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Cytotoxic Activity of *Aquilaria malaccensis* Ethanolic Leaves Extract on Human Chronic Myeloid Leukaemia K-562 Cell Line

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Abstract

Aquilaria malaccensis, also known as "Pokok Karas" in Malaysia, has various benefits and widely used in Chinese herbal medicines. Natural products such as medicinal plant have been found to be effective anti-cancer agents, killing cancer cells with fewer side effects than chemotherapy. Therefore, the purpose of this study was to evaluate the cytotoxic activities of ethanolic extract of *A. malaccensis* on the human chronic myeloid leukaemia K-562 cell line. In this study, cytotoxic activities of ethanolic extract of *A. malaccensis* leaves on K-562 cells was determined by using (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT) assay. K-562 cells were treated with 70% ethanolic extracts and incubated for 24, 48 and 72 hours. The half-maximal inhibitory concentration (IC50) of ethanolic extract of *A. malaccensis* leaves was observed at 72 hours of incubation; 90 µg/mL for 70% ethanolic extract. Moreover, the morphological changes of K-562 cells were observed by using acridine orange/ propidium iodide (AO/PI) dual staining assay. The results showed that the ethanolic extract of *A. malaccensis* leaves induced K-562 cells into apoptotic cell death mode manner. This finding suggested that the ethanolic extract of *A. malaccensis* has the potential to become an anti-cancer agent for chronic myeloid leukaemia K-562 cell line.

Keywords: *Aquilaria malaccensis*, Chronic Myeloid Leukaemia, K-562, Cytotoxic, AO/PI Double Staining, Apoptosis

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Introduction

Leukaemia is a blood disease that induced by overproduction and aggregation of irregular white cells in the bone marrow, and the blood causes room for normal cells to decrease dramatically, leading to bone marrow loss and organ invasion. Chronic myeloid leukaemia (CML) is a slow-progressing leukaemia characterised by increased granulocytic cell proliferation without loss of differentiation potential1. It emerges 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[1].

The discovery of a reliable and safer therapeutic drugs option for hematologic cancer may become worldwide interest. The traditional herbs can be demonstrated as a powerful source of natural antioxidants that are a good choice for anti-cancer studies. In developing new medication, medicinal plants play a big role even though in few fields such as combinatorial chemical structure, chemical synthesis and molecular modelling become particular interest by any pharmaceutical company. *Aquilaria malaccensis*, one of the species that easily can be found in Malaysia's ecological system. This precious tree contains numerous green leaves throughout the year that, like the bark, may provide numerous benefits. The heartwood of *A. malaccensis* can generate a valuable residue which is a resin marinate. *A. malaccensis* act as astringent, stimulant, tonic herb that helps in relieving spasms throughout the digestive, respiratory, and endocrine systems, as well as lowers fevers[2]. It is used as a sedative in China to treat abdominal pain, diarrhoea, and asthma[3]. The plant has been used in Chinese traditional medicine for a long time ago[4]. *Aquilaria malaccensis* extract has a wide-ranging activity such as antibacterial, antioxidant, antifungal, antiviral, anticancer, analgesic, hypotensive, anti-inflammatory, and immune-boosting properties[2]. This resin is the result of a natural immunological reaction to a fungal infection[5]. Alkaloids, tannins, phenols, terpenoids, quinones, and flavonoids also have been extracted from the *A. malaccensis* plant[6]. The non-infected leaves extract of *A. malaccensis* have antioxidant activity. The highest antioxidant activity was found in the combined fraction of 1:3 chloroform: methanol from methanol extract[7]. Many scientific publications on *A. malaccensis* have been published in recent years, focusing on its pharmacological action, which includes anti-diabetic, anti-arthritic, anti-cancer, antioxidant, anti-fungal, lipid-lowering, anti-tumor, and hepatoprotective properties[8]. However, anti-cancer activity of *A. malaccensis* has not been reported yet in chronic myeloid leukaemia (CML) K-562 cell in vitro. Thus, the present study was conducted to investigate the effects of ethanolic extract of *A. malaccensis* on K-562 cells.

Materials and Methods

Chemicals

Rosewell Memorial Park Institute 1640 (RPMI) medium, Foetal bovine Serum (FBS), Dimethyl Sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) and both Acridine Orange (AO) and Propidium Iodide (PI) were obtained from Sigma-Aldrich Co.

Plant materials

The leaves samples of *Aquilaria malaccensis* were collected from the Forest Research Institute Malaysia (FRIM) Forest Reserve in Merchang, Terengganu. The random sampling technique was applied, which was young, and old leaves were mixed. The leaves were washed to remove the impurities before being oven-dried at the temperature 40ºC for 24 hours. The sample was ground into the powder. It was kept in a glass vial and was stored at the -80ºC freezer until further used.

Plant extraction

The extraction was carried out using 100% and 70% ethanol (EtOH) and the powder sample was macerated in the solvent with the ratio 1:50 sample to solvent at room temperature (22-24ºC). The mixture was soaked for 24 hours before was sonicated for 30 minutes at the temperature 40ºC to facilitate the extraction process. The mixture was centrifuged at 10,000 rpm for 10 minutes before filtered through Whatman Filter No.1. The extraction steps were repeated three times and the filtrates were pooled together before was evaporated to dryness using rotatory evaporator (Heidoph, Germany). The extract was stored in -20ºC freezer prior to further use.

Cell line

The human chronic myeloid leukaemia K-562 cells (ATCC) was cultured on RPMI 1640 media, supplemented with 10% FBS, 100I.U/mL penicillin (ATCC) and 100 ng/mL streptomycin (ATCC) and incubated in 90% humidified incubator with 5% CO_2 at 37ºC for several days waiting for the cells to grow in T25 culture flask.

MTT cytotoxic assay

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The MTT assay was carried out in the 96-wells plate. Briefly, a volume of 1 x 10^5 K-562 cells were seeded each well of 96-wells flat bottom microtiter plate (Nunclon, USA) and incubated overnight. The extract was aliquoted into wells in triplicate, serially diluted and cells were seeded in each well for 72 hours in CO_2 incubator. After 72 hours incubation, a volume of 20 µL of MTT solution (5 mg/mL) was added into each well and incubated for 4 hours. The culture medium was removed and 100 µL of 100% DMSO solution were added to each well to solubilize the formazan formed. The plates were read using the plate reader (Tecan, USA) at 570 nm with reference wavelength at 630 nm wavelength. A dose response curve of the percentage of cell viable versus extract concentration was plotted. The percentage of proliferation was calculated using the following formula:

Percentage of cell viability = (OD $_{sample}$/OD $_{control}$) X 100%

AO/PI Double Staining Assay

The mode of cell death was determined by staining the K-562 cells with acridine orange (AO) and propidium iodide (PI). The preparation of AO was done by diluting 2mg AO in 1mL of DMSO. A volume of 25µL of AO stock solution was added into 975µL of Phosphate-Buffered Saline (PBS). Then, the preparation of PI was done by diluting the PI solution (2mg/mL) into PBS. A volume of 25µL of PI solution was added into 975µL of PBS. Final concentration for working solutions of AO and PI were 50µg/mL. For cell preparation, a volume of 2 × 10^5 of cells were seeded in a 6 well plate and treated with extract by using IC_{50} values from MTT assay. After incubation at 24, 48 and 72 hours, the cells were harvested and spun at 1500 rpm for 10 minutes at 21ºC. A volume of 20µL of each AO and PI dyes were added into the cells pellet. The stained cells were then mounted on slides and examined under a fluorescence microscope.

Statistical analysis

The analysis was done by using Microsoft Excel 2016. Results were expressed as Mean ± Standard Error (S.E.M)

Results

Cytotoxicity of Ethanolic Extract of A. malaccensis Leaves on K-562 cells

Cytotoxicity effects of the *A. malaccensis* ethanolic extract towards K-562 cells were determined by measuring the cell viability using MTT assay after 72-hour treatment with the different concentrations of extract. The IC_{50} value was obtained from the plot between the concentrations of extract versus percent of cell viability. The value was used to describe the degree of cytotoxicity of the extract towards cell lines. Figure 1 shows that IC_{50} of the extract for the K-562 cells was 89µg/mL. The graph also gave the concentration of the extract that reduced 25% of the cell population (IC_{25}) at 40 µg/mL and for 75% reduction (IC_{70}) the concentration was more than 500µg/mL. In this study, doxorubicin, a commercial drug for the treatment of certain types of leukaemia was used as a positive cytotoxic control compound. The IC_{50} of doxorubicin for K-562 cells was 2.17 µg/mL (Table 1).

Table 1. IC_{50} values of K-562 Cells after 24, 48 and 72 hours of treatment by doxorubicin and ethanolic extract of *A. malaccensis*

Incubation Period (Hours)	Mean Average Values of IC_{50} (µg/mL) * ± S.E.M	
	Doxorubicin	70% Ethanolic Extract of *A. malaccensis*
24	2.63 ± 0.88	120.00 ± 5.77
48	2.37 ± 0.88	100.33 ± 2.60
72	2.17 ± 0.12	89.00 ± 2.08

*IC50 values were determined from the dose response curves that correlate between the cell viability and extract concentration. The IC50 values were expressed as Mean ± S.E.M determined from three independent experiment that were performed in three replicates

Mode of Cell Death Induced by A. malaccensis Leaves on K-562 cells

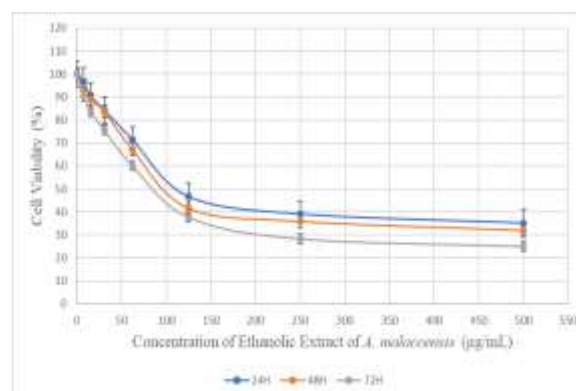

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Figure 1. Cytotoxic effect of ethanolic extract of *A. malaccensis* leaves on K-562 cells at 24, 48 and 72 Hours.

Acridine orange (AO) and propidium iodide (PI) staining was considered the appropriate method for evaluating the mode of cell death induced by any compound. In this study, AO/PI was used to differentiate the viable, apoptotic, and necrotic cells under fluorescence microscope. Figure 2 is the representative picture of the untreated and treated K-562 cells after 24, 48 and 72 hours of treatment with IC_{50} value from ethanolic extract of *A. malaccensis*, staining with dual staining AO/PI. In general, AO/PI double staining clearly showed the induction of apoptosis in K-562 cells after treatment with *A. malaccensis*. Majority of the untreated cells emitted green fluorescence and intact nuclear structure (V), indicating healthy viable cells (Figure 2A, C and E). Meanwhile, the treated cells exhibited either green flourescense or mixed bright orange stain and green. The early apoptotic (EA) cells showed green flourescence with chromatin condensation, nuclear fragmentation, and membrane blebbing (Figure 2B) while most cells at late-stage apoptosis (LA) appeared in mixed orange and green colour as a result of co-staining with both AO and PI (Figure 2D and F). Besides the colour from the staining, the relative number of green cells, the morphology, number, and size of cells gradually decreased indicating apoptosis event.

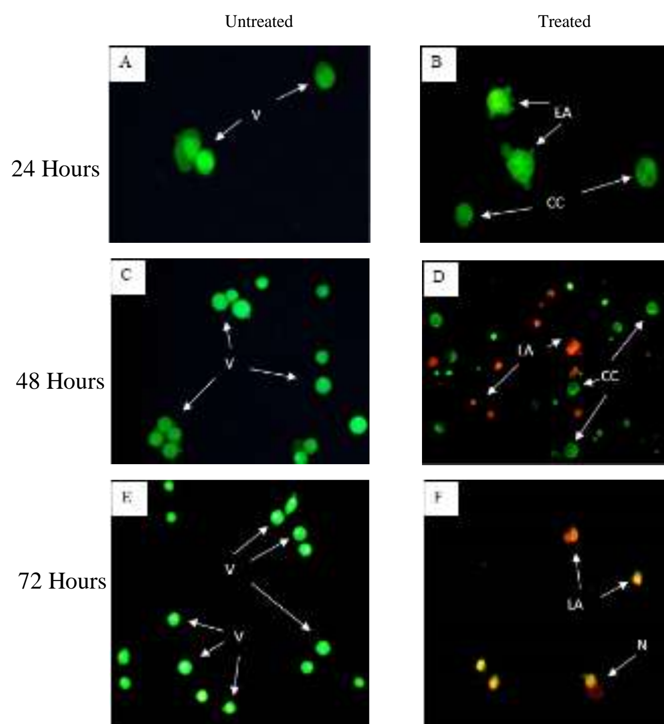

Figure 2. The morphology assessment of K-562 cells at 24, 48 and 72 hours. Cells were incubated (A,C,E) without any treatment by *A. malaccensis*. (B,D,F) were incubated with treatment by 70% ethanol extract of *A. malaccensis* at 90µg/mL for 24, 48 and 72 hours in culture plates. Cells that have intact membrane and stained green express as viable cells (V); cells that have the appearance of chromatin condensation (CC), nuclear genome fragmentation and membrane blebbing indicate the early apoptosis process (EA); cells that are stained in orange colour and contain fragmented DNA represent the late apoptotic (LA) while cells that stained red in colour show the necrotic cells (N).

Discussion

Leukaemia remains as a major global health concern even though there are recent advances in the diagnostic and treatment of the disease[9]. The cancer rate is steadily increasing globally, as are concerns about cancer therapy. Due to the emergence of resistance, harmful effects, and expensive prices, the use of several anti-cancer drugs in clinical practise has been constrained. As a result, most of the research today focuses on discovering novel bioactive chemicals to address these issues. Natural products as anti-cancer agents, particularly those derived from local or medicinal plants, have been shown to be a promising and novel anti-cancer treatment[10]. There are numerous natural compounds in plants that can cause apoptosis, including alkaloids, phenylpropanoids, terpenoids, and anthraquinone[11]. Plant screening for anti-cancer activity has lately become a major area of research, resulting in the commercialization of several cancer medicines.

The MTT assay is based on a colorimetric reaction in which dehydrogenase enzymes present only in the mitochondria of live cells reduce the tetrazolium salt from yellow to purple formazan crystal[12]. In the present study, the cytotoxicity of ethanolic extract of *A. malaccensis* leaves on K-562 cell was assessed using an MTT assay. The ethanolic extract of *A. malaccensis* exhibited the cytotoxic effect against K-562 cells. The *A. Malaccensis* extract was considered to be weak cytotoxic activity towards K-562 cells. According to Ali *et al.*[13], compounds which demonstrated the IC_{50} value more than 25µg/mL were considered to be weak in cytotoxicity. Within the first three days of the experiment, the treatments of ethanolic extract of *A. malaccensis* resulted in 50% growth inhibition. Consequent on that, when the incubation duration is increased, the ethanolic extract of *A. malaccensis* has a considerably much potent cytotoxic impact, allowing the extract component to pass the K-562 cells barrier more easily. Ethanolic extract of *A. malaccensis* also have a cytotoxic effect on other cancer cell lines such as MCF-7 which the IC_{50}

recorded is 8µg/mL[14]. Previously, the compounds isolated from *Aquilaria sp.* were shown to possess cytotoxicity towards some cancer cell lines such as Eagle's carcinoma of the nasopharynx[15].

The staining of apoptotic cells with fluorescent dyes such as acridine orange (AO) and propidium iodide (PI) are thought to be the suitable method for assessing changes in nuclear morphology. AO/PI double staining procedure also allows use in distinguishing some sub-populations of apoptotic cells from the viable, early apoptotic membrane, late apoptotic and necrotic cells populations. In the case of the AO/PI staining, many cells were found to be undergoing apoptosis, as evidenced by formation of apoptotic bodies, cell shrinkage, membrane loss, membrane blebbing, chromatin condensation, nuclear fragmentation and nuclear margination[16–18]. Furthermore, the development of the early phase into apoptosis was identified after prolonged incubation with the extract. This reveals that the 72-hour incubation of IC_{50} resulted in the best result observed among the three incubations[19].

Conclusion

This finding suggested that *Aquilaria malaccensis* leaves has the promising outcomes as anti-proliferative agents towards K-562 cells that were shown by cytotoxicity and the morphological changes when the cells undergo treatments. Additional experiments and improvements needed to further investigate the possible therapeutic mechanisms and pathways.

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