FUNDAMENTAL STUDIES OF STEVIA REBAUDIANA BERTONI ON BREAST CANCER CELL COMBINED WITH LOW VOLTAGE PULSED ELECTRIC FIELD

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Abstract. Plant extract has found to be an effective and useful chemo preventive agents in various type of cancer. Plants extracts part, that most includes fruits, vegetable and spies. This extract can be used to prevent inflammation, wounds and infection. And recently, bioactive regent has shown to display anti-microbial, anti-oxidant, anti-inflammatory and anti-cancer properties. For this study, the chosen plant extract was Stevia rebaudiana Bertoni. It has known to be an effective treatment for allergies, treating cancer and cholesterol. While pulsed electric field has known to be an effective way to influence an opening pore on the cell membrane. It also had been used as one of the cancer treatment method in clinical practice nowadays. But, if these two methods combined, it might enhance more reduction in time taken for the cancer treatment process and less harmful/after side effect to the cancer patients. The most used range of pulse electric field are 1 – 1000 V/cm. the concentration will be known by finding the IC₅₀ of stevia on breast cancer cell. The effectiveness of these method will be known by monitoring the cell proliferation, viability and antiproliferation of cancer cell. These methods might be an effective way to treat cancer without harming or left any after side effect to the cancer patients.

Keywords: Stevia Rebaudia Bertoni, Pulsed Electric Field, Breast cancer cell, Cancer treatment, Bioactive compounds

1. Introduction
Stevia Rebaudia Bertoni is one of plant extract that recently focuses worldwide as the sugar replacement or supplement. It is rich within terpenes and flavonoids. The phytochemicals that present in stevia is beta-carotene, austrinullin, dulcoside, rebaudioxides, nicalin, riboflavoin, steviosides, sreviol and thiamine. The main part of stevia that attracts most on researcher is the leaves of this plant, due to their extreme sweet nature with the presence of main active constituents rebaudioside and steviosides [1] It had been widely used in different commercial sectors such as pharmaceutical, food and chemical industries [2]. stevia rebaudiana Bertoni is known to be effective as it has anti-tumorous, anticancer, anti-inflammatory and cytotoxic properties antihypertensive, antihyperglycemic, anti-diarrheal, anti-oxidant, prevents dental caries and having enzyme inhibitory activities [3]. The anti-
tumour potential has been evaluated by several researchers by using the in-vitro method on HEP2 (Human Laryngeal epithelioma cells) [1], gastrointestinal cancer [4] and ovarian cancer cell [5]. The finding showed that the stevia is non-toxic to normal cells but has anti-cancer and anti-proliferation activities against cancer cell. Nowadays, finding the new anti-cancer agents with lesser toxicity have been always urged due to drug resistance associated with overused chemotherapy agents.

Electroporation is a non-invasive technique that had been a well-known technique in medical field, nanotechnology and microfabrication. This method is known as one of the popular physical membrane-penetrating delivery methods other than this is called carrier-mediated delivery. It is a membrane disruption modality which is classified as direct penetration and permeabilization. Permeabilization is to make a cell permeable to a substance when apply disruption on the cell membrane are sufficient to allow any substance trough the membrane. Most of permeabilization are required to set a specific condition such as, voltage, pulse duration, frequency and amplitude. Comparing with other permeabilization method. This method had better control of membrane disruption effect depends on parameter selected. Thus, it is important to find a balance of membrane disruption effect by optimizing the effective parameter for both membrane damage and cell treatment conditions. These method are still in process of expanding in biological applications, where electroporation systems are considerable importance for intracellular delivery [6] due to the feasible control and efficiency in desirable delivery from small molecule to large molecules and has been deem as a promising methods.

Breast cancer is a 2nd leading cause of death among woman in Malaysia [7]. Type of Breast cancer line used in this study are MCF-7 by using in vitro study. It has been commonly used in cancer research for over than 40 years. It was first taken in 1970 from a 69-year-old Caucasian woman. The provided treatment for breast cancer now a days is surgery, and essential treatment are hormonal therapy, chemotherapy, radiation therapy and targeted therapy. Breast cancer cell had a metastatic property, it can spread outside the original organ which is bone, brain, lung and liver. It is complimentary to find the best treatment for the breast cancer cell due to high number of deaths among patients. The alternative combination of pulsed electric field and plant extract might produce a novel technique for cancer treatment with less or no side effect on cancer patients.

2. LITERATURE REVIEW

2.1 Cell
A cancer cell defines that the cell that can divides relentlessly, forming a solid tumour or swamping blood with abnormal cells. Where, the normal process of repair and growth that used by the body is called cell division. The growth rate of cancer cells is faster compared to normal cells. The more positive cells there are, the more quickly there are dividing and forming new cells. For example, breast cancer rapid by 20% compared to normal cells. The causes of a cancer cell are due to uncontrollable cell growth. Changes in the gene can cause cancer to accelerate in cell division or inhibiting normal controls on the system, such as cell cycle arrest or programmed cell death. A mess of cancerous cells growth, can lead to developing a tumour. The characteristic of a cancer cell is it grow and divide abnormally fast, poorly differentiate and have irregular membranes, cytoskeleton proteins and morphology. The abnormality of the cell can be formed from normal cells to benign tumours and malignant tumours. Cancer cells can be metastasis from the primary site through the blood and lymph systems.

2.2 Breast cancer cell (MCF-7)
Breast cancer is a common disease among woman in the world. based on statistic done by the international agency for research on cancer (WHO), new cases for breast cancer in Malaysia 2018 is 17.3% (7,593) out of Malaysia popularity (as shown in Figure 1), which rank first in top ten among
other cancer with 11.0% (2,894) death in a year [8]. Treatment for cancer is a multidisciplinary effort. The common cancer treatment includes surgery, and supported with radiotherapy, chemotherapy, immune therapy, hormonal therapy and symptomatic and supportive therapy. Patient that trough chemotherapy has been shown to improvement of survival, but a big concern to the patients is that their immune system might have a side effect with chemotherapy.

![Figure 1. Number of cases, Females, all ages](image)

2.3 Stevia Rebaudiana Bertoni
Stevia leaves as shown on Figure 2 has been used as a medicinal plant and is still commonly used in oriental medicine. In China it is considered that stevia can be used for the treatment of allergies, to increase the defences, in treating cancer and cholesterol [9]. In the west, stevia is mainly used as a natural sweetener alternative to sugar. Besides being a sugar replacement and other simple carbohydrates, it is believed that it also can be useful for remedy in the treatment of other conditions such as diabetes, hypertension, indigestion and constipation with acceptable daily intake was 0-4mg/kg per body weight [10], [11]. On several previous studies, stevia compound has been applied on pancreatic cancer [12], breast cancer [13], [14] and gastrointestinal cancer [15]. Remedy for external use also can be applied as it contains vulnerary, anti-inflammatory and antibacterial properties that primarily give its tannin content. These properties are useful for treating skin and hair diseases such as; eczema, skin spots, seborrhoea, acne and dandruff. All of these beneficial properties are significantly depending on the drying method in obtaining the active compound. Based on the study by Halim et al. (2019), the microwave dried stevia preserved the antioxidant compound and gave the lowest IC50 against radical. In this study, the potential of dried stevia extract as an anti-cancer agent will be investigated. Where one of the major steviol glycosides in stevia which is stevioside is believed to be as enhancer towards cell apoptosis (or cell death) [16] as shown on table 1.

| Author | Extract | Cell | Result |
|--------|---------|------|--------|
| [15]   | Steviol | Gastrointestinal cancer cells | + inhibit proliferation |
| [17]   | Stevia rebaudiana (acetone extract) | Human laryngeal epithelioma cells (Hep2) | Anti-cancer and anti-proliferative to activities against cancer cells |
| [18]   | Stevia rebaudiana (ethanolic extract) | Erlisch's Ascites carcinoma | Good anti-cancer activity at dose level 300mg/kg |
| [19]   | Stevia | Skin carcinogenesis | Inhibit tumor promotion/reduction tumor formation |
2.4 Electroporation

Electroporation is the effective and simple technique for the intracellular delivery of molecules in living tissue [6], [22] thus, lead to various medical application such as treatment of tumours, genetics, immunology [23]–[26] and focal ablation of defective tissue. parameters play an important role for the efficiency of this technique, which includes pulsed duration, amplitude and frequency of applied electrical pulses [27]. the pulsed electrical field induces cause a transient reversible interruption on the cell membrane due to the electrical energy applied reach is more that is needed on the cell membrane. resulted in the formation of pores. the pores form can either be permanent or temporary. the size and formation of the pores depend on the duration of time applied [28], [29] there two types of electroporation; reversible (temporary) means cell survive after the electric field cut off and irreversible (permanently) can lead cell death due to cell lysis [30].

2.5 Intracellular delivery

Intracellular delivery is a process of cargo and transportation (as shown in figure 3). It's delivery in the variable in size, source, physicochemical properties and organization. Such a variety of transfer could make a great influence on delivery approaches and intracellular applications. Based on the various properties and applications of cargos there are two types of current techniques for intracellular delivery, which is carrier-mediated delivery and membrane-penetrating delivery. Carrier mediated delivery involves various biochemical assemblies, from molecular to nanoscale dimensions. Though membrane disruption is more to a physical effect, including the temporary interruption in the plasma membrane by mechanical, thermal, electrical, acoustics or optical means. This study is more on electrical inducement to disrupt the membrane layer to enhance the formation of pore on the cell membrane surface [6].

2.6 Permeabilization

Permeabilization is one of the primary physical methods for membrane-disruption technique, the other one is direct penetration strategies (as shown on figure 3), where the method of direct injection was done by an external force. Basically, permeabilization approaches is to permeabilize the cell membrane with the substance when the disruptions to the membrane are of sufficient to allow any substance through the membrane. Practically this process approaches need to have specific parameter, such as temperature and electric field to initiate permeabilization, delivery and subsequently facilitate cell retrieval [6]. Most of commonly used tools for permeabilization are electroporation and laser-based delivery. Compared to the direct penetration approaches, permeabilization can control stably
membrane interruption effect with the parameter of different conditions. Basically, optimizing both the membrane damage and cell treatment conditions are the key balance of the membrane interruption effect in permeabilization strategies.

![Figure 3. Membrane disruption-mediated intercellular delivery [31]](image)

2.7 Combination of Electroporation and plant extract
Combination of electroporation and plant extract is a promising method on cancer treatment. Electroporation had been widely used in medical applications as it was able to make cell-permeable to any substances as an electrical inducement was done. While plant extract is a potential anti-cancer agent that been proven by the previous researcher as shown in table 1. Combination of these two techniques will give an impact on cancer treatment without drug dependent. The previous researcher had shown a promising result regarding the combination of these techniques.

| Author | Type of Cell | Type of Compound  | EP | Response |
|--------|--------------|-------------------|----|----------|
| [32]   | HeLa         | Neolamarckia Cadamba | 600V/cm, 5ms | +        |
| [33]   | HT29         | Luffa Acutangula  | 600V/cm | +        |
| [34]   | MCF-7        | Curcumin          | 1200 V/cm 600 V/cm 100µs, 1500 V/cm 100µs, | +        |
| [35]   | MCF-7        | Cisplatin         | 1008 V & 1280 V 20µs | +        |
3. Methodology

![Diagram of flow of work]

**Figure 4.** Flow of work

### 3.1 Electroporation Setup

The electroporation method configuration used in this research is seen in Figure 5. The electrical field cell influence was observed by the Nikon inverted TS100 microscope attached to the Dino Camera and the Dinocapture 2.0 software. This experimental setup cannot be demonstrated for real-time visualization. Thus, a few seconds after the procedure taking the sample out from the safety stand and placing it under the microscope should be taken into count. 4 mm size of the cuvette was used because it is suitable for the mammalian cell. While the parameter that set into the pulse generator was 100-1 kV/cm of electric field intensity, 30-100µs of pulse duration and a single number of pulse [22], [36], [37].

![Diagram of electroporation in vitro method]

**Figure 5.** Electroporation in vitro method by using cuvette

### 3.2 Cell Culture Preparation

The cell line of breast cancer is a well-known line of cells that is used mostly for scientific research. It is the oldest and most commonly used line of human cells. The primary benefit of this cell is that it can be split into an infinite amount of occasions as long as the fundamental cell survival requirements are met. Breast cancer cell was therefore used as the main form of cell. These samples of breast cancer
cells were attained from the animal laboratory cell cultures (Kulliyyah of Allied Health Sciences, IIUM).

Figure 6. Breast cancer cells

MCF7 breast cancer cells will be investigated via in-vitro techniques. The following procedures are performed under sterile condition. Cells will grow until it covers the surface area about 80 – 90%. Old RPMI media was discarded from the culture flask, and then PBS (Gibco BRL., UK) were used to washed cells twice to remove all dead cell and unnecessary medium. Then, cell was detached by using 1-2 mL of TrypLE™ express (Gibco BRL., UK) and incubated for 2 – 15 min depending on cell characteristics. The harvested cells were then centrifuged at 1500 rpm for 8 –10 minutes inside 15 mL centrifuge tube. Resulting supernatant removed. Then, 2 mL of complete growth medium was added to the pellet. The cells were pipetted and transferred into new six well plate containing 4 mL of culture media (as shown on figure below). After 24 hours of incubation, cell confluence was observed under the inverted microscope and incubated to observe the initial cells growth rate prior to electric field exposure experiments.

Figure 7. New culture cell will be put onto six well plate and keep in Incubator

3.3 Extract preparation
Fresh stevia leaves were washed and oven dried (37ºC) for three days up to a week until it totally dried. Then, the dried leaves were ground into powder form. Extract was prepared into a stock solution of 1.0 mg/ml. Then, the dried powder was dissolved in sterile deionized water by stirring for 18 hours using magnetic stirrer in a sonicator. The stock solution was further diluted with complete growth media immediately prior to use. After removal of solvents under reduced pressure and freeze dried, extract was stored at -20ºC until use. In this experiment, MCF7 cell will be treated with the extract at different concentrations of 10.0μg/ml - 160.0μg/ml from 1.0 mg/ml of stock solution [38], and it will be added into the MCF7 cell to observe the anti-proliferation of the cell.
4. PRELIMINARY RESULTS

4.1 Morphological Changes

The pulsed electrical field causes the destruction of the cell membrane as seen in Figure 8 above. The destruction of the membrane causes the formation of pore on the membrane layer due to higher induced electrical energy than that which occurs in the membrane layer. It also causes the cell to form irregularly during an opening pore. It survived after cutting off cells. The electrical inducer affects the difference in the diameter of the MCF7 cell with and without the exposure of the electric field pulse. It has been shown that the cell expands due to cell swelling after pulsed electrical field induction and continues to grow and proliferate this experiment has shown the Reversible Electroporation (RE) effect. As discussed above, one of the direct penetrating methods of the physical type is electrical cell induction. The opening pore may increase the absorption of plant extract into the cell. This may result in either an increase in cell proliferation or anti-proliferation that will contribute to the in vitro anticancer treatment study.

![Figure 8. MCF7 cells morphological changes (a) cells with PEF (b) cells without PEF](image)

4.2 Low voltage on MCF7

This study was conducted in order to detect a cell response with a low range electric pulse field. The parameter selected for this analysis is 100 – 500 V/cm electrical field with pulse length of 30μs and single pulse. Cell proliferation was tracked and assessed for 48 hours as seen in Table 3.

| Veff (V) | V set (V) | %  |
|----------|-----------|----|
| C        | C         | 100|
| 100      | 40        | 58 |
| 200      | 80        | 83 |
| 300      | 120       | 91 |
| 400      | 160       | 57 |
| 500      | 200       | 40 |
Figure 9. Low range pulse electric field effect on MCF7 cell proliferation

The graph in Figure 9 shows the percentage of cell proliferation of MCF7 after inducing with several low electric fields in the range of 100 to 500 V/cm after 48h incubation. The result showed that the cell with the highest percentage of proliferation was 300 V/cm induced compared to the other with 91% percent cell proliferation, which shows the highest number of cells after the pulse induced by the electric field. It may be due to the pulsed electrical field inducement that has opened enough pore to induce more nutrients from the media to be ingested into the cell that stimulates the cell proliferation of MCF7. This may lead to the enhancement of the uptake of plant extracts into the cell for further therapy. The lowest level of cell proliferation was 500 V/cm with 40% cell proliferation. This may be due to the pulsed electrical field inducement sufficiently to suppress the cell and activate an anti-proliferative of the MCF7 cells. Inhibition of pulse electrical field produces an increase in the permeability of cell membranes, which, if the voltage stimulates more than the threshold value, results in the opening of pores to cell membranes that can be used to move small to large molecules into the cell.

5. Conclusion
The use of the bioactive compound such as stevia plant in cancer study are still under investigations. Where finding the less toxicity in cancer treatment is at the urge. The researcher nowadays is trying to find effective ways or methods for the cancer treatment without leaving any side effect to the cancer patients. This idea which is the combination of extract effects on cancer cell plus with pulse electric field inducement might be an effective method for cancer treatment. Some researcher found that plant extract is a potential anti-cancer agent and had been tested on several cancer cells such as, breast cancer, gastrointestinal cancer and pancreatic cancer. This is due to the plant extract consist of carotenoids and polyphenols and positively responds towards normal cell growth, proliferation and differentiation and also degenerate cancer-related epigenetic disfunctions, preventing metastasis, reducing tumorigenesis and/or increasing chemo and radiotherapy efficiency. While the pulse electric field is an established technique used in the medical field that used for the membrane penetrating delivery methods. Thus, the electrical inducement that causes permeabilization of cell membrane may enhance the uptake of plant extract onto the cancer cell. Which may result in enhancement of the anti-proliferative activity of the cancer cell. The combination of these two techniques needs to be further studied as in vitro or in vivo to proof up to clinical studies. This combination is a promising technique in the biomedical application and may have a great impact in future anti-cancer treatment without having leave any side effect on the cancer patients.
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