Original research article

Ethyl acetate extract of *Clausena excavata* induces growth inhibition of non-small-lung cancer, NCI-H460, cell line via apoptosis

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Abstract

**Context:** *Clausena excavata* Burm. f is a plant used in folklore medicine for the treatment of various ailments in South East Asia. The plant parts contain chemical components that are cytotoxic to many cancer cells.

**Objective:** The study investigated the cytotoxic effects of ethyl acetate, methanol and chloroform *C. excavata* leaf extracts on the non-small-lung cancer, NCI-H460, cell line.

**Methods:** Based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, among extracts, ethyl acetate *C. excavata* leaf extract (EACE) was the most potent anti-NCI-H460 cells, with IC₅₀ value of 47.1 ± 6.1 μg/ml. The effects of EACE on NCI-H460 cells were also determined by clonogenic, 4', 6-diamidino-2-phenylindole (DAPI), and annexin-V-fluorescein isothiocyanate/propidium iodide-PI flow cytometric assays. Reactive oxygen species (ROS) production and apoptotic gene expressions was determined via flow cytometry and real-time quantitative PCR, respectively.

**Results:** EACE-treated NCI-H460 cells after 48 h underwent apoptosis as evident by loss of cell viability, cell shrinkage, and chromatin condensation. The results also showed EACE mediated increase in ROS production by the NCI-H460 cells. After 48 h treatment, EACE increased the pro-apoptotic BAX and decreased the anti-apoptotic Bcl-2, Survivin and c-Myc gene expressions.

**Conclusions:** EACE is a potential anti-lung cancer by increasing cancer cell ROS production and apoptosis.

**Keywords:** Apoptosis; Cytotoxicity; ROS; Survivin

**Highlights:**
- Our study evaluated the mechanism of action of ethyl acetate extract on the non-small lung cancer, NCI-H460, cell line.
- Ethyl acetate *C. excavata* leaf extract inhibits lung cancer proliferation by increasing production of ROS and activation of apoptosis.
- Ethyl acetate *C. excavata* leaf extract is a potent anticancer agent.

**Abbreviations:** ATCC – American Type Culture Collection; Bax – Bcl 2 associated X protein; BCL2 – B cell lymphoma-2; CH – Chloroform; DAPI – 4, 6-diamidino-2-phenylindole; DCFH-DA – dichlorofluorescein; DMSO – dimethyl sulfoxide; EA – ethyl acetate; EACE – ethyl acetate extract of *Clausena excavata*; ELISA – enzyme-linked immunosorbent assay; FITC – fluorescein isothiocyanate; GAPDH – Glyceraldehyde-3-phosphate dehydrogenase; ICCBS – International Center for Chemical and Biological Sciences; MOH – Methanol; MTT – 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NCI-H460 – Non-small-lung cancer cell lines; PCMD – Dr. Panjwani Center for Molecular Medicine & Drug Research; PI – propidium iodide; PT – Petroleum ether; ROS – Reactive oxygen species; RPMI – Roswell Park Memorial Institute

**Introduction**

Cancer, one of the major causes of death, is on the increase in the last few decades. Lung cancers are among the most prevalent worldwide accounting for 31 to 57 cases of every 100,000 individuals (World Cancer Research Fund, 2019). Among lung cancers, non-small-lung cell cancer (NSCLC) accounts for approximately 85% of all cases. NSCLC is associated with smoking (Townsend et al., 2017). Like all cancers, the treatment of advanced NSCLC with surgery and chemotherapeutic drugs often failed because of drug resistance and cytotoxicity. There-
fore, it is necessary to discover safer and more efficacious anticancer therapeutic agents (Albaayit, 2020; Ling et al., 2016).

Plants are among the most common sources of drugs. Recently, research to determine the potential of plant-derived compounds for the cancer treatment has flourished with the discovery of many new drug candidates (Albaayit and Ozaslan, 2019; Greenwell and Rahman, 2015). The anticancer effects of these natural products are attributed to several mechanisms including apoptosis and necrosis (Albaayit et al., 2020a; Ullah et al., 2014).

_Clausena excavata_ Burm. f. (Family: Rutaceae) is one of the species widely distributed in Southeast Asia that has been used in traditional medicine in Thailand for the treatment of cancers (Perry and Metzger, 1980; Waziri et al., 2016). This plant parts are also used to treat numerous disorders such as wounds, stomachaches, fevers, abdominal pains, and cancers.

In Malaysia it’s locally known as “Kemantu hitam”, “Chemama” and “Cherek hitam”. The plant contains many potent compounds shown to have immune-modulatory, anti-inflammatory, and anti-tumor, and wound healing properties (Albaayit et al., 2014; 2016; 2020c; Huang et al., 2017; Waziri et al., 2018).

_C. excavata_ parts have been shown to be rich in polyphenol compounds such as alkaloids, flavonoid carbazole glycosides, and coumarins. The coumarins are among the main components of the _C. excavata_ extracts reported to exhibit anticancer, antibacterial, and antimalarial effects. Among the coumarins, furanocoumarins are antiproliferative towards cancer cells (Albaayit et al., 2021; Brunii et al., 2019). Other components of _C. excavata_ extracts, including norterpen, xanthoxylitin, clausarin, and denticin C are cytotoxic to hepatoma, prostate cancer, and human promyelocytic leukemia cell lines (Huang et al., 2017). Currently, there is no scientific evidence suggesting that this plant is therapeutically efficacious towards lung cancers. Thus, we investigated the _in vitro_ effect of _C. excavata_ leaf extracts on the non-small-lung cancer, NCI-H460, cell line.

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**Materials and methods**

**Preparation of plant material**

The leaves of _C. excavata_ plant obtained from Pendang, Kedah, Malaysia (5° 59’ N, 100° 28’ E) on December 2010 and were identified by Dr. Shamsul Khamis (Resident Botanist) at the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia (5° 59’ N, 100° 28’ E) on December 2010 and were identified by Dr. Shamsul Khamis (Resident Botanist) at the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia, voucher specimen (TI-013201-CE). Fresh leaves were dried at room temperature, powdered and extracted according to the method described in our previous study (Albaayit et al., 2014). A mixture composed of 1:5 ratio of dried plant by weight to petroleum ether (PT) by volume was subject to the extraction. The filtrate was collected and the residues subjected to further extraction with chloroform (CH), ethyl acetate (EA), and methanol (MOH). The filtrates were again collected and evaporated in a rotary evaporator under reduced pressure at 45–50 °C to obtain stock crude extract. Only the methanol and ethyl acetate _C. excavata_ extracts were sent for LCMS/MS analysis to screen for the presence of phytochemicals (Advance Chemistry Solution, GHOD Sdn Bhd ACD/Labs Inc., Malaysia). The stock extracts were dissolowed in 0.1% dimethyl sulfoxide (DMSO) to obtain the respective extract concentrations used in the study.

**Cell maintenance and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay**

The human non-small-lung cancer, NCI-H460, cell line, a tumor of NSCLC subtype, was obtained from cell culture bio bank (PCMD, ICCBS) from American Type Culture Collection (ATCC), was cultured and passaged using RPMI medium (Albaayit et al., 2019; Zehira et al., 2019). The cytotoxic effect of the solvent extracts on the NCI-H460 cell lines was investigated using the MTT assay (Sajid et al., 2018). 5 × 10^3 NCI-H460 cells/well were placed in each well in a 96-well plate and incubated for 24 h at 37 °C, before treatment with 12.5–100 μg/ml of the MOH, EA, CH, and PT fractions, while the control was treated with 0.1% DMSO for 48 h. 20 μl of MTT (5 mg/ml) reagent was added to each well and the plate incubated for 4 h at 37 °C. The purple formazan crystals formed were dissolved with 150 μl DMSO and the absorbance measured at 570 nm using an ELISA plate reader (Tecan, California, USA). The cancer cell growth inhibition was expressed as IC_{50} (half-maximal inhibitory concentration) value.

** Colony formation assay**

The NCI-H460 cells were seeded at a density of 1 × 10^4 cells/well/1 ml in a 6-well plate overnight to confluence, treated with 47 (IC_{50}) and 100 μg/ml EACE, and the plate incubated for 21 days at 37 °C. At the end of the incubation period, 2 ml 0.5% (w/v) of crystal violet in 25% ethanol was then added to each well and the plate incubated for another 30 min at room temperature. After removal of excess stain, the cells washed with ddH_2O and the colonies formed immediately photographed and counted (Sajid et al., 2018).

** Apoptosis**

**Annexin-V-propidium iodide assay (PI)**

The early and late apoptosis in NCI-H460 cells were determined using the PI staining technique (Thermo Fisher Scientific). Briefly, 1 ml of 1 × 10^6 NCI-H460 cells suspension was seeded into each well of a 6-well plate and the plate was incubated for 24 h at 37 °C, treated with EACE at concentration either 47 or 100 μg/ml to the respective wells and the plate incubated for 48 h. After the treatments, the cells were harvested by trypsinization, washed thrice with phosphate buffered saline (PBS), and stained with 5 μl fluorescein isothiocyanate (FITC)- conjugated annexin-V and 5 μl PI for 15 min at room temperature in the dark. The percentage of cells in apoptosis and necrosis were determined by flow cytometry on FACSCalibur™ (Becton Dickinson).

**4,6-diamidino-2-phenylindole assay (DAPI)**

DAPI staining is one of the most commonly used fluorescent-based assays for the determination of the effect of treatments on cell morphology (Rahman and Hussain, 2015). The NCI-H460 cells were seeded into each well at a density of 1 × 10^6 NCI-H460 cells/well/ml in a 6-well plate for 24 h at 37 °C, treated with either 47 or 100 μg/ml EACE in Roswell Park Memorial Institute Medium, and the plate incubated for 48 h at 37 °C. 100 μl of DAPI (1: 1000) was added to each well and the plate incubated for 1 h at room temperature. The cells were observed for fluorescence, fragmentation, and nuclear condensation under Nikon fluorescence microscopy at 200× magnification.

** Reactive oxygen species (ROS)**

Flow cytometry was used to determine production of ROS by treated NCI-H460 cells (Ahamed et al., 2013). 500 μl of 1 × 10^6 NCI-H460 cells were seeded into each well of 24-well plate and treated with either 47 or 100 μg/ml EACE and the plate incubated for 48 h. Non treated cells served as negative while cells treated 50 μl (50 M) hydrogen peroxide (H_2O_2) were the positive control. The cells were then detached by trypsin-
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**Quantitative Real-time polymerase chain reaction (RT-qPCR)**

RT-qPCR was used to determined expression of apoptosis-related genes in treated NCI-H460 cells. The NCI-H460 cell were seeded into each well of a 6-well plate at a density of $1 \times 10^6$ NCI-H460 cell/well/ml and treated 47 μg/ml EACE for 48 h. Total RNA was isolated from harvested cells using the TRizol® Reagent (Bio basic BS410A, Canada). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using cDNA synthesis kit (Thermo Fisher Scientific K0221). Then, 1 μl of cDNA was amplified by real-time PCR using primers listed in Table 1. The amplification of genes was performed using SYBR Green Master Mix (Thermo Fisher Scientific). LightCycler® 480 Gene Scanning Software (Agilent Technologies Stratagene Mx 3000P, Santa Clara, CA) was used to generate the results and calculate number of transcripts relative to the normal control. GAPDH was used as the housekeeping gene control (Albaayit et al., 2020b, c; Hidayat et al., 2016).

### Statistical analysis

Data are expressed as mean ± SD and level of significance at $P < 0.05$ determined by one-way analysis of variance using SPSS (Version 19.0; IBM Corporation, Armonk, NY, USA). All experiments were performed in triplicates.

### Results

The yield (wt. of crude extract / wt. of fresh plant) of the petroleum ether, chloroform, ethyl acetate, and methanol extracts of *C. excavata* leaf were of 1.56, 2.57, 0.38, and 0.94%, respectively.

**LCMS/MS screening**

EACE has high contents of phenolic acid, furocoumarin-based compounds, and 8-geranyloxy psoralen and its isomer (Fig. 1 – on the next page).

**Cytotoxicity assay**

Among extracts, the ethyl acetate *C. excavata* extract (EACE) was most active against cancer cells, showing the lowest mean IC$_{50}$ of 47 μg/ml (Table 2) against NCI-H460 cells, and hence was chosen for subsequent studies.

After 21 days of incubation, 47 and 100 μg/ml EACE inhibited NCI-H460 colony formation by 73 and 99%, respectively, relative to the control (Fig. 2).

**Table 1. Genes and primer sequences**

<table>
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<tr>
<th>Gene</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL2-associated X (BAX)</td>
<td>AAGAGGCTGACCGATGTC</td>
<td>GGCCCCACGGTAAGTTC</td>
</tr>
<tr>
<td>B cell lymphoma-2 (BCL2)</td>
<td>GGCTATTCAATGACCTGACAT</td>
<td>AGTCATGGCCTTGAGAAC</td>
</tr>
<tr>
<td>Survivin</td>
<td>CAGAGTCCGCCCTCCTCTAC</td>
<td>GGTCATGCGGCTGTGCTA</td>
</tr>
<tr>
<td>C-Myc</td>
<td>CACACACAAACTCTCTCACAG</td>
<td>GGTGCAATTCTGGTGTTGGTC</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
<td>CCAGAACATCATCACTCCGTCT</td>
<td>CCAGAACATCATCCCTGCCT</td>
</tr>
</tbody>
</table>

**Table 2. IC$_{50}$ values of *Clausena excavata* extracts treatment on NCI-H460 after 48 h**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>IC$_{50}$ (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum ether</td>
<td>$&gt;100$</td>
</tr>
<tr>
<td>Chloroform</td>
<td>55.16 ± 9.10</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>47.10 ± 6.10</td>
</tr>
<tr>
<td>Methanol</td>
<td>$&gt;100$</td>
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**NCI-H460 cell apoptosis**

Under DAPI staining, the EACE treated NCI-H460 cells showed morphological changes (Fig. 3). Treated cells underwent apoptosis as evident by cell shrinkage and condensed nucleus. On the contrary, the non-treated control cells were mostly intact. Upon treatment of NCI-H460 cells with EACE at 100 μg/ml, late apoptosis phase increased up to 77.3%, which was much higher as compared to 47 μg/ml which increased up to 24.5% (Fig. 4).
Fig. 1. LCMS/MS characterizations of ethyl acetate *C. excavata* leaf extract showing its high contents of (A) phenolic acid, (B) furocoumarin, and (C) 8-geranyloxy psoralen isomer.
Fig. 3. Ethyl acetate *C. excavata* leaf extract (EACE)-treated non-small cell lung cancer, NCI-H460, cell line after 4, 6-diamidino-2-phenylindole (DAPI) staining. After treatment with (B) 47 and (C) 100 μg/ml of EACE, the cells show shrinkage and condensed chromatin. The effect was more prominent at the higher EACE treatment dose. (A) Control (Magnification 200×).

Fig. 4. Flow cytometric analysis of non-small cell lung cancer, NCI-H460, cell line. Cells treated with 47 and 100 μg/ml ethyl acetate *C. excavata* leaf extract (EACE) for 48 h. Control show low number of apoptotic cells. Significantly high apoptotic cells treated EACE especially at the higher dose.

Fig. 5. mRNA expression of BAX, BCL2, survivin and c-Myc genes in non-small cell lung cancer, NCI-H460, cell line treated with 47 μg/ml (IC50) ethyl acetate *C. excavata* leaf extract (EACE). Values are mean ± standard deviation. * Mean significantly different (p < 0.05) from the respective nontreatment control mean.
Expression of apoptotic and anti-apoptotic genes in NCI-H460 cell
After 48 h-treatment, the NCI-H460 expression of BAX gene was significantly ($p < 0.05$) up regulated by ~1.7-fold, while that of Bcl-2, surviving and c-MYC genes down-regulated by ~3.1-, 1.6- and 5.7-fold, respectively, in comparison with the control (Fig. 5).

Reactive oxygen species (ROS) production by NCI-H460 cells
After treatment with 47 and 100 µg/ml EACE for 24 h, ROS production by NCI-H460 cells increased significantly by 55.5 and 79.5%, respectively. The positive ($H_2O_2$) control cells showed significant increase in ROS production while the non-treated cells did not generate ROS (Fig. 6).

Discussion
Over the last decade the field of ethno-pharmacology had steadily gained the interest of researchers, especially those in search of novel natural compounds with potent anticancer properties (Zehra et al., 2019). Now, it is known that many medicinal plants possess anti-proliferative properties for various types of cancer. C. excavata is one of the plants with parts that contain several compounds with a wide array of biological activities, including anticancer, antitumor, antimalarial, anti-inflammatory, and antiviral properties (Huang et al., 2017; Waziri et al., 2016). We subjected the leaves of C. excavata to the extraction process using various solvents to determine the extract with potentially the best anticancer properties. Among these extracts, EACE exhibited the most potent anti-proliferative effect towards the NCI-H460 cells. The leaves of C. excavata are especially high in phenolic contents that could be main contributor to the cancer cell apoptotic effect of the extract (Huang et al., 2017; Waziri et al., 2016). In the development of therapeutics, compounds that could specifically cause cancer cell apoptosis would be ideal candidates as anticancer drugs (Che et al., 2018; Huang et al., 2017). In this study, the apoptosis-inducing properties were investigated using the annexin V/propidium iodide staining of the EACE-treated NCI-H460 cells. It was shown that EACE induced significant NCI-H460 cell late apoptosis after 48 h treatment. The apoptotic effect of EACE was also evident by the condensed chromatin and loss of normal cellular morphology. The study also showed that EACE caused significant up-regulation of the pro-apoptotic BAX gene, while the anti-apoptotic survivin, Bcl-2, and c-Myc genes were down-regulated (Kim et al., 2014; Sivakumaran et al., 2018). Since increase in BAX expression and down-regulation of survivin, Bcl-2, and c-Myc genes are mitochondria-mediated (Losuwannarak et al., 2018; Yao et al., 2017), it is suggested that at least the intrinsic apoptosis pathway is involved in the anti-proliferative effect of EACE on the NCI-H460 cells.

There are numerous evidences that showed ROS has both tumor-promoting (Reczek and Chandel, 2018) and tumor-suppression effects (Wang et al., 2017), presumably dependent on the concentration of ROS in the tumor microenvironment and state of tumor cells (de Sá et al., 2017). It was proposed that response of tumors to ROS follows a threshold concept, where the cancer cells enter an adaptive state to increasing ROS and eventually perish as the ROS level exceeds a certain limit (Redza-Dutordoir and Averill-Bates, 2016). Excessive ROS is toxic to cells and can cause apoptosis and necrosis (Albaayit and Maharjan, 2018; Al-Bahrani et al., 2020; Mileo and Miccadei, 2016). Our study showed that EACE induced increased
ROS generation by the NCI-H460 cells to a level that exceeded the capacity of the cellular anti-oxidant defense mechanism, resulting in damage to the mitochondrial membrane and tumor cell death (Avenaondo and Menendez, 2015; Hu et al., 2014). Evidently, the cancer cell killing effect of ROS occurs through both the intrinsic and extrinsic apoptosis pathways (Essafi Rhouma et al., 2019).

Phenols from plant, including C. excavata, have anticancer, antioxidant, and pro-oxidant properties (Essafi Rhouma et al., 2019). The EACE, in particular, has high phenolic content and it is proposed that these compounds are responsible for excessive ROS production resulting in the anti-proliferation of NCI-H460 cells (Albaayit et al., 2014). Phenols can cause cell arrest and premature aging, facilitating their anticancer effects (Mileo and Miccadei, 2016). It was suggested that phenolic compounds inhibit the stress-activated NF-κB and AP-1 signaling cascade. Phenolics also enhance the immune system in the destruction of cancer cells, inhibit angiogenesis, and reduce the metastatic potential of tumors (Mohammad and Mahdi, 2017). Based on the evidence in this study and those reported earlier, the EACE has potential to be developed into an anticancer agent.

Conclusions

In conclusion, the results collectively provided evidence that the EACE is toxic to the lung cancer, NCI-H460, cells and the anticancer effect of the extract is through the activation of apoptosis-induction with concomitant inhibition of the anti-apoptosis mechanisms. Therefore, the findings show that EACE is a promising and novel chemotherapeutic agent for the treatment of lung cancers.

Conflict of interests

We wish to confirm that there is no known conflict of interests associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Author contributions

SFAA and MAK conceptualized, designed and conducted the experiment. SFAA, RA and MMNH interpreted the data and wrote the manuscript draft. All authors have read and approved the final manuscript.

References


