

POTENTIAL USE OF CERI TERENGGANU FOR FUNCTIONAL FOOD: A PRELIMINARY STUDY ON ITS CYTOTOXICITY ASPECT

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ABSTRACT

Ceri Terengganu (Lepisanthes fruticosa) is a kind of indigenous fruit in Malaysia. It is a non-season fruit species that produces fruits throughout the year. Recently, the use of this fruit is gaining attention due to its high antioxidant value. These fruits have the potential to be used as functional food for maintaining good health. Thus, the safety status of Ceri Terengganu needs to be assessed scientifically for human consumption. This preliminary study was conducted to evaluate the cytotoxicity effects of different forms of Ceri Terengganu samples on the different cell lines (normal and cancerous cells) by using MTT assay. As in vitro screening assays and toxicity studies become increasingly important in chemical risk assessment. Results found that all samples (aqueous extract and oven-dried) did not inhibit the normal cell growth of selected cells (3T3, Vero and MRC5) as the IC50 were more than 500 µg/ml. Interestingly, an oven-dried of ripe and unripe Ceri Terengganu strongly managed to suppress the cancer cells (MCF-7 and CRL1739 cells) with an IC50 at/below 50 µg/ml. This indicates that Ceri Terengganu is safe on normal cells and has potential to inhibit the proliferation of certain cancer cells. These results are important for the promotion use of Ceri Terengganu in the development of functional food in future.

Key words: *Lepisanthes fruticosa*, Ceri Terengganu, Functional Food, Cytotoxicity, MTT Assay

INTRODUCTION

Malaysia is one of the countries which has a rich diversity of underutilized fruits that grow wild in the region of Peninsular Malaysia, Sabah and Sarawak. Some of these underutilized fruits are rarely eaten, unknown and unfamiliar. Based on the broad spectrum of their flesh and skin color, these underutilized fruits may have potential benefits to human health. In addition, some of these fruits have the potential to be used and processed as food products for local consumption. Based on ethnobotanical studies, many of the underutilised fruits and traditional vegetable species are the source of food and medicine for some communities in Malaysia. These species have great potential however, they are under exploited and not fully utilised. Research and development (R&D) in Malaysian Agricultural Research and Development Institute (MARDI), especially on the bioprospection of underutilised fruits, indicated several potential species, such as Ceri Terengganu, dabai, kebayau Sentul and tengkawang (Shukri et al., 2011).

One of the underutilized fruits which gaining attraction among the researchers in Malaysia is Ceri Terengganu or its scientific name *Lepisanthes fruticosa* (Roxb) Leenh. It is a non-seasonal underutilised fruit species that can be found in South East Asia which comprise Malaysia, Myanmar, Indo-China, Thailand, Philippines and Indonesia (Lim, 2013; Mirfat & Salma, 2015). The species is found growing naturally in the forests and mostly found growing as a home garden plant. A rare attractive tree with young purple leaves turning to green and purple-red flower clusters. In Malaysia, this species is widely distributed in Johor and the East Coast of Peninsular Malaysia (Mirfat & Salma, 2015). Based on the ethnobotanical studies, Ceri Terengganu is usually consumed as food source and also used in traditional medicine by rural folks. The fruit is a globose berry, about 2 to 3cm in diameter. It grows in large clusters of 20 fruits that are a shiny, deep red with a pointed tip. Sweet but a little tart with 3 seeds per fruit. Flesh is yellow, sweet and crunchy and the skin can be astringent if not fully mature. Meanwhile, the seed is eaten

roasted and the root is used in a compound poultice to relieve itching and to lower temperature during fever (Mirfat & Salma, 2015). It was reported that Ceri Terengganu root has antipyretic properties and the ripe fruit has anti-diarrhoea properties (Wetwitayaklung et al., 2012).

Recently, interest in Ceri Terengganu fruits has arisen due to its strong antioxidant capacity as compared to a number of underutilized fruits and commercial fruits (Mirfat & Salma, 2015). The ripe fruits showed the highest free radical scavenging activity and had a great source of total phenolic contents among many other fruits tested. It was also reported that the maturity index of Ceri Terengganu fruits had influenced the antioxidant activity. The lower maturity (unripe) stages exhibited the strongest potential (Mirfat et al., 2017). Figure 1 shows the different maturity index of Ceri Terengganu which have been studied and developed by MARDI.



Figure 1: Different of maturity index of Ceri Terengganu fruits (from green to red colour)

The proximate analysis also been studied by Umi Kalsum & Mirfat (2014) on Ceri Terengganu and other underutilized fruits. It was found that Ceri Terengganu had the highest ash content which indicated the highest mineral content among others. It was also had low calorie and high moisture content that suitable to produce it into drink or juice for health.

The other important aspect of Ceri Terengganu is the safety status of its fruit before can be eaten for human consumption. To date, no toxicological evaluation of this fruit is carried out elsewhere. A risk assessment to identify the adverse health effects is a pre-requisite before taking forward the development of new drugs, cosmetics and foods from certain plants. Thus, the study was conducted for the first time, to evaluate the safety profile of Ceri Terengganu. The toxicity evaluation study was carried out on various forms of Ceri Terengganu such as in dried, extract and fermented. The ripe as well unripe fruits as shown in Figure 1 were used in this study.

Cytotoxicity tests (*in vitro*) were performed in normal as well as cancerous cells. Cancer is a complex multifactorial cell disease characterized by abnormal cellular proliferation. Cancer development is normally caused by oncogene, tumor suppressor gene, and microRNA gene alterations (Burstein, 2008). In Malaysia, cancer is the fourth leading cause of death; it is the second leading cause of death in developed countries after cardiovascular diseases. The use of MCF-7 cells (human breast adenocarcinoma cell line) for this screening test due to the breast cancer is one of the active cancers reported in Malaysia. The Ministry of Health of Malaysia has reported that deaths from breast cancer rank among the top 10 cancer-related deaths in the country including stomach cancer (CRL1739) as reported by Zainal et al (2014) and Azizah et al (2016).

Thus, the objective of this study was to determine the cytotoxicity activity of Ceri Terengganu against the breast cancer cells (MCF-7), gastric/stomach cancer (CRL 1739), normal fibroblast cell (3T3 cells), normal kidney cells (Vero cell) and normal lung cells (MRC5). The results will be beneficial to the development of functional product from Ceri Terengganu.

MATERIALS AND METHODS

Preparation of Ceri Terengganu samples and cells line

There were 5 forms of samples prepared from Ceri Terengganu (CT), which were Sample A: CTi8 (ripe/aqueous extract); Sample B: CTi3 (unripe/aqueous extract); Sample C: CT ripe/dried; Sample D: CT unripe/dried and Sample E: CT ripe/fermented.

Aqueous Extract of Ceri Terengganu

The ripe (index 8) and unripe (index 3) of Ceri Terengganu fruits were sampled from MARDI fruit genebank in Serdang, Selangor. To prepare the aqueous extract (Sample A and B), fruits were washed with running tap water and let it air dried. The edible portion of fruits were separated from the seeds, cut into smaller pieces and dried in an oven at 40°C for 48 hrs. The fruits were dried until the moisture content achieved less than 10%. After that, the dried fruit were ground and were sieved with 1 mm mesh. The fruit powder was extracted with deionised water (10 volume) by shaking on orbital shaker at room temperature for 12 hours. Afterwards, the slurry was filtered through thin cloth and the separated solid were extracted two more times. The aqueous solutions were combined and concentrated at 40 °C using a rotary evaporator to give concentrated crude extracts. Maltodextrin were added to concentrate crude extract at final content which was not more than 40% in solid crude extract. This mixture was lyophilized to powder and the extract powders were sealed in plastic bag before stored in a freezer (-20 °C).

Dried and fermented of Ceri Terengganu

The other three types of Ceri Terengganu (CT) samples prepared for the analysis were Ripe CT (index 7-8) powder (Sample C), Unripe CT (index 4-5) powder (Sample D) and Ripe Fermented CT (index 7-8) powder (Sample E). Fruits (ripe and unripe) that have been collected were washed, cut and deseeded. After that, the fruits were ground and dried in the oven at 55 °C for 48 h. The fruits were dried until the moisture content achieved less than 10%. The dried samples were once again being ground into powder form. The samples were kept at 4 °C until further analysis. For preparation of Sample E, 15% of Sample C was fermented with *Saccharomyces cerevisiae* 7013 IRNA Naborn, DSM from German Collection of Microorganism and Cell Cultures. The strain was initially cultured in potato dextrose broth (PDB) for 24 hr at 30 °C before inoculation into sample medium. Sample medium was prepared by mixing 15% ripe CT powder (Sample C) with sugar (15%) and water. After that, the medium was pasteurized and cooled to room temperature. The medium was then inoculated with yeast strain (3%) and incubated aerobically at 30 °C for 7 days with static condition. After fermentation, the medium was centrifuged and the pellet was dried in the oven at 55 °C for 48 hr. The dried pallet was ground into powder form and kept at 4 °C until further analysis.

Cell lines for cytotoxicity studies

The type of cells that used in this study were MCF-7 cells (cancer: human breast adenocarcinoma), 3T3 cells (normal: Mouse Embryonic fibroblast), CRL1739 (cancer: human gastric adenocarcinoma), Vero cell (normal: green monkey kidney epithelial cells) and MRC5 (normal: human lung fibroblast). All cells were purchased from Universiti Putra Malaysia (UPM). Cytotoxicity is the killing ability of synthesized chemicals, naturally occurring toxins or immune-mediator cells. One of the parameter to determine cytotoxicity is by using MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). Cell culture with the concentration of 2×10^3 cells/ml was prepared and was plated (100 µl/well) onto 96-wellplates. The diluted ranges of sample extracts were added to each well with identified concentrations; 500, 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 µg/ml further incubated for 24 hr, 48 hr and 72 hr. MTT solution was added by the end of incubation samples (triplicate each samples) to the cells and continued for incubation in incubator for 3 hours. After solubilization of the purple formazan crystals using DMSO were completed, the Optical Density (OD) of the plants extract was measured using an ELISA reader at a wavelength of 570 nm. The cytotoxicity was recorded as the drug concentration causing 50% growth inhibition of the tumour cells (IC₅₀ value) using the formula given below:

$$\text{Cell viability} = \frac{\text{Absorbance sample (mean)}}{\text{Absorbance control (mean)}} \times 100\%$$

After the determination of the percentage of cell viability, graphs were plotted with the percentage of cell viability against their respective concentrations.

RESULTS

Table 1 summarizes the results of different samples of Ceri Terengganu on different cell lines used in this study. As in cancer cells, Figure 2 demonstrates the value of IC₅₀ (50% inhibition) of Sample D: CT unripe/dried on MCF-7 cells after 72 hr exposure occurs at 13 µg/ml. Figure 3 demonstrates the value of IC₅₀ (50% inhibition) of Sample C: CT ripe/dried on MCF-7 cells after 72 hr exposure occurs at 30 µg/ml. Figure 4 demonstrates the value of IC₅₀ (50% inhibition) of Sample A: CTi8 on MCF-7 cells after 72 hr exposure occurs at 40 µg/ml. Figure 5 demonstrates the value of IC₅₀ (50% inhibition) of Sample C: CT ripe/dried on CRL1739 cells after 72 hr exposure occurs at 30 µg/ml. Lastly, Figure 6 demonstrates the value of IC₅₀ (50% inhibition) of Sample D: CT unripe/dried on CRL1739 cells after 72 hr exposure occurs at 50 µg/ml.

Table 1: IC50 of different Ceri Terengganu samples on different cell lines

No	Sample Name	Type of cells	Results (IC ₅₀)
1.	Sample A: CTi8 (ripe/extract) Sample B: CTi3 (unripe/extract) Sample C: CT (ripe/dried) Sample D: CT (unripe/dried) Sample E: CT (ripe/fermented)	a) MCF-7 cells (human breast adenocarcinoma cell line) (Cancer cell)	Sample A: 40 µg/ml Sample B: 275 µg/ml Sample C: 30 µg/ml Sample D: 13 µg/ml Sample E: 35 µg/ml
2.	Sample A: CTi8 (ripe/extract) Sample B: CTi3 (unripe/extract) Sample C: CT (ripe/dried) Sample D: CT (unripe/dried) Sample E: CT (ripe/fermented)	b) CRL1739 (human gastric adenocarcinoma cell line) (Cancer cell)	Sample A: 65 µg/ml Sample B: 60 µg/ml Sample C: 30 µg/ml Sample D: 50 µg/ml Sample E: 495 µg/ml
3.	Sample A: CTi8 (ripe/extract) Sample B: CTi3 (unripe/extract) Sample C: CT (ripe/dried) Sample D: CT (unripe/dried) Sample E: CT (ripe/fermented)	c) 3T3 cells (Mouse embryonic fibroblast cell line) (Normal cell)	Sample A: No IC ₅₀ up to 500 µg/ml Sample B: No IC ₅₀ up to 500 µg/ml Sample C: No IC ₅₀ up to 500 µg/ml Sample D: No IC ₅₀ up to 500 µg/ml Sample E: No IC ₅₀ up to 500 µg/ml
4.	Sample A: CTi8 (ripe/extract) Sample B: CTi3 (unripe/extract) Sample C: CT (ripe/dried) Sample D: CT (unripe/dried) Sample E: CT (ripe/fermented)	d) Vero cell (green monkey kidney epithelial cell) (Normal cell)	Sample A: No IC ₅₀ up to 500 µg/ml Sample B: No IC ₅₀ up to 500 µg/ml Sample C: No IC ₅₀ up to 500 µg/ml Sample D: No IC ₅₀ up to 500 µg/ml Sample E: No IC ₅₀ up to 500 µg/ml
5.	Sample A: CTi8 (ripe/extract) Sample B: CTi3 (unripe/extract) Sample C: CT (ripe/dried) Sample D: CT (unripe/dried) Sample E: CT (ripe/fermented)	e) MRC5 (human lung fibroblast) (Normal cell)	Sample A: No IC ₅₀ up to 500µg/ml Sample B: No IC ₅₀ up to 500µg/ml Sample C: No IC ₅₀ up to 500µg/ml Sample D: No IC ₅₀ up to 500µg/ml Sample E: No IC ₅₀ up to 500µg/ml

Figure 2: Effect of Sample D: CT Unripe/Dried on MCF-7 cells after 72 hr exposure (IC₅₀ at 13µg/ml).

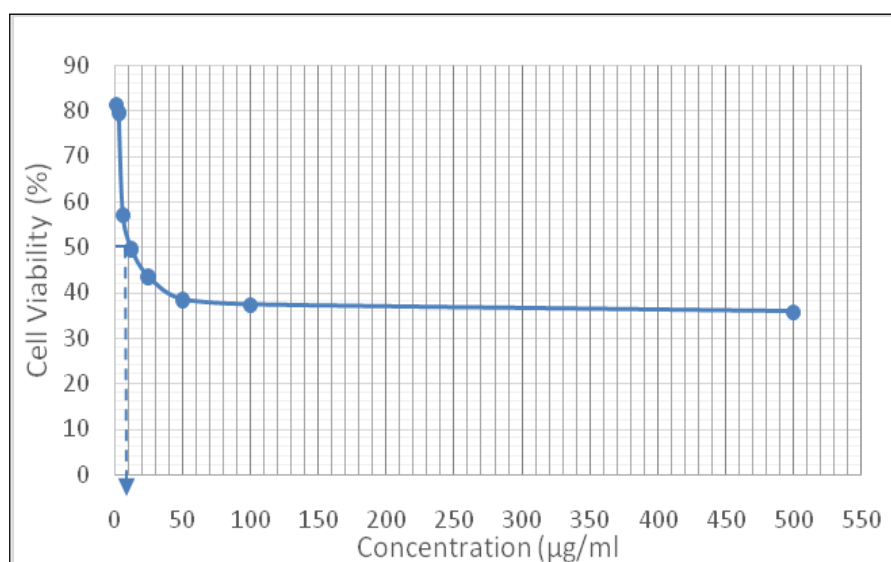


Figure 3: Effect of Sample C: CT Ripe/Dried on MCF-7 cells after 72 hr exposure (IC50 at 30µg/ml).

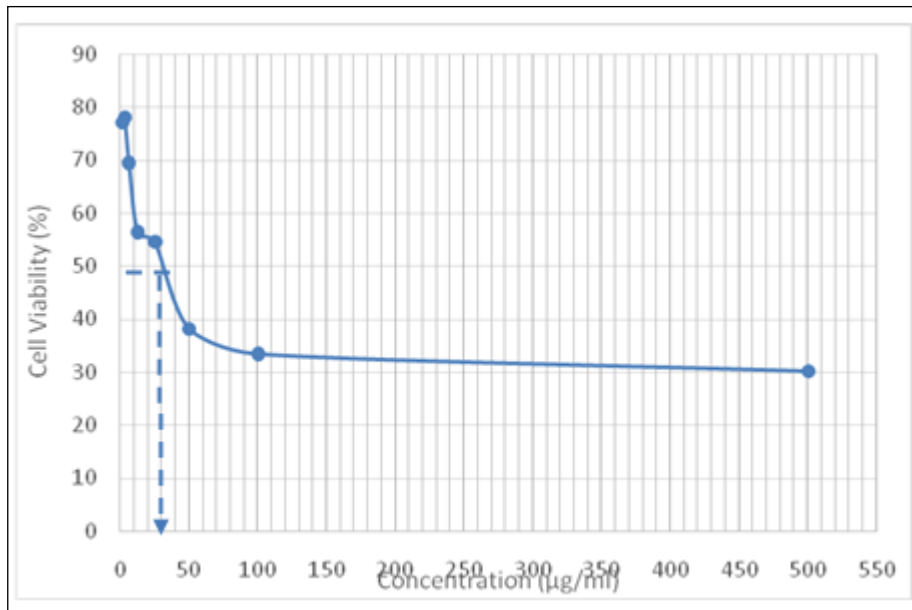


Figure 4: Effect of Sample A: CTi8 (Ripe/Extract) on MCF-7 cells after 72 hr exposure (IC50 at 40µg/ml).

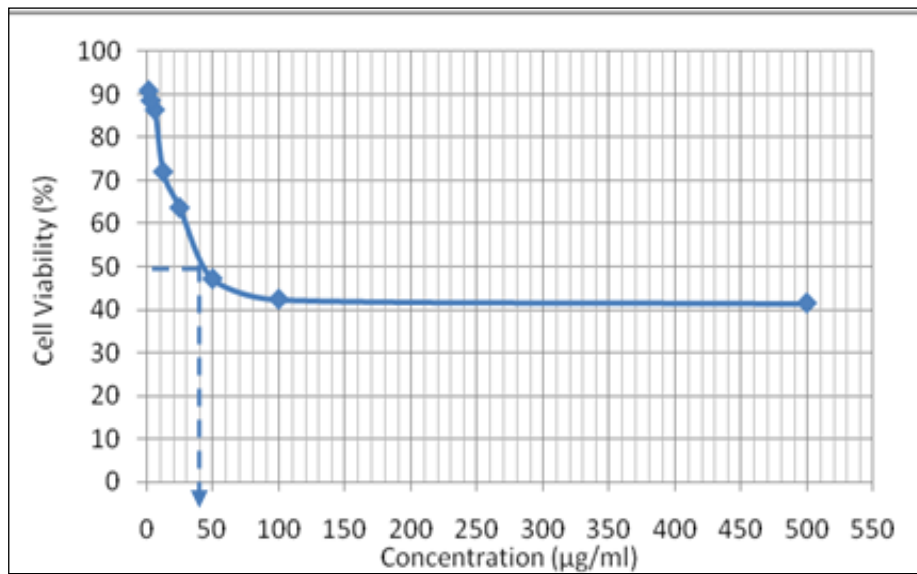
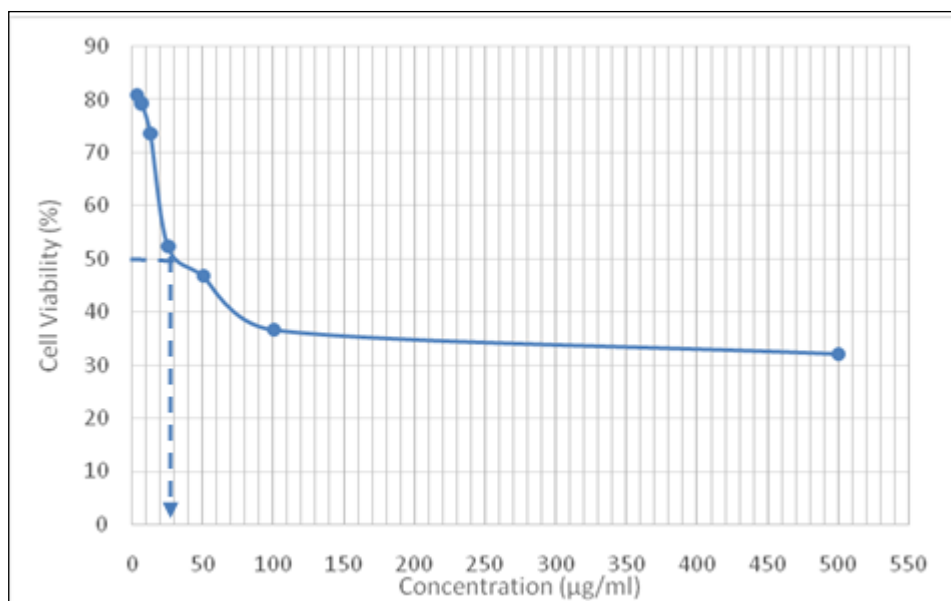
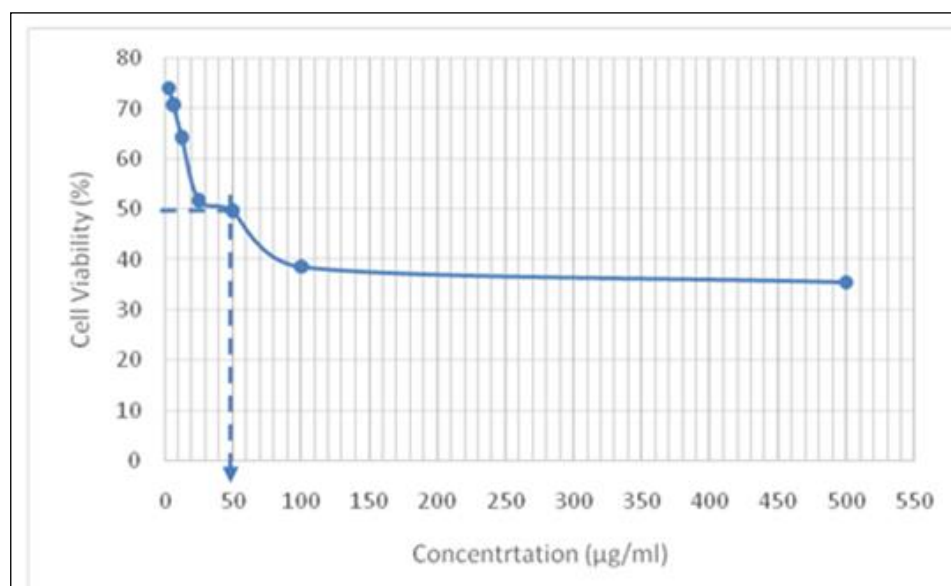


Figure 5: Effect of Sample C: CT Ripe/Dried on CRL1739 cells after 72 hr exposure (IC₅₀ at 30µg/ml).Figure 6: Effect of Sample D: CT Unripe/Dried on CRL1739 cells after 72 hr exposure (IC₅₀ at 50µg/ml).

DISCUSSION

Currently, research and development activities on underutilised fruits varieties have become priority areas in Malaysia. Many of these fruits were eaten locally, having a broad range of flavours and colours with potential health benefits (Ikram et al. 2009). However, the scientific information and knowledge about them is limited including the safety effects (Gruere et al. 2009). Some of the underutilised fruits which have potential to be exploited for commercial production are the Ceri Terengganu (*Lepisanthes fruticosa*). Hence, this study was carried out to investigate the cytotoxicity activities of Ceri Terengganu on normal as well as cancer cells to determine their potential effects. In this study, a wide range of maturity index of Ceri Terengganu which was from unripe to ripe fruits has been used.

The cytotoxicity or also known as antiproliferative activities of the Ceri Terengganu samples were preliminarily screened by MTT assay. The percentage viability curves of treated cells were plotted against the sample concentrations, and the IC₅₀ as compared to that of untreated cells was determined. In the present study, results presented in Table 1 suggest that there was no cytotoxic effect of all samples (five treated Ceri Terengganu samples) have been observed when treated on normal cells (3T3 cells, Vero cell and MRC5 cells) up to the identified concentrations (maximum at 500 µg/mL). Hence, there is no IC₅₀ were determined on these

samples. It was suggested that Ceri Terengganu is safe on normal cells and not show any sign of toxicity towards the growth of normal cells.

Interestingly, Ceri Terengganu managed to suppress the human cancer cells (MCF-7 and CRL1739 cells) with an IC_{50} at/below than 50 $\mu\text{g/ml}$ as shown in Figure 2, 3,4,5 and 6. This indicates that Ceri Terengganu is safe on normal cells and has potential to inhibit the proliferation of certain cancer cells. According to the report by Atjanasuppat et al. (2009), the antiproliferative activities of the samples were categorized according to the median inhibitory concentration (IC_{50}) into four groups: ≤ 20 $\mu\text{g/mL}$, active; >20 – 100 $\mu\text{g/mL}$, moderately active; >100 – 1000 $\mu\text{g/mL}$, weakly active; and >1000 $\mu\text{g/mL}$, inactive. In addition, the United States National Cancer Institute plant screening program, stated that a crude extract is generally considered to have *in vitro* cytotoxic activity if the IC_{50} is < 100 $\mu\text{g/mL}$ against human cancer cell lines (Oskoueian et al 2011).

From our study, Sample D (unripe/dried) had the most potent anti-cancer activity against breast cancer - MCF-7 cell (IC_{50} at 13 $\mu\text{g/ml}$) followed by Sample C (30 $\mu\text{g/ml}$), Sample E (35 $\mu\text{g/ml}$), Sample A (40 $\mu\text{g/ml}$) and the weakest effect was from Sample B (275 $\mu\text{g/ml}$). On the other hand, the different effects were observed on stomach cancer (CRL 1739 cell). The most active was Sample C (IC_{50} at 30 $\mu\text{g/ml}$), followed by Sample D (50 $\mu\text{g/ml}$), Sample B (60 $\mu\text{g/ml}$) and Sample A (65 $\mu\text{g/ml}$). Meanwhile, Sample E showed only weakly effect against CRL 1739 cell (IC_{50} at 495 $\mu\text{g/ml}$). As noted in this study, the different compounds from different Ceri Terengganu samples might be play an important role towards the cytotoxicity activities.

Al-Rashidi et al. (2011) noted that low toxicity towards normal cells and high toxicity towards cancer cells indicates that a plant extract has good anti-cancer constituents, and shows that the plant extract has a cytotoxic effect on cancer cells without causing toxicity in normal cells. Plants have a long history in both traditional and modern cancer treatments and have been used to treat human diseases for centuries (Nisa et al 2011; Caamal-Fuentes et al 2011). A total of 32 Malaysian plant species also have been reviewed for their cytotoxicity effects against breast cancer cell lines (Nurhanan et al 2008). Previous study indicated that another rare fruit in Malaysia, which is Bambangan (*Mangifera pajang*) also been studied for antiproliferative activity against breast cancer and the compound, methyl gallate is responsible for this positive property (Che Rahim et al. 2019). Thus, it is possible that underutilized plants such as Ceri Terengganu can serve as potential sources for developing new drugs and more effective anti-cancer agents for future therapy.

In future, the active compounds from several Ceri Terengganu extracts need to be determined and quantitated. This is important to correlate and verify the mechanism of action of each compound in selected cancer cell lines (MCF-7 and CRL 1739 cells). In addition, the efficacy of Ceri Terengganu extract through *in vivo* study is also recommended to validate its use and potential as functional food. These studies need to be conducted before the functional product from rare fruits can be registered and accepted by Ministry of Health for human consumption.

CONCLUSION

This present study indicates that Ceri Terengganu samples exhibited cytotoxic effect in cancer cells, but not in normal cells, which is good to explore its anticancer potential for health benefits. From this study, we can conclude that Ceri Terengganu is safe for consumption. These results are important for the promotion of use of Ceri Terengganu in the development of functional food. Planned future investigations will involve the purification, identification, determination of the mechanisms of action, and molecular assay of different extract of Ceri Terengganu. Moreover, extension study of Ceri Terengganu can be explored against other human cancer cells too. Further verification study also need to be carried out on unripe Ceri Terengganu as it shows the most active antiproliferative against breast cancer cells which is the top cancer incident in Malaysia. Potential bioactive compounds that contribute to this effect are worth to be further identified.

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