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Phyllanthus debilis Methanolic Extract Reduces the Viability of Human Colorectal Adenocarcinoma (HT-29) Cells and Increases *LINE-1* and *Alu* DNA Methylation

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ABSTRACT

Phyllanthus debilis was shown to have a strong anti-proliferative effect on cancer cells with less effect in normal cells. However, its mechanism on the epigenetic mechanism at repeat sequences is unknown. This study was carried out to determine the effect of *P. debilis* extract on long interspersed nuclear element-1 (*LINE-1*) and *Alu* DNA methylation. The anti-proliferative effect of *P. debilis* methanolic extract on human colorectal adenocarcinoma (HT-29) at 24 hours was done using trypan blue assay. *LINE-1* and *Alu* methylation measurement on the HT-29 cell line was done after 72 hours of treatment using Pyrosequencing. The effect of *P. debilis* methanolic extract at 24 hours on the viability of HT-29 cells was dose-dependent with the half-maximal inhibitory concentration (IC_{50}) concentration of 0.1 mg/mL. Treatment with *P. debilis* methanolic extract showed significantly higher *Alu* DNA methylation when compared with the untreated HT-29 cells (37.0 ± 2.5% vs 32.3 ± 4.3%, $p<0.05$). Similarly, treatment with 5-aza-2-deoxycytidine also significantly increased the *Alu* DNA methylation compared with the untreated HT-29 cells (46.0 ± 2.3% vs 37.0 ± 2.5%, $p<0.05$). For *LINE-1*, there was a significant increase of *LINE-1* methylation when treated with *P. debilis* extract (80.3 ± 1.3% vs 76.3 ± 2.1%, $p<0.05$) and with 5-aza-2-deoxycytidine (81.8 ± 4.3% vs 76.3 ± 2.1%, $p<0.05$) when compared with untreated cells. In conclusion, treatment of *P. debilis* methanolic extract on HT-29 cell line reduces the viability of HT-29 cells and increases the methylation of *Alu* and *LINE 1*. Similar changes in methylation were also seen in the 5-aza treatment. These epigenetic changes by *P. debilis* methanolic extract may contribute to its anti-cancer properties.

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INTRODUCTION

Aberrant DNA hypomethylation, which was frequently seen in the cancer cells, can induce activation of oncogenes and loss of imprinting (Sharma et al., 2010). Overall genomic methylation in carcinogen-induced cancer cells was reduced by 20–60% compared to normal cells, and demethylation of repetitive sequences' methylation accounts for 20–30% of the human genome (Ehrlich, 2002). Hypomethylation of the genome occurs early in the development of cancer. It accumulates throughout all tumorigenic steps, from benign proliferation to invasive cancer (Fraga et al., 2004). Although gene-specific demethylation occurs in the context of global DNA hypomethylation, many of the effects are thought to be caused by the activation of transposable elements and endogenous retroviruses found in the human genome as by loss of imprinting (Whitelaw & Martin, 2001). Potentially, the reactivation of the strong promoters associated with transposable elements can globally modify the expression levels of transcription factors and/or the gene expression levels of the growth regulatory genes in which these factors reside (Whitelaw & Martin, 2001). Human DNA comprises a significant number of transposable elements. The most studied sequences were *LINE-1*, and *Alu* repeats. *LINE-1* and *Alu* repeat sequences are highly methylated in somatic tissues (Ehrlich, 2002). These repeat genetic elements are sequences that can change places on a chromosome and be exchanged between chromosomes that need to be repressed by methylation to prevent disruption to the genetic sequences and transcriptome (Rodić & Burns, 2013). Studies have shown that changes in methylation at these repeat sequences affected the changes of genome-wide methylation, and these changes can be measured as a surrogate marker for epigenetic changes in genome-wide methylation status (Lisanti et al., 2013).

Phyllanthus debilis, a less common *Phyllanthus* species, is usually used to substitute other popular *Phyllanthus* species, such as *Phyllanthus amarus* (Kumaran & Karunakaran, 2007). This herb shows anti-inflammatory, anti-microbial, anti-diabetic, anti-cancer, and antihepatotoxic properties (Ahmed et al., 2009; Chandrashekar et al., 2005). The aqueous extract of the plant shows an antihyperglycemic property (Wanniarachchi et al., 2009). *Phyllanthus debilis* has been shown to possess higher antioxidant activity than *Phyllanthus amarus*, *Phyllanthus maderaspatensis*, *Phyllanthus urinaria*, and *Phyllanthus virgatus* (Kumaran & Karunakaran, 2007). Many bioactive compounds had been found in *P. debilis*, such as β-sitosterol, debelalactone, and phyllanthin. These compounds were shown to have anti-inflammatory, antihepatotoxic, and anti-cancer (Sarin et al., 2014). Based on traditional usage but limited scientific evidence, *Phyllanthus* sp. could be explored for their potential anti-cancer activity through an epigenetic mechanism in colorectal cancer cells line (HT-29).

METHODS

Sample Preparation

Herbal Specimen Collection and Identification. *Phyllanthus debilis* was collected from a local collection at Tasek Gelugor, Penang, Malaysia. The species was identified by the herbarium unit, School of Biology, Universiti Sains Malaysia. The voucher specimen was deposited at the Universiti Sains Malaysia herbarium unit (*Phyllanthus debilis* specimen number: 11623)

Sample Extraction

The whole sample of *P. debilis* was cleaned and washed before being dried in the oven at 50 °C for three days. The dried sample was then ground and prepared in powder form. First, a five-gram sample was extracted with 100 mL of methanol (Fisher Scientific, USA) in an ultrasonic bath (Power-Sonic 405 Model, Hwashin, Korea) for 20 min and then filtered. The procedure was carried out twice more with the remaining residual extract. Finally, a rotary evaporator was used to dry the extract (Rotary Evaporator RII, Büchi, Switzerland). Prior to use, the dried extracts were kept at a temperature of -20 °C.

Cell Culture for *Phyllanthus debilis* Methanolic Extract on HT-29 Cells Viability

Passage 19 of human colorectal adenocarcinoma (HT-29) was used in this study and was grown in the Roswell Park Memorial Institute (RPMI)-1640 medium. The medium was supplemented with 10% fetal bovine serum (FBS) (Gibco™, USA) and 1% penicillium-streptomycin (10,000 U/mL, Gibco™, USA) to ensure cell growth and viability. The cells were incubated in a humidified atmosphere with 5% carbon dioxide at 37 °C for growth and maintenance.

Viability of HT-29 Cell Lines

Cells were seeded at their optimal cell density (0.05 x 10^6 cells/well) into a 24-well plate and were incubated in 5% carbon dioxide at 37 °C for 36 to 48 hours to allow cell attachment and make them reach the growth of 80% confluency. The cells were then treated *P. debilis* methanol extracts at 6 different concentrations (0.03125, 0.0625, 0.125, 0.25, 0.5, and 1 mg/mL). The experimental control was prepared with only cells and cultured medium without treatment with *Phyllanthus* extract. The experimental plates were incubated at 37 °C, 5% carbon dioxide for another 24 hours.

The cells were then washed using phosphate-buffered saline (PBS) to remove any floating cells and then incubated with trypsin for 5 min to detach the cells from the well. Next, the medium was added to the well to deactivate the trypsin and thoroughly mixed. Once mixed, the cell suspension was then mixed with 0.4% trypan blue solution in the ratio of 1:1. The cells in 10 µL of this suspension were then directly counted on a haemocytometer. The average cell count of four fields represented the number of cells per mL of cell solution and determined the total number of cells from each well.

The number of cells was counted in each of the four quadrants by using the following formula:

$$\text{Number of cell} = \left(\frac{A + B + C + D}{4}\right) \text{x } 10^4 \text{ x 2 x sample dilution}$$

The viability of the cell is determined by comparing viable cells for the treatment with the untreated cell:

$$\text{Percentage of cell viability (\%)} = \left(\frac{\text{Number of viable cells from treatment}}{\text{Number of viable cell from experimental control}}\right) \text{x } 100$$

The percentage of cell inhibition is determined by using the following formula:

$$Percentage\ of\ cell\ inhibition\ (\%) = 100 - Viability\ of\ cell\ (\%)$$

The percentage of cell inhibition (%) versus treatment concentration (mg/mL) was plotted based on the data obtained. The concentration that inhibits 50% of the cells (IC_{50}) was obtained by extrapolating the graphs constructed on the viability test.

Treatment of Cells with *Phyllanthus debilis* for Determination of DNA Methylation at *LINE-1,* and *Alu* Repeats Sequence

HT29 cells were seeded at their optimal cell density (0.4 x 10^6 cells/well) in a 6-well plate in RPMI-1640 medium (Gibco™, USA) supplemented with 10% fetal bovine serum (Gibco™, USA) and 1% penicillin-streptomycin (Gibco™, USA). Cells were maintained at 37 °C in a 5% carbon dioxide (CO_2) atmosphere. IC_{50} of the *P. debilis* (0.1 mg/mL) was used to treat the HT-29 cell line. Stock solution (0.1 mg/mL) was prepared by diluting the crude extracts in RPMI for treatment. 5-aza-2-deoxycytidine (Acros, USA) was used as a positive control in methylation study as the drug act as an epigenetic modulator. 5-aza-2-deoxycytidine stock solution (4.382 mM) was prepared by dissolving 1 mg of 5-aza-2-deoxycytidine (molecular weight = 228.208 g/mol) in 1 mL dimethyl sulphoxide (DMSO) (Sigma Aldrich, USA). The stock solution was then diluted into 100 µM in PBS (Gibco™, USA). Then, the stock was diluted with cultured medium to the concentration of 0.5 µM. The concentration of 5-aza-2-deoxycytidine at 0.5 µM used in this study was shown previously to demethylate genes at genome-wide scale (Ishiguro et al., 2007; Khamas et al., 2012). The working solutions of the drug were freshly prepared before usage. When the cells reached 80% confluency, the cells were then treated with *P. debilis* extract and 5-aza-2-deoxycytidine at the concentration

of 0.1 mg/mL and 0.5 µM, respectively. Treatments were continued for three days (72 hours) while replacing the RPMI-1640 medium and the treatment daily. Cells were harvested after completing 72 hours of treatments.

Bisulfite Modification of DNA

Genomic DNA of the HT-29 cells was isolated using Wizard SV Genomic DNA purification kit (Promega, USA) following manufacturer protocol with slight modifications. The cells were then bisulfite modified by treating them to sodium bisulfite, which selectively converts unmethylated cytosine to uracil. Still, the 5-methylcytosine is protected from deamination and is preserved in the downstream reactions. The bisulfite modification was conducted using EZ DNA Methylation-Gold™ Kit (Zymo Research, USA) by following the manufacturer's protocol with slight modification. Briefly, 500 ng of DNA was incubated with CT conversion reagent at the following temperatures: 98 °C for 10 min, 64 °C for 2.5 h, held at 4 °C. Once completed, the DNA was transferred to a spin column, washed, and desulphonated. It was further purified using wash buffer before being eluted in 20 µL deionised water. The bisulfite modified DNA was kept at 4 °C for further use.

Polymerase Chain Reaction (PCR) and Pyrosequencing

HT-29 cells were assayed at the repeat sequences of *Alu* and *LINE-1*. Each sample was biologically and technically replicated four times. *LINE-1* and *Alu* sequences and primers followed Lisanti et al. (2013). Two microliters (2 µL) of bisulfite modified DNA were used in a polymerase chain reaction (PCR) containing a total volume of 25 µL reaction. The reaction includes 12.5 µL GoTaq Green master mix (Promega, USA), 400 nM forward primer, and 400 nM biotin-labelled reverse primer. The biotinylated labelled reverse primer was used to capture a single-stranded DNA template for the Pyrosequencing assay later. PCR condition was carried out using the following a three-step protocol: an initial denaturation step at 95 °C for 15 min, then 50 cycles of 95 °C for 15 s and annealing temperature 50 °C for 30 s, followed with 72 °C for 30 s, and final elongation step at 72 °C for 5 min. About 5 µL of PCR product was subjected to gel electrophoresis to confirm the amplicon size of *LINE-1* and *Alu*.

The biotin-labelled PCR product was then captured with Streptavidin Sepharose beads (Qiagen, Germany) using a Pyrosequencing Vacuum Prep Tool (Qiagen, Germany). It was made into single-stranded DNA. The single-stranded DNA was then annealed to the sequencing primer and was heated to 80 °C for 5 min and allowed to cool to room temperature. Pyrosequencing was then carried out on a PyroMark ID (Qiagen, Germany). The percentage of methylation at the target cytosine-guanine dinucleotide (CpG) sites was quantified using PyroMark Q96 2.5.8 software (Qiagen, Germany). The primers used in PCR and Pyrosequencing and sequence to analyse *LINE-1* and *Alu* were shown in Tables 1, 2, and 3.

Table 1

The primers sequences of LINE-1 *and* Alu *repeat sequences used in the PCR reaction and Pyrosequencing*

Assay	Forward primer	Biotinylated labelled reverse primer
LINE-1	5'-TTTTGAGTTAGGTGTGGGATATA-3'	5'-AAAATCAAAAAATTCCCTTTC-3'
ALU	5'-TTTTTTTTTAAAGGTTATG-3'	5'-TCTATCCCTAAAATTAAAA-3'

Table 2

The sequencing primer of LINE-1 *and* Alu *repeat sequences used in the Pyrosequencing assay*

Assay	Sequencing primer
LINE-1	5'-AGTTAGGTGTGGGATATAGT-3'
Alu	5'-TTTTTTTTTAAAGGTTATG-3'

Table 3

Sequence to analyse for PyroMark CpG assays

Assay	Sequence to analyse
LINE-1	5'-TTC/TGTGGTGC/TGTC/TG-3'
Alu	5'-TC/TG-3'

Statistical Analysis

Comparison between 2 groups of treatments was made using student *t*-test (SPSS Version 24). All the data presented as mean ± standard deviation (SD).

RESULTS

The Effect of *Phyllanthus debilis* Methanolic Extract Towards Viability of HT-29 Cell Line

The effect of *P. debilis* methanolic extract on the viability of HT-29 was evaluated. Figure 1 shows the results of the effect of *P. debilis* methanolic extract on the viability of HT29 cells. The effect of *P. debilis* methanolic extract on the viability of HT-29 cells was dose-dependent manner. The IC_{50} concentration of *P. debilis* methanolic extract was 0.1 mg/mL.

Measurement of *Alu* and *LINE-1* DNA Methylation in HT29 Cells

The methylation changes of *Alu* and *LINE-1* repeat elements as a surrogate marker for global methylation were measured. DNA methylation was measured at one CpG site in the *Alu* sequence (Figure 2) and 3 CpG sites in the *LINE-1* repeat sequence. For *LINE-1*, data were presented as mean methylation of all 3 CpG sites (Figure 3). The methylation changes at targeted CpG sites of the untreated cells were compared with the methylation changes of the treated cells with *P. debilis* and 5-aza-2-deoxycytidine for 72 hours.

For *Alu* methylation, treatment with 5-aza-2-deoxycytidine showed higher DNA methylation when compared with the untreated HT-29 cells (46.0 ± 2.3% vs 32.3 ± 4.0, $p<0.05$). Similarly, treatment with *P.*

debilis also significantly increased the DNA methylation compared with the untreated HT-29 cells (37.0 ± 2.8% vs 32.3 ± 4.3%, $p<0.05$).

For *LINE-1*, compared with untreated cells, the average DNA methylation showed a significant increase of *LINE-1* methylation when treated with 5-aza-2-deoxycytidine (81.8 ± 4.3% vs 76.3 ± 2.1%, $p<0.05$) and with *P. debilis* (80.3 ± 1.3% vs 76.3 ± 2.1%, $p<0.05$).

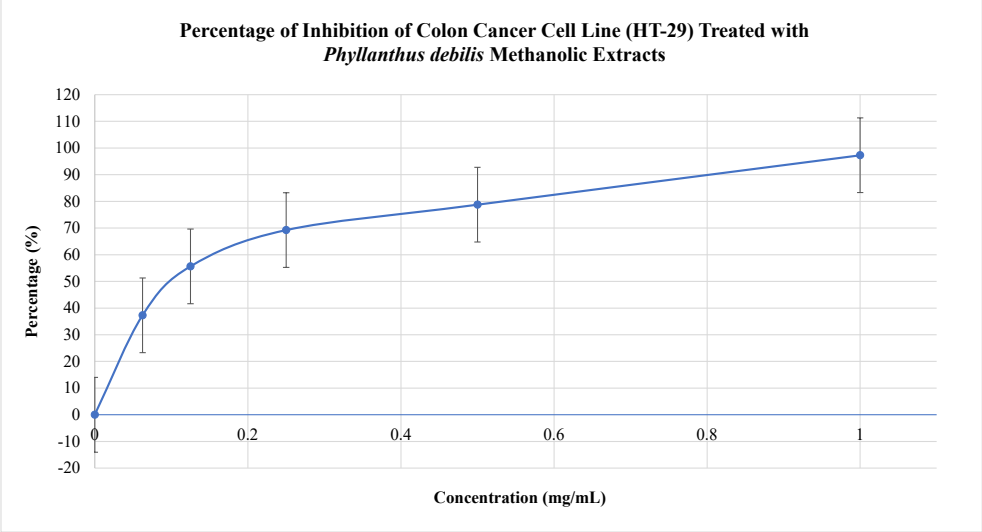

Figure 1. The inhibition of *Phyllanthus debilis* methanolic extract on HT-29 cell line. HT-29 were treated with different concentrations of *P. debilis* methanolic extract for 24 hours

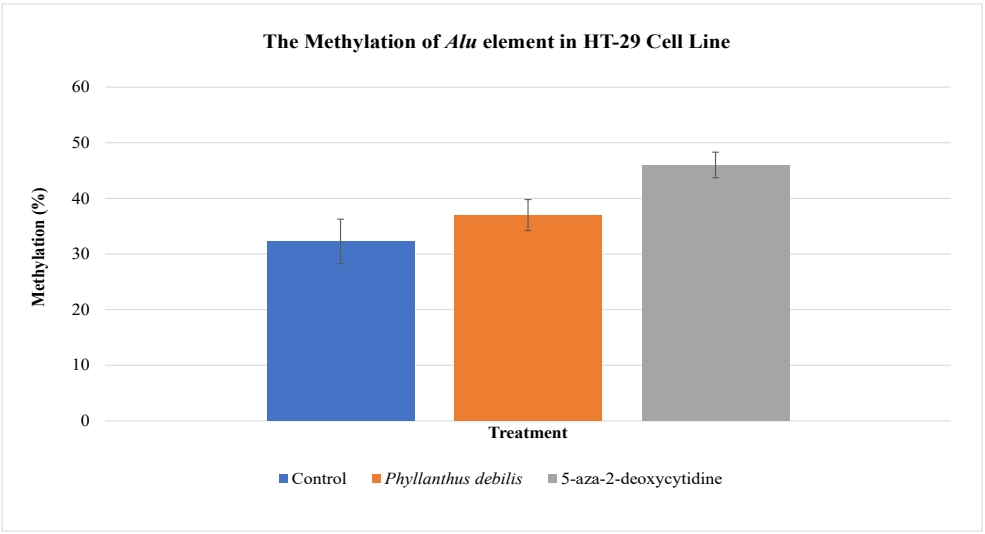

Figure 2. The methylation of *Alu* element in the untreated cells and cells treated with *Phyllanthus debilis* and 5-aza-2-deoxycytidine

Note. * Denotes significant difference in DNA methylation of *Alu* when compared with untreated cells ($p<0.05$)

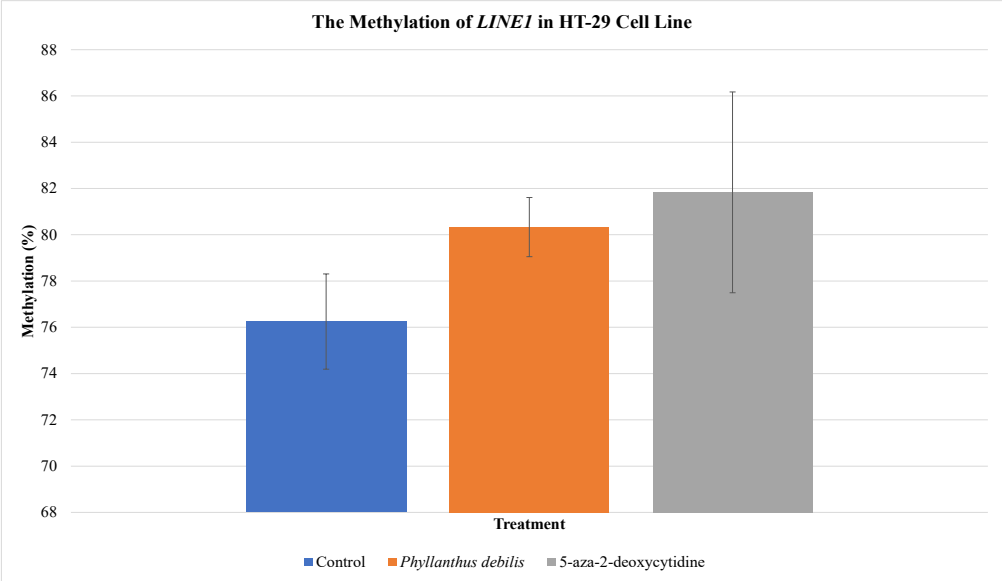

Figure 3. The methylation of *LINE-1* in the untreated cells and cells treated with *Phyllanthus debilis* and 5-aza-2-deoxycytidine

Note. * Denotes significant difference in DNA methylation of *LINE-1* when compared with untreated cells (p<0.05)

DISCUSSION

Genome-wide DNA hypomethylation plays an important role in epigenomic and genomic instability and colorectal carcinogenesis (Natsume et al., 2008). Human DNA contains large numbers of non-coding repeat sequences, the most studied sequences being *LINE-1* and *Alu* repeats. *LINE-1* repeat sequences constitute about 17% of the human genome, while *Alu* repeats constitute about 11% of the human genome (Lander, 2001; Sellis et al., 2007). Changes in methylation at these repeat sequences may affect genome-wide methylation changes, and these changes could be monitored and measured as a surrogate marker for genome-wide methylation status (So et al., 1996). A simple method was used to measure global DNA methylation using bisulfite PCR of DNA repetitive elements, and pyrosequenced was carried out by Lisanti et al. (2013). Lisanti et al. (2013) showed a strong correlation between *Alu* and *LINE-1* methylation with global methylation, analysed by high-performance liquid chromatography (HPLC), in which HPLC was considered a gold standard in measuring genome global DNA methylation.

Genomic hypomethylation demonstrated by downregulation of methylated CpG dinucleotides, which disperse throughout the genomes in non-coding repetitive sequences, has been recognised as a common epigenetic change during cancer development (Feinberg & Tycko, 2004; Sugimura & Ushijima,

2000). It has been proposed that genome hypomethylation contributes to oncogenesis by activating oncogenes, such as *c-Myc* and *H-RAS75*, activating latent retrotransposons, or causing chromosome instability (Das & Singal, 2004). Global hypomethylation in colon, liver, bladder, oesophagus, head and neck, prostate, stomach, breast, and lung carcinomas tissues was common observations compared to their normal tissue counterparts (Chalitchagorn et al., 2004).

Global DNA methylation, measured by HPLC, *LINE-1* and *Alu* assays were lower in colorectal tumour tissue than in paired normal tissue (Natsume et al., 2008). Hypomethylation of *Alu* and *LINE-1* has been reported as early events in the multistep carcinogenesis of colorectal cancer (Chalitchagorn et al., 2004; Kwon et al., 2010; Lee et al., 2009). Hypomethylation of transposable elements, such as *Alu* and *LINE-1*, causes transcriptional activation, resulting in transposable element retrotransposition, chromosome alteration, and thus genomic instability (Bae et al., 2012; Saito et al., 2010). On the other hand, CpG sites within *Alu* and *LINE-1* are usually methylated in normal cells, thus maintaining transcriptional inactivation and inhibiting retrotransposition (Yoder et al., 1997).

This study showed that the treatment with *P. debilis* induces anti-proliferation to HT-29 cells at 0.1 mg/mL with an observable small significant increase in DNA methylation of *LINE-1* and *Alu* element in HT-29 cells compared with the untreated cells. Furthermore, the changes observed due to the treatment of *P. debilis* were also seen when the HT-29 cells were treated with 5-aza-2-deoxycytidine at a low dose (0.5 µM), which showed a similar increase in DNA methylation of *LINE-1* and *Alu* but with higher methylation when compared with *P. debilis*.

Although 5-aza-2-deoxycytidine is a drug that can demethylate global and gene-specific regions (Momparler, 2013), this study showed a reverse pattern of methylation where it was found that 5-aza-2-deoxycytidine at the dose of 0.5 µM increase the *LINE-1* and *Alu* methylation. Whether the observations made on the effect of 5-aza-2-deoxycytidine at a low dose and *P. debilis* methanolic extract happened only in HT-29 cells or whether the findings were specifically at the *LINE-1* and *Alu* target sequences in this study need further evaluation.

A previous study on breast cancer cell lines and normal cell lines showed that *P. debilis* methanolic extract targets the cancer cells while sparing the normal cells (Omar & Zain, 2018). Furthermore, at the gene-specific level, *P. debilis* methanolic extract was able to reduce the *TAC1* DNA methylation (Zain & Omar, 2020). Thus, the specific target of *P. debilis* methanolic extract on cancer cells, and the increasing level of global methylation as observed in this study with increasing *LINE-1* and *Alu* methylation may reduce the proliferative ability of the colorectal cancer cells. Whether these changes may sensitise the cell to undergo apoptosis should further be tested and studied.

CONCLUSION

Treatment of *Phyllanthus debilis* methanolic extract on HT-29 cell line induced anti-proliferation of HT-29 cells with an increase of the methylation of *Alu* and *LINE-1*. Furthermore, increased methylation of *Alu* and *LINE-1* was also seen in the 5-aza-2-deoxycytidine treatment. Thus, the methylation changes in *Alu* and *LINE-1* by *P. debilis* methanolic extract may contribute to its anti-cancer properties, and its regulation on the DNA methylation should be further studied.

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