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CYTOTOXICITY EFFECT OF *AZADIRACHTA INDICA* EXTRACT ON VERO CELLS

By

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This is to certify that we have read this research paper entitled "Cytotoxicity Effect of *Azadirachta indica* Extract" by Chin Xin Yee (D20B0055), and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirements for the course DVT 55204- Research Project.

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DEDICATIONS

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List of Abbreviations

BHK-21	Baby Hamster Kidney Fibroblast
CC50	Cytotoxic Concentration 50%
CP	Crude protein
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal Bovine Serum
MTS assay	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetra-zolium
PTTH	Prothoracicotropic Hormone
TDP	Total Digestible Proteins
VEGF	Vascular Endothelial Growth Factor

CYTOTOXICITY EFFECT OF *AZADIRACHTA INDICA* EXTRACT

ABSTRACT

Neem, or scientifically known as *Azadirachta indica*, is a fast growing evergreen plant that can be found in hot temperature countries like Malaysia, India and Pakistan, has its significant value as folk medicine. Previous research found that *A. indica* components make it a very good candidate for antifungal, anti-parasitism, antibacterial and anticancer. This study was conducted to determine the cytotoxic effects of this plant against normal mammalian cells such as Vero cells which are kidney cells derived from African Green Monkey. The study is aimed to have a better understanding on the safety profile of this plant against healthy mammalian cells. Cell culture was conducted on Vero cells, whereby the cells were treated with *A. indica* hexane extract at concentration ranging from 3.125 µg/mL to 100 µg/mL. The cultured cells incubated for a total of 72 hours, and accessed with MTS assay to assess the cell viability. The results of this study revealed that *A. indica* hexane extract showed no cytotoxic towards Vero cells, with MTS value of 11.13% at the concentration of 100 µg/mL against the normal mammalian cells, Vero cells,. This indicates that *A. indica* hexane extract is not cytotoxic towards normal healthy mammalian cells, which will not cause adverse effects to the normal functioning cells of the body, albeit possess a great anticancer property.

Keywords: Neem, *Azadirachta indica*, hexane, evergreen, cytotoxic effects

KESAN SITOTOKSIK EKSTRAK *AZADIRACHTA INDICA*

ABSTRAK

Neem, dengan nama saintifiknya *Azadirachta indica,* merupakan pokok hijau yang cepat tumbuh yang mudah dijumpai di negara panas termasuk Malaysia, India dan Pakistan. Pokok hijau ini mempunyai nilai tinggi sebagai ubat tradisional. Kajian sebelum ini memaparkan komponen-komponen *A. indica* yang menjadikan ianya sebagai calon terunggul untuk antikulat, antiparasitisme, antibakteria dan antikanser. Kajian ini berhasrat untuk menentukan kesan sitotoksik *A. indica* terhadap sel Vero. Sel Vero ialah sel ginjal Monyet Hijau Afrika. Dengan menentukan kesan sitotoksik terhadap sel mamalia yang normal, margin keselamatan pokok hijau boleh diketahui sebelum digunakan dalam bidang perubatan kemudian. Sel kultur yang tersedia di dalam makmal ditambah dengan ekstrak heksana *A. indica* dengan kepekatan 3.125 µg/mL hinge 100 µg/mL*,* lalu diinkubasi selama 72 jam dan diakses dengan ujian MTS untuk menentukan kebolehmandirian sel. Keputusan kajian ini menunjukkan bahawa ekstrak hexane *A. indica* tidal mempunyai sifat sitotoksik terhadap sel mamalia yang normal, iaitu sel Vero,walaupun dengan kepakatan 100 µg/mL, dibuktikan dengan niai MTS 11.13%. Oleh sebab itu, sel Vero tidak bersifat sitotosik terhadap sel Amalia yang sihat, dan tidak akan menyebabkan kesan negatif terhadap sel badan normal walaupun sifat antikansernya yang baik.

Kata kunci: Neem, *Azadirachta indica,* heksana, pokok hijau, kesan sitotoksik

1.0 INTRODUCTION

1.1 Research Background

Azadirachta indica or commonly known as Neem leaves have been used for centuries in traditional medicine for their potent medicinal properties. These leaves are rich in bioactive compounds such as nimbin, nimbidin, and azadirachtin, which have been shown to possess a wide range of therapeutic effects including antimicrobial, antifungal, antiviral, and anti-inflammatory properties. In veterinary medicine, neem leaves have gained increasing attention for their potential benefits in managing various health conditions in animals. (Osunwoke, et al., 2013) The bioactive compounds within Neem are the key factors to various cancer management. They governs the signaling pathways such as apoptosis (bcl2, bax); transcription factors (e.g. NF-κB), angiogenesis (VEGF), and tumour suppressor gene (e.g., p53, pTEN).

According to Akbar et al. (2011), neem leaves have been traditionally used in the treatment of various ailments in livestock such as gastrointestinal parasites, respiratory infections, skin conditions, and wound healing. Furthermore, studies have shown that neem leaves can also help in boosting the immune system, improving overall health and well-being in animals.

By exploring the diverse applications of neem leaves in animal health, it is possible to focus the light on the potential benefits of *A. indica* into veterinary care practices as anti angiogenic agents, antioxidant and antifungal.

1.2 Research Problem Statement

Neem leaves has been used for centuries in curing disease both in humans and animals. However, lack of scientific works documented the cytotoxic effects of the plants. Thus, necessary works need to be conducted to verify the effects against Vero cells.

1.3 Research Questions

What is the cytotoxic effects of *A. indica* extracts against Vero cells?

1.4 Research Hypothesis

A. indica extracts show measurable cytotoxic effects against Vero cells.

1.5 Research Objectives

To determine the cytotoxic effects of *A. indica* extracts against Vero cells.

2.0 LITERATURE REVIEW

2.1 Bioactivity of *Azadirachta indica*

Azadirachta indica or neem offers various biological benefits, ranging from its antioxidant property, antihelminthic, antifungal properties, antibacterial effects, cytotoxic effects to antiviral effects. These can be supported by the numbers of studies conducted that demonstrated the antiviral activity against viruses like dengue (Ichsyani, 2017; Parida et al., 2002), Foot and mouth disease (Deshpande and Chaphalkar, 2013), Newcastle Disease in poultry (Jayati et al., 2013). *A. indica* has a pivotal role in fighting the free radical generation and prevention of disease pathogenesis (Alzohairy, 2016).

The livestock industry can benefit from the usage of *A. indica* from the economic aspect in treating helminthiasis infestation, it can even be prescribed without any process, to the livestocks. These can be achieved by curbing the release of morphogenic peptide, prothoracicotropic hormone (PTTH) and allatotropins from the brain-corpus cardiacum complex. In short, Neem can prevent the insect from molting, suppress chitin formation that further prevent normal metamorphosis hence cause immature death of the worms. (Tibebu, A., Haile, G., and Kebede, A., 2017)

2.2 The role of A. indica in the anticancer studies

Ethanol A. Indica leaves extract have the ability to induce and enhance apoptosis in MCF-7 breast cancer cellular line, this is further supported by Othman et al., 2012, the team successfully proved the ability of the extract to down regulate 4T1 breast cancer cells. Research conducted by Arumugan et al., 2014, concluded that tumour volume and progression of tumour growth may be suppressed by the administration of neem leaves extracts towards the rat induced with breast cancer, acted upon by increasing Bcl-2-associated death promoter protein (Bad) caspases, p53, phosphatase and tensin homolog gene (PTEN), B cell lymphoma-2 protein (Bcl-2)-associated X protein (Bax), and c-Jun

N-terminal kinase (JNK). The bioactive ingredients of Nimbolide is an effective anticancer therapeutic agent as it has been proven to pose anticarcinogenic effect on human breast cancer cells. As stated by Elumalai et al., 2012, MCF-7 and MDA-MB-231 human breast cancer cell lines shown proapototic activity when induced by this bioactive agent, however, it is time and dose dependent.

2.3 Cytotoxicity of *A. indica*

Research focused on the use of neem leaves extract to laboratory animals and baby hamster kidney fibroblast (BHK-21) proved its cytotoxic effects, when used in a specific concentration (Qurni et al., 2017). Another study done by Wong et al. (2014), revealed the cytotoxic effects of *A. indica* using brine shrimp lethality assay, yielded the result that proved *A. indica* can cause potent cytotoxic effects.

To further discuss the cytotoxic effects of *A. indica*, it has to be established the understanding of cytotoxic studies. According to Kuete (2014), cytotoxicity studies determine the potential toxicity of a test substance which in this case, covers the plant extracts used or the biologically active compounds isolated from plants. While cytotoxicity refers to the potential and degree of damage incurred at the cellular level, it may result in the form of necrosis, apoptosis, autophagy and decreased cell proliferation. In light of cytotoxic effects of certain drugs as the fundamental research and drug discovery, it is the indicator of the effectiveness of the drugs in cancer therapeutics. Studies conducted in human cancer studies proved that active components of *A. indica*, namely azadirachin, nimbolinin, nimbin, nimbidin, nimbidol, sodium nimbinate, gedunin, salannin, and quercetin (Alzohairy, 2016), warrant an apoptotic effects towards common cancers, that included leukemia (Kikuchi et al., 2011), cervical cancer (Mahapatra et al., 2011), prostate cancer (Priyadarsini et al., 2010) and mammary cancer (Elumalai et al., 2012). Tumour growth inhibition is more likely to be achieved by Neem leaves because of the important anti angiogenic agents that hinder the growth of new blood vessels prelimentally. (Lavanya et al., 2015)

In a study carried out by Majapatra et al. (2012), ethanol extract of neem leaves were found to be able to decrease the stimulatory effects of vascular endothelial growth factor (VEGF) originating from endothelial cells of human umbilical vein, concluding that neem leaves have strong antiangiogenic effects.

2.4 The cell line used in this cytotoxicity study

2.4.1 Vero cell

Vero cells are the mammalian continuous cell lines which is mainly made up of the epithelial cell line. It is isolated from the African green monkey kidney cells which are commonly used in the research of microbiology, and molecular and cell biology. The reason that Vero cells were used in this research is due to the fact that it is a well established non cancerous mammalian cell lines, that is sensitive to various microbes, chemical complexes and toxins. (Siddiqui et al., 2019). Vero cells act as the control of normal cell line in cytotoxic effect studies, cancer studies and natural product screening tests. (Siddiqui et al., 2019; Mashjoor et al., 2015; Sit et al., 2018; Kumarihamy et al., 2019). Vero cells need to be supplemented with a medium called Dulbecco's Modified Eagle's Medium (DMEM) as a growing agent as well as 5% fetal bovine serum (FBS). Cell survivability can be evaluated and counted using MTS assay, 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetra-zolium. (Masaeli, E., et al., 2012).

3.0 MATERIALS AND METHODOLOGY

3.1 Sample collection

Azadirachta indica leaves were collected from a 20 year-old Neem tree in a village located in Bachok, Kelantan, Malaysia.

3.2 Sample preparation and extraction

The leaves of *A. indica* were removed from the stalks. They were dried in the oven dryer in the laboratory at 40℃ for three days. The dried leaves were then ground into powder using an electric mill and dissolved in hexane (brand: Merck) at the ratio of 200 gram of dried leaves into 1000 ml of the solvent respectively for 24 hours, at the concentration of 1ppm. Upon dissolution of the leaves, the solution was filtered via filter paper (Whatman No. 1) to obtain the particle free extract. Finally, the extracts were concentrated under reduced pressure using a rotary evaporator and a paste-like consistency extract obtained.

3.3 Cell culture procedure

3.3.1 Preparation of cell line

Cell culture preparation was conducted according to Ammerman et al. (2008). Growth medium from the confluent monolayer of Vero cells was removed and incubated at 37°C for 2 to 3 minutes. The cells were detached from the flask and transferred to a sterile 15 mL conical tube. The desired dilution of cells was prepared in a total of 12 to 20mL Dulbecco's modification of Eagle medium (DMEM) with 10% FBS and add to 75cm^2 cell culture flasks with vented caps. The flasks were incubated in 37°C incubator with 5% carbon dioxide supplementation.

3.4 Cell counting

The cells were detached and resuspended using Trypsin and placed into a centrifuge tube with 20 to 200µL of DMEM and stained with 0.4% Trypan Blue. Upon centrifuge at 2500rpm for 10 minutes, the supernatant was discarded and the precipitate was used in cell counting with a haemocytometer. A total of 10µL of stained precipitate was pipetted into the haemocytometer chamber and observed under 10X magnification using an inverted microscope.

3.5 Treatment

The cells were seeded onto a 96-well plate and incubated for 24 hours at 37℃ in 5% carbon dioxide supplementation. A 2-fold dilution method using *A. Indica* hexane extract was initiated on the 96-well plate, starting with the highest concentration of 100µg/mL to the lowest concentration of 3.125µg/mL, using micropipette (brand: Eppendorf).

As Hexane extracts of *A. indica* were titrated to the cultured cells, the cells were incubated at 37℃ for 72 hours in 5% carbon dioxide supplementation. Cell viability was evaluated using MTS assay. A total of 20 µl of MTS were added to each well and incubated for 3 hours wrapped in aluminium foil. After completing with incubation, the plate was read at 517 nm using a spectrophotometer.

3.6 Cell viability calculation

Calculation of percentage of cell viability from MTS value for the wells treated with *A. indica* hexane extract were determined using the formula:

MTS value: (negative control- reading control)/ negative control X 100%

4.0 RESULTS

The mean percentage (%) of cell viability of Vero cells (96-well plate) after 72 hours treatment with *A. indica* hexane extract as in Table 4.1.

Table 4.1 Percentage (%) of the cell viability of Vero cell line treated with *A. indica* hexane extract.

Concentration (µg/mL)	3.125	6.25	25	100
MTS %	4.01%	5.46%	11.61%	11.13%

The mean percentage (%) of cell viability of Vero cells after 72 hours treatment with *Azadirachta indica* extract dissolved in hexane showed in Figure 4.1. It depicted that at concentration of 3.125µg/mL has the lowest MTS percentage with 4.01%, the cell viability increases as the concentration increases to 6.25µg/mL and 25µg/mL, however, a slight drop in cell viability was achieved as the concentration peaks at 100µg/mL. It is also noted that cytotoxic concentration 50% (CC50) is not achieved in the treatment plate. However, it showed that the extract tested at various concentration promote cell growth, instead of inducing cytotoxicity.

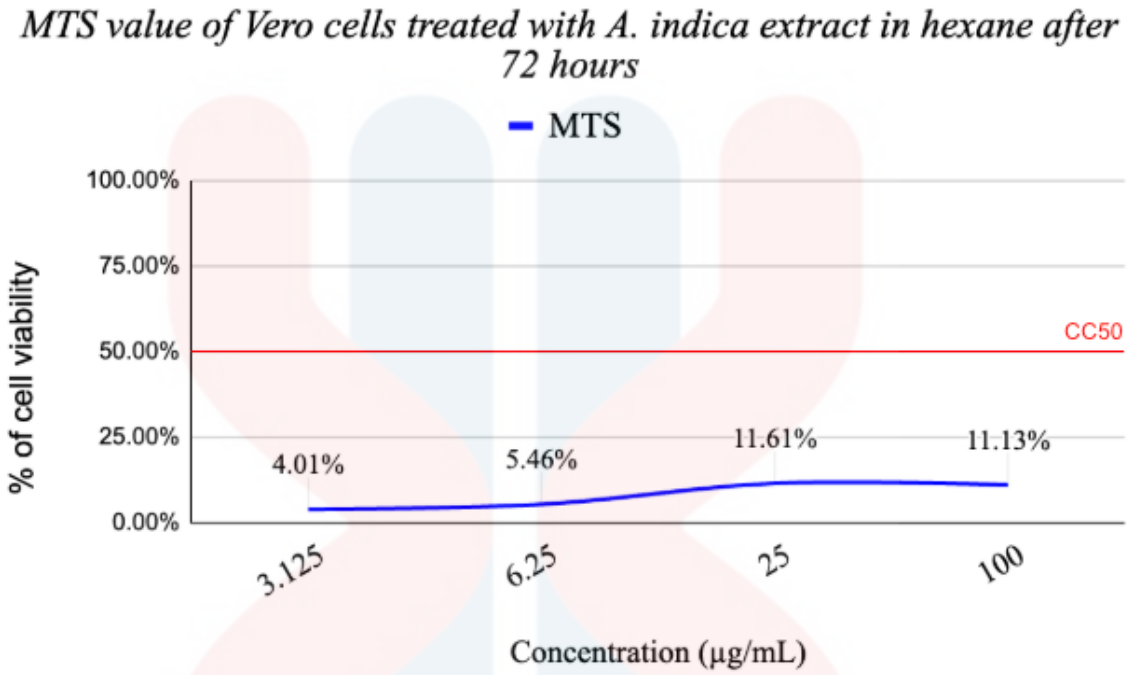

Figure 4.1: Percentage of cell viability, depicted in MTS, of Vero cells after 72 hours treatment with *A. indica* extract dissolved in hexane.

5.0 DISCUSSION

This study was aimed to investigate the cytotoxicity activity of *Azadirachta indica* extract dissolved in hexane, against the normal, non-cancerous mammalian cells, namely Vero cells, which are derived from the kidney of African Green Monkey. *A. indica* is known to have cytotoxic effects towards cancerous cells, such as the breast cancer cells, which in turns expressing beneficial values for its anticancer properties to humans. Studies conducted revealed that the 9 flavonoids isolated from *A. indica* have shown their potential cytotoxic effects against various cell lines, which include lungs, leukemia, breast and stomach cancer cell lines, with IC_{50} value of 4.2 to 100µM (Amer et al., 2010; Jadari et al., 2013; Kitdamrongtham et al., 2014). These prevalent studies are further supported by research conducted by Fadhel et al. (2023) that depicts the effects of *A. indica* in inhibiting rapidly dividing cells, such as breast cancer cell lines, whereby it only causes minimal cytotoxicity to the vital organs, conducted with laboratory mice. Interesting results obtained by Fadhel et al., 2023, discovered the potential of *A. indica* to become alternatives for drug resistance associated with malignancies in breast cancer studies.

In this context, understanding the potential cytotoxic effects of *A. indica* extract towards the normal mammalian cells become vital as it is to determine the risk and benefits of the extract in anticancer therapy studies. The experimental study conducted by Moga et.al., 2021, tested *A. indica* extract dissolved in various solvent towards Vero cells, cervical cancer cells and prostate cell lines. The findings shown that the methanol extracts of this plant can significantly cause cytotoxic effects towards the tested cancer cell lines.

Vero cells as the control cell line, were then tested with the same concentration, which yielded the results of highest CC50 value for Vero cells, it concluded that albeit being cytotoxic towards cancerous cells, *A. indica* extracts are not cytotoxic towards Vero cells.

A similar anticancer study was conducted by Trivedi et al, 2018, which incorporate *A. indica* extract at physiological pH 7.4 towards cancer cell lines and used Vero cells as a control cell line. It was shown that the IC50 value for *A. indica* extract is more than 1600 µg/mL, concluded that the extract can be safely utilised in anticancer treatment without exerting harmful effects towards normal healthy, non-cancerous mammalian cells.

According to an unpublished study conducted in Malaysia in 2024, phosphoric acid, trimethyl ester comprised of the largest component, at 61.36% of *A. indica* dissolved in Hexane, which can highly be suggested as the main component that contribute to the cytotoxicity of the plant extract.

In this study, the cell survivability was evaluated and counted using 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetra-zolium (MTS) assay. The results showed that only mild cytotoxic effect was achieved in the tested cell line, which is Vero cells, whereby the lowest loading dose of 3.125µg/mL can has a MTS percentage of 4.01%, and tested increments in the concentration of *A. indica* hexane extract does not show any significant harmful effects towards Vero cells. This means that the CC_{50} is more than 100µg/mL. Thus, it can also be concluded that *A. indica* does not possess cytotoxic effects towards the Vero cells, derived from the kidney of African Green Monkey.

The fact that *A. indica* is selectively cytotoxic towards cancerous cells has been proven in various studies conducted. The apoptosis-inducing effects of *A. indica* along with its anti-proliferative is deemed to be exclusive for tumour cells only, whereas normal cells should only experience minimal cytotoxic effects. By discovering this, we can now have an idea that *A. indica* extract has a rather lowered toxicity during cancer therapy as the normal cells will have a higher cell viability as to answering the side effects of this drug choice. (Hao, et al., 2014).

As stated by Kamath (2019), the application of *A. indica* in anticancer studies concluded that it can specifically target growing and proliferating tumour cells. The proteins that play the main role in cell cycle control and cellular process would be specifically targeted, one example is regulated genes induced by gedunin, which can decrease the proliferation of pancreatic and ovarian cancer cells. In addition, the combination of gedunin and cisplatin can produce synergistic effect up to 50% in ovarian cancer treatment alone.

The results of this study shows that *A. indica* hexane extract is not posing cytotoxic effects against the tested cell line, Vero cells. The findings emphasize *A. indica* hexane extract is harmless towards the normal mammary cells, namely the African Green monkey kidney cells. The cytotoxicity studies of *A. indica* hexane extract against Vero cells, is believed to be beneficial in terms of anticancer studies to determine possible detrimental health effects as stated in the above mentioned.

6.0 CONCLUSION AND RECOMMENDATIONS

The findings indicated that *Azadirachta indica* hexane extract, may possess no cytotoxicity activity against the Vero cell line, which is extracted from the kidney of African Green Monkey, even at a higher concentration, at 100µg/mL. MTS value of Vero cells treated with *Azadirachta indica* extract dissolved in hexane after 72 hours incubation was 4.01% at the concentration of 3.125µg/mL, with the increment of concentration to 6.25µg/mL, MTS value was 5.46%. At the concentration of 25µg/mL, MTS value increased to 11.61%, when it achieved its highest concentration of 100µg/mL in this experiment, MTS value dropped to 11.13%. Linear relationship between the concentration of *Azadirachta indica* hexane extract and the MTS value. It shown that the cells were viable even after the 72 hours of incubation period, and *Azadirachta indica* hexane extract is not cytotoxic to the normal non-cancerous Vero cells extracted from African Green Monkey's kidney. Thus *Azadirachta indica* hexane extract is safe towards the selected cell line, and safe for future consumption as well as application. This research paper's information can serve as a valuable reference and guideline for future cytotoxicity studies for *Azadirachta indica* hexane extract in Malaysia as there is currently lack of cytotoxicity study done on this *Azadirachta indica* extract in Malaysia. Hence, further comprehensive and specific cell culture using different cancer cell lines need to be done to determine its cytotoxic effects and cell viability in anticancer treatment research.

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8.0 APPENDIX

Figure 8.1: Water bath of the frozen cells at 37°C for 30 minutes

Figure 8.2: Precision Water Bath (N-Biotek) used for water bath of medium wash solution.

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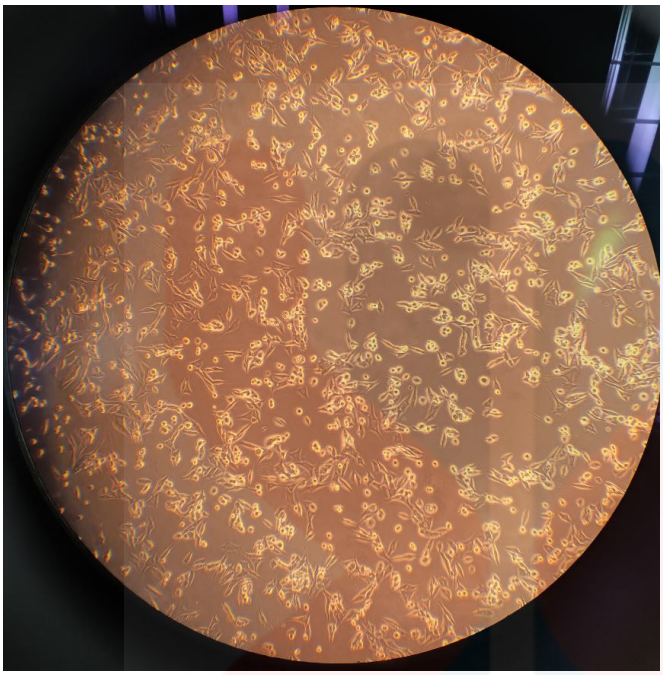

Figure 8.3: Vero cells that achieved confluent, viewed under inverted microscope.

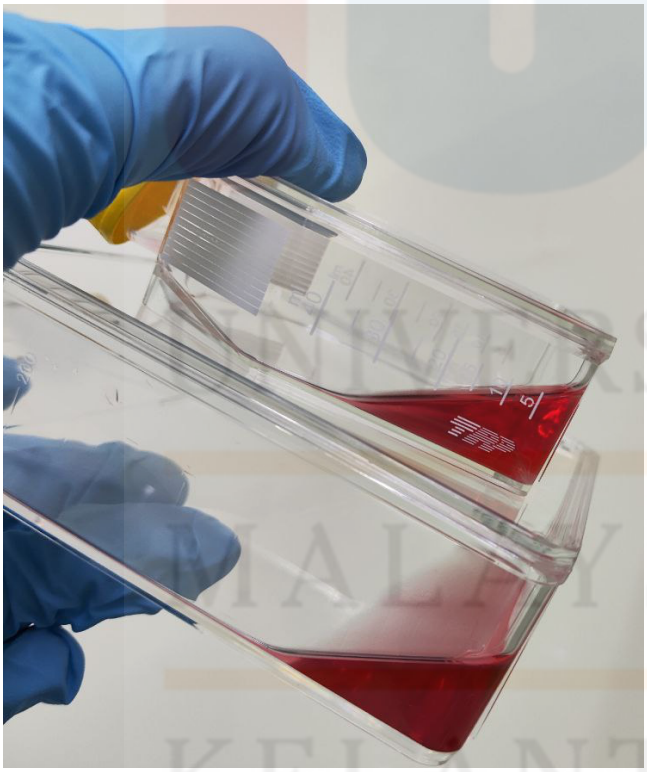

Figure 8.4: Viable Vero cells in DMSO solution after 24 hours of incubation.

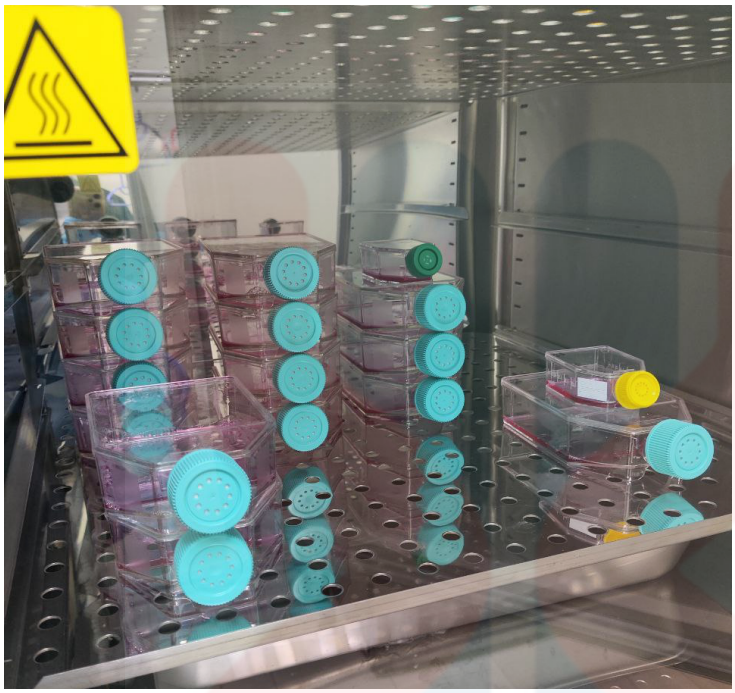

Figure 8.5: Cell culture flasks incubated at 37°C for 24 hours, under 5% carbon dioxide.

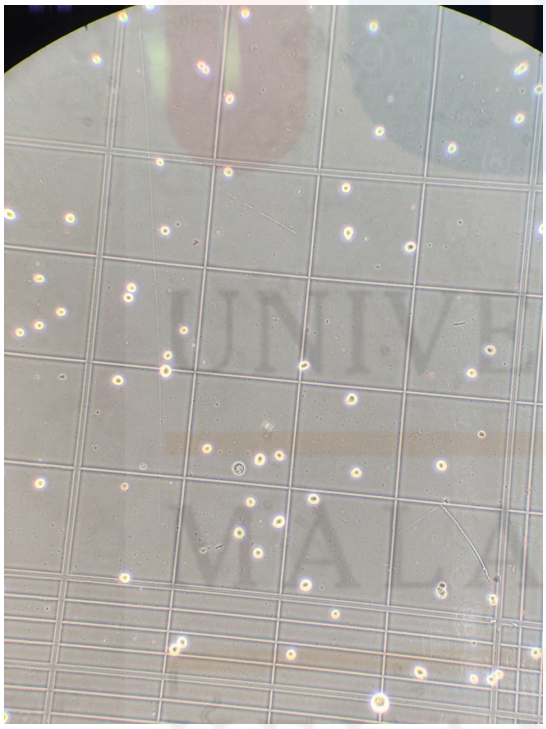

Figure 8.6: Cell counting using haemocytometer, observed under inverted microscope.

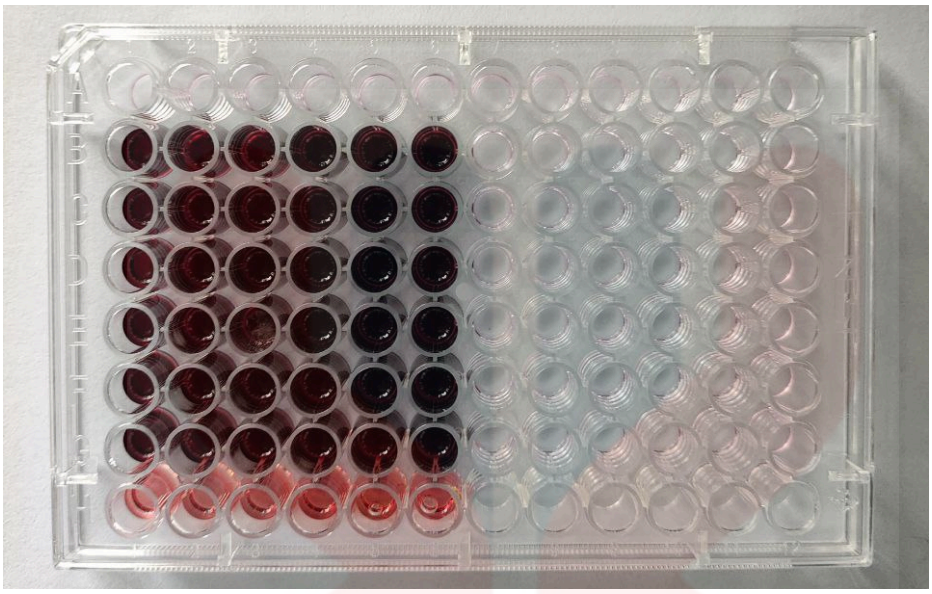

Figure 8.7: Vero cells in 96-well plate, treated with *A. Indica* hexane extract, added with MTS, before reading by 517 nm using a spectrophotometer.

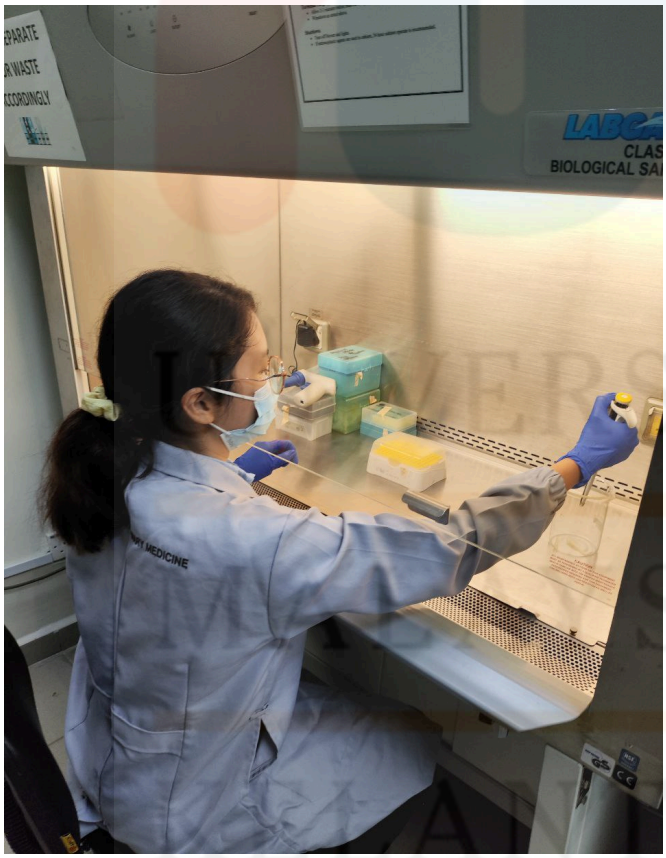

Figure 8.8: Cell culture laboratory work taken place in Virology Laboratory, UMK.