EURYCOMANONE INDUCES APOPTOSIS THROUGH ACTIVATION OF CASPASE-9 ON HUMAN CERVICAL CANCER (HeLa) CELLS

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Abstract

Background. *Eurycomanone is a quassinoid derivative extracted from the root of Eurycoma longifolia Jack. Previous studies had noted its cytotoxicity effect against various cancer cell lines by inducing apoptosis through up regulation the p53 tumor suppressor protein. The present study investigated the cytotoxic effect mechanism of eurycomanone on human cervical carcinoma (HeLa) cells.*

Methods. *Eurycomanone was extracted as white crystal and identified as eurycomanone by comparison of its NMR spectral data with published value. The cytotoxic mechanism were studied by western blotting.*

Results*. This study found that after the up regulation of p53 protein and increasing the Bax:Bcl-2 ratio, the caspase-9 were activated as shown by decreasing procaspase-9 bands without the induction of Apaf-1. The activation of caspase-9 were induced after increasing Bax:Bcl-2 ratio lead to cytochrome c release. Complex apoptosome consisting of cytochrome c, Apaf-1 and ATP activated procaspase-9 with proteolytic mechanism. Active caspase-9 will activate the executioner caspase in caspase cascade.*

Keywords : *eurycomanone, apoptosis, caspase-9.*

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INTRODUCTION

Eurycoma longifolia Jack is one of the popular folk medicines of South East Asia including Myanmar, Indochina, Thailand, Laos, Cambodia and Malaysia (Kuo et al. 2004). Several classes of compounds have been isolated and identified, including quassinoids (Darise et al. 1982; Morita et al. 1990; 1992; Itokawa et al, 1992; 1993; Chan et al. 1991; 1992; Ang et al. 2000; 2002) canthin-6-one alkaloids (Mitsunaga et al. 1994), â-carboline alkaloids (Kardono et al. 1991) triterpene tirucallane type (Itokawa et al. 1992), squalene derivatives [Morita et al. 1993) and biphenylneolignan (Morita et al. 1992).

Previous study of methanolic extract showed induction of apoptosis on human breast cancer cell lines (MCF-7) via decreasing of Bcl-2 proto-oncogene (Tee & Azimahtol 2005). Eurycomanone isolated from *E. longifolia* was proven to inhibit the growth of MCF-7 by triggering apoptosis involving down regulation of the anti-apoptotic protein Bcl-2 but the pro apoptotic Bax remained at the basal level. It is relatively non toxic on non cancerous breast cell lines (MCF-10A) (Cheah & Azimahtol 2004). On the cervical cancer cells (HeLa), eurycomanone was reported to induce apoptotic cell death through the up regulation of p53 tumor suppressor protein. Following the up regulation of p53, the ratio of Bax:Bcl-2 was increased and leading the cells to enter apoptosis (Nurkhasanah & Azimahtol 2008). Eurycomanone also was found to relatively non toxic on non cancerous cells compare to cancerous cells (Nurkhasanah & Azimahtol 2007).

The objective of this study was to investigate the mechanism of cell death in human cervical carcinoma (HeLa) cells and the role of caspase in apoptosis death induce by eurycomanone.

METHOD

Plant materials

E. longifolia roots were provided by Prof Dr. Azimahtol Hawariah Lope Pihie (National University of Malaysia).

Compound extraction

Eurycomanone was extracted from the root of *E. longifolia* as previously described (Darise et al. 1982). The root of the plant was dried, grounded and extracted with methanol. The methanol extract was then concentrated to dryness. A suspension of the dry extract in water was separated with diethyl ether using separating funnel. The water fraction was then separated with n-buthanol saturated with water. The buthanol layer was then evaporated to dryness and the residue was subjected to column chromatography over silica gel using a mixture of ethyl acetate-ethanol-water (100:10:1) as the mobile phase.

Cell culture conditions

All cell lines (CaOv-3, HeLa, HepG2, HM3KO, MCF-7, Vero, and MDBK) were obtained from American Type Culture Collection (ATCC). The cell lines were maintained in DMEM medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin streptomycin. The cells were grown at 37^{o}C in humidified atmosphere of 5% CO_2.

Western blotting

Equal amounts (20 µg) of protein extract from treated and untreated HeLa cells were separated on 12% SDS-polyacrylamide gels. After electrophoresis, the proteins were blotted onto polyvinyl-difluoride (PVDF) membranes. The membranes were dried, preblocked with 5% non-fat milk in phosphate-buffered saline and

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0.1% Tween-20, then incubated with primary antibody for caspase 9, and Apaf-1 diluted 1:1000, and detected with horseradish peroxidase-labeled antibodies. Protein band were detected by ECL system (Perkin Elmer). The membranes were reprobed with β-actin as an internal control. Relative band intensities were determined by quantitation of each band with an image analyzer (Alpha Imager).

RESULTS AND DISCUSSIONS

Cervical cancer is an important health problem world-wide, being the second most common cancer among women and first ranking in many developing countries. The cancer treatment including chemotherapy, radiotherapy and surgery are still unsatisfied. Moreover, the cancer cells could be resistant against chemotherapy. The need to develop more effective anti tumor drugs has prompted investigators to explore new sources of pharmacologically-active compounds, especially from natural products.

The ability to induce apoptosis is an important property that should be owned by the agent anti cancer Apoptosis is physiologically controlled process having important role in development of several diseases including cancer. Apoptosis is characterized by typical morphological and biochemical hallmarks including cell shrinkage, nuclear DNA fragmentation and membrane blebbing (Lawen 2003). Proteolytic enzymes such as caspases play an important role as effector molecules in apoptosis (Fulda & Debatin, 2003).

The p53 plays an important role in normal cell proliferation by controlling cell cycle progression and inducing apoptosis (Levine 1997). The activation of p53 can result in cell cycle arrest, presumably to allow DNA repair to occur before replication or mitosis. p53 activation can also result in apoptosis, as means of eliminating irreparable damaged cells (Vousden 2002). Following eurycomanone treatment, it was found that the p53 level significantly increased (Nurkhasanah & Azimahtol 2008).

Previous study found that the up-regulation of p53 was followed by the increasing of pro-apoptotic protein Bax and decreasing of anti-apoptotic protein Bcl-2 expression. p53 can transcriptionally repress the Bcl-2 expression and stimulate the Bax expression (Miyashita *et al.* 1994). Thus, the effect of p53 on eurycomanone induced apoptosis may be mediated, in part, through its effect on the expression of Bcl-2 and Bax (Nurkhasanah & Azimahtol 2008).

Caspases activation

In this study, we investigate the influence of increasing Bax:Bcl-2 ratio on eurycomanone treated HeLa cells to the activation of caspase-9. As shown in Figure 1, the level of procaspase-9 in eurycomanone treated HeLa cells were decreased. Thus indicate that procaspase-9 was activated with cleavage to active caspase-9. However, the level of Apaf-1 that involved on procaspase-9 activation was stay at basal level (Figure 2).

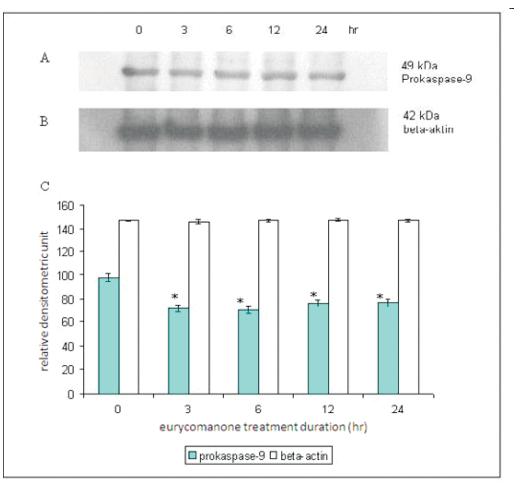

Figure 1. Western blotting and densitometric analysis of procasapse-9 on eurycomanone treated HeLa cells. Western blotting of procaspase-9 (A) and beta-actin as internal standard (B). Densitometric analysis of procaspase-9 lane.

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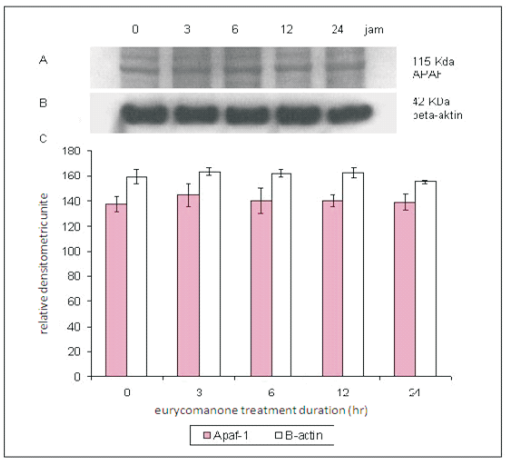

Figure 2. Western blotting and densitometric analysis of Apaf-1 on eurycomanone treated HeLa cells. Western blotting of Apaf-1 (A) and beta-actin as internal standard (B). Densitometric analysis of Apaf-1 and beta actin (C).

After cytochrome c release from mitochondria, following the apoptotic signal the cytochrome c will recruite Apaf-1 with involvement of ATP. The bonding of Apaf-1 and cytochrome c increased the affinity of recruiting dATP/ATP and influence to oligomerisation and apoptosom complex formation (Gewies 2003) (Figure 3). The ratio of procaspase-9 and Apaf-1 was 1:1 (Zou et al. 1999). Active caspase-9 will activate the executioner caspase and apoptosis occure. mitochondria. Cytochrome c induce apoptosom formation and caspase-9 activation. Cytochrome c, dATP, Apaf-1 form oligomer complex called apoptosom.

In short, the increasing level of p53 in eurycomanone induced apoptosis on HeLa cells were followed by pro caspase-9 activation but did not affect on Apaf-1 level.

CONCLUSIONS

Eurycomanone induced apoptosis on HeLa cells were involved the increasing caspase-9 activation, without affect to Apaf-1.

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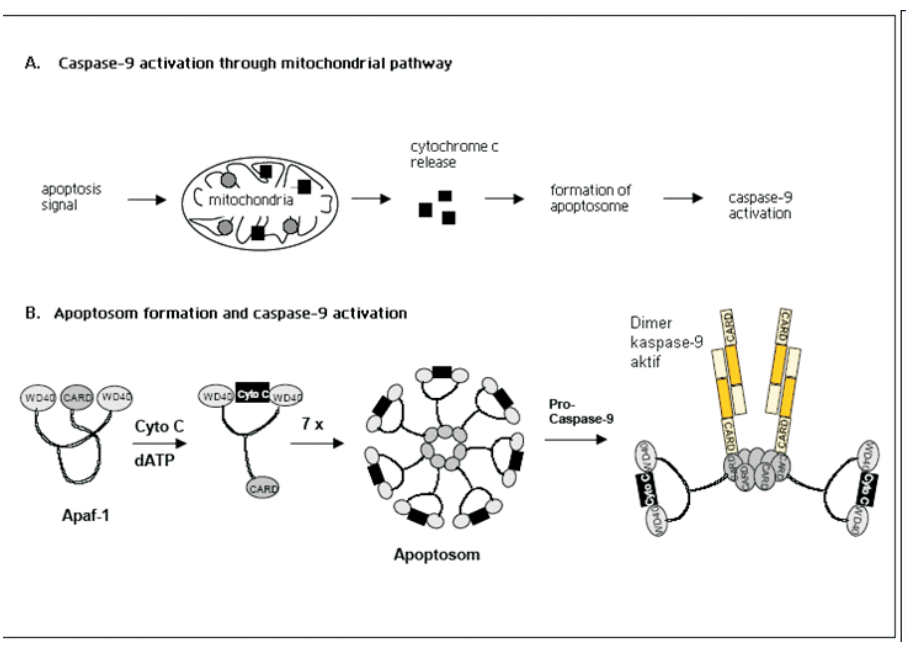

Figure 3. Caspase-9 activation. Apoptosis signal induce cytochrome c release from mitochondria. Cytochrome c induce apoptosom formation and caspase-9 activation. Cytochrome c, dATP, Apaf-1 form oligomer complex called apoptosom.

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