Cytotoxicity Effect and Morphological Changes of *Chrysanthemum morifolium* Methanolic Extract against Chronic Myeloid Leukaemia K-562 Cell Line

Muhamad Tarmizi bin Muhammad Noor¹, Hudaa Zulfaa binti Mohd Zainuddin¹, Muhammad Yusran bin Abdul Aziz¹, Syed Ahmad Tajudin bin Tuan Johari², Wan Nurfarahin binti Wan Osman*¹

¹School of Biomedicine, Faculty of Health Sciences, Universiti Sultan Zainal Abidin (UniSZA)
²Faculty of Medicine, Universiti Sultan Zainal Abidin (UniSZA)

* nurfarahinosman@unisza.edu.my

**Abstract**

*Xanthemum morifolium*, also known as “Bunga kekwa” in Malaysia, has various benefits and widely used in Chinese herbal medicines. The plant extract was reported to have significant biological activities, such as anti-inflammation, anti-tumour, antioxidant, and anti-cancer. Nonetheless, its anti-cancer potential on chronic myeloid leukaemia has remained elusive. The main goal of this study is to evaluate the cytotoxic effect of *C. morifolium* buds and flowers in methanolic extracts on chronic myeloid leukaemia malignancy K-562 cell lines. The bud and flower of *C. morifolium* were macerated for 72 hours in 100% methanol then were concentrated under reduced pressure using a rotary evaporator and oven-dried to obtain crude extracts. K-562 cells were treated with six different concentrations 400, 200, 100, 50, 25, and 12.5 µg/ml and incubated for 24, 48 and 72 hours. The in vitro cytotoxic activity was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test and was quantified using a microplate reader at 570 nm. Acridine orange and propidium iodide (AO/PI) staining were used to assess morphological alterations. MTT assays results showed moderate toxicity of both extracts. The lowest maximal half inhibitory concentration (IC50) value was observed at 72 hours of incubation; 182 ± 4.04 ug/ml for BM and 161 ± 7.88 ug/ml for flower extract (FM). However, there was a significantly different IC50 value (p<0.05) between the incubation periods of both treatments where the IC50 value at 24 hours was 301.33 ± 8.51 ug/ml 301 µg/ml in BM, 216 ± 10.79 ug/ml 216 µg/ml in FM and at 48 hours was 227 ± 12.25 ug/ml 227 µg/ml in bud extract (BM), 174 ± 11.92 ug/ml 174 µg/ml in FM. The morphological changes evidence was shown in AO/PI staining by the appearance of a mixed population of cells, early apoptosis, late apoptosis and necrotic cells. These findings suggested that methanolic *C. morifolium* extracts showed moderate cytotoxic effect on chronic myeloid leukaemia K-562 cells. Further study needed to identify the mode and mechanism of cell death in K-562 cells treated with the *C. morifolium* extracts.

**Keywords:** Anti-cancer; *Chrysanthemum morifolium*; K-562; Methanol extract; Bud; Flower; Cytotoxicity activity; AO/PI staining

*Author for Correspondence*


DOI: [https://doi.org/10.37231/ajmb.2021.5.2.446](https://doi.org/10.37231/ajmb.2021.5.2.446)
Introduction

Leukaemia is a type of cancer that involves the blood system. Leukaemia affects the blood system, which causes body failure to fight infections. Chronic Myeloid Leukaemia (CML) is slow-progressing leukaemia defined by the increased proliferation of the granulocytic cell line without losing its capacity to differentiate. It occurs when a mutation involving chromosome 9 and chromosome 22 undergo a reciprocal translocation t (9; 22) (q34; q11) and produces a chimeric oncogene BCR/ABL. CML can occur at any age but is most common between 40 and 60 years and slightly higher in males than females (1.4:1). Incidence increases with age between 40 – 60 years. Up to 30% of CML patients are aged more than 60 years.

In Malaysia, common CML cases are younger age groups between 20 – 50 years. Several treatment choices in CML include chemotherapy, targeted therapy using tyrosine kinase inhibitor (TKI), hydroxyurea, and allogenic stem cell transplantation. However, TKI treatment has been linked to cardiovascular disease, such as dasatinib and bosutinib, related to the higher risk of cardiac failure. At the same time, nilotinib was correlated with the most increased reports for ischemic heart disease, QT prolongation, and cardiac arrhythmias.

Chinese heritage in herbal medicine is awe-inspiring. According to Chinese culture, herbs’ properties and actions are fundamental in medicine. Secondary metabolites are known for the small organic molecules that have no responsibility for a plant’s development but are crucial in studying plants’ pharmacological effect on cancer cells. Chrysanthemum morifolium is one of the famous herbal medicinal plants enriched with various secondary metabolites such as flavonoids, terpenoids, and phenols. C. morifolium has many benefits, such as an anticancer activity shown on human prostate cancer DU145 cells. In C. morifolium, an established CML cell line known as K-562 is used in this study. Thus, this study was intended to uncover further the cytotoxic activity of C. morifolium plants in methanol extract against human CML, K-562 cells.

Materials and Methods

1.1 Reagents and materials

Sample of flowers and buds were purchased from Happy Organic market in Kuala Terengganu. The flowers were distributed from Oasis Wellness Sdn. Bhd. Rosewell Memorial Park Institute 1640 (RPMI) medium was purchased from Gibco, Fetal bovine Serum (FBS) and Dimethyl Sulfoxide (DMSO) were obtained from Sigma-Aldrich Co. Meanwhile, 3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) was gained from Molecular Probes Life Technologies Corp. Both Acridine Orange (AO) and Propidium Iodide (PI) were purchased from Biotium.

1.2 Extraction

The samples were ground to form a fine and delicate texture using Waring 8010S/G Two-Speed laboratory blender for 15 minutes. Next, each bud and flower petal weighed on the analytical balance to an actual 10g sample. The samples were then macerated with 100 ml of 100% methanol for 72 hours at room temperature covered with aluminium foil. Then, samples were filtered several times using restrain cloth and Whatman No.1 filter papers until no visible residues were observed. The methanol was removed using Stuart RE300 rotary evaporator under the condition of reduced pressure at 150hPa at 41°C until the crude extracts were obtained. The extracts were transferred to oven drying for several days. After drying, the extracts were weighed again to get the percentage of extracts yielded before keeping them in a refrigerator at 4°C.

1.3 Cell culture

Human chronic myeloid leukaemia K-562 cells (ATCC CCL-243) was cultured on RPMI 1640 medium that was supplemented with 10% FBS and incubated in the incubator with a condition of 5% CO₂ at 37°C for several days to wait for cells to grow. 100uL of cells with a density of 1 x 10⁶ cells/well were loaded into the 96 well microplate. Cells were treated with 100uL of BM and FM extracts. Six different concentrations of extracts were used, ranging from 400, 200, 100, 50, 25 and 12.5ug/ml loaded into triplicate wells for each type of extract. The untreated cells were left without giving any extracts treatments (0µg/ml). All plates were incubated at 24, 48 and 72 hours.

1.4 Cytotoxicity assay

The viability of the cells was measured by performing an MTT assay. 20uL of MTT solution with 5mg/ml concentration was added to each well on the microplate. The 96 well plates then were wrapped with aluminium foil before incubating at 37 ± 0.4°C in a humidified, 5% CO₂ atmosphere for 3 hours. After the incubation, the media was removed. 100uL of DMSO was added to each well and incubated at room temperature for around 10 minutes. The absorbance at 570nm was recorded using an Infinite M2000 Pro microplate reader. The percentage of cell
viability and cell inhibition was calculated using the Microsoft Excel 2010. The IC_{50} value was determined by plotting the graph of cell inhibition (%) against the extract concentration (µg/ml) [14]. Experiments were repeated for three independent sets of results.

1.5 AO/PI staining

Acridine Orange (AO) and Propidium Iodide (PI) dyes were prepared beforehand. 2mg of AO powder was weighed and diluted with 1ml of DMSO [15]. As for PI, it came in diluted liquid form, so no dilution is needed. Next, before using the dyes, working solutions were prepared by mixing the dyes with PBS. 25uL of diluted dyes from the stock solution were mixed with 975uL of PBS to produce 1ml of working solution. For cell preparation, 1 x 10^5 of cells/well were seeded in a six-well plate and treated with extract using IC_{50} values from MTT assay [15]. After incubation at 24, 48, and 72 hrs, cells were collected and spun at 1500rpm for 10 minutes at 21°C. 20uL of each Acridine Orange (AO) and Propidium Iodide (PI) dyes were added with the cells pellet. The dyed cells then were fixed on the slides and were viewed under the fluorescence microscope.

1.6 Statistical analysis

The analysis was performed by using IBM SPSS Statistics version 25 and Microsoft Excel 2010. Data were expressed as the mean ± standard error of the mean (SEM). One Way ANOVA test was used to compare cytotoxicity differences from different incubation phases (24, 48, and 72 hrs) in each type of extract. The significant differences of p-value < 0.05 were defined as the statistical significance compared to control.

Results

Extraction yielded

The crude extracts were weighed before and after the oven drying process to obtain the percentage of extraction yielded. BM extract yielded 4.94% while FM 5.91%.

Cytotoxicity effect of BM and FM extract on K-562 cells

Significant reduction in K-562 cells in viability in both treatments started at a concentration of 100, 200 and 400µg/ml. IC_{50} value for BM extract were 301.33 ± 8.51µg/ml, 227 ± 12.25ug/ml, and 182 ± 4.04ug/ml at 24, 48, and 72 hours respectively (Table 1). Meanwhile, for FM extract, the IC_{50} values were 216 ± 10.79ug/ml, 174 ± 11.92ug/ml, and 161 ± 7.88ug/ml at 24, 48, and 72 hours respectively (Table 1). There were significant differences between the incubation phases in both extracts proved by p-value <0.05.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Time (h)</th>
<th>IC_{50} (µg/ml)</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bud</td>
<td>24</td>
<td>301.33 ± 8.51</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>227 ± 12.25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>182 ± 4.04</td>
<td></td>
</tr>
<tr>
<td>Flower</td>
<td>24</td>
<td>216 ± 10.79</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>174 ± 11.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>161 ± 7.88</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. The IC_{50} values at 24, 48 and 72 hrs. Data are expressed as mean ± S.E.M from three independent experiments. Statistics performed by one way ANOVA test with significant p-value (p<0.05)

*Morphological changes of cells in AO/PI staining

Results showed the morphological changes of cells accessed by the color of the stained cells. Viable cells (VC) were stained as green stained with the intact nucleus. The presence of green cytoplasm and an orange-ish nucleus showed that the cell experienced early apoptosis. In late apoptosis, both the cytoplasm and the nucleus are dominantly orange due to nuclear envelope breaking, resulting in nuclear death (Figure 4 and Figure 5). Meanwhile, necrotic cells (NC) had a compacted shape and a homogenous orange to red nucleus. The characteristics of apoptosis and necrosis were also observed with the appearances of apoptotic bodies (AB), cells-blebbing (BL), nuclear margination (NM), chromatin condensation (CC), and membrane loose (ML), and nuclear fragmentation (NF).

Discussion

The global cancer rate is constantly rising, along with the advent of cancer treatment concerns. Many anticancer compounds’ use in clinical practice has been restricted due to the advent of resistance, toxic effects, and exorbitant prices. As a result, most research is now centred on developing novel bioactive compounds to resolve these concerns. Plant screening for anticancer activity has recently been a significant area of research that has culminated in the launch of many cancer therapies in the sector.

So far, there is little information about C. morifolium anticancer activity on myeloid cancer amid its use in traditional medicine for cancer treatment. The in vitro cell based cytotoxicity assay has been a valuable, dependable, and fast technique of evaluating anticancer and chemotherapeutic action [16]. According to the American National Cancer Institute (NCI) criteria, the effectiveness for cytotoxicity in crude extracts is based on its IC_{50}, which must be less than 30µg/mL, especially after 48 and 72 hours of the incubation period. While a crude extract
Figures 4. The morphology of untreated K-562 cells (UT) and treated K-562 cells (T) at 24, 48, and 72 hrs under BM extract at concentration of 301, 227, and 182μg/ml respectively. The photomicrograph of morphological changes in K-562 cells taken by using fluorescence microscope under 40X magnification. Pictures showing viable cells (VC), early apoptosis (EA), late apoptosis (LA), cell blebbing (BL), necrotic cells (NC), nuclear fragmentation (NF), secondary necrosis (SN), membrane loose (ML), apoptotic bodies (AB) and chromatin condense (CC).
Figures 5. The morphology of untreated K-562 cells (UT) and treated K-562 cells (T) at 24, 48, and 72 hrs under FM extract at concentration of 216, 174, and 161μg/ml respectively. The photomicrograph of morphological changes in K-562 cells taken by using fluorescence microscope under 40X magnification. Pictures showing viable cells (VC), early apoptosis (EA), late apoptosis (LA), cell blebbing (BL), necrotic cells (NC), nuclear fragmentation (NF), secondary necrosis (SN), membrane loose (ML), apoptotic bodies (AB) and chromatin condense (CC).
with an IC₅₀ greater than 30g/mL is considered lowly cytotoxic to cancer cells [17]. This study found that bud methanol (BM) and flower methanol (FM) extracts of the C. morifolium plant showed a moderate cytotoxicity effect on K-562 cells (Figure 1 and Figure 2). However, there were significant differences between the incubation periods in each extract (Table 1) which leads to significant morphological changes in the cells were seen due to the extracts' high toxicity at a specific IC₅₀ dosage.

A previous study has demonstrated that extraction with occasional shaking is the best method [18]. In this study, the plant samples were soaked together with methanol and incubated at lower room temperature for 72 hours without shaking, leading to the incomplete process of breaking the cell walls to release the soluble bioactive compounds of the C. morifolium plant. In addition, the extraction process suggested to use the soxhlet apparatus followed by the purification of the active compound of C. morifolium to synthesize the silver nanoparticle (AgNPS). The cytotoxic activity of C. morifolium extract on lung cancer cells line A549 reported was improved using that method [19]. In this study, the oven-drying method was used to remove moisture from the samples. However, the drying process on extract content may affect the bioactive compounds, suggesting the sensitivity of the compounds to temperature exposure.

Determination of the apoptosis induction involving the treated K-562 cells was assessed by AO/PI staining. Based on the finding, the cells appear green fluorescence while PI appears red-orange fluorescence in damaged cells. In this study, after 24 hours of treatment with IC₅₀ from BM and FM extract, apoptosis in the treated cells began to be observed. The number of cells experiencing early and late apoptosis rose when the treatment period was extended. Treated cells demonstrated clear morphological changes as evidenced by their characteristics such as membrane blebbing, fragmentation of nuclei, membrane loss, nuclear margination, cell shrinkage, chromatin condense, and formation of apoptotic bodies (Figure 3 and Figure 4).

This finding is consistent with a previous study that uses kenaf seed oil on the K-562 cells [15].

Conclusion

C. morifolium has promising outcomes as antiproliferative agents towards K-562 cells as shown by the morphological changes of cells under treatments even the cytotoxic effects are moderate. Improvement is needed to further clarify the antiproliferative activity on the cells.

Acknowledgments

We would like to thank Universiti Sultan Zainal Abidin (Grant no. UniSZA/LABMAT/2018/06 – R0044-R006) for the research grant and the facilities provided.

References


