The potency of *Hibiscus tiliaceus* leaves as antioxidant and anticancer agents via induction of apoptosis against MCF-7 cells

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**Abstract.** *Hibiscus tiliaceus* is one of the herbal medicines that have been used as traditional medicine for a long time. Antioxidant and anticancer potencies of this plant have reported by some researchers. However, there are no studies reported on antioxidant and anticancer potencies of *H. tiliaceus* leaves collected from Terengganu-Malaysia, especially against breast cancer cells (MCF-7). Different solvents in the extraction process and different sampling areas were chosen in this study compared to other studies. These could affect the chemicals content of *H. tiliaeus* leaves as well as on their bioactivities. Hence, the objectives of this study were to investigate the antioxidant and anticancer potencies of *H. tiliaceus* leaves from Terengganu, Malaysia, against MCF-7. The sample was extracted by solvent-solvent partitioning using hexane and ethyl acetate. Antioxidant and cytotoxicity properties were carried out by DPPH free radical scavenging activity and MTT, respectively. The morphological features were stained by Annexin- V/PI and DAPI. Results revealed that *H. tiliaceus* leaves exhibited strong DPPH free radical scavenging and cytotoxic activities against MCF-7. Morphological features showed the cells were put to death by both early and late apoptosis. Our results found that *H. tiliaceus* leaves have potency as antioxidant and anticancer agents against MCF-7 cells.

**1. Introduction**

Breast cancer is one kind of cancer diseases that becomes a leading cause of death among women worldwide. An estimated of 2.09 million new cases of breast cancer and deaths were estimated to occur among women in 2018. Nearly 70% of deaths from cancer occur in low and middle-income countries [1]. Cancer is one of the situations where too little apoptosis occurs, subsequent in malignant cells that will not die [2]. The mechanism of apoptosis is complex and involves many pathways. Regardless of being the cause of problem, apoptosis shows a significant role in the treatment of cancer as it is a common goal of many treatment strategies [2]. Current cancer treatments, chemotherapy and chemo therapeutic drugs have adverse side effects to the patients, which may be divided into temporary and permanent effects. According to Komen, temporary effects include languishment, soreness in mouth,
throat and skin, hair loss, frequent urination and signs of anorexia. On the other hand, poor salivary function, menopause, sterility and nerve damage have been recognized as permanent effects which are also equally rare [3].

Some herbs are known to have anticancer properties [4–5] that can be easily obtained or grown at one’s own localities, inexpensive and gives minimal side effects [6] to patients treated with the herbs as therapeutic agents. Besides, herbs are also recommended to be given to cancer patients who develop side effects during or after mainstream therapies like chemotherapy and immunotherapy. According to Lobo et al. [7], a high amount of antioxidant found in vegetables and fruits have the capability to reduce oxidative damage caused by free radicals and reactive oxygen molecules. Antioxidant inhibits oxidation process by preventing or slowing down damage to cells due to production of free radicals that exist as unstable molecules with unpaired electron and are highly reactive. Free radicals have been reported to naturally produce and build up in the body but hasten with exposure to ultraviolet light, tobacco smoke, alcohol, pesticides and pollutions that leads to oxidative stress [8]. Oxidative stress happens when free radicals are produced in excess and may establish a deleterious process by which it may alter or damage cellular structures and functions [9–11]. Antioxidants also naturally exist as vitamin E and phenolic compounds such as flavonoids and alkaloids that contributes to its renowned ability to fight cancer. Antioxidant prevents carcinogenesis by inhibiting the initiation of the cancer cell formation by reactive radicals [12].

One of potential plants is Waru Laut (H. tiliaceus). It has long been used as a traditional medicine for treatment of coughs and fevers, bronchitis and skin diseases (figure 1) [13]. Its methanol leaves extract is reported to have good antioxidant potential, and some chemical constituents such as phenolics, flavonoids, tannins, steroids, and terpenoids [14–15]. Those chemical constituents have been correlated to the antibacterial property of the H. tiliaceus [14,16–17], and they could be responsible to its antimicrobial activity [14].

**Figure 1. Hibiscus tiliaceus** leave and flower.

Hibiscus can be commonly found in Asia and they are extensively diversified in species, for instance H. rosa-sinensis and H. tiliaceus are the two of the many favored species among botanists and flower lovers. In the current study H. tiliaceus is selected to investigate antioxidant and anticancer potential due to its medicinal properties that mostly the bark and leaves of the plant are traditionally used to treat many diseases for years long [18]. It has been evidenced that the aqueous extract from the wood and flowers of H. tiliaceus has also been used to treat skin diseases [19]. Previous studies have also reported that this species has high antioxidant properties determined in AEAC (ascorbic acid equivalent antioxidant capacity) [20].

Furthermore, other researchers used ethanol extract and proved that it has antioxidant property [21]. Samsuadin et al. [12] using 3 different solvents in extraction process also reported that H. tiliaceus exhibited very good antioxidant and antibacterial activities. From the outcome of these two researchers, it is proven that the H. tiliaceus is a good candidate for the antioxidant studies. Due to the fact that there
is a high radical scavenging activity presence in this plant [20], *H. tiliaceus* was also used for cytotoxicity study, such as colon cancer and gastric cancer. The chemical constituents that present in these plants contributed to the various activities of the samples. Rahman and Khan [22] had carried out a research to identify the anti-cancer potential of South Asian plants. One of the species that was listed is *H. tiliaceus*. Phytochemicals presented in this plant are quercetin, kaempferol and B-sitosterol. Even some studies have reported on cytotoxicity activity of *Hibiscus* species against some cancer cells [18, 23], however, there is no study reported on the cytotoxicity of *H. tiliaceus* leaves (collected from Terengganu, Malaysia) and the morphological features of MCF-7 cells death after treated by *H. tiliaceus* leaves. Thus, it is eagerly needed to discover the potency of *H. tiliaceus* as anticancer and antioxidant potential agents.

2. Methodology

2.1 Sample collection and extraction process
Sample of *H. tiliaceus* leaves was collected around coastal area of UMT, Terengganu, Malaysia in April, 2019. The voucher specimen of sample has been deposited at Institute of Marine Biotechnology, Universiti Malaysia Terengganu with the voucher number of TER0619001. The part of leaves was cut into a small pieces, and then put into the -80 °C for further drying using freeze dryer for 3 days. Dried sample was then ground to be fine powder and used for extraction process. Extraction by cold maceration using methanol solvent was done first to yield methanol extract. Solvent-solvent partitioning was continued on methanol extract using hexane and ethyl acetate, subsequently. Total samples obtained from the extraction process were two, namely hexane (HF) and ethyl acetate (EF) fractions.

2.2 Antioxidant activity: DPPH free radical scavenging assay
Antioxidant capability was achieved by DPPH free radical scavenging assay based on Kumaran and Karunakaran [24]. Quercetin and DMSO were used as a positive control and negative control, respectively. Two-fold serial dilution of EF fraction in DMSO (10, 5, 2.5, 1.25, 0.625, 0.313, 0.156 mg/ml) was prepared in 96 well plates. Two hundred microliter of methanolic DPPH solution (6 x 10^5 M) was loaded into all wells and left in a dark room under room temperature for 30 minutes. The absorbance of the sample was measured with ELISA reader (Multiskan ascent, Thermo Electron Corporation) at 517 nm. Free radical scavenging activity in the sample was then calculated.

2.3 Cytotoxicity activity: MTT assay
MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). All organic solvents, media and other reagents were of analytical grade and purchased from Sigma or Merck. Cytotoxicity properties of *H. tiliaceus* leaves samples against MCF-7 cells were determined by MTT assay (Adapted from Andriani et al.) [15]. The cells were incubated at 37 °C in an atmosphere of 5% CO2 until cells confluence reached at least 80% to proceed with MTT assay. The cells were trypsinised and counted using hemocytometer. 100 µl of 1x10^5 cells were seeded into each wells of 96-well plate and the plate was placed in the incubator for 24 hours. After the incubation period, culture medium in each wells are discarded completely and added a 100 µl mixture of EF diluted with culture medium by two-fold serial dilution of six different concentrations (60; 30; 15; 7.5; 3.75; 1.875 µg/ml). The concentration of positive control was prepared same as sample. Furthermore, 96- well plate was incubated for 72 h then 20 µl of MTT (5 mg/ml dissolved in PBS) and was incubated for 72 h followed by addition of 20 µl of MTT (5 mg/ml dissolved in PBS) into each well and incubated for another 4 hours. After the incubation, all wells are completely emptied and replaced with 100 µl of DMSO and incubated for 10 more minutes. Enzyme linked immunosorbent assay (ELISA) reader is used to measure the absorbance at 570 nm. IC50 value is determined from the calculation of percentage of cell viability using the following formula:

\[(\text{Absorbance sample}/\text{Absorbance blank}) \times 100\] 

2.3.1 Morphological features of cells death (Adopted from Andriani et al.) [25]
The concentration of samples used in this part was determined based on the IC50 value obtained in
cytotoxicity analysis. Cells were seeded at $5 \times 10^3$ cells/well in 96-well plate and incubated for 24 h. Subsequently, medium was discarded and replaced with new medium containing EF fraction of *H. tiliaceus* leaves at the concentration of 10 µg/ml and incubated for 24 h. Vincristine sulfate was used as positive control and vehicle as negative control. After 24 h incubation, medium was discarded and Annexin-V/PI/DAPI reagent was added to each well. Cells were then incubated for 10 - 15 min at 15-25 °C. The morphological feature of cells death was analyzed by ImageXpress Micro XLS Widefield High-Content Analysis System (HCS) (Sunnyvale, USA).

3. Results and discussion

3.1. Antioxidant property

Based on figure 2, EF of *H. tiliaceus* leaves exhibited strong DPPH free radical scavenging activity (IC$_{50}$ < 2 µg/ml) compared to the hexane fraction (HF) with low DPPH free radical scavenging activity (IC$_{50}$=10 µg/ml). The DPPH radical scavenging assay was used in this study since it has been long used to determine and measure compounds of free radical scavenging activities to evaluate antioxidant properties of natural products mostly plant samples [26]. The DPPH is a stable free radical that is able to react with compounds to donate a hydrogen atom [27].

A high antioxidant property of EF could be correlated the chemicals constituent content in this fraction. The EF fraction was obtained from extraction process using ethyl acetate solvent. Bata et al. [28] suggested that ethyl acetate is an effective solvent for the extraction of phenols and flavonoids compounds group. Even different solvent has used in the extraction process (hexane, dichloromethane, ethyl acetate, and methanol), ethyl acetate fraction of *H. tiliaceus* leaves still showed strong antioxidant property [15]. They also reported that this fraction was rich by phenolic compounds. Phenolic compounds like flavonoids and phenols have been reported on their effective ability to exhibit potent anti-cancer activities as well as combat various diseases associated with oxidative stress [29]. Since EF has strong antioxidant activity (figure 2), it was selected for cytotoxicity test against MCF-7 cells (figure 3).

![Figure 2](image-url)  
**Figure 2.** DPPH free radical scavenging activity of HF and EF of *H. tiliaceus* leaves as compared to the Quercetin (Q), respectively. All data were calculated in 3 replicates and black arrow indicates the IC$_{50}$ value.

3.2. Cytotoxicity

Cytotoxic activity of the *H. tiliaceus* was tested using MTT assay that is interpreted by the expression
of IC\textsubscript{50} value measuring the inhibition of cell proliferation by 50% obtained by linear regression of concentration against percentage of cytotoxicity activity [30]. Cytotoxicity effect was exhibited by the decrease in the number of viable cells and their morphology changes after being treated with the sample. MTT assay is colorimetric test that measures the intensity of the colour produced due to the metabolic activity of living cell that appears as coloured product. The MTT is reduced to formazan catalysed by mitochondrial succinate dehydrogenase of living cells. The colour intensity of the formazan that is measured using the ELISA reader gives a proportional value of the number of viable cells after treated with the sample of different concentrations by which in this experiment was 60, 30, 15, 7.5, 3.75, 1.875 µg/ml. Cytotoxicity study was carried out using ethyl acetate extract of \textit{H. tiliaceus} on human breast cancer cell line, MCF-7. Our result showed that there was a dose-dependent inhibition as the cells were treated with higher concentrations (figure 3).

According to Andriani \textit{et al.} [31], sample extracts that possess less than 30 µg/ml of IC\textsubscript{50} is considered to be a potential anticancer agent. Our result also showed that the sample has moderate cytotoxic property against MCF-7 cells with the IC\textsubscript{50} value=10 µg/ml (figure 3). Cytotoxic property of EF in this study (figure 3) could be correlated to its antioxidant activity (figure 2). Sample with antioxidant activity can inhibit the propagation of the oxidative chain reaction. Propagation stage is mainly caused by the presence of free radicals [32], a component that causing cancer [12]. Thus, samples with antioxidant properties can be used to prevent the form of cancer cells by free radicals.

Several studies on the cytotoxic effect of \textit{H. tiliaceus} against cancer cell line have been conducted in the past years. A study was carried out by Artanti \textit{et al.} [30] indicated that there is a correlation between antioxidant and cytotoxicity activity based on the IC\textsubscript{50} value obtained from the study when the ethanolic extract of \textit{Hibiscus rosa-sinensis L.}, \textit{Hibiscus tiliaceus L.}, and \textit{Malvaviscus arboreus Cav.} was introduced to T47d cell line, which is another type of breast cancer cell line. Other study that demonstrates the cytotoxicity effect of \textit{H. tiliaceus} includes the crude extract of leaves and bark that showed the presence of cytotoxic compounds and suggested that both the extracts of different parts of \textit{H. tiliaceus} (leaves and bark) have the ability to kill cancer cells and potential to be tested for anti-tumour and anti-cancer properties. Uddin \textit{et al.} [33] demonstrated cytotoxicity properties of 32 Bangladeshi plant extracts including methanolic extract of \textit{H. tiliaceus} that was tested against healthy mouse fibroblast and three human cancer cell lines (gastric, colon and breast cancer cells), however the sample only showed selective cytotoxicity effect on breast cancer cells (IC\textsubscript{50}=1.1–1.6 mg ml\textsuperscript{-1}) and proposed that it could be used as a source or lead to designing anti-cancer drug in the future.

![Figure 3. Cytotoxicity property of HF and EF of \textit{H. tiliaceus} leaves as compared to the hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), respectively. All data were calculated in 3 replicates and black arrow indicates the IC\textsubscript{50} value.](image)

\textbf{3.2.1.} \textit{Morphological features of cells’ death}. 

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Our findings showed that the cells died by both early and late apoptosis after the cells treated by EF (figure 4c) compared to the positive control (figure 4b). Furthermore, DAPI stain positive (blue colour) was presented for both life and dead cells. The result with Annexin V/PI negative and DAPI positive specify the viable cells (Figure 4a). In general, Annexin V/PI method was successfully applied in the current study to exhibit induction of apoptosis in MCF-7 cells after treated with EF. MCF-7 cells treated with EF showed clear apoptotic features after treatment period (24 hours incubation). Most cells remained intact but they still didn’t lose their shape. Similar to the Anexin/PI staining, DAPI also indicates cells that experience apoptotic cell death from colour changes, which is in the current study upon treated with EF. Bright blue colouration with intact nuclear structure showed viable cells for control which is in contrast to the cells that were stained bright red or pink in colour in the nucleus that indicates the presence of dead cells or those of that has undergone apoptosis. Interestingly, a significant phenomenon was observed after 24 hours of incubation with EF where chromatins appeared shorter or has undergone chromatin condensation (nuclear pyknosis) which is one of the known hallmarks of apoptosis and blabbing of the MCF-7 cell membrane. It showed similar morphology features compared to the control drug, vincristine sulfate. Hence, our results demonstrate that EF is proven to have potencies in inducing apoptosis against MCF-7 cells (figure 4c).

![Image](image1)

![Image](image2)

![Image](image3)

Figure 4. Apoptotic cell death of MCF-7 cells among group of negative control (a), vincristine sulfate (b), and upon treatment with EF of *H. tiliaceus* leaves (c) (Magnification = 10x).

According to Hingorani *et al.* [34], reported that the determination of apoptotic cell death with
Annexin V method is indicated from the staining of the cells whereby cells that undergo early apoptosis upon treatment appears green (PI negative) meanwhile red (PI positive) indicates that the cells undergo late apoptosis. In addition, Vermes et al. [35] stated that PI is widely used to determine if cells are viable, apoptotic, or necrotic in conjunction with Annexin V through differences in plasma membrane integrity and permeability. Usually, combination of the Annexin V/PI is used for studying apoptotic cells as described by Cornelissen et al [36]. The integrity of the nuclear membranes and plasma will be decreased in late apoptotic and necrotic cells [37–38]. Decreasing of membrane integrity allowing PI to pass through the membranes, into nucleic acids, and shows as red fluorescence [35,39].

4. Conclusion
Our results provide evidence that H. tiliaceus leaves have potency as antioxidant and anticancer potential agents against MCF-7 cells. However, further study at molecular levels using various incubation time, and using different staining such as tunnel, annexin V-FITC, caspase, and could be explored deeply.

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