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Cytotoxic Activity of *Tinospora crispa* Crude Extracts (Stem) Against K562 Human Leukemia Cells

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Abstract: *Tinospora crispa* (*T. crispa*) is traditionally used as an herbal medicine for treatment of gout and is an analgesic, anti-inflammatory and antihyperuricemic agent. Cytotoxicity of hexane, dichloromethane, ethanol and aqueous extracts of *T. crispa* stem against K562 cells was assessed. Crude extracts of *T. crispa* stem were obtained by sonication and maceration methods. *T. crispa* stem powder was macerated and sonicated with hexane, dichloromethane, ethanol and aqueous (1:10 w/v). Extraction via sonication method yielded higher product compared to maceration method in which the yield percentage of crude extract was 0.97%, 1.92%, 5.27% and 12.80% for hexane, dichloromethane, ethanol and aqueous respectively. The yield percentage obtained from maceration method for hexane, dichloromethane, ethanol and aqueous was 0.91%, 1.71%, 3.01% and 12.05% respectively. The cytotoxicity of the crude extracts were tested against K562 cells using 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide assay (MTT) for 24, 48 and 72 h with the highest concentration of 200 µg/mL. The MTT assay result showed that T.crispa stem crude extract significantly reduced cell viability ($p<0.005$) at the concentration more than 200ug/ml for 72 h treatment using hexane, dichloromethane, ethanol and aqueous extract at concentrations 200 µg/mL, while 100 µg/mL for aqueous extract only."4). The IC_{50} value for dichloromethane and ethanol extracts of *T. crispa* was 158±1.33 µg/mL and 172±6.00 µg/mL respectively. On the other hand, 72 h treatment caused significant effects on the viability of cells treated with hexane and ethanol extracts of *T. crispa*. Hence, this study indicated that dichloromethane and ethanol extracts of *T. crispa* were able to inhibit cell proliferation based on the results from the MTT assay. Further study should be conducted to elucidate the potential of *T. crispa* crude extract before it can be developed as a new anti-leukemic agent.

Keywords: *Tinospora crispa*, Sonication, Maceration, K562 Leukemic Cell, Cytotoxic

Introduction

Cancer is the second largest cause of death in the world and caused about 9.6 million deaths in 2018 (WHO, 2017). About 4.5% of total cancer cases in Malaysia for 2006, (539 cases) are myeloid leukemia and 433 cases are lymphatic leukemia (Omar *et al*., 2006). Leukemia cases in males are slightly higher compared to those in females, where the ratio are 1.7:1 for lymphatic leukemia and 1.1:1 for myeloid leukemia. Along with that, Omar *et al*. (2006) also reported that leukemia is the fourth highest cancer occurrence among men and the seventh among women.

The treatment received by leukemia patients varies according to the type of leukemia, age and condition of the patient. Leukemia patients are usually given chemotherapy, radiation therapy, interferon therapy and targeted therapy as their treatment (Christian Nordqvist,

2017). To be more specific, chemotherapy is given after the patient has undergone surgery. Clinical treatments are often associated with side effects and toxicity due to the high dose given. Chemotherapy can affect the entire body, while targeted therapies only involve the specific cancer cell. Various alternative treatments have been proposed to treat cancer, such as plant products and others treatments that are less toxic toward normal cells (Desai *et al*., 2008). Natural medicine is often used as a treatment traditionally but the medicine has a slow effect. Each plant has a certain biological activity that is believed to have its own anticancer properties (Greenwell and Rahman, 2015). Most herbal plants have the potential to be a source of nutritious food and for medical uses (Zubairi and Jaies, 2014).

Herbal medications have valuable curative properties due to the presence of various complex chemical substances of different compositions (Al-Alusi *et al*., 2010). *T. crispa* is normally known as 'patawali' or 'akar patawali' and is a member of the *Menispermaceae* family, which is a family of flowering plants (Hipol *et al*., 2012). *T. crispa* originates from India and grows abundantly in Southeast Asia. *T. crispa* leave are boiled with oil and used as massage oils by traditional massagers (Pathak *et al*., 1995). In addition, every part of the *T. crispa* plant can be used as a traditional medicine. Several studies have been conducted on *T. crispa* extract and more than 65 biological active compounds have been extracted and identified as lactone, steroids, flavonoids, lignans and alkaloids (Ahmad *et al*., 2016). A previous study by Kadir *et al*. (2011) found that *T. crispa* extracts have high antioxidant and radical scavenging activity potential *in vitro*.

In this study, *T. crispa* stem were specifically selected due to its potential to be a source of natural antioxidants and nutrients, besides having a moderate anti-proliferative effect on MCF-7, HeLa 165, Caov-3 and HepG2 (Zulkhairi *et al*., 2008). The cytotoxicity of *T. crispa* stem extract against K562 human leukemia cell line was assessed. K562 human leukemia cells were cultured and treated with hexane, dichloromethane, ethanol and aqueous extracts of *T. crispa* stem.

Materials and Methods

Plant Material

T. crispa stem powder was purchased from Yuda Trading Sdn. Bhd.

Preparation of Crude Extract

Maceration

Maceration extraction was conducted based on method by Kadir *et al*. (2011) with some modifications. About 15 g of *T. crispa* stem powder was macerated in 150 mL (1:10 w/v) hexane, dichloromethane, ethanol and aqueous respectively for 72 h at room temperature. The mixtures were occasionally shaken using an orbital shaker. The protocol was repeated twice. The extracts were filtered using Whatman paper No. 1. After filtration, each mixture was evaporated using a rotary evaporator and resulting of liquid concentrated dark gummy-green crude extracts. *T. crispa* stem crude extracts were weighed to obtain the percentage of yield. The crude extracts were stored at −18°C.

Sonication

Sonication extraction was performed according to Balouiri *et al*. (2014), with some modifications. Flasks containing 15 g of *T. crispa* stem powder dissolved in 150 mL hexane, dichloromethane, ethanol and aqueous respectively were immersed in an ultrasonic bath. Sonication was performed with ultrasound frequency for 30 min. After incubation, the extracts were filtered using Whatman paper No. 1 and each mixture was evaporated using a rotary evaporator and resulting in concentrated dark gummy-green extract. The extracts were weighed to obtain the percentage of yield. The extracts were stored at −18°C.

Preparation of Stock Solution

T. crispa crude extracts were dissolved in Dimethyl Sulfoxide (DMSO), diluted in culture media and used to treat K562 cells. The percentage of DMSO present in any well was less than 0.1%. Each crude extract (200 mg) was dissolved in 1 mL DMSO to produce a stock solution. The stock solution was further diluted with Iscove's Modified Dulbecco's Medium (IMDM) culture media to produce the highest working solution of 200 µg/mL.

Cell Culture and Maintenance

K562 human leukemic cell line was purchased from the American Type Culture Collection (ATCC) and cultured at Biocompatibility Laboratory, Faculty of Health Sciences, UKM Kuala Lumpur. K562 cells were cultured in IMDM media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. K562 cells were cultured in 75 cm^2 flasks in a humidified atmosphere containing 5% CO_2 incubator at 37°C. The cells were grown to confluence before treatment with *T. crispa* stem extract.

Assessment of Cytotoxicity using MTT Assay

Cytotoxic assessment of *T. crispa* stem extract was performed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The assay was conducted as described by Mosmann (1983) with slight modifications. K562 cells were treated with four different crude extracts of *T. crispa* stem with concentrations of 6.25, 12.5, 25, 50, 100 and 200 µg/mL.

Briefly, cells were seeded in 96-well plate at density of 1×10^6 cells/mL. Then, the extracts were added into each well at different concentrations as described. The plate was then incubated for 24, 48 and 72 h. After the incubation period, 20 µL of MTT was added into the 96-well plates and they were further incubated at 37°C for 4 h. In metabolically active cells, MTT is reduced by the mitochondrial enzyme succinate dehydrogenase to form insoluble purple formazan crystals that are subsequently solubilized. After incubation, 180 mL of supernatant was carefully removed from each well and 180 mL of DMSO was added to dissolve the formazan crystals. The plates were agitated using an orbital shaker for 3 to 5 min to ensure complete dissolution of crystals. The Optical Density (OD) of each well was measured at 570 nm wave length using iMark Microplate Reader (BioRad, USA). The inhibitory concentration that killed 50% of cell population (IC_{50}) was calculated and used as a parameter to compare the relative cytotoxicity of each extract. Menadione (MD) was used as a positive control and untreated cells as a negative control.

Statistical Analysis

Data for cytotoxic assessment of *T. crispa* stem crude extract was analyzed using the computer software Statistical Package for the Social Sciences (SPSS) version 21.0. The data was expressed as mean ± standard deviation. Normality test was conducted to determine the distribution of data. One-way ANOVA test (posthoc: Dunnett) test was used in this study to compare the viability of K562 cells in different extracts. In addition, one-way ANOVA (posthoc: Dunnett) test statistic was also used to compare the percentage of cell viability and periods of treatment for 24, 48 and 72 h.

Results and Discussion

Yield Percentage of Crude Extract

Extraction via sonication method (Fig. 1) gave higher product compared to maceration method in which yield percentage of crude extract was 0.97%, 1.92%, 5.27% and 12.80% for hexane, dichloromethane, ethanol and aqueous respectively. While the yield percentage obtained from maceration method (Fig. 2) for hexane, dichloromethane, ethanol and aqueous were 0.91%, 1.71%, 3.01% and 12.05% respectively.

This study indicated that sonication method was more effective than maceration method due to its additional help of high frequency with the range of 20 kHz to 2000 kHz. Sonication method help in producing higher crude extract (Dhanani *et al*., 2013). Sonification was good in extracting herbal plant (Vinatoru *et al*., 1996). Result obtain from both extraction method show that aqueous extraction produced highest crude extract. Percentage of crude extract mostly depend on polarity of solvent and bioactive compound that presence in the plant (Chikezie *et al*., 2015). Others factor such as type of solvent, temperature and time also effect the production of crude extract. Therefore, different solvents will result in different percentage of crude extracts. Aqueous has the highest polarity followed by ethanol, dichloromethane and hexane. High polarity of the solvent will produced higher percentage of crude extracts. Percentage of crude extracts are directly proportional to the polarity of the solvent (Harwoko and Choironi, 2016).

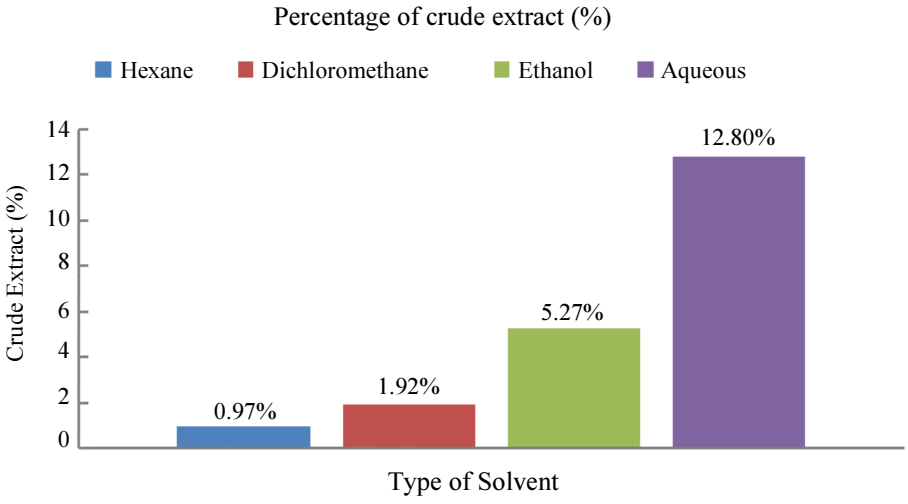

Fig. 1: The percentage of crude extracts obtained using sonication extraction technique

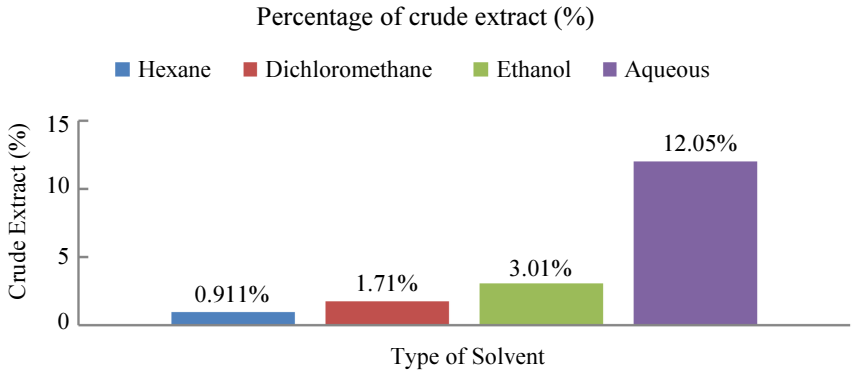

Fig. 2: The percentage of crude extracts obtained using maceration extraction technique

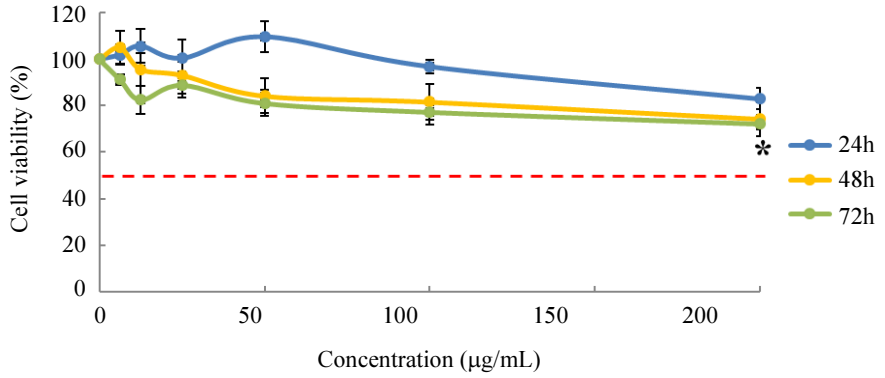

Fig. 3: Percentage cell viability of leukemia cell K562 after treatment with hexane extract after incubation period for 24 h, 48 h and 72 h. The data shows the cell viability (%) ± S.E.M obtained from three separate repeated experiments. *Significant difference ($p<0.05$) compared to negative control

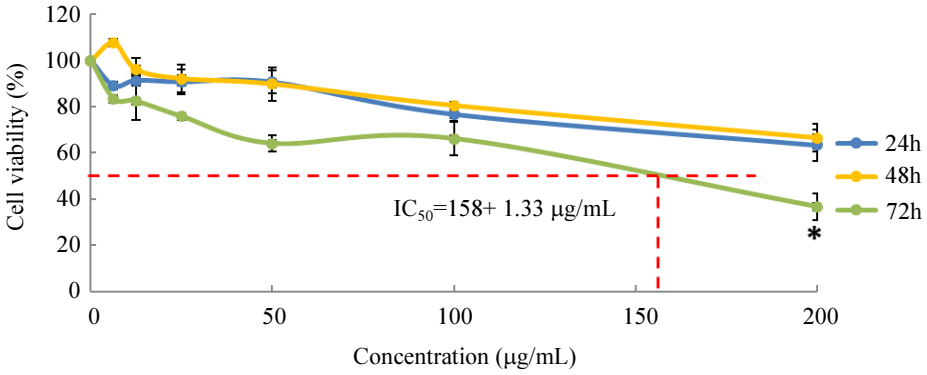

Fig. 4: Percentage cell viability of leukemia cell K562 after treatment with dichloromethane extract after incubation period for 24 h, 48 h and 72 h. The data showed the cell viability (%) ± S.E.M obtained from three separate repeated experiments. *Significant difference ($p<0.05$) compared to negative control

Cytotoxicity Screening

Hexane Extract

The graph in Fig. 3 showed the cytotoxic effects of hexane extract on K562 cell for 24 h, 48 h and 72 h. Based on the graph, there was no IC50 value obtained. However, it can be seen that hexane extract were able to reduce the viability of K562 cell. The cell viability (%) for hexane extract at highest concentrations 200 µg/mL were 82.94±4.56% for 24 h incubation, 74.29±7.57% for 48 h incubation and for 72 h incubation were 72.0818±2.50%.

Dichloromethane Extract

The graph in Fig. 4 showed the cytotoxic effects of dichloromethane extract on K562 cell for 24 h, 48 h and 72 h. The cell viability demonstrated a decrease in dose-dependent manner for all treatment periods. The percentage of cell viability for dichloromethane extract at highest concentration was 63.42±0.87, 66.55±5.93 and 36.64±5.61% for 24, 48 and 72 h treatment period respectively. The IC_{50} value can be observed in 72 h treatment at concentration of 158 µg/mL.

Ethanol Extract

The graph for the cytotoxic effects on K562 cells after 24 h, 48 h and 72 h treatment using ethanol extract is shown in Fig. 5. Based on the figure, it was found that ethanol extract was able to reduce the viability of K562 cells. The viability of the cells treated with ethanol extract at different treatment period 24, 48 and 72 h were 89.08±4.06, 71.86±3.30 and 40.79±7.32% respectively for the highest concentration 200 µg/mL. The IC_{50} values obtained from the K562 cells induced by ethanol extract were 172±6.00 µg/mL for 72 h incubation.

Aqueous Extract

The graph in Fig. 6 showed the cytotoxic effects on K562 cells upon induction with aqueous extract after 24 h, 48 h, 72 h treatment duration. Based on the graph, it can be seen that aqueous extract were able to reduce the viability of K562 cell but not shown IC_{50} value for all different incubation period. The cell viability for water extract at highest concentrations at 200 µg/mL were 72.04±6.63% for 24 h incubation, 68.34±3.51% for 48 h incubation and for 72 h incubation were 66.57±0.75%.

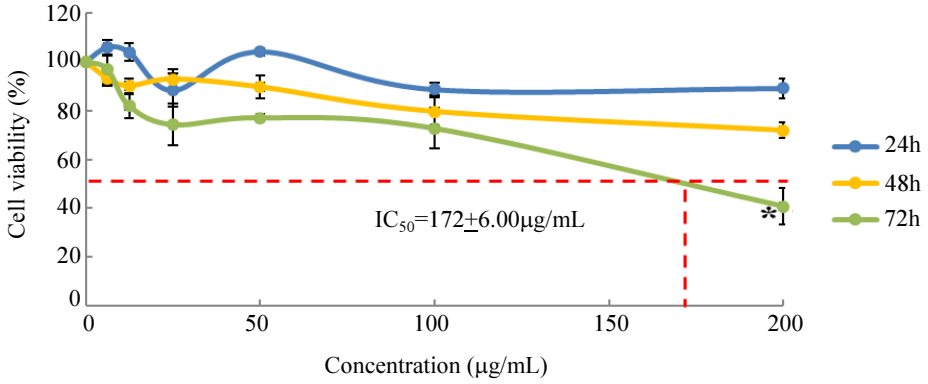

Fig. 5: Percentage cell viability of leukemia cell K562 after treatment with ethanol extract after incubation period for 24 h, 48 h and 72 h. The data shows the cell viability (%) ± S.E.M obtained from three separate repeated experiments. *Significant difference ($p<0.05$) compared to negative control

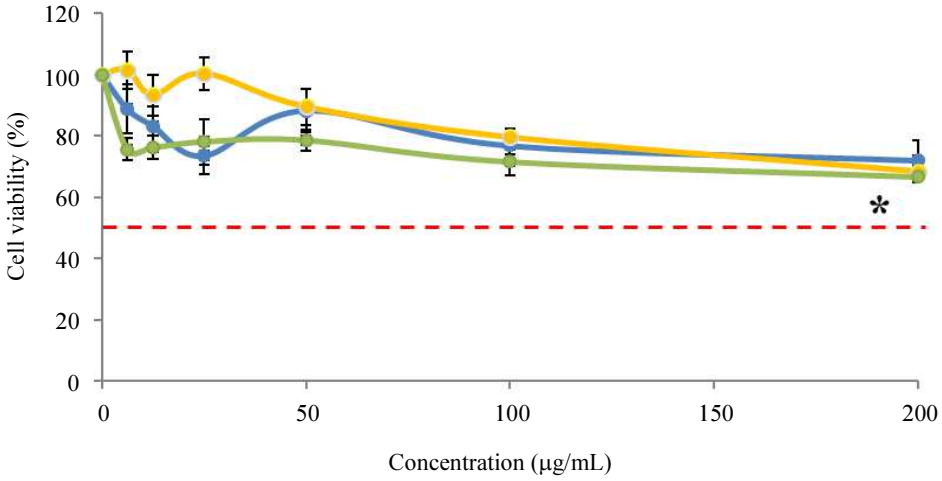

Fig. 6: Percentage cell viability of leukemia cell K562 after treatment with aqueous extract after incubation period for 24 h, 48 h and 72 h. The data shows the cell viability (%) ± S.E.M obtained from three separate repeated experiments. *Significant difference ($p<0.05$) compared to negative control

Table 2: IC_{50} values of *T. crispa* extract on K562 cell for different incubation period

	IC_{50} values (µg/ml)		
Extract	24 h	48 h	72 h
Hexane	> 200	> 200	> 200
Dichloromethane	> 200	> 200	158±1.33
Ethanol	> 200	> 200	172±6.00
Aqueous	> 200	> 200	> 200

In general, *T. crispa* stem extracts were able to reduce the cell viability of K562 cells with different incubation period. From the previous study, it was found that *T. crispa* stem extract reduced the cell viability of different panel of cell lines such as MCF-7, HeLa, HN22 and HSC with the highest concentration of 100 µg/mL for MCF-7 and HeLa (Ibahim *et al*., 2011; Phienwej *et al*., 2015). These studies emphasized the potential of *T. crispa* stem extracts have potential cytotoxic effect on K562 cell but not reducing until 50% of the cell viability. Based on the data, extract from dichloromethane and ethanol was able to inhibit half of cell proliferation as the IC_{50} values were observed for both extracts of *T. crispa*. Hexane and water extract did give reduction to cell viability but not exceed IC_{50}, this show that hexane and water extract has less toxic compound in *T. crispa* compare to dichloromethane and ethanol extract. Hexane and water extract nee longer incubation time and higher concentration causes reduced cell viability by 50%. Table 2 shows the cytotoxic effect of *T. crispa* extract on K562 cell for different incubation period.

Statistical Analysis

The statistical analysis showed that there is significant difference (p<0.05) in terms of viability for the K562 cells treated with hexane, dichloromethane, ethanol and aqueous extract at concentrations 200 µg/mL, while 100 µg/mL for aqueous extract compared to that of the untreated cells. However, the statistical analysis in terms of incubation period of hexane and ethanol extract for 72 h has significant different with cell viability.

Conclusion

Sonication method produced higher yield percentage of crude extract than maceration method. Maceration method exhibited the same trend where the aqueous extract had the highest yield. This study showed that the aqueous extract produced the highest yield percentage of crude extract for both methods.

Meanwhile, cytotoxic assessment showed that the dichloromethane and ethanol extracts exhibited cytotoxic effect toward K562 cells after treatment for 72 h. The IC_{50} value obtained was 158±1.33 µg/ml for dichloromethane extract and 172±6.00 µg/ml for ethanol extract on K562 cells for an incubation period of 72 h. Hence, this study indicated that dichloromethane and ethanol *T. crispa* crude extracts were able to inhibit cell proliferation based on the results from MTT assay.

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Author's Contributions

All authors had given their contribution in preparation, development and publication of this manuscript.

Ethics

This article is originally from the authors works. The corresponding author confirms that all of the other authors have read and recognized the manuscript.

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