ORIGINAL ARTICLE

Chemoprevention Effects Through Polyamines Modulation Induced by *Clinacanthus nutans* in Human Lung Adenocarcinoma Cells (A549)

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ABSTRACT

Introduction: Polyamines involves in cellular proliferation, maintenance and transformation which was discovered to be dysregulated in many types of carcinogenesis by which high intracellular bioavailability represent the promotion of tumour growth, invasion and metastasis. While high polyamines types of foods suggested to be portion-controlled in cancer patients, traditional herbal plants have also been investigated for its efficacies in controlling polyamine synthesis thus making it to be suitable chemoprevention agents targeting to mitigate the risk of cancer occurrence and recurrence. This study aimed to determine the cytotoxicity effect and modulation of polyamine synthesis induced by Sabah Snake Grass (*Clinacanthus nutans*) on human lung adenocarcinoma cells, A549. **Methods:** The antiproliferative effect of *C. nutans* was investigated using trypan blue exclusion assay. The intracellular polyamines content was quantified using High Performance Liquid Chromatography (HPLC) while gene expression analysis was done using quantitative PCR. **Results:** The IC_{50} values for *C.nutans* was 20 µg/ml and it has been demonstrated that *C. nutans* hamper the A549 cell's growth after 24 hours of exposure. Depletion of polyamines level after 24 hours to 96 hours of exposure were observed and aligned with a significant gene expression changes of spermidine/spermine-N1-acetyltransferase (SSAT), antizyme (AZ1) and ornithine decarboxylase (ODC) activities. **Conclusion:** *C. nutans* has a potential as chemopreventive agents since they demonstrated to reduce cell proliferation and decrease polyamines intracellularly. The reduction of polyamines reflects by increase catabolic enzymes, SSAT and decreasing activity in biosynthesis pathway which involves AZ1 and ODC. Further investigation is warranted to evaluate the mechanism and pathway of cell death and the impact of *C.nutans* on normal cells.

Keywords: Polyamines; Ornithine decarboxylase; Antizyme; Spermidine/spermine N-1 acetyltransferase; *Clinacanthus nutans*

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INTRODUCTION

According to World Health Organization (WHO), lung cancer ranks as the second most commonly diagnosed cancer and responsible for the highest cancer-related mortality, accounting for one in five cancer deaths (1,2). Meanwhile, Malaysian National Cancer Registry also reported lung cancer as a significant public health issue with increasing incidence of lung cancer at only 9.0% of 5-years relative survival rate due to a rise in smoking rates, exposure to environmental pollutants, and aging population (3). The most prevalent type of lung cancer, accounting for 85% of occurrences, is non-small cell lung cancer (NSCLC), which has three distinct types: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Several risk factors for developing lung cancer involving lifestyle, environmental and occupational exposure are identified where cigarette smoking is the most prominent factor (4). As there are high probability of recurrent lung cancer despite treatments with chemotherapy, radiation and surgery, it has been suggested that the application of chemoprevention is crucial to intercept the development of post-therapeutic recurrence of cancer. Different strategies of chemoprevention should be executed according to the category of either primary, secondary or tertiary. Correspondingly, every strategy of chemoprevention could use either blocking or suppressing agents to

inhibit the initiation stage of tumorigenesis or to decrease the proliferative capacity of initiated cells (promotion and progression) respectively (5,6).

Furthermore, polyamine pathways concerning oncogenes have directly or indirectly played an important role in determining the effectiveness of chemopreventive agents and therefore further findings should be elucidated properly in lung cancer chemoprevention. The elevation of polyamines levels was significantly identified in cancer patients, hence targeting polyamines pathways has been executed for the favourable outcome of chemoprevention and chemotherapy (7,8). Polyamines synthesis could also deprive the antineoplastic immune functions, and enhance the malignancy of cancer along with the invasion and metastasis of cancer cells. Overall, polyamines pathways including metabolism or biosynthesis are mainly depending on the rate-limiting enzyme of ornithine decarboxylase (ODC) to produce putrescine, spermidine, and spermine from ornithine making it ubiquitously available thus enhancing tumour cell growth. Meanwhile, spermidine/spermine-N1-acetyltransferase (SSAT) is the essential rate-limiting enzyme in polyamine catabolism by degradation of spermine to spermidine and vice versa, therefore, lowering the bioavailability of polyamines for proliferation leading to cell death (7,9,10). The polyamines putrescine, spermidine, and spermine are crucial for cell proliferation and functional differentiation through influencing gene expression and protein synthesis. The enzyme antizyme 1 (AZ1), which binds to and helps break down ornithine decarboxylase (ODC), negatively regulates polyamine production (6). AZ1 has been connected to a number of cellular activities because of its effect on polyamine metabolism. It is abundantly dispersed in all tissues (5).

Natural products have been scrutinized for its anti-cancer properties as it has great resources of bioactive compounds with therapeutic potential since half century ago (11). The utmost benefits of natural products as anti-neoplasm are because of low toxicity, minimum side-effects and off-targets effects. Hence, natural products had it prominence as chemo-protective effect in terms of reducing chemotherapy-associated side effects and enhancing the therapeutic efficacy (12). Sabah Snake Grass (*Clinacanthus nutans*) are among promising Malaysian herbs due to its chemopreventive potential where previously it showed anti-proliferative and apoptotic effects on breast (MCF-7), liver (HepG2), colon (LS-174T), and cervical (HeLa) cancer cells (13). The herbal plant have been use traditionally as anti-microbial, anti-inflammatory, anti-viral against herpes simplex virus, anti-oxidant, anti-cancer, and possess hepatoprotective effects (14–17). The promising phytochemical properties in *C. nutans* was speculated to be effective as chemoprevention are catechin, quercetin, luteolin, gallic acid, squalene, kaempferol, flavonoids, and vitamin E (18,19). However, its effects on A549 cells proliferation through polyamine pathways is not fully elucidated yet. Therefore, the purpose of this study was to determine how *C. nutans* affected the proliferation of A549 cells and metabolic enzymes for polyamines (AZ, ODC and SSAT).

MATERIALS AND METHODS

Plant material

Fresh *C. nutans* plants or Malay name Belalai Gajah were bought from Taman Pertanian Jubli Perak Sultan Haji Ahmad Shah in Kuantan, Pahang. The botanical vouchering of the plants was done at Kulliyyah of Pharmacy, International Islamic University Malaysia with voucher specimen number is PIIUM0238.

Preparations of plant extracts

The collected leaves of *C. nutans* were rinsed with tap water, then rinsed with distilled water and oven dried (50 °C). The leaves were then put through an electronic blender and pulverised to powder. The extraction method used was maceration where the powdered leaves was macerated for 48 hours in distilled water at weight to volume ratio of 1:5. The extract were then filtered twice and frozen at -80°C. Samples were then freeze-dried. The extract powder was kept at room temperature until used for experimental procedures. The extraction protocol was adapted from (20). The IC_{50} concentration of *C. nutans* extract (20 µg/ml) which diluted in distilled water were prepared fresh before used for treatment on cells.

Cell culture maintenance and treatment

The Dulbecco's Modified Eagle Medium (DMEM) was used for the cultivation of A549 human lung cancer cell lines, CCL-185 (ATCC, Manassas, VA, USA), which were also supplemented with 10% One Shot™ Fetal Bovine Serum (FBS) and 1% Penicilin-Streptomycin. Cells were grown and maintained at 37°C in an environment with 5% CO_2 and humidity. The concentration treatment with *C. nutans* extract used was 20 µg/ml where the results obtained from IC_{50} concentration from our published results (20).

Cell Growth Inhibition

A549 cells were seeded on 5 cm2 cell culture plate at seeding density of 1.96 x 10^5 cells/ml. After 48 hours, the cells were exposed with IC50 concentration of *C. nutans* obtained MTT assay from our previously published results (21). Negative control was treated sterile distilled water (solvent used to dilute *C. nutans* extract) while positive control was treated with etoposide dissolved in dimethyl sulfoxide (DMSO) and later diluted in distilled water. Cells were harvested and counted using trypan blue at different time points (every 24-hour until 144-hours). Viability

of cells were counted using haemocytometer under EVOS inverted microscope using the protocol described by (22).

Measurement of Intracellular Polyamine level

The intracellular level of polyamines of A549 cells were then determined at several time points after treatment where the treatment started after 48-hour of cell seeding and intracellular polyamines levels were observed every 24-hour from 24 to 96 hours of exposure with the plants using a pre-column derivatization method for high-performance liquid chromatography (HPLC) determination according to a modification method by (21,23). A549 cells were harvested after trypsinization with 0.5 ml of Trypsin EDTA and centrifuged at 5,000 x g for 5 minutes at 5°C. The supernatant were decanted and tube were gently tapped to loosed the cell pellet. Cell suspension was then transferred into clean Eppendorf tubes after the pellet had been resuspended in 1 ml of PBS. The procedure followed by centrifugation at 7,500 x g for 5 minutes and removed the supernatant. The pellet was then resuspended in 300 µl of 0.2 M HClO4 and kept on ice for 20 minutes. Samples were then centrifuged once more for 5 minutes at 7,500 x g and the supernatant containing the polyamine fraction was transferred into microcentrifuge tube and was kept at -20 °C until HPLC analysis.

HPLC analysis were executed by dansylating of samples at 25 °C for 8 to 10 hours where the samples were then extracted in toluene and dried by nitrogen stream before reconstituted in 200 µl of acetonitrile. The samples were then subjected to a 3-minute, 7,500-gav centrifugation. Reverse-phase HPLC was used to analyse the reconstitute polyamine samples on an HIRPB-2922 column employing a gradient of 60% deionized water to 40% methanol, 40:60 (v/v ratio).

Gene expression analysis using quantitative Polymerase Chain Reaction (qPCR)

After being exposed to *C. nutans* aqueous extract for a variety of time periods (0–48 h), A549 cells were washed twice with PBS and then treated with TRIzol reagent for the extraction of total RNA in accordance with the manufacturer's (TRIzol) instructions. The absorbance at 260/280 nm was used to gauge the yield and quality of total RNA. According to the manufacturer's recommendations (Iscript supermix, Bio-Rad, California, USA), reverse transcription was performed using 1 g of total RNA and 0.5 g of the random primers. The acquired cDNA was diluted to a volume of 100 ul in water that had been treated with diethylpyrocarbonate and used as a template for real-time PCR. In a summary, PCR primers were generated with melting temperatures (Tm) ranging from 65 to 95 °C. 50–150 bases made up an amplifying unit. To prevent amplification of genome sequences, forward and reverse primers spanning exon-exon junctions were chosen; OCD, 5′-AAAACATGGGCGCTTACACT (forward primer) and TGGAATTGCTGCATGAGTTG-3′ (reverse primer); SSAT, 5′-CACCCCTTTTACCACTGCCT (forward primer) and TGCCAATCCACGGGTCATAG-3′ (reverse primer); for AZ1 5′-TGTACTCCGACGAGCGGCTG-3′ (forward primer) and 5′-GTGACCTGCTTGGCCTCCGT-3′(reverse primer). Two housekeeping genes used were; ACTB, 5′-AGTCCTGTGGCATCCACGAAA (forward primer) and GTCATACTCCTGCTTGCTGA-3′ (reverse primer); and GAPDH, 5′-TCCCTGAGCTGAACGGGAAG (forward primer) and GGAGGAGTGGGTGTCGCTGT-3′(reverse primer).

qPCR analysis for gene expression

The threshold cycle value (Ct), or the moment at which a significant rise in fluorescence is first noticed, was used to calculate quantitative values. Each sample was normalised based on the amount of β -actin present, which was quantified as an internal RNA control. After that, each sample's relative gene expression level was standardised against the control. The average Ct value of a target gene was subtracted from the corresponding Ct value of the β -actin gene to arrive at the final results, which were expressed as an n-fold difference in gene expression in relation to β -actin and calibrator.

Statistical analysis

GraphPad Prism 5.0 was used to conduct the statistical analysis. Data were analysed using either a two-way ANOVA with Bonferroni's post-test or the Student's unpaired t-test, and are shown as mean±SEM. When a *p*-value less than 0.05 was found, the results were considered significant.

RESULTS

Anti-proliferative effect

Figure 1 and 2 showed the increasing pattern of cell number from 0 to 120 hours for untreated A549 cells while the treated A549 cells with *C.nutans* presented decreasing cell number starting from the exposure at 48 hours. By comparing the differences in cell number and percentage of viable cells between untreated and treated A549 cells (labelled as * in Figures 1 and 2), statistical analysis using Two way ANOVA with Bonferroni's post-test revealed *p*-value less than 0.05.

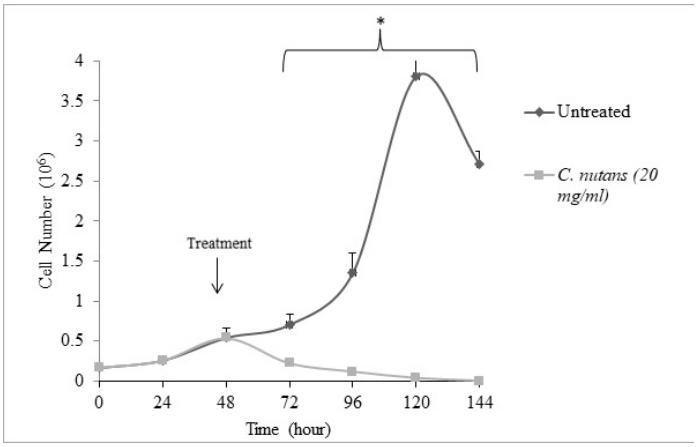

Figure 1 : The A549 cells number over time for untreated and treated samples (* is where *p*-value < 0.05 when the difference in cell number was compared between *C. nutans* treated cells and untreated).

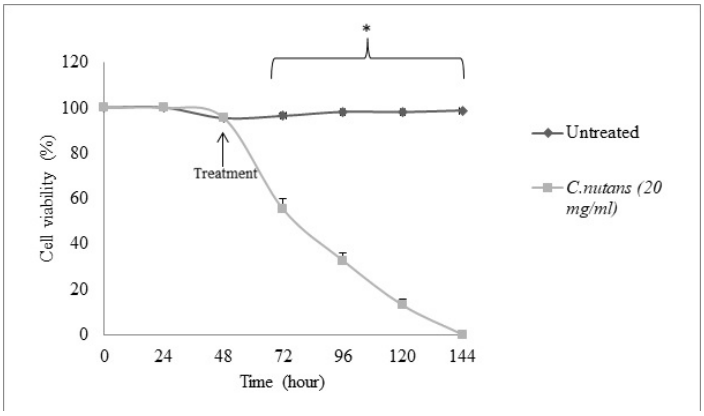

Figure 2 : The viability of A549 cells between untreated and treated cells with *C. nutans* extract (* is where *p*-value < 0.05 when the difference in percentage of cell viability was compared between *C. nutans* treated cells and untreated).

Determination of intracellular polyamines changes

To confirm the roles of polyamines in chemoprevention, the intracellular changes of polyamines induced by *C. nutans* in A549 cells were quantified as time-response analysis. The study found that the intracellular polyamines levels increased and fluctuated in untreated A549 cells over 144 hours of observation whereas there were gradual decrease in intracellular polyamines levels in A549 cells treated with plants extract starting from treatment at 48-hours (*p-value < 0.05) as shown in Figure 3.

ODC, Antizyme-1, and SSAT gene expression analysis

In A549 cells, the polyamines gene expression level was plotted against time in culture where ODC expression level illustrates the downregulation of cells treated with *C. nutans* at 24-hour exposure as compared to untreated cells (Figure 4). Moreover, the expression of AZI expression significantly different between treated and untreated cells. The AZI gene in untreated

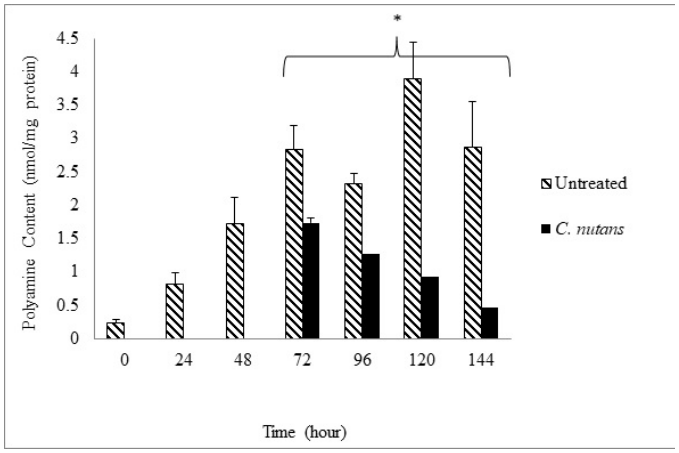

Figure 3 : Time-response of intracellular polyamines changes between untreated and treated A549 cells (* is where *p*-value < 0.05 when polyamine content significantly reduced in *C. nutans* treated cells as compared to untreated).

cells increased significantly from 0 to 48-hour while AZI expression did not increase starting from the exposure with *C. nutans* at 0 hour and reduced at 48-hour exposure (Figure 5). Meanwhile, it has been shown that the SSAT gene expression was increased with the treatment of C. nutans. The *C. nutans* induced an increase of 50% of SSAT gene expression after 24 h exposure compared to untreated cells (Figure 6). The expression remained high at 48-hour with overall upregulation between 50-60% for both treatments.

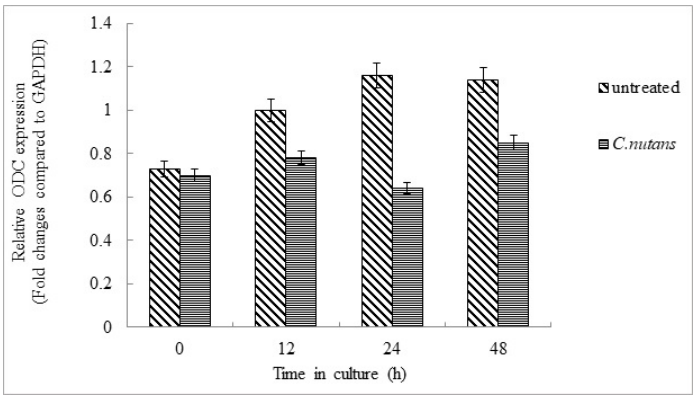

Figure 4 : The relative changes of ODC expression at different time points on A549 cells between treated and untreated cells with *C. nutans* (* is where *p*-value < 0.05 when ODC expression significantly reduced in *C. nutans* treated cells as compared to untreated).

DISCUSSION

Chemoprevention are influential strategies in suppressing, reversing, and preventing the carcinogenic advancement to invasive cancer through utilisation of chemical agents including natural compounds from medicinal plants (24). As current chemotherapy causes side effects without complete cure, risks of cancer recurrence and chemotherapeutic drug resistance,

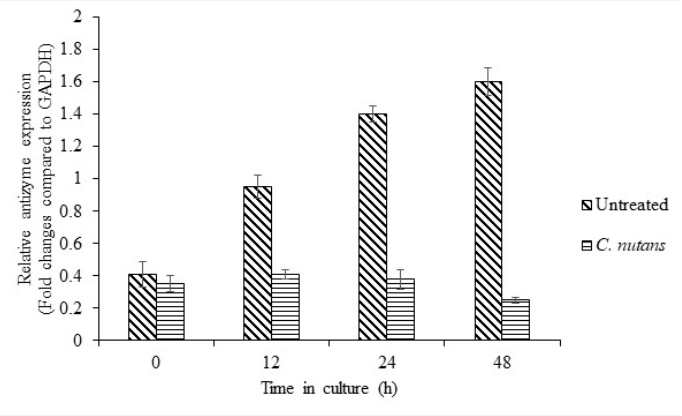

Figure 5 : The relative changes of AZI expression at different time points on A549 cells between treated and untreated cells with *C. nutans* (* is where p-value < 0.05 when AZI expression significantly reduced in *C. nutans* treated cells as compared to untreated).

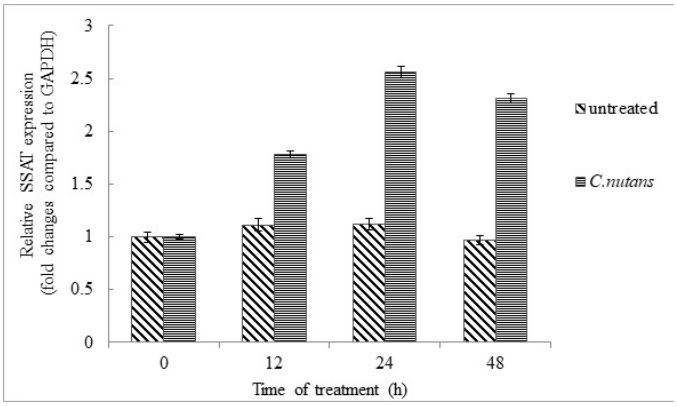

Figure 6 : The relative changes of SSAT expression at different time points on A549 cells between treated and untreated cells with *C. nutans* (* is where *p*-value < 0.05 when SSAT expression significantly increased in *C. nutans* treated cells as compared to untreated).

natural compounds have been widely investigate as central play for alternative treatment strategies. Traditional Malay herbs have been receiving much attention as healing properties for many types ailments since long time ago as scripted from ancient Malay Medical Manuscript. *C. nutans* that have been used to relief inflammatory conditions also showed cytotoxicity, anti-proliferative, antimicrobial and antioxidant against cervical (HeLa), liver (Hep-G2), leukimia (K562), colon (HT-29), breast (MDA-MB-231 and MCF-7), and stomach (CRL 1739) cancer cell lines (25–28). The exponential growth of cancer cells without treatment reflected the cell proliferation and limitless replicative potential while inhibition of cell numbers marked the anti-proliferative effects and inhibit further growth of cells caused by the *C. nutans* as presented in Figure 1 and 2. While our study use aqueous extract of *C. nutans* (IC_{50} = 20 µg/ml) there are previous findings investigated the antiproliferative effects of *C. nutans* on lung cancer, A549 cells where methanol, hexane and chloroform extracts showed cytotoxicity at IC_{50} of 300, 164.1, and 74 µg/ml respectively (29). Anti-proliferative and antioxidant activity imparted on cancer cells showed the ability of the plant to limit the progression of cancer cells leading to apoptosis (26).

Since elevated levels of polyamines are seen in eukaryotes and have been shown to have a significant impact on the development of cancer cell lines, including those that cause lung, prostate, colon, and skin cancers, it has repeatedly been discovered that the rate-limiting enzymes alter polyamine biosynthesis and catabolism. Hence, targeting polyamine synthesis for chemoprevention is crucial to investigate the carcinogenesis as increasing polyamines levels signify the high metastasis rate while depletion levels of polyamines showed low proliferation of cancer cells (30). While ornithine decarboxylase (ODC), a short-lived protein, converts ornithine to putrescine to increase the bioavailability of polyamines, antizyme (AZ1) on the other hand connects with ODC by sensitively controlling ODC activity and degradation. Antizyme can decrease the levels of polyamines through three mechanisms; 1) disruption of active ODC homodimer; 2) interruption of polyamines transport system; or 3) 26S proteasome-mediated, ubiquitin-independent degradation of ODC (31,32). Polyamine homeostasis is complex and is controlled at the level of biosynthetic and catabolic enzymes, as well as transport. The extent of this regulation was seen in the present study because ODC expression is lower in treated cell as compared to untreated cells. Evidence possibly linking the reduction in spermidine content to changes in polyamine metabolic enzymes activity has been obtained. Moreover, linkage between polyamine metabolic enzymes, spermidine depletion and induction to apoptosis has been revealed in this study. The depletion of polyamines occurred in conjunction with the induction of SSAT in A549 cells treated with *C. nutans* as compared to untreated A549 cells (Figure 4).

Another novel finding to note is that *C. nutans* plants stimulate an inhibition of ODC and AZI activity while upregulating the SSAT activity in A549 cells. Elevated of ODC activity is linked to the increases of polyamine content in the cells. It has been found that the over-expression of ODC is associated with cancer cells transformation (33,34). Therefore, inhibition of ODC has an excellent therapeutic potential in many cancers (34,35). This study illustrates that ODC inhibition results in reduction of putrescine and spermidine concentration in A549 cells (Figure 5). Moreover, the reduction of AZI expression also resonates the reduction of ODC because AZI responsible in regulating the homeostasis of polyamines by controlling the ODC activity (8,10,36). On the other hand, SSAT gene plays a major role in the polyamine's catabolism pathways by degradation

of spermine and spermidine trough acetylation into N1-acetylspermine and N1-acetylspermidine respectively (30,37). Increase of SSAT expression in A549 cells with the treatment of *C. nutans* explained the increase catabolism rate in lowering the production of endogenous polyamines pool of lung cancer cells thus concludes the reduction of A549 cells number and percentage of A549 cell viability. Interestingly, this study has shown that *C. nutans* are an excellent candidate as chemo preventive agents targeting polyamine synthesis in lung adenocarcinoma through restriction of ODC expression and upregulation of SSAT gene. It should also be emphasised that the process for cells to commit apoptosis can involve the suppression of ODC function. The results of the current investigation on the molecular processes of A549 caused by changes in ODC, AZI, and SSAT expression were able to add new understanding about the use of Malay herbs as chemoprevention agents.

CONCLUSION

In conclusion, *C. nutans* were able to show the anti-proliferative effect and showed gene expression changes in ODC and SSAT on A549 lung cancer cells. Thus, *C. nutans* is among potential alternatives or complementary medicine for chemoprevention of lung cancer.

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