Recognition of Phosphatidylserine Externalisation on Human Chronic Myeloid Leukaemia (K562) Cells Induced by *Chrysanthemum morifolium* Methanol Extract

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**Abstract**

*Chrysanthemum morifolium* is one of the common plants used in traditional Chinese herbal medicine and currently being discovered as an invaluable source for treatment of cancerous diseases. Conventional approach to leukaemia treatment such as chemotherapeutic agents result in good overall survival (OS) in patients, but issues of multi-drug resistance (MDR) and disease heterogeneity can make chemotherapy less effective. Thus, there is a constant need for alternative therapeutic strategy such as developing new anticancer drug from natural products. The present study is carried out mainly to detect phosphatidylserine externalisation as an apoptosis indicator on human chronic myeloid leukaemia (K562) cells induced by *Chrysanthemum morifolium* methanol extract. K562 cells were treated with indicated IC<sub>50</sub> values of bud and flower methanol crude extracts for 24 hours. Phase contrast microscopy was used to observe morphological changes in K562 cells post-treatment. Annexin V-FITC/PI apoptosis assay via flow cytometry was done to investigate apoptosis phenomena by detecting phosphatidylserine (PS) externalisation in treated K562 cells. Morphological observations using phase contrast microscopy showed distinct apoptotic characteristics in K562 cells treated with both bud and flower methanol extracts. Annexin V-FITC/PI assay demonstrated a significantly high apoptotic cell population and apoptotic rate (p < 0.05) in treated K562 cells compared to control cells. The study outcomes presented apoptotic inducing ability of *Chrysanthemum morifolium* towards chronic myeloid leukaemia cancer cells.

**Keywords**

Phosphatidylserine externalisation; *Chrysanthemum morifolium*; Methanol extract; K562 cells; Flow cytometry; Annexin V-FITC/PI assay, Apoptosis, Chronic myeloid leukaemia

**Introduction**

Genetic mutations that promote myeloid blast proliferation, induce self-renewal and/or inhibit haematopoietic differentiation are the hallmarks of the heterogeneous disorder known as myeloid malignancies [1]. They include myeloproliferative neoplasms (MPNs), myelodysplastic syndrome (MDS),...
and acute myeloid leukemia (AML). Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm characterized by the overproduction of granulocytes [2]. It is a disease associated with the occurrence of the Philadelphia (Ph) chromosome, caused by a reciprocal chromosomal translocation between chromosome 22 and chromosome 9. The BCR-ABL1 fusion oncogene is produced as a result of this DNA interchange, a 210 kDa protein crucial for the unregulated myeloid cell proliferation and eventually leading to CML [3]. CML accounts for 15% of leukaemia incidences in the Western hemisphere and the approximate annual incidence is 1-2 per 100000 persons per year [4]. It is estimated that 30% of adult leukemia incidents are of CML and it affects people with median age of 50 years in Africa and Asia with a slight male prevalence [5,6].

Chemotherapy, radiation therapy and stem cell transplant are among the main treatment options in leukaemia [7]. People with leukaemia are usually treated with chemotherapy as most of the patients, especially adults are unsuited for bone marrow transplantation [8]. Chemotherapies for leukaemia include anti-metabolites (such as hydroxyurea), topoisomerase II inhibitors (such as doxorubicin), and alkaloids (such as vincristine). Targeted therapies are used for particular types of leukaemia, such as BCR-ABL positive leukaemia that responds to tyrosine kinase inhibitors (such as imatinib). Still, dangerous side effects associated with chemotherapy drugs such as immunosuppression, liver dysfunction, neuropathy and cardiac disease causes poor adherence to the course of therapy and poor prognosis [9]. Although combining anticancer drugs is an effort to activate different mechanisms and to improve therapeutic after-effects, normal tissue toxicity and multidrug resistance continues to be a major setback [10]. One potential means to lower toxicities or side effects and enhance the efficacy of anti-cancer drugs is to develop traditional medicines, especially from natural products [11].

Chrysanthemum is a species of perennial plant from family Asteraceae which includes about 40 species. Chrysanthemum has been used for hundreds of years in Chinese medicine. The Chinese Pharmacopoeia have recorded the medicinal use of Chrysanthemum morifolium for the treatment of respiratory and cardiovascular diseases [12]. Recent studies have shown that Chrysanthemum morifolium has many pharmacological activities, such as anti-inflammatory, anti-oxidation, anti-cancer, prevention of cardiovascular diseases and hepatoprotective properties [13]. As flavonoids, caffeoylquinic acids and essential oils are the major bioactive components found in Chrysanthemum morifolium, these natural agents contribute to the therapeutic activities of this plant [14,15]. These plant metabolites are valuable sources of anticancer drugs and play an important role in cancer by preventing damage to DNA and increasing antioxidant levels in the body with minimal adverse effects [16].

In vitro investigations suggested that anticancer activities of natural products such as flavonoids are associated with inducing cell differentiation, cell proliferation inhibition, cell cycle arrest and apoptosis [17,18]. Organic compounds that can induce apoptosis are also found to trigger externalisation of a cell membrane phospholipid called phosphatidylserine (PS) to attract macrophages for phagocytosis and promote clearance of apoptotic debris [19,20]. A reliable method to identify apoptosis is using Annexin V binding assay. This technique utilises flow cytometry to detect the attachment of Annexin V-FITC, a specialised probe for PS recognition, coupled with a dye called propidium iodide, that can penetrate cells with impaired membranes [21]. This is an important characteristic for anti-cancer drug development, a valuable tool for apoptosis detection and a golden opportunity for developing potential cancer biomarker. Thus, the focus of the present research is to detect phosphatidylserine externalisation as an apoptosis indicator on human chronic myeloid leukaemia (K562) cells induced by Chrysanthemum morifolium methanol extract to facilitate its application as a nutraceutical and drug candidate.

Materials and Methods

Reagents and materials

Roswell Park Memorial Institute (RPMI) 1640 medium was procured from Nacalai Tesque (Kyoto, Japan). Foetal bovine serum (FBS) was acquired from Tico Europe (Netherlands). Phosphate buffer saline (PBS)
and absolute methanol were obtained from Sigma-Aldrich Corporation (St. Louis, USA). Doxorubicin hydrochloride was bought from Abcam (UK). BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I was purchased from Becton, Dickinson and Company (USA). Edible Chrysanthemum morifolium in the form of dried bud and flower were procured from a local organic food store in Kuala Terengganu, Terengganu, Malaysia, originating from China (Tongxiang city, Zhejiang province).

Preparation of extract
Extraction was performed at Postgraduate Laboratory, UniSZA according to the method as previously described [22]. Dried bud and flower were pulverised into fine texture using Waring Commercial Blender (Model 7010S). The fine powder of bud and flower (10 mg) was macerated in absolute methanol (100 ml) by 1:10 (w/v) ratio at room temperature, dark place for 72 hours. The extract was filtered using cloth strainer and Whatman No.1 filter paper until no residue appeared. Methanol was removed by rotary evaporation (Stuart RE300 rotary evaporator, 150hPa, 41°C) until the crude was obtained. The crude extract was further oven-dried at 40°C to form a honey-like consistency. The extract was prepared as a stock solution in 100% dimethyl sulphoxide (DMSO) at a concentration of 10 mg/ml (w/v).

Cell culture, treatments, and cell morphology analysis
Cell culture, treatment and morphology analysis were done in CERIDB, UniSZA. Human chronic myeloid leukaemia K562 cells (ATCC CCL-243) were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS). Antibiotics were not added to the medium to prevent development of antibiotic resistance environment. The cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C. K562 cells seeded with a density of 5 × 10⁵ cells/well in sterile round-bottomed 6-well culture plates were treated with the IC₅₀ values of bud and flower methanol extracts (300 μg/ml and 216 μg/ml respectively) determined from the previous study [22] and incubated for 24 hours. K562 cells treated with doxorubicin hydrochloride (1 μg/ml) were prepared as positive control and K562 cells without treatment were used as a negative control. After 24-hours incubation period, control and treated K562 cells were observed for morphological changes using phase contrast inverted microscope (Olympus CKX41) and photomicrographs were taken from each treatment group for cell morphology analysis.

Apoptosis assay
Apoptosis assay was carried out in Faculty of Science and Marine Environment, Universiti Malaysia Terengganu. BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I was used to detect phosphatidylserine externalisation and apoptosis phenomena of treated K562 cells at 24-hours incubation period. Cells from the control and treatment groups were harvested, centrifuged and washed twice in 1X PBS. 100 μl of 1X Annexin V binding buffer was added to resuspend the cells, along with 5 μl of Annexin V-FITC conjugate and 5 μl of propidium iodide solution for 15 minutes’ incubation in the dark at room temperature. Next, the cells were added with 400 μl of 1X Annexin V binding buffer and analysed using BD Accuri™ C6 Plus flow cytometer (Becton, Dickinson and Company, USA). 10,000 events were recorded for each sample, and positive FITC and/or PI cells were quantified using the BD Accuri C6 Plus software (Becton, Dickinson and Company, USA). The results of three different readings were generated as dot plot graphs.

Statistical Analysis
Three repeated tests for each method yielded all of the data. All experiment values were denoted as mean ± SEM. GraphPad Prism version 9.5.1 was used to generate statistical analysis. The comparison between treatment groups were studied using ANOVA (One-way or Two-way depending on the number of independent variables) with Tukey post hoc test. P-values (p < 0.05) were considered significant.

Results
Effect of Chrysanthemum morifolium methanol crude extracts on K562 cell morphology

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To observe the alterations in cell appearance caused by *Chrysanthemum morifolium* methanol crude extracts and doxorubicin as positive control drug, K562 cells were treated with the IC\textsubscript{50} values of extract for 24 hours before being evaluated via phase contrast microscopy (Figure 1). Untreated cells displayed normal characteristics, such as spherical and uniform nuclei (Figure 1A). The usual hallmarks of apoptosis, namely shrinkage of cells, blebbing of membrane and formation of apoptotic bodies, were evident in treated cells (Figure 1B-1D, arrows).

**Figure 1:** Morphological alterations in K562 cells post 24-hours treatment. Control K562 cells (A) retains normal features. Treated K562 cells (B – doxorubicin; C – bud methanol extract; D – flower methanol extract) exhibited cytotoxic morphological changes consistent with apoptosis as shown with arrows (1 – apoptotic bodies, 2 - membrane blebbing, 3 – cell shrinkage), 100× magnification.

**Apoptotic effect of Chrysanthemum morifolium methanol crude extracts on K562 cells**

Annexin V-FITC/PI apoptosis assay was carried out to demonstrate the apoptotic activity of *Chrysanthemum morifolium* methanol crude extracts on K562 cells. Dual fluorescence stain, Annexin V-
FITC/PI was applied to differentiate between live, necrotic, and apoptotic cells. Dual parameter dot plots were generated based on the intensity of fluorescence produced by FITC and PI. Live cells (Annexin V-FITC negative/PI negative), early apoptosis (Annexin V-FITC positive/PI negative), late apoptosis (Annexin V-FITC positive/PI positive), and necrotic cells (Annexin V-FITC negative/PI positive) were separated into four quadrants. Figure 2, 3 and 4 showed the flow cytometry dot plot graphs, cell population and apoptosis rate after exposure to the crude extracts and doxorubicin for 24 hours. K562 cells treated with bud and flower methanol extracts have a significantly higher apoptosis population and rate compared to the control group (p < 0.05). Doxorubicin induces prominent necrosis in K562 cells with 27.8% of cells are necrotic.

Figure 2: Flow cytometry dot plot graphs of control and treated K562 post 24-hour exposure. The four quadrants represented populations of live cells (lower left, LL), early apoptotic cells (lower right, LR), late apoptotic cells (upper right, UR) and necrotic cells (upper left, UL). Each quadrant value is represented in percentages.
Figure 3: The viable, early-apoptotic, late-apoptotic, and necrotic cell populations of control and treated K562 cells are shown in a bar chart. The findings are presented as mean ± SEM of three separate trials, with * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001 denoting significant differences from control cells.

Figure 4: The apoptosis rate (early and late apoptosis) in control and treated K562 cells are depicted in a bar chart. The data are shown as mean ± SEM of triplicates. Significant differences from control cells are indicated as * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001.
Discussion

A crude extract is usually regarded as having in vitro cytotoxic effect if the IC\textsubscript{50} value is less than 30-40 µg/ml, in accordance with the United States National Cancer Institute plant testing protocol [23]. The anti-proliferative effects of extracts were classified into four categories based on the median inhibitory concentration: active (less than or equals to 20 µg/ml); moderately active (20-100 µg/ml); weakly active (100-1000 µg/ml) and inactive (more than 1000 µg/ml) [24]. A previous investigation on bud and flower methanol extracts of the Chrysanthemum morifolium plant revealed a moderate cytotoxicity effect on K562 cells [23]. Methanol, a polar solvent is used in this study as it is discovered to be effective in recovering polyphenols from plant materials [25, 26]. Previous studies have shown that plant methanol extracts contain a variety of polyphenolic compounds and these plant extracts have demonstrated cytotoxic abilities towards cancer cell lines [27, 28, 29]. Plant-derived anti-cancer drugs have a wide range of different modes of action, but the majority of them cause apoptotic cell death via extrinsic or intrinsic routes, caspase-mediated and/or p53 independent or dependent mechanisms [30].

Phase contrast microscopic observation of treated K562 cells with IC\textsubscript{50} values of Chrysanthemum morifolium methanol extracts indicated cytotoxic morphological changes akin to apoptosis. The term apoptosis is often used interchangeably with programmed cell death. When a cell dies through apoptosis, certain morphological alterations occur, such as shrinkage of cells, condensation of chromatin, disintegration of nucleus and blebbing of membrane [31]. Shrinkage of cells is a major morphological feature of practically all apoptotic cell death caused by aberrant changes in cellular water content [32]. According to a previous study, leukaemia cells respond negatively to extract exposure at higher concentrations than control cells, as evidenced by the leukaemia cells’ altered shape [33].

The apoptosis activity in K562 was also detected by Annexin V-FITC/PI apoptosis assay, which quantifies externalization of phosphatidylserine (PS) in apoptotic cells using flow cytometry. When a population of cells is stained with Annexin V-FITC and propidium iodide (PI) in the binding buffer, apoptotic cells fluoresce green, dead cells fluoresce red and green, and living cells fluoresce little to none. The aforementioned cell groups can be easily identified by a flow cytometer that uses argon-ion laser’s 488 nm spectral line for excitation. K562 cells exposed to the IC\textsubscript{50} concentrations of methanol crude extracts for 24 hours showed significant increase in apoptotic cell population and apoptotic rate. The findings are in support of a report that clearly indicates K562 cells are chemically sensitive to the components in crude extract, albeit to variable degrees [34]. The phytochemical combinations in Chrysanthemum morifolium, which are normally responsible for the plant’s biological activity, may have contributed to the cytotoxic activity in this study. This is corroborated by earlier research that identified various anti-cancer compounds in Chrysanthemum morifolium extracts such as caffeoylquinic acids and flavonoids especially luteolin, quercetin and diosmetin [14, 15, 35]. Previous cytotoxicity studies also showed the efficacy of Chrysanthemum morifolium in inhibiting gastric (MKN45) and colon (Colon205) cancer cell lines [35, 36].

PS is usually restricted to the inner leaflet of the plasma membrane under normal circumstances. On the other hand, PS externalises on the exterior layer of cells during cellular stimulation and/or initiation of cell death [37]. PS exposure is a common event during apoptosis event, which occurs when DNA damage and membrane leaks occur [38]. Given that Annexin V belongs to the phospholipid-binding annexin family which binds most efficiently to the negatively charged PS, Annexin V-FITC was developed as an effective probe for early detection of apoptosis [39]. Staining cells simultaneously with PI dye aids in detecting cells in early and late apoptosis phase. PI only penetrates membranes of dead or damaged cells. Therefore, cells that are both Annexin V-FITC and PI negative are viable cells. Cells in the late apoptosis phase are both Annexin V-FITC and PI positive. Exposure of PS triggered by pro-apoptotic stimuli is an evolutionary characteristic of mammalian and bacterial cells [21]. While a wide range of biochemical techniques may be used in the molecular analysis of key apoptosis regulators, microscopy and immunostaining utilises straightforward, affordable techniques that offers an extensive amount of information on the characteristics of apoptosis.
Based on the general observations of cytotoxic morphological alterations and apoptotic events in K562 cells treated with bud and flower methanol extracts, bud extracts are found to be more severe in damaging K562 cells compared to flower extracts. The dissimilarity could be contributed by the different levels of bio-compounds found in the bud and the flower head of Chrysanthemum morifolium. Studies have presented variation of polyphenol and flavonoid levels in different stages of flower development in safflower and Camellia sinensis flowers [40,41]. In a prior study on the development of Chrysanthemum morifolium florets, flavonoid concentrations reached its highest in the half-open flower and subsequently dropped significantly as the florets matured. It is possible that this could be a result of the flowers’ exposure to sunlight and hot temperatures [42]. It should be taken into consideration that the present study was based on crude extracts and in-depth investigation should be carried out to isolate the bioactive compounds from Chrysanthemum morifolium, that is responsible for the cytotoxicity and apoptosis effect on K562 cells.

**Conclusion**

Chrysanthemum morifolium methanol extract induces apoptosis in K562 cells as shown in phase contrast microscopy and quantification of phosphatidylserine externalisation in flow cytometry apoptosis assay. More detailed investigations are needed to further explore the anti-cancer ability of Chrysanthemum morifolium, in which could serve as one of complementary and alternative medicine (CAM) for chronic myeloid leukaemia treatment.

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**Conflict of Interest Disclosure**

None to declare.

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