

## *International Journal of Scientific Research and Reviews*

### **Aqueous and methanol extracts of roots of *Asparagus racemosus* induced anticancer activity against non-small-cell lung cancer A549 cells**

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#### **ABSTRACT**

**Background:** Non-small-cell lung cancer (NSCLC) is one of the most commonly found lung cancer accounting for 80% to 85% of total cases. Targeted therapies have improved the treatment of patients but have their limitations. Therefore, the development of new chemotherapeutic agents has become an urgent need of the hour. Medicinal plants can be a good source of such agents that can prove to be beneficial to the patients because of minimal side effects and natural origin.

**Objective:** Evaluation of anticancer activity of aqueous and methanolic extracts of *Asparagus racemosus* root.

**Materials and Methods:** Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,6-diphenyltetrazolium bromide [MTT] assay. Wound healing assay was done to determine inhibition of migration. The morphological assay was done to find out the morphological changes after treatment. Colony forming assay was done to determine change in colony forming potential after treatment.

**Results:** In cell viability assay, *Asparagus* water extract was found to be reducing the cell viability of A549 cells better in comparison to *Asparagus* methanol extract. Relative cell migration was better reduced by aqueous extract (55.6%) than methanolic extract (34.7%) after 48 hours of treatment. The morphological assay showed rounding of cells after treatment. In colony forming assay, it was found that both the extracts reduced the number of colonies almost equally; aqueous (30%) and methanolic (27.8%).

**KEYWORDS:** Non-small cell lung cancer, cytotoxicity, cell cycle assay.

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## INTRODUCTION

Among all types of cancer, lung cancer remains the cause of highest mortality worldwide <sup>1</sup>. Non-small-cell lung cancer (NSCLC) is the most common (85 %) histological subtype of lung cancer<sup>2</sup>. Most of the patients are diagnosed at an advanced stage and so they survive hardly for a few months<sup>3</sup>. Moreover, survival rates and a cure remains very low too <sup>2</sup>.

*Asparagus racemosus* is an important medicinal plant in Ayurveda <sup>4</sup>. It is commonly known as Shatavari and belongs to the Asparagaceae family. It is a thorny woody climber found in the tropical and subtropical regions <sup>5</sup>. Roots are the most important part of the plant having most of the pharmacological activity. It is used as lactagogue <sup>6</sup>, Candida infection <sup>7</sup>, in diarrhoea, nervous disorder, dysentery, etc <sup>8</sup>. There are only limited studies related to the anticancer activity of *Asparagus racemosus* <sup>9-12</sup>.

However, the studies regarding the anticancer activity of *Asparagus racemosus* in lung cancer is limited. So, in this study, we have determined the anticancer activity of aqueous and methanol extracts of *Asparagus racemosus* roots in A549 cells to assess their potential for the treatment of NSCLC patients

## MATERIALS AND METHODS

### *Chemicals and Reagents*

For cell viability assay, MTT was obtained from Invitrogen (Life Technologies, Oregon, U.S.A.) and DMSO was obtained from Himedia (Mumbai, India). For cell cycle analysis Propidium Iodide was obtained from Sigma-Aldrich (St. Louis, U.S.A.) and RNase from Genei (Bangaluru, India). For apoptosis analysis, Annexin V Recombinant FITC antibody was obtained from BD Pharmingen™ (BD Biosciences, Singapore). For Hoechst 33258 staining, bisbenzimidazole (Hoechst 33258) was obtained from Himedia (Mumbai, India).

### *Plant material*

*Asparagus racemosus* was obtained from National Institute of Ayurveda, Jaipur (Rajasthan, India). The plant was identified by Dr Amit Kotia (Assistant Professor, Department of Botany, University of Rajasthan) and submitted in herbarium in the Department of Botany with herbarium number RUBL211670.

### *Plant extract preparation*

*Asparagus racemosus* roots were collected and washed thoroughly with tap water. Roots were then cut into small pieces and were allowed to dry in the shade until all moisture evaporated. Small pieces of roots were finely ground into powder form in a mixer grinder. The powder obtained was weighed, mixed with organic solvents [Water and Methanol] and introduced into Soxhlet apparatus round bottom flask for preparation of the extracts.

### **Cell line and cell culture**

The human NSCLC cell line A549 was purchased from NCCS, Pune. The cell line was cultured in Dulbecco's modified eagles medium, supplemented with 10% (v/v) FBS and 1% antibiotic-antimycotic solution incubated in a humidified atmosphere of 5 % CO<sub>2</sub> at 37 °C.

### **Cell viability assay**

Cell viability of the *Asparagus racemosus* root extracts was checked on A549 cell line using MTT (3-[4, 5-dimethyl-2-thiazolyl]-2, 5 diphenyltetrazolium bromide) assay as per Chen *et al.*<sup>13</sup>. In this assay, 4 x 10<sup>4</sup> cells were seeded into the flat bottom 96-well plate and next day incubated with the water and methanol extract of roots for 24 h and 48 h in CO<sub>2</sub> incubator with concentration ranging from 7.81 µg/ml to 500 µg/ml for each extract. At the end of extract treatment, 20 µl of MTT (Life Technologies, Oregon, U.S.A.) was added to each well and the plate was incubated for 4 h at 37°C. The supernatant was removed; blue formazan produced was dissolved in 100 µl of DMSO. OD was measured at 570 nm using a plate reader. The experiment was performed in triplicates (n=3). Cell viability percentage was calculated with formula O.D. of drug-treated sample/ O.D. of non treated sample × 100.

### **Cell migration assay**

Cell migration assay was done to access cell-cell interaction *invitro* migration studies as per Wang *et al.*<sup>14</sup>. Briefly, A549 cells were allowed to grow till 80% confluency was reached. On the next day, the monolayer was scratched with the help of 200 µl pipette tip and then washed twice with PBS to remove suspended cells. Cells were then treated with different concentrations of extract for 24 h and 48 h. Wound closure was observed and pictures were taken at 10X magnification.

### **Morphological analysis**

Morphological analysis was done to determine the morphological changes, like cell shrinkage, membrane blebbing, etc, as per Srivastava *et al.*<sup>15</sup>. Briefly, A549 cells were seeded and the next day the cells were treated with water and methanol extract for 24 h and 48h. The effect of the extract on the morphology of A549 cells was assessed under an inverted phase-contrast microscope after 24 h and 48 h (at 10X magnification). The cells which were not treated with plant extract served as control.

### **Colony forming assay**

A549 (300 cells/well) cells were seeded in 6-well plate and their potential for colony formation was analyzed as described previously<sup>16-18</sup>. Cells were then treated with IC<sub>50</sub> concentration of plant extracts for 3 days (*Asparagus* water- 36 µg/ml and *Asparagus* methanol – 71 µg/ml). After 3 days, the treated medium was discarded and complete medium was added. Cells were then allowed to grow for 7 days in complete medium. At the end of 7 days, the medium was removed and cells

were washed with PBS. Then after, colonies were stained with 2 ml/well of staining solution [glutaraldehyde 6.0 % (vol/vol), crystal violet 0.5% (wt/vol) in H<sub>2</sub>O] for 30 min. Finally, remove the staining solution and rinse with tap water and allow colonies to dry. The colonies are then counted.

### ***Statistical analysis***

The half maximum inhibitory concentration (IC<sub>50</sub>) was calculated using Graph Pad Prism 5 software. Data was represented as Mean ± SD of 3 independent experiments. P-value was calculated using one-way ANOVA test in Graph Pad Prism 5 software.

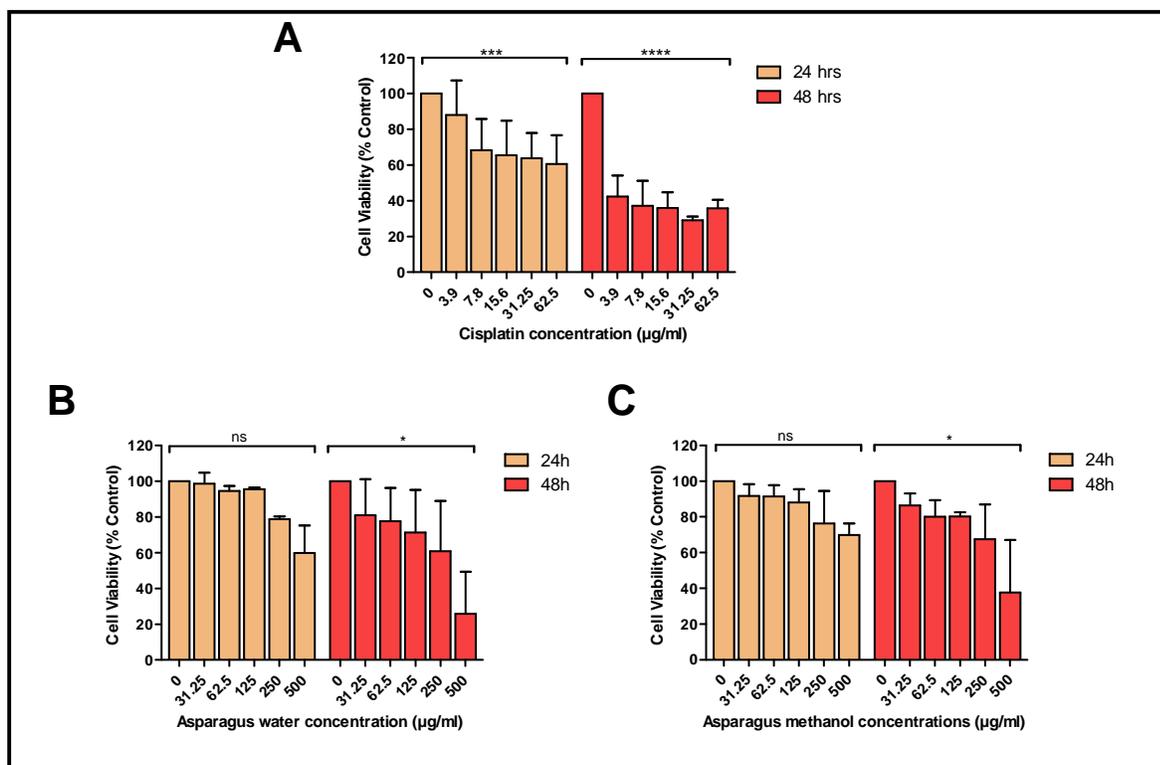
## **RESULTS AND DISCUSSION**

Lung cancer takes millions of deaths in India and worldwide <sup>19,20</sup>. There is only meagre benefit from the current radio and/or chemotherapy <sup>21</sup>, so there is an urgent need to find alternative therapeutic agents that can prove to be more beneficial to the patients and that can improve their survival without any side effects.

There are various medicinal or herbal plants with anticancer activities. Currently, most of the drugs in use like taxol, vinblastine, vincristine, etc are derived from the plants and some are under trials <sup>22</sup>. Drugs derived from plants are safer, more effective and have no side effects <sup>23</sup>. Traditionally, in India herbs have been in use since ancient times <sup>24</sup> and are also used in the treatment of cancer <sup>25</sup>.

*Asparagus racemosus* is an important medicinal plant in ayurveda and have got various medicinal properties. The roots are an important part of the plant and it is used for nervous disorders, tumours, inflammation, diarrhoea, tumour, dysentery, bronchitis, cough, etc <sup>26</sup>. There are limited reports that showed the anticancer activity of *Asparagus racemosus in-vitro*<sup>9-12</sup>. So, here we have determined its anticancer activity against NSCLC.

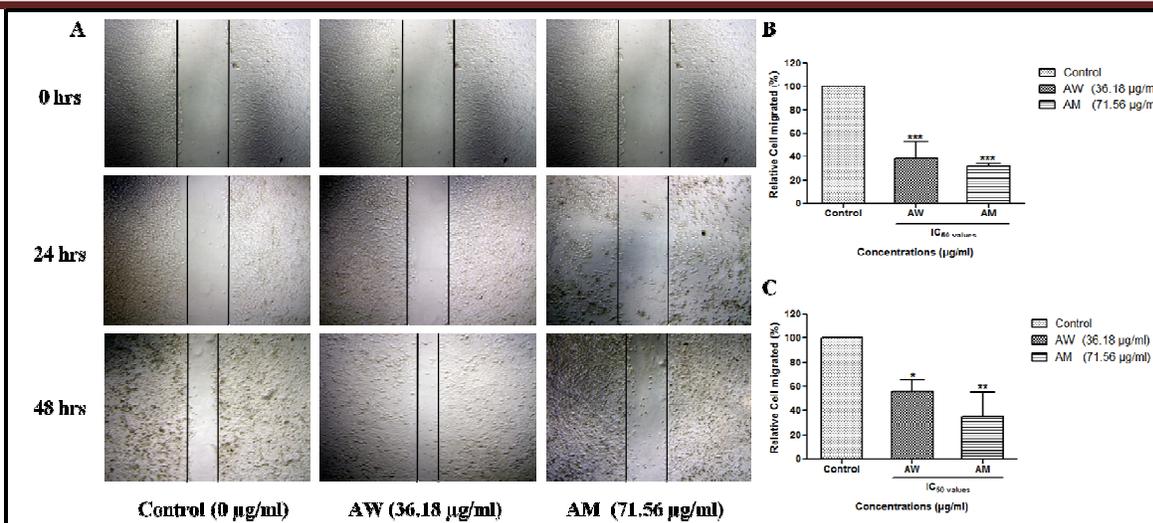
The cell viability was examined by MTT assay in A549 cells. Several studies have reported anticancer activity of plants of *Asparagus* family <sup>10,11,27-33</sup>. But there are only two reports in the literature which has showed anticancer activity of *Asparagus racemosus* root extracts. In 2010, Bhutani *et. al.* determined that steroidal compounds isolated from an n-butanolic extract of *Asparagus racemosus* roots were cytotoxic to colon carcinoma HCT-116 cells <sup>10</sup>. While, Mitra *et. al.* in 2012 found that steroidal compounds present in the ethyl acetate (insoluble fraction) of ethyl acetate were cytotoxic to breast cancer MCF-7 cells, kidney cancer A498 cells and colon carcinoma HT-29 cells <sup>11</sup>. In our study, we found that *Asparagus* water (AW) extract was found to be reducing the cell viability of A549 cells better in comparison to *Asparagus* methanol (AM) extract after 48 hours [Figure.1]. This is the first study done to determine anticancer therapy against non-small-cell lung cancer A549 cells.



**Figure 1. In vitro effect of Cisplatin and *Asparagus racemosus* root extracts on cell viability in human lung cancer A549 cells.**

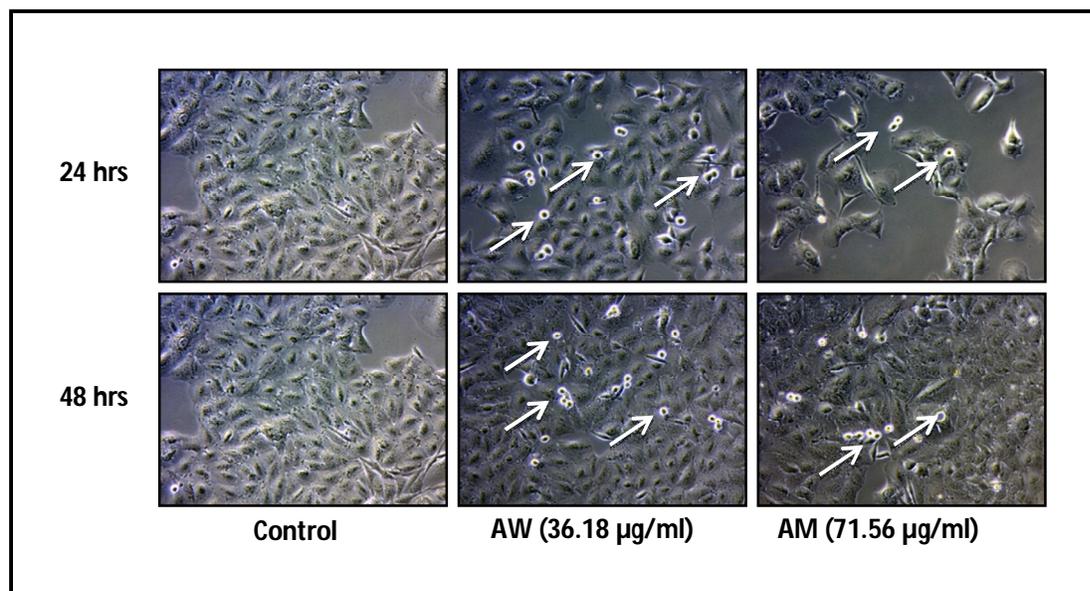
(A) A549 cells were treated with increasing concentration of Cisplatin (B, C) A549 cells were treated with increasing concentration of *Asparagus racemosus* root Water and Methanol extracts. Cell viability was determined after 24 and 48 hours of treatment. Control cells received no treatment. Data are shown as mean  $\pm$  SD of three independent experiments in technical triplicates (\*\*\*\* $P < 0.0001$ ; \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ ; ns: not-significant)

Inhibition of migration is an important aspect to explain the cause of malignancy. In our study, we found that relative cell migration after treatment with *Asparagus* methanol extract was (34.7 %) better than *Asparagus* water (55.6 %) extract after 48 hours of treatment [Figure.2]. In 2012, Wang *et. al.* found that saponins isolated from the stem of *Asparagus officinalis* L. reduced the migration of MDA-MB-231 breast cancer cells<sup>27</sup>.



**Figure 2.** *In vitro* effect of *Asparagus racemosus* root extracts on the migration of human lung cancer A549 cells. (A) A549 cells were treated with  $IC_{50}$  values of AW and AM (B) Relative cell migration rate (%) of A549 cells after treatment with  $IC_{50}$  values of Asparagus Water (AW) and Asparagus Methanol (AM) after 24h. (C) Relative cell migration rate (%) of A549 cells after treatment with  $IC_{50}$  values of Asparagus Water (AW) and Asparagus Methanol (AM) after 48h. Control cells received no treatment. Data are shown as mean  $\pm$  SD of three independent experiments (\*\*\*\* $P < 0.0001$ ; \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ ; ns: not-significant)

Changes in the morphology are an indication of cell death. In our study, we found that after the treatment with both the extracts cells became rounded and this is the first study done [Figure.3].



**Figure 3.** *In vitro* effect on the morphology of A549 cells after treatment with  $IC_{50}$  values of Asparagus Water (AW) and Asparagus Methanol (AM) extracts after 24 and 48 hours.

Control cells received no treatment. White arrows indicate the cell shrinkage and cell rounding (Magnification 20x)

Colony forming assay is done to determine the reduction in colony forming potential after treatment. In our study, we found that after treatment number of countable colonies gets reduced

after treatment almost equally in both extracts [Figure.4]. In one of the previous study, it was found that Asparifioside B treatment made colonies be smaller in size in comparison to the control group.<sup>32</sup>

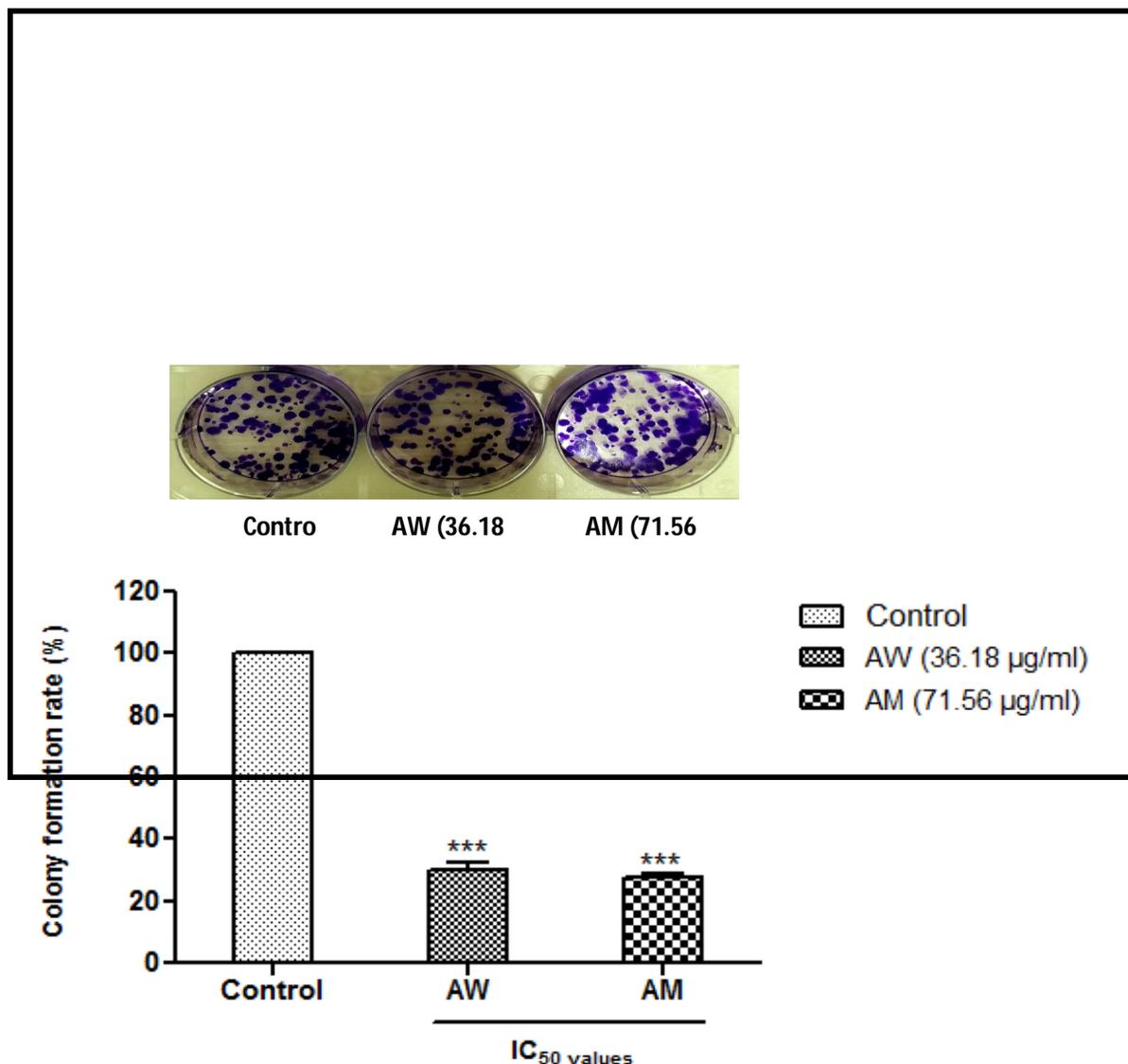


Figure 4. Effect of *Asparagus* root extracts on clonogenic potential of A549 cells. Control cells received no treatment. Data are shown as mean  $\pm$  SD of three independent experiments (\*\*\*\* $P < 0.0001$ ; \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ ; ns: not-significant)

Limitation of our study was that we were not able to isolate the active anticancer compounds from the crude extracts and their mechanism of action. In future, combination studies (with standard chemotherapeutic drug) will be done to enhance the anticancer activity of the extracts.

## CONCLUSION

In this study, we found that cell viability was reduced more in the presence of *Asparagus* water extract in comparison to *Asparagus* methanol extract. Surprisingly, migration was inhibited more with *Asparagus* methanol extract in comparison to *Asparagus* water extract. Both the extracts

were able to induce a change in cell morphology and reduced colony formed almost equally. However, the mechanism with which cell death occurred needs to be studied. As the effect of extracts alone was not significant, so combination studies with the known standard chemotherapeutic drug can also be done in future.

## **ACKNOWLEDGEMENT**

The author would like to thank ICMR for providing Senior Research Fellowship for conducting the research work. The author would also like to acknowledge Dr. Pankaj Goyal (HOD, Department of Biotechnology, Central University of Rajasthan, Bandarsindri) for providing support in cell culture work and cell cycle analysis and Dr. Pradeep Kumar Sharma (Scientist, Division of Food, Drug and Chemical Toxicology, CSIR-IITR, Lucknow) for providing the facility of flow cytometry for Annexin V FITC assay and in performing Hoechst 33258 staining.

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