 and 2 were performed using plate incorporation and pre-incubation methods, respectively. Based on the DRF data, concentrations for the Phase II experiments were selected and provided in the following Table 16;

| Phase II               | Concentration of test item solution(mg/mL) | Volume of test item solution per culture (μL) | Final concentration (mg/plate) |
|------------------------|-------------------------------------------|---------------------------------------------|--------------------------------|
| Mutagenicity assay 1   | 50                                        | 100                                         | 5                              |
| (Plate incorporation method) | 25                                        | 100                                         | 2.5                            |
|                        | 12.5                                      | 100                                         | 1.25                           |
| Mutagenicity assay 2   | 6.25                                      | 100                                         | 0.625                          |
| (Pre-incubation method) | 6.25                                      | 50                                          | 0.313                          |

Final concentrations represented up to 3 decimals only. In order to obtain all test doses within the validated bracketed range of 5 to 50 mg/mL for dose formulation analysis, test volume was adjusted to 50 μL of 6.25 mg/mL to achieve 0.313 mg/plate.
CRediT Author Statement

Siti Nurazwa Zainol: Investigation; Anis Fadhлина: Writing- Original draft preparation; Sri Vijaya Rentala: Formal analysis, Investigation; Manjula Yalaka: Formal analysis, Investigation; Leela Krishna Vatsavai: Project administration; Renuka Pillai: Formal analysis, Investigation; Hassan Fahmi Ismail: Review & editing; Fadzilah Adibah Abdul Majid: Conceptualization, Supervision.

Declaration of Competing Interest

The 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. The following authors; Siti Nurazwa Zainol and Fadzilah Adibah Abdul Majid are affiliated to Proliv Life Sciences SDN. BHD. The following authors; Sri Vijaya Rentala, Manjula Yalaka, Leela Krishna Vatsavai and Renuka Pillai are affiliated to Aurigene Pharmaceutical Services Limited. All authors confirm that the results of this experiments are not influenced by the authors’ affiliation to the stated companies.

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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.2021.107075.

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