[BIO04] Eurycomanone exerts antiproliferative activity via apoptosis upon MCF-7 cells

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Introduction

E. longifolia Jack belongs to the Simaroubaceae family and is abundant in South East. From the roots of *E. longifolia* Jack, used as a folk medicine, a highly oxygenated quassinoid, named eurycomanone was isolated. Eurycomanone, a C_{20} quassinoid is a cytotoxic bioactive ingredient found in *E. longifolia* Jack with a molecular formula of $C_{20}H_{24}O_9$ (Chan et al., 1986) and was isolated as white cubic crystals. Eurycomanone demonstrated a general cytotoxic response with all of the epithelial cell types: KB, vincristine resistant KB (KB-VI), fibrosarcoma, melanoma, lung cancer, colon cancer and breast cancer cell lines. In addition, the most intense response was observed with the fibrosarcoma cell line, implying selectivity. Eurycomanone has demonstrated significant antimalarial activity as judged by studies conducted with on cultured *Plasmodium falciparum* strain (Chan et al., 1986; Kardono et al., 1991).

In the present study, we tested semi-purified eurycomanone induced apoptotic activity and examined the effects of semi-purified eurycomanone on BCL-2 family proteins, caspases and caspases targeted proteins in MCF-7 breast cancer cells.

Materials and Methods

Cell culture

MCF-7 and MCF-10A cells were obtained from the American Type Culture Collection (ATCC, USA). MCF-7 cells was cultured in Dulbecco's Modified Eagle's medium (Gibco BRL) was supplemented with 5% fetal bovine serum and penicillin-streptomycin, fungizon and gentamycin. Whereas, MCF-10A cells were maintained in MEGM (Clonetics) supplemented with 100 ng/ml Cholera toxin.

Cell proliferation assay

Semi-purified eurycomanone was isolated from the roots of *E. longifolia* Jack, as previously described (Darise et al. 1982). MCF-7 cells were seeded into 96 well plates at an initial cell density of approx. 1 X 10^5 cells/ml. After an overnight incubation for cell attachment, the medium was removed and replaced with fresh medium. Semi-purified eurycomanone was first dissolved in DMSO and the cells were treated at increasing concentrations to evaluate its *in vitro* antiproliferative activity. Control wells received only DMSO. The assay was terminated on day 4. Cell survival on day 4 was determined by the procedure using methylene blue as described by Lin & Hwang (1991). Cell viability was determined at 660 nm employing the ELISA reader. EC_{50} values were then calculated from the graph plotted for percentage of viable cells (%) versus log concentration (mg/ml) of each test.

TUNEL assay

Cells were grown on slides in petri dishes and treated with semi-purified eurycomanone, DMSO and tamoxifen respectively and incubated overnight. DNA fragmentation that is characteristic of apoptotic cells was quantified by Tdt-mediated dUTP nick end labeling (TUNEL) with the Apoptosis Detection Kit, Fluorescein (Promega) according to the manufacturer's instruction. Cells were observed under the fluorescence microscope at 520nm. To calculate the percentage of TUNEL positive cells, we counted all of the cells from four random microscopic fields at x1000 magnifications.

Western blotting analysis

20µg of protein from both the treated and untreated cells were size fractionated under denaturing conditions on 12 % SDS-polyacrylamide gels. After electrophoresis, the resolved proteins were transferred onto polivynyl-difluoride membranes (Nen Life Science). The membranes were dried, preblocked with 5% non-fat milk in phosphate-buggered saline and 0.1% Tween-20, then incubated with a primary antibody with either anti-BAX monoclonal, BCL-2,

caspase-6, 7, 8, 9; BID, FADD, Lamin and anti-PARP monoclonal (1: 2000 dilution; PharMingen) antibodies for 2 h at room temperature. This was followed by incubation with anti-mouse (1:30, 000 dilution) IgG conjugated with horseradish peroxidase. Probed immunoreactive protein bands were visualized with ECL (Nen Life Science). Following exposure on a Kodak OMAT x-ray film. Protein bands were quantified by densitometry analysis was performed with GS 670 Imaging Densitimeter with the Molecular Analyst Software (BioRad, Hercules, USA).

Results and Discussion

Antiproliferative assay

According to the National Cancer Institute in United States of America, plant extract that confer cytotoxicity at EC_{50} value exceeding 20 µg/ml can be categorized as a anticancer or cytotoxic potential (Wall et al., 1987). In these studies we attempted to determine the effect of semi-purified eurycomanone on the growth of cancerous mammary cells, MCF-7 and non-cancerous breast cells, MCF-10A. Tamoxifen was used on these cells lines for positive control.

EC_{50} value of semi-purified eurycomanone and tamoxifen on MCF-7 cell is 2.2±0.18 µg/ml and 4.79±0.04 µg/ml respectively (Figure 1). These findings suggest that semi-purified eurycomanone as a potential antiproliferative agent on MCF-7 cells since the EC_{50} value is less than 20 µg/ml. The antiproliferative activity of semi-purified eurycomanone in this research is effective on MCF-7 cancer cells and this is in concert with the results obtained with pure eurycomanone (EC_{50} 1.1 µg/ml) on mammary cancer cells (Kardono et al., 1991). Some biological researchers show that certain chemical compounds from *E. longifolia* Jack have significant biological activity. For example, quassinoids were reported to have anticancer activity (Kardono et al., 1991). Tamoxifen imposed an inhibitory effect on estrogen receptor positive MCF-7 cells with an EC_{50} value of 4.79±0.04 µg/ml. Tamoxifen is effective towards MCF-7 cells because of its antiestrogenic properties (Lippman & Huff, 1986). Tamoxifen interacts directly with the estrogen receptor and forms an inactive complex (Rochefort et al., 1983) thus disabling estrogen from binding estrogen positive tumor cells. These complexes prevent cancerous cells from entering G1 phase in cell cycle (Jordon et al., 1980). Therefore, the population of the cancer cells diminishes.

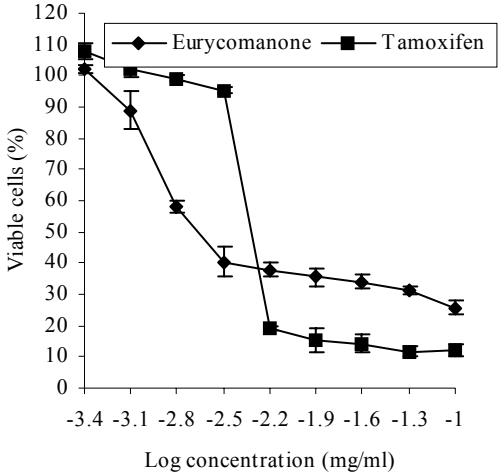

FIGURE 1 Antiproliferative effects of the semi-purified eurycomanone and tamoxifen on cancerous breast cells (MCF-7). EC_{50} values are 2.2±0.18 and 4.79±0.04 µg/ml respectively.

EC_{50} value of semi-purified eurycomanone and tamoxifen on MCF-10A cell is 30.90±0.99 µg/ml and 2.59±0.11 µg/ml respectively (Figure 2). Cytotoxicity test conducted on MCF-10A using semi-purified eurycomanone; a high EC_{50} value exceeding 20 µg/ml was obtained, 30.90±0.99 µg/ml and this showed that semi-purified eurycomanone is cytoselective towards cancerous cells but does inhibit the proliferation of MCF-10A cells. Tamoxifen, however disposed strong inhibitory effects which inhibits the proliferation of MCF-10A cells. Tamoxifen is not a selective anticancer drug since it inhibited the proliferation of both non-cancerous cells (EC_{50} = 2.59 ± 0.1 µg/ml) and cancerous cells (EC_{50} = 4.79 ± 0.04 µg/ml) at low concentrations.

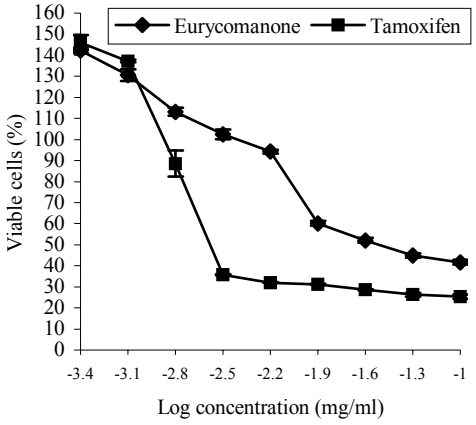

FIGURE 2 Antiproliferative effects of the semi-purified eurycomanone and tamoxifen on non-cancerous breast cells (MCF-10A). EC_{50} values are 30.90±0.99 and 2.59±0.11 µg/ml respectively.

In cancer treatment, tumour cell loss may be contributed to a high frequency of apoptosis due to the effect of anticancer agents (Kerr et al., 1994) and the antiproliferative activity shown by semi-purified eurycomanone could possibly be due to the induction of apoptosis. To test this hypothesis, methods detecting different end-points of apoptosis were employed.

***TUNEL* assay**

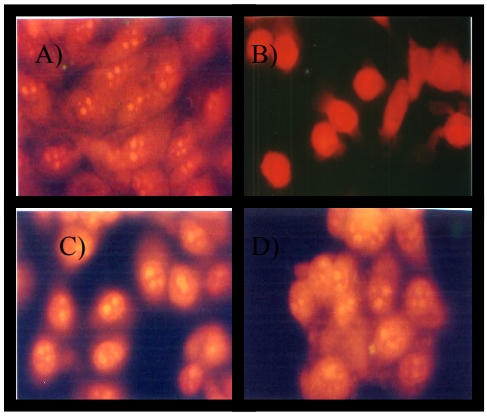

FIGURE 3 *TUNEL* Assay: MCF-7 cells treated with A) DNase 1 (positive control), B) DMSO (negative control), C) semi-purified eurycomanone at 2.5 µg/ml and D) Tamoxifen at 4.8 µg/ml at 24 hours.

A set of characteristic changes that make apoptotic cell death readily identifiable: cells shrinkage; chromatin condensation; membranes blebbing and nuclear fragmentation. The morphological changes in apoptosis have been extensively reviewed elsewhere (Wyllie et al. 1980). Figure 3 shows nuclear DNA fragmentation, a hallmark of apoptosis. In the positive control which was treated with DNase 1, 100% of MCF-7 cells underwent apoptosis (Figure 3a) and almost all nuclei of MCF-7 were seen to be fluorescent. Negative control using DMSO (< 1%) showed little fluorescense in the nucleus (Figure 3b). DMSO by itself does not cause cells to undergo apoptosis. Hence, apoptosis signal is less due to less DNA fragmentation and less fluorescent TdT-binding to DNA. MCF-7 cells treated with eurycomanone at 2.5 µg/ml (Figure 3c) showed extensive apoptosis activity whereby intense fluorescence was seen in nuclei of cells undergoing DNA fragmentation. Cells treated with semi-purified eurycomanone were proven to induce apoptosis in MCF-7 cells. Previous reports have suggested that Tamoxifen induced characteristic morphological changes consistent with apoptosis, including condensation of cytoplasm and convolution of nuclear and internucleosomal DNA fragmentation in breast cancer cells.

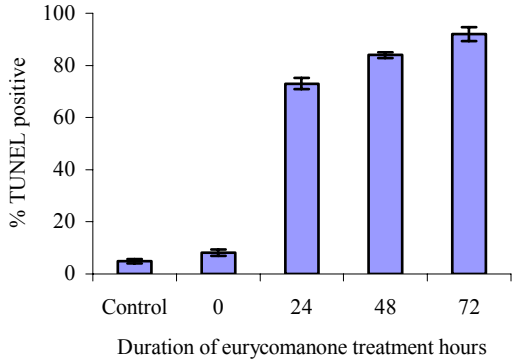

FIGURE 4 Semi-purified eurycomanone treatment (2.5 µg/ml) significantly increased the level of apoptosis in MCF-7 cells in contrast to untreated controls. Results are presented as the mean ± SD of 3 independent experiments.

To confirm whether semi-purified eurycomanone-treated cells underwent death via apoptosis; the extent of DNA fragmentation was analyzed in a time-course manner. When MCF-7 cells were treated with 2.5 µg/ml of eurycomanone, TUNEL-positive cells were first detected at 24 hours of treatment with >70% of cells score as apoptotic, and this number increased to >80%

by 48 hours, and then to >85% at 72 hours of eurycomanone treatment. Untreated control cells only recorded <5% apoptotic cells. When treated with semi-purified eurycomanone for 72 h, the cells exhibited a 8-fold higher apoptosis rate than untreated cells ($P < 0.001$). Eurycomanone treatment significantly increased the level of apoptosis of MCF-7 cells in contrast to untreated controls.

Cancer cell death via apoptosis has been discerned in chemotherapeutic treatment of cancerous cells. Chemotherapeutic drug that can induce apoptosis in cancerous cell are tamoxifen, taxol and meltafan (Thompson 1995). The precise nature of the death pathway silenced by semi-purified eurycomanone still needs to be studied.

Effects of semi-purified eurycomanone on the BCL-2 and BAX proteins expression

To determine the effects of semi-purified eurycomanone on the expression of BCL-2 and BAX proteins in MCF-7 cells, we performed Western blotting analysis was performed. BCL-2 protein expression decreased as early as 2 hours following semi-purified eurycomanone treatment, while BAX levels were not altered and remained low throughout the experiment. 2.5 µg/ml of semi-purified eurycomanone caused 55% reduction in BCL-2 protein expression following 24 hours of treatment compared with non-treated cells (Figure 5B). Semi-purified eurycomanone down-regulated BCL-2 protein levels in a time-dependent manner (Figure 5*B*). Furthermore, this down-regulation in BCL-2 protein correlated with apoptosis detected by *TUNEL* assay (Figure 3). These observations suggest that down-regulation of BCL-2 plays a critical role in deregulation of apoptosis inhibition and also triggers apoptosis by eurycomanone.

We determined the effects of semi-purified eurycomanone on p53 protein expression by Western blotting analysis. No differences in p53 protein levels were found between untreated cells and those treated with 2.5 µg/ml for up to 24 h (data not shown).

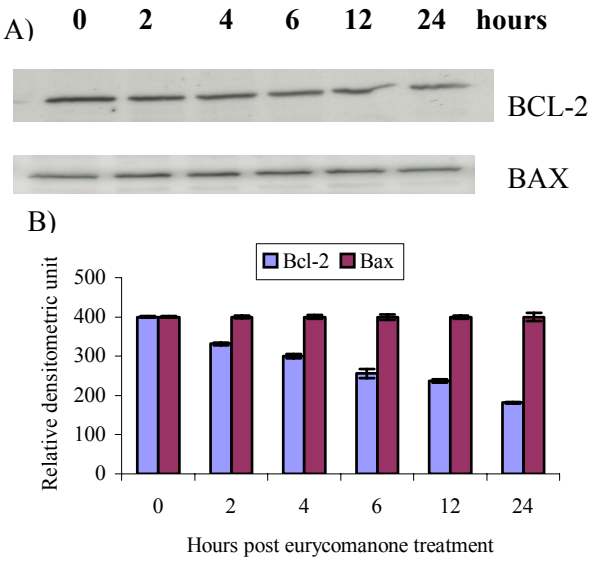

FIGURE 5 Effects of eurycomanone on BCL-2 and BAX protein in MCF-7 cells. (A) MCF-7 cells were treated with semi-purified eurycomanone at the concentrations 2.5 µg/ml for up to 24 hours. (B) Eurycomanone-induced BCL-2 and BAX protein expression decreased in a time-dependent manner as seen in Western Blot. BCL-2 and BAX bands were quantified using Imaging Densitimeter. Data are shown as the means of three separate experiments; *bars*, SD.

Effects of semi-purified eurycomanone on the expression of caspases

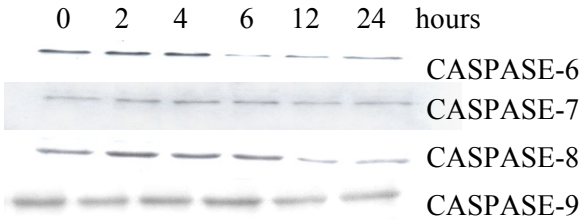

FIGURE 6 Following semi-purified eurycomanone (2.5 µg/ml) treatment on MCF-7 cells, the caspase-6 pro-enzyme (34 kDa), caspase-7 pro-enzyme (35 kDa), caspase-8 pro-enzyme (50 kDa) and caspase-9 pro-enzyme (49 kDa) was cleaved.

Caspases, a family of cysteine proteases, play a critical role in the execution phase of apoptosis and are responsible for many of the biochemical and morphological changes associated with apoptosis. It has been proposed that "initiator" caspases, such as caspase-8 and -9 either directly or indirectly activates "effector" caspases, such as caspases-6, and -7. Semi-purified eurycomanone caused reduction in

procaspase-8 protein expression after 24 hours of treatment compared with non-treated cells. These observations suggest that active caspase-8 which is the cleaved product of procaspase-8 plays a critical role in triggering the apoptotic machinery via the death pathway.

Effects of semi-purified eurycomanone on BID and FADD proteins expression

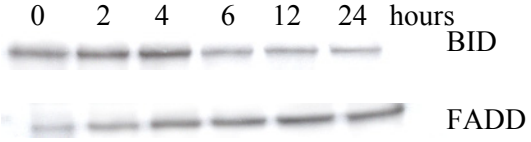

FIGURE 7 Following semi-purified eurycomanone treatment on MCF-7 cells (2.5 µg/ml), the Bid (23 kDa) was cleaved generating a catalytically active tBid and the FADD (24 kDa) was being express.

To establish that caspase-8 was being activated, the cleavage of the caspase-8 substrate Bid was examined by immunoblot. Caspase-8 cleaves and activates effector caspases but also processes the Bcl-2 family member Bid to generate a proapoptotic fragment termed truncated Bid (tBid). tBid translocates to mitochondria, inserts in the outer membrane, and, in cooperation with Bax or Bak, brings about Cytochrome *c* release.

Caspase-8 is a 55 kDa cytosolic protein with homology to the CD95/Fas-associated signal transducer, FADD. FADD therefore acts as an adapter molecule, allowing caspase-8 to become recruited to the cytoplasmic region of Fas following receptor activation.

Effects of semi-purified eurycomanone on the expression of caspases-targeted proteins

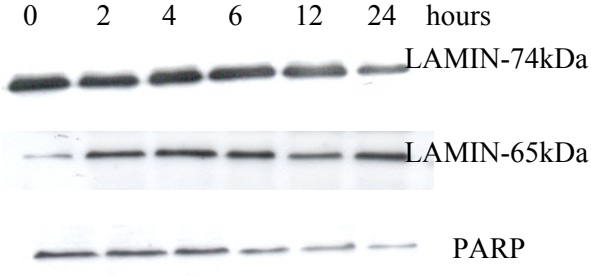

FIGURE 8 Following semi-purified eurycomanone (2.5 µg/ml) treatment on MCF-7 cells, the lamin (74 kDa) was cleaved generating a catalytically active 65-kDa fragment of lamin and the PARP (116 kDa) was cleaved.

Caspases have been recognized as the central executioners of apoptosis. Once activated, effector caspases target cellular proteins for proteolysis, leading eventually to cell death. Caspase-6, appears to cleave only the nuclear lamins during apoptosis. Lamin is an important nuclear membrane protein in maintaining normal cell functions such as cell cycle control, DNA replication and chromatin organization. Lamin serves as a marker for caspase-6 activation. The cleavage of lamins results in nuclear disregulation and cell death.

PARP is a target of the caspase protease activity (caspase-7) associated with apoptosis. During apoptosis, PARP is cleaved from its proform of 116 kDa. On the other hand, PARP is always cleaved by a caspase regardless of the death stimulus or cell type. PARP cleavage is believed to attenuate its ability to carry out DNA repair and so hastens apoptosis

In a nutshell, semi-purified eurycomanone of *E. longifolia* Jack displays cytoselectivity which inhibits the growth of cancerous cells, MCF-7 with an EC_{50} value 2.2 µg/ml but not non-cancerous cells, MCF-10A. The antiproliferative effect of semi-purified eurycomanone on MCF-7 cells was depicted via apoptotic cell death. Semi-purified eurycomanone treatment resulted in apoptotic cell death of MCF-7 breast cancer cells involving down-regulation of BCL-2; activation of caspases-6, 7, 8 and 9 leading to activation of tBID and FADD followed by cleavage of PARP and Lamin.

Acknowledgements

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References

Chan, K.L., O'Neill, M.J., Phollopson, J.D. & Warhust, D.C. 1986. Plants as sources of antimalaria drugs. *Planta Med*. 52:105.

Darise, M., Kohda, H., Mizutani & Tanaka, O. 1982. Eurycomanone and eurycomanol, quassinoids from the roots of *Eurycoma longifolia*. *Phytochemistry*. 21(8): 2091-2093.

Jordon, V. C., Allen, K. E. & Dix, C. J. 1980. Pharmacology of tamoxifen in laboratory animal. *Cancer Trap. Rep*. 64: 745.

Kardono, L.B.S., Angerhofer, C.K., Tsauri, S., Paadmawinata, K., Pezzuto, J.M. & Kinghorn, A.D. 1991. Cytotoxic and anti-malarial constituents of the roots of Eurycoma longifolia. *J. of Nat. Prod*. 54(5): 1360-1367.

Lin, L. & Hwang, P.L. 1991. Antiproliferative effects of oxygenated sterols: positive correlation with binding affinities for the antiestrogen binding sites. *Biochemica-et-Biophysica-Acta* 1082: 177-187.

Lippman, M.E & Huff, K. 1986. The effect of estrogen and antiestrogen on hormone-responsive human breast cancer in long term tissue culture. *Nature* 256: 592-593.

Rochefort, H., Borgna, J.L. & Evans, E. 1983. Cellular and molecular mechanism of action of antiestrogen. *Journal of Steroid Biochem*. 19: 69-74.

Thompson, C. B. 1995. Apoptosis in the pathogenesis and treatment of disease. *Chem*. 35(4): 1456-1461.

Wall, M. E., Taylor, H. & Wani, M. C. R. 1987. Plans antitumor agents 24 rapids 9-KB Assay. *J. Nat. Prod* 50 (4): 764-766.

Wyllie, A.H., Kerr, J. F. R. & Currie, A.R. 1980. Cell death: The significance of apoptosis. *Int. Rev. Cytol*. 68: 251-306.