THE EFFECT OF *Goniothalamus umbrosus* EXTRACTS ON HELA CELL

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By

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ABSTRAK

Kanser serviks merupakan tempat keempat penyebab utama kematian akibat kanser dalam kalangan wanita di seluruh dunia. Kadar insiden dan kematian adalah lebih tinggi di negara membangun termasuk Malaysia. Walaupun taraf hidup pesakit telah meningkat oleh kemajuan pesat kemoterapi neoadjuvan, dan pembedahan atau kedua-duanya, tetapi masih mengakibatkan kadar kematian yang ketara. Selain itu, kebanyakan rawatan semasa tidak menunjukkan peningkatan dan mengakibatkan kesan sampingan kepada pesakit. Pada masa ini, pendekatan kepada strategi kemo-pencegahan menggunakan bahan semulajadi menjadi tumpuan dalam kalangan penyelidik. Kajian ini bertujuan untuk mengkaji potensi ekstrak daun *Goniothalamus umbrosus* (Kenerak) terhadap kesan kemo-pencegahan ke atas sel kanser serviks, HeLa. Kaedah MTS dan AlamarBlue menunjukkan dos IC_{50} bagi ekstrak daun *G. umbrosus* adalah 45.31 µg/ml dan 48.44 µg/ml masing-masing. Perubahan morfologi menunjukkan proses apoptosis boleh diperhatikan bagi rawatan dengan ekstrak daun *G. umbrosus* seperti pengecutan sel, pembulatan sel, penvakuolan dan pemanjangan sel. Analisis pembiakan sel yang dijalankan selama 72 jam telah menunjukkan bahawa ekstak daun *G. umbrosus* mempunyai kesan anti-proliferatif. Secara keseluruhan, data yang terkumpul dari kajian ini membuka ruang baru terhadap ekstrak daun *G. umbrosus* sebagai agen kemo-pencegahan terhadap kanser serviks.

ABSTRACT

Cervical cancer is the fourth leading cause of cancer death in female worldwide. The rate of incidence and mortality is higher in developing countries including Malaysia. Although the survival of the patients had increased due to the rapid advancement of neoadjuvant chemotherapy and surgery or both, but there still results in substantial morbidity. Moreover, most of the cases for current treatments do not show improvement and results side effects to the patients. Currently, chemoprevention approach using natural products is one of great interact among researchers. This study evaluated the potentially of the *Goniothalamus umbrosus* (Kenerak) leaves extracts in exhibiting chemopreventative effects on cervical cancer cell (HeLa cells). The IC_{50} of the extracts were determined with MTS and AlamarBlue assay lies in 45.31 µg/ml and 48.44 µg/ml respectively. Morphological changes displaying apoptosis can be observed upon treatment with *G. umbrosus* leaves extracts, including cell shrinkage, rounding of cells, vacuolization and cell elongation. The 3 days cell proliferation assay indicated that *G. umbrosus* leaves extracts has an anti-proliferative effects. Overall, the data collected provide new insight of using *G. umbrosus* leaves extracts which can be used as chemopreventive agent on cervical cancer.

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CHAPTER 1

INTRODUCTION

Cancer disease was reported over 6 million cases per year and became the second cause of death after the cardiovascular disease (Srivastava et al., 2005). Cervical cancer is the third most frequent diagnosed cancer and the fourth most frequent cause of cancer among female, the report in the year 2008 indicated 9% (529800) of the total new cancer cases and 8% of the total cancer deaths among female (Jemal et al., 2011).

According to American Cancer Society, a new analysis estimated that within six minutes, a gynaecological cancer is diagnosed with the majority of cervical cancer (American Cancer Society, 2010). A study in Malaysia, Omar and Tamin (2011), reported that the cervical cancer is the third most common cancer among female, with the total of 847 issue cases diagnosed. The data indicated that the rate of incidence and mortality is higher in developing countries including Malaysia.

The uses of natural product especially from plant derived products as alternative therapies is growing an interest among researchers (Goldfrank et al., 1982; Vulto and Smet, 1988; Mentz and Schenkel, 1989). Thus, in modern era, the research on medicinal plants for development of new drugs are needed for the needs of the pharmaceutical market (Elisabetsky, 1987a; Calixto, 1996)

In December 1967, the record on plant dependent anti-cancer drug from published and unpublished works were first published by Hartwell with monumental information of 3000 species of plants with anticancer property (Graham et al., 2000). The National Cancer Institute ratified 114,000 anticancer plant extracts from 35,000 plant species from 20 countries (Shoeb et al., 2005).

Goniothalamus is the second largest genus of Annonaceae and in Asia, the *Goniothalamus* has been widely used for traditional practices in Asia (Nasir et al., 2004)

Goniothalamus umbrosus, or well known as 'kenerak', is possibly one of the interesting medicinal plants of the East coast of the Peninsular Malaysia. It is grown by generations of traditional practitioners mostly for healthcare. Based on the previous study, the *Goniothalamus* has a very special compound that can only be found within the family of Annonaceae, known as styrylpyrone goniothalamin. One of its bioactive fraction is styryl-lactones , that is predominantly restricted to the genus *Goniothalamus*. Goniothalamin is the first styryllactone (Nasir et al., 2004)

The bioactive styryl-lactone has been reported by the previous study that it can show medicinal properties against many diseases and anti-cancer that will cause apoptosis of various human tumours and animal cell lines (Nasir et al., 2004).

The present investigation was carried out to determine the anticancer potential of medicinal plant extracts of *G. umbrosus.* Understanding of the anti-cancer activity of extracts may provides useful information for developing anti-cancer treatment in future.

1.1 OBJECTIVE

Main Objectives

- To determine the effects of *G. umbrosus* extracts on HeLa cell lines.

Specific Objectives

- To determine the viability of HeLa cell lines treated with *G.umbrosus* extracts
- To determine morphology changes of the HeLa cell lines treated with *G. umbrosus*

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer is the worst public health problem in the United States and many other parts of the world. One in four deaths in the United States is due to cancer (Siegel et al., 2014).

Cancer is a disease that is defined as the uncontrollable cell proliferation that increases the number of abnormal cells. Cancer is a progressive disease which involved several stages, including the initiation caused by carcinogenic inducer, the initiation of development tumor cells, the progression into malignant cells and lastly by invasion and the metastasis of the cancer cells to other body parts (Metha et al., 2010). Cancer is a malignant disease caused by a fast and uncontrollable proliferation of abnormal cells that will also accumulate together to form a solid tumor and proliferate throughout the body which can cause death. Cancer cells are able to grow and invade the neighbouring tissue and may also metastasize to other organs through the lymphatic system or bloodstream (Knudson, 2001).

According to a previous study in advanced cancer research in the last decade that was highlighted, there are at least 5 cancer-causing factors. These include are cytotoxic and/or inflammatory carcinogenic substances, spontaneous replication errors in DNA, genotoxic (direct DNA injurious) and chronic inflammatory. These factors are the key point in promoting tumor proliferation and angiogenesis that causes tumor growth (Coussens and Werb, 2002).

The National National Institute of Health, USA had estimated an overall of $209.9 billions were invested throughout the world in 2005 and had been a big challenge for the economical aspects.

Cancer is usually treated by chemotherapy, surgery, immunotherapy, radiation therapy, monoclonal antibody therapy, or combinations of these methods. The treatment methods can be different depending on the type of cancer and location, disease stage, as well as the general health status of the patient (Coseri., 2009).

Natural products represent one of the most valuable sources for new drugs development that still needs investigation of their specific function in medicinal field (Coseri, 2009). Many compounds originated from plants are frequently used in cancer therapy such as vincristine, camptothecin, paclitaxel, and etoposide (Rocha et al., 2001). These natural compounds have been chemically modified to make them more active, more safe, and more soluble (Srivastava et al., 2005).

2.2 Cervical cancer

The cervical cancer formation can be triggered via several factors such as genetic and environmental aspects. These include Human Papillomavirus (HPV) infection, sexual partners, smoking, age, HIV infection, family history of cervical cancer and long-term use of oral contraceptive (Schiffman and Brinton, 1995).

The International Agency for Research on Cancer estimated 493,000 new cases and 274,000 deaths worldwide in the year 2003 (Bachtiary et al., 2006). The disease disproportion affects the poorest regions and more than 80% of causes found in Latin America, Sub-sahara Africa and India (Petignant and Roy, 2007). Recurrent of cervical cancer is almost always incurable and less than 5% of patients developed recurrence (Petignant and Roy, 2007).

2.3 HeLa cell lines

HeLa (ATCC®CCL-2™) cell line is a cervix epithelial adenocarcinoma cell, an attached cell. The HeLa cells is the "immorrtal cell line" that is first successfully grown in a laboratory condition. A researcher from John Hopkins University, Otto obtained another cells sample from a black women patient, Henrietta Lacks who was undergoing treatment in that hospital (Spencer, 2009). T he sample cells was reported to contain HPV 18 strain, positive keratin by immunoperoxidase staining containing low level of p53 expression and normal level of pRB expression as referred from ATCC® (American Type Culture Collection) CCL-2™ product sheet.

2.4 Medicinal plants

Plants have developed complex mechanisms for their life such as space. Thus, some plants produce toxic substances, such as terpenes and alkaloids which will inhibit the growth of surrounding plants (Ramawat and Merillon., 2008).

Plant-derived compounds over the years have been a very good source for medicinal use; many have been used as drugs, either in their original or semi-synthetic forms (Ramawat and Merillon., 2008)

High in effectiveness and safety have always been the gold standard for the discovery of new drug programs. The potential natural product from plants or others natural sources need to undergo screening to discover their pharmaceutical agents. Most of the time, the drug originated from natural sources show a significant segment of pharmaceutical market compared to randomly synthesized compounds ((Efferth and Koch., 2011)

Therapeutic efficacy of phytotherapy is based on the combination action of a mixture of constituents, and since many diseases are multi-factorial in nature, it provides an effective treatment strategy (Efferth and Koch., 2011)

In addition, the plant derived therapies usually have high effectiveness in their safety profiles. For example, the adverse reaction shown in chinese herbal medicine appears to relatively safe compared to prescription drug use (Shaw., 2012)

Historically, plants with known therapeutic potential have been used for a long time to cure many diseases. As an example, a morphine is a drug from a plant product that was discovered in 1861 as an analgesic agent. Later, the Cinchona bark that was isolated in 1820 has an active compound known as quinine and functions as an effective antimalarial drug (Rebecca., 2004; Gary and Bryn., 2003).

The scientist continues to demonstrate many researches involving plants as the potential sources of treatment for complicated diseases. Nowadays, natural products for the treatment of Alzheimer's disease have attracted an interest among researchers (Park., 2010)

Plant derived compounds are also good in common health problems such as diabetes, hypertension and obesity. Recently, a number of randomized, controlled intervention trials have shown that drinking green tea catechins (270 mg to 1200 mg/day) may reduce body weight and fat (Rain et al., 2011)

There are some plant-based natural products which are commonly used in the treatment of hematological malignancies such as vinca alkaloids, which are used to treat lymphomas and acute lymphoblastic leukemias (Karon et al., 1966; Dancey and Steward., 1995), and podophyllotoxin derivatives, which is useful to treat acute myeloid leukemias, Hodgkin’s and non-Hodgkin’s lymphomas (Hande, 1998).

Other than that, there are several plant-based natural products that have been under clinical trials and shows good potentials to treat several haematological malignancies such as flavopiridol (Byrd et al., 2007) and meisoindigo (Wang et al., 2005; Weng et al., 2005). Meanwhile, there are also the plant-based natural products that are still under preclinical studies for haematological malignancies treatment such as combretastatins and honokial (Fang et al., 2007; Petit et al., 2008; Battle et al., 2005)

2.5 *Goniothalamus umbrosus*

2.5.1 Botanical description

Goniothalamus species has uniform and simple characteristics, with few-leaved and the leaves are simple and alternate. The shrub is smooth and cylindrical. The blade is thick (Wiart., 2006). The flowers are easy to recognize by the calyx consists of three sepals which is commonly membranous or valvate shaped. The fruits are stalked or sessile one to two seeded ripe carpels (Saunders, 2003). The genus of *Goniothalamus* have been suggested to have been colonized in earth during the post-Permian early Cretaceous time (Wiart., 2007a)

The synonym name for *Goniothalamus umbrosus* species is a *Goniothalamus tapis* Miq (Sinclair., 2003).

2.5.2 Traditional Medicinal Uses

G.umbrosus plants has been used for a long time ago in traditional medicinal Asian practices such as for abortion and and fever (Wiart., 2007b). The roots of the *G. umbrosus* was used as abortifacient during the early months of pregnancy (Burkill.,

1953) and the typhoid fever was treated in Java, Indonesia by an infusion of the roots of *G. umbrosus*.

However, none of the medicinal properties of *G. umbrosus* has been substantiated yet via strict pharmacological experimentation but these species have been studied for their chemical compounds (Wiart., 2007a)

2.5.3 Anticancer Studies

The National Cancer Institute had collected about 35,000 plant samples from 20 countries and around 114,000 extracts were screened for their anticancer property (Shoeb, 2005).

Twenty two species (13.7%) out of 160 species, in the genus *Goniothalamus*, have been phytochemically investigated including the *G. umbrosus*. These studies have resulted in two very distinct classes of lipophilic secondary metabolites: acetogenins and styryl-lactones, both possessing complex stereochemistry and existing in different stereoisomeric forms (Motoyuki et al., 2000).

The research test for these chemical cytotoxity activity showed that styryl-lactones and acetogenins are toxic for several human tumors cell lines (Yang and Yu., 2000). These findings can be the evidence for the present anticancer, antibacterial and antiviral agents in the genus *Goniothalamus* and further consistent and systematic research on this genus will lead to the discovery of antineoplastic and antimicrobial agents (Wiart.,2007a).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant materials

Plant material used in this study was Kenerak (*Goniothalamus umbrosus*) leaves. The plants materials were obtained from Machang, Kelantan.

3.1.2 Cell line

Cell line used in this study were human cervix adenocarcinoma cell line (HeLa), HeLa cell line was obtained commercially (ATCC, USA or AllCells, USA).

3.1.3 Chemicals and reagents

Chemicals and reagents that were used in this study were listed in Table 3.1.

Table 3.1: List of chemicals and reagents

Name	Source
Trypan Blue Solution	Sigma-Aldrich, USA
Gibco® Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen Inc, USA
Gibco® Fetal Bovine Serum (FBS)	Invitrogen Inc, USA
Gibco® Penicilin-Streptomycin	Invitrogen Inc, USA
Gibco® Phosphate Buffered Saline Solution (PBS)	Invitrogen Inc, USA
Gibco® 0.25% Trypsin-EDTA	Invitrogen Inc, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, USA
CellTiter 96® AQueous One Solution Cell Proliferation Assay	Promega, USA
alamarBlue® Cell Viability Assay	Life technology, USA

3.1.4 General commercials kits and consumables.

Commercials kits and consumables that were used in this study were listed in Table 3.2.

Table 3.2: List of commercials kits and consumables.

Kits and consumables	Sources
Filter (0.2 µm)	Milipore, Singapore
6-well plates	Nunc, Denmark
Cell culture flask T25 cm^2 and T75 cm^2	Nunc, Denmark
Cell culture tube	Becton-Dickson, USA
Centrifuge tubes, 15 ml and 50 ml	Falcon Labware, USA
Pipette tips	Appendorf, USA
50 ml syringe	Becton-Dickson, USA
Whatman no.1 filter paper	Whatman Inc., New Jersey

3.1.5 Laboratory apparatus and equipment

Laboratory apparatus and equipment. used in this study were listed in Table 3.3.

Table 3.3 : List of laboratory apparatus and equipments.

Name	**Source**
Blender grinder	Panasonic, Malaysia
Centrifuge 5415R	Eppendorf, Germany
Class II Biohazard safety cabinet	Esco Micro Pte. Ltd. Singapore
CO_2 Incubator	Thermo Fischer Scientific, USA
Freezer (-20^{o}C)	Hitachi, Japan
Freezer (-80^{o}C)	Thermo Fischer Scientific, USA
Phase contrast microscope	Nikon Instrument Ins, USA
Analytical balances	Sartorious, Germany
Rotary evaporator	Eyela, N1100, USA
Water bath for rotary evaporator	Eyela, OSB2100,USA

3.2 Methods

3.2.1 Collection and preparation of plant extracts

G. umbrosus leaves were obtained from Machang, Kelantan. The leaves were collected and washed thoroughly using distilled water in order to remove dust, soil and insects. The fresh leaves were then allowed to dry at room temperature (RT). The dried leaves were weighed and recorded before were grinded using a electrical blender.

3.2.2 Extraction of plant extract

The methanol aqueous extracts were performed by grinding the fresh leaves with methanol and distilled water in ratio of 4:1. The mixtures were incubated on the shaker for 3 days at 250 rpm, each one days the mixtures were filtered with 125 mm filter paper, after the filtration process the filtered extracts were stored in -4^{o}C, then the supernatants were be added with methanol and distilled water in ratio of 4:1, then the mixtures were incubated on shaker at 250 rpm for 24 hours. The process was continued for 3 times in 3 days. After the 3 times process of the filtration of the extracts, the filtered extracts were then pooled, concentrated and dry using freeze dryer for one week. The crude extracts were then stored at -20^{o}C until further use.

3.2.3 Preparation of stock extracts

The 0.06 g crude extracts were dissolved in the 1000 µl sterile distilled water, Then, the extracts were sterilised using with 0.2 µm syringe filter and 50 µl of extracts was aliquoted and was dissolved in 3000 µl of medium at concentration of 1000 µg/ml . The excess filtered were kept in a falcon tube and stored at -20^{o}C.

3.2.4 Cell culture

3.2.4.1 Cell lines

HeLa cell lines was obtained commercially (ATCC, USA or AllCells, USA). The cells was cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin). The cultures was incubated at 37°C with 5% CO_2 in 25 cm^2 flask. The medium were replaced every 72 hours and the cells was cultured until they reach 70% to 80% confluence. Only cell lines from passage 4 to passage 6 were used throughout the experiment.

3.2.4.2 Thawing frozen cells

The frozen cells stored in the -80°C were retrieved and rapidly thawed by immersed in water bath at 37°C a few minutes. The cells suspension from cryotube then was transferred into a 15 ml falcon tube and 3 ml pre-warmed medium added into the cell suspension. The mixtures then was centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and 1 ml of medium was added to pellet. The cell suspension was transferred to the culture flask with 4 ml of medium then incubated and maintained at 37°C under humidified atmosphere, with 5% CO2 and 95% air. The medium were replaced every 72 hours and the cells was cultured until they reach 70% to 80% confluence.

3.2.4.3 Preparation of cell suspension

For cell growth in monolayer culture, the 1 ml of 0.25% trypsin/EDTA was used to detach the cells from surface of culture flask and incubated for around 5-15 minutes in incubator at 37°C. Then, 3 ml of complete medium was added to neutralise and stop the reaction of trypsin. To completely detach the cells the wall of flask was flushed for a

few times. The cell suspension in flask was collected by using 15 ml falcon tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and cells were diluted in 1 ml complete medium and resuspend its to obtain uniform suspension. Hundred µl of DMSO was aliquoted, and 900 µl of the cell suspension was resuspend in the 2 ml cryotube for storage and were slowly frozen from 4°C to 20°C before transferred to -80°C freezer. Meanwhile, the cells suspended in 1 ml medium were ready to be cultured again in culture flask

3.2.4.4 Subculturing cell lines

The cell cultures were propagated in large culture flasks until a confluent monolayer had formed. When the cells reached confluent 80% to 90%, the medium was removed and rinse once with phosphate-buffer saline (PBS). The cell were detached from the cell culture flask using trypsin, collected in 5 ml falcon tube and centrifuged at 1000 rpm for 5 minutes. After removing supernatant, the cell pellet was subsequently resuspended in 3 ml fresh complete medium. To subculture the cells in new flask, 500 µl of the cell were aliquot into new cell culture flask containing fresh complete medium. The culture flask were shook gently to distribute the cell evently around the flask. The cell when incubated at 37°C under humidified atmosphere, with 5% CO2 and 95% air.

3.2.4.5 Cryopreservation of cells lines

Briefly, the confluent cells in the flask were trypsinised, collected, centrifuged, and supernatant was removed. After that, the cell pellet was suspended with 1 ml of fresh complete medium before aliquot 900 µl of the cell suspension and 100 µl of DMSO into labelled cryotube and sealed. To prevent cell undergo cool shock, the cryotube were secured with tissue paper, then were frozen in -80°C freezer.

3.2.5 Determination of inhibitory concentration 50% (IC_{50})

3.2.5.1 Leaf extracts preparation

G. umbrosus leaves extracts concentration were calculated based on the following formula (Ahmad et al., 2014):

$M_1V_1 = M_2V_2$

Where,

M_1 = initial concentration of *G. umbrosus* leaves extracts stock solution.

V_1 = initial volume of *G. umbrosus* leaves extracts stock solution.

M_2 = new concentration of *G. umbrosus* leaves after dilution.

V_2 = new volume of *G. umbrosus* leaves after dilution.

3.5.5.2 Treatment of HeLa cells with *G. umbrosus* leaves extracts

When the cells reaching confluent, the old medium was discarded and washed once with PBS, then 1 ml of trypsin was used to detach the cells, then incubated for around 5-15 minutes. Then, 3 ml of complete medium was added to neutralise and stop the reaction of trypsin. To completely detach the cells the wall of flask was flushed for a few times. The cell suspension in flask was collected by using 15 ml falcon tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and cells were diluted in 1 ml complete medium and resuspend its to obtain uniform suspension.

Total number of 5 x 10^3 cell was pipette into each of the well with 100 µl of fresh medium in each well of 96-well plate,. After 24 hours, old medium was discarded and replaced with mixture of 100 µl of fresh complete medium with *G. umbrosus* with varying range. The untreated cells or control cells were exposed with complete medium without *G. umbrosus* leaves extracts. The cultures was incubated at 37°C with 5% CO_2. After 72 hours of incubation times, then cells were undergo MTS and AlamarBlue assay method for determined the viability of cells.

Graphs of cell viability against *G. umbrosus* leaves extracts concentration plotted and the percentage of inhibitation of cells were compared with the untreated cells group. The IC_{50} of the extracts was determined as concentration of extracts that killed 50% of the viable cells.

3.2.5.3 Cell count using trypan blue

Cell count was carried out according to the manufacturer protocol, with slight modification on the volume and dilution factor. Briefly, from 1 ml cell suspension, 50

µl of cells were aliquot into 1.5 ml eppendorf tube and mixed with 50 µl of trypan blue dye in 1:1 ratio. The mixtures were mixed well by resuspend using a pippete. After incubation of 1 minute, 10 µl of cell suspension diluted in trypan blue solutions was transferred into the cell counter slide and was read by a cell counter machine.

3.2.6 Microscopic observation of morphological changes

The human cervix adenocarcinoma cell lines, HeLa cells were seeded at 5 x 10^4 cells in a 6-well plate. After 24 hours growth, the old medium from 6-well plates was removed and replace with the varied concentration of the mixtures from fresh medium and extracts. Then, the cell cultures treated with extracts was incubated for 72 hours at 37^{o}C with 5% CO_2 humidified atmosphere. For the control untreated cells, the cell culture was exposed to medium without any extracts. Morphological changes of the cells were observed under phase contrast microscope. Morphology changes of the cell were observed according to varying of concentration applied. The features related to apoptosis or cell death mechanism were identified on the HeLa cells treated with extracts. The pictures of cell cultures were captured in 40x and 100x magnification with Olympus Xcam_Alpha camera attached to the phase contrast microscope.

3.2.7 Cell proliferation assay

3.2.7.1 Cell proliferation assay using MTS assay method

Cell proliferation assay was done with total number of 5 x 10^3 cell was pipette into each of the well with 100 µl of fresh medium in each well of 96-well plate. After 24 hours, old medium was discarded and replaced with mixture of 100 µl of fresh complete medium with *G. umbrosus* with varying range. The untreated cells or control cells were exposed with complete medium without *G. umbrosus* leaves extracts. The cultures was incubated at 37°C with 5% CO_2. After 72 hours of incubation period, cells were treated

with varying concentration of *G. umbrosus* leaves extracts in triplicates, then 20 µl of AlamarBlue assay were added in each of 100 µl of mixture of fresh complete medium with extracts in varying concentration. Then the mixture were incubated between 2 to 4 hours before the absorbanced were calculated using 96-well ELISA reader at 460 nm of wavelength.

3.2.7.2 Cell proliferation assay using AlamarBlue assay method

Cell proliferation assay was done with total number of 5 x 10^3 cell was pipette into each of the well with 100 µl of fresh medium in each well of 96-well plate,. After 24 hours, old medium was discarded and replaced with mixture of 100 µl of fresh complete medium with *G. umbrosus* with varying range. The untreated cells or control cells were exposed with complete medium without *G. umbrosus* leaves extracts. The cultures was incubated at 37°C with 5% CO_2. After 72 hours of incubation times, cells were treated with varying concentration of *G. umbrosus* leaves extracts in triplicates, then 20 µl of AlamarBlue assay were added in each of 100 µl of mixture of fresh complete medium with extracts in varying concentration. Then the mixture were incubated between 2 to 4 hours before the absorbanced were calculated using 96-well ELISA reader at 460 nm of wavelength.

CHAPTER 4

RESULTS

This study was carried out to determine the potential of *G. umbrosus* as promising chemopreventation agents against cervical cancer cells. The *G. umbrosus* leaves extracts were tested on cervical cell cancer cells (HeLa cells).

4.1 Extraction Yield

The mass of *G. umbrosus* leaves before and after aqueous 80% methanol extraction was shown in Table 5.1. The yield of aqueous 80% methanol extraction of *G. umbrosus* leaves showed 4.45% yield of extraction.

Table 4.1 : Mass of *G. umbrosus* leaves

Items	**Leaves (g)**	**Methanol : water (ml) (4:1)**
Before extraction (fresh leaves)	88.22	400 : 100
After extraction (concentrated & powdered)	3.93	-
% yield extraction	4.45	-

4.2 Cellullar growth inhibition of *G. umbrosus* and the determination of inhibitory concentration 50% (IC_{50}) on HeLa cells.

The evaluation of *G. umbrosus* leaves extracts in inhibiting the cells growth and cell proliferation on HeLa cells upon treatment for 72 hours were conducted. Therefore, *G. umbrosus* leaves extracts were screened with different concentration ranges and the inhibitory concentration 50% (IC_{50}) for extracts were determined against untreated HeLa cells using AlamarBlue and MTS method.

Table 4.2 (a) and table 4.2 (b) showed HeLa cells concentration and cell viability after treated with *G. umbrosus* leaves extracts by using AlamarBlue and MTS method respectively. In addition, Figure 4.1 (a) and Figure 4.1 (b) showed the *G.umbrosus* leaves have reduced the HeLa cells growth as compared to untreated cells in a dose-dependent manner respectively which were analyses by AlamarBlue and MTS. Whereas Figure 4.2 (a) and Figure 4.2 (b) showed the percentage of viable cell upon treatment with *G. umbrosus* leaves extracts using the AlamarBlue and MTS. The concentration of *G.umbrosus* leaves extracts ranges from 31.25 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, and 1000 µg/ml. The IC_{50} value was determined as in between range of 31.25 µg/ml and 62.5 µg/ml respectively. The experiment was conducted in triplicate.

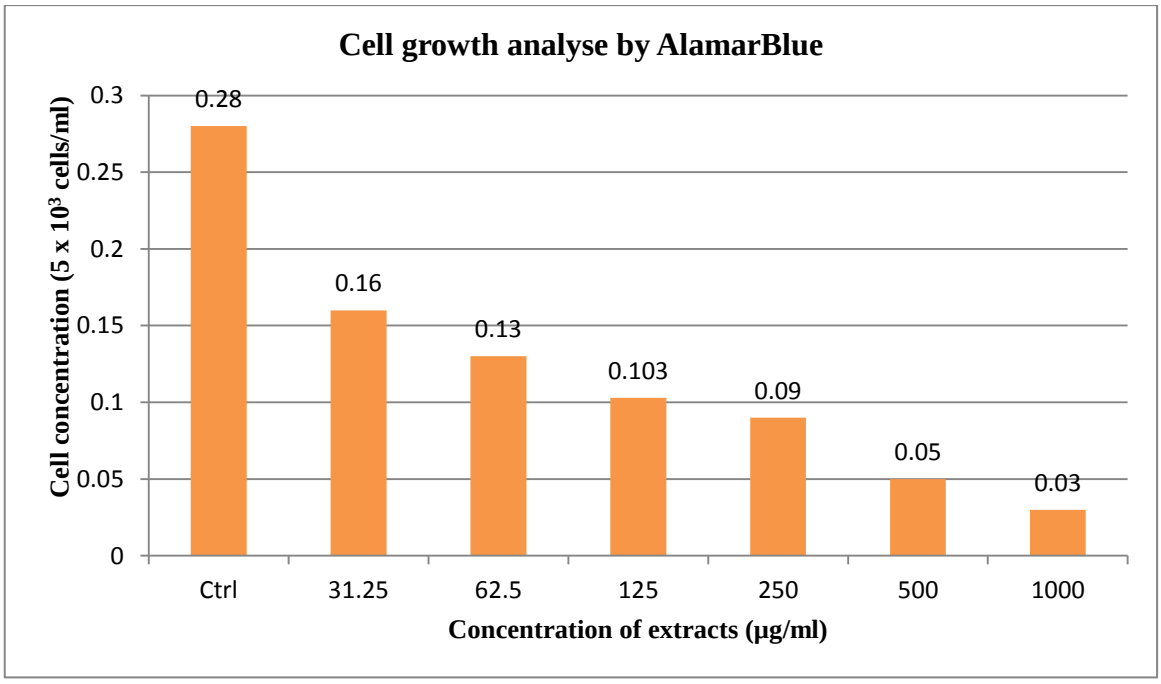
Table 4.2 (a) : HeLa cell concentration and cell viability treated with *G. umbrosus* leaves extracts analyses by using AlamarBlue.

Concentration of leaves extract (µg/ml)	**Cell concentration (5 x10^3 cells/well)**	**Cell viability (%)**
Ctrl	0.23	100
31.25	0.16	57.14
62.5	0.13	46.43
125	0.103	36.79
250	0.09	32.14
500	0.05	17.86
1000	0.03	10.71

Table 4.2 (b) : HeLa cell concentration and cell viability treated with *G. umbrosus* leaves extracts analyses by using MTS.

Concentration of leaves extract (µg/ml)	**Cell concentration (5 x10^3 cells/well)**	**Cell viability (%)**
Ctrl	1.162	100
31.25	0.696	59.87
62.5	0.526	45.27
125	0.279	24.01
250	0.236	20.31
500	0.161	13.86
1000	0.127	10.93

(a)



(b)

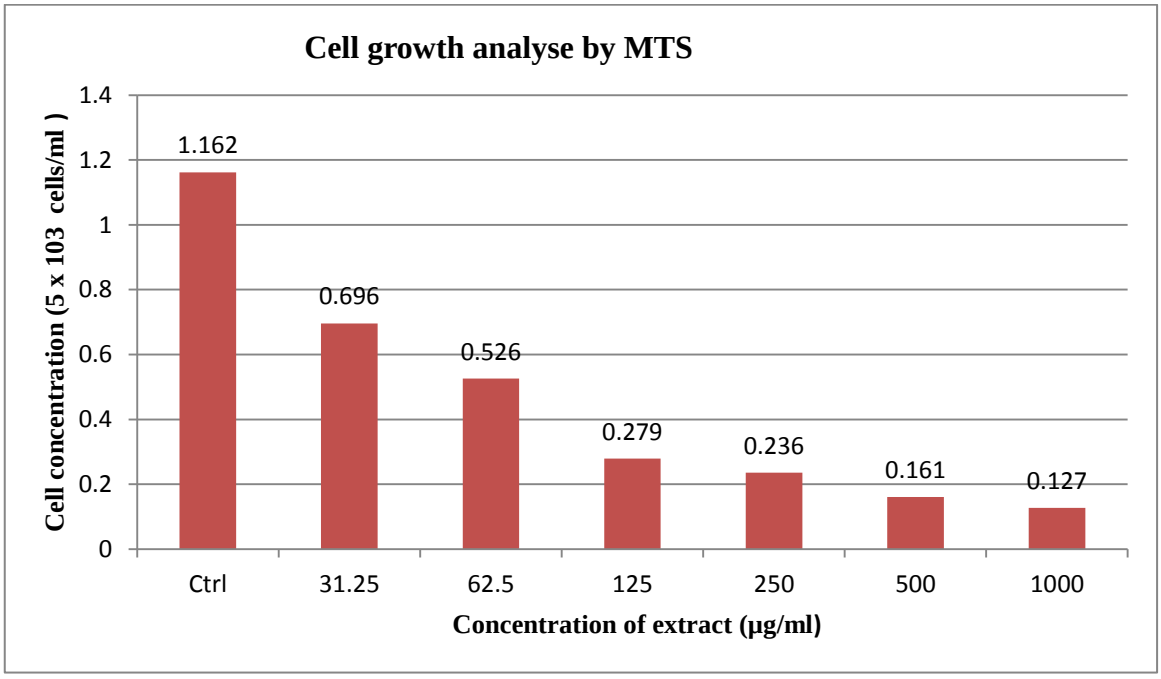


Figure 4.1 : Cell growth of HeLa cells treated with *G. umbrosus* leaves extracts (a) Analyses by AlamarBlue (b) Analyses by MTS. Values were presented in manner of the triplicates.