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Anticancer effect of underutilized fruits

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Abstract

Plants, particularly fruits and vegetables, have many phytochemicals that possess various bioactivities, including antioxidant and anticancer properties. In this study, the aim was to investigate the antiproliferative properties of *Syzygium* fruits, namely water apple (*Syzygium aqueum*), milk apple (*Syzygium malaccense*), and malay apple (*Syzygium malaccense* L.) against two types of cancer-origin cells, namely MCF-7 (hormone dependent breast cancer cell line) and MDA-MB-231 (nonhormone-dependent breast cancer cell line). Two solvent methods were prepared using aqueous and methanol extraction. Antiproliferation activities of these extracts were evaluated by employing colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)2,5 diphenyltetrazolium bromide) assay through time periods of 24, 48, and 72 hours. The result showed that extracts from the three fruits had no significant effects for 24 and 48 hours time periods ($p > 0.05$) but extracts of Water apple and Malay apple displayed antiproliferation effects on MCF-7 cell lines ($p < 0.05$) in 72 hours, also there were no effects on the non-cancer origin cell line. The methanolic extracts of the malay apple was more significant with 79% cell viability in the case of MCF7 (IC_{50} =632.3 µg/ml). However, extracts of the milk apple did show antiproliferation effects on both cancer origin and non cancer-origin cell lines ($p < 0.05$) in 72 hours. This finding revealed that fruits extract exhibit antiproliferative activity against MCF-7, which is strongly estrogen-dependant, probably due to extract compound responsible for its anticancer properties. Many studies had shown that plant polyphenols may prevent the metastasis of breast cancer cells through common pathway.

Introduction

Recent studies focusing on the exploitation of natural compounds from fruits for medicinal purposes has drawn much attention to the effective extraction of the desired bioactive ingredients from natural products. Plants, particularly fruits and vegetables, have many phytochemicals that possess various bioactivities, including antioxidant and anticancer properties. Fruits can add important vitamins, minerals, and other bioactive compounds to the human diet (Vasco *et al*., 2008). Some promising, but very under-utilized fruits belonging to *Syzygium* genus of the Myrtaceae family found in Malaysia have been recognized as having the potential to be more useful for nutritional and medical purposes.

Syzygium is a genus in the Myrtaceae family that includes a number of popular species cultivated for their colorful, edible fleshy fruit. The genus name *Syzygium* is derived via Latin from the Greek word 'syzygos', meaning yoked together, possibly referring to the paired leaves (Janick and Paull, 2008). Their fleshy fruit are eaten as such or added to fruit salads, or are cooked or preserved in various ways for home use (Wong and Lai, 1996). The three fruits focused on water apple, *Syzygium aqueum*, which is also called *jambu air* by the local community, milk apple, *Syzygium malaccense* also known as *jambu susu* or *jambu tetek* and lastly, the malay apple, *Syzygium malaccense* L. which is commonly referred to as *jambu bol* or *jambu agung*. According to the study by Khoo *et al*. (2008), milk apple contains a low level of total carotene content of (about 3.35 mg/100 g), however beta-carotene was not found in milk apple.

In addition, *Syzygium* species possess antibacterial activity (Chattopadhyay and Sinha, 2000). Also, the bark of the malay apple tree has a variety of interesting biological activities. It inhibited four species of

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viruses, three species of fungus and provides experimental verification for its use in traditional medicine (Locher *et a*l., 1995). *Syzygium* species has the potential to be more useful for nutritional and medical purposes.

Breast cancer is one of the major causes for the increasing mortality among women. In Malaysia, there has been an increased admission rate of patients diagnosed with breast cancer in government hospitals (Abdullah and Yip, 2003). Breast cancer is the most common cancer among females in all ethnic groups and all age groups in females from the age of 15. It is also the most important cancer regardless of sex in Peninsular Malaysia (Zainal *et al*., 2006).

According to the study done by Norsa'adah *et al*. (2005), the main factors associated with high risk of breast cancer in women are nulliparity (the condition of not bearing offspring), overweight/obesity, family history of breast cancer, and oral contraceptives (birth control pills) usage. Abdominal obesity has been shown to be correlated with breast cancer risk in the Klang Valley, Malaysia (Rabeta *et al*., 2007) and estrogen hormone modifying breast cancer risk (Oldenburg *et al*., 2007).

Many evidences from researches have demonstrated that many natural products isolated from plant sources possess antitumor properties (Wu *et al*., 2002). A few studies have already examined the antioxidant properties of these fruits. The main objective of this study was to investigate the anti proliferative properties of selected underutilized fruits namely water apple, milk apple, and malay apple against cancer-origin MCF-7 (hormone dependent breast cancer cell line), MDA-MB-231 (nonhormone-dependent breast cancer cell line) and noncancer origin HS27 (human foreskin fibroblast cell line).

Materials and Method

Water apple, milk apple, and malay apple were harvested in October 2010. HS27 (ATCC® CRL-1634™, human foreskin fibroblast cell line), MCF-7 (ATCC® HTB-22™, hormone-dependent breast cancer cell line) and MDA-MB-231 (ATCC® HTB-26™, non hormone-dependent breast cancer cell line) were purchased from the American Type Culture Collection (ATCC), USA. Phosphate Buffer Solution (PBS) tablets were obtained from AMRESCO INC, Cleveland, Ohio, USA. The media used was Dulbecco's Modified Eagle Medium (DMEM with low glucose, and high glucose) and Foetal Bovine Serum (FBS), penicillin–streptomycin and trypsin were from Gibco®, InvitrogenTM, USA. MTT labelling reagent was obtained from Molecular Probes®, InvitrogenTM, Oregon, USA.

Sample preparation

The fruits were harvested from Kuala Kurau, Perak, Malaysia. The fruits were identified by the Herbarium Unit of the Forest Research Institute Malaysia (FRIM) in Kepong, Selangor. Fruits of water apple, milk apple and malay apple were cut into small pieces and dried by using a freeze drier (ALPHA Freeze Drier Model 1-2 LD plus, Vacuubrand, Germany) for four days. Ground fruits were kept in -20°C prior to extraction. This was done at the School of Industrial Technology, USM Penang, Malaysia.

Hot aqueous extraction

Based on the method proposed by Huang *et al*. (2003), ground fruits were extracted with boiling distilled water in the proportion of 1:20 (w/v) for 4 hours. The resulting crude extracts were filtered with Whatman filter. The filtrate was lyophilized down to dry powder by using freeze drier. The dried extracts were kept in -20°C.

Methanol extraction

Methanol extraction of the plant was performed according to the method described by Wicaksono *et al*. (2009). Firstly, we had use 100 g of ground fruits. The sample were weighed and then soaked in 300 mL absolute methanol for 24 hours. Subsequently the crude extract was filtered with Whatman filter. Residual solvent of methanolic extract was removed under reduced pressure at 40°C using a rotary evaporator (EYELA Rotary Evaporator Model N-1000, Tokyo Rikakika Co., Ltd, Japan). Evaporation was continued by storing the methanolic extract at room temperature for 2 days. The extract was diluted in PBS before assays. Final dilution was made in DMEM containing 20% FBS.

Cell subculture

This was carried out at the Institute for Research in Molecular Medicine (INFORMM), USM based on method from Freshney (1994). The cells were observed under inverted phase-contrast microscope and split using trypsin-EDTA after incubation at 37°C in 5% CO_2 incubator for 5 mins. Cell suspension of 10^3 cells /ml was added into the T-25 flask containing complete media. The cells were checked daily under inverted microscope and the subculture was fed by removing the existing media and replenished with fresh complete media.

Cell plating

Cell growth was observed under inverted phase-contrast microscope. Firstly, the cells were trypsinized and then centrifuged at 1000 rpm for 4 mins. Subsequently, the supernatant was discarded and the cells were resuspended with 1 mL PBS. Again, the cells were centrifuged at 1000 rpm for 4 mins. Once the supernatant was discarded, the cells were resuspended with 3 mL of incomplete media (incomplete media = basal medium + 1% penicillin-streptomycin). Then, the cell suspension was well mixed and 15 µL of cells was added to 15 µL of trypan blue in a small vial for cell counting using the hemocytometer. The cells were diluted to obtain 3000 cells in each 60 µL using incomplete media. Cells of 60 µL were dispensed into each well of a 96-well microtitre plate and incubated at 37°C and 5% CO_2 (Freshney, 1994).

MTT assay

Each cancer cell line was grown in a 96-well microtiter plate (Nunc, Denmark) in a final volume of 120 µL culture medium per well. Each well contained 3 x 10^3 cells/well and was incubated for 24 hours in a 5% CO_2 incubator at 37°C. The cells were then treated with extracts of the fruits at doses of 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL and maintained at 37°C with 5% CO_2 for 24,48 and 72 hours. After the incubation period, 0.5 mg/ml of MTT labelling reagent was added to each well. The microtiter plate was then incubated again for 4 hours at 37°C with 5% CO_2. Then, the formazan crystals were solubilised with 100 µL of acidified-isopropanol. One hundred microlitre distilled water was added into each well for further colour development. Absorbance of viable cells was measured using a spectrophotometric plate reader (Multiskan spectrum, Thermo Electron Co., Waltham, Massachusetts, USA) at 570 nm (Freshney, 1994).

Statistical analysis

Results for percentage cell viability were reported as means ± standard error from triplicate determinations. Significant differences for multiple comparisons were determined by one-way analysis of variance (ANOVA) followed by Duncan test by SPSS statistical package (ver.17.0). The p value less than 0.05 were considered as statistically significant.

Results and Discussion

Growth inhibition with water apple extract

Figures 1 and Figure 2 showed the effects of water and methanol extracts in water apple using MCF-7 and MDA-MB-231 cell lines and non cancer-origin cells at 72 hours, respectively. In examining the antiproliferative effect of aqueous extract from water apple, it was found to have the strongest inhibitory effect when non cancer-origin cell lines were compared with cancer-origin cell lines. It was observed that the viability of MCF-7 and MDA-MB-231 cells were reduced in extracts at high concentration, the viability of MCF-7 cells were significantly ($p < 0.05$) reduced in methanol extract.

Based on results obtained, it is apparent that water apple possesses inhibiting activity on two breast cancer origin cell lines, especially MCF-7 that is hormone/estrogen dependent breast cancer cell line, and its activity is more prominent after 72 hours incubation with the extract. Some studies reported a strong correlation between phenolic content and antioxidant activity in fruits, vegetables and grains (Ismail *et al*., 2004; Dasgupta and De, 2007; Osman *et al*., 2009). From the study by Ling *et al*. (2010), water apple contains natural antioxidants.

Studies also showed that the volatile oils isolated from *Syzygium* species by vacuum distillation contain a high percentage of terpenoids and γ-terpinene. Terpenoids may act by affecting the farnesylation of ras gene product in premalignant and malignant cells (Smith and Yang, 1994). Limonene has been found to be effective in inhibiting the promotion or progression stage of carcinogenesis and significant ($p < 0.05$) in inhibiting rat mammary tumors. Limonene has also been found to cause inhibition to primary differentiated mammary tumors by 7,12-Dimethylbenz(a)anthracene (DMBA) which is a carcinogen.

Results from this study indicate that the water apple has antiproliferative effects on MCF-7. This may be due to the involvement of polyphenols on estrogen metabolism, hence inhibiting the proliferation of MCF-7 which is strongly estrogen-dependant. Furthermore, it has also been suggested that polyphenols may act as estrogen agonists or antagonists in different contexts. Thus, several factors may play a role in determining the effect of polyphenols on breast cancer cell growth (Hakimuddin *et al*., 2008).

Growth inhibition with milk apple extract

The percentage of cell viability of MCF-7, MDA-MB-231 and HS-27 cell line for treatment with different concentrations of aqueous and methanol extract of milk apple in 72 hours are presented in Figure 3 and Figure 4, respectively.

According to the figures, there is a significant increase ($p < 0.05$) in cell viability for HS27 cell and

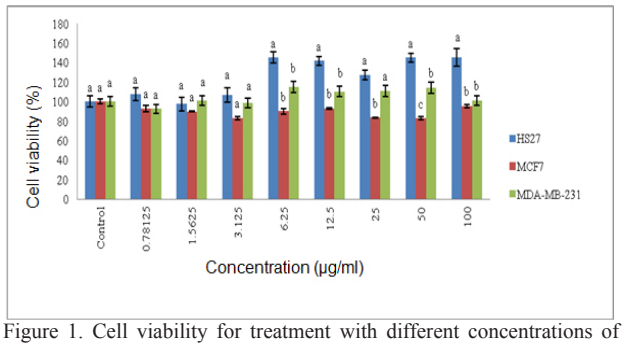

Figure 1. Cell viability for treatment with different concentrations of aqueous extract of water apple for 72 hours. Values are expressed as mean ± standard error (SE) of triplicate measurements.
a-c represents means for each concentration labelled with different letters were significantly different at $p<0.05$.

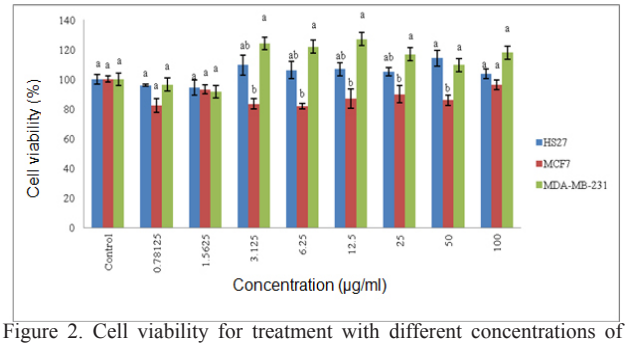

Figure 2. Cell viability for treatment with different concentrations of methanol extract of water apple for 72 hours. Values are expressed as mean ± standard error (SE) of triplicate measurements.
a-b represents means for each concentration labelled with different letters were significantly different at $p<0.05$.

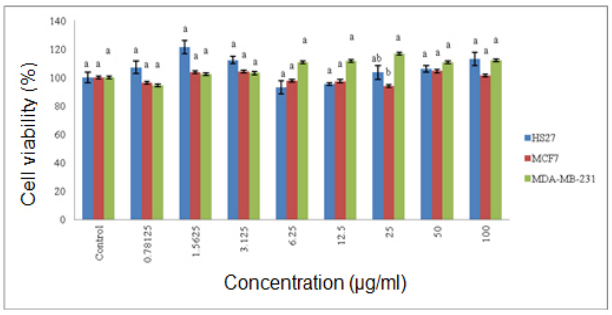

Figure 3. Cell viability for treatment with different concentrations of aqueous extract of milk apple for 72 hours. Values are expressed as mean ± standard error (SE) of triplicate measurements.
a-b represents means for each concentration labelled with different letters were significantly different at $p<0.05$.

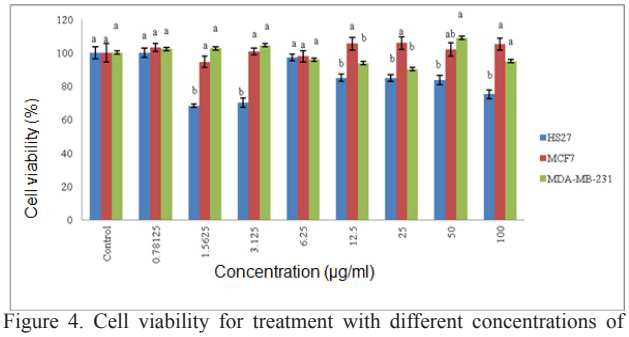

Figure 4. Cell viability for treatment with different concentrations of methanol extract of milk apple for 72 hours. Values are expressed as mean ± standard error (SE) of triplicate measurements.
a-b represents means for each concentration labelled with different letters were significantly different at $p<0.05$.

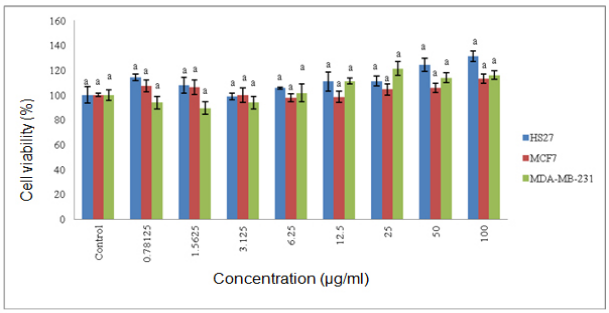

Figure 5. Cell viability for treatment with different concentrations of aqueous extract of malay apple for 72 hours. Values are expressed as mean ± standard error (SE) of triplicate measurements.
a represents means for each concentration were not significantly different at $p<0.05$.

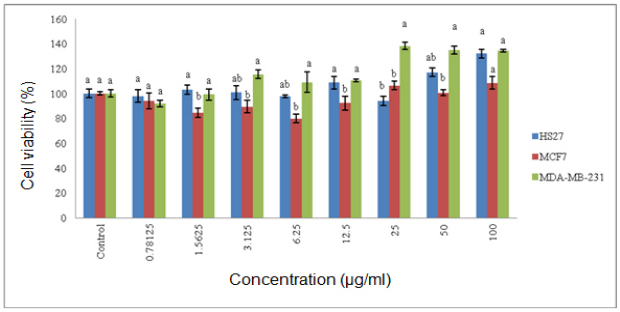

Figure 6. Cell viability for treatment with different concentrations of methanol extract of malay apple for 72 hours. Values are expressed as mean ± standard error (SE) of triplicate measurements.
a-b represents means for each concentration labelled with different letters were significantly different at $p<0.05$.

decrease in cell viability for MCF-7 and MDA-MB-231 cells at low concentration in 72 hours incubation with aqueous extract of milk apple. However, after incubation with methanol extract of milk apple for 72 hours, the cell viability of noncancer-origin cell lines (HS-27) showed significant decrease ($p < 0.05$) starting from a concentration of 1.56 µg/mL, while cell viability for cancer-origin cell lines does not show significant changes ($p>0.05$).

Based on the results obtained, it can be concluded that milk apple possesses antiproliferation effects against HS27 cell and growth-promoting effects against MCF-7 and MDA-MB-231 cells. This study can supports the finding by Khoo *et al*. (2008) that milk apple does not contain beta-carotene, which acts as an anticancer compound as discussed in the previous section. This can be explained by the whitish colour of milk apple, as beta-carotene is mostly present in fruits with yellow-orange colour.

Treatment with malay apple extract

The effect of aqueous and methanol extracts of malay apple was tested against MCF-7 and MDA-MB-231. Figures 5 and 6 exhibit the effect of these

extracts in increasing concentrations during a 72 hours period.

Both figures indicated that for both examined cancerous cell lines, the viability of cells reduced constantly by increasing malay apple concentration. Cell viability for HS-27 showed significant increase ($p<0.05$) starting from concentration 25 µg/mL; while MDA-MB-231 did not show significant changes ($p>0.05$) after treatment with methanol extract.

Based on the results obtained, the methanol extract of malay apple displays antiproliferative effects against MCF-7 cell line. The effect is more obvious after 72 hours of incubation (Figure 6). This is corroborated by the findings of Wongwattanasathien *et al*. (2010) that showed the antiproliferative effect of malay apple on MCF-7 cell line with The methanolic extracts of the malay apple was more significant with IC_{50} value of 632.3 µg/ml. The polyphenilic effect of malay apple on estrogen metabolism is presumed to be the antiproliferative cause of MCF-7, which is known to be strongly estrogen dependent (Hakimuddin *et al*., 2008).

Conclusion

Based on results obtained, the water apple, malay apple, and milk apple possess antiproliferative activity against cancer origin cell lines of MDA-MB-231 and MCF-7. The results of the study demonstrated that both water and methanolic extracts of the fruits decrease the viability of the mentioned cancerous cell lines. However, the influence of the methanol extracts of the Malay apple was more significant with 79% cell viability in the case of MCF7 at 72 hours (IC_{50}=632.3 µg/ml) that is categorized as a hormone/ estrogen dependent breast cancer cell line. Extracts of the three fruits did not exhibit significant effects for 24 and 48 hour periods ($p>0.05$). Furthermore, it can be concluded that the fruits have anticancer activity as demonstrated in previous studies on different cancerous cell lines. However, knowing the exact compound responsible for its anticancer properties will help in making appropriate formulations that can be used as anticancer agents in future.

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