Synthesis and Proapoptotic Activity on Cervical Cancer Cell of Ester Eugenol 1-(3-Methoxy-4-hydroxy)phenyl-2-propylmethanoate

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Abstract. Proapoptotic activity of ester eugenol, 1-(3-methoxy-4-hydroxy)phenyl-2-propylmethanoate, which synthesized from eugenol is reported. Eugenol as starting material in the synthesis of ester eugenol was obtained from fractional distillation of clove oil with the yield of 70.66%. Synthesis of ester eugenol was carried out by addition-esterification reaction through reaction between eugenol and formic acid with mol ratio of 1:27 and reaction time for 11 h. GC-MS analysis showed ester eugenol was afforded purity of 92.42% and the yield in of 93.34%. UV spectra of ester eugenol was observed the formation of carbonyl group at \( \lambda_{\text{max}} \) 290 nm and supported by FT-IR analysis at 1714.60 cm\(^{-1}\) (carbonyl group), 1193.65 cm\(^{-1}\) (C-O-C ester group) and the absence of vinyl group in eugenol structure at region 914.20 and 995.20 cm\(^{-1}\). Mass spectra showed ion molecule at \( m/z \) 210 was accordance with molecular weight of ester eugenol. Afterward, HeLa cell culture media was prepared for cervical cancer antiproliferative test. The result which showed in histogram indicated that LC\(_50\) of ester eugenol was reached at concentration below 0.01% while eugenol was up to 0.01% that observed cervical cancer cell apoptotic activity. LC\(_50\) value of ester eugenol was obtained at concentration 48.73 ppm. This research reported that natural product modified its structure has potency to cure cervical cancer.

Keyword: Eugenol, cervical cancer, apoptotic, antiproliferative

1. Introduction

Cervical cancer is a disease which is generally suffered by woman and attributes the highest human death in Indonesia. Annually, about 500,000 new patients all over the world especially in developing country [1]. In 2013, 98,692 people were diagnosed with cervical cancer and becomes the highest prevalence level in Indonesia which increased 0.8% [2]. Cervical cancer is caused by human papilloma virus (HPV) which responsible for all of cervical cancer cases. Cervical cancer is vicious tumor that grows in women reproductive organs which located in upper genital tract between uterus and vagina [3]. Persistant infection of oncogenic HPV types explicitly assigned as causing agent of vicious process in the cervix epithel lesions. Women who is infected persistant HPV may indicated increased risk to the cervical cancer [4].

Therapy provision like operation, radiotherapy, and chemotherapy may lead to double disadvantages into patients, such as lower immune systems, high cost treatment due to import medicine. Therefore, it
requires novel innovation to provide medicines from natural products which spread over in Indonesia
and has antiproliferative agent toward cancer cell. A compound possesses antiproliferative activity and
easy to find is eugenol (4-allyl-2-methoxyfenol) which comprised in clove oil (80-90%) with strong
aroma [5]. Eugenol has been applied for antiseptic and anesthesia in medical science [6]. Eugenol also
has pharmacology effect such as antihelminthic, antiinflammatory, antiviral, antibacteria, analgesic,
antioxidant, anticancer, and antimutagenic [7].

Eugenol has been reported going to tumor growth by 24.35% with dose of 100 ppm [8]. Eugenol also
reveals its significant effect toward hepar cancer cell growth. HCT 15 and HT 29 cell was suppressed
by eugenol with IC50 value 300 and 500 μM, respectively [9]. Eugenol possesses good reducibility and
deployment of antioxidant activity against DPPH and lipid peroxidation [10]. Moreover, DNA oxidation
process which exposed by hydroxyl radical that afford fenton reaction can be protected by eugenol.
Those findings support eugenol becomes one of candidate for disease prevention which induced by
stress oxidative effect. Thus, eugenol has good prevention effect toward diseases such as cancer,
coronary heart, inflammatory disorder, and neurologic degeneration [11]. This obviously revealed that
natural antioxidant which contained phenolic structure, such as eugenol plays an important role in
protecting tissues toward free radical damages [12].

Antiproliferative activity of eugenol toward cancer cell is corresponding to the aromatic ring in
eugenol structure. Methylated eugenol has ability to inhibit cervix cancer [13]. This ability can be
improved by modifying allylic group which bonded with aromatic ring of eugenol to be an ester group
that useful for increasing radical scavenging ability [14] and reduce the flavor of eugenol. Allylic group
of eugenol converted to carbonyl group by addition reaction gave ester eugenol, 1-(3-methoxy-4-
hydroxy)phenyl-2-propylmethanoat, that expected to enhance antiproliferative effect toward cervix
cancer cell. Ester group in the molecule such as caffeic acid phenylethyl ester (CAPE) which contained
in propolis extract gives strong antioxidant activity [15].

Assymmetric alkene addition of eugenol which utilized asymmetric reactant principally occur
regiospecifically which means reactant involving addition to the double bond in one direction. In this
reaction, it prevailing the Markovnikov rules that addition of positive charges, such as H+, reactant will
bond double bond C atom which contain more hydrogen atom, thus it forms the stable carbocation.
Therefore, according to observation only one product is abundant from two of obtained products [16].

\[ \text{CH}_3\text{O} \quad \text{HO} \quad \text{CH}_3\text{O} \quad \text{HO} \quad \text{OO} \quad \text{H} \quad \text{HCOH} \]

\[ \text{O} \quad \text{HCHO} \rightarrow \text{CH}_3\text{O} \quad \text{HO} \quad \text{O} \quad \text{O} \quad \text{H} \]

**Figure 1.** Synthetic route of ester eugenol through addition reaction

Cytotoxicity test toward cancer is a general basic test for anticancer drug and chemopreventive
compounds. One of method that used generally for in vitro cytotoxicity test is MTT method [17]. This
method is based on reduction reaction of MTT reagent (3-(4,5-dimethylthiazole-2-yl) 2,5-
diphenyltetrazolium bromide) which catalyzed by dehydrogenase succinate enzyme in human cell. This
test is initiated by transfer of 104 test cell into complete culture media which consist of FBS as a main
cell nutrition, penicillin-streptomycin as a bacteria contaminant prevention, amphoterizin-B as a fungi
contaminant prevention and RPMI 1640 as carrier media which is each volume of 100 μL) into 96-well
plate and incubated in CO₂ incubator 5% overnight. Afterward, test samples were given using yield
series and replicated 3 times, and then reincubated overnight. In the third test day of MTT reagent
addition, it forms formazan crystal after 4 h in living cell. And then, it was added SDS stopper to quench
the MTT reaction. The absorbance was recorded using ELISA reader at wavelength of 595 nm. Death
level apoptotically can be analyzed using flow cytometry methods [18].
2. Material and Methods

2.1. Material
Sample materials used in this research is clove leaves oil collected from distillers. Chemical used in this research, was distilled water, sodium hydroxide (Merck), sodium sulfate anhydride (Merck), hydrochloric acid (Merck), dichloromethane (Merck), formic acid (Merck), diethyleter (Merck), sodium carbonate (Merck), eugenol (Merck). Media Rosewell Park Memorial Institute (RPMI 1640) (Gibco), Fetal Bovine Serum (FBS) (Sigma), Penicillin–streptomycin (Sigma), Amphotericin B (Sigma), dimethyl sulfoxide (DMSO), trypsin-EDTA (Sigma) (trypsin 0.25%), Trypan Blue (Sigma), Sodium Dodecyl Sulfate (SDS) and Phosphat Buffer Saline (PBS) (Invitrogen).

2.2. Methods

2.2.1. Eugenol isolation from clove oil
150 mL of clove oil was distilled in vacuum at 100 mmHg. Distillate was collected at 80-82°C. The density was also measured and analyzed using Infra-Red spectrophotometer and GC-MS.

2.2.2. Synthesis of ester eugenol
Method was modified from synthesis of ester methyleugenol [19]. 248.40g of formic acid 98% (203.90 mL, 5.40 mol) was put into 2-neck flask 500 mL which set by condenser, stirrer, and dropper funnel which contained 32.80 g of eugenol (30.83 mL, 0.20 mol). Eugenol was added into the flask stirred refluxed for 11 h at 130°C. Afterward, distillation was conducted under 100-105°C (1 atm) to obtain a residual reaction of formic acid. The residue washed using concentrated NaHCO₃ until pH reached neutral and then was extracted using 30 mL of diethyl ether for 2 times. Organic phases was collected and dried by anhydrous Na₂SO₄. The organic phases was filtered and concentrated. The result was analyzed by UV-Vis spectrophotometer, Infra-Red spectrophotometer, and GC-MS.

2.2.3. Apoptotic test toward HeLa cervix cancer cell
Anticervical cancer test was modified from MTT method [20]. 100 μL of cervical cancer cell suspension (HeLa) using 3x10⁴ cell/100 μL media was distributed into 96-well plate and incubated for 24 h. After incubation, 100 μL of test solution in various concentrations was added into the plate. For positive control, 100 μL of culture media was added, and then 100 μL of cisplatin in various concentrations was added into the plate. For cell control, 100 μL of culture media was added into 100 μL of cell suspension and for solvent control, 100 μL of DMSO was added into culture media 100 μL and 100 μL of cell suspension with proper dilution which is equivalent with dilution of test solution concentration. And then, the solution was incubated for 24 h with CO₂ flow 5% and O₂ flow 95% at 37°C.

In the final incubation, flowcytometry procedure was conducted by washing cell for 2 times using cold cell staining buffer then cell re-suspension was conducted in annexin v binding buffer in concentration of 0.25-1.0 x 10⁷ cell/mL. Afterward, 100 μL of cell suspension was transferred into 5 mL reaction tube and was added 5 μL of FITC annexin v and 10 μL of propidium iodide solution with concentration of 20 μg/mL. cell was rotated slowly and was incubated for 15 mins under room temperature (25°C) in dark container. Finally, 400 μL of annexin v binding buffer in each tube was analyzed by flowcytometer with proper machine setting. Data was analyzed by Cell Pro Quest software. Afterward, uncolored cell using FITC annexin v and PI will not recorded as apoptotic, cell colored using FITC annexin v will be measured as early apoptotic, cell positively colored using FITC annexin v and PI will be recorded as late apoptotic or necrotic.

3. Result and Discussion

3.1. Synthesis of ester eugenol
Synthesis of ester eugenol was carried out by reacting eugenol from fractional distillation and formic acid using mol ratio of eugenol:formic acid = 1 : 27 for 11 h. The result tabulated in Table 1.

Table 1. Physical properties of the product

| No | Physical parameter | Eugenol  | Ester Eugenol |
|----|-------------------|----------|---------------|
| 1. | Color             | colorless| brown         |
| 2. | Odor              | spicy    | scent         |
| 3. | Density           | 1.065 g/mL| 1.26 g/mL   |

A specific chromatogram peak in $t_R$ 19.31 mins which indicated the purity level of ester eugenol reached 92.42%. This result based on the mass spectra in Figure 4 which observed molecule ion at m/z 210 was accordance with molecular weight of ester eugenol. According to the purity grade, the yield percentage of ester eugenol was determined as high. This result indicated that reaction time treatment for 11 h showed high efficiency level compared to the earlier report for reaction of methyleugenol and formic acid with reaction time for 21 h [19].

Figure 2. Mass spectra in $t_R$ 19.31 mins

Ester eugenol was successfully synthesized by analyzing using UV-Vis which observed the carbonyl group ($\text{C}=\text{O}$) according to the absorbance at $\lambda_{\text{max}}$ of 290.40 nm. This result also corresponds to the FT-IR spectra (Figure 3) which showed the carbonyl group with high intensity at 1714 cm$^{-1}$ and C-O-C ester at 1193 cm$^{-1}$ which indicated the formation of ester eugenol. It also supported the absence of absorbance at 914.20 which indicated the absorbance from out of plane of terminal alkene in the eugenol.

Figure 3. Infrared spectra overlay of ester eugenol and eugenol
3.2. Apoptotic test of ester eugenol toward cervical cancer HeLa cell

Apoptotic test of ester eugenol toward cervix cancer cell was conducted by preparing the HeLa cell media culture in certain time and compared to treatment of 0.1% ester eugenol. Figure 4 showed the observation result of HeLa cell growth after left for 2 days.

\[ \text{Figure 4. Cervical cancer HeLa cell growth: (A) without treatment (B) treatment with 0.1% ester eugenol.} \]

Cell death was observed in ester eugenol treatment which indicated the decrease of cell rigid compared without treatment. It indicated that ester eugenol has toxic effect toward cervix cancer cell. Moreover, inhibition effectivity of ester eugenol can be analyzed from LC\textsubscript{50} of each ester eugenol and eugenol toward cervix cancer cell as shown in Figure 5.

\[ \text{Figure 5. Comparison of eugenol and ester eugenol effect toward cervix cancer cell HeLa apoptotic} \]
According to the Figure 5, it observed qualitatively that LC50 value of ester eugenol was below than 0.01% (→), while eugenol was up to 0.01% (→). It showed that ester eugenol ability in the cervix cancer HeLa cell was greater than eugenol. It showed that vinyl group (alkene) of eugenol which converted to ester contain carbonyl group that affected significantly to enhance apoptotic toward cervix cancer cell besides its ability as radical scavenger to inhibit cancer cell growth. Moreover, the test result of LC50 determination of ester eugenol toward cervix cancer cell below 0.01% (after compared to the control and solvent effect) can be seen in Figure 6.

![Figure 6. Correlation between ester eugenol concentration and cervix cancer HeLa cell death](image)

Figure 6 showed linear correlation between ester eugenol concentration and cervix cancer cell apoptotic level. According to equation of $y = 10.259x$, LC50 of ester eugenol was determined at concentration 48.73 ppm. It indicated that ester eugenol ability toward cervix cancer HeLa cell apoptotic was very strong in concentration below 50 ppm that was able to induce cervix cancer cell apoptotic until 50%.

4. Conclusion
Ester eugenol was successfully synthesized from eugenol isolated from clove oil mol ratio of eugenol : formic acid = 1:27 for 11 h purity 92.42% and yield of 93.34%. Ester eugenol showed apoptotic antiproliferative level toward cervix cancer cell greater than eugenol with LC50 value at concentration 48.73 ppm.

Acknowledgement
Authors are grateful to Ministry of Research, Technology and Higher Education of the Republic of Indonesia for for their financial support to fund this research.

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