The cytotoxicity activity of Borneo-cultivated mulberry against breast cancer cell (MCF-7): the influence of maturity stages and extraction solvents

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Abstract

Breast cancer is one of the leading causes of death in a part of Borneo, which is Sabah, Malaysia, and worldwide. The anticancer activity of white mulberry (Morus alba Linnaeus) has been widely reported. However, cultivation locations, maturity levels, and extraction solvents could influence their phytochemical and pharmacological activities. The lack of anticancer investigation has hampered the development and potential use of Borneo-grown mulberry as an anticancer agent or source. This study investigated the cell cytotoxicity activity of Malaysia-grown mulberry at two maturity stages (fruits: brackish black fully ripe and red mature; leaves: young and mature) extracted using 70% (v/v) methanol, 60% (v/v) ethanol, and 65% (v/v) acetone, against a human breast cancer cell line (MCF-7). As a result, fruits demonstrated maturity-dependent cytotoxicity decrement as the red mature fruits in 70% (v/v) methanol exerted the strongest cytotoxicity (IC_{50}=26.83 mg/mL). Meanwhile, the cytotoxicity of leaves revealed maturity-dependent increment as mature leaves in 60% (v/v) ethanol exhibited the strongest cytotoxicity against MCF-7 (IC_{50}=2.45 mg/mL). Their cytotoxicity is possibly correlated to the anticancer-possessing phenolic acids and flavonoids in fruits; and the anticancer-possessing alkaloids and terpenes in leaves. Overall, Sabah-grown mulberry possesses substantial cytotoxicity against MCF-7, suggesting its anticancer potential as a complementary treatment for breast cancer.

1. Introduction

Morus alba Linnaeus, also known as white mulberry, is one of the medicinal plants with renowned therapeutic effects. The multi-functionality of mulberry fruits, leaves, roots, branches and barks have been well documented in the Chinese Pharmacopoeia and British Herbal Pharmacopoeia (Khan et al., 2013; Younus et al., 2016; He et al., 2018). Mulberry contains abundant metabolites, including macronutrients, micronutrients, phenolic acids, flavonoids, flavonols, anthocyanins, vitamins and volatile aromatic compounds (Sánchez-Salcedo et al., 2015a; Sánchez-Salcedo et al., 2015b). Its fruits and leaves contain significant amounts of phytochemical compounds, including ferulic acid, gallic acid, chlorogenic acid, protocatechuic acids, quercetin, rutin, apigenin, and others, that have been proven to exhibit excellent antioxidant activity. The potent antioxidant activity of mulberry fruit was seen from DPPH (IC_{50} of 0.518 mg/mL), FRAP (IC_{50} of 0.522 mg/mL) and lipid peroxidation inhibition in the liver (45.5%), microsomes (42.8%), and mitochondria (39.4%) (Sánchez-Salcedo et al., 2015a; Natić et al., 2015). While the leaves had FRAP activity of 3.35–4.37 mg GAE/g DW and ascorbic acid equivalent antioxidant activity of 5.08–9.70 mg ascorbic acid/g DW (Eric et al., 2012). Aside from being antioxidative, the compound richness of mulberry is also the main contributor to its various pharmacological effects, including its anticancer effects against various cancers (Dat et al., 2010).

Cancer is the second leading cause of death worldwide. It is a disease characterized by the abnormal growth rate of body cells that can occur in any tissue or organ and is capable of spreading to other organs. According to World Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN) 2020, a total of 19.3 million new cancer cases and 10 million cancer deaths, with 50.6 million 5-year prevalent cases, were publicized (Sung et al., 2021). Moreover, an increase to a total of 28.4 million new cancer incidents and 16.2 million cancer-related deaths is estimated in 2040. In Malaysia, cancer is the top four most prominent cause of death. In Malaysia, the Malaysia National Cancer Registry

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(MNCR) 2012-2016 reported a total of 115,238 new cancer cases, with 44.7% among men and 55.3% among women (Azizah et al., 2019). Also, according to the Malaysia Ministry of Health (MOH) hospitals, an increase of 2.64% in cancer-related deaths has occurred from 2004 (9.54%) to 2019 (12.18%) (Ministry of Health Malaysia, 2021). Out of these cancer cases, breast cancer was reported as the highest cancer case in Malaysia, with a rate of 19% in MNCR 2012-2016 and 32.9% in Malaysia GLOBOCAN 2020 (Azizah et al., 2019; Sung et al., 2021). In Sabah alone, the western state of Malaysia, MNCR 2012-2016 recorded a total of 8,818 cancer incidences with a 45.4% occurrence in men and 54.6% in women, and breast cancer accounted for the highest cancer incidences (13.8%) in Sabah (Azizah et al., 2019). Details indicate a total of 1206 (25.1%) breast cancer cases in Sabahan women and only 12 (0.3%) cases in Sabahan men. Moreover, a study in Sandakan City, Sabah has identified a total of 115 breast tumour cases from 2016–2018, with invasive ductal carcinoma (4.82%) as the highest case, followed by invasive lobular carcinoma (2.9%) (Wong et al., 2021).

Free radicals, especially reactive oxygen species (ROS), are the hallmark of cancer’s aetiology (Ríos-Arrabal et al., 2013). It is due to their ability to damage and mutate DNA through the rearrangement of DNA sequence, miscoding DNA lesions, amplification of genes, modification of bases, breakage of single or double-strand, and activation of oncogenes (Ríos-Arrabal et al., 2013; Pourahmad et al., 2016). These events can cause deleterious effects and loss of cell cycle control, inducing the initiation and development of several cancers. Moreover, the extra ROS that is created through the metabolism of carcinogens (Pourahmad et al., 2016), macrophages, mitochondria, and peroxisomes, might play a role in cancer proliferation (Pucci et al., 2019). Hence the importance of antioxidants to maintain redox homeostasis. Endogenous antioxidants aside, exogenous antioxidants are needed to boost the body’s homeostasis, and natural antioxidants are gaining greater attention due to their safer side effects than synthetics. Plant phytochemicals are excellent natural antioxidants. Several clinical trials have been conducted on phenolic acids, flavonoids, alkaloids and vitamins for their preventative and therapeutic drug abilities (Singh et al., 2016; Chikara et al., 2018). This is due to their proven antioxidant, and anti-inflammatory properties as well as their significant in vitro and in vivo anti-proliferative and pro-apoptotic properties (Chikara et al., 2018). As a result, some of these compounds have been introduced as complementary cancer therapies.

In the East of Malaysia, a three-hectare mulberry plantation at Tudan village, Tuaran, Sabah is being underutilized. Previous studies showed that the fruit and leaves of this Sabah-cultivated mulberry contain high levels of phytochemicals and antioxidant activity that are influenced by maturity levels and extraction solvents (Centhyea et al., 2020; Chen et al., 2022). As reported in various studies, mulberry contains an abundant amount of bioactive compounds whose anticancer activity has been validated against various cancers (Dat et al., 2010). However, this Sabah-grown mulberry has not been subjected to any anticancer studies, which hampered its growth and possible application as an anticancer source or agent. Accordingly, variations in species, development levels, cultivation locations, environments, and handling or processing techniques could influence the biosynthesis of metabolites in plants (Radojkovića al. et al., 2012; Lee and Hwang, 2017; Li et al., 2020). These variations would consequently influence the chemical and biological activity of plants, including their anticancer activity. For example, a study on Sabah tea leaves under the influence of different maturity stages, fermentation stages, and extraction solvents revealed that the ethanol (EtOH) extracted green tea exerted higher cytotoxicity against human lung (A549) (IC50 = 61.0), breast cancer (MDA-MB-231) (IC50 = 34.0), and cervical cancer cell lines (HeLa) (IC50 = 30.0), compared to the black tea, compost, and waste tea leaves (Izzreen et al., 2020). In a different study, the n-hexane and dichloromethane fractions of mulberry fruits demonstrated the highest cytotoxicity against colon (HCT116) (100%) and breast cancer cells (MCF-7) (99.4%) compared to other solvent extracts (El-Baz et al., 2017). Meanwhile, a study on mulberry leaves showed that the 50% (v/v) methanol (MeOH) and 100% MeOH leaf extracts exerted the best inhibition of hepatocellular carcinoma’s (HepG2) proliferation via NF-xB suppression and biomarker modulation (Fathy et al., 2013). Hence, the distinction between maturity stages and extraction solvents is important to obtain the optimal anticancer activity of a plant. Therefore, in this study, the local Sabah highland-cultivated mulberry fruit and leaves at different maturity levels (fruits: red mature (RF) and blackish black fully ripe (BF); leaves: mature (ML) and young (YL)) and extracted in different solvents (70% (v/v) methanol (MeOH), 60% (v/v) ethanol (EtOH), and 65% (v/v) acetone) were analyzed for their cytotoxicity activity against MCF-7, which is the highest cancer incident in Sabah, Malaysia, and worldwide. This research will be the first step to finding out the anticancer potential of Sabah-cultivated mulberry in the pharmaceutical industry.

2. Materials and methods

2.1 Sample collection and preparation

Both the mulberry (Morus alba Linnaeus) fruit and
leaf samples were harvested from Tudan Village, Tuaran, Sabah, East Malaysia (Borneo). The fruits at maturity index 4 (red and mature) and 5 (brackish black and fully ripe) (Lee et al., 2016) were collected in September and October of 2018 and 2019. For the leaves, young (the 1st to 4th leaves from the shoot) and mature leaves (the 5th to 8th leaves from the shoot) (Wulandari et al., 2019) were collected between July and August 2020.

2.2 Preparation of mulberry extracts

The collected brackish black fully ripe fruits (BF), red ripe fruits (RF), young leaves (YL), and mature leaves (ML) were washed, frozen in a -80°C freezer (New Brunswick Scientific U410, Germany) for 24 hrs, and freeze-dried (Labconco, USA) at -40°C and 0.5 Pa for 72 hrs. Next, samples were blended to powder using a laboratory blender (Waring 8010S, USA), set to 18,000 rpm for 5 mins. The powdered mulberry fruit and leaves were respectively extracted using 70% (v/v) MeOH (Tan et al., 2015), 60% (v/v) EtOH (Radojkovića et al., 2012), and 65% (v/v) acetone (Jeszka-Skowron et al., 2014), with a ratio of 1: 30 (m/v) at 60°C in a shaking water bath (Daihan MaXturdy 30, Korea), set at 120 rpm for 4 hrs (Radojkovića et al., 2012; Kim et al., 2014). The samples were filtered through Whatman paper No.1 and the filtrates were rotary evaporated at 40°C (Heidolph Laborota 4000, Germany) until dry. Samples were further dried using a freeze-dryer (Labconco, USA) at -40°C and 0.5 Pa for 48 hrs, then re-dissolved in sterilized distilled water (dH2O).

2.3 Cell culture

The human breast cancer (MCF-7) cell line (Elabscience Biotechnology Inc., USA) was cultured in a T25 flask with 5 mL of complete media consisting of Dulbecco’s modified Eagle’s medium (DMEM) (Nacalai Tesque, Japan), 10% fetal bovine serum (FBS) (Tico Europe, Netherlands) and 1% of 100 U/mL of penicillin-streptomycin mixed solution (Nacalai Tesque, Japan). The cells were incubated for growth at a 37°C incubator supplemented with 5% purified carbon dioxide (CO2). Cells were sub-cultured upon reaching 70%-80% confluence.

2.4 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The MTT assay was conducted according to Deepa et al. (2013) with modifications. A total volume of 90 μL of cells and media mixture was seeded into a 96-well microplate to culture 1×10^4 cells/well. The MCF-7 cells were cultured for 24 hrs in a 37°C incubator supplemented with 5% purified CO2 to reach 60% confluence. After that, the old media was replaced with a fresh 90 μL of complete media and the cells were treated with mulberry fruit and leaf extracts for another 24 hrs in a 37°C incubator supplemented with 5% purified CO2. Next, 80 μL of 0.5 mg/mL MTT (Nacalai Tesque, Japan) dissolved in 1X PBS was added to each well and incubated for 4 h in a 37°C incubator supplemented with 5% purified CO2. The supernatant was then removed from each well and 100 μL of dimethyl sulfoxide (DMSO) (Nacalai Tesque, Japan) was added. The mixture was then incubated for 20 mins before being measured at 570 nm via the Tecan Infinite M200 plate reader (Tecan, Switzerland). The final results were given in the form of IC50 by plotting the graft percentage (%) of live MCF-7 cells against the fruit and leaf concentrations via the formula (1).

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\text{Cell viability} (%) = \left(\frac{A_{\text{test}}}{A_{\text{control}}}\right) \times 100 \tag{1}
\]

Where A0: abs of control: 1 × 10^4 cells/well + 10 μL of sterilized dH2O and A1: abs of sample: 1 × 10^4 cells/well + 10 μL of BF/RF/YL/ML in sterilized dH2O.

2.5 Data analysis

All experiments were performed in triplicate. All data is expressed as the mean ± standard deviation (SD). A two-way Analysis of Variance (ANOVA) (IBM SPSS Statistics 23.0, IBM Corp., Armonk, New York) was performed to determine the effect of two independent factors (maturation stages and extraction solvents) on dependent factors (cytotoxicity against MCF-7). Upon the significant difference (p < 0.05) of two-way ANOVA, a one-way ANOVA and Tukey’s HSD Comparison Test at a 95% significance level (p < 0.05) were conducted to see the significant difference among samples (IBM SPSS Statistics 23.0, IBM Corp., Armonk, New York).

3. Results and discussion

3.1 The cytotoxicity of mulberry fruits against MCF-7

The cytotoxicity of mulberry fruits against MCF-7 is shown in Table 1. Mulberry fruits showed a maturity-dependent increment of cytotoxicity, indicating the stronger cytotoxicity of RF than BF against MCF-7. RF showed 64.2% stronger cytotoxicity activity against MCF-7 (IC50 = 26.83–31.41 mg/mL) than BF (IC50 = 77.06–85.73 mg/mL). This difference can also be seen from Figure 1 which shows the condition of MCF-7 after the treatment of BF and RF. The two-way ANOVA revealed a significant relationship (p < 0.05) between m*es on the cytotoxicity of mulberry fruits against MCF-7.

The obtained data is seen to be more cytotoxic than the cytotoxicity of mulberry fruits in Gug (2012) (IC50 =
and induce apoptosis via G2/M cell arrest, up-regulation of the tumour suppressor genes; p53 and PTEN (by 1.52 and 13.47-fold, respectively), and cell cycle-associated genes; p21 and CDK1 (by 1.28 and 11.15 fold, respectively) (Hasani et al., 2018). Hence, the stronger anticancer ability of RF can be associated with its higher level of chlorogenic acid and rutin than BF.

In addition, studies have reported the higher presence of other anticancer-possessing phenolics and compounds in less ripe fruits. Examples of these compounds are protocatechuic acid, caffeic acid, ferulic acid and ellagic acid (Lee and Hwang, 2017). According to various studies, ferulic acid has an IC₅₀ of 75.4 g/mL against MCF-7 and induces apoptosis via caspase-3 and -9 elevation (ElKhazendar et al., 2019), whereas ellagic acid induces cell cycle arrest and apoptosis via the transforming growth factor-β (TGF-β)/Smad3 signalling pathway (Chen et al., 2015). Likewise, Lee et al. (2016) discovered higher levels of aconitate, phenolics (neochlorogenic acid, cryptochlorogenic acid, and chlorogenic acid), and vitamins (δ-tocopherol and ascorbic acid) in red mature mulberry fruits than in brackish black fully ripe fruits. The cytotoxicity of ascorbic acid was seen through its dose-dependent MCF-7 inhibition and ability to enhance the release of lactate dehydrogenase to induce MCF-7 apoptosis (Ganash, 2021).

Besides, the mature mulberry fruits were also revealed to contain a higher concentration of polyunsaturated fatty acids (PUFA) than the fully ripe fruits (Lee et al., 2016; Jelled et al., 2017). Reports showed that docosahexaenoic acid (DHA) can reduce the size of breast tumours in mice, increase the expression of the BRCA1 tumour suppressor gene by 60%, and induce apoptosis by activating peroxisome proliferator-activated receptors γ (PPARγ) (Berquin et al., 2008). Meanwhile, omega-3 PUFA have been shown to have anticancer activity, improve chemotherapy efficacy, and reduce cancer incidence and mortality (Berquin et al., 2008;
Jóźwiak et al., 2020). Therefore, the higher cytotoxicity activity of RF against MCF-7 in this study could be associated with their higher content of phenolic compounds, including chlorogenic acid and rutin, vitamins, and PUFA, that contain strong anticancer activity.

For the assessment among solvents, some inconsistency was seen in BF and RF. In BF, the 65% (v/v) acetone extract exhibited the highest cell inhibitory activity against MCF-7 (IC$_{50} = 77.06$ mg/mL), which was 5.16% better than 70% (v/v) MeOH extracts and 10.1% better than 60% (v/v) EtOH. This data is consistent with its highest quantified chlorogenic acid value (4.49 mg CGAE/g DW) in our previous study (Chen et al., 2022). However, in RF, the 70% (v/v) MeOH extract exerted the highest inhibitory action (IC$_{50} = 26.83$ mg/mL), which was 7.6% better than 60% (v/v) EtOH and 14.6% better than 65% (v/v) acetone extracts. This data is also consistent with its highest quantified rutin value (4.93 mg RE/g DW) in our previous study (Chen et al., 2022). Based on this, it can be concluded that chlorogenic acid and rutin have a positive correlation with the cytotoxicity activity of fruits against MCF-7. Hence, the anticancer activity of the fruit extracts depended on the solubility rate of chlorogenic acid and rutin in the solvents.

The variation of anticancer ability among the three solvents could also be influenced by the solubility rate of other anticancer compounds. For instance, ellagic acid was reported to be sparingly soluble in alcohol such as methanol (671 µg/mL) but poorly soluble in water (9.7 µg/mL) (Bala et al., 2006). This is based on the higher ellagic acid content found in the MeOH extract of the chestnut shell (12.57 mg/g), compared to its EtOH extract (4.07 mg/g) (Jung et al., 2016). Next, p-Hydroxybenzoic acid revealed better solubility in MeOH, followed by EtOH, acetone and other lower polarity solvents (Gracin and Rasmussen, 2002). A study of protocatechuic acid also revealed its greater solubility in MeOH than acetone, evidenced by its higher solvation free energy and higher number of formed hydrogen bonds (Gurina et al., 2017). Further, it was reported that ascorbic acid has better solubility in more polar solvents, indicating the solubility order of: Water > MeOH > EtOH > Acetone (Nemdili et al., 2022). Therefore, the higher cell inhibition activity of RF 70% (v/v) MeOH could be due to the higher solubility of ellagic acid, p-Hydroxynamic acid, and ascorbic acid in it.

3.2 The cytotoxicity of mulberry leaves against MCF-7

Table 1 reveals the cytotoxicity of mulberry leaves against MCF-7 in a maturity-dependent increment manner. ML showed 70.0% higher cytotoxicity (IC$_{50} = 2.45$–4.55 mg/mL) than YL (IC$_{50} = 10.62$–14.69 mg/mL) against MCF-7. This difference can also be seen from Figure 2 which shows the condition of MCF-7 after being treated with YL and ML. The two-way ANOVA revealed a significant relationship ($p < 0.05$) between ms*es on the cytotoxicity of mulberry leaves against MCF-7. This finding was supported by Hyun et al. (2013), which mature and senescent yellow Korean Dendropanax leaves had greater cell inhibitory and anti-migratory effects against hepatocellular carcinoma cells (Huh-7) than the non-senescent leaves at sample concentrations of 100 µg/well and 200 µg/well.

Figure 2. MCF-7 after treated with YL and ML at 12.5 mg/mL under 4× magnification using inverted microscope. (A) YL 70% (v/v) methanol; (B) YL 60% (v/v) ethanol; (C) YL 65% (v/v) acetone; (D) ML 70% (v/v) methanol; (E) ML 60% (v/v) ethanol; (F) ML 65% (v/v) acetone.

Similarly in leaves, changes in MCF-7 cell shape from polygonal-spindle shape to round shape were observed upon treatment with YL and ML, indicating a possible apoptotic or necrosis reaction. Moreover, a higher amount of these possibly-apoptotic cells was seen under the treatment of ML compared to YL at the same concentration (12.5 mg/mL). The better cell inhibitory effect of ML against MCF-7 is speculated to be due to the higher quantity of 1-Deoxynojirimycin (DNJ) in mature mulberry leaves (0.16–0.27 mg DNJ/g leaves) than in the young leaves (0.07–0.08 mg DNJ/g leaves) (Wulandari et al., 2019). One study revealed that 0.02-0.1% of DNJ-diets have significantly reduced the incidence of colorectal cancer in mice and dose-dependently suppressed the growth of cancer (Yamamoto et al., 2017). Meanwhile, a derivative of DNJ has down-regulated the expression of cyclooxygenase 2 and arrested the cell cycle of MCF-7 at the S phase (Zhang et al., 2021). This derivative compound also inhibited the migration of MCF-7 and induced cell apoptosis via a mitochondrial-mediated pathway. Both DNJ and the derivative decreased the
expression of anti-apoptotic Bcl-2 but increased the expression of pro-apoptotic Bax (Yamamoto et al., 2017; Zhang et al., 2021). All of these have validated the anticancer activity of DNJ. Hence, the stronger cytotoxicity of ML in this study could be due to its higher DNJ value.

Wulandari et al. (2019) also reported a higher presence of simple terpenes, such as phytol and limonene, in mature mulberry leaves (8.16% and 6.33%, respectively) than in young leaves (0.69% and 1.12%, respectively). One study reported that phytol showed the strongest cytotoxicity against MCF-7 (IC_{50} = 8.79 µM) compared to the cervical, colon, lungs and other cell lines (Pejin et al., 2014). Furthermore, a daily 8 g/m² dose of D-limonene showed a partial positive response in a pharmacokinetics study of advanced breast cancer (Sun, 2007). This diet has managed to stabilise the axillary and supraclavicular lymph node carcinoma and reduce more than 50% of the supraclavicular lymphadenopathy before the spread of cancer to the bones after 11 months of treatment. Besides, Wulandari et al. (2019) also identified 1.87% of citronellal in mature leaves but not in young leaves. Citronellal was proven to exert cytotoxicity (IC_{50} = 1.41 nM) and reduce cell proliferation and migration of MDA-MB-231 cells (Ho et al., 2020). Citronellal also arrests cells at the G2/M phase and induces 13.92% apoptosis via a caspase-dependent pathway, increment of the Bax gene, and reduction of the Bcl-2 gene. Based on the strong anticancer ability of phytol, limonene, and citronellal, the stronger anticancer ability of ML in this study might be due to their higher presence in mature mulberry leaves than in young leaves.

In addition, the anticancer properties of mulberry leaves and their anticancer-contributing compounds have been extensively reviewed by Chan et al. (2020). However, based on the lower bioactive compounds, chlorogenic acid, and rutin content of ML in our previous study (Chen et al., 2022), as well as the higher phenolic acid and flavonoid compounds in He et al. (2019), it could be speculated that the anticancer ability of leaves has a better correlation with other secondary metabolites such as alkaloids and terpenes instead of phenolic acid and flavonoid compounds.

For the assessment among solvents, inconsistency of data was also seen in the leaves (Table 1). In YL, the 70% (v/v) MeOH extract revealed the highest inhibition activity against MCF-7 (IC_{50} = 10.69 mg/mL). Meanwhile, in ML, the 60% (v/v) EtOH exhibited the highest cell inhibition against MCF-7 (IC_{50} = 2.45 mg/mL). The obtained data from ML is similar to the higher cytotoxicity of EtOH-extracted Cichorium intybus against MCF-7 than the MeOH extract at 250 ppm and 750 ppm (Kandil et al., 2019). In contrast to fruits, only the ML 60% (v/v) EtOH demonstrated consistency with the highest chlorogenic acid content (8.78 mg CGAE/g DW) in our previous study (Chen et al., 2022). Nonetheless, its lower chlorogenic acid value than YL (8.93–30.73 mg CGAE/g DW) indicates its low correlation.

On the other hand, the different cytotoxicity of solvents is correlated to the presence of compounds possessing anticancer properties. For instance, Themelis et al. (2018) mentioned that the high polarity of DNJ has allowed its extraction using various solvents, including water, electrolytes water, and/or their mixture with MeOH or EtOH. In fact, the 70% (v/v) EtOH in their study extracted the highest DNJ value (84%) and was used for their subsequent assays. Next, the preferable solubility of phytol in EtOH was seen by the higher amount of phytol in the chromatographic separation of the EtOH fraction than the MeOH extract (Chakraborty et al., 2021). Furthermore, D-limonene is reported to be partially soluble in water but miscible in hydrocarbons and alcohol solvents, except MeOH (Ngema et al., 2012). Therefore, the interchanging miscibility of these compounds in both EtOH and MeOH could be the reason for the highest cytotoxicity of ML at 60% (v/v) EtOH, followed by 70% (v/v) MeOH, and 65% (v/v) acetone. Unfortunately, further discussion of other possible compounds has been hampered by the lack of studies on the presence of anticancer compounds in different maturities of mulberry leaves.

4. Conclusion

In conclusion, Sabah-cultivated mulberry possesses a substantial cytotoxic activity against the human breast cancer cell line (MCF-7), which is significantly affected by the maturity levels and extraction solvents. The cytotoxicity of mulberry fruits occurs in a maturity-dependent decrement manner, as the red mature mulberry fruits exert higher cytotoxicity compared to the brackish black fully ripe fruits. Among the solvents in fruits, the 70% (v/v) methanol of red mature fruits displayed the strongest cytotoxicity. Meanwhile, the cytotoxicity of mulberry leaves against MCF-7 shows a maturity-dependent increment. The mature leaves of mulberry exhibit stronger cytotoxicity than the young leaves and, among the solvents, the 60% (v/v) ethanol of mature leaves exhibited the strongest cytotoxicity against MCF-7. The cytotoxicity of fruits is possibly correlated to their anticancer-possessing phenolic acids and flavonoids, whereas the cytotoxicity of leaves is conceivably correlated to anticancer-possessing alkaloids and terpenes. The cytotoxicity variation of the three
solvent extracts is due to the rate of solubility of anticancer-possessing compounds in different solvents. Overall, the red mature fruits and mature leaves of the mulberry are significantly more cytotoxic against MCF-7. This indicates their potential anticancer activity and functionality as a complementary treatment for breast cancer. Nevertheless, the screening of compounds responsible for their anticancer activity, apoptotic reaction and cytotoxicity against normal cell lines should be the focus of future studies before delving into their in vivo and clinical trials.

Conflict of interest
The authors declare no conflict of interest.

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