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Antiproliferative and Apoptosis-Inducing Effects of Ethanolic Extract of Morus Alba Leaves on Human Chronic Myeloid Leukaemia K-562 Cell Lines

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Abstract

The rate of occurrence and prevalence of chronic myeloid leukaemia (CML) are increasing globally, despite it being a rare type of cancer. Morus alba also known as mulberry, has been used in Chinese traditional medicine for a long time to treat various conditions and diseases like hypertension and arthritis. Modern scientific studies have also recognized its medicinal properties, including antioxidant, antidiuretic, antidiabetic, antimicrobial and anticancer. However, its antiproliferative effects on specifically human CML K-562 cell lines remain poorly elucidated. This study aims to determine the antiproliferative effects of M. alba on K-562 cells. In vitro cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Acridine orange and propidium iodide (AO/PI) dual staining were then employed to assess the method of cell death. The ethanolic extract of M. alba leaves exhibited significant antiproliferative activity, with the lowest maximal half inhibitory concentration (IC50) value observed at 72 hours being 23 µg/mL. Notably, AO/PI staining revealed significant morphological changes, such as membrane blebbing, nuclear fragmentation, and indicator of early apoptosis, and late apoptosis. These results demonstrated that ethanolic extract of M. alba leaves induces apoptotic cell death in K-562 cells. Overall, these findings suggested that ethanolic extract of M. alba leaves exerts potent antiproliferative effects on human CML K-562 cells a in time- and dose-dependent manner.

Keywords
MorusA; Chronic Myeloid Leukaemia; Cytotoxic; AO/PI Double Staining Assay; Apoptosis

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Introduction

Cancer ranks as a prominent global cause of mortality, second only to heart disease.[1] The Malaysia National Cancer Registry Report reveals that a total of 111,238 new cancer cases were identified in Malaysia between 2012 and 2016. Leukaemia ranked as the sixth most prevalent cancer among the top ten cancers in Malaysia, following breast, colorectal, lung, lymphoma, and nasopharyngeal cancers. It accounted for 4,273 cases, which represented approximately 3.7% of the total cancer cases in Malaysia. This corresponds to 3.7% of the total 111,238 cancer cases reported. Among the 4,273 patients diagnosed between 2012 and 2016, 56.5% were male and 43.5% were female. Interestingly, most cases were observed in children aged between 0 and 14 years.[2]

Chronic Myeloid Leukemia (CML) is characterized by a gradual progression and the excessive presence of myeloblasts in both the bone marrow and blood. This condition is caused by a reciprocal translocation known as t(9;22)(q34;q11) involving chromosome 9 and chromosome 22. This translocation leads to the formation of the BCR/ABL chimeric oncogene.[3] CML can occur at any age, but it is most observed between 40 and 60 years of age, with a slightly higher incidence in males compared to females (1.4:1). The frequency of CML cases increases with age within the 40-60 age range, and up to 30% of CML patients are over 60 years old. However, in Malaysia, CML is more prevalent among younger age groups, particularly individuals between 20 and 50 years old.[2]

Morus alba, commonly known as mulberry or white mulberry, is a plant belonging to the Moraceae family. It is referred to as 'Sang Shen' in China and 'Oddi' in Korea.[4] In India, it is known as 'Tut' in Hindi and 'Musukette' in Tamil.[5] In Malay, the plant is known as 'besaram', 'malberi', or 'daun ulat sutera'. M. alba has a long history of use in traditional medicine and Ayurveda, owing to its numerous health benefits.[6] The plant contains natural bioactive compounds that exhibit significant biological activity and have been found to have pharmacological benefits for various illnesses.[6–10]

Various parts of the plant contain a wide range of bioactive compounds, including phenolic acids, flavonoids, flavonols, anthocyanins, macronutrients, vitamins, minerals, and volatile aromatic compounds.[6,11] Extracts and isolated components obtained from the fruits, leaves, stem, and root of M. alba have demonstrated antioxidant, antibacterial, antiviral, and neuroprotective properties.[8] A limited number of compounds have been identified from the root bark and leaf extract of M. alba, and their potential anticancer effects have been investigated in relation to breast, cervical, liver, and colon cancer, as well as human acute myeloid leukaemia.[12–15] However, no research has been conducted specifically on chronic myeloid leukaemia. Therefore, the present study aims to assess the cytotoxic activity of the ethanolic extract derived from M. alba leaves against the human chronic myeloid leukaemia K-562 cell line.

Materials and Methods

Methodology

Plants collection

Leaves of M. alba were collected from the districts of Kuala Nerus and Kuala Terengganu in Terengganu. Plant authentication was performed by a competent botanist from the Universiti Sultan Zainal Abidin. The voucher number is UniSZA00286. Only fresh, green leaves were selected for the study.

Preparation of plant materials

The leaves were dried using an oven-drying method at a temperature of 40˚C. Subsequently, the dried leaves were cut and ground into a powder. The powdered M. alba leaves were then soaked in 70% ethanol, maintaining a sample-to-solvent ratio of 1:50. This maceration process was conducted at room

temperature, allowing the ground leaves to soak for one week. After the maceration period, the mixture then underwent centrifugation at 1780 rpm for 5 minutes at a temperature of 20˚C. Next, the extract was collected and filtered through Whatman No. 1 filter paper. To concentrate the extract and remove the ethanol, a Stuart RE300 rotary evaporator was utilized, operating at a reduced pressure of 175 hPa and a temperature of 40˚C. The ethanolic M. alba extracts were transferred into falcon tubes and stored in a chiller at 4 ˚C before proceeding with the freeze-drying process to obtain the extract in solidified form. The final product of the extract was weighed, and the percentage of extraction yield was then calculated using the formula below:

$$\text{Extraction yield (\%)} = \frac{weight\ of\ extract\ after\ evaporating\ solvent\ and\ freeze\ drying\ (g)}{dry\ weight\ of\ sample\ (g)} \times 100$$

Cell lines and cell culture preparation

Human chronic myeloid leukaemia K-562 cell lines were purchased from ATCC (Manassas, VA, USA). The cells were cultured in RMPI-1640 media (Nacalai Tesque, Japan), supplemented with 10% FBS (Gibco, USA), and antibiotics (100.0 units/mL penicillin and 100.0 µg/mL streptomycin) (Gibco, USA), and incubated in the incubator with a condition of 5% CO2 at 37°C. K-562 cells were sub-cultured every 3 days by splitting the culture 1: 2 in the new flask with fresh growth medium. Cell confluency of above 95% was used throughout this study.

Cytotoxicity test-MTT assay

The MTT assay was performed using a 96-well flat-bottom microtiter plate (Eppendorf, Germany). Etoposide was utilized as a positive control in this assay because of its known anti-tumor activity and its ability to induce apoptotic cell death in tumor cells.16 Initially, a volume of 100 µL of complete RMPI-1640 media was added into each well in rows B to H of the 96-well plate. Next, a volume of 200 µL of either M. alba extract or etoposide drug was aliquoted into wells in rows A to B of the 96-well plate in triplicate, and serial dilutions were performed from row B to G. Wells of row H were left untreated to serve as a negative control. Subsequently, a volume of 100 µL of 1 x 105 cells/mL K-562 cells were seeded into all 96-wells plate and incubated for 24, 24 and 72 hours in a CO2 incubator. After consecutive hours of incubation, 20 µL of MTT solution (5 mg/mL) was added into each well and incubated for 4 hours. Then, the culture medium was removed and 100 µL of 100% DMSO solution was added to each well to solubilize the formazan formed. The plates were read using the plate reader (Tecan, USA) at 570 nm with a reference wavelength of 630 nm wavelength.17 A dose-response curve of the percentage of cell viability versus extract concentration was plotted using the formula below:

$$Cell\ viability\ (\%) = \left(\frac{ODsample}{ODcontrol}\right) X\ 100$$

Acridine orange/propidium iodide double staining assay

Morphological changes and mode of cell death of K-562 cells were assessed using Acridine Orange (AO) and Propidium Iodide (PI) dyes. Initially, a volume of 3 mL containing 2 X 105 K-562 cells were seeded in a 6-well plate (Eppendorf, Germany). Subsequently, the K-562 cells were treated with M. alba extract by using the IC50 value obtained from the MTT assay. Each treatment was performed in triplicate, while cells without treatment served as a negative control. After 24-, 48- and 72-hour incubation, the cells were harvested and pelleted at 1500 rpm for 10 minutes at a temperature of 21°C. The cell pellets were washed

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with PBS (Nacalai Tesque, Japan) by the centrifuging process. Next, the pellets were resuspended in 50 µL of acridine orange (10 µg/mL) and 50 µL of propidium iodide (10 µg/mL) for 5 minutes. Subsequently, 20 µL of stained cells were pipetted onto a glass slide and covered with a cover slip. The stained cells were then observed under the inverted fluorescent microscope (Nikon TE2000-U, Nikon, Japan).[18]

Statistical analysis

The analysis was done by using Microsoft Excel 365 Apps for the enterprise. Results were expressed as mean ± standard error of mean (S.E.M).

Results

Extraction yield

The percentage of extraction yield was calculated and obtained by measuring the weight of dried M. alba leaves before the extraction process and the weight of the final product after extraction with ethanol. Table 1 shows the percentage extraction yield of ethanolic extract of M. alba leaves is 11.89%.

Table 1: The percentage of extraction yield of ethanolic extract of M. alba leaves.

Sample	Extraction yield (%)
Ethanolic extract of *M. alba* leaves	11.89

Effects of ethanolic extract of M. alba leaves on K-562 cell line

The cytotoxic effects of the ethanolic extract of *M. alba* leaves towards K-562 cells were determined by measuring the cell viability using MTT assay following a 72-hour treatment with the various concentrations of the extract. Table 2 shows the cytotoxicity activity of the ethanolic extract of *M. alba* leaves on the growth of K-562 cells. The IC_{50} value of the extract for the K-562 cells after 72 hours was 23 ± 2.08 µg/mL. In comparison, the IC_{50} value of etoposide for the K-562 cells after 72 hours was found to be 4.6 ± 0.12 µg/mL. In Figure 1, the IC_{50} of the *M. alba* leaves ethanolic extract for the K-562 cells was observed to be 23 ± 2.08 µg/mL. Additionally, the graph also gave the concentration of the ethanolic extract of *M. alba* leaves that resulted in a 25% reduction in the cell population (IC_{25}) at 45 µg/mL and for a 75% reduction (IC_{70}), the concentration was determined at 11 µg/mL.

Table 2: The IC_{50} values of ethanolic extract of *M. alba* leaves and etoposide on K-562 cells after 24, 48 and 72 hours.

Incubation Period (hrs)	Mean Average Values of IC_{50} (µg/mL)	
	Etoposide	*M. alba*
24	44.8 ± 0.88	54.5 ± 1.03
48	7.7 ± 0.78	38.2 ± 2.60
72	4.6 ± 0.12	23 ± 2.08

*IC50 values were determined from the dose responses curve that correlates between the cell viability and extract concentration. The IC50 values were expressed as Mean ± S.E.M determined from three independent experiments that were performed in three replicates.

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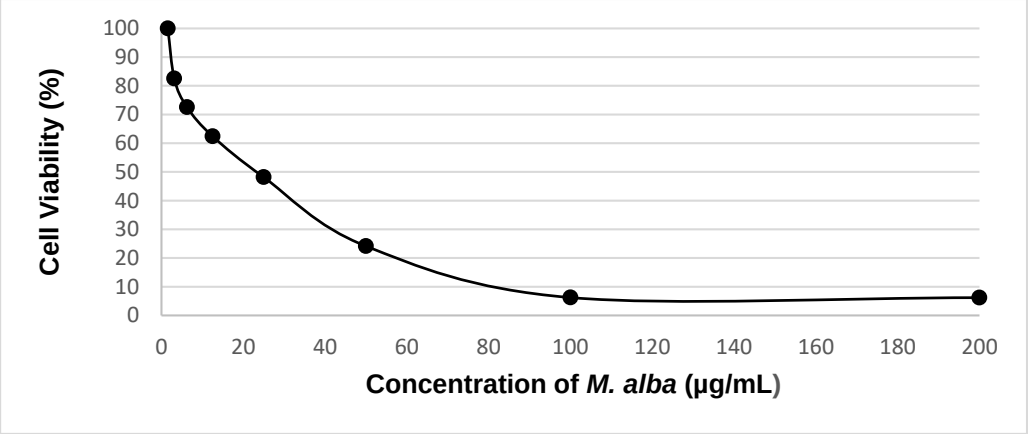
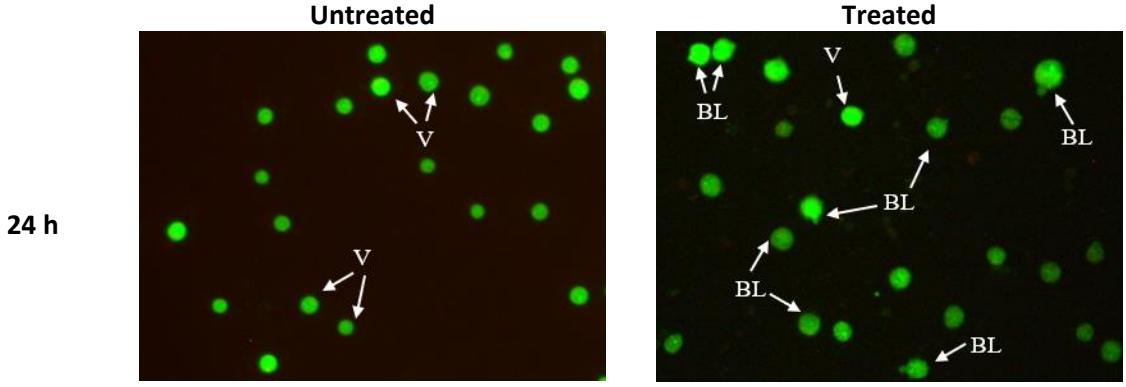


Figure 1. Cytotoxic effect of ethanolic extract of *M. alba* leaves on K-562 cells at 72 hours.

Mode of cell death induced by ethanolic extract of M. alba leaves

Detailed morphological changes and the mode of cell death were assessed by utilizing acridine orange (AO) and propidium iodide (PI) dyes. AO emits green-coloured fluorescence and can permeate to both viable living and non-viable dead cells upon being stained. In contrast, PI emits red fluorescence and is only permeable to dead-nucleated cells while remaining impermeable to viable living cells.[19] Both dyes serve as nuclear dyes. Consequently, the concurrent application of AO and PI enables clear differentiation and evaluation of cellular viability, cellular morphology, and cellular damage under a fluorescence microscope. For this assay, K-562 cells were treated with 23 µg/mL of *M. alba* leaves ethanolic extract and incubated for 24, 48 and 72 hours before being stained with AO/PI dyes to investigate and determine the method of cell death induced by the extract. At 24 hours incubation period, the K-562 cells exhibited morphological changes, such as blebbing of the cell membrane, suggesting the extract's impact on the cells. Additionally, certain K-562 cells displayed nuclear fragmentation. However, since all K-562 cells still emitted green luminescence and no orange-to-red luminescence was detected, it implied that the K-562 cells remained viable without any signs of cell death as depicted in Figure 2. At the 48-hour incubation period, the morphological changes in K-562 cells became more pronounced, particularly with distinct alterations in cellular membrane integrity. Based on Figure 2, observed morphological changes included blebbing of the cell membrane, early apoptosis, and necrosis. Nuclear fragmentation was evident in the treated K-562 cells exposed to the ethanolic extract of *M. alba* leaves. Furthermore, early apoptosis and necrosis were identified as a few K-562 cells emitted green and orange-to-red luminescence. During the 72 hours incubation period, further morphological changes were observed in the treated K-562 cells. Based on Figure 2, the observed morphological changes included blebbing of the cell membrane, early apoptosis, late apoptosis, and necrosis. The number of apoptotic and necrotic cells increased at the 72 hours mark compared to 48 hours.



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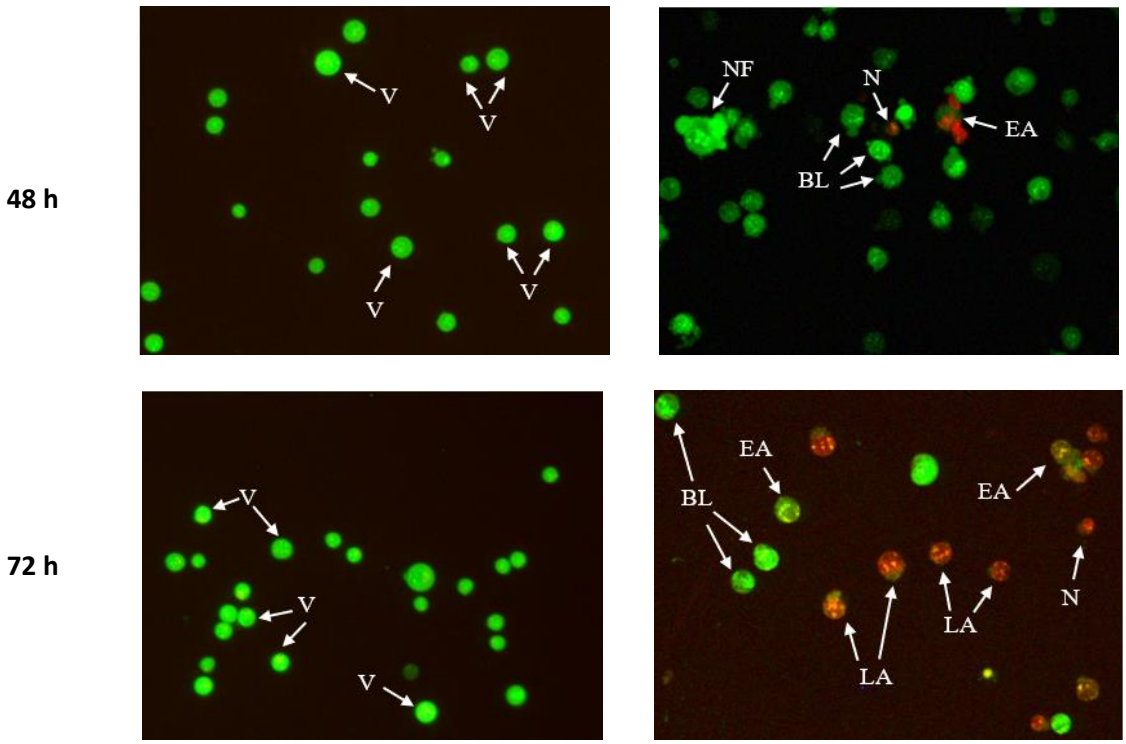

Figure 2: The morphology of untreated and treated K-562 cells at 24, 48 and 72 hours under ethanolic extract of *M. alba* leaves at concentration of 23 µg/mL. The photomicrograph of morphological changes in K562 cells was taken by using a fluorescent microscope under 20X magnification. Arrows indicate viable cells (V), blebbing of the cell membrane (BL), nuclear fragmentation (NF), early apoptosis (EA), late apoptosis (LA) and necrosis (N).

Discussion

The global incidence and prevalence of chronic myeloid leukaemia (CML) are increasing despite its classification as a rare form of cancer. Medicinal plants, including *M. alba*, possess significant potential for the development of anticancer drugs due to their abundant reservoir of beneficial phytochemical constituents. These phytochemicals have been shown to have various biological activities, such as anti-inflammatory, anti-proliferative, and antioxidant properties, which make them a potential source of therapeutic agents to treat cancer.[11] However, there is limited information available regarding the anticancer activity of *M. alba* on human CML, despite its traditional use in cancer treatment. Therefore, the primary objective of this study was to investigate the potential antiproliferative effects of the ethanolic extract derived from *M. alba* leaves, with a specific focus on targeting K-562 cells.

Crude extracts obtained from medicinal plants are valuable resources for the development of anticancer drugs and natural, nutritious meals for cancer treatment.[20] In this study, crude *M. alba* leaf extract was obtained to explore its medicinal properties. Ethanol was selected as the solvent due to its well-established safety, practicality, and ability to consistently produce reliable results.[8] Ethanol effectively dissolves both polar and nonpolar compounds, making it suitable for extracting a wide range of biologically active components with potential pharmacological benefits.[8] Additionally, ethanol has been shown to yield higher quantities of extracts compared to other solvents.[21] In this study, a 70% ethanol solution was utilized, resulting in an approximate extraction yield of 11.89% from *M. alba* leaves. Another study by Chaiyana et al employing 95% ethanol as the solvent, reported an average extraction yield of 15% for *M. alba* leaves.[22] Slight variations in the percentage yield can be attributed to factors such as solvent concentration, which can influence the results.[21]

The MTT assay is a widely employed colorimetric method in cell biology and drug discovery research, primarily utilized for measuring cell viability and assessing cellular metabolic activity.[23] In this study, the cytotoxicity of the ethanolic extract of *M. alba* leaves on K-562 cells was evaluated using the MTT assay. The results presented in Table 2 indicate that the ethanolic extract of *M. alba* exhibited cytotoxic effects against K-562 cells. According to the guidelines provided by the American National Cancer Institute (NCI), a crude extract is considered potent if the IC_{50} value is below 30 µg/mL after 72 hours of exposure to the extract.[24] Highly cytotoxic extracts have IC_{50} values below 20 µg/mL.[24] In our study, the IC_{50} value for the ethanolic extract of *M. alba* leaves after 72 hours of incubation was found to be 23 µg/mL, falling within the range established by the NCI for substances classified as potential anticancer agents. Thus, the ethanolic extract of *M. alba* leaves exhibited potent antiproliferative and cytotoxic effects on K-562 cells. Furthermore, the extract's potency depended on the incubation period, as the IC_{50} values decreased with longer incubation times.

Comparisons with other studies revealed varying IC_{50} values for *M. alba* extracts on different cell lines. For example, Deepa et al reported a more potent IC_{50} value of 13.8 µg/mL for a methanolic extract of *M. alba* leaves on HCT-15 cells.[13] Similarly, Naowaratwattana et al found an IC_{50} value of 33.1 µg/mL for an organic extract of *M. alba* leaves using pure methanol on HepG2 cells.[25] These discrepancies may be attributed to differences in the extraction solvent, solvent strength, and phenolic contents, which can influence the antiproliferative effects of the extract. Additionally, *M. alba* leaf extracts may exhibit varying antiproliferative effects on different cancer cell lines. Yang et al demonstrated lower IC_{50} values for two phytochemicals derived from the ethanolic extract of *M. alba* leaves, with IC_{50} values of 6.4 µg/mL and 9.4 µg/mL on HCT-8 human adenocarcinoma cell line.[26] The same study also showed cytotoxic effects on the BGC823 human gastric cancer cell line, with IC_{50} values of 5.7 µg/mL and 8.3 µg/mL.[26]

The ethanolic extract of *M. alba* leaves significantly influenced the morphological changes in K-562 cells, indicating signs of apoptosis, as shown in Figure 2. According to T-Johari et al,[27] apoptosis is characterized by cell shrinkage, blebbing of the plasma membrane, chromatin condensation, and fragmentation into membrane-bound apoptotic bodies. On the other hand, irreversible cellular damage such as cytoplasmic enlargement, cell membrane damage, organelle disintegration, and release of cellular material into the surrounding media are typically associated with necrosis. Hence, it can be inferred that the ethanolic extract of *M. alba* leaves exerted antiproliferative activity and induced apoptosis. This finding aligns with a study by Fathy et al., where mulberry leaf extracts inhibited cell proliferation and induced apoptosis in hepatocarcinoma cell lines.[14] Similarly, Katsube & Yamasaki demonstrated a dose-dependent inhibition of human acute myeloid leukaemia HL-60 cell lines by the ethanolic extract of *M. alba* leaves, which was associated with the activation of caspase-3, a key enzyme involved in apoptosis .[28]

Conclusion

This study demonstrated the cytotoxicity of the ethanolic extract of *M. alba* towards K-562 cells, primarily inducing apoptotic cell death. The *M. alba* leaves ethanolic extract exhibited a significant dose- and time-dependent effect, highlighting its potency in inhibiting cell growth and inducing apoptosis in K-562 cells. However, the cytotoxicity of the extract was relatively lower compared to etoposide, a plant-derived anticancer treatment. Further research is recommended to elucidate the specific apoptotic pathway involved, whether it is the intrinsic or extrinsic pathway, as well as to determine the stage of the cell cycle at which the ethanolic extract of *M. alba* leaves induces cell cycle arrest.

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Conflict of Interest Disclosure

None to declare

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