RESEARCH ARTICLE

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# Anti Proliferative and Apoptotic Effect of Soluble Ethyl Acetate Partition from Ethanol Extract of *Chromolaena Odorata* Linn Leaves Against HeLa Cervical Cancer Cell line

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## Abstract

**Background:** Cervical cancer is the leading cause of cancer death in women. Chemotherapy is one of the treatment modalities with many side effects. This study aimed to evaluate the anticancer activity of soluble ethyl acetate partition of *Chromolaena odorata* (*C. odorata*) leaves on HeLa cells of cervical cancer. **Material and methods:** The cytotoxicity activity of the soluble ethyl acetate extract of the *C. odorata* leaves was analyzed by MTT colorimetry assay. The apoptosis activity was determined by double staining and flow cytometry techniques. Doubling time assay was used to observed HeLa cells proliferation. **Results:** The $IC_{50}$ of soluble ethyl acetate partition of this plant was 82.41± 6.73 µg/ml against HeLa cells. The apoptosis activity showed that HeLa cells underwent morphological changes in dose-dependent manner while the highest number of dead cells was observed after treatment with 500 µg/ml of the partition. Flow cytometry analysis showed that treatment with $IC_{50}$ and 2x $IC_{50}$ resulted in death of more than 97% cells (p-value <0.05 in both comparisons). Proliferation of HeLa cells was also inhibited following treatment with ½ IC50, IC50, and 2x$IC_{50}$ in the first 24 hours (p-value <0.05 in all comparison). **Conclusion:** The findings of this study suggested that the soluble ethyl acetate partition from ethanol extract of *C. odorata* leaves had cytotoxic and antiproliferative properties against HeLa cells.

**Keywords:** Cervical cancer- chromolaena odorata- doxorubicin- proliferation

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## Introduction

Cervical cancer is the fourth most frequently diagnosed cancer and known as the leading cause of cancer death in women. It is estimated that 604,000 new cases of cervical cancer will be diagnosed tile 2020 along with 342,000 fatal cases worldwide. Cervical cancer is the leading cause of cancer death in 36 countries, including sub-Saharan Africa, Melanesia, South America, and South Eastern Asia (Sung et al., 2021). Human papillomavirus (HPV) is a known cause of cervical cancer and it is also found that HPV 1 is carcinogenic to humans. HPV consists of 12 oncogenic strains (Humans, 2007). Other sexually transmitted diseases (i.e. HIV and Chlamydia), smoking, high number of childbirth, and oral contraceptive use are cofactors associated with cervical cancer(Thun et al., 2017). The incidence and mortality rate of cervical cancer have been declined for the past few decades due to HPV vaccine, screening, and prevention measures(Organization, 2017; Palmer et al., 2019). Depends on the stage of the cancer, patient with cervical cancer are treated surgically or with chemoradiation (Eifel, 2006; Maher and Denton, 2008). However, side effects associated with the cervical cancer treatments include urinary and sexual dysfunction. They may have substantial negative impact on the patients' quality of life (Frumovitz et al., 2005; Korfage et al., 2009). On the other hand, treatment failure due to drug resistance to chemotherapy drugs, such as cisplatin, doxorubicin, and melphalan, has been also reported (Izquierdo et al., 1996; Ke et al., 2021).

Therefore, cancer research in the last decades has focused on natural compounds to develop effective and selective cytotoxicity agents for the treatment of many cancers, including cervical cancer.

The effect of *Chromolaena odorata* (*C. odorata*) on breast cancer(Yusuf et al., 2021c), colorectal cancer (Yusuf et al., 2021b), hepatocellular carcinoma(Prabhu and Ravi, 2012), lung cancer (Adedapo et al., 2016), and skin cancer (Yajarla et al., 2014a) was investigated in previous studies. *C. odorata* is known to have several compounds that are cytotoxic and can enhance the apoptosis (Kouame et al., 2013). Kaempferide, one of *C. odorata* compounds was proven to be pharmacologically safe for normal cell while inducing apoptosis in cervical cancer cell line (HeLa)

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(Nath et al., 2015). Triterpene that is extracted from fresh leaves of *C. odorata* showed cytotoxicity effect on HepG2 cell line(Prabhu and Ravi, 2012). Acacetin and luteolin, both isolated from the flower of *C. odorata*, exhibited a moderate cytotoxicity against small cell lung cancer(Suksamrarn et al., 2004). The antiproliferative properties of *C. odorata* on breast cancer and cervical cancer were studied previously (Yusuf et al., 2021c; Rusdi et al., 2020). However, no study has yet compared the anticancer properties of *C. odorata* on cervical cancer cell line (HeLa). Therefore, in this study, we investigated the antiproliferative and apoptotic properties of *C. odorata* on HeLa cells.

## Materials and Methods

*Extraction of C. odorata Linn*

Ten kg of dry *C. odorata* leaf powder was extracted by maceration using ethanol as a solvent, because ethanol has a high degree of polarity so that it can dissolve polar, semipolar, and nonpolar compounds from the compounds found in *C. odorata* leaves. Following maceration, a liquid extract was obtained, then the extract was concentrated using a vacuum rotary evaporator at a temperature of 50 °C until a thick extract was obtained. Next, the extract was dried and weighed. The extraction process led to production of an ethanol extract of *C. odorata* leaves weighing 220.15 gr.

*Preparation of partition the solved compound of ethyl acetate from C. odorata Linn leaves ethanol extract*

Initially, 220.15 gr thick extract of the leaves of *C. odorata* was prepared through maceration. The extract was then partitioned into n-hexane and ethyl acetate to obtain compounds with different polarity in the solid-liquid system. To do so, the partition was carried out by dissolving the thick extract with n-hexane, then the liquid extract obtained was set aside and dried. The insoluble residue was further partitioned with ethyl acetate and the filtrate obtained was dried. The result of partitioning with n-hexane was 20.50 gr, soluble ethyl acetate partition was 70.90 gr and insoluble ethyl acetate partition was 65.10 gr.

*Preparation of HeLa cells*

HeLa cells (ATCC®CCL-2™) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified eagle medium supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) penicillin-streptomycin, then incubated at 37°C for 24 hours in a humidified 5% $CO_2$ incubator. Cells were harvested when the number of cells were sufficient (70–80% confluent) and the cells were ready to be used.

*Comparison of cytotoxic activity of ethyl acetate partition of C. odorata Linn with doxorubicin on HeLa cell*

The cytotoxic activity test was adapted from previously described method using MTT colorimetric assay in 96 wells microplate (Yusuf et al., 2020). HeLa cell was used as experimental sample, and well seeded with only growth media acting as blank sample. The ethyl acetate partition of *C. odorata* (EACO) was divided into 6 concentrations, namely: 500,250, 125,62.5, 31.25, and 15.625 µg/ml. Doxorubicin (DOXO) was divided into 100, 50, 25, 12.5, 6.25, and 3.125 µg/ml concentrations. HeLa cell was suspended in growth media. Two microplates were seeded with HeLa and growth media, then incubated at 5% $CO_2$ incubator at 37°C for 24 hours. The medium then was discarded, and plates were washed with PBS. Then, 100 µL of EACO and DOXO in serial concentration were added to each plate for 24 hours incubation. The solution in the plates was then removed and washed with PBS. Afterwards, 100µl of fresh media and 10µL MTT (5 mg/mL) were added to each well and incubated for 4 hours at 37°C until formazan salts were formed. The reaction was stopped by adding 10% SDS stopping solution in 0.1N Hidrochoride acid and incubated in a dark place overnight. These experiments were repeated in triplicate. The absorbance of the plates was then read by a microplate ELISA reader at λ 595 nm and converted into the percentage of viable cells by using the following formula:

% Viable cells = (treated cell absorbance – medium absorbance )/(control cell absorbance – medium absorbance) x 100%

Low percentage of viable cells showed more cancer cell death in a specific concentration. The percentage of viable cell was used to determine the $IC_{50}$ value. The potency of anticancer activity was represented by $IC_{50}$ value given that this concentration of the EACO can produce 50% cell deaths. The $IC_{50}$ value was calculated by using nonlinear regression analysis in Graph Pad Prism v.8.0.2 (GraphPad Software, CA, USA).

Double staining apoptosis assay Initially, 200 µL HeLa (60- 80% confluent) with density of 5 x $10^4$ cells/well and growth media were seeded on 24-well plates separately and incubated for 24 hours. Then, these cells were treated with 1mL of EACO and DOXO in serial concentration over 24 hours incubation at 37°C. At the end of incubation, the plates were washed with PBS, and the cover slip was carefully removed and placed on a glass object. Then, 10 µL of acridine orange-ethidium bromide was dripped on the cover slip and allowed to settle for 5 minutes. The cover slip was then immediately observed under a fluorescence microscope (Zeiss MC 80) using 10 x 40 magnification. All changes in the cells were recorded such as normal cells, dead cells and cell undergone membrane-like changes blebbing, nuclear condensation and other features of apoptosis and cytoplasmic and nuclear changes. These images were documented with digital cameras (CCRC, 2009). Cells with bright green nuclei and intact structure were identified as viable cells had cells, while cells showed nuclear chromatin condensation and with dense green patches or fragments were identified as early apoptotic cells. Late apoptotic and necrotic cells were identified by uniform labelling of cell nuclei, but apoptotic cells contained condensed chromatin or fragmented chromatin and were orange colored, and the necrotic cells showed orange nuclei with intact structures (Ciniglia et al., 2010).

*Apoptosis induction test using flow cytometry*

HeLa cells (5 X $10^5$ cells/well) and growth media were filled into 6-well microplate and then incubated for 24 hours. Then, cells were treated with EACO with a concentration of $IC_{50}$ and 2x $IC_{50}$, then incubated overnight. The floating and attached cells were collected by giving 0.25% trypsin. They were then transferred into a conical tube. Cells were washed twice with PBS and centrifuged at 600 rpm for 5 min. Afterward, the cells were stained with the Propidium Iodide and RNAse in PBS (Roche, Mannheim, Germany) according to the manufacturer's recommendation. The samples were assessed for apoptosis by using the BD FACS Calibur Flow cytometer (Becton Dickinson, California, US) and analysed by Modfit Lt. 3.0 (CCRC, 2014).

*Cell proliferation test (doubling time)*

First, 100 µL HeLa (70- 80% confluent) with density of 1.5 x 104 cells/well and growth media were seeded into 96-well plate and incubated for 24 hours. The media was then discarded, and the plate was washed with PBS. Then, the cell was treated with 100 µL of ½ $IC_{50}$, $IC_{50}$, and 2x $IC_{50}$ of EACO and incubated for 72 hours. The plate was observed every 24 hours and data was recorded. The solution on the plate was discarded and plate was washed with PBS. Afterwards, 100 µL MTT 0.5 mg/mL was added to the plated and incubated for 4 hours until purple formazan crystal formed. To stop the reaction, 10% SDS in in 0.1N Hidrochoride acid was added and incubated in a dark place overnight. These experiments were performed in triplicate. The absorbance of the plates was then read by a microplate ELISA reader at λ 550-600 nm. The result for each 24 hours was then converted to viable cells percentage by using previously mentioned formula.

*Statistical analysis*

Experiments were repeated at least three times and the results were expressed as mean ± SD. To determine any significant differences between treatments, data were analyzed by analysis of variance (ANOVA) followed by a Tukey posttest. P-values <0.05 were considered to be significant. All statistical analyses were performed using Graph Pad Prism v.8.0.2 (GraphPad Software, CA, USA).

## Results

*Comparison of cytotoxic activity of ethyl acetate partition of C. odorata Linn with doxorubicin on HeLa cell*

The results on the cytotoxicity effect of EACO and DOXO in serial concentration against HeLa cells are

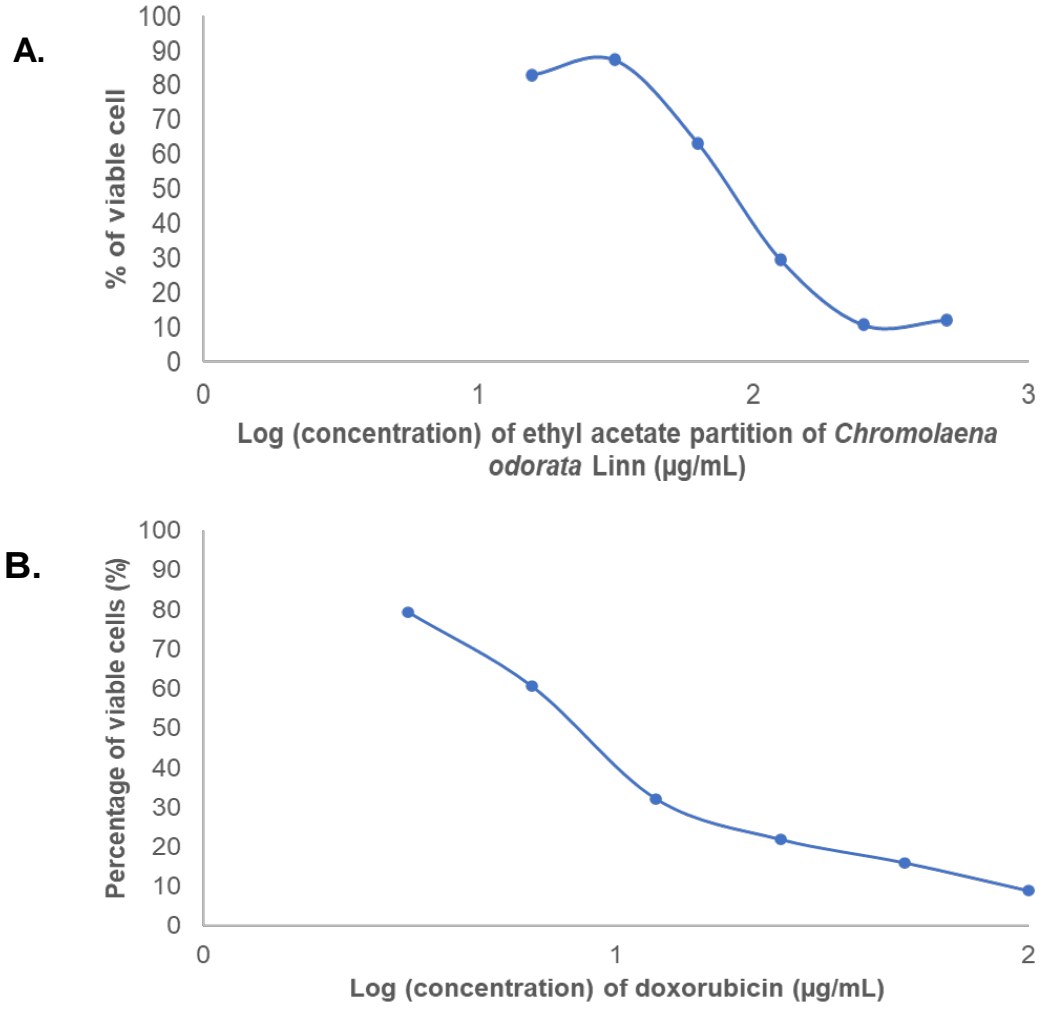


Figure 1. The Cytotoxic Activity of Ethyl Acetate Partition of Chromolaena Odorata Linn (A) Compared to Doxorubicin (B) on HeLa Cells.

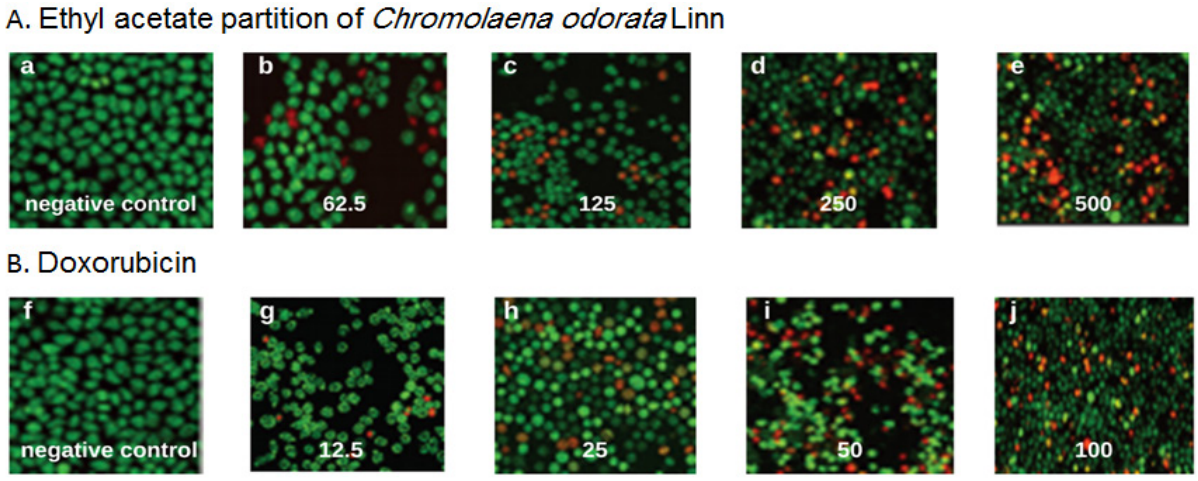


Figure 2. The Changes of Morphology in HeLa Cells after Treatment with Ethyl Acetate Partition of *Chromolaena Odorata* (EACO) and Doxorubicin (DOXO) in Serial Concentration for 24 hours (Magnification 400x).

presented in Figure 1. The $IC_{50}$ of EACO and DOXO against HeLa cell were found to be 82.41± 6.73 µg/ml and 7.96±0.83 µg/ml, respectively.

*Double staining apoptosis assay*

The apoptosis effect of EACO and DOXO on serial concentration was also evaluated by using double staining method (Figure 2). Viable cells (negative control, no treatment) with green nuclei and regular intact structures were observed (Figure 2a and Figure 2f). Figure 2b and Figure 2g show cells underwent apoptosis process with loss of nuclear shape, membrane blebbing, and some dead cells (orange cells). Early apoptosis stage can be observed in Figure 2c and Figure 2h. However, more dead cells (orange-stained cells) were found in HeLa treated with 125 µg/ml EACO(Figure.1c) compared to HeLa treated with 25 µg/ml DOXO. Disintegration of apoptotic bodies with orange-stained nuclei was observed on HeLa cells treated with 250 µg/ml of EACO (Figure.2d) , but not on HeLa cells treated with 50 µg/ml DOXO. According to Figure 2e, there were more apoptotic cells with fragmented nucleus after HeLa cells were treated with 500 µg/ml of EACO than when they were treated with 100 µg/ml of DOXO.

*Apoptosis induction test using flow cytometry*

Figure 3 shows the results of apoptosis analysis on HeLa cells using flow cytometry at the concentration

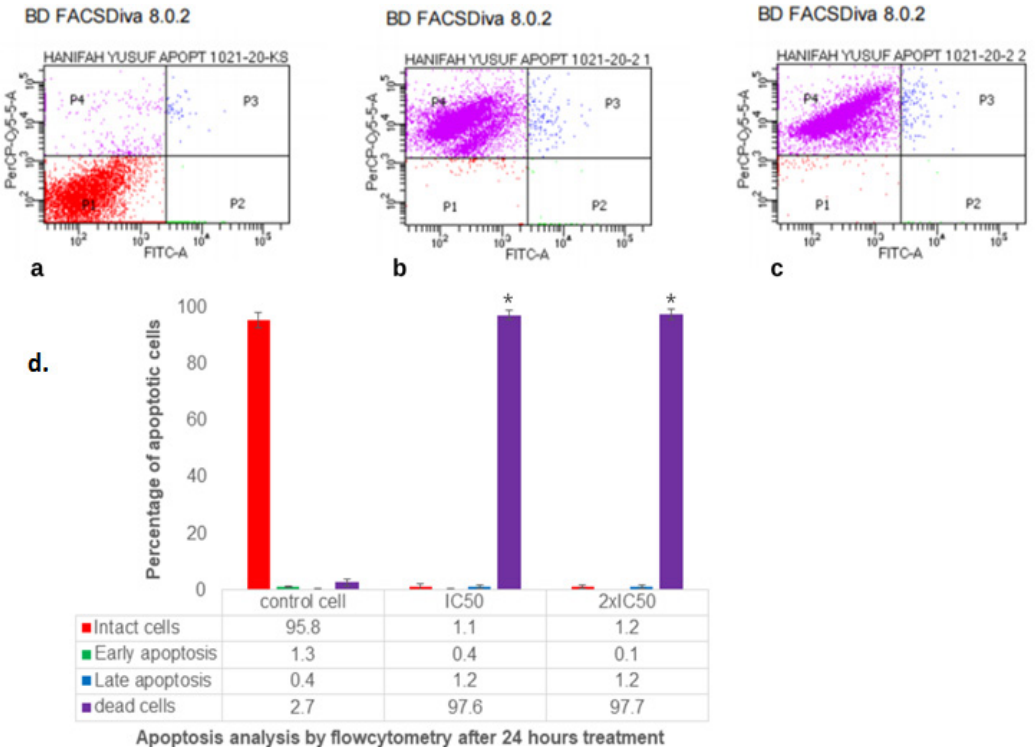


Figure 3. The Result of Flowcytometry Test. a, HeLa cell control without treatment; b, HeLa cells that was treated with $IC_{50}$ of EACO; c, HeLa cells that was treated with 2x $IC_{50}$ of EACO; d, The graphic of HeLa cells undergone apoptosis in each treatment; P1) viable cells; P2) early apoptosis; P3) late apoptosis; P4) dead cells.

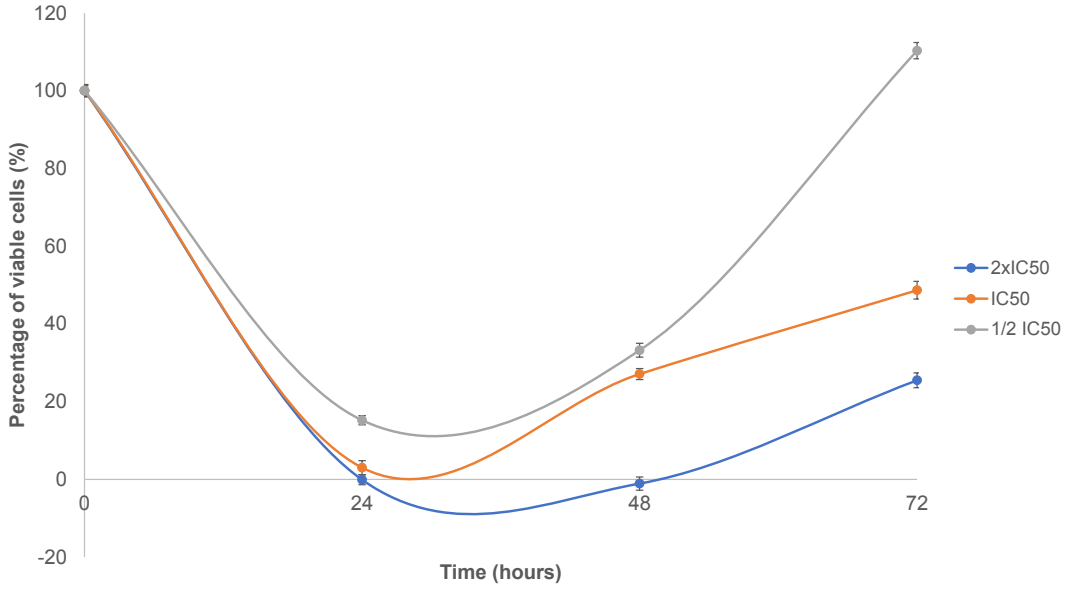


Figure 4. The Proliferation of HeLa Cells after 24 hours, 48 hours and 72 hours Treated with 2x$IC_{50}$, $IC_{50}$ and ½ $IC_{50}$ of ethyl acetate partition of *Chromolaena odorata*.

of $IC_{50}$ and 2x $IC_{50}$. The majority of control cells (HeLa cells without treatment) were seen on P1 (Figure 3a) area, which indicated intact structure of cells (95.8±2.8%). Meanwhile, most of HeLa cells treated with $IC_{50}$ and 2x $IC_{50}$ were seen on P4 areas (Figure 3b and Figure 3c), which indicated a significant increase in number of dead cells when compared to controls (97.6±1.6% versus 97.7±1.9%, p-value <0.05).

*Cell proliferation test (doubling time)*

Based on Figure 4, after administration of 2x$IC_{50}$, $IC_{50}$, and ½ $IC_{50}$ EACO, a significant decline in number of viable cells on HeLa cells were observed in the first 24 hours. Afterwards, the number of viable cells were started to increase within 48 hours and 72 hours. The highest decline after 24 hours was seen after treatment with 2x$IC_{50}$ EACO with 100.1±1.3% inhibition (p-value <0.05), followed by 101.09±1.7% in 48 hours, and 74.51±1.9% in 72 hours.

Treatment with $IC_{50}$ and ½ $IC_{50}$ EACO were also decreased the number of cells with intact structure by 96.9±1.8% and 84.78±1.2% inhibition effect, respectively, after 24 hours (p-value <0.05 in all comparisons). However, after 48 hours of incubation, both groups treated with $IC_{50}$ and ½ $IC_{50}$ EACO showed a decline in their inhibitory effect on HeLa cells proliferation with 27.10±1.4% and 33.23±1.8% normal cells , respectively. The number of viable cells were significantly increased after 72 hours of treatment with $IC_{50}$ and ½ $IC_{50}$ EACO (48.67±2.3% and 110.32±2.1%, respectively) with p-value <0.05 in all comparisons.

## Discussion

*C. odorata* has been extensively studied in the last decade. N-hexane fraction of this plant has antiproliferative activity against MCF-7, MDAMB-468, and CAL51 breast cell lines, while ethanol extract of its leaves can suppress the growth of Lewis lung carcinoma cell (LLC) and HL-60 human leukemia cell lines (Ohiagu et al., 2021). The essential oil of this plant (pentacyclic triterpenoid) could decrease the viability of HepG2 cells by 90% after incubation with $IC_{50}$ value of 206 µg/ml (Prabhu and Ravi, 2012).

To date, this study was the first one investigating the antiproliferative activity of soluble ethyl acetate partition of *C. odorata* Linn on HeLa cells. We found that treatment with $IC_{50}$ of 82.41 µg/ml resulted in 97.6±1.6% of dead cells .In line with this study, a previous research showed that ethyl acetate extract of this plant with $IC_{50}$ of 40 µg/ml altered the morphology (broken cell membrane) of A431 cell (skin cancer cell line) and exerted antiproliferative property in using flow cytometry analysis of cell cycle (Yajarla et al., 2014b).

The selective activity of *C. odorata* against cancer cell line was also reported by Kouamé et al. They found that the viability of Cal51, MCF-7, and HeLa cells declined following treatment with 20 µM of the extract, however, no cytotoxicity was observed on KB, BC, and NCI-H187 cell lines even with >58µM of the extract (2013). The anticancer activity of ethyl acetate extract of this plant may be also enhanced by its antioxidative property, resulted in 79% DPPH inhibition effect (Kusuma et al., 2014). The ethyl acetate extract was also rich in phenolic and flavonoid content with 110mg and 380 mg, respectively (Yajarla et al., 2014b). Overexpression of LC3A protein was also used in determining the autophagy property of this plant. The findings of a previous study revealed morphological changes on MCF-7 cells after $IC_{50}$ treatment (133.9 µg/ml) of EACO leading to membrane blebbing (small protrusions of the membrane) after 24 hours (Harun et al., 2012). This finding was aligned with our result suggesting that HeLa cells lost its nuclear shape during the apoptosis process with some dead cells (orange cells). The phytochemical analysis of ethanol extracts of *C. odorata* was reported in our previous articles (Yusuf et al., 2021a; Yusuf et al., 2021c).

*Study limitations*

The limitation of this research was that phytochemical analysis of ethyl acetate partition used in this study was not conducted. So, further analysis on compound acting as antiproliferative is needed to be tested from this partition .

In conclusion, the findings of the present study suggested that soluble ethyl acetate partition of *C. odorata* Linn had potent cytotoxic activity against HeLa cells. The findings also showed that the mechanism underlying cell death by this partition was due to growth inhibition and its the antiproliferative activity. Further studies on the active components for proper assessment of their chemotherapeutic properties and possible development as promising anticancer drugs is warranted.

## Author Contribution Statement

Conceptualization, HY; Methodology, Hy; MF, CM; Software, MF, CM; Validation, HY, MF; Formal Analysis, MF, CM; Investigation, HY, MF; Resources, HY; Data Curation, HY, MF; Writing – Original Draft Preparation, HY, MF, CM; Writing – Review & Editing, HY, MF; Visualization, MF; Supervision, HY.

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*Ethical approval*

The research was approved by the Ethics Commission, Faculty of Medicine, Lambung Mangkurat University, Banjarmasin, Indonesia (No. 239/KEPK/FK UNLAM/VI/2020).

*Conflict of interest*

The authors declare no competing interests.

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