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Antioxidant And Cytotoxicity Properties of *Clitoria ternatea* Extract Towards K562 Leukaemia Cells

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Abstract
The local medicinal plant has been shown to be effective against cancer. Chronic myeloid leukaemia (CML) is a type of hematopoietic stem cell cancer that accounts for 15% to 20% of all leukaemia cases. The conventional treatment is expensive, and long-term use of tyrosine kinase inhibitors (TKI) causes treatment-related side effects. *Clitoria ternatea* is a local medicinal plant with the potential for cancer treatment that is still underutilised due to its economic and pharmacological value. This study aimed to assess the antioxidant and cytotoxicity of *C. ternatea* leaves and flower extract toward the CML, K562 cell line. The total phenolic quantification, in vitro antioxidant DPPH assay and in vitro cytotoxicity MTT assay for each aqueous and 50% methanolic extract of leaves and flower were performed at various concentrations of the extracts. The total phenolic content showed a direct relationship with antioxidant activity. The 50% methanolic flower extract contributed the highest antioxidant activity at an IC_{50} value of 40.703 ± 6.1339 µg/mL. Moreover, 50% methanolic flower was shown to exert more cytotoxicity toward K562 at IC_{50} value of 461.2 ± 13.555, 319.27 ± 152.19, and 278.2 ± 162.62 µg/mL at treatment periods 24, 48, and 72 hours respectively. Due to the compromisation of the microplate reading by the strong extract colouration, the MTT quantitative result aligned with the morphological differences of the treated group revealed that necrosis was induced on K562 at 600 µg/mL for 24, 48 and 72 hours, respectively. Thus, *C. ternatea*'s antioxidant activity was attributed to the phenolic content. The radical scavenging activity in 50% methanolic flower extract was due to the presence of anthocyanin. These antioxidant properties further contribute to the radical scavenging activity toward the reactive oxygen species (ROS) produced by the K562 cell leading to a decrease in cell viability over time.
Keywords: K562; *Clitoria ternatea*; total phenolic content; DPPH assay; MTT assay; IC50 value

Introduction

Malaysia was known for its rich biodiversity, with 1,300 medicinal plant species have been documented in Peninsular Malaysia (Abu et al., 2018). Local medicinal plants have been extensively used to address mild and chronic illnesses as well as proven to be useful against acute and more severe health issues, such as cancer by focusing on their preventive, palliative, and curative as well as dealing with the side effects of conventional treatments (Gras et al., 2022). Chronic myeloid leukaemia (CML) was one of the malignant disorders of hematopoietic stem cells that probably appear in 15% to 20% of all leukaemia cases in adults. The CML was a result of the abnormal reciprocal translocation between chromosomes that led to the formation of oncogene fusion BCR-ABL (Flis & Chojnacki, 2019). The development of tyrosine kinase inhibitors (TKIs), for instance, imatinib last 22 years has become an outstanding revolution in the treatment of CML and a paradigm for targeted chemotherapy of CML (Bonifacio et al., 2019).

Local medicinal plants become an additional and traditional treatment of cancer when there were obstacles in conventional treatments. The medicinal plant has a vast amount of natural anticancer compounds comprising phenolic derivatives, flavonoids, alkaloids, saponins, terpenoids, quinones, carotenoids, and steroids (Dehelean et al., 2021). These compounds become adjuvants in

cancer treatment and chemosensitizers help to promote the tumoricidal effect and cytotoxic effect of conventional chemotherapeutic drugs thus increasing the drug efficacy at a lower dose (Dehelean et al., 2021). The bioactive compounds in the medicinal plant not only serve as a chemotherapeutic agent that induced cytotoxicity in the cancer cell but at the same time act as chemopreventive agents by exerting antioxidant activity in cancer prevention (Siddiqui et al., 2022).

The previous study has shown that the preliminary phytochemical screening of ethanol and ethyl acetate extract of *C. ternatea* has four constituents which are glycosides, flavonoids, tannins and triterpenoids that exhibit beneficial medicinal properties (Arsianti et al., 2022). It has also been proposed that the antioxidant activity of the components of the plant origin can be mainly ascribed to the presence of the phenolic compounds due to their high radical scavenging activity (Taviano et al., 2017). Furthermore, it was proven that ethanol and ethyl acetate extract of *C. ternatea* possesses very active antioxidant activity and a high cytotoxicity activity towards the T47D breast cancer cell line (Arsianti et al., 2022). Moreover, one of the studies involving the cytotoxicity assay on the acute promyelocytic leukaemia HL60 cell line showed that methanolic leaves extract of *C. ternatea* exerts slight to severe cytotoxic reactivity to HL60 cells was proportional to the concentration of the extracts (Das et al., 2020).

Based on these assumptions that *C. ternatea* was also a potential source of treatment for chronic myeloid leukaemia (CML), especially for those patients bearing high costs of maintenance of therapy with TKIs and the treatment-associated adverse effects. *C. ternatea* has the ability on becoming economically as well as a pharmaceutically valuable medicinal plant in the future. However, investigation on antioxidant activity and cytotoxicity activity of *C. ternatea* polar extracts on CML is still limited. Therefore, the present study was designed to define and compare the total phenolic content, antioxidant and cytotoxicity properties of the polar extracts obtained from the leaves and flowers of *C. ternatea* toward the K562 cell line. In addition, further comparisons on cytotoxicity were carried out with normal cell line HSF1184, especially on the morphology of the cells.

Materials and methods

Approximately 200 g of the leaves of the blue variety of the *C. ternatea* were collected from the backyard of the T02, Department of Bioscience, Faculty of Science (Longitude: 1.5649° N, Latitude: 103.6538° E). The leaves were air-dried in the shade for two weeks while 200 g of the dried flower of the blue variety of *C. ternatea* was purchased from an online store (SF FOOD Trading, Selangor, Malaysia. Both dried leaves and flowers were powdered by using an electric grinder (WARING COMMERCIAL, USA) and sieved using a sieve at a particle size of ≤ 20 mm separately. The processed sample was at -40℃ in the freezer in dark conditions.

5 g of powdered leaves and flowers were weight by using a precision balance (SARTORIUS, Goettingen, Germany) and extracted using 100 ml of 50% methanol (Merck KGaA, Darmstadt, Germany) and distilled water separately via dark maceration. Extraction using distilled water was carried out by gently shaking of 250 mL conical flask (SCHOTT Glass, Penang, Malaysia) in the water bath (Yamato Scientific, California, USA) at a temperature of 50℃ for an hour. Meanwhile, 50% methanol extraction was carried out with constant shaking of a 250 mL conical flask (SCHOTT Glass, Penang, Malaysia) with a circular motion at a speed of 100 rpm in the incubator shaker (PROTECH, Balakong, Selangor) at a temperature of 25℃ for 3 hours. The extract was filtered using Whatman No. 1 (Merck KGaA, Darmstadt, Germany) and concentrated separately by using a vacuum rotary evaporator (IKA® Works, Rawang, Selangor) at 45℃ at a speed of 100 rpm. The extract was freeze-dried at -80℃ and stored at the same temperature. The weight of the extract was measured, and of yield was determined.

The freeze-dried extract was dissolved in distilled water to a concentration of stock solution of extract at 1 mg/mL. Gallic acid was used as the standard, and a calibration curve was established within the range of 0 to 100 µg/mL. The 0.1 mL extract or gallic acid was added to 0.75mL Folin-Ciocalteu reagent (Millipore, Shah Alam, Malaysia) that was previously tenfold diluted with distilled water and mixed thoroughly to allow the mixture to react in darkness for 3 minutes. Sodium carbonate (0.75 mL, 7.5% w/v) was added to the mixture and shaken. The mixture was allowed to stand in darkness for 30 minutes at room temperature. The absorbance of the mixture was measured using a UV-Vis spectrophotometer (Beckman Coulter, Indiana, US) and a microplate reader (Beckman

Coulter, Indiana, US) at a wavelength of 760nm. Three independent measurements were recorded and TPC was expressed as mg gallic acid equivalent/g dry weight of extract (mg GAE/g DE extract) using the slope equation of the gallic acid calibration curve obtained.

A methanolic solution of DPPH (2,2-Diphenyl-1-picrylhydrazyl) at 100μM was prepared. The triplicate of each extract was prepared at twelve different concentrations ranging from 0 – 2000 μg/mL. 300 μL of DPPH solution was added to 300 μL of extract. The mixture was vortexed (SCIENTIFIC ZX3, Usmate, Italy) and left to stand for 30 minutes in the dark at room temperature, followed by absorbance measurement at a wavelength of 517 nm. 600 μL of DPPH prepared with distilled water and methanolic DPPH solution respectively use as the negative control. A positive control or standard was prepared by mixing 300 μL of DPPH solution with 300 μL ascorbic acid (Acros Organics, Geel, Belgium) at the same concentration range of plant extract as well as the solvent without DPPH used as blank. The radical scavenging activity or percentage of inhibition was calculated by using the equation (1) below:

$$Scavenging\ activity\ (\%) = \frac{(OD\ of\ control\ -\ OD\ of\ sample)}{(OD\ of\ Control)} \times 100 \quad (1)$$

The graph of the percentage of the scavenging activity was plotted against the concentration of the extracts and ascorbic acid. IC_{50} was determined through the graphical method through the best-fit curve plotted. The antioxidant intensity for the extract was recognized according to the power levels of antioxidant by DPPH method: Highly active antioxidant activity (IC_{50} <50 μg/mL); Active antioxidant activity (50 μg/mL < IC_{50} <100 μg/mL); Moderate antioxidant activity (101 μg/mL < IC_{50} <250 μg/mL); Weak antioxidant activity (250 μg/mL < IC_{50} <500 μg/mL); and Inactive antioxidant activity (IC_{50} >500 μg/mL) (Marjoni et al., 2017).

For the cytotoxicity testing, the K562 as the chronic myeloid cancer cell obtained from the Institute for Medical Research (IMR), Malaysia and the human fibroblast cell HSF1184 as a normal cell were used to compare cytotoxicity. K562 cells and HSF1184 cells were cultured in the Roswell Park Memorial Institute (RPMI) medium (Gibco, Scotland, UK) and Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Scotland, UK) respectively. Both mediums were supplemented with 10 % fetal bovine serum (FBS) (Sigma-Aldrich, Selangor, Malaysia), and 1% penicillin-streptomycin (Gibco, Scotland, UK). The cells were grown in the prepared medium in a humidified atmosphere with 95% air and 5% CO_2 at 37℃.

The cytotoxicity activity of the extract was assayed according to the published method (Rajput & Bithel, 2022) with some modifications. The inhibitory effect of extracts was analysed via MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. The extracts must be sterile filtered with a syringe filter 0.22 μm nylon filter membrane. 40μL of cell suspension at the density of 1×10^5 cells per mL cultured and grown to the log phase was seeded in 96-well plate wells and observed under the microscope followed by overnight incubation in a humidified incubator. Subsequently, 40μL of various concentrations of extract ranging from 50 – 2000 μg/mL in the sterile medium was added and incubated for 24, 48 and 72 hours respectively. After incubation, the 10μL of MTT reagent at a concentration of 5mg/ml was mixed with the reaction mixture following incubation for 4 hours. The MTT reagent was prepared by mixing 20 mg of MTT with 4 mL of sterile PBS until vortexing dissolved, sterile filtered using a syringe filter 0.22 μm nylon filter membrane and stored at -40℃ in a freezer.

The cells before the addition of the MTT reagent were observed for the morphological view of the cell under an inverted microscope as the qualitative analysis. After incubation with the MTT reagent, the formazan crystal was solubilized in 100μL 0.01N SDS-HCL for cell lysis and incubated for 18 hours. The absorbance was recorded at a wavelength of 570nm. 0.01N SDS-HCL was preferred rather than DMSO as a solvent for dissolving the formazan crystal in the MTT assay due to the stable formazan solution (Benov, 2019). On the other side, using 0.01N SSD-HCl also can prevent the formation of protein precipitation (Septisetyani et al., 2014). The non-treated cell culture with 0.01N SDS-HCL was taken as the negative control and sterile complete media alone was taken as blank (Rajput & Bithel, 2022). Due to the interference of *C. ternatea* flower extract at 570nm on the reading of the microplate reader, background interference was deducted by taking the reading of

extracts in media alone. The graph of percentage cell viability against extract concentration was constructed, and the IC_{50} value was identified. The percentage of cell viability was calculated by using the following equation (2) as shown below:

$$\%\ Cell\ Viability = \frac{OD\ of\ Treated\ cells - OD\ of\ Blank}{OD\ of\ control\ cells - OD\ of\ Blank} \times 100 \quad (2)$$

The statistical data analysis was carried out following the method published by Taviano et al., (2017) due to the similarity of the goal of the researcher with the objective of this study. All the quantitative data will be reported as the mean ± standard deviation of independent triplicates (n=3). Results were subjected to a one-way analysis of variance (ANOVA) followed by a post-hoc Tukey-Kramer multiple comparison test for total phenolic content and DPPH assay. Meanwhile, the cytotoxicity assay data was analysed by one-way ANOVA followed by Dunnett's multiple comparison test since there are control groups. The *P*-values lower than 0.05 were considered statistically significant. All these analyses were carried out using GraphPad Prism version 9.5.1.

Results and discussion

The setup of the extraction condition parameter was the most crucial part, especially when the extract was used in subsequent analysis or assay. The type of solvent, the concentration of the solvent, temperature, extraction duration and solid-to-liquid ratio can significantly affect the extraction yield of bioactive compounds from *C. ternatea* (Dumbrava et al., 2019). The choice of the solvent and the condition of the extraction influence the quantification of the total phenolic content and antioxidant activity of the extracts in the subsequent steps (Karle et al., 2021). Based on Table 1, the extractable weight of the crude extracts from a total of 5.0 g of the powdered *C. ternatea* sample used in maceration was nearly the same for both plant portions within different types of extracts.

According to Zulkamal et al., (2023), the yield of the *C. ternatea* methanolic and aqueous flower extract was 9.1% and 8.1%, respectively, which was lower than the extraction yield of flower extract shown in Table 1. The extraction yield of methanolic leaves extract also does not deviate a lot from a study by Yun & Pa'ee, (2022), in which the extraction yield obtained was 20.29% when using a solid-to-liquid ratio of 1:10. On the other side, the extraction yield shown by Jeyaraj et al., (2021) for 100% methanolic extract of leaves at 6.5% which was less than the yield contributed by 50% methanolic leaves extract at 21.67% as shown in Table 1 even though the extraction time was prolonged to 12 hours by the researcher. This suggests that an increase in the polarity of the solvent results in higher extraction yields. Thus, the solid-to-liquid ratio and solvent polarity that reflects the solvent types used strongly influence the extraction yields.

Table 1 The weight and extraction yield of *C. ternatea* crude extracts for different types of extracts and plant body parts.

***C. ternatea* Extracts**		**Extraction Yields**	
		Weight of Crude Extract (g)	**Percentage of Yield (%)**
Leaves	Aqueous	1.1583	23.17
	50% Methanolic	1.0834	21.67
Flower	Aqueous	2.0356	40.71
	50% Methanolic	2.1186	42.37

Quantification of total phenolic content was carried out using two different instruments, which were the UV-Vis spectrophotometer and the microplate reader. Figure 1 and 2 shows the construction of the gallic acid calibration curve using both types of instruments respectively. The *R*-squared value nearer to value 1 on both curves in Figures 1 and 2 indicates a strong linear relationship between the gallic acid concentration and the measured response, as mentioned by Toledo et al., (2016). However, both gallic acid standard curves differ in the slope gradient value as shown by the equation

y = 0.007460x + 0.01464 in Figure 1 and y = 0.004276x + 0.009636 in Figure 2. This difference in slope gradient and precision of the slope influenced the accuracy of the slope estimates (Solomon et al., 2021). Hence the accuracy of the total phenolic content estimation was affected by the type of instrument used.

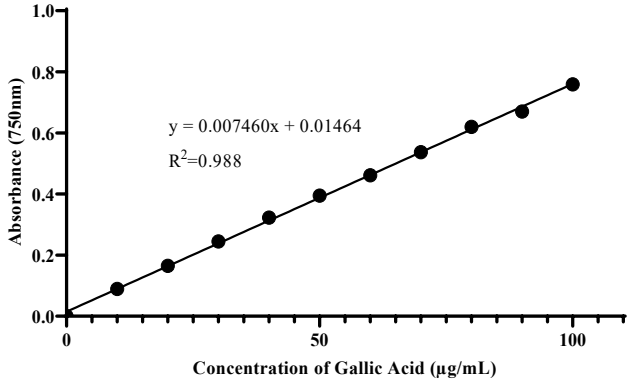


Figure 1 The standard gallic acid curve constructed from TPC assay using a UV-Vis spectrophotometer.

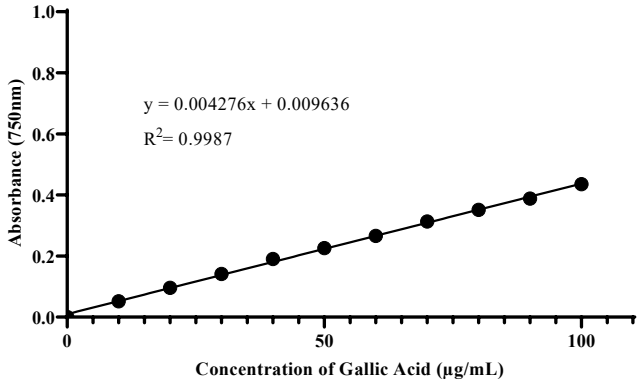


Figure 1 The standard gallic acid curve constructed from TPC assay using a microplate reader.

The quantification of total phenolic content by using the gallic acid curve constructed via UV-Vis spectrophotometer was more accurate and precise due to the significant comparison between the total phenolic content of the extracts based on Figure 3. When compared to the total phenolic content shown in Figure 4, the estimation of total phenolic content using the microplate reader resulted in the insignificant value of the total phenolic content in between some types of extract. A lot of previous researchers agreed that generally, UV-Vis spectrophotometer has higher sensitivity compared to that of the microplate reader in which UV-Vis spectrophotometer can measure the absorbance at the wide range of wavelengths that allows the precise measurement of analyte in sample that results in higher resolution and better signal-to-noise ratio (Lewis & Gibney, 2023; Upadhyayet al., 2021; and Beck et al., 2016). Considering the estimation using UV-Vis spectrophotometer was more accurate and reliable, the total phenolic content of leaves extracts in which was 52.422 ± 0.89254 mg GAE/g DE and 62.475 ± 0.50750 mg GAE/g DE for aqueous and 50% methanolic leaves respectively was higher compare to that of the total phenolic content of the flower extract in which 43.172 ± 0.50750 mg GAE/g DE and 48.266 ± 0.50749 mg GAE/g DE for aqueous and 50% methanolic flower respectively based on Table 2.

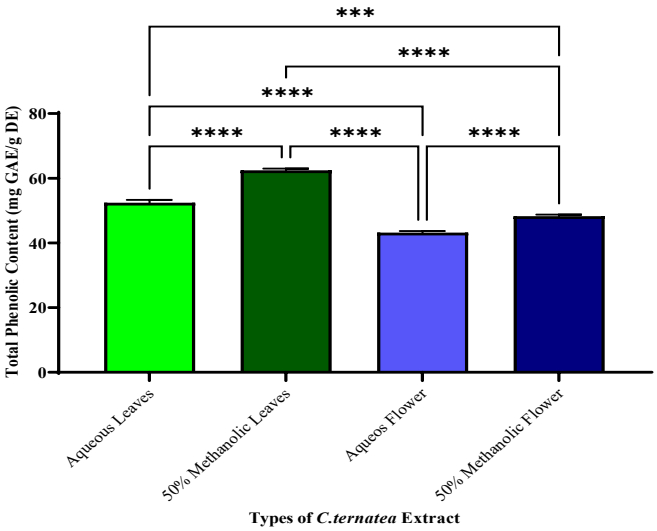


Figure 2 The comparison between the total phenolic content within each extract using UV-Vis spectrophotometer is statistically significant if the *p*-value is less than 0.05. *p* value less than 0.05 is represented by an asterisk "*", where *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ (One-way ANOVA followed by a Tukey-Kramer multiple comparison test).

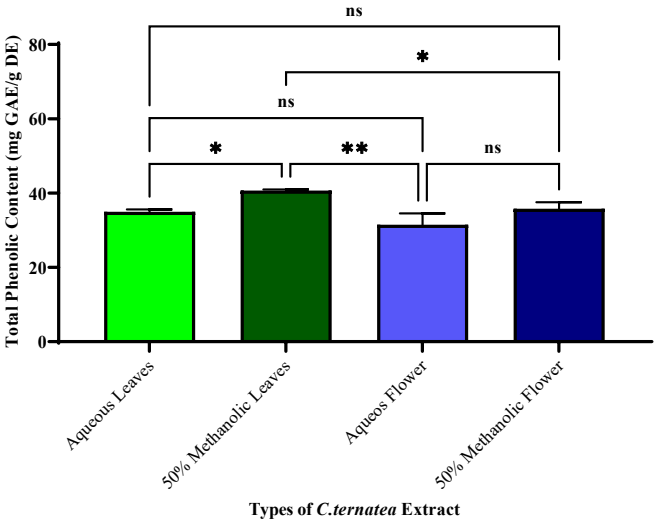


Figure 3 The comparison between the total phenolic content within each extract using a microplate reader is statistically significant if the *p*-value is less than 0.05. *p* value less than 0.05 is represented by an asterisk "*", where *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ (One-way ANOVA followed by a Tukey-Kramer multiple comparison test). *p* higher than 0.05 is represented by "ns" (non-significant)

Table 1 The total phenolic content of *C. ternatea* extract quantified using different types of extract.

***C. ternatea* Extracts**		**Total Phenolic Content (mg GAE/ g DE)**	
		UV-Vis Spectrophotometer	**Microplate Reader**
Leaves	Aqueous	52.422 ± 0.89254	34.989 ± 0.619874
	50% Methanolic	62.475 ± 0.50750	40.680 ± 0.27004
Flower	Aqueous	43.172 ± 0.50750	31.481 ± 3.0671
	50% Methanolic	48.266 ± 0.50749	35.769 ± 1.7708

Even though these result does not align with the finding by Havananda & Luengwilai, (2019) that the *C. ternatea* flower has higher total phenolic content than the leaves extract, some other researcher has found that *C. ternatea* leaves have high total phenolic content compared to the flower on both solvents used (Durga et al., 2014 ; Rabeta & Nabil, 2013; and Pengkumsri et al., 2019). Besides that, when compared in terms of the solvent, the methanolic extract showed higher total phenolic content than that of the aqueous extract. This was due to the solubility of the phenolic content in organic solvents being higher compared to the non-organic solvent such as distilled water (Dibacto et al., 2021; Zuorro et al., 2019). However, some study also proposed that the solubility of the phenolic content was dependent on the specific phenolic compound in which finding by Zhao et al., (2018) and Sireen & Anbumalarmathi, (2020) in their study using different sample shows that higher total phenolic content was mainly attributed by aqueous extract compared to the methanolic extract.

According to the power levels of antioxidants of the DPPH method by (Marjoni et al., 2017), 50% methanolic flower has highly active antioxidant activity at IC_{50} value 40.703 ± 6.1339 µg/mL compared to three other types of extract, which is 57.473 ± 6.7546 µg/mL, 947.07 ± 77.033 µg/mL and 549.37 ± 14.368 µg/mL respectively for aqueous flower, aqueous leaves and 50% methanolic leaves extract which has lower IC_{50} value as shown in Table 3 and Figure 5. Both types of leaf extract have inactive antioxidant activity due to IC_{50} values of more than 500 µg/mL. 50% methanolic flower extract has highly active antioxidant intensity same as ascorbic but antioxidant intensity for aqueous flower extract was only active. Since the lower the IC_{50} value of the extract, the higher it is antioxidant activity, there was a significant relationship between the total phenolic content and the antioxidant activity. The total phenolic content for both leaves and flower extract was inversely proportional to the IC_{50} value of the DPPH assay. From this relationship, it can be deduced that the phenolic compound was the major compound contributing to the antioxidant properties of the used plant.

Table 2 The IC_{50} value of *C. ternatea* plant extracts in DPPH antioxidant assay. Power levels of antioxidants: Highly active antioxidant activity (IC_{50} <50 µg/mL); Active antioxidant activity (50 µg/mL < IC_{50} <100 µg/mL); Moderate antioxidant activity (101 µg/mL < IC_{50} <250 µg/mL); Weak antioxidant activity (250 µg/mL < IC_{50} <500 µg/mL); Inactive antioxidant activity (IC_{50} >500 µg/mL).

***C. ternatea* Extracts**		**DPPH Assay**	
		IC_{50} Value (µg/mL)	**Antioxidant Intensity**
Standard (Ascorbic Acid)		2.718 ± 0.33692	Highly Active
Leaves	Aqueous	947.07 ± 77.033	Inactive
	50% Methanolic	549.37 ± 14.368	Inactive
Flower	Aqueous	57.473 ± 6.7546	Active
	50% Methanolic	40.703 ± 6.1339	Highly Active

The antioxidant activity of *C. ternatea* flower using both methanolic and aqueous extract exert a higher percentage of DPPH scavenging activity than the leaves extract. This was supported by the findings by Gamage et al, (2021b) that flower extract exerted high antioxidant activity due to the presence of anthocyanin, the coloured pigment in flower that plays its role in the radical scavenging activity compared to that of the leaves of *C. ternatea*. Besides that, this finding as well aligns with the general claim by most researchers that the coloured flower of plants exerts high antioxidant activity than leaves due to the presence of anthocyanin (Naing & Kim, (2021); Park et al., (2019); Chepel et al., (2020). In terms of solvent used, the methanolic extract for both types of flower and leaf extracts has high antioxidant activity compared to that of the aqueous extract.

Since methanolic extract resulted in high total phenolic content in both flower and leaves extract, it can be concluded that in the methanol extracted sample, the higher TPC resulted from the solubility nature of plant phenolics that was enhanced by the organic solvent, which facilitates the solubilisation through penetration in plant cell structure (Rabeta & Nabil, 2013). Another study used

the same type of solvents and *C. ternatea* leaves as well stating that methanolic extract has higher radical scavenging activity compared to that of the aqueous extract (Jayanthi et al., 2021). When comparing the antioxidant activity between types of extract based on the plant part, the flower extracts IC_{50} values were not significant between both types of solvent but the leaf extract has IC_{50} values significant between both types of solvent as shown in Figure 6. Both aqueous and methanolic flower IC_{50} values are not significant to standard ascorbic acid based on Figure 6.

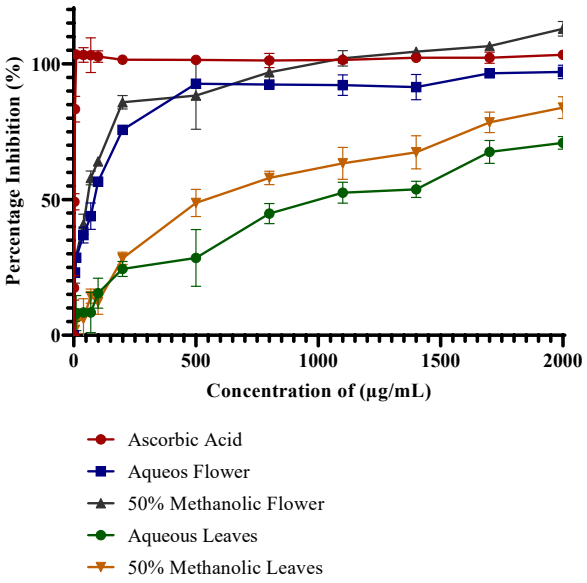


Figure 4 The curve on the radical scavenging activity exerted by different types of *C. ternatea* plant extract.

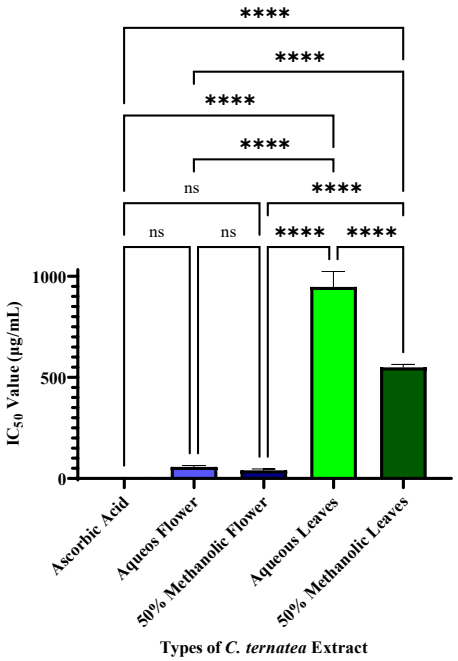


Figure 5 The comparison between the IC_{50} value obtained in the DPPH assay within each extract using a microplate reader as statistically significant if the *p-value* is less than 0.05. *p* value less than 0.05 is represented by an asterisk "*", where *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$ (One-way ANOVA followed by a Tukey-Kramer multiple comparison test). *p* higher than 0.05 is represented by "ns" (non-significant)

The main principle of the MTT assay was based on the conversion of MTT into purple colour formazan crystal by active mitochondrial dehydrogenase in living cells (Meerloo et al., 2011). The amount of formazan was proportional to the number of viable cells in the treatment sample. Based on Figure 7 as well as Table 4, the cytotoxicity of the *C. ternatea* extract increase with the increase of the incubation time and increasing on the concentration of the extracts. This type of cytotoxicity was said to be in a dose-dependent manner in which a decrease in cell viability indicates an increase in cytotoxicity (Talib & Mahasneh, 2010). The lowest IC_{50} value for incubation within 24, 48 and 72 hours are mainly by 50% methanolic flower extract with respective IC_{50} values of 461.2 ± 13.555 µg/mL, 319.27 ± 152.19 µg/mL and 278.2 ± 162.62 µg/mL as shown in Table 4. These showed that 50% methanolic flower extract has high cytotoxicity toward K562, followed by the aqueous flower extract. The highest IC_{50} value, which was 1363 ± 308 µg/mL, 1003.6 ± 111.8 µg/mL and 913.6 ± 110.85 µg/mL, was attributed to the 50% methanolic leaves extract, respectively for 24, 48 and 72 hours of incubation. This indicates that 50% methanolic leaves extract exert the lowest cytotoxicity among other *C. ternatea* extract.

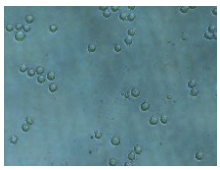
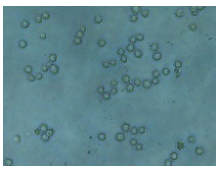
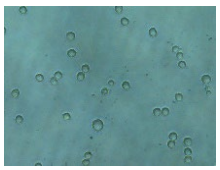
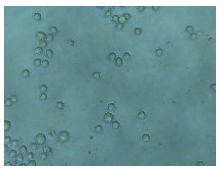
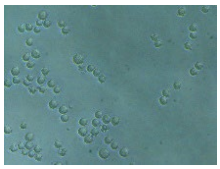
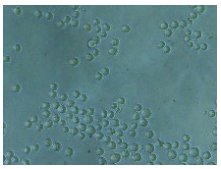
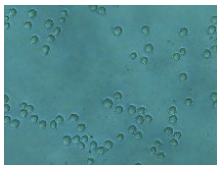
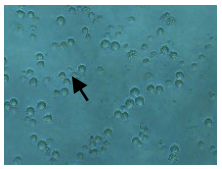
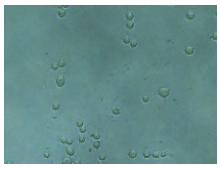
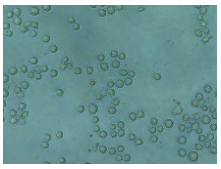
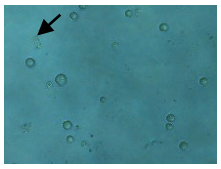
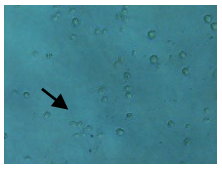
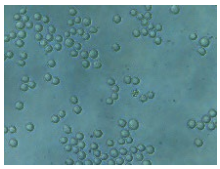
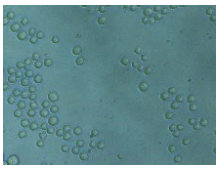
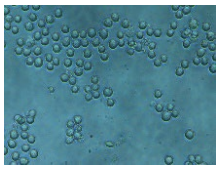
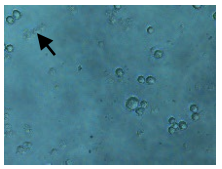
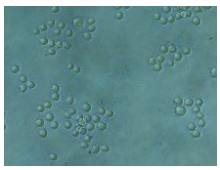
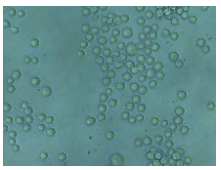
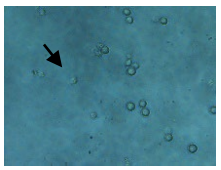
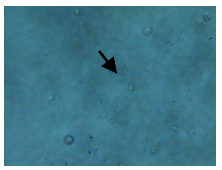
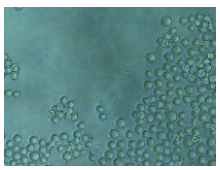
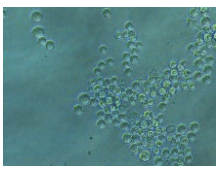
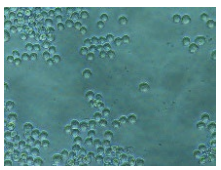
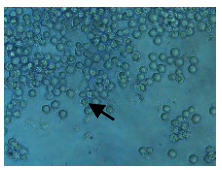
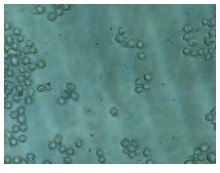
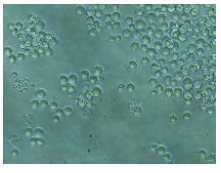
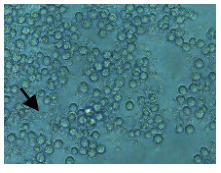
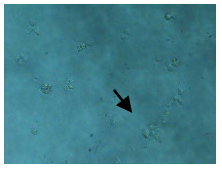
Table 3 The IC_{50} value of *C. ternatea* plant extracts cytotoxicity on the K562 cell line within 24, 48 and 48 hours.

***C. ternatea* Extracts**		**MTT Assay IC_{50} Value (µg/mL)**		
		24 Hour	**48 Hour**	**72 Hour**
Leaves	Aqueous	964.07 ± 172.3	893.37 ±131.44	839.77 ± 149.22
	50% Methanolic	1363 ± 308	1003.6 ± 111.8	913.6 ± 110.85
Flower	Aqueous	465.97 ±4.7596	325.83 ± 159.91	372.27 ± 146.95
	50% Methanolic	461.2 ± 13.555	319.27 ± 152.19	278.2 ± 162.62

However, in terms of the *C. ternatea* body part, both types of flower extract exert the highest cytotoxicity compared to that of the leaf extract. To support this result, the morphology of the K562 cell treated with 50% methanolic flower extract started to lose its membrane integrity starting from a concentration of 600 µg/mL for 24, 48 and 72 hours as in Table 5. While for aqueous flower extract at 1000 µg/mL for both 24 and 48 hours as well as at 1500 µg/mL for 72 hours as shown in 5, the cell membrane integrity started to lose. For both leaves extract treated K562 cells, there are no obvious changes in the membrane integrity for all three incubation times throughout the different concentrations. According to Brassolatti et al., (2022) and Chota et al., (2022), a high concentration above the IC_{50} value can cause the cell to encounter death through necrotic mechanisms in which cell death was characterized by loss of membrane integrity and release of cellular content. Hence, by defining the actual meaning of necrosis, cell death occurs altogether due to extreme environmental conditions compared to apoptosis which occurs only in specific cells (Brassolatti et al., (2022); Chen et al., (2022)). Thus, it can be concluded that the viability of the cell at the necrosis level started to fall to 0.00 %, which aligns with the result in Figure 7 for flower extract.

By referring to the National Cancer Institute (NCI), the criteria used for categorized the cytotoxicity of the plant extracts against cancer in which $IC_{50} \leq 20$ µg/mL are highly cytotoxic, IC_{50} values within the range 21 to 200 µg/mL are considered as moderately cytotoxic, IC_{50} value within the range 201 to 500 µg/mL are weakly cytotoxic and $IC_{50} > 501$ µg/mL considered as no cytotoxicity (Al-saraireh et al., 2020). Hence, it can be concluded that both *C. ternatea* leaves extracts were not cytotoxic, while both *C. ternatea* flower extracts exert weak cytotoxic effects. To align with the DPPH assay carried out, the higher antioxidant activity of the *C. ternatea* extracts especially the flower extracts does contribute to the cytotoxicity of the extracts toward the K562 cell line. According to Gorrini et al., (2013) and Sies, (2015), the cancer cells have elevated ROS levels that were balanced by an increased antioxidant capacity. The presence of antioxidants was able to mount an antioxidant defence and modulate the ROS levels that gave rise to therapeutic implications.

Table 4 The microscopic view of the K562 cell morphology under an inverted microscope at 20X magnification for an incubation time of 24, 48 and 72 Hours at certain concentrations. Arrows indicate loss of membrane integrity and cell shrinkage.

Changes in Cell Morphology of K562 at Different Incubation Time	*C. ternatea* Extract			
	Aqueos Leaves	50% Methanolic Leaves	Aqueous Flower	50% Methanolic Flower
0µg/mL (Control)				
24 Hours (600µg/mL)				
24 Hours (1000µg/mL)				
48 Hours (600µg/mL)				
48 Hours (1000µg/mL)				
72 Hours (600µg/mL)				
72 Hours (1500µg/mL)				

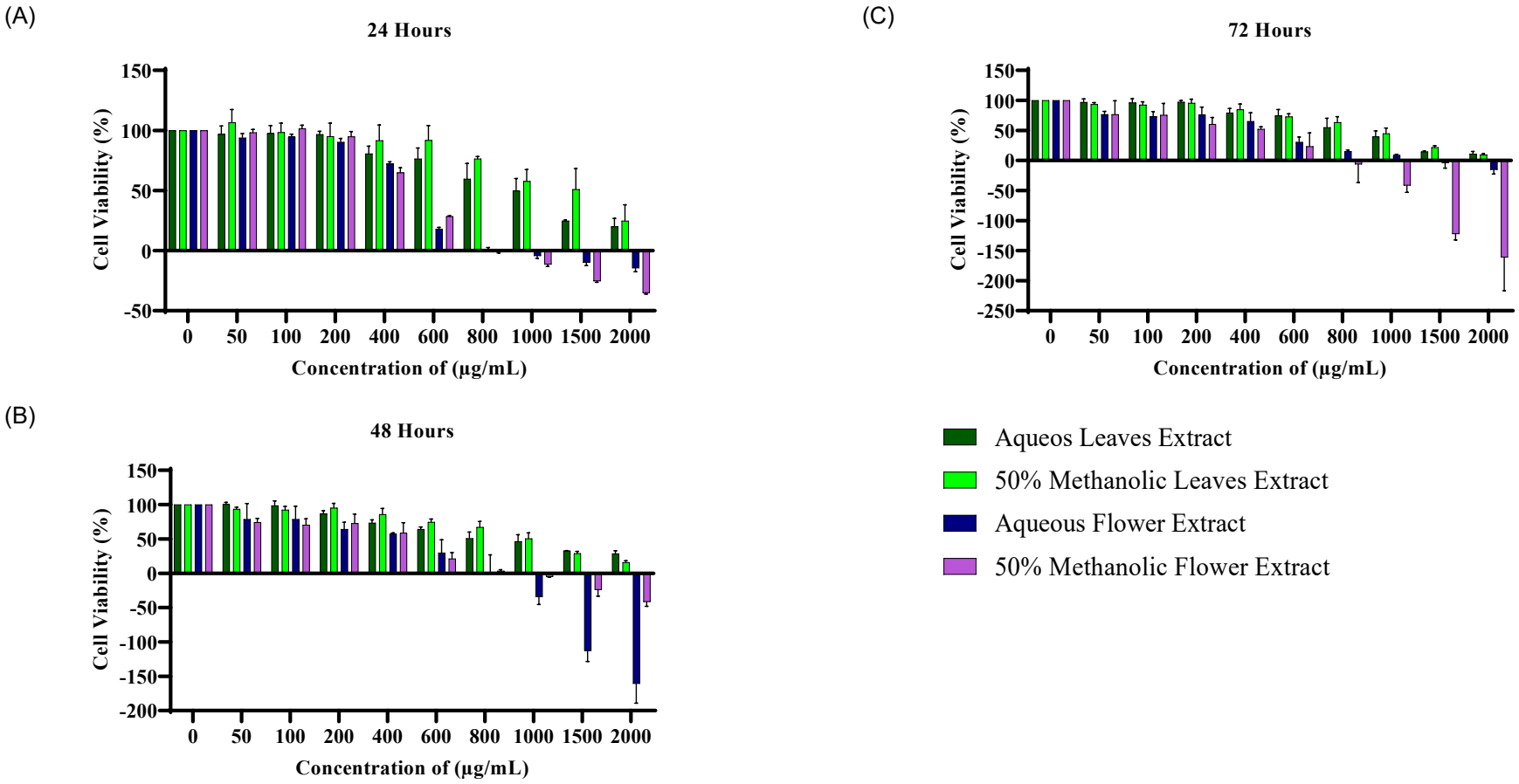


Figure 6 Bar graph showing the percentage of cell viability of the K562 cell line at different types of *C. ternatea* extracts. A) Treated in 24 hours. B) Treated in 48 hours. C) Treated in 72 hours.

The cytotoxicity of *C. ternatea* extract on K562 was compared with normal cell HSF1184. However, due to the reading by the microplate being compromised with the colour of the extracts and DMEM media colour, this led to inaccurate results as shown in Figure 8, in which the cell viability increases and IC_{50} value was undetermined. This result was opposite to the qualitative observation on the microscopic view of the morphology of the HSF1184 within 24, 48 and 72 hours of incubation, as shown in Table 6. Based on the morphology of HSF1184 in Table 6, the cell undergoes necrosis with loss of membrane integrity and attachment for both types of flower extract starting at a concentration of 800 µg/mL for 24. The same goes for the 48 hours of incubation, in which the concentration of flower extract for the cell to undergo loss of membrane integrity and detachment are same as 24 hours of incubation which was 800 µg/mL. However, for 72 hours of incubation, the cell undergoes necrosis at 600 µg/mL of both flower extracts based on Table 6. There are no significant differences in the morphology of HSF1184 in the treatment using both types of leaf extracts, especially the cell attachment impairment. Hence, it can be concluded that the *C. ternatea* extracts also exert cytotoxicity toward HSF1184 cells and estimating that the IC_{50} value of HSF1184 on both flower extracts are lower than 800 µg/mL for 24 and 48 hours as well as less than 600 µg/mL for 72 hours.

Last but not least, previous research on *C. ternatea* flower on other normal cell lines have mentioned that *C. ternatea* flower extracts exert cytotoxicity differently on normal cell based on the specific cell line. In the case of the IMR90 cell line which was the human fibroblast isolated from the normal lung tissue, the *C. ternatea* flower extract exhibited no cytotoxicity in which the IC_{50} value was more than 900 µg/mL (Escher et al., 2020). There was also a study on the cytotoxicity of both aqueous and organic solvent extract on the Hs27 fibroblast cell line in which both extracts were not toxic up to 100 µg/mL (Jeyaraj et al., 2022).

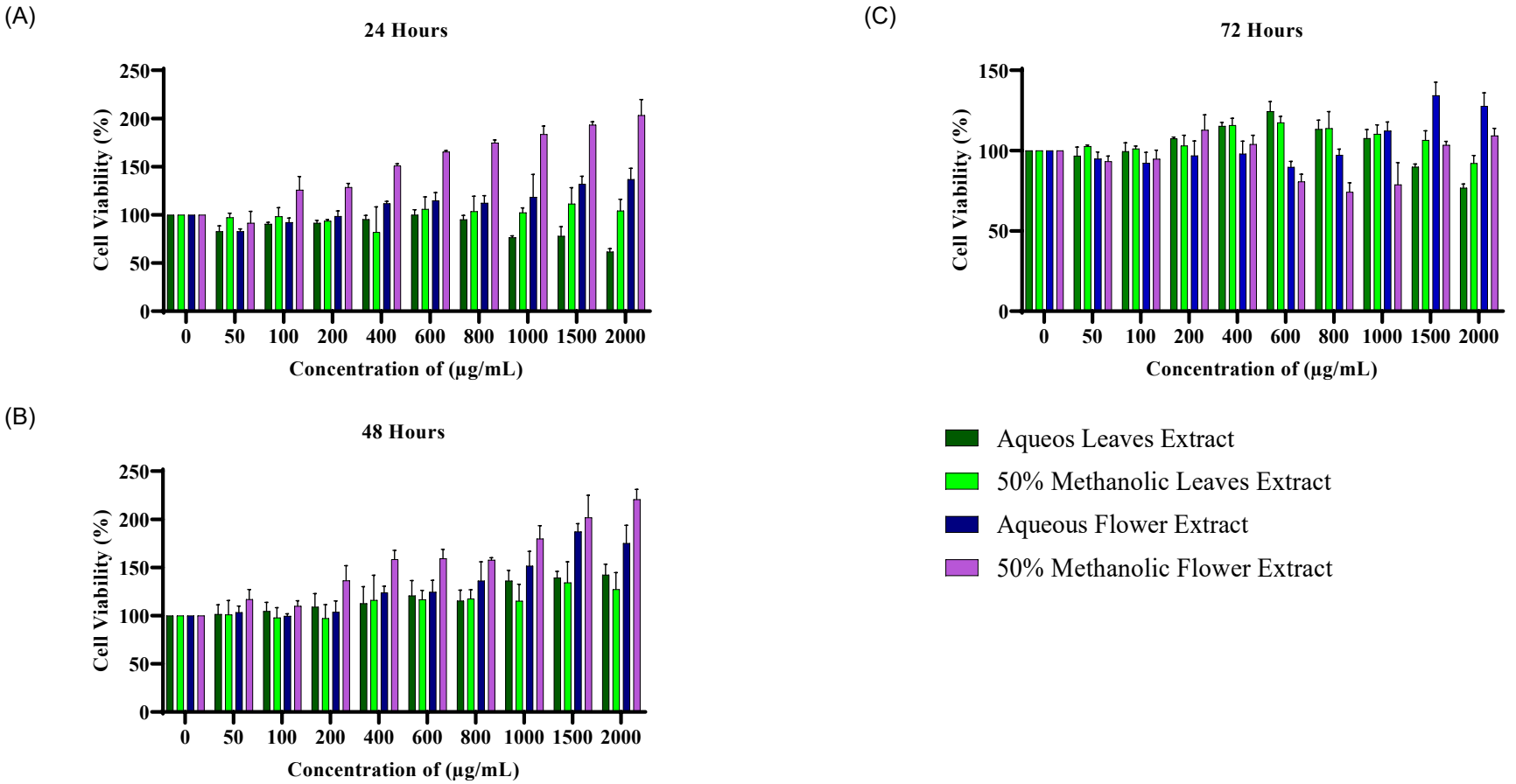
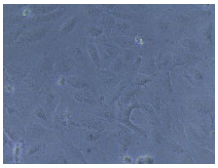
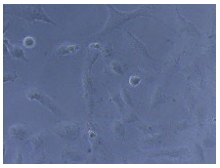
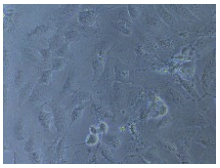
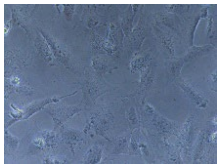
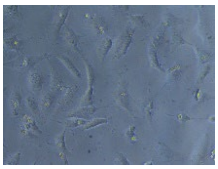
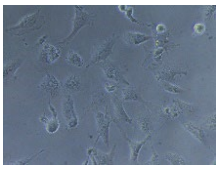
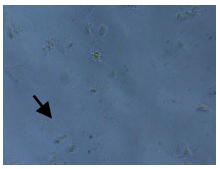
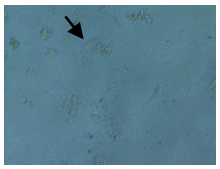
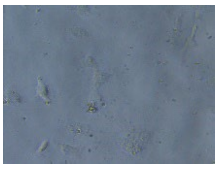
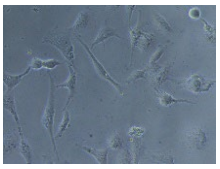
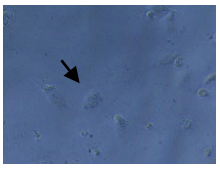
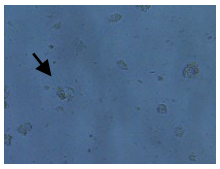
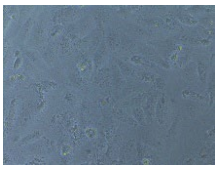
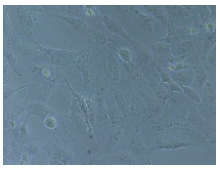
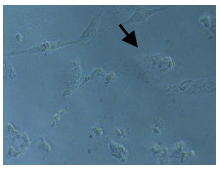
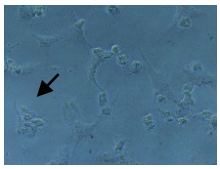
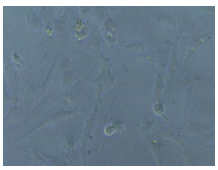
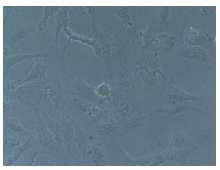
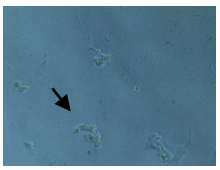
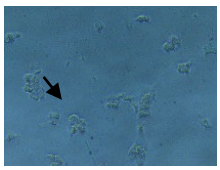
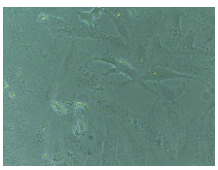
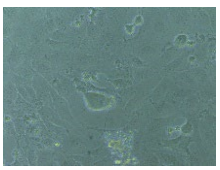
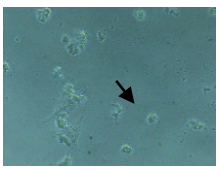
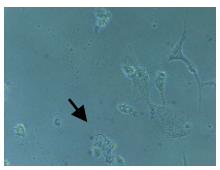
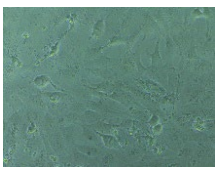
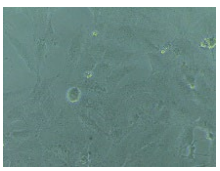
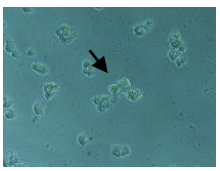
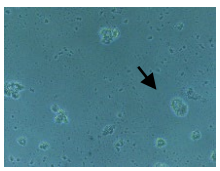


Figure 7 Bar graph showing the percentage of cell viability of HSF1184 cell line at different types of *C. ternatea* extracts. A) treated in 24 hours. B) treated in 48 hours. C) treated in 72 hours.

Table 6 The microscopic view of the HSF1184 cell morphology under an inverted microscope at 20X magnification for an incubation time of 24, 48 and 72 Hours at certain concentrations. Arrows indicate loss of membrane integrity and cell shrinkage.

Changes in Cell Morphology of K562 at Different Incubation Time	***C. ternatea* Extract**			
	Aqueos Leaves	**50% Methanolic Leaves**	**Aqueous Flower**	**50% Methanolic Flower**
0µg/mL (Control)				
24 Hours (800µg/mL)				
24 Hours (1000µg/mL)				
48 Hours (800µg/mL)				
48 Hours (1000µg/mL)				
48 Hours (600µg/mL)				
48 Hours (1000µg/mL)				

Conclusion

This study demonstrated that *C. ternatea* has significant antioxidant activity that was attributed to the phenolic content of the extract derived from the different portions of the plant and extraction solvent polarity. The flower of *C. ternatea* exerts high antioxidant activity than the leaves due to the presence of anthocyanins in the flower part of *C. ternatea*. Furthermore, the methanolic extract increases the solubility of the phenolic compounds and promotes extraction efficiency and antioxidant activity more than the aqueous solvent. Besides, the cytotoxicity of *C. ternatea* toward the K562 cell line showed there was a correlation with the antioxidant activity of the extracts depicting that the mechanism that induced the K562 cell death corresponded with the radical scavenging activity of the extracts toward the ROS released by the cancer cell. The extract cytotoxicity effect on K562 was in a dose-dependent manner, and at the very high dose, the necrosis was triggered based on the morphological result. In summary, 50% methanolic *C. ternatea* flower extract exhibits the highest antioxidant activity and cytotoxic effect toward K562. In future studies, it is recommended to identify the potential major constituent of the *C. ternatea* leaves and flowers that induce K562 cell death. This knowledge would provide clearer insight into the actual mechanism of K562 cell death by the extracts. Moreover, the cytotoxic assay can be improved by using different tetrazolium salts such as MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) or XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), which are less susceptible to interference from plant extracts strong colouration and polyphenols that absorb same wavelength as in the MTT assay. Despite that, normal blood cells such as macrophages and natural killer cells can be used on the cytotoxicity assay to get a reliable comparison with the K562 cells as K562 itself was a blood cell.

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