Investigation on Anti-Proliferation Properties of Porcupine Bezoar (Hystrix Brachyuran) Extracts Exposed on Hela Cells Lines Combined with Electroporation Technique

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***Abstract*—Electroporation (EP) is a technique whereby the biophysical changes on the cells that induced external high-intensity electrical field pulses in order to enhance applications in the medical field. It is a molecular biology technique in order to create pores through a cell wall membrane, boost the permeability of the cell membrane, and support chemicals, drugs or DNA to be imported into Hela cells. While by combining electroporation (EP) technique with porcupine bezoar (PB) extract might reduce the proliferation of HeLa cells because this compound extract has the ability of anti-proliferation and also anti-angiogenesis properties for controlling cancer cell growth. This research concentrate on reviewing and analyses the basic concepts and methods of combining electroporation and porcupine bezoar (PB) extract as applied in cancer treatment application. The combination of this technique might be a new alternative for anti-cancer treatment. The combination of this technique might be a new way for anti-cancer treatment.**

***Index Terms*—Cell Anti-Proliferation; Cell Growth; Electroporation; Porcupine Bezoar (PB) Extract.**

I. INTRODUCTION

This paper briefly describes techniques of electroporation, the use of porcupine bezoar (PB) extract as an anti-cancer treatment and some study on research literature review. Nowadays, cancer is a dominant factor of death around the world and also now the 4th leading cause of death in Malaysia. Studied showed that cancer is caused by many factors such as an external factor likes the chemicals used, sun radiation exposed, and infectious organism .In addition, an internal factor such as genetic mutations, immune condition and human hormones that exposed to sedentary lifestyles like smoking, poor diet, and inactive physical activities also contributed to the risk of cancer.

There were many treatments provided in order to help a cancer patient, for instances, radiation therapy, chemotherapy hyperthermia, hormone treatment and much more. Unfortunately, advances in cancer therapy will lead and resulted in highest numbers of long-term survivors who are then will leave to handle the consequences of their treatments.

Therefore, this research is to discover an alternative treatment which has minimal or almost no side effect by using Electroporation and porcupine bezoar (PB) extract, that focused on a new alternative agent which can suppressing, delaying or reversing carcinogenesis by pharmacologic intervention with naturally occurring or synthetic agents even though there is still no research or work had been done by combining this two method so far.

A. Electroporation

In the most recent decades, the use of electromagnetic devices in medicine in modern science and technology is very aggressive day by day. For example, the process taking measurements which induced by the internal electric fields and also an electric simulation of volatile tissue utilized to assist life, rehabilitation damages and to improve the general life's element [1]. Moreover, Electroporation (EP) was later termed after some detected an alteration occurred in wall membrane permeability in sequential of creation of hydrophilic pores [2].

Electroporation (EP) is a feasible and applicable physical mechanism, where it is in huge intensity and sharp duration pulses are exposed to create temporary pores in the wall plasma membrane of the cells. The point is to permit transport of restorative materials for example medication drugs and genes (DNA) [3].In this phenomenon, the cell electroporation in-vitro is utilized for the most part for transfection by DNA presentation, however numerous different mediations are conceivable, including microbial killing. In-vivo electroporation of tissues upgrades sub-atomic transport through tissues and into their constitutive cells whilst ex-vivo electroporation gives control of cells that are reintroduced into the body to provide treatment. Moreover, tissue electroporation by shorter, smaller pulses is under scrutiny for biomedical engineering applications utilizations of therapeutic treatment for gene therapy, cancer treatment, and transdermal drug conveyance.

There are many advantages of electroporation technique such as this technique does not change the natural biological structure and function of the target cells, and it is safer, high efficiency and less immunologic [4].

Therefore, by applying electric pulse field crosswise over cells will give an assortment of results; from zero impact to reversible electroporation or to irreversible electroporation. Reversible electroporation will permit transient molecular transport through the pores and following a couple of minutes, cell membrane layer will reseal and cell functions

are reestablished [5].Conversely, irreversible electroporation demonstrated the capability to kill undesirable tissues in the body and could be connected as without chemo drug to kill the cancerous cells.

B. *Porcupine Bezoar (PB) Extract*

In this study, the use of porcupine bezoar (PB) extract will be explored to reduce the proliferation of HeLa cells through electroporation. This is achieved by combining phenomenon of electroporation with the animal extract such as porcupines. Specific bioactive compounds from porcupine bezoar (PB) which also well-known as "prince of antidotes" have the ability of anti-proliferation and also anti-angiogenesis properties for cancer growth.

Porcupine bezoar (PB) or porcupine stone extract from porcupines that are rodents with a coat or sharp quills which will protect and also camouflage themselves from predators. There are 29 species of porcupine in the world and species that mostly found in Malaysia is Malayan porcupine (Hystrix Brachyuran) or commonly known as "Landak Raya" or "Landak Borneo" by locals. Thus, a bezoar is retained concretions of undigested foreign material that amass and mix inside the gastrointestinal tract, most normally in the stomach or gallbladder [6]. Back to history, Arabic medical literature had been referring to bezoar since the 8th century, mainly as alexipharmic and YuhannaMasawayh was the one of the first who mentioned its use. Moreover, history recorded that porcupine bezoar (PB) can be used to treat the poisonous wound, poisonous bite, plague, quartan fever, severe febrile disease, contagious disease, measles, smallpox, jaundice and renal problem. During 7th until the end of the 17th century, bezoar was appreciated as antidotes by the Arabic, European, West and Chinese authors. In the 18th century, the establishment of more effective therapies and the chemical revolution led to the abandonment of bezoar and it becomes beautiful relics of the past [7].

As conventional treatment leads to many sides effect to the cancer survival, therefore porcupine bezoar (PB) could be an alternative treatment as it poses naturally alternative and minimal or no side effect.

C. *Anti-cancer study*

Since ancient times, natural sources have been an important element in medicines field. For example, medicinal mushrooms have been utilized as remedial agents also known as FunaliaTrogii. Unyayarmentioned FunaliaTrogii which also known as TrametesTrogii recently showed anti-cancer properties. Experiment in 2006 reported the cytotoxic and mutagenic impacts of F. Trogii and C. Versicolor extracts on HeLa cells and ordinary human fibroblast cells. These impacts were contrasted with the impacts delivered by the anti-cancer agent mitomycin C. Moreover, a cold water extract of F. Trogii elicits a defensive impact on deltamethrin-induced liver toxicity in rats detailed by Mazmanci [8].Extracts of both fungi hindered the proliferation of HeLa cancer cells and normal fibroblasts in a dosage subordinate way. Both extracts had a more grounded inhibitory impact on cancer cells contrasted with their impact on fibroblasts.

Further, alternatives of anti-cancer have been developed by using curcumin, commonly known as turmeric.Curcumin (diferuloylmethane) is a polyphenol gotten freshly from the Curcuma longa plant. Moreover, Curcumin has been utilized widely in Ayurvedic medicine treatment for quite a long time, as it is non-toxic and has an assortment of therapeutic properties such as cancer prevention agent, pain relieving, antiseptic activity, and anti-inflammatory.

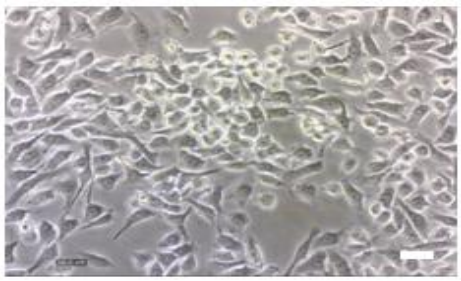
Recently, it has been discovered to have anti-cancer activities via its impact on an assortment of natural biological pathways required in mutagenesis, cell cycle regulation, tumor genesis and metastasis and it demonstrated an antiproliferative impact in numerous cancers.

II. TECHNIQUES

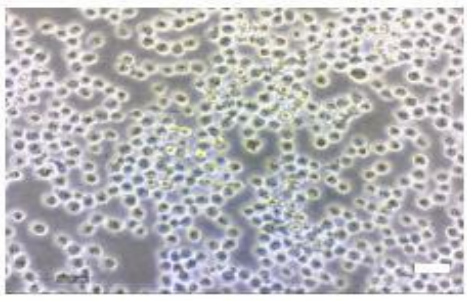
A. *Cell culture*

In this experiment, HeLa cells were utilized for doing every one of the trials. Firstly, the HeLa cells line was cultured in RPMI1640 (plus L-glutamine) as a monolayer. Next, The RPMI 1640 was upgraded with 10% fetal bovine serum (FBS) plus with 1% antibiotic (penicillin and streptomycin). Later, the cultured cells were incubated in a humidified incubator containing 5% CO2 at 37°C condition. The cells were sub-cultured every three to five days and will be monitored until it reached 80% to 90% confluent.

For subculture process, the old medium was initially disposed of by aspiration. Next, 2ml of phosphate buffer saline (PBS) was used in purpose for washing the cells. Then, the additional PBS was aspirated, and 2ml of trypsin express solution was added for cells separation or also known as cell detachment. Later, the cells with the addition with the trypsin express were incubated for ten until fifteen minutes at 5% CO2 at 37 °C condition. This is on account of the trypsin express function admirably in a warm environment so the HeLa cells line will have strong adherent to the monolayer.



(a) HeLa cells before on detached condition by using trypsin express *(Scale bar = 20µm)*



(b) The cell starts appeared rounded and fully detached from the flask surface *(Scale bar = 20µm)*

Figure 1: Comparison between the HeLa cells condition before detached and (b) After trypsinized with trypsin and start to detach from the surface

Based on Figure 1 above, when the cells were completely detached, an equivalent volume of full complete growth medium was added to stop the detachment or separation of the cell. Unfortunately, the too long time taken for the cell

treatment with trypsin express may cause irreversible cell damage occurred. Later on, 0.5ml of the cells suspension is then reseeded in another 25cm2 flask containing 7ml of complete growth media.

B. Electroporation (EP) Technique

Electroporation (EP) is the system that uses of applying high pulse electric field (thousands of V/cm) to instigate permeability increment in the wall cell membrane, short duration pulses length to open up pores, allowing the passage of chemo drugs that are normally impermeable or less permeable through the cell plasma membranes. Electroporation (EP) generally depends on upon the intensity of the electric field, the duration of each pulse, the number of pulses and the interval between them.

In this research, the electroporation device (ECM830) from BTX Harvard Apparatus (see Figure 2) was utilized for electroporating the cells line in suspension.

Figure 2: ECM®830 square wave pulse generator

In addition, the ECM830 electroporation device has two methods of operation: Firstly, a low voltage mode with output voltage going begin from 5V to 500V and pulses duration of 10ms to 999ms (1ms resolution). Next, is the high voltage mode with output voltage extending from 501V to 3KV and pulses duration of 10µs to 600µs (1µs resolution).

C. Experimental setup for Electroporation System

The general of the exploratory setups of Electroporation (see Figure 3) is to produce a pore which either temporarily open or permanently open. In Figure 3, the experiment is handled in order to embed natural biological specimen into the cell (Reversible Electroporation) and also electroporation irreversibly to discharge their intercellular substance for further natural biological investigations. This experiment setup for electroporation (EP) technique that needs the huge suspension volumes and commercially available of Electroporation cuvette size with electrode gaps of 1, 2, and 4mm and also have the volume of 100µl, 200µl and 400µl.In this research, the size of 4mm cuvette is used because it is suitable for the mammalian cell while 1mm is suitable for bacteria and yeast, and 2mm for all cell types (see Figure 4).

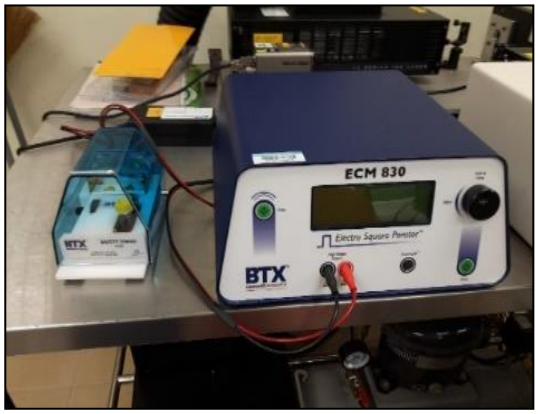


Figure 3: Electroporation system

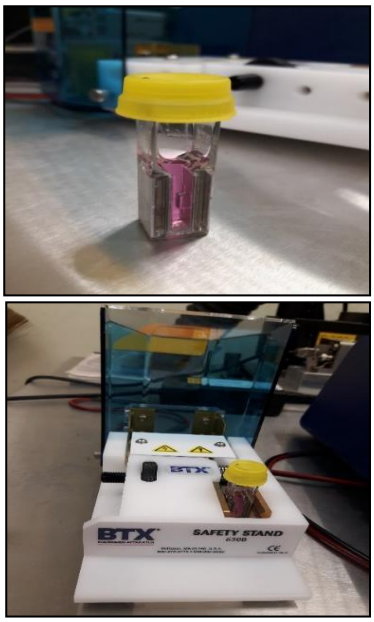


Figure 4: Cuvette (electrode gap 4mm) with electric field stimulation.

The important of electroporation process was to identify the experimental setup for electroporation event in order to make sure that the process taken followed the procedure and controlled. For electroporation system without real-time visualization, the equipment used as shown in Figure 5. Due to electroporation event is handle inside of the cuvette, this experimental setup is used. The effect of the cell with the electric field is monitored under the Nikon inverted microscope that connected with Dino Camera and Dinocapture2.0 Software (see Figure 5).

Figure 5: Nikon TS100 inverted microscope with Dino camera and Dinocapture2.0 Software

In this experiment, for the purpose of this study, pulse duration of 30µs (constant) was selected and the field strengths was varied (200 V/cm, 400 V/cm, 600 V/cm, 800 V/cm, and 1000 V/cm) to obtain the proliferation for growth rate of HeLa cells.

D. Porcupine Bezoar (PB) Extract Preparation

PB extract was prepared into a stock solution of 1.0 mg/ml and PB powder was dissolved in sterile deionized water. Next, the stock solution was further diluted with complete growth media immediately prior used.Later, HeLa cells were treated with PB extracts at different concentrations of 5.0, 10.0, 20.0, 40.0, and 80.0µg/ml and it will be added to the electroporation (EP) cell to see the anti-proliferation rate of the cell after combining these two techniques.

III. Results and Discussion

A. Results of Pulse Electric Field on HeLa cells

In order to do the splitting process, HeLa cells must be confluence at 80 – 90% in 25cm^2 flask as shown in Figure 6.

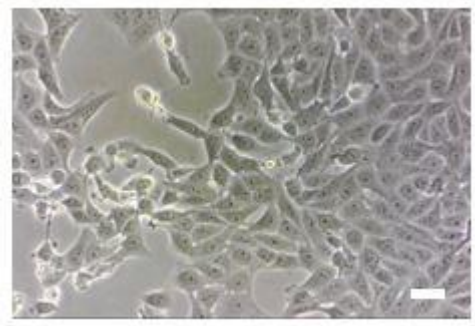
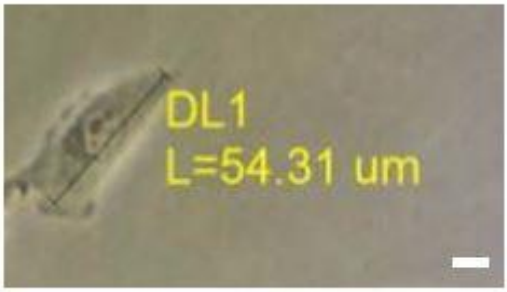


Figure 6: 80-90% confluency of HeLa cells in 25cm^2 flask *(Scale bar = 20µm)*

The result in Figure 7 shows the effect on HeLa cell length when induced by 200 V/cm. The pulse duration is 30µs and the number of pulses is single shown it is the best parameters of electroporation (EP) effect on the HeLa cells. The imaging technique is the phase contrast microscopy with the 20X objective. The results are shown in Figure 7 (a) demonstrate HeLa cells average length is 54.31µm without PEF exposure. After being exposed to PEF it can be seen that HeLa cells length expanded, an increased as in Figure 7 (b). Figure 7 shows the differences in cell lengths of HeLa cells with and without exposed with electroporation (EP). This situation confirms that physical changes indicated by the HeLa cell and the response encountered in this study named as reversible EP.



(a) HeLa cells without EP *(Scale bar = 20µm).*

(b) HeLa cells with EP after 6 hours *(Scale bar = 20µm).*

Figure 7: HeLa cells size comparison (a) without EP (length: 54.31µm) and (b) With EP (Length: 80.93µm)

Therefore, the result has shown how the PEF exposure does have an effect on HeLa cells. The result from this experiment shows an effect of reversible electroporation (RE). The size of HeLa cells expanded when it was induced with electroporation (EP) compared to HeLa cells without electroporation (EP) which has a smaller size. This process also called cell swelling. This is due to HeLa cells with EP have absorbed more nutrients on media and grow faster when swelling compared to HeLa cells without electroporation (EP).

Finally, the result demonstrated how does pulse electric field exposure effect HeLa cells proliferation and growth rate, where the exposure of electric field permeabilize the cell membrane thus, increasing cell growth for the cell migration activities. However, these are the preliminary finding of the study in order to show the correct experimental setup has been achieved.

IV. Conclusion

The fundamental ideas and methods of electroporation and the combination with porcupine bezoar (PB) extract as anti-proliferation were highlighted. Both of the technique was found to be related to anti-cancer proliferation as well as the optimal Electroporation conditions of various pulse parameter lengths and width as applicable to HeLa cells. Investigation on electroporation (EP) with porcupine bezoar (PB) extract proved that the two techniques could be combined in-vivo studies to observe the cell response thereby increasing the yield of compound extract to the anti-proliferation of HeLa cells. To date, the initial stage of testing the pulse electric field effect has been achieved and the next will be to look at the potential combination of electroporation (EP) and the porcupine bezoar (PB) extract. The outcome of the result may open the door to reveal new target and new electrochemical pathways for cancer treatment and with this new discovery, the health of cancer patients will be maintained and consequently, there will be an improvement in the general life expectancy.

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