ANTI-PROLIFERATIVE ACTIVITY OF PILOT SCALE PRODUCTION OF ANTI-BREAST CANCER PRODUCT (COALEX-1) FROM COCOA LEAVES EXTRACT

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ABSTRACT - *To solve the increased global burden issue due to breast cancer disease, extract from Theobroma cacao has been used as an alternative therapy for improving its efficacy against breast cancer and minimizing toxicity. The objective of this study is to investigate the anti-breast cancer activity of Theobroma cacao leaves extract (CoaLeX-1) against human breast cancer (MCF-7) cells. The CoaLeX-1 was extracted using a pilot-scale extraction process. The anti-breast cancer activity of MCF-7 cells was evaluated by MTT assay and different time activities. The morphology phase contrast and different dosage assay were observed by a light inverted microscope. The results showed that CoaLeX-1 can inhibit the proliferation of human breast cancer cells, with* IC_{50} value *of 4.17 0.48 g/mL after 48 hours of treatment at different concentrations. Percentage cell viability at a time point of 48 hours inhibits the cell proliferation in the time-dependent manner of extracts. The morphology features of apoptosis were observed on the cell by showed the formation of shrinking, membrane blebbing, and membrane-bounded vesicles after 48 h of treatment. In the addition, CoaLeX-1 also exerted significant cytotoxic effect against MCF-7 cells by inducing marked morphological changes associated with apoptosis and a decrease in the number of cells in a time and dose-dependent manner after 2 days treatment at a concentration of 5 µg/ml. In conclusion, the present study demonstrated that CoaLeX-1 possesses anti-breast cancer potential against MCF-7 cells either used alone or as an adjunct to the standard chemotherapeutic drugs.*

***Key words*:** Anti-breast cancer, leaves extract, *Theobroma cacao*, MCF-7 cells, cocoa

INTRODUCTION

According to the World Health Organization (WHO) in 2016, cancer is defined as the uncontrolled growth of cells that can spread to other parts of the body which normally broadened to more distant organs such as the bone, liver, lung and brain in a process known as metastasis (Poobalan et al., 2018). Epidemiological and laboratory data indicate that cancer is related not only to genetics but also to lifestyle, including dietary, harmful chemicals and environmental exposure that can stimulate the alteration of genetic and DNA injury (Elsamanoudy et al., 2016).

Breast cancer is the most commonly diagnosed cancer and leading cause related-deaths in women worldwide. In the year 2018, GLOBOCAN estimated about 18 million new cases and 9,555,027 deaths which 11.6 % obtained from breast cancer cases were reported over the world in both ages and sexes (Globocan, 2018). As a result of this drawback of current treatment methods that may cause serious side effects such as immune system compromise, neutropenia as well as the destruction of a normal cell, natural products have been used as an alternative therapy for improving its efficacy against cancer and inducing cell toxicity (Singh *et al*., 2019). Historically, natural products play as lead molecules for drug development with unique novel pharmacophores as compared to synthetic products. It also shows excellent biological activities and has been a major source of therapeutic agents (Chen et al., 2015).

It has been reported that several experimental models from natural products have resulted as anticancer agents (Anggraini *et al* 2019) and play a significant role in treating cancer by the ability to induce apoptosis in cancer cells (Singh *et al*., 2019). One of the potential natural plants that are being investigated in our laboratory is *Theobroma Cacao*, which is also known as cacao or cocoa, belongs to the family *Sterculiaceae*, order Malvales. Based on the scientific name of *Theobroma*, it is taken from the Greek words: *Theo* is meaning "god" and *Broma* is meaning "drink". People from Olmec and Mayan believed that cacao as "a drink of the Gods" (Ettinger *et al*., 2017).

Studies have demonstrated that cocoa can reduce the growth and proliferation of breast carcinoma cells (MCF-7). Based on Ishaq *et al*. (2017), phytochemical studies on cocoa leaves from *Theobroma cacao* showed the presence of flavonoids, procyanidins, methylxanthines, fibers, and various phenolic compounds which gives anti-proliferative activity against breast cancer cell line. Flavonoids and polyphenols in the seed of cocoa may contribute to the control of NADPH oxidase activity and decreased NF-κB activation (Ettinger et al,. 2017). Similarly, cocoa induces positive effects on blood pressure, insulin resistance, and vascular function by increases production of nitric oxide (NO), delayed oxidation of low-density lipoprotein (LDL) cholesterol and inhibiting ultraviolet-induced DNA oxidation (Montagna et al., 2019).

Previous studies reported MTT assay has been used to evaluate the anti-cancer activities and the effects of the cocoa extract on MCF-7 cell viability. It has been shown that cocoa leaves extract contributes to the prevention and anti-cancer agent activity (Baharum *et al*., 2016). Based on Ariza *et al*., 2015, the cytotoxic potential of cocoa extract was much lower than doxorubicin which is the IC_{50} value was 0.777 µg/ml (MCF-7) and 0.082 µg/ml (T47D). It is shown, bioassay using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and cytotoxic method is very important for evaluating the anti-cancer activities. The objective of this study is to investigate the anti-breast cancer activity of cocoa leaves extract (CoaLeX-1) from *Theobroma cacao* against human breast cancer (MCF-7) cells.

MATERIALS AND METHODS

General chemicals

Roswell Park Media Institute (RPMI) 1640, penicillin/streptomycin (100x), foetal bovine serum mycoplex (FBS) and trypsin-EDTA (1x) and Thiazolyl Blue Tetrazolium Bromide (MTT) were obtained from Nacalai Tesque (Japan). Dimethyl sulfoxide (DMSO), Trypan blue (0.4 %) and phosphate-buffered saline (PBS) were purchased from Sigma Chemical Co. USA. Tamoxifen tablets (20mg, Nolvadex D) were purchased from AstraZeneca UK.

Plant material preparation

Fifteen (15) kg of fresh cocoa leaves were collected at cocoa smallholder field in several areas such as Durian Tunggal in Melaka, Bagan Datuk in Perak, Bagan Lalang in Selangor and Ranau Sabah during peak season of cocoa fruiting in middle year 2019. A voucher specimen (SK 2434/14) was deposited at the Bioscience Institute of Universiti Putra Malaysia. The leaves were harvested, cleaned and washed with distilled water before being air dried in the shade at room temperature. After this period, the leaves were grounded using a heavy duty blender with sizes of filter 2 mm and until it became powder form. The powder was kept in a clean plastic container for preservation from light, heat and moisture until further use.

Extract preparation

Cocoa leaves were sent to Bio Aromatic Research Center of Excellence Universiti Malaysia Pahang (UMP), Gambang for the extraction process. Cocoa leaves went through the pilot scale process of water distillation, filtering, concentrating and spray drying. An amount of 15 kg of dried powdered cocoa leaves were added into 200 L of distilled water and extracted by using a multipurpose extraction machine for 2 hours at 100 ℃. The extract was

then filtered using a clean muslin cloth and followed by the 100 micron filter paper to ensure no powder left. The cocoa leaves extract (CoaLeX-1) was concentrated using a vacuum concentrator and then undergo to 6 hours of spray dry for dryness. The CoaLeX-1 was successfully extracted from the fresh cocoa leaves with 0.85 % of yield. Finally the CoaLeX-1 was stored at 4˚C until further analysis.

Cell culture preparation

Human breast cancer (MCF-7) cells were grown in Roswell Park Media Institute (RPMI) 1640 supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin as a complete growth medium. The complete media was preheated in water bath for 30 minutes at 37 ℃ before used. The cells were recovered using thawing method and were kept in a humidified incubators with 5 % CO_2 at 37 ◦C. Cell Culture and preparation were evaluated according to the method described by (Chaudhary *et al.*, 2015) with some modifications. The cells were maintained by sub-cultured when the cells achieved about 80-90 % confluence. Old media was discarded and washed with PBS solution for twice. After removing PBS solution, 1 mL of trypsin-EDTA solution were added and incubated for 5 minutes at 37 ℃. Next, the culture flask was tapped and observed under inverted microscope to ensure cells were fully detached from the surface of the flask. 2 mL of complete media were added to deactivate the trypsin activity then were dispensed into another flask and were kept in a humidified incubators with 5 % CO_2 at 37 ◦C.

Anti-proliferative assay

The inhibition of proliferation of MCF-7 cells were tested by MTT assay *in vitro*. The principle of this method is reacting the bioactive compound with cancer cells. Conversion of tetrazolium salt (MTT) into formazan blue is found only in cells that are still alive and the amount of formazan produced is proportional to the number of existing living cells. Thus MTT assay was used to test potential anti-proliferative activity of the extract (Nisa *et al*., 2017). In this assay, about 80-90 % confluent cells at exponential phase were harvested by trypsinization for cell viability assay. Before starting the MTT assay, about $1x10^5$ cells of MCF-7 were seeded into 96-well plates containing 100 µl of media. After 24 hours of incubation, cells were treated with CoaLeX-1 at different concentrations from 1000 μg/ml to 0.001 μg/ml with three replications with incubation time of 48 hours at 37◦C and followed by addition of 20 μl of MTT reagent and incubated for 4 hours at 37◦C. The medium was removed and 100 μl of DMSO was added to the 96 well plates. After solubilizing the purple formazan, absorbance was measured using a Thermo Scientific Varioskan Flash multimode reader, USA at wavelength of 540 nm. Cytotoxic activity was recorded as IC_{50}, which is the concentration necessary to reduce the absorbance of treated cells by 50 % compared to the control (untreated cells).

Different time point assay

Cells viability and cytotoxic of MCF-7 cells at different time points were evaluated using MTT assay according to the method described by (Wang *et al*., 2019) with some modifications. MCF-7 cells were trypsinized and collected in a conical tube. The number of cells were calculated and seeded into 96-well plates with the cell concentration ($1\ x\ 10^5$ cells/well). After 24 hours of incubation, cells were treated with CoaLeX-1 and Tamoxifen of different concentrations from 1000 μg/ml to 0.001 μg/ml with technical replicates and incubated for 15, 24, 48 and 72 hours. The test compound containing media was added with 20 μl of MTT reagent and incubated for 4 hours at 37◦C. The medium was removed and 100 μl of DMSO was added to the 96 well plates. After solubilizing the purple formazan, absorbances were measured using a microplate reader at wavelength of 540 nm. Cytotoxic activity was recorded as IC_{50}, which is the concentration necessary to reduce the absorbance of treated cells by 50 % compared to the control (untreated cells) and was defined as the concentration required to inhibit cell viability by 50 %.

Cell morphology assay

In this assay, cell morphology changes of MCF-7 cells were evaluated using an inverted light microscope with

phase-contrast according to the method described by (Khazaei *et al*., 2017) with some modifications. About 80-90 % confluent cells at exponential phase were harvested by trypsinization for cell viability assay. Human breast cancer (MCF-7) cells were seeded into 6 well plates, in which each well contains a total of 2 mL media. The plate was incubated for 24 hours with 5 % CO_2 at 37 °C. After 24 hours of incubation, cells were treated with CoaLeX-1 at different concentrations at IC_{25} (585.0 μg/ml), IC_{50} (4.71 μg/ml), and IC_{75} (0.83 μg/ml). The treated cancer cells were incubated with 5 % CO_2 at 37 °C for 48 hours. The untreated cells were applied as the negative control. After 48 hours incubation time, the untreated and treated cells were observed for morphological changes and for characteristic of apoptosis under Leica DM IL LED inverted light microscope with phase-contrast (Leica Microsystems, Germany) at 10x and 20x magnification.

Different dosage assay

Assay of cell morphology changes for MCF-7 cells treated in a time-dose dependent manner were evaluated using an inverted light microscope with phase-contrast according to the method described by (Razak *et al*., 2019) with some modifications. About 80-90 % confluent cells at exponential phase were harvested by trypsinization for cell viability assay. Human breast cancer (MCF-7) cells were seeded into 6 well plates, in which each well contains a total of 2 mL. The plate was incubated for 24 hours with 5 % CO_2 at 37 °C. After 24 hours of incubation, old media of cells were discarded and treated with CoaLeX-1 at different doses which were 50, 250, 500, 750, 1000 μ g/ml continuously every 24 hours for 4 days and incubated with 5 % CO_2 at 37ºC. The untreated cells were applied as the negative control. The untreated and treated cells were observed for morphological changes under Leica DM IL LED inverted light microscope with phase-contrast (Leica Microsystems, Germany) at 10x and 20x magnification.

Statistical analysis

Anti-proliferative studies were performed in three replicates and the results were expressed as percentage growth inhibition of control. An IC_{50} value for growth inhibitions was derived from a nonlinear regression model (curve fit) based on a sigmoidal dose response curve (variable) and computed using GraphPad Prism (La Jolla, CA, USA). Data are given as the mean ± SEM. Data obtained were analyzed using SPSS software version 16.0. Statistical evaluation was performed using one way analysis of variance. Confidence limit of $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Extraction and yield

Extraction is the first step in the utilization of bioactive compounds in the natural plants. Water extraction is considered as ‘‘green techniques’’ as they provide an environmentally friendly technology, reduce derivatives and catalysis, better extraction process, improving the mass transfer efficiency of the extracts and maintaining its biological activities from natural products especially under high temperature and high pressure (Zhang *et. al.*,2019). In this study, the CoaLeX-1 extracts was prepared by extracting 169.4658 g of dry powdered cocoa leaves in water for 2 hours at 100 ℃, producing 1.1298 % of yield using the water extraction method. Taparia *et al*., 2016 reported that non-alkalized cocoa powder was used as source material to enrich procyanidin compounds and prevent loss in the polyphenol content during ditching or alkalization of the cocoa powder. Based on Oreopoulou *et al*. (2019), extraction time from 1 to 10 h gives an efficient time for the quantitative recovery of phenolic compounds through conventional extraction from aromatic plants. This showed that 2 hours of extracting CoaLeX-1 increases the efficiency of the extract. In this study also resulted in an amount of 15 kg of dry powdered cacao leaves were grinded using a heavy duty blender with sizes of filter 2 mm until it became powder form before added into 200 L of distilled water and extracted can increase the mass transfer between the solvent and raw materials. This can be proven by the previous study stating that particle size of the raw material is a major factor to enhance

quantitative recovery at shorter extraction time as the high temperature affects diffusivity of the phenolic compounds (Oreopoulou *et al*., 2019). Based on Zhang *et al*. (2019), water extraction was called as an environmentally friendly method which ensures safe and superior extracts due to non-toxic, readily available, non-flammable mediums and does not produce greenhouse gases and wastes. In addition, water extraction also can easily separate polar, moderately polar, low-polar and non-polar compounds with less expensive instrumentation with the continuous operation. Those above results together proved that water extraction is an effective method for extracting chemical constituents from CoaLeX-1.

Antiproliferative activity

In this study, cytotoxicity of *Theobroma cacao* leaves extracts (CoaLeX-1) and Tamoxifen was tested against breast cancer cell lines (MCF-7) for 48 hours using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in 96 well plates. Seven concentrations of serial dilution were used: 1000, 100, 10, 1, 0.1, 0.01 and 0.001 g/mL. Based on the result shown in Figure 1, the water extract of CoaLeX-1 achieves the value IC_{50} of 4.17 ± 0.48 g/mL while Tamoxifen against MCF-7 cells with the IC_{50} value of 3.05±0.28 g/mL. The results demonstrate that there was no significant difference in values of the CoaLeX-1 and Tamoxifen treatment against MCF-7 cells which indicates that these cocoa leaves extract (CoaLeX-1) has similar activity with commercial drugs. According to the American National Cancer Institute (NCI), plant extracts are usually known to have a significant cytotoxic effect if it has a value equal to or lesser than 20 g/mL. As shown, for cocoa leaves extract (CoaLeX-1) IC_{50} values is less than 20 g/mL, it's considered highly cytotoxic. These findings were supported by Vijayarathna *et al*., 2012 stated that the value was found to be lower than specified by NCI, therefore the categorization of the pure compound as an anticancer agent. Similar with Lalitha *et al*., 2015 demonstrated that cytotoxic effect of biosynthesized silver nanoparticles studied by MTT assay against breast cancer cells (MCF-7 cell line) showed significant cytotoxic activity with a value 3.04 µg/ml. The cytotoxicity of the CoaLeX-1 extracts on the MCF-7 cells showed inhibitory and ability to prevent the proliferation against cancer indicating that the extracts could be alternatives of conventional anti-breast cancer natural products.

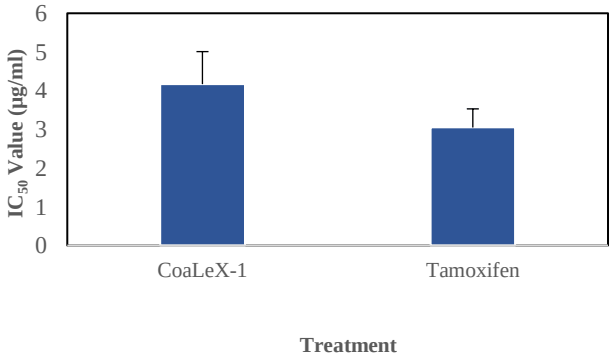


Figure 1: Mean IC_{50} values of CoaLeX-1 and tamoxifen treatment against MCF-7 cells. Values are the means of triplicate samples (n = 3). Data presented as the mean ± SEM.

Different time point activity

MTT assay was used to determine the viability of the cancer cells and the potential anti-proliferative activity of the extract. To determine the changes in the cell number, cell proliferation was measured at a different time point

after incubation begins. The inhibition of proliferation of breast cancer (MCF-7) cells was tested with *Theobroma cacao* leaves extracts (CoaLeX-1) and Tamoxifen for 15, 24, 48 and 72 hours using MTT assay *in vitro*. Seven concentrations of serial dilution were used: 1000, 100, 10, 1, 0.1, 0.01 and 0.001 g/mL. As shown in Table 1, CoaLeX-1 at 15 h and 24 h are not considered as cytotoxic with IC_{50} values of 681.33±31.87 µg/mL and 49.47±9.84 µg/mL. This followed by CoaLeX-1 treatment at 48 h with IC_{50} values approximately 7.22±0.55 µg/mL which considered to be significantly higher cytotoxic compared to others. However, at 72 h treatment, the IC value was estimated approximately >1000 µg/mL due to the longer time of incubation that degrades the CoaLeX-1 compound. In contrast, CoaLeX-1 extract was recorded with a highly cytotoxic effect to the MCF-7 cells which exhibit lower than 20 µg/mL at 48 h. This result shows that the incubation time also plays an important role as it might affect anti-proliferative activity against MCF-7 cells. These results, supported by Razak et al., 2019, stated that more suspension of dead cells was observed when the eupatorin treatment against MCF-7 cells was extended to 48 h. This finding similar to Khazaei *et al*., 2017 which reported that Flower *Allium atroviolaceum* for 24 and 48h resulted in more percentages of early apoptotic cells rather than 72 h which resulted in more cells present in the late apoptosis stage than early apoptosis. This data shows that the percentage of cell viability at 48 hours inhibits the cell proliferation in the time-dependent manner of extracts. Subsequently, the value of CoaLeX-1 at 48 h was selected to treat MCF-7 in further experiments.

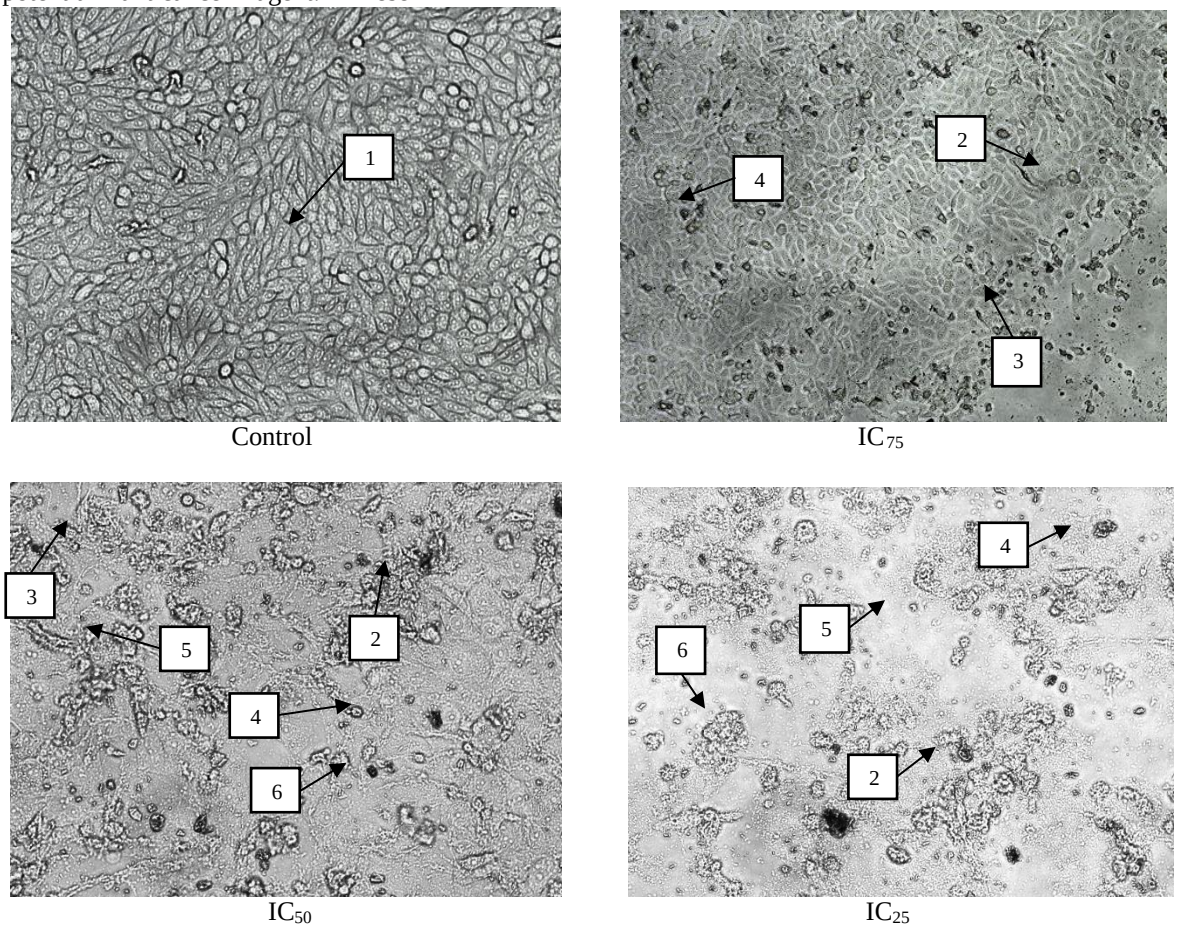
Table 1: Mean IC_{50} value of CoaLeX-1 treatment against MCF-7 cells. Values are the means of triplicate samples (n = 3). Data presented as the mean ± SEM.

CoaLeX-1	15 H	24 H	48 H	72 H
$\boldsymbol{IC_{50}}$ value ($\boldsymbol{\mu g/mL}$)	681.33±31.87	49.47±9.84	7.22±0.55	>1000

Morphology phase contrast activity

Apoptosis is a mechanism used as a criterion for discovering new anticancer agents. Apoptosis was investigated through morphological and biochemical changes of cells under inverted light microscopy such as cytoplasmic cell shrinkage, membrane blebbing, chromatin condensation, nuclear condensation, chromatin cleavage, and formation of pyknotic bodies of condensed chromatin (Nisa *et al*., 2017). Figure 2 shows the observation of the morphological changes in MCF-7 cells after treatment with CoaLeX-1. After 48 hours, our data demonstrated that the CoaLeX-1 treatment showed clear apoptosis characteristics such as nuclear fragmentation, cell shrinkage, membrane blebbing, apoptotic bodies and pyknotic bodies were observed in the comparison to control cells. Visualization of the control (untreated) cells showed that the cells maintained their original morphology and remained confluent. At an IC_{75}, it's shown that the CoaLeX-1 activity was started to react with the MCF-7 cells while at an IC_{50}, showed signs of detachment from the surface of the walls denoting cell death, reduction in cell volume and gives typical apoptotic features. An increased rate of cell death was shown in the IC_{25} corresponding with the concentration increment of the extract treatment regarding observation. This finding is similar to a previous study of Khazaei *et al*. (2017), that demonstrated the features of apoptosis were observed on the cell by showed the formation of shrinking, membrane blebbing, and membrane-bounded vesicles after 48 h of treatment. This finding also can be supported by Chaudhary *et al*. (2015), reported that MCF-7 cells treated with *Nardostachys jatamansi* extracts exhibit characteristic apoptosis changes such as cell shrinkage, nuclear condensation and formation of round apoptotic bodies cause cell death by inducing apoptosis which is highly desirable as anticancer agents. A previous study reported that anticancer drugs capable of inducing selective apoptosis of cancer cells with a minimal side effect on normal cells (Sivakumaran *et al*., 2018). This

result supported by Singh et al., 2019 stated that drugs that have an apoptosis-inducing property in cancer cells are considered as the potential anticancer agent. These findings confirmed that the results of CoaLeX-1 compounds are parallel and able to induce apoptosis in MCF-7 cells.



Control IC_{75}

IC_{50} IC_{25}

Figure 2: Representative photomicrograph shows morphological changes of breast cancer cells (MCF-7). Cells were treated with CoaLeX-1 for 48h and imaged by inverted phase contrast microscope (magnification 20x). Arrows show (1) control cells, (2) nuclear fragmentation, (3) cell shrinkage, (4) membrane blebbing, (5) apoptotic bodies and (6) pyknotic body.

Different dosage activity

To understand the characteristic of the cytotoxicity effect of the CoaLeX-1 extract on the cells, the cancerous MCF-7 cell lines were selected to measure the morphology features which triggered apoptosis of the cell in a dose-dependent manner. Complete dose-response against MCF-7 cells with CoaLeX-1 treatment were generated by phase-contrast microscopy in 4 days at different doses of treatment. As shown in Figure 3, the activity rate of the cell death on day 3 with 5 µg/ml of CoaLeX-1 extract treatment started to show morphology effect and reduced number of the viable cells compared to day 2. While at day 4 with the 5 µg/ml of CoaLeX-1 extracts treatment, the cells show detachment on the cells wall and the increasing number of cells dead. It was observed that as the dose of the tested compound was increased, cells undergoing apoptosis also resulted in other types of morphological changes such as echinoid spikes

on the surface of the apoptotic cell, apoptotic bodies and decrement of cell numbers. These results similar to the previous studies on *Corydalis govaniana* wall roots stated that the MCF-7 cells treated with higher doses of the extract showed marked changes in morphological associated with late stage apoptosis (Sivakumaran *et al*., 2018). Khazaei *et al*. (2017), also reported that time and dose-dependent manner of extracts is an important factor to inhibit the proliferation of the MCF-7 cells. These results were supported by Lalitha *et al*. (2015), who demonstrated that higher cytotoxic activity of plant-mediated nanosilver against MCF-7 cells results in the dose- and time-dependent. The nanosilver plant also proves to be a promising drug for chemotherapeutic treatment due to the increased cytotoxicity, decreased viability and proliferation which results in apoptosis through induced programmed cell death. Therefore our data demonstrated that CoaLeX-1 at a concentration of 5 µg/ml exerted significant cytotoxic effect against MCF-7 cells by inducing marked morphological changes associated with apoptosis and decrease in the number of cells in a time and dose-dependent manner after 2 days treatment continuously.

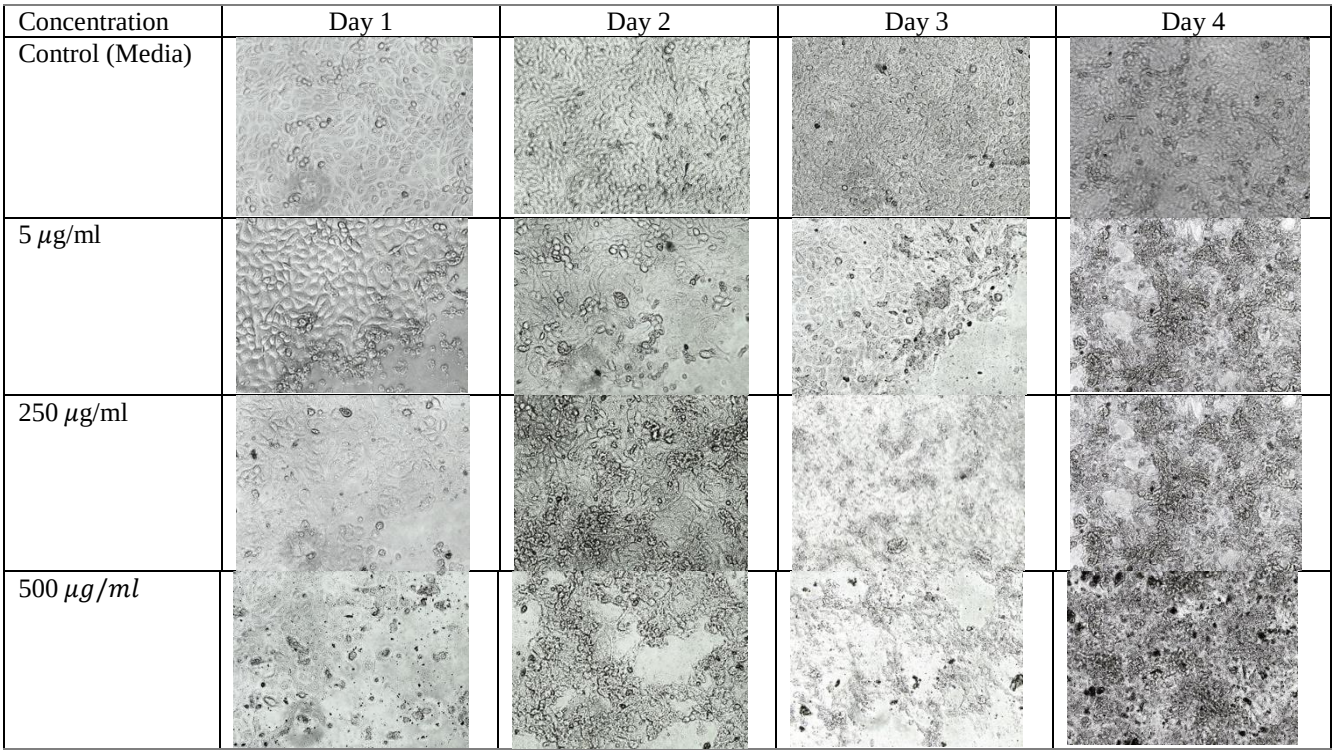


Figure 3: Representative photomicrograph shows morphological changes of breast cancer cells (MCF-7). Cells were treated with CoaLeX-1 for 4 days at different concentrations and imaged by inverted phase contrast microscope (magnification 20x).

CONCLUSIONS

Pilot scale product of CoaLeX-1 has been clearly shown to be an effective anticancer agent. This study showed that *in-vitro* cytotoxic activity and apoptotic effect of CoaLeX-1 were preliminary investigated by assessing the cells viability and cell morphology against breast cancer (MCF-7) cells. Our data demonstrated that CoaLeX-1 extract exerted higher cytotoxic effect by inducing marked morphology changes associated with apoptosis and death of the cells in the time and dose dependent manner. Hence, it can be concluded that CoaLeX-1 could be a potential preventative and/or therapeutic agent for breast cancer, either used alone or as an adjunct to the standard chemotherapeutic drugs.

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