

In Vitro Cytotoxic Effect of Dichloromethane Extract of *Prismatomeris glabra* in Human Breast Cancer Cells

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Abstract

Breast cancer is the most prevalent cancer cases worldwide contributing 15% of fatality of all cancer deaths altogether. The discovery of natural product able to act as an anti-cancer agent has led to the opportunity to kill cancer cells with fewer side effects compared to chemotherapy. *Prismatomeris glabra* (P.glabra) is a medicinal plant found in Southeast Asia, used to treat various human disease and might contain anti-cancer properties. The purpose of this study was to investigate the cytotoxic effect of P.glabra on the MCF-7 human breast cancer cell line. Dichloromethane extraction method was used to extract the crude of P.glabra roots and leaves. Cytotoxicity of P.glabra on MCF-7 cells was evaluated by MTT assay treated with various concentrations of extract to determine the IC50 with the concentration of 500, 250, 125, 62.5, 31.2, and 15.6 µg/mL for a treatment time of 24, 48 and 72 hours in 37°C CO2 incubator. The most significant cytotoxic effect of P.glabra in MCF-7 cells was a half-maximal inhibitory concentration (IC50) of 64.5±2.1µg/mL treated with the leaves of P.glabra extract at 72 hours treatment time. P.glabra showed in vitro cytotoxic effects in MCF-7 cells with dose- and time-dependent manner as demonstrated by MTT assay. The leaves extract of P.glabra can induce apoptosis and act as an alternative to anti-cancer treatment. Further analysis on gene expression is recommended to elucidate the functionality of cytotoxicity effect.

Keywords: MCF-7, breast cancer, cytotoxicity, MTT assay, *Prismatomeris glabra*, Ajisamat

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Introduction

Breast cancer has been reported to be the most prevalent cancer cases in the world and this includes in the Malaysian population¹. Deaths reported due to breast cancer had exceeded 15% of all cancer cases together in 2018². Current chemotherapy treatment generates many unwanted side effects and the risk of developing multidrug-resistant has posed a challenge in lowering the mortality rate³⁻⁶. The discovery of bioactive compound from the medicinal plant that possesses ability as anti-cancer prompting research in finding the alternative cancer treatment. Phytochemicals extracted from the plants can positively impact the health by stabilizing metabolic pathway into a normal physiological level and some even can be a potential anti-cancer treatment as the molecules can elicit apoptosis, inhibit cell proliferation, affect both angiogenesis and cancer metabolism^{4,6}.

Prismatomeris glabra (*P.glabra*), a medicinal plant also known as Ajisamat, widely used by the locals for wellness remedies such as increase stamina, combating tropical diseases and boosting freshness⁷. It is a type of Rubiaceae family plant, distributed throughout tropical and subtropical area in Southeast Asia⁷. Rubiaceae plant has been noted for its richness in anthraquinone compound^{8,9}. Phytochemical anthraquinone was reported able to induce apoptosis in cancerous cells^{10,11}. This preliminary study was done to investigate the cytotoxic effect of dichloromethane extract of *P.glabra* in inducing apoptosis in human breast cancer cells, MCF-7, by using MTT assay and eventually act as an alternative treatment replacing the conventional chemotherapy treatment for cancer. MTT assay method is a quantitative colourimetric assay that able to determine the cell viability after it had undergone certain treatment^{12,13}.

Materials and methods

P.glabra dichloromethane extraction

The plant leaves and roots were obtained from the Faculty's herbarium with a voucher code of PG0001. The dried plant materials were grounded to crude powder. Seven grams of leaves and 20g of roots were macerated overnight in 70mL and 200mL of dichloromethane respectively. The mixture was then filtered by using a Whatman No.1 filter paper. The filtrate was let evaporated under reduced pressure at 19°C by using a rotary evaporator. The products were left evaporated to dryness at room temperature to obtain crude extract which was then collected and stored at -20°C for further use and test. The yield of the crude extract was determined by subtracting the weight of crude extract in a container with the weight of the empty container.

Cell growth and maintenance

Human breast cancer cell line, MCF-7 was originally obtained from Kampus Perubatan, Universiti Sultan Zainal Abidin, Terengganu, Malaysia which was purchased at American Type Culture Collection (ATCC). The cell line was derived from breast cancer of a 69 years

old Caucasian female¹⁴. MCF-7 cells were grown and maintained in a T₂₅ culture flask filled with RPMI-1640 and supplemented with 10% foetal bovine serum (FBS). The cells were cultured and incubated in a 37°C with 95% humidity supplemented with 5% of a CO₂ incubator. A 70% confluence cells were taken into subculture into another flask containing the new culture media of the same constituent. The media was changed every 2-3 days to optimise cell growth.

Cell viability test

P.glabra roots and leaves extract were tested for *in vitro* cytotoxicity on MCF-7 cells by utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay¹³. Cells were seeded in a 96-well microtitre plate at a density of 1x10⁴ cells/well from row A to row G (triplicate) with each well constitute of RPMI 1640 and 10% FBS (100µL). Row H was left as blank with no added cell. The cells in the microtitre plate were then left incubated for overnight. Next, 100µL of diluted roots and leaves extract were added in row A and row B. A serial dilution was performed from row B until row F by using a micropipette. The excessive 100µL from row F were discarded. The final volume in each well was made until 200µL. The steps were done as illustrated in Figure 1. The procedure was repeated for 3 plates following three treatment times (24, 48 and 72-hour). The plates were incubated at 37°C in a humidified 5% CO₂ with 95% humidity as according to the allocated treatment time. After the incubation time completed, MTT (20µL of 5mg/mL) was added into each well and incubated for another 4 hours to allow the formation of visible purple coloured formazan precipitate. Once incubation completed, the solution in the wells was aspirated out and 100µL of DMSO was added into each well and was left incubated for another 10 minutes at 37°C incubator. The absorbance was then measured with the wavelength of 570nm using a microplate reader to obtain the cell viability percentage. The following formula was used to calculate the cell viability percentage:

$$\text{Percentage of cell viability(\%)} = \frac{OD_{\text{treated}}}{OD_{\text{control(untreated)}}} \times 100\%$$

The data from these results were used to find the medium inhibitory concentration (IC₅₀) through plotting in the Graphpad Prism 8 software.

Statistical Analysis

All statistical analysis was done by using GraphPad Prism 8. The results were expressed as mean values with ± standard deviation of the mean. All data were conducted in triplicates and analysed using the two-way ANOVA followed by Bonferroni multiple comparison tests, where differences $p \leq 0.05$ were considered significant. The two independent factors were concentration and treatment time in categorical with the outcome of cell viability in numerical justifying the use of two-way ANOVA.

Results

The crude extract of dichloromethane of P.glabra extract yield

The dichloromethane extract of *P.glabra* exhibited a powdery and sticky appearance for roots and leaves part respectively. The roots of *P.glabra* extract recorded yield of 10.4% while the leaves of *P.glabra* extract recorded yield of 14.7%. This resultant yield indicated the number of phytochemicals or the active compound of the extract.

Cell viability percentage and IC₅₀ determination

P.glabra in vitro cytotoxic effect on MCF-7 viability was evaluated using tetrazolium assay (MTT). Various concentration of dichloromethane extract of *P.glabra* roots and leaves were used. Figure 2 was the results of MCF-7 cell viability after being treated with *P.glabra* extract roots and leaves respectively at three (3) treatment times. A similar trend can be observed from the figures in which a decreasing in cell viability as the time of treatment and concentration increases. From these results, the IC₅₀ was determined for each hour for both roots and leaves extract. Table 1 shows the IC₅₀ for each extract at different treatment time. The cytotoxic level of the crude extract was determined as according to the U.S National Cancer Institute with IC₅₀ of crude extract <20mg/mL is highly cytotoxic, 20-100mg/mL is mildly cytotoxic and >100mg/mL is poor cytotoxic¹⁵. It was found that the lowest concentration to be effective against MCF-7 for roots extract of *P.glabra* was 125µg/ml at 48 and 72 hours treatment time with significant value of $p=0.0155$ and $p<0.0001$ respectively. Meanwhile, the lowest concentration of *P.glabra* leaves extract effective against MCF-7 was 62.5µg/ml at 48 and 72 hours treatment time with significant value of $p=0.0232$ and $p<0.0001$ respectively.

Discussion

The urgency of finding an alternative treatment for breast cancer is due to numerous undesirable side effects suffered by the patient from the use of current chemotherapy treatment that impacted their quality of life¹⁶. Additionally, the emergence of multidrug-resistant among breast cancer patient from the utilization of chemotherapy drug has posed a challenge to the society in combating the high mortality rate due to breast cancer^{3,4}. Research on natural product as an anti-cancer agent especially from the local or medicinal plant has been proven to act as a promising and novel anti-cancer treatment¹⁷. There are abundant of natural compound in plants that can cause apoptosis such as alkaloids, phenylpropanoids, terpenoids and anthraquinone^{17,18}. To mention a few, Tualang honey that contain flavonoid has been reported to induce apoptosis in cancerous cells while generating less unfavourable side effects as compared to Tamoxifen¹⁹.

In this preliminary study, MCF-7 cell line was selected to investigate the cytotoxic effect of *P.glabra* roots and leaves dichloromethane extract by measuring the percentage of cell viability through the utilization of MTT assay. *P.glabra* was first extracted using dichloromethane

through maceration technique and the resultant yield indicated a considerably amount of phytochemicals, its active compound. The amount of extracted yield is directly proportional to the amount of phytochemicals extracted²⁰. These crude extracts of roots and leaves of *P.glabra* were then made into various concentration. In this study, *P.glabra* extract demonstrated a mild cytotoxic towards human breast cancer cell, MCF-7 as evident by its IC₅₀ value. Moreover, the cell viability of MCF-7 was also affected by different treatment time. This indicated that the treatment from both of the extracts was dose and time dependent. The effect was analysed with two-way ANOVA followed by Bonferroni multiple comparison tests and was found that the p-value is highly significant ($p<0.0001$). Furthermore, *P.glabra* leaves extract exhibited a more potent cytotoxic effect compared to *P.glabra* roots suggesting that different parts of the plant play a significant role in cytotoxic level, could be due to its nutritional content or macromolecules composition.

The finding in this study is in contrast with the previous study that found *P.glabra* had a poor cytotoxic effect with an IC₅₀ value of >1000mg/mL²¹. The plausible explanation for the difference in the effect might be due to the different in solvents used during extraction in which previous study used an aqueous extraction method while in this study used dichloromethane extraction method. The cytotoxic effect by *P.glabra* can be explained by the presence of anthraquinone as discovered by Azlan et. al.²². Anthraquinone compound was proven to induce apoptosis^{10,23}. This compound is best to be extracted by a polar aprotic solvent²⁴, for instance, dichloromethane that was used in this study²⁵. Other explanation that contribute to the different of effect compared to the previous study might be due to the type of active compound extracted, in this case, the anthraquinone. A study by Osman et. al. reported 1,2-dimethoxy-6-methyl- 9,10-anthraquinone has better cytotoxic effect on cancer cell compared to 1-hydroxy-2-methoxy-6-methyl-9,10-anthraquinone that has no significant cytotoxic activity in which they only differ in their C1 structure²⁶. Therefore, it is possible that numerous type of anthraquinone existed in *P.glabra* was able to be extracted through dichloromethane method and yielded a different outcome. In conclusion, dichloromethane extract of *P.glabra* roots and leaves did show cytotoxic effect against MCF-7 cells as observed in this current study. A further investigation to assess the mode of cell death accomplished by *P.glabra* extract is much needed to develop its full potential as a promising alternative anti-cancer treatment.

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Table & Figures

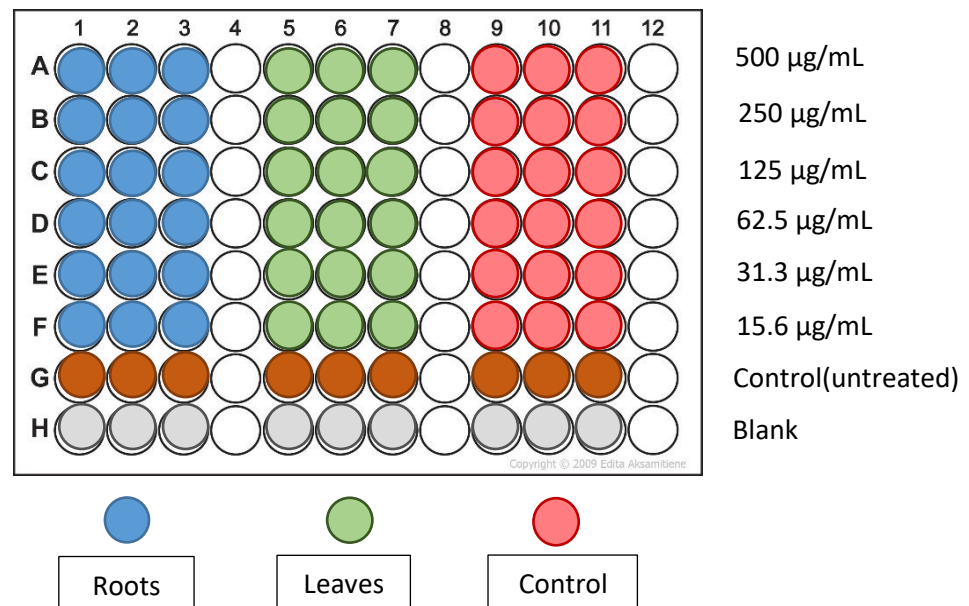


Figure 1: The arrangement of *P. glabra* extract treatment on MCF-7 breast cancer cell line on 96-well culture plate.

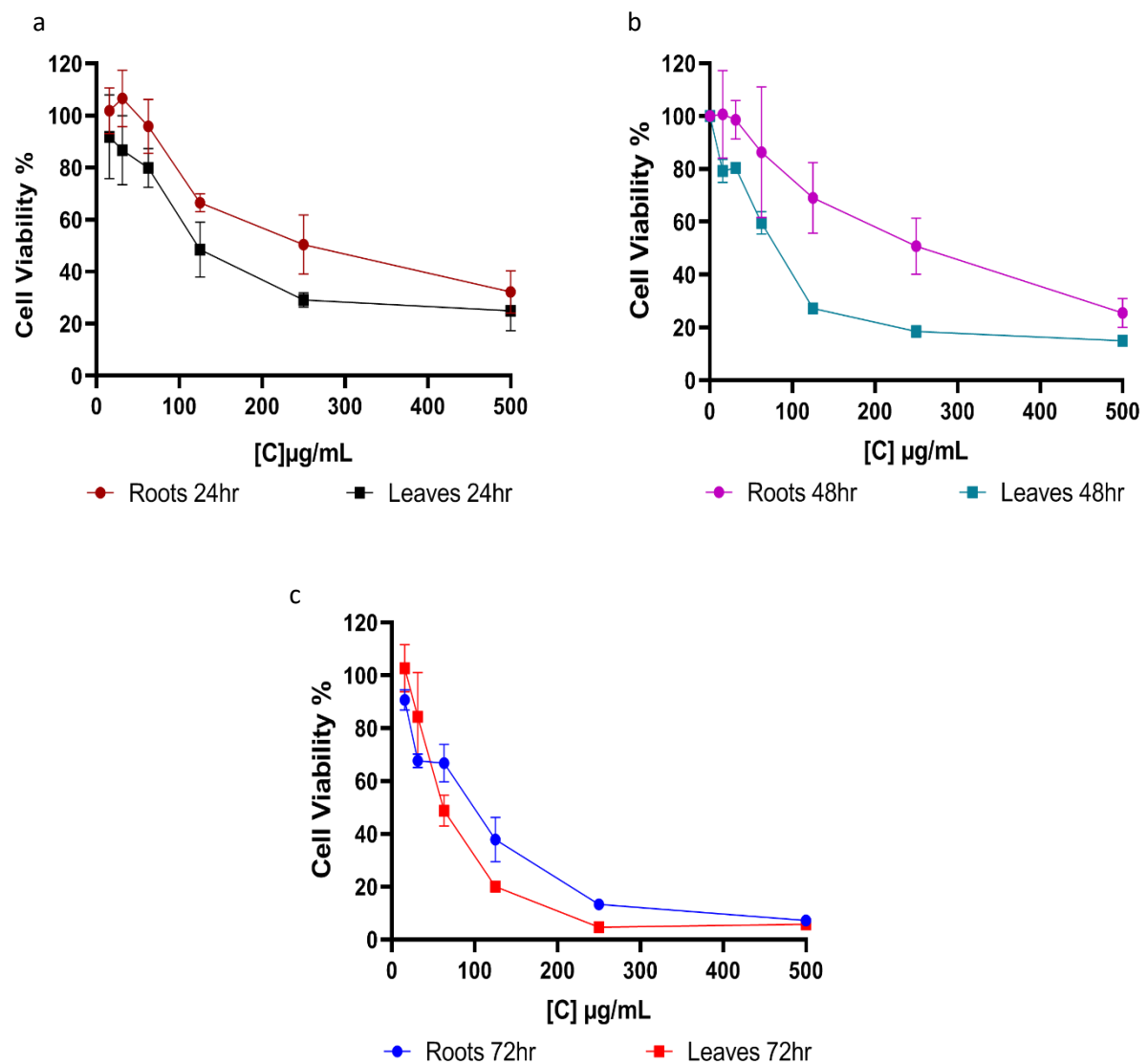


Figure 2: MCF-7 cells viability percentage (%) after (a)24, (b)48 and (c)72 hours of exposure towards different concentration of *P. glabra* roots and leaves of dichloromethane extract (µg/mL).

Table 1: The IC₅₀ value measured for each treatment time of *P.glabra* roots and leaves

Extract	Treatment time (hours)	IC ₅₀ (mg/mL)
Roots	24	200.2±18.2
	48	236.6±65.3
	72	93.5±35.8
Leaves	24	127.8±8.4
	48	75.6±3.7
	72	64.5±2.1