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Antioxidant activity and cytotoxicity property of extracts from various coastal plants against HepG2 cell lines

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ABSTRACT

Reports have shown an upward trend for liver cancer in recent years. It is also one of the deadliest cancers globally due to its complexity in detection and treatment. Hence, there is an urgency to develop the anti-liver cancer agent from natural resources which is highly effective with minimum side effects. This study aimed to evaluate the antioxidant activity and cytotoxicity property of extracts with different polarity solvents (hexane and methanol) obtained from coastal plants collected from Merang, Terengganu. Three species (*Melaleuca leucadendra, Terminalia catappa,* and *Rhodomyrtus tomentosa*) were chosen due to their abundance and lacking anticancer studies performed on them. Green leaves were collected directly from the trees and extracted using hexane and methanol successively. Preliminary phytochemical tests (phenolic, flavonoid, terpenoid, saponin, alkaloid and glycoside) were performed on the extracts, followed by an antioxidant test based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Methanol extracts with high antioxidant property were continued for cytotoxicity study on HepG2 cell lines by 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results showed that all methanol extracts from all plants showed high antioxidant property (IC_{50}: 0.83-1.62 mg/ml) and moderately cytotoxic activity (IC_{50}: 24.7-28 µg/ml) against HepG2 cell lines. In contrast, hexane extracts showed very weak antioxidant activities. The highest activity was obtained by methanol extract of *M. leucadendra* (MLM), followed by *T. catappa* (TCM) and *R. tomentosa* (RTM), respectively. These promising results indicated that MLM could be a potential candidate for further study related to the antioxidant and cytotoxicity on HepG2 cell lines, such as for anti-liver cancer agent.

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1. Introduction

Since ancient times, human has been desperately trying to find the treatment for all the diseases that they have encountered. They studied and explored the mountains and oceans in searching for disease medication. Besides looking for drugs from natural resources, some even turn to magical rituals. Among all of these different ways of curing disease, plants have been proven to be the best remedy. Plants have been used for medicinal purposes before the prehistoric period. Plants were the only medicines used before 2500 years ago based on ancient records. Plant-derived medicines are popular around the world until now, they are about 75-80% of the world population that still rely on them (Kamboj, 2000). Besides being popular as traditional medicine among rural people, herbal medicines are also gaining global fame in modern medicine practices. Even after over thousands of years of exploitation, herbal drugs still have a huge market and continually expanding every year. The estimated global market for over the counter plant medicine products in 1997 was 10 billion US Dollars and had an annual growth rate of 6.5% (Soldati, 1997). The plant will always be the main source of finding new medicines because of their minimal toxicity, fewer side effects to humans, ease of collecting samples and low-priced (Andriani et al., 2011). Plants are also superior to other medicine sources such as animal sources as plants are rich in secondary metabolites. A single plant species can contain over 1000 of these unique chemical entities which have a huge potential in becoming the next medicine in the future (Mitra et al., 2007). It is reported that more than 3000 species of plants have been used for cancer treatment (Cragg and Newman, 2005) and estimated that plants currently made up of over 60% of global are used as anticancer agents (Bhanot et al., 2011).

Cancer comprises a group of malignant tumours that grow and spread rapidly and can affect almost all parts of the human body. Cancer is the second leading cause of death globally according to World Health Organization (WHO). About 1 in 6 deaths is due to cancer. In 2015 alone, cancer took out 8.7 million lives around the world (Fitzmaurice et al., 2017). This figure will continue to rise as a result of rapid global population growth and the adoption of various cancer-associated lifestyle choices. According to Lim (2002), cancer is the fourth leading cause of death in Malaysia. Although intensive researches have been carried out on developing anticancer drugs, cancer remains a major obstacle to global public health due to the complexity of cancers. Among the many types of cancer, liver cancer is one of the deadliest. Being the main malignant hepatobiliary disease, hepatocellular carcinoma (HCC) is the third cause of cancer-related deaths globally (Jemal et al.,

2011). Every year, more than half a million new cases of HCC are reported. This matter even worse because, prognosis and treatment even more difficult than in HCC compared to other cancer types as it is often associated with cirrhosis and hepatic dysfunction in 80% of patients (Sengupta and Siddiqi, 2012). Hence, the HepG2 cell lines were chosen in our study as it is a type of liver cancer cells responsible for causing HCC in human.

To combat cancer, research on cancer treatments have become more enterprising during the last 20 years. According to Redd et al. (2001), modern cancer treatments such as chemotherapy, surgery and radiation therapy have many aversive side effects on patients. Therefore, alternative treatment with minimal side effects is needed. One of the reasons why plant medicine is widely used to combat cancer is because plant-derived anticancer agents are highly effective inhibitors against many cancer cells while having minimal toxic side effects towards human cells (Greenwell and Rahman, 2015). Besides that, plants are also known for their significant antioxidant activities due to their abundance of phenolic compounds.

In response to various factors such as environmental pollutants, chemicals and radiation, the cells of living organisms generate free-radicals as a result of pathophysiological and biochemical processes. These free-radicals cause an imbalance in the formation and neutralization of pro-oxidants. As a consequence, these free radicals undergo electron pairing with biological macromolecules such as DNA, proteins and lipids to achieve stability, leading to oxidative stress in the physiological system. This cellular failure brings several negative effects to the body such as aging and some chronic diseases like atherosclerosis, cancer, diabetes, inflammation, and other degenerative diseases in humans (Nagmoti et al., 2012). Antioxidants are a particular group of phytochemicals in the natural extracts that inhibit or delay the oxidation of other molecules by suppressing the initiation or propagation of the oxidizing chain reactions. As such they are important active alternatives in complementary medicine (Al-Abd et al., 2015). Many studies have shown that there is a correlation between antioxidant and anticancer activities. Phenolic content in plants which are known to exhibit high antioxidant is also found out to have incredible effects in the prevention of oxidative stress associated disease such as cancer (Roleira et al., 2015) and shows that plants are still very much needed in the quest of finding new anticancer drugs. We can propel Malaysia into a major global source of finding new anticancer meidicines as Malaysia houses a vast diversity of plants.

There is a big of diversity of flora and fauna available in Malaysia. Besides being listed as one of the top 17 ‘megadiversity’ countries in the world, Malaysia has the world oldest and most complex tropical rain forest ecosystem which has evolved untouched for over 100 million years (Napis et al., 2001). According to Jantan (1998), more than 3200 species of plant in Malaysia have been identified to have medicinal value and have been used for generations in traditional medicine among locals. All of these show that Malaysia has huge natural resources that scientist can tap into making future anticancer drugs. It is interesting to note that it is different between the medicinal properties of inland forest and coastal plants. According to Gerwick and Moore (2012), the marine environment is a very rich source of biologically active natural products and many of them have unique structural features that are not found in terrestrial natural products due to several factors such as higher competition and harsher living conditions in the marine environment. Being popularly known as the land of turtles and beautiful beaches, Terengganu has one of the longest coastlines on Peninsular Malaysia. Therefore, there are high diversity and abundance of coastal plants available in Terengganu which researchers can utilize to find new herbal medicines. *Melaleuca leucadendra, Terminalia catappa* and *Rhodomyrtus tomentosa* are some of the coastal plants that abundance in the coastal area of Merang, Setiu. They are reported to have various biological activities, such as antifungal, antibacterial, anti-HSV-1, antigenic (Bandaranayake, 2002); anti-cancer (Almosnid et al., 2018), and antioxidant activities (Anand et al, 2015; Thanh and Dai, 2019; Siddique et al., 2020). Despite their reported antioxidant and cytotoxicity activities, there is lacking study investigated on these species from Setiu, Terengganu especially on their antioxidant and cytotoxicity properties against HepG2 cell lines. Hence, this study aimed to evaluate the antioxidant activity and cytotoxicity properties of *M. leucadendra*, *T. catappa* and *R. tomentosa* from this area.

2. Materials and methods

2.1. Materials

Sample of *M. leucadendra*, *T. catappa* and *R. tomentosa* leaves were obtained from Merang Jetty area, Setiu, Terengganu, Malaysia and collected on March 2017. HepG2 cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). Minimum Essential Medium (MEM) media was purchased from Nacalai (Nacalai Tesque, Kyoto Japan). While Fetal Bovine Serum (FBS), 1% non-essential amino acid, 1% sodium pyruvate, and 1% penicillin were purchased from GibcoDiagnostics (Madison, WI, USA). While, DPPH, Quercetin, and other chemicals, solvents and reagents were of analytical grade and purchased from Sigma Aldrich (Steinheim, Germany).

2.2. Methods

2.2.1. Sample preparation

The leaves of *M. leucadendra, T. catappa* and *R. tomentosa* were collected around Merang Jetty, Setiu. Only green leaves were chosen and collected. Leaves were air-dried for 3-6 days depending on its drying rate. After drying, the leaves were pulverized using a sterile electric blender to obtain a powdered form. The powdered form of the leaves was weighted before they were stored into an airtight glass container until further use. Herbarium voucher specimens of *M. leucadendra* (TER01217002), *T. catappa* (TER01817001), *and R. tomentosa* (TER02017002) have been deposited in Institute of Marine Biotechnology (IMB), Universiti Malaysia Terengganu (UMT), Terengganu, Malaysia.

2.2.2. Extract preparation

The extracts from the leaves were obtained by performing successive extraction (modified from Andriani et al., 2019). The powdered form of the leaves (400 g) were placed into a large conical flask and extracted sequentially using n-hexane and methanol to yield hexane and methanol crude extracts. The samples were then soaked in the solvents for 24 hours at room temperature and subsequently filtered through filter paper. The extracts were then concentrated using a rotary evaporator under reduced pressure and finally, the extracts were kept in a vial and stored at 3°C until further use. The extractions were carried out continuously until the solvents turn colourless.

2.2.3. Phytochemical screening

Phytochemicals analysis on phenolics, flavonoids, alkaloids, terpenoids, glycoside and saponins were adapted from Yadav and Agarwala (2011) as follows.

2.2.3.1. Test for phenols

Amount of 2 ml of ferric chloride solution (2%) was added to the crude extracts. The presence of phenols will result in the change of colour of the mixture to a blue-green or black colour.

2.2.3.2. Test for flavonoids

Amount of 2 ml of sodium hydroxide solution (2%) was added to the crude extract. The presence of flavonoids will first result in the formation of an intense yellow colour, which will then become colourless on the addition of a few drops of diluted acid.

2.2.3.3. Test for alkaloids

Amount of 2 ml of HCl solution (1%) was added to the crude extract and the mixture was heated gently. Mayer's and Wagner's reagent were then further added to the mixture. The presence of alkaloids will result in the formation of turbidity precipitate in the test tube.

2.2.3.4. Test for terpenoids

The crude extract was dissolved in 2 ml of chloroform and evaporated to dryness. Then, 2 ml of sulphuric acid was added and the test tube was heated for about 2 min. The presence of terpenoids will result in the change of colour of mixture to a greyish colour.

2.2.3.5. Test for glycosides

Amount of 2 ml of glacial acetic acid containing 1-2 drops of ferric chloride solution (2%) was mixed with the crude extract. The mixture was then poured into another test tube containing 2 ml of sulphuric acid carefully. The presence of cardiac glycosides will result in the formation of brown ring interphase on top of the mixture.

2.2.3.6. Test for saponins

The crude extract was diluted with 5 ml of distilled water in a test tube. The mixture was shaken vigorously. The presence of saponins will result in the formation of stable foams.

2.2.4. Antioxidant test

The antioxidant test was carried out based on the DPPH free radical scavenging assay (modified from Kanski et al., 2002) using Quercetin as the positive control and dimethyl sulfoxide (DMSO) as the negative control. Firstly, the DPPH solution ($6x10^{-5}$ M) was prepared in methanol while stock samples were prepared in DMSO (10 mg/ml). Sample tested were prepared in varying concentration using 2-fold serial dilution method with concentration of 10, 5, 2.5, 0.65, 0.313 and 0.156 mg/ml. Then, 20 µl of dimethyl sulfoxide (DMSO) was added to all wells except row A (Fig. 1). Subsequently, the stock sample was added to the wells (row A and row B). After that, the 2-fold dilution was done from row B to row G. 20 µl of the extract was discarded from row G to yield the same volume of sample for all wells (A-G).

	Quercetin	Sample 1	Sample 2	Sample 3	Concentration (mg/ml)
A	○ ○ ○	○ ○ ○	○ ○ ○	○ ○ ○	10.000
B	○ ○ ○	○ ○ ○	○ ○ ○	○ ○ ○	5.000
C	○ ○ ○	○ ○ ○	○ ○ ○	○ ○ ○	2.500
D	○ ○ ○	○ ○ ○	○ ○ ○	○ ○ ○	1.250
E	○ ○ ○	○ ○ ○	○ ○ ○	○ ○ ○	0.625
F	○ ○ ○	○ ○ ○	○ ○ ○	○ ○ ○	0.313
G	○ ○ ○	○ ○ ○	○ ○ ○	○ ○ ○	0.156
H	○ ○ ○	○ ○ ○	○ ○ ○	○ ○ ○	0 (DMSO)

Fig. 1. The concentration of samples after 2-fold dilution in 96-well plate.

Furthermore, 200 µl of the prepared DPPH solution was then added into all wells and the mixture was left to incubate for 30 min in the dark at room temperature. Finally, the absorbance was measured at 517 nm by enzyme-linked immunosorbent assay (ELISA) microplate reader (Multiskan ascent, Thermo Electron Corporation). The DPPH free radical scavenging activity was calculated according to the following equation:

$$Free\ radical\ scavenging\ activity\ (\%) = \frac{A_c - A_s}{A_c} \times 100\% \quad (1)$$

where A_s is the absorbance of the sample. A_c is the absorbance of the negative control.

A graph of the percentage of antioxidant against the concentration of samples was then plotted to calculate the IC_{50}. To determine the antioxidant activity of the samples, their IC_{50} values were compared with the IC_{50} value of positive control, quercetin.

2.2.5. Cell culture and treatment

HepG2 cells were acquired from Institute of Marine Biotechnology, Universiti Malaysia Terengganu. The cells were cultured in 25 cm^2 tissue culture flask containing M added with 10% fetal bovine serum, 1% non-essential amino acid, 1% sodium pyruvate and 1% penicillin-streptomycin (modified from Hamid et al., 2017). The HepG2 cells were then incubated and maintained at 37°C in 5% CO_2 incubator for a few days or until the cells confluence rate reach 80% or more of the culture flask. 1ml of 0.25% (v/v) trypsin was added into the culture flask to detach the cells from the flask wall. After adding the trypsin, the flask was incubated for 5-10 min in 37 °C in 5% CO_2 incubator. Then, 3 ml of complete culture medium was added to the cells as soon as the cells appeared to be rounded and solitary under the inverted phase-contrast microscope. The cells suspension was then transferred to a 15 ml centrifuge tube and then centrifuged at 1000 rpm and 20°C for 5 min. Later, the cells were resuspended in 1 ml fresh media and cell viability was determined by dyeing the cells with trypan blue before plating.

2.2.6. Cytotoxicity test

In this part, only samples which showed strong antioxidant activity were chosen to undergo MTT assay to access their cytotoxic effect on HepG2 cells. Similar to DPPH assay, quercetin (Q) was used as a positive control for MTT assay. The MTT assay was used to check for the cell viability against HepG2 cells after 72 hours of incubation with the selected sample and Q. The cytotoxicity capacity of HepG2 cell was obtained by carrying out MTT assay (adapted from Andriani et al., 2019). Each well in the 96 well plates was seeded with 8 x 10^4 ml cell/well with 100 µl of an aliquot of counted live HepG2 cell. The plate was incubated for 24 hours in 5% CO_2 incubator at 37 °C. After 24 hours, the cultured medium was discarded and 100 µl of crude extract diluted using culture medium (MEM media) with six different concentrations (60, 30, 15, 7.5, 3.75, 1.875 µg/ml) that were prepared by two-fold serial dilution and then added to the 96 well plates. A blank and positive control (quercetin) was also prepared. The quercetin had the same concentrations as the sample. The 96-well plate with samples was then incubated for 72 hours. Then, MTT was dissolved in PBS (5 mg/ml) and each well was loaded with 20 µl of the dissolved MTT. The plates were then incubated for another 4 hours. After 4 hours, all solution in each well was discarded and 100 µl of DMSO was added into each well. The plates were then incubated for another 10 min. The plates were shaken after incubation and the absorbance was measured by ELISA microplate reader (Multiskan Ascent, Thermo Electron Corporation) at 570 nm. The percentage of cell viability was then calculated using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{absorbance of sample}}{\text{absorbance of blank}} \text{x} 100\% \quad (2)$$

A graph of cell viability against the concentration of samples was plotted to calculate the IC_{50}. To determine the cytotoxicity properties of the samples on HepG2 cells, their IC_{50} values were compared with the IC_{50} value of standard quercetin.

2.3. Data analysis

All the experiments were conducted in triplicate and the data are presented as mean values ± standard deviation. The Pearson coefficient correlation between antioxidant and cytotoxicity property among methanol extracts of each plant were performed at the level P-value of <0.01 to evaluate the significance of differences between values. Statistical analyses are performed using SPSS 20.0 version.

3. Results and Discussion

3.1. Extraction yields

In this study, the dry powder of the three different leaves was extracted using two different solvents with increasing polarity: hexane and methanol. Most of the phytochemicals that are known to exhibit high antioxidant and cytotoxicity activities are usually found in polar fraction (Bekir et al., 2013). Bioactivity of samples will be disrupted if the samples extracted directly using methanol. It is probably caused by antagonistic effect between non-polar and polar constituents. Hence, in this study, the samples were extracted with hexane and methanol solvent, successively. Generally, the yield of methanolic extracts was much higher compared to hexane extracts. The highest extraction yield was obtained in methanolic extract of *M. leucadendra* (27.78%) while the lowest extraction yield obtained was in the hexane extract of *T. catappa* (1.32%). Among all three methanol extracts, the highest extraction yield was found out to be from *M. leucadendra* (27.78%) while the lowest was from *R. tomentosa* (10.75%). Among all three hexane extraction, the highest extraction yield was found out to be from *M. leucadendra* (2.44%) while the lowest was from *T. catappa* (1.32%). Overall, *M. leucadendra* has the highest combined extraction yield at 30.22% while *R. tomentosa* has the lowest combined extraction yield at 12.12% only.

3.2. Phytochemical screening

A total of six different phytochemical studies were carried out on all six plant extracts to find out the presence of chemical constituents such as alkaloids, phenolics, terpenoids, flavonoids saponins and glycosides (Table 1).

Table 1. Phytochemical test of all six plant extracts

Phytochemicals	Plant samples					
	MLH	TCH	RTH	MLM	TCM	RTM
Phenols	-	-	-	+	+	+
Flavonoids	-	-	-	+	-	+
Terpenoids	+	-	-	+	-	-
Alkaloids	+	-	-	+	-	-
Saponins	-	-	-	+	+	+
Glycosides	+	-	+	-	+	-

+: Positive result, -: negative result, MLH: *M. leucadendra* hexane extract, TCH: *T. catappa* hexane extract, RTH: *R. tomentosa* hexane extract, MLM: *M. leucadendra* methanol extract, TCM: *T. catappa* methanol extract, RTM: *R. tomentosa* methanol extract.

Based on the findings, methanolic extracts generally contain more variety of phytochemicals when compared to hexane extracts. Both phenolic and saponin compounds were detected only in the methanolic extracts of all three plants and none are found in the hexane extracts. Flavonoid compounds were only detected in methanolic extracts of *M. leucadendra* and *R. tomentosa* (MLM and RTM). Glycoside was found in the hexane extract of *M. leucadendra* and *R. tomentosa* (MLH and RTH) and methanolic extract of *T. catappa* (TCM). On the other hand, terpenoid and alkaloid were presented in both hexane and methanolic extracts of *M. leucadendra* (MLH and MLM) and were not detected in the other two plants which showed that *M. leucadendra* have the richest phytochemicals among the three tested plant specimens as it contained all six types of phytochemicals group tested. Overall, MLM has the richest phytochemical, containing five out of the six tested phytochemicals.

Phytochemical analysis conducted on *M. leucadendra* leaves extracts revealed that the leaves contained all six phytochemicals group tested (Table 1), showing the plant has the richest phytochemical compounds. *M. leucadendra* hexane extract contained terpenoids, alkaloids and glycosides while methanolic extract contained phenolics flavonoids, terpenoids, saponins, alkaloid and glycoside. The results showed similarity with a phytochemical characterization study done on methanolic leaves extract of *M. leucadendra* by Al-Abd et al. (2015). According to Al-Abd et al. (2015), the methanol extract predominantly contains phenolic compound and is unique with a rich source of different classes of terpenoids. It is interesting to note that both terpenoid and alkaloid were only found in *M. leucadendra* and not the other two plants. The presence of terpenoid and alkaloid could explain why its methanolic extract can outperform the other plants in terms of antioxidant activity (Fig. 1) and cytotoxic properties (Fig. 2).

Table 2. The IC_{50} value of antioxidant activity against DPPH radicals

Sample	IC_{50}, mg/ml	Percentage of inhibition at maximum concentration, %
Q	0.314	90.39 ± 0.62
MLH	-	41.84 ± 0.61
TCH	-	22.54 ± 1.4
RTH	5.47	67.41 ± 0.88
MLM	0.83	87.61 ± 0.48
TCM	1.62	83.32 ± 0.78
RTM	1.01	80.56 ± 1.05

Q: Quercetin, MLH: *M. leucadendra* hexane extract, TCH: *T. catappa* hexane extract, RTH: *R. tomentosa* hexane extract, MLM: *M. leucadendra* methanol extract, TCM: *T. catappa* methanol extract, RTM: *R. tomentosa* methanol extract. Data are mean ± SD values of triplicate determinations.

The result was found differently on *T. catappa* leaves extracts. Phytochemical screening performed on *T. catappa* showed that it was the poorest plant in terms of phytochemical richness compared to other plant extracts (Table 1). Only three groups of compounds were found in its methanol extract while no compounds were detected in its hexane extract. The three compound groups found in the methanol extract of *T. catappa* were phenolic, saponin and glycoside. The flavonoid, terpenoid and alkaloid were absent in the plant. This finding is similar to another phytochemical screening performed by Muhammad and Mudi (2011) on *T. catappa* leaves from Nigeria. However, they reported the presence of saponin in their hexane extract which was absent from our sample. This might be due to the difference in geographical condition as modern theories have established that secondary metabolites are expressed as a result of external stimuli. Based on this theory, completely different groups of metabolites can be produced in an organism depending on the environmental conditions, duration and intensity of stress, composition and genetic plastic of plants (Zhao et al., 2005).

Furthermore, a phytochemical study was also conducted on *R. tomentosa* leaves extracts, which found 4 phytochemicals compound group in its extracts (include both hexane and methanol

extracts). Glycoside was found in its hexane extract, while phenolic, flavonoid and saponin were found in its methanol extract (Table 1).

3.3. Antioxidant properties

A quantitative analysis using radical scavenging DPPH assay was used to identify the antioxidant activities of all six plant extracts. Quercetin was selected as the standard for the experiment and the result obtained from the DPPH assay was shown in Fig 2 and Table 2. Fig. 2 shows the percentage inhibition of free radical by plant extracts due to hydrogen donation from the antioxidant while Table 2 shows the respective IC_{50} value and percentage of inhibition at the maximum concentration of the samples. For the visual confirmation of results, extract with high antioxidant activity will significantly reduce the colour of the DPPH reagent from purple to yellow.

Generally, all methanolic extracts showed antioxidant activity of more than 50%, while only one hexane extract (RTH) showed antioxidant activity of more than 50%. For methanolic extracts, all sample showed IC_{50} of less than 2 mg/ml and lower than 1 mg/ml for MLM. By comparing IC_{50} values with the standard quercetin, which is 0.314 mg/ml, all extracts could be considered to have high antioxidant activity. For hexane extracts, only one extract (RTH) was able to achieve antioxidant activity of more than 50%. However, the sample was considered to have low antioxidant activity as its IC_{50} value was higher than 5 mg/ml. Overall, MLM demonstrated the strongest antioxidant activity of all six samples, achieving an IC_{50} value of lower than 1 mg/ml while the weakest antioxidant activity was displayed by TCH. It only managed to achieve 22.54 ± 1.4% antioxidant activity even when it is at the maximum concentration of 10 mg/ml. The order of antioxidant activity based on their respective IC_{50} value was Q>MLM>RTM>TCM>RTH>MLH>TCH. From the results, it showed that the plant which has the strongest antioxidant activity is *M. leucadendra* while the weakest is *T. catappa.*

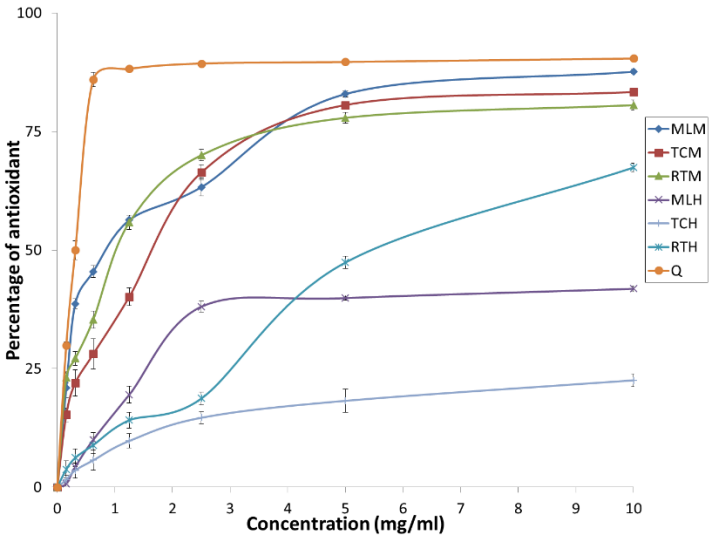

Fig. 2. The antioxidant property of all samples compared to standard (Q, quercetin). MLM: *M. leucadendra* methanol extract, TCM: *T. catappa* methanol extract, RTM: *R. tomentosa* methanol extract, MLH: *M. leucadendra* hexane extract, TCH: *T. catappa* hexane extract, RTH: *R. tomentosa* hexane extract. Each data in the graph is stated as means ± standard deviations.

According to Grassmann (2005), terpenoid is a highly effective antioxidant due to its ability to exert synergistic effects with other antioxidant and also among its class. In this study, even though the methanol extract of *M. leucadendra* ranked first among the samples in the DPPH assay, its IC_{50} was lower when compared with a previous study (Al-Abd et al., 2015). This reduction in antioxidant activity might be due to the difference in the extraction methods. In this study, the leaves were first extracted by hexane and followed by methanol, effectively separating its rich class of terpenoids into hydrophobic and hydrophilic groups. Hence, it might reduce the original synergistic effect of the terpenoid. Being the plant with the richest variety of phytochemicals, it is no doubt that it will have the strongest cytotoxic effect on HepG2 cells among the samples. *M. leucadendra* extract had previously been described as having high cytotoxicity towards a wide range of cancer cells crediting it to its rich phytochemicals (Almosnid et al., 2018). However, there is currently no study done to identify the cytotoxicity on HepG2 cell lines by methanolic extracts of the plant.

Furthermore, for the *T. catappa* leaves, TCH exhibited the lowest antioxidant activity due to the lack of phytochemicals in its hexane extract, However, its methanol extract possessed high of antioxidant activity based on the DPPH assay performed (Fig. 2). Many previous studies have highlighted the strong DPPH scavenging action and antioxidant activities possessed by leaves extract of *T. catappa* (Ko et al., 2002; Chyau et al., 2002; Kinoshita et al., 2007). According to Kinoshita et al. (2007), its high antioxidant activities were largely attributed to the phenolic compounds found in the plant. Six different phenolic compounds were isolated from a high antioxidant activity aqueous extract of the plant, showing its rich amount of phenolic content (Chyau et al., 2002). When the results from the DPPH assay of this study were compared with another similar study performed on the plant (Chyau et al., 2002), it revealed that our sample has weaker antioxidant activity. Again, this might be due to the difference in geographical conditions as both samples were collected in a different area.

Moreover, the antioxidant property of *R. tomentosa* leaves extract was shown in Fig. 2. It is found that *R. tomentosa* leaves extracts have high antioxidant activity. Flavonoids and saponins were found in its methanol extract could be correlated to this activity. The methanol extract showed an IC_{50} of 1.01 mg/ml, very close to the IC_{50} achieved by methanol extract of *M. leucadendra*. A previous DPPH assay done on a sample from Sarawak showed that various extracts of the leaves part also displayed a very promising antioxidant activity (Rosli et al., 2017). The strong antioxidant activity displayed by *R. tomentosa* leaves extract in both *in vitro* and *in vivo* studies indicated that it can act as a potent antioxidant (Lavanya et al., 2012). The most unique antioxidant feature of the plant is from its hexane extract. Based on our study, only hexane extract showed IC_{50} value in the DPPH assay. Its antioxidant activity was much higher than the phytochemical-rich hexane extract of *M. leucadendra*, suggesting it contains either very potent glycosides or other effective phytochemicals. Further works should be carried out to substantiate this finding and also isolate and characterize the compounds responsible for the bioactivity of this plant.

3.4. Cytotoxicity properties

Since only methanolic extracts of samples showed strong antioxidant activity, they were chosen to undergo MTT assay to access their cytotoxic effect on HepG2 cells. The IC_{50} achieved by the samples and quercetin are shown in Table 3. Results showed that all tested samples were cytotoxic to HepG2 cells (IC_{50} less than 30 µg/ml) and were able to induce the decrease of cell viability in a concentration-dependent manner. The standard quercetin has shown high cytotoxic activity against HepG2 cells (IC_{50} value of 7.5 µg/ml), while the samples all showed moderately cytotoxic with similar IC_{50} at lower than 30 µg/ml. According to Andriani et al. (2011), s sample with an IC_{50} value of higher than 30 µg/ml can be considered as non-cytotoxic categories to the cells. Hence, all samples can be considered as cytotoxic towards HepG2 cells. Among the methanolic samples, MLM displayed the greatest cytotoxic effect for HepG2 cells and it was able to achieve an IC_{50} of 24.7 µg/ml. On the other hand, RTM displayed the weakest

cytotoxic effect with an IC_{50} of 28 µg/ml. The result from MTT assay and DPPH assay showed high similarity and this further suggests that *M. leucadendra* is potentially to have better medicinal properties among the three plant samples.

Table 3. The IC_{50} value of MTT assay

Sample	MLM	TCM	RTM	Q
IC_{50}, µg/ml	24.7	26.6	28	7.5

Q: Quercetin, MLM: *M. leucadendra* methanol extract, TCM: *T. catappa* methanol extract, RTM: *R. tomentosa* methanol extract.

The cytotoxic activity against HepG2 cells displayed in this study might be due to the major phenolic and terpenoid compounds in *M. leucadendra*. Previous studies found that the major compound in essential oil from *M. leucadendra* leaves were 1,8-cineol, a type of terpenoid (Burits et al., 2001; Pujiarti et al., 2012). According to Rodenak et al. (2014), 1,8-cineol were able to significantly inhibit cell viability of HepG2 cell by having a synergistic antiproliferative effect with linalool. This might explain the high cytotoxic effect of *M. leucadendra* extract in this study as linalool has also been previously detected in the plant's essential oil (Pino et al., 2010). Based on the results from the extraction yield, *M. leucadendra* displayed a very high percentage of the recovery. This showed that *M. leucadendra* has enormous potential to be developed into commercial drugs as it will be very economical to extract it. Given its high antioxidant and cytotoxicity against HepG2 cells, there is a huge potential for it to be developed into future anti-liver cancer drugs. Future research can be focused on a more detailed study on how HepG2 cells react under *M. leucadendra* extract such as its morphological change. Studies can also be carried out to identify the major compounds that are responsible for the high anticancer properties of the plant. *M. leucadendra* is found to have various medicinal properties such as antifungal, antibacterial, anti-HSV-1, antigenic, inhibitors of induced histamine release (Bandaranayake, 2002). Also, for anticancer potencies, *M. leucadendra* showed the highest cytotoxicity against four different cancer cells (MCF7, A549, BT20 and U2OS) with an IC_{50} range of 3.1-32.7 µg/ml. Extract from the plant showed the highest cytotoxicity on BT20 cells while the lowest on MCF7 cells (Almosnid et al., 2018).

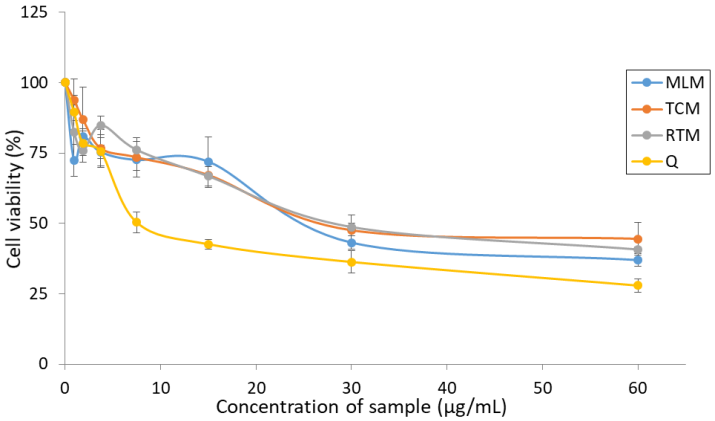

Fig. 3. Cytotoxicity property of methanolic extracts of samples compared to standard (quercetin) toward HepG2 cells. MLM: *M. leucadendra* methanol extract, TCM: *T. catappa* methanol extract, RTM: *R. tomentosa* methanol extract, Q: Quercetin. Each value in the graph is expressed as means ± standard deviations.

Many previous studies have shown *T. catappa* leaves extract possesses hepatoprotective activity (Gao et al., 2004; Kinoshita et al., 2007). Due to this, very few studies were done to access the cytotoxicity properties of *T. catappa* against human hepatoma cells as the plant is known more popularly as having hepatoprotective effects instead of damaging liver cells. A cytotoxicity study of *T. catappa* leaves performed on a type of liver cancer cell (Huh 7) showed weak cytotoxicity activity (Ko et al., 2003). Based on Andriani et al. (2011), *T. catappa* demonstrated moderately cytotoxic activity against HepG2 cells in this study (Fig. 3). Overall, *T. catappa* in this study was found to have fewer phytochemicals content and lowest antioxidant activity when compared with other previous studies. This might be due to geographical factors. Therefore, a detailed phytochemical screening should be carried out on local *T. catappa*. This can eventually help researchers to identify the compounds that are responsible for the plant's high antioxidant activity in other international samples by comparing the chemical constituents from local and international samples.

Table 4. Antioxidant activity and cytotoxicity property of methanol extracts of each selected plant (MLM, TCM, RTM) and quercetin.

Sample	Concentration of sample, mg/ml	Antioxidant activity, % DPPH	Concentration of sample, mg/ml	Cytotoxicity property, % MTT**
MLM	10	87.61±0.48	100	36.97±2.12
	5	82.90±0.63	50	43.17±2.36
	2.5	63.27±1.89	25	71.92±8.77
	0.125	56.32±1.03	12.5	72.53±3.86
	0.625	45.46±1.25	6.25	75.32±2.33
	0.313	38.68±1.042	3.125	80.83±2.85
	0.156	20.95±2.041	0.156	72.37±5.63
	0	0.00±0.00	0.00	100.00±0.00
TCM	10	83.32±0.78	100	44.50±5.78
	5	80.59±0.72	50	47.57±5.29
	2.5	66.37±1.65	25	67.17±4.38
	0.125	40.15±1.84	12.5	73.45±7.08
	0.625	28.08±3.18	6.25	76.59±6.63
	0.313	21.97±2.76	3.125	86.87±11.47
	0.156	15.27±1.48	0.156	93.86±7.33
	0	0.00±0.00	0.00	100.00±0.00
RTM	10	80.56±1.05	100	40.73±0.89
	5	77.88±1.16	50	48.70±1.25
	2.5	70.01±1.21	25	66.76±3.46
	0.125	55.84±1.51	12.5	76.11±3.08
	0.625	35.34±1.82	6.25	84.85±3.33
	0.313	27.13±1.42	3.125	75.74±3.96
	0.156	23.16±1.17	0.156	82.51±9.45
	0.00	0.00±0.00	0.00	100.00±0.00
Q	1	90.39±0.62	100	27.92±2.40
	0.5	89.67±0.39	50	36.29±3.97
	0.25	89.34±0.51	25	42.59±1.76
	0.013	88.20±0.29	12.5	50.35±3.76
	0.063	85.92±1.50	6.25	75.48±5.06
	0.031	49.96±1.97	3.125	78.47±4.42
	0.016	29.94±0.70	0.156	89.41±5.98
	0.00	0.00±0.00	0.00	100.00±0.00

**Significant correlation between antioxidant and cytotoxicity values at $p<0.01$. MLM: *M. leucadendra* methanol extract, TCM: *T. catappa* methanol extract, RTM: *R. tomentosa* methanol extract, Q: Quercetin. Each data is the mean of three replicates. Each data was stated as the mean±standard deviation (SD).

Result of MTT assay from this study characterized the cytotoxicity of *R. tomentosa* against HepG2 cell as moderately cytotoxic activity (Fig. 3). A previous study found that methanol root extract of *R. tomentosa* possesses high anti-proliferation effect on HepG2 cells and suggested that the effect might be due to the abundance of flavonoid and phenolic content in its extract (Hamid et al., 2017). It is recommended that more detailed study such as deep and systematic phytochemical investigation and its mechanism of toxicity action to be carried out on the extracts of this study to figure out what caused the difference in cytotoxicity displayed by the leaves and root extract of the plant. In recent years, there are considerable phytochemical studies aimed at exploring the relatively unknown *R. tomentosa* leaves and have resulted in

the isolation of several unique phloroglucinols which are all novel to science (Hiranrat and Mahabusarakam, 2008; Liu et al., 2016). However, their cytotoxicity is yet to be tested and more can be done in the future to determine their bioactivities. According to (Hamid et al., 2017), while *R. tomentosa* displayed excellent antioxidant properties, there are only a small number of toxicity studies conducted for the plant. The results from this study might provide a reference for future toxicity profiling of *R. tomentosa* crude extract on human hepatoma cells as most of the current cytotoxicity studies were focused on the root and fruit of the plant. The results also showed that *R. tomentosa* has the potential of being developed into a natural antioxidant additive in food due to its high antioxidant activity and relatively low cytotoxicity to human cells. Table 4 showed the significant correlation between antioxidant activity and cytotoxicity property of each methanol extracts at $p<0.01$. This data showed that increasing the sample concentrations followed by increasing of antioxidant activity as well as cytotoxicity property against HepG2 cells. Study on the correlation between antioxidant and cytotoxicity properties have been reported by Sammar et al. (2019). They revealed that the extracts of plants that exhibit a high of the free radical scavenging show a degree of enrichment toward increased cytotoxicity.

4. Conclusion

All methanol extracts of samples possessed high antioxidant activity and mild cytotoxicity against HepG2 cell lines. Among the plants, *M. leucadendra* was proven to be the plant with the highest potential of being developed into future anti-liver cancer drug due to its high antioxidant property and cytotoxic activity against HepG2 cells. It also showed the highest percentage of yield from the extraction process. Further study of bioactive compounds isolation can be done on this plant to assess their ability and investigate their mechanisms of action as potential agents in treating cancer.

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Conflict of Interest

The authors stated no conflict of interests in this research.

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