Expression of Matrix Metalloproteinase-13 in Human Skin Melanoma Cancer Treated by *Baccaurea angulata in vitro*

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Abstract: This study aims to explore the cytotoxicity effects of *B. angulata* whole fruits and berries as well as to identify the fruit's probable role on the level of MMP-13 protein expressions in human cancer cells. Cytotoxicity effects of *B. angulata* were evaluated *in vitro* using skin melanoma (A375) through treatment with novel *B. angulata* fibers (whole fruit and berries) via direct contact method. The growth inhibitions of the samples were evaluated through Methylene Blue Assay (MBA) with incubation time of the fibers on cells at 24, 48 and 72 hours. The results showed significant inhibition of growth in all samples with *B. angulata* whole fruit exhibiting the highest level of inhibition at 72 hours (87.69 %) while its berries showed a reading of 86.16 %. Consequently, this study indicates that both whole fruit fibers and berries of *B. angulata* may have cytotoxic effects against human skin melanoma. The quantitative expressions of MMP-13 in A375 cell lines which were subjected to the specified amount of fibers were evaluated through ELISA analysis. The results showed no expression of MMP-13 proteins in A375 cells for both whole fruit fibers (-1217.9 pg/ml) and berries (-1222.9 pg/ml). Furthermore, the results of the ELISA analysis depict a probable regulative effect of the fibers toward MMP-13 protein expressions in cancer cells. Hence, it can be concluded that *B. angulata* fruit has the potential to be used as a new source of natural substitute for anticancer treatment. Moreover, further study is needed in order to find the specific bioactive compounds involved in the anticancer properties which may have been influential in the regulation of MMP-13 proteins that could be fundamental in future endeavors for prospective therapeutic applications.

INTRODUCTION

Cancer is described as the uncontrolled or uninhibited growth of cells and it is a major public health dilemma in Malaysia and many other parts of the world. Unfortunately, cancer deemed to be the main leading cause of death worldwide, as in the United States alone one in four deaths is due to cancer. World Health Organization estimates that 84 million deaths will occur between 2005 and 2015 without intervention. Over the years, the most common approaches being used for cancer treatments are radiation therapy, surgery and chemotherapy. Each of these treatment methods has significant limitations. However, over time, cancer cells may develop resistance against chemotherapy treatments which is one of the most challenging problems in cancer treatment. Consequently, as stated by Kashkar (2010), a common reason for such resistance is due to the defects in induction of cancer cell apoptosis in response to anticancer treatment. In this regard, the search for new medicinal plants offers enormous prospects for discovering new compounds with potential therapeutic potentials.

Nevertheless, many tropical underutilized fruits such as *Baccaurea angulata* or locally known as belimbing dayak contain high nutritional value as well as various classes of organic compounds of medicinal interest. The natural habitat for the species *Baccaurea angulata* (Figure 1.1) is in the tropical primary and secondary riverine and non-riverine rain forest in the island of Borneo, Indonesia. The tree of the fruit might elevate to 10 m long occurs in sandstone or lateritic soil (Voon and Kueh, 1999). The name *Baccaurea angulata* is the scientific name of the fruit, whereas the common English name is Red Angled Tampoi, while in Malaysia

the fruit has several traditional names, for instance, Belimbing Dayak, Tampoi Belimbing and Rambai Belimbing.

The fruit *B. angulata* is edible; the berries (Figure 1.2) and pericarp taste sour to acid sweet. The characteristics of the tree are: leathery leaves, turn yellowish from green when it is dry, the cross section of fruit is star-shaped (Voon and Kueh, 1999). The fruit was reported to contain proteins, carbohydrates, fiber, minerals and vitamin C. The color of the flower is yellow and the fruit can come in red to purple.

However, the past two decades of biomedical research have yielded an enormous amount of information on the molecular events that take place during carcinogenesis and the signaling pathways participating in cancer progression. The molecular mechanisms of the complex interplay between the tumor cells and the tumor microenvironment play a pivotal role in this process (Kessenbrock *et al.*, 2010). Studies conducted over more than 40 years have revealed mounting evidence supporting that extracellular matrix remodeling proteinases, such as Matrix Metalloproteinases (MMPs), are the principal mediators of the alterations observed in the microenvironment during cancer progression (Kessenbrock *et al.*, 2010 and Page-McCaw *et al.*, 2007).

Matrix Metalloproteinases (MMPs) are proteases that belong to endopeptidases of zinc-dependent family. They are implicated in a variety of physiological processes and various micromolecular activity including wound healing, vascularization and carcinogenesis. Previous studies found that MMPs are expressed in high level during malignant tumor, thus the supposed role of MMPs on tumor invasion is mainly based on this observation. Accordingly, finding a potential inhibitor for MMP-13 is crucial.

This study was aimed at investigating the cytotoxic activity of different parts of *Baccaurea angulata.* One of the main objectives of this research is to assess and evaluate the potential cytotoxic effect of *Baccaurea angulata*'s berries and whole fruit fibers against human skin melanoma cancer cell line (A375). Thus, *in vitro* cytotoxicity test was carried out and cells viability was evaluated by Methylene blue assay (MBA).

Figure 1.1: *Baccaurea angulata* (Basri, 2012)

Figure 1.2: Berries of *Baccaurea angulata*

MATERIALS AND METHODS

Cell lines

Human skin cancer cell lines (A375) obtained from American Type Cell Culture (ATCC, USA) were cultured in DMEM medium supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 100µg/mL of 1% (v/v) Penicillin-Streptomycin (PenStrep), sodium pyruvate and HEPES. The cells were maintained in a humidified atmosphere provided with 5% CO_2 at 37°C.

Plant materials

The relevant parts of the fruit *B. angulata* were obtained from Dr. Mohamed Ibrahim, Department of Nutrition Sciences, Kulliyyah of Allied Health Sciences, International Islamic University Malaysia, Kuantan, Pahang, Malaysia. The average weight of each berry sample was 2 mg whereas the whole fruit sample weighed 1 mg.

Media preparation

The culture medium containing Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, USA) supplemented with 10% Fetal Bovine serum (FBS) (GIBCO, USA) and 1% Penicillin-Streptomycin (GIBCO, USA) was used for cell maintenance in a humidified atmosphere containing 5% CO_2 at 37 °C. All processes from preparation of the medium to the handling of the cells were carried out under strictly aseptic technique inside the type II Biohazard Safety Cabinet.

Cell subculture and cell maintenance

The medium was ready for the growth and expansion of the A375 skin cancer cell line. Upon reaching confluence, the cultured cells were subcultured. Upon cells reached confluence, the old culture medium was

discarded. On the way to eliminate the serum inside of the cells that could disrupt the action of trypsin, the cells were then washed three times with 1 mL Phosphate Buffer Saline (PBS) successively. One milliliter of trypsin was then added to the T-flask that containing the A375 cells in order to detach the cells of the base. The flask then incubated for 2-3 minutes at 37 ºC. Once cells were observed floated under the inverted microscope, 10 mL of fresh medium was added since FBS will inhibit any further action of trypsin. The suspension was then transferred into 50 mL Falcon tube and the cells were centrifuged at 1500 rpm, at 25 ºC for 5 minutes. The supernatant was discarded and the pellet was mixed with 10 mL of complete medium and then transferred into the new flask. The flask was then incubated in a humidified atmosphere provided with 5% CO_2 at 37°C. The sub-culturing was done occasionally and cells were maintained throughout the experiment.

Cell counting

According to Doyle and Griffiths (2000), to ensure that the cells are growing significantly, the percentage of cell viability and the percentage of cell death must be determined. The most widespread and routine method known to count fresh cells is the use of a hemacytometer; a counting chamber with a depth of 0.1 mm and a ruling cover to eases counting. According to Doyle and Griffiths (2000), dead cell stains blue, while living cell stains pale yellow.

In the biosafety hood, the hemacytometer and its cover slip were sanitized thoroughly with 70% ethanol. The cover slip was positioned centrally over the counting area and across the groove of the hemacytometer. Then a pipette was used to mix an amount of 100µ of the cell suspension with 100µ of Trypan Blue stain. The combination was then mixed very well to separate any cell clumps. The ratio of the mixture (1:1) set up the dilution factor (DF).
After that, a 20 µL was withdrawn from the mixture and introduced to fill the chamber via allowing the tip of the pipette to rest at the junction between the counting chamber and the cover slip. The cells were counted in a chosen number of ruled squares using a light microscope at low magnification. The number of the cells per milliliter of suspension was calculated using the following formula (Doyle and Griffiths, 2000)**.**

$$\text{No. of cells/ml} = \text{No. of live cells per } 0.1\mu l \times 10^4 \times DF$$

Using the cell counts, percentage viability was then calculated using the formula:

$$\%\ \text{viability} = (\text{viable cell count}/\ \text{total cell count}) \times 100\%$$

Plating

After the cells reached confluence, cell suspension was transferred into a Petri dish. Using multichannel pipette 100 µL of the cell suspension were then seeded into 96-well plates. The plates were then incubated in a humidified atmosphere containing 5% CO_2 at 37°C for about 24 to 48 hours to allow the cells to reach confluence. Afterward, the old medium was discarded after the cells reached the confluence of 70-80% and then replaced by new medium that contain only 0.5% (v/v) FBS. Approximately about four hours of incubation, cells were treated with the samples.

***In vitro* Cytotoxicity test**

After four hours of incubation in a humidified atmosphere containing 5% CO_2 at 37°C, cells were treated carefully by adding the samples (B1, B3, B5, W1, W3, and W6). Samples were incubated for 24, 48 and 72 hours in 96-well plates. Methylene Blue Assay was performed to determine the percentage of cell death. Briefly, 96-wells were taken out of the incubator followed by removing of the old medium. The wells were then washed with PBS for three times to remove unattached cells. Glutaraldehyde was then added to each well to a final concentration of 2.5% (v/v) and the viable cells were fixed for 15 minutes. The previous solution was then discarded and the plate was washed with 0.15 M sodium chloride (NaCl). Then the plate was dried and cells were stained with 100 µl of 0.05% (w/v) methylene blue solution for 15 minutes. The plate was then washed with 0.15 M sodium chloride (NaCl). Finally, after removing of the dye with 200 µl of 0.33 M hydrochloric acid (HCl) the absorbance was then read at 650 nm using Vmax Kinetic Microplate Reader (TECAN, Germany).

The percentage of cell death was counted as follow:

$$\%\ \text{Cell death} = ((\text{Abs. Control} - \text{Abs. Sample})/\ \text{Abs. Control}) \times 100$$

Note: Abs. = Absorbance.

Enzyme-linked Immunosorbent assays (ELISA)

For quantification of MMP-13 level in the tissue culture supernatant, A375 cells were plated in triplicate in 96-well plates for 24 hours. Then fresh media was added to allow the confluence of the cells. The next day, the culture medium was replaced with fresh medium (200 µl/well) and cells were then treated with the specific amount of samples. After 24 hour of incubation, supernatants were collected and stored at −80°C for future analysis by ELISA Kit. Human MMP-13 ELISA kits were purchased from Calbiochem, EMD Chemicals, Inc (Germany). A standard curve was performed by serial dilution and generated by plotting the mean absorbance (y axis) against pg/ml standard (x axis). Standard concentrations were 0, 32, 63, 125, 250, 500, 1000 and 2000 pg/mL.

Statistical analysis
The experiment was performed in triplicate. Statistics were assessed using 2-tailed Paired sample test. Data

were expressed as mean ± standard deviation (SD) analyzed by SPSS version 20 and Microsoft Excel 2007. When *p*-value is < 0.05, the result considered to be significant.

RESULTS AND DISCUSSION

Methylene blue stain was used in this study to evaluate the viability of the cells. Methylene blue is a basic dye that is positively charged at pH8-5. It binds electrostatically to negatively charged groups within cells, predominately phosphate moieties of nucleic acids and some charged groups in proteins (Oliver *et al.*, 1989). This explanation gives a clear elaboration of the variation in dye bound by cells from different species as well as by different cell types within the same species. The techniques usually rely on the capacity of the living cells to encourage the formation of a product that is noticeable by colorimeter. Adding HCl to the solvent causes acidic groups to protonate, liberating the Methylene blue into the elution solvent by means of pH below 2. The demonstration that an elution solvent of 0-1M HC1 (1:1) gives a single absorption peak for Methylene blue at 650 nm. This is important because it means absorption at this wavelength is an accurate and reproducible reflection of the number of cells stained by the dye (Horobin, 1982).
We used Methylene blue assay to stabilize and fix A375 cells to the wall of the 96-well in order to stain of the cells. The wells containing the cells were washed gently (and not directly pipetting to the bottom of the wells) to avoid cells detachment. The fixative agent Glutaraldehyde provides faster penetration and good crosslinking of proteins and reduces the extraction of cellular components by autolysis.
According to Mosman, (1983), colorimetric techniques are increasingly being used to assess cell number in assays of cell proliferation, particularly in studies of the effects of cytotoxic drugs on tumour cells. This shows that both MTT (tetrazolium salt) assay and Methylene blue assay share the same concept. It is a measurement of viable cells by micro-plate reader such as spectrophotometer which read the absorbance based on the amount of cells that have been colored.

Therefore, in our experiment we used Methylene blue assay because it is very suitable and cost-cutting measurement for cell culturing especially in 96-well plates where it can accurately quantifies the small number of cells present in the cultures. This permits the generation of growth curves and dilution analysis to quantify growth factors, and is ideal for the testing of large numbers of samples. The assay is also reproducible, rapid, inexpensive and easy to perform. A potential problem of the assay is that proliferating cultures could be underestimated because cells round up in metaphase can be lost during the washing procedures.

Malignant melanoma is a serious skin cancer that the survival rate for the five-year is less than 10%. To date, there is still no effective therapeutic treatment for malignant melanoma, only loco-regional treatment therapies such as surgery or radiation.

In this study, human melanoma skin cancer cell lines (A375) were used. We found this line very sensitive to *B. angulata* treatment in culture mainly because it is hypotriploid with a modal number of 62 chromosomes, and 9 marker chromosomes that are commonly found in each cell, and normal N2, N6, and N22 are present at one copy per cell (ATCC). A375 were treated with number of *B. angulata* berries and whole fruit samples for 24, 48 and 72 hours and analyzed by Methylene Blue assay. Both samples exhibit significant growth inhibition toward A375 with *B.angulata* whole fruit exhibiting the highest level of inhibition at 72 hours (87.69%) while its berries showed a reading of 86.16%. Percentage inhibitory values are summarized in Table 3.1.

However, the search for new biologically active compounds from natural sources has always been of great interest. New investigative techniques for the evaluation of natural products with biological activity require the implementation of a large-scale screening system. *B. angulata* has not been screened yet for its fine chemicals which are potentially responsible for its cytotoxic activities. Therefore, the investigation of cytotoxic potential of *B. angulata* fractions is an initial step. Previous studies have shown that PLX4032 (RO5185426; Plexxikon/Roche, Berkeley, CA) is a potential drug for melanoma treatment and could effectively inhibit the *v-raf* murine sarcoma viral oncogene homolog B1 (BRAF). Flaherty *et al*. (2010) concluded that treatment of metastatic melanoma with PLX4032 in patients with tumors that carry the BRAF mutation resulted in complete or partial tumor regression in the majority of patients. But, despite the fact that the early reaction to PLX4032 seems to happen dependably, responsive tumors can develop resistance to the treatment. Consequently, the search for more effective drugs for the treatment of malignant melanoma is still in demand. Our study demonstrated that *B. angulata* significantly suppressed the survival of A375 cells. This suggests that *B. angulata* has the capacity to confer the anti-cancer effect in melanoma cells. Moreover, Kapadia (1996) reported that an *in vivo* anti-tumor promoting activity evaluation of *Beta vulgaris* (beet) root extract against the mice skin and lung bioassays revealed a significant tumor inhibitory effect, suggesting that beet-root ingestion can be one of the useful means to prevent cancer. Another study by Wang *et al.* (2011) demonstrated that α-mangostin, γ-mangostin, and 8-desoxygartanin isolated from the pericarp of mangosteen exhibited significant anti-cancer effect on human melanoma (SK-MEL-28) cell line. However, no information is available on the anti-skin cancer activity of these plant extract in detailed as well.

Table 3.1: Percentage inhibition of whole fruit and berries on A375 cell lines after 24, 48 and 72 hours of treatment

	Berries			**Whole fruit**		
Duration	**B1**	**B3**	**B5**	**W1**	**W3**	**W6**
24 hours	24.51±0.12	67.58±0.004	69.17±0.018	23.73±0.030	43.08±0.144	63.88±0.266
48 hours	32.01±0.016	73.07±0.005	73.20±0.019	26.23±0.037	50.88±0.027	76.80±0.009
72 hours	42.93±0.018	85.54±0.0026	86.16±0.00329	39.81±0.291	60.12±0.024	87.69±0.0012

Note: values have been expressed as mean ± standard deviation (n=3)

On the other hand, the underlying mechanism of *B. angulata* antiproliferative activity remains unclear as *B.angulata* has not been fully exploited and little is known regarding the scientific and anticancer properties of the fruit. But according to recent studies reported on phytochemistry, *B. angulata* was found to consist of various organic compounds such as flavonoids and anthocyanins. On the same hand, studies conducted on fruits of the same genus, *B. motleyana* (rambai) and *B. polyneura* (jentik-jentik) contain high phenolic contents and antioxidant activity (Ikram *et al*. 2009). Interestingly, Muhammed *et al*. (2011) reported that the fruit contains a high level of flavonoids and anthocyanins.

Accordingly, flavonoids has found to be interfering in several of the steps that lead to the development of malignant tumors, including protecting DNA from oxidative damage, inhibiting carcinogen activation, and activating carcinogen-detoxifying systems (Galati *et al*., 2000, Birt *et al*., 2001 and Ren *et al*., 2003). Flavonoids which are constituents of fruits, vegetables, and nuts and plant-derived beverages such as tea and wine as well as components present in a plethora of herbal-containing dietary supplements (Galati, 2004), have often been linked to their ability to act as antioxidants. Besides, they may prevent tumor development by inducing tumor cell apoptosis by inhibiting DNA topoisomerase II and p53 downregulation or by causing mitochondrial toxicity, which initiates mitochondrial apoptosis (Galati, 2004). A study conducted by Miean *et al.* (2001) on more than 62 edible tropical plants revealed that the highest total flavonoids content was in onion leaves, followed by Semambu leaves, bird chili, black tea, papaya shoots, and guava.

Another good organic compound present in *B. angulata* is anthocyanins which belong to the flavonoid class of compounds, and are responsible for the attractive colors, varying from red to blue, found in flowers and fruits (Strack and Wray, 1989). It is natural colorant that is now an extensive and active area of investigation due to the growing interest of substituting synthetic colorants with toxic effects in humans. Another significant property of anthocyanins is their antioxidant activity, which plays a vital role in the prevention of neuronal and cardiovascular illnesses, cancer and diabetes, and many others. For example fruits such as: blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts can inhibit growth and stimulate apoptosis of human cancer cells *in vitro* (Seeram *et al.*, 2006).

Those results are in agreement with our current result as *B. angulata* fruit has the ability to inhibit melanoma cell growth and it may achieved that by stimulating apoptosis. However, further studies are needed in order to clarify this point. Taken together these implications contribute to the understanding of our findings and it also associates the activity of *B. angulata'*s berries and whole fruit fibers toward melanoma cells.

Our findings have shown a significant reduction on the level of Metalloproteinase-13 (MMP-13) after human melanoma skin cancer cell line (A375) has been treated by *B.angulata*'s whole fruits and berries. Figure 3.3 demonstrate the level of MMP-13 before and after treatment. The evaluation of its level was carried out by ELISA analysis.

Generally, Matrix Metalloproteinases are believed to mediate tumor invasion through activation of other enzymes, processing of matrix components to modulate cell migration, and release of bioactive factors mainly bound to the ECM (Zigrino *et al*., 2009). However, of

all collagenolytic enzymes only expression of MMP-1 and MMP-13 mRNA has been found to correlate with melanoma progression and early metastasis (Airola *et al*., 1999; Nikkola *et al*., 2001; Kuivanen *et al*., 2005), and expression of MMP-13 is believed to promote tumor development in other type of cancers (Zhang *et al*., 2008). The cellular source and role of MMP-13 appears to be different depending on the type of tumor analyzed. For example, in breast cancer, Zhang *et al*. (2008) suggested that only tumor-derived, but not stromal fibroblast-derived, MMP-13 correlated with aggressive tumor phenotypes. In agreement, in invasive and metastatic human skin tumors, MMP-13 expression was predominantly confined to the tumor cells, whereas its expression was absent in normal dermis and benign pigmented lesions (Airola *et al*., 1999). Conversely, in mouse models for squamous-cell carcinomas and in breast cancer xenografts, a prominent upregulation of MMP-13 was observed in the host stroma (Lafleur *et al*., 2005). However, Corte *et al*. (2005) have detected MMP-13 in tumor cells in 30% of *in situ* melanomas. Thus, it is possible that expression of MMP-13 by stromal cells may be vital to direct toward a malignant phenotype in a particular and limited timeline.

Table 3.2: The level of MMP-13 in A375 cell line treated with whole fruit and berries

Samples/Control	MMP-13 (pg/ml)	Overall MMP-13 (pg/ml)
Whole fruit	-1217.9	-7307.5
Berries	-1222.9	-7337.5
Control	1286.7	7720

Consistent with these conclusions, we could show a strong expression of human MMP-13 in melanoma skin cancer cell line (Table 3.2). The amount of MMP-13 analyzed by ELISA in A375 melanoma was 1286.7pg/ml. This high level of expression may suggest a marked role for MMP-13 toward melanoma cells. The proposed role of MMPs in tumor invasion is mainly based on the observation of high-level expression of distinct MMPs in invasive malignant tumors (Westermarck and Kahari, 1999). Further studies may reveal the underlined regulatory function of MMP-13 in melanoma cells. Our results is also in total agreement with previous studies done by Giambernardi (1998) concluded that high level of MMP-13 (collagenase-3) mRNA expression was found in 13 cell lines of different origins including A375 cell line. The results reported here suggest that further investigation of MMP-13 expression in other tumor types is warranted.

On the other hand, Figure 3.3 show that A375 cell lines which subjected to the specified amount of *B. angulata'*s berries and whole fruit fibers resulted in no expressions of MMP-13 proteins for both whole fruit fibers (-1217.9pg/ml) and berries (-1222.9pg/ml). As mentioned above, MMPs degrade extracellular matrix components and contribute to angiogenesis. To verify the result, quantitative real-time RTPCR and western immunoblotting should be performed. The observed reduction in MMP-13 level is largely believed to be due to flavonoids and/or another active compound within the fruit *B. angulata* that is able to inhibit the protein MMP-13. According to Mojzis (2008), flavonoids and chalcones regulate expression of vascular endothelial growth factor (VEGF), Matrix Metalloproteinases (MMPs), epidermal growth factor receptor (EGFR) and inhibit nuclear factor kappa-B (NFκB) signalling pathways, thereby causing strong antiangiogenic effects.

Furthermore, Nikkola *et al*., (2005) has found active MMP-13 in serum of melanoma patients. Serum levels of MMP-13 have been associated with severity of systemic sclerosis (Asano *et al*., 2006). Nonetheless, whether this may stand for a clearing way for depletion of the enzyme or a distribution way is presently unclear. Further studies are continuing to elucidate whether the secretion of MMP-13 may contribute to metastasis formation. Accordingly, Zijlstra *et al*. (2004) have identified MMP-13 as one of the collagenases responsible for collagen remodeling associated with angiogenesis, thus implying that MMP-13 in angiogenesis may be necessary for cellular migration rather than participating in the VEGF/VEGFR cascade. The crucial role of MMP-13 in promoting and maintaining angiogenesis is further supported by the localization of MMP- 13 in close proximity to newly formed blood vessels (Zigrino *et al*. 2009). In addition, macrophages have also been shown to produce MMP-13, which they use to dissolve the interstitial matrix and successfully remodel the fibrotic liver (Fallowfield *et al*., 2007). Other cells implicated in MMP-13 production are neutrophils, which were shown to secrete MMP-9 and MMP-13 thereby modulating angiogenesis, by remodeling the ECM (Obermueller *et al*., 2004).

In this research, we only used melanoma skin cancer cell line (A375) and we suggest future studies to be including various types of cell lines. Taken these data together, we could conclude that our finding implicates MMP-13 in melanoma and indicates a probable regulative effect of the *B. angulata'*s fractions toward MMP-13 protein expressions in melanoma cells. The fruit may inhibit tumor growth and metastasis

formation. Ongoing proteomic analysis may unravel the mechanisms of this finding.

Conclusion

In conclusion, this research provided supporting evidence on the cytotoxic effects of *B. angulata*. The study also indicated that all samples demonstrated a time-dependent and also dose-dependent relationship toward human skin cancer cell line when assayed using Methylene blue assay (MBA). *B.angulata* fruit has the potential to be used as a new source of natural substitute for anticancer treatment. In addition, the high level of MMP-13 in human melanoma skin cancer was dramatically reduced when treated by *B. angulata* fractions.

Based on the mechanism of cell death such as apoptosis, further studies are needed in order to assess the cytotoxic effect of the fruit as well as to test the fruit on different types of cell lines. Also, the fruit contains a variety of bioactive compounds which may have been influential in the regulation of MMP-13 proteins as it could be used in future endeavours for prospective therapeutic applications. Further on MMP-13 regulation pathway, the use of Western blot in combination with Immunohistochemistry will confirm the presence of the protein as well as the quantity.

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