Full Length Research Paper

Induction of apoptosis in MCF-7 via the Caspase pathway by longilactone from *Eurycoma longifolia* Jack

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The effects of longilactone isolated from *Eurycoma longifolia* Jack were studied in human breast cancer cell line MCF-7. The research aim was to investigate the possible molecular mechanism of action of longilactone on MCF-7. The SRB assay showed that longilactone exerts a strong cytotoxic activity on MCF-7 with an IC₅₀ of 0.53 ± 0.19 µg/ml. Hoechst 33342 staining assay and TEM analysis showed that longilactone induced apoptosis in MCF-7 as evidenced by nuclear condensation, fragmentation and margination; apoptosis induction occurred in a time-dependent manner. Western blot analysis indicated that longilactone activated the caspase-7,-8 and poly (ADP-ribose) polymerase, but failed to activate caspase-9. The basal levels of Bcl-2 and Bax were also not influenced by longilactone. Results indicate that longilactone induced apoptosis in MCF-7 via an extrinsic pathway, but not through an intrinsic pathway. We conclude that longilactone is a promising cytotoxic chemotherapeutic agent for treating breast cancer after further evaluation.

Key words: Longilactone, *Eurycoma longifolia*, MCF-7, apoptosis, caspase.

INTRODUCTION

Worldwide, cancer is one of the major causes of human death (Jemal et al., 2009). The total number of deaths from cancer in 2007 was 7.6 million and an estimated 17.5 million peoples will die due to cancer annually by 2050 (Pan et al., 2010). The growth and survival of a cancer cells in hosts is linked to a malfunction of programmed cell death (apoptotic) machinery (Waxman and Schwartz, 2003). Thus, cytotoxic agents including vinca alkaloids (vinblastine and vincristine), epipodophyllotoxins (etoposide), paclitaxel, and camptothecins which promote apoptosis in cancer cells are currently used as anticancer chemotherapeutic agents (Balunas and Kinghorn, 2005; Bailly, 2009). Apoptotic cells are characterized by typical morphological hallmarks including cell shrinkage, chromatin condensation, nuclear DNA fragmentation, membrane blebbing and the formation of apoptotic bodies (Harmon et al., 1998). The process of apoptosis is controlled by various biochemical routes, but finally converges to the caspase pathways. Caspases (cysteine-aspartic proteases) is a family of cytokine proteases and its activation is accepted as a biochemical hallmark of apoptosis (Holdenrieder and Stieber, 2004). As such, caspase pathways are commonly exploited for anticancer drug discovery and target (Waxman and Schwartz, 2003). For instance, anticancer drugs such as paclitaxel (Ofir et al., 2002), tamoxifen (Obrero et al., 2009), doxobubicin (Fulda et al., 1998), etoposide, teniposide (Kasibhatla et al., 1998) and cisplatin (Seki et al., 2000) induce apoptosis in cancerous cells via caspase pathways.

Plant secondary metabolites remain a reasonable source of novel compounds for treatment of cancer, as...
evidenced by successful plant-derived anticancer agents such as taxol, vinblastine, vincristine, topotecan and etoposide (Nobili et al., 2009). Moreover, several plant-derived compounds are undergoing clinical trials including curcumin, phenoxodiol and mixture of homoharringtonine/harringtonine (Pan et al., 2010). *Eurycoma longifolia* Jack (Simaroubaceae) is a famous medicinal plant, indigenous to South-east Asian countries like Malaysia, Indonesia, Thailand and Vietnam. The roots of *E. longifolia* have been claimed to be antipyretic, antihelmintic, antimalarial, antihistamine and to increase males’ sexual drive. The leaf decoction is used to wash pruritus and to reduce fever. The fruit is effective for treating dysentery and the bark is recommended to relieve lumbago and arthralgia (Compendium of Medicinal Plants used in Malaysia, 2002). In Malaysia and Indonesia, a water and ethanol extracts of roots of *E. longifolia* are widely used as health supplements and beverages to increase libido and sexual powers (Ang and Sim, 1998; Ang et al., 2004). In this plant, more than 70 compounds were identified and have various pharmacological effects including cytotoxic, antiplasmodial, antidiabetic, aphrodisiac and antibacterial activities (Bhat and Karim, 2010). Recently, Zakaria et al. (2009) has reported that eurycomanone from root of *E. longifolia* was cytotoxic on HepG2 cells by inducing apoptosis through the up-regulation of p53 and Bax, and down-regulation of Bcl-2.

In the present work, we successfully purified and identified the bioactive constituent longilactone from root of *E. longifolia*. We studied the *in vitro* anticancer activity of longilactone on MCF-7 by determining the cytotoxicity, apoptosis induction and effects on key proteins in apoptotic caspase pathways. We demonstrated that longilactone appears to be a promising cytotoxic chemotherapeutic agent for treating breast cancer.

**MATERIALS AND METHODS**

**Chemicals**

DMEM/F12 medium, trypsin-EDTA, antibiotic-antimycotic solution and foetal bovine serum were obtained from GIBCO BRL Life Technologies Inc. Trichloroacetic acid, Dimethyl sulfoxide (DMSO), sulforhodamine B (SRB) and Trizma base were obtained from Sigma Chemical. All antibodies and reagents for Western blot were purchased from Millipore Inc. USA.

**Cell culture**

Human breast cancer cell line, MCF-7, was purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA and cultured in DMEM/F12 medium supplemented with 10% foetal bovine serum, 0.5% antibiotic-antimycotic solution and incubated at 37°C in 5% CO₂.

**Plant sample and extraction**

The material of the root of *E. longifolia* was provided by Forest Research Institute of Malaysia (FRIM), Selangor, Malaysia. A voucher specimen of the plant (voucher number: IMR/M0014/01) was kept at Herbal Medicine Research Centre, Institute for Medical Research, Kuala Lumpur. The dried root was powdered using an electric grinder. In soxhlet extractor, 500 g of powdered root of *E. longifolia* was defatted with 5 L petroleum ether for 4 h and repeated 3 times. Then, extracted with 5 L methanol for 4 h and repeated 3 times. The total of methanol extract was dried under reduced pressure and yielded 63.5 g of extract.

**Thin layer chromatography**

Thin layer chromatography (TLC) was performed on glass-backed silica gel (20 × 20 cm, Merck, Germany) using ethyl acetate-methanol (70:30) as mobile phase and vanillin-sulphuric acid as detection reagent.

**Low pressure liquid chromatography**

The low pressure liquid chromatography (LPLC) system consists of solvent pump (Gilson 205, Gilson Inc.), glass column (5 × 60 cm, SIGMA, USA) and packed with silica (850 g, 40 to 63 μm, Merck) and fraction collector (Fraction Collector 2, Waters Inc.). Sample (5 g) was pre-absorbed in silica (5 g) and loaded as dried slurry. The mobile phase was ethyl acetate-methanol (70:30) and the flow rate was 5 ml/min.

**Preparative HPLC**

The preparative HPLC system consists of solvent pump (Gilson 205, Gilson Inc.), preparative column C₁₈ (4 um, 2.22 × 25 cm, Jones Chromatography, UK), UV-Vis detector with preparative cell path (Shimadzu SPD-10A, Shimadzu, Japan) and chart recorder (Linear Model 1101, Altech Associates, Inc.). The UV wavelength was monitored at 254 nm. The mobile phase was acetonitrile-water (15:75) and flow rate was 5 ml per min. Sample (100 mg) was dissolved in 5 ml of acetonitrile-water (50:50), filtered through C₁₈ solid phase extraction (1 g, Altech Associates, Inc.) and was manually loaded using 5 ml sample loop.

**Cytotoxic assay**

The cytotoxic activities of test samples were performed as described previously (Vichai and Kirtikara, 2006). Briefly, cells in 100 µl of medium per well were seeded in 96-well flat-bottomed microtiter plate. After 24 h incubation, the cells were fixed by adding 50 µl of 50% TCA for 30 min at room temperature, washed with tap water and stained with 0.4% SRB (in 1% acetic acid) for 30 min. The plates were then washed four times with 1% acetic acid to remove unbound dye, air-dried and solubilised in 100 µl of 10 mM unbuffered tris base solution. The plates were read in micro-plate reader at 490 nm. Results were expressed as the dose that inhibited 50% control growth after the incubation period (IC₅₀). The values were estimated from plot of drug concentration (µg/ml) against the percentage of viable cells compared to control. The tests were repeated in at least three independent experiments.

**Hoechst 33258 assay**

MCF-7 cells were plated at a density of 3 × 10⁵ cells per well onto
cover slips in 6-well plates. After incubation for 24 h, the cells were treated with the 5 µg/ml longilactone and further incubated for 48 h in CO₂ incubator. The cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were washed in PBS and stained with 1 µg/ml Hoechst 33258 for 10 min. The cells were washed three times with PBS and analysed via fluorescent microscope using texas red filter.

For quantitative assays, MCF-7 cells were incubated with the 5 µg/ml longilactone for 24, 48 and 72 h. At least 800 cells were counted per experiment, and the percentage of apoptotic cells was defined as (number of apoptotic cells/total cell number) × 100. Normal nuclei was identified as non-condensed chromatin dispersed over the entire nucleus and apoptotic nuclei was identified as condensed chromatin, contiguous with the nuclear membrane and/or fragmented nuclei.

Ultrastructure analysis in MCF-7 after treatment with longilactone

MCF-7 cells were seeded in 25 cm² tissue culture treated flasks at 10.5 × 10⁶ cells per flask in DMEM growth medium. After 24 h incubation at 37°C to allow attachment, the cells were treated with 5 µg/ml longilactone, and further incubated for 72 h. Cells in suspension were collected and pelleted. While cells attached cells were trypsinized and pelleted. These samples were processed separately. The pellets were washed in 0.2 M phosphate buffer (pH 7.2) and fixed for 2 h in a 0.2 M phosphate buffer with 2.5% glutaraldehyde (pH 7.2) and embedded in low melting point agarose gel and cut into 1 mm³ cubes. The cube-containing cells were then washed three times with 0.2 M phosphate buffer (pH 7.2) and processed as described previously (Harmon et al., 1998). Stained sections were viewed using a transmission electron microscope (Philips CM10).

Assay for caspase-8 and caspase-9 activity

MCF-7 cells (2.5 × 10⁵) in a 75 cm² flasks were treated with 5 µg/ml longilactone for 0, 12, 24, 48 and 72 h. The activity of caspase-8 and caspase-9 were determined by FLICE/Caspase-8 colorimetric assay kit (BioVision, USA) and Caspase-9 colorimetric assay kit (BioVision, USA), respectively. The detailed protocol is as described in the manufacturer’s protocol. Results were represented as the caspase activity as measured by optical density (OD) in 150 µg of total protein.

Western blotting

MCF-7 cells (2.5 × 10⁵) were seeded in 75 cm² flasks. After 24 h of incubation, the cells were treated with 5 µg/ml longilactone for 0, 12, 24, 48 and 72 h. The cells were washed twice with ice-cold PBS and suspended in 100 µl mammalian cell extraction kit (BioVision, USA) supplemented with protease inhibitor cocktail (BioVision, USA) and 0.1 M DTT. The suspensions were incubated on ice for 10 min, vortexed for 5 s and centrifuged at 15,000 rpm for 5 min. Total protein content was determined by Bio-Rad protein assay reagent (BioRad Laboratories, Inc.) using BSA as standard. The protein extracts were mixed with 6X sample buffer (0.35 M Tris-HCL pH 6.8, 0.35 M SDS, 30% (v/v) glycerol, 0.6 M DTT, 0.175 mM bromophenol blue) and heated at 95°C for 4 min. Equal amounts of protein were transferred to a PVDF membrane. Membranes were block with 5% skim milk (BioRad Laboratories, Inc.) in TBST for 1 h and subsequently incubated with the primary antibodies. They were then incubated with a horseradish peroxidase-conjugate goat anti-rabbit or anti-mouse antibody at 1:40,000 for 90 min. Antibody-bound proteins were detected by the Immobilon Western Chemiluminescent HRP substrate (Millipore) and exposed to x-ray film. The experiment for each antigen was done in at least three independent assays.

Statistic analysis

All data values were presented as the mean ± standard deviation (SD). Analyses of variance (ANOVA) were performed using BioStat 2008 software. Statistical significance was defined as P<0.05.

RESULTS

Isolation of longilactone by bioassay-directed fractionation

A total of 15.78 g (that is, 5.26 g × 3 times) of methanol extract was subjected to LPLC and yielded 20 fractions. The fractions were analysed using TLC after spraying with vanillin-sulphuric acid. Fractions with similar profiles were pooled together to give five pooled fractions F1, F2, F3, F4 and F5. The cytotoxicity of fractions were tested on MCF-7 and results indicated that F2 was the most potent fraction (IC₅₀ = 3.8 ± 1.2 µg/ml) when compared to other fractions (IC₅₀ > 20 µg/ml). F2 was subjected to preparative HPLC and the major peak that eluted at retention time 11 to 13 min was collected (Figure 1) and freeze-dried to give a white crystalline compound. The structure of compound from major peak was determined and elucidated by EI-MS and ¹H NMR. The mass spectra data and proton NMR data showed the compound to be similar to longilactone as described by Morita et al. (1990) and Itokawa et al. (1992), and we concluded it to be longilactone. Figure 2 shows the summary of bioassay-directed fractionation and the molecule structure of longilactone.

Effect of longilactone on cell viability

To examine the in vitro anticancer activity of longilactone, cell lines were exposed to various concentration of longilactone for 72 h (Figure 3A) and then subjected to SRB assay. For comparison, we have used the anticancer agent, taxol as a positive control (Figure 3B). The IC₅₀ for longilactone and taxol on MCF-7 were 0.53 ± 0.19 µg/ml and 0.047 ± 0.023 µg/ml, respectively.

Longilactone induced apoptosis in MCF-7

To evaluate whether the cytotoxicity of longilactone on MCF-7 was associated with apoptosis, we examined its effect on nuclear morphology of MCF-7 by subjecting 5 µg/ml longilactone for 48 h and stained with Hoeschst 33342. The effect of longilactone on the nuclear morphology of MCF-7 is demonstrated in Figure 4. We
observed that longilactone treatment caused the typical apoptotic nuclear morphology changes such as nuclear fragmentation, nuclear hypercondensation and nuclear shrinkage. Furthermore, we quantified the number of MCF-7 that underwent apoptotic nuclear morphology changes after incubation with 5 µg/ml longilactone for 0, 12, 24, 48 and 72 h. As shown in Figure 5, longilactone significantly increased the percentage of apoptotic cells over the period of incubation. After 72 h incubation, about 74.3 ± 6.6% of longilactone-treated MCF-7 were apoptotic; and ultrastructure analysis by TEM (Figure 6) indicated that these cells showed nuclear fragmentation and margination, vacuolization of cytoplasm and formation of apoptotic bodies. Untreated cells showed integrated nuclear membrane and homogeneous chromatin.

Longilactone-induced apoptosis in MCF-7 via caspase activation

Activation of caspase is a common apoptotic mechanism by anticancer agents in induction of cell death (Waxman and Schwartz, 2003). In order to study the involvement of caspase in longilactone-induced apoptosis, we investigated the expression or activation of some key regulator proteins associated with caspase pathways in MCF-7 upon exposure to 5 µg/ml longilactone. Enzyme assay indicated that the caspase-3 activity (Figure 7A) in MCF-7 cells treated by longilactone was increased significantly in a time-dependent manner, but the caspase-9 (Figure 7B) was not affected. Figure 8 shows Western blots of several proteins in MCF-7 upon exposure to longilactone at various incubation times. Results indicate that longilactone increased the pro-caspase-8 (55 kDa), active caspase-8 (29 kDa), active caspase-7 (20 kDa) and cleaved subunit of poly (ADP-ribose) polymerase (PARP 89 kDa) in a time-dependent manner. In contrast, the expressions of Bax and Bcl-2 after the cells were treated with longilactone remained at a basal level. In the case of caspase-9, we did not detect an active subunit of caspase-9 in longilactone-treated MCF-7 (data not shown).

DISCUSSION

In the present study, we have isolated longilactone from methanol extract of E. longifolia root by employing cytotoxic-based bioassay guided isolation. The isolation and bioactivities of longilactone are also reported by several authors (Guo et al., 2005; Jiwadinda et al., 2001; Morita et al., 1990). Kou et al. (2004) has reported that longilactone exhibits cytotoxicity on human lung cancer cell line A-549 and human breast cancer cell line MCF-7. However, to the best of our knowledge, there are no further reports on mechanism of action on MCF-7 and on other cancer cell lines. Therefore, we further explored the possible mechanism of action of longilactone on MCF-7 by focusing on its ability to induce apoptosis and effects on key regulator proteins in caspase pathways.

We re-evaluated the cytotoxicity of longilactone on MCF-7 along with positive control taxol. Our results indicate that longilactone exerts a strong cytotoxic activity
on MCF-7 with \( IC_{50} \) was 0.53 ± 0.19 µg/ml, but is less potent than the established anticancer agent taxol, that is, \( IC_{50} \) was 0.047 ± 0.023 µg/ml. According to National Cancer Institute, USA, pure compounds that exhibit a cytotoxic activity at \( IC_{50} \) less than 4 µg/ml are considered as active substances that should be further evaluated for mechanisms of action on human cancer cell lines and antitumor activity in rodent models (Boyd, 2004; Boyd and Paull, 1995).

Apoptosis is a vital mechanism that is exerted by cytotoxic anticancer agents in reducing tumor volumes upon cancer treatment (Waxman and Schwartz, 2003). Thus, in order to determine whether or not the cell growth inhibitory effect of longilactone was mediated by apoptosis, we employed the Hoechst 33342 staining assay on MCF-7 exposed to 5 µg/ml longilactone for 48 h. As evidenced by typical apoptotic nuclear morphology changes, including nuclear fragmentation and shrinkage (Hacker, 2000; Harmon et al., 1998), it appears that apoptosis might be the major mechanism for cells growth inhibition in the presence of longilactone. Furthermore, we found that 5 µg/ml longilactone induced apoptosis on MCF-7 in a time-dependent manner, which the number of apoptotic cells reached more than 70% upon exposure up to 72 h. We also confirmed that the cell death upon 72 h exposure to longilactone was associated with apoptosis.
Figure 3. Cytotoxic activity of longilactone (A) and taxol (B) on MCF-7. The cells viability was measured using SRB assay. Data points presented are means ± SD (n = 4).

Figure 4 Longilactone induced apoptosis in MCF-7. Apoptotic morphology changes in MCF-7 after induced by 5 µg/ml longilactone for 48 h. Cells morphology was observed under fluorescence microscope via texas red filter by staining with Hoechst 33342. A: Shows untreated cells with normal nuclei. B: Shows longilactone-treated cell. Arrows indicated apoptotic cells with fragmented nuclei and nuclear shrinkage. Each assay was performed in triplicate. Magnification ×200.
Figure 5. Induction of apoptosis in MCF-7 after exposed to longilactone. MCF-7 was exposed to 5 µg/ml longilactone for indicative time and stained with Hoechst 33342. Cell with fragmented nuclei and chromatin condensation were scored as apoptosis (Figure 3). Data points presented are means ± SD (n = 3). The asterisk indicates a significant difference between untreated and longilactone-treated cells, P<0.05.

Figure 6. TEM analysis of MCF-7 pre-stained with uranyl acetate and osmium tetroxide and counter stained with lead acetate. A, representative untreated control: Shows intact nuclear and cytoplasm. B, representative longilactone-treated cell (cell was detached by trypsinization after treated with longilactone): Shows margination of nuclear chromatin and cytoplasm vacuolization. C, longilactone-treated cell (suspension cell in growth medium): Shows nuclear fragmentation, cytoplasm vacuolization and apoptotic bodies. D, magnification of area from C as indicate by red rectangle: Shows apoptotic bodies with intact membrane.
Apoptosis is an irreversible process which is mainly control by a cascade of caspase proteins through two pathways, that is, extrinsic pathway (Sun et al., 1999) and intrinsic pathway (Ashkenazi and Dixit, 1998). The activation of extrinsic and intrinsic pathways is triggered by activation of initiator caspase-8 and caspase-9, respectively. Activation of pro-caspase-9 to active caspase-9 is triggered by formation of apoptosome complex in cell cytoplasm which contains pro-caspase-9, cytochrome c, Apaf-1 and ATP. The complex of apoptosome occurs in the presence of cytochrome c from mitochondrial (Cain et al., 2002; Chinnaiyen, 1999). Mitochondrial cytochrome c release and apoptosome formation are regulated by the Bcl-2 family which consists of proapoptotic members, such as Bax and Bid, overexpression of which promotes mitochondrial release of cytochrome c; and anti-apoptotic members, such as...
Bcl-2, Bcl-XL, and Mcl-1, overexpression of which blocks release of cytochrome c (Reed,1998). Both pathways converge to activate the effector caspases, that is, capase-3,-6 and -7 and in turn activate poly (ADP-ribose) polymerase. Activation of PARP triggers an activation of various enzymes for degradation of cell cytoskeleton and nucleoproteins during process of apoptosis as a main functions are to cut a cell into small pieces to form apoptotic bodies (Blajeski and Kaufmann, 1999). In the present work, we demonstrated that longilactone activated caspase-8, caspase-7 and poly (ADP-ribose) polymerase but did not activate caspase-9 and also did not alter the basal levels of Bax and Bcl-2. These results suggest that the apoptotic activity of longilactone in MCF-7 cells was associated with caspase extrinsic pathway activation but not the intrinsic pathway.

In brief, this work demonstrated that longilactone exerted cytotoxic activity on MCF-7 associated with apoptotic cell death. The apoptotic mechanism of longilactone on MCF-7 is via activation of an extrinsic or receptor-mediated pathway but does not involve intrinsic or mitochondria pathway. We conclude that longilactone could be a promising antitumor agent and further evaluation is required.

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REFERENCES


