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Cytotoxicity and Antioxidant Properties of *Diplazium Esculentum* Towards K562 Cells

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Abstract
The National Cancer Institute (NCI) stated that leukemia is a type of cancer disease which specifically affect a person's blood cells that often occurs in adults of above 55 years of age and children below 15years of age which has taken approximately 1,480 lives in Malaysia in the year 2020. One type of leukemia that has been a concern to health institutes is Chronic Myelogenous Leukemia (CML) which derives from the K562 cell line. The chemotherapy of CML was associated with chronic side effects tothe patient's overall health such as heart and lung problems according to American Society of Clinical Oncology (ASCO). Several medicinal plants were found to have anticancer capabilities towards cancer.However, there was a lack of information regarding the anticancer capability of the *Diplazium esculentum* towards the K562 cells. Thus, this study aimed to examine the antioxidant activity, quantifythe phenolic compound, and determine the cytotoxicity effect of the *D. esculentum* extract towards K562 cells. Ethanolic and methanolic extracts of *D. esculentum* were used in this study. The antioxidant activity, total phenolic compound, and cytotoxicity effect were analysed using DPPH, TPC assay, and MTT assay respectively. The IC50 value for DPPH and MTT assays for both extracts were undeterminedwhile total phenolic compound was calculated to be higher in methanolic extract with 9.73 mg GAE/g DE compared to ethanolic extract with 8.73 mg GAE/g DE. Generally, methanolic extract of *D. esculentum* exhibited better activity in all assays although IC50 value for DPPH and MTT assays for both extracts were undetermined.
Keywords: K562; *Diplazium esculentum*; DPPH assay; TPC assay; MTT assay.

Introduction

According to the World Health Organization (WHO), nearly 10 million of human lives were deceased due to cancer diseases in 2020 alone. The Global Cancer Observation (GCO) reported that almost 30,000 of those deaths were the people of Malaysia in the year 2020. Furthermore, Malaysia is also estimated to have over 90% increase of deaths caused by cancer from the year 2020 to 2040 (29,500 to 57,300 deaths). The most common causes of cancer risks are due to obesity, infections (vertical transmissions), UV radiation, and alcohol. According to the National Cancer Institute (NCI), cancer is achronic disease which a person's body cells proliferate uncontrollably which then spread throughout thebody forming tumors that can be life threatening especially when it occurs in the brain. Cancer is considered a chronic disease as it affects the physical health condition by certain restrictions or ongoingtreatment requirements for more than 1 year (Bernell & Howard, 2016).

The National Cancer Institute (NCI) stated that leukemia is a type of cancer disease which

2040 as reportedby the Global Cancer Observation (GCO). As of today, there are approximately 1,905 cases of leukemia which caused approximately 1,481 deaths. This placed leukemia disease as the top 5 cause of death between cancer patients in Malaysia. The National Cancer Institute (NCI) explained that the CML is a disease in which the bone marrow makes too much white blood cells that often leads to feeling tired, loss of weight, night sweats, fever, and pain on the left ribs.

One type of leukemia that has been a concern to health institutes is Chronic Myelogenous Leukemia which derives from the K562 cell line. According to the National Cancer Institute (NCI), the standard treatments for CML disease include targeted therapy, chemotherapy, immunotherapy, high-dose chemotherapy, donor lymphocyte infusion, and surgery. However, the choice treatments differ according to the phases (chronic, accelerated, blastic, relapsed) of the CML disease.

The *D. esculentum* is locally known as “paku pakis” and commonly consumed by the locals asa side dish. Its morphology appears as dark green in colour with lanceolate shape, smooth fracture, herbaceous texture, and a palatable taste. The *D. esculentum* has a quite wide range of size of between20 to 50 cm (Bohara et al., 2020). It is also mostly known as a medicinal plant used by the traditional people to treat various illnesses from minor to severe (Bohara et al., 2020). Thus, the *D. esculentum* has made its reputation locally which makes it easy to find in every food related stores.

Materials and methods

Approximately 1750 g of *D. esculentum* plant was bought from a local shop (Gemilang Frozen, Johor, Malaysia). The *D. esculentum* leaf was plucked by hand and washed with running water before being oven-dried at 45°C for 72 hours. The dried sample was then pulverized into powder using a blender and was soaked with methanol (Merck Millipore, Darmstadt, Germany) and ethanol (Merck Millipore, Darmstadt, Germany) with the ratio of 1:20 for the extract preparation. In relation to that, approximately10 g of the powdered leaf sample was soaked into 200 mL of methanol and ethanol in separate Schottbottles. Then, the mixture was incubated in the incubator shaker at 37°C at 105 rpm for 48 hours beforefiltration of the mixture using a Whatman filter paper. The filtrate was evaporated until constant weight using rotary evaporator at 60°C. The extract yield was stored in the fridge at 4°C until further use.

The K562 cells was cultured in Roswell Park Memorial Institute (RPMI-1640) media (Gibco™ by Thermo Fisher Scientific, Massachusetts, United States) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco™ by Thermo Fisher Scientific, Massachusetts, United States), and 1% (v/v) penicillin-streptomycin (Sigma-Aldrich, St. Louis, Missouri). The K562 cells were grown in the completeRPMI-1640 media at 5% CO2 at 37°C.

For the cell thawing, the K562 cell sample was thawed in the water bath at 37°C for 2 minutes. The thawed cells sample was centrifuged at 300 g for 3 minutes before resuspending the cell pellet obtained in 5 mL of the freshly made complete RPMI-1640 media. Finally, the suspended K562 cell sample was transferred into a 25 cm^2 (T25) culture flask and incubated at 5% CO2 at 37°C until furtheruse. After 24 hours of incubation at 5% CO2 at 37°C, the old culture medium of the K562 cells must berenewed with freshly made complete RPMI-1640 media. In relation to that, a total of 3 mL of fresh complete RPMI-1640 media was added into the culture flask.

Cell counting was done using haemocytometer to count the density of the viable and dead cells.1 mL of the K562 cell sample was transferred into an Eppendorf tube after gently swirling the culture flask until homogenize. Then, 40 µL of the K562 cell sample was suspended with 40 µL of the TrypanBlue (Nacalai Tesque Inc., Kyoto, Japan) solution into a new Eppendorf tube (2 dilution factor). 12 µLof the Trypan Blue-treated K562 cell sample was used for the cell counting by haemocytometer under an inverted microscope. The percentage of viable cells and the cell density were calculated using the formula in Equation 1 and 2 respectively.

Equation 1:

$$Percentage\ of\ viable\ cells\ (\%) = \frac{Total\ viable\ cells}{Total\ cells} \times 100\%$$

Equation 2:

$$Cell\ density\ (\frac{cells}{mL}) = \frac{Total\ viable\ cells}{Total\ box} \times Dilution\ Factor \times 10^4$$

The cell subculture of the K562 cell sample was done when the cell density of the K562 cells reached 1 x 10^6 cells/mL. In relation to that, the initial volume of the K562 cell sample was transferred into a new T75 culture flask before resuspending the cell pellet into the complete RPMI-1640 media tostart the new culture at 2 x 10^5 cells/mL in the T75 culture flask using the formula in Equation 3 below.

Equation 3:

$$M1V1 = M2V2$$

M1 = initial cell count of the K562 cells M2 = desired cell count of the K562 cells
V1 = initial volume of the K562 cell sampleV2 = final volume of the K562 cell sample

Cell cryopreservation was to preserve the viability of the K562 cells by freezing the cells in thefreezer storage at -80°C. For this purpose, the cells were centrifuged at 300 g for 3 minutes. The cell pellet was then resuspended in a freezing culture medium containing 0.5% of DMSO and RPMI-1640 media with a cell density of 3 x 10^6 cells/mL. The aliquot was transferred into the cryogenic storage vials with 1 mL for each vial. The K562 cell sample was frozen in the freezer storage at -80°C. The K562 culture was cryopreserved until next use.

The DPPH assay measured the antioxidant activity of the *D. esculentum* extract by the ability of the antioxidants inside the extract to scavenge the DPPH reagent when mixed as reported previously (Sachett et al., 2021). 0.24 mg/mL of DPPH reagent (Sigma-Aldrich, St. Louis, Missouri) was used for this purpose. To prepare the samples for the DPPH assay, both 1 mg/mL of the methanolic and ethanolic plant crude extract was prepared and directly diluted to 25, 50, 75, 100, 250, 500, and 1000 µg/mL into separate 96-well plate in triplicate. The sample were then incubated in the dark for 30 minutes before reading the absorbance at 517 nm. The ascorbic acid (Sigma-Aldrich, St. Louis, Missouri) was used as the positive control while methanol and ethanol respectively were used as the blank for the DPPH assay. The obtained absorbance values were used to calculate the percentage of inhibition of the DPPH reagent using the formula in Equation 4 below.

Equation 4:

$$Percentage\ of\ Inhibition\ (\%) = [\frac{ODc - (ODs - ODb)}{ODc}] \times 100\%$$

ODs = absorbance of sample
ODb = absorbance of blank
ODc = absorbance of control

The TPC assay quantified the total phenolic compounds in both extracts by the absorbance of the oxidised form of the Folin-Ciocalteu reagent as reported by Bohara et al., (2020). 40µL of different concentrations of gallic acid (25, 50, 75, and 100 µg/mL) were prepared and incubated with the 0.2 mLof the FolinCiocalteu reagent mixed with 3.16 mL of distilled water and 0.6 mL of 7.5% sodium bicarbonate (Na_2CO_3) aqueous solution in different test tubes in triplicate. The samples were incubatedfor 90 minutes before reading the absorbance at 765 nm to construct the linear regression model for the gallic acid standard. The experiment was repeated for the methanolic and ethanolic extracts of *D. esculentum* and ascorbic acid but only at a concentration of 1 mg/mL.

The MTT assay measured the cytotoxicity of the *D. esculentum* extract by determining the viability andproliferation of treated K562 cells. The protocol for the MTT assay was adapted from Kumar et al., (2018) and Chai et al., (2015) but with slight modifications. 5 mg/mL of MTT reagent was used for this purpose which was stored in the dark at -40°C. To prepare the samples for the MTT assay, 1 mg/mL ofboth methanolic and ethanolic plant crude extract were prepared by diluting in the complete culture media and were directly diluted to 100, 200, 300, 400, and 500 µg/mL into separate 96-well plate in triplicate. In relation to that, 50 µL for each plant samples were then incubated with 50 µL of the K562cells for 48 hours before further 4 hours of incubation with 10 µL of the MTT reagent. 100 µL of acidified dodecyl sulfate (SDS-HCl) which was prepared by dissolving 1 g of dodecyl sulfate (SDS) (Fisher Scientific, Loughborough, England) in 10 mL of 0.01N hydrochloric acid (HCl) was then added into eachwell was left for 18 hours in the dark at 37°C before reading the absorbance at 570 nm. The experimentwas repeated with 72 hours of incubation of the of plant extracts with the K562 cells. From the absorbance obtained, the percentage of cell viability was calculated by using the formula as shown in Equation 5 below.

Equation 5:

$$Percentage\ of\ Cell\ Viability\ (\%) = \frac{(ODs - ODb)}{(ODc - ODb)} \times 100\%$$

ODs = absorbance of sample
ODb = absorbance of blank
ODc = absorbance of control

All data were presented by mean. The standard curve for gallic acid in TPC assay was constructed andanalyzed by using Microsoft Excel. The linear regression and correlation analysis were constructed using GraphPad Prism software. The value of IC_{50} for both antioxidant activity assay and cytotoxicity screening assay were generated by the GraphPad Prism 9.0.0 software. Finally, IBM SPSS Statistics 27 software also was used to analyse the data by one-way ANOVA test and means of significant differences of variance at 0.05 level of significance.

Results and discussion

The *D. esculentum* obtained had bipinnate leaves with long brownish petioles. Its frond was around 30cm in length and its pinna (leaf) was around 15 cm and 3 cm in length and width respectively (Srivastava& Rao, 2019). The plant fiddlehead (shoot) and the fern leaf are as shown in Figure 1. The leaf samplewas oven-dried mainly to remove the moisture trapped inside the leaf sample and was able to preservethe main bioactive compounds as stated in the research by Şahin et al., (2018).

A. **B.**

Figure 1 (A) The morphology of the *D. esculentum* shoot. (B) The leaf sample from the *D.esculentum* plant.

The pulverized sample powder was soaked into distilled water at 1:20 ratio (weight:volume) which produced a 200 mL of mixture and incubated for 48 hours to further extract the main bioactive compounds from the *D. esculentum* plant into the methanol and ethanol solvents (Rajha et al., 2019). Absolute methanol and ethanol solvent were used for extract preparation as the solvents were known to yield better quantity of the main bioactive compounds from the *D. esculentum* such as flavonoids andphenols (Bohara et al., 2020). The mixture was then subjected to filtration by Whatman filter paper which obtained less than 200 mL of the solvent containing the bioactive compounds from the *D. esculentum* plant. The loss of volume was due to the quantity of mixture which cannot be recovered from the filter paper (Nichols, 2021). The filtrate was then treated to rotary evaporator at 60°C to furtherdry out the solvent from the mixture until a constant weight was reached.

Based on the regression model shown in Figure 2, both of the methanolic and ethanolic extracts of the *D. esculentum* did not exhibit any antioxidant activity as both of the extracts did not scavenge a percentage of the DPPH reagent. This could be due to the loss of antioxidants during the *D. esculentum* extract preparation as reported in the study by Stagos, (2019) that the overall extract preparation time was the greatest factor to the loss of phenolic compounds and followed by the extract preparation temperature used. With that, it could explain why both of the extracts did not exhibit such activity towards the DPPH reagent as there could be a major loss of antioxidant due to the fact that the phenolic compounds are the most significant antioxidants which exist inside of a plant including *D. esculentum* because of their major role in plant defence and survival systems (Tsimogianis, & Oreopoulou, 2019).

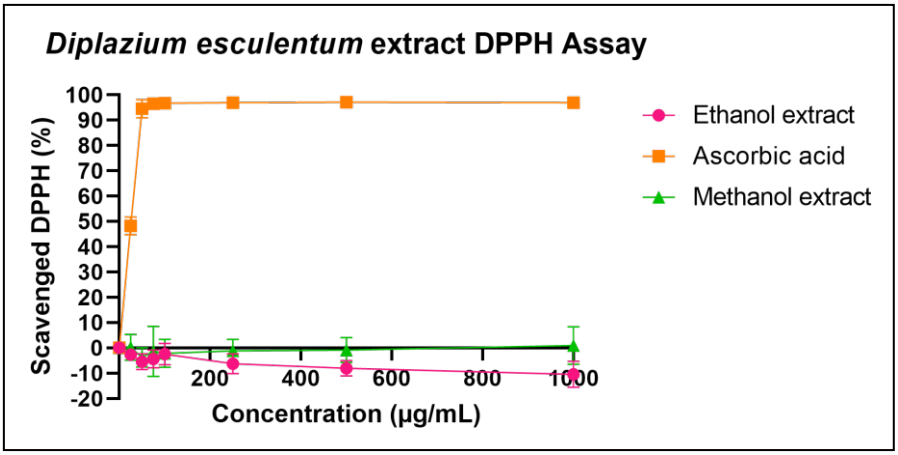

Figure 2 The regression model of DPPH assay.

From the regression model, the IC_{50} value was then calculated to compare the potency of the antioxidant activity in comparison with ascorbic acid which was known for its strong antioxidant activityagainst the DPPH reagent. The IC_{50} value for the ascorbic acid positive control was calculated to be
25.06 µg/mL. However, the IC_{50} value for both of the extracts were not able to be calculated due to thelack of activity towards the DPPH reagent. The summary for the IC_{50} values calculated from DPPH assay was tabulated in Table 1.

Table 1: The IC50 value calculated from DPPH assay.

Sample	DPPH assay IC_{50} (µg/mL)
Methanolic extract	Undetermined
Ethanolic extract	Undetermined
Ascorbic acid	25.06

As shown in Table 1, the ascorbic acid had a potent DPPH scavenging activity and both of themethanolic and ethanolic extracts of the *D. esculentum* were lacking of the DPPH scavenging activity. Thus, the potency of both methanolic and ethanolic extracts were not able to be compared

to each other as their IC_{50} values were undetermined at the end of this assay analysis. However, based on theslope from the regression model in Figure 2, the methanolic extract had a better slope compared to theethanolic extract which implies that the methanolic extract had the slight edge in the antioxidant activity.Similar situation was observed in the previous study by Bohara et al., (2020) which reported that methanol was the best solvent that exhibit the best DPPH scavenging activity compared to ethanol, aqueous, and chloroform as the solvents.

Construction of linear gallic acid standard curve was based on the absorbance obtained as shown in Figure 3. From the standard curve, the R^2 value obtained was 0.985, thus generated the equation: y=0.003x-0.0102 for the calculation of the total phenolic compounds in the methanolic and ethanolic extracts of the *D. esculentum*. The subsequent absorbance obtained from both extracts and ascorbic acid as the positive control (1 mg/mL) were substituted into the y value to calculate the total phenolic compounds residing in the test subjects.

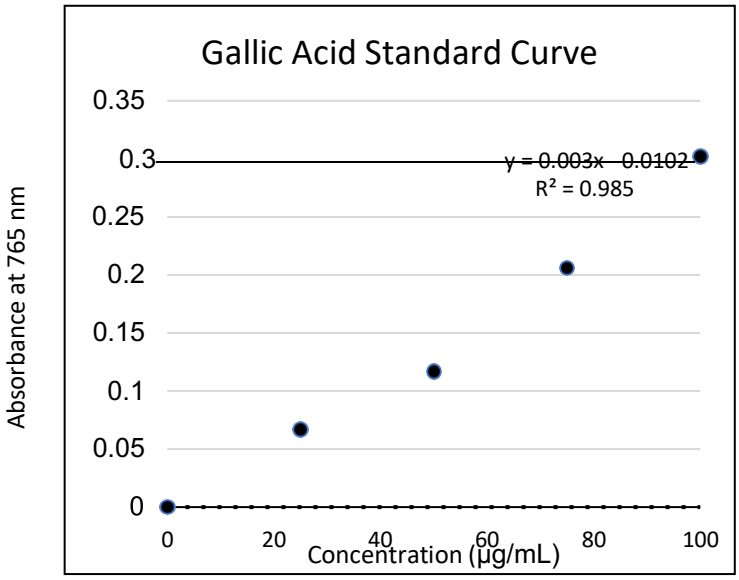

Figure 3 Gallic acid standard curve.

By using the equation (y=0.003x-0.0102) generated, the total phenolic content was calculatedto be the highest for the ascorbic acid positive control with 66.40 mg GAE/g DE. However, a tiny quantityof phenolic compounds was calculated for both of the methanolic and ethanolic extracts with 9.73 and
8.73 mg GAE/g DE respectively as shown in Table 2 which could explain the negative result observed in the DPPH assay. Despite the report in the previous study by Stagos (2019), this result was also explained in the previous study by Nur et al. (2018) which stated that the overall extraction temperature,time, and water volume were the factors that affect the yield of phenolic compounds.

Table 2: Total phenolic compound calculated from TPC assay.

Sample	Total Phenolic Content (mg GAE/g DE)
Methanolic extract	9.73
Ethanolic extract	8.73
Ascorbic acid	66.40

As shown in Table 2, the ascorbic acid scored the highest total phenolic content and both of the methanolic and ethanolic extracts of the *D. esculentum* were lacking of the total phenolic content inequivalence to the gallic acid standard. However, the methanolic extract had a slightly better quantity of content of the phenolic compounds that was able to be extracted compared to the ethanolic extract

which suggests that the methanolic extract had the slight edge in the antioxidants extracted. Similar situation was observed in the previous study by Bohara et al., (2020) which reported that methanol wasthe best solvent that extracted the best yield of phenolic compounds compared to ethanol, aqueous, and chloroform as the solvent.

Based on the regression model shown in Figure 4, both of the methanolic and ethanolic extracts of the *D. esculentum* exhibited cytotoxicity effects towards the K562 cells as the slopes were in the decreasing trend. The cytotoxicity effects for both extracts were also observed to be directly proportional to the period of exposure of the K562 cells with the *D. esculentum* extracts. Similar situation was observed inthe previous study by Chai et al., (2015) where the aqueous extract of *D. esculentum* was showing cytotoxicity effects towards the K562 cells. Even with the negative results in both DPPH assay and TPCassay, both of the methanolic and ethanolic extract were still able to exhibit such effects towards the K562 cells. Hence, indicated that there was another bioactive compound with no antioxidant propertieswhich led to the result as observed in the MTT assay.

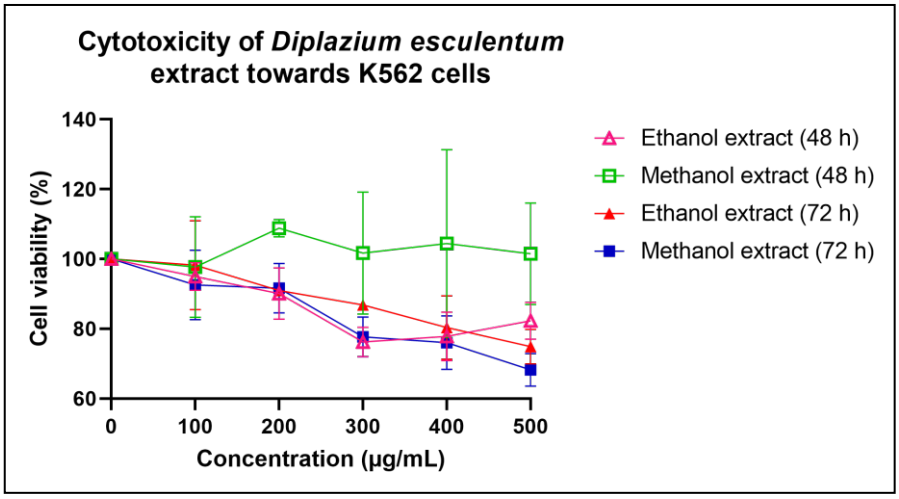

Figure 4 The regression model of MTT assay.

From the regression model, the percentage of K562 cell viability exposed with the methanolic and ethanolic extracts for 48 hours were calculated to be 103.85% and 75.93% respectively. In additionto that, the cell viability was calculated to decrease for both of the extracts with 68.50% and 75.47% ofcell viability respectively in the 72 hours of exposure. However, both of the extracts were lacking in thecytotoxicity effects towards the K562 cells with the chosen concentration. Thus, the cytotoxicity effectsof both extracts were not enough to calculate the IC_{50} value for the MTT assay due to the lack of activitytowards the K562 cells. The results obtained from this MTT assay was as summarized in Table 3.

Table 3: Percentage cell viability and IC50 value calculated from MTT assay.

Sample	Incubation period (h)	Percentage cell viability (%)	MTT assay IC_{50} (µg/mL)
Methanolic extract	48	103.85	Undetermined
	72	68.50	
Ethanolic extract	48	75.93	Undetermined
	72	75.47	

As shown in Table 3, both of the methanolic and ethanolic extracts of the *D. esculentum* werelacking of the cytotoxicity effects towards the K562 cells. Thus, the potency of both methanolic and ethanolic extracts were not able to be compared to each other as their IC50 values were undeterminedat the end of this assay analysis. However, based on the regression model in Figure 5, the methanolicextract was observed to have better cytotoxicity effects towards the K562 cells in 72 hours period of exposure as compared to the ethanolic extract as the cell viability were calculated

to be 68.50% and 75.47% respectively which implies that the methanolic extract had the slight edge in the cytotoxicity effects towards K562 cells within the 72 hours of exposure period. Hence, the bioactive compounds which exhibited such effects towards the K562 cells extracted using methanol as the solvent had betteryield as compared to using ethanol as the solvent as observed in this MTT assay analysis. This provedonce more that methanol was the better solvent as compared to the ethanol.

Conclusion

In conclusion, both of the *D. esculentum* methanolic and ethanolic extract did not exhibit antioxidant properties as observed in DPPH assay even though through the observation from the regression model, the slope for the methanolic extract had the slight edge as compared to the slope for the ethanolic extract as shown in the Figure 3. The lack of antioxidants was then proven in the TPC assay where both of the extracts were only containing a tiny quantity of phenolic compounds even though the methanolic extract did have the slight edge on the total phenolic compound extracted compared to theethanolic extract with 9.73 and 8.73 mg GAE/g DE respectively based on gallic acid equivalence of TPCassay. For the MTT assay, both of the extracts did exhibit cytotoxicity activity towards the K562 cells. However, the IC_{50} value for both of the extracts were not able to be calculated due to the lack of activity. Overall, antioxidants present in the *D. esculentum* did not play a role in exhibiting the cytotoxicity activitytowards the K562 cells. Thus, the presence of another bioactive compound of the *D. esculentum* whichwas better extracted using methanol as the solvent and had no antioxidant properties was the cause for such activity towards the K562 cells.

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