

Proapoptotic activity of essential oils from *Syzygium aromaticum*, *Melaleuca cajuputi*, and *Cymbopogon nardus* on HeLa human cervical cancer cells

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ABSTRACT

Cancer develops through an imbalance in the cell cycle regulators, resulting in persistent proliferation and escaping apoptosis. Essential oils (EOs) are gaining popularity as a potential natural-based anticancer agent. This research aimed to evaluate cytotoxic activity by induction of apoptosis in *Syzygium aromaticum*, *Melaleuca cajuputi*, and *Cymbopogon nardus* EOs against the HeLa human cervical cancer cell lines. HeLa cell cultures were treated with EOs from three plants separately at 30, 60, and 120 µg/ml and 0 µg/ml as control. The trypan blue exclusion test was conducted to examine cell viability and determine the inhibitory concentration (IC₅₀) value. Cytotoxicity activity was evaluated using Annexin V (AV) and propidium iodide (PI) flow cytometry. The EOs of the three herbs significantly decrease HeLa cells' viability at 24 and 48 hours after treatment in comparison to the control. AV-PI analysis displayed significant induction of late-stage apoptosis in a dose-dependent relationship. The 48 hour IC₅₀ values of EOs were 18.06 µg/ml (*S. aromaticum*), 26.25 µg/ml (*M. cajuputi*), and 28.03 µg/ml (*C. nardus*). The EOs of *S. aromaticum*, *M. cajuputi*, and *C. nardus* have potential anticancer activity with their cytotoxic effects on HeLa cells and could be explored further as a cervical cancer treatment.

INTRODUCTION

Cervical cancer is an uncontrolled growth from the tissue of the cervix of the female reproductive organ, which is strongly linked to persistent infection with oncogenic types of the human papillomavirus (HPV) (Ibeanu, 2011). In 2018, it was considered the second most prevalent cancer seen in women worldwide, with about 885,193 new cases and over 311,365 deaths annually (Aoki *et al.*, 2020). Globally, the incidence and mortality of cervical cancer are as high as 13.1 and 6.9 per 100,000, respectively (Setiawan *et al.*, 2020). Furthermore, in

recent years, the incidence of cervical cancer has been more alarming in developing countries due to a lack of effective prevention measures such as HPV vaccines and cervical screenings, which are considered expensive. On the other hand, currently, there is no effective treatment for cervical cancer, especially in the advanced stages (Hu and Ma, 2018).

The search for effective cervical cancer treatment is currently challenging owing to its complex pathogenesis (Bhat *et al.*, 2018). The carcinogenesis of cervical cancer was driven primarily by high-risk HPV infection, which expresses oncoproteins E6 and E7 (Vandermark *et al.*, 2012). The high-risk E6 and E7 oncoproteins can degrade p53 proteins and inhibit the retinoblastoma protein (pRb), thus inhibiting the activation of apoptotic proteins such as caspases and escaping normal cell cycle control in G₁ (Jung *et al.*, 2013). The imbalance of proapoptotic and antiapoptotic proteins leads to sustained proliferation and resistance of apoptosis, leading to malignancy (Zhou *et al.*, 2015).

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Toxic side effects are a common issue of conventional chemotherapy and radiotherapy, which could reduce patients' quality of life (Marjanovic *et al.*, 2019). Antineoplastic agents' poor selectivity results in a proliferation inhibitory effect, targeting the cancer cells and rapidly growing healthy cells, such as hair cells, epithelial cells, and hematopoietic precursors (Blowman *et al.*, 2018). The undesirable effects include loss of appetite, diarrhea, nausea and vomiting, reduced white blood cell count, and hair loss (Rodenak-Kladniew *et al.*, 2014). Molecular biology's current advances have introduced the development of new anticancer drugs with enhanced cancer-cell-killing function that cause less alteration to the normal cells (Sampath *et al.*, 2018).

Medicinal properties from plant-derived natural products provide a broad range of potential sources for drug discovery and cancer chemoprevention (Bayala *et al.*, 2014). Inexhaustible sources of natural chemical constituents, suitable chemical characteristics, and various biological and pharmacological activities have been attributed to phytochemical-based-therapeutic agents (Blowman *et al.*, 2018; Sampath *et al.*, 2018). Cancer therapy has widely employed phytochemicals with potential antitumor activities (Khazir *et al.*, 2014; Murata *et al.*, 2013). Previous research has shown essential oils (EOs) anticancer properties with various mechanisms of action. Three EOs are currently gaining research attraction due to their growing evidence of anticancer properties, namely clove oil, eucalyptus oil, and citronella oil (Blowman *et al.*, 2018). These EOs have plants of origin that are grown abundantly in tropical regions, including Indonesia, Vietnam, Thailand, Sri Lanka, India, and Malaysia in the Asiatic regions, and also Madagascar and Tanzania in African regions (Cortés-Rojas *et al.*, 2014; Kaur *et al.*, 2021; My *et al.*, 2020).

Eugenol (4-allyl-2-methoxyphenol-2-methoxy-4-prop-2-enyl phenol) is an aromatic phenylpropanoid phenol which is the major bioactive constituent in clove (*Syzygium aromaticum*) (Bezerra *et al.*, 2017). While well known for its culinary use for its flavor and aroma, eugenol has been documented to have medicinal properties, such as analgesic, anesthetic, antiseptic, antiviral, antimicrobial, antioxidant, anti-inflammatory, and anticancer activities (Barboza *et al.*, 2018; Batiha *et al.*, 2020). A recent study reveals eugenol cytotoxicity against cervical carcinoma (HeLa cell lines) that can be explored in advance as an antineoplastic agent (Das *et al.*, 2018; Permatasari *et al.*, 2021).

1,8-Cineole (1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane), which is also known as eucalyptol, is a cyclic ether monoterpene as a major constituent of eucalyptus EO. Traditional usages of eucalyptol are food flavoring agents, airway diseases' symptom relievers, and aromatherapy (Rodenak-Kladniew *et al.*, 2014). Eucalyptol is known to have diverse biological and pharmacological applications, such as antibacterial, antifungal, anti-inflammatory, antioxidant, and hypolipidemic (Rodenak-Kladniew *et al.*, 2020). Recently, 1,8-cineole has been shown to possess anticancer properties (Sampath *et al.*, 2018).

Another EO with potential as an anticancer agent is citronella oil. *Cymbopogon nardus* or citronella oil's main components are citronellal (3,7-dimethyl-6-octenal) and geraniol ((E)-3,7-dimethyl-2,6-octadien-1-ol), monoterpene aldehydes, and monoterpene alcohols (Bayala *et al.*, 2020). Previously, citronella oil was particularly known for its antioxidant, anti-inflammatory, and antimicrobial properties (De Toledo *et al.*, 2016; Kačániová

et al., 2017; Pontes *et al.*, 2019). Recent studies have shown that citronella oil exhibits antiproliferative properties against cancer cells (HeLa, LNCaP, and MDA-MB-231 cell lines) *in vitro*, which are thought to be derived from the activity of citronellal and geraniol (Bayala *et al.*, 2020; Ho *et al.*, 2020; Kim *et al.*, 2012; Zhang *et al.*, 2018).

Resisting apoptosis, sustaining the proliferation cycle, and evading cellular arrest are some of the cancer hallmarks (Hanahan and Weinberg, 2011). Therefore, the search for anticancer therapeutics is focused on promoting cancer cells' apoptosis and induced cellular arrest. This research aimed to evaluate antitumor activities of three EOs, i.e., *S. aromaticum*, *Melaleuca cajuputi*, and *C. nardus*, toward cervical carcinoma (HeLa cell line) viability.

MATERIALS AND METHODS

HeLa cell culture preparation

HeLa (Henrietta Lacks) human cervical carcinoma cell lines were obtained from the American Type Culture Collection (ATCC) and stored in the Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya (Malang, East Java, Indonesia). Cells were then incubated at 5% CO₂ at 37°C, pH 7.2–7.4. The culture was made in Dulbecco's modified Eagle's medium (Invitrogen, MA, USA) with 10% (v/v) fetal bovine serum and antibiotics (100 IU/ml penicillin, 100 µl/ml streptomycin) (Das *et al.*, 2018). HeLa cells were grown and routinely harvested with a trypsin-ethylenediaminetetraacetic acid (EDTA) [CH₂N(CH₂CO₂H)₂]₂ solution (Permatasari *et al.*, 2021).

EO preparation

Syzygium aromaticum, *M. cajuputi*, and *C. nardus* plant samples were obtained from PT, Jeeva Aroma Nusantara Institute (Surabaya, East Java, Indonesia), in July 2021. The aerial parts of the samples were grounded and subjected to Soxhlet extraction. EOs were extracted in a Soxhlet by diluting 10 g of each grounded plant with a solvent of 150 ml *n*-hexane at 150°C (Butt *et al.*, 2019). The extraction process was continued until the mixture returned to its original solvent color. EOs were collected, and the solvent was removed by a Soxhlet extractor at 150°C.

Cell viability assay

HeLa cells were seeded in 24-well plates and treated with different concentrations of each EO from three plants (*S. aromaticum*, *M. cajuputi*, and *C. nardus*) at doses of 0, 30, 60, and 120 µg/ml. The 0 µg/ml dose served as a negative control for each group. Cells were incubated at 37°C for 24 hours treatment in RPMI-1640. The concentration determination was based on the previous research, with some modifications. The current research used different delivery methods, of which the EOs were encapsulated (Permatasari *et al.*, 2021).

Trypan blue exclusion assay

Cytotoxic activity of the EOs was examined by the trypan blue exclusion test (Sinha *et al.*, 2011). The trypan blue assay aimed to estimate the number of viable and dead cells within a population. After 24 hours of treatment, the cell culture medium was discarded from each plate. Trypsinization was performed by administering 250 µl of trypsin-EDTA 0.25% for 10 minutes. Cells were then suspended, 20 µl of which was taken. Subsequently,

cells were stained with 250 μ l 0.1% trypan blue dye. Next, 10 μ l of cell suspension was subjected to a hemocytometer for cell quantification and observed under a microscope to calculate its viability. Viable cells did not get stained nor emit fluorescence under a light microscope. Conversely, dead cells were stained dark blue. Cell calculations were carried out at four repetitions of each sample to obtain the average value. The half-maximal inhibitory concentration (IC₅₀) was derived from the cell calculation (Gupta *et al.*, 2019; Muthukrishnan *et al.*, 2018).

Cell apoptosis assay

The number of cells that undergo apoptosis induced by *S. aromaticum*, *M. cajuputi*, and *C. nardus* EOs was measured by the flow cytometry method using Annexin V (AV)–fluorescein isothiocyanate (FITC)–propidium iodide (PI) staining (Sigma). First, the cells were washed twice with a cold cell staining buffer and then resuspended in an AV binding buffer at a concentration of 0.25–1.0 \times 10⁷ cells/ml. After that, 100 μ l of cell suspension was transferred to a 5 ml test tube, and a mixture with a concentration of 20 μ g/ml was made by adding 5 μ l of FITC AV and 10 μ l of PI solution. The cells were then vortexed slowly and incubated for 15 minutes at room temperature (25°C) in the dark. Finally, 400 μ l of AV binding buffer was added to each tube and then analyzed with a flow cytometer according to the standard machine settings. The data were analyzed using the Cell Pro-Quest software on a computer connected to a flow cytometer to measure the percentage of apoptosis in the HeLa cells (Crowley *et al.*, 2016; Rieger *et al.*, 2011).

Statistical analysis

One-way analysis of variance (ANOVA) test, followed by *post-hoc* Tukey's multiple comparison test, was adopted for the statistical evaluation of the result's data. Cytotoxic activity was presented as a percentage and calculated using the following formula of Sithole and Mukanganyama (2017):

$$\text{Viable cell (\%)} = \frac{(n) \text{ viable cells treated with EOs}}{(n) \text{ viable cells in negative control}} \times 100.$$

To evaluate the correlation of EO doses and HeLa cells' viability, Pearson's correlation test was also performed. Significant differences were established at $p \leq 0.05$. Statistical analysis and graphical representation were accomplished using GraphPad Prism 9 (GraphPad Software, San Diego, USA).

RESULTS

Cytotoxic activity in a dose-dependent pattern

To investigate the cytotoxic ability of three EOs from *S. aromaticum*, *M. cajuputi*, and *C. nardus* against human cervical cancer cells, HeLa cells received treatment of three different EOs for 24 hours at doses of 30, 60, and 120 μ g/ml, with the negative control of that of 0 μ g/ml. Following 24 hours of treatment, a trypan blue exclusion test was performed to determine the viability of the HeLa cell line. Figure 1 shows the microscopic view of the aftertreatment HeLa cell line. The viability of the HeLa cell lines decreased after treatment with the three EOs following a dose-dependent pattern, shown by the increasing clear zone. Three EOs showed potent antiproliferative activity, as depicted by a significant decline in HeLa cells' viability at doses of 30, 60, and 120 μ g/ml, at 24 and 48 hours after treatment compared

to the control (p -value < 0.0001). At the maximum dose of 120 μ g/ml, the administration of all three EOs was able not only to inhibit proliferation but also to reduce the HeLa cells number into a minimum amount, of which almost no cancer cells were viable at the end of 48 hours. In brief, all three EOs caused a significant reduction in the number of viable HeLa cell lines, indicating their potent cytotoxic effects. Figure 2 shows the cytotoxic activity of the three plant EOs.

Comparison of all three EOs' efficacy to reduce HeLa cells' viability

The efficacies of the three EOs were compared at doses of 30, 60, and 120 μ g/ml at 24 and 48 hours after treatment. Figure 3 shows the comparison of treatment using three EOs toward viable cells number after 24 hours at a similar dose. At the lowest administration dose of 30 μ g/ml, the three EOs had an insignificant difference in viable cell number ($p = 0.4468$). Meanwhile, when the cells were treated with a dose of 60 μ g/ml, *S. aromaticum* showed the highest efficacy, while *M. cajuputi* had the lowest. At the maximum dose of 120 μ g/ml, the EOs from *S. aromaticum* also displayed the highest cytotoxic activity, as demonstrated by the least viable cell contrast to that of the other plants.

At 48 hours after treatment, *S. aromaticum* at a dose of 30 μ g/ml also showed the highest efficacy, while the efficacy of *M. cajuputi* was similar to that of *C. nardus* (Fig. 4). At doses of 60 and 120 μ g/ml, *S. aromaticum* was still the highest and comparable to *M. cajuputi*, while *C. nardus* still had the lowest efficacy. We can see that the *S. aromaticum* EO demonstrated the most potent efficacy at higher doses of 60 and 120 μ g/ml when compared to the other two EO sources.

IC₅₀ determination

The half-maximal IC₅₀ was extracted to measure the efficacy of the plants' EOs as an anticancer agent to induce 50% population death of HeLa cells. Table 1 summarizes the IC₅₀ values of the three Eos' extract (*S. aromaticum*, *M. cajuputi*, and *C. nardus*) on the HeLa cervical cancer cell line. All three EOs showed potent activity in inhibiting the proliferation of cancer cells. The *S. aromaticum* extract yielded lower IC₅₀ values after 48 hours incubation, which represented a more potent inhibitory effect after longer treatment. In addition, *S. aromaticum* EO was most potent in inducing cell death, as it exhibited the lowest 48 hours IC₅₀ among the others. On the other hand, treatment with *M. cajuputi* and *C. nardus* suppressed the HeLa cell line with lower IC₅₀ values at 24 hours in comparison to after 48 hours of incubation. The IC₅₀ values of all three EOs were below the lowest dose used in the current research of 30 μ g/ml. Therefore, future research should be performed at lower than 30 μ g/ml to examine the optimized dose of each EO.

EOs' proapoptotic activity

The proapoptotic activity measurement of the three EOs was examined using flow cytometry with AV and PI following the administration of four doses EOs (30, 60, and 120 μ g/ml and 0 μ g/ml as the control). Cells that failed to express both AV and PI (AV–/PI–) were regarded as healthy cells. Cells only expressing AV but not PI (AV+/PI–) were measured as cells that underwent early apoptosis. On the contrast, cells expressing both AV and PI (AV+/PI+) were measured as cells that underwent late apoptosis or

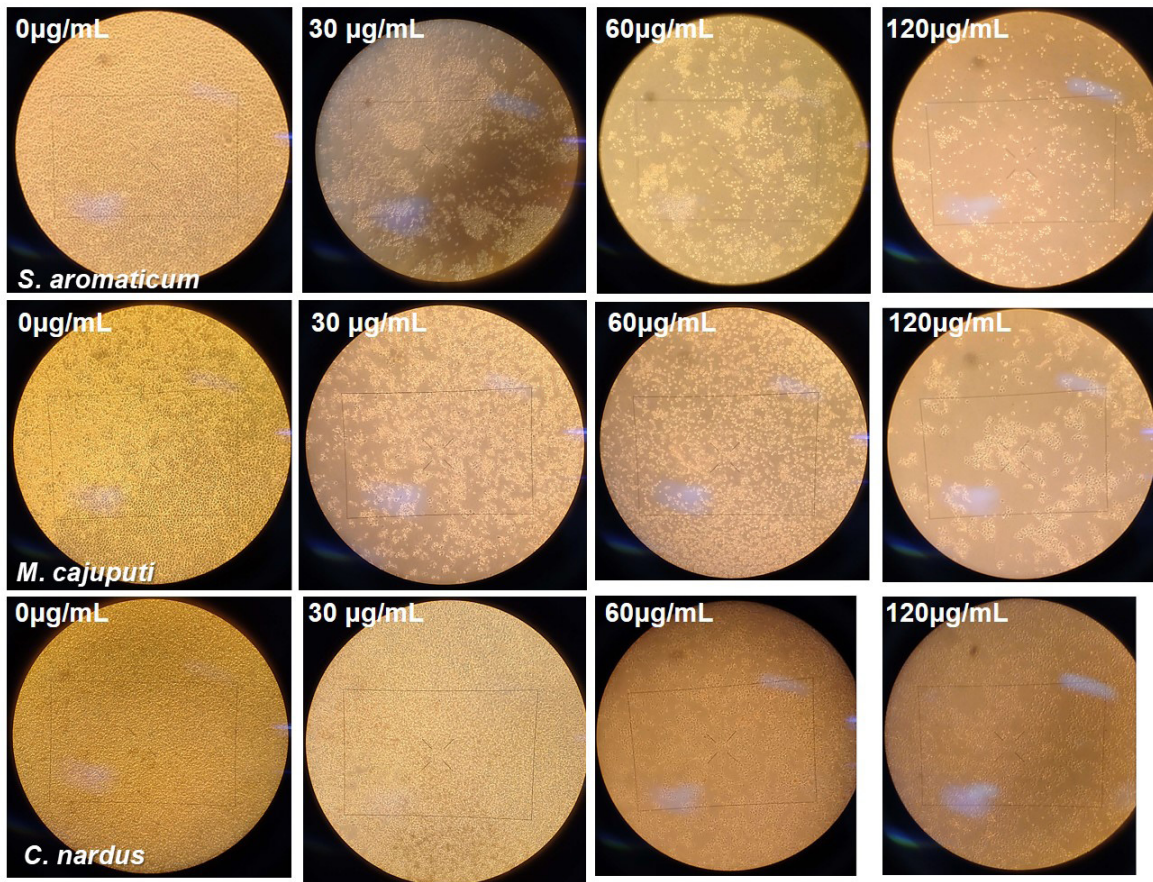


Figure 1. Microscopic feature of cytotoxic activity assessment from the treatment at 0, 30, 60, and 120 µg/ml of *S. aromaticum*, *M. cajuputi*, and *C. nardus* under a light microscope. Escalating concentration was associated with a reduced number of viable cervical cancer cells.

necrosis due to the three EOs. Table 2 presents the AV/PI analysis as a percentage of viable, early, and late apoptotic cells.

The AV/PI assay scatter plots are shown in Figure 5. The negative control displayed negative results for both Annexin and PI (AV⁻, PI⁻), which indicated that the cancer cells were still viable as they did not receive any treatments. Meanwhile, at the lowest dose of 30 µg/ml, the populations treated with EOs from *M. cajuputi* and *C. nardus* still dominated with viable cells: negative results for both probes (AV⁻/PI⁻) as of the negative control. Meanwhile, increasing dosage shifted the expression into positive results of both AV and PI. Cells treated with *S. aromaticum* EOs consistently demonstrated AV⁺/PI⁺. Here, we can see that EOs' treatment induced the necrotic cell stage of the HeLa cancer cells.

Syzygium aromaticum EO treatment significantly decreased viable cell population (AV⁻/PI⁻ HeLa cells) ($p < 0.0001$), with a noticeable increment in both early apoptotic (AV⁺/PI⁻) and late apoptotic (AV⁺/PI⁺) HeLa cells ($p < 0.0001$; two ANOVA tests) at a dose of 30 µg/ml compared to control. Afterward, the percentage of AV⁻/PI⁻ HeLa cells had a slight decrease trend although not significant at the 60 µg/ml dose, followed by a slight increase at the 120 µg/ml dose. Likewise, there was a decline in a dose-dependent relationship of the percentage of AV⁺/PI⁻ HeLa ($p < 0.01$; 30 µg/ml compared to 120 µg/ml). On the other hand, there was a rise in the late apoptotic cell percentage (AV⁺/PI⁺) reciprocal to the dose increment ($p < 0.001$), with the

exception of a slight increase between the doses of 60 µg/ml and 120 µg/ml (Fig. 6A).

A distinct trend can be inferred from the administration of *M. cajuputi* EO. The percentage of AV⁻/PI⁻ HeLa cells was diminished significantly in respect to the dose ($p < 0.0001$), with a special case of an insignificant decrease between the doses of 60 and 120 µg/ml. On the other hand, there was a positive trend in the percentage of AV⁺/PI⁻ and AV⁺/PI⁺ correspondingly to the dose (Fig. 6B).

Comparatively, *C. nardus* EO significantly decreased the number of viable HeLa cells (AV⁻/PI⁻) following dose escalations ($p < 0.05$, two-way ANOVA test). Dose increments of *C. nardus* EO increased the percentage of AV⁺/PI⁺ significantly ($p < 0.05$, two-way ANOVA test), while there was insignificance in the percentage of AV⁺/PI⁻ HeLa cells (Fig. 6C).

DISCUSSION

Cervical cancer progression was driven primarily by high-risk HPV infection, which expresses oncoproteins E6 and E7 (Ibeanu, 2011). The E6 and E7 oncoproteins have the ability to degenerate p53 proteins and hinder the expression of pRb, thus suppressing the activation of apoptotic proteins such as caspases and escaping normal cell cycle control in G₁ (Wang *et al.*, 2004).

Cancer therapy conventional modalities are surgery, radiotherapy, chemotherapy, and combinative treatment (Shahneh

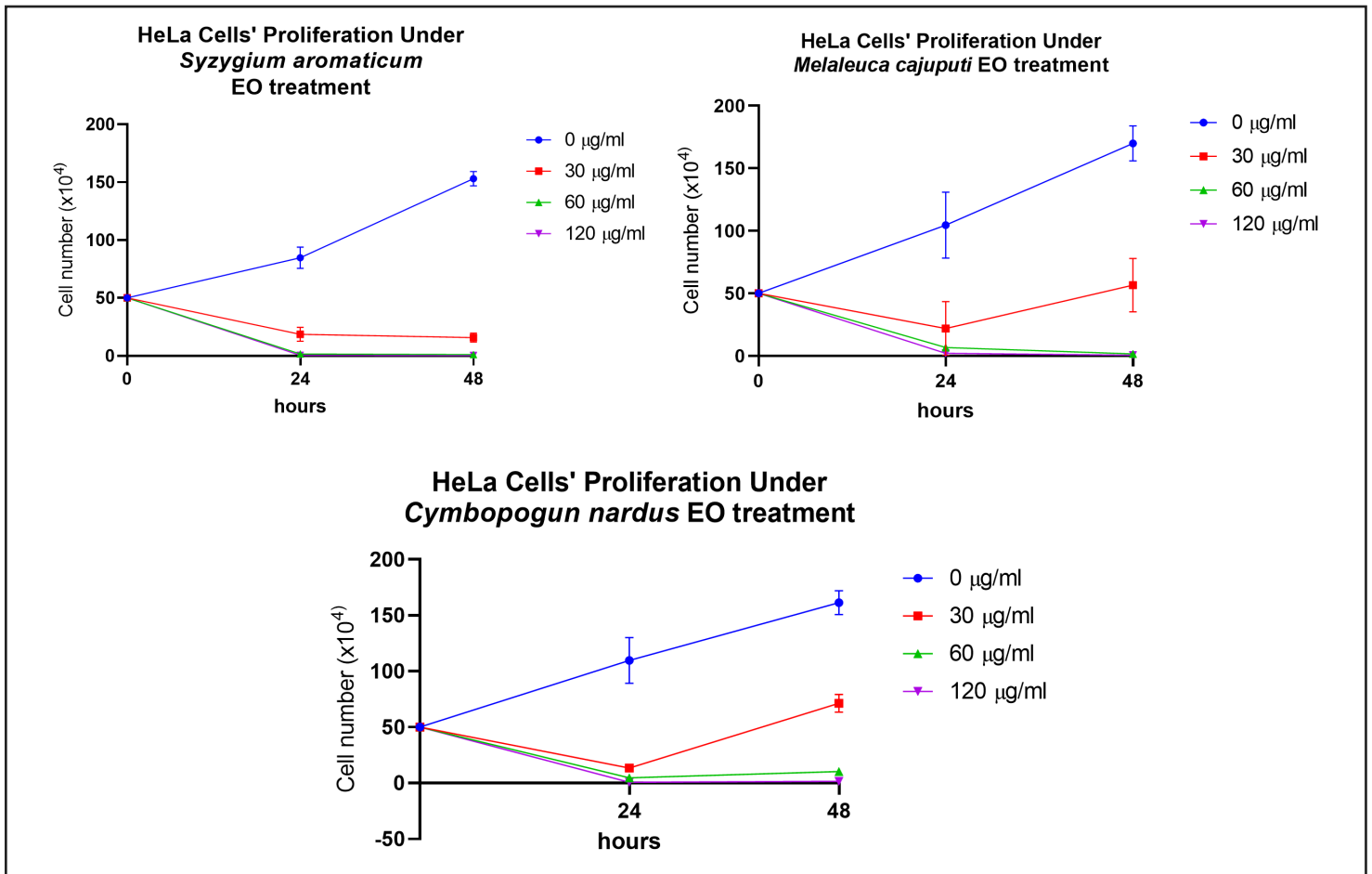


Figure 2. Comparison of the antiproliferative effects of A) *M. cajuputi*, B) *S. aromaticum*, and C) *C. nardus* EOs in the HeLa cell line at 0, 30, 60, and 120 µg/ml. Values are presented as the mean \pm SD from eight replications.

et al., 2013). Apoptosis, a programmed cell death mechanism, plays a role as the protective means against cancer development (Gong *et al.*, 2019). Nonetheless, cancer hallmarks include evasion and resistance to apoptosis (Fouad and Aanei, 2017). The imbalance of proapoptotic and antiapoptotic proteins leads to sustained proliferation and resistance of apoptosis, leading to malignancy (Zhou *et al.*, 2015). Apoptosis disruption due to oncogenic mutations leads to initiation and progression of tumors (tumorigenesis) and also drug resistance, which results in chemotherapy failure (Gong *et al.*, 2019; Shahneh *et al.*, 2013). Therefore, a promising approach in the development of cancer therapies is to induce apoptosis and interfere with the cell cycle (Sampath *et al.*, 2018).

EOs refer to secondary metabolites that plants generate to protect themselves against pests and predators and increase appeal to pollinators or promote seed dissemination. These oils can be obtained from numerous parts of plants, for instance, roots and aerial parts: stems, leaves, flowers, fruits, and seeds, according to their species (Mugao *et al.*, 2020). EOs have been known for their anticancer properties with a wide range of mechanisms, such as their role in cancer chemoprevention and the ability to interfere with established tumor cells and interact with the tumor microenvironment (Bayala *et al.*, 2014; Blowman *et al.*, 2018). Several plant-derived antineoplastic agents have been documented to cause cell cycle arrest and induce both intrinsic and extrinsic

apoptosis pathways. Moreover, cancer cell target specificity and less toxicity toward healthy cells have been observed in EOs application as a single agent (Blowman *et al.*, 2018).

This study established the potential anticancer activity of EOs from three plants of *S. aromaticum*, *M. cajuputi*, and *C. nardus* for human cervical cancer. The HeLa cervical cancer cell line was treated with one plant EO for 24 and 48 hours before being assessed for death and viable cells calculation. The cytotoxic activity, represented by the IC_{50} value, was examined at two time points, at 24 and 48 hours, since the EOs may exert different half-lives. In addition, the IC_{50} was further evaluated at 48 hours owing to the average doubling time of HeLa cells which is approximately in the range of 33–35 hours (Sato *et al.*, 2016). All EOs explored in this research manifested cytotoxic activity to suppress the proliferation and viability of cervical cancer cells, as depicted by the significant reduction of HeLa cell viability correspondingly to the EO dose augmentation. *Syzygium aromaticum* EO demonstrated a more potent inhibitory effect after a longer incubation period, and it showed the lowest IC_{50} in 48 hours treatment. The two other EOs displayed lower IC_{50} in 24 hours compared to 48 hours treatment. The EO from *C. nardus* exhibited the lowest IC_{50} after 24 hours incubation, showing the most potent activity compared to the other two.

The cell death phase, subsequent to the exposure to anticancer activity from the EOs, is portrayed by flow cytometry

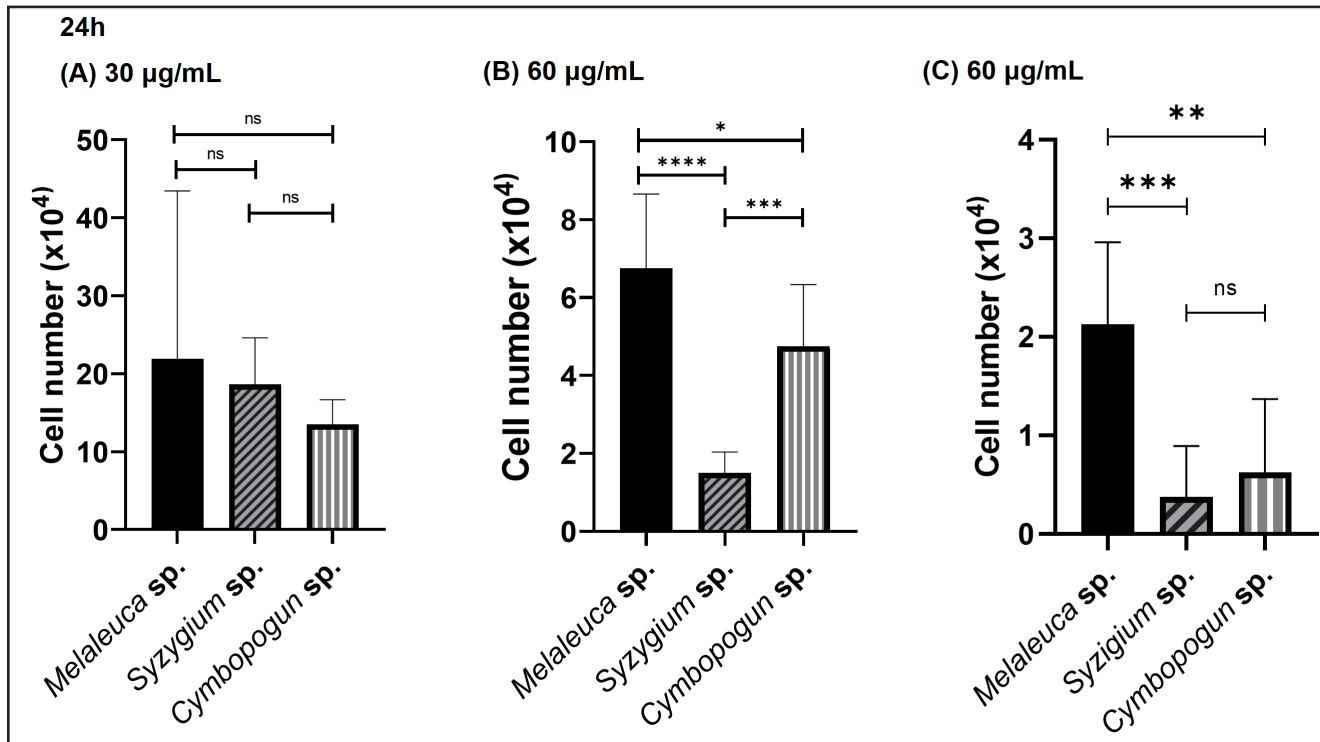


Figure 3. Comparison of cytotoxic effects of *S. aromaticum*, *M. cajuputi*, and *C. nardus* EOs at 24 hours after treatment against the HeLa cervical cancer cell line investigated by incubating HeLa cells with each extract in 12-well plates for 24 hours. (A) 30 µg/ml, (B) 60 µg/ml, and (C) 120 µg/ml. Values are presented as the mean ± SD from eight replications. Statistical one-way ANOVA and Tukey's multiple comparison test were carried out. ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

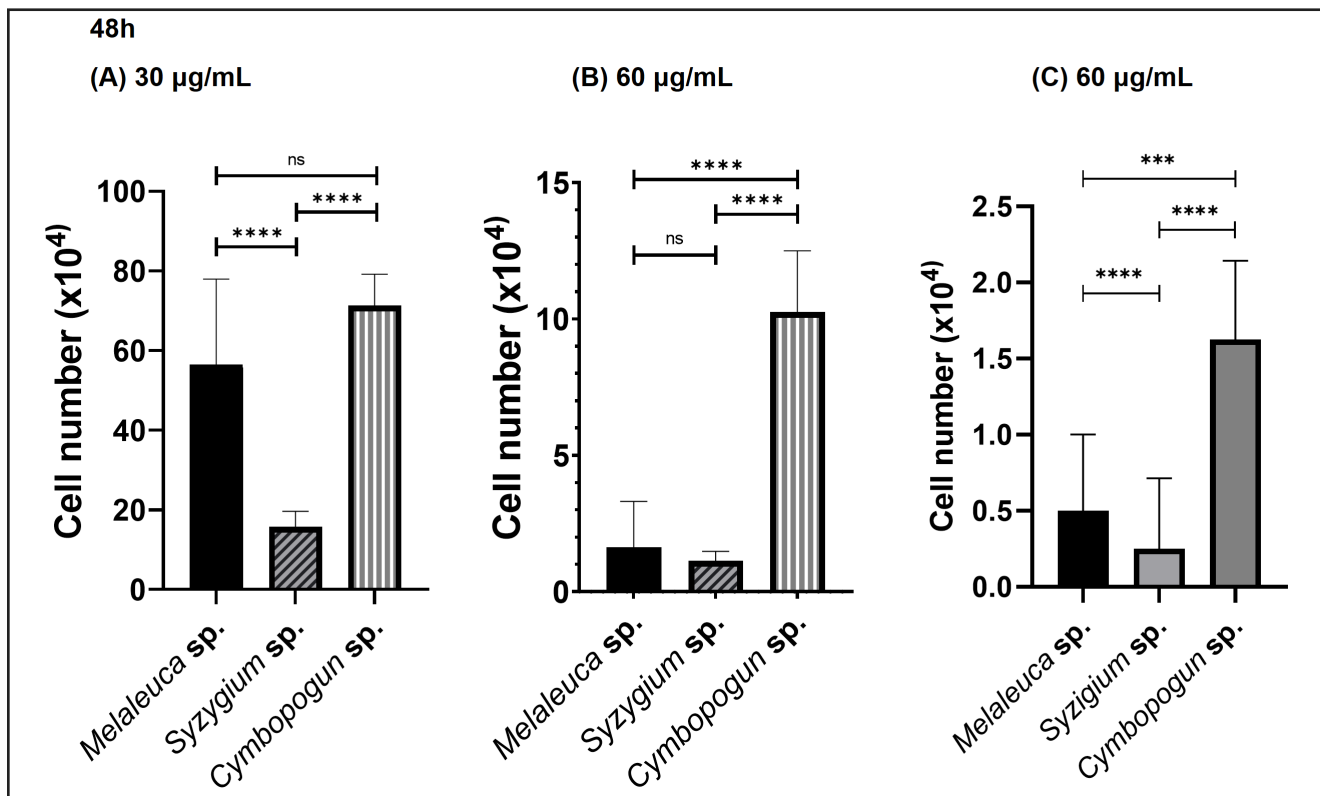


Figure 4. Comparison of cytotoxic effects of *S. aromaticum*, *M. cajuputi*, and *C. nardus* EOs at 48 hours after treatment against the HeLa cervical cancer cell line investigated by incubating HeLa cells with each extract in a 12-well plate for 48 hours. (A) 30 µg/ml, (B) 60 µg/ml, and (C) 120 µg/ml. Values are presented as the mean ± SD from eight replications. Statistical one-way ANOVA and Tukey's multiple comparison test were performed. ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Table 1. The EOs dose to cause half-maximal inhibitory effect (IC₅₀) on HeLa cervical cancer cell line in 24 and 48 hours.

EOs	IC ₅₀ (µg/ml)	
	24 hours	48 hours
<i>S. aromaticum</i>	22.33	18.06
<i>M. cajuputi</i>	25.89	26.25
<i>C. nardus</i>	11.80	28.03

analysis of AV/PI staining. Cells in the viable-, early-, and late-stage apoptotic states were identified using AV/PI. Membrane permeability affects PI's ability to enter a cell. Cells in the viable or in the early apoptotic state with the plasma membrane in an intact state prevent the PI staining penetration inside. On the contrary, a decrease in plasma and nuclear membrane integrity enables PI penetration into the nucleus and DNA staining of late apoptotic and necrotic cells (Chothihipirat *et al.*, 2019; Rieger *et al.*, 2011). During the late phase of apoptosis, such as secondary necrotic cells, there is an increase in cell permeabilization (Poon *et al.*, 2009).

There is an identical outcome of the administration of three EOs to the cervical cancer cells: exposure to EO treatment decreased the population of viable cervical cancer cells (AV-/PI-). In comparison to the control group, all HeLa cervical cancer cell cultures incubated with EOs underwent early- and late-stage apoptosis, as displayed by the increment of cells exhibiting AV+/PI- and AV+/PI+. Late apoptotic, necrotic cells, with double-positive AV/PI (AV+/PI+), dominate the population of all three EOs, and fewer cells appear in early apoptotic cells. The number of late-stage apoptotic cells increased following the increase in concentration exposure of *S. aromaticum*, *M. cajuputi*, and *C. nardus*. Therefore, the EOs demonstrated a potent activity in inducing late-stage apoptosis in a dose-dependent relationship of the cervical cancer cell. This study discovered the potent anticancer activity of the three EOs.

The exploration of anticancer treatment is currently concentrated on agents that induce cell apoptosis. Nevertheless, as tumor cells develop various strategies to evade apoptosis during tumorigenesis, chemotherapeutic development which enhances necrotic cell death has been appreciated (Yu *et al.*, 2020). Necroptosis is considered to be a novel necrotic cell death programmed form, which resembles the apoptosis mechanism and necrosis morphology. Necroptosis came up as a novel phase to be targeted in the search for anticancer therapy (Gong *et al.*, 2019). These research findings suggested the potential antineoplastic agent of EOs with late apoptotic/necrotic activity for cervical cancer.

Previous research revealed apoptosis-inducing activity against human cervical cancer of eugenol from *S. aromaticum* EO with the proposed mechanism from the expression upregulation of proteins related to cell cycle arrest and apoptosis such as caspase-3 and p53 (Permatasari *et al.*, 2019). Eugenol has demonstrated potent activity in altering the epithelial-mesenchymal transition pathway in metastasis development by repressing transcription factors and mesenchymal cell markers. At the same time, it also enhances the expression of epithelial cell markers of HeLa cervical cancer, which indicates a less invasive property (Permatasari *et al.*, 2021). In breast cancer, eugenol-treated cancer cells displayed a

Table 2. AV/PI staining flow cytometry results to evaluate the HeLa cells after treatment with EOs.

EOs	<i>S. aromaticum</i>			<i>M. cajuputi</i>			<i>C. nardus</i>			
	Control	30 µg/ml	60 µg/ml	120 µg/ml	30 µg/ml	60 µg/ml	120 µg/ml	30 µg/ml	60 µg/ml	120 µg/ml
% viable cells (AV-/PI-)	73.92	7.60	0.61	1.18	58.06	6.77	2.95	55.48	10.76	10.61
<i>p</i>		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0021	<0.0001	<0.0001
% early apoptotic cells (AV+/PI-)	1.45	22.72	19.23	10.48	3.14	10.74	14.96	2.81	13.17	7.52
<i>p</i>		<0.0001	<0.0001	0.0497	0.6743*	<0.0001	<0.0001	0.99*	0.0673*	0.5385*
% late apoptotic cells (AV+/PI+)	24.09	63.74	80.05	88.13	37.20	56.74	80.89	37.50	51.70	79.55
<i>p</i>		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.03	<0.0001	<0.0001

* Not significant.

p < 0.05 by using ANOVA multiple comparison test.

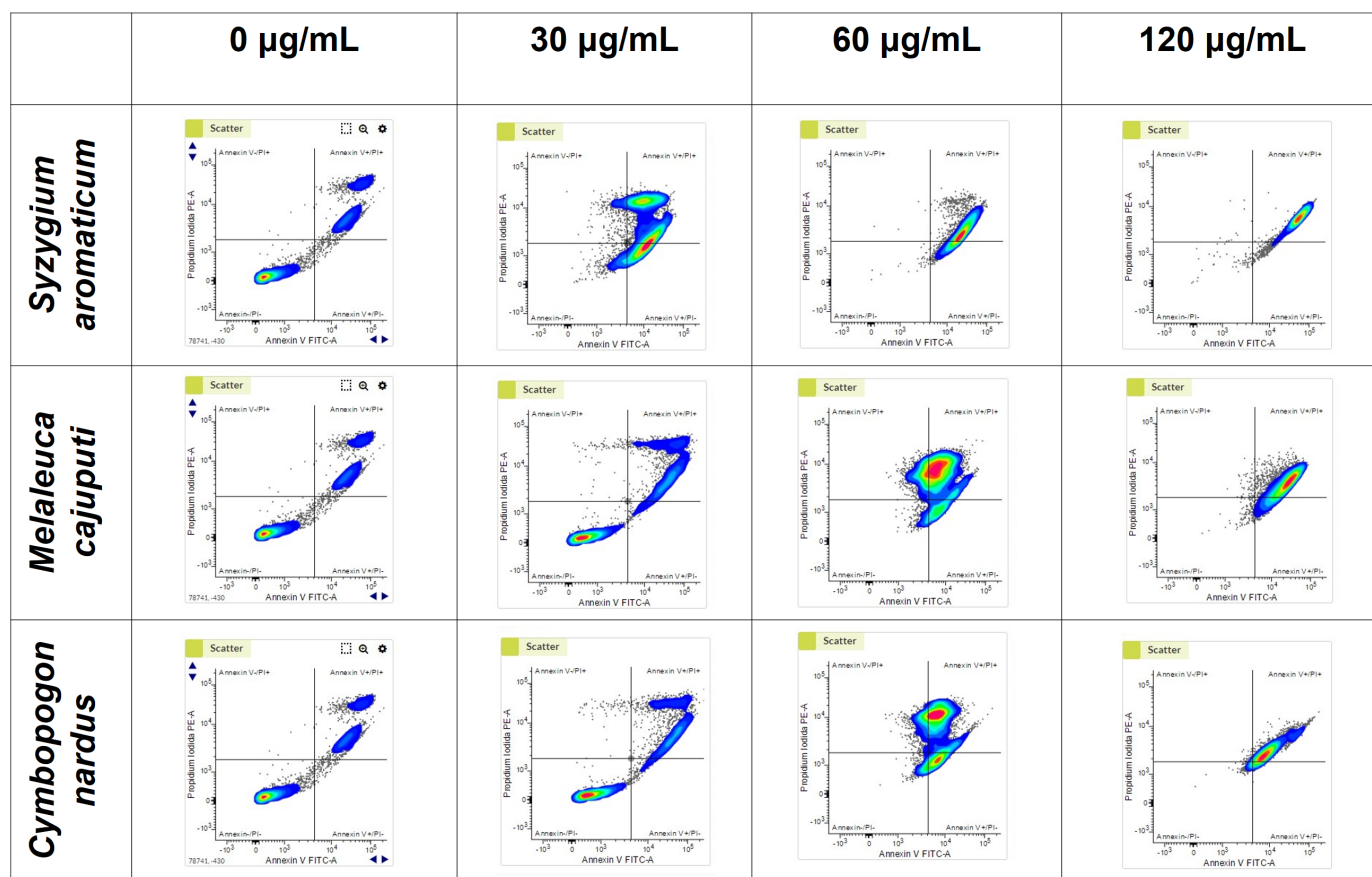


Figure 5. Flow cytometry scatter plots of AV/PI assays. HeLa cells were incubated separately with the EOs of *S. aromaticum*, *M. cajuputi*, and *C. nardus* at concentrations of 0, 30, 60, and 120 µg/ml.

greater expression of proteins involved in cell death apoptosis: caspase-3, caspase-7, and caspase-9. Therefore, eugenol can serve as a potent cytotoxic compound with the ability to trigger caspase-mediated cell death in breast cancer cells (Abdullah *et al.*, 2018). Moreover, eugenol, incorporated into the other active agents, displays a synergistic antitumor activity. Amarogentin and eugenol combined impeded cells from proliferating and forming colonies, while also inducing apoptosis. The antiproliferative mechanism was proposed through the means of downregulating the expression of cyclinD1 and upregulating cell cycle inhibitors (Pal *et al.*, 2018).

Anticancer activity of eucalyptol, the main component of *M. cajuputi*, has been observed in other types of malignancies. Eucalyptol induced apoptosis in human skin carcinoma (A431) cell lines by mitochondrial apoptotic proteins activation. Cells receiving eucalyptol treatment demonstrated a shift of expression of the mitochondrial pro- and antiapoptotic proteins in proportion to the dose, such as Bcl-2 and mitochondrial Cyt-C downregulation with upregulation of proapoptotic factors such as Bax, p53, caspase (Sampath *et al.*, 2018). Furthermore, eucalyptol also displayed a potent ability to suppress human colorectal cancer proliferation by means of inducing apoptosis. Administration of eucalyptol was linked with silencing survivin and Akt. It promotes the expression of p38, which subsequently leads to cleaved caspase-3 and ultimately causes apoptosis (Murata *et al.*, 2013). Eucalyptol also showed antineoplastic potential effects when

combined with other drugs. In combination with antisense drugs to treat hepatocellular cancer, eucalyptol induces G0/G1 arrest and apoptosis in HepG2 cells by oxidative stress and the other pathways. Eucalyptol also enhances the sensitivity of cancer cells toward antisense drugs (Rodenak-Kladniew *et al.*, 2020). Together with simvastatin, eucalyptol increased G0/G1 cell cycle arrest in human small cell lung cancer (Rodenak-Kladniew *et al.*, 2020).

Citronellal is the major constituent of *C. nardus* EOs. Citronellal and geraniol, monoterpene alcohol, and monoterpene aldehyde, respectively, were recognized for their antiproliferative activities (Bayala *et al.*, 2020). Citronellal demonstrates a potent ability to promote transient calcium signaling and activate human olfactory receptor OR1A2, which results in phosphorylation of p38 MAPK and hinders cell proliferation (Maßberg *et al.*, 2015). Conversely, geraniol is able to restrain AKT signaling and stimulate AMPK signaling, which consequently leads to mTOR inhibition, thus cell apoptosis and autophagy (Kim *et al.*, 2012). It also enhances the expression of Bax and suppresses Bcl-2 expression, causing DNA damage, and cell cycle arrest at the G2/M phase in cancer cell lines (Zhang *et al.*, 2018).

Study limitation

There are some aspects that need further attention in the application of EOs. Being highly volatile, EOs' application differs from a common plant crude extract. As a result, EOs need some

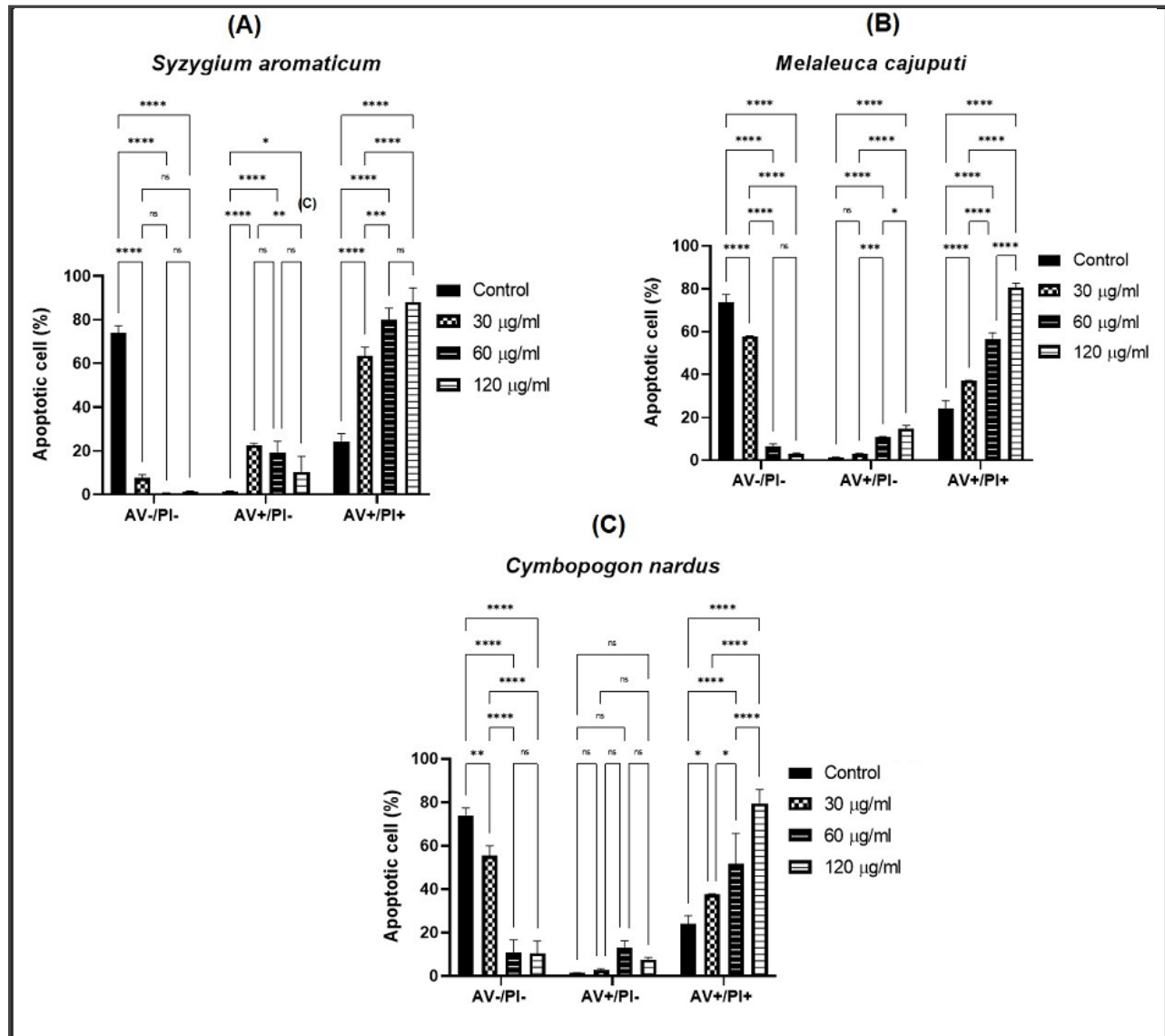


Figure 6. Flow cytometric analysis of AV/PI. EOs of (6A) *S. aromaticum*, (6B) *M. cajuputi*, and (6C) *C. nardus* induce apoptosis in the HeLa cervical cancer cell line. AV(-) PI(-), AV(+) PI(-), and AV(+) PI(+) cells served as viable cells, early apoptotic cells, and late apoptotic/necrotic cells, respectively.

modification regarding their drug delivery, such as developing an encapsulation method, to optimize their utilization in the targeted cells. This study made use of whole EOs rather than isolation of specific bioactive compounds from each EO. Exploration of the constituents of the volatile compounds from the EOs of each plant still has not been conducted. Isolating individual compounds is considered to be essential as they may possess distinct uses and medicinal properties. Therefore, the authors recommend the analysis of chemical composition and isolating specific compounds.

This experimental study explored the cytotoxic activity of the EOs on a small population of HeLa cells. Hence, the authors recommend continuing research on these three plant EOs in a larger HeLa cell population. It was also necessary to study the efficacy of the EOs in animal model studies to explore the pharmacology of their bioactive compounds.

In the instance of study significance, this study revealed the potential of the three aforementioned EOs to halt the proliferation and induce apoptosis of HeLa cell cultures. While

prior research on the other types of cancer has revealed some possible mechanisms for the cytotoxic activity of each EO, this study has not yet explored possible pathways and mechanisms in HeLa cells due to the absence of mechanism investigation and an *in vitro* adverse drug reaction diagnostic test. Further study is required to fill the gap. There is an inadequate exploration of factors contributing to the reduced viability of HeLa cells. For that reason, the toxic side-effect possibilities could not be assessed extensively. Upcoming research may focus on the evaluation of the expression of proteins linked with apoptosis and cell cycle arrest in the cervical cancer cell lines and exploration of any possible toxic side effects from treatment with EOs.

CONCLUSION

This study reveals the cytotoxic activity of EOs from three plants, *S. aromaticum*, *M. cajuputi*, and *C. nardus*, to induce death in the HeLa human cervical cancer cell line. EOs' treatment significantly decreased the number of viable HeLa cells, with a rise in cytotoxic activity respective to the dose increment. At 24 hours

of treatment, *C. nardus* EOs stand as the most potent EO, while *S. aromaticum* EOs are the most potent at 48 hours. Flow cytometry analysis indicated EOs treatment's positive effect in triggering late-stage apoptosis in cervical cancer cells proportionately with the concentration. These findings suggest these EOs have potential anticancer properties that could be further explored as a combination with antineoplastic drugs for cervical cancer therapy.

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CONFLICTS OF INTEREST

The authors disclosed no conflicts of interest.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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DATA AVAILABILITY

All data generated and analyzed are included within this research article.

AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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