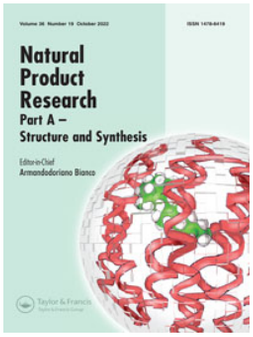

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SHORT COMMUNICATION

Eurycomanol and eurycomanone as potent inducers for cell-cycle arrest and apoptosis in small and large human lung cancer cell lines

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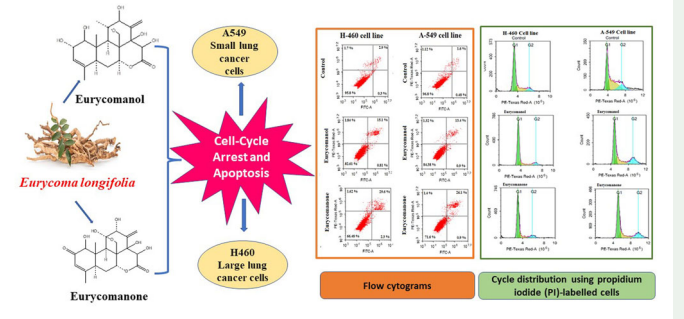
ABSTRACT

Eurycoma longifolia Jack is one of traditional herbal medicines in South-East Asia. This study evaluated the anticancer, cell-cycle arrest, and apoptotic induction potentials of eurycomanone (EONE) and eurycomanol (EOL), highly oxygenated quassinoids previously isolated from its roots, against large (H460) and small (A549) lung cancer cells. EOL and EONE exhibited IC_{50} of 386 and 424 µg/mL on normal human lung cell line. EONE exhibited higher anticancer activity with an IC_{50} of 1.78 µg/mL and 20.66 µg/mL than EOL which exhibited an IC_{50} of 3.22 µg/mL and 38.05 µg/mL against H460 and A549, respectively. Both reduced the viability of H460 and A549 and arrested G0/G1 phase. The increase in the apoptotic rates was mainly in the percentage of late apoptosis. Moreover, they inhibited A549 by inducing the accumulation of S and G2/M phases. This study revealed EOL and EONE potential as novel leads exhibiting cell-cycle arrest and apoptosis induction potentials.



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1. Introduction

Lung cancer is currently the highest mortality worldwide, mostly because it goes undetected until there is significant progression in the disease, resulting in a significant reduction in the patient's quality of life (Klastersky 2022). Cell apoptosis is a natural physiological process in which cells die in a controlled and ordered manner to keep the internal environment of the whole organism stable. There is evidence that apoptosis is related to cell cycle arrest (Kanipandian et al. 2019). Compounds that can cause cell cycle arrest and apoptosis are believed to be promising anticancer drugs (Kim et al. 2020). Several plants have been shown to have promising anti-metastasis activities by suppressing key molecular features upholding cell aggressiveness in lung cancer cells (Chanvorachote et al. 2016). *Scutellaria barbata* D. Don. ethanol extract greatly inhibited A549 cell growth via inhibition of cell apoptosis (Yin et al. 2004). The ethyl acetate extracts of *Bridelia ovata* Decne., *Croton oblongifolius* Roxb., and *Erythrophleum succirubrum* Gagnep., and the ethanolic extract of *E. succirubrum* exhibited toxic effects on A549 cells (Poofery et al. 2020). The green tea extract induced actin remoulding associated with increased cell adhesion and decreased motility in A549 lung cancer cells through altering the levels of many proteins involved in growth, motility and apoptosis of A549 cells (Lu et al. 2009).

Many plants derived secondary metabolites exhibited potent anti-lung cancer potentials. Erianin, isolated from *Dendrobium chrysotoxum* Lindl., exhibited lung anticancer activity by inducing cell death and inhibiting cell migration in cancer cells (Chen et al. 2020). Secondary metabolite mapping of lung cancer growth inhibitors in *Scutellaria baicalensis* Georgi root crude extract revealed that baicalin, baicalein and wogonin showed IC_{50} comparable to that of the antineoplastic cisplatin (Gao et al. 2010).

Eurycoma longifolia Jack (Tongkat Ali) is a flowering plant in the Simaroubaceae family and one of Southeast Asia's most popular herbal folk remedies. *E. longifolia* roots are used in folk medicine as an aphrodisiac, antibiotic, appetite stimulant and health supplement, as well as it is used for sexual dysfunction, cancer, leukaemia, malaria, aches, anxiety, diabetes, osteoporosis, syphilis and stress (Kuo et al. 2004; Miyake et al. 2009; Fiaschetti et al. 2011; Ezzat et al. 2019)

Different classes of bioactive compounds are present in *E. longifolia* like quassinoids, canthin-6-one alkaloids, β-carboline alkaloids, squalene derivatives, bioactive steroids triterpene-type tirucallane, and biphenyl *neo*-lignans (Mahfudh and Pihie 2008; Miyake et al. 2009; Bhat and Karim 2010; Tran et al. 2014).

Numerous earlier studies have shown that certain fractions of *E. longifolia* extract inhibit cell proliferation in a variety of human cancer cell lines, including MCF-7 cells (4.40 ± 0.42 to 20.00 ± 0.08) (Tee and Azimahtol 2005), liver cancer, HepG2 cell (45 ± 0.15 μg/ml) (Zakaria 2009), cervical carcinoma, Hela cells (Tran et al. 2014), lung cancer, and leukemic, K-562 cells (Al-Salahi et al. 2014), while few studies clearly demonstrate their mode of action (Thu et al. 2018; Zou et al. 2018).

Therefore, we explored EONE and EOL antiproliferative mode of action of large and small lung cancer cells by studying their effect on cell cycle regeneration and apoptosis.

2. Results and discussion

Extraction and isolation processes of EONE and EOL from the aqueous extract of *E. longifolia* roots were described briefly in the supplementary file, and spectroscopic features of these compounds were reported in our previous publications (Ezzat et al. 2019).

2.1. Cytotoxicity and antitumor activity of EOL and EONE

The cytotoxic effect of EOL and EONE was evaluated by determining the effect of different concentrations (400–1.56 μg/mL) on the viability of normal fibroblast WI-38 cells using SRB assay. The cell viability decreased in dose dependent manner (Figure S1A). The half-maximal inhibitory concentrations (IC_{50}) of EOL and EONE were 386 μg/mL (940.55 μM) and 424 μg/mL (1038.20 μM), respectively. Thus, they were considered non-toxic ($IC_{50} > 90.00$ μg/mL) according to the Special Programme for Research and Training in Tropical Diseases (WHO – Tropical Diseases). Concentration less than 200 μg/mL of EOL and EONE was selected to study their anticancer property against large (H460) and small (A549) lung cancer cell lines proliferation. EOL and EONE gradually decreased the survival percentage of H460 and A549 cells in dose dependent manner (Figures S1B and S1C). Both EOL and EONE exhibited significant anticancer activity on both cell lines. EONE is more potent than EOL. It exhibited IC_{50} of 1.78 μg/mL (4.36 μM) and 20.66 μg/mL (50.59 μM) against H460 and A549, respectively, accompanied by cell shrinkage, poor cell adhesion, rounded cell appearance, and reduction in the cell number. Similar morphological changes were observed in H460 and A549 cells after treatment with EOL which showed its anticancer activity at an IC_{50} of 3.22 μg/mL (7.85 μM) and 38.05 μg/mL (92.71 μM), respectively. Changes in cell morphology induced by EOL and EONE in A549 and H460 cell lines were illustrated in (Figure S2).

2.2. EOL/EONE-arrested cell cycle at G0/G1 phase

Treatment of H460 with 2 x IC_{50} of EOL and EONE for 48 h led to significant ($^*P < 0.05$) increase in the G0/G1 population from 65.33 ± 3.9% in the control cells to 73.06 ± 7.8% and 72.77 ± 6.9% in treated cells, respectively (Table S1). The S population bite decreased from 23.12 ± 2.6% in the control cells to 17.3 ± 3.6% and 14.2 ± 2.1% in EOL and EONE treated H460 cells, respectively. These results showed that EOL and EONE arrested H460 at the G0/G1 phase (Figures S3 and S4). On the contrary, treatment of A549 cells with the tested compounds for 48 h led to increase in the G1 population from 36.83 ± 3.5% in the control cells to 53.60 ± 4.1% and 59.70 ± 2.8% in EOL and EONE treated cells, respectively. EOL decreased S populations significantly to 24.32 ± 3.0% in A549 treated cells while, EONE decreased S populations significantly to 20.75 ± 2.0% compared to 41.09 ± 2.8% in control cells. Also, A549 cells treated with EOL and EONE showed an accumulation in G2/M phase with 20.3 ± 3.2% and 17.7 ± 2.3%, respectively, of the cells population compared to untreated cells 7.72 ± 2.1% (Table S1 and Figure S3). Thus, EONE inhibited cell proliferation by triggering the accumulation of S phase, G0/G1 and G2/M phases. These results matched that

reported by (Khari 2014) about the ability of EONE to induce cell death in HepG2 cells via apoptosis induction. It was noticed that G1 and S phases became the dominant phase in cells treated with EOL and EONE. The G1 phase is marked by the production of mRNA and protein, which are necessary for DNA replication. The cell proliferation is controlled by the G1-S phase checkpoint. At this checkpoint, cells combine and transmit many complex intrinsic and extrinsic signals to determine whether to divide, undergo programmed cell death, or enter the G0 phase (Kastan and Bartek 2004). The cell cycle distribution diagram (Figure S3) revealed that EOL and EONE efficiently suppressed cell growth and decreased proliferation activity by arresting H460 and A549 at the G0/G1 phase and preventing them from transforming to the S and M phases, where DNA doubles.

2.3. EOL/EONE-induced apoptotic cell death

The nature of cell cycle progression in H460 and A549 cells under EONE and EOL treatment was further investigated using the flow cytometry approach. The inhibition effect of both EOL and EONE on cell proliferation via apoptosis was evaluated using annexin V-FITC/PI apoptosis detection assay. As indicated in Figure S4, in untreated cells, only 2.9% and 1.6% of apoptotic cells were detected with 0.3% and 0.48% of late apoptotic cells in H460 and A549 cell lines, respectively. Both H460 and A549 cells were still viable with percentage of 95.8% and 96.8% in the control untreated cell lines, respectively. Thus, the untreated cell lines continuously grew and an apoptosis process was just observed as a normal process in every cell life. Significant differences in the levels of apoptosis between H460 and A549 cells lines were detected in response to EOL/EONE ($P < 0.01$). EOL induced apoptosis in both H460 and A549 and cells, after 48 h of exposure, only 82.61% and 84.38% cells were alive in H460 and A549 cell lines, respectively. Significant ($P < 0.01$) increase in late apoptotic cell to 15.1% and 13.4% along with increased in early apoptosis to 0.81% and 0.90% in H460 and A549 cell lines, respectively, was observed. Nonsignificant necrosis was observed after 48 h treatment with EOL (Figure S4). EONE showed a similar result. EONE induced apoptosis in both H460 and A549 cells. After 48 hours of exposure, only 66.48% and 71.6% cells were alive revealing that almost all of cells go through apoptosis (Figure S4). Significant ($P < 0.01$) increase in late apoptotic cell to 29.6% and 26.1% along with increased in early apoptosis to 2.3% and 0.9%, respectively, was noticed. Nonsignificant necrosis was observed after 48 h treatment. These results confirm that EOL and EONE were capable of inducing cell death in H460 and A549 cells via apoptosis process.

The observed superior activity of EONE over that of EOL could be attributed to the α, β-unsaturated ketone group in EONE. Compounds containing α,β-unsaturated ketone group are well documented to exert higher biological activity than their corresponding analogues lacking this group (Amslinger 2010; Arshad et al. 2017; Ezzat et al. 2019; Ezzat et al. 2019). EONE α,β-unsaturated ketone group was previously reported to be responsible for inhibition of the nuclear factor κB signalling pathway of human leukaemia cell lines (K562 and Jurkat) by inhibiting the phosphorylation of nuclear factor IκBα (Hajjouli et al. 2014). Tumour cells are characterized by molecular changes

causing constitutive increase of nuclear factor κB activation which in turn result in the expression of genes being involved in apoptosis resistance (Prasad et al. 2010). Therefore, it induces apoptosis via inhibiting NF-κB signalling pathway.

Many recent studies reported phytochemicals as potential lead for management of wide varieties of diseases (Abdel-Baki et al. 2022; El Gaafary et al. 2022). It is highly recommended to conduct further detailed studies beside those conducted by (Low et al. 2011) and (Ahmad et al. 2018) on EONE and EOL pharmacokinetics and bioavailability following oral and intravenous administration.

3. Experimental

See the Supplementary Material.

4. Conclusion

The present study demonstrates that the antiproliferative activity of Eurycomanol and Eurycomanone is mediated by inducing G0/G1 phase arrest and apoptosis in A549 and H460 cell lines cancer cells. These findings suggest that EONE and EOL can serve as a potential lead candidate for developing cancer chemotherapeutic agents from *E. longifolia* quassinoids.

Disclosure statement

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