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EUGENOL-LOADED CHITOSAN NANOPARTICLE INDUCES APOPTOSIS, INHIBITS CELL MIGRATION AND EPITHELIAL TO MESENCHYMAL TRANSITIONPROCESS IN HUMAN CERVICAL CANCER *CELL LINE* HELA CELLS.

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Abstract

Eugenol is a phenylpropanoid group compound found in cloves, nutmeg, cinnamon, and bay leaves. Apart from being used as a cosmetic, perfume, and food ingredient, eugenol is known to have an antioxidant, antibacterial, anti-inflammatory, and anti-cancer profile. Eugenol has therapeutic potential by increasing reactive oxygen species formation, decreasing anti-apoptotic protein Bcl-2, increasing the release of cytochrome c that leads to apoptosis in cancer cells, and inhibit the epithelial to mesenchymal transition (EMT) process that could reduce the cell ability to migrating.

We synthesized eugenol loaded chitosan nanoparticles (Nano-EU) by ionic gelation method to overcome its shortcoming which is volatile and to increase its bioavailability. The nanoparticles were characterized by using Dynamic Light Scattering (DLS). Anticancer activity of Nano-EU was investigatedin cervical cancer HeLa cell line by flow cytometry using Annexin-V/PI staining, and by measuring cleaved-caspase-3 protein expression which is the executor of the apoptosis process by immunofluorescence.

The results of the study evidenced that Nano-EU inducing apoptosis and increasing activated caspase-3 expression in HeLa cells. Nano-EU could also inhibit cell migration by reducing vimentin and Snail as mesenchymal markers leading to inhibition of the EMT

process. Further research is still needed to investigate the anticancer potential of Nano-EU in HeLa cells to in vivo and clinical studies.

Keywords: Eugenol, Nanoparticles, Chitosan, Apoptosis, EMT, HeLa cells

Introduction

Cervical cancer is a disease that arises due to abnormal cell growth in the cervical area of female organs[1]. Cervical cancer is the most common cancer after breast cancer in women worldwide and is one of the leading causes of cancer death in developing countries[2]. In 2012, a total of 528,000 women in the world were diagnosed with cervical cancer, and an estimated 266,000 women die from cervical cancer each year[3]. The percentage of cervical cancer cases is 4% of all cancers diagnosed worldwide, with about 84% of cervical cancer cases in developing countries[4]. In Southeast Asia, Indonesia ranks fourth with cervical cancer after Cambodia, Myanmar, and Thailand[5]. In Indonesia alone, based on the Indonesian Ministry of Health, a total of 98,692 women developed cervical cancer in 2013 and estimated that there are 100 new cases for every 100,000 population per year[6].

Ninety-five percent of cervical cancer cases are caused by persistent infection by the carcinogenic human papillomavirus (HPV)[7]. When infection occurs, viral genes will integrate with the genome of the host cell so that it changes normal cell function and promotes the replication of viral particles and transformation of malignancy[8]. The two main oncoproteins belonging to HPV are E6 and E7, E6 can combine with the cellular protein ubiquitin-protein ligase E3A (UBE3A) to initiate degradation of the tumor suppressor gene p53. This degradation will lead to reduced apoptosis mediated by genes through caspase activation and termination of the p21 gene-mediated cell cycle[9]. The molecular process through inhibition of apoptosis creates an imbalance between the proliferation and apoptosis processes so that cancer cells continue to grow indefinitely. If it is not immediately protected, cervical cancer will continue to proliferate and be able to attack distant organs through the metastasis process[10].

The oncoproteins E6 and E7 will reduce p53 protein and influence the endothelial growth factor (EGF) signaling. Cancer cells are easily stimulated by EGF because there is an upregulation of EGFR. In this circumstances,Snail transcriptional factor will be activated. Snail transcriptional factor will activate the cell epithelial to mesenchymal transition (EMT) program leading to cell transformation, from the epithelial cell to mesenchymal cell. The mesenchymal marker such as vimentin, an intermediate filament, will give the cancer cell an ability to migrate and invade other organs leading to metastasis[11]. If metastasis has occurred, curative therapy cannot be done, and only palliative therapy is performed Therefore, a therapeutic agent that can provide cytotoxicity effects through selective induction of apoptosis is needed to prevent and treat cancer[12].

One of the compounds that have anti-cancer properties and are easy to obtain is the eugenol compound (4-allyl-2-methoxy-phenol), which is an active compound in the clove plant, apart from being found in basil, nutmeg, cinnamon, and bay leaves[13]. Apart from its use in cosmetic and food products, eugenol is also traditionally used as an antiseptic, analgesic, and antibacterial agent. Eugenol is proven to have antioxidant, antimutagenic, anti-inflammatory, and anti-cancer properties[11,14]. This suggests that eugenol can be a candidate to be developed as an alternative therapy for cervical cancer.The use of eugenol as a drug will

certainly go through various processes in the body before it can reach target cells so that it will affect the effectiveness of this drug. The pharmacodynamic and pharmacokinetic properties of drugs can be improved, one of which is the nanoparticle system[15]. Nanoparticles are a drug delivery system that makes it possible to enter medicinal compounds into them[16]. Since eugenol as a plant-derived bioactive compound is highly volatile and susceptible to the oxidation process, its effectiveness will decrease before eugenol reaches the target cells. So, we need a method to increase its availability in the body. Nanoparticles are particles with sizes in the range of 1 to 1000 nanometers, so that this size facilitates the administration of drugs and provides the ability to penetrate barriers in the body[17-18]. Encapsulation of eugenol into nanoparticles canenhance its stability, protect against oxidation, reduce toxic side effects, increase the water solubility of hydrophobic materials, increase efficacy, controllable release, and increase the bioavailability[15].

One of the good materials used for nanoparticles is chitosan, a natural polysaccharide compound which is mainly obtained from chitin in the cuticles of arthropods, the endoskeleton of cephalopods, and the cell walls of fungi[19]. Chitosan is currently being developed for encapsulation / as a carrier for bioactive compounds because of its biocompatibility, non-toxic and biodegradable properties[20]. Studies show that chitosan can trap protein and peptide drugs, then protect it from hydrolysis by proteolytic enzymes so that it can last longer in the body[21]. Also, the manufacture of chitosan nanoparticles has little effect on the bioactive substances that are included in it[22]. Through the process of eugenol encapsulation in chitosan nanoparticles, it is expected that its bioavailability, cytocompatibility, thermal stability, and anticancer potential can be increased[19]. Therefore, initial research is needed regarding the benefits of eugenol encapsulated by chitosan as an alternative method of treating cervical cancer (in this case in HeLa cervical cancer cell culture) on the apoptosis and EMT process so that an effective and efficient drug can be developed to treat cervical cancer cells.

Materials and Methods

2.1. Ethical approval

The current study was approved by Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Brawijaya, Indonesia (approval number 173/EC/KEPK/10/2020).

2.1. Materials

Eugenol for synthesis (99% purity), Tween 60 was purchased from Merck Chemical Company (Germany), chitosan (50,000-190,000 Da, Sigma-Aldrich, USA), dimethyl sulfoxide (DMSO) (78.13 g/mol, Sigma-Aldrich, USA), penicillin and streptomycin (Gibco, USA), DAPI, sodium tripolyphosphate (TPP), glacial acetic acid (100%) were procured from Sigma-Aldrich (St Louis, MO, USA), Fetal Bovine Serum (FBS) was purchased from Himedia Laboratories (Mumbai, India), Dulbecco's modified eagle medium and trypsin-ethylenediaminetetraacetic acid solution (Gibco, USA). Triton® X-100 (pro GC-Merck), 1:100 rabbit polyclonal anti-cleaved caspase-3 antibody (ab2302, Abcam),1:100 mouse polyclonal anti-Snail antibody (ab167609, Abcam), 1:100 rabbit polyclonal anti-vimentin

antibody (ab137321, Abcam), phosphate buffered saline, RIPA buffer, NaCl, paraformaldehyde (PFA) 4%, distilled water were supplied from Biomedical Department and Biochemistry Department, Medical Faculty of Universitas Brawijaya, Indonesia. Annexin V-FITC apoptosis detection kit with propidium iodide (Biolegend Inc., USA). Cervical cancer cell line (HeLa) were purchased from *American Type Culture Collection* (ATCC) which was then cultured in Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya (Malang, Indonesia).

2.2. Preparation of chitosan nanoparticles

Eugenol-loaded chitosan nanoparticles were prepared according to Woranuch and Yoksan by a two step method, i.e. oil-in-water (o/w) emulsion and ionic gelation of chitosan with TPP. Chitosan solution (1.2% w/v) was prepared by agitating chitosan in acetic acid solution (1% v/v) overnight. Tween 60 was added to the chitosan solution (40 mL), and the mixture stirred at 50^{o}C for 30 min. Eugenol was gradually dropped into the stirred mixture, and agitated for 20 min. TPP solution with 0.5% w/v (40 mL) then dropped into an o/w emulsion slowly while stirring at ambient temperature, and agitated for 30 min. The formed particles were collected by centrifugation at 5,000 rpm for 30 min at 25^{o}C. The obtained particles kept at 4 ^{o}C.

2.3. Particle size analysis

To measure the size of the nanoparticles, dynamic light scattering (DLS) method was performed using Delsa^{TM} Nano C (Beckman Coulter, USA).

2.4. Cell culture

HeLa cells were obtained from the Department of Biomedical (Faculty of Medicine, Universitas Brawijaya, Indonesia), were cultured in RPMI-1640 media, supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µl/ml streptomycin at 37°C in a 5% CO_2 incubator. HeLa cells are routinely grown and harvested with Trypsin-EDTA solution. Subconfluent cell cultures were used.

2.5. Measurement of Cell Migration with Scratch test

The cells are fixed on the object-glass before observed. Then, manually scratch it using a pipette tip p10. The eroded cells were then washed using 1ml of phosphate buffer saline (PBS). To obtain the same field of view during the shooting, a reference point is made using a permanent marker. The preparation is then placed on a phase-contrast microscope, and making part of the external reference point outside the field of view, the image is taken immediately after the streak is made. Cells then returned to the incubator at 37^{o}C, and the next picture will be taken in the next 24 hours[23].

2.6. Apoptosis detection using flow cytometry

Apoptosis induction by eugenol loaded nanoparticle was evaluated by double staining of annexin V-FITC and propidium iodide (PI) using Apoptosis Detection Kit with PI cat#640914 (Biolegend, USA) according to manufacturer instruction. After HeLa cells were

treated with 50, 100, 200 μM Nano-EU, and 200 μM eugenol (positive control), in DMEM medium containing 0.2% FBS in 24-well plates for 24 h, the cells were harvested, washed twice with cold PBS, and assayed for apoptosis by the double staining of annexin V-FITC and PI. Then, 5×10^5 cells were resuspended in a binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl, 2.5 mM $CaCl_2$), stained with 5 μl annexin V-FITC for 10 min, and then stained with 5 μl PI for 15 min. The cells were then immediately analyzed with a flow cytometer (FACScan; BD Biosciences, California, USA).

2.7. Immunofluorescence assay

Cells were seeded into 24-well plates (2×10^5 cells / well) overnight and incubated with different concentrations of Nano-EU at 37℃ for 24 h. Cells then washed with PBS two times, and fixed in 4% paraformaldehyde in PBS form 15 min at room temperature, then wash with PBS twice. After that, cells were permeabilized by incubating with 2 ml 0.1% Triton X-100 in PBS for 15 minutes on ice, then wash cells three times with PBS. Cells were blocked for 1 hr in a blocking buffer consisting of 10% goat serum, 2% BSA, 0.2% Triton-X. Primary antibodies (rabbit cleaved caspase-3 primary antibody 1:100, rabbit vimentin primary antibody 1:100, mouseSnail primary antibody 1:100) were diluted in blocking buffer and then incubated in the dark overnight at 4^oC. Whole mounts or sections were washed at least 5 times with PBS. Then, incubate sample with 1 μg/ml DAPI. Mount the sample by mounting medium. The analysis was performed on inverted fluorescence microscope Olympus IX71 in a dark room.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 8.0 (GraphPad Software Corporation, La Jolla, CA). ANOVA, Tukey's multiple comparison test was used to compare the differences between the control and drug dose groups. P value less than 0.05 is the effective significant difference.

Result

3.1. Size and polydispersity index of Nano-EU

In this study, eugenol is encapsulated into chitosan nanoparticles with an ionic gelation method using sodium tripolyphosphate as a cross-linking agent. The result of particle size analysis using DLS shows that the average size of Nano-EU is 250 nm with a polydispersity index of 0.312 (Table 1), in accordance with the criteria that nanoparticles are measuring less than 1000 nanometers[24]. This shows that eugenol is successfully modified into a nanoparticle. The DLS result also showed that Nano-EU has a polydispersity index of 0.312, where a polydispersity index value close to zero indicates a homogeneous size dispersion[25]. This shows that our synthesized Nano-EU has high homogeneity.

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Table 1.
Size and polydispersity index of Nano-EU

Sample	Average Size (nm)	Polydispersity Index
Nano-EU	250	0.312

3.1. Nano-EU inhibited cell viability

After the nanoparticles had been formed, the HeLa cells were revived, which were placed in medium and incubated overnight at 37°C and 5% CO2. After the cells had become sub-confluent, i.e., 70-80% confluence (Fig. 1), we analyzed them using both qualitative and quantitative assays. For the qualitative assay, we observed the morphological changes of the HeLa cells after 48-hour treatment with eugenol and Nano-EU. Normal HeLa cells had a regular polygonal shape that appeared homogenous, and there were also some short antennae with a few round cells (Fig. 1A). However, after eugenol and Nano-EU treatment, the cells shrank and mainly appeared as round cells. Moreover, the nuclei appeared different, indicating the possibility of the occurrence of apoptosis. Apart from that, the HeLa cells population decreased dose-dependently following the Nano-EU therapy (Fig. 1D–G).

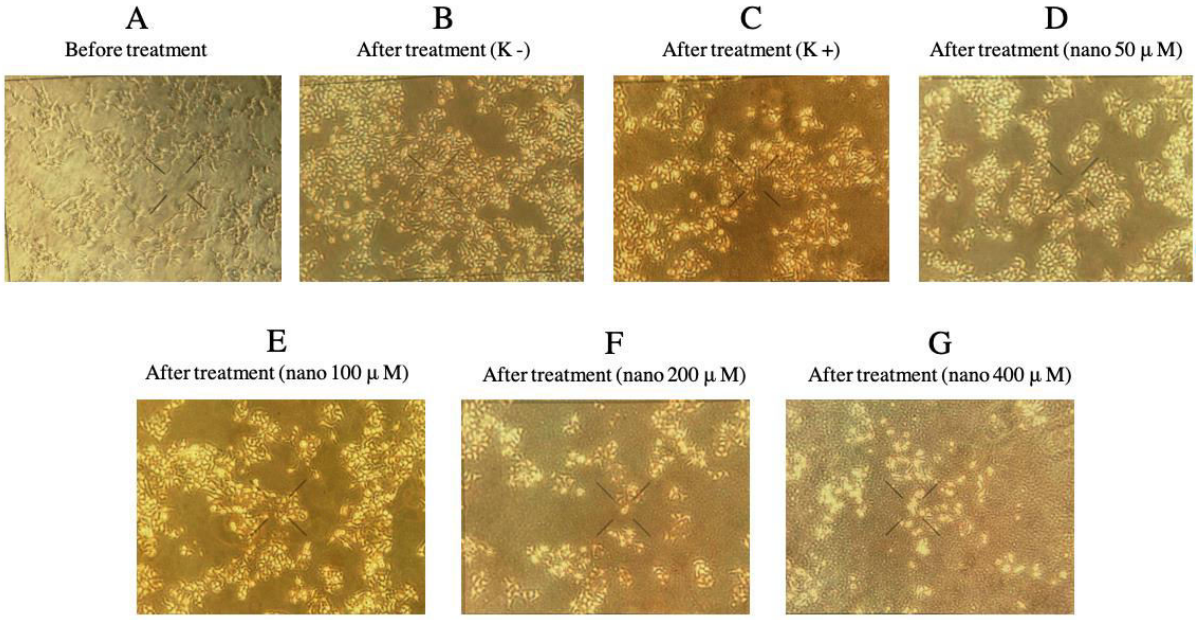

Fig. 1. HeLa cells showing morphological changes from homogeneous polygonal to decreased population size, followed by an increase in the number of circular cells (light microscopy with ×10 magnification). (A) Image shows pre-therapy cells. (B) The negative control at 48 hours. (C) to (G) Morphology of HeLa cells after 48-hour therapy with (C) 200 µM eugenol; (D) 50 µM Nano-EU; (E) 100 µM Nano-EU; (F) 200 µM Nano-EU; (G) 400 µM Nano-EU.

3.2. Nano-EU induced apoptosis in HeLa cell lines

To investigate whether HeLa cells apoptosis was affected by various concentrations of Nano-EU (0, 100, 200, and 400 M) for 24 h, Annexin-v/PI staining and flow cytometry was used to measure the number of apoptotic cells. We noticed that Nano-EU treatment

increased apoptosis rate significantly compared with the control (Fig. 2A, B) ($p < 0.001$). It shows that the mean percent of apoptosis increases following an increase in the concentration of Nano-EU. The HeLa cells that undergo apoptosis are elevated from 6.16% (control) to 56.2% after treated with 400 µM Nano-EU. At the 50 M Nano-EU, the mean percent apoptosis of HeLa cells was 11.09%, at the 100 µM dose was 12.75%, at the 200 µM dose was 24.28%, and 56.2% at the 400 µM. These results indicate that Nano-EU induces apoptosis in HeLa cells.

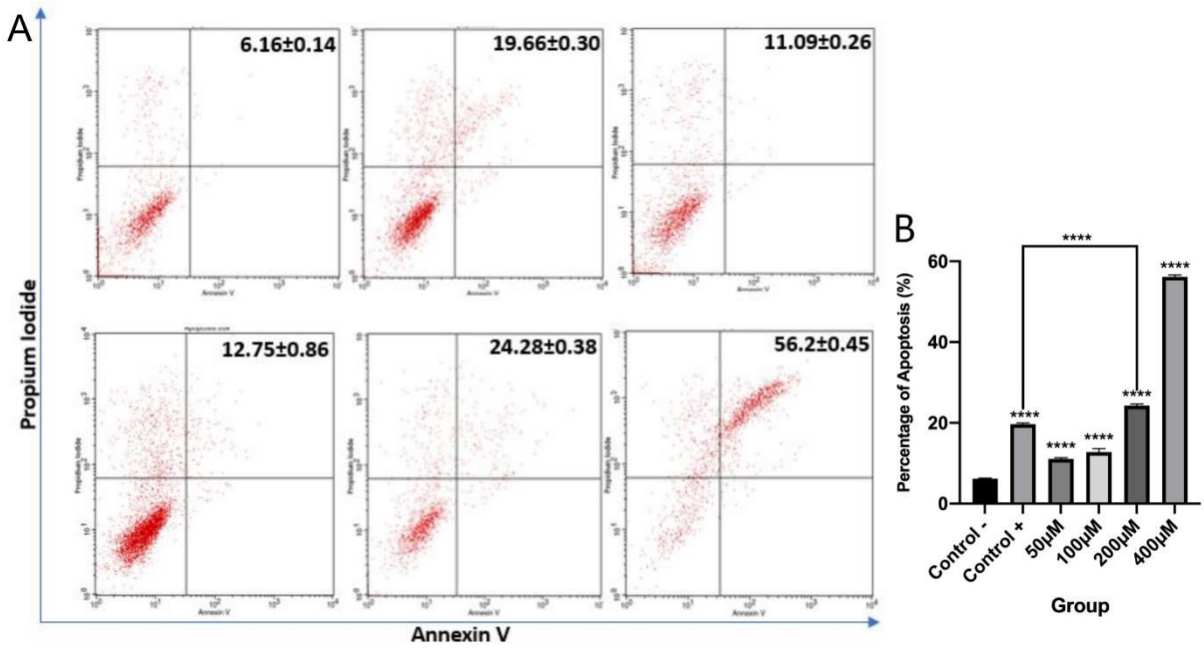

Fig. 2. Nano-EU promoted apoptosis in HeLa cell lines. **(A, B)** The apoptosis of HeLa cell lines were analyzed using flow cytometry after annexin-v/PI staining. Compared to the control group, *** $p < 0.001$, **** $p < 0.0001$ (ANOVA, Tukey's multiple comparison test).

3.3. Cleaved-caspase-3 expression in HeLa cells induced by Nano-EU

The cell signaling pathway of Nano-EU in activation of the apoptotic pathway was assessed after 24 h of treatment. In order to explore the role of executor caspases in the process of Nano-EU induced apoptosis, we examined the effect of Nano-EU on the activation of caspase-3. The expressions of cleaved caspases-3, was increased significantly in Nano-EU-treated HeLa cells compared to control through immunofluorescence analysis (Fig. 3A, B). The result suggest that Nano-EU significantly stimulated the activation of caspase-3 in HeLa cells.

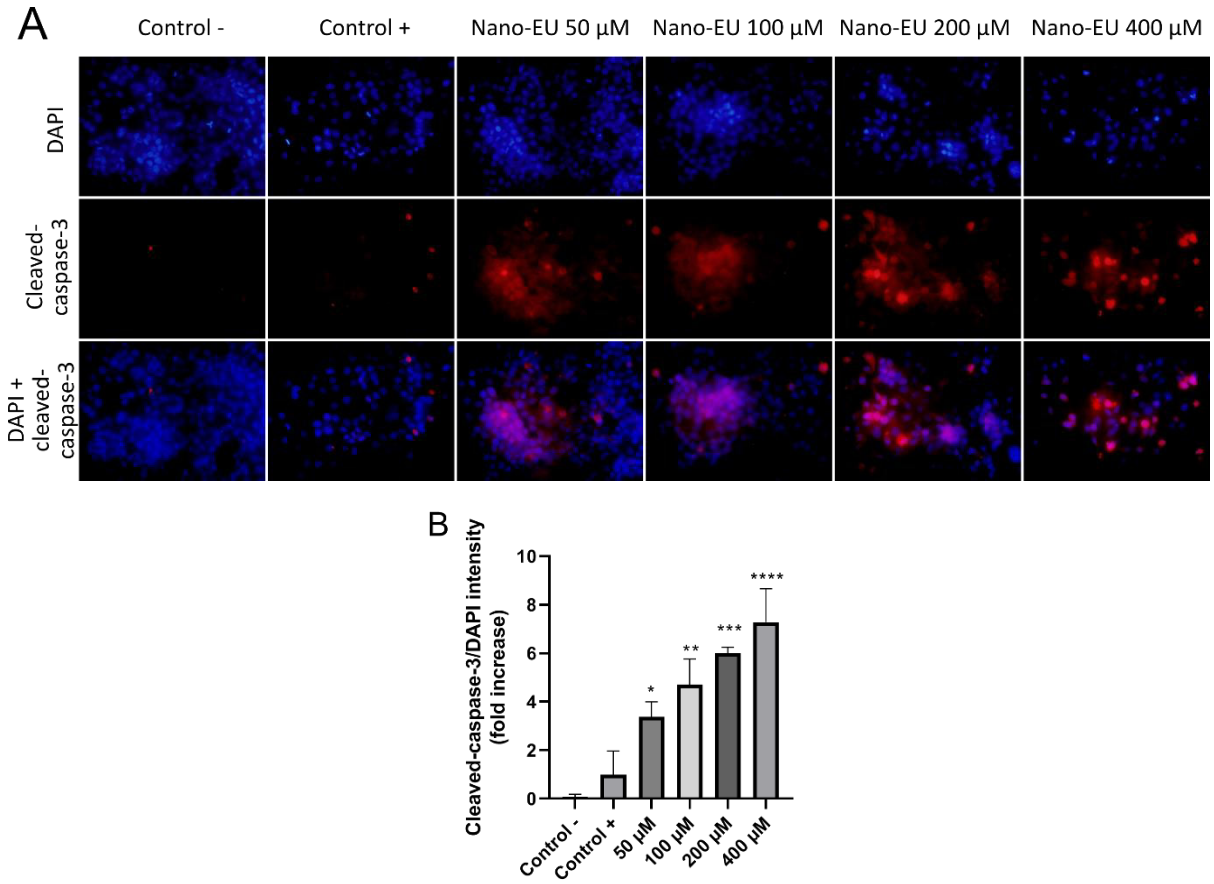

Fig. 3. Nano-EU promoted apoptosis through caspase-3 signaling pathway in HeLa cell lines. **(A, B)** Expression of cleaved-caspase-3 as executor caspase in HeLa cells were analyzed using immunoflourescence. Compared to the positive control group, * $p < 0.05$,*** $p < 0.001$, **** $p < 0.0001$ (ANOVA, Tukey's multiple comparison test).

3.4.Nano-EU inhibits Vimentin and Snail as mesenchymal marker in EMT Process

To determine the expression of vimentin and Snails, which are mesenchymal markers of EMT (epithelial to mesenchymal transition), indirect immunofluorescence staining was performed on HeLa cervical cancer cell cultures.Then the results will be observed using a dark field microscope.From the measurement of the levels of vimentin expression, it was found that the treatment group with doses of 100 µM, 200 µM, and 400 µM were able to suppress vimentin expression significantly more than the positive control group. In the measurement of Snail expression, it appears that the Snail expression decreased significantly at the 200 µM and 400 µM doses compared to the positive control group. These results illustrate that with the same dose as the positive control, administration of 200 µM Nano-EU was able to significantly suppress mesenchymal markers more than 200 µM pure eugenol.

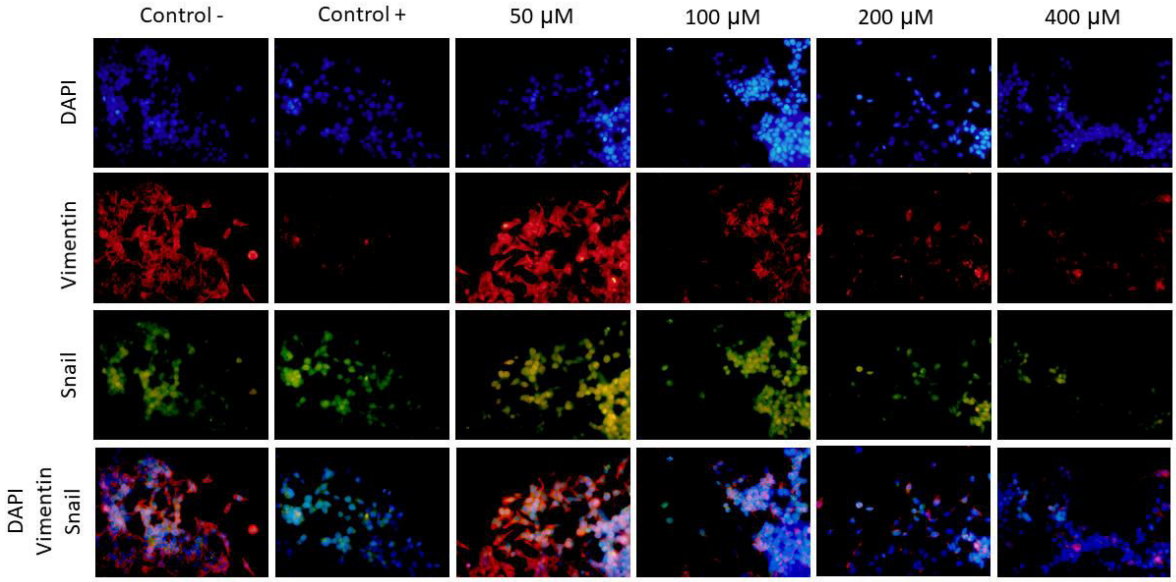

Fig. 4. The expressions of vimentin (red) and Snail (green) analyzed using immunofluorescence in Hela cells showed qualitative changes.The expression of vimentin and Snail was seen to be reduced compared to negative controls, except in the 50 µM group which was not significantly different from negative controls.Respectively, the expression of vimentin and Snail seemed to decrease at the Nano-EU dose of 100 µM, 200 µM, and 400 µM.

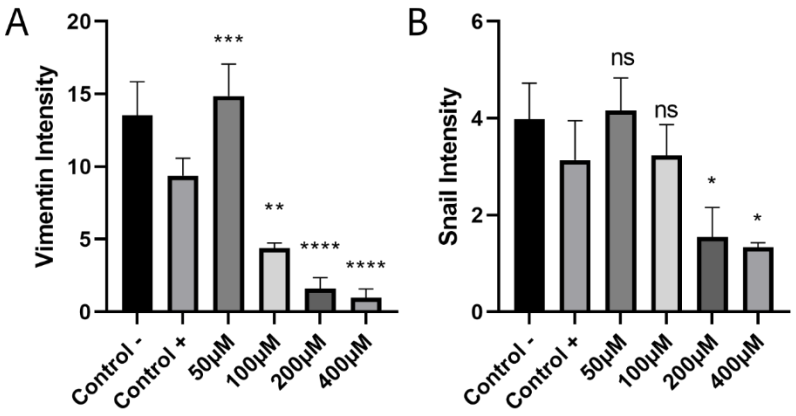

Fig. 5. Nano-EU inhibits vimentin and Snail in HeLa cell lines. **(A, B)**Both vimentin and Snail expression were observed by immunofluorescencein HeLa cells, compared with positive control group, * p< 0.05, ** p< 0.01, *** p< 0.001, **** p<0.0001 (ANOVA, Tukey's multiple comparison test).

3.5.Nano-EU inhibits cell migration in scratch test

In order to explore the role of Nano-EU in the cell migration process, scratch test is performed after 24-hour of treatment.After 24-hour post scratch test, we found that the distance between the cell are closer than before. But, the Nano-EU group has only a slightly different distance from before than the negative control group.The result of the scratch test has shown us there is inhibition of the cell migration in the Nano-EU group with 200 µM and

400 µM doses. Where they have a significantly better ability to inhibit cell migration than the positive control group ($p < 0.05$). The result also showed us that the dose of 50 µM dan 100 µM Nano-EU has already given us the same ability to inhibit cell migration as the 200 µM pure eugenol. This indicates that with the same dose, Nano-EU could give us a better result to inhibit cell migration than the pure eugenol.

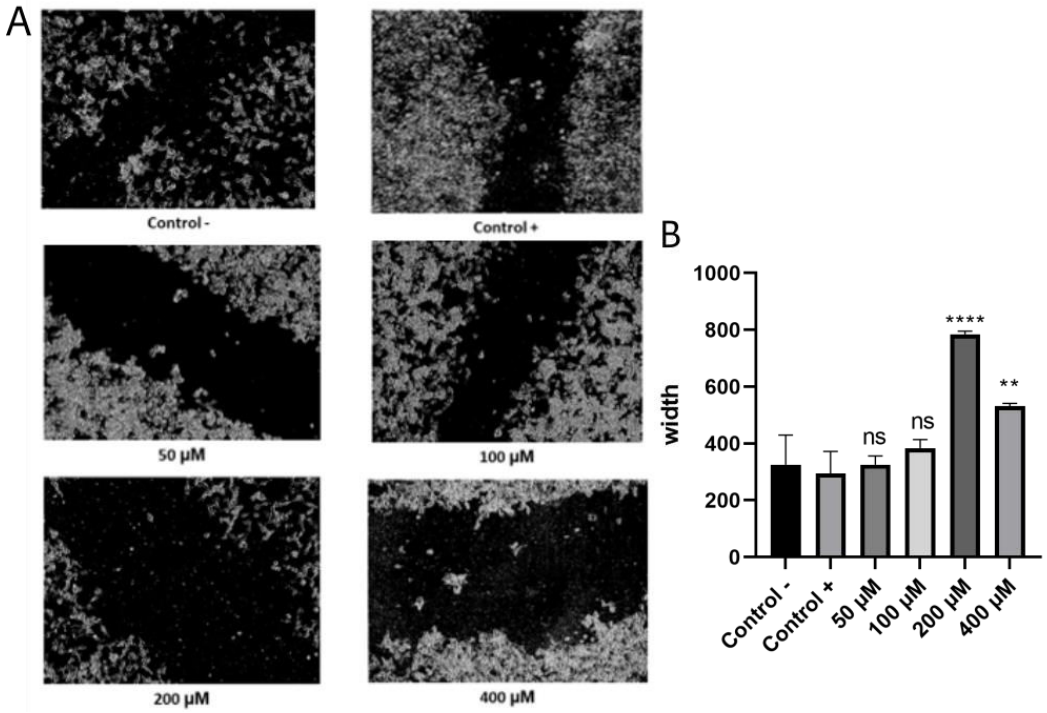

Fig. 6. Nano-EU inhibits cell migration in HeLa cell lines. **(A, B)**Nano-EU at 200 µM and 400 µM dose could inhibit cell migration better than the positive control group ($p < 0.05$). Meanwhile the 50 µM and 100 µM of Nano-EU have given us the same result as the positive control group does ($p > 0.05$).

Discussion

Eugenol (C10H12O2; 4-allyl-2-methoxy-phenol) is a member of the phenylpropanoids class of chemical compounds that are currently being studied because of their potential as an anticancer agent[14]. Eugenol was previously known to have a pro-apoptosis effect against a different types of cancers[26-28]. In this study, eugenol was loaded in chitosan nanoparticles to investigate its mechanism as a potential anticancer agent in the human cervical cancer cell line.

We made morphological observations on each well to qualitatively prove the effect of Nano-EU. Typically, HeLa cells have regular polygonal arrangement, have short cell antennae, and some cells are round[29]. In this case, eugenol therapy could cause morphological changes on the cell surface that were dose- and time-dependent. Normal cells became increasingly shrunken and circular. This was due to deformation of the cell plasma membrane. Moreover, there were differences in nucleus formation, which is theoretically due to the fragmentation of chromatin, which is then associated with the apoptosis process[30]. Das et al. examined the potential of eugenol in cervical cancer. They found that eugenol affected

changes in the cellular architecture of not only HeLa cells, but also that of MCF-7, SiHA, and SK-MEL-28 cells. This is due to a disturbance in the cytoskeletal dynamics leading to cytoplasmic retraction[31]. Subsequently, shrinkage, picinosis, and cell release occurs, culminating in anoikis (a cell death program caused by the release of cells from the extracellular matrix)[32].

In the present study, we did not measure the tumor-suppressor genes or the associated signaling pathways to determine the advanced mechanism. However, the morphological change in the form of chromatin fragmentation in the nucleus followed by microscopic cell shrinkage is closely related to apoptosis. Apoptosis is generally regulated by two important roles, namely pro-apoptosis and anti-apoptosis. High Bcl-2 expression can mediate resistance to the cytotoxic effects of chemotherapy agents[33]. In addition, apoptosis can be induced by modulating the cell survival signaling pathways. One such pathway is the Akt pathway, which is the key signaling molecule of the route. Increased activity of Akt and PI3K (phosphatidylinositol 3-kinase) and the mutation of PTEN (a negative regulator of Akt) are strongly associated with the occurrence of malignancy and induction of apoptosis. Accordingly, the inhibition of this pathway by eugenol will contribute to apoptosis. In addition, another fundamental mechanism of eugenol influencing apoptosis is via the mitochondrial pathway. This pathway can increase Bax and p53[34].

Apoptosis, programmed cell death, is a complex process that involves many functional molecular pathways in the cells. Apoptosis itself is one of the main routes targeted in various types of cancer therapy (Dutta and Chakraborty, 2018). The apoptotic pathway will lead to activation of the cysteine-dependent aspartate-specific protease (caspase). Caspase in the apoptosis process consists of two groups, the upstream initiator which plays a role in initiating cell death such as caspase-2, -8, -9, and -10, and the downstream effector which cuts substrates such as caspase-3, -6, and -7[35]. In HeLa cells treated with a various dose of Nano-EU for 24 h, apoptotic cell percentage showed a dose-dependent increase via annexin V and PI double staining (Fig.2), which is consistent with previous studies using eugenol[36]. We also investigate the expression of active caspase-3, cleaved-caspase-3, using the immunofluorescence method. Our results indicate that Nano-EU significantly increased the expression of cleaved-caspase-3 which leads to apoptosis (Fig.3).

Epithelial to mesenchymal transition (EMT) is a biological process that involves the polarization of epithelial cells. It causes various biochemical changes that allow epithelial cells to have a mesenchymal cell phenotype[37]. EMT allows epithelial cells to move and invade surrounding tissue[38]. Snail and vimentin are mesenchymal markers that play an important role in the EMT process. Snail is the main transcription factor that controls the EMT program. Snail belongs to the Zinc-finger protein class along with Slug and Smuc. These transcription factors all play a role in suppressing the expression of the E-cadherin gene and regulating the function of other genes that lead to EMT[39]. When HPV E6 degrades p53 protein, this will also reduce miR-34a levels which play a role in inhibiting Snail transcription factor. So that the degradation of p53 protein will lead to the activation of the Snail transcription factor[40]. Also, the activity of the Snail transcription factor is also supported by the presence of excessive EGF stimuli. This is because cervical cancer cells have a greater number of EGFRs than normal cells. These EGF stimuli result in the

inactivation of GSK-3β and stabilize the nuclear expression of the Snail transcription factor[41].Meanwhile, vimentin is a mesenchymal intermediate filament. Vimentin is one of the mesenchymal markers in the EMT process[42]. When Snail is expressed in cancer cells, it will induce the expression of vimentin as a mesenchymal marker[43]. The expression of vimentin will increase the motility of cancer cells so that cells can migrate and invade the surrounding tissue[44].

In this study, the results of immunofluorescence (Fig. 4) show that the expression of Snail and vimentin can be inhibited by eugenol and Nano-EU treatment. From Fig. 5, we can conclude that administration of Nano-EU at the same dose as eugenol gives far better results in inhibiting vimentin and Snail expression. Even the expression of Snail was significantly inhibited at the 100 µM Nano-EU. With the inhibition of Snail and vimentin as mesenchymal markers, it indicates that the EMT process in HeLa cervical cancer cells is inhibited, and it leads to the cell migration process being greatly suppressed. The results obtained are in accordance with the theory previously mentioned, that eugenol can give better results to inhibit the cell migration process by adding nano-capsule technology, as a drug delivery technology[15,17,19]. This of course makes Nano-EU has the potential to be a candidate for therapy in inhibiting the progression of cervical cancer which can metastasize and attack other organs in patients so that the patient's prognosis can be improved.

Metastasis in cancer is a complex process and has various stages in the process. One of the markers of the metastasis process is the migration of primary tumor cells into circulation[45]. To be able to invade the surrounding tissue, cancer cells need the ability to migrate. However. In cervical cancer, cells that experience malignancy is epithelial cells that have strong bonds between cells and are attached to the basal membrane[38]. Epithelial cells are cells that are fixed and have strong bonds between cells, so they generally do not have the ability to migrate. However, certain conditions allow epithelial cells to migrate. It is known that epithelial cells can migrate collectively or through the EMT process and migrate individually in the form of mesenchymal cells [46]. EMT can promote metastasis by increasing the movement of cells collectively. EMT can facilitate cell migration from primary tumor cells by maintaining epithelial features that maintain cell clusters and acquiring mesenchymal features that promote cell invasion and migration[47]. With the inhibition of EMT, migration from cancer cells will also be inhibited. In this study, the inhibition of cell migration was evidenced by using a scratch test. Scratch test or wound-healing assay is the easiest method to do. This method is useful for assessing the migration ability of the whole-cell mass[48].

In this study, interesting results were obtained on the scratch test as shown in Fig. 6. Where Nano-EU treatment at low doses (50 µM & 100 µM) does not give much difference from eugenol 200 µM as a positive control group. Meanwhile,Nano-EU with higher doses (200 µM & 400 µM) was able to provide significantly better results in inhibiting cell migration compared to the control group. This study shows the appropriate results to the theory, where Nano-EU can inhibit cell migration through inhibition of the EMT process. Nano-EUalso gives better results compared to eugenol at 200 µM, which is the optimal dose of eugenol in inhibiting cervical cancer development[36]. However, unfortunately, the results of this study do not include what cytotoxic effects can be produced by administering Nano-EU or eugenol.

In conclusion, the results of this study indicate that Nano-EU inhibits the growth of human cervical cancer cells by inducing cell apoptosis via activation of caspase-3 as the executor caspase. Nano-EU also inhibits Snail and vimentin as an important markers of the EMT process for cell migration in cervical cancer cells, better compared to eugenol.The mechanism of action of Nano-EU in cervical cancer is reported for the first time and is expected to be a potential therapeutic agent against human cervical cancer cells.

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