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Original Research Article

In vitro Anti-proliferative and Apoptotic Activities of *Eurycoma longifolia* Jack (Simaroubaceae) on HL-60 Cell Line

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Abstract

Purpose: *To investigate the anti-proliferative, apoptotic and differentiating activities of Eurycoma longifolia root extracts on HL-60 leukemic cells.*
Methods: *HL-60 cells were treated with different partially purified sub-fractions (F1 – F3) derived from the resin chromatography of the crude methanol root extract of E. longifolia roots, at different doses and time points. The anti-proliferative activity of E. longifolia was assessed via cell counting and trypan blue exclusion. Apoptosis was evaluated via Annexin-V FITC/IP and Hoechst staining. Flow cytometry and Wright staining were used to assess its differentiation activities.*
Results: *F1 showed unremarkable growth inhibition rate while F2 and F3 showed growth inhibitory effects with median inhibitory concentration (IC_{50}) values of 15.2 and 28.6 µg/ml, respectively. Treatment with F2 and F3 (100 µg/ml) for 96 h increased cell death from 3.3 to 95.5 and 76.3 %, respectively. Treatment with F2 (50 µg/ml) induced apoptosis by 14, 19.5 and 25 % after 24, 48 and 72 h, respectively. No differentiation activity was observed, as indicated by absence of myeloid maturation and a non-significant CD14 positivity ($p > 0.05$).*
Conclusion: *E. longifolia extract (F2) showed promising anti-leukemic activity and can be a candidate for the development of a drug for the treatment of acute promyelocytic leukemia (APL).*

Keywords: *Acute promyelocytic leukemia (APL), HL-60 cells, Eurycoma longifolia, Apoptosis, Antiproliferation, Differentiation*

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INTRODUCTION

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia, accounting for approximately 10 – 15 % of all cases of this disease [1]. The treatment of APL with current chemotherapy is successfully achieved with a complete remission of 90 % [2]. Apoptosis is genetically regulated and morphologically characterized by cell shrinkage, the formation of apoptotic bodies, and DNA fragmentation in dying cells [3]. Methods employing the differentiation of leukemic blast cells have also been used as therapeutic approaches for the last three decades [4].

The emergence of resistance to currently used drugs as well as high relapse rate create difficulties in the treatment of APL [5]. Therefore, studies on new compounds that can induce the

differentiation and/or apoptosis of APL cells are necessary. Medical herbs used in traditional medicine for cancer treatment are promising sources for such compounds.

Eurycoma longifolia Jack (Tongkat Ali) is a slender, evergreen flowering tree from the Simaroubaceae family present in Southeast Asia [6]. The plant is rich in various bioactive compounds, such as eurycomanole [7], eurycomanone, eurycomalactone, 13α (21)-epoxyeurycomanone, 9-methoxycanthin-6-one, canthin-one alkaloids, β-carboline alkaloids, squalene derivatives, biphenylneolignans, 14,15 β-dihydroxyklaineanone and tirucallane-type triterpenes [8]. The anti-cancer activities of *E. longifolia* on various solid tumors, including lung [9], breast [10] and cervical cancers [11] have been reported. However, studies on the antileukemic activity of this plant have not been conducted. Accordingly, the present study aims to investigate, the anti-proliferative, apoptotic, and differentiation activities of *E. longifolia* on the HL-60 cell line *in vitro*.

EXPERIMENTAL

Plant material

The plant and root samples of *E. longifolia* Jack were identified by Professor Ahmad Latif from Universiti Kebangsaan Malaysia (UKM). The plant samples were collected in 2003 from Perak, Malaysia. A voucher specimen (no. 785-117) was deposited in the herbarium of Penang Botanical Garden, Penang, Malaysia.

The F1, F2 and F3 were partially purified sub-fractions derived from resin chromatography of the crude methanol extract of *E. longifolia* roots as previously reported [12]. Briefly, powdered roots of *E. longifolia* Jack were extracted with 95 % methanol at 60 °C for 6 hours per day. It was filtered and the residue further extracted consecutively with two fresh portions of solvent for 5 days. The combined filtrate was then concentrated to dryness under partial vacuum at room temperature of 24 - 27 °C to yield a dark brown residue. The combined extract was next fractionated by column chromatography, using Diaion HP 20 column with a H2O–MeOH (1:0–0:1) gradient, to yield 4 fractions of the desired quassinoid-rich *E. longifolia* extract. Before the treatment, stock solutions, 5 ml each, of F1, F2 and F3 were prepared at a concentration of 10 mg/ml. F1 was dissolved in dimethyl sulfoxide (DMSO) while F2 and F3 were dissolved in water. All the fractions were sterilized by filtration using a syringe filter (0.2 µm, Sartorius, Germany).

Cells and culture medium

The human promyelocytic leukemia cell line HL-60 was obtained from American Type Culture Collection (ATCC). Cells were maintained in RPMI 1640 medium (Sigma) containing 10 % fetal bovine serum (Sigma), 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin (Hyclon). The cells were cultured at 37 °C in a humidified incubator containing 5 % CO_2. RPMI 1640 medium was used in all experiments.

Assessment of anti-proliferation

HL-60 cells (3×10^5/ml) were suspended in 2 ml culture medium in 24-wells cell culture plates and treated with F1 , F2 and F3 at various concentrations (6.25 – 100 µg/ml) and time points (0 – 96 h). HL-60 cells also were treated with vincristine (positive control) at the concentrations of 0.0625, 0.125, 0.25, 0.5 and 1µg/ml at the same time points. Untreated cells acted as a negative control. At each time point, the cell suspensions were mixed at a ratio of 1:1 with 0.4 % trypan blue solution (Sigma) and incubated for 3 - 5 min. The viable cells were counted using a hemocytometer chamber. Cell growth inhibition (CGI, %) at each concentration was calculated according to Eq 1.

$$CGI\ (\%) = (1 - Ct/Cu)100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where Ct is the viable count of treated cells and Cu the viable count of untreated cells. Inhibitory concentration (IC_{50}) values were calculated by plotting growth inhibition rate versus concentration. Trend line was drawn and a suitable calibration curve with correlation coefficient (r^2) > 0.95 was determined and applied appropriately. The results are reported as mean ± standard deviation (SD, n = 3).

Morphological changes

HL-60 cells (3×10^5/ml) were treated with F2 and F3 for 24, 48, 72 and 96 h at concentrations of 25, 50 and 100 µg/ml. The treated and untreated cells were examined and photographed under an inverted microscope (IX51 Olympus). For the assessment of chromatin condensation, slide smears of the treated and untreated HL-60 cells at the indicated time points were prepared, air-dried, fixed with absolute methanol, and stained with 10 µg/ml Hoechst 33258 dye for 20 min at 37 °C in the dark. The incubated slides were washed with phosphate-buffered saline (PBS), allowed to dry in a dark chamber, and then examined under a fluorescence microscope (Olympus BX51).

Measurement of apoptosis via flow cytometry

The HL-60 cells were exposed to F2 at various concentrations and time points (24, 48, and 72 h). After each time point, the cells were harvested, washed, and re-suspended at 1×10^5 in cold PBS. Apoptotic cells were identified using Annexin V/PI Apoptosis Detection kit (BD Pharmingen™) according to the manufacturer's instructions. Flow cytometric analysis was performed within 1 h of staining. The number of early apoptotic and late apoptotic/necrotic cells were determined as the percentage of Annexin V+/PI− and Annexin V+/PI+, respectively. Data acquisition and analysis were performed using a Becton Dickinson flow cytometer with CellQuest software.

Assessment of differentiation

HL-60 cells (2×10^5/ml) were treated with F2 (20, 50 and 100 µg/ml) for up to 7 days (24 h interval) to determine the maturation process. Untreated HL-60 cells were also maintained in culture medium at the same time intervals. The treated and untreated cells were then harvested via centrifugation and washed three times in PBS. The HL-60 cells were smeared onto glass slides, air-dried, stained with Wright stain, and examined under a light microscope. Differentiation was also assayed with regard to the HL-60 cells, treated for 7 days at various concentrations of F2, via flowcytometric analysis of CD14 expression. As previously reported [13], treated and untreated HL-60 cells were incubated with anti-CD14 antibody (BD Biosciences) for 30 minutes at 4 ^{0}C followed by washing (twice) with cold PBS. Flow cytometric analysis was performed on them within 1 h.

Statistical analysis

The results are presented as mean ± SD and differences between groups were compared by Student's *t*-test using SSPS, version 11.5. Data were considered significant at $p < 0.05$.

RESULTS

Antiproliferation

F2 and F3 inhibited cell growth in a time- and dose-dependent manner (Figure 1). Mean IC_{50} values for F2 and F3 were 15.2 and 28.6, µg/ml, respectively. Vincristine strongly inhibited the growth of HL-60 cell with IC_{50} of 0.3 µg/ml. HL-60 cells treated with F1 showed a similar growth rate to the untreated cells at all concentrations used. Based on the anti-proliferative findings, F2 was used for further experiments.

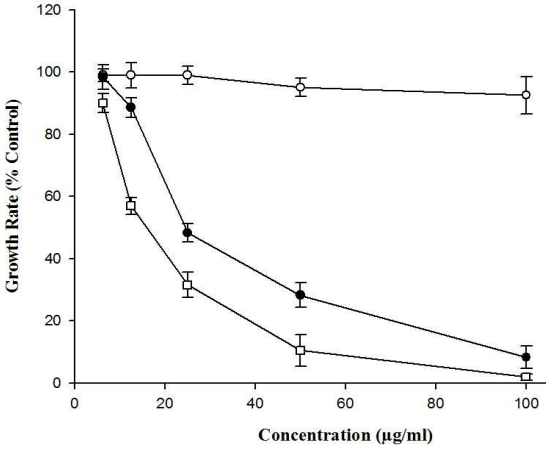

Figure 1: Effect of *E. longifolia* extracts (F1- F3) on HL-60 cell growth rate (GR). HL-60 cells treated with various concentrations of F1, F2 & F3 for 96 h. Results are expressed as mean ± SD, (n = 3). (○ = F1; □ = F2; ● = F3).

Death (%) was also dose- and time-dependent. Table 1 shows cell death after 96 h.

Morphological changes

Nuclear condensation was dose and time-dependent and clearly noted in the HL-60 cells treated with F2 (100 µg/ml) after 48 - 72h (Figure 2). Shrinkage and fragmentation of the treated cells were also observed during examination of treated cells in the cell culture plates. Photographs of treated and untreated cells, taken at the indicated time points, showed that morphological changes were time and dose-dependent. Figure 3 shows the morphologically changed HL-60 cells after 48 h of treatment with F2 (100 µg/ml). Early changes (3 h) were observed in the cells treated with more than 400 µg/ml of F2 (data not shown).

Table 1: Death of the HL-60 cells treated with various fractions of F2 and F3 at zero time and 96 h

	F2 (µg/ml)					F3 (µg/ml)					Control
	6.2	***12.5***	***25.0***	***50.0***	***100.0***	***6.2***	***12.5***	***25.0***	***50.0***	***100.0***	
Death (%) *at 0 h	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3
Death (%) at 96 h	8.0	17.0	30.0	73.3	95.5	8.4	10.9	20.9	31.2	76.3	8.5

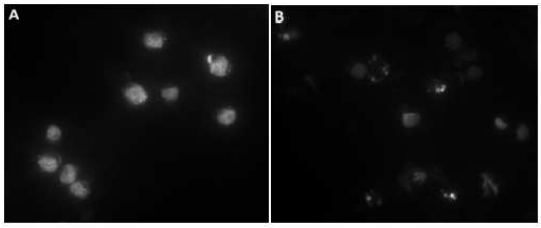

Figure 2: Apoptotic nuclear changes in (**A**) non-treated (control) and (**B**) F2-treated (100 µg/ml) HL-60 cells after 72 h cultivation. HL-60 cells stained with Hoechst 33258 (10 µg/ml) were observed under a fluorescence microscope (×40)

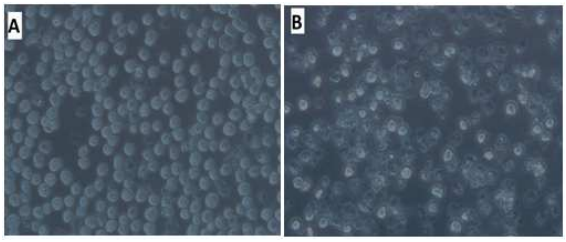

Figure 3: Morphology of (**A**) untreated and (**B**) F2-treated (100 µg/ml) HL6 cells after 48 h cultivation. The cells were observed under an inverted microscope at x40.

Differentiation

Wright stain of HL-60 cells incubated with and without F2 at different time points showed that the cells remained in the blast stage despite the morphologic changes (nuclear condensation and membrane blebbing) observed in a number of the treated cells; that is, none of the cells differentiated into mature cells (Figure 4). CD14 expression on the treated HL-60 cells was not statistically significant, compared with the untreated cells ($p > 0.05$)

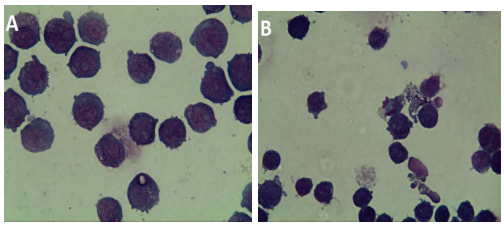

Figure 4: Morphologic changes in (**A**) untreated (control) and (**B**) F2-treated HL-60 cells after 48 h cultivation. The cells were harvested, washed with PBS, stained with Wright stain, and then examined under a light microscope (×100).

Apoptosis

Treatment with the extract resulted in induction of apoptosis in a time- and dose-dependent manner. Treatment of the HL-60 cells with F2 (50 µg/ml) increased early apoptotic cells from 14 % after 24 h to 19.5 and 25 % after 48 and 72 h, respectively while 100 µg/ml increased early apoptotic cells to 20 % after 24 h ($p < 0.05$).

DISCUSSION

The main characteristic feature of APL is that immature cells are blocked at an early stage of development and they fail to differentiate into functional cells [14]. Therapeutic strategies based on induction of differentiation and apoptosis is currently the standard method for APL treatment [4]. Although terminal differentiation and complete remission has been achieved in a majority of APL patients treated with all-trans retinoic acid (ATRA) [15], the inability to eliminate leukemic cells and the emergence of resistance [16] have led to combining this drug with other drugs (e.g., arsenic).

The results of the present study clearly show that *E. longifolia* has a potential anti-leukemic activity, as indicated by IC_{50} values 15.2 and 28.6 µg/ml for F2 and F3, respectively, as well as increased death rates. The difference in sensitivities of the two fractions might be due to their different chemical constituents and/or variation in the concentration(s) of the active compound(s). Eurycomanone is the major constituent of F2 and represent about 20 w/w% [6, 17]. Other compounds isolated from this fraction include 13,21-dihydroeurycomanone, 13α(21)-epoxyeurycomanone and 2,3-Dehydro-4a-hydroxylongilactone with lower concentrations [6,12]. The cytotoxcity (IC_{50} of 45 ± 0.15 µg/ml) of *E. longifolia* crude extract towards HepG2 cells has been reported [18]. Eurycomanone showed potential cytotoxic effect on CaOv-3, HeLa, HepG2, HM3KO and MCF-7 cancer cells with IC_{50} around 5 – 10 µM [11]. F2 induced cell membrane changes and caused phosphatidylserine externalization, as indicated by Annexin V positivity. Nuclear changes (chromatin condensation) were also dose- and time-dependent. These findings suggest induction of apoptosis which is a therapeutic target for many chemotherapeutic agents. In a previous study, Nurkhasanah and Azimahto [11] found that eurycomanone induced apoptosis in cervical cancer Hela cells with up regulation of p53 and Bax and down regulation of Bcl-2 genes. Induction of apoptosis in MCF-7 breast cancer cell with methanol *E. longifolia* root extract was also reported by Tee *et al* [9].

The absence of cell maturation and CD14 positivity in the HL60 cells after treatment with F2

indicates that this plant has not differentiation activities on this cell line.

The findings of this study showed that the E. longifolia root extracts caused the cytotoxic effect via the apoptotic mechanism and this effect is most likely due to the eurycomanone.

CONCLUSION

E. longifolia root extract (F2) has a potential anti-proliferation activity on HL60 cells, which occurs through the induction of apoptosis rather than induction of differentiation.

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REFERENCES

1. *Dimov ND, Medeiros LJ, Ravandi F, Bueso-Ramos CE. Acute promyelocytic leukemia at time of relapse commonly demonstrates cytogenetic evidence of clonal evolution and variability in blast Immunophenotypic features. Am J Clin Pathol 2010; 133: 484-490.*
2. *Ohno R, Asou N, Ohnishi K. Treatment of acute promyelocytic leukemia: strategy toward further increase of cure rate. Leukemia 2003; 17: 1454-1463.*
3. *Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol 2007; 35: 495-516.*
4. *Nowak D, Stewart D, Koeffler HP. Differentiation therapy of leukemia: 3 decades of development. Blood 2009; 113: 3655-3665.*
5. *Degos L, Wang ZY. All trans retinoic acid in acute promyelocytic leukemia. Oncogene 2001; 20: 7140-7145.*
6. *Wernsdorfer WH., Sabariah I, Chan KL, Congpuong K, Wernsdorfer G. Activity of Eurycoma longifolia root extract against Plasmodium falciparum in vitro. Wien Klin Wochenschr 2009; 121: 23-26.*
7. *Chan KL, Choo CY, Noor RA, Zakiah I. Antiplasmodial studies of Eurycoma longifolia Jack using the lactate dehydrogenase assay of Plasmodium falciparum. J Ethnopharmacol 2004; 92: 223-227.*
8. *Kuo PC, Damu AG, Leeb KH, Wua TS. Cytotoxic and antimalarial constituents from the roots of Eurycoma longifolia. Bioorg Med Chem 2004; 12: 537-544.*
9. *Tee TT, Cheah YH, Azimahtol-Hawariah LP. F16, a fraction from Eurycoma longifolia jack extract, induces apoptosis via a caspase-9-independent manner in MCF-7 Cells. Anticancer Res 2007; 27: 3425-3430.*
10. *Ueda Jy, Tezuka Y, Banskota AH, Tran QL, Tran QK, Harimaya Y, Saiki I, Kadota S. Antiproliferative activity of Vietnamese medicinal plants. Biol Pharm Bull 2002; 25: 753-760.*
11. *Nurkhasanah M, Azimahtol-Hawariah LP. Eurycomanone induces apoptosis through the up-regulation of p53 in human cervical carcinoma cells. J Cancer Mol 2008; 4: 109-115.*
12. *Teh CH, Morita H, Shirota O, Chan KL. 2,3-Dehydro-4a-hydroxylongilactone, a novel quassinoid and two known phenyl propanoids from Eurycoma longifolia Jack. Food Chem 2010; 120: 794-798.*
13. *Mosieniak G, Sliwinska M, Piwocka K, Sikora E. Curcumin abolishes apoptosis resistance of calcitriol differentiated HL-60 cells. FEBS Lett 2006; 580: 4653-4660.*
14. *Sirulnik A, Melnick A, Zelent A, Licht J D. Molecular pathogenesis of acute promyelocytic leukaemia and APL variants. Best Pract Res Clin Haematol 2003; 16: 387-408.*
15. *Tsiftsoglou AS, Pappas IS, Vizirianakis IS. Mechanisms involved in the induced differentiation of leukemia cells. Pharmacol Ther 2003; 100: 257 - 290.*
16. *Zhou GB, Zhao WL, Wang ZY, Chen SJ, Chen Z. Retinoic acid and arsenic for treating acute promyelocytic leukemia. PLoS Med 2005; 2: 3-12.*
17. *Purwantiningsih, Abas H, Chan KL. Herb drug interaction studies of Eurycoma longifolia extract F273 on the metabolism of rosiglitazone, an antidiabetic drug. Asian J Pharm Clin Res 2011; 4: 88-92.*
18. *Yusmazura Z, Asmah R, Azimahtol-Hawariah LP,Noor-Rain A, Peter JH. Eurycomanone induce apoptosis in HepG2 cells via up-regulation of p53. Cancer Cell Int 2009; 9: 1-21*