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• **Research Article**

# Ultra-highly diluted plant extracts of *Hydrastis canadensis* and *Marsdenia condurango* induce epigenetic modifications and alter gene expression profiles in HeLa cells *in vitro*

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## ABSTRACT

**OBJECTIVE:** Methylation-specific epigenetic process and gene expression profiles of HeLa cells treated with ultra-high dilutions (HDs) of two plant extracts, *Hydrastis canadensis* (HC-30) and *Marsdenia condurango* (Condu-30), diluted $10^{60}$ times, were analyzed against placebo 30C (Pl-30) for alterations in gene profiles linked to epigenetic modifications.

**METHODS:** Separate groups of cells were subjected to treatment of Condu-30, HC-30, and Pl-30 prepared by serial dilutions and succussions. Global microarray data recorded on Affymetrix platform, using 25-mer probes were provided by iLifeDiscoveries, India. Slides were scanned with 3000 7G microarray scanner and raw data sets were extracted from Cel (raw intensity) files. Analyses of global microarray data profile, differential gene expression, fold change and clusters were made using GeneSpring GX12.5 software and standard normalization procedure. Before microarray study, concentration of RNA (ng/μL), RIN value and rRNA ratio for all the samples were analysed by Agilant Bioanalyzer 2100. Reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative RT-PCR were done for analyzing SMAD-4 expression. Fluorescence-activated cell sorting study was further made to elucidate fate of cells at divisional stages. Methylation-specific restriction enzyme assay was conducted for ascertaining methylation status of DNA at specific sites.

**RESULTS:** HDs of HC-30 and Condu-30 differentially altered methylation in specific regions of DNA and expression profiles of certain genes linked to carcinogenesis, as compared to Pl-30. Two separate cut sites were found in genomic DNA of untreated and placebo-treated HeLa cells when digested with McrBC, compared to a single cut observed in Condu-30-treated genomic DNA. SMAD-4 gene expression validated the expression pattern observed in microarray profile. Methylation-specific restriction enzyme assay elucidated differential epigenetic modifications in drug-treated and control cells.

**CONCLUSION:** HDs triggered epigenetic modifications and alterations in microarray gene expression profiles of many genes associated with carcinogenesis in HeLa cells *in vitro.*

**Keywords:** plant extracts; homeopathy; reactive oxygen species; apoptosis; gene expression; epigenesis, genetic; Smad4 protein

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## 1 Introduction

In the development of pharmacological drugs, some of the most common barriers include poor pharmacokinetics, insufficient therapeutic activity, drug toxicity, and poor bioavailability[1]. Given these issues, complementary and alternative medicines (CAMs), which often use plant extracts and other natural products that are relatively less cytotoxic, have gained increasing popularity. Various scientific methods are now being applied to learn about their efficacy, proper dosage, and mechanisms of action. Homeopathy, a popular branch of CAM, uses both crude and ultra-highly diluted (potentized) forms of remedies. Homeopathy is often criticized for its failure to explain the mechanism of action of the ultra-high dilutions (HDs) and therefore needs closer examination with a modern scientific approach[2]. An exhaustive survey in Britain suggests that 70% of all oncology departments employ at least one form of CAM treatment in cancer care[3]. More recently, the National Center for Complementary and Alternative Medicine (NCCAM), under the National Institutes of Health (NIH), USA, approved homeopathy as an alternative medicinal approach under the regulation of Food, Drug and Cosmetic Act (FDCA)[4]. However, the dearth of information on homeopathy's scientific mechanisms of action makes its acceptance rather difficult. In recent years, the documentation of the existence of nano-particles[5,6] of the original drug substance(s) in some highly diluted remedies used in homeopathy has given a new impetus for conducting more rigorous research utilizing state-of-the-art techniques[7–9].

Other studies have used *in vitro* and *in vivo* conditions in models like *Escherichia coli*, yeast, bacteriophage and plant-based experimental models to understand exact molecular mechanisms of highly diluted homeopathic remedies[10–16]. Human tumor-derived cell line models used in cancer therapeutics programs are effective for the evaluation of potential new therapeutic agents. These cell line-based models are integrated with efforts to identify biomarkers of cancer progression and mechanisms of drug action[17].

Human cervical cancer cell line HeLa belongs to HPV-18-positive cell line, which carries approximately 10–50 viral integrated copies[18]. This is an ideal model to study genetic and epigenetic modifications by therapeutic agents. The epigenetic phenomenon is defined as a heritable reversible phenotype, resulting from changes in a chromosome without alteration in the DNA sequence. DNA methylation, histone modifications, nucleosome positioning, micro-RNAs and non-coding RNAs are the hallmark traits of epigenetics[8,9,19].

Previously, the notable hypotheses put forth to explain the efficacy of homeopathic remedies include molecular imprints or memory of water, similia principle, hormesis, nano-silicon principle, electromagnetic transfer through DNA wave principle, thermo luminiscence, and gene regulatory and epigenetic hypotheses[9,11,20–26]. Of these, the gene regulatory hypothesis of Khuda-Bukhsh[11] is gradually gaining wide attention for its ability to explain the biological action of HDs in all living organisms, from prokaryotes through human beings; this hypothesis is being studied at the molecular level.

This study has been designed to examine the cervical cancer HeLa cell line, a suitable *in-vitro* model, to get insight into whether the HDs of two plant extracts, *Hydrastis canadensis* (HC-30) and *Marsdenia condurango* (Condu-30), can manifest any demonstrable changes in the global microarray profiles of HeLa cells by induction through epigenetic alterations. We also conducted experiments on functional validation of SMAD4 biomarker in cancer and methylation specific restriction enzyme (RE) digestion in order to make differential gene expression analysis of two HDs generally used against cancer, namely, HC-30 and Condu-30, versus placebo 30C (Pl-30), the vehicle of the HDs, and if epigenetic alteration is one of the main pathways for bringing about necessary changes in gene expression profiles, to elucidate underlying mechanisms of action of the highly diluted homeopathic remedies at the molecular level.

## 2 Materials and methods

### 2.1 Chemicals and reagents

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), trypsin and ethylene diamine tetra-acetic acid (EDTA) were purchased from Gibco BRL (Carlsbad, CA, USA). Penicillin-streptomycin-amphotericin (PSA) antibiotic and Hipur A RNA-Xpress reagent were obtained from Himedia (Mumbai, India). Tissue culture plastic wares were obtained from Tarsons (Kolkata, India). Propidium iodide (PI) was purchased from Sigma Chemical Co. (St Louis, USA). Oligonucleotide primers were obtained from Imperial Life Sciences, India. Agarose was procured from Lonza, USA. Taq DNA polymerase, deoxynucleoside triphosphates (dNTPs) and reverse transcriptase enzymes were procured from Chromous Biotech (Bangalore, India). Methylation-specific restriction enzyme McrBC was procured from New England Biolabs, USA. All other chemicals used were procured either from Sigma, USA or from Merck, Germany, if not mentioned otherwise.

### 2.2 Preparation of HC-30, Condu-30 and Pl-30

Crude ethanolic root extract of *Hydrastis canadensis* and *Marsdenia condurango* were dynamized to the 30th potency by following the standard serial dilution and succussion method as advocated in the European

Homeopathic Pharmacopeia (http://www.echamp.eu/news/newsletter/newsletter-archive/2013/september/homeopathy-in-the-european-pharmacopoeia.html; see Khuda Bukhsh[27] for further detail by Boiron Laboratories (Lyon, France)). In brief, 1 mL of the crude ethanolic plant extract is mixed with 99 mL of 70% ethanol and given 10 mechanical jerks (succusions) of equal magnitude and the potency 1 is obtained. Again 1 mL of potency 1 is mixed with 99 mL of 70% ethanol and given 10 jerks to produce the potency 2 and so on. We procured both the HDs (diluted $10^{-60}$), HC-30 and Condu-30, as well as the Pl-30, in which the same stock of ethanol as in drugs was used, from Boiron Laboratories, Lyon, France.

**2.3 Cell culture and treatment**

HeLa cells were obtained from National Centre for Cell Science (NCCS), Pune, India. Cells were routinely maintained in DMEM supplemented with 10% FBS and 1% antibiotic at 37 ℃ in a humidified incubator containing 5% $CO_2$. Cells were treated with 4% (v/v) of either HC-30, Condu-30 or Pl-30 and incubated for 48 h in $CO_2$ incubator. Cells without any treatment were considered as negative control.

**2.4 Microarray experiment**

Separate groups of cells were subjected to the treatment of Condu-30, HC-30, and Pl-30. Cells were sent to iLifeDiscoveries, Gurgaon, India for providing us global microarray data conducted on Affymetrix platform, using 25-mer probes. The total number of probes detected for the experiment was 49 395; hybridization was done at 45 ℃ for 16 h at $60\times g$.

Slides were scanned with 3000 7G microarray scanner and raw data sets were extracted from the Cel (raw intensity) files. Microarray data analysis, differential gene expression analysis, fold change analysis and cluster analysis were performed using GeneSpring GX12.5 software.

**2.5 Experimental grouping**

For microarray gene expression study, samples of untreated HeLa cells, and HC-30-, Condu-30- and Pl-30 -treated series were termed as control, SET I, SET II and SET III respectively. All sets were taken in triplicates.

**2.6 RNA quality control before microarray experiment**

Before the microarray gene expression study, concentration of RNA (ng/μL), RIN value and rRNA ratio for all the samples were analysed by Agilant Bioanalyzer 2100 .

**2.7 Data pre-processing and normalization**

All the original microarray data (CEL files) for the experiment were pre-processed using robust multichip average (RMA) algorithm that consists of three steps: a background adjustment, quantile normalization and finally summarization. All above procedures were done by selecting RMA algorithm in GeneSpring GX12.5. Genes of low-intensity information content in each data set were filtered as follows: the probes of intensities less than the 10.0 percentile in the raw data were excluded from the analysis. The step used in pre-processing and normalization is given below.

**2.7.1 Raw signal values**

The term "raw" signal values refer to the linear data after thresholding and summarization. Summarization is performed by computing the geometric mean.

**2.7.2 Normalized value**

"Normalized" value is the value generated after log transformation and normalization (scale) and baseline transformation.

**2.7.3 Treatment of control probes**

The control probes were included while performing normalization. However, there should be an exact match between the control probes in the technology and the sample for the probes to be utilized.

**2.7.4 Sequence of events**

The sequence of events involved in the processing of the data files was: thresholding > summarization (summarization is performed by computing the geometric mean) > log transformation > normalization > baseline transformation.

**2.7.5 Baseline to median of all samples**

For each probe the median of the log summarized values from all the samples was calculated and subtracted from each of the samples.

**2.8 Box whisker and profile plot**

The box-whisker plot presents the normalized microarray expression data visualization summary. Further data are also distributed on conditions in the active interpretation with respect to the active entity or gene list in the experiment. The box-whisker plots are created between normalized intensity values and all probes.

The profile plot describes the summary of overall expression patterns of microarray experimental data.

**2.9 Principle component analysis plot**

After data normalization, quality control (QC) on samples was performed to remove the unreliable data from further analysis. The QC results are shown in the form of principle component analysis (PCA) in a 3D scatter plot. The scores are used to check data quality. It shows one point per array and is colored by the experiment factors or conditions. This allows viewing of separations between groups of replicates. Ideally, replicates within a group should cluster together but be separate from arrays in other groups.

**2.10 Hybridization and correlation plot**

Hybridization quality was checked by comparison with "hybridization control". Hybridization controls were composed of a mixture of biotin-labelled cRNA transcripts of bioB, bioC and bioD, and prepared in staggered concentrations (1.5, 5, 25, and 100 parts per million (PPM), respectively). This mixture was spiked into the hybridization cocktail.

BioB was at the level of assay sensitivity and should be considered at least "50% present" at the time. BioC, bioD and cre must be "present" all of the time and must appear in increasing concentrations.

**2.11 Gene expression analysis**

A total number of 1 345 genes were found to be oppositely expressed in SET I (HC-30) *vs.* control and SET III (Pl-30 or placebo) *vs.* control. Number of genes with ≥ 1.5-fold differential expression was found to be 23. The 1.5-fold change was used because during the fold change analysis, 1 out of 3 of the compared conditions was considered valid. This means if any gene showed up-or down-regulation ≥ 1.5 in any 1 out of the 3 conditions, this would be included in the fold change results. This is important for the gene regulation pattern identification. If one gene is expressed below 1.5 in two conditions and the same gene has expressed more than 1.5 fold in 1 condition, the gene could be considered as showing a change in expression. If we selected the condition 3 out 3 then we would filter out only those genes that have consistence in fold change ≥ 1.5 in all three compared conditions. So fold change 1.5 in 1 out of 3 was deemed optimal. This parameter was also suggested for time series, dose and drug response microarray experiments. How this particular analysis was carried out includes the chance that we may have missed identifying some important gene information from our data, because of the possibility that some gene expressions were instantly decreased/increased in any conditions (less than 1.5 fold) after drug treatment, and these were not counted.

A total number of 650 genes were identified as oppositely expressed between SET II (Condu-30) *vs*. control and SET III (ethanol-30 or placebo) *vs.* control. The number of genes with ≥ 1.5-fold differential expression was found to be 12.

A total number of 1 182 genes were identified to be oppositely expressed between SET I (HC-30) *vs.* control and SET II (Condu-30) *vs.* control. The number of genes with ≥ 1.5-fold differential expression was found to be 36.

**2.12 Hierarchical clustering**

Cluster analysis was performed for the identification of similar type of experiments or co-expressed gene sets across the sample for the differentially expressed genes. Clustering can group the genes having similar type of expression. Unfortunately, because of the limited number of experiments run (largely due to prohibitive costs), the cluster analysis did not yield significant results. But gene alteration using heatmap image of hierarchical clustering was more revealing, showing more clear differential expression of genes for individual experiment sets.

Hierarchical clustering is one of the simplest and widely used unsupervised clustering techniques for the analysis of gene expression data. The method follows an agglomerative approach, where the most similar expression profiles are joined together to form a group. These profiles are further joined in a tree structure, until all data form a single group. The dendrogram is the most intuitive view of results of this clustering method. There are several important parameters, which control the order of merging entities and sub-clusters in the dendrogram. The most important of these is the linkage rule. After two most similar entities (clusters) are clubbed together, this group is treated as a single entity and its distances from the remaining groups (or entities) have to be recalculated.

**2.13 Qualitative reverse transcriptase-polymerase chain reaction and quantitative reverse transcriptase-polymerase chain reaction analysis of SMAD4**

For quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis the method described by Saha and Khuda-Bukhsh[28] was followed. Equal amounts of total RNA extracted with RNA expression reagent (Himedia, Mumbai, India) were reverse-transcribed using random hexamer primer and then subjected to PCR with enzymes and reagents of the reverse transcription system using Techne PCR system (Staffordshire, UK). Sequences of primers used in the study are given in Table 1.

Quantitative measure of SMAD4 gene expression analysis was further done by qRT-PCR on ABI 7900HT by relative quantification using the comparative $C_T$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as an internal control for normalization. For all the samples, the median $C_T$ values for the target genes and GAPDH were taken and the expression of target genes was normalized with that of GAPDH. The primer used in the assay is of the same sequence as used in qualitative RT-PCR study.

**2.14 Epigenetic study by RE digestion assay**

In order to determine methylation status of genomic DNA of HeLa cells in HC-30-, Condu-30-and Pl-30-treated and untreated series, the RE digestion method was employed. First, DNA samples were prepared for RE study by following the rapid isolation method[29] consciously to

**Table 1** Primer sequences for qualitative RT-PCR and quantitative RT-PCR analyses of SMAD4

| Primer | Forward 5′–3′ | Reverse 5′–3′ |
|---|---|---|
| SMAD4 | TCCTGTGGCTTCCACAAGTC | TCCAGGTGGTAGTGCTGTTATG |
| GAPDH | CAGCCTCAAGATCATCAGCA | TGTGGTCATGAGTCCTTCCA |

RT-PCR: reverse transcriptase-polymerase chain reaction; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

avoid the use of phenol-chloroform. In brief, cells were harvested and dissolved in ice-cold lysis buffer containing 10 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA pH 8.0 and 0.1% SDS with proteinase K (20 mg/mL) followed by incubation at 55 ℃ for 3 h. The digest was then incubated further at 37 ℃ for 1 h with RNase (4 mg/mL). Next, potassium acetate solution (60 mL of 5 mol/L potassium acetate, 11.5 mL of glacial acetic acid and 28.5 mL of water) was added and mixed by vortexing. Protein-SDS complex was precipitated by centrifugation at 8 000 × *g* for 15 min. The supernatant was transferred to a fresh tube containing isopropanol. It was then mixed and again centrifuged at 8 000 × *g* for 15 min. The DNA pellet was washed with 70% ethanol. The semi-dried DNA was dissolved in TE buffer at pH 7.0. Further quality checking was carried out by measuring optical density (OD) at 260/280 nm (ratio maintained at 1.6–1.8) to confirm that DNA was free of protein contamination. After confirmation of quality, DNA was used for further experiments. Methylation-dependent enzyme McrBC (New England Biolabs) was used in the study. Genomic DNA (1.0 μg) was digested with 10 units of RE, in 50 μL total reaction mixtures supplemented with reaction buffers and accessory reagents for overnight at 37 ℃. The digested DNAs were electrophoresed on 1.5% agarose gel followed by ethylene bromide staining to observe differential band patterns, if any.

### 2.15 DNA content or ploidy analysis in terms of cell cycle distribution by flow cytometry

HeLa cells were grown at an equal density of 2 × $10^6$ cells in 90 mm culture plate and treated with HC-30, Condu-30 and Pl-30. The cells were harvested and fixed in ice-chilled 80% ethanol with constant vortexing and stored at −20 ℃ for future use. For flow cytometric analysis, cells were separated from the fixative by centrifugation and washed in staining buffer (1× phosphate buffered saline, 2% FBS, 0.1% sodium azide at pH 7.1–7.4). RNase (100 μg/mL) was added and the cells were incubated at 37 ℃ for 2 h. After incubation 50 μg/ml PI was added to the material and kept for 30 min at 4 ℃. Cell cycle analysis was performed using the BD FACS Verse™ system and data were analyzed using the BD FACS Diva™ software. For each sample equal numbers of cells (10 000) were counted. Cell cycle distribution analysis was done by gating the cell population[30].

### 2.16 Blinding

The observer was kept "blinded" during the main part of observation, after which the codes were deciphered for data analysis.

## 3 Results

### 3.1 RNA quality analysis data

All samples showed RIN values above 6.0, considered highly satisfactory for microarray analysis. These samples were therefore further processed for gene expression microarray experiment.

### 3.2 Box-whisker and profile plot data

The total number of probe sets detected for the experiment was 49 395. After data pre-processing, normalization and quality control on data, 40 678 probe sets remained out of 49 395. Baseline transformation was performed by taking median of all probe sets. Transformation was shifted from 20% to 75% for raw intensity data in GeneSpring tool on the basis of median value. The box-whisker plots were created between normalized intensity values and all probes. The box-whisker plots showed the median in the middle of the box, the 25th quartile and the 75th quartile. Box whisker plots had been generated for all samples for expression pattern visualization. The same replicate experiment set had similar expression pattern in the whisker plot (Figures 1A and 1B). All experiment entities (probe sets and genes) were represented separately in the form of line graphs. The experiment name is represented at X-axis, and Y-axis shows the normalized intensity value for the experiment (Figure 1C).

### 3.3 PCA plot

The PCA components, represented in the X, Y and Z axes, are numbered 1, 2 and 3, according to their decreasing significance (Figure 2).

### 3.4 Hybridization and correlation plot

The X-axis in this graph represents the controls and the Y-axis, the log of the normalized signal values. The correlation plot shows the correlation analysis across arrays. It finds the correlation coefficient for each pair of arrays and then displays them in a textual form as a table as well as in the form of a heatmap. The correlation coefficient is calculated using pearson correlation coefficient (Figures 3A and 3B).

### 3.5 Cluster analysis

In the current study hcl (average linkage) was performed on "conditions" and "genes" to explore the co-expression or co-regulation of genes in three groups. First cluster analysis performed on all experiment sets (SET I, SET II, SET-III and control) tried to explore biological similarity among experiment sets. The hcl expression image was presented in the form of a dendrogram. The expression image tree was further characterized on the basis of color. The red color shows over-expression, blue color shows under-expression and yellow color shows normal expression of genes. In the condition tree, experiments having similar expression profiles clustered adjacently in the tree. The genes having similar type of expression profiles also clustered adjacently or on the same tree node (Figure 4).

### 3.6 Functional validation of SMAD4 gene

There was down-regulation observed in Pl-30-, HC-30- and Condu-30-treated series compared to the untreated

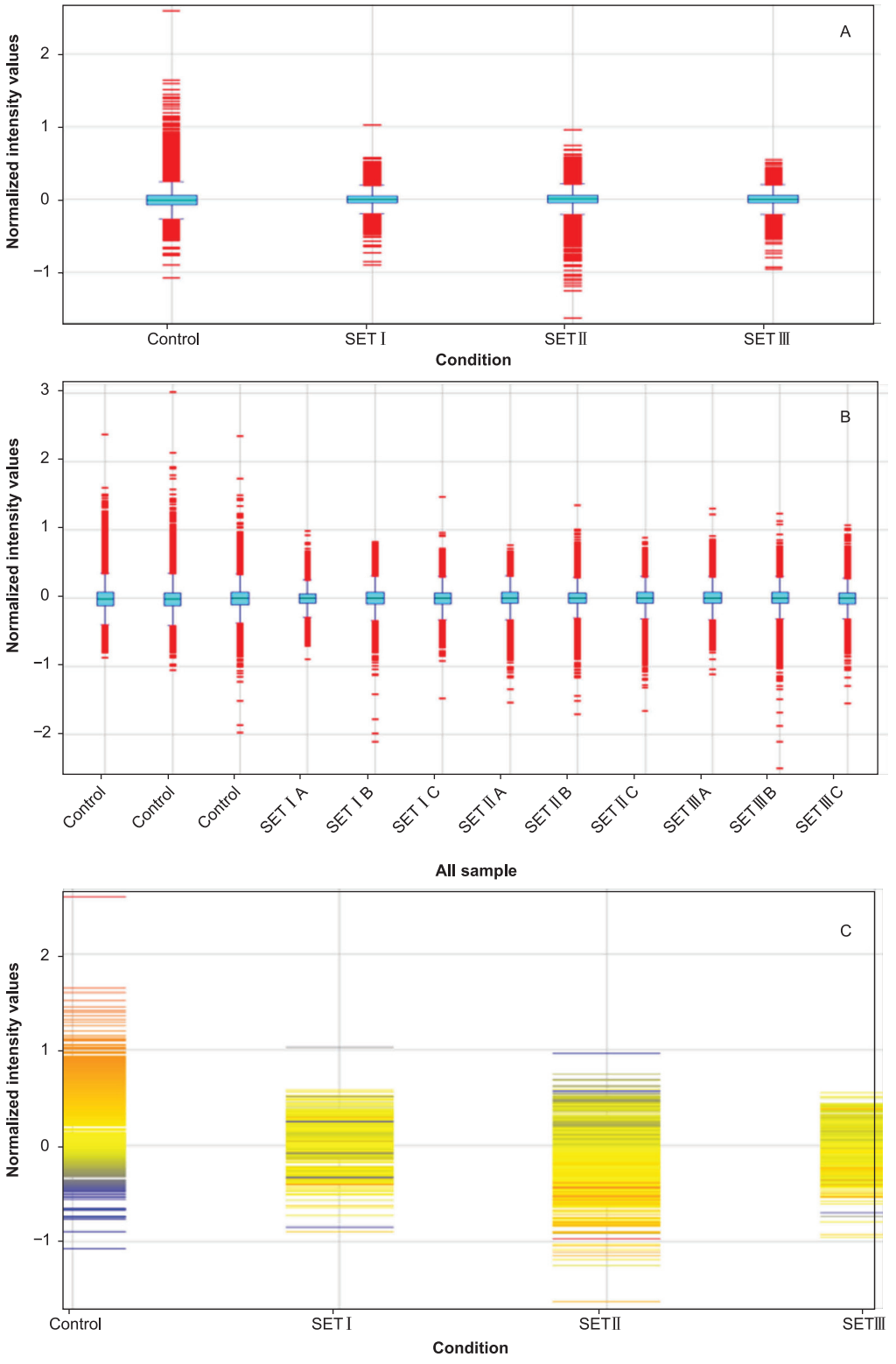


**Figure 1** Box-whisker and profile plot

(A) Box-whisker plot of different groups. (B) Box-whisker plot of individuals. Data are robust multichip average normalized from all experiment sets (data align well from baseline to median, and microarray experiment was successful). It is also clear from the image that same replicate experiment has closely related expression pattern. (C) The profile plot shows over all expression patterns of genes. The Y-axis shows normalized ratio and X-axis shows experiment set name. The normalized expression value >0 shows over-expressed genes and <0 shows under-expressed genes.

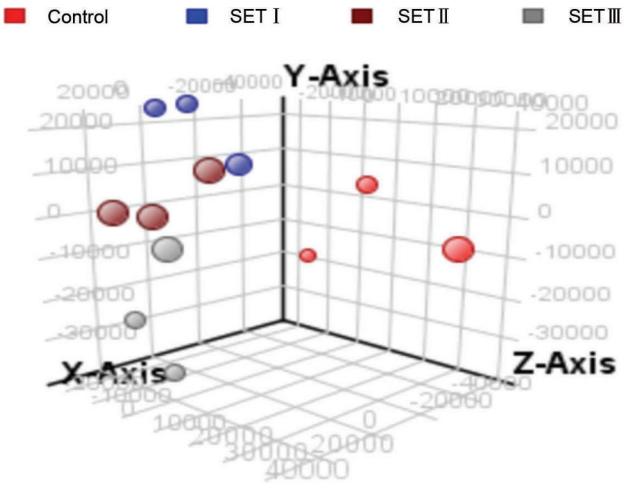


**Figure 2** Principle component analysis plot

control. Fold change values were as follows: for placebo it was 1.76, for HC-30C the fold change was 3.867 and for Condu-30C it was 4.72 (Figures 5A and 5B).

### 3.7 Alteration in methylation status: an event of epigenetic alteration

Alteration of methylation-status of DNA was analyzed using methylation-dependent enzyme McrBC digestion. There appeared to be two separate cut sites in genomic DNA obtained from untreated and placebo-treated HeLa cells when digested with McrBC, in contrast to a single cut observed in DNA of Condu-30-treated genomic DNA after proper normalization (Figure 5C).

Changes observed were marginal when DNA was digested with HC-30 used for the methylation-dependent enzyme McrBC digestion (Figure 5C).

### 3.8 Cell cycle distribution analysis

There was increased accumulation of cells at $G_0/G_1$ population by 10.25% in HC-30-treated HeLa cells, compared to 6.62% in untreated HeLa cells and 4.59% in Pl-30-treated HeLa cells (Figure 6). In case of HC-30-treated HeLa cells the $G_0/G_1$ population was found to be 2.83%.

## 4 Discussion

Our previous study[7] showed that the expression profiles of certain genes of HeLa cells treated with HC-30 and Condu-30, respectively, were significantly different from that of the Pl-30-treated cells. Both the drugs and placebo differed in their ability to trigger gene responses, some of which were implicated in cancer. Thus, an analysis of data obtained in this global microarray study would be able to demonstrate whether the HDs can trigger gene responses in a cascade of reactions consistent with the hypothesis first proposed by Khuda-Bukhsh[11,27]. Ideally, the microarray data would have been tested by making qualitative and quantitative analyses of expressions of many candidate genes, but due to financial and resource constraint, we could validate the microarray data by RT-PCR and qRT-PCR studies of only one important cancer-related candidate gene, SMAD4. Incidentally, Belavitte *et al*[29] also obtained evidence through transcriptome analysis that HDs of *Gelsemium semperverens* could alter expression profiles of genes in neurocytes. Very recently, Bigagli *et al*[30] also demonstrated the effects of homeopathic *Apis mellifica* preparations on altered gene expression profiles of human prostate cells. The ability of HDs to trigger gene expression profiles is not limited to animal models. Marotti *et al*[31] documented clear evidence of change in expression profiles of wheat seedlings in the plant kingdom following treatment with ultra-high dilutions of arsenic trioxide used as a homeopathic remedy against symptoms of arsenic poisoning. Thus substantial recent evidence on microarray analysis, which is an accepted modern tool of studying large scale gene expression profiles, clearly depicts that homeopathic remedies in ultra-high dilutions can trigger altered gene expressions presumably through epigenetic modifications, validating the "gene regulatory hypothesis" first advocated by Khuda-Bukhsh[11,27].

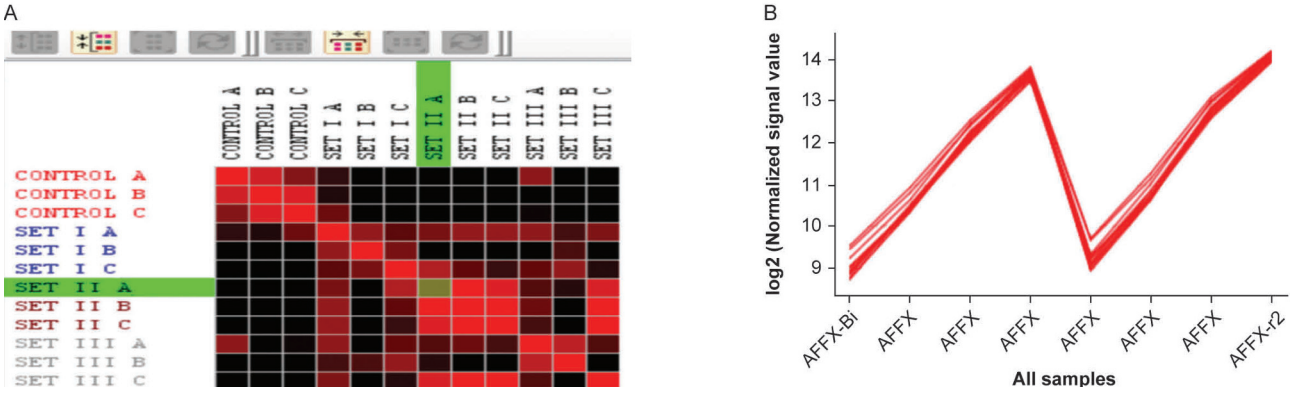


**Figure 3** Hybridization and correlation plot
(A) Pair-wise correlation plot for each experiment; (B) Hybridization plot with control probes.

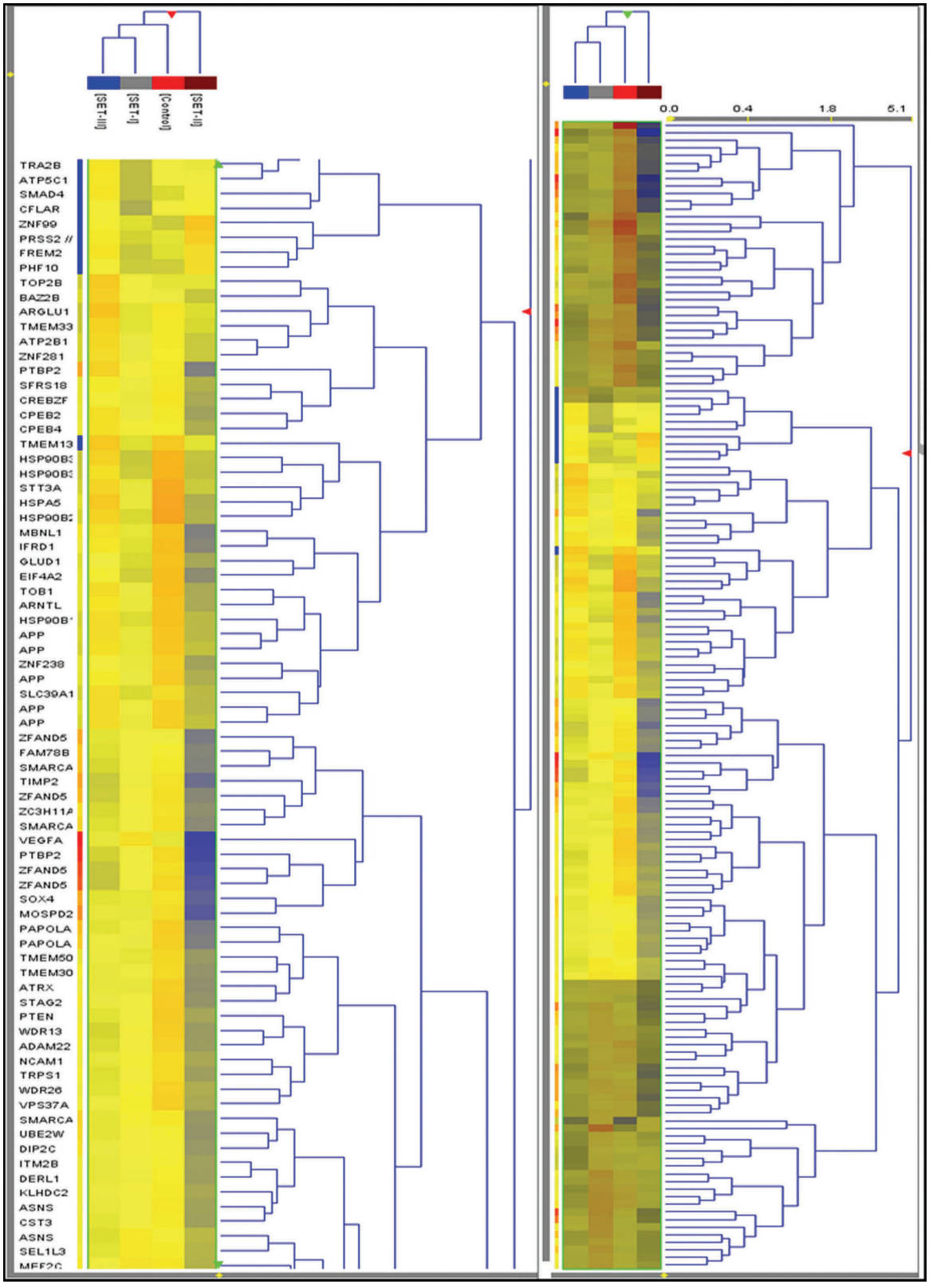


**Figure 4** Cluster analysis

The snap shot of the heatmap image on experiment conditions and genes. The heatmap image was generated on the experiment conditions and classified on the basis of gene expression. Red color shows over-expressed genes (>0) and blue color shows under-expressed genes (<0).

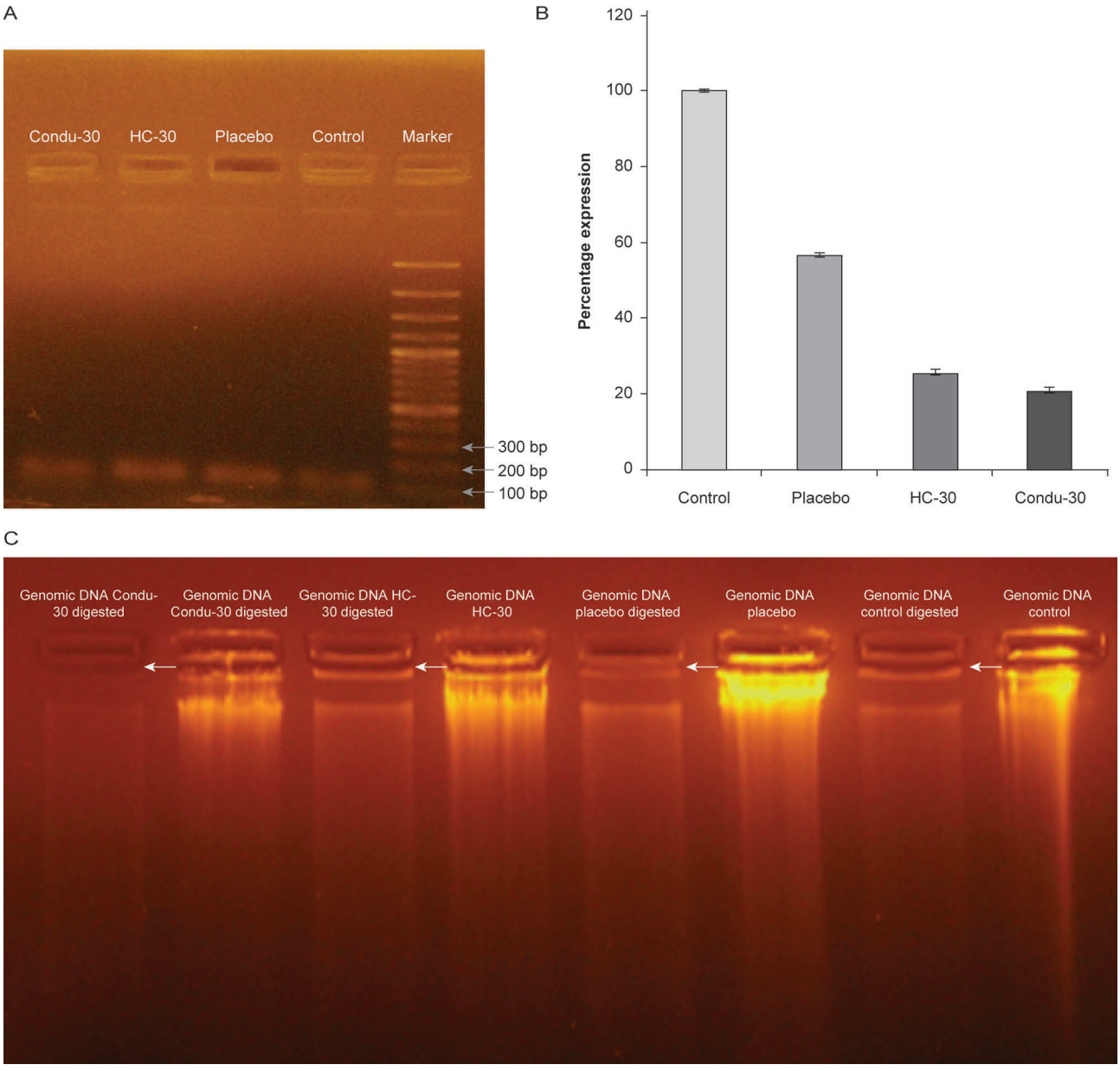


**Figure 5** Functional validation of SMAD4 and methylation-dependent restriction enzyme digestion
(A) Qualitative RT-PCR study of SMAD4; (B) Quantitative RT-PCR study of SMAD4; (C) Methylation-dependent McrBc digestion of genomic DNA obtained from untreated, HC-30-, Condu-30- and placebo-treated HeLa cells. RT-RCR: reversed transcriptase-polynerase chain reaction.

Epigenetic modifications are a hallmark of cancer, and a large number of genes remain in modified state of expression in cancer cells. Our present study of alteration in methylation status of DNA by Condu-30 by McrBc RE supports the previous findings obtained by Bishayee *et al*[8]. Additionally, the results of the present study would further lend support to the gene regulatory hypothesis[11,27,32] that can effectively be achieved by the epigenetic modification triggered by the HDs. Epigenetic modification is one way through which the expression of genes can be strongly influenced and regulated as per need and condition of the organism; it is particularly useful at abnormal physiological states or in disease states, when the regulatory systems are often error-prone.

Our present study revealed the increase in $G_0/G_1$ population by HC-30-treated set compared against untreated control and placebo-treated HeLa cells. Increase in $G_0/G_1$ population is an indication of apoptosis induction. The present findings thus confirm the apoptosis-inducing ability of Condu-30 observed in HeLa cells and H460 lung

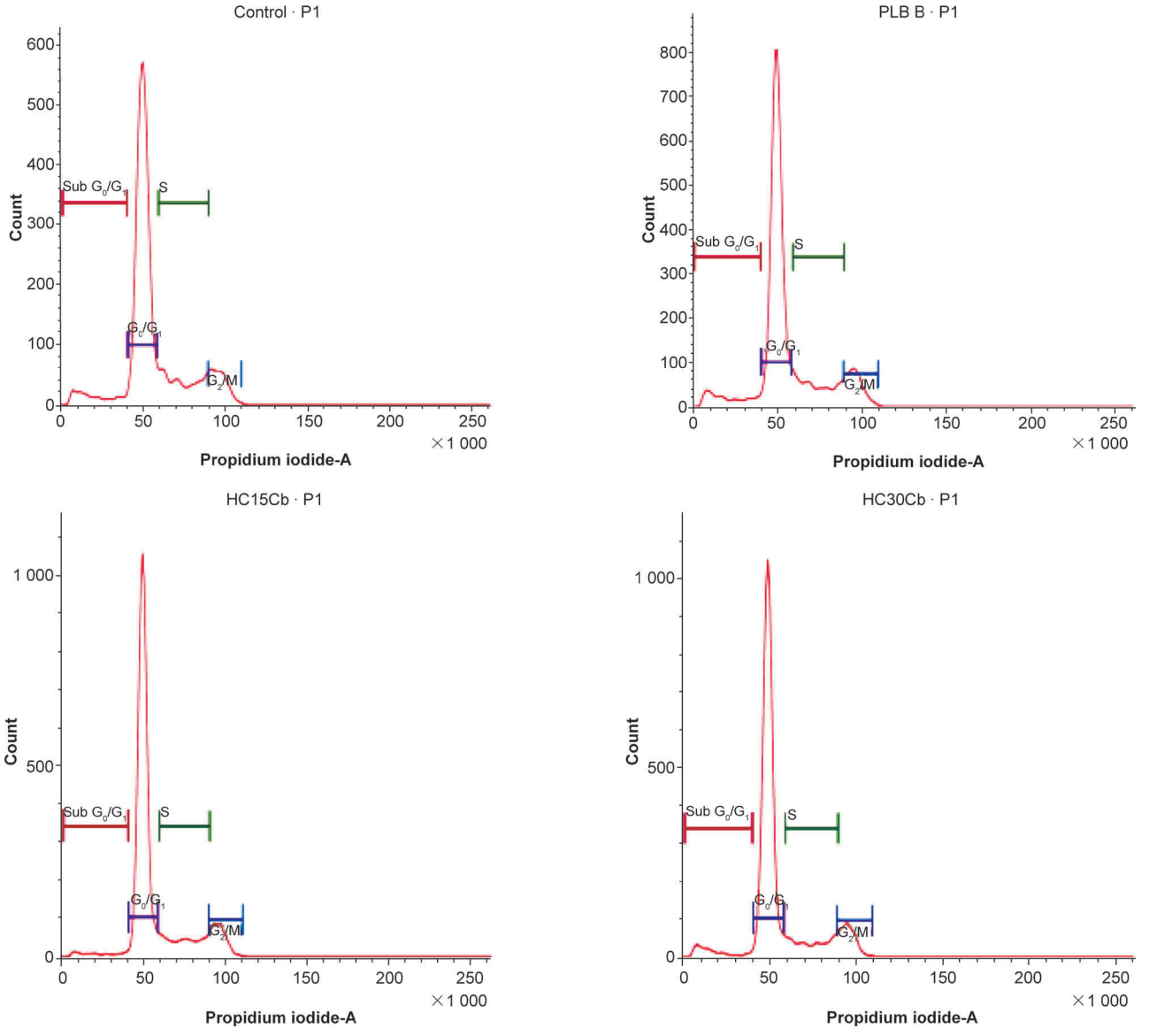


**Figure 6** Cell cycle distribution study

cancer cells, also previously reported by our group[8,9]. As cell cycle events are very much related to the structural and functional states of DNA, and the ability of the HDs to induce changes in DNA methylation status adds an important clue in the induction of apoptosis[28,33],thus, various mechanisms of action shown by the HDs indicate the need for further in-depth studies to see if these phenomena can also be observed in other *in vitro* and *in vivo* experimentations. Our results give further credence for the gene regulatory hypothesis, which can explain the molecular mechanism of action of the HDs in a scientifically validated way. The ability of the HDs in modulation of various genes in the cell cultures indicates that they can have direct influence on the expression of relevant genes, particularly when the HDs have been reported to elicit responses in unicellular organisms such as yeast[34], bacteria and bacetriophages[12–14].

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## 6 Competing interests

The authors declare that there are no competing interests.

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